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ORIGINAL ARTICLE

Circulating miRNAs and tissue iron overload in transfusion-dependent β-thalassemia major: novel predictors and follow-up guide

Nashwa El-Khazragy[1] · Safa Matbouly[2] · Demiana H. Hanna[3] · Nievin Ahmed Mahran[4] · Sally Abdallah Mostafa[5] · Bassam A. Abdelrehim[6] · Yasmeen K. Farouk[6] · Soha Abuelela[7]

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Abstract

Tissue iron overload is a life-threatening scenario in children with transfusion-dependent β-thalassemia major, miRNAs that are involved in iron hemostasis could serve as therapeutic targets for control of iron overload. We aimed to find out the association between three iron-related miRNAs "miR-let-7d, miR-122, and miR-200b" and excess iron in tissues, in transfusion-dependent β-thalassemia major patients. Circulating miRNA expressions are measured in peripheral blood (PB) samples using qPCR of transfusion-dependent (TDT) β-thalassemia patients ($n=140$) and normalized to non-transfusion-dependent (NTDT) β-thalassemia ($n=45$). Results revealed that plasma expression levels of miR-let-7d and miR-200b were significantly downregulated in TDT patients; however, miR-122 was upregulated. In terms of tissue iron load, aberrant expression of miRNAs was significantly associated with increased—iron accumulation in hepatic and cardiac tissues. We concluded that circulating miRNAs are strong candidates that associate iron hemostasis in transfusion-dependent β-thalassemia major patients. And by extension, targeting miR-let-7d, miR-122, and miR-200 might serve as novel sensitive, specific and non-invasive predictor biomarkers for cellular damage under condition of tissue iron excess.

Keywords β-Thalassemia · Iron overload · Blood transfusion · Circulating miRNAs

Introduction

β-Thalassemia is a diverse group of inherited hemoglobinopathies, characterized by mutations in the β-globin gene [1]. Frequent blood transfusion is still the standard management of β-thalassemia major; however, excess iron deposition in different organs is associated with transfusion-dependent (TDT) and non-transfusion-dependent thalassemia's (NTDT), which further increase the mortality rate [2, 3]. Two mechanisms explain the process of iron overload in thalassemia patients; they include the following: the increased iron burden in TDT and enhanced intestinal absorption in case of NTDT patients [4]. The iron overload is associated with the production of labile plasma iron in abnormal red blood cells with a subsequent formation of reactive oxygen species and cell damage [5, 6]. The iron overload, through the promotion of free radicals' production, can cause serious adverse effects including sexual maturity delay/dysfunction, liver pathologies (cancer and hepatitis), cardiomyopathies, and metabolism irregularities (thyroid/parathyroid disorders, diabetes, adrenal dysfunction, and hypogonadism) [7–11]. Notably, myocardial

✉ Nashwa El-Khazragy
nashwaelkhazragy@med.asu.edu.eg

1 Clinical Pathology-Hematology Department, Faculty of Medicine Ain Shams Research Institute (MASRI), Ain Shams University, Abbassia 11566, Cairo, Egypt

2 Pediatrics Hematology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

3 Biochemistry Department, Faculty of Science, Cairo University, Cairo, Egypt

4 Biochemistry Department, Faculty of Dentistry, Sinai University, Kantara, Egypt

5 Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

6 Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Giza, Egypt

7 Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

iron accumulation leads to cardiac mortality in 71% of the β-thalassemia patients [12, 13].

Although iron chelators represent the standard therapy for chelation of excess iron, several limitations are addressed regarding their efficacy, safety, and associated side effects such as gastrointestinal disturbances, rash, elevated liver enzymes, and renal insufficiency [14].

With the development of high throughput sequencing technologies, non-coding RNAs have been discovered. MicroRNAs (miRNAs), short single-stranded RNAs, that are not coded for protein are significant molecules involved in cell development/proliferation, apoptosis, and differentiation [15, 16]. It has demonstrated that a single transcript is targeted by numerous miRNAs; it regulates gene expression by post-transcriptional silencing [17, 18]. Recent studies highlight the role of miRNAs in pathogenesis of iron overload [19]

The circulating miRNAs are considered a useful non-invasive diagnostic, prognostic, and therapeutic biomarkers for diseases [20–22]. In thalassemia, the erythroid-specific miR-451 was found to be upregulated in erythroid progenitors, and its level is associated with severity [23, 24]. Moreover, significantly higher levels of plasma miR-451 and miR-155 are found in β-thalassemia/hemoglobin E, compared to their healthy controls [25]. During erythroid differentiation, abnormal expression of transferrin receptor gene, in the part regulated by miR-210, leading to increased levels of α-globin, γ-globin, and fetal hemoglobin (HbF), in thalassemia patients [26]. Furthermore, different miRNAs were associated with iron metabolism including, iron uptake, systemic regulation, and storage [27, 28].

