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Response to hydroxyurea treatment in Iranian transfusion-dependent β-thalassemia patients

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A B S T R A C T

Background and Objectives. Hydroxyurea (HU) is known to increase γ-globin chain expression in postnatal life. The efficacy of HU treatment in thalassemia patients is still unclear. The aim of this study was to monitor treatment of a large cohort of patients with β-thalassemia major in order to establish the response to HU and the associated elements.

Design and Methods. HU therapy was started in 133 patients diagnosed with transfusion-dependent β-thalassemia in 1999. The molecular background of the disease, the polymorphisms of the promoter region of the genes, the haplotype of the β-globin gene cluster, the α-gene deletions and the HS2 polymorphism at the locus control region (LCR) of the β-globin gene cluster were studied.

Results. We were able to classify three categories of response: a *good* response (61%) in patients who shifted from monthly blood transfusion dependency to a stable transfusion-free condition at an average Hb level of more than 10 gm/dL; a *moderate* response (23%) in patients who remained transfusion dependent but at longer intervals (6 months or more), and *non response* in patients who, after one year of treatment, remained at the same level of transfusion dependency. The correlations with the molecular defects were found to be secondary to the presence of the (C→T) at -158 of the Gγ gene (XmnI polymorphism). The T allele, in linkage to the haplotype I (+----) and to the internal β-globin gene framework 2, was the most significant modulating factor involved.

Interpretation and Conclusions. The good response to HU treatment that a significant number of southwest Iranian patients with β-thalassemia patients had seems to correlate with particular haplotypes. This indicates that HU treatment is a sensible option for transfusion-dependent β-thalassemia patients with a favorable molecular background.

Key words: thalassemia, hydroxyurea, XmnI, Iran.

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Fetal hemoglobin (HbF=α2/γ2) persisting after birth and the minor HbA_2 fraction (α2/δ2) are the only functional Hb tetramers in β-thalassemia major. In the severe forms of β-thalassemia major, the HbF fraction does not compensate for the deficiency of HbA (α2/β2) and the patient becomes transfusion-dependent. Patients with severe transfusion-dependent forms usually start becoming transfusion dependent between one and two years of age. Mild thalassemia intermedia may be caused by less severe mutations (β^+) with residual HbA expression, by modulating factors such as α-thalassemia or by genotypes associated with continuous production of fetal Hb in childhood and adulthood. Therefore, the switch from fetal to adult hemoglobin has been intensively studied[1] in an attempt to develop therapeutic methods that could increase HbF expression. In 1982 Ley *et al.*[2] published their experience on the effect of drugs increasing γ-globin expression in postnatal life. Since that time 5-azacytidine, erythropoietin, butyrates and hydroxyurea (HU) have been widely experimented and the last has become the alternative of choice. HU is a well-tolerated cytostatic agent that inhibits the ribonucleotide reductase pathway and increases HbF expression in genetically predisposed patients.[2] Several authors hhave published their experience on patients affected with sickle cell disease (SCD) treated with HU. Favorable effects of high HbF levels, a reduction of severe crises,[3,4] a 40% decline in mortality,[5] and an association with a lower incidence of rejection in stem cell transplantation[6] have been reported. Clinical trials on thalassemic patients have also shown that a beneficial effect is derived from the partially restored balance between non-α and α-globin chains.[7,8]

HU treatment in SCD and β-thalassemia has been carefully studied in the clinical setting[9,5,4] and also in *in vitro* experiments for a possible long-term mutagenic effect. Experimentally, it was shown that HU has a mutagenic effect inducing chromosomal and teratogenic aberrations.[10-14] However, two independent clinical studies did not find an increase in mutagenic or chromosomal aberrations.[15,16] Similarly, no evidence of increased mutagenesis, carcinogenic events or chromosomal aberration were found, even after long-term exposure to the HU.[17,18]

In this study, a large number of patients diagnosed with transfusion-dependent β-thalassemia attending the Thalassemia Centers of Bandar Abbas and Shiraz were carefully treated and followed-up over the past 5 years. The aims of this study were: (i) to test the efficacy of the drug in reducing the need for blood transfusions, while preserving normal growth, reducing secondary iron overload and other blood transfusion-related complications; (ii) to study which molecular factors are accountable for a good response to HU treatment.

