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تقييم طفرات جين PAH في المناطق الاكسونية ومناطق وصل الاكسون–انترون لدى عائلات مصابة بالبيلة الفينيل كيتون الكلاسيكية (PKU) في البصرة، العراق

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المستخلص:

البيلة الفينيلية الكيتونية (PKU) هي اضطراب وراثي نادر يؤثر على طريقة استقلاب الجسم للحمض الأميني فينيل ألانين(Phe) ، وهو مكوّن موجود في العديد من الأطعمة الغنية بالبروتين. ينجم هذا الاضطراب عن نقص أو غياب تام في نشاط إنزيم فينيل ألانين هيدروكسيلاز(PAH) ، والذي يقوم عادةً بتحويل الفينيل ألانين إلى الحمض الأميني الآخر تيروزين. وعند غياب النشاط الكافي لهذا الإنزيم، يتراكم الفينيل ألانين في الجسم ليصل إلى مستويات سامة، مما يؤدي إلى أضرار خطيرة وغالباً ما تكون غير قابلة للعكس في الدماغ والجهاز العصبي. وقد يترتب على ذلك إعاقات عقلية ومضاعفات عصبية أخرى ما لم يتم التشخيص المبكر والتدخل العلاجي المناسب.وفي إطار السعي لفهم الطفرات الجينية المرتبطة بمرض PKU ضمن السكان المحليين، أُجريت دراسة وصفية مستعرضة شملت ١٥ مريضاً من محافظة البصرة، العراق. تم جمع عينات دم من جميع المشاركين، واستخلاص الحمض النووي (DNA) منها للتحليل. ركزت الدراسة على تسلسل كل من الإكسونات والإنترونات (المناطق المشفرة والمحيطة بها) في جين PAH باستخدام تقنية سانغر للتسلسل، والتي لا تزال تُعد معياراً ذهبياً في الكشف عن الطفرات النقطية. وقد أظهرت نتائج التحليل وجود عدة طفرات متكررة، كانت أبرزها الطفرة c.782G>A في الإكسون ٧، والتي ظهرت لدى ٤٠% من المرضى. تلتها الطفرة c.1066–11G>A في الإنترون ١٠ (٣٣.٣%)، ثم c.168+5G>C في الإنترون ٢ (١٣.٥%)، وأخيراً c.926C>A في الإكسون ٩ (١٣.٥%).

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وتشير هذه الطفرات إلى إمكانية تأثيرها السلبي على الوظيفة الطبيعية لإنزيم**PAH** ، مما يساهم في الخلل الأيضي لدى هؤلاء المرضى. تسلط هذه النتائج الضوء على التنوع الجيني لطفرات **PKU** حتى ضمن عينة صغيرة نسبياً، مما يشير إلى ضرورة إجراء دراسات جينية أوسع تشمل مختلف المجموعات العرقية في العراق، بهدف استكشاف العلاقة بين الطفرات الجينية المحددة والمظاهر السريرية المصاحبة لها. وقد يكون لمثل هذه الأبحاث دور مهم في تحسين سبل التشخيص المبكر والعلاج والإرشاد الوراثي للعائلات المتأثرة بهذا الاضطراب.

الكلمات المفتاحية: البيلة الفينيل الكيتونية، فينيل الانين هيدروكسيلاز، الطفرات الجينية، الاعراض السريرية.

