ARTICLE

Hydroxyurea differentially modulates activator and repressors of γ-globin gene in erythroblasts of responsive and non-responsive patients with sickle cell disease in correlation with Index of Hydroxyurea Responsiveness

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ABSTRACT

Hydroxyurea (HU), the first of two drugs approved by the US Food and Drug Administration for treating patients with sickle cell disease (SCD), produces anti-sickling effect by re-activating fetal γ-globin gene to enhance production of fetal hemoglobin. However, approximately 30% of the patients do not respond to HU therapy. The molecular basis of non-responsiveness to HU is not clearly understood. To address this question, we examined HU-induced changes in the RNA and protein levels of transcription factors NF-Y, GATA-1, -2, BCL11A, TR4, MYB and NF-E4 that assemble the γ-globin promoter complex and regulate transcription of γ-globin gene. In erythroblasts cultured from peripheral blood CD34$^+$ cells of patients with SCD, we found that HU-induced changes in the protein but not the RNA levels of activator GATA-2 and repressors GATA-1, BCL11A and TR4 correlated with HU-induced changes in fetal hemoglobin (HbF) levels in the peripheral blood of HU high and low responders. However, HU did not significantly induce changes in the protein or RNA levels of activators NF-Y and NF-E4. Based on HU-induced changes in the protein levels of GATA-2, -1 and BCL11A, we calculated an Index of Hydroxyurea Responsiveness (IndexHU-3). Compared to the HU-induced fold changes in the individual transcription factor protein levels, the numerical values of IndexHU-3 statistically correlated best with the HU-induced peripheral blood HbF levels of the patients. Thus, IndexHU-3 can serve as an appropriate indicator for inherent HU responsiveness of patients with SCD.

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Introduction

Sickle cell disease (SCD) is a common, genetic disorder of adult β-hemoglobin, which affects millions of people of diverse racial groups worldwide, including approximately 100,000 Americans, mostly of African descent. Hydroxyurea (HU) is the first of two US Food & Drug Administration (FDA)-approved drugs for treating SCD. In contrast to the recently approved Endari (L-glutamine), HU is shown to ameliorate the SCD symptoms by re-activating the fetal γ-globin gene to produce fetal hemoglobin (HbF) with anti-sickling activity,[1-10] although HU also provides beneficial effects in decreasing adhesion of sickle erythrocytes to vascular endothelial cells, thus reducing complications of vaso-occlusion and infarction.[11,12] However, approximately 30% of SCD patients do not respond to HU therapy in

increasing HbF levels to ameliorate the SCD symptoms.[3-10] The molecular basis of HU non-responsiveness is largely unknown.

The fetal γ-globin gene is silenced in adult erythroid cells but can be re-activated through mechanisms that include the signal-transduction pathway.[13] Thus, the cGMP pathway provides a potential mechanism of γ-globin gene re-activation by HU: HU and/or the nitric oxide generated by HU binds to and activates soluble guanylyl cyclase to synthesize cGMP;[14,15] cGMP in turn activates cGMP-dependent protein kinase PKG to phosphorylate and activate p38 MAPK,[16,17] whose downstream targets ultimately impinge on the γ-globin promoter to activate synthesis of γ-globin mRNA and HbF to produce anti-sickling effect.[13,18] However, the nuclear targets of the HU-induced signaling pathway, the transcription factors (TFs) that bind to γ-globin promoter and activate transcription of γ-globin gene, have not been clearly identified.

A number of TFs bind to the proximal γ-globin promoter and regulate transcription of γ-globin gene. These TFs could be the ultimate nuclear targets of HU in re-activating γ-globin gene in adult erythroid cells. For example, NF-Y binds to the tandem CCAAT motifs in the γ-globin promoter to serve as a pioneering TF in recruiting other TFs to assemble the proximal γ-globin promoter complex and activate transcription of γ-globin gene (Figure 1).[19-21] CoupTFII and dimeric TR2/TR4 compete with NF-Y for binding to DNA motifs overlapping the distal CCAAT box and repress γ-globin gene;[22-25] GATA-1, and -2 bind to the GATA motif in γ-globin proximal promoter to respectively repress and activate γ-globin gene[21,26,27] NF-E4/CP2 dimer binds to its cognate DNA motif near the TATA box to activate γ-globin gene[28] (Figure 1). In addition, BCL11A and MYB are involved in γ-globin gene regulation, since their genetic variants are associated with elevation of HbF levels.[29,30] BCL11A can bind to DNA motifs distal to the γ-globin promoter and act over distance to indirectly repress transcription of γ-globin gene,[31,32] although BCL11A as well as MYB also binds directly to the γ-globin promoter to repress γ-globin gene (Figure 1).[21,33,34] Thus, the inactive γ-globin promoter in adult erythroid cells can bind both a repressor hub of BCL11A/GATA-1/CoupTFII/TR2/TR4 and an activator hub of NF-Y/GATA-2/NF-E4 (Figure 1).[21] The poised state of the γ-globin promoter suggests that pharmacological compounds including HU can modulate the levels of the TFs in the activator and repressor hubs to re-activate the silenced γ-globin gene in adult erythroid cells.

Here, we report that, in erythroblasts cultured *ex vivo* for ten days from peripheral blood CD34⁺ cells of HU high responsive SCD patients, HU increased the protein level of activator GATA-2 and drastically decreased the protein levels of repressors GATA-1, BCL11A, TR4 and MYB to activate transcription of γ-globin gene and synthesis of HbF to produce corresponding anti-sickling effect. In cultured erythroblasts of HU low/non-responders, such HU-induced differential changes in protein levels of the key activator and repressors were not observed. Our findings indicated that HU-induced changes in protein levels, but not RNA levels, of key TFs in the γ-globin promoter complex were strong modulators of HU responsiveness of the SCD patients. Thus, IndexHU-3, based on combined, HU-induced changes in protein levels of GATA-2, -1 and BCL11A, could serve as a strong indicator for inherent HU responsiveness of the SCD patients.

