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Research paper

Structural and Functional Analysis of an HBB Variant (p.Leu4Gln) in β-Thalassemia and Its Impact on Heme Binding Using Molecular Docking

Fasiha Bibi[1], Samrina Muskan[1], Muhammad Affnan[1]*

1. Center of Biotechnology and Microbiology, University of Peshawar, 25120, Khyber Pakhtunkhwa, Pakistan

Abstract: β-thalassemia is an inherited hemoglobin disorder caused by mutations in the *HBB* gene. This study investigated the structural and functional effects of the *HBB* p.Leu4Gln mutation on β-globin and its interaction with heme. **Methodology:** The pathogenic variant NC_000011.10:g.5227020A>T (p.Leu4Gln) was identified through UniProt and evaluated using MutationTaster. Wild-type and mutant β-globin structures were modeled with AlphaFold2, and protein–heme docking was performed using MOE. **Results:** The mutant protein exhibited a less favorable docking score (−9.8716 kcal/mol) than the wild type (−10.6160 kcal/mol) and a higher RMSD value (1.8171 vs. 1.2387 Å), indicating reduced binding affinity and stability. Interaction analysis revealed the formation of a metal coordination bond and a novel π-H interaction with His64 in the mutant complex, suggesting alteration of the heme-binding pocket. **Conclusion:** The p.Leu4Gln mutation disrupts heme binding and alters the structural integrity of β-globin, potentially contributing to impaired hemoglobin function in β-thalassemia. These findings provide molecular insights into disease pathogenesis and potential therapeutic targets.

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Corresponding author
Muhammad Affnan[1]*
Afnanmarwat710@gmail.com

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Introduction

Hemoglobinopathies are a group of monogenic inherited disorders which are caused by mutations in genes encoding globin chains, with higher prevalence in low- and middle-income countries [1]. β-thalassemia is the most common and clinically significant form of hemoglobinopathies, resulting from the absence or reduced synthesis of β-globin chain of hemoglobin [2], which is a tetrameric protein consisting of two α and two β globin chains. The HBB gene is located on chromosome 11p15.5 within the β-globin gene cluster. This gene is composed of three exons and two introns, mutations within which decrease or halt β-globin production, giving rise to excess unpaired α-chains [2]. These unstable α-chains form precipitates within erythroid precursors, causing ineffective erythropoiesis, premature RBC destruction, chronic hemolytic anemia and in severe cases may lead to transfusion dependency [2]. β-thalassemia has a high frequency in the Mediterranean, Middle East, South Asia, and Sub-Saharan Africa [3]. Pakistan has recorded carrier rates ranging from 5-8% of the general population, with thousands of infants being born with thalassemia major, annually [5]. High consanguinity rates in the country reaching as high as about 70% significantly contribute to the prevalence of this disorder by increasing the chances of inheriting pathogenic variants from both parents [5]. So far, more than 200 pathogenic variants have been identified in the HBB gene, most of which are point mutations, small insertions or deletions, or splice-site alterations [4]. The persistence of these variants is likely favored by evolution in malaria-endemic regions because they offer a survival advantage to heterozygotes, providing partial protection against

severe Plasmodium falciparum malaria [2]. New advances in computational biology have enabled detailed studies of pathogenic mutations. Tools such as MutationTaster, AlphaFold2, and molecular docking platforms allow the study of the impact of mutations on protein function by predicting pathogenicity, 3D structures, and ligand-binding affinities. The present study used these methods to evaluate HBB gene mutation with the aim of supporting future therapeutic research [11,12,13].

Methodology

Gene selection

GeneCards and DisGeNET databases were employed using the search term "thalassemia" to identify genes strongly associated with β-thalassemia [7,6]. Gene lists from both the tools were retrieved and compared using Venny 2.0 [8]. Overlapping genes HBB and HBA2 were identified, with HBB being selected due to its established role in β-thalassemia.

Variant Identification

Variant data for HBB gene was obtained from UniProt [9]. The human protein entry was selected, and the Variant Viewer section was examined to identify disease-related mutations. Information regarding mutation type, amino acid substitution, and positional information was recorded for further analysis.

Pathogenicity Prediction

MutationTaster was utilized to confirm the pathogenicity of the mutation and predict whether it was disease-causing or harmless [10]. The tool also provides wild-type and mutant protein sequences.

Protein Structure Prediction

The 3D structures of wild-type and mutant HBB proteins were generated using the AlphaFold2 server [11,12]. Each sequence was separately submitted to create structural models. The outputs were downloaded in the form of compressed files, which were then extracted using WinRAR and converted from CIF format to PDB format to make them compatible with the subsequent molecular analysis tools.

