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Profiling circulating microRNA and regulatory pathways in transfusion-dependent thalassemia and thalassemia trait compared to healthy controls: a preliminary study

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Abstract: **Background**: Thalassemia is a genetic blood disorder characterized by abnormal hemoglobin production. MicroRNAs (miRNAs) regulate gene expression and are implicated in thalassemia pathogenesis. This study aimed to profile circulating miRNAs in transfusion-dependent (TD), Thalassemia trait (TT), and non-thalassemic individuals, and elucidate their functional pathways. **Methods**: Serum samples were collected from TD thalassemia patients (n = 4), thalassemia trait (n = 4), and healthy controls (n = 4). Total RNA was extracted and miRNA expression analyzed using NanoString nCounter assays. The nCounter Human v3 miRNA panel consisting of 800 miRNAs was used to scan and quantify miRNA levels. Differentially expressed miRNAs between the three groups were identified through statistical analysis. Bioinformatics analysis using DIANA-miRPath was then conducted on the top differentially expressed miRNAs to identify associated molecular pathways and gene targets. **Results**: Three miRNAs (miR-4435, miR-566, miR-219a) were upregulated while miR-485-5p was downregulated in both TD and TT groups versus controls. miRNA profiles were also compared between TD and TT groups. Initial pathway analysis revealed involvement of upregulated miRNAs in hematopoietic, erythroid differentiation, and AMPK signaling pathways. **Conclusion**: Distinct circulating miRNA profiles exist between TD, TT, and healthy controls. miR-4435, miR-566, and miR-219a are consistently upregulated while miR-485-5p is downregulated, suggesting their functional significance.

1. Introduction

Thalassemia, a health concern, is one of the most common genetic disorders worldwide. According to the World Health Organization (WHO) 5.2% of the population carries abnormal hemoglobin genes, which aligns with their estimation that around 7% of people worldwide are carriers of these abnormal genes. This prevalence is particularly high, in regions such as the Middle East, Mediterranean, Central Asia, Southern China and the Indian subcontinent. Moreover it has been observed that globally there are between 300,000 and 500,000 children born each year with hemoglobin disorders [1]. Out of these cases over 40,000 infants are diagnosed with β thalassemia annually. Among them 25,500 suffer from transfusion β thalassemia. Southeast Asia has the largest number of newborns affected by β thalassemia followed by the Eastern Mediterranean region and Europe along, with parts of the world [2].

Thalassemia is characterized by anemia, which occurs when there is a decrease or absence of hemoglobin due, to problems in the production of blood cells. This condition also involves imbalances in the globin chain and disturbances in the synthesis of molecules involved in hemoglobin production. MicroRNAs (miRNAs) play a crucial role in post-transcriptional gene regulation, particularly in the context of hemoglobin synthesis. Specific miRNAs such as miR-144-3p have been identified as significantly increased in individuals with beta thalassemia, where it inhibits the expression of gamma globin and affects processes like erythropoiesis and apoptosis [3]. Additionally, miRNAs like miR-486-3p, miR-26b, miR-199b-5p, miR-210, and miR-34a are involved in increasing the expression of gamma globin, while others such as miR-96, miR-146a, miR-223-3p, miR-144, and miR-451 play a role in inducing alpha, beta, and gamma globin expression [4]. Furthermore, microarray analysis has demonstrated the up-regulation of 12 miRNAs targeting BCL11A in patients with beta-thalassemia, shedding light on the intricate regulatory network involving miRNAs in hemoglobin disorders [5].

The role of miRNAs in erythropoiesis and hemoglobin disorders has been extensively studied, peeling light on the physiological and pathological implications of post-transcriptional regulation machinery in erythropoiesis [6]. The dysregulation of miRNAs has been identified as potential markers involved in erythroid differentiation, offering promising prospects in blood disease. Moreover, the epigenetic effects of miRNAs have been recognized as potential therapeutic targets in β-thalassemia, with miRNAs being considered as prospective biomarkers for disease identification, stage, and prognosis [7].

Thalassemia manifests in various forms, primarily categorized into transfusion-dependent (TD) thalassemia and thalassemia trait (TT). Transfusion-dependent thalassemia, also known as thalassemia major, is a severe form requiring regular blood transfusions to manage anemia and prevent complications associated with ineffective erythropoiesis. Patients with TD thalassemia often present early in life with severe anemia, requiring lifelong transfusion therapy and iron chelation to mitigate the risks of iron overload [8]. In contrast, thalassemia trait, also known as thalassemia minor, is a milder form where individuals typically carry one mutated gene and one normal gene. These individuals are usually

asymptomatic or present with mild anemia that does not require regular transfusions. TT individuals are often identified through routine blood tests or genetic screening, especially in regions with high thalassemia prevalence [9].

The study of microRNAs (miRNAs) in different thalassemia groups is crucial due to their role in hemoglobin switching, specifically in the regulation of HbF. Targeting miRNAs for therapeutic intervention offers a novel approach to managing β-thalassemia, especially considering the significant limitations of current treatments such as blood transfusions and bone marrow transplantation [10]. Understanding the role and interaction of miRNAs in hemoglobin switching and gene regulation can provide insights into novel therapeutic strategies, potentially improving the quality of life for β-thalassemia patients. This study aims to profile differentially circulating miRNAs among thalassemia individuals with transfusion-dependent (major), thalassemia trait (minor), and healthy non-thalassemic individuals and try to elucidate their functional pathways. By achieving these objectives, we hope to contribute to a deeper understanding of miRNA-mediated regulation in thalassemia and open avenues for the next innovative therapeutic approaches.

2. Methods

2.1. Design

This preliminary study was a cross-sectional analysis conducted among three groups: transfusion-dependent thalassemia patients, Thalassemia trait, and healthy non-thalassemic control individuals.

2.2. Subjects Characteristics

Transfused-dependent thalassemic patients were recruited from previous genetics study [11,12]. The carrier or thalassemia trait group included individuals who were identified as carriers of thalassemia through a screening program. The healthy group consisted of volunteers who did not have thalassemia. All participants were between the ages of 10 and 30, had no health conditions except for thalassemia in the case of patients and being healthy for individuals, in the other groups.

2.3. RNA Isolation

Serum samples were collected from 10 mL of total blood by centrifugation at 1500 × g. The samples were then stored at −80 ℃ until further use. Total RNA was extracted from the plasma of each sample using a miRNeasy Serum/Plasma Kit (Qiagen USA, City, State, Country) according to the manufacturer's instructions. The quality of the RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific™ USA, City, State, Country). The miRNAs isolated for this study were derived from extracellular vesicles present in the serum.