In the current study, three miRNAs, miR-let-7d, miR-200b, and miR-122, were measured; the selection was based on their cross association with iron overload [28]; however, the association with excess tissue iron deposition in transfusion-dependent β-thalassemia's have not been investigated. The systemic iron regulation has been controlled by several miRNAs; the expression of divalent metal transporter-1 (Dmt1), which controls the release of endosomal iron from transferrin (Tf), is suppressed by miR-let-7d. In addition, the cytosolic iron storage is enhanced by the suppressive effect of miR-let-7d on Bach1-mediated transcriptional suppression of ferritin, whereas the miR-22, miR-320, and miR-200a regulate the expression of transferrin receptor (TfR1) [19]; the ferritin heavy (FtH1) and light (FtL) chains were regulated by miR-200b [29]. The liver-specific miR-122 comprises around 70% of the total liver miRNAs [30]. MiR-122 regulates the systemic iron storage by indirect suppression of hepcidin gene [31]; the process is mediated by direct suppression of miR-122 via targeting the hemochromatosis (HFE) and hemojuvelin (HJV) genes [31].

Since miRNAs have been implicated in iron hemostasis by post-transcriptional regulation of systemic iron-associated genes, several possibilities exploiting their therapeutic and protective potential in iron-related disorders through alternating their expression have been elicited [28]. Recently, the association between the selected miRNAs, miR-let7-5p [32], miR-200 [28], and miR-122 [31], and iron overload have been established with comprehensive description of their biologic role in iron metabolism. The expression level and the mechanistic pathways were proved via miRNA in vivo experiments. In addition, specific miRNAs mimic and inhibitors models were used to evaluate their therapeutic potential in iron overload disorders. The main objective of the current study is to find out whether the aberrant expression of three iron-related miRNAs is significantly linked to excess tissue iron in TDT patients. In order to illustrate its prediction value, the level of miRNA expression was linked to the level of tissue iron and serum ferritin as a second objective. Accordingly, alternation of miRNA function might have a therapeutic benefit against iron overload, and in fact, they could be targeted for attenuating the iron overload in the TDT patients referred to their confirmed roles on iron metabolism by previous studies.

Subjects and methods

Ethical approval

The study was approved by the ethical committee of the Faculty of Medicine, Ain Shams University. The study was conducted according to the World Medical Association Declaration of Helsinki. Written consent was obtained from all participants because this was an observational study and all diagnoses and management followed standard of care. Participants were assured that all information generated in this study remained confidential. An exclusive identification number was assigned to each patient at the time of enrolment.

Subjects

A case–control observational study was conducted, and peripheral blood samples were collected from TDT "β-thalassemia major" patients ($n = 140$) and 45 NTDT "β-thalassemia intermedia" who represent the negative controls. Both groups were age and sex-matched. The eligible criteria include the following: β-thalassemia major phenotype, aged 3–17 years, and being transfusion-dependent, by fulfilling one of the following criteria: (1) receive at least 10 transfusions and (2) did not start iron chelation therapy, due to lack of the main indicators for start iron chelation therapy which include the following: serum ferritin level below 1,000 μg/l, transferrin (Tf) level ≤ 70%, and or receiving less than 10 transfusions. All samples were collected

before starting iron chelation therapy, then patients were followed up 6 months later for the development of excess iron deposition in hepatic and cardiac tissues. Patients who receive blood transfusion less than ten times, or start iron chelation therapy before sampling, or develop tissue iron overload and patients with inborn errors in iron metabolism were all excluded.

The diagnosis of β-thalassemia major phenotype was confirmed through a cytogenetic analysis to measure the red blood cell indices that reveal microcytic hypochromic anemia, and nucleated red blood cells on a peripheral blood smear. In addition, hemoglobin electrophoresis was done by isoelectric focusing technology to detect the decreased amounts of HbA and increased amounts of hemoglobin F (HbF) after age 12 months, and the clinical severity of anemia. Furthermore, molecular genetic testing was done for the identification of biallelic pathogenic variants in HBB (the gene encoding the hemoglobin subunit beta). Meanwhile, β-thalassemia major phenotype represents the TDT patients; the β-thalassemia intermedia represents the transfusion-independent thalassemia's syndromes; patients with other β-thalassemia phenotypes such as β-thalassemia minor and β-thalassemia-associated with hemoglobinopathies and β-thalassemia receiving blood transfusion for less than two years or irregular transfusion and patients with inborn errors in iron metabolism were all excluded.

