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A comprehensive in silico analysis of the mutation spectrum of the PAH gene in the Iranian population: Designing a novel workflow for predicting putative mutations in the Iranome database

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Abstract

Background: Phenylketonuria (PKU) is the most prevalent inborn error of metabolism, resulting from a malfunction of the phenylalanine hydroxylase (PAH) enzyme. The diversity and high rate of consanguinity in the Iranian population provide an apt study sample for autosomal recessive disorders.

Methods: In this study, we investigated 154 mutations in the PAH gene reported in Iran using various computational approaches. We predicted the pathogenicity and stability of genetic variants through the use of the American College of Medical Genetics and Genomics (ACMG) criteria, Functional Analysis through Hidden Markov Models (Fathmm), Combined Annotation Dependent Depletion (CADD), Sorting Intolerant from Tolerant (SIFT), Polymorphism Phenotyping version 2 (POLY PHEN 2), Mutation Taster, MUpro, and I-Mutant 2.0. Additionally, we performed an analysis of amino acid conservation using Clustal Omega and Consurf web servers. Molegro Virtual Docker (MVD), an integrated platform, was also utilized to perform protein-ligand docking simulations. This research presents a novel method for predicting pathogenic variants in the Iranome database and examines the pathogenicity of mutations in the PKU gene, enhancing the understanding of the genetic landscape of PKU in Iran.

Results: The results of this study showed that 80.8% of mutations occur in conserved regions, especially the catalytic domain. About half of all reported mutations were missense. This research introduces a novel workflow that predicts the pathogenicity of variants present in the Iranome database. Furthermore, docking studies revealed that this variant exhibits a critical loss of a catalytic site residue within the catalytic domain. Also, the most common genetic test was polymerase chain reaction (PCR) sequencing, which accounts for 71.5% of cases.

Conclusion: This study delineates future directions for functional studies, genetic counseling, and the development of diagnostic tools (e.g., strip assay kit).

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Highlights

What is current knowledge?

Phenylketonuria (PKU) is the most common inborn metabolic disorder caused by phenylalanine hydroxylase (PAH) enzyme deficiency. Iran's population diversity and high consanguinity rate make it a strategic sample for studying autosomal recessive disorders such as PKU. Over 154 PAH gene mutations have been reported in Iran, but their structural and functional impacts remain poorly characterized.

What is new here?

The novelty of our study lies in the introduction of a comprehensive computational workflow that integrates multiple pathogenicity prediction tools, conservation analysis, and protein-ligand docking to evaluate PAH mutations reported in Iran.

Introduction

Phenylalanine hydroxylase (PAH) is an enzyme that catalyzes the conversion of phenylalanine to tyrosine. Dysfunction of PAH causes phenylketonuria (PKU), a common hereditary metabolic disorder inherited in an autosomal recessive manner. PKU manifests in three clinical types: Classic PKU (cPKU, 62%), mild PKU (mPKU, 22%), and mild hyperphenylalaninemia (HPA, 16%) (1-3). Tetrahydrobiopterin (BH4) serves as a vital cofactor in this metabolic pathway. Mutations in genes involved in BH4 synthesis, including GTP cyclohydrolase (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), dihydropteridine reductase (DHPR), and pterin-4a-carbinolamine dehydratase (PCD), can also lead to PKU (2). Accurate molecular diagnosis is crucial for differentiating PAH gene defects from BH4-related metabolic abnormalities. Early diagnosis and treatment with a low-phenylalanine diet and phenylalanine-free supplements can prevent severe neurological and dermatological complications (4,5).

The PAH gene is located on chromosome 12 (12q22-q24.2), spans approximately 90 kb, and comprises 13 exons. It encodes a 452-amino acid protein (~52 kDa) (6). The severity of PKU correlates with residual enzyme activity, which depends on the mutation type and its impact on protein function. Globally, the incidence of PKU averages about 6.002 per 100,000 births but varies widely by region; for instance, European countries report rates near 1 in 10,000 births, whereas certain Middle Eastern populations, such as Iran, experience higher prevalence due in part to consanguinity and founder effects (2,3,7). In Iran, the PKU prevalence is approximately 1 in 1,000 live births, reflecting these genetic and demographic factors (5).

Despite this relatively high prevalence, studies exploring PAH gene mutations in the ethnically diverse Iranian population - comprising Persians (65%), Azeris (16%), and others (8) - are limited. To address this gap, we performed an in-silico analysis of PAH mutations in Iranian PKU patients. This study aims to characterize the frequency of variants registered in the Iranome database and evaluate their pathogenicity using multiple computational prediction tools and three-dimensional protein structural modeling. Furthermore, we introduce a novel bioinformatics workflow that integrates sequencing data analysis, variant annotation, and protein modeling to improve the accuracy of pathogenic variant prediction in this population. Our findings provide valuable insights into the genetic landscape of PKU in Iran and offer a framework for future research and clinical decision-making.

Methods

Variant identification and selection

We thoroughly searched for phenylalanine hydroxylase (PAH) gene variants in Iranian patients. The Scientific Information Database (SID) and several search engines, including PUBMED, Web of Science, Scopus, Cochrane Library, and Google Scholar, were utilized for this purpose. In both English and Farsi, the keywords "phenylketonuria," "Iran," "PAH gene," and "PKU" were used in the search strategy. Duplicate articles, studies involving non-Iranian patients, and papers lacking genetic testing were excluded from the collected data.

Predicting pathogenicity and stability of variants

Bioinformatics methods, such as the American College of Medical Genetics and Genomics (ACMG) criteria (https://franklin.genoox.com/clinical-db/), Combined Annotation Dependent Depletion (CADD; https://cadd.gs.washington.edu/), Sorting Intolerant From Tolerant (SIFT; https://sift.bii.a-star.edu.sg/), Polymorphism Phenotyping version 2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/), Functional Analysis through Hidden Markov Models (Fathmm; http://fathmm.biocompute.org.uk/), I-Mutant 2.0 (https://sls.cup.edu.in/imutant2/), and MUpro (https://mupro.proteomics.ics.uci.edu/) were used to study how pathogenic genetic changes can affect the stability of proteins in Iranian individuals with phenylketonuria.

Predicting putative pathogenic variants using the Iranome database

We used the Iranome Genomic Database (http://www.iranome.ir/) to search for possible detrimental changes in the PAH gene. Whole exome sequencing (WES) was conducted on 800 healthy individuals from eight major Iranian communities: Persians, Kurds, Lurs, Azeris, Baluchs, Iranian Arabs, Persian Gulf Islanders, and Turkmen. Among the more than 1.5 million variants identified in the database, over 300,000 were found to be unique (9). Six methods were employed to evaluate pathogenicity, including SIFT, PolyPhen-2, Mutation Taster, Mutation Assessor, FATHMM, and FATHMM MKL. The predictions for each variant are available on the Iranome website.

In this study, we developed a comprehensive workflow employing computational methods optimized for the Iranome database to investigate variants in the phenylalanine hydroxylase (PAH) gene associated with phenylketonuria (PKU). The workflow included the following steps:

1. Exclusion of variants: To ensure statistical significance, we excluded variants with fewer than 10 heterozygous occurrences.
2. CADD score threshold: Variants scoring below 20 on the CADD scale were eliminated, since this score signifies the potential harmful effects of a variant.
3. Predictor consensus: Variants identified as pathogenic by fewer than 3 out of 6 web server predictors were eliminated to enhance the reliability of the findings.

By applying these criteria, we assessed the variants found in the Iranome database to identify those likely to be pathogenic and observable in patients.

