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In Vivo Evaluation of Bcl-xl, Foxo3, ARG1, and miR-144/451 Genes in Response to Oxidative Stress Induced by Hydroxyurea in β-Thalassemia Major Patients

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Abstract

Introduction: Use of HU among beta-thalassemia patients show different clinical outcomes and about 25% of patients do not response to this agent; the objective of this study was to evaluate the possible role of expression of anti-oxidative genes in response to HU.

Materials and methods: For miRNAs and mRNAs, RT-PCR was done by using specific primer for each miRNA and random hexamers for mRNAs.

Results: In responders, we observed a higher expression of Bcl-xl, Foxo3, ARG1, and miR-451 in comparison to non-Responders. For miR-144, we did not find any difference between the two groups.

Conclusion: As Hu can induce production of HbF, activation of genes involved in regulation of oxidative stress in responders may have a critical role in the survival of erythroid progenitors and maturation. So, it can cause to increase Hb levels in responders in comparison to non-responders

Key Words:

Hydroxyurea; Oxidative stress; miR144/451; Foxo3; Bcl-xl; ARG1

Introduction

Several chemical agents have been found to induce high levels of fetal hemoglobin (HbF) in patients, and Hydroxyurea (HU) treatment has gained wide acceptance for the treatment of Sickle Cell Anemia (SCA) and beta thalassemia (BT) [1,2]. Since 1994 using HU showed vary degrees of success in different reports [3]. It is a ribonucleotide reductase inhibitor, and several reports confirmed the HbF augmenting effect of HU both *in vitro* and *in vivo* and partially correcting α and non-α globin chains imbalance, thus decreasing the hemolytic symptoms of these patients [4-7]. HbF response in BT and SCD patients upon HU treatment is variable, which approximately 25% of these patients being poor or non-responders (NR) [8].

Along with adverse effects of HU on erythroid cells in BT, high level of free cellular iron in BT erythroid precursors, and accumulation of unpaired hemoglobin chains could initiate self-amplifying redox reactions that simultaneously deplete cellular reduction potential [9]. So, it seems an adequate concentration of intracellular anti-oxidant levels need to protect cells from HU-induced oxidative damage [10].

In fact, HU is a toxic agent and could cause cytopenia, so it is possible that variation of the hematologic response to HU in patients may correlate with bone marrow toxicity [11]. In some patients, the threshold for acceptable toxicity may be reached before a favorable hematologic response and HU therapy must thus be discontinued. So, differences in pharmacokinetics and pharmacodynamics as well as in genetic background among patients should be investigated [12].

In order to find the possible differences between BT major patient's responders (R) and non-responders to HU along with Bcl-xl, Foxo3, and ARG1 expression three known genes involved in cell survival, oxidative stress, and HU metabolism, respectively is investigated the expression of miR-144/451, two miRNAs involve in regulation of oxidative stress.

Materials and Methods

Blood collection and reticulocyte purification

The study was conducted in accordance with the Declaration of Helsinki for Human Research and the protocol was accepted by the Local Ethics Committee at Iranian Blood Transfusion Organization (IBTO). All the subjects signed the informed consent before enrolling in the study. Among patients newly registered in Zafar Adult Thalassemia Center (belong to Iranian Blood Transfusion Organization, Tehran), whom voluntary participated in HU therapy project conducted by center under observation of two pediatric

oncologists, were followed up for six months with using average 14 Mg/Kg/day HU.

By definition, " responders" were patients on regular blood transfusions who did not need transfusion after HU, or decreasing blood demand, and "non-responders" were defined as no decline in the amount of needed transfusions [13].

After six months, patients who became transfusion independent, categorized in R group (5 patients), and in those patients who HU therapy hadn't any significant effect in elevation of Hb, and likewise was transfusion dependent, categorized in NR group (5 patients). All selected patients had not any other hemoglobinopathies and alpha thalassemia deletions.

