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Original Article

Evaluation of Genes and Molecular Pathways Involved in Pathogenesis of Sickle Cell Anemia: A Bioinformatics Analysis and Future Perspective

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Abstract

Background: Sickle cell disease (SCD) is one of the hematological disorders characterized by a defect in the structure and function of globin chains. Hereditary factors play an important role in the pathogenesis of SCD. We aimed to investigate the genes and pathways related to the pathogenesis of SCD.

Methods: Microarray dataset was downloaded from the Gene Expression Omnibus (GEO) database. LIMMA package of R-software was used to detect UP and Down regulations between SCD and control subjects. Enrichment analysis and Protein-protein interaction (PPI) networks were performed using GeneCodis4 software and GeneMANIA database, respectively. PrognoScan database was used to evaluate the relationship between the hub genes and patients' survival.

Results: Overall, 447 DEGs were identified in SCD patients compared to control subjects. Out of 447 DEGs, 345 genes were up-regulated and 102 genes were down-regulated. Effective hub genes in SCD pathogenesis include *SLC4A1, DTL, EPB42, SNCA,* and *TOP2A*. In addition, hub genes had a high diagnostic value.

Conclusion: Evaluation of hub genes in SCD can be used as a diagnostic panel to detect high-risk patients. In addition, by identifying the UP and Down stream pathways, treatment strategies in the monitoring and treatment of patients can be designed.

Keywords: Sickle cell; Gene; Molecular pathway; Pathogenesis

Introduction

Sickle cell disease (SCD) is an autosomal recessive genetic disorder characterized by a mutation in the globin chain. In SCD mutations occur in the gene encoding the β globin chain (1). About 176,000 people die each year from SCD complications. The clinical symptoms of the disease in patients are heterogeneous and vary from mild to severe, depending on whether the mutation occurs in one or two β globin chain genes (2, 3). So far, many studies have been conducted in relation to the factors involved in the SCD pathogenesis; however, the main cause of the disease has not been identified. It is an inherited blood disorder caused by a genetic mutation. It affects hemoglobin, a protein that carries oxygen in red blood cells. The genetic factors responsible for the occurrence and progression of SCD have been extensively studied (4, 5). The genetic mutation that causes SCD is a single nucleotide substitution that changes the amino acid sequence of the beta-globin gene. Individuals who inherit two copies of this mutated gene (one from each parent) develop SCD, while those who inherit only one copy are carriers of the disease (6, 7).

One of the main genetic factors that affect the progression of SCD is the type of hemoglobin that an individual produces. The mutated hemoglobin (HbS) is less stable than normal hemoglobin, leading it to form long, rigid fibers, causing red blood cells to become sickle-shaped (8).

Variations in the *HoxB5* gene were associated with increased severity of SCD. Individuals with certain variations of the gene had more frequent pain crises and poorer lung function (5). A study identified a new genetic risk factor for SCD. Individuals with certain variations in the *MYH9* gene were more likely to develop SCD and have more severe disease. Other study found that genetic variations in the BCL11A gene were associated with a milder form of SCD. Individuals with certain variations of the gene had higher levels of fetal hemoglobin, which can help protect against the effects of SCD. Overall, these studies and others have helped to increase our understanding of the genetic factors that contribute to the occurrence and progression of SCD (5, 9). Based on studies, in most cases family history and genetic factors lead to disease (10). On the other hand, some genes and molecular pathways are responsible for the occurrence and progression of the disease. Identifying these genes can be helpful in designing diagnostic panels and using preventive and therapeutic strategies (11, 12). In this regard, systems biology approach is helpful (13, 14).

We aimed to provide valuable insights into the genetic basis of sickle cell disease and may pave the way for new treatments and therapies in the future.

Materials and Methods

Data achievement and analysis

In this study, GSE72999 Dataset was downloaded with GPL13497 (Agilent-026652 Whole Human Genome Microarray 4x44K v2) from Gene Expression Omnibus (GEO). The examined samples in this dataset included 7 samples with SCD steady and 6 samples with healthy controls. The transcriptome was compared between the HbSS SS vs HbAA study groups; the peripheral blood specimens were used.