Protein Preparation and Molecular Docking

Molecular Operating Environment (MOE) was used for molecular docking. The PDB structures of wild-type and mutant proteins were docked against the HEM ligand, selected based on its established role as the natural iron-containing cofactor of hemoglobin. The structure of the ligand was obtained from PubChem in the sdf format [13]. Before docking, the PDB structures of proteins were separately imported into MOE and prepared by removing water molecules and heteroatoms, followed by quick preparation and energy minimization for ensuring structural stability. The ligand was then directly imported into MOE. Dummy atoms were placed to define the active site, and docking was performed using the Induced Fit docking model. The conformation that exhibited the most favourable binding energy score was selected and used for ligand interaction analysis, which produced a 3D model of protein-ligand complex and a 2D interaction diagram highlighting key binding residues. In addition, MOE generated a detailed report of binding energy values and interaction profiles, which were used for further comparing wild-type and mutant complexes.

Visualization and Data Analysis

The docking complexes were visualized in BIOVIA Discovery Studio to create 2D and 3D interaction diagrams. The amino acid interactions and ligand-binding orientations of the wild-type and mutant complexes were examined and compared to assess the structural impact of the mutation.

Results

Gene Overlapping Analysis and Selection of Target Gene

Venny 2.0 identified two overlapping genes between GeneCards and DisGeNET datasets, HBB and HBA2, which are associated with β-thalassemia. Among the two, HBB gene was selected for further investigation because of its established role in thalassemia pathogenesis in thalassemia.

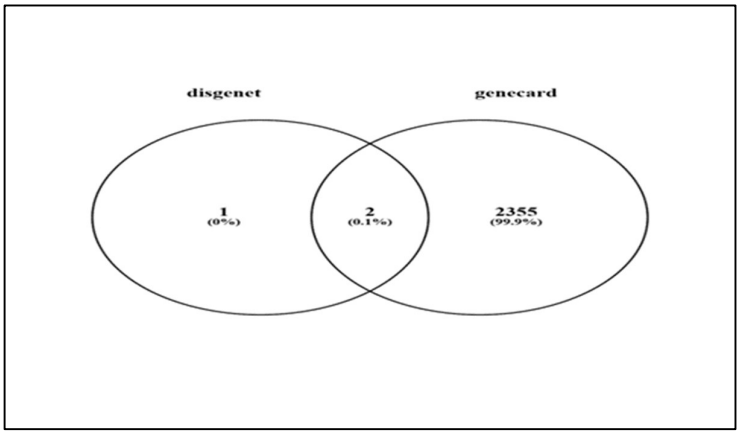


Figure 1: Gene overlap analysis between DisGeNET and GeneCards dataset using venny 2.0

Variant Identification and Pathogenicity Assessment

In UniProt, Variant Viewer analysis revealed that several pathogenic mutations are linked with the HBB gene. The variant NC_000011.10:g.5227020A>T was selected for further analysis [9]. MutationTaster confirmed the variant to be disease-causing [10]. An allele frequency of approximately 6.27×10^{-7} for the T allele was recorded in the gnomAD database, indicating it to be extremely rare in the general population [14]. The wild-type and mutant protein sequences generated by MutationTaster are as follows:

Protein Structure Preparation

The structures of both wild-type and mutant HBB protein were successfully modeled, and then prepared, and were optimized to obtain a stable conformation suitable for docking analysis.

Ligand Preparation

The HEM ligand (PubChem CID: 25245619) was obtained from PubChem and prepared for docking, resulting in a stable 3D conformation suitable for interaction analysis.