Evaluation of PAH Gene Mutations in Exonic and Exon-Intron Junction Regions in Families with Classical PKU in Basrah, Iraq

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Abstract

Phenylketonuria (PKU) is a rare inherited disorder that affects how the body processes the amino acid phenylalanine (Phe), a component found in many protein-rich foods. The condition stems from a deficiency or complete inactivity of the enzyme phenylalanine hydroxylase (PAH), which normally converts Phe into another amino acid, tyrosine. Without sufficient PAH activity, Phe accumulates in the body to toxic levels, leading to serious and often irreversible damage to the brain and nervous system. This can result in intellectual disabilities and other neurological complications if not managed early. In an effort to better understand the genetic mutations associated with PKU in the local population, a descriptive cross-sectional study was conducted involving 15 patients from Basrah, Iraq. Blood samples were taken from each participant, and DNA was extracted for analysis. The study focused on sequencing both the exons and introns (regions within and surrounding the coding areas) of the PAH gene using Sanger sequencing, which remains a gold-standard method for detecting point mutations. The

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analysis revealed several recurring mutations. The most frequent was c.782G>A located in exon 7, found in 40% of the patients. This was followed by c.1066-11G>A in intron 10 (33.3%), c.168+5G>C in intron 2 (13.5%), and c.926C>A in exon 9 (13.5%). Each of these mutations could potentially interfere with the normal function of the PAH enzyme, contributing to the metabolic dysfunction observed in these patients. The findings highlight the genetic diversity of PKU mutations even within a relatively small sample group. This suggests a need for broader genetic studies across Iraq's different ethnic groups to further explore the relationship between specific genetic mutations and clinical outcomes. Such research could be crucial in refining approaches to early diagnosis, treatment, and genetic counseling for families affected by PKU.

Keywords: phenylketonuria, Phenylalanine hydroxylase, Mutation, Clinical symptoms

1. Introduction

Phenylketonuria (PKU) is a metabolic disorder caused by a deficiency of phenylalanine hydroxylase in the liver (Kenneson et al., 2021). It follows an autosomal recessive inheritance pattern. Due to this enzyme deficiency, Phe is not converted to tyrosine, leading to elevated levels of Phe in the blood. Infants with PKU appear normal at birth, but Phe accumulation in the brain causes severe intellectual impairment in the first year of life if left undetected (Kenneson et al., 2021; de Groot et al., 2010). PKU affects one out of every 10,000 to 20,000 live births depending on the area. The prevalence of this disease also varies from 1 in 4,000 births in Northern Ireland to 1 in 71,000 births in Finland (Karamifar et al., 2010; Shoraka et al., 2020).

A mutation on chromosome 12q23.2 disrupts the function of the PAH gene, which encodes the enzyme phenylalanine hydroxylase (PAH). Deficiency or malfunction of this enzyme results in the development of PKU. As a consequence, phenylalanine is not effectively converted into tyrosine, leading to an abnormal accumulation of phenylalanine in the blood (Volgina et al., 2017; Ribas et al., 2011). This excess phenylalanine can

exceed 1000 µmol/L in untreated PKU patients (Ghadbeigi et al., 2013). The elevated levels of phenylalanine interfere with brain enzymes such as pyruvate decarboxylase, contributing to neurotoxicity and impaired neurotransmitter synthesis (Bilder et al., 2016). Consequently, untreated PKU patients may present with severe behavioral disorders, including intellectual disabilities, autistic features, and aggressive behaviors (Brumm et al., 2010; Burlina et al., 2019).

A newborn with PKU should be treated immediately to avoid a rise in blood Phe levels. As a result, early detection of this disorder in the earliest days of life is essential. Screening for the disorder using newborn screening tests is a social health strategy that has been used in several countries over the last 50 years (van Spronsen et al., 2021; Borrajo et al., 2019; Wiedemann et al., 2021).

Recent studies have demonstrated that Sanger sequencing remains a highly accurate and reliable method for detecting mutations and confirming genetic variations, particularly in monogenic disorders. Its high specificity and sensitivity make it a gold standard for identifying known and novel mutations, ensuring precise genetic diagnosis (Neissi et al., 2024; Neissi et al., 2025). Given this evidence, we chose to investigate mutations in the PAH gene in patients with classical PKU in Basrah, Iraq. Since mutations in the PAH gene vary among different populations and ethnic groups, studying these variations in the Basrah population is crucial for understanding the genetic basis of PKU in this region and improving diagnostic and therapeutic strategies.