In order to find and compare levels of expression of these genes and miRNAs in a steady state condition *vs.* under stress erythropoiesis, we also investigated the variables in the same population of the healthy individual (H) with no hemoglobinopathies and any history of using oxidative agents. Stress erythropoiesis defines as a state of increased red cell production in response to an enhanced rate of synthesis and secretion of Erythropoietin (EPO), which may be associated with malformation of RBC in illness [14].

In R patients along with using HU, and in NR patients before stopping HU, blood was obtained by venipuncture, and collected into sodium citrate-containing tubes. For harvesting reticulocyte, we used Anti-CD71 conjugated magnetic beads (Miltenyi Biotec, CA) as described by company. Briefly, 107 cells were suspended in 70 μL of cold separation buffer (phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA; both from Sigma-Aldrich), incubated for 15 minutes at 4°C with 20 μL of anti-CD71 Microbeads per 70 μL of cell suspension, washed by adding 2 mL of separation buffer and centrifuged at 300 × g for 10 minutes. Cells were suspended in 500 μL of separation buffer and applied onto a MACS Column (LS type; Miltenyi Biotec) placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The flow-through was collected as the unlabeled negative fraction. The column was then washed three times with 500 μL of separation buffer and the retained magnetically labeled cells were flushed out with 1 mL of separation buffer as the positive fraction. This fraction contains CD71 positive cells.

Flowcytometric analysis

For evaluation of purity of isolated CD71 positive cells, we used CD71-FITC (Miltenyi Biotec) and CD235a (Miltenyi Biotec), as described by company. After adjusting cell number to 107/100 μL, the cell, the suspension should be centrifuge at 300 × g for 10 minutes. Aspirate supernatant completely. Up to 107 nucleated cells per 100 μL should resuspend in buffer. Add 10 μL of the antibody. Mix well and incubate for 10 minutes in the dark in the refrigerator (2-8°C). Washing cells by adding 1-2 mL of buffer and centrifuge at 300 × g for 10 minutes. Aspirate supernatant completely. Resuspend cell pellet in a suitable amount of buffer for analysis by flowcytometry.

RNA extraction and RT-PCR

Total RNA was extracted by YTA kit (Yekta Tajhiz Azma, IRAN) according to the manufacturer's instructions. For microRNAs quantification, poly-A addition and specific primers for each miRNA were obtained from Parsgengan, Iran. Reverse transcription (RT) reactions for both miRNAs and mRNAs, were performed using hyper script first strand synthesis kit (pishgam biotech, Iran), using specific primer for each miRNA and random hexamers for mRNAs.

The primers sequences used for RT-PCR analysis of Bcl-xl, Foxo3 and ARG1 mRNAs are here reported: Bcl-xl forward primer, 5'-ACCTGAATGACCACCTAGAGC-3', Bcl-xl reverse primer, 5'-CAGCGGTTGAAGCGTTCC-3', Foxo3 forward primer, 5'-CGTTGCGTGCCCTACTTC-3', Foxo3 reverse primer, 5'-CTCTTGCCAGTTCCCTCATTC-3', ARG1 forward primer, 5'-CAAGAAGAACGGAAGAATCAGC-3', ARG1 reverse primer, 5'-CCAGATGACTCCAAGATCAGG-3', GAPDH forward primer, 5'-GCCAAAAGGGTCATCATCTC -3', GAPDH reverse primer, 5'-GGTGCTAAGCAGTTGGTGGT -3'.

The real-time PCR was performed using the Rotor Gene 6000 instrument (Corbett, Life Science, Australia). For each reaction 12.5 μL of SYBR Premix Ex Taq II master mix (Takara, Japan), 10 pmol of each forward and reverse primers, and 2 μL of cDNA were applied in final volume of 25 μL. The PCR program for miRNA was 30 sec at 95°C followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C and 30 sec at 72°C and for mRNA was 1 minute at 95°C followed by 40 cycles of 15 sec at 95°C and 30 sec at 60°C.

Relative expression was calculated using the comparative cycle threshold method [15]. As internal control (reference gene) miR-152 microRNA and GAPDH gene mRNA were used to normalize RNA samples [16,17].