2.4. MicroRNA expression profiling

The expression profiles of 798 miRNAs from serum samples were analyzed using the NanoString nCounter Human v3 miRNA Panel (NanoString Technologies, City, State, USA) according to the manufacturer's instructions, and were done in Genetica Science Laboratory, Jakarta, Indonesia. Data were analyzed and normalized using nSolver™ Software Analysis (NanoString Technologies, City, State, USA). NanoString data were used to obtain the miRNA expression in each sample as a fold-change value, with a fold change of ±2 considered significant. After background subtraction, the data were normalized to the geometric mean of the top 100 miRNAs. Statistical significance was calculated using paired Student's t-tests and analysis of variance.

Advanced data analysis was performed using ROSALIND software (https://www.rosalind.bio/, Version XX, accessed on Day Month Year) with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA, USA). The analyzed data were downloaded from ROSALIND. Volcano plots of differential expression data were generated using the −log10 (P-value) and log2 fold change in GraphPad Prism (Version 9, GraphPad Software, San Diego, CA, USA). Heatmaps were also constructed in GraphPad Prism. Venn diagrams were created using Venny 2.1.

2.5. Bioinformatics Analysis

The DNA intelligent analysis (DIANA)-miRPath online software suite was used to identify potentially dysregulated pathways by up- and down-regulated miRNAs. This software links miRNAs to target genes from Tarbase, v7.0 [13], and identifies the targeted KEGG pathways. The “pathway union” option of the miRPath software was used. Fisher’s exact test was used as an enrichment analysis method to obtain the p-values, and the Benjamin and Hochberg method was used to estimate the false discovery rate (FDR).

2.6. Statistical Analysis

The data is presented as the mean ± standard deviation (SD). Study used hierarchical clustering with Euclidean distance and average linkage clustering to generate a heatmap and clusters for the samples. To compare the differential expression of miRNAs between the two groups, we employed two-sample t-tests or nonparametric tests. We considered two-sided p-values less than 0.05 as statistically significant. The statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, City, State, USA).

2.7. Ethical approval and Informed consent information

This research was carried out following guidelines and received approval, from the Ethical Committee of the Medical Faculty, at Jenderal Soedirman University (Reference Number; 042/KEPK/PE/III/2023). Before participation all individuals provided written consent. The consent document explained the study purpose, procedures, discussed risks and benefits and

highlighted participants' rights. Participants were assured that their involvement were voluntary and that they could opt out of the study at any time without facing consequences or losing any advantages.

3. Results

The results of our Nanostring assay analysis are presented in this section. The raw data, consisting of Reporter Code Count (RCC) and Reporter Library File (RLF) data, have been deposited in the Figshare dataset repository for transparency and reproducibility. These data are publicly accessible under the Digital Object Identifier (DOI) 10.6084/m9. figshare. 26073439.

3.1. All significant alterations in circulating miRNAs in thalassemia

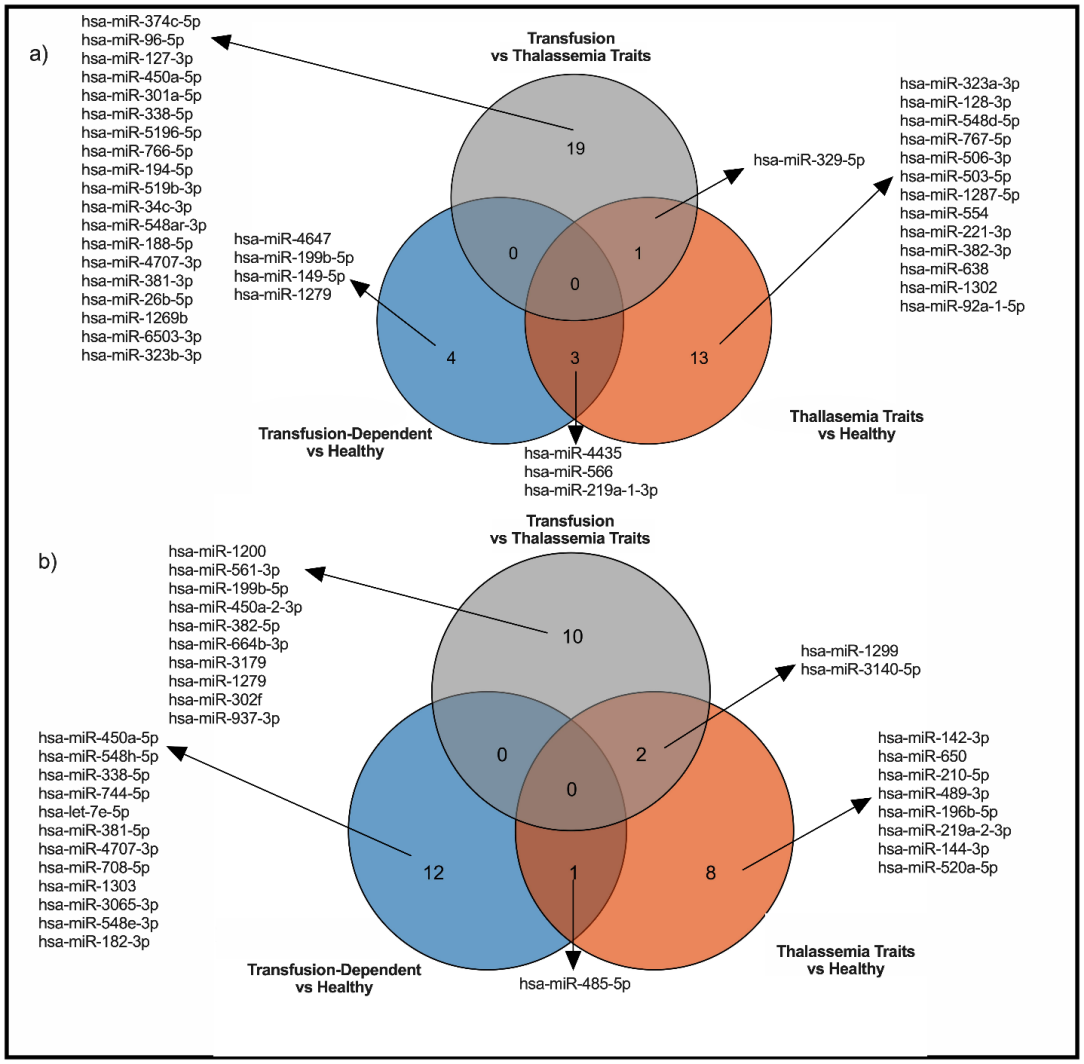

Figure 1. Venn Diagram analysis of significantly differentially expressed miRNAs in transfusion dependent (TD) patients, thalassemia trait (TT) individuals, and healthy individuals. (**a**) Upregulated miRNAs, which found should be include miR-4435, 566, and 219a-1-3p, (**b**) Downregulated miRNAs, which found miR-485-5p consistently, respectively.