Amino acid conservation analysis

A conservation analysis of the PAH protein was performed using two computational tools: The European Bioinformatics Institute (EBI) Clustal Omega Tool and ConSurf (ConSurf Server). PAH amino acid sequences from different species, including *Gallus gallus*, *Macaca mulatta*, *Oryctolagus cuniculus*, *Homo sapiens*, *Bos taurus*, *Mus musculus*, *Felis catus*, *Equus asinus*, *Rattus norvegicus*, and *Pongo abelii*, were obtained from UniProt (https://www.uniprot.org/). These sequences were uploaded to Clustal Omega to perform multiple sequence alignment, allowing evaluation of the evolutionary conservation of amino acids in the PAH protein across different species. The evolutionary conservation of amino acid and nucleotide residues was subsequently assessed using the ConSurf tool, which provides conservation ratings ranging from 1 (Denoting variable areas). High-scoring exposed residues are regarded as functional, whereas high-scoring buried residues are considered structural.

Molecular docking simulation

The impact of the detected variant on the function of the encoded protein, specifically the mutated form c.688G>A corresponding to V230I in the protein, was evaluated through docking simulations using Molegro Virtual Docker (MVD) version 6.0.1 software. The interaction between the phenylalanine hydroxylase enzyme and its substrate, phenylalanine, was examined. The Moldock scoring algorithm, implemented in MVD software, applies a piecewise linear potential and a re-ranking procedure to identify the most optimal protein-ligand complexes; a higher preference for these complexes is indicated by lower energy scores. For structural analysis, the crystal structure of the active site of the phenylalanine hydroxylase enzyme (PDB: 1PAH) and a modeled structure of the mutated protein were utilized. The mutated protein structure was generated using homology modeling via the SWISS-MODEL online platform (https://swissmodel.expasy.org/interactive). The quality of the homology model was evaluated using the Global Model Quality Estimation (GMQE) score provided by SWISS-MODEL, which ranges from 0 to 1 and predicts the expected accuracy of the modeled structure; higher GMQE scores indicate higher reliability. This metric was used to confirm the suitability of the mutant protein model for subsequent docking simulations.

Results

Variant identification and selection

Initial keyword research conducted across six search engines resulted in the identification of 8,414 manuscripts. After applying the inclusion criteria and removing duplicate studies, it was found that 23 of these papers reported homozygous variants in Iranian patients with phenylketonuria (PKU). We compiled a total of 154 variants from the filtered manuscripts, which included 76 missense mutations, 8 nonsense mutations, 5 silent mutations, 40 splice site mutations, 2 duplications, and 23 indel mutations. A comprehensive list of mutations, classified by marriage type, patient count, and genetic testing, is provided in Supplementary 1. Furthermore, the range of PAH mutations in Iran is graphically depicted in Figure 1.

Pathogenicity and stability of variants

The pathogenicity and stability of all missense variants were predicted using seven different bioinformatic tools. Table 1 displays the results of the pathogenicity and stability analysis of missense mutations. Furthermore, Table 2 describes the documented impacts of splice site, deletion, and duplication mutations on the protein.

Putative pathogenic variants revealed by the Iranome database

By searching for the phenylalanine hydroxylase (PAH) gene in the Iranome database, we were able to identify all variants, including deletions, duplications, splice region, intronic, and single nucleotide polymorphisms (SNPs). A total of 20 missense variants were discovered, with their respective maximum and minimum allele frequencies reported across various populations in Iran. Additionally, information on pathogenicity predictions and the number of heterozygotes can be found in Supplementary 2. After designing and implementing this workflow, we tested the variant c.688G>A to assess whether our criteria and workflow could predict its occurrence in the Kurdish population. The results demonstrated that our criteria functioned effectively, and the workflow successfully predicted that the variant c.688G>A would be observed in this population. This variant has been particularly identified among Kurdish patients, and its presence is supported by a 2019 study that reported a patient with phenylketonuria

(PKU) carrying a homozygous mutation of c.688G>A. This prior documentation serves as a strong validation of our predictive criteria and underscores the reliability of our methodology in genetic assessments. The structural consequences of this mutation are illustrated in Figure 2.

Conservation study of amino acids

We performed a multiple sequence alignment of the PAH protein in H. sapiens with other species using Clustal Omega. This alignment allowed us to determine the corresponding positions in the PAH proteins of the ten species relative to those in the human PAH. Our findings, illustrated in Figure 3, reveal that 77.63% (n = 59) of the reported missense variants are conserved across the ten species analyzed. This conservation indicates significant evolutionary stability of these positions, suggesting that these mutations may play a crucial role in maintaining protein function across different organisms. Furthermore, the assessment of PAH amino acids utilizing the Consurf web server corroborated our results from Clustal Omega, reinforcing the reliability of our findings. These data are visually represented in Figure 4. Additionally, the Consurf grade scores for each amino acid are detailed in Supplementary 3, providing further insights into the conservation of these residues.

Molecular docking findings

Figure 5 illustrates the docking study of both the intact protein and its mutated form (c.688G>A, corresponding to V230I in the protein) with phenylalanine serving as the ligand molecule. Significant involvement of residues SER349, ARG270, and SER350 from the wild-type protein, along with ARG270 and SER349 from the mutated protein, in hydrogen bond interactions with the ligand has been observed (10). The assessment of the ligand's binding affinity to the target protein was determined based on their MolDock scores. A comparative analysis of the results from both protein types is shown in Table 3. The MolDock scores for binding to phenylalanine differed slightly between the mutated and normal PAH proteins. It is noteworthy that the mutated protein showed a lower binding affinity for phenylalanine in comparison to the wild-type protein, leading to disrupted interactions and diminished catalytic activities of the PAH protein (11).