Statistical analysis

Statistical significance of difference between various groups was determined by one-way analysis of variance followed by the post hoc test by SPSS 16 software. Curves provided by using GraphPad Prism 6 (GraphPad Inc.).

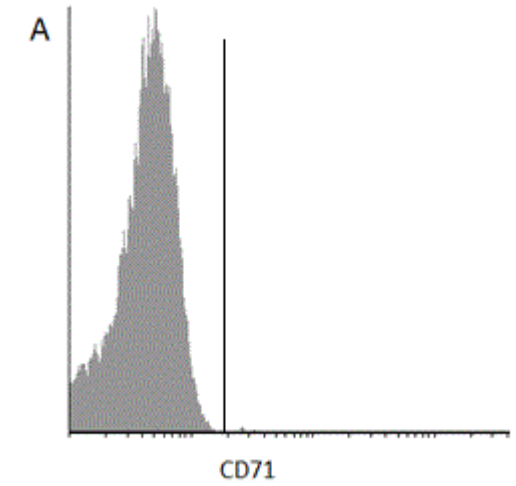
Results

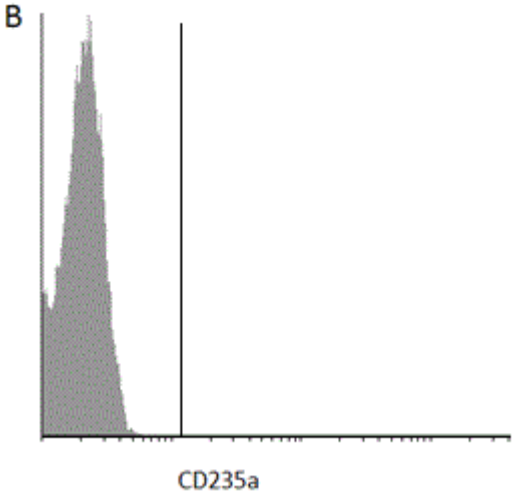
Of 5 patients involved in each group, three were male and two were female. Average ages of patients were 18 ± 3.3 and 19 ± 2.6 year in R and NR group, respectively. The results of hematologic parameters after 3-month treatment showed in Table 1 and indicated that increasing of Hb in the R group was significantly higher than the NR group.

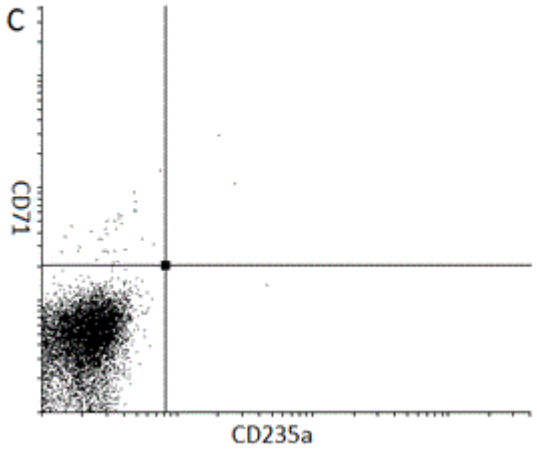
Parameters	WBC	Hb (g/dL)	MCV (fl)	MCH (pg)	Platelet
Responders	7.5 ± 1.4	10.2 ± 1.1	81 ± 4.9	18.1 ± 2.1	489 ± 48
Non-Responders	6.8 ± 1.8	7.2 ± 0.8	79 ± 7.2	17.9 ± 2.8	355 ± 39
fl=femtoLiter, pg=picogram					

Table 1: Hematologic parameters after HU treatment in groups under study.

