Whole Blood miR-210, miR-122, miR-223 Expression Levels and Their Relationship With Iron Status Parameters and Hypercoagulability Indices in Children With Iron Deficiency Anemia

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Summary: MicroRNAs have the potential to regulate systemic and cellular iron homeostasis at multiple points. In iron deficiency anemia (IDA), hypoxia, platelet reactivity, and potentially microRNAs play a role in the development of hypercoagulability. A total of 57 children diagnosed with IDA between October 2016 and October 2017 and 48 healthy children were included in this cross-sectional study. Blood count parameters, serum iron, transferrin saturation, ferritin level, maximum clot firmness (MCF), and clot formation time index, which are indicators of hypercoagulability in rotational thromboelastometry test, of the IDA and control groups obtained in our previous study were recorded. miR-210, miR-122, and miR-223 levels were analyzed. There was no difference in the miR-210, miR-122, and miR-223 levels between the IDA and control groups. Patients with hemoglobin (Hb) <8 g/dL had higher miR-210 levels than patients with Hb > 8 g/dL ($P < 0.05$). There was a negative correlation between miR-210 and Hb and ferritin levels, a positive correlation between miR-122 and ferritin levels, and a negative correlation between miR-223 and MCF index. In IDA, there is a close relationship between the severity of anemia and miR-210, and miR-210 expression is slightly increased in those with severe anemia. miR-210 and miR-122 collectively play a role in maintaining the iron balance. The correlation between miR-223, a platelet function regulator, and the MCF index, suggested that miR-223 has a role in the development of hypercoagulability in IDA.

Key Words: iron deficiency anemia, iron status, hypercoagulability, microRNA

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Iron deficiency is the most common micronutrient deficiency across the world. It affects ~25% of the world's population.[1] Iron is critic for oxygen transport, DNA synthesis, adenosine triphosphate production, and cellular proliferation. The physiologic adaptation mechanisms responsible for the preservation of iron, which is necessary for the maintenance of vital functions, in iron deficiency are not yet fully understood.[2]

Transferrin-bound iron enters cells that require iron, mainly through a transferrin receptor (TfR) 1-mediated mechanism. During cellular iron deficiency, TfR1 expression increases, whereas during iron excess, TfR1 expression decreases. Erythroblasts, which require large amounts of iron for hemoglobin (Hb) synthesis, also express very high levels of TfR1.[3] TfR1 expression is regulated at both transcriptional and posttranscriptional levels. At the transcriptional level, in conditions of hypoxia or iron deficiency, the expression of hypoxia-inducible factors (HIF-1α and HIF-2α) increases, and these proteins increase TfR1 transcription by binding to the hypoxia response element in the TfR gene promoter.[4] At the posttranscriptional level, another complex mechanism called the iron regulator protein (IRP)—iron responsive element system plays an important role.[5] miR-210 plays a role in regulation at the posttranscriptional level. The specific roles of TfR2 in erythropoiesis are not entirely elucidated, observations support that TfR2 may play a role in the regulation of erythrocyte numbers by interacting with erythropoietin (EPO) receptor, especially under conditions of iron deficiency.[3]

miR-210 is highly expressed in late-stage erythrocytes and is thought to play a critical role in erythrocyte maturation.[6] It is the major microRNA induced under hypoxic conditions and contributes to cellular adaptation to the hypoxic environment.[7] TfR and iron-sulfur cluster scaffold protein (ISCU) are the target molecules of miR-210.[8] Under hypoxic conditions and in iron deficiency, miR-210 is induced by HIF-1α. miR-210 suppresses ISCU. The suppression of ISCU leads to the activation of IRP.[8] miR-210-mediated IRP activation accelerates the binding of IRP1's TfR to the iron responsive elements located in the mRNA's 3′-untranslated region. This binding stabilizes the TfR mRNA and increases TfR1 protein expression. This causes an increase in the uptake of iron ions into the cell.[3] If the miR-210 is excessively elevated, TfR is directly suppressed and iron uptake into the cell is decreased.[8]

miR-122 is primarily synthesized in the liver and is also called liver-specific microRNA.[9,10] It plays a role in cholesterol and fatty acid metabolism, apoptosis, hepatocellular carcinoma growth, and hepatitis C virus propagation. miR-122 is released into the bloodstream, and it is suggested that changes in its blood levels are a predictive marker for viral, alcohol, and chemical-induced liver damage.[10] miR-122 regulates the expression of genes involved in maintaining iron metabolism (hemochromatosis, hemojuvelin, bone morphogenetic protein receptor type 1A, and hepcidin).[9] It plays a role through EPO synthesis in the development of chronic inflammation anemia.[10]