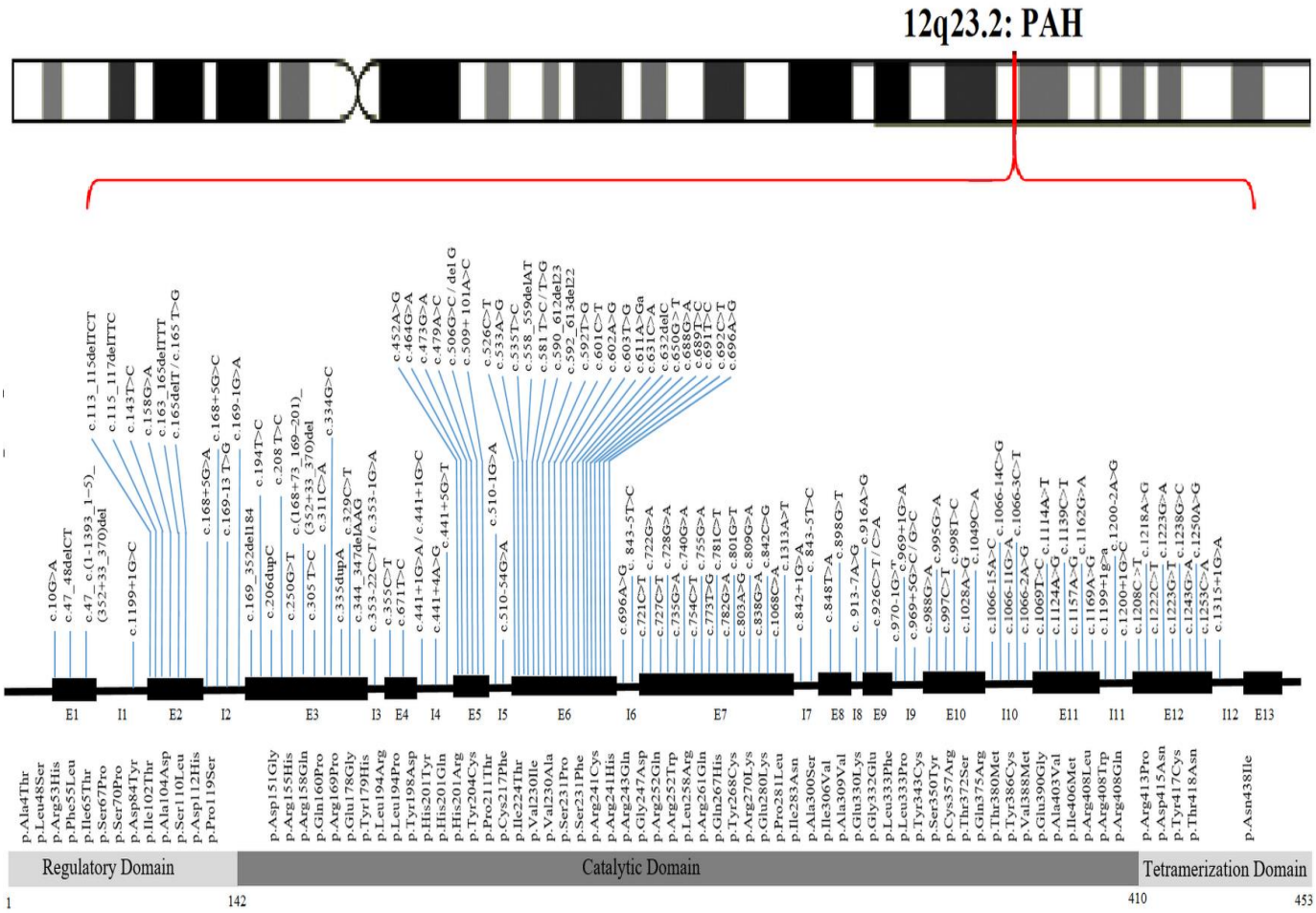

Figure 1. Graphical illustration of the mutation spectrum of the PAH gene in the Iranian population

Table 1. prediction of pathogenicity and stability of missense mutations in PAH gene in Iranian population

Variant	Protein	ACMG	Fathmm	CADD	SIFT	POLY PHEN 2	Mutation taster	MUpro	I-Mutant 2.0
c.143T>C	p.Leu48Ser	Pathogenic	Damaging (-6.67)	26.5	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.92)	Decrease (DDG: -2.59)
c.838G>A	Glu280Lys	Pathogenic	Damaging (-6.64)	46	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -0.85)	Decrease (DDG: -0.26)
c.782G>A	p.Arg261Gln	Pathogenic	Damaging (-6.71)	32	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.14)	Decrease (DDG: -1.11)
c.842C>G	p.Pro281Leu	Pathogenic	Damaging (-7.90)	32	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.17)	Decrease (DDG: -1.10)
c.755G>A	p.Arg252Gln	Pathogenic	Damaging (-6.71)	31	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.73)	Decrease (DDG: -0.78)

Table 1 (Continued)

c.728G>A	p.Arg243Gln	Pathogenic	Damaging (-6.71)	31	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -0.98)	Decrease (DDG: -0.53)
c.848T>A	p.Ile283Asn	Pathogenic	Damaging (-6.30)	29	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.63)	Decrease (DDG: -1.22)
c.1069T>C	p.Cys357Arg	Likely pathogenic	Damaging (-6.23)	24	Damaging	Probably damaging (0.991)	Disease causing	Decrease (DDG: -1.29)	Decrease (DDG: -0.55)
c.691T>C	p.Ser231Pro	Pathogenic	Damaging (-6.64)	26.3	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -0.99)	Decrease (DDG: -1.61)
c.754C>T	p.Arg252Trp	Pathogenic	Damaging (-6.77)	13.64	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.71)	Decrease (DDG: -0.63)
c.1124A>G	p.Gln375Arg	Likely pathogenic	Damaging (-6.25)	23.8	Damaging	Probably damaging (0.991)	Disease causing	Decrease (DDG: -0.43)	Decrease (DDG: -1.27)
c.1162G>A	p.Val388Met	Pathogenic	Damaging (-6.68)	21.4	Damaging	Probably damaging (0.998)	Disease causing	Decrease (DDG: -1.21)	Decrease (DDG: -0.59)
c.506 G > C	p.Arg169Pro	Likely pathogenic	Damaging (-6.25)	24	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -1.28)	Decrease (DDG: -1.40)
c.898G>T	p.Ala300Ser	Pathogenic	Damaging (-7.00)	32	Damaging	Probably damaging (0.999)	Disease causing	Decrease (DDG: -1.02)	Decrease (DDG: -0.10)
c.1313A > T	p.Asn438Ile	Likely pathogenic	Damaging (-6.20)	23	Damaging	Benign (0.361)	Disease causing	Decrease (DDG: -0.51)	Increase (DDG:1.41)
c.721C>T	p.Arg241Cys	Pathogenic	Damaging (-6.17)	24.5	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -0.45)	Decrease (DDG: -0.63)
c.988G>A	p.Glu330Lys	Likely pathogenic	Damaging (-6.93)	55	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG: -0.79)	Decrease (DDG: -1.32)
c.1223G>T	p.Arg408Leu	Likely pathogenic	Damaging (-6.72)	25.9	Damaging	Probably damaging (1.000)	Disease causing	Increase (DDG:0.09)	Decrease (DDG: -0.40)
c.199T>C	p.Ser67Pro	Pathogenic	Damaging (-5.58)	26.6	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.22)	Decrease (DDG: -2.19)
c.997C>T	p.Leu333Phe	Pathogenic	Damaging (-7.24)	21.1	Damaging	Probably damaging (0.996)	Disease causing	Decrease (DDG:-0.71)	Decrease (DDG: -0.32)
c.194T>C	p.Ile65Thr	Pathogenic	Damaging (-5.67)	28.9	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.54)	Decrease (DDG: -2.05)
c.1222C>T	p.Arg408Trp	Pathogenic	Damaging (-6.77)	11.14	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.54)	Decrease (DDG: -1.28)
c.473G>A	p.Arg158Gln	Pathogenic	Damaging (-6.71)	27.4	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG- 1.22)	Decrease (DDG: -0.87)
c.926C > A	p.Ala309Val	Pathogenic	Damaging (-6.66)	29.4	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-1.01)	Decrease (DDG: -0.40)
c.671T>C	p.Ile224Thr	Likely pathogenic	Damaging (-6.23)	28	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-2.27)	Decrease (DDG: -3.74)
c.1169A> G	p.Glu390Gly	Pathogenic	Damaging (-6.46)	25.3	Damaging	Probably damaging (0.998)	Disease causing	Decrease (DDG-1.60)	Decrease (DDG: -2.00)
c.10G > A	p.Ala4Thr	VUS	Damaging (-5.77)	7.05	Tolerated	Benign (0.002)	Polymorphism	Decrease (DDG:-1.22)	Decrease (DDG: -0.91)
c.1208C > T	p.Ala403Val	Pathogenic	Damaging (-6.21)	26.5	Damaging	Probably damaging (0.997)	Disease causing	Decrease (DDG-0.92)	Decrease (DDG: -0.12)
c.650G > T	p.Cys217Phe	Likely pathogenic	Damaging (-6.28)	29.5	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-0.61)	Decrease (DDG: -0.17)
c.998T > C	p.Leu333Pro	Pathogenic	Damaging (-7.30)	29.7	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-1.64)	Decrease (DDG: -2.07)
c.479A > C	p.Gln160Pro	Likely pathogenic	Damaging (-6.07)	22.5	Damaging	Probably damaging (0.834)	Disease causing	Decrease (DDG-1.81)	Decrease (DDG: -1.05)
c.581T>G	L194R	Likely pathogenic	Damaging (-6.61)	23.5	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-1.76)	Decrease (DDG: -1.84)
c.601C>T	p.His201Tyr	Pathogenic	Damaging (-6.60)	24.9	Damaging	possibly damaging (0.750)	Disease causing	Decrease (DDG-0.38)	Increase (DDG:1.08)
c.535T>C	p.Tyr179His	Pathogenic	Damaging (-7.19)	27.8	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-1.68)	Decrease (DDG: -1.03)
c.603T>G	p.His201Gln	Pathogenic	Damaging (-6.60)	5.625	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG-0.46)	Decrease (DDG: -0.83)
c.688G>A	p.Val230Ile	Pathogenic	Damaging (-6.21)	26.5	Tolerated	Benign (0.030)	Disease causing	Decrease (DDG:-0.62)	Increase (DDG:0.11)
c.740G>A	p.Gly247Asp	Likely pathogenic	Damaging (-7.02)	28.2	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.13)	Decrease (DDG: -0.66)
c.809G>A	p.Arg270Lys	Pathogenic	Damaging (-8.41)	29.6	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.79)	Decrease (DDG: -1.90)

