

Study on hydroxyurea response in hemoglobinopathies patients using genetic markers and liquid erythroid cultures

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Abstract

Increased expression of fetal hemoglobin (HbF) may ameliorate the clinical course of hemoglobinopathies. Hydroxyurea (HU) is the only inducer approved for the treatment of these diseases able to stimulate HbF production but patients' response is highly variable indicating the utility of the identification of pharmacogenomic biomarkers in order to predict pharmacological treatment efficacy. To date few studies to evaluate the role of genetic determinants in HU response have been conducted showing contradictory results. In this study we analyzed *BCL11A*, *GATA-1*, *KLF-1* genes and γ -globin promoter in 60 alleles from 30 hemoglobinopathies patients under HU treatment to assess the role of these markers in HU response. We did not find any association between these genetic determinants and HU response. Before treatment started, the same patients were analyzed *in vitro* using liquid erythroid cultures in a test able to predict their response to HU. The results of our analysis confirm the absence of pharmacogenomic biomarker associated to HU response indicating that, the quantification of γ -globin mRNA fold increase remains the only method able to predict *in vivo* patients response to the drug.

Introduction

Hemoglobinopathies are inherited disorders characterized by anomalies of structure, function or production of globin chains that lead to a low or absent production of hemoglobin (Hb) with a consequent wide clinical and phenotypic heterogeneity.¹ Increased expression of fetal Hb (HbF) during adult life may ameliorate the clinical course of these disorders. In sickle cell

disease (SCD), the beneficial effects of HbF are the inhibition of sickle Hb (HbS) polymerization, and dilution of the HbS concentration in the red blood cells while in β -thalassemia, γ -globin chains can substitute for β -globin chains and prevent the excess of α -globin chains from damaging the red blood cells membrane.¹ Multiple pharmacological agents able to increase HbF production have been investigated and hydroxyurea (HU) is the only inducer approved for the treatment of adult patients affected by sickle cell disease² which entered into clinical practice for β -thalassemia intermedia (β -TI).³ However, there is a great variability in the response of patients after HU therapy, in fact some patients are good responders while others exhibit little or no change in HbF levels.^{4,5} Moreover, in responder patients, a decrease in the efficacy during long-term treatment was observed.^{6,7}

Liquid erythroid cultures, a system reproducing erythropoiesis *in vitro*, are a useful model to study human erythroid cells. It was previously shown that the γ -globin mRNA fold increase in cultured erythroid progenitors exposed to HU, correlates to the *in vivo* HbF fold increase observed in patients after HU therapy.⁶ This approach is used as an *in vitro* test, the only current method able to predict *in vivo* patients response to the drug.

Several genetic factors can influence the severity of haemoglobinopathies; some of them act by reducing the degree of α/β imbalance (co-inheritance of α -thalassemia) while others act by increasing HbF levels.

There is a broad range of basal HbF levels among individuals; single nucleotide polymorphisms (SNPs) or deletions within the beta globin cluster account for a portion of this variation. Moreover genetic studies are being done to detect the determinants influencing HbF levels by trans-acting factors not linked to the β -globin cluster. Genome-wide association studies (GWAS) have identified quantitative trait loci (QTLs) that affect baseline HbF levels in patients with Hb disorders. Several SNPs in these loci seem to be responsible for an estimated 15 to 20% of individual variation in HbF levels.⁸ A well-known HbF QTL is the XmnI polymorphism (rs7482144), a C T substitution at position -158 of the γ globin gene promoter.⁹ Early reports and GWAS studies show that the presence of the XmnI T allele correlates with higher HbF levels in β -thalassemia and SCD patients.¹⁰⁻¹²

Another HbF QTL is the *BCL11A* gene, that codify for a γ -globin gene repressor.¹³ Several GWAS studies have associated SNPs in intron 2 of the *BCL11A* gene (e.g. rs11886868 and rs4671393) with higher HbF levels in patients with hemoglobinopathies.^{12,14} In particular, a strong association between rs11886868 and HbF level was highlighted in the Sardinian population.¹⁵ High HbF levels have been also

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Contributions: SS and AP conducted the study and drafted the manuscript. VA and AT performed the laboratory works and collected data. RDM recruited patients and collected clinical data. MS performed the statistical analysis. AM coordinated the study. ED and RD designed the study and edited the manuscript. They contributed equally.