Sample's collection

Samples were collected from the Pediatric and Clinical Hematology Department in Ain Shams University hospitals, following the ethical rules of Helsinki. For each participant, two samples are collected; whole blood sample in K2 EDTA Vacutainer tubes used for miRNA extraction, hemoglobin electrophoresis, and cytogenetic analysis; in addition, a serum sample is used for measurement of serum ferritin level.

Assessment of iron overload and dyserythropoietic status

The iron status was quantitatively determined within two hours of sample collection, serum ferritin levels were estimated on serum samples using an immune enzymatic assay on Abbott Architect $i2000_{SR}$ analyzer (Abbott Diagnostics, USA). The tests were conducted according to the manufacturer's specifications and following the laboratory standard operating procedures.

Estimation of myocardial and liver iron using T2* MRI to assess both/either liver or cardiac iron load. All T2* MRI results were obtained using a gradient-echo T2* MRI technique according to extension-myocardial iron overload in thalassemia (E-MIOT) protocol.

Bioinformatics analysis

First, the genes related to iron metabolism were extracted from previous literature, and the related miRNAs and its related molecular functions were extracted from six miRNA database including the Human microRNA Disease Database (HMDD) and miRNet, miRBase, miRTarBase, Tools4miRs, and miRDB databases, and the selected miRNAs "miR-let-7d, miR-122, and miR-200b" were selected based on their significant association with excess iron overload.

Quantitative assay for miRNA expression levels by quantitative real-time PCR (qPCR)

miRNA was extracted from whole blood using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, then RNA quality and concentration were validated by measuring the ratio of the optic density at 260/280 nm using UV spectrophotometry (Eppendorf, Germany). The cDNA was synthesized by reverse transcription reaction using the miScript II RT Kit (Qiagen, Hilden, Germany). The serum expression levels of miR-let-7d, miR-200b, and miR-122-5p were performed using the SYBR-Green fluorescent-based primer assay [hsa-let-7d-3p, ID: MIMAT0004484, sequence: 5′CUAUACGACCUGCUG CCUUUCU], [hsa-miR-200b-3p, ID: MIMAT0000318, sequence: 5′UAAUACUGCCUGGUAAUGAUGA],and [Hs_miR-122-5p, ID: MIMAT0000421, sequence: 5′UGG AGUGUGACAAUGGUGUUUG], and the Hs_ snoR78-2_1 miScript Primer Assay, ID: MS00078679 primer assay was used as a housekeeper gene for normalization. The qPCR was performed in the 5-plex Rotor-Gene PCR System (Qiagen, Hilden, Germany). The 20-µl reaction mixture/reaction consists of 2 × miScript SYBER Green PCR Master Mix, 10 × miScript universal primer, 2 µl primer assay, and 50 pg–3 ng cDNA. Both targets were amplified in duplicates for each sample. The thermal protocol consists of 15 min for HotStarTaq DNA Polymerase activation at 95 °C followed by 40 cycles of denaturation at 94 °C for 15 min, primer annealing for 30 s at 55 °C, and extension at 70 °C for 30 s. To quantify the miRNA expression levels, the $2^{\Delta\Delta Ct}$ method was conducted using snoR78-2_1as an endogenous reference control for normalization purposes. All samples were amplified in duplicates.

Statistical analysis

The analysis of data and figures was performed on GraphPad Prism software statistics version (V.9). Descriptive statistics of various studied parameters were expressed as percentages for dichotomous variables, median (*M*), and range for continuous variables. Circulating miRNA expression were constructed data were reported as mean and 95% CI. Clinical

and sociodemographic data of the patients were expressed in number and percentage (%). For continuous variables among case and control groups, the Mann–Whitney test was used as appropriate. For categorical variables, Fisher's exact test or the chi-square test was used as appropriate. The receiver operating characteristic (ROC) curves and area under the curve (AUC) were used for evaluating the diagnostic and predictive values of the circulating miRNAs for increase tissue iron. Cox regression analysis was conducted to access the association between the expression of miRNAs and increase probability for iron deposition in tissues following repeated blood transfusions. All values are *p*-values were two-sided and p-value < 0.05 was considered statistically significant.