Methods

Isolation of CD34⁺ cells from peripheral blood samples and *ex vivo* culture of CD34⁺ cells in the presence or absence of HU

CD34⁺ cells were isolated from peripheral blood (30 mL) of homozygous HbS/HbS SCD patients seen at the pediatric and adult sickle cell clinics at Medical College of Georgia (MCG), Augusta University, USA and from aphoresed mononuclear cells of normal donors obtained anonymously from MCG Tumor Cell Bank, using protocol approved by the institutional review board (HAC #1009064). The isolated CD34⁺ cells were cultured to days 10-12 in appropriate medium as described,[21,35] in the absence or presence of 50 μM HU, which was the lowest concentration of HU that activated γ-globin mRNA to a high level (see *Online Supplementary Methods* and *Online Supplementary Figure S1*).

RNA and protein analyses by qRT-PCR, RNA-seq and Western blots

The numerical values of HU-induced fold changes, the +HU/-HU values, in the RNA and protein of each TF were calculated as the ratios of the levels of the RNA or protein normalized to the RNA and protein levels of β-actin in cells cultured with HU over the normalized levels of the respective RNA and protein in cells cultured without HU (*Online Supplementary Methods*).

Calculation of IndexHU

IndexHU-3 was calculated according to the formula: IndexHU-3= (FcGATA-2)/(FcGATA-1)x(FcBCL11A), where Fc was the fold change of the respective TFs induced by HU, i.e. the +HU/-HU ratio obtained as shown above. IndexHU-4 was calculated with (FcTR4) included in the denominator and IndexHU-5 with both (FcTR4) and (FcMYB) included in the denominator.

Statistical analysis

Correlation between HU-induced changes in RNA and protein levels of the TFs and HU-induced HbF levels was performed using Prism 5 linear regression analysis program. Pearson correlation coefficient was calculated between peripheral blood HbF levels and IndexHUs. A 2-sample *t*-test was performed to compare HU-induced changes in each of the TFs and to compare the IndexHUs between high- and low/non-responders. Paired *t*-test was used to analyze equivalence of HU-induced changes in HbF levels in cultured erythroblasts and in peripheral blood of the SCD patients. The statistical tests were two-sided at 0.05 significance levels with SAS 9.4.0 (SAS InstituteInc., Cary, NC, USA).

Hypoxia condition to induce sickling of SCD erythrocytes

Day 12 erythrocytes were isolated from *ex vivo* cultured day 12 cells (erythroblasts mixed with erythrocytes) of peripheral blood CD34⁺ cells by Ficoll-Paque gradient as described (*Online Supplementary Methods*). The day 12 erythrocytes were deoxygenated in a hypoxia chamber (Coy Laboratory Products) at 2% oxygen for 6 hours. The deoxygenated cells were fixed with 3.7% formaldehyde on ice for 15 minutes in the hypoxia chamber before removal to atmosphere for microscopic observation and imaging (EVOS fluorescent cell imaging system).

Results

HU increased HbF levels and produced anti-sickling effect in *ex vivo* cultured erythroid cells

In order to unambiguously analyze the molecular basis

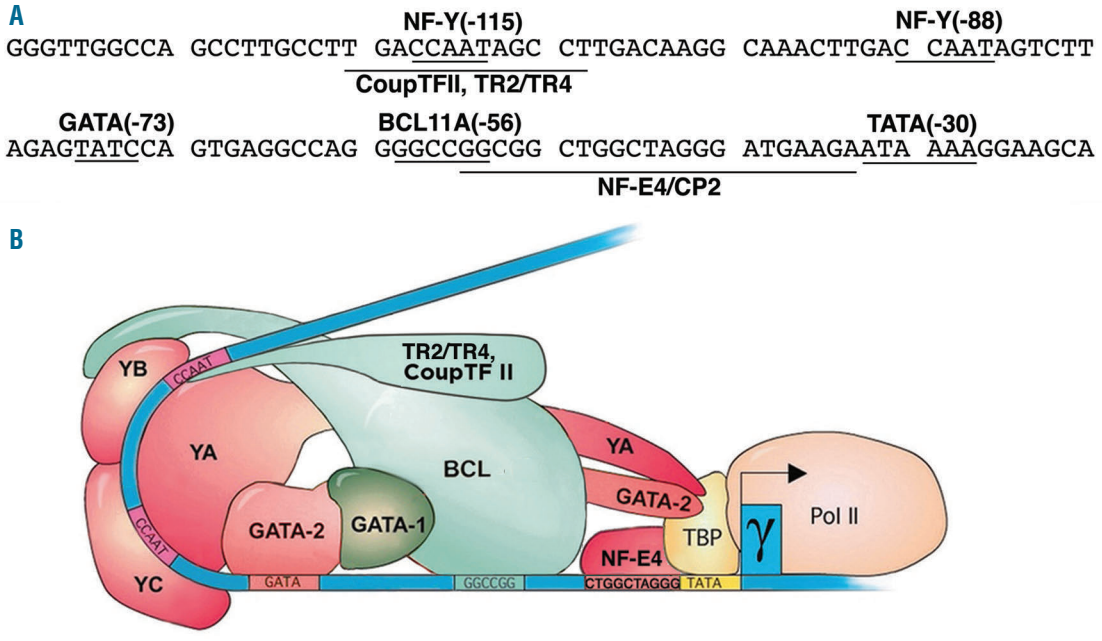

Figure 1. Molecular assembly of the key transcription factors (TFs) in proximal γ-globin promoter complex. (A) Sequence of the proximal γ-globin promoter. Underlined bases: DNA motifs that bind transcription factors as marked. Numbers in parentheses: first base positions in the motifs relative to the transcription start site of γ-globin mRNA at +1 located 25 bases 3' of the TATA box. The MYB binding site CAATG at -181[33] was not shown. (B) Proximal γ-globin promoter complex. Blue ribbon: promoter DNA containing transcription activator-binding motifs (red bars) and repressor-binding motifs (light green bars), which respectively bind transcription activators, NF-Y, GATA-2 and NF-E4, marked in red and transcription repressors, BCL11A, GATA-1, CoupTFII and TR2/TR4, marked in green. Blue rectangle with angled arrow: γ-globin gene and transcriptional direction of γ-globin mRNA. NF-Y, composed of subunits YA, YB and YC, binds to each of the tandem CCAAT motifs and bends the DNA by approximately 70° to form the pocket to recruit and interact with other TFs in assembling the proximal promoter complex (adapted from Zhu *et al.*[21]). MYB protein was not shown, as its interaction with TF partner(s) in the proximal γ-globin promoter complex was not known.