In order to screen differentially expressed genes (DEGs) between SCD and the control group, the LIMMA package of R software was used. The criteria regarded for the selection of DEGs were considered based on |logFC| greater than 1 and adjusted *P*-value lower than 0.05.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

GO analysis for selected DEGs was executed using GeneCodis4 software. For this purpose, selected DEGs were investigated based on the molecular functions (MF), cellular component (CC), and biological process (BP). In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was analyzed to evaluate the

relationship of molecular pathways with DEGs using GeneCodis4 software. The *P*-value lower than 0.05 was regarded as statistical significance for these analyses.

Investigation of gene-disease association

In order to evaluate the relationship between DEGs and human disease, the DisGeNET database linked to GeneCodis software was used. The DisGeNET database is one of the most exhaustive databases of genes and variations linked to human illnesses. A *P*-value lower than 0.05 was regarded as statistical significance.

Designing the PPI network and identification of hub genes

Protein-protein interaction (PPI) is used to evaluate the relationship between DEGs at the protein level. To achieve PPI, the STRING database was utilized. Interaction between proteins was visualized using Cytoscape software. In the next step, the Cytohubba plugin of Cytoscape was employed to identify hub genes based on overlapping among the three methods (degree, closeness, and betweenness).

ROC curve analysis

Receiver operating characteristic (ROC) and area under the curve (AUC) analysis were calculated via GraphPad prism 8.0 to evaluate the diagnostic significance of hub genes. Criteria for hub genes included AUC greater than 0.90 and *P*-value lower than 0.05.

Evaluation of associations between hub genes with TFs and miRNAs

The interactions between the hub genes and related microRNAs (miRNAs) were assessed using the miRNet software. MiRNAs that had the most interaction with hub genes were considered as the key miRNAs. Moreover, the interactions between hub genes and associated transcription factors (TFs) were evaluated using the ChEA database linked to Network Analyst **s**oftware. TFs that had the most interaction with hub genes were considered as the key TFs.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. All the procedures performed in the studies involving human participants were in accordance with ethical standards of Local Ethics Committee of Iran University of Medical Sciences (IR.IUMS.REC.1401.607), as well as 1964 Helsinki Declaration.

Results

Identification of DEGs

Overall, 447 DEGs were identified in SCD patients compared to the control subjects. Out of 447 DEGs, 345 genes were up-regulated and 102 genes were down-regulated. The list of DEGs and additional information is shown in supplementary file 1.

GO and KEGG enrichment analyses

GO enrichment analysis was used to evaluate the function of up- and down-regulated DEGs. In terms of BP, the DEGs were mainly involved in ankyrin binding, nucleoside monophosphate kinase activity, TPR domain binding, phosphatase binding, and calcium-dependent protein serine/threonine phosphatase activity (Fig. 1). In terms of CC, they were mainly involved in endocytic vesicle lumen, spectrin-associated cytoskeleton, intrinsic component of the cytoplasmic side of plasma membrane, endocytic vesicle membrane and platelet alpha granule membrane (Fig. 1). In terms of MF, they were mainly involved in the positive regulation of I-kappa B kinase/NF-kappa B signaling, nucleoside triphosphate metabolic process, nucleoside phosphate biosynthetic process, positive regulation of nucleic acid-template transcription and negative regulation of transcription by RNA polymerase II (Fig. 1). Furthermore, in terms of the KEGG pathway, they were mainly involved in mitophagy, NOD-like receptor signaling pathway, ferroptosis, PPAR signaling pathway and lipid and atherosclerosis (Fig. 2).