Results

Demographic and clinicopathological characteristics

A total of 140 TDT and 45 NTDT patients matched for age and sex were enrolled in this study. Boys represent 60% of the TDT patients in contrast to 56% in the NTDT. The mean age of the TDT and NTDT patients was 12.3 ± 2.4 (7–17) and 11.0 ± 1.8 (8–15), respectively. Regarding the TDT patients, 42% had received frequent blood transfusion for more than 20 months, and 32% of them started blood transfusion at the age of 6 months or earlier. Splenectomy was performed in 29% of patients, while splenomegaly was detected in 59% of cases. For different iron measures in the TDT patients, 22% of them had a serum ferritin > 1000 µg/l. Liver iron overload (LIC) was detected in 30 patients, whereas 26 cases had excess cardiac iron concentration (T2*) (Table 1). The TDT patients were classified into two subgroups according to the level of iron concentration as detected by MRI. The LIC level > 7 mg/g dw indicates increased risk for complications related to iron overload, and cardiac T2* values < 10 ms predict a higher risk of symptomatic heart failure and mortality [4] (Table 2).

Table 1 Demographic and clinical characteristics of enrolled participants

Group	TDT ($n=140$)	NTDT ($n=45$)
Age: mean ± SD (range)	12.3 ± 2.4 (7–17)	11.0 ± 1.8 (8–15)
Gender: *n* (%)		
Boys	84 (60)	25 (56)
Girls	56 (40)	20 (44)
Age of start BT		
≤ 6 months	45 (32)	
> 6 months	95 (68)	
BT frequency		
Every 2 weeks	60 (42)	
> 2 weeks	80 (58)	
Splenectomy		
Yes	41(29)	
No	99 (71)	
Splenomegaly		
Negative	57 (41)	
Positive	83 (59)	
Serum ferritin		
≤ 1000 µg/l	109 (78)	
> 1000 µg/l	31 (22)	
Iron deposition in tissue		
Negative	84 (60)	
Positive	56 (40)	
T2*		
Negative	124 (84)	
Positive	26 (16)	
LIC		
Negative	110 (79)	
Positive	30 (21)	

TDT transfusion-dependent β-thalassemia, *NTDT* non-transfusion-dependent β-thalassemia, *T2** cardiac iron overload by magnetic resonance imaging, *LIC* liver iron concentration by magnetic resonance imaging; the cut-off of ferritin level was calculated as median value of ferritin among the β-thalassemia patients. *n* = number of cases, %: percentage of cases

Expression pattern miR-let-7d-5p, miR-200b-3p, and miR-122-5p in β-thalassemia patients

There was a highly significant difference (p-value = 0.001) in the three evaluated miRNA expression in TDT compared to matched NTDT; miR-let-7d-5p (Fig. 1a) and miR-200b-3p (Fig. 1b) are significantly downregulated 1.6- and 6.5-fold, respectively, in TDT patients compared to NTDT. On the contrary, miR-122-5p expression was increased twofold (Fig. 1c). Moreover, patients who received frequent blood transfusion for more than 20 months showed a significantly lower expression of miR-let-7d-5p and miR-200b-3p, whereas miR-122-5p expression was significantly higher. Aberrant expression of miR-let-7d-5p and miR-122-5p were significantly associated with higher serum ferritin (> 1000 µg/l), whereas miR-200b-3p did not (Table 2). We further measure the expression of miRNAs in 10 healthy children (data are not presented); the obtained results showed a significant difference between the expression levels of each miRNA in healthy children and the NTDT patients ($p < 0.05$), which means that the interested miRNAs are related to thalassemia.

