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 hemoglobinopathy 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. 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. 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 disease which entered into clinical practice for β-thalassemia intermedia (β-TI). 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. Moreover, in responder patients, a decrease in the efficacy during long-term treatment was observed.

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 vitro HbF fold increase observed in patients after HU therapy. 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 cβ 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 variability. 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. A well-known HbF QTL is the Xmnl polymorphism (rs7482144), a C T substitution at position -158 of the γ3 globin gene promoter. Early reports and GWAS studies show that the presence of the Xmnl T allele correlates with higher HbF levels in β-thalassemia and SCD patients. Another HbF QTL is the BCL11A gene, that codify for a γ-globin gene repressor. Several GWAS studies have associated SNPs in intron 2 of the BCL11A gene (e.g. rs1188868 and rs4671393) with higher HbF levels in patients with hemoglobinopathies. In particular, a strong association between rs1188868 and HbF level was highlighted in the Sardinian population. High HbF levels have been also associated with rs4671393 SNP (AA genotype) in two different SCD cohorts (African American Cooperative Study of Sickle Cell Disease and SCD cohort from Brazil).

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. 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.

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

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 Xmnl 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 βS/βS patient, 14 βS/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 tetra-acetic 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 μL) contained 200 ng of genomic DNA, 1.5 μL of 10 pmol primers, 5 μL of buffer 10X, 1.5 μL of MgCl2 50 mmol, 1 μL of 40 mmol dNTP and 2.5 μL of Taq polymerase enzyme (Invitrogen Corp., Carlsbad, CA, USA). 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 Xmn1 genotype was obtained by Xmn1 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 × g for 15 min. The nucleated cells were first cultured in a minimal essential medium (MEM) supplemented with 1 μg/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), 1μM 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 μM HU (Teofarma Srl, Pavia, Italy), a dose that corresponds to the serum HU concentration during in vivo treatment at 20 mg/kg/day. 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 μM HU dosage, erythroid progenitors express the maximum potential of γ-mRNA expression, in that, when lower concentrations were tested (75 μM), 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 glycoporphin A antigen (GPA-FITC) (Immunotech) as previously described.27 Cells were analyzed using the Cytomics FC 500 (Beckman Coulter Inc., Fullerton, CA 92635).

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 μg 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’-GGCAACCTGTCCCTCGCTC-3’; γ-globin reverse primer, 5’-GAATGAGATTGCCCAAACGG-3’; γ-globin probe, 5’-FAMCAAGCTCTGGGAAATGTGGTCTGG-TAMRA-3’.27 As endogenous control the human glyceroldehyde-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 (ΔCT) 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±SD. χ2 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
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 (β039/β0), 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 rs1188668 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.54T>C) was found in heterozygous in 3 patients.