Evaluation of purity of isolated CD71 positive cells by flowcytometry



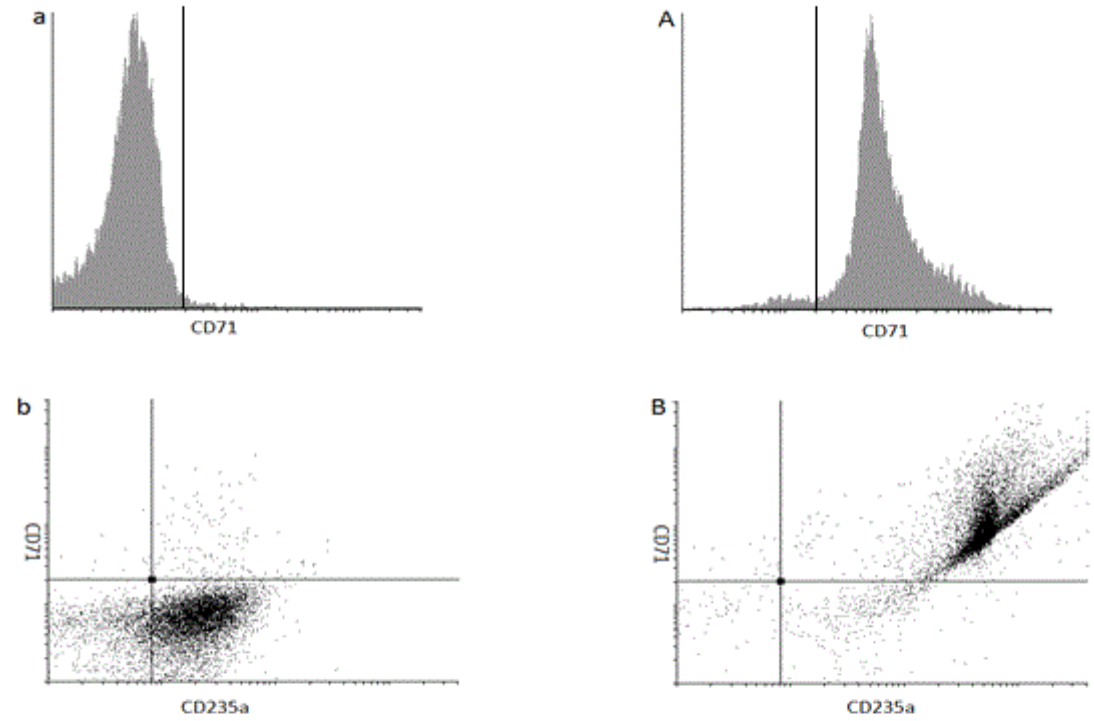




D

	Number of events	% of visible events
Visible events	10000	100
Upper Left (CD71-FITC)	32	0.32
Upper Right (double positive)	2	0.02
Lower Left (double negative)	9964	99.64
Lower Right (CD235a-PE)	2	0.02

Figure 1: Flow cytometry evaluation of erythroid precursor cells isolated by MACS method with isotype control. More than 99% of cells are in negative zone for both CD71 and CD235a. Part C and D shows the number of events in each zone.