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associated with rs4671393 SNP (A/A genotype) in two different SCD cohorts (African American Cooperative Study of Sickle Cell Disease and SCD cohort from Brazil).¹²

Biological variability in HbF levels is also influenced by genes involved in γ -globin gene expression such as *KLF-1* and *GATA-1*. Certain mutations within the *KLF-1* transcription factor gene result in persistent high-level of HbF (HPFH) after birth.¹⁶ In a Maltese family a single mutation located in *KLF-1* exon 2 (K288X) causing HPFH was shown to produce the lack of a DNA binding domain.¹⁷

Moreover the identification of a mutation in *GATA-1* gene (R216W) in a child affected by congenital erythropoietic porphyria and β -thalassemia showing increased HbF level, suggests also an important role for *GATA-1* in globin chain switching.¹⁸

The identification of pharmacogenomic biomarkers is important to predict pharmacological treatment efficacy. To date few studies have been conducted to evaluate the role of the genetic determinants in HU response.

Some studies have shown an association between the XmnI T/T and *BCL11A* rs 766432

markers and response to HU.^{14,19,20} Conversely, other studies have failed to detect the correlation between XmnI genotype and HU response.²¹⁻²³

The aim of this study was to analyze *BCL11A*, *GATA-1*, *KLF-1* genes and γ -globin promoter in 60 alleles from 30 hemoglobinopathies patients to assess if there is a significant association between these genetic determinants and the efficacy of HU treatment. Furthermore in the same patients the response to HU was evaluated *in vitro* in a liquid erythroid culture system.

Materials and Methods

Cohort description

Thirty patients (12 males and 18 females) affected by SCD and thalassemia (1 β S/ β S patient, 14 β S/ β -thalassemia patients and 15 β -TI patients) were studied. Molecular studies for genotype detection were performed according to procedures reported elsewhere.²⁴ The patients characteristics are shown in Table 1. All the patients were treated with hydroxyurea. The mean dosage of HU administered was 16.4 mg/kg (range 9.2-21), and the mean follow-up time was 111 months (range 5-180).

Genotyping

Peripheral blood in ethylene diamine tetraacetic acid-containing (EDTA) collection tubes was used to extract genomic DNA (standard phenol-chloroform method). DNA was amplified using the polymerase chain reaction (PCR): PCR mix (50 μ L) contained 200 ng of genomic DNA, 1.5 μ L of 10 pmol primers, 5 μ L of buffer 10 \times , 1.5 μ L of MgCl₂ 50 mmol, 1 μ L of 40 mmol dNTP and 2.5 U of Taq polymerase enzyme (Invitrogen Corp., Carlsbad, CA, USA). PCR products were analyzed on SYBR-Safe 3% agarose gel and displayed to the ultraviolet lamp. PCR products were sequenced bidirectional directly using Big-Dye terminator 3.1 cycle sequencing kit and run on ABI PRISM 3130 DNA analyzer (Applied Biosystems, Foster City, CA). Primers used for PCR and sequencing were designed in our laboratory (Table 2). Mutations in *KLF-1* were detected by DNA sequencing of exon 2; mutations of *BCL11A* were found by sequencing of the 5 exons and the two intronic regions containing SNP rs11886868 and rs4671393. For the analysis of *GATA1* gene the entire coding region (6 exons) was sequenced. Also the XmnI genotype was obtained by XmnI enzymatic restriction. The PCR conditions will be made available upon request.

