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Effect of hydroxyurea on *SP1*, *LIN28B*, *IGF2BP3*, *COL4A5*, *BCL2*, gamma globin genes expression: an in vitro study

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Abstract

Background In some β -thalassemia intermedia patients, hydroxyurea (HU) increases hemoglobin and HbF levels. However, HUs' effects molecular mechanism is still unclear.

Methods In this study, a weighted gene co-expression network analysis was conducted on the GSE109186 dataset. The genes *LIN28A*, *COL4A5*, *SP1*, *BCL2*, and *IGF2BP3* were identified as hub genes involved in the γ -gene switching process. The effect of HU treatment at doses of 50, 100, and 150 μ M for 12, 24, and 48 h on the K562 cell line was examined by using qRT-PCR to measure the expression levels of these hub genes.

Results *SP1* gene expression decreased after treatment with 50, 100, and 150 μ M HU for 12, 24, and 48 h. The expression of the *LIN28A* gene was tripled at three concentrations of 50, 100, and 150 μ M for 12 h. The *IGF2BP3* gene expression was doubled after 24 and 48 h at a concentration of 150 μ M HU. Regarding *COL4A5* gene expression, except at 12 h after treatment at a concentration of 50 μ M, a significant increase was observed in other concentrations and times. The *BCL2* gene expression pattern at all concentrations decreased significantly after 12 h. The γ gene showed a significant increase compared to the control group after 24 and 48 h at the different concentrations.

Conclusion The results showed that in HU-treated cells, changes in the expression of *LIN28A*, *COL4A5*, *SP1*, and *IGF2BP3* genes were accompanied by an increase in γ -gene expression. By elucidating precisely the mechanism of γ -to- β gene switching, we can hope for less complicated drugs.

Keywords Thalassemia, Hemoglobin F, Gene expression, Hydroxyurea

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Introduction

Hemoglobinopathy is a group of disorders related to the abnormal structure or production of the hemoglobin molecule. More than 330,000 babies are born with these disorders each year in developing countries [1]. These disorders affect various organs and are associated with high morbidity and mortality, making them a severe public health problem. Regular blood transfusions with iron chelation and bone marrow transplantation are practical therapeutic approaches in beta-thalassemia (β -thal) [2, 3].

Today, pharmacologically induced human γ -globin gene expression in the intermediate β -thal treatment is a new therapeutic strategy [3, 4]. Drugs that reactivate



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the γ -globin gene can reduce the complications of ineffective erythropoiesis by increasing γ -globin and fetal hemoglobin (HbF) synthesis [1, 4, 5]. To date, drugs such as HU, demethylating agents such as 5-azacitidine, and short-chain fatty acids have been studied to reactivate fetal hemoglobin [3, 5]. In clinical practice, HU promotes HbF production in intermedia β -thal. In addition, previous research has shown that the HU's beneficial effects on HbF production diminish over time due to its possible side effects [6]. Modulation of soluble guanylate cyclase, cyclic adenosine monophosphate (cGMP)-dependent protein kinase (PKG), among other signaling pathways, are associated with HU-regulated γ -globin production [7–9].

cGMP suppresses the activity of cAMP-specific phosphodiesterase 3 (PDE3), which occurs when there is an increase in cAMP levels inside cells. This leads to the activation of PKA, which acts as a negative control for the expression of the γ -globin gene [7]. In K562 cells, the p38 mitogen-activated protein kinase (MAPK) pathway and ERK/MAPK pathway function as activating and inhibitory mechanisms, respectively, for inducing γ -globin gene expression through histone deacetylase inhibitors like butyrate [7, 8]. Hyroxyurea (HU) inhibits erythroid growth by inducing c-Jun expression in transcriptional and post-transcriptional stages [8]. HU has cytotoxic effects on the bone marrow and reduces the absolute number of neutrophils, reticulocytes, and platelets [5]. Success in treating beta-thalassemia with HU has different consequences in different populations. Still, it is usually associated with reducing the need for blood transfusions and preventing side effects such as viral infections and iron deposition in tissues [6, 10]. However, the specific process by which HU triggers an increase in HbF levels is still unclear.