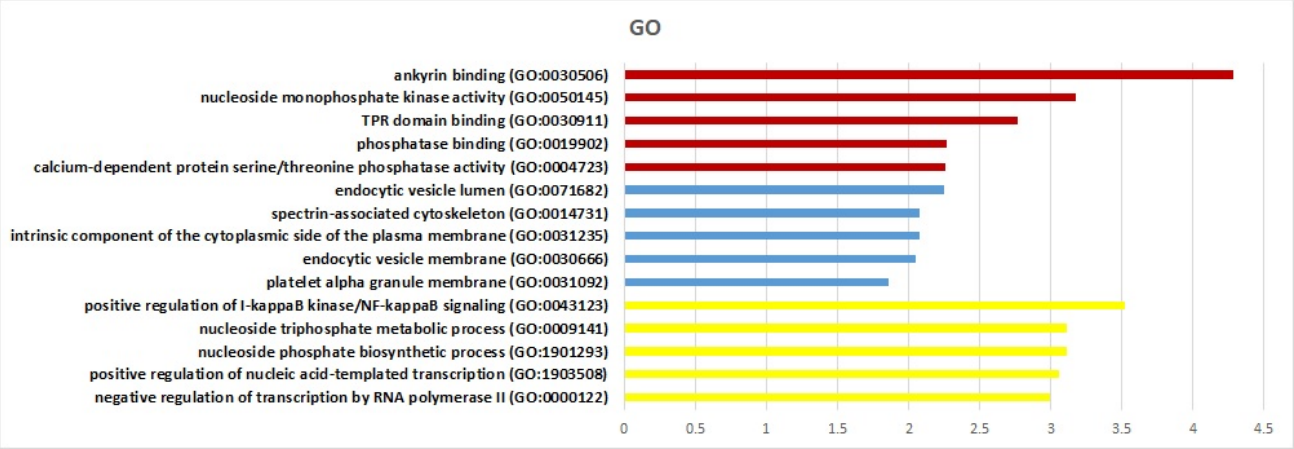

Fig. 1: The results of GO analysis. The red lines represent BP terms, the blue lines represent CC terms and the yellow lines represent MF terms. The x-axis indicates the logarithm (base 10) of the *P*-value and the y-axis indicates the GO terms (BP, CC, and MF)

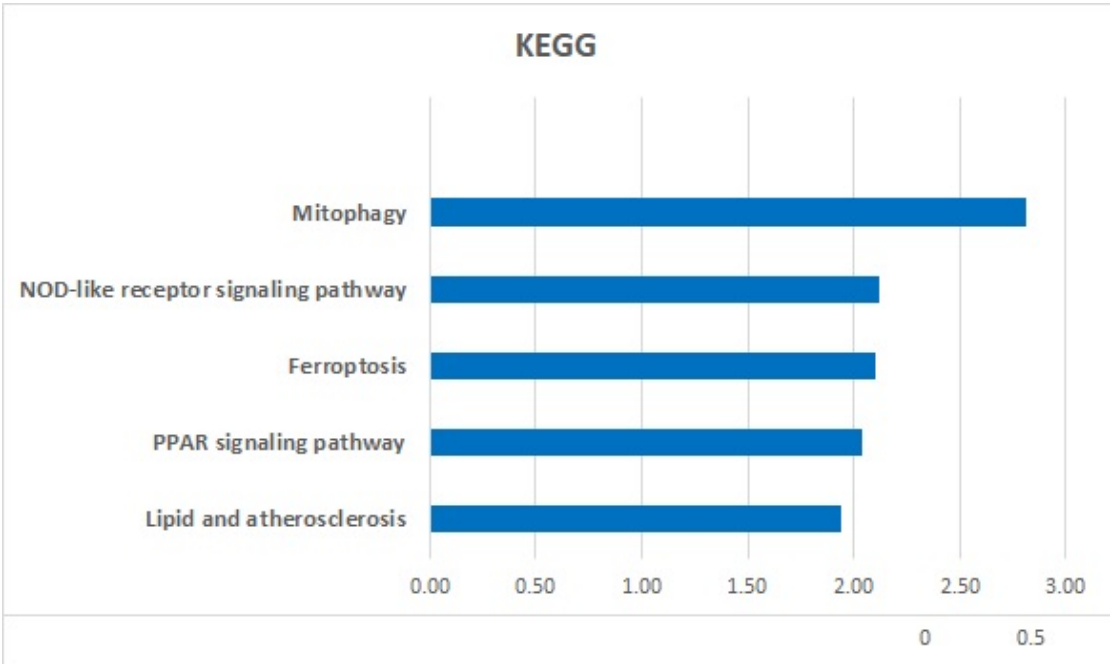

Fig. 2: The results of KEGG analysis. The x-axis indicates the logarithm (base 10) of the *P*-value and the y-axis indicates the KEGG pathway terms

Investigation of gene-disease association

According to the DisGeNET database, DEGs are critical factors in the structure of red blood cells, including reticulocyte count, red blood cell distribution (RDW), and spherocytosis (Fig. 3).