A Venn diagram represents of differentially significant expressed all miRNAs transfusion dependent (TD) patients and trait individuals, while the healthy control group as a reference (seen in Figure 1). It was revealed that three miRNAs were significantly upregulated in transfusion dependent (TD) patients and thalassemia trait individuals (miR-4435, miR-566, and miR-219a), while miR-485-5p was significantly downregulated in both transfusion dependent (TD) patients and thalassemia (TT) individuals.

3.2. Significant alterations in expression in transfusion dependent (TD) patients

The expression levels of all significantly differentially expressed miRNAs in transfusion dependent (TD) patients compared to healthy control non-thalassemia patients (Figure 2). In the heatmap, the miRNAs are clustered based on their expression patterns, and the color intensity of each square represents the expression level of the corresponding miRNA. Red squares indicate high expression, while blue squares indicate low expression. Besides, the Graph shows the fold change in expression of the top 10 most differentially expressed miRNAs in transfusion dependent (TD) patients thalassemia patients compared to healthy control non-thalassemia patients. The fold change is calculated by dividing the expression level of the miRNA in major thalassemia patients by the expression level in control non-thalassemia patients. The most upregulated miRNAs include miR-1279, miR-4435, miR-566, miR-149-5p, and miR-199b-5p. The most downregulated miRNAs include miR-450a-5p, 485-5p, miR-4707-3p, miR-744-5p, and miR-1303.

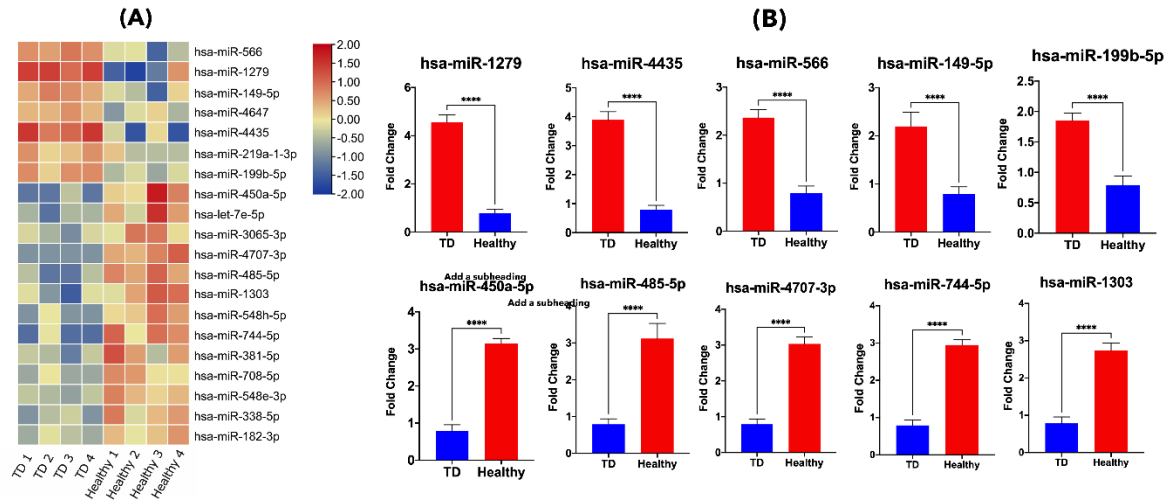

Figure 2. Significant differentially circulating expressed miRNAs in Transfusion-Dependent (TD) thalassemia patients compared to healthy control, non-thalassemia individuals. (**a**) Heatmap all miRNAs found alterations significant in TD patients and normal control individuals (red; high expression and blue; down expression), (**b**) the top ten significant over and down expression miRNAs among TD patients and healthy control non-thalassemia individuals.

3.3. Significant alterations and expressions of thalassemia trait (TT)

The heatmap and Graph in Figure 3 show that a number of miRNAs are differentially expressed in minor thalassemia patients (TT) compared to control non-thalassemia patients. The top 10 most differentially expressed miRNAs in TT group compared to healthy control non-thalassemia subjects. The fold change is calculated by dividing the expression level of the miRNA in TT individuals by the expression level in healthy control non-thalassemia subjects. The most upregulated miRNAs include miR-566, miR-4435, miR-92a-1-5p, miR-382-3p, and miR-503-5p. The most downregulated miRNAs include miR-144-3p, miR-219a-2-3p, miR-485-5p, miR-1299, and miR-198b-5p.

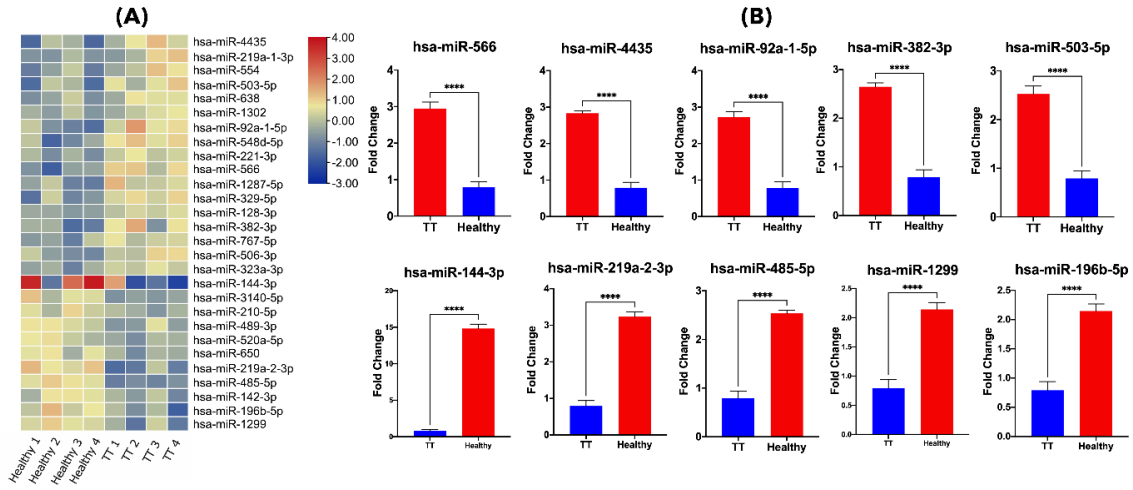

Figure 3. Differentially circulating expression of miRNAs in thalassemia trait (TT) patients compared to healthy control, non-thalassemia subjects. (**A**) all alterations circulating miRNAs performed with heatmap in TT and normal control individuals (red; high expression and blue; down expression), (**B**) top ten significant expression TT and normal control individuals.