Table2 Expression level of miRNAs in whole blood of β-thalassemia major children

Group	*N* [%]	miRNA expression [$\log_{10}$] median [range]					
		miR-let-7d-5p	Statistics	miR-200b-3p	Statistics	miR-122-5p	Statistics
Subjects							
TDT	140	9.6 (4.5–24)	$U=1099$	0.7 [0.14–2.6]	$U=0$	83 (62–116)	$U=56$
NTDT	45	15.4 (11–31)	$p=0.001$	4.6 [2.6–6.4]	$p=0.001$	41 (21–70)	$p=0.001$
No. of transfusions in TDT							
≤ 20 months	95 [68]	11 (4.5–24)	$U=829$	0.9 [0.2–2.6]	$U=908$	77 (62–106)	$U=764$
> 20 months	45 [32]	6.8 (4.0–14)	$p=0.0001$	0.5 [0.1–1.4]	$p=0.0001$	93 (63–116)	$p=0.0001$
Serum ferritin							
≤ 1000 µg/l	109 [78]	11.2 (4.5–24)	$U=696$	0.7 [0.2–2.6]	$U=1588$	77 (62–98)	$U=1225$
> 1000 µg/l	31 [22]	7.0 [4–11]	$p=0.001$	0.6 [0.1–2.5]	$p=0.62$	86 (74–116)	$p=0.03$
LIC (mg/g dw) ($n=30$)							
< 7	12 [40]	8 (5.2–11)	$U=43$	0.9 [0.6–2.6]	$U=17.5$	72 (63–93)	$U=33.5$
> 7	18 [60]	5.6 (4.2–9)	$p=0.008$	0.5 [0.2–0.9]	$p=0.0001$	85 [70–98]	$p=0.001$
Cardiac T2*ms ($n=26$)							
> 10	17 [65]	8 (5.2–11)	$U=5$	0.9 [0.7–2.6]	$U=0.5$	70 (63–79)	$U=2$
< 10 ms	9 [35]	5 (4.2–6)	$p=0.0001$	0.4 [0.2–0.7]	$p=0.0001$	86 (78–98)	$p=0.0001$

TDT transfusion-dependent β-thalassemia, *NTDT* non-transfusion-dependent β-thalassemia, *T2** cardiac iron overload by magnetic resonance imaging, *LIC* liver iron concentration by magnetic resonance imaging; the cut-off of ferritin level was calculated as median value of ferritin among the β-thalassemia patients. *dw* dry weight, *ms* milliseconds. LIC values > 7 mg/g dw are used to indicate increased risk for complications related to iron overload in TDT. T2* technique in milliseconds used for quantification of the cardiac iron concentration, when T2* gets shorter indicates that myocardial iron concentration increases, *U* Mann–Whitney *U* value

The association between circulating miRNAs and iron overload in transfusion-dependent β-thalassemia patients

There was a statistically significant difference in the evaluated miRNA expression according to the status of iron deposition in tissues; lower expression levels of miR-let-7d-5p and miR-200b-3p were significantly associated with excess liver iron concentration (LIC > 7 mg/g dw) (Fig. 1d), and increased cardiac iron concentration (T2 < 10 ms) (Fig. 1e). On the other hand, miR-122-3p was significantly upregulated in TDT patients with increase LIC > 77 mg/g dw (Fig. 1f) and excess cardiac iron concentrations (T2 < 10 ms) (Fig. 1g); however, overexpressed miR-122-3p was significantly associated with excess LIC > 7 mg/g dw (Fig. 1h) and T2 < 10 ms (Fig. 1i). The median expression level of miRNAs is presented in Table 2.

Circulating miRNAs are significantly associated with iron hemostasis in transfusion-dependent β-thalassemia major patients

To investigate the potential of circulatory miRNAs (miR-let-7d-5p, miR-200b-3p, and miR-122-5p) as predictor biomarkers for iron overload in TDT patients, ROC curve analysis was performed (Fig. 2). At an optimum cut-off value < 13.5, the AUC is 0.82 (95% CI: 0.8–0.9); the miR-let-7d-5p can early predict tissue iron hemostasis in TDT patients. The calculated biomarker sensitivity and specificity were 72% and 87%, respectively. Similarly, miR-200b-3p and miR-122-5p also are strong predictors for excess tissue iron, the obtained area under the curve (AUC) of 0.9 (95% CI: 0.87–0.96) and 0.9 (95% CI: 0.8–0.9) at a cut-off of < 1.2 and > 62 l, respectively. The calculated biomarker sensitivities are 78% and 92%, specificities are 91% and 82% for miR-200b-3p and miR-122-5p, respectively.

Circulating miRNAs are associated with increase probability for iron deposition in transfusion-dependent β-thalassemia patients

The association between circulatory miRNAs (miR-let-7d-5p, miR-200b-3p, and miR-122-5p) and the risk of increased tissue iron is summarized in Table 3. The logistic regression analysis revealed that tissue iron overload was more probable with lower expression levels of miR-let-7d-5p, miR-200b-5p, and overexpression of miR-122-3p.