Rotational thromboelastometry is used to determine procoagulant conditions in many diseases.[11–14] Clot formation time (CFT) and maximum clot firmness (MCF) index, which are among rotational thromboelastometry parameters, are the most commonly used variables to evaluate hypercoagulability, and MCF is closely associated with platelet count and function.[15,16] It is a direct marker of the maximum dynamic

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properties of GPIIb/IIIa-mediated fibrin and platelet aggregation.[17] Increased platelet reactivity in iron deficiency anemia (IDA) is a factor contributing to the development of thrombosis. miR-223 is highly expressed in platelets and megakaryocytes.[18] miR-223 potentiates platelet aggregation induced by thrombin and other agonists and plays an important role in thrombus formation and stability.[19–21] It can regulate the $P2Y_{12}$ receptor.[22] Studies[23,24] have reported that there is a close relationship between platelet miR-223 and platelet reactivity index and that the inappropriate activation of platelets plays a role in the pathogenesis of a wide variety of important diseases, including stroke and myocardial infarction.[18]

This study investigated the relationship of miR-210 and miR-122 with iron status and anemia, and the relationship of miR-223 with CFT and MCF index, which are indicators of hypercoagulability, in children with IDA.

MATERIALS AND METHODS

A total of 57 children diagnosed with IDA between October 2016 and October 2017 (IDA group) and 48 healthy children (control group) were included in the study. Complete blood count, serum iron, transferrin saturation, ferritin levels, intrinsically activated thromboelastometry (INTEM) and extrinsically activated thromboelastometry CFT, MCF indices of the IDA, and control groups were obtained from our previous study.[25]

Serum or plasma is considered a microRNA reservoir in most of the studies. However, microRNA isolation from serum or plasma samples requires additional processing, and microRNA expression patterns may be sensitive to slight variations during these processing steps.[26] In addition, since cellular microRNAs are present in very low amounts in the extracellular environment, even the slightest contamination of serum or plasma samples with blood cells significantly alter their microRNA expression profiles.[26,27] Considering the hematopoietic origin of miR-210[28] and miR-223,[29] the higher concentration thereof in whole blood compared with serum, and the difficulties in microRNA profiling in serum or plasma, microRNA analyses were performed in whole blood.

Whole blood samples were stored at −80°C in our previous study to analyze microRNA levels. miR-210, miR-122, and miR-223 expression levels were analyzed using reverse transcription (RT)-polymerase chain reaction (PCR). The IDA and control groups were compared in terms of microRNA levels. The correlations between miR-210 and miR-122 and Hb, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW), serum iron, and transferrin saturation and ferritin levels were examined. The correlations between CFT and MCF indices and miR-223 were examined to investigate the role of microRNAs in the development of hypercoagulability.

The study was approved by the ethics committee (ethic approval no: 80558721/25) and was conducted in accordance with the principles of Helsinki. Written informed consent was obtained from the legal guardians of the participating children.

Collection of Blood Samples

For microRNA, 3 mL blood samples were collected into blood RNA tubes (Tempus, Austin, TX) containing 6 mL stabilizing reagent and stored at −80°C until the day of analysis.

MicroRNA Analysis

MicroRNA Isolation Procedures

MicroRNA isolation was performed using a microRNA isolation kit (RTA, Turkey) as per the study protocol. After the tubes were thawed on ice, 350 μL lysis buffer and 20 μL proteinase K solution were added to 200 μL whole blood in accordance with the isolation protocol. It was mixed gently by pipetting and incubated for 30 minutes at 60°C and then centrifuged for 1 minute at 11,000*g*. The resulting supernatant was transferred to a different tube. Then, 20 μL of DNase I working buffer and 10 μL of DNase I were added and incubated for 15 minutes at room temperature; 350 μL binding buffer was added to the liquid in the tube and mixed by pipetting. The entire lysate was transferred to the microRNA Spin column and centrifuged for 1 minute at 11,000*g*. The filtered column was placed in a different collection tube. After adding 500 μL wash buffer to the column and centrifuging for 1 minute at 11,000*g*, it was placed in a 1.5 μL Eppendorf tube. Next, 50 μL Elution buffer, previously incubated at 65°C, was transferred to the filter of the column and incubated for 3 minutes at room temperature. After incubation, microRNA was precipitated into the Eppendorf tube by centrifuging for 1 minute at 8000*g*. Nucleic acid measurements of the microRNA specimens of the samples were made using a fluorescent spectrophotometry device (Colibri Microvolume Spectrometer; Berthold Titertek Instruments Inc., Germany).

