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ORIGINAL ARTICLE

Genetic determinants related to pharmacological induction of foetal haemoglobin in transfusion-dependent HbE-β thalassaemia

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Abstract

Thalassaemia are the most common inherited autosomal recessive single gene disorders characterized by chronic hereditary haemolytic anaemia due to the absence or reduced synthesis of one or more of the globin chains. Haemoglobin E-β thalassaemia is the genotype responsible for approximately one half of all severe beta-thalassaemia worldwide. This study proposes to evaluate the effect of various molecular parameters on the response of hydroxyurea. Hydroxyurea was started at an initial dose of 10 mg/kg of body weight/day on 110 transfusion-dependent HbE-β thalassaemia patients. HbF level was measured by HPLC analysis. β-Thalassaemia mutations, XmnI and five other SNPs, and α-globin gene deletions and triplications were detected by ARMS-PCR, RFLP-PCR and Gap-PCR, respectively. Based on the factors for evaluating hydroxyurea-response, 42 patients were responders as they showed an increment of Hb from a mean baseline value of 6.45 g/dl (± 0.70) to 7.78 g/dl (± 0.72) post-therapy. Based on increase in HbF above the median value (14.72%) post-therapy, 78 patients were found to be responders. All the 78 responders showed mean decrease in transfusion of 74.26% (± 8.32) with a maximum decrease of 98.43%. There was a significant correlation between decrease in transfusion and increase in HbF level for all 78 responders. XmnI polymorphism showed the strongest association ($p < 0.0001$) with increase in HbF levels and Hb levels. Patients with α-globin gene deletions were better responders. It was concluded that hydroxyurea treatment is effective in transfusion-dependent HbE-β thalassaemia patients and the response is best in patients having both XmnI polymorphism and α-deletion.

Keywords HbE-β thalassaemia · Hydroxyurea · Foetal haemoglobin (HbF) · Alpha-deletion · Xmn1 polymorphism · Transfusion

Introduction

Thalassaemia are the most common inherited autosomal recessive single-gene disorders characterized by the absence or reduced synthesis of one or more of the globin chains. This results in chronic hereditary haemolytic anaemia due to imbalance in the ratio of alpha/non-alpha globin chains. Haemoglobin E-beta thalassaemia (HbE/β-thalassaemia) is the genotype responsible for approximately one half of all severe beta-thalassaemia worldwide [1, 2]. The highest frequencies of HbE/β-thalassaemia are observed in India, Bangladesh and throughout Southeast Asia. In the Indian subcontinent, HbE is mostly restricted to North-Eastern states, i.e., West Bengal, Assam, Nagaland, Manipur, Tripura and Meghalaya with an average allele frequency of 10.9% [3]. The ICMR study showed that HbE was mainly seen in Assam (23.9%) and in Kolkata, West Bengal (3.92%) [4]. It is well established that the incidence of HbE gene in the North Eastern region of India is one of the highest in the world [5]. HbE is caused by a substitution of glutamic acid by lysine at codon 26 of the β-globin gene, producing a structurally abnormal haemoglobin [6]. This mutation also activates a cryptic mRNA splice site which results in abnormal messenger RNA (mRNA) processing [7].

Phenotypic presentation of HbE-β thalassaemia is highly variable ranging from those who never require blood transfusion to those who are dependent on it for survival [8], and this is influenced by foetal haemoglobin concentration (HbF). Patients with higher HbF levels show much less severity of the disease with less number of transfusion requirements.

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Foetal haemoglobin (HbF) is the main haemoglobin component throughout foetal life and at birth, accounting for approximately 80% of total haemoglobin in newborns [9]. After birth as the expression of ϒ-globin is progressively silenced and β-globin expression is activated, HbF is gradually substituted by HbA in the peripheral blood [10, 11]. In normal individuals, HbF comprises only 5% of the total haemoglobin by the age of 3 to 6 months and falls below 1% in adults [12].

Pharmacological induction of foetal haemoglobin synthesis by hydroxyurea has been widely used and has shown to reduce the clinical complications and transfusion requirements in sickle cell disease as well as in β-thalassaemia intermedia patients [13, 14]. Since the patients with intrinsically higher HbF levels are found to exhibit a milder disease phenotype, there have been attempts to induce HbF expression by pharmacological means [15].

