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Predicting the Structural Impact of the Hemoglobin Constant Spring Mutation on HBA2

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Background: Thalassemia is a disease caused by the impaired production of globin chains. The most prevalent non-deletional α-thalassemia variants in Indonesia include Hemoglobin Constant Spring (Hb CS), Hb Adana, and codon 22 (GGC→GGT). However, structural bioinformatics studies on Hb CS remain limited. This study aims to predict the three-dimensional structure of Hb CS and its comparison to normal HBA2 globin chain structures.
Materials and methods: The reference HBA2 nucleotide sequence was retrieved from NCBI and translated to amino acid using ExPASy Translate. The three-dimensional structure of Hb CS was predicted using Iterative Threading Assembly Refinement (I-TASSER). In addition, physicochemical properties, as well as structural and functional predictions, were analyzed using the ProtParam and PredictProtein servers.
Results: I-TASSER generated five structural models of Hb CS, all showing high similarity to HBA2 (81.98% sequence identity; RMSD 0.322–0.396 Å). Compared with HBA2, Hb CS exhibited increased molecular weight, higher theoretical pI, and a higher instability index. The additional 31-amino-acid C-terminal segment was predicted to form a solvent-exposed intrinsically disordered region predominantly composed of coil structures. The additional amino acid segment was predicted to form a solvent-exposed intrinsically disordered region, suggesting reduced structural stability. Some gene ontology terms of Hb CS were found to be consistent with those of functional hemoglobin.
Conclusion: Hb CS mutation causes an extension of hydrophobic amino acids and forms the IDP region, thereby reducing its stability in the cytoplasm compared to HBA2. This mutation also triggers the REMD mechanism that accelerates mRNA degradation due to excessive translation into the 3'-UTR region.

Keywords: *α-thalassemia, HBA2, Hb CS, in silico*

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Introduction

Thalassemia is a disorder caused by impaired globin chain production, leading to reduced or imbalanced hemoglobin synthesis.[1-2] Thalassemia is classified into α-thalassemia and β-thalassemia. α-Thalassemia is characterized by reduced production of α-globin, whereas β-thalassemia is primarily caused by mutations in the β-globin (HBB) gene.[3-4] In the Special Region of Yogyakarta, the most common β-thalassemia and α-thalassemia mutations are IVS-1-5 and the -α3.7 deletion, respectively.[5-6] The common forms of non-deletional α-thalassemia in Indonesia are Hemoglobin Constant Spring (Hb CS), Hb Adana, and codon 22(GGC→ GGT).

Changes in DNA sequences may affect gene expression and protein synthesis, resulting in various disorders, including congenital heart disease, increased obesity risk, and thalassemia.[7-9] These alterations may occur as substitutions, insertions, or deletions in coding or non-coding regions of DNA. Hb CS is caused by a transition mutation in the termination codon 142 TAA → CAA.[10] TAA encodes a stop codon, whereas CAA encodes glutamine. The Hb CS mutation results in the addition of 31 amino acids to the α-globin chain.[11] C-terminal elongation may disrupt normal protein folding pathways, expose hydrophobic residues, increase susceptibility to proteolytic degradation, alter intermolecular interactions, and affect assembly of the αβ hemoglobin tetramer. Certain non-deletional α-thalassemia variants, including Hb CS, may produce more severe clinical manifestations than deletional α-thalassemia variants because of their combined effects on globin synthesis and protein stability.

Three-dimensional structures of deletional α-thalassemia variants have previously been predicted. The deletion results in structural changes to the protein because it produces shortened polypeptides. In addition to these structural changes, the globin protein also loses its ability to form a hemoglobin complex.[12] Despite extensive studies on the molecular diagnosis, epidemiology, clinical manifestations, and therapeutic correction of Hb CS, little is known about the structural consequences of the characteristic nonstop mutation. In particular, the impact of the 31-amino-acid C-terminal extension on α-globin folding, stability, and potential interactions within the hemoglobin complex remains poorly understood. Although Hb CS has been extensively investigated from molecular diagnostic, epidemiological, clinical, and therapeutic perspectives, little is known about how the characteristic nonstop mutation affects the three-dimensional organization of the α-globin chain. Recent advances in protein structure prediction and experimental structural biology have improved our understanding of protein variants associated with human diseases; however, structural characterization of Hb CS remains largely unavailable. Therefore, a comparative structural analysis of wild-type HBA2 and Hb CS may provide mechanistic insights into the instability and functional alterations associated with this clinically important α-thalassemia variant. This study aims to analyze the three-dimensional structure of Hb CS and compare it with the globin chain structure of normal HBA2 using an in silico approach. The novelty of this study lies in the structural characterization of the Hb CS mutant and the prediction of how the characteristic C-terminal extension influences protein architecture and stability.