Design and Methods

Patients

One hundred and thirty-three patients from the Hormozgan (n=57) and the Fars province (n=76) in the south-west part of Iran were randomly enrolled. They all had the typical phenotype, which is the most common in these regions, of *transfusion-dependent β-thalassemia*. Thus, all patients had (i) anemia, splenomegaly and retarded development; (ii) started to be transfusion dependent between 2 and 3 years of age; (iii) had transfusion therapy when their Hb levels became lower than 6 g/dL; (iv) had undetectable HbA on electrophoresis prior transfusion; (v) required transfusions at least monthly to maintain the Hb level at 10 g/dL; (vi) had received at least 5 years of chelation therapy (Desferal). Furthermore, all patients were at least 8 years old at the beginning of the HU treatment.

The age at which the β-thalassemia patients treated in Bandar Abbas and Shiraz initiate transfusion therapy ranges between 2 and 5 years. As said above, all our patients became transfusion dependent between 2 and 3 years of age. We did not include in our study patients in need of occasional transfusion at a later age, who could be classified as having thalassemia intermedia, in our study. To avoid inhibitory effects of iron load on HU therapy all patients with ferritin levels higher than 3000 ng/mL were excluded from our study. The thalassemia genotypes were characterized at the molecular level and all pairs of parents were diagnosed as carriers. The age of the patients ranged from 8 to 31 years, with the average being 17.1(±5.2). The gender ratio was equal (69 F: 67 M). Sixty-nine patients (45 F and 24 M) older than 6 years had undergone splenectomy before starting HU therapy because their lowest Hb levels could not be maintained above 6 g/dL in spite of monthly transfusions.

Hydroxyurea treatment

HU therapy was started after parents/guardians had received thorough information and granted full consent and in agreement with the local research commission authorities. HU was supplied as an oral preparation of commercially available 500 mg capsules (Verwend Bar, Switzerland). Patients were followed at regular intervals to monitor their tolerance of the drug. During the first 2 months of treatment, the transfusion regimen was continued in order to maintain the Hb level at 10 g/dL. From two months onwards, transfusion was continued only when the hemoglobin level dropped below 8 g/dL. The median therapy duration was 42 months (range 24-60 months) and the median HU dosage was 10 to 15 mg/day/kg. The dosage was obtained by distributing the appropriate number of capsules in a week, which in most cases resulted in one capsule a day. Patients whose Hb level remained at 8 g/dL or more were also treated with Desferal (Novartis, Switzerland) until their serum ferritin level was lower than 1000 ng/mL.

Both hematologic and clinical toxicity were monitored. Criteria for defining hematologic toxicity were: (i) total white blood cells < 3,000/μL or absolute neutrophil count < 1,500/μL, (ii) platelets < 100,000/μL. Side effects such as nausea, vomiting, diarrhea, rashes, and malaise, experienced during the first 6 hours after taking the HU were considered as clinical toxicity.

Hematology

The hematologic data were obtained by automatic analysis (Sysmex K800 or Sysmax K1000). Hb fractions were estimated on alkaline cellulose acetate electrophoresis. An attempt to estimate HbF before and during therapy was made using a modified Betke method. The original Betke method is sufficiently accurate only in the range 1-12%,[19] therefore, a 1 to 10 mixture of equally concentrated lysates from the patient and normal individuals was used. As expected, the decreasing presence of HbA derived from donor blood or the absence of HbA after interruption of transfusion made the HbF evaluation inconsistent. Patients becoming transfusion free only expressed HbF, maintaining their Hb level above 9.5 g/dL. Equally only HbF but lower Hb levels were measured in patients remaining transfusion dependent but at longer intervals. The pattern in patients not responding to HU was a mixed one created by the transfused blood. Conse-

quently, the only significant value for monitoring the increase in HbF (the only endogenous fraction produced) was the total Hb concentration during treatment although high performance liquid chromatography (HPLC) has recently been introduced in our protocol to monitor the presence of HbA traces in patients who have become transfusion free after HU therapy.