Poly (A) Polymerase Procedure

For the 0.1 μg/μL total microgram microRNA specimens obtained from blood samples, 3′ adenine tails were created using a poly (A) polymerase tailing kit (ABM, Canada) before the complementary DNA (cDNA) procedure. A mixture of 0.2 μg total microRNA, 0.5 mM adenosine triphosphate (10 mM), 1× poly (A) polymerase reaction buffer (5×), 2.5 mM $MnCl_2$ (25 mM), and 1 U poly (A) polymerase (1 U/μL) was prepared, the final volume was diluted (to volume) with 25 μL nuclease-free distilled water (dH_2O) on ice, and the mixture was incubated for 20 minutes at 37°C. The reaction was terminated at 20 minutes at 65°C and the poly (A) tail-added microRNA products were stored at −80°C for further application.

RT Procedure

Poly (A) hairpin RT primers (Diagen, Turkey) designed for each region were used for the RT procedures. Viva cDNA Synthesis Kit (Vivantis, Malaysia) was used to create cDNA from 0.01 μg/μL total microRNA specimens, which were spiked with poly (A) tail, by RT. In the first stage, to bind the RT primers and the 3′-identification regions (seed regions) of the microRNAs, 10 μL reaction was prepared by adding 1 mM dNTP mixture (10 mM), 1 mM RT primer (10 mM), and 0.01 μg total poly (A) tailed microRNA, and the final volume was diluted (to volume) with nuclease-free dH_2O. This mixture was incubated for 10 minutes at 65°C to bind the primers. The mixture was kept on ice for 5 minutes after the procedure, and 7.5 μL nuclease-free dH_2O, 1× m-MuLV buffer (10×), and 100 U M-MuLV RT (200 U/μL) enzyme were added and mixed. Then, 20 μL microRNA component in total was incubated for 1 hour at 42°C and the resulting enzyme was inactivated for 20 minutes at 65°C. The prepared cDNA samples were stored at −20°C.

MicroRNA Hydrolysis Probe Quantitative PCR Gene Expression Procedure

Optimized hsa-miR-122-5p (Diagen), hsa-miR-210-3p (Diagen), and hsa-miR-223-3p (Diagen) were used for real-time

TABLE 1. The Sequences of Probes Used for miRNA Assays

miRNA	Sequences 3′-5′	
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA	
hsa-miR-210-3p	CUGUGCGUGUGACAGCGGCUGA	
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	
SNORD47 NR_002746	AACCAAUGAUGUAAUGAUUCUGCCAAAUGAA AUAUAAUGAUAUCACUGUAAAACC GUUCCAUUUUGAUUCUGAGGUU	
RNU6-1 RNU6A	GUGCUCGCUUCGGCAGCACAUAUAC UAAAAUUGGAACGAUACAGAGAAGAUUAGCAU GGCCCCUGCGCAAGGAUGACACGCAAAUUCGU GAAGCGUUCCAUAUUUUU	
miRNA design code	Primer codes	Sequences
RT primer_miR223_S-loop	S-loop_miR223_RT	GCTACCAGAAGCCATTAGTGTCCAA GTACTGCAATGAGAAGTTCGTCCTGCCGG GTAGCTTTTTTTTTTGGGGTA
Forward primer	S-loop_miR223_F	GAGCTGTGTCAGTTTGTCA
RT primer_miR122_S-loop	S-loop_miR122_RT	GCTACCAGAAGCCATTAGTGTCCAAGTACT GCAATGAGAAGTTCGTCCTGCCGGGTAG CTTTTTTTTTCAAACA
Forward primer_miR122	S-loop_miR122_F	GATGTTTGGAGTGTGACAATG
RT primer_ miR210-loop	S-loop_miR210_3pRT	GCTACCAGAAGCCAT TAGTGTCCAAGTACTGCAATGAGAAGTTC GTCCTGCCGGGTAGCTTTTTTTTTTCAGCCG
Forward primer_ miR210	S-loop_miR210_3pF	GATTCTGTGCGTGTGACAG
RT primer_SNORD47 NR_002746	SNORD47_RT primer	GCTACCAGAAGCCATTAGTGTCCAAGTAC TGCAATGAGAAGTTCGTCCTGCCGGG TAGCTTTTTTTTTTAACCTC
Forward primer_SNORD47 NR_002746	SNORD47_F	GTTGCCAATGATGTAATGATTCTG
RT primer_RNU6A_S-loop	S-loop_ RNU6A_RT	GCTACCAGAAGCCATTAGTGTCCAAGTACTG CAATGAGAAGTTCGTCCTGCCGGGTAGCTTT TTTTTTTAAAAATAT
Forward primer_RNU6A	S-loop_RNU6A_F	GCAAATTCGTGAAGCGTTC
UniV primer_S-loop	S-loop_UniV_R	AGAAGCCATTAGTGTCCAAG
UniV probe_S-loop (F-specific)	S-loop_UniV_P	5′-6-FAM-GGCAGGACGAACTTCTCATTG-BHQ-1-3′

miRNA indicates microRNA; RT, reverse transcriptase; UniV, universal.