Table1 (Continued)

c.1223G>A	p.Arg408Gln	Pathogenic	Damaging (-6.71)	26.2	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.66)	Decrease (DDG: -1.72)
c.122T>C	p.Leu41Pro	Likely pathogenic	Damaging (-5.56)	26.4	Damaging	Probably damaging (0.639)	Disease causing	Decrease (DDG:-2.29)	Decrease (DDG: -1.93)
c.926C>T	p.Ala309Val	Pathogenic	Damaging (-6.66)	27.6	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.01)	Decrease (DDG: -0.40)
c.1114A>T	p.Thr372Ser	Pathogenic	Damaging (-6.20)	22.9	Damaging	Possibly Damaging (0.582)	Disease causing	Decrease (DDG:-0.47)	Decrease (DDG: -0.56)
c.1049C>A	p.Ser350Tyr	Pathogenic	Damaging (-6.81)	32	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.41)	Increase (DDG:0.19)
c.803A>G	p.Tyr268Cys	Pathogenic	Damaging (-6.78)	28.1	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.60)	Increase (DDG:0.05)
c.334G>C	p.Asp112His	Likely pathogenic	Damaging (-5.83)	25.7	Tolerated	Benign (0.080)	Disease causing	Decrease (DDG:-2.13)	Decrease (DDG: -0.69)
c.1028A>G	p.Tyr343Cys	Pathogenic	Damaging (-7.06)	27.1	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.86)	Increase (DDG:0.30)
c.1250A>G	p.Tyr417Cys	Pathogenic	Damaging (-6.53)	27.1	Damaging	Probably damaging (0.995)	Disease causing	Decrease (DDG:-0.50)	Increase (DDG:0.33)
c.533A>G	p.Glu178Gly	Pathogenic	Damaging (-6.39)	24.9	Damaging	Probably damaging (0.962)	Disease causing	Decrease (DDG:-1.35)	Decrease (DDG:-0.73)
c.722G>A	p.Arg241His	Pathogenic	Damaging (-6.15)	24.8	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.66)	Decrease (DDG:-0.27)
c.1139C>T	p.Thr380Met	Pathogenic	Damaging (-6.25)	24.2	Damaging	Probably damaging (1.000)	Disease causing	Increase (DDG:0.35)	Increase (DDG:0.27)
c.305T>C	p.Ile102Thr	Likely pathogenic	Damaging (-6.78)	23.2	Damaging	Benign (0.037)	Disease causing	Decrease (DDG:-1.79)	Decrease (DDG:-1.71)
c.158G>A	p.Arg53His	VUS	Damaging (-5.54)	24.7	Damaging	Probably damaging (0.976)	Disease causing	Decrease (DDG:-1.28)	Decrease (DDG:-2.12)
c.311C>A	p.Ala104Asp	Pathogenic	Damaging (-4.84)	20.8	Damaging	Benign (0.000)	Disease causing	Decrease (DDG:-0.95)	Decrease (DDG:-0.34)
c.464G>A	p.Arg155His	Pathogenic	Damaging (-6.74)	29.9	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.57)	Decrease (DDG:-1.85)
c.689T>C	p.Val230Ala	Pathogenic	Damaging (-6.49)	26.9	Damaging	Probably damaging (0.999)	Disease causing	Decrease (DDG:-1.62)	Decrease (DDG:-1.45)
c.1238G>C	p.Arg413Pro	Pathogenic	Damaging (-6.36)	17.64	Damaging	Probably damaging (0.999)	Disease causing	Decrease (DDG:-1.28)	Decrease (DDG:-1.45)
c.250G>T	p.Asp84Tyr	Pathogenic	Damaging (-5.91)	24.7	Damaging	Probably damaging (0.931)	Disease causing	Decrease (DDG:-0.96)	Increase (DDG:0.58)
c.581T>C	p.Leu194Pro	Pathogenic	Damaging (-6.62)	23.5	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.79)	Decrease (DDG:-1.51)
c.916A>G	p.Ile306Val	Pathogenic	Damaging (-6.43)	23.5	Damaging	Probably damaging (0.976)	Disease causing	Decrease (DDG:-0.55)	Decrease (DDG:-1.01)
c.611A>G	p.Tyr204Cys	Pathogenic	Damaging (-6.09)	21.6	Damaging	possibly damaging (0.905)	Disease causing	Decrease (DDG:-0.61)	Decrease (DDG:-0.32)
c.165T>G	p.Phe55Leu	Pathogenic	Damaging (-5.64)	12.62	Damaging	Probably damaging (0.984)	Disease causing	Decrease (DDG:-0.85)	Decrease (DDG:-2.16)
c.329C>T	p.Ser110Leu	Pathogenic	Damaging (-5.82)	38	Damaging	possibly damaging (0.847)	Disease causing	Decrease (DDG:-0.42)	Decrease (DDG:-0.76)
c.452A>G	p.Asp151Gly	Likely pathogenic	Damaging (-6.77)	27.9	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.65)	Decrease (DDG:-1.96)
c.592T>G	p.Tyr198Asp	Likely pathogenic	Damaging (-6.33)	22.9	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.82)	Decrease (DDG:-1.68)
c.602A>G	p.His201Arg	Pathogenic	Damaging (-6.58)	25.2	Damaging	Probably damaging (0.996)	Disease causing	Decrease (DDG:-0.48)	Decrease (DDG:-0.59)
c.631C>A	p.Pro211Thr	Pathogenic	Damaging (-6.10)	28.3	Damaging	Probably damaging (0.989)	Disease causing	Decrease (DDG:-1.45)	Decrease (DDG:-2.30)
c.692C>T	p.Ser231Phe	Pathogenic	Damaging (-6.65)	28.1	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.14)	Increase (DDG:0.08)
c.773T>G	p.Leu258Arg	Likely pathogenic	Damaging (-6.89)	28.5	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.28)	Decrease (DDG:-2.06)
c.801G>T	p.Gln267His	Pathogenic	Damaging (-6.55)	10.21	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.98)	Decrease (DDG:-1.52)
c.1157A>G	p.Tyr386Cys	Pathogenic	Damaging (-6.90)	25.1	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-1.25)	Increase (DDG:0.34)
c.1218A>G	p.Ile406Met	Pathogenic	Damaging (-6.40)	9.678	Damaging	Probably damaging (0.998)	Disease causing	Decrease (DDG:-0.58)	Decrease (DDG:-1.68)