Materials and methods

Sample Retrieved and Preparation

The reference HBA2 nucleotide sequence was retrieved from GenBank (AAK61215.1) and translated using ExPASy Translate (https://web.expasy.org/translate) (Swiss Institute of Bioinformatics, Swiss). The Hb CS mutant sequence was generated by substituting the termination codon at position 142 (TAA→CAA), resulting in a predicted protein of 172 amino acids. Deoxy hemoglobin (2DN2) macromolecules were retrieved from RCSB PDB to prepare the HBA2 structure.

Hb CS Modeling

The three-dimensional structure of Hb CS was predicted using I-TASSER (Iterative Threading Assembly Refinement) (Zhang Lab, Singapore). The predicted structure was visualized using VMD 1.9.3 (Theoretical and Computational Biophysics Group, US). The result was aligned with HBA2 by using MultiSeq in VMD 1.9.3 (Theoretical and Computational Biophysics Group, US). The Hb CS–heme interaction predicted by I-TASSER was visualized using BIOVIA Discovery Studio 2021 Client (Dassault Systèmes, France) and compared with the HBA2–Heme interaction. The stereochemical quality of the predicted structure was assessed using Ramachandran plot analysis generated with VMD, which evaluates the distribution of backbone dihedral angles (φ and ψ) and identifies residues located in favored, allowed, and disallowed regions.

Physicochemical Properties, Structural, and Functional Prediction of Hb CS

The physicochemical properties of HBA2 and Hb CS were analyzed using the ProtParam ExPASy server (Swiss Institute of Bioinformatics, Swiss).13 Parameters, including molecular weight, isoelectric point (pI), amino acid composition, extinction coefficient (ε280), instability index (II), aliphatic index (AI), and grand average of hydropathicity (GRAVY), were observed. The structure and function of the Hb CS mutant were predicted. Specifically, the solvent accessibility (SA) value, secondary structure, intrinsically disordered protein (IDP), gene ontology (GO), and protein binding site were predicted using the PredictProtein server (Rostlab, Germany), which could be accessed for free at PredictProtein server (https://predictprotein.org). Predictions were interpreted according to the reliability scores provided by PredictProtein. Only predictions with moderate-to-high confidence were considered during functional interpretation.

Results

Hb CS Retains the Native HBA2 Fold with C-terminal Variation

The I-TASSER prediction generated five models (Models 1–5). The aligned three-dimensional structures of the Hb CS models and HBA2 are shown in Figure 1. The QH values, root mean square deviation (RMSD), and percentage identity are presented in Figure 2. All aligned globin chains showed similarly high QH values. Hb CS shared 81.98% sequence identity with HBA2. Figure 1 presents the MultiSeq alignment of all five predicted Hb CS models with HBA2. The proteins were predominantly composed of α-helical structures. The predicted models showed different conformations in the additional amino acid region.

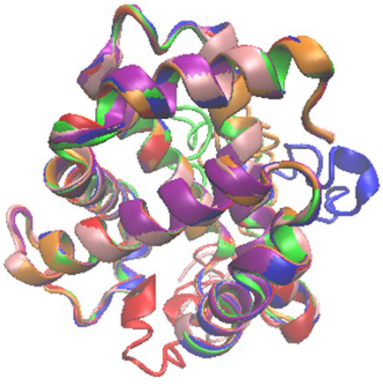


Figure 1. MultiSeq alignment of HBA2 dan Hb CS. Color legend: purple (Color ID 11): HBA2; orange (Color ID 3): model 1; red (Color ID 1): model 2; green (Color ID 7): model 3; pink (Color ID 9): model 4; dan blue (Color ID 0): model 5.

In Ramachandran plots, the blue regions represent the most favored conformational regions, while the green regions indicate the additionally allowed regions. White areas correspond to disallowed regions, where residue conformations are generally considered sterically unfavorable. Comparison of the predicted Hb CS models revealed that Models 1, 2, 3, and 5 displayed residue distributions similar to that of HBA2, with most residues located within the favored and allowed regions of the Ramachandran plot. In contrast, model 4 showed a higher proportion of residues located outside these regions, suggesting lower stereochemical quality relative to the other models (Figure 3).