of HU-responsiveness of SCD patients, we focused on two groups of patients with widely separated peripheral blood HbF levels induced by HU therapy: the HU low/non-responsive patients with post HU HbF levels of ≤10% and the HU high responsive patients with post HU HbF levels of 20-30%[3,9] (*Online Supplementary Table S1*). We isolated CD34$^+$ cells from peripheral blood samples of the SCD patients and cultured the CD34$^+$ cells in erythroid differentiation medium for ten days in the presence or absence of HU. To validate these *ex vivo* cultured day 10 erythroblasts as an appropriate cell system for dissecting the molecular basis of HU responsiveness, we compared the HbF levels induced by HU in cultured day 10 erythroblasts and in peripheral blood of the SCD patients. We found that in day 10 erythroblasts of HU high responders SCD 01 and 02 and low responder SCD 04, HU increased HbF levels by 2.5-, 2.1- and 2-fold, from 8% to 20%, 14% to 29% and 4% to 8%, respectively (Figure 2A). In the peripheral blood of these 3 SCD patients, the clinical records showed that HU at maximum tolerated dose (MTD) increased HbF levels by 2.1-, 2.7- and 2-fold, from 12% to 25%, 11% to 30% and 3% to 6%, respectively (Table 1 and *Online Supplementary Table S1*). The comparable fold changes in HbF levels induced by HU in cultured erythroblasts and in initial HU trials at maximum tolerated dose (MTD) in peripheral blood of both HU responsive and low/non-responsive SCD patients (see *Online Supplementary Table S2* for paired *t*-tests) indicated that successive transfusions in SCD04 and other low/non-responsive patients on blood exchange (*Online Supplementary Table S1*), which could blunt subsequent HbF induction by HU in the patients, did not exert lasting genetic effects on patient CD34$^+$ cells to suppress HU-induced HbF levels in cultured day 10 erythroblasts. Thus, the *ex vivo* cultured patient erythroblasts could serve as an appropriate cell system for designing bioassays to dissect the *in vivo* molecular basis of HU responsiveness of the SCD patients.

Since the HU low responsive SCD04 patient required blood exchange from normal donors to ameliorate the SCD symptoms, HbA expressed by donor erythrocytes, in addition to patient HbS, was detected by HPLC in the exchanged peripheral blood of the patient (Figure 2A, right 2nd panel). However, the donor HbA was not detected by HPLC in the cultured day 10 erythroblasts (Figure 2A, right 3rd and 4th panels). This was expected, since the transfused, donor blood did not contain nucleated progenitor cells including CD34$^+$ cells, which were removed prior to transfusion. Thus, the day 10 erythroblasts were derived only from the patient CD34$^+$ cells and expressed only HbS. In HPLC analysis of HbF levels in patient erythrocytes after blood exchange, HbF% was calculated as HbF/HbF+HbS without including HbA (see *Online Supplementary Methods*), since HbA was expressed in separate donor erythrocytes. However, the calculated HbF% of 6% for SCD04 (Figure 2A, right 2nd panel) could be overestimated slightly, since the HbF peak in HPLC contained also approximately 0.2-0.5% of HbF contributed by the normal donor erythrocytes (Table 1).

Hypoxia chamber assay of cultured day 12 erythrocytes showed that HU treatment reduced sickled erythrocytes of HU high responder, SCD 02, from 38% to 20%, and HU low responder, SCD 04, from 80% to 58% (Figure 2B). Thus, HU-induced increase in HbF levels correlated with

HU-induced reduction in sickling of the erythrocytes (Figure 2C). The cell sickling assays together with the HPLC analysis (Figure 2A) showed that cultured patient erythroblasts provided an appropriate *ex vivo* cell system for dissecting the molecular basis of HU-responsiveness of the SCD patients.

HU slowed down the cell cycle but did not delay *ex vivo* erythropoiesis of cultured patient erythroblasts

As HU is an inhibitor of DNA synthesis,[37] it could slow down cell division and delay *ex vivo* erythropoiesis.[2,38] Thus, it could be argued that the HU treated day 10 erythroblasts, as compared to the untreated day 10 erythroblasts, contained more abundant earlier stage erythroblasts, which expressed higher levels of γ-globin gene and thus higher HbF levels. Therefore, HU-induced HbF production did not directly involve TF-mediated nuclear events on transcription of the γ-globin gene.

To investigate this possibility, we analyzed by FACS the day 10 cells stained with antibodies against erythroid dif-