Molecular analysis

Samples were collected in Na-EDTA and DNA was isolated as previously described.[20] The common point mutations, the 619 bp deletion and the internal frame of the β genes[21] were detected by direct DNA sequencing. The 5' β cluster haplotype [ε-*Hin*cII, Gγ and Aγ *Hin*dIII, Ψβ and δ *Hin*cII] was examined by polymerase chain reaction (PCR)[22,23] and numerically classified according to Flint *et al.*[24] The HS-2 polymorphisms[25] and α-gene deletions were identified as previously described.[26,27] The Aγ 4 nucleotide deletion and the presence of the –369 (C→G), –309 (A→G) and –158 (C→T) polymorphism of the Gγ gene promoter were examined by direct sequencing using the following standard primers:

M13F-Gγ: 5'-CGACGTTGTAAAACGACGGCCAGTGAACTGTTGCTTTATAG GAT-3, M13F Aγ: 5'-CGACGTTGTAAAACGACGGCCAGTACTGTGGTCTTTATGAAA ATTGT-3 and M13Rγ: 5'-CAGGAAACAGCTATGACCATGAGCTTGTGATAG TAGCC TTGTC-3.

PCR was done in a reaction volume of 50 μL containing 50 ng of genomic DNA, 100 μmol/L of each dNTP and 10 pmole/μg of each primer. Amplifications were performed using 1 unit of AmpliTaq DNA polymerase (Perkin Elmer Foster City, CA 94404, USA). The PCR conditions were 35 cycles at 92°C for 60s, 58°C for 60s and 72°C for 120s. Direct DNA sequencing was performed on an ABI PRISM® 3700 DNA Analyzer (P-E Biosystems, Foster City, CA, USA) using the ABI PRISM® Big Dye Terminator.

Results

Response to HU therapy

During monthly clinical and laboratory controls we were able to define three categories of patients according to their response to HU. The category of *good responders* (GR) was the largest (61%). These patients had shifted, after 4 months of treatment, from monthly blood transfusion dependency to a stable transfusion-free condition at a Hb level above 9.5 g/dL (average10.3 g/dL). Most of these patients showed a lower degree of hemolysis (improved levels of serum bilirubin, SGOT and SGPT values). Traces of HbA (between 1 and 5%) were observed in some of the patients with β^+ mutations who became transfusion free after HU therapy. Erythroblast counts increased on average from 40 to 150 cells/100 WBC.

Cardiac function during exercise was monitored and the general physical condition of most patients improved, indicating that the increased HbF level could deliver sufficient oxygen to the tissues. We noted that liver and spleen size did not increase even in patients treated for a longer time and that the skeletal as well as hormonal conditions did stabilized. Hormone levels (testosterone, FSH, LH and TSH) were monitored and showed a better balance and growth and sexual development improved in both sexes. The emotional burden of recurring transfusions was reduced but the psychological problems associated with chelation did remain until this treatment was suspended at ferritin levels lower than 1,000 ng/mL.

A *moderate response* (MR) was achieved by 23% of the patients. These patients remained transfusion dependent but at intervals of 6 months or longer, maintaining Hb levels between 7.5 and 9.5 g/dL (average 8.85 g/dL). The improvements described for good responders were much less evident in this category. However, there were psychological and clinical advantages (less iron overload and less transfusion-related risks) associated with lower transfusion requirements. Sixteen percent of the patients were *non-responders* (NR). These were patients who, after one year of treatment, could not maintain Hb levels above 7.5 g/dL. They remained transfusion dependent without any apparent benefit from HU therapy.

No significant differences in response to HU were observed between splenectomized and non-splenectomized patients.

Since the monitoring of the Hb pattern during treatment is inconsistent we have summarized the average significant hematologic indices measured in patients in the three response categories in Table 1.