2.Methodology

Samples collection

In this descriptive cross-sectional study, 15 patients with PKU in Basrah, Iraq, after confirmation by biochemical testing were included in the study. Then, blood samples were obtained at a rate of 2-5 ml from each patient, and 5 ml Falcons containing 300 µl and 0.5 M EDTA were used as anticoagulants to prevent blood coagulation. All patients filled out consent papers.

DNA extraction

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The Qiagen Blood DNA Extraction Mini Kit (Qiagen, Germany) and the manufacturer's instructions were used to extract the DNA content of the samples. The extracted DNA was analyzed using Nanodrop after DNA extraction and before PCR reaction to determine the quantity of nucleic acid (NanoDrop, 2000C, Thermo scientific, USA). The samples were also electrophoresed on a 1% agarose gel to confirm the quality of the extracted DNA.

Polymerase chain reaction (PCR)

The PCR reaction was carried out by Analytik Jena Thermocycler (Germany) as following: 12.5 µL Master Mix 2X (Taq Mix Red, PCR bio, UK), 1 µL DNA (100 ng), 0.5 µL forward primer. 0.5 µL reverse primer, and H2O up to a final volume of 25 µL. The PCR reaction was performed as follows: initial annealing temperature of 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 69°C for 30 seconds, 72°C for 30 seconds, and the final elongation temperature was 72 ° C for 5 minutes. Table 1 lists the properties of the primers utilized in this reaction. Using the Blast NCBI tool, the specificity of primers for the required sequences was also tested. Bioneer, a South Korean business, synthesized the primers. After that, 3 µl of reaction products (patient samples) and a 100 bp marker were loaded on a 1 % agarose gel and electrophoresed to ensure the amplification of the desired component and its quality, as well as the non-proliferation of non-specific products.

Table 1. The primers that were utilized in this study

PAH-IVS10-11	**Forward: GGGTAGAACCTAGTATGTGC**
	Reverse: GGGAGAGAAACTGTCTATGG
PAH-IVS2+5	**Forward: GTTCATGCTTGCTTTGTCC**
	Reverse: CTGTTGAAACTGACAAGGC
PAH-Exon7	**Forward: GGTGATGAGCTTTGAGTTTT**
	Reverse: TGGATCACAGGATGACCAAA
PAH-Exon9	**Forward: TCATGAGCAAGTCACTTCCC**
	Reverse: TGGTGGGTTCAAGATACTGC

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Sanger Sequencing

The sequencing was conducted using the ABI 3730 DNA Analyzer (Macrogen, South Korea) following the chain termination method pioneered by Sanger, which relies on labeled dideoxynucleotides to generate DNA fragments of varying lengths for size-based separation and analysis. Post-sequencing data was processed using CLC Central Workbench 5 and Chromas software to visualize chromatograms, perform base calling, and align sequences for comparison with the PAH gene's coding and intronic regions. Rigorous sequence quality assessment was performed to ensure accurate variant detection, including careful inspection of chromatogram peaks and exclusion of low-quality reads.

Sequencing and Bioinformatics Analysis

To identify the known mutation, researchers examined databases like ENSEMBL (https://useast.ensembl.org/index.html) and double-checked that the mutation found had previously been reported as pathogenic polymorphisms or novel mutations. The Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/ac/index.php), a one-of-a-kind resource for precise information on human disease mutations in genetics and human research, was also utilized. Additionally, protein-protein interaction (PPI) analysis was performed using STRING (https://string-db.org/) to predict how the variant might disrupt molecular interactions relevant to the observed phenotype.

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3. Results and discussion

Patient demographic information

This study enrolled 15 children with classic PKU, comprising 9 females (60%) and 6 males (40%) whom a pediatrician had examined. Patients were separated into three groups before therapy depending on their Phe levels (Table 2). Patients ranged in age from 1 to 21 years. All of the patients' parents were related.

Table 2. The demogheraphic information of patients.