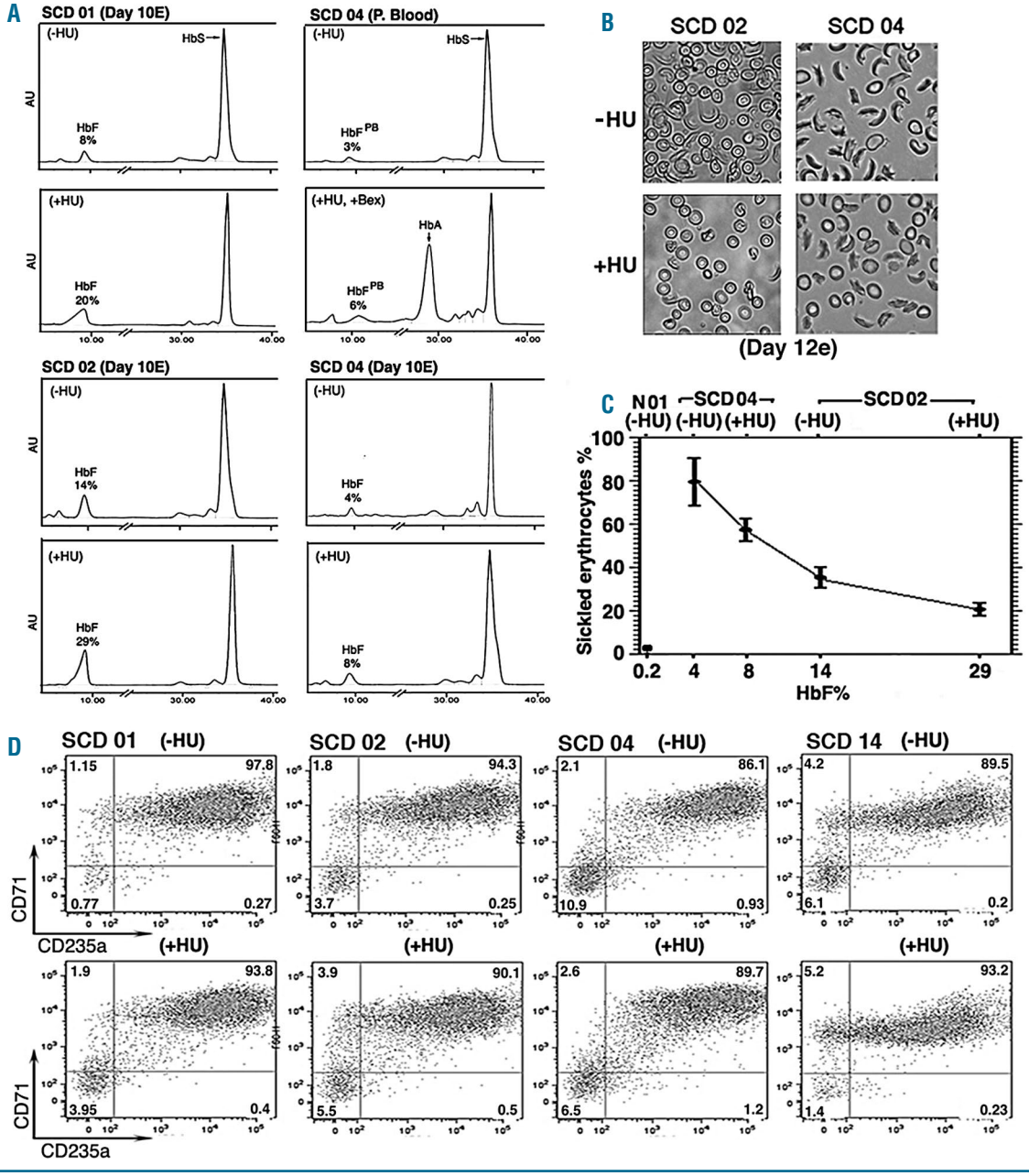

Figure 2. Hydroxyurea (HU) increased fetal hemoglobin (HbF) levels and produced anti-sickling effect in cultured erythrocytes of sickle cell disease (SCD) patients. (A) HPLC analysis of HbF levels in day 10 erythroblasts (Day 10E) and HbF^{PB} in peripheral blood (P.blood) of SCD patients. (-HU) and (+HU): day 10 erythroblasts cultured without and with HU, or patient peripheral blood obtained before HU therapy and after HU and blood exchange (Bex) therapies. HbF, HbS and HbA elution peaks were as marked. y-axis: absorption units (AU) at 410 nm of the eluted hemoglobins; x-axis: time in minutes when the hemoglobins were eluted from the HPLC column. (B) Day 12 erythrocytes (Day 12e) of HU high and low responders, SCD02 and 04, respectively, cultured without and with HU, and subjected to hypoxia to induce cell sickling. Images of peripheral blood erythrocytes of SCD04 without and with HU therapy were similar to those of cultured erythroblasts (*data not shown*). (C) Percentages of sickled erythrocytes [among 400 counted cells in images in (2B)] plotted against HU-induced HbF levels of Day 10 erythroblasts from the same patients in (2A). (D) FACS analysis of Day 10 erythroblasts cultured without and with HU from HU high responders SCD 01, 02, and low responders SCD04, 14. Cells were stained with erythroid markers CD71 and CD235a, respectively.

ferentiation markers transferrin receptor and glycophorinA to determine if HU-treated day 10 cells contained more abundant early stage erythroblasts. We found that day 10 cells cultured in the presence or absence of HU showed similar staining patterns for both HU high and low responders (Figure 2D and *Online Supplementary Figure S2*), indicating that the HU-treated erythroblasts did not contain a higher proportion of earlier erythroid progenitors. However, the cell numbers of the day 10 erythroblasts grown in the presence of HU were 1/2 to 1/4 of those grown in the absence of HU for both HU high and low responsive patients. This indicated that HU slowed down the cell culture by 1-2 cell divisions without apparently delaying the *ex vivo* erythropoiesis of the day 10 ery-

Table 1. Hydroxyurea (HU)-induced changes in RNA and protein levels of transcription factors (TFs), γ-globin (γ-glob) and fetal hemoglobin (HbF) levels in day 10 erythroblasts of 18 sickle cell disease (SCD) patients (SCD #1-18) and 3 normal donors (Normal #1-3).