PCR analyzes, and small RNA Snord47 (Diagen) forward primers were used as reference genes. For complementary sequence, universal reverse primers and hydrolysis probe (Diagen) compatible with RT primers were used. Lyophilized primers were dissolved in nuclease-free dH_2O to obtain 100 mM. Diag 2× master mix (Diagen) was used for the quantitative PCR procedure. Then, 1× master mix (2×), 0.5 mM forward and reverse primers (10 mM), 0.4 mM probe (10 mM), 2 μL cDNA, and 0.2 μL Taq DNA polymerase (Vivantis) and nuclease-free dH_2O were mixed, and the volume was diluted to 20 μL by stirring. The Stem-loop RT-quantitative PCR method was used to verify the specificity of the probes used in microRNA analysis.[30] The sequences of probes used for microRNA assays are presented in Table 1. MicroRNA threshold cycle (C_t) values were calculated for each sample, and the fold change of relative expression of each microRNA was calculated by the $2^{-\Delta\Delta Ct}$ method.[31,32]

Statistical Analysis

Statistical analyzes were performed using SPSS for Windows, version 15.0 software (SPSS Inc., Chicago, IL). Descriptive statistics were expressed in mean ± SD for normally distributed variables and in median and interquartile range for other variables. Categorical data were compared using the χ^2 test. The Kolmogorov-Smirnov test was used to analyze the normality of distribution for continuous variables. Normally distributed continuous variables were compared using the independent *t* test, whereas the Mann-Whitney *U* test was used to compare abnormally distributed variables. To assess the relationship between the 2 variables, the Spearman correlation test was applied. Differences were considered statistically significant with *P*-values < 0.05.

RESULTS

There was no difference in age and sex distribution between the IDA and control groups ($P > 0.05$; for both). The Hb, MCV, MCH levels of the IDA group were lower than those of the control group ($P < 0.001$; for all), and their platelet count and RDW levels was higher ($P = 0.003$ and $P < 0.001$, respectively). Serum iron, transferrin saturation, and ferritin levels of the IDA group were lower than those of the control group ($P < 0.001$; for all). The INTEM CFT index of the IDA group was lower than that of the control group ($P = 0.025$), and the extrinsically activated thromboelastometry MCF and INTEM MCF indexes of the IDA group were higher than those of the control group ($P < 0.001$; for both).[25] The clinical characteristics and laboratory parameters of the IDA and control groups are presented in Table 2. There was no difference between the IDA and control groups in terms of the expression levels of miR-210, miR-122, and miR-223 ($P > 0.05$; for all) (Table 3).

According to the World Health Organization (WHO) 2011 "Hemoglobin concentrations for the diagnosis of

TABLE 2. Clinical Characteristics and Laboratory Parameters of the IDA and Control Groups[25]

	Median (IQR)		
	IDA Group (N = 57)	Control Group (N = 48)	*P*
Age (y)	11.0 (2-14)	9.9 (8-13)	NS
Female/male	37/20	23/25	NS
Hb (g/dL)	8.4 (6.8-9.4)	12.80 (12.0-13.2)	< 0.001
MCV (fL)	58.20 (54.35-62.75)	79 (77-81)	< 0.001
MCH (pg)	17.90 (16.05-19.45)	26 (24.7-27.55)	< 0.001
RDW (%)	20.20 (18.75-22.15)	13.40 (12.97-15.80)	< 0.001
Platelet ($\times 10^9$/L)	350.50 (280-436.25)	285.50 (242.25-370.25)	0.003
Serum iron (μg/dL)	20 (15-27.5)	37.50 (29-61)	< 0.001
Transferrin saturation (%)	4.66 (3.48-6.30)	16.6 (11.19-21.22)	< 0.001
Ferritin (ng/mL)	5.50 (3.65-9.70)	28.24 (22.25-36.90)	< 0.001
EXTEM CFT (s)	87 (73-107)	90.5 (78.25-101.0)	NS
EXTEM MCF (mean ± SD) (mm)	69.64 ± 0.73	63.93 ± 0.6	< 0.001
INTEM CFT (mean ± SD) (s)	69 (59-80.50)	77.50 (67.25-86)	0.025
INTEM MCF (mean ± SD) (mm)	67.05 ± 0.58	62.14 ± 0.60	< 0.001

References values: Hb: 11.5 to 13.5 g/dL, MCV: 77 to 86 fL, MCH: 25 to 29 pg, platelet: $300 \pm 50 \times 10^9$/L, serum iron: 73 ± 3.4 (23 to 123) mg/dL; transferrin saturation: 25 ± 1.2% (7% to 43%), ferritin: 7 to 140 mg/mL.