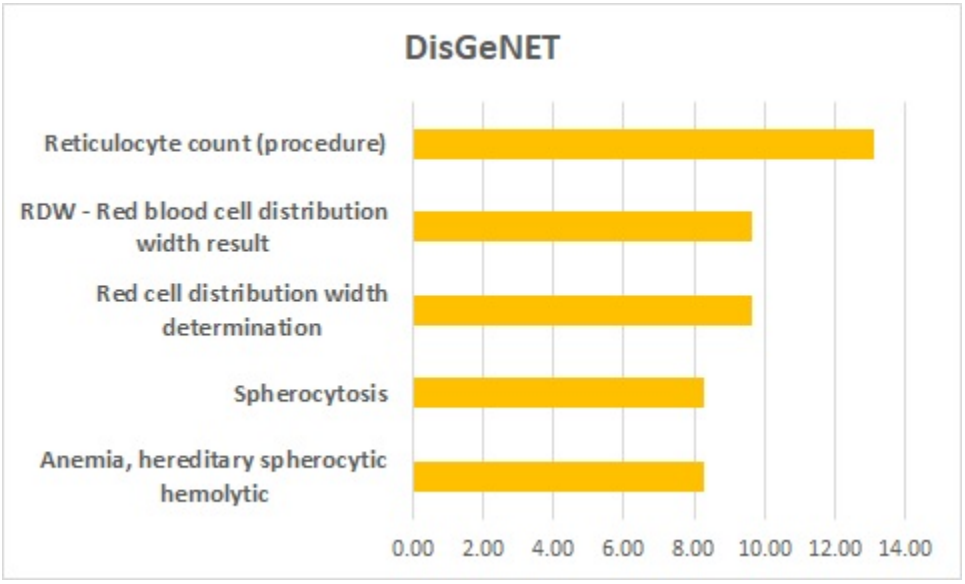

Fig. 3: The results of gene-disease association analysis; they represent the association of DEGs with human diseases. The x-axis indicates the logarithm (base 10) of the *P*-value and the y-axis indicates the disease terms.

Design the PPI network and identification of hub genes

Using STRING and as well as Cytoscape the PPI of DEGs was evaluated. PPI consisted of 297 nodes and 613 edges (Fig. 4). Using the Cytohubba plugin of Cytoscape and overlap overlapping between degree, closeness, and betweenness methods, 5 following genes were considered as the hub genes including, *SLC4A1*, *DTL*, *EPB42*, *SNCA*, and *TOP2A* (Fig. 5).

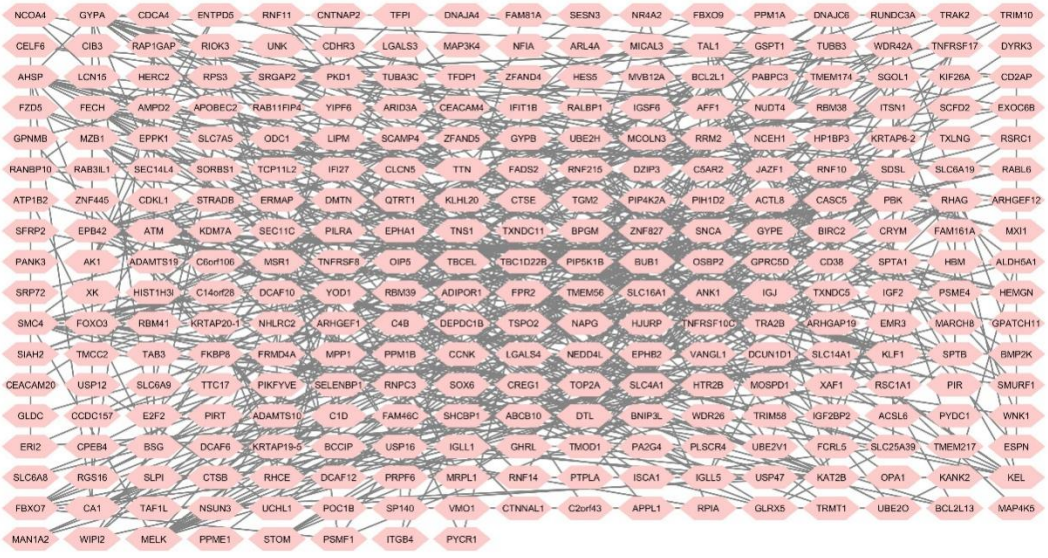

Fig. 4: The results of the PPI analysis. The pink octagons represent proteins and the black lines represent interactions between them