Table1 (Continued)

c.1243G>A	p.Asp415Asn	Pathogenic	Damaging (-6.06)	26.1	Damaging	Benign (0.002)	Disease causing	Decrease (DDG:-0.89)	Decrease (DDG:-1.82)
c.1253C>A	p.Thr418Asn	Likely pathogenic	Damaging (-6.57)	26.6	Damaging	Probably damaging (0.999)	Disease causing	Decrease (DDG:-0.61)	Decrease (DDG:-1.19)
c.208 T>C	p.Ser70Pro	Pathogenic	Damaging (-6.68)	23.9	Damaging	Probably damaging (0.999)	Disease causing	Decrease (DDG:-0.61)	Decrease (DDG:-1.70)
c.355C>T	p.Pro119Ser	Pathogenic	Damaging (-4.93)	26.5	Damaging	Probably damaging (0.999)	Disease causing	Decrease (DDG:-0.70)	Decrease (DDG:-0.60)
c.995G>A	p.Gly332Glu	Likely pathogenic	Damaging (-8.79)	29.2	Damaging	Probably damaging (1.000)	Disease causing	Decrease (DDG:-0.52)	Decrease (DDG:-0.80)

Table 2. Protein effects of splice site, deletion, and duplication mutations in PAH gene in Iranian PKU patients

Variant	ACMG	Reported Protein Effect
c.168+5G>A	Pathogenic	IVS2+5G>A
c.168+5G>C	Pathogenic	IVS2+5G>C
c.632delC	Pathogenic	p.P211>Hfs
c.969+1G>A	Pathogenic	IVS9+1G>A
c.969+5G>A	Pathogenic	IVS9 +5G>A
c.1199+1G>C	Pathogenic	IVS11+1G>C
c.526C>T	Pathogenic	p.R176X
c.781C>T	Pathogenic	p.R261X
c.1066-11G>A	Pathogenic	IVS10-11G>A
c.1068C>A	Pathogenic	Y356X
c.727C>T	Pathogenic	R243X
c.592_613del22	Pathogenic	p.Y198_E205>Sfs
c.1200-2A>G	Pathogenic	IVS11-2A>G
c.1124A>G	Likely pathogenic	Q375Rx
c.1197A > T	Pathogenic	p.Val399Val
c.45_46delCT	Pathogenic	p.Ser16Ter
c.115_117delTTC	Pathogenic	p.F39delTTC
c.1155C > G	Benign	p.Leu385Leu
c.1089_1089delG	Pathogenic	p.Lys363AsnfsTer37
c.1200+1G>C	NA	IVS11+1G>C
c.696A>G	NA	p .Q232Q
c.735G>A	Benign	p .V245V
c.706+36T>G	NA	IVS6+36 T>G
c.706+44T>G	NA	IVS6+44T>G
c.1066-15A>C	VUS	IVS10-15A>C
c.353-22C>T	Benign	IVS3-22C>T
c.509+ 101A>C	Benign	-
c.970-195G>A	Benign	-
c.1065+97G>A	Benign	-
c.335dupA	Likely pathogenic	p.Asp112fs
c.510-54G>A	Benign	IVS5-54G>A
c. 843-5T>C	Pathogenic	IVS7-5T>C
c. 913-7A>G	Pathogenic	IVS8-7A>G
c.1090-1092delctt	NA	l364del
c.441+5G>T	Pathogenic	IVS4+5G>T
EX3del4765	NA	deletion could interfere with regulatory domain of *PAH* enzyme's proper function
c.1199+1g>a	Pathogenic	IVS11nt1
c.113_115delTCT	Pathogenic	p.Phe39del (p.F39del)
c.(168+73_169–201)_(352+33_370)del	NA	-
c.165delT	Pathogenic	p.Phe55LeufsTer6 (p.F55Lfs*6)
c.1068C>A	Pathogenic	p.Tyr356Ter (p.Y356*)
c.510-1G>A	Pathogenic	p. IVS5-1G>A
c.970-1G>T	Pathogenic	p. IVS9-1G>T
c.1092_1094delTCT	Pathogenic	p.Leu365del (p.L365del)

Table 2 (Continued)

c.590_612del23	Likely pathogenic	p.Ser196_Leu197insTer
c.441+1G>C	Pathogenic	p.IVS4+1G>C
c.169-13 T>G	Pathogenic	p. IVS2-13 T>G
c.632DelC	Pathogenic	p.Pro211HisfsTer130 (p.P211Hfs*130)
c.969+5G>C	VUS	p. IVS9+5G>C
c.47_48delCT	Pathogenic	p.Ser16Ter (p.S16*)
c.169-1G>A	Pathogenic	p. IVS2-1G>A
c.331C>T	Pathogenic	p.Arg111Ter (p.R111*)
c.707-96A>G	NA	p. IVS6-96A>G
c.1066-2A>G	Likely pathogenic	p.IVS10-2A>G
c.353-1G>A	Pathogenic	p.IVS3-1G>A
c.558_559delAT	NA	p.Trp187GlyfsTer12 (p.W187Gfs*12)
c.1066-14C>G	Pathogenic	p.IVS10-14C>G
c.1106_1107insAGCT	Likely pathogenic	p.Glu370AlafsTer25 (p.E370Afs*25)
c.1245delC	Likely pathogenic	p.Pro416HisfsTer36 (p.P416Hfs*36)
c.1315+1G>A	Pathogenic	p.IVS12+1G>A
c.206dupC	pathogenic	p.Ser70PhefsTer7 (p.S70Pfs*7)
c.842+1G>A	pathogenic	p.IVS7+1G>A
c.506G>C	Likely pathogenic	p.Lys115ThrfsTer79 (p.L115Tfs*79)
c.344_347delAAGA	pathogenic	p.Lys115ThrfsTer79
c.1147C>T	pathogenic	p.Gln383Ter (p.Q383*)
c.169_352del184	Likely pathogenic	p.Ex3del4765
c. (1-1393_1–405) (352+33_370) del	NA	p.Ex1_3del
c.506delG	Likely pathogenic	p.Arg169ProfsTer26 (p.R169Pfs*26)
c.707-1G>C	NA	p. IVS6-1G>C
c.1066-3C>T	Likely pathogenic	p. IVS10-3C>T
c.1177_1178InsT	pathogenic	p.Asn393IlefsTer2 (p.N393Ifs*2)
c.441+4A>G	VUS	p.IVS4+4A>G
c.163_165delTTT	pathogenic	p.Phe55del (p.Phe55>Lfs)
c.510-54G>A	Benign	IVS5-54G>A
c.441+1G>A	pathogenic	IVS4+1G>A
c.441+5G>T	pathogenic	IVS4+5G>T
c.842+1G>A	pathogenic	IVS7+1G>A
c.843-5T>C	pathogenic	IVS7-5 T>C