CFT indicates clot formation time; EXTEM, extrinsically activated thromboelastometry; Hb, hemoglobin; IDA, iron deficiency anemia; INTEM, intrinsically activated thromboelastometry; IQR, interquartile range; MCF, maximum clot firmness; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; NS, nonsignificant; RDW, red cell distribution width.

From Lanzkowsky et al.[33]

anemia and assessment of severity" criteria, the IDA group was divided into 2 groups as severely anemic (Hb < 8 g/dL) and moderately anemic patients (Hb > 8 g/dL).[34] The Hb, MCV, MCH, serum iron, transferrin saturation, and ferritin levels of patients with Hb < 8 g/dL were lower than those of patients with Hb > 8 g/dL ($P < 0.001$, <0.001, <0.001, <0.001, $P = 0.001$, and 0.001, respectively), and their RDW value were higher ($P < 0.001$). There was no difference between the 2 groups in terms of platelet count (Table 4).

TABLE 4. Clinical Characteristics and Laboratory Parameters of Patients With Hb <8 g/dL and Patients With Hb > 8 g/dL

	Median (IQR)		
	Hb < 8 g/dL (N = 27)	Hb > 8 g/dL (N = 30)	*P*
Hb (mean ± SD) (g/dL)	6.71 ± 0.87	9.44 ± 0.68	< 0.001
MCV (fL)	54.70 (52.65-58.05)	60.70 (58.20-66.10)	< 0.001
MCH (pg)	16.05 (15.15-17.28)	19.2 (17.9-21)	< 0.001
RDW (%)	21.50 (20.18-23.15)	19 (17.5-20.5)	< 0.001
Platelet (mean ± SD) ($\times 10^9$/L)	358.60 ± 127.61	359.58 ± 142.55	NS
Serum iron (μg/dL)	17 (13-20.25)	24 (18-42)	< 0.001
Transferrin saturation (%)	3.82 (3.10-4.84)	5.4 (4.20-11.70)	0.001
Ferritin (ng/mL)	4 (2.82-5.96)	8.4 (4.1-12)	0.001

Hb indicates hemoglobin; IQR, interquartile range; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; NS, nonsignificant; RDW, red cell distribution width.

Expression level of miR-210 was higher among patients with Hb < 8 g/dL compared with patients with Hb > 8 g/dL (21.86 [−19.91 to 63.64] vs. 5.41 [0.17-10.65], $P = 0.024$). There was no difference between patients with Hb <8 g/dL and patients with Hb > 8 g/dL in terms of the expression levels of miR-122 and miR-223 ($P > 0.05$; for all) (Table 5).

There was a negative correlation between miR-210 level and Hb ($r = -0.310$, $P = 0.018$), MCV ($r = -0.328$, $P = 0.012$), MCH ($r = -0.351$, $P = 0.007$), and ferritin ($r = -0.284$, $P = 0.031$); a positive correlation between miR-210 level and RDW ($r = 0.371$, $P = 0.004$); a positive correlation between miR-122 level and ferritin ($r = +0.305$, $P = 0.032$); and a negative correlation between miR-223 level and INTEM MCF ($r = -0.295$, $P = 0.027$). There were no correlation between miR-122 and miR-223 levels and blood count parameters. Correlation graphs between miR-210 level and Hb, MCV, MCH, and ferritin are provided in Figure 1, correlation graph between miR-122 level and

TABLE 3. miR-210, miR-122, miR-223 Expression Levels in Whole Blood in IDA and Control Groups

	Median (IQR)		
	IDA Group (N = 57)	Control Group (N = 48)	*P*
miR-210 (FC)	12.91 (−5.75 to 31.59)	7.28 (−4.59 to 19.16)	NS
miR-122 (FC)	1.20 (1.01-1.39)	1.13 (0.94-1.32)	NS
miR-223 (FC)	16.13 (−10.60 to 42.88)	1.56 (0.92-2.21)	NS

FC indicates fold change; IDA, iron deficiency anemia; IQR, interquartile range; NS, nonsignificant.