Discussion

Iron overload in transfusion-dependent β-thalassemia patients is affecting patients' quality of life and different strategies have been implemented for the prevention and treatment of this pathology, which have not yielded the expected results. Magnetic resonance imaging (MRI) remains the gold standard method for assessment of excess tissue iron. Thus, determining circulating biomarkers for

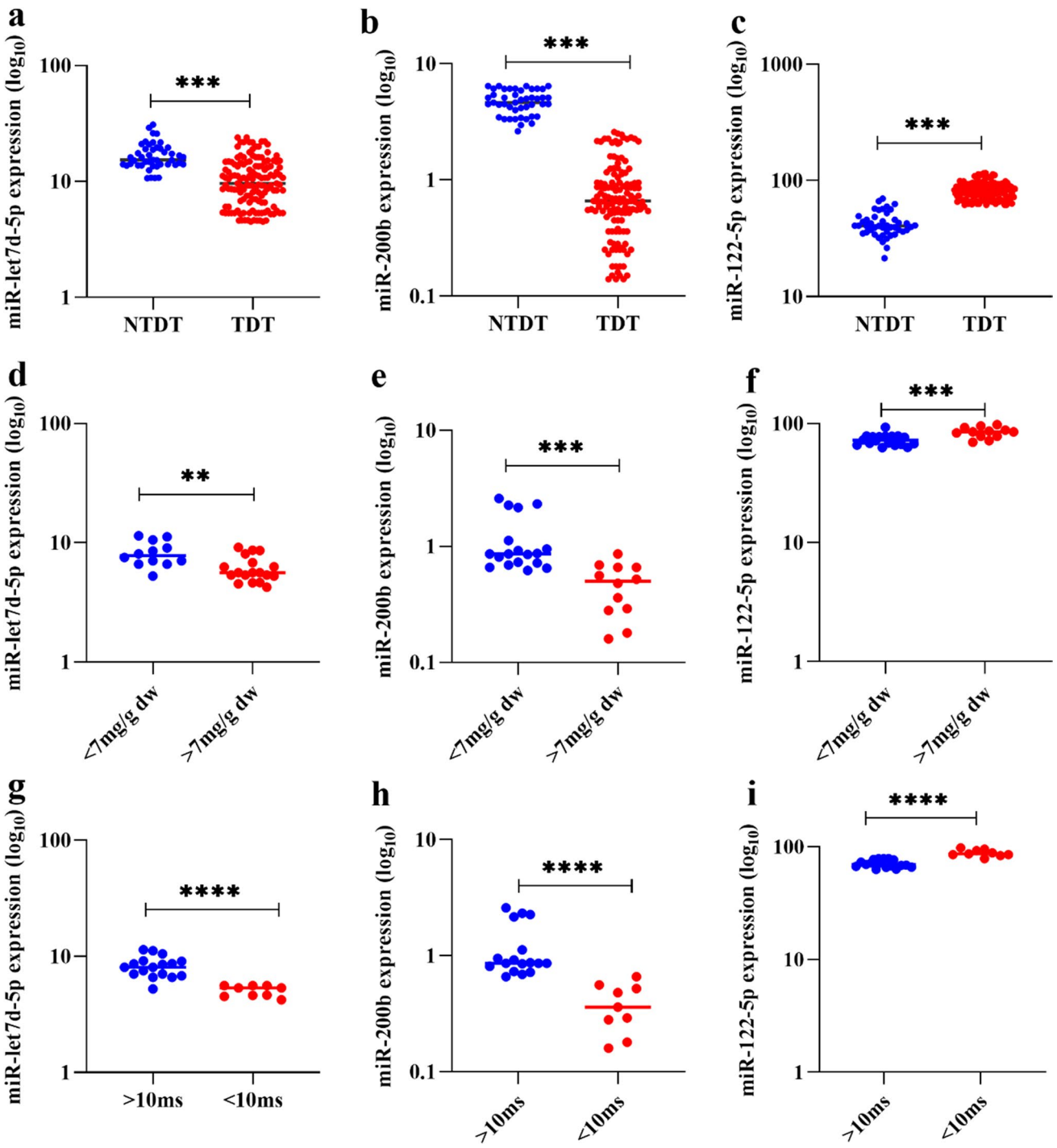

Fig. 1 Expression level of miR-let-7d-5p, miR-200b-3p, and miR-122-5p in TDT and NTDT patients (**a**, **b**, **c**), in TDT patients with excess LIC (**d**, **e**, **f**), and TDT patients with excess cardiac iron concentration T2 (**g**, **h**, **i**). TDT transfusion-dependent β-thalassemia, NTDT non-transfusion-dependent β-thalassemia, $T2^*$ cardiac iron overload by magnetic resonance imaging, LIC liver iron concentration by magnetic resonance imaging, dw dry weight, ms milliseconds. LIC values > 7 mg/g dw are used to indicate increased risk for complications related to iron overload in TDT. T2* technique in milliseconds used for quantification of the cardiac iron concentration, when T2* gets shorter indicates that myocardial iron concentration increases. ***p-value = 0.001, ****p-value = 0.0001

iron overload is important to guide changes in iron chelation therapy instead of the MRI and provide a therapeutic target to inhibit iron deposition in tissues, which would be useful in TDT patients. Being quantifiable and sufficiently stable, the circulating microRNAs can serve as clinical biomarkers [22, 33, 34]. The present work evaluated the

