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The Potential Role of Increased MiR-144 Expression in Egyptian Sickle Cell Disease Patients

Mona F. Sokkar[1*], Mona Hamdy[2], Niveen Salama[2], Khalda S. Amr[3]

[1]*Molecular Genetics and Enzymology Department, Human Genetics and Genome Research Institute, National Research Centre (NRC), Cairo, Egypt*

[2]*Department of Pediatrics, Faculty of Medicine, Cairo University, Cairo, Egypt*

[3]*Medical Molecular Genetics Department, Human Genetics and Genome Research Institute, National Research Centre (NRC), Cairo, Egypt*

Abstract

Sickle cell disease (SCD) is a recessively inherited blood disease. Although being monogenic, the phenotypes are markedly diverse. Different studies have confirmed that the deregulation of microRNAs (miRNAs) boosts the clinical severity or has a modulatory effect in SCD. MiR-144 has been claimed to be a contributing factor for the impaired oxidative stress tolerance and aggravated anemia in SCD. Thus, the present investigation intends to assess the relative expression of miR-144 in 40 SCD patients to validate its role in relation to clinical phenotype and HbF production. Forty SCD cases and 40 healthy individuals were studied in the current research. Patients were genotyped by direct sequencing of the *HBB* gene. The expression of miR-144 was assessed by real-time PCR (qRT-PCR). Thirty cases (75%) were SS and 10 cases (25%) were S/β. MiR-144 was upregulated in SCD cases in relation to controls (P = 0.015). MiR-144 was positively correlated to the frequency of painful episodes (p = 0.0001). It was also associated with vertigo in the examined cases. Concerning HbF, an inverse correlation was observed in regards to the miR-144 expression but without achieving the significance level. In conclusion**,** miR-144 could be a prospective genetic marker of disease outcome and targeted therapy in sickle cell disease. MiRNA mimics and antagomirs may be future therapeutic agents stimulating HbF production and thus potentially alleviating the clinical sequalae of SCD.

***Keywords*:** Sickle cell disease; microRNAs; miR-144; HbF; hydroxyurea

1. Introduction

Sickle cell disease (SCD) is a recessively inherited hemoglobin disorder brought on by substitution in the β-globin chain (*HBB*), replacing the glutamic acid by valine at the 6th position **[1].** In Egypt, carrier rates range from 9% to 22.17% with a heterogeneous distribution **[2].** SCD is inherited either in homozygosity for the S allele (SS) or with another hemoglobin variant (compound heterozygosity), such as HbC or β-thalassemia **[3].** The sickling events induce intravascular hemolysis and recurring vaso-occlusion, vascular inflammation, and endothelial activation [4]**.** The frequently encountered causes of mortality in SCD cases are vaso-occlusive crises (VOCs), acute chest syndrome (ACS), and occlusive stroke **[5, 6].** Despite being a monogenic illness, the manifestations and clinical sequalae of SCD vary among the diseased individuals, and understanding the genetic diversity of SCD is essential for developing novel treatment strategies **[7].** Recent reports shed light on the therapeutic potential of microRNAs (miRNAs) to alleviate the severity of hemoglobinopathies, including SCD, by targeting globin genes or their regulatory pathways enhancing HbF production **[8].** MiRNAs are short non-coding nucleotide sequences modulating gene expression via messenger RNA degradation or translation repression **[9].** Several miRNAs contribute to the regulation of different stages of erythropoiesis, including proliferation, differentiation, and maturation **[10].** Erythroid microRNAs could serve as genetic

*Corresponding author e-mail: monasokkar@yahoo.com.; (Mona F. Sokkar).

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modifiers of HbS-related anemia that might provide novel insights into the clinical diversity in SCD and offer attractive molecular tools for new therapeutic approaches **[11, 12]**. MiR-144 is one of the erythroid-specific microRNAs, and it has been known to modulate oxidative stress in sickle cell cases **[13]**. It targets nuclear factor erythroid 2-related factor 2 *(NRF2*), which is a transcription factor that activates *γ-globin* transcription and promotes the expression of antioxidant proteins. It adheres to the antioxidant response element (ARE). The attachment of *NRF2* to ARE is essential for the expression of antioxidant enzymes **[14].** MiR-144 expression in HbSS reduces *NRF2* and attenuates the antioxidant capacity in these cells, leading to hemolysis and a more severe phenotype [**12**]. Additionally, overexpression of miR-144 diminishes antioxidant protein levels, including glutamate-cysteine ligase, catalytic /modifier subunit (GCLC/M), and superoxide dismutase 1 (SOD1), as confirmed by **Sangokoya et al. [15]**. Thus, the manipulation of miR-144 levels could offer a promising tool to alleviate the disease severity in SCD **[16].**

Our research intended to investigate the differential expression of miR-144 in Egyptian sickle cell cases to make a correlation with clinical outcome and HbF levels.