3.4. Alterations expressions of transfusion dependent (TD) vs thalassemia trait (TT)

Figure 4 shows the expression levels of all significantly differentially expressed miRNAs in major thalassemia patients (TD) compared to minor thalassemia patients (TT). The miRNAs are clustered based on their expression patterns, and the color intensity of each square represents the expression level of the corresponding miRNA. Graph showing the fold change in expression of the top 10 most differentially expressed miRNAs in TD patients compared to TT subjects. The fold change is calculated by dividing the expression level of the miRNA in TD patients by the expression level in TT patients. The most upregulated miRNAs include miR-329-5p, miR-519b-3p, miR-450a-5p, miR-6503-3p and miR-1269b. The most downregulated miRNAs include miR-561-3p, miR-302f, miR-3179, miR-1299, and miR-1200.

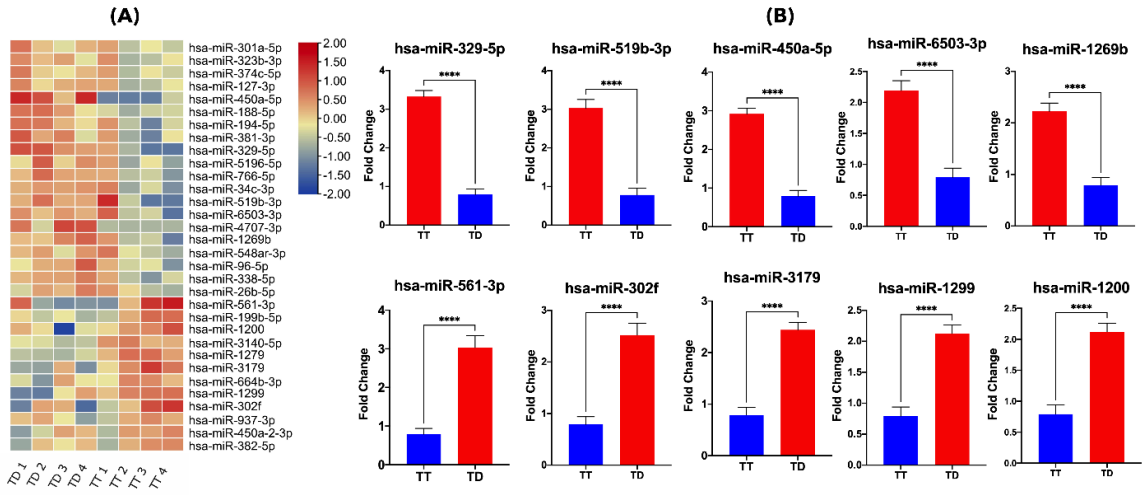

Figure 4. Heatmap and Graph of differentially significant circulation expression of miRNAs in transfusion dependent (TD) and thalassemia trait (TT) patients ($p<0.05$). (**A**) Heatmap of miRNAs in TD and TT (red; high expression and blue; down expression), (**B**) Top ten significant over and down expression among TD and TT patients.

3.5. Functional pathway analysis of significant alterations in miRNA expression

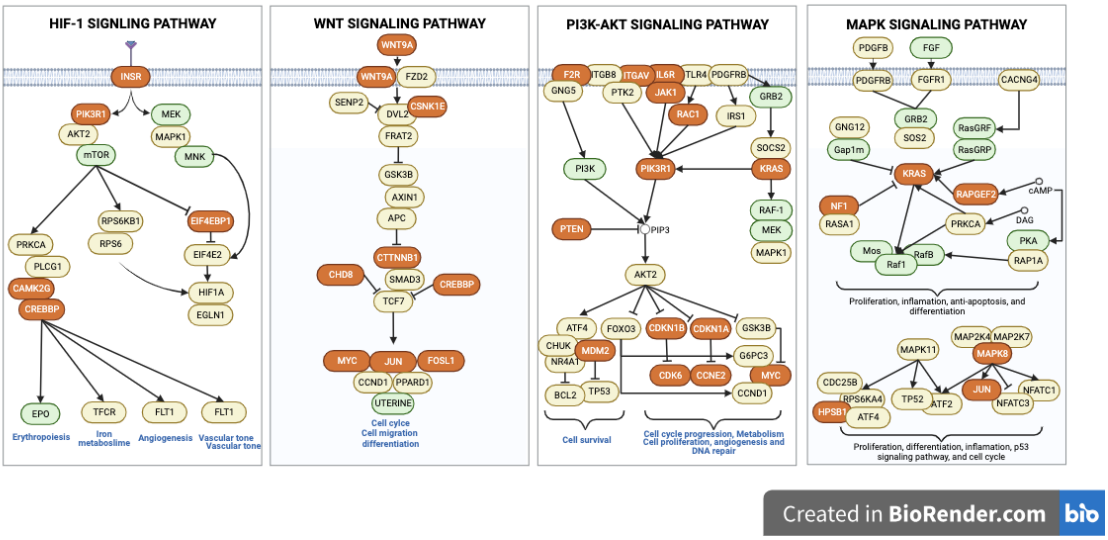

Figure 5. MiRNA regulation in thalassemia disease constructed using Biorender online software. The figure should illustrate the involvement of miRNAs in regulating the PI3K, MAPK, HIF-1, and WNT signaling pathways in the context of thalassemia.

To investigate the functional mechanisms and biological processes associated with the differentially expressed miRNAs, we performed a KEGG pathway analysis using the top 20 miRNAs that were significantly upregulated or downregulated in transfusion dependent (TD) patients compared to healthy control non-thalassemia patients, as depicted in Table 1 (due to large table, the table is placed in repository database with DOI access number http://dx.doi.org/10.6084/m9.figshare.26073442) [14,15]. The pathway analysis construction

results showed using online software (www.biorender, accessed on Day Month Year)[16] that these miRNAs were involved in various pathways, including the hematopoietic cell lineage pathway, the erythroid differentiation pathway, and the AMPK signaling pathway (Figure 5). Pathways analysis of alterations expression of miRNA showed in Table 2 http://dx.doi.org/10.6084/m9.figshare.26073442. Analysis interaction of miRNA using miRTARbase and miRbase online software [17–20].

