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Investigation of miR-144-3p expression levels in HbSS cases with high and normal HbF



[HbF'i normal ve yüksek olan HbSS'li olgularda miR-144-3p ekspresyon düzeylerinin araştırılması]

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Abstract

Background: Sickle cell anemia (HbSS) is a hereditary blood disease that affects millions of people worldwide. Increased the HbF levels affects the clinical course of HbSS positively. The aim of this study was to investigate the association between miR-144-3p and HbSS.

Materials and methods: In this study 84 cases (44 HbSS/HbS combination and 40 HbAA) were performed. The expression of miR-144-3p was determined by RT-PCR. Statistical analysis was performed by the Mann-Whitney U test (SPSS 20.00 for Windows and $p<0.005$).

Results: The miR-144-3p expression levels were higher in the HbSS cases ($p\leq 0.001$). Additionally, it was determined that the expression of miR-144-3p was higher in the cases with $HbF<3$ ($p=0.043$).

Discussion: In our study, the increase in the miR-144-3p levels in low HbF levels may be associated with the severity of the disease.

Conclusion: Considering these results, suppressing miR-144-3p may be considered as a new treatment option in HbSS.

Keywords: fetal hemoglobin; miRNA; sickle cell anemia.

Öz

Amaç: Dünya'da milyonlarca insanı etkileyen orak hücreli anemi (Hb SS) bir kalıtsal kan hastalığıdır. Hb F düzeyinin yüksekliği Hb SS'in kliniğini pozitif yönde etkilemektedir. Bu çalışmadaki amacımız miR-144-3p ve HbSS'in birliktelığını araştırmaktır.

Gereç ve Yöntemler: Bu çalışmada 84 olgunun (44 HbSS/HbS birlikteliği ve 40 HbAA) verileri değerlendirilmiştir. miR-144-3p'nin ekspresyonu RT-PCR ile belirlenmiştir. İstatistiksel analizler Mann-Whitney U testi (Windows SPSS 20.00 ve $p<0.005$) ile değerlendirilmiştir.

Sonuçlar: HbSS olgularında miR-144-3p'nin ekspresyonunun seviyesi daha yüksek olduğu belirlenmiştir ($p \leq 0.001$). Buna ilaveten, $Hb F < 3$ olan olgularda miR-144-3p'nin ekspresyon düzeyinin daha yüksek olduğu saptanmıştır ($p = 0.043$).

Tartışma: Çalışmamızda Hb F düzeyi düşük olan olgularda miR-144-3p ekspresyonunun yüksekliği hastalığın şiddeti ile orantılı olabileceği gözlenmiştir.

Sonuç: Bu sonuçlar göz önünde bulundurulduğunda, miR-144-3p ekspresyonunun baskılanması Hb SS'nin tedavisinde yeni bir yaklaşım olabileceği düşünülmüştür.

Anahtar Kelimeler: fetal hemoglobin; miRNA; orak hücreli anemi.

Introduction

Sickle cell anemia (HbSS) is one of the most common blood diseases in the world, which is an autosomal recessive disorder of hemoglobin. It is caused by the substitution of glutamic acid (Glu) by valine (Val) at the sixth position of

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the β globin chain [HbS (β (6) GTG→GAG)] [1]. This single amino acid replacement leads to large changes in molecular stability and solubility. Erythrocytes that carry HbS that polymerizes deoxygenated conditions, leads to flexion in the red blood cells and it takes a sickle-shape. These cells have a short lifespan and lead to disruption in normal blood flow and local hypoxia by clogging the capillary blood vessels [2]. In HbSS, the β^s globin chain is carried in the homozygous form. Clinical features of this disease include anemia, increase in the severity of some infections, tissue infarctions that may lead to organ damage and recurring pains. HbA and HbS are found together in the erythrocytes of individuals who carry the HbS gene in the heterozygous form (HbA/S). Blood cell morphologies, physical development, activities and lifespan of individuals with sickle cell trait (HbAS) are generally normal. While HbS/ β^0 double heterozygous picture shows a clinical similarity to homozygous HbSS, HbS/ β^+ has a double heterozygous characteristic with mild or severe clinical signs. HbF (2 α 2 γ), which is the major hemoglobin of fetal life and newborn, is the most significant modulator of the clinical and hematological features of HbSS. The combination of HbS/hereditary persistence of fetal hemoglobin (HbS/HPFH) leads to quite mild clinical symptoms [1, 3]. The fetal hemoglobin production of some cases with HPFH continues in adulthood. It has been reported that higher HbF levels not only prevent the polymerization of HbS but also play an important role in reducing rate of various complications of sickle cell disease (SCD) such as acute painful sickle cell episodes, leg ulcers and osteonecrosis [1, 3, 4]. Therefore, in recent years most researchers focus on agents that would increase the HbF values as effective treatments for HbSS [5].