Analysis of β, γ and α genes

A total of 136 patients were studied at the molecular level for their β globin gene mutations and for the presence of the -158 (C→T) polymorphism on the Gγ gene promoter. One of the two β-gene defects could not be characterized in 3 patients. These cases were treated with HU and were in good responders but were excluded from the study.

As expected, the predominant β-thalassemia defect was the IVSII-1 (G→A), the most frequent β^0-thal mutation in the area. This mutation was found in 39.4% of the GR patients and in 15.2% and 10% of the MR and NR patients, respectively. The second most represented defect was the β^+-thalassemia IVSI-5 (G→C) mutation. In spite of the mild classification, this mutation was predominant between MR (54.3%) and NR (35.0%) categories and infrequent (16.2%)

Table 1. Average Hb, MCV and MCH measured in the 3 categories of patients during HU treatment.

	Sex	*Hb (g/dL)*	*MCV (fl)*	*MCH*
Good Response	F=41, M=40	10.3±0.6	76.3±9.9	24.3±3.1
Moderate Response	F=17, M=14	8.85±0.4	78.02±9.5	23.8±2.5
No Response	F=11, M=10	6.75±0.5	76.68±5.9	21.3±1.6

among GR patients. The distribution of the genotypes among the GR, MR and NR categories is summarized in Table 2. The C→T polymorphism at position -158 of the Gγ promoter was the most significant parameter correlating with HU response. Only 19% of the GR category had the wild type C/C genotype while 81% of the GR had either C/T or T/T alleles. The T polymorphism in the homozygous state was found in only 4.8% of the non-responders (NR). These data are summarized in Table 3.

In order to define on which allele the polymorphic sites of the β-globin gene cluster are located one would need to analyze the propositus and both parents. Since the parents were not always available, we selected 51 patients homozygous for the same mutation to increase the chance of homozygosity for the entire allele. In these patients we analyzed the HS-2 domain, the 4bp deletion (-222 to -225) on the Aγ gene promoter, and the polymorphic sites -369 (C→G), -309 (A→G) of the Gγ gene promoter.

None of them had the 4bp deletion. All but two of them were C/C and A/A homozygous wild type at position -369 and -309. Two patients homozygous for the IVSI-5 (G→C) β^0-thalassemia mutation showed C/G and A/G at -369 and -306, respectively, and C/C at –158. Neither of these patients responded to HU.

Out of these 51 patients 15 were randomly selected for β-haplotype analysis. IVSII-1 (G→A) homozygosity associated with T/T homozygosity at -158 of the Gγ gene promoter was found in 10 of them while 5 were homozygous IVSI-5 (G→C) and homozygous C/C at the -158 position. All of them were good responders. While most of the IVSII-1 (G→A) (8 out of 10) had haplotype I (+----) all IVSI-5 (G→C) had haplotype XI (+---+) and the rest had haplotype III (-+-++).

To study whether the LCR haplotype (tandem AT repeat) correlated with HU response, the HS-2 domain of the LCR was analyzed by bi-directional DNA sequencing, on the same 51 patients with identical mutations on both alleles. In spite of the expected high level of homozygosity we were not able to configure the tandem AT repeats and define the HS-2 haplotype.

The β-gene framework was analyzed in all 133

Table 2. β-thalassemia genotypes and HU response categories.