Systems biology has added a fresh perspective to pathophysiology by examining the levels of gene expression related to particular diseases [11]. Genes with different expressions (DEG) and their expression levels and regulatory mechanisms can be identified through transcriptome analysis [12]. This research utilized the weighted gene co-expression network analysis (WGCNA) algorithm to identify and assess the levels of gene expression in cells treated with HU. The likely path of HbF expression change was explored using bioinformatics studies and online databases, and essential genes involved in this process were found. Finally, changes in the target genes expression have been studied to investigate the action of HU on erythroid cells.

Materials and methods

Transcriptome analysis of β-thal RNA-seq dataset

This study was approved by the ethics review board of Birjand University of Medical Sciences and also followed the ethical guidelines of the Declaration of Helsinki (IR. BUMS.REC.1400.022). For the in silico study, we used the GSE90878 RNA-seq gene expression dataset from the Gene Expression Omnibus (GEO) database (https:// www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE90 878). This dataset contains raw data from 12 fetal liver erythroblast and 12 bone marrow erythroblast. First, differential gene expression (DEG) analysis was conducted and are considered as DEGs; they have the following criteria: FDR < 0.001 and $|\log_2 FC| \ge 2.89$. Next, the gene co-expression network of fetal liver erythroblast and adult bone marrow erythroblast (control group) was reconstructed using the WGCNA algorithm. The expression profiles for each module were condensed into eigenvectors that were then compared to the first principal component of the overall expression matrix. This was done using the module eigengene (ME) technique, which helped identify modules that displayed strong correlations with certain clinical features. Individual genes' associations with the β -thal were calculated using gene significance (GS) values. In this study, the membership of each module was determined using both the ME correlation profile and gene expression. The genes that showed higher levels of GS and MM compared to the control sample were considered hub genes.

Cell culture and viability determination

The K562 cell line, a type of CML (chronic myelogenous leukemia) human cells, was grown in RPMI-1640 medium (Gibco-Invitrogen, USA), containing 1% penicillin/streptomycin and 10% heat-inactivated fetal calf serum (provided by Gibco-Invitrogen, USA), at a temperature of 37°C with 5% CO2. The cells were passaged every three days. Cell viability and growth kinetics were evaluated using a hemocytometer and a trypan blue dye exclusion method. An equivalent volume of cell suspension with 4% trypan blue solution were mixed, and the cells were counted using a hemacytometer under a light microscope. The cells were passaged twice a week and then were divided into control (untreated) and HUtreated groups. The cells were cultured at 50, 100, and 150 µM HU concentrations for 12, 24, and 48 h at 37 °C with 5% CO_2 in three replications.

MTT assay

MTT assay was performed to evaluate the cell viability after treating HU at different concentrations. K562 cells were seeded in a 96-well plate at a density of 10×10^3

cells/well in 50, 100, and 150 μ M HU for 12, 24, and 48 h in five replications and incubated at 37°C with 95 percent humidity and 5% CO₂. Next, 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was introduced into each well and left to incubate at 37°C for four h. After removing the supernatant, 100 μ l of DMSO was added to dissolve the formazan crystals. Using a microplate reader, the optical density at wavelengths of 570–630 nm was measured for each well (Bio-Rad Laboratories, Inc.).

RNA extraction and cDNA synthesis

After cell harvesting, total genomic RNA was extracted using the Gene All kit (Hybrid R-South Korea) according to the manufacturer's procedure. The isolated RNA's optical density (OD 260/280) was measured using spectrophotometry to determine its quantity (BioTek, EpochUSA). The extracted RNA was stored in a - 80 °C freezer. Complementary DNA (cDNA) was synthesized according to a commercial kit (Pars Tous manufacturer, Iran). The thermal cycle conditions (Eppendorf Germany) were as follows: 10 min at 25 °C, 60 min at 47 °C, 5 min at 85 °C, and holding at 4 °C. To perform qPCR, the generated cDNA was stored at 20 °C.