TABLE 5. miR-210, miR-122, miR-223 Expression Levels in Whole Blood in Patients With Hb <8 g/dL and Patients With Hb > 8 g/dL

	Median (IQR)		
	Hb < 8 g/dL (N = 27)	Hb > 8 g/dL (N = 30)	*P*
miR-210 (FC)	21.86 (−19.91 to 63.64)	5.41 (0.17-10.65)	0.024
miR-122 (FC)	1.21 (0.88-1.54)	1.19 (0.95-1.44)	NS
miR-223 (FC)	31.33 (−28.93 to 91.60)	3.39 (1.12-5.65)	NS

Hb indicates hemoglobin; FC, fold change; IQR, interquartile range; NS, nonsignificant.

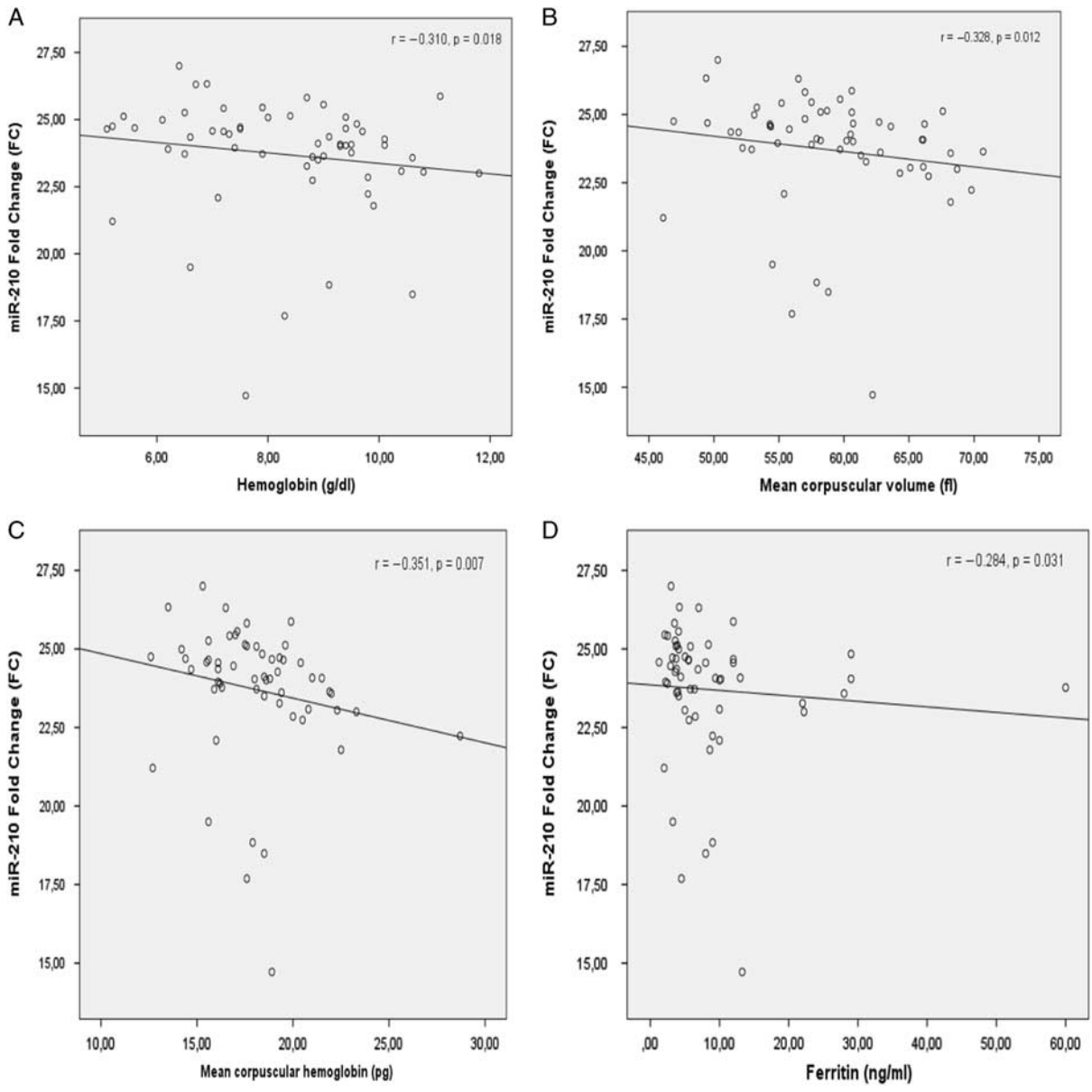

FIGURE 1. Correlation between whole blood miR-210 and anemia markers. Negative correlations between whole blood miR-210 and hemoglobin level (A), mean corpuscular volume (B), mean corpuscular hemoglobin (C), and ferritin (D) in children with iron deficiency anemia.

ferritin in Figure 2, and correlation graph between miR-223 expression level and INTEM MCF index in Figure 3.