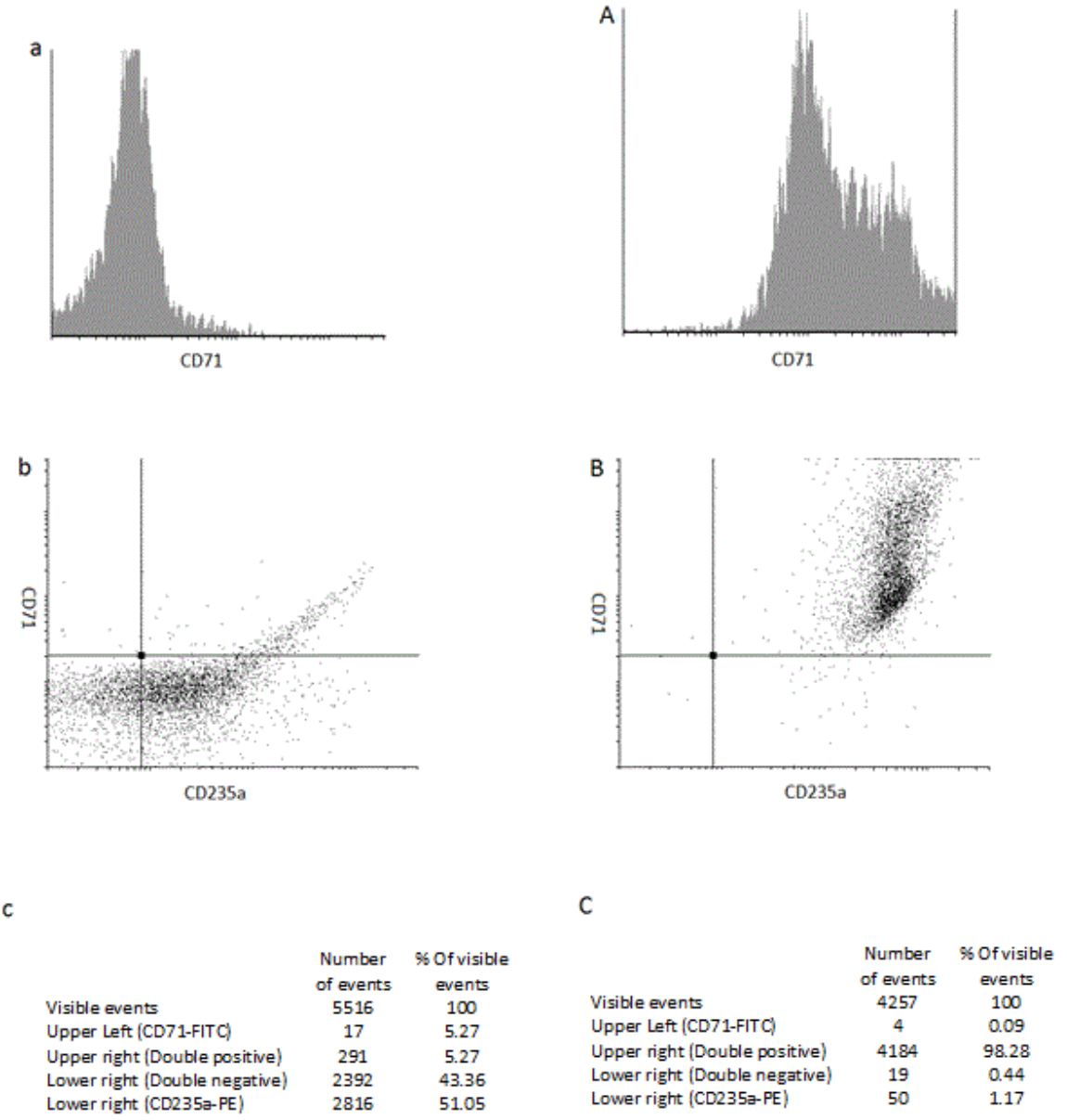
c

	Number of events	% Of visible events
Visible events	10000	100
Upper Left (CD71-FITC)	21	0.21
Upper right (Double positive)	173	1.73
Lower right (Double negative)	2925	25.25
Lower right (CD235a-PE)	6881	68.81

C

	Number of events	% Of visible events
Visible events	10000	100
Upper Left (CD71-FITC)	21	0.21
Upper right (Double positive)	9426	94.26
Lower right (Double negative)	124	1.24
Lower right (CD235a-PE)	429	4.29

Figure 2: Flow cytometry evaluation of erythroid precursor cells isolated by MACS method by CD71-FITC in a healthy sample before (a) and after (A) MACS isolation. More than 90% of cells are on double positive zone (b) and (B). Part (c) and (C) shows the number of events in each zone.



c

	Number of events	% Of visible events
Visible events	5516	100
Upper Left (CD71-FITC)	17	5.27
Upper right (Double positive)	291	5.27
Lower right (Double negative)	2392	43.36
Lower right (CD235a-PE)	2816	51.05

C

	Number of events	% Of visible events
Visible events	4257	100
Upper Left (CD71-FITC)	4	0.09
Upper right (Double positive)	4184	98.28
Lower right (Double negative)	19	0.44
Lower right (CD235a-PE)	50	1.17

Figure 3: Flow cytometry evaluation of erythroid precursor cells isolated by MACS method by CD71-FITC in a thalassemia major sample before (a) and after (A) MACS isolation. More than 90% of cells are on double positive zone (b and B). Heterogenic expression of CD71 in thalassemia sample *vs.* it`s homogeny expression in healthy sample can indicate existence of immature progenitors with different amount of CD71 marker in thalassemia patients. Part c and C shows the number of events in each zone.