MicroRNAs (miRNA) have a length of 19–25 nucleotides and are small non-coding RNAs that regulate gene expression by cleaving the target mRNA or preventing translation [6]. miRNAs play an important role in several cellular functions such as cell proliferation, differentiation, reproduction, apoptosis, oxidative stress and DNA repair. Furthermore, miRNAs have contributed to our understanding of the biology and pathophysiology of many diseases and epigenetic changes in the expression of genes [7].

The study conducted by Chen et al. which was carried out on mice for the first time showed that different miRNAs were expressed in hematopoietic cells [8]. In later years, it was shown that miRNAs are found in granulocytes, monocytes, lymphocytes, thrombocytes and reticulocytes of human beings [9, 10]. Additionally, it has been demonstrated that miRNAs also played a role in the hematopoietic cell differentiation, and the expression level of some miRNAs decreased or increased depending on the type of

differentiated cells [11]. In some studies, it was reported that different types of miRNAs were found in matured erythrocytes and leukocytes and there was a statistically significant correlation between the severity of the SCD and the high level of leukocytes [8, 12, 13].

In this study, we aimed to investigate the changes in miR-144-3p expression associated with HbF induction in patients with HbSS/HbS combination.

Materials and methods

Sample selection

Ethical approval for the study was granted on by the Ethics Board of the Faculty of Medicine at Cukurova University. Blood samples were collected from patients visiting the Hereditary Blood Diseases Association of Adana and Departments of Pediatric Hematology and Medical Biochemistry at Cukurova University in Turkey. The study was conducted with participation of 44 HbSS/HbS combination (patient group) and 40 HbAA (control group). Additionally, patient group was divided into two groups according to their HbF levels: 11 cases with HbF<3% (group 1) and 20 cases with HbF>9% (group 2) to assess the effects on HbF levels of miRNA-144-3p. The interviews with the patient and control groups were conducted to collect data regarding their demographics. The mean age of the patient group was 31.66 years (18–65 years), the mean age of the control group was 23.66 years (6–48 years). The patient group was composed of 25 male (56.8%) and 19 female (43.2%). The control group was composed of 19 male (47.5%) and 21 female (52.5%). The information about gender and average age of the control and patient groups is given in Table 1.

A total of 5 mL of blood, 2.5 mL in EDTA tube and 2.5 mL in RNALater tube (PaxGene, Qiagen), was received from each case. The samples collected in the RNALater tubes were stored at -20 °C. The same procedure was applied for all cases at all stages of the experiment.

Hemoglobin typing

Hematological parameters were determined by a Sysmex-KX-21 N analyzer. Sickling test, cellulose acetate electrophoresis (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and high-performance liquid chromatography [HPLC (Agilent 1100)] were performed for identities of hemoglobin types. DNAs were isolated by using

Table 1: Demographics of patients and healthy controls.

	Patients n=44 n (%)	Control Groups n = 40 n (%)
<i>Gender</i>		
Male	25 (56.8)	19 (47.5)
Female	19 (43.2)	21 (52.5)
<i>Age (years)</i>		
Medium	31.66	23.66
Range	18–65	6–48

n: number of cases.

MagNAPure LC Automated DNA Isolation Device (Roche Diagnostics, the Netherlands BV) and stored at +4 °C. Hemoglobin mutations were characterized by the ARMS and Gap-PCR.

miRNA isolation

The RNAlater tubes were left at room temperature and homogenized. The erythrocytes were lysed and leukocytes were obtained by using a Red Blood Cell Lysis Buffer kit (Roche Diagnostics). A High Pure miRNA Isolation Kit (Roche Diagnostics) was used to obtain miRNA from the leukocytes. All samples were stored at -80 °C until use [14, 15].

cDNA synthesis

Complementary DNA (cDNA) was synthesised from the isolated RNA samples in 10 µl final reaction volumes using a miRCURY Universal cDNA Synthesis Kit (Exiqon). All reactions were performed as specified in the protocols of the manufacturer: 2 µL total RNA was added to 8 µL of the master mix consisting of 5x reaction buffer (2 µL), nuclease free water (4.5 µL), enzyme mix (1 µL), spike in (0.5 µL). The reactions were incubated at 42 °C for 60 min (1 cycle), at 95 °C for 5 min (1 cycle) and storage at +4 °C or -20 °C [16].