DISCUSSION

Iron is the main component of the oxygen-binding heme molecule in Hb. Iron deficiency causes anemia, and anemia reduces the oxygen-carrying capacity of the blood, thereby leading to cellular hypoxia.[35] Body oxygen level is an important factor that affects erythropoiesis. Low levels of oxygen lead to the activation of erythropoiesis through the release of EPO. In addition, the oxygen level in the bone marrow microenvironment controls the interaction between erythroid progenitor cells and stromal cells, which promotes erythroid differentiation.[36] Changes in oxygen levels also change the expression of globin genes.[37–39] Several microRNAs play a role in the regulation of erythropoiesis. miR-210 is associated with a high fetal γ-globin level in K562 cells induced with mithramycin.[40] In addition, miR-210 levels increase during in vitro mouse fetal liver erythroid cell differentiation.[6] Therefore, miR-210 is likely to play a role in the link between erythropoiesis and hypoxia.[41] Sarakul et al[41] have shown that in β-thalassemia/HbE progenitor cell cultures, α-globin, β-globin, γ-globin mRNA levels increase under hypoxic conditions compared with normoxic conditions, that early erythroid precursors decrease, and that mature erythroid precursors and miR-210 levels significantly increase. It has been demonstrated that in the presence of

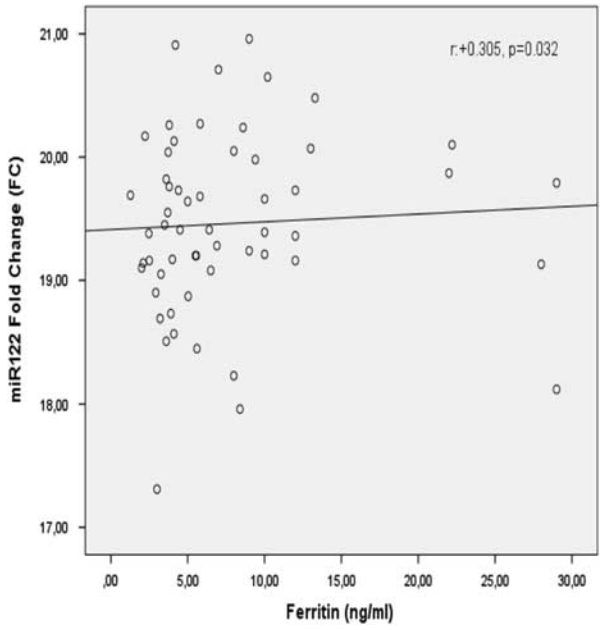

FIGURE 2. Correlation between whole blood miR-122 and ferritin.

anti-miR-210, α-globin and γ-globin mRNA expression is decreased, maturation of erythroid progenitors is delayed, and therefore miR-210 contributes to erythroid differentiation induced by hypoxia. In a study on β-thalassemia/HbE patients,[35] it was shown that plasma and erythrocyte miR-210 levels were higher than those in the control group, that there was a negative correlation between miR-210 and Hb and hematocrit levels, and that higher expression of miR-210 may be the result of hypoxia due to low Hb level, which supports the results of the study by Sarakul et al.[41] On the basis of the results of our study, patients with Hb < 8 g/dL had higher levels of miR-210 than patients with Hb > 8 g/dL. This result demonstrates that miR-210 levels are

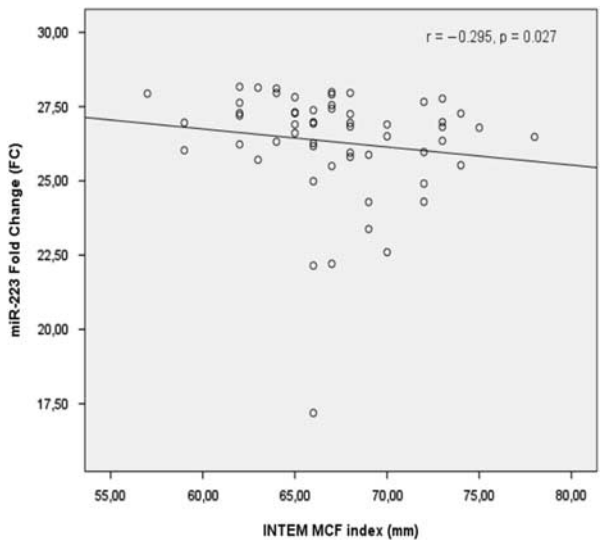

FIGURE 3. Correlation between whole blood miR-223 and intrinsically activated thromboelastometry (INTEM) maximum clot firmness (MCF) index.

affected in moderate and severe IDA. The negative correlation between miR-210 and ferritin level suggests that ferritin may be a miR-210 regulator. The negative correlation between Hb level and miR-210 can be explained by miR-210 levels, which increase in parallel with the increased tissue hypoxia as the anemia deepens.