For evaluation of purity of isolated erythroid progenitors by MACS, we evaluated the percentage of these cells before and after isolation, by flow cytometry. The results showed that the purity of isolated cells was more than 90% (Figures 1-3)

For confirming responding or non-responding to HU, we evaluated the γ-globin expression by molecular analysis of erythroid precursors of all patients (Figure 4A). After isolation of desire cells by flowcytometry, we evaluated the expression of genes and miRNAs by PCR methods. Investigation of Bcl-xl as a cell survival gene shows, in R group expression of this gene was significantly higher than both H and NR group (p=0.001, p=0.008, respectively), although between NR and H group this was not significant (Figure 4B).

Similarly, ARG1 gene expression had a similar pattern to Bcl-xl gene, and higher amount of gene expression was observed in R group

vs. NR (p=0.006) and H group (p=0.001), and NR *vs.* H didn't show any difference (Figure 4C).

Evaluation of Foxo3 gene showed that in R it was significantly higher than NR (p=0.008), but between R and H, it didn't show any significant difference. Comparison of expression of this gene between NR and H also was not significant (Figure 4A).

For further study, along with Foxo3, we also evaluated miR-144 and miR-451. The results of evaluation of miR-144 didn't show any significant difference between groups (Figure 4B). It means this miRNA in H group and both R and NR group had similar expression. But miR-451 expression in R group was significantly higher in comparison to NR (p=0.000) and H (p=0.000). Between NR and R group also, no significant differences were observed (Figures 4C-4F).