SCD	NFY	GATA2	GATA1	BCL	TR4	MYB	NFE4	γ-glob	HbF%	HbF%^{PB}	IndexHU-3
1 RNA^{a}		4.7	1.3	0.9				2.8			
Protein		2.86	0.195	0.175				3.51	8/20	12/25	83.8
2 RNA^{a}		4.4	0.9	0.83				2.5			
RNA^{b}	0.93	4.1	0.97	0.65	0.8	1.38		2.09	14/29	11/30	101.6
Protein		2.9	0.172	0.166							
8 RNA^{a}	1.1	3.7	0.97	0.86	0.87	0.57	0.95	2.0			
RNA^{b}	1.11	2.47	1.04	0.75	1.03	0.44		2.44			
Protein	0.93	1.21	0.25	0.10	0.23	0.29	0.90	1.81	5.3/16.4	6.2/17	42
9 RNA^{a}	1.13	3.88	1.38	0.61	0.5	0.53	1.1	2.38			
Protein	1.11	1.42	0.18	0.08	0.14	0.09	1.17	2.94	6/18	7/22	98.6
10 RNA^{a}	0.99	2.67	0.89	0.78	0.87	0.61	0.95	2.53			
Protein	1.10	1.74	0.21	0.10	0.18	0.2	0.82	3.86		12/23.6	83
3 RNA^{a}		4.1	1.2	1.2				1.8			
Protein		0.99	0.53	0.61					4.2/9	4.5/8.8	3.0
4 RNA^{a}		2.9	1.2	1.1				1.25			
Protein		1.9	0.53	0.49					4/8	3/6	7.3
5 RNA^{a}		2.7	0.7	0.8				1.2			
Protein		2.24	3.2	0.34					2.2/5.3	1.5/4.9	2.1
6 RNA^{a}	0.94	2.5	0.89	0.78	0.83	0.63	1.0	1.87			
Protein	0.95	1.0	0.52	0.68	1.16	0.2	0.93	1.23		4/10	2.8
7 RNA^{a}	0.95	1.96	1.0	0.95	0.96	1.1	0.99	1.51			
Protein	1.12	1.05	0.78	0.58	0.95	1.0	0.98	1.06	5.1/9.6	4.7/11.5	2.3
11 RNA^{a}		3.25	1.2	0.7				1.75			
Protein		0.87	0.95	0.99					3/4	3.2/3.1	0.9
12 RNA^{a}		3.49	1.21	0.72				1.63			
Protein		0.76	0.4	0.4						1.2/2.9	4.7
13 RNA^{a}		4.8	1.16	0.5				0.46			
Protein		0.42	0.48	0.65						2.1/3.5	1.4
14 RNA^{a}		3.39	0.61	0.66				0.53			
RNA^{b}	0.86	2.64	0.57	0.4	0.98	0.85		0.51			
Protein		0.54	0.77	1.17						1.1/1.8	0.6
15 RNA^{a}		2.78	1.06	0.79				1.12			
Protein		0.67	0.41	0.95						1.4/2.9	1.7
16 RNA^{a}		3.79	0.74	0.82				1.16			
Protein		1.7	0.93	0.64						2.6/3.8	2.9
17 RNA^{a}	1.10	1.87	1.38	0.87	1.10			1.27			
Protein	1.05	1.06	1.04	1.13	0.95					0.7/1.0	0.9
18 RNA^{a}	0.97	1.53	0.82	0.9	0.75	0.58		1.36			
RNA^{b}	1.05	2.56	0.96	0.98	0.63	0.7		1.89			
Protein	1.14	0.91	0.78	1.47	0.86	0.49		1.16		2.1/3.7	0.8

Normal	NFY	GATA2	GATA1	BCL	TR4	MYB	NFE4	γ-glob	HbF%	HbF%PB	IndexHU-3
1 RNA^{a}	0.98	1.91	0.98	1.13	0.91	0.75		1.41			
Protein	1.0	1.65	1.0	1.23	0.24	0.39		1.28		0.2	1.3
2 RNA^{a}		1.79	0.86	1.34				1.1			
Protein		1.1	1.07	1.06						0.3	1.0
3 RNA^{a}		1.63	0.9	0.65							
Protein		1.6	0.91	0.62						0.5	5.5

RNA^{a} and RNA^{b}: hydroxyurea (HU)-induced changes in RNA levels in total cellular RNAs determined by qRT-PCR and RNA-seq, respectively. Protein: HU-induced changes in protein levels determined by Western blots. Numbers: HU-induced fold changes, +HU/-HU, in RNAs and proteins. HbF%^{PB} : fetal hemoglobin (HbF) levels in peripheral blood (PB) of the sickle cell disease (SCD) patients; two numbers separated by a slash: HbF levels in day 10 erythroblasts before and after HU treatment or HbF levels in peripheral blood of SCD patients pre- and post-HU treatment at maximum tolerated dose (MTD) recorded in the clinic. IndexHU-3: quantitative estimates of HU responsiveness calculated from HU-induced changes in the protein levels of GATA2, GATA-1 and BCL11A. CoupTFII was not analyzed as its RNA was not detected by RNA-seq on day 10 erythroblasts cultured either with or without HU (*Online Supplementary File 2*), nor was TR2, as TR4 with lower RNA level than TR2 RNA level (*Online Supplementary File 2*) appeared to be the limiting partner in the TR2/TR4 heterodimer. See *Online Supplementary Files S1* and *S2* for data and calculations.

throblasts. One explanation could be that, in our cell culture condition, HU was added on day 4, by which time the erythroid differentiation program of the cultured cells could have already been set up. Therefore, HU did not appreciably delay the differentiation of the cultured erythroblasts, indicating that the cultured erythroblasts were appropriate for subsequent studies to dissect the molecular basis of HU responsiveness of the SCD patients.

HU-induced changes in protein but not RNA levels of key TFs correlated with HU-induced HbF levels in cultured erythroblasts of SCD patients

To determine the HU-induced changes in the RNA and protein levels of activators NF-Y, GATA-2 and NF-E4 and repressors GATA-1, BCL11A, TR4 and MYB that assemble the γ-globin proximal promoter complex (Figure 1), we used qRT-PCR, RNA-seq and Western blots to analyze the day 10 erythroblasts cultured with or without HU. To achieve statistical significance for the bioassays, we analyzed a total of 18 homozygous SCD patients (7 pediatric and 11 adult patients) among whom 5 were HU high responders (SCD 01, 02, 08, 09 and 10) and 13 were HU low responders (*Online Supplementary Table S1*). Although HU low/non-responsive patients comprise approximately 30% of the SCD patients, approximately 70% of the blood samples examined in this study were from low/non-responsive patients, because blood samples in large volumes of 30 mL required for the bioassays were more readily available from HU low/non-responsive patients undergoing blood exchange (see *Online Supplementary Methods*).

RNA analysis by qRT-PCR was performed for all 18 SCD patients and 3 normal donors; genome-wide RNA-seq analysis was performed for HU high responders (SCD 02 and 8), and HU low responders (SCD 14 and 18). The results showed that HU significantly and universally increased GATA-2 RNA levels by 200-500% in day 10 erythroblasts of both HU high and low responders and the normal donors (Table 1, Figure 3A, and *Online Supplementary Files 1* and *2*), in agreement with an earlier report.[39] In contrast, HU mildly decreased or increased by ≤50% the RNA levels of repressors GATA-1, BCL11A, TR4 and MYB in erythroblasts of all the SCD patients and normal donors (Table 1 and *Online Supplementary Files 1* and *2*). These results were consistent with earlier reports that HU treatment modulates the RNA levels of BCL11A and MYB.[40,41] However, CoupTFII RNA was not detected by either RT-PCR or RNA-seq (*Online Supplementary File 2*) in day 10 erythroblasts and was not further analyzed.