4. Discussion

In the current investigation, we embarked on a comprehensive analysis of the microRNA (miRNA) profiles in patients with thalassemia, specifically focusing on those who are transfusion dependent (TD) and those with minor or thalassemia trait (TT) forms of the disease. The primary objective of this study was to unearth potential circulating miRNA biomarkers that could shed light on the pathophysiological variances between these two patient cohorts.

MicroRNAs (miRNAs) have been increasingly recognized for their significant involvement in pathogenesis and potential therapeutic targets in thalassemia. Several studies have highlighted the role of specific miRNAs in thalassemia, such as miR-326, let-7, miR-96, miR-144, miR-451, and miR-150, which have been shown to modulate the expression of α-, β- and γ-globin [21]. Additionally, miRNAs have been implicated in malarial pathogenesis and anti-plasmodial defense in thalassemia, suggesting their potential as targets for understanding and managing thalassemia-related complications [22]. Furthermore, studies have demonstrated abnormal regulation of miRNAs in pediatric β‐thalassemia, emphasizing the relevance of miRNA dysregulation in the disease [3]. Moreover, investigations into the expression of specific miRNAs, such as miR-15a, miR-16-1, miR-96, and miR-486-3p, have been conducted to correlate their levels with HbF levels in thalassemia patients, indicating their potential role in hematopoiesis and as therapeutic targets [23].

Our data revealed a complex landscape of miRNA expression, where both similarities and distinctive patterns emerged between TD and TT thalassemia patients. In TD patients, we identified a significant alteration in the expression levels of a subset of miRNAs, including miR-4435, miR-566, miR-219a, miR-4647, miR-548e-3p, miR-485-5p, miR-146a-5p, miR-182-3p, and miR-1303. These miRNAs emerged as potential biomarkers or therapeutic targets, offering insights into the molecular underpinnings of transfusion dependency in thalassemia. Conversely, in the TT cohort, we observed a differentially expressed set of miRNAs comprising miR-4435, miR-566, miR-219a, miR-4647, miR-3140-5p, miR-485-5p, miR-146a-5p, miR-182-3p, and miR-489-3p. This study found the overlap in some upregulated miR-4435, miR-566, and miR 219a-1-3p between the two groups suggests shared molecular pathways, while the unique profiles indicate distinct regulatory mechanisms at play in TT thalassemia.

This data replicate a previous study which found that miR-4435, was significantly upregulated in pediatric patients with β‐thalassemia [3]. Based on previous data, miR-4435 has been implicated in various biological processes and diseases, including diabetic

retinopathy (DR), colorectal cancer, melanoma, non-small cell lung cancer, Alzheimer's disease, and pregnancy progression [24–27]. Its regulatory role in various cellular processes and diseases underscores the importance of further investigating its specific mechanisms of action and downstream targets to gain a comprehensive understanding of its functional significance, including in thalassemia context.

There is evidence of a potential relationship between miR-566 and hematology regulation through BCL11A pathways. A study suggests that miR-566 can reduce BCL11A expression by directly binding to the 3′UTR region or the coding sequence of the BCL11A gene [28]. BCL11A is a key regulators, which represses the genes encoding HbF and plays a crucial role in the regulation of human hemoglobin switching during development [29]. The other context of hematopoietic regulation, references by and discussed the association of miR-566 with Down syndrome and its potential implications for hematopoiesis and myeloproliferative disorders [30]. Furthermore, and highlighted the role of specific miRNAs, including miR-566, in granulopoiesis and hemopoietic cells, indicating its potential involvement in hematopoietic processes [31].

Additionally, demonstrated that upregulated miR-219a-5p inhibits the expression of CCNA2 and CACUL1, further regulating Akt/Foxo3a and p53/Bcl-2 signaling pathways, leading to neuronal apoptosis [32]. Furthermore, linked the upregulation of miR-219a-2-3p to anti-inflammatory responses, it has been associated with the modulation of the NF-κB signaling pathway, leading to the suppression of cell proliferation and the promotion of apoptosis including in hematopoietic cells and thyroid [33]. These findings suggest that miR-4435 and miR-219a-1-3p may be involved in thalassemia and neuroprotective responses, respectively. miR-219a-1 has been reported to imply various pathologies, especially in tumor progression, inhibit proliferation. For instance, reported that miR-219a-1 is significantly downregulated in the peripheral blood of patients with colon cancer, and its inhibition leads to increased proliferation and invasion of colon cancer cells [34]. Finding of miR-219a-1 in this study express the concern of rule of regulation in hematopoietic tissue. Overall, the data from the referenced studies indicate that miR-4435, miR-566, and miR-219a-1-3p are involved in various pathophysiological processes, including β‐thalassemia.

Regulation of miR-485-5p, which dowregulated in this stsudy, has been extensively studied in various biological contexts, cardiovascular diseases, diabetes, and skin wound healing. In cardiovascular diseases, miR-485-5p was shown to down-regulate the expression of endogenous Corin, affecting the morphology and function of the cardiovascular system [35]. Additionally, miR-485-5p has been implicated in hepatocellular carcinoma malignancy by mediating the miR-485-5p/LSM4 axis [36]. In small cell lung cancer, miR-485-5p was demonstrated to suppress proliferation, migration, and invasion of cancer cells by targeting flotillin-2 [37]. In diabetic nephropathies, miR-485-5p was found to attenuate the proliferation, inflammation, and oxidative stress of high glucose-induced human mesangial cells by regulating the miR-485-5p/YAP1 pathway [38]. However, We did not find literature examining the effects of miR-485-5 in hematological systems. This suggests that further research is needed to investigate the role of this miRNA.

5. Functional pathway analysis

miRNA-mRNA target interactions that were identified in the DIANA miRPath analysis [39]. miR-4435 targets KLF1: miR-4435 is upregulated in major thalassemia patients, and KLF1 is a transcription factor involved in erythroid differentiation. miR-4435 binding to KLF1 mRNA inhibits its translation, which leads to a decrease in KLF1 protein levels. This decrease in KLF1 protein levels disrupts erythroid differentiation and contributes to the pathogenesis of major thalassemia. miR-485-5p targets AKT1: miR-485-5p is downregulated in major thalassemia patients, and AKT1 is a kinase that is involved in the PI3K-Akt signaling pathway. miR-485-5p binding to AKT1 mRNA inhibits its translation, which leads to a decrease in AKT1 protein levels. This decrease in AKT1 protein levels disrupts cell growth and survival, contributing to major thalassemia pathogenesis. miR-182-3p targets MAPK1: miR-182-3p is upregulated in major thalassemia patients, and MAPK1 is a kinase involved in the MAPK signaling pathway. miR-182-3p binding to MAPK1 mRNA inhibits its translation, which leads to a decrease in MAPK1 protein levels. This decrease in MAPK1 protein levels disrupts cell growth and differentiation, contributing to major thalassemia's pathogenesis. The pathway analysis results suggest that miRNAs play a role in the pathogenesis of thalassemia by disrupting the development and differentiation of red blood cell's energy metabolism and cellular growth.