Genotype	*β/β*	*GR*	*MR*	*NR*
IVSII-1(G→A)/IVSII-1 (G→A)	0/0	22	4	–
IVSII-1(G→A)/IVSI-5 (G→C)	0/+	11	–	3
IVSII-1(G→A)/cd8(-AA)	0/0	5	–	–
IVSI-5 (G→C)/IVSI-5 (G→C)	+/+	3	12	6
IVSI (-25nt) 3'/IVSII-1 (G→A)	0/0	3	2	–
cd36/37 (-T)/CD82/83 (-G)	0/0	2	–	–
cd39 (C→T)/IVSII-1 (G→A)	0/0	2	–	–
cd36/37 (-T)/cd36/37 (-T)	0/0	2	–	–
cd8 (-AA)/cd8 (-AA)	0/0	2	–	–
IVSI (-1) (G→A)/IVS1-5 (G→C)	0/+	2	–	–
IVSI-1 (G→A)/IVSII-1 (G→A)	0/0	2	3	–
IVSI-110 (G→A)/IVSII-1 (G→A)	+/0	2	–	–
IVSI-128 (T→G)/IVSII-1 (GvA)	+/0	2	–	–
-28 (A→C)/cd22 (-AAGTTGG)	+/0	2	–	–
-28 (A→C)/IVSII-1 (G→A)	+/0	1	–	–
-88 (C→A)/cd44 (-C)	+/0	1	–	–
cd 28/29 (-G)/IVSI-1 (G→A)	0/0	1	–	–
cd22 (-AAGTTGG)/IVSI-5 (G→C)	0/+	1	–	–
cd28/29 (-G)/IVSII-1 (G→A)	0/0	1	–	–
cd39 (C→T)/cd44 (-C)	0/0	1	–	–
cd44 (-C)/IVSII-1 (G→A)	0/0	1	–	1
cd5 (-CT)/IVS1-5 (G→C)	0/+	1	–	–
cd8/9(+G)/IVSII-1G→A)	0/0	1	–	–
cd44 (-C)/cd44 (-C)	0/0	1	–	–
IVSI-128(T→G)/IVSI-128(T→G)	+/+	1	–	–
IVSI(-25)3'end/IVSI(-25)3'end	0/0	1	2	2
Init. (ATG→ACG)/IVSII-1(G→A)	0/0	1	–	–
IVS1-110 (G→A)/IVS1-130 (G→A)	+/0	1	–	–
IVS1-5 (G→C)/cd44 (-C)	+/0	1	–	–
IVSI-110 (G→A)/cd39 (C→T)	+/0	1	–	–
IVSI-5 (G→C)/cd 39 (C→T)	+/0	1	–	–
IVSII-1(G→A)/cd82/83 (-G)	0/0	1	–	–
IVSII-1(G→A)/IVSII-745 (C→G)	0/+	1	1	–
IVSI-110 (G→A)/IVSI-128 (T→G)	+/+	–	–	1
IVSI-6 (T→C)/IVSI-110 (G→A)	+/+	–	–	1
IVSI-5 (G→C)/IVSI-110 (G→A)	+/+	–	–	1
IVSI-5 (G→C)/IVSI (-25nt) 3'	+/0	–	–	1
Init. (ATG→ACG) Init. (ATG→ACG)	0/0	–	–	1
cd8 (-AA)/cd39 (C→T)	0/0	–	–	1
cd39 (C→T)/cd45(-T)	0/0	–	–	1
IVSI-5 (G→C)/cd44(-C)	+/0	–	–	1
cd39 (C→T)/IVSI-110 (G→A)	0/+	–	–	1
cd36/37 (-T)/IVSII-1 (G→A)	0/0	–	1	–
Init. (ATG→ACG)/IVSI-5 (G→C)	+/0	–	1	–
IVSI-6 (T→C)/IVSI-5 (G→C)	+/0	–	1	–
IVSI-5 (G→A)/cd44 (-C)	+/0	–	1	–
cd15 (TGG→TAG)/cd15 (TGG→TAG)	0/0	–	1	–
cd15 (TGG→TAG)/IVSI-5(G→A)	0/+	–	1	–
Init. (ATG→ACG) Init. (ATG→ACG)	0/0	–	1	–

Table 3. Frequency of the XmnI polymorphism in correlation to the three response categories to HU treatment.

Patients	*n*	*C/C (%)*	*C/T (%)*	*T/T (%)*
Good response	81	19.1	42.5	38.4
Moderate response	31	65.5	31.0	3.5
No response	21	61.9	33.3	4.8

Table 4. Frequencies of the β-gene framework among 266 alleles in the patients grouped according to response to HU treatment.