Quantitative RT-PCR (Q-RT-PCR)

Table 1 displays the primer sequences utilized for measuring the expression of γ -globin, *BCL2*, *SP1*, *IGF2BP3*, *LIN28A*, and *COL4A5* genes. The qRT-PCR technique was used to evaluate the levels of gene expression. All samples were performed in triplicate. qRT-PCR was carried out in a reaction volume of 20 µl containing: 10 µl of Master Mix SYBR Green (Amplicon A325402 2X qRT-PCR Master Mix Green high Rox), 7µl DEPC, one µl (10 pm concentration) each of forward and reverse genespecific primers (Table 1), and one µL of cDNA sample. Thermal cycling conditions for 40 cycles are presented in

Table 1 Sequences of the primers used in real-time PCR assay

Genes	Sequence primers	Tm
Col4A5	F: ACTATGCCAACTCCTACAGC	55
	R: TTCATGCACACTTGACATCGG	
SPI	F: CTCCTTCAGCCCTTATTACCAC	58
	R:ATTGCCACTGATATTAATGGACTGC	
IGF2BP3	F:ATTCACAGAAGAGATCCCCTTGA	58
	R: ATTCCTGCAATGGAGATATCGTGA	
LIN28A	F: AAGGGTCTGGAATCCATCCGTGT	61
	R: AGACCTCCACAGTTGTAGCACC	
BCL2	F: CGACGACTTCTCCCGCCGCTA	62
	R: GACATCTCCCGGTTGACGCTCT	

Gene Step 1 Step 2 (30 cycles) Step 3 Step 4 SP1 95 °C; 5 min 95 °C-60 s 72 °C; 5 min 60 °C; 1 min 58 °C-45 s 72 °C-45 s IGF2BP3 95 °C-60 s 58 °C-45 s 72 °C-45 s LIN28A 95 °C-60 s 61 °C-30 s 72 °C-30 s COL4A5 95 °C-60 s 55 °C-20 s 72 °C-30 s BCL2 95 °C-60 s 62 °C-30 s 72 °C-30 s

Table 2. The ABI StepOne software was used to analyze the cycle threshold (Ct), amplification plot, and melting curve of each product. The effectiveness of each gene primer was assessed by utilizing the standard curve from a series of diluted cDNAs that were synthesized. We used the *GAPDH* primers for normalizing the data in a real-time instrument (Applied Biosystem, StepOne, USA). The $2^{-\Delta\Delta Ct}$ technique was used to calculate the relative fold change in gene expression in the treatment groups compared with the untreated control group.

Statistical analysis

Statistical analysis was conducted in GraphPad Prism 8.4.3.686 to examine alterations in gene expression, using a one-way ANOVA test and Tukey's test for the mean \pm standard deviation data (p < 0.05).

Results

Transcriptome analysis using WGCNA

In 93 published studies, there was an evaluation of DEG differences between fetal liver and bone marrow erythroblasts, with 65 genes showing increased activity and 28 genes showing decreased activity. These DEGs were subsequently used to identify hub genes. According to the range of expression values, WGCNA included a total of 4000 genes. The findings from WGCNA showed that the blue module (r=0.99, p=1.00 e-200) had the strongest positive correlation with erythroblast evaluation. Genes that had the highest MM and GS scores in the blue module were subsequently compared to the DEG list. Any genes that displayed similarities were identified as ultimate hub genes. Finally, the *LIN28A*, *COL4A5*, *SP1*,

Table 2 Programs used to measure the expression of different genes by real-time PCR



Fig. 1 Effects of HU on K562 cell survival. **A** After 12 h of treatment (p > 0.05) (ns = non-significant) ($p = 0.02^{*}$), **B** after 24 h of treatment ($p = < 0.0001^{****}$), **C** after 48 h of treatment ($p = < 0.0001^{****}$)

BCL2, and *IGF2BP3* genes were selected as the hub genes in the γ -globin gene switching process.