The HU-induced changes in TF RNA levels did not consistently correspond to HU-induced changes in protein levels of the respective TFs. Thus, despite the HU-induced, universal increase in GATA-2 RNA levels in all SCD patient erythroblasts, HU increased GATA-2 protein level only in HU high responders SCD 01, 02, 08-10, whereas HU appeared to randomly decrease or increase GATA-2 protein level in the HU low responders (Table 1 and Figure 3B). Similarly, despite the HU-induced mild changes in RNA levels of repressors GATA-1, BCL11A, TR4 and MYB in the 18 SCD patient erythroblasts, HU drastically decreased by 70-80% the protein levels of these repressors only in high responders SCD 01, 02, 08, 09 and 10; but HU did not reduce or mildly reduced by ≤50% the repressor protein levels in the erythroblasts of HU low/non-responders SCD 03-7 and 11-18, with the exception that MYB protein level was significantly reduced by ≥50% also in HU low responders SCD 06 and 18 (Table 1 and *Online Supplementary File S1*). Statistical analysis by 2-sample *t*-tests confirmed that HU differentially modulated the protein levels, but not the RNA levels, of the repressors in HU high *versus* HU low responders; in contrast, in both HU high and low responders, HU did not induce significant changes in either the protein or the RNA levels of NF-Y and NF-E4, indicating that these two activator TFs did not mediate HU responsiveness of the SCD patients (*Online Supplementary Table S3*).

In HU high responders, HU drastically decreased the repressor protein levels and mildly increased the protein level of activator GATA-2, which in combination lead to activation of γ-globin RNA transcription, synthesis of γ-globin protein and HbF (Table 1, and *Online Supplementary Table S3* and *Online Supplementary Figure S3A*), and consequently production of anti-sickling effect in both the cultured erythroblasts and peripheral blood of the SCD patients (Figure 2B and C). In HU low responders, HU mildly decreased the repressor protein levels and inconsistently increased GATA-2 protein level, which in combination did not sufficiently activate γ-globin gene and synthesis of HbF to produce significant anti-sickling effect in these patients.

We next used scatter plots to statistically and graphically correlate the HU-induced changes in protein and RNA levels of the TFs with HU-induced changes in HbF levels in peripheral blood of the 18 SCD patients. Regression analysis showed that HU-induced changes in GATA-1 and BCL11A protein levels, but not RNA levels, significantly correlated with HU-induced changes in peripheral blood HbF levels of the patients, while the correlation between GATA-2 protein levels and HbF levels was less significant (Figure 4A and B). The negative slopes of the correlation graphs of GATA-1 and BCL11A (higher HU-induced repressor protein levels correlating with lower HU-induced HbF levels) were consistent with GATA-1 and BCL11A being repressors of γ-globin gene; the positive slope of the GATA-2 graph was consistent with GATA-2 being an activator of γ-globin gene. Scatter plots also indicated that the HU-induced changes in TR4 and MYB protein levels correlated less strongly with the HU-induced changes in HbF levels, as compared to HU-induced changes in GATA-1 and BCL11A protein levels (compare *Online Supplementary Figure S3B* and *C* with *F* and *G*). In contrast, HU-induced changes in GATA-2 protein level correlated weakly but NF-Y and NF-E4 protein levels correlated not at all with the HU-induced changes in HbF levels (*Online Supplementary Figure S3D* and *E*). Thus, the protein levels of repressors GATA-1 and BCL11A were strong modulators and the protein level of activator GATA-2 was a weak modulator of the HU responsiveness of the SCD patients.

Index of HU responsiveness, IndexHU-3, calculated from HU-induced changes in protein levels of GATA -1, BCL11A and GATA-2, as a numerical indicator for HU responsiveness of SCD patients

To quantify the cumulative effects of HU-induced changes in the protein levels of the transcription activator and repressors, we calculated the IndexHU-3 according to the formula: *IndexHU-3= (FcGATA-2)/(FcGATA-1)x(FcBCL11A)*, where Fc was the HU-induced fold changes in the protein levels of GATA-1, -2 and BCL11A

(*see Methods section*). The underlying rationale for the formulation was that HU-induced changes in activator protein level in the numerator together with HU-induced decrease in the repressor protein levels in the denominator would produce numerical values of IndexHUs quantitatively correlating with HU-induced changes in HbF levels and thus HU responsiveness of the SCD patients. Indeed, IndexHU-3s had numerical values of 40-100 for HU high responders and ≤10 for HU low/non-responders (Table 1). Correlation analysis by scatter plots showed that IndexHU-3 calculated from HU-induced protein levels, but not RNA levels, correlated strongly with the HU-induced HbF levels in peripheral blood of the SCD patients, with R^2=0.9, as compared to the correlation between HU-induced changes in the protein levels of the individual TFs, with R^2=0.3-0.6 (Figure 4B and C). Thus, IndexHU-3 could serve as a quantitative indicator/predictor for the inherent HU responsiveness or non-responsiveness of the SCD patients (*Online Supplementary Table S3*).

As HU also induced a drastic reduction in the protein levels of TR4 and MYB in HU high responders, we calculated IndexHU-4 and -5 to include HU-induced (FcTR4) and (FcMYB) in the denominator. However, plotting IndexHU-3, -4 and -5 with respect to HU high *versus* HU low responders showed that IndexHU-4 and -5 did not improve the power to distinguish between HU high and low responders (Figure 4D). Thus, IndexHU-3 could serve as a reliable indicator to predict HU responsiveness of the SCD patients.