Clinical responses	*I N (%)*	*II N (%)*	*III N (%)*	*Asian N (%)*
Good response	56 (34.5)	83 (51.4)	15 (9.2)	8 (4.9)
Moderate response	31 (50.0)	21 (33.9)	4 (6.5)	6 (9.7)
No response	23 (54.8)	8 (19.0)	4 (9.5)	7 (16.7)

patients. The distribution did not differ significantly among the three response groups except, perhaps, for the Asian haplotype. The HU responses versus framework are summarized in Table 4. In conclusion, correlations with molecular defects were found to be secondary to the presence of the (C→T) at -158 of the Gγ gene (XmnI polymorphism). The T allele, in linkage with the haplotype I (+----) and with the internal β-globin gene framework 2, was the most significant modulating factor involved.

Discussion

Several genetic, non-genetic and pharmacological factors have been reported to influence HbF expression and the severity of β-thalassemia major and sickle cell disease; these factors include age and gender,[27] α globin gene expression[27,28] and the molecular background of the β globin gene cluster.[28,29] A 4-bp deletion 5' to the Aγ allele has been reported as an element acting in-*cis* which increases the expression of both β-globin genes.[30] The (C→T) polymorphism at -158 upstream of the Gγ globin gene, detectable by the restriction enzyme *Xmn*I, has been shown to be responsible for high HbF levels in β-thalassemia and sickle cell disease patients. During erythropoietic stress, adults heterozygous or homozygous for the -158(C→T) polymorphism have higher HbF expression than individuals with the wild type -158 C.[31] An increase of HbF has also been documented in the presence of this polymorphism during pregnancy,[32] in Fanconi's anemia[33] and in leukemia.[34] The effects of other polymorphisms on HbF expression have been previously studied.[25,35,36]

HU is a myelosuppressive agent that may increase HbF levels. In culture, concentrations of about 50 μM/L have been shown to increase the percentage of HbF.[37] Erythroid cultures from different thalassemia patients showed different results in the presence of HU. The number of HbF-cells and the HbF content per cell increased in some individuals, while in others, only the HbF-cell number increased with a moderate rise in HbF content per cell, whereas in yet other studies only a minimal effect was observed.[38] This variation in response to HU suggested mechanisms involving one or more genetic factors. Moreover, several studies have reported data suggesting that response to HU is also dosage dependent.[37] In the clinic, a serum concentration of 100 μM/L can be obtained by a 20 mg/kg intravenous infusion.[39] The same amount of drug given orally results in a serum concentration of about 80 μM/L, with a peak (C_{max}) at 1.22 hours after ingestion.[40] A study on a limited group of β-thalassemia patients from central Iran treated with HU reported that the *Xmn*I polymorphism and the IVSII-1 (G→A) mutation were associated with good response to treatment.[41]

In our study we monitored the response to HU treatment in Iranian β-thalassemia patients in relation to their specific molecular background and coexisting modulating factors. The most significant observation of our study is that after only four months treatment 61% of our patients had shifted from monthly blood transfusion dependency to a stable transfusion-free condition at an average Hb level of 10.3 g/dL. Moreover 23% of our patients, although still transfusion dependent, went from a monthly regime to transfusions at intervals of 6 months or longer, maintaining an average Hb of 8.85 g/dL.

The common IVSII-1 (G→A) mutation was present, either in homozygous or heterozygous form, in 46.9% of the good responders while it was present in 16% of the moderate responders and in 14% of the non-responders. Moreover, 52.4% of the patients not responding to HU treatment had the IVSI-5 (G→C) β-thal defect and 61% of them had the wild type C/C polymorphism at -158, the *Xmn*I polymorphism of the Gγ gene promoter.

The response to HU was equal in males and females and the age distribution was not significantly different in the three response categories. Similarly, no significant difference in response was found between splenectomized and non-splenectomized patients.