Variable	Category	Number of patients (n=15)	Percentage (%)
Sex	Female	9	60.0%
	Male	6	40.0%
Age at diagnosis	1–5 years	7	46.7%
	6–10 years	5	33.3%
	>10 years	3	20.0%
Parental relationship	Consanguineous	15	100%
Phenotype distribution	cPKU (severe)	2	13.3%
	mPKU (moderate)	7	46.7%
	mHPA (mild)	6	40.0%
Phe concentration (µmol/L)	Mean ± SD	1,353 ± 290	—
	Range	1,060 – 1,800	—

Abbreviations: cPKU: severe phenylketonuria; **mPKU:** moderate phenylketonuria; **mHPA:** mild hyperphenylalaninemia; **Phe:** phenylalanine.

Discovery of Homozygous Missense Mutation in Exon 9 of the Chromosomal Sequence

The product was sequenced after electrophoresis of PCR products and verifying the formation of a specific band with a length of 550 bp. In 15 patients with classical PKU, nucleotide sequences of exons 9 and 7 and surrounding introns showed two homozygous missense mutations (13.5%) in the chromosomal sequence in exon 9 (NM_000277.3) (c.926C>A). This mutation, located at hg38: chr12-102846938-G-T, results in a change from alanine (Ala) to aspartic acid (Asp) at position 309 (p.Ala309Asp), altering the protein's structure. This change may significantly impact the protein's

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function or stability (Figure 1). Table 3 also includes all of the details about the detected mutation. The relevance and role of this mutation in pathogenicity have been determined using bioinformatics approaches (Table 4).

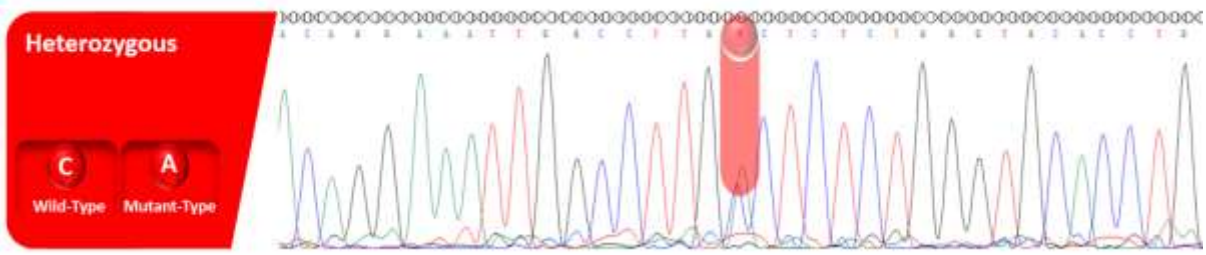

Figure 1. Sequencing results showing the c.926C>A missense mutation in patients

Table 3. Summary of detected PAH gene mutations in PKU patients.

Mutation (HGVS)	Location (Exon/Intron)	Nucleotide change	Protein change	Mutation type	ACMG classification	Inheritance
NM_000277.3: c.926C>A; p.Ala309Asp	Exon 9	c.926C>A	p.Ala309Asp (A309D)	Missense	Pathogenic	Autosomal Recessive
NM_000277.3: c.782G>A; p.Arg261Gln	Exon 7	c.782G>A	p.Arg261Gln (R261Q)	Missense	Pathogenic	Autosomal Recessive
NM_000277.3: c.1066-11G>A (IVS10-11G>A)	Intron 10	IVS10-11G>A	—	Splicing mutation	Pathogenic	Autosomal Recessive
NM_000277.3: c.168+5G>C (IVS2+5G>C)	Intron 2	IVS2+5G>C	—	Splicing mutation	Pathogenic	Autosomal Recessive

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Abbreviations: ACMG: American College of Medical Genetics and Genomics.