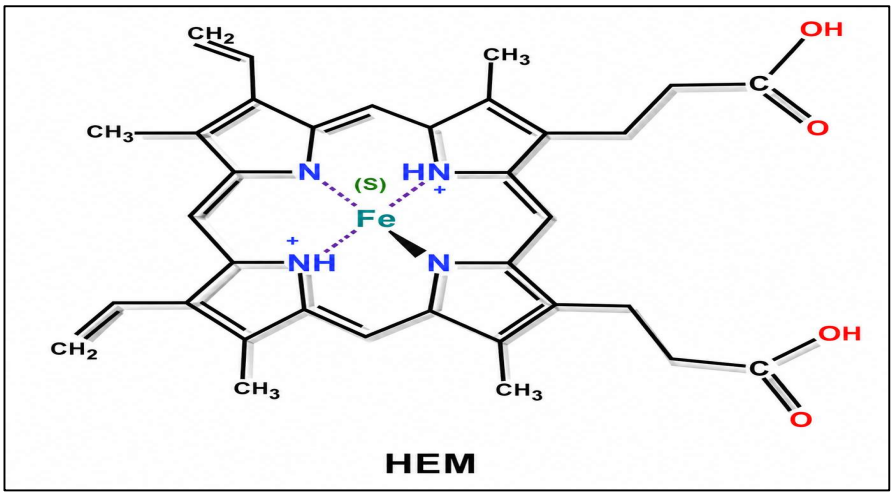
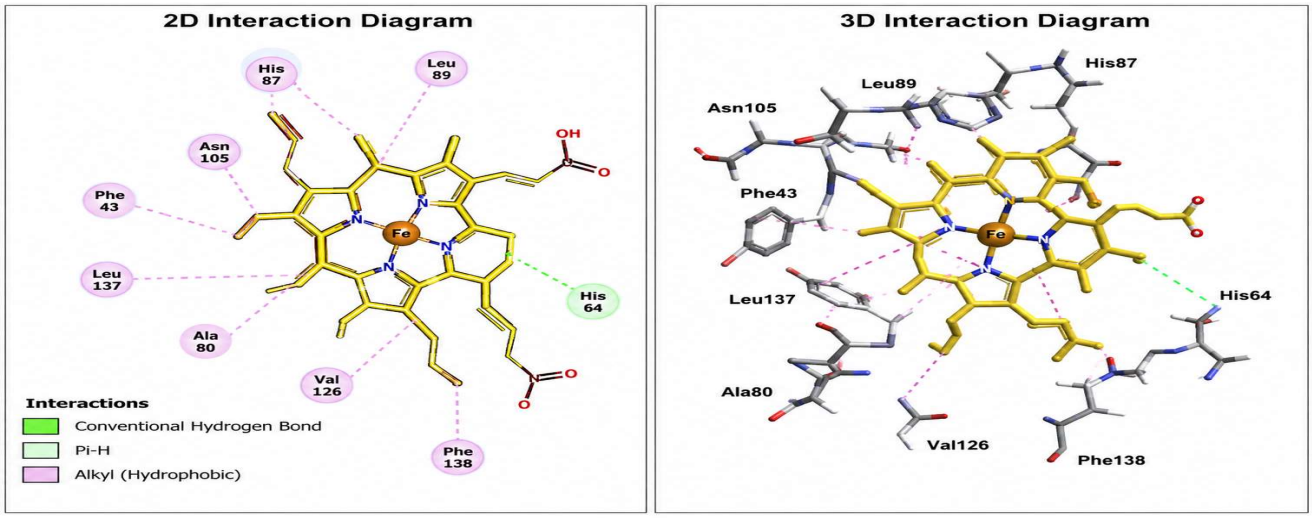


Figure 2: 2D structure of ligand retrieved from PubChem database (CID: 25245619)

Molecular Docking — Wild-Type HBB

The molecular docking of HEM ligand with the active site of wild-type HBB protein revealed that the binding was favorable as the docking score (E_score2) was −10.6160 kcal/mol. The RMSD refine value was low (1.2387 Å), supporting a stable binding conformation of the dock complex. Moreover, interaction analysis identified a key interaction involved in heme binding which is a metal coordination bond between the iron atom of the heme group and the NE2 atom of His93 of the HBB protein at a distance of 2.16 Å. The binding pockets were mostly hydrophobic in nature and

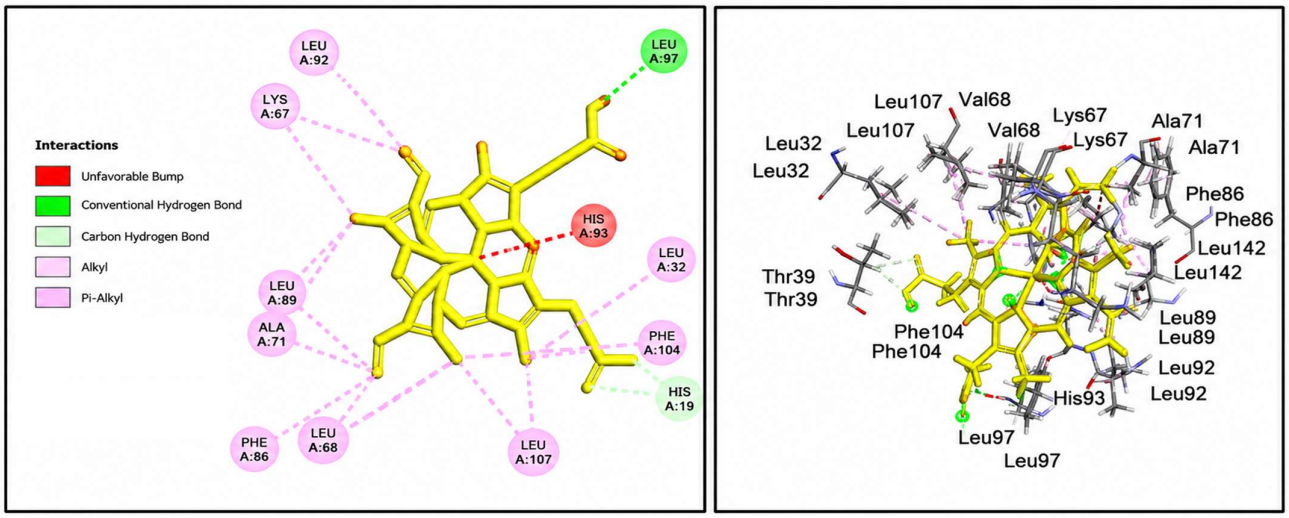
residues such as Phe42, Phe43, Phe72, Leu29, Leu89, and Val68 were their components. Additional polar contacts were also observed with residues including His64, Lys67, Thr38, and Asn103. The placement, refinement, and conformational energy values were −96.6033, −56.7541, and −259.5479 kcal/mol, respectively.