Hb CS Displays Increased Molecular Weight, Basicity, and Reduced Predicted Stability Compared with HBA2

The physicochemical properties of Hb CS and HBA2 were calculated based on their amino acid sequences. Hb CS exhibited physicochemical properties different from those of HBA2 (Table 1). Hb CS was more basic and contained more positively charged residues than HBA2. The values of molecular weight, II, ε280, AI, and GRAVY were higher in Hb CS than in HBA2. The Hb CS–heme and HBA2–heme interactions were visualized using BIOVIA Discovery Studio 2021 Client. The results showed that Hb CS and HBA2 shared the same heme-binding region, spanning residues 38–136 (Figure 4).

Figure 5 shows the secondary structure and solvent accessibility (SA) of Hb CS. The predicted protein-binding site is shown in Figure 6, whereas the intrinsically disordered protein (IDP) regions and relative B-values are presented in Figure 7. The additional amino acids (residues 142–172) in Hb CS were predominantly arranged in a coiled conformation. These additional residues were predicted to be IDP regions and were exposed to the cytoplasmic environment. The predicted protein-binding site overlapped with the Hb CS–heme-binding region, which spanned residues 38–136. Several gene ontology (GO) terms of Hb CS, including oxygen transport (GO:0015671), hemoglobin complex (GO:0005833), oxygen carrier activity (GO:0005344), oxygen binding (GO:0019825), and heme

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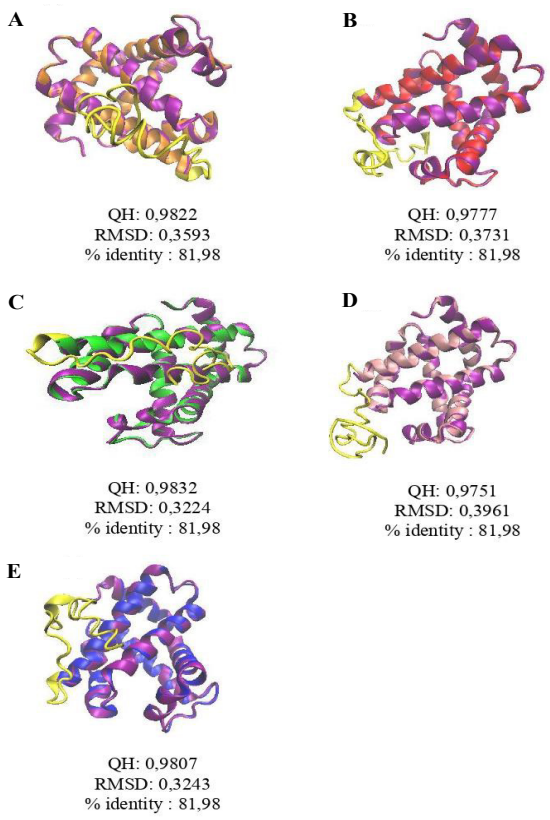


Figure 2. MultiSeq alignment of HBA2 dan Hb CS. Color legend: purple (Color ID 11): HBA2; orange (Color ID 3): model 1; red (Color ID 1): model 2; green (Color ID 7): model 3; pink (Color ID 9): model 4; dan blue (Color ID 0): model 5.

binding (GO:0020037), were consistent with the functional properties of normal hemoglobin (Table 2).

Discussion

The Hemoglobin Constant Spring (Hb CS) mutation is classified as a nonstop (antitermination) mutation in which the translation termination codon is replaced by a sense codon, allowing translation to continue beyond the normal stop codon. In Hb CS, the stop codon (TAA) is replaced by glutamine (CAA), resulting in the addition of 31 amino acids to the α-globin chain and extension of translation into the 3′-UTR.14 A hallmark of nonstop mutations is the marked reduction of mutant mRNA abundance, as reported in several disorders caused by nonstop mutations, including Factor X deficiency, anterior segment dysgenesis, and pan-cancer associated with mutations in the F10, FOXE3, and SMAD4 genes, respectively.[15–17] These findings indicate that reduced transcript abundance is a fundamental molecular consequence of nonstop mutations.