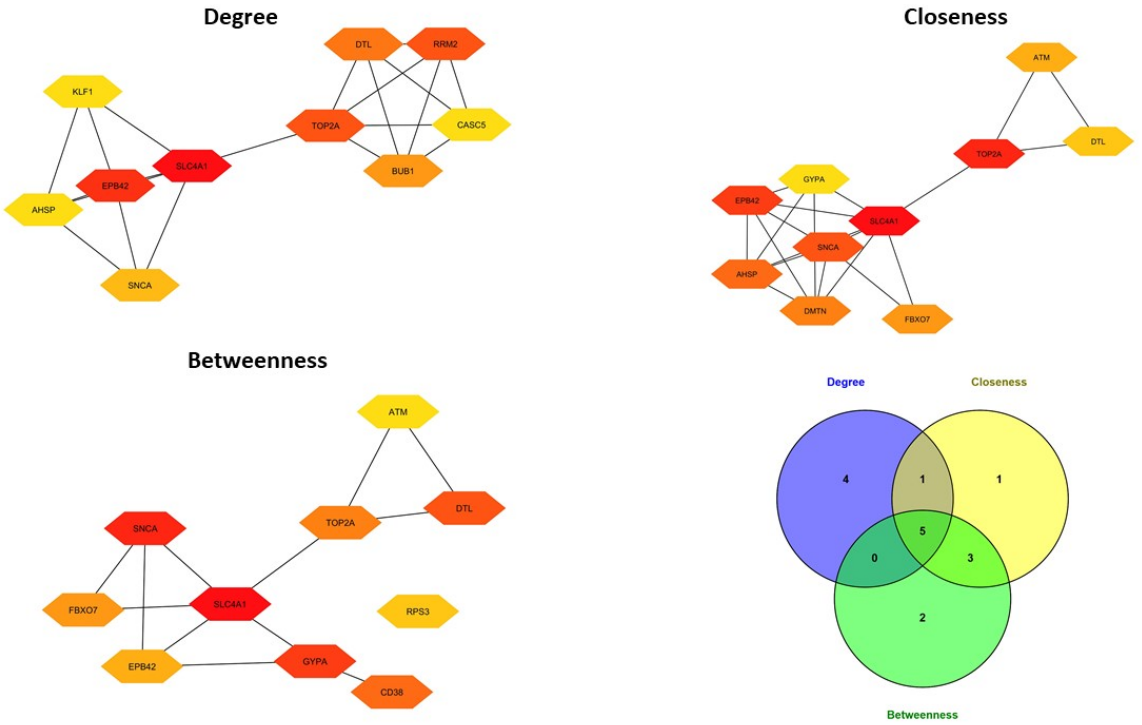

Fig. 5: The results of hub genes identification. Hub genes, including *SLC4A1*, *DTL*, *EPB42*, *SNCA*, and *TOP2A* were determined through the intersection between degree, closeness, and betweenness methods. The color red is associated with the hub genes that have the most interactions with other proteins

ROC curve analysis

ROC curve analysis was employed to assess the diagnostic value of hub genes. All hub genes have AUC greater than 0.90, which indicates good diagnostic value. The values of AUC and further information are shown in supplementary file 2.

Evaluation of associations between the hub genes with TFs and miRNAs

Using miRNet software, miRNAs targeting hub genes were identified (Fig. 6). After the investigations, the results showed that among miRNAs, hsa-mir-34a-5p had the most interactions with hub genes. Moreover, using the ChEA database of NetworkAnalyst, TFs targeting hub genes were identified (Fig. 7). After the investigations, the results showed that some TFs such as MYC and GATA1 had the most interaction with hub genes.