The liver is the main iron storage site and an endocrine organ responsible for the regulation of systemic iron homeostasis. Mutations in hemochromatosis (*Hfe*), hemojuvelin (*Hjv*), *TfR2*, or hepcidin genes cause hereditary hemochromatosis characterized by low hepcidin activity and iron overload. In mice, miR-122 has been found to be critical for the control of systemic iron homeostasis and to target *Hfe* and *Hjv*, which encode proteins that are crucial to the response of the hepcidin hormone to systemic iron availability.[9] The fact that miR-122 was not downregulated in $HFE^{-/-}$ mice that were experimentally loaded with iron but downregulated in wild-type mice is explained by the inhibitory effect of miR-122 on the Hfe gene.[9] As a result, the downregulated hepcidin protein interacts with ferroportin, an iron exporter, and inhibits systemic iron uptake and iron release.[42] It has been reported that miR-122 depleted mice had reduced systemic iron that caused inadequate iron delivery to the erythron, thereby leading to slightly impaired hematopoiesis.[9] Specifically, miR-122 inhibition leads to increased reticulocyte count and decreased MCV and reticulocyte Hb content. In addition, iron content in the liver, plasma, and spleen is reduced in mice with miR-122 inhibition. With this experimental study, it has been shown that miR-122 inhibition leads to iron deficiency.[9] However, it is not clear how miR-122 expression changes in conditions of iron deficiency. The results of our study showed that miR-122 levels did not change in the IDA group compared with the control group; however, there was a weak positive correlation between miR-122 and ferritin. This finding suggested that miR-122 plays a role in maintaining iron homeostasis. As far as is known, miR-122 is the only tissue-specific microRNA that is continuously released into circulation. It can be easily detected in the serum and plasma due to its abundant production in the liver.[43] miR-122 is found in much higher amounts in the serum than in whole blood.[28] If the level of miR-122 had been measured in the serum rather than in whole blood, a stronger correlation with ferritin could have been demonstrated. However, it is noteworthy that there are no standard and generally accepted experimental, analytical, and normalization guidelines for detecting and measuring microRNAs in biofluids.

Thrombocytosis, microcytosis, blood viscosity, changes in oxidative balance, and increased platelet aggregation are among the factors contributing to the development of thrombosis in IDA.[44–46] In our previous study, we suggested that platelet activation and microRNA profile may play a role in the pathophysiology of thrombotic tendency.[25] Available data indicate that microRNAs are associated with the regulation of platelet functions and show that platelets contain significant levels of microRNA.[22,47] miR-223, miR-146a, miR-495, and miR-107 are highly expressed in platelets, and these microRNAs regulate the genes that are involved in platelet activation,[48] platelet shape changes, and circadian platelet reactivity.[24] A study has found a negative correlation between platelet miR-223 and P-selectin, an indicator of platelet activation, and demonstrated that the loss of miR-223 and miR-146a expressions may be a risk factor for ischemic stroke in patients with diabetes mellitus.[24] Compared with wild-type mice, mice with miR-223 deficiency have been shown to have larger thrombi, a delayed clot retraction, and an increased aggregation

response of platelets to thrombin and collagen.[49] Low platelet miR-223 levels result in greater $P2Y_{12}$ receptor activity and higher platelet reactivity.[18] miR-223 targets a 3′-untranslated region of human $P2Y_{12}$ platelet receptor mRNA. $P2Y_{12}$ receptor is important in platelet activation and is the target of many drugs used to inhibit platelet function.[18] Our study has shown a negative correlation between miR-223 and MCF. The negative correlation between MCF, which is known to reflect platelet and fibrinogen function, and miR-223 is considered to be an indirect indicator that miR-223 is associated with platelet reactivity.

The limitations of our study are that our patient population was small, and platelet aggregation tests were not performed.

In conclusion, miR-210 is closely associated with the severity of hypoferritinemia and anemia in IDA. Iron balance is maintained by highly complex mechanisms involving miR-122 and miR-210. The correlation between miR-223 and MCF index suggests that miR-223, a regulator of platelet function, may play a role in the development of hypercoagulability in IDA. Further studies are needed to understand the functions of microRNAs in iron deficiency, hypoxia, erythropoiesis, and hypercoagulability, which are all interconnected.

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