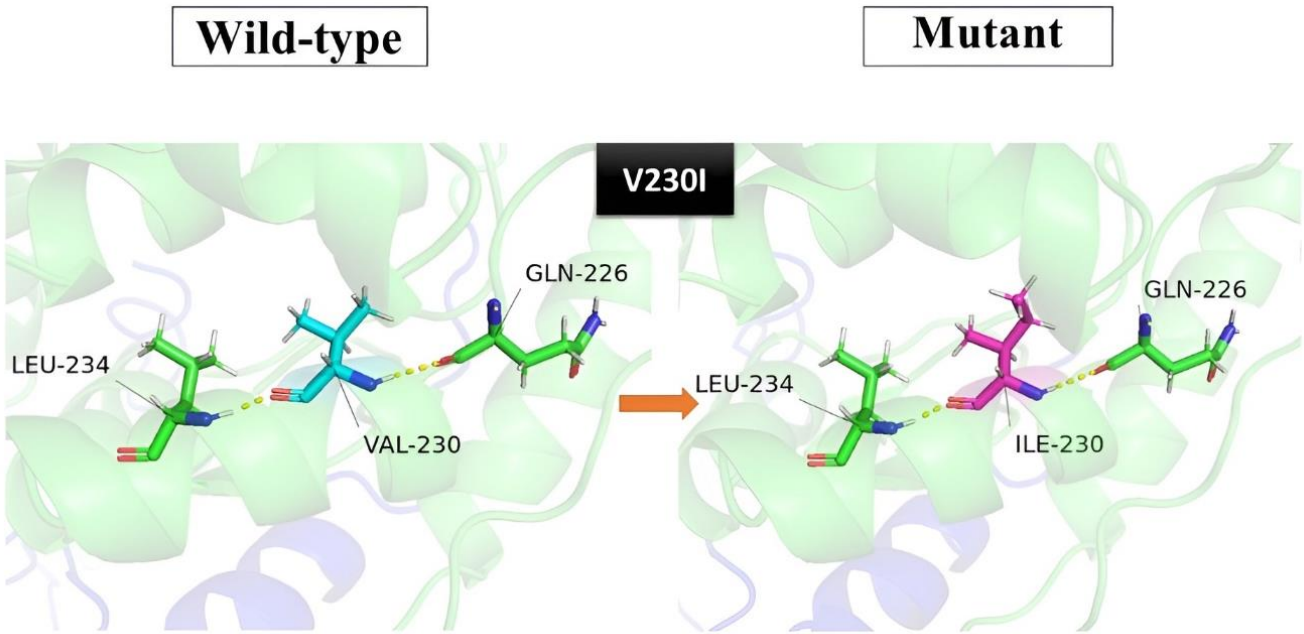

Figure 2. Tertiary structure of the c.688G>A (p.Val230Ile) mutation

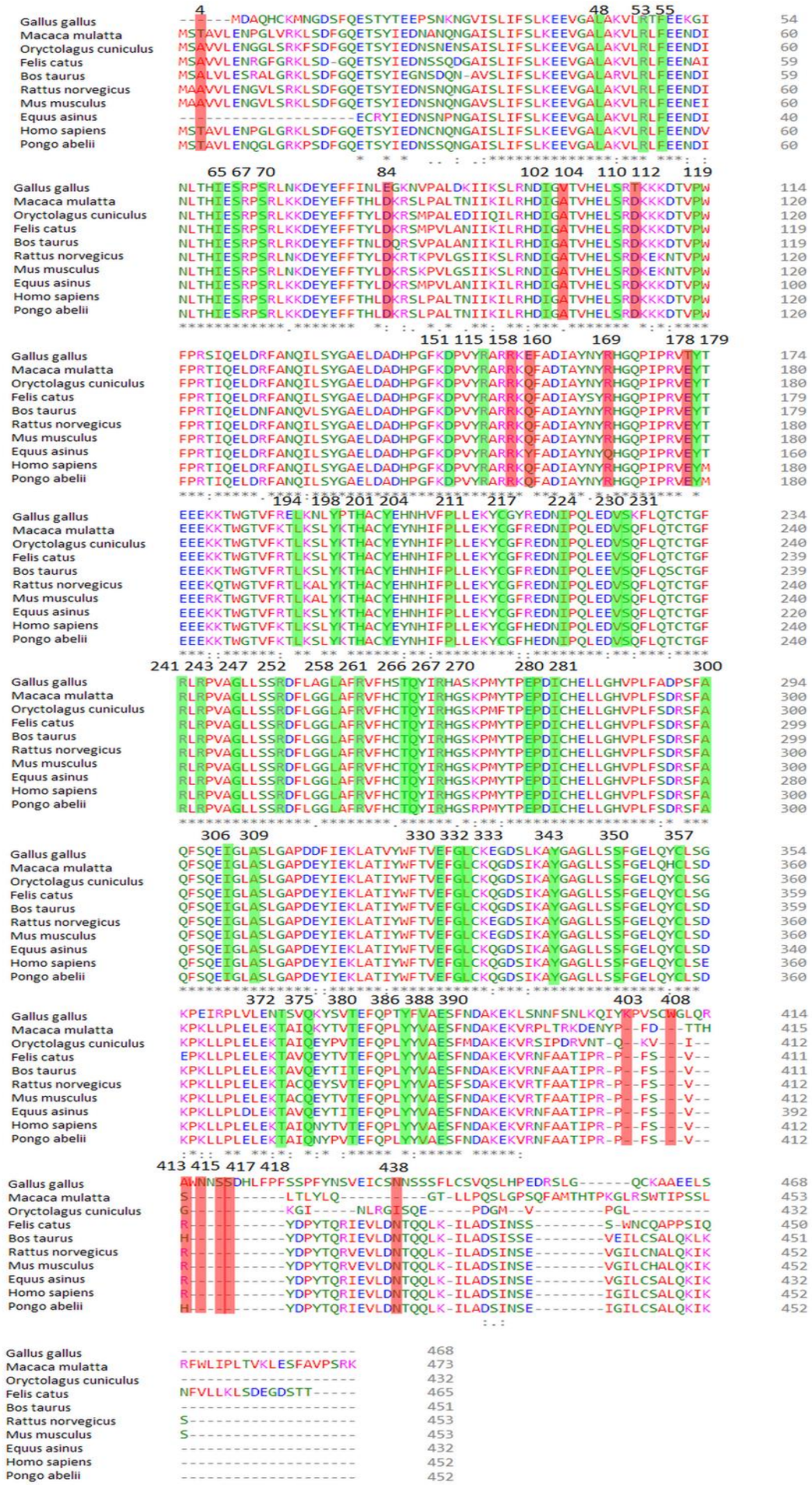

Figure 3. Amino acid conservation study of the PAH protein sequence using Clustal Omega across 10 different species (Green and red indicate conserved and non-conserved variants, respectively)

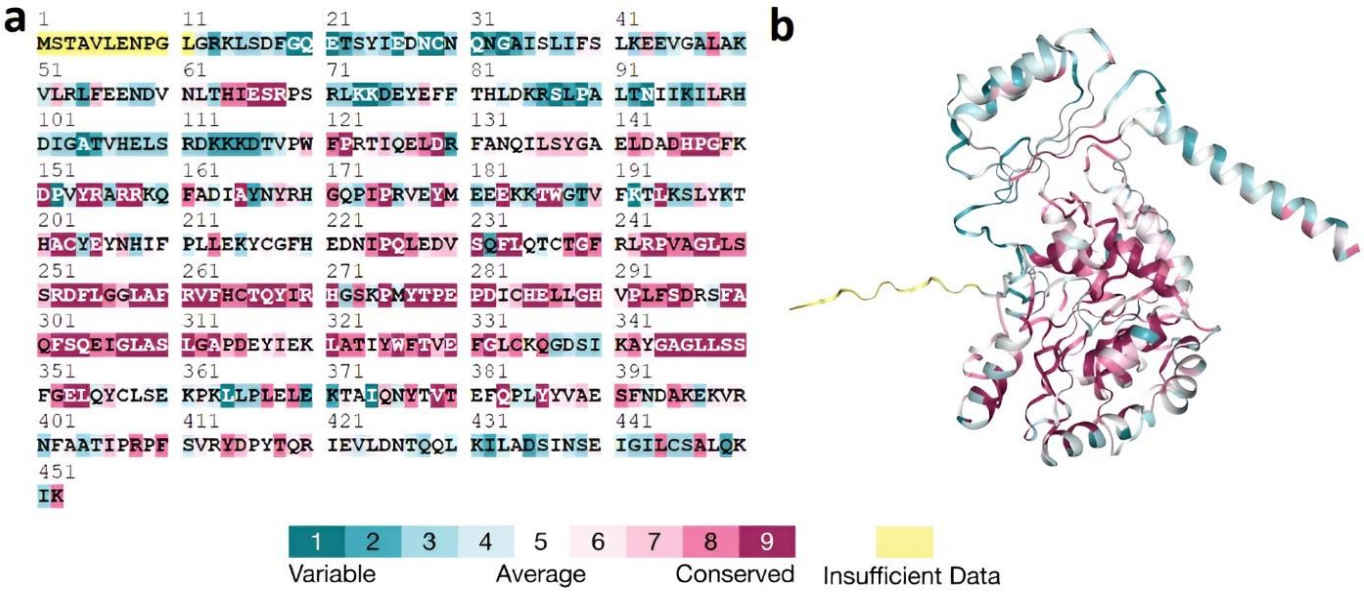

Figure 4. Evolutionary conservation profile of the PAH protein according to the Consurf web server, shown by sequence (a) and by three-dimensional structure (b)