RT-PCR (real time PCR) analysis

The cDNAs that were obtained from all cases were used as a template and for each cDNA sample, the gene expression of miR-144-3p (UACAGUAUAGAUGAUGUACU) and the reference gene (U6) was analyzed by using a Roche LightCycler®480 Real-Time PCR instrument (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Firstly, U6 and target gene (miR-144-3p) were diluted separately by using 220 µl of nuclease-free water. Reactions were performed in a final volume of 10 µl containing 5 µl of PCR SYBR green Master Mix, 1 µl of specific PCR primer (Exiqon), and 4 µl of cDNA template. Reactions were run on a Light Cycler 480 Real-Time PCR in 96-well optical plates. The cycle conditions were denaturation at 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 10 s and 60 °C for 60 s, melting curve at 95 °C for 30 s, 40 °C for 60 s, 85 °C for continued and cooling at 40 °C for 60 s. Calculation of quantification cycle (Cq) values for relative quantification were performed using Light Cycler 480 Software, Version 1.5 (Roche Diagnostics) [16]. The reference gene was used for the normalization of target gene expression. The fold change was calculated with the equation $2^{-\Delta\Delta C_t}$.

Statistical analysis

The miRNA expression levels of the control and patient groups were calculated by the formula of $2^{-\Delta\Delta C_t}$. Differences in the data were determined by Mann-Whitney U test. The analyses were carried out by using SPSS 20.0 for Windows Program, and the statistically significant level was accepted as p<0.05. Additionally, the relationship between 11 cases with HbF<3% and 20 cases with HbF>9% and miR-144-3p levels were analyzed by using Mann-Whitney U test.

Results

Hemoglobin typing was carried out for all cases and was determined that 40 cases with HbAA, 35 cases of whom with HbSS, 7 cases with a combination of HbSS and -3.7α/AA, 1 case with a combination of HbSS and -20.5-/AA, and 1 case with the genotype of S/IVS1-110 by using molecular methods (Table 2).

The expression levels of the miR-144-3p target gene of the cases were determined by RT-PCR in all cases (Figure 1). The expression levels were calculated by the method of $2^{-\Delta\Delta C_t}$ and the significance level based on the Mann-Whitney U test was found as p<0.001.

Additionally, when the expression levels in patient group were compared between 11 cases with HbF<3% and 20 cases with HbF>9% in order to determine the effects of miR-144-3p on HbF levels, the significance value was found as p=0.043.

Discussion

Point mutations, insertions and deletions that take place in the exon parts or other parts of the genes that code the polypeptide chains of the hemoglobin molecule cause abnormal hemoglobin. Among abnormal hemoglobin changes, HbS [(β (6) GTG →GAG)] is known as the most prevalent hereditary blood disease in both Turkey and the world [17, 18]. HbA/S, HbSS, other abnormal hemoglobins and thalassemia mutations have been combined with HbS and this combination results in variable clinical manifestations [19]. There are several studies in the Human miRNA Disease Database (HMDD) related to miR-144 and human diseases [20]. In this study, it was observed that the expression levels of miRNA-144-3p were higher in the patient group at the steady state in comparison to those with control group (p<0.001). The study conducted by Swem et al. investigated expression of miRNA-144 of 20 HbSS steady state, 20 crisis state, 20 HbAA controls and 20 HbAS controls. According to their analyses, there was no

Table 2: Hemoglobin typing in all cases.

Hemoglobin types	Numbers
HbSS	35
HbSS/- ^{3.7} α/αα	7
HbSS/- ^{20.5} /αα	1
HbS/IVS1-110	1
HbAA	40

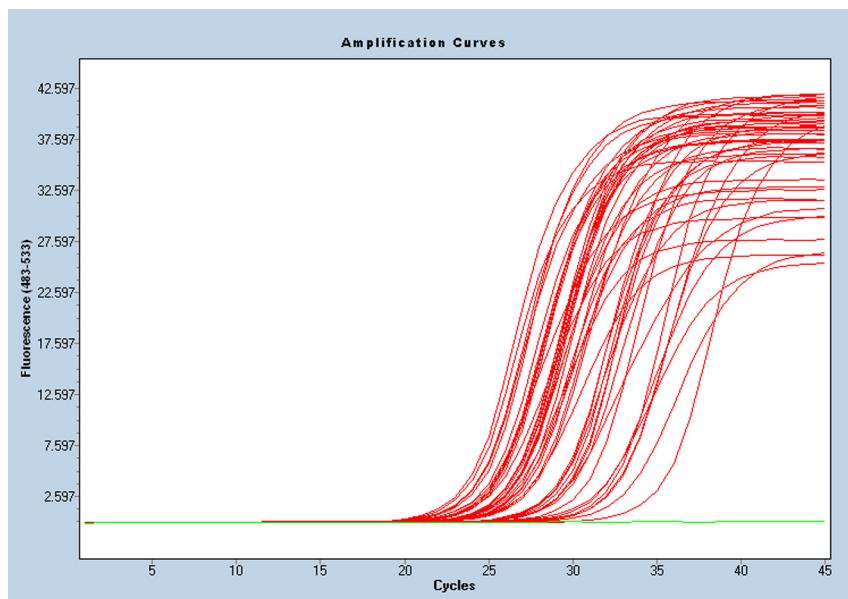


Figure 1: Determination of miR-144-3p gene (target) expression level by RT-PCR in all cases.

significant difference ($p>0.05$) in the miRNA-144 levels between the HbSS cases at the crisis state and those at the stable state, while miRNA-144 showed a statistical significant difference between HbSS steady state and HbAS control; HbSS steady state and HbAA; HbSS crisis state and HbAA control ($p<0.001$) [21]. This study shows that a high level miRNA-144 in HbSS compared to HbAS and HbAA. These results are similar to our findings.