The high structural similarity between Hb CS and HBA2 indicates that the characteristic nonstop mutation does not substantially alter the conserved α-globin core. Instead, the principal structural consequence is confined to the elongated C-terminal region, suggesting that the pathological effects of Hb CS are more likely to arise from

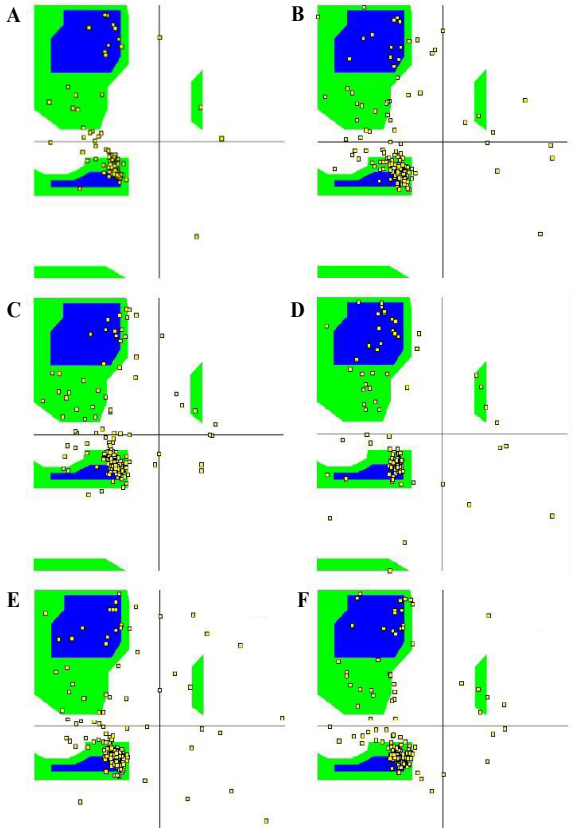


Figure 3. Ramachandran plot analysis of HBA2 and the five predicted Hb Constant Spring (Hb CS) structural models. The Ramachandran plots illustrate the distribution of backbone dihedral angles (φ and ψ) for each structure. Blue regions represent the most favored conformations, green regions indicate additionally allowed conformations, and white regions correspond to disallowed conformations. Residues located within the favored and allowed regions indicate acceptable stereochemical quality, whereas residues outside these regions suggest conformational strain or reduced structural reliability. (A) HBA2. (B) Hb CS Model 1. (C) Hb CS Model 2. (D) Hb CS Model 3. (E) Hb CS Model 4. (F) Hb CS Model 5.

Table 1. Results of mean analysis, normality test, homogeneity test and difference test more than 2 groups on TNF-α levels in various treatment groups.

Physicochemical Properties	α-Globin Chain	
	HBA2	Hb CS
Molecular weight (Da)	15,126.36	18,481.26
Theoritical pI	8.73	9.30
Negatively charged residues (Asp + Glu)	12	13
Positively charged residues (Arg + Lys)	14	17
Instability Index (II)	6.95	19.54
ε280($M^{-1}cm^{-1}$)	9,970	15,470
Aliphatic index (AI)	91.42	92.56
Grand average of hydropathicity (GRAVY)	0.035	0.069

the acquired extension than from disruption of the native globin fold. Ramachandran plot analysis further supported this observation. Most residues in HBA2 and Hb CS Models 1, 2, 3, and 5 were located within the favored and allowed regions, indicating acceptable stereochemical quality and preservation of the native globin fold despite the C-terminal extension. In contrast, Model 4 contained more residues outside these regions, consistent with the absence of helical elements in the extended region and suggesting greater conformational flexibility. The increased occurrence of nonfavored conformations in Model 4 may reflect structural instability associated with the elongated C-terminal segment, which was also predicted to contain intrinsically disordered residues and exposed hydrophobic amino acids. Overall, the acceptable stereochemical quality of most predicted models supports the reliability of the structural predictions and the subsequent physicochemical and functional analyses, whereas the poorer stereochemical quality of Model 4 most likely reflects the intrinsic flexibility of the elongated C-terminal segment rather than instability of the conserved globin core.