2. Subjects and Methods:

Forty SCD cases were enrolled in the study after the ethical approval of the Medical Ethics Research Committee (MERC) of the National Research Centre (NRC) according to the Helsinki Declaration 1975, Approval No. 16 096. Written informed consent was signed by all participants or their guardians. Cases were recruited from the Hematology clinic, Abo El-Rish Pediatric Hospital, Cairo University. Forty healthy volunteers matched for age and sex with a normal complete blood picture (CBC), Hb electrophoresis, and no history of any hemoglobinopathy were also included.

2.1. Clinical Evaluation

SCD participants were exposed to history-taking, pedigree analysis and clinical examination. Computed tomography (CT) scan was done for testing of vascular complications. Complete blood picture (CBC) and Hb electrophoresis were done to all cases, and the diagnosis was proven by sequencing of the *HBB* gene. Serum ferritin, lactate dehydrogenase, and liver enzymes (AST and ALT) were also measured. Blood specimens were withdrawn in EDTA vacutainers for molecular studies.

2.2. Molecular Analysis:

2.2.1. Genotyping of sickle cell disease cases

DNA was obtained from 200 µL of blood using ZYMO DNA extraction Miniprep (ZYMO, USA) according to the manufacture protocol and subjected to PCR using primers designed by the primer3 tool **[17]** to cover exons and intron-exon junctions of the *HBB* gene. Purification of amplified PCR was done using an Exonuclease 1-Sahrimp Alkaline Phosphatase (EXO-SAP) purification kit (Neb, USA). Following purification, samples were then run through an ABI Prism 3500 Genetic analyzer (Applied BioSystems, USA) for direct sequencing using the Big Dye Terminator v3.1 Cycle Sequencing kit. The sequences were then subjected to a blasting procedure at the URL https://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.2.2. MiRNA isolation

Blood samples taken on EDTA were centrifuged at 7000 rpm for 15 min, and the supernatant plasma was used for miRNA isolation. MiRNA extraction was done utilizing Qiagen miRNA extraction kit following the manufacturer's guidelines. MicroRNA purity and concentration were measured using a Thermo Scientific NanoDrop spectrophotometer (USA).

2.2.3. cDNA synthesis

The process of reversing miRNA into single stranded complementary DNA (cDNA) was achieved in 20 µl reaction volume using the miScript II RT Kit (Qiagen). All reactions were done as specified in the protocols of the manufacturer: 2 µL 10x nuclear mix buffer, 4 µL 5x miScript buffer, 2 µL reverse transcriptase (RT) mix, and 100 ng of the miRNA. The reactions were incubated at 37 °C for 1 hour, 95 °C for 5 min, and then kept at −20 °C.

2.2.4. Quantitative real time PCR (qRT-PCR)

cDNAs from all cases and healthy subjects were used as templates. Expression analysis of miR-144 and the reference miRNA (U6) was evaluated by a Roche LightCycler 480 Real-Time PCR instrument (Roche Diagnostics, Germany) using the miScript SYBR Green PCR kit. Reactions were carried out in a total volume of 25 µL containing 12.5 µL of SYBR green Master Mix, 2.5 µL of each specific PCR primer and universal primer (contained with the kit), and 5 µL of cDNA template. The reference miRNA (U6) was used for the normalization of target miRNA expression **[18].**

The cycle conditions were: denaturation at 95 °C for 15 min, followed by 40 amplification cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s, melting curve at 95 °C for 30 s, 40 °C for 30 s, 85 °C continuous, and cooling at 40 °C for 30 s. Ct, ΔCt,

ΔΔCt were determined, and the fold change represented as relative quantification (RQ) was determined using the $2^{-\Delta\Delta Ct}$ method.