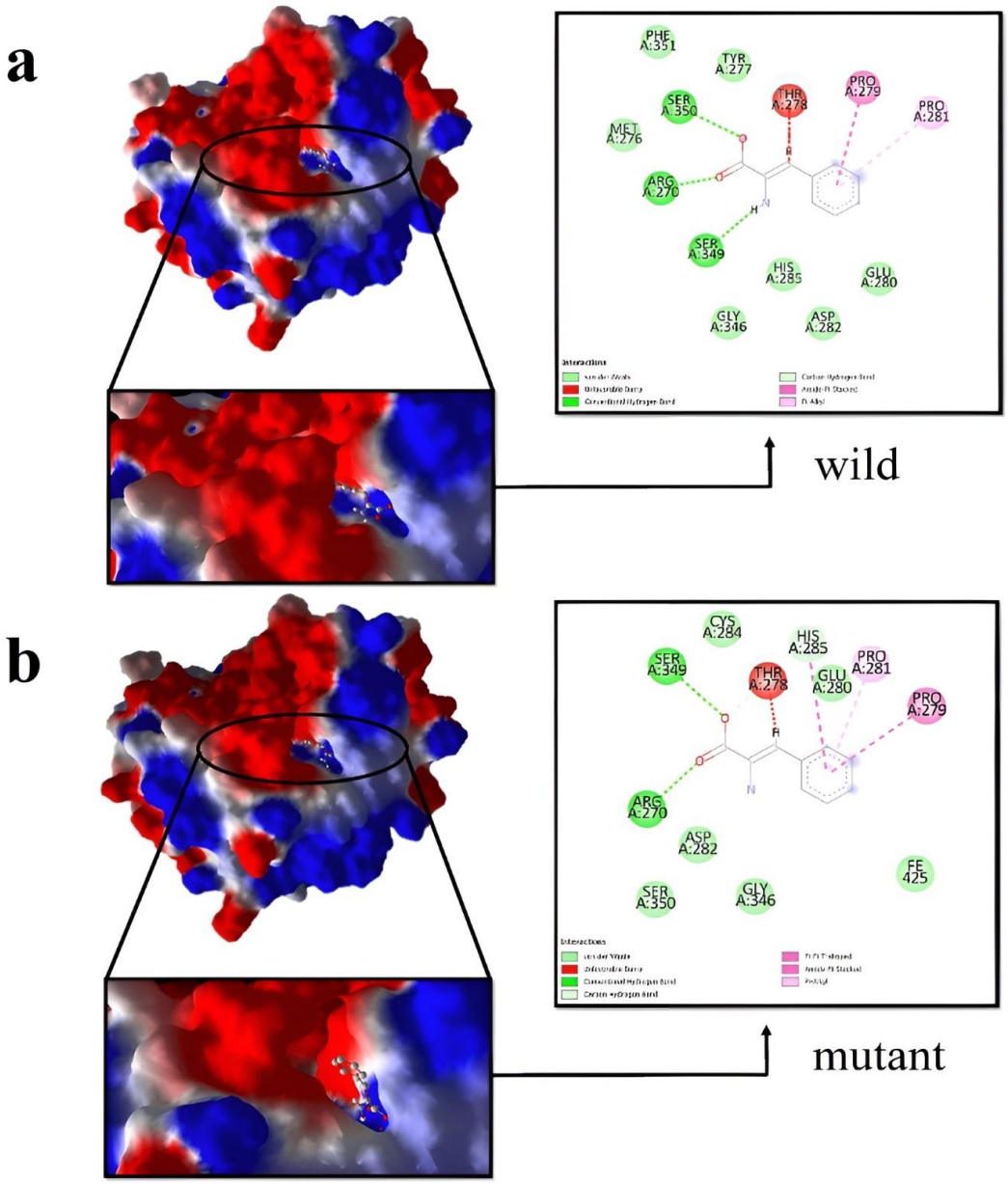

Figure 5. Molecular docking simulation for wild-type PAH (a) and p.Val230Ile-mutant PAH (b)

Table 3. The docking prediction results for the mutation in PAH gene using molegro virtual docker software

wild			Mutant (V230I)		
MolDock score (kcal/mol)	Rerank score (kcal/mol)	ResiduesFormingH-bondswiththePose	MolDock score (kcal/ mol)	Rerank score (kcal/mol)	ResiduesFormingH-bondswiththePose
-60.9723	-59.664	SER349, ARG270, SER350	-60.1076	-38.0429	ARG270, SER349

Discussion

The phenylalanine hydroxylase (PAH) gene encodes phenylalanine hydroxylase, an essential enzyme in the metabolism of phenylalanine that plays a vital role in multiple biochemical pathways, including tyrosine degradation, biogenic amine synthesis, and L-phenylalanine catabolism. This enzyme consists of three domains that regulate its activity; mutations - particularly in the catalytic domain - disrupt enzyme structure and function, thus leading to phenylketonuria (PKU). The catalytic domain is critical for the proper folding and stability of PAH, and mutations in the hotspot exon 6 can significantly reduce enzymatic activity (12). Our bioinformatics analysis with Clustal Omega and Consurf indicated that this domain is highly conserved across species, emphasizing its functional importance. Supporting this, over 80.82% of PAH mutations identified in Iranian PKU patients were found in these conserved regions.

In silico analyses provide valuable insights by predicting the impact of mutations on protein function, offering a rapid and cost-effective alternative to experimental methods (13). This study aimed to conduct an in-silico analysis of the PAH gene mutation profile in Iranian PKU patients. A total of 154 variants from 685 patients were examined, with 31.67% of cases involving consanguineous marriages, highlighting the significance of genetic screening in this population. Polymerase chain reaction (PCR) sequencing was the primary method in 71.5% of cases. Among 76 missense variants analyzed using six prediction programs, 63 were consistently classified as deleterious, indicating a high likelihood of pathogenicity. Heatmap analysis (Figure 6) revealed that most mutations were missense, many classified as pathogenic or likely pathogenic, implying significant potential harm to affected individuals.

Our data strongly suggest that a high proportion of the mutations are deleterious missense variants, predominantly in the catalytic domain, affecting the tetrahydrobiopterin (BH4) cofactor binding site. Key mutations included the addition of three Gly-Leu-Gln amino acids between exons 10 (Yellow region) and 11 (Light blue region), p.Pro 281Leu, p.Arg 261X, and p.Arg 261Gln (14,15). The Iranome database identified 20 missense variants in PAH. Supplementary 2 shows that the variant c.688G>A has the highest number of heterozygotes (13 individuals) and the highest frequency (0.03) in the Kurdish population. This specific mutation (c.688G>A, p.Val 230Ile) was selected for detailed docking analysis because it exhibited the highest frequency in the Kurdish population according to the Iranome database and fulfilled all three pathogenicity criteria in our workflow. Furthermore, previous reports have suggested its potential pathogenic role in Iranian PKU patients. Considering both its relatively high prevalence and predicted deleterious effect on enzyme function, this variant was chosen as a representative model for structural analysis.