Two phase liquid primary erythroid cultures

After informed consent was obtained, 20 mL

of peripheral blood was withdrawn from 30 patients before HU treatment. Primary cell cultures were performed as previously described.²⁵ Mononuclear cells from peripheral blood were isolated by centrifugation over Ficoll-Hypaque (1.077 g/mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 1200 \times g for 15 min. The nucleated cells were first cultured in a minimal essential medium (MEM) supplemented with 1 μ g/mL cyclosporine A (Novartis Basilea, Switzerland), 10% fetal calf serum (FCS, Invitrogen, Carlsbad, California, USA) and 10% conditioned medium collected from cultures of the human bladder carcinoma 5637 cell line. After 6 days of incubation in phase I culture, the non-adherent cells were harvested, washed, and resuspended in phase II medium composed of α -MEM, 30% FCS, 1% deionized bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA), 10 mM 2-mercapto-ethanol (Sigma), 1.5 mM glutamine (Euroclone), 1 mM dexamethasone (Laboratorio Farmacologico Milanese, MI, Italy), 1U/mL recombinant erythropoietin (Janssen-Cilag, Leiden, The Netherlands), 10 ng/mL Stem Cell Factor (Sigma), and 0.3 mg/mL human holo-transferrin (Sigma). Cells were harvested at day 10 of phase II culture. We performed at least two different primary cultures for each patient.

Hydroxyurea treatment *in vitro*

At day 6 of phase II, cells were washed with α -MEM, and the cell culture was split. Half of the culture was exposed to 100 μ M HU (Teofarma Srl, Pavia, Italy), a dose that corresponds to the serum HU concentration during *in vivo* treatment at 20 mg/kg/day.²⁶ As a control, the other half of the culture was grown without the drug. At day 10, the cells were harvested and analyzed. At the 100 μ M HU dosage, erythroid progenitors express the maximum potential of γ -mRNA expression, in that, when lower concentrations were tested (75 μ M), the same γ -mRNA increase was obtained. Higher doses cause the cell death.

Flow cytometric analysis

The phase II cultured cells were monitored for erythroid differentiation by measuring cellular expression of transferrin receptor (CD71-PE) (Immunotech, Marseille Cedex 9, France) and glycophorin A antigen (GPA-FITC) (Immunotech) as previously described.²⁷ Cells were analyzed using the Cytomics FC 500 (Beckman Coulter Inc., Fullerton, CA 92835).

Real-time quantitative polymerase chain reaction

RNA was isolated with TRIzol® reagent (Invitrogen, Carlsbad CA 92008, USA) according to manufacturer's instructions. cDNA synthesis was carried out using High Capacity cDNA Reverse Transcription kit (Applied

Biosystems) from 1 μ g of total RNA. Quantitative real-time PCR assay of transcripts was then performed with the use of gene-specific double fluorescently-labeled probes in a 7900 Sequence Detector (Applied Biosystems, Norwalk, CT). All samples were assayed using TaqMan Universal PCR Master Mix (Applied Biosystems Foster City, CA, USA). The following primer and probe sequences were used: γ -globin forward primer, 5'-GGCAACCTGTCCTCTGCCTC-3'; γ -globin reverse primer, 5'-GAAATGGATTGCCAAAACGG-3'; γ -globin probe, 5'-FAM-CAAGCTCCTGGAAATGTGCTGGTG-TAMRA-3'.²⁷ As endogenous control the human glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) (Pre-Developed Taqman assay control kit, Applied Biosystem) was used. The comparative threshold cycle (Ct) method was used to determine the difference (Ct) between the Ct of treated samples and the Ct of the untreated samples. Before subtraction, the Ct was normalized by the Ct of the endogenous reference gene, *GAPDH*. The experiments were performed in triplicate to ensure the reproducibility of results.

Statistical analysis

Data are presented as mean \pm SD. χ square test was used to assess if there is statistical significance of the differences in the frequency of genetic variants among the hemoglobinopathies groups analyzed. R software and Microsoft Excel were used for the statistical analysis.