2. 3. Statistical analysis

All statistical analysis was conducted using the SPSS software 20. Quantitative data was represented in terms of mean and standard deviation (SD). Qualitative data was represented as numbers and percents. The normality assumption was tested with the Shapiro–Wilk test. T-test was used for comparing two parametric groups and Mann–Whitney test for comparing two nonparametric groups. The receiver operating characteristic (ROC curve) was used to evaluate the diagnostic accuracy of miR-144 expression, and the area under curve (AUC) was estimated with a 95% confidence interval (CI). Relationships between various variables were done using the spearman correlation coefficient (R) with graphic representations using linear regression. A probability value (p value) less than 0.05 was considered significant.

3. Results

3.1. Clinical Results

The study was conducted on 40 SCD participants (21 males and 19 females) with an average age of 13.25 ± 7.84 years old. Forty healthy individuals matched for age and sex were also included, with an average age of 15.46 ± 8.65.

37.5% of cases were descended from consanguineous marriage, and 65% of cases have similarly affected family members. The age of first presentation was 3.36 ± 2.39 years, and the average rate of transfusion was 5.62 ± 4.04 times per year.

62.5% of cases had splenectomy. The commonly encountered complication in our cases was painful VOCs in 36 cases (90%) followed by ACS in 17.5%, vertigo in 10%, transient ischemic attacks (TIA) in 7.5%, and acute avascular necrosis of the femoral head (AVN) in 5%. The average number of painful vaso-occlusive crises was 7.76 ± 4.72 episodes per year. All data and investigations of cases are represented in **Table 1.**

Table 1: Descriptive and laboratory findings of SCD cases

Data	Mean ± SD
Age (years)	13.25 ± 7.84
Age of onset (years)	3.36 ± 2.39
Rate of transfusion (times / year)	5.62 ± 4.04
Hb g/dl	8.76 ± 1.74
Hct %	25.67 ± 4.75
MCV fl	79.13 ± 10.94
MCH pg	27.21 ± 4.09
MCHC g/dl	33.08 ± 6.025
HbF%	13.003 ± 11.30
HbS%	77.27 ± 15.21
HbA%	8.004 ± 6.86
HbA2%	2.40 ± 0.61
Ferritin ng/ml	1158.52 ± 957.93
Lactate Dehydrogenase U/L	654.28 ± 453.83
ALT U/L	60.522 ± 34.35
AST U/L	72.82 ± 60.05
Vaso-occlusive painful crises (episodes / year)	7.76 ± 4.72
Data	**No of cases (%)**
Vaso-occlusive painful crises	36 (90%)
Acute chest syndrome (ACS)	7 (17.5%)
Vertigo	4 (10%)
Transient ischemic attacks (TIAs)	3 (7.5%)
Acute avascular necrosis of femoral head (AVN)	2 (5%)
Stroke	1 (2.5%)

SD: standard deviation, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration.

3.2. Molecular results:

Sequencing of the *β-globin* gene revealed that 30 cases (75%) were homozygote for the S allele (SS), and 10 participants (25%) were S/β. The genotyping results of SCD cases were illustrated in **Figure 1.**

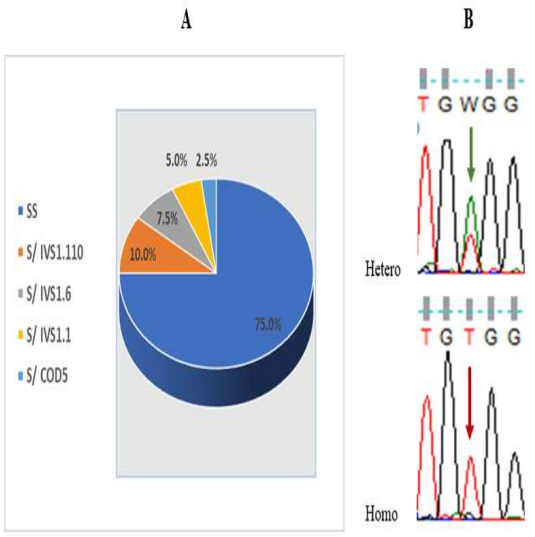

Figure 1: A: Pie chart showing the genotyping of SCD cases. B: Partial nucleotide sequence of the *HBB* gene showing heterozygous S allele (green arrow) and homozygous S allele (red arrow). Hetero for heterozygous and Homo for homozygous

Results of miR-144 expression:

The fold change (RQ) of miR-144 was significantly upregulated in patients (3.589 ± 2.056) in comparison to healthy volunteers (1.003 ± 0.426) (P = 0.015) **Figure 2.** No significant variation was observed between miR-144-fold change in SS and Sβ groups (P = 0.308).