This variant met all three criteria established in our pathogenicity workflow. Given the high rate of consanguineous marriages in Iran, we designed the workflow to assess its capability in identifying previously reported pathogenic mutations. Validation with c.688G>A showed accurate identification of this variant's pathogenicity, consistent with its documented prevalence in Kurdish patients (15-17). This mutation leads to a critical loss of a catalytic site residue within the catalytic domain (Figure 1), resulting in reduced binding affinity for phenylalanine and diminished catalytic activity (18,19).

Among the reported variants in the Iranome database, there are 20 missense variants. As indicated in Supplementary 2, the variant c.688G>A has the highest number of heterozygotes (13 heterozygotes) and the highest frequency (0.03) in the Kurdish population. Notably, c.688G>A meets the three criteria upon which our workflow was designed. Given the high rate of consanguineous marriages in Iran, we designed this workflow to test its efficacy in identifying previously reported mutations that are confirmed to be pathogenic. To validate our algorithm, we examined the mutation c.688G>A and found that our workflow accurately identifies it as pathogenic. According to a study published in 2014, this mutation has been observed in patients from the Kurdish population (18), which aligns with the population in the Iranome database that exhibits the highest frequency. This mutation leads to a critical loss of a catalytic site residue within the catalytic domain (Figure 1), reducing binding affinity with phenylalanine and consequently decreasing catalytic activity (11).

Remarkably, despite these pronounced effects, the difference in the MolDock score between the wild-type and mutant proteins remains imperceptible. This subtle impact can be attributed to the missense mutation involving a single nucleotide alteration. Additionally, hydrogen bonds play a pivotal role in maintaining protein stability (20). To explore this, we assessed the number of hydrogen bonds present in both the mutant and wild-type PAH structures. The mutant structure exhibits a disruption in its intramolecular hydrogen bond network, with fewer hydrogen bonds observed compared to the wild-type. This reduction likely contributes to the overall loss of stability (Table 3). Therefore, the decrease in hydrogen bonds associated with the V230I mutation signifies a compromised protein stability resulting from the substitution of valine with isoleucine at the 230th residue of the PAH protein (21).

As phenylketonuria (PKU) is an autosomal recessive disorder, consanguineous marriages increase the likelihood of children inheriting two copies of a pathogenic variant in the PAH gene, potentially resulting in PKU. If both parents carry such a variant, the risk of their offspring developing the disorder increases due to consanguinity. This underscores the importance of genetic counseling for affected families.

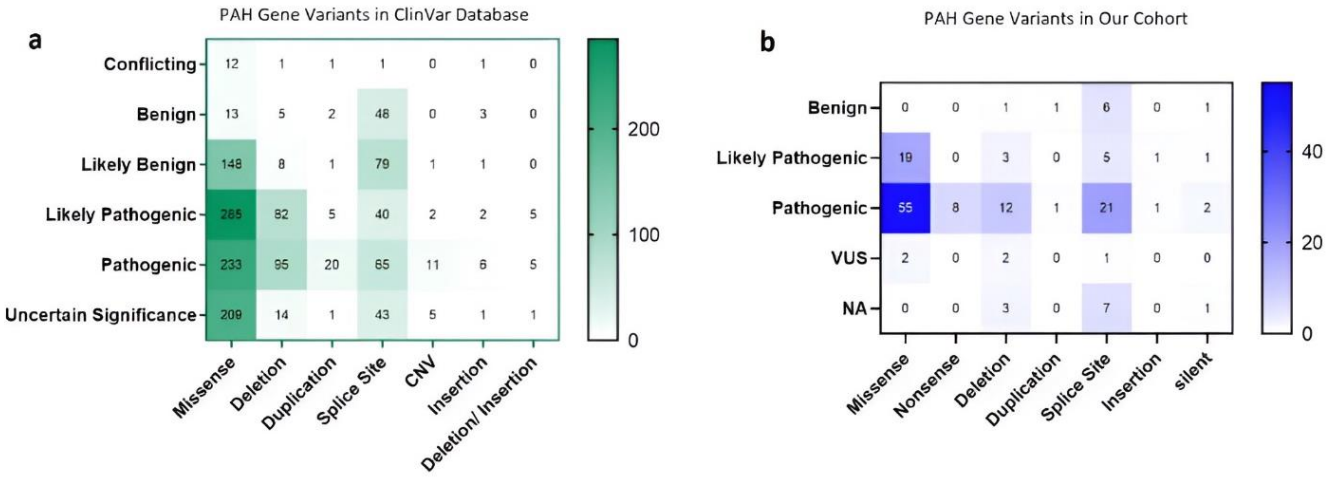

Figure 6. Heatmaps illustrating genetic variations in the PAH gene based on the ClinVar database (a) and the findings of this study (b)

Conclusion

This study provides a comprehensive in silico analysis of PAH gene mutations among Iranian patients with PKU, highlighting the exceptional genetic diversity and frequency of consanguinity within this population. Using integrated computational approaches - including the American College of Medical Genetics and Genomics (ACMG) criteria, Functional Analysis through Hidden Markov Models (Fathmm), Combined Annotation Dependent Depletion (CADD), Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping version 2 (PolyPhen-2), Mutation Taster, MUpro, and I-Mutant 2.0, alongside conservation analysis with Clustal Omega and Consurf - the pathogenicity and stability of 154 reported PAH variants were systematically evaluated. Our workflow enabled rapid, cost-effective screening and accurate prioritization of potentially deleterious mutations for further functional investigation.

Key findings reveal that 80.8% of PKU-causing mutations cluster in conserved regions, especially the catalytic domain, and that approximately half are missense variants. The rate of consanguineous marriage in the analyzed cohort was high (31.67%), underscoring the need for tailored genetic counseling programs. Docking analyses demonstrated that recurrent variants such as c.688G>A critically impair catalytic residues and destabilize the enzyme's structure. Notably, the prevalence and impact of this variant were validated against the Iranome database, reinforcing its relevance across multiple ethnic groups.

While in silico approaches offer valuable predictions regarding the molecular and clinical effects of genetic variation, these findings require further confirmation through laboratory-based functional studies to establish direct impacts on phenotype and treatment outcomes. Building upon the compiled catalog of common PAH mutations, the development of rapid diagnostic tools - such as strip assay kits incorporating high-frequency variants like c.688G>A specific to regional populations - can facilitate efficient genetic screening and contribute to precision medicine in PKU care. Ultimately, integration of computational and experimental methods will enable clinicians to prescribe optimal treatment, improve prognoses, and reduce unnecessary interventions, particularly in populations with elevated consanguinity rates (22).

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Ethical statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Golestan University of Medical Sciences (Code: IR.GOUMS.REC.1401.522).

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Fatemeh Vaghefi: Methodology, Investigation, Formal analysis, and Writing-Original draft; Farzaneh Motallebi: Methodology, Investigation, Formal analysis, and Writing-Original draft; Niloufar Moradi: Methodology and Writing-Original draft; Teymoor Khosravi: Visualization and Writing; Akram Vahidi: Writing-Review and Editing; Morteza Oladnabi: Conceptualization, Supervision, Writing-Review, Editing, Validation, and Data curation; Nahid Rezaie: Methodology.

Data availability statement

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Use of Artificial Intelligence

AI-based tools were utilized only to assist with language editing. They played no role in data analysis, drafting the scientific content, interpreting the results, or making any scientific decisions.

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