Discussion

In this study, we investigated the molecular basis of HU responsiveness of SCD patients to ascertain the underlying mechanism(s) of why approximately 30% of SCD patients could not respond to HU therapy in enhancing HbF levels to produce an anti-sickling effect and to ameliorate the SCD symptoms. We first validated the appropriateness of the *ex vivo* cultured patient erythroblasts for the bioassays to dissect the *in vivo* molecular basis of HU responsiveness. We found that HU similarly induced HbF

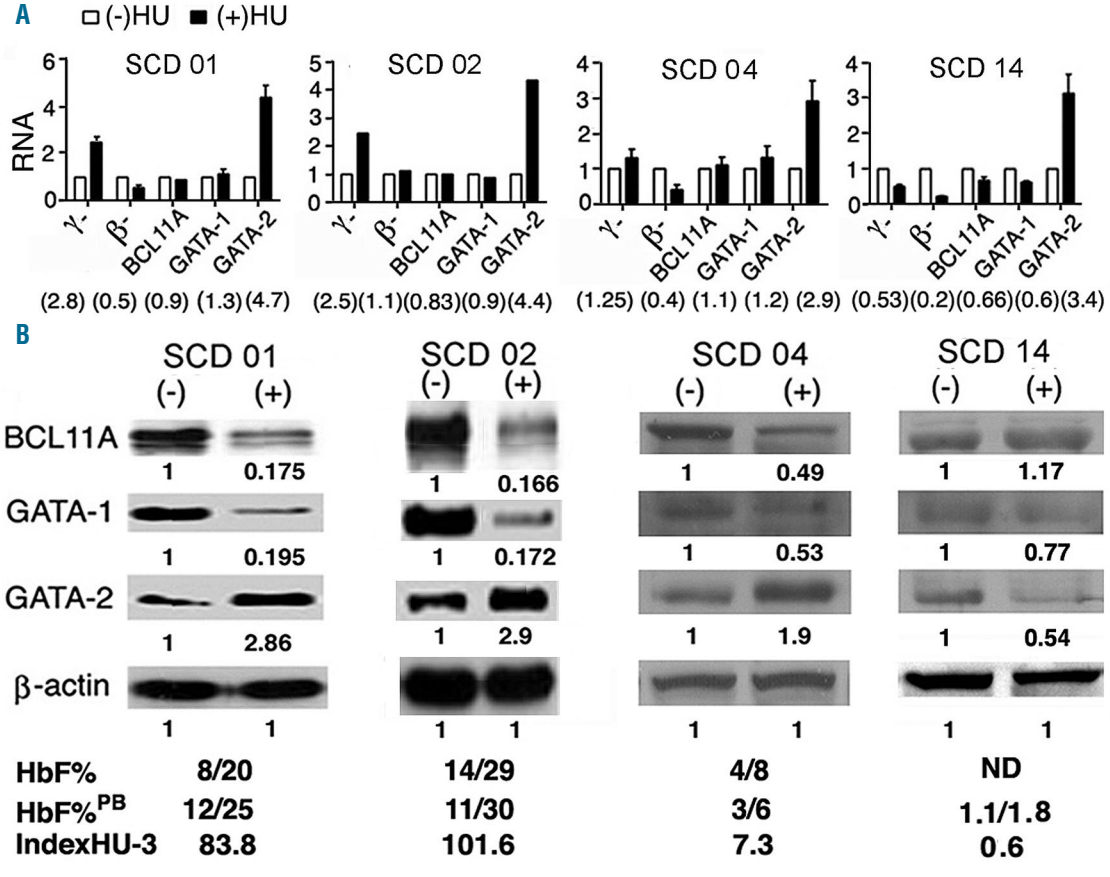

Figure 3. Hydroxyurea (HU)-induced changes in RNA and protein levels of GATA-2, -1 and BCL11A in day 10 erythroblasts of HU high and low/non-responsive sickle cell disease (SCD) patients. (A) Total cellular RNAs isolated from day 10 patient erythroblasts cultured without and with HU and analyzed by qRT-PCR. For SCD 02, 04 and 14, the RT-PCR results shown were means of technical triplicates from two independent RNA preparations normalized with respect to the RNA level of β-actin; for SCD 01, the RT-PCR results were means of technical triplicates. y-axis: the +HU/-HU ratios of normalized RNA levels of genes in cells treated with HU over the normalized RNA levels of the same genes in control cells not treated with HU, which were set at 1. Numbers in parentheses: numerical values of +HU/-HU ratios of each of the genes. (B) Western blots of protein levels of GATA-1, -2 and BCL11A in day 10 erythroblasts cultured without and with HU, (-) and (+), respectively, from the same SCD patients as in (A). Numbers underneath the blots: +HU/-HU ratios of the protein levels of the TFs normalized with respect to the protein level of β-actin in erythroblasts treated with HU over the normalized protein levels of the same TFs in control erythroblasts without HU. HbF% and HbF%^{PB}: Fetal hemoglobin (HbF) levels in day 10 erythroblasts determined by HPLC and in peripheral blood of the SCD patients obtained from the clinic; ND: not done; IndexHU-3: Index of HU responsiveness calculated from HU-induced fold changes in protein levels of GATA-2, -1 and BCL11A.

levels in cultured patient erythroblasts as in peripheral blood of the SCD patients on HU and/or blood exchange therapies (Table 1 and Figure 2). It has recently been reported that, in erythroblasts cultured from peripheral blood CD34+ cells of a group of SCD and β-thalassemia patients who were initially not on HU therapy but were subsequently put on prospective HU therapy, HU-induced changes in HbF levels either before or after HU therapy are similar.[42] This finding, together with our results, indicates that HU and blood exchange therapies did not exert lasting genetic effects on bone marrow CD34+ cells of the patients to significantly change HU-induced HbF levels in patient erythroblasts cultured from the CD34+ cells.

Protein and RNA analyses of the cultured patient erythroblasts showed that HU-induced changes in the protein levels of repressors GATA-1 and BCL11A were strong modulators, and activator GATA-2 a weak modulator of HU-induced HbF levels, and hence HU responsiveness of the SCD patients. In HU low/non-responsive patients with post-HU HbF levels ≤10%, HU did not drastically decrease the protein levels of the repressor TFs nor consistently increase the protein level of activator GATA-2 (Table 1 and Figure 3) to sufficiently activate transcription of γ-globin RNA and synthesis of HbF to produce significant anti-sickling effect in cultured erythroblasts and peripheral blood (Figure 2). Since HU-induced changes in the RNA levels of the key TFs did not correlate at all with HU-induced peripheral blood HbF levels (Figure 4A), HU-induced changes in the RNA levels of the TFs could not serve as appropriate indicators of HU responsiveness of the SCD patients.