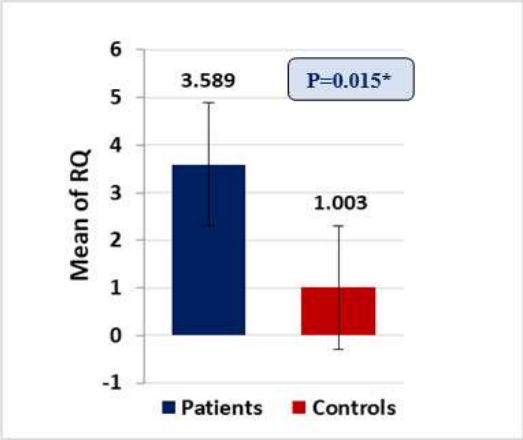

Figure 2: A: Bar chart illustrating the comparison of RQ of miR-144 expression presented as mean between cases and control groups.

Using the ROC curve, the diagnostic accuracy of miR-144 expression was found to be excellent, with the AUC being 0.887 (95% CI: 0.814–0.960). The P value was statistically significant (P < 0.001). The best cutoff value of miR-144 expression was 1.025 with sensitivity = 100% and specificity = 75% **Figure 3.**

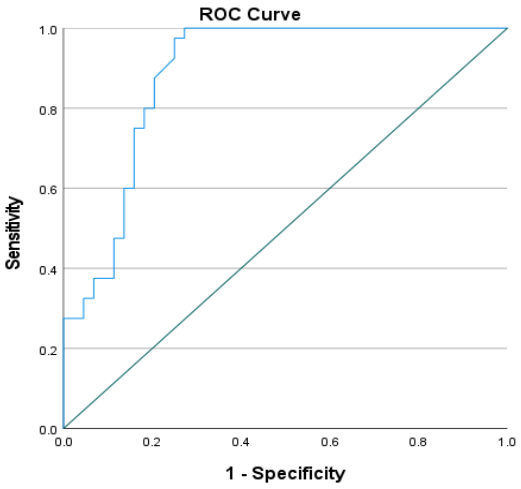

Figure 3: ROC Curve of RQ for cases according to controls.

Comparison of RQ of miR-144 with clinical manifestations revealed a substantial positive correlation between fold change of miR-144 and the frequency of painful crises per year (r = 0.822, P = 0.0001) **Figure 4.**

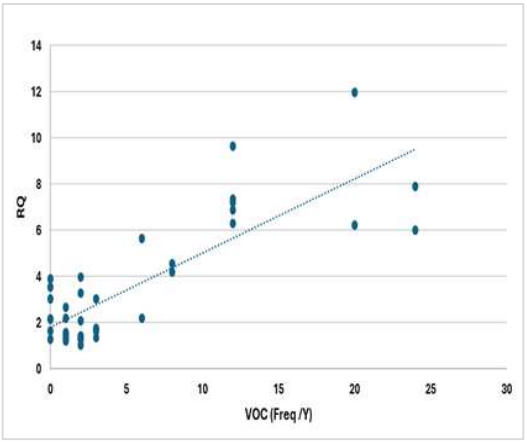

Figure 4: Scattered chart representing significant positive correlation between RQ and VOC episodes.

Additionally, a statistically considerable association was identified in regards to the occurrence of vertigo, as the patients suffering from vertigo had a higher RQ of 3.70 ± 2.43 vs. 2.25 ± 1.64 in the other cases (P = 0.048) **Figure 5.**

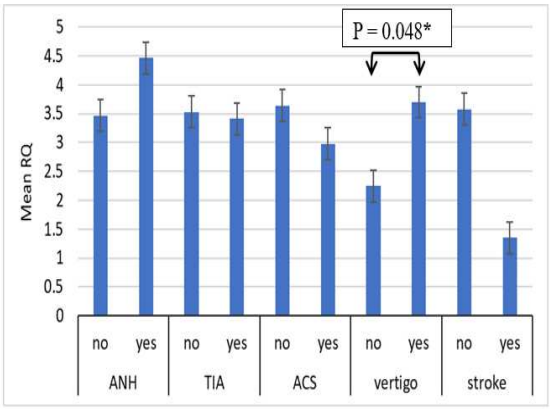

Figure 5: Bar chart representing association between RQ and clinical complications.