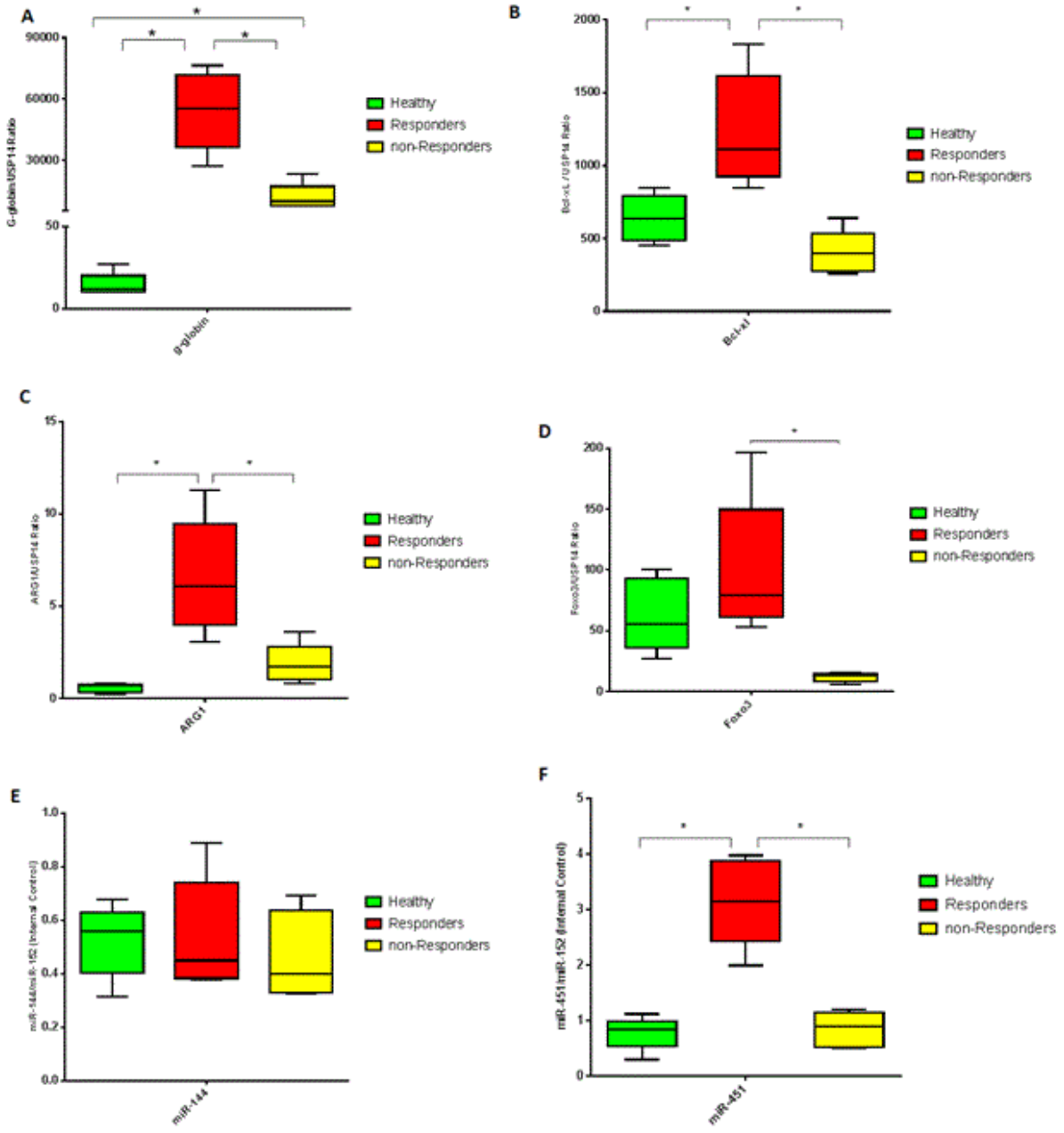


Figure 4: Pattern of expression of γ-globin gene (A), Bcl-xl (B), ARG1 (C), Foxo3 (D), miR-144 (E), and miR-451 (F) in groups under study. In pattern of expression of γ-globin gene (A), confirmed that responders had higher expression of γ-globin gene than non-responders and healthy controls.

Discussion

In this study, we evaluated the expression of some mRNAs and miRNA, thought to have a role in anti-oxidative response in erythroid cells. Higher expression of Bcl-xl, Foxo3, ARG1, and miR-451 in R group can indicate the role of the anti-oxidant process to access to desired therapeutic response in the application of toxic agents such as HU.

Our results in expression of Bcl-xl levels were in agreement with Pourfarzad et al., [18] that R-cells have higher levels of BCL-xL than NR-cells, so R-cells can be protected from apoptosis due to increased BCL-xL anti-apoptotic activity, and much stronger growth inhibition upon HU treatment and greater cell death in NR-cells than R cells was observed. Also, it showed that BCL-XL down-regulation *via* Bcl-xl siRNA exposure leads to decreased cell growth and apoptosis [19]. Recently, it has been demonstrated *in vitro* that Bcl-xL prevents apoptosis predominantly at the end of erythrocyte maturation (reticulocyte), thus the ability of R group in expressing higher levels of Bcl-xl can be consider as a compensatory mechanism in survivals of erythroid cells in late erythropoiesis, so observing not-similar pattern in NR group can be interpreted that after exposure to oxidative stress, NR cells could not produce proper levels of Bcl-xl to rescue the cells from apoptosis [20,21]. This result was confirmed by Lee et al. [22]. They found that Bcl-XL expression effectively blocked serum deprivation- and Romo1-triggered ROS generation and suggest that increased Bcl-XL expression, which is observed in many cancer cells, confers resistance to oxidative stress in the cancer cells by suppressing Romo1-mediated oxidative stress.