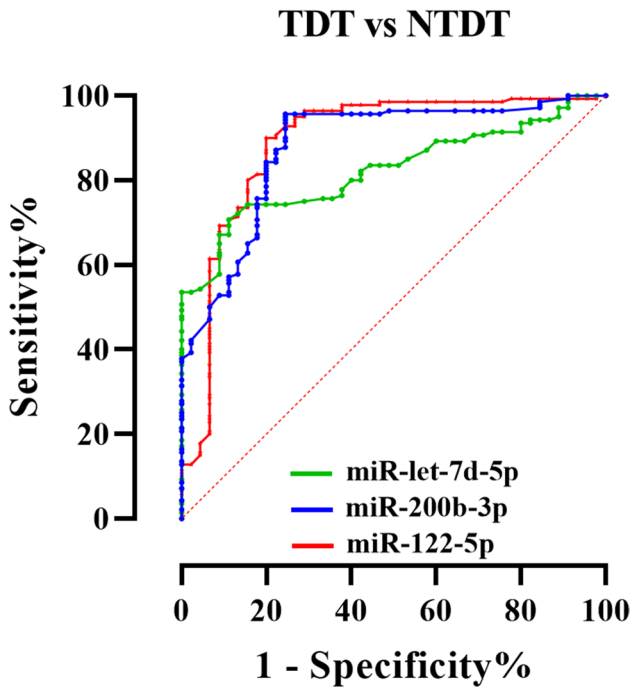

Fig. 2 ROC curve illustrates the significant potential of miRNAs to discriminate between TDT patients with low vs high tissue "LIC and or T2" iron concentration

Table 3 Logistic regression analysis using miR-let-7d-5p, miR-200b-3p, and miR-122-5p gene expression as predictors for increased risk for excess liver and cardiac iron concentration in TDT patients

Variable	LIC (mg/g dw) OR (95%CI)	T2*(ms) OR (95%CI
miR-let-7d-5p ($\log_{10}$)	1.9(0.9–1.0), $p=0.002$	1.2 (0.8–0.9), $p=0.02$
miR-200-3p ($\log_{10}$)	1.7(1.2–2.4), $p=0.003$	1.4(1.2–1.6), $p=0.001$
miR-122-5p ($\log_{10}$)	1.9(1.2–2.9), $p=0.005$	1.4(1.1–1.7), $p=0.006$
Model	2 log likelihood:17.4 χ^2:95.4, $p=0.001$	2 log likelihood:50.0 χ^2:76.7, $p=0.001$

OR adjusted odds ratio, *CI* confidence interval; *LIC* liver iron concentration, *T2** cardiac iron overload by magnetic resonance imaging

value of miR-let-7-5p, miR-200b-3p, and miR-122-5p in assessment for iron overload in transfusion-dependent β-thalassemia major.

The current study showed a significant difference in the level of the circulating miRNA between TDT and NTDT patients. Moreover, the frequency of blood transfusion had a significant effect on miR-let-7d-5p and miR-200b-3p expression with lower values, and higher expression levels of miR-122-5p in patients with more frequent transfusions. With regard to iron load, miRNAs were able to predict hepatic and cardiac iron accumulation. Moreover, regression analysis revealed that aberrant expression of measured miRNAs was substantially related to increased iron hemostasis in cardiac and hepatic tissues.