The physicochemical analysis further suggests that the C-terminal extension alters the biochemical properties of Hb CS. Compared with HBA2, Hb CS exhibited greater molecular weight, a higher theoretical pI, and an increased instability index, although both proteins remained within the range of stable proteins. The relatively higher instability index is consistent with previous observations that Hb CS protein abundance is substantially lower than expected despite the presence of an intact α-globin gene, suggesting that reduced protein stability contributes to its pathophysiology in addition to decreased mutant mRNA levels. The higher pI of Hb CS is consistent with its greater proportion of basic amino acids relative to acidic residues.[18-20]

The low instability index (II) values (below 40) of Hb CS and HBA2 indicate that both proteins are stable. HBA2 had a lower II value and was therefore more stable than Hb CS. The ε280, AI, and GRAVY values of Hb CS were higher than those of HBA2. A positive GRAVY value indicates that a protein is hydrophobic. The higher GRAVY value of Hb CS may be attributed to its greater proportion of hydrophobic amino acids compared with HBA2. However, the hydrophobicity of Hb CS was not pronounced, as its GRAVY value was close to 0. The GRAVY value was calculated solely based on the linear amino acid sequence and does not account for the protein's three-dimensional structure.[21]

Secondary-structure prediction indicated that the additional 31 amino acids were predominantly arranged as solvent-exposed coil structures. Notably, 21 of these

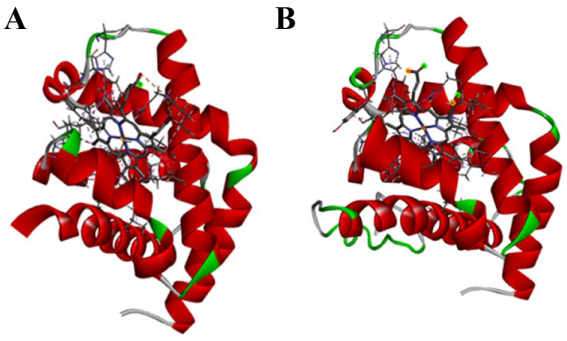


Figure 4. Comparison of the predicted heme-binding interactions between HBA2 and Hb Constant Spring (Hb CS). The α-globin backbone is shown in cartoon representation, with α-helices displayed in red, β-strands in green, and loops in gray. The heme prosthetic group and interacting amino acid residues are shown in stick representation. (A) Predicted heme-binding interactions in the native HBA2 structure. (B) Predicted heme-binding interactions in the Hb CS model. The predicted heme-binding region is conserved in both proteins (residues 38–136).

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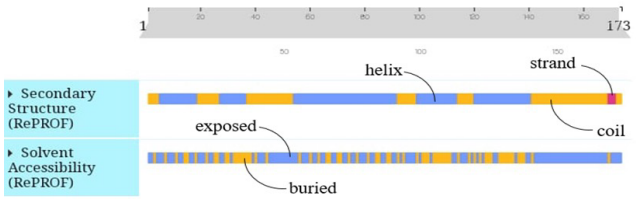


Figure 5. Predicted secondary structure and solvent accessibility of Hb CS. The upper panel shows the distribution of α-helices (blue), coils (orange), and β-strands (pink) along the Hb CS sequence, whereas the lower panel indicates buried (blue) and solvent-exposed (orange) residues. The elongated C-terminal segment generated by the Hb CS nonstop mutation is predominantly composed of coil structures with increased solvent accessibility, supporting the prediction that this region is structurally flexible and prone to intrinsic disorder.

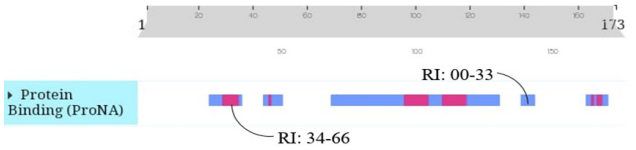


Figure 6. Predicted protein-binding sites of Hb Constant Spring (Hb CS). Blue and magenta bars represent predicted binding sites with low (RI = 0–33) and moderate (RI = 34–66) confidence, respectively. The principal predicted binding region coincides with the conserved heme-binding domain, indicating that the C-terminal extension does not substantially alter the predicted protein interaction site.

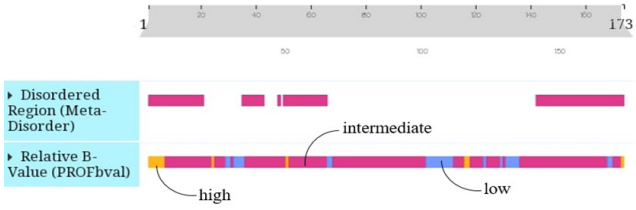


Figure 6. Predicted intrinsically disordered regions (IDRs) and residue flexibility of Hb CS. The upper panel identifies residues predicted to lack a stable three-dimensional structure (magenta), whereas the lower panel displays residue flexibility based on predicted relative B-values, with yellow, magenta, and blue indicating high, intermediate, and low flexibility, respectively. The elongated C-terminal segment generated by the Hb CS nonstop mutation is predominantly predicted to be intrinsically disordered and structurally flexible, providing a plausible structural basis for the reduced stability of the Hb CS protein.