Table 4. In silico prediction of the effects of the missense variant (c.926C>A; p.Ala309Asp)

Gene/variant	Polyphen2 HDIV score	SIFT score	Mutation taster
ENST00000553106, Ala309Val	(Probably 1.000 damaging)	0(Damaging)	Disease-causing

Discovery of Homozygous Missense Mutation in Exon 7 of the Chromosomal Sequence

As shown in Figure 2, a homozygous missense mutation in exon 7 (NM 000277.3) was detected as homozygous (c.782G>A). This mutation, located at hg38: chr12-102852875-C-T, results in a nucleotide substitution from guanine (G) to adenine (A) at position 782. Consequently, this change alters the codon from CGA to CAA, leading to the replacement of arginine (Arg) with glutamine (Gln) at position 261 (p.Arg261Gln). Arginine is a positively charged, hydrophilic amino acid, while glutamine is a polar but uncharged amino acid (Table 3). Also, in silico prediction of the missense, variant effects has been shown in Table 5.

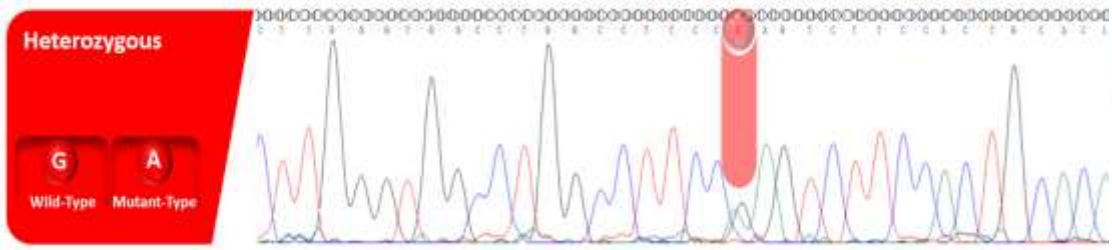

Figure 2. Sequencing results showing the c.782G>A missense mutation in patients

Table 5: In silico prediction of the effects of the missense variant

Gene/variant	Polyphen2 HDIV score	SIFT score	Mutation taster

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ENST00000553106, p.Arg261Gln	**(Probably 1.000 damaging)**	**0.001, 0 (Damaging)**	**Disease-causing**

Discovery of Homozygous Splicing Mutation in Intron 10 of the Chromosomal Sequence

Splicing mutations in chromosomal sequences in intron 10 (NM_000277.3) were detected as homozygous (c.1066-11G>A), as shown in Figure 3. This mutation, located at hg38: chr12-102843790-C-T, results in a substitution of guanine (G) with adenine (A) at position -11 relative to exon 11. Since this variant occurs within the intronic region near the splice acceptor site, it may disrupt normal splicing by weakening or altering the recognition of the splice site. Consequently, this can lead to aberrant exon skipping, retention of intronic sequences, or activation of a cryptic splice site, ultimately causing a frameshift or introducing premature stop codons. Such splicing defects may produce a truncated or nonfunctional protein, affecting enzyme activity and contributing to disease pathology (Table 3).

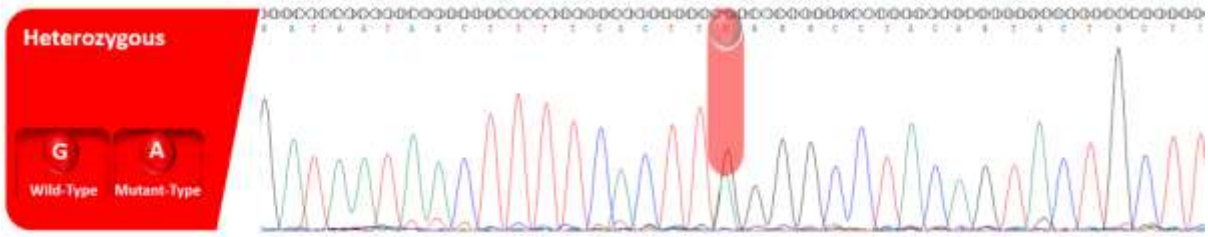

Figure 3: Sequencing results showing the c.1066-11G>A splicing mutation in patients