Regarding the hematological and biochemical parameters, RQ of miR-144 was inversely correlated to HbF level but without reaching the significance value (r = - 0.371, P = 0.067) **Figure 6.**
No statistical variation was detected in regards to other parameters or Hydroxyurea (HU) treatment (all p > 0.05). The correlation between RQ and different parameters is listed in **Table 2.**

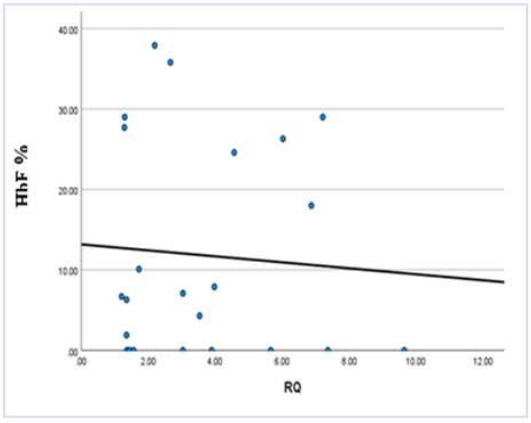

Figure 6: Scattered chart representing negative correlation between RQ and HbF%.

4. Discussion

Sickle cell disease (SCD) is a common genetic disease worldwide. Transfusion, hydroxyurea, and pain management are the treatment options for SCD. Allogeneic bone marrow transplantation (BMT) is the other alternative treatment**.** Nevertheless, not all patients can find compatible donors **[19]**. Recently published studies have focused on post-translational mechanisms mediated by miRNA molecules to block hemoglobin switching **[20].** Accumulating data suggests that miRNAs play a vital function in globin gene regulation, and different researches have confirmed that dysregulation of miRNAs is correlated with the alteration of HbF production and enhances the clinical severity of SCD **[12].** MiR-144 is one of the erythroid-specific miRNAs. It has been reported to be linked to severe anemia and diminished redox potential in individuals having SCD **[21]**.

In our research, we investigated the relative expression of miR-144 in our sickle cell disease cases to explore its impact on HbF level and patient' clinical outcome.

Our results demonstrated upregulated miR-144 in patients (SS and Sβ) compared to controls (P = 0.015)**.** Similarly, **Kargutkar et al.** examined thirty homozygote SS patients and indicated high miR-144 expression **[22]**. He also found that miR-144 showed the highest expression following hydroxyurea therapy. **Sangokoya et al.** also noticed that miR-144 was overexpressed in all HbSS erythrocytes in comparison to normal erythrocytes **[15]**. Additionally**, Guzelgul et al.** investigated 84 SCD cases and reported higher expression of miR-144-3p in cases than healthy participants **[23]**. Increased generation of miR-144 in SCD cases was explained by the fact that hemolysis and anemia trigger erythropoietin response and stress erythropoiesis that leads to increased expression of the erythropoietin-dependent *GATA-1,*which subsequently stimulates the downstream effector, miR-144/451 locus **[24, 25]**.

Table 2: Correlation between RQ of miR-144 and different parameters

Parameter	RQ	
	R	P value
Age of onset / y	-0.129	0.428
Age of Ist transfusion / y	0.021	0.908
Rate of transfusion / month	-0.300	0.114
VOC (frequency / year)	0.822	0.0001*
Hb g/dl	0.055	0.744
Hct %	0.228	0.175
HBF%	-0.371	0.067
HBS%	-0.182	0.418
HBA2%	-0.183	0.416
HBA%	-0.108	0.633
Ferritin ng/ml	0.086	0.606
LDH U/L	-0.157	0.231
AST U/L	-0.216	0.213
Hydroxyurea intake in years	0.019	0.918

VOC: Vaso-occlusive Crises, *Significant correlation at P < 0.05.

The commonly occurring complication in our cases was the painful VOC, accounting for 90% of cases, followed by ACS in 17.5% and vertigo in 10%. TIA and stroke were identified in 7.5% and 5% of cases, respectively. In line with our results, VOC was the highly reported complication in many previous analyses **[26-28].** These vaso-occlusive pain episodes were the primary presenting illness associated with SCD and the major cause of hospitalization in these cases**.** It is induced by hemoglobin polymerization, inflammation, adhesion, and accumulation of sickled cells, endothelial cells, and other blood cells as described by **Darbari and colleagues [29].**

Our research further compared the miR-144 expression with different clinical and hematological parameters and revealed a statistically positive correlation between RQ and the frequency of painful occlusive crises (P = 0.0001). Moreover, relative quantification of miR-144 was significantly correlated with vertigo occurrence in sickle cell cases (p = 0.048). These findings could highlight the possible

role of miR-144 as a biomarker of clinical complications in SCD.