Experimental validation of Silico analysis

MTT assay was conducted in order to determine the survivability of cells treated with HU at 50, 100, and 150 μ M. After 12 h, there was no notable difference in cell viability between the control group and those treated with 50 or 100 μ M of HU (p > 0.05). However, there was a significant decrease in viability at the concentration of 150 μ M (p < 0.05). Evaluation of the viability of K562 cells in the different concentrations of HU after 24 and 48 h showed a significant change compared to the control group (p < 0.05) (Fig. 1).

Genes expression levels

To assess the impact of HU on the reactivation of fetal hemoglobin, we examined the levels of *SP1, LIN28A, IGF2BP3, COL4A5, BCL2,* and γ -globin genes in K562 cells treated with varying concentrations of HU. The expression of these genes was measured at three concentrations of 50, 100, and 150 μ M HU at three time points of 12, 24, and 48 h. *SP1* gene expression levels at 50, 100, and 150 μ M HU after 12, 24, and 48 h were significantly decreased compared to the control group (p < 0.05) (Fig. 2) (reduce by half).

LIN28A gene expression pattern at 50, 100, and 150 μ M HU after 12 h revealed a threefold increase compared to the control group (p < 0.05) (Fig. 3). There were no significant differences at 50 and 100 μ M HU concentrations after 24 and 48 h compared to the control group (p > 0.05). But at 150 μ M, it increased significantly compared to the control group (Fig. 3).

The results did not show a significant difference in the expression pattern of the *IGF2BP3* gene at 50, 100, and







Fig. 3 Evaluation of *LIN28A* gene expression in K562 cells treated with concentrations of 50, 100, and 150 μ M HU at three times of 12, 24, and 48 h. The data are presented as a relative expression with the internal control gene *GAPDH* and repeated three times (*p*=0.002^{**}, *p*=0.003^{*}) (ns=non-significant)



Fig. 4 Evaluation of *IGF2BP3* gene expression in K562 cells treated with concentrations of 50, 100, and 150 μ M HU at three times of 12, 24, and 48 h. The data are presented as a relative expression with the internal control gene *GAPDH* and repeated three times (ns = non-significant, $p = 0.02^*$)



Fig. 5 Evaluation of *COL4A5* gene expression in K562 cells treated with concentrations of 50, 100, and 150 μ M HU at three times of 12–24-48 h. The data are presented as a relative expression with the internal control gene *GAPDH* and repeated three times (ns = non-significant, $p = 0.004^{**}$, $p = 0.0002^{***}$, $p < 0.0001^{****}$)

150 μ M HU concentrations after 12 h, also at 50 and 100 μ M HU after 24 and 48 h compared to the control group. In contrast, the expression level of the *IGF2BP3* significantly increased at 150 μ M HU concentration after 24 and 48 h (p < 0.05) (Fig. 4).

There was no significant difference in *COL4A5* gene expression pattern at 50 μ M HU concentration after 12 h compared to the control group (p > 0.05), while at 100 and 150 μ M HU increased significantly (p < 0.05). In the expression pattern at 50, 100, and 150 μ M HU concentrations after 24 and 48 h, a significant increase was observed compared to the control group (p < 0.05) (Fig. 5).

BCL2 gene expression pattern at all concentrations decreased significantly after 12 h (p < 0.05). There was no significant expression at 50 μ M HU and significantly increased expression at concentrations of 100 and 150



Fig. 6 Evaluation of *BCL2* gene expression in K562 cells treated with concentrations of 50, 100, and 150 μ M HU at three times of 12, 24, and 48 h. The data are presented as a relative expression with the internal control gene *GAPDH* and repeated three times (ns = non-significant, *p* < 0.0001****, *p* = 0.004***, *p* = 0.02*, *p* = 0.004***)



Fig. 7 Evaluation of γ -globin gene expression in K562 cells treated with concentrations of 50, 100, and 150 μ M HU at three times of 12, 24, and 48 h. The data are presented as a relative expression with the internal control gene *GAPDH* and repeated three times (ns = non-significant, $p < 0.0001^{****}$, $p = 0.0009^{***}$, $p = 0.02^*$, $p = 0.008^{**}$)

 μ M after 24 h. It showed a decrease at 50 and 100 μ M HU after 48 h (p < 0.05) (Fig. 6).