The pathway analysis results showed that the differentially expressed miRNAs were involved in various pathways. Pathways analysis of alterations expression of miRNA showed four mechanisms that have impact significantly in thalassemia disease (Figure 5) and (Table 2). The PI3K/Akt and HIF-1 signaling pathways play crucial roles in hypoxia-induced responses, including the regulation of glucose metabolism and angiogenesis [40,41]. These pathways are also involved in stabilizing HIF-1α, a key regulator of metabolic adaptation to hypoxia [42]. In thalassemia, a condition characterized by chronic hypoxia, the expression of HIF1α and the glucose transporter 1 receptor is altered [43,44]. Additionally, the PI3K/Akt pathway, which is activated by insulin-like growth factor-I (IGF-I), has been shown to regulate proliferation and apoptosis in multiple myeloma cells and also hematopoietic cells [45,46]. These findings suggest that the PI3K/Akt and HIF-1 signaling pathways may be dysregulated in thalassemia, potentially contributing to the pathophysiology of the disease.

In thalassemia, dysregulation of various signaling pathways has been implicated in the pathogenesis of the disease. The MAPK signaling pathway, involved in cell proliferation and differentiation, is considered overactive, contributing to the development of ineffective erythropoiesis. Conversely, the PI3K-Akt signaling pathway, responsible for cell survival and growth, is believed to be underactive in thalassemia, potentially leading to the development of iron overload. Furthermore, the Wnt signaling pathway, crucial for cell migration and differentiation, is also thought to be dysregulated in thalassemia, which may contribute to ineffective erythropoiesis. Additionally, the HIF-1 signaling pathway, which responds to hypoxia, is thought to be upregulated in thalassemia, potentially leading to ineffective erythropoiesis and iron overload [47]. Moreover, studies have shown that specific

microRNAs, such as miR-190b-5p, are dysregulated in pediatric β-thalassemia and may play a role in the disease pathogenesis by targeting genes like BCL11A, which is involved in hemoglobin switching [3,47].

The hypoxia-inducible factor 1 (HIF-1) pathway plays a crucial role in mediating the cellular response to low oxygen levels, and its activation has been implicated in various downstream effects in the context of thalassemia. Under hypoxic conditions, HIF-1α stabilizes and translocates to the nucleus, where it dimerizes with HIF-1β, leading to the activation of target genes involved in erythropoiesis, iron metabolism, and angiogenesis [48]. In the context of thalassemia, chronic hypoxia and the consequent activation of the HIF-1 pathway can lead to several downstream effects. Erythropoiesis is stimulated by the HIF-1 pathway through the production of erythropoietin (EPO), which drives the formation of red blood cells. However, in thalassemia, this increased erythropoiesis is ineffective due to the genetic defect, leading to an expansion of the bone marrow and associated complications [49].

HIF-1 activation can also lead to increased intestinal iron absorption and mobilization of iron from stores, exacerbating iron overload, a common complication in thalassemia patients due to repeated blood transfusions [50]. Furthermore, the hypoxic environment and HIF-1 activation may promote angiogenesis, contributing to the abnormal bone development seen in thalassemia patients [51]. Recent publications have explored the intricacies of HIF-1 in thalassemia, including studies on how HIF-1α polymorphisms might influence the severity of thalassemia, as certain variants may lead to a more pronounced hypoxic response [52]. Other research has focused on therapeutic approaches to modulate HIF-1 activity, potentially offering a way to alleviate some of the complications associated with thalassemia [53].

These findings underscore the complex molecular mechanisms involved in thalassemia and highlight the potential significance of microRNAs in the disease process. Further research in this area may provide valuable insights into the development of targeted therapeutic interventions and diagnostic approaches for thalassemia. Further research is needed to fully understand the implications of these microRNAs in thalassemia and their potential as therapeutic targets or diagnostic markers [54,55].

This study has several limitations that should be acknowledged in this preliminary study. The sample size is relatively small, which may impact the statistical power and reliability of our findings. This recognizes the need for validation with a larger, independent cohort of samples to confirm our results. While our study includes a functional pathway analysis of the identified miRNAs, it is based solely on bioinformatics approaches. Study have not yet conducted experimental validation of these pathways. Future studies should include in vitro and in vivo experiments to elucidate the functional roles of the identified miRNAs in thalassemia. Such experiments will provide a deeper understanding of the biological mechanisms involved and validate the bioinformatics predictions. Conducting quantitative PCR (qPCR) experiments are crucial for strengthening the robustness and reliability of our initial findings.

6. Conclusion

The study found there were upregulation of the miR-4435, miR-566, and miR-219a, while miR-485-5p was significantly downregulated in both transfusion dependent (TD) patients and minor or Thalassemia traits (TT). The involvement of miRNAs in the context of thalassemia, are following the regulation of the PI3K, MAPK, HIF-1, and WNT signaling pathways.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical statement

The study was performed in accordance with the Declaration of Helsinki and approved by the name of the Ethical Committee of the Medical Faculty, at Jenderal Soedirman University (approval date, approval Number; 042/KEPK/PE/III/2023). All individuals provided written consent before participation.

References

[1] Williams T.N, Weatherall D.J. World Distribution, Population Genetics, and Health Burden of the Hemoglobinopathies. *CSH. Perspect. Med.* 2012, 2(9):a011692.

[2] Kattamis A, Kwiatkowski J.L, Aydinok Y. Thalassaemia. *Lancet* 2022, 399(10343):2310–2324.

[3] Wang H, Chen M, Xu S, Pan Y, Zhang Y, *et al*. Abnormal regulation of microRNAs and related genes in pediatric β‐thalassemia. *J. Clin. Lab. Anal.* 2021, 35(9):e23945.

[4] Li B, Zhu X, Ward C.M, Starlard-Davenport A, Takezaki M, *et al*. MIR-144-mediated NRF2 gene silencing inhibits fetal hemoglobin expression in sickle cell disease. *Exp. Hematol.* 2019, 70:85–96.e5.