residues were hydrophobic, whereas hydrophobic amino acids are normally buried within the protein core during protein folding.[22] Exposure of these residues to the aqueous cytoplasmic environment may therefore compromise structural integrity. One of the most biologically relevant findings of this study is the prediction that the elongated C-terminal segment forms a solvent-exposed intrinsically disordered region. Intrinsically disordered regions frequently promote protein instability, abnormal molecular interactions, and accelerated degradation, providing a plausible structural explanation for the reduced intracellular abundance of Hb CS observed experimentally.[23]

The predicted protein-binding regions suggest that Hb CS retains the potential to participate in protein–protein interactions despite the presence of the C-terminal extension. Hb CS and HBA2 also shared the same predicted heme-binding region. Despite the structural alterations introduced by the C-terminal extension, the preservation of GO terms associated with oxygen binding, oxygen transport, oxygen carrier activity, and heme binding suggests that the mutation does not abolish the core molecular function of α-globin. Instead, the pathogenic mechanism of Hb CS is more likely attributable to impaired protein stability and reduced expression than to complete loss of oxygen-binding capacity.

Extension of translation into the 3′-UTR becomes destabilizing when the elongating peptide reaches the poly(A) tail, interfering with poly(A)-binding proteins and triggering ribosome extension-mediated decay (REMD). REMD accelerates deadenylation, followed by exonucleolytic degradation of the mutant transcript through both the 5′–3′ and 3′–5′ pathways.[24,25] Hb CS is synthesized at a rate comparable to that of normal α-globin; however, its abundance decreases markedly during erythroblast differentiation. These findings indicate that the Hb CS phenotype results from two complementary mechanisms: post-transcriptional reduction of mutant mRNA through REMD and structural destabilization of the elongated α-globin protein.

To our knowledge, this study represents one of the first comprehensive structural bioinformatics analyses of Hb CS and provides preliminary mechanistic insights into how the characteristic C-terminal extension influences α-globin stability while preserving its conserved globin fold. Nevertheless, this study has several limitations. First, all structural and functional predictions were generated

computationally and were not validated experimentally. Second, molecular dynamics simulations were not performed, preventing assessment of conformational stability under physiological conditions. Third, interactions between Hb CS and β-globin chains were not evaluated. Future studies integrating molecular dynamics simulations, protein interaction analyses, and experimental validation will be important to confirm these predictions and further elucidate the molecular pathogenesis of non-deletional α-thalassemia.

Collectively, these findings provide structural evidence that the Hb CS nonstop mutation primarily affects protein stability rather than disrupting the conserved α-globin fold. The predicted formation of a solvent-exposed intrinsically disordered C-terminal region offers a plausible molecular mechanism linking the characteristic elongation mutation to the reduced protein abundance observed in patients with Hb CS. These findings further support the concept that the clinical phenotype of Hb CS results from the combined effects of REMD-mediated transcript depletion and structural destabilization of the elongated α-globin protein rather than loss of its conserved globin architecture.

Conclusion

The Hb CS mutation causes an extension of 31 hydrophobic amino acids and forms the IDP region, thereby reducing its stability in the cytoplasm compared to HBA2. This mutation also triggers the REMD mechanism that accelerates mRNA degradation due to excessive translation into the 3'-UTR region. Although the heme-binding function is predicted to remain normal, further experimental validation is needed to fully elucidate the biological implications of this mutation. In particular, molecular dynamics simulations can help visualize how this mutation alters the conformational stability of HBA2 and its interaction with heme. This approach can contribute to a deeper understanding of the molecular mechanisms underlying the Hb CS variant.

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Authors' Contributions

AS and NS were involved in conceiving and designing the research. AS performed data acquisition, data analysis, and designed the figures and tables. AS and NS interpreted the results and prepared the manuscript. NS contributed to the critical revision of the manuscript. All authors approved the final version of the manuscript.

Ethical Statement

No primary data were collected, and no human participants, human biological materials, or animals were involved in this study. Therefore, ethical approval from an institutional review board or ethics committee was not required.

Conflict of Interest

All authors declare that they have no conflict of interest.

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