Evaluation of γ -globin gene expression showed no significant difference at 50, 100, and 150 μ M HU after 12 h compared to the control group (p > 0.05). But, the expression pattern at 50, 100, and 150 μ M HU concentrations showed a 15-fold increase after 24 and 48 h compared to the control group (p < 0.05) (Fig. 7).

Discussion

Thalassemia is a prevalent genetic disorder worldwide that results from abnormal production of α/β globin due to mutations during mRNA translation [1]. The unpaired globin is seen in erythrocytes as cytoplasmic inclusion bodies and causes the shortening the erythrocytes'

lifespan and occurrence of chronic hemolytic anemia. In intermediate β -thal and some hemoglobinopathies, elevation of HbF levels in adult erythroblasts is an important therapeutic target [1, 13, 14]. The mechanism by which HU generates her HbF is unknown. The discovery of associations between genes affected by HU and those involved in HbF switching may help explain some of these mechanisms.

In this study, using transcriptome analysis by WGCNA method, six genes were selected and the expression of these genes (IGF2Bp3, LIN28A, COL4A5, BCL2, and SP1) under HU treatment and their relationship with γ -globin switching were evaluated.

According to the findings, the level of expression for the LIN28A gene was elevated following HU treatment. The discovery of this gene initially occurred in the Caenorhabditis elegans nematode, and it is responsible for regulating the timing and progression of developmental processes [15]. In humans, two similar versions of the LIN28 gene, known as LIN28A and LIN28B, have been identified. These genes are involved in promoting pluripotency in stem cells and the differentiation of skeletal muscle cells. They also contribute to various physical developments such as changes in height, puberty timing, and age at menopause [15]. The LIN28 gene alters the let-7 family of miRNAs, and its expression plays a role in blocking let-7 [15]. In mature red blood cells, LIN28 protein has been found to reduce let-7 miRNA levels and potentially increase HbF expression [16]. Lee et al. confirmed that a higher level of *LIN28B* expression in mature erythroid cells can lead to down-regulation of let-7 expression and a significant increase in HbF expression level. Furthermore, they found that increased expression of the HbF in mature CD34+cells coincided with a decrease of mature globin expression [17]. These findings align with Lee et al.'s results that LIN28A, homologous to LIN28B, also increases its expression under the influence of HU.

The results showed that *SP1* gene expression decreased with the time series and dose in HU-treated cells. In other studies, they have been shown that the effect of some microRNAs, such as miR-23a and miR-27a, on *SP1* mRNA, inhibits γ -to- β switching [18, 19]. Moreover, HU inhibits the ERK pathway by activating the MAPK pathway, inhibiting SP1 and fetal hemoglobin increases [20]. The ERK pathways are highly regulated cascades responsible for fundamental cellular processes including cell proliferation and differentiation [21]. The normal maturation of erythrocytes is linked to heightened levels of transcriptional activators *GATA-1* and *SP1* [22]. *GATA-1* plays a significant role in promoting the development of red blood cells by regulating various genes specific to this process, such as globin. Additionally, research has demonstrated that *GATA-1* and *SP1* collaborate at the γ -globin promoter [22]. Mnika et al. showed that SP1 is a main target of miR-23a, which inhibits switching γ -globin to β -globin. *KLF3*, a negative regulator of erythrocytes, is also inhibited explicitly by miR-27a [23]. The results of this study are in line with the study of Mnika et al. which showed that HU prevents gamma-to-beta switching by inhibiting SP1.