Uwaezuoke et al. evaluated the relationship between the severity of SCD and expression of miR-221 by leukocytes and 73 HbSS and 25 HbAA cases were analyzed. According to the findings of the study, leukocyte miR-221 was a strong correlate of SCD severity, and a candidate biomarker for assessing the disease severity at clinic [12].

Chen et al. examined the expression levels of miRNA-144 that were isolated from the erythrocytes of 12 HbSS and 7 HbAA cases and found that the miRNA-144 expression levels were higher in the HbSS cases [8]. Dore et al. investigated the function of miRNA-144 and miRNA-451 in hematopoietic cells. They reported that miRNA-144 and miRNA-451 acted as regulators of erythropoiesis by affecting the hematopoiesis transcription factor GATA-1 [22]. Keller et al. conducted a study on various types of cancer for determining the biomarker characteristics of miRNA-144. They reported that miRNA-144 and miRNA-20b levels were significantly lower in the cases with disease and $AUC=0.771$ (95% CI 0.721–0.821) was found through the ROC curve analysis [23].

The levels of fetal hemoglobin (HbF) are highly variable. It has high levels in the fetus and is produced at normal levels on adults with Hb S/A. HbF levels of adults

with HbSS range is between %1 and %20. High HbF levels prevent sickling and causes less severe clinical course of HbS by inhibiting polymerization [24]. Because of these advantages, treatment methods that would increase HbF levels are utilized. Agents including hydroxyurea, epigenetic modifications (e.g. inhibition of γ globin gene promoter methylation or deacetylation with thalidomide and sodium butyrate) and miR-based regulation may be used for the purpose of treatment in SCD patients [25, 26].

Several studies have been conducted on the use of miR-144 in the treatment of hemoglobinopathies. Fu et al. asserted that erythroid specific miRNA-144 in zebra fish is specifically expressed during the developmental processes in embryogenesis, and it is a negative regulator of the α -globin gene by targeting KLF4. When the expression of miRNA-144 in zebra fish was inhibited, an increase was observed in the synthesis of the α -globin gene. Accordingly, the researchers aimed to develop a new treatment method for thalassemia cases by using miRNA-144 [27, 28].

Some studies have shown the relationship between miR-144 expression profiles in reticulocytes and erythrocytes in oxidative stress conditions [29–31]. According to Sangokoya study, miR-144 directly targets Nuclear related factor 2 (NRF2) which is the main factor in oxidative stress response and cellular antioxidant defense system. Higher levels of miR-144 expression in HbSS decrease the level of NRF2. Higher miR-144 expression is associated with severity of anemia and it results in hemoglobin/hematocrit cells to decrease. Moreover, increased values have been reported to cause the deficiency of antioxidant proteins

such as glutamate-cysteine ligase, catalytic/modifier subunit (GCLC/M) and superoxide dismutase 1 (SOD1) [29].

Several studies have indicated that miR-144 and NRF2 expression were associated with oxidative stress, redox potential and anemia in SCD. Li et al. found out that miRNA-144 was highly expressed in SCD patients with high and low HbF levels. These results demonstrated that oxidative stress and inflammation play an important role in the pathophysiology of SCD, so drugs that induce HbF and reduce oxidative stress have a greater impact on clinical status [32].

Increased HbF levels causes more moderate clinical course of HbSS cases. HbSS cases were divided into groups based on their HbF levels to assess the usability of miRNA-144-3p in treatment. Considering the results of our analysis, it was found out that miR-144-3p had higher expression levels in the cases with HbF<3% ($p=0.043$).

Conclusion

In this study, we wanted to evaluate whether the expression levels of miR-144-3p could be used as potential treatments by determining the expression levels of miR-144-3p. The increased miRNA-144-3p expression in the patients group with low HbF values might be related to the severity of the disease. Suppressing miRNA-144-3p might be considered as a new treatment strategy for a milder clinical picture in HbSS cases. However, considering one miRNA could control multiple genes, and one gene could be controlled by multiple miRNAs, it was needed to conduct more comprehensive studies on usage of miRNAs in treatment and prognosis.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: Ethical approval for the study was granted on by the Ethics Board of the Faculty of Medicine at Cukurova University(03.05.2012). The study was done with informed consent forms from humans.

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