2D: Schematic of heme (HEM) interactions with protein residues 3D: 3D representation of heme (HEM) interactions in the protein active site

Molecular Docking — Mutant HBB

Molecular docking of HEM ligand with mutant HBB protein showed a weaker binding as compared to wild-type protein, with a less favorable docking score (E_score2) of −9.8716 kcal/mol. The mutant complex also showed lesser structural stability, with less favorable refinement and conformational energy values (E_refine = −46.6821 kcal/mol and E_conf = −243.1005 kcal/mol, respectively). Furthermore, the higher rmsd_refine value of 1.8171 Å suggests that the position of the HEM ligand is comparatively less stable in the mutant binding site. Interaction analysis showed that heme remained coordinated to His93 through two metal coordination interactions at distances of 1.95 Å and 2.16 Å. In addition, a pi–H interaction involving His64 was detected, an interaction that was absent in the wild-type complex. This change in the interaction of heme with the surrounding amino acids suggests that the shape or arrangement of the binding pocket has been altered due to the mutation. Although the same hydrophobic nature of the binding pocket is retained, the specific amino acids involved in ligand binding have changed.



[A] 2D interaction of HBB mutant protein with HEM [B] 3D interaction of HBB mutant protein with HEM

Comparative Molecular Docking and Interaction Analysis of HEM with Wild-Type and Mutant HBB Protein

The molecular docking analysis revealed differences in binding affinity, docking energies, and interaction patterns between the wild-type and mutant HBB proteins when complexed with the HEM ligand. The wild-type HBB exhibited a more favorable overall docking score (E_score2 = −10.6160 kcal/mol) and lower RMSD refinement value (1.2387 Å) compared to the mutant protein (E_score2 = −9.8716 kcal/mol; RMSD = 1.8171 Å), indicating a more stable ligand-binding conformation. Energy decomposition analysis demonstrated variations in conformational, placement, and refinement energies between the two complexes. Interaction profiling showed that the wild-type protein formed a single metal coordination bond between the heme iron (Fe) and His93 residue, whereas the mutant protein established two metal coordination interactions and an additional π–H interaction with His64. Despite exhibiting a higher total interaction energy (−5.9 kcal/mol), the mutant complex displayed reduced docking stability relative to the wild type, suggesting that the mutation alters the binding environment and structural dynamics of the HEM-binding pocket.

Table 1: Comparative molecular docking parameters and ligand interaction profile of HEM with wild-type and mutant HBB proteins.

Parameter	Wild-Type HBB	Mutant HBB
E_score2 (kcal/mol)	−10.6160	−9.8716
E_score1 (kcal/mol)	−11.4341	−12.1232
E_refine (kcal/mol)	−56.7541	−46.6821
E_place (kcal/mol)	−96.6033	−103.6680
E_conf (kcal/mol)	−259.5479	−243.1005
RMSD refinement (Å)	1.2387	1.8171
Metal coordination (Fe–His93)	1 interaction, 2.16 Å, −3.3 kcal/mol	2 interactions, 1.95 Å (−2.8) and 2.16 Å (−2.4 kcal/mol)
π–H interaction (His64)	Absent	Present, 4.57 Å, −0.7 kcal/mol
Total interaction energy (kcal/mol)	−3.3	−5.9