[5] Zakaria N.A, Islam A, Abdullah W.Z, Bahar R, Mohamed Yusoff, A.A, *et al*. Epigenetic Insights and Potential Modifiers as Therapeutic Targets in B–Thalassemia. *Biomolecules* 2021, 11(5):755.

[6] Li Y, Zhang H, Hu B, Wang P, Wang W, *et al*. Post-Transcriptional Regulation of Erythropoiesis. *Blood Sci.* 2023, 5(3):150–159.

[7] Guo C, Li X, Liu S, Sun M. MicroRNAs as Potential Markers Involved in Erythroid Differentiation: A Systematic Literature Review. *Sci. J. Clin. Med.* 2021, 10(2), 16–29.
[8] Betts M, Flight P.A, Paramore L.C, Tian L, Milenković D, *et al.* Systematic Literature Review of the Burden of Disease and Treatment for Transfusion-dependent β-Thalassemia. *Clin. Ther.* 2020, 42(2):322–337.
[9] Hasanshahi F, Khanjani N. Investigating the reasons for marriage among couples with thalassemia minor, in Iran. *J. Community Genet.* 2021, 12(4):507–513.
[10] Li L, Yi H, Liu Z, Long P, Pan T, *et al.* Genetic correction of concurrent α- and β-thalassemia patient-derived pluripotent stem cells by the CRISPR-Cas9 technology. *Stem Cell Res. Ther.* 2022, 13(1):102.
[11] Rujito L, Basalamah M, Mulatsih S, Sofro A.S.M. Molecular Scanning of β-Thalassemia in the Southern Region of Central Java, Indonesia; a Step Towards a Local Prevention Program. *Hemoglobin* 2015, 39(5):330–333.
[12] Susanto Z.A, Siswandari W, Rujito L. Cd60 (GTG > GAG)/Hb Cagliari mutation was found in scanning of β-thalassemia alleles from patients of East Kalimantan, Indonesia. *Mol. Genet. Metab. Rep.* 2020, 22:100550.
[13] Vlachos I.S, Paraskevopoulou M.D, Karagkouni D, Georgakilas G, Vergoulis T, *et al.* DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res.* 2015, 43(D1):D153–D159.
[14] Hsu J.B.K, Chiu C.M, Da Hsu S, Huang W.Y, Chien C.H, *et al.* MiRTar: An integrated system for identifying miRNA-target interactions in human, *BMC Bioinf.* 2011, 12:1–12.
[15] Wan Z, Zhang X, Luo Y, Zhao B. Identification of Hepatocellular Carcinoma-Related Potential Genes and Pathways through Bioinformatic-Based Analyses. *Genet. Test. Mol. Biomarkers* 2019, 23(11):766–777.
[16] S. Aoki, BIORENDER, Biorender (2017).
[17] Kozomara A, Birgaoanu M, Griffiths-Jones S. MiRBase: From microRNA sequences to function. *Nucleic Acids Res.* 2019, 47(D1):D155–D162.
[18] Griffiths-Jones S, Saini H.K, Van Dongen S, Enright A.J. miRBase: Tools for microRNA genomics. *Nucleic Acids Res.* 2007, 36(suppl_1):D154–D158
[19] Kalaigar S.S, Rajashekar R.B, Nataraj S.M, Vishwanath P, Prashant A. Bioinformatic Tools for the Identification of MicroRNAs Regulating the Transcription Factors in Patients with β-Thalassemia. *Bioinf. Biol. Insights* 2022, 16:11779322221115536.
[20] Huang H.Y, Lin Y.C.D, Li J, Huang K.Y, Shrestha S, *et al.* MiRTarBase 2020: Updates to the experimentally validated microRNA-target interaction database. *Nucleic Acids Res.* 2020, 48(D1):D148–D154.
[21] Bravo Vázquez L.A, Moreno Becerril M.Y, Mora Hernández E.O, de León Carmona G.G, Aguirre Padilla M.E, *et al.* The Emerging Role of MicroRNAs in Bone Diseases and Their Therapeutic Potential. *Molecules* 2021, 27(1):211.
[22] Papadopoulos K.I, Sutheesophon W, Manipalviratn S, Aw T.C. A Southeast Asian Perspective on the COVID-19 Pandemic: Hemoglobin E (HbE)-Trait Confers Resistance Against COVID-19. *Med. Sci. Monit. Basic Res.* 2021, 27:e929207–1.

[23] Eltaweel N.H, ElKamah G.Y, Khairat R, Atia H.A.E, Amr K.S. Epigenetic effects toward new insights as potential therapeutic target in B-thalassemia. *J. Genet. Eng. Biotechnol.* 2021, 19(1):51.

[24] Zhu X, Hao M, Yu X, Lin W, Ma X, *et al.* Dissecting the Pathogenesis of Diabetic Retinopathy Based on the Biological ceRNA Network and Genome Variation Disturbance. *Comput. Math. Methods Med.* 2021, 2021(1):9833142.

[25] Hong J.W, Kim J.M, Kim J.E, Cho H, Kim D, *et al.* MiR-4435 Is an UQCRB-related Circulating miRNA in Human Colorectal Cancer. *Sci. Rep.* 2020, 10(1):2833.

[26] Yu H, Li S. Role of LINC00152 in Non-Small Cell Lung Cancer. *J. Zhejiang Univ. Sci. B* 2020, 21(3):179–191.

[27] Vashukova E.S, Kozyulina P.Y, ИлларионовР.А, Yurkina N.O, V Pachuliia O, *et al.* High-Throughput Sequencing of Circulating MicroRNAs in Plasma and Serum During Pregnancy Progression. *Life* 2021, 11(10):1055.

[28] Yin J, Xie X, Ye Y, Wang L, Che F. BCL11A: A Potential Diagnostic Biomarker and Therapeutic Target in Human Diseases. *Biosci. Rep.* 2019, 39(11):BSR20190604.

[29] Basak A, Munschauer M, Lareau C.A, Montbleau K.E, Ulirsch J.C, *et al.* Control of Human Hemoglobin Switching by LIN28B-mediated Regulation of BCL11A Translation. *Nat. Genet.* 2020, 52(2):138–145.

[30] Wong J.J, Ritchie W, Gao D, Lau K.A, Gonzalez M, *et al.* Identification of nuclear-enriched miRNAs during mouse granulopoiesis. *J. Hematol. Oncol.* 2014, 7:1–15.