In addition to Bcl-xl, EPO can induce activation of multiple major signaling cascades such as Foxo3, a critical physiological regulator of oxidative stress in mammalian cells [23]. We showed that R cells expressed significantly higher amount of Foxo3 gene than NR cells. Foxo3 nuclear activity by inducing cell cycle arrest, repair of damaged DNA, and apoptosis *via* up-regulation of genes that control these processes, responds to cellular stress (including oxidative stress) by sharing several down-stream anti-stress targets [24-26]. In Foxo3-deficient mice showed that loss of Foxo3 led to rapid death of the animals in response to an oxidative stress [27]. Foxo3 by up-regulating scavenging enzymes such as catalase and peroxiredoxin-2, let to cyto-protective systems enabling the pathological erythroid precursors to resist the oxidative damage and continue to differentiate.

As whereas evaluation of expression of Foxo3 gene cannot shows it`s nuclear or cytoplasmic accumulation, we evaluated the miR144 and miR-451, two miRNAs involve in oxidative stress response which thought to be essential in accumulation of Foxo3 in nucleus [28]. Our results did not find any difference in expression of miR-144 between groups, but it was significant in miR-451 expression, which indicated miR-451 more than miR-144 are engaged in regulation of sensitization to oxidative stress and over-expression of miR-451 but not miR-144 in miR-144/451 mutant zebrafish rescues erythropoiesis [29-31].

One important direct target gene down-modulated by miR-451 is 14-3-3ζ, a phospho-serine/threonine binding protein that inhibits nuclear accumulation of FoxO3, which the excess 14-3-3ζ protein causes a partial re-localization of FoxO3 from nucleus to cytoplasm and reduce expression of genes that encode the important anti–oxidant proteins catalase and glutathione peroxidase 1 [32]. Patrick et al., [33], by observing the loss of miR-451 alone confers susceptibility to phenyl hydrazine-induced hemolysis, suggested that this miRNA has the predominant role in protection against oxidant stress in the miR-144/451 deficient mice. In agreement, Patrick et al., and Yu et al., [29] demonstrated that over-expressed 14-3-3ζ, as observed upon loss of miR-451, could inhibit FoxO3 nuclear accumulation. So, in our study, simultaneous increasing Foxo3 and miR-451 in R group can interpret as Foxo3 nuclear localization. Thus miR-451–14-3-3ζ–FoxO3 regulatory axis can be potentially interesting relevant implications, especially beyond stress erythropoiesis.

Arginase 1 (ARG1) acts as a regulator of HU in erythroid cells and inhibit nitric oxide (NO) production *via* competition with nitric oxide synthesize (NOS) for the substrate L-arginine [34]. In our study ARG1 expression in R group was significantly higher than NR and H group, but between NR and H the difference was not significant. Pourfarzad et al., [18] reported that the ARG1 genes are strongly activated in R-cells before and after HU administration and reported to be significantly associated with response to HU treatment [35]. HU increases NO production through phosphorylation and activation of NOS [36]. High NO concentrations promote apoptosis, while low NO concentrations result in resistance to apoptosis. So, it can conclude that higher expression of ARG1 in R group may protects erythroid progenitor cells against the excessive amounts of NO after HU treatment, to prevent apoptosis, and so scavenge the extra NO at the later stages of differentiation.

Conclusion

Management of toxic effects of agents such HU can be a milestone of achieving their optimum clinical benefits. As, the mutation in beta-globin gene seems to be the sole genetic difference between thalassemia patients and healthy individual, so the environment that the cells are living there, and their interaction to stresses induces by the environment, can be an essential topic in optimum responsiveness to HU. A wide network of growth factors and cytokines also can effect on cell fate, to promote or inhibit their ability to respond to stress. So, resistance or sensitivity to oxidative stress cannot regard as the subsequent defect of thalassemia mutation, so it should be considered as the independent ability of cells to consistent to stress, whether it is BT patient or healthy individual.

In this study, we just focused on possible differences between responders and non-responders to HU. So, recognizing the mechanisms which make the non-responder be sensitive to toxic agents, like HU, therefore activating the compensatory mechanism, may resolve the problem not only in thalassemia patients but also in any patients with same problems. So, the individual medical should be taken into consideration in all cases in the future.

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