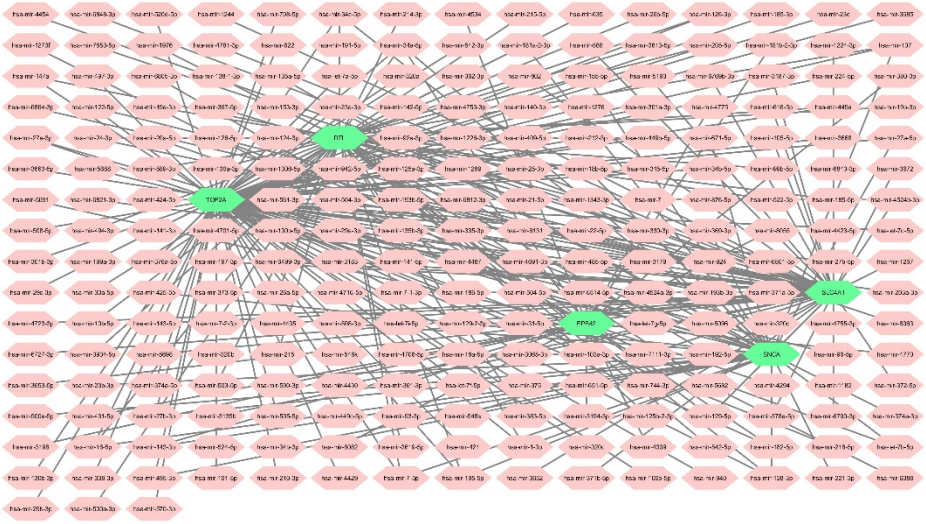

Fig. 6: The hub genes-miRNAs association analysis. The green octagons represent hub genes and the pink octagons represent their related miRNAs. The color green is associated with the hub genes that have the most interactions with other proteins

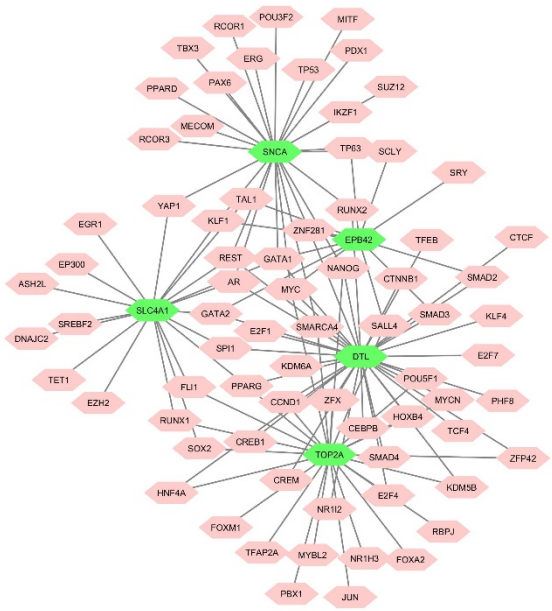

Fig. 7: The hub genes-TFs association analysis. The green octagons represent hub genes and the pink octagons represent their related TFs. The color green is associated with the hub genes that have the most interactions with other proteins.

Discussion

Today, many attempts are made to identify the molecular targets that can modulate γ-globin expression including microRNAs that work via post-transcriptional gene regulation. Increasing the expression of miR-34a leads to increase the fetal hemoglobin levels consistent with silencing a repressor of the γ-globin gene. Furthermore, miR-34a promotes cell differentiation by increasing the expression of KLF1, glycophorin A, and erythropoietin receptors (15).

MiR-34a is a tumor suppressor gene that is regulated through p53; it increases the production of