IndexHU-3, calculated from combined HU-induced changes in the protein levels of GATA-2, -1 and BCL11A, correlated strongly (R^2=0.9) with HU-induced peripheral blood HbF levels of the patients and, therefore, could serve as a strong indicator of HU responsiveness (Figure 4B and C, and *Online Supplementary Table S3*). It has been shown recently that HU-induced fold changes in γ-globin RNA levels in cultured erythroblasts of a group of SCD and β-thalassemia patients are the best indicator so far to predict HU responsiveness for these patients.[42] Indeed, HU-induced fold changes in γ-globin RNA levels correlated with HU-induced γ-globin protein and HbF levels in cultured patient erythroblasts (Figure 2 and *Online Supplementary Figure S3A*) and showed a significant difference between HU responsive and non-responsive patients (*Online Supplementary Table S3*). However, the HU-induced fold changes in γ-globin RNA levels were in a narrow range of 1.2-1.9 for HU low/non-responders and 2-2.8 for HU high responders (Table 1 and Figure 3). IndexHU-3 with numerical values of

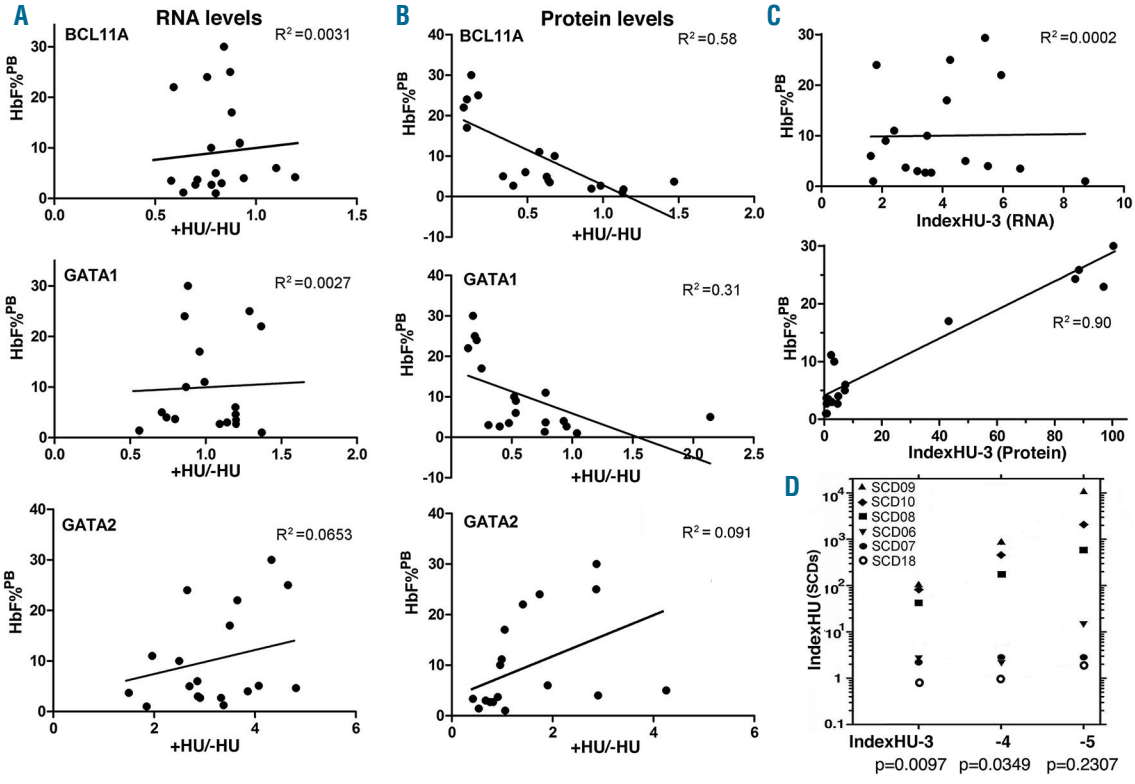

Figure 4. Statistical correlation analysis by scatter plots of hydroxyurea (HU)-induced fold changes in RNA and protein levels of GATA-2, -1 and BCL11A and of Index HU with respect to HU-induced changes in peripheral blood HbF levels of the 18 sickle cell disease (SCD) patients. (A and B) Scatter plots of HU-induced fold changes in RNA and protein levels of BCL11A, GATA-1 and -2 with respect to HU-induced peripheral blood HbF levels of the 18 SCD patients. (C) Scatter plots of IndexHU-3s calculated from HU-induced fold changes in the RNA and protein levels of GATA-2, -1 and BCL11A plotted against HU-induced peripheral blood HbF levels of the 18 SCD patients. (D) IndexHU-3, -4 and -5 calculated from the HU-induced changes in protein levels of 3, 4 and 5 TFs in 6 SCD patients. SCD 08, 09 and 10: HU high responders; SCD 06, 07 and 18: HU low responders. y-axis: numerical values of the respective IndexHUs; *P*-values: significance of the separation in the numeric values of IndexHU-3, -4 and -5 of the HU high *versus* low responsive groups.

40-100 for high responders and <10 for low/non-responders (Table 1 and Figure 3), therefore, provided a much wider numeric range for more accurate assessment of HU responsiveness of the SCD patients.

Hydroxyurea could modulate the protein levels of the key TFs by modulating translational efficiency and/or stability of the TF proteins. Thus, genetic variations in HU low/non-responders, such as quantitative trait loci (QTL) identified by single nucleotide polymorphisms (SNPs) to associate with HU response in SCD patients,[43] could impair critical steps in the HU-mediated protein translation and degradation pathways of the key TFs, resulting in low HU responsiveness of the patients. Recent studies on translational initiation and ribosome profiling show that the translation efficiency of key erythroid mRNAs, including BCL11A and γ-globin mRNAs, is dynamically controlled during erythropoiesis and could be subject to regulation by HU.[44,45] In addition, HU through regulating specific miRNA levels,[41,46] could differentially block or unblock translation of the activator and repressor TF proteins. During erythropoiesis, GATA-1 protein has been shown to be post-transcriptionally phosphorylated and subsequently degraded through the ubiquitin-proteasome pathway.[47-49] These HU-downstream pathways that regulate protein synthesis and stability of key TFs in the γ-globin promoter complex, as well as other γ-globin modulators such as lysine specific demethylase 1 (LSD1) and GPC1 that could regulate γ-globin through pathways independent of HU,[13,50] may provide targets for designing new SCD drugs to ameliorate the SCD symptoms of HU low/non-responsive patients.

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