Discovery of Homozygous Splicing Mutation in Intron 2 of the Chromosomal Sequence

A chromosomal splicing mutation in intron 2 (NM_000277.3) was found to be homozygous (c.168+5G>C), as illustrated in Figure 4. This mutation, located at hg38: chr12-102912786-C-G, results in a guanine (G) to cytosine (C) substitution at position +5 of the intron, a critical region for proper splicing. Since the +5 position is part of the conserved splice donor site, this change may weaken or disrupt normal spliceosome recognition, leading to aberrant splicing outcomes such as exon skipping, partial intron retention, or activation of cryptic splice sites. These splicing errors can alter

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the reading frame, potentially generating a truncated or dysfunctional protein, which may significantly impact enzymatic activity or stability. The complete details of this mutation are provided in Table 3.

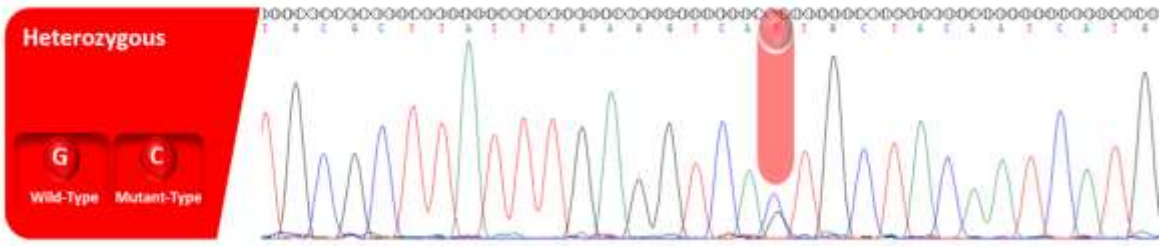

Figure 4: Sequencing results showing the c.168+5G>C splicing mutation in patients

Protein-Protein Interaction (PPI) Network Analysis of PAH

The PPI network analysis of the PAH gene was conducted to identify its functional associations with other proteins involved in Phe metabolism. Using the STRING database, the interaction network revealed that PAH has strong associations with multiple enzymes, including QDPR, SPR, PCBD1, PCBD2, DDC, GOT1, GOT2, GOT1L1, TAT, and TYR (Figure 5). These interactions suggest a coordinated role of PAH with tetrahydrobiopterin (BH4)-dependent enzymes and transaminases, which are crucial for amino acid metabolism. The network exhibited high connectivity, indicating that PAH is central in maintaining Phe homeostasis. The presence of multiple interaction edges with varying confidence scores further highlights PAH's involvement in complex biochemical pathways. Notably, interactions with genes like PCBD1 and SPR, which participate in BH4 regeneration, emphasize the importance of cofactor availability in PAH function. This analysis provides a deeper insight into potential compensatory mechanisms and secondary metabolic disruptions that could arise due to PAH mutations in PKU.