HbF in cells. The high level of miR-34a expression causes induction of HbF production and reduces STAT3 phosphorylation in erythroid cells; this process results in the improvement of clinical signs and symptoms of SCA patients (15).
Recent studies show the role of the transcription factor GATA-1, its cofactor FOG-1, and NuRD-associated chromatin remodeling complex in silencing HBG1 and HBG2 gene expression. Moreover, the hematopoietic transcription factor GATA-1 has been recommended to help globin genes silencing (16, 17). Unexpectedly, NuRD is vital for FOG-1-based activation of adult globin gene expression in vivo (16).
EPO receptor (EpoR) and GATA-1 signaling pathway regulate erythroid cells differentiation, proliferation, survival and maturation. Stimulation of EpoR through the PI3-kinase/AKT signaling pathway leads to GATA-1 phosphorylation in fetal erythroid liver progenitors, which is important for the induction of fetal erythroid cell maturation (18, 19).
The expression of nuclear factor-erythroid 2 (NFE2) is activated through CREB1 and inhibited via MYC. NFE2 is affected by 5 rSNPs (regulatory single nucleotide polymorphism), expected to disrupt numerous MYC motifs but do not affect CREB1 binding affinity (20). These rSNPs with altered MYC binding cause NFE2 overexpression, found in SCD patients; furthermore, elevated expression of NFE2 leads to the production of the pro-inflammatory cytokine IL-8 in myeloid cells and induces excessive neutrophil counts, and neutrophilia is related to SCA severity (21).
Dysregulation of c-Myc and overexpression of hypoxia-inducible factor (HIF) generally developed in a few malignancies. HIF inhibits c-Myc activity, but in hypoxic situations the oncogenic regulation of c-Myc via the HIF-1 signaling pathway performs a critical role in adapting to the tumorigenic microenvironment (22). HIF influences the secretion of VEGF and angiogenic activity within the bone marrow microenvironment. Similarly, in hypoxemic situations like SCA the proliferation of endothelium cells leads to more severity of disease (22).
Renal problem is one of the most dangerous complications in sickle cell anemia patients (23, 24). *TOP2A* (DNA Topoisomerase II Alpha) is a nuclear enzyme that controls DNA topological shape and cell cycle (25). The increase in *TOP2A* expression leads to increased proliferation and reduction of its expression via the activation of the ERK/JNK/p-P38/CHOP signaling pathway causing improvement of the disease (26, 27).
The protein encoded through *SLC4A1* (solute carrier family 4 members 1) is a part of the anion exchanger (AE) own family and is expressed within the plasma membrane of erythrocytes, it acts as a chloride/bicarbonate exchanger involved in the delivery of carbon dioxide from the lungs. This protein contains areas that might be structurally and functionally distinct. The N-terminal area of 40 kDa is positioned within the cytoplasm and binds to the red blood cell skeleton (28). Many mutations in this gene were recognized, which result in the instability of the red blood cell membrane and defective kidney acid secretion, and distal renal tubular acidosis (29).
-lnc-*SLC4A1*-1 alters trophoblast function by activating the immune responses and via regulating the NF-k B/CXCL8 axis. lnc-*SLC4A1*-1 recruits NF-kB and binds to the CXCL8 promoter, resulting in expanded CXCL8 activity. Increased CXCL8 activity exacerbates the inflammatory response and impacts trophoblast characteristics by inducing TNF-α and IL-1, which may also have an effect on the immune responses and ultimately results in pregnancy loss because of placental vascular modifications (30). This inflammatory response and vascular modifications may additionally play a role in the pathogenesis of SCA (30).
Denticleless E3 Ubiquitin Protein Ligase Homolog (*DTL*) expression causes infiltration of type 2 T helper cells and memory B cells and inversely is associated with the infiltration of memory CD4+ T cells and macrophages. Furthermore, *DTL* gene transcription levels have been associated with markers of CD8+ T cells, T cells (general), monocytes, DC cells, Treg cells, and T cell exhaustion (31). CD8+ T cells release Perforin and Granzyme B via the Fas/FasL pathway and re-

lease IFN-g and TNFa, which play a protective role in the immune system. Besides, patients with excessive *DTL* expression may benefit more in prolongation of overall survival from treatment with checkpoint inhibitors (31, 32). Likely, the accumulation of inflammatory CD8+ T cells aggravate the signs and symptoms of the disease (32).

DTL is also called retinoic acid-regulated nuclear matrix-related protein (RAMP) or DNA replication factor 2 (CDT2). It is related to the cell proliferation, cell cycle arrest, and cell invasion. *DTL* functions as a key regulator of the cell cycle and genomic stability. It is also a substrate receptor for the CRL4 ubiquitin ligase (33). Together with the substrate receptor *DTL*, it causes the CRL4 ubiquitin ligase-based degradation of numerous proteins and is crucial for cell cycle progression as well as for DNA replication and repair. *DTL* expression level is known as a prognostic biomarker in diseases (33). Elevated expression of *DTL* is associated with the activation and infiltration of M1 macrophages, neutrophils, and CD4+ memory T cells. It has an inverse relationship with the activation of memory B cells.