The relationship between miR-144 and the clinical sequalae in SCD is sparsely investigated. **Swem and co-authors.** compared the miR-144 levels between HbSS individuals during crises and in the steady state, but no significant variation was observed. He supposed that various factors could impact the crises' severity **[30]**. According to the study of **Santos et al.** the circulating miR-144 contributed to the occurrence and clinical evolution of sickle leg ulcers **[31]**. Moreover, **Sangokoya et al.** linked the higher relative quantification of miR-144 to the degree of anemia and decreased the hemoglobin/hematocrit in SCD cases **[15]**.

Considering HbF in our study, a negative linear correlation was also observed in regards to RQ of miR-144 but without reaching the significance value (P = 0.067), indicating the inverse impact of miR-144 on HbF. Likewise, **Li and colleagues.** identified an eightfold increase in miR-144-3p and miR-144-5p in cases having reduced HbF in contrast to those with high HbF levels **[32]**. The same finding was also detected by **Guzelgul et al.,** who studied 84 SCD cases in Turkey and found significant higher expression of miR-44-3p in cases having HbF less than 3% (P = 0.043) **[23]**.

Herein, no significant variance was noted between HU treatment and the relative quantification of miR-144 in the evaluated cases. In contrast, **Kargutkar et al.** reported an elevation in miR-144 level following HU therapy without any association with the HbF levels **[22]**. This disparity could be explained by varied genetic control of HbF and variable HU response in SCD cases **[33, 34]**.

MiR-144 targets *NRF2*, the key role of the oxidative stress response. Increased expression of miR-144 promotes *NRF2* gene silencing and suppresses foetal hemoglobin expression. It also reduces cellular glutathione levels, rendering cells more vulnerable to oxidative stress, and exposes HbSS to hemolysis, and more severe anemia **[35, 36].** Other studies also emphasized the inverse effect of miR-144 on the *NRF2* expression and activity **[37].** Furthermore, **Penglong et al.** confirmed by functional studies in the K562 cell line the ability of miR-144 in the regulation of ***γ-globin*** gene expression through *NRF2* and proved that the inhibition of miR-144 induced a fold change of the *NRF2* gene, increasing ***γ-globin***. He also reported that overexpressed miR-144 inhibited *α-globin* and *ζ-globin* gene expression, which is correlated with lower expression of erythroid Kruppel-Like Factor (*KLF1)* **[14]**.

It has also been recorded that miR-144 expression negatively controls the α/β globin gene in β-thalassemia children, improving the clinical complications by reducing excess *α-globin* precipitation **[36].** Additionally, miR-144 impacts human erythropoiesis by limiting the *RAB14* expression, which modulates the function of receptors implicated in erythropoiesis, such as the transferrin receptor **[20]**. Thus, miRNA mimics or inhibitors could be used as future treatment strategies in SCD and other hemoglobinopathies.

5. Conclusion

To date, the role of miRNAs in SCD has not yet been investigated in Egypt. Our research displayed overexpression of miR-144 in SCD cases that was positively correlated with the painful VOCs and inversely correlated with HbF level, illustrating the potential role of miR-144 as a genetic marker of disease outcome and foetal hemoglobin expression in SCD that could be a target for future therapies.

Understanding the genetic and epigenetic variables, including miRNAs implicated in *γ-globin* regulation, is crucial to predict innovative ways for HbF stimulation and discover targeted therapeutic options for SCD. Hence, conducting more comprehensive studies on varied microRNAs and their usage in prognosis and treatment is highly encouraged.

6. Abbreviations

ACS: acute chest syndrome
ARE: antioxidant response element
AUC: area under curve
BMT: bone marrow transplantation
CI: confidence interval
CT: computed tomography
HU: hydroxyurea
KLF1: Kruppel-Like Factor
NRF2: nuclear factor erythroid 2-related factor 2
MiR: MicroRNA
ROC: receiver operating characteristic
RQ: Relative quantification
SCD: sickle cell disease
TIA: transient ischemic attacks
VOC: vaso-occlusive crises
AVN: acute avascular necrosis.

Declaration of competing interest:

The authors declare that they have no conflict of interest.

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