[31] Pacifici R. Osteoimmunology and Its Implications for Transplantation. *Am. J. Transplant.* 2013, 13(9):2245–2254.

[32] Yan J, Bu X, Li Z, Wu J, Wang C, *et al.* Screening the expression of several miRNAs from TaqMan Low Density Array in traumatic brain injury: miR‐219a‐5p regulates neuronal apoptosis by modulating CCNA2 and CACUL1. *J. Neurochem.* 2019, 150(2):202–217.

[33] Yang C, Zhang S, Chang X, Huang Y, Cui D, *et al.* MicroRNA‐219a‐2‐3p modulates the proliferation of thyroid cancer cells via the HPSE/cyclin D1 pathway. *Exp. Ther. Med.* 2021, 21(6):1–8.

[34] Xu K, Shi J, Mo D, Yang Y, Fu Q, *et al.* miR-219a-1 inhibits colon cancer cells proliferation and invasion by targeting MEMO1. *Cancer Biol. Ther.* 2020, 21(12):1163–1170.

[35] Zhang D, Nie Q, Liu Y, Li Z, Xie W, *et al.* Mir-485-5p on the Morphology and Function of Cardiovascular System, *Cell. Mol. Biol.* 2022, 68(1):117–123.

[36] Sun Z P, Tan Z G, Peng C. Long noncoding RNA LINC01419 promotes hepatocellular carcinoma malignancy by mediating miR‐485‐5p/LSM4 axis. *Kaohsiung J. Med. Sci.* 2022, 38(9):826–838.

[37] Gao F, Wu H, Wang R, Guo Y, Zhang Z, *et al.* MicroRNA-485-5p suppresses the proliferation, migration and invasion of small cell lung cancer cells by targeting flotillin-2. *Bioengineered* 2019, 10(1):1–12.

[38] Wan H, Wang Y, Pan Q, Chen X, Chen S, *et al.* Quercetin attenuates the proliferation, inflammation, and oxidative stress of high glucose-induced human mesangial cells by regulating the miR-485-5p/YAP1 pathway. *Int. J. Immunopathol. Pharmacol.* 2022, 36:20587384211066440.

[39] Vlachos I.S, Zagganas K, Paraskevopoulou M.D, Georgakilas G, Karagkouni D, *et al.* DIANA-miRPath v3.0: Deciphering microRNA function with experimental support. *Nucleic Acids Res.* 2015, 43(W1):W460–W466.

[40] Zhang Z, Yao L, Yang J, Wang Z, Du G. PI3K/Akt and HIF-1 signaling pathway in hypoxia-ischemia (Review). *Mol. Med. Rep.* 2018, 18(4):3547–3554.

[41] Norbitt C.F, Kimita W, Ko J, Bharmal S.H, Petrov M.S. Associations of Habitual Mineral Intake with New-Onset Prediabetes/Diabetes after Acute Pancreatitis. *Nutrients* 2021, 13(11):3978.

[42] Zhou J, Schmid T, Frank R, Brüne B. PI3K/Akt Is Required for Heat Shock Proteins to Protect Hypoxia-inducible Factor 1α from pVHL-independent Degradation. *J. Biol. Chem.* 2004, 279(14):13506–13513.

[43] Ferro E, Visalli G, Currò M, La Rosa M.A, Piraino B, *et al.* HIF1α and Glut1 receptor in transfused and untransfused thalassemic patients. *Br. J. Haematol.* 2016, 174(5):824–826.

[44]Kontoghiorghes G, Kontoghiorghe C. Iron and Chelation in Biochemistry and Medicine: New Approaches to Controlling Iron Metabolism and Treating Related Diseases. *Cells* 2020, 9(6):1456.

[45] Qiang Y.W, Kopantzev E, Rudikoff S. Insulinlike growth factor-I signaling in multiple myeloma: Downstream elements, functional correlates, and pathway cross-talk. *Blood* 2002, 99(11):4138–4146.

[46] Wang L, Lin N, Li Y. The PI3K/AKT signaling pathway regulates ABCG2 expression and confers resistance to chemotherapy in human multiple myeloma. *Oncol. Rep.* 2019, 41(3):1678–1690.

[47] Chen M, Wang X, Wang H, Zhang M, Chen L, *et al.* Down-Regulated hsa-miR-190b-5p is Correlated With BCL11A Expression in Pediatric β -Thalassemia. *Res. Pre-Print* 2021, 1–12.

[48] Kim J, Barsoum I.B, Loh H, Parél, Siemens D.R, *et al.* Inhibition of hypoxia-inducible factor 1α accumulation by glyceryl trinitrate and cyclic guanosine monophosphate. *Biosci. Rep.* 2020, 40(1):BSR20192345.

[49] Parisi S, Finelli C, Fazio A, De Stefano A, Mongiorgi S, *et al.* Clinical and Molecular Insights in Erythropoiesis Regulation of Signal Transduction Pathways in Myelodysplastic Syndromes and β-Thalassemia. *Int. J. Mol. Sci.* 2021, 22(2):827.

[50] Manolova V, Nyffenegger N, Flace A, Altermatt P, Varol A, *et al.* Oral ferroportin inhibitor ameliorates ineffective erythropoiesis in a model of β-thalassemia. *J. Clin. Invest.* 2020, 130(1):491–506.

[51] Garcia J.A, Chen R, Xu M, Comerford S.A, Hammer R.E, *et al.* Acss2/HIF-2 signaling facilitates colon cancer growth and metastasis. *PLoS One* 2023, 18(3):e0282223.

[52] Li H, He T, Zha Y.J, Du F, Liu J, *et al.* HIF-1α rs11549465 C>T polymorphism contributes to increased cancer susceptibility: Evidence from 49 studies. *J. Cancer* 2019, 10(24):5955.

[53] Saad H.K.M, Abd Rahman A.A, Ab Ghani A.S, Taib W.R.W, Ismail I, *et al.* Activation of STAT and SMAD Signaling Induces Hepcidin Re-Expression as a Therapeutic Target for β-Thalassemia Patients. *Biomedicines* 2022, 10(1):189.

[54] Li Z, Rana T.M. Therapeutic targeting of microRNAs: current status and future challenges. *Nat. Rev. Drug Discov.* 2014, 13(8):622–638.

[55] Patil K.M, Chen G. Recognition of RNA Sequence and Structure by Duplex and Triplex Formation: Targeting miRNA and Pre-miRNA. Jurga S, Erdmann (Deceased) V, Barciszewski J, Eds. In Modified Nucleic Acids in Biology and Medicine. Cham: Springer, 2016, pp. 299–317.