Neutrophil infiltration in SCA disease might be related to more severity of the disease and signs and symptoms associated with vascular occlusion (33). *DTL* is a new goal of T-cell-mediated autoimmune or inflammatory sicknesses, as its complex is an essential mediator controlling M-phase exit in the activated T cells (34). The higher the expression of *DTL*, the more the decrease in the protein level of genes including PDCD4, NKX2-1, and PRKAA1. Notably, PDCD4 acts through inhibiting PI3K/Akt, MAPK, and TGF-beta signaling pathways (35).

Erythrocyte Membrane Protein Band four.2 (*EPB42*) an ATP-binding protein performs a vital role in conserving the structural integrity of the cytoskeleton protein ankyrin within the red blood cell membrane. Insufficient level of *EPB42* results in anemia and hemolysis. The integrity of the red blood cell membrane can deliver sufficient oxygen to the skeletal muscle, which facilitates exercise ability increment in vascular diseases (35). This gene is specifically involved in cytokine and cytokine receptors, signaling pathways, IL-17, and TLR. TLR7 is extensively expressed in respiratory system including epithelial cells and smooth muscle cells. TLR7 together with the TLR signaling pathway performs a crucial role in the innate and adaptive immune response and may motivate the production of inflammatory cytokines (36). This problem might be associated with the velocity of disease progression and the exacerbation of signs and symptoms in vascular occlusion crisis in patients with sickle cell anemia (36).

EPB42 and SCL4A1 may also play a functional role in the incidence and development of ISR (instent restenosis), however, there is still inadequate research on this subject (21, 37). According to current studies, these genes are impressive in the composition of maturation, stability, cell transport, and diffusion of hemoglobin in red blood cells (37). It has been proven down expression of *EPB42* and *SLC4A1* genes leads to hemolysis, an increase in blood viscosity followed by an increase in blood flow resistance and a decrease in blood flow velocity, which results in ischemia, hypoxia, and the accumulation of numerous metabolites within the tissue. It could be one of the mechanisms leading to ISR, which leads to SCA severity and vascular occlusions crisis via vascular blockage caused by endothelial destruction (20).

α-synuclein (*SNCA*) is expressed and secreted in endothelial cells (ECs). *SNCA* levels are correlated with blood pressure and age, suggesting the involvement of *SNCA* in age-related endothelial disorder (37). Exogenous treatment with recombinant *SNCA* (r*SNCA*) activates the Akt-eNOS axis and elevates the nitric oxide (NO) production in ECs. Treatment with r*SNCA* additionally suppressed TNF-α- and palmitic acid-triggered NF-kB activation, leading to suppression of vascular cell adhesion molecule1 (VCAM-1) and restoration of reduced eNOS expression in ECs. *SNCA* silencing results in reduced eNOS, increased β-gal activity, decreased Sirt1 expression, and elevated expression of the p53 signaling pathway in ECs, impairs normal endothelial integrity and function (37). Exogenous and endog-

enous *SNCA* in ECs can also physiologically preserve vascular integrity, and age-related endothelial disorder can be in part resulting from the lack of *SNCA* function in ECs (28). Likely, endothelial disorder performs a role in the pathogenesis of SCA with the same mechanism (20).

Conclusion

In general, evaluation of hub genes in SCD can be used as a diagnostic panel to detect high-risk patients.Following genes were considered as the hub genes including, *SLC4A1*, *DTL*, *EPB42*, *SNCA*, and TOP2A. In addition, by identifying the UP and Down stream pathways, treatment strategies can be designed in the monitoring and treatment of patients. In future studies, we suggest more emphasis on understanding the precise molecular pathways and mechanisms that contribute to SCD pathogenesis. This can be achieved using advanced genomics, proteomics, and bioinformatics techniques. Furthermore, it is important to identify environmental and lifestyle factors that contribute to SCD incidence and progression. Future studies focused on understanding these mechanisms and identifying new therapeutic targets will be crucial in improving the clinical management of this disease and ultimately finding a cure.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Data availability

Supplementary files may be asked from the corresponding author for reasonable application.

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None.

Conflict of interest

The authors declare that there is no conflict of interests.

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