Results

Primary erythroid cultures from peripheral blood hematopoietic stem cells of 30 patients (15 SCD and 15 β -TI) were performed. Flow cytometric analysis of erythroid markers (CD71 and GPA) did not show any difference during erythroid differentiation between cultures derived from HU responder and non-responder patients. For each sample, the effect of HU in terms of γ -globin mRNA fold increase respect to untreated cells was measured by mRNA relative quantification using a fluorescence-based quantitative real-time PCR assay. At least two different primary cultures for each sample were performed. The results of this analysis, predictive of patients' response to HU treatment, have been summarized in Table 1. After the *in vitro* study, all the patients started HU treatment *in vivo*. Nine β -TI patients attained an increase of their transfusion interval while 6 of them stopped the treatment since they did not show an increase in Hb or an appreciable change in blood transfusion intervals. In SCD patients HU treatment determined increase in HbF and total Hb and

decrease in white blood cell counts. Moreover a reduction of vaso-occlusive crisis, acute chest syndromes and the other complications of the disease were observed. Only 1 SCD patient did not respond and stopped the treatment. After one to two years of treatment, when the *in vivo* response was stabilized, HbF fold increase was calculated and compared to γ -globin mRNA fold increase measured *in vitro*. For all patients a very good correlation between the *in vivo* and the *in vitro* HU response was observed (Pearson correlation=0.95, P=0.05). In fact, in all patients studied, the *in vitro* γ -globin mRNA fold increase was very similar to the *in vivo* HbF fold increase confirming the predictive value of the *in vitro* test. All the patients were studied for the presence of some genetic determinants responsible of high level of fetal hemoglobin production (*BCL11A*, -158 γ -globin gene promoter, *GATA-1* and *KLF-1*). Genotyping results are summarized in Table 1. No association

between these genetic markers and HU response was found. Moreover, in exon 2 of *BCL11A* gene of 1 patient (n. 14) a new mutation (L45R/N) not previously described was detected. This mutation does not appear to be associated to HU response since the patient's brother (n. 13) that presents the same thalassemia genotype ($\beta 039/\delta\beta$), the same basal HbF level and the same response to HU treatment does not show the mutation.

A not previously described T insertion located 74 bp downstream to rs11886868 SNP of *BCL11A* gene was identified always associated to the T allele.

The XmnI polymorphism was found in heterozygous only in two responder patients (n. 3 and n. 18) while R216W mutation in the exon 4 of *GATA-1* gene and K288X in *KLF-1* gene were not found in any patient. A point mutation in exon 2 of *KLF-1* gene causing the aminoacid substitution F182L (c.544T>C) was found in heterozygous in 3 patients.

Discussion and Conclusions

Hemoglobinopathies, such as SCD and thalassemia are among the most common human genetic disorders. HbF increase is of enormous clinical relevance given its role in ameliorating the severity of these diseases. Among the pharmacological agents able to induce HbF production, HU is the drug of choice for the treatment of hemoglobinopathies but patients' response to this drug varies considerably. For this reason the ability to predict HbF response to HU treatment would be helpful for the selection of responders to prevent potential adverse drug reactions in non-responder patients.

The regulation of HbF level is complex and involves elements and factors cis- and trans-acting to the β -globin cluster such as -158 γ -globin gene promoter, *KLF-1*, *BCL11A*, *GATA-1* and probably other regulatory loci; epigenetic and cellular factors could also have regulatory

Table 1. Genotyping results and correlation between *in vitro* and *in vivo* response.