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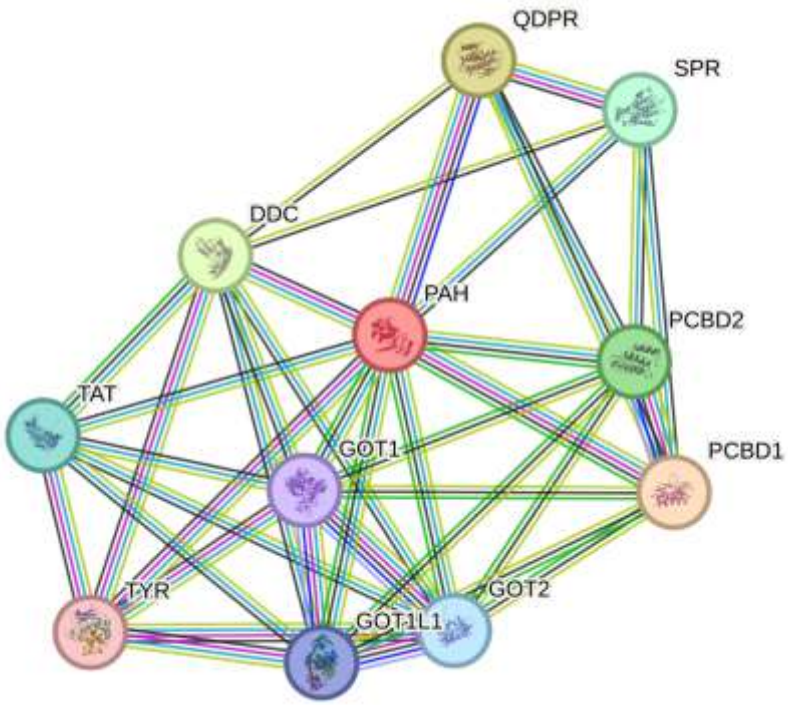

Figure 5. PPI network of the PAH gene generated using the STRING database. The network displays PAH interactions with proteins involved in Phe metabolism and BH4 biosynthesis, including QDPR, SPR, PCBD1, PCBD2, DDC, GOT1, GOT2, GOT1L1, TAT, and TYR.

٤.Discussion

Genetic disorders, can cause significant emotional and financial burdens in human communities. PKU is an autosomal recessive disorder that affects amino acid metabolism and presents with various clinical and biochemical symptoms. In untreated patients, it also causes severe intellectual impairment. unless they are maintained on a phenylalanine (Phe)-restricted diet. Because of the high frequency of PKU, newborns are now routinely screened and treated with a Phe-restricted diet in many developed countries (Oussalah et al., 2020).

The PAH gene, located on chromosome 12q23.2, contains 13 exons and 12 introns. Over 800 mutations have been documented in the PAH locus database, including nonsense variants, insertions/deletions, and splicing defects. Since PAH mutations

account for nearly all cases of PKU, comprehensive analysis of this gene is critical for effective disease management (Morovatdar et al., 2014).

In the present study, 15 patients with classical PKU from Basrah, Iraq, were evaluated. Preliminary evaluation showed that the cohort included all three phenotypes: severe (cPKU), moderate (mPKU), and mild (mHPA); with parental consanguinity observed in all cases. The most frequently affected regions were exon 7 (40%), intron 10 (23.3%), intron 2 (13.5%), and exon 9 (13.5%).

Only 2 patients (13.3%) exhibited the severe cPKU phenotype in homozygous form. mHPA may also result from younger patient age or impaired liver function, in addition to genetic mutations. In such cases, conventional mutation screening may fail to detect variants due to these confounding factors, therefore, high-resolution approaches such as whole-exome sequencing, as performed in this study, are recommended (Yan et al., 2019).

Our findings demonstrate significant heterogeneity in PKU-associated mutations among patients. This is consistent with findings by Enacán et al. (2019), who reported exon 7 as the most frequently detected mutation. Their study also revealed considerable genetic heterogeneity among European patients. Although their study was conducted in Europe, the distribution differed from that observed in Central Europe and aligned more closely with Mediterranean hereditary patterns (Enacán et al., 2019).

The intron 10 mutation (c.1066-11G> A) was shown to be the second most prevalent in our study, comparable to that discovered by Eduardo Vieira Neto and colleagues in Brazil. According to the findings of this study, the intron 10 mutation is one of the most prevalent mutations discovered (Vieira Neto E et al., 2018). 13 exons were evaluated in a thorough analysis done in 2019 to discover the most frequent PKU mutations in Iran. This investigation discovered 34 mutations with various symptoms, with the most prevalent intron 10 and exon 7 alterations (Esfahani et al., 2019).