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

| ID | Gender | Genotype | BCL11A rs4671393 | BCL11A | KLF-1 -158 | GATA-1 | γ-globin mRNA increase | In vivo response |
|----|--------|----------|------------------|--------|------------|--------|-----------------------|----------------|
| 1  | M      | IVS, 110-87 | G/G             | T/T +ins | w.t.       | F182L/N | C/C | 1.3±0.2 | YES (1.24 fold increase) |
| 2  | F      | IVS 111-101 | A/G             | C/C     | w.t.       | w.t.    | 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 | 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 | 1.5±0.2 | NO (1.89 fold increase)   |
| 5  | M      | IVS 1,6/IVS 1,6 | A/G           | C/T +ins | w.t.       | w.t.    | C/C | 3±0.1   | NO (2.58 fold increase)   |
| 6  | F      | IVS 1,6/IVS 1,6 | A/G           | C/T +ins | w.t.       | w.t.    | C/C | 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 | 2±0     | NO (2.15 fold increase)    |
| 8  | F      | β039/IVS 1,6 | G/G           | C/T +ins | w.t.       | C/C     | 1.8±0.1 | YES (1.59 fold increase)  |
| 9  | F      | β039/IVS 1,6 | A/G           | C/C     | w.t.       | C/C     | 1±0.2   | NO (11.0 fold increase)    |
| 10 | M     | β039/IVS 1,6 | A/A           | C/C     | w.t.       | w.t.    | C/C | 1±0.2   | NO (12.0 fold increase)    |
| 11 | M     | β039/IVS 1,6 | A/G           | C/T +ins | w.t.       | C/C     | 1.8±0.3 | YES (1.58 fold increase)  |
| 12 | M     | βLepore/βLepore | G/G          | C/T +ins | w.t.       | C/C     | 1.4±0.2 | YES (1.15 fold increase)  |
| 13 | M     | β039/β039   | A/A           | C/C     | w.t.       | C/C     | 1.3±0.2 | YES (1.29 fold increase)  |
| 14 | F     | β039/β039   | A/G           | C/T +ins | L45R/N     | C/C     | 2.2±0.3 | YES (1.87 fold increase)  |
| 15 | M     | cod8/β039   | A/G           | C/T +ins | F182L/N    | w.t.    | 1±0   | YES (1.17 fold increase)  |
| 16 | M     | βS/β039    | G/G           | T/T +ins | w.t.       | C/C     | 1.3±0.2 | YES (1.5 fold increase)    |
| 17 | F     | βS/β039    | A/G           | C/T +ins | w.t.       | C/C     | 2.7±0.3 | YES (3 fold increase)      |
| 18 | M     | βS/β039    | G/G           | T/T +ins | w.t.       | C/C     | 1.2±0.2 | YES (1.86 fold increase)  |
| 19 | F     | βS/β039    | G/G           | T/T +ins | F182L/N    | C/C     | 1.7±0.2 | YES (1.85 fold increase)  |
| 20 | F     | βS/β039    | G/G           | C/T +ins | w.t.       | C/C     | 1.4±0.1 | YES (1.51 fold increase)  |
| 21 | F     | βS/β039    | G/G           | T/T +ins | w.t.       | C/C     | 2.2±0.3 | YES (1.87 fold increase)  |
| 22 | F     | βS/β039    | G/G           | T/T +ins | w.t.       | C/C     | 3±0   | YES (3.04 fold increase)  |
| 23 | M     | βS/IVS, 110 | A/G           | C/C     | w.t.       | C/C     | 2.5±0.2 | YES (2.56 fold increase)  |
| 24 | F     | βS/IVS, 110 | A/A           | C/C     | w.t.       | C/C     | 2.3±0.2 | YES (2.83 fold increase)  |
| 25 | F     | βS/IVS, 110 | A/A           | C/C     | w.t.       | C/C     | 1±0   | YES (1.11 fold increase)  |
| 26 | F     | βS/IVS, 110 | A/G           | C/T +ins | w.t.       | C/C     | 1.6±0.2 | YES (1.81 fold increase)  |
| 27 | F     | βS/IVS II, 74S | A/G       | C/T +ins | w.t.       | C/C     | 1.7±0.2 | NO (1.79 fold increase)   |
| 28 | M     | βS/β0    | A/G           | C/T +ins | w.t.       | C/C     | 1±0   | YES (1.22 fold increase)  |
| 29 | M     | βS/βS    | G/G           | T/T +ins | w.t.       | C/C     | 1.2±0.1 | YES (1.24 fold increase)  |
| 30 | F     | βS/IVS 1,5c | G/G           | T/T +ins | w.t.       | C/C     | 1.3±0.2 | YES (1.35 fold increase)  |

### 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
roles. 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, other studies did not find any association of such polymorphism with high HbF levels upon HU treatment. 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 HbF 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, SAL1) have been reported. 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.

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

| Allele       | Transcription | Primers sequence (5’<3’)            |
|--------------|---------------|-------------------------------------|
| rs 4671138   | Forward       | CACTAGCTGAGAAATGGACCT               |
|              | Reverse       | ACCCTTCTAAACAGCGTCC                 |
| rs 11886868  | Forward       | AGTCATGGACAGACAGTCC                 |
|              | Reverse       | GTCCATTGACGATGTTGTC                 |
| KLF-1 exon 2b| Forward       | AGACCTGTGGCCGATATGCT                |
|              | Reverse       | AGAGGGCACTGACTCTAGA                 |
| BCL11A exon 1| Forward       | GGA TGT CA AAG GCA CTG ATG          |
|              | Reverse       | TCCTTTTACCTGACTCTCG                 |
| BCL11A exon 2| Forward       | TTACATGATGGTGTTGGAAT                |
|              | Reverse       | CTCCTCAGATGAGTGTCTG                 |
| BCL11A exon 3| Forward       | CTGGAATAATCTCACCCTCT                |
|              | Reverse       | GCTCAGTGACTAGTGAATGGA               |
| BCL11A exon 4| Forward       | GGCTGAGGTTGGAACATAA                 |
|              | Reverse       | GCTCAGTGACTAGTGAATGGA               |
| BCL11A exon 5| Forward       | CATCTGTTAGATGCCAGTGG                |
|              | Reverse       | AAAAAATGCGGCTGGCAGGCC               |
| GATA-1 exon 1| Forward       | CAAGAGTGCTCCCAACAGCAT               |
|              | Reverse       | GACACTTTGGGGACCAGCTGT               |
| GATA-1 exon 2| Forward       | AGAAATAGTGAGACCTAGGTG               |
|              | Reverse       | ATCTCTACAGTTGATCTGGA                |
| GATA-1 exon 3| Forward       | TGGAGGCTGGAGAATCTT                  |
|              | Reverse       | TCAGCTCAGCTATTAGGTGG                |
| GATA-1 exon 4| Forward       | CCGTAGTGGAGAAATCCAA                 |
|              | Reverse       | GCAGATCTCTTGGATGTC                  |
| GATA-1 exon 5| Forward       | TCTACGCTGACCCTCACCTCT               |
|              | Reverse       | GAGTAGGACAGCAAAGACGAGAG             |
| GATA-1 exon 6| Forward       | TGAAGAAGTGGGTAGAGAG                 |
|              | Reverse       | AGAGGAGCCAGCGGAGCTTTA               |

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