ID	Gender	Genotype	BCL11A rs4671393 rs11886868	BCL11A	KLF-1	-158	GATA-1	γ -globin mRNA increase	<i>In vivo</i> response	
1	M	IVS1, 110/-87	G/G	T/T +ins	w.t.	F182L/N	C/C	w.t.	1.3±0.2	YES (1.24 fold increase)
2	F	IVS 1,1/-101	A/G	C/C	w.t.	w.t.	C/C	w.t.	1.2±0.2	NO (1.29 fold increase)
3	F	IVS 1,6/IVS 1,6	G/G	T/T +ins	w.t.	w.t.	T/C	w.t.	1.5±0.1	YES (1.31 fold increase)
4	M	IVS 1,6/IVS 1,6	A/A	C/C	w.t.	w.t.	C/C	w.t.	1.5±0.2	NO (1.69 fold increase)
5	M	IVS 1,6/IVS 1,6	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	3±0.1	NO (2.68 fold increase)
6	F	IVS 1,6/IVS 1,6	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	2±0	NO (2.3 fold increase)
7	M	IVS 1,6/IVS 1,6	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	2±0	NO (2.15 fold increase)
8	F	$\beta 039$ /IVS 1, 6	G/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1.8±0.1	YES (1.59 fold increase)
9	F	$\beta 039$ /IVS 1, 6	A/A	C/C	w.t.	w.t.	C/C	w.t.	1±0.2	NO (1.10 fold increase)
10	M	$\beta 039$ /IVS 1, 6	A/A	C/C	w.t.	w.t.	C/C	w.t.	1±0.2	YES (1.20 fold increase)
11	M	$\beta 039$ /IVS 1, 6	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1.8±0.2	YES (1.57 fold increase)
12	M	β Lepore/ β Lepore	G/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1.8±0.3	YES (1.58 fold increase)
13	M	$\delta\beta$ / $\beta 039$	A/A	C/C	w.t.	w.t.	C/C	w.t.	1.4±0.2	YES (1,15 fold increase)
14	F	$\delta\beta$ / $\beta 039$	A/G	C/T +ins	L45R/N	w.t.	C/C	w.t.	1.3±0.2	YES (1.29 fold increase)
15	F	cod6/ $\beta 039$	A/G	C/T +ins	w.t.	F182L/N	C/C	w.t.	1±0	YES (1.17 fold increase)
16	M	βS / $\beta 039$	G/G	T/T +ins	w.t.	w.t.	C/C	w.t.	1.3±0.2	YES (1.5 fold increase)
17	F	βS / $\beta 039$	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	2.7±0.3	YES (3 fold increase)
18	M	βS / $\beta 039$	G/G	T/T +ins	w.t.	w.t.	T/C	w.t.	1.2±0.2	YES (1.06 fold increase)
19	F	βS / $\beta 039$	A/G	C/C	w.t.	F182L/N	C/C	w.t.	1.7±0.2	YES (1.85 fold increase)
20	F	βS / $\beta 039$	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1.4±0.1	YES (1.51 fold increase)
21	F	βS / $\beta 039$	G/G	T/T +ins	w.t.	w.t.	C/C	w.t.	2.2±0.3	YES (1.87 fold increase)
22	F	βS / $\beta 039$	G/G	T/T +ins	w.t.	w.t.	C/C	w.t.	3±0	YES (3.04 fold increase)
23	M	βS /IVS1, 110	A/G	C/C	w.t.	w.t.	C/C	w.t.	2.5±0.2	YES (2.56 fold increase)
24	F	βS /IVS1, 110	A/A	C/C	w.t.	w.t.	C/C	w.t.	2.3±0.2	YES (2.83 fold increase)
25	F	βS /IVS1, 110	A/A	C/C	w.t.	w.t.	C/C	w.t.	1±0	YES (1,11 fold increase)
26	F	βS /IVS1, 110	G/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1.6±0.2	YES (1.81 fold increase)
27	F	βs /IVS II, 745	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1.7±0.2	NO (1.79 fold increase)
28	M	βS / $\delta\beta$	A/G	C/T +ins	w.t.	w.t.	C/C	w.t.	1±0	YES (1.22 fold increase)
29	M	βS / βS	G/G	T/T +ins	w.t.	w.t.	C/C	w.t.	1.2±0.1	YES (1.24 fold increase)
30	F	βs /IVS I, 5c	G/G	T/T +ins	w.t.	w.t.	C/C	w.t.	1.3±0.2	YES (1.35 fold increase)

Table 2. Primer used for amplification and detection of the single nucleotide polymorphisms and alleles.