The results demonstrated that IGF2BP3 gene expression increased after HU treatment. The IGF2BP3 gene is highly expressed in the early stages of development of most tissues and declines after birth. This protein induces erythroblast differentiation and maturation. Also, IGF2BP3 plays an important role in the differentiation process at the early stages of embryogenesis, by binding to three IGF2 leader mRNA and blocking its expression [15]. Jaira et al. showed that induction of IGF2BP1 expression increased y-globin gene. IGF2BP3 is evolutionarily regulated in human erythroid tissues by being silenced during fetal-adult transition. However, the effect of IGF2BP3 on HbF levels was lower than that of IGF2BP1 in cultured adult erythroblasts [24]. The results of this study are consistent with Jaira et al.'s study that the expression of IGF2BP3, like IGF2BP1, increases γ-globin gene expression [24].

The results demonstrated that COL4A5 gene expression was increased in K562 cells exposed to HU. Hydroxyurea is known to induce the expression of γ -globin by miRNA-mediated silencing of BCL11A, a gene involved in switching from γ to β globin gene expression during erythropoiesis in adulthood. The gene expression modulating effects of HU can be mediated through three main mechanistic actions, including epigenetic modifications, post-transcriptional effects, and signaling mediators, including MAPK, PI3K/Akt, and MYB [25]. In this study, we demonstrated that HU treatment can be associated with the up-regulation of COL4A5 along with the induction of y-globin expression. It is currently unknown whether a similar HU-mediated regulatory pathway controls the expression of COLA45 and y-globin, warranting more research in this area. It has been noted that COL4A5 can activate phosphatidylinositol 3-kinase PI3K/protein kinase B (AKT) signaling pathway [26], contributing to the expression of γ -globin [27]. There is a need for further evaluations to divulge other possible roles of COL4A5 in HU-mediated cellular and molecular functions.

The results demonstrate that *BCL2* gene expression decreased significantly after 12 h, but increased again at 24 and 48 h. *BCL2* proto-oncogene inhibits the apoptosis pathway. HU is a compound that acts on ribonucleoside diphosphate reductase and inhibits DNA synthesis. Caspase-3 activation is an important factor in the activation

of the apoptosis cascade. HU increased caspase-3 activity 20 to 30 times higher than the untreated cells. Therefore, there is a correlation with this reagent-induced apoptosis [28]. In a study, it was reported that HU induced apoptotic death of K562 cells through *BCL2* protein down-regulation and Bax protein up-regulation and degraded the PARP protein in a p53-independent pathway [29]. The results are consistent with other studies showing that HU induces apoptosis through down-regulation of the anti-apoptotic protein *BCL2*, inhibiting apoptosis and keeping cells alive.

The findings demonstrated that HU notably increased the expression of the y-globin gene after 24 and 48 h. Pule et al. found that when HU down-regulated BCL11A, MYB, and KLF-1, it resulted in an increase in y-globin expression. Their study revealed a connection between the expression patterns of miR-15a, miR16-1, miR-210, miR-29a, miR-96, and the genes MYB, BCL11A, and KLF-1 in response to HU treatment. They proposed a direct interaction between miR-26b and the MYB 3'-UTR as a possible explanation for the up-regulation of γ -globin through the MYB/KLF-1/BCL11A pathway [30]. Consequently, blocking the c-MYB pathway results in an increase of HbF production. According to Barbarani and colleagues, a heightened HbF expression in cultured red blood cells was linked to decreased c-MYB levels [31]. Although these results may help clarify the molecular mechanism of the y-globin gene switching process, further in vivo and clinical studies are still needed to find alternative therapies for β -thal patients.

Conclusion

The results showed that changes in the expression of *LIN28A*, *COL4A5*, *SP1*, and *IGF2BP3* genes were associated with an increase in the γ -gene in HU-treated cells. By elucidating the mechanism of more precise switching from the γ - gene to β -gene, we can hope for new, less complicated treatments for thalassemia patients.

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Author contributions

E.A., H.S., and E.M.M. designed the experiments. A.A.F and E.A. performed the experiments. A.A.F. and H.S. carried out the data analysis. A.A.F, E.A., and H.S. wrote the manuscript. E.M.M. revised the manuscript. All authors reviewed, considered, and approved the manuscript.

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

This study was approved by the ethical review board of Birjand University of Medical Sciences and followed the ethical guidelines of the Declaration of Helsinki (IR.BUMS.REC.1400.022).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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