Discussion

This study aimed to understand the effect of a pathogenic mutation in the HBB gene on the structure and function of the protein. The variant NC_000011.10:g.5227020A>T, corresponding to the amino acid substitution p.Leu4Gln (L4Q), was identified through UniProt's Variant Viewer [9] and confirmed as disease-causing using MutationTaster [10]. The 3D structures of both mutant and wild-type HBB protein were generated by AlphaFold2 and docked against HEM using MOE 2019.01 to compare the changes in heme binding at the molecular level [11,12]. The docking scores (wild-type vs mutant) suggest that HEM binds more strongly to the wild-type protein. The more favorable refinement and conformational energies further imply that the wild-type HEM complex is more stable, although the mutant protein shows slightly better E_place and E_score1 values. These are values from earlier stages of docking and are less reliable than the final docking scores. The mutant protein, with an increased RMSD (1.8171 Å vs 1.2387 Å), indicates positional instability of HEM within the mutated pocket. The formation of a new metal coordination bond and a pi-H interaction with His64 in the mutant protein suggest a possible geometric distortion within the binding site, where the heme iron now interacts with the surrounding residues in an altered manner, potentially disrupting the normal physiological function of hemoglobin. Several previous studies have shown that mutations in HBB can alter the protein and affect how it interacts with heme. Ejaz et al. used in-silico analysis to investigate various sites and frequencies of mutations linked with β-thalassemia in Southern Punjab, Pakistan, by screening 49 β-thalassemia major patients and 49 carriers. They demonstrated that pathogenic HBB variants including c.47G>A (p.W16*) result in premature translation termination, causing the β-globin protein to lose its function [19]. Our docking results are consistent with this study, as the wild-type protein showed a stable metal coordination bond between the iron of the heme and His93 at 2.16 Å — a well-established critical proximal histidine bond essential for normal hemoglobin function [15]. In contrast, the mutation produced a reduced docking score, higher RMSD, and a new pi-H interaction with His64, changes that have previously been linked to disruption of heme binding and reduced oxygen transport in β-thalassemia patients [16,17]. Similarly, Qadah and Jamal performed a computational analysis of structural changes caused by mutations in the HBB gene. Their data demonstrated that mutations in HBB alter the normal function of the protein and give rise to anoma-

lies such as hemolysis, chronic anemia, and ineffective erythropoiesis, highlighting the importance of computational approaches in studying the molecular basis of β-thalassemia [18]. The present study similarly used computational methods to understand the effect of a pathogenic HBB mutation on protein structure and heme binding. Carlice-dos-Reis et al. used the 1,000 Genomes database to investigate HBB variations across world populations, revealing that approximately 8.3% of phenotypically healthy individuals carry some mutation in the HBB gene [20]. They estimated that 14 out of every 10,000 individuals worldwide will have a hemoglobinopathy in the next generation, highlighting the importance of understanding HBB mutations at a molecular level. Even a single nucleotide change can alter the β-globin chain, leading to imbalanced globin production and the characteristic symptoms of β-thalassemia. The computational evidence presented here suggests that mutations in this gene may alter the geometry of the heme-binding pocket [21]. The importance of the current study lies in the fact that it directly compares the structures of wild-type and mutant heme–protein complexes using induced fit docking, revealing not only a reduction in binding affinity but also qualitative changes in the interaction profile, specifically the novel appearance of an additional metal coordination interaction and distal histidine engagement in the mutant protein. This provides a possible molecular explanation for the functional impact of this variant in greater detail than what has been previously documented in computational studies. This study is limited to computational analysis, and experimental validation through techniques such as X-ray crystallography would be highly useful in confirming these findings in a real biological setting. Moreover, only one variant and one ligand were studied, so expanding the analysis to multiple HBB variants and potential therapeutic ligands would strengthen the conclusions and allow for a more concrete understanding of how mutations affect heme binding.

Conclusion

This study demonstrated that the HBB p.Leu4Gln variant induces structural and functional alterations in β-globin that affect its interaction with heme. Molecular docking analysis revealed reduced binding affinity, increased RMSD, and altered interaction patterns in the mutant protein compared with the wild type, indicating decreased complex stability. The observed changes in the heme-binding pocket suggest a potential disruption of normal hemoglobin function, providing molecular insight into the pathogenic role of this variant in β-thalassemia. Further experimental studies are needed to validate these computational findings.

Data Availability Statement

All data generated and analyzed during this study are included in this manuscript. The protein structures used in this study were obtained from the AlphaFold2 server (https://alphafoldserver.com/). The HEM ligand structure was retrieved from PubChem (Compound ID: 25245619). Molecular docking analysis was performed using MOE 2019.01 software. Variant information was obtained from UniProt (https://www.uniprot.org/). The data and analysis scripts are available from the corresponding author upon reasonable request.

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