Allele	Transcription	Primers sequence (5'<3')
rs 4671393	Forward	CACTAGCTCAGAAATGGACTT
	Reverse	ACCTTCCTTTAATCAGCTTCC
rs 11886868	Forward	ATCAGATACAGAACACGTCC
	Reverse	GTCCATTGTAGCACTGTTTAT
KLF-1 exon 2b	Forward	AGACTCTGGGCGCATATGCT
	Reverse	AGGAGGCCACTCACTCTCAGA
BCL11A exon 1	Forward	GGA TGT CAA AAG GCA CTG ATG
	Reverse	TCTCTTTTACCTCGACTCTCG
BCL11A exon 2	Forward	TTACATGATGTGGTGGGATGG
	Reverse	GCTGTGATGGACAGTACATA
BCL11A exon 3	Forward	CTGGGAAATAACTCACCCTC
	Reverse	GCTGCCAAGTGAGTAATGGAA
BCL11A exon 4	Forward	GGCTGGAGGTGGAACATAAAT
	Reverse	TCTCTCGATACTGATCCTGGT
BCL11A exon 5	Forward	TCATGCTTAAGATGCCAGTGG
	Reverse	AAATAAATGGCGCTGCAGGC
GATA-1 exon 1	Forward	CAAGAGGTGCCCCACAAGCAT
	Reverse	GACACTTTGGGGACCAGCTGT
GATA-1 exon 2	Forward	AGAAATATGGAGACTGAGGTG
	Reverse	ATCCTCACAGTGGTATTCTGA
GATA-1 exon 3	Forward	TGTGGAGCTGGGAACCTTG
	Reverse	TCAGCTCAGCTTTACTTTGGG
GATA-1 exon 4	Forward	CCCTGTGGTGGAAATTCAAA
	Reverse	GCACTAGCTTCCGTGAATCAT
GATA-1 exon 5	Forward	TTCAACCTGACCCTCACTTCT
	Reverse	GAGGTAGAACAGGAACAGAGT
GATA-1 exon 6	Forward	TGAAAGAAGTGGGGTAGAGAG
	Reverse	AGAGAGCCACAGGCATTGTTA

roles.²⁸ It is possible that these and also other genetic determinants modulate HbF response. The correlation of genetic determinants with high HbF levels after HU treatment was studied yielding contradictory results. In fact, although the presence of Xmn I polymorphism is more often associated with better response to HU therapy,^{19,20} other studies did not find any association of such polymorphism with high HbF levels upon HU treatment.²² In the present study, we examine the possible association of some HbF QTLs with the response to HU in 30 hemoglobinopathies patients. No association between these genetic markers and HU response is established, even if could be useful to extend this analysis to a larger cohort of patients to confirm these results. The response to HU therapy is complex and polymorphisms in many genes modulating γ -globin gene expression, HU metabolism and erythroid progenitor proliferation might affect patients' response. Statistically significant associations of HbF response to HU with multiple SNPs in several genes (*ARG2*, *FLT1*, *HAO2*, *NOS1*, *KLF10*, *SALL2*) have been reported.^{29,30} To date the use of genetic determinants to predict the effect of HU is still not possible. For this reason human liquid erythroid culture system, an efficacious but complex and time consuming approach, at the moment remains

the only method able to predict patients' response to HU.

Nevertheless recent advances in genomic analysis technologies, including next generation sequencing and microarrays will provide revolutionary opportunities allowing to analyze a large number of genes and patients simultaneously for the search of genetic determinants that could help to trace a genetic profile rather than single mutations that may be predictive of pharmacological response.

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