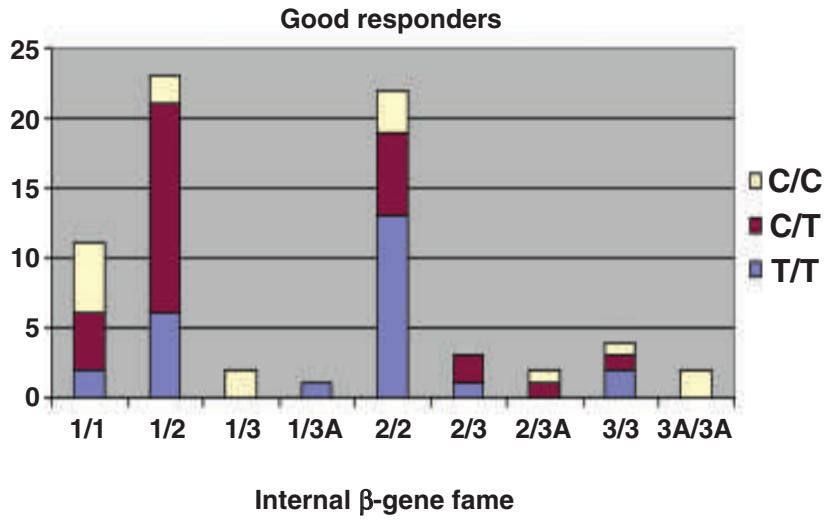

Figure 1. Good responders to HU treatment in correlation with *Xmn*I polymorphisms and internal β-globin gene frame 2.

Less than 40% of the splenectomized patients were good responders, while a larger share was found in the moderate responders. The most favorable molecular background to HU response, irrespective of the IVS II-1 (G→A), IVS I-5 (G→C) or other β^0 or β^+ thalassemia mutations, was T/T homozygosity for the *Xmn*I polymorphism ($p < 0.0001$). As many as 82% of the patients with this genotype responded fully to HU therapy, whereas 40% of the C/T heterozygotes had a full response and only 12% of the patients with wild type C/C polymorphism were good transfusion free responders, suggesting that other factors may contribute to the positive response to HU.

Almost all good responders with IVSI-5 (G→C) and C/C polymorphism homozygosity were located on β-gene framework I, suggesting that other elements associated with this framework could be related to good response to HU. Conversely, 18% of the T/T homozygotes responded only partially or not at all to HU therapy. We are currently verifying whether the high prevalence of G6PD deficiency in the area (up to 29%)[42] could be associated with the persisting high hemolysis and lack of response in this group.

The coexistence of α-thalassemia is a known modulating factor in β-thalassemia patients. The beneficial effect of α-thalassemia is due to a partial reduction of free α chains, hemolysis and ineffective erythropoiesis. In spite of the fact that α^+ thalassemia is frequent in Iran[43] we found heterozygosity for the frequent $-\alpha^{3.7}$ allele ($-\alpha/\alpha\alpha$) in only two females, both homozygous IVSI-5 (G→C), both splenectomized and both moderate responders to HU therapy. α^+ thalassemia alleles, especially homozygous ($-\alpha/-\alpha$) in the presence of *Xmn*I polymorphisms may generate non-transfusion-dependent intermediate phenotypes. Due to the fact that the selected cohort was transfusion dependent, these patients were *a priori* excluded from this study, which explains the few α-thalassemia traits found in our study.

We may assume that a large proportion of the thalassemia patients in south-west Iran have a molecular background favorable to HU response, represented mainly by the -158 C→T polymorphism strongly associated with the IVS-II-1 (G→A) mutation in linkage with haplotype I (+----) and internal β-globin gene framework 2 (Figure 1). Therefore, carriers of this genetic background can be expected to have a beneficial effect from HU therapy ($p<0.01$). Finally, during the 5 years of treatment we did not observe any significant problems regarding drug compliance and no myelogenic or clinically adverse events occurred. This in agreement with other long-term clinical trials which reported no significant increases in secondary malignancy following HU therapy.[9,15,16,44]

MJ and MK are the Iranian physicians who treated and followed the patients in Bandar Abbas and Shiraz, respectively. MY, who graduated in medical laboratory sciences, studied the molecular background of all patients at Leiden University Medical Center (The Netherland) where he is preparing his PhD thesis. MY was assisted by EB, CLH and PCG in the molecular studies and preparation of the manuscript.

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