Since the discovery of miRNAs, they have been sorted as the biggest subclass of non-coding RNA and are thought to regulate 30–60% of all protein-coding genes and regulate the iron acquisition process [35]. For instance, the overexpression of miR-210 and/or miR-320 decreases the abundance of transferrin receptor (TfR) protein in MCF7 cells (human breast adenocarcinoma cell line) and plasma membrane, respectively [36, 37]. Moreover, lactoferrin, the iron-binding protein, in human cancer cells is mainly regulated by miRNAs (miR-214 and miR-584) [38, 39]. The miRNAs also regulated iron export, where the cellular exporter ferroportin (FPN) was found to be targeted by miR-485-3p, affecting iron homeostasis [40]. Consistent with our results, the systemic regulation of iron hemostasis is influenced by the expression of miR-122 (plasma and liver iron) [31], miR-200b (iron storage) [41, 42], and miR-let-7d (ferritin expression and synthesis) [32, 43, 44]. On the other hand, the cytosolic iron showed the ability to promote miRNA precursors processing through poly(C)-binding protein 2 [45].

Previous reports linked miR-200b and iron storage. The intracellular iron storage is regulated via the ferritin heavy chain FtH1 ferroxidase activity which has been regulated by miR-200b [41]. By consistency, lower expression of miR-200b is significantly correlated with high FtH1 concentrations. It has been demonstrated that in vitro transfection with miR-200b improves the sensitivity to doxorubicin in breast cancer. Additionally, increase aggressiveness of cancer and resistance to therapy was observed in patients with lower expression levels of miR-200b, in turn emphasizing the clinical relevance of miR-200b on iron regulation [29].

Exclusively, miR-122 is expressed in the liver. In the current study, circulating miR-122-5p expression was significantly associated with excess liver and cardiac iron concentration in TDT patients. It has been demonstrated that in vivo depletion of miR-122 lower transferrin iron-binding capacity, reduces both serum and tissue iron contents, and resulted in impaired hematopoiesis [31]. Furthermore, miR-122 directly targets the human hemojuvelin (HJV) and hemochromatosis (HFE) protein. In hereditary hemochromatosis, the depression of HJV and HFE genes expression levels are contributed to depletion of miR-122 [19]. These mechanisms are consistent with our results which indicates that the reduction in miR-122-3p and overexpression of miR-200-5p results in iron precipitation of iron in tissues of iron overload, trying to maintain iron hemostasis and getting rid of extracellular iron.

The divalent metal transporter 1 (DMT1) is a metal-iron transporter gene which encodes for four mRNA transcripts variants, which differ from each other by lacking or containing an iron regulatory element (IRE) [46]. In erythroid cells, it has been demonstrated that miR-let-7d regulates the

DMT1-IRE expression at mRNA and protein levels during differentiation process. The trend of expression of miR-let-7d is inversely correlated with the CD34 expression [47]. In the current study, lower expression level of miR-let-7d was significantly associated with excess liver and cardiac iron. By consistency, downregulation of miR-let-7d increases the DMT1-IRE expression level thus resulted in accumulation of iron in endosomes and finally impairs erythroid differentiation. In addition, miR-let-7d contributes to the uptake of non-transferrin-bound iron through the regulation of the DMT1 gene [48].

The potential of miRNA to serve as therapeutic targets for the treatment of excess iron disorders has recently been investigated through alternating the functions of the miRNAs. In vivo transgenic miRNAs mimic and knock-out approaches were used to modulate miRNAs function in iron overload disorders in order to access their value as novel therapeutic targets. In vitro mimic of miR-let-7 protects hepatocyte and neural tissue from oxidant injury induced by iron overload via indirect suppression of HMOX1 [5, 32] and DMT1, respectively [47]. In addition, miR-122 might target for therapeutic intervention in excess iron disorders [28].

The current study has some limitations that may suggest further large-scale validation of the suggested biomarkers and selective phenotype of β-thalassemia patients.

Conclusion

The aberrant expression of miR-let-7d, miR-122, and miR-200b in major patients with transfusion-dependent β-thalassemia reflects disturbed iron hemostasis. Therefore, they are sensitive, specific, and non-invasive biomarkers that can predict excessive cellular damage at an early stage and guide clinicians on the iron status in TDT patients.

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Declarations

Ethics approval The study protocol number FMASU 28115/2017 was approved by the research ethics committee of the Faculty of Medicine, Ain Shams University. All participants were informed of the objectives of the study and signed informed-consent forms.

Conflict of interest The authors declare no competing interests.

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