Mutation IVS10-11G> A in intron number 10 has been documented as the most prevalent dominant mutation in the Mediterranean region and the second most common PKU mutation in the PAH database, which is referred

to as the "Mediterranean" mutation owing to its prevalence in the Mediterranean region (Okano et al., 1991). In the mutation IVS10-11 G> A, a new split site in intron 10 is generated, resulting in the placement of nucleotide number 9 between introns 10 and 11. The deposition of three amino acids (Gly-Leu-Glu) results in losing PAH's catalytic activity. The Mediterranean mutation was one of the most prevalent variants in Iran individuals with typical PKU (Nemati et al., 2021).

The PPI network analysis of PAH underscores its pivotal role in Phe metabolism, interacting with multiple enzymes essential for amino acid catabolism and BH4-dependent pathways (Flydal et al., 2019). The presence of pathogenic mutations within PAH, such as p.Ala309Asp and p.Arg261Gln, likely disrupts these interactions by altering protein stability, enzymatic efficiency, or cofactor binding, ultimately leading to impaired phenylalanine hydroxylation. Additionally, splicing mutations (c.1066-11G>A and c.168+5G>C) may cause aberrant mRNA processing, resulting in truncated or misfolded PAH proteins with diminished function. These molecular alterations not only reduce PAH activity but may also have cascading effects on associated proteins within the metabolic network, potentially leading to compensatory or maladaptive responses. Given PAH's high connectivity in the PPI network, its dysfunction could contribute to broader metabolic disruptions, exacerbating PKU severity beyond Phe accumulation. Understanding these network-level consequences is crucial for developing targeted therapies that address both direct enzymatic deficits and secondary metabolic disturbances in PKU patients.

One of the most important uses of patient genetic testing in predicting the response rate of patients to sapropterin dihydrochloride (BH4). This treatment has been used as a single therapy or as part of a treatment regimen in patients for more than ten years in Europe and North America, depending on the response rate to each patient's treatment. Recent research has proven that it is useful in people with mPKU and HPA. Mild phenotypes are caused by mutations (c.1066-11G>A) in intron 10 and (c.926C>A) in exon 7. Because the findings of our study demonstrate that the highest frequency is associated with these two mutations, it can be assumed that the use of

sapropterin dihydrochloride can offer a suitable response to therapy in the Iraqi population. More research, however, is required to achieve this. Other therapies for classical PKU, other from the diet, are not available to all patients in Iraq; therefore, determining the patient profile allows patients to be classified and classic PKU cases, which are the most vulnerable populations, to be identified. Exon 9 mutations (c.926C>A) and intron 2 mutations (IVS2+5G>C) were the least common in the study population. Other Exon 9 mutations found in research by E Vieira Neto and colleagues in Brazil include (c.967_969delACA) and a novel variant (c.934G>T) (Vieira Neto et al., 2018).

Despite the valuable insights gained, this study has an important limitation related to its small sample size (15 patients). Such a limited number of participants restricts the statistical power of the findings and reduces the ability to generalize the results to the broader Iraqi population or to other ethnic groups. Therefore, caution should be exercised when interpreting these results. Future studies should include larger cohorts from different regions of Iraq and neighboring countries to provide a more comprehensive understanding of PAH gene mutations and their clinical correlations. Expanding the sample size will also allow for better genotype–phenotype correlation analysis, improve the reliability of the results, and contribute to developing more precise diagnostic and therapeutic strategies.

5.Conclusion

Findings from this and other studies indicate, there is a wide variety of PKU mutations that are strongly dependent on ethnicity; hence, it appears that many mutations are exclusive to specific populations. Given the significant variation across groups, further ethnic-based research in Iraq is warranted to investigate the relationship between genotype and phenotype. Identifying PAH gene variations and their link to patient phenotype could provide valuable insights in prenatal genetic counseling and prenatal diagnosis.

Ethical approval and consent to participate

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and

with the 1964 Helsinki declaration and its later amendments or compare ethical strand.

Consent for publication

Written informed consent was obtained from the patients for this publication.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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