Point mutations at the promoter of the γ-globin gene and deletions within the β-globin gene cluster can result in increased levels of HbF [16–18]. It is known that the coinheritance of genetic factors like C → T polymorphism at − 158 base pair upstream Gγ gene (XmnI polymorphism) may affect the HbF production in patients with β-thalassaemia major or thalassaemia intermedia [19, 20].

HbF levels are also affected by genetic factors outside of the β-globin gene cluster. Genetic association studies have identified SNPs in major loci that are associated with the variation of HbF levels in patients with sickle cell disease (SCD) or β-thalassaemia and in healthy adults. These loci are the *BCL11A* gene on chromosome 2 (2p16.1), the *HBS1L-MYB* intergenic region on chromosome 6 (6q23.3) [21, 22] and polymorphisms on the *HBBP1* gene [23–25].

An association of α-thalassaemia (α deletion) minimizes the excess of α-globin chains and produces a less severe β-thalassaemia condition [26]. The majority of the α-thalassaemias worldwide are mainly caused by a few common deletions, for example $-\alpha^{3}.7$ and $-\alpha4.^{2}$ [27]. Also, the coexistence of α-globin gene triplication (ααα) is another important modulator, exacerbating the severity of β-thalassaemia, by causing more globin chain imbalance [19, 20]. Generally, two types of triplicated alleles can be generated, αααanti3.7 and αααanti4.2.

In this study, we tried to evaluate the effect of various molecular parameters on the response of hydroxyurea.

Materials and methods

Patient recruitment and sample collection

This was a cross-sectional study conducted over a period from May 2015 to August 2017. Previously diagnosed transfusion-dependent HbE-β thalassaemia patients attending OPD, Thalassaemia Clinic and Day Care of IHTM, Medical College, Kolkata, were recruited for the study. The study was approved by the Institutional Ethical Committee. Blood samples were collected from the recruited patients. Informed written consent was obtained from all patients and parents of paediatric patients. All patients had to complete at least 3 months of therapy to be eligible for analysis considering the fact that the median time to response in hydroxyurea therapy is 3 months [28]. Accordingly, 133 consecutive HbE-β thalassaemia patients attending OPD, Thalassaemia Clinic and Day Care of IHTM, Medical College, Kolkata, were recruited for the study. However, 18 patients who consumed the drug for less than 3 months and 5 patients who became positive for HBsAg, HCV and HIV were excluded from the study. Finally, 110 patients, who adhered to the study protocol, were included in the analysis. Detail history of the patients at the time of recruitment was taken regarding their age, height, weight, age of diagnosis, age of first blood transfusion, frequency of blood transfusion, serum ferritin level, facial deformities, pubertal development, spleen size, baseline Hb and complete haemogram, which were followed up at regular monthly intervals for 28 months. Echocardiography and electrocardiography were carried out for the patients at the time of recruitment and during treatment. Due to regular transfusions, the patients showed severe iron overload (transfusional iron), which was measured on a regular basis by serum ferritin level. At the time of recruitment, the patients were put off transfusion for 2–3 weeks. Baseline Hb is the Hb recorded after the patients were put off transfusion for a period of 2–3 weeks. While on hydroxyurea treatment, the patients were assessed carefully on a regular basis to alleviate the clinical symptoms (such as lethargy, fatigue, loss of energy, loss of appetite, complains of physical unwellness, etc.) and to assure adequate growth in children [29], which indicated the needs of transfusion.

Sample design

Inclusion criteria:

1. Patients with a Hb-HPLC proven HbE-β thalassaemia
2. Who are naïve with respect to hydroxyurea or other HbF-Induction therapies

Exclusion criteria:

1. Patients < 2 years of age
2. Patients maintaining Hb 6 g% without transfusion
3. Pregnant, planning to become pregnant, or lactating
4. Hypersensitivity to hydroxyurea
5. SGOT/SGPT—any of them > three times than the upper range of normal
6. Serum bilirubin > 3 mg%
7. Positive for HBsAg/HCV/HIV

Sample size

Sample size was calculated at the beginning of the study using Fleming's model. Previous studies have evaluated the effect of hydroxyurea in patients with HbE-β Thalassaemia. The mean increase in Hb was 1.3–1.5 g/dl [28, 30]. It is also known that 1 g Hb increment may change the phenotype from TDT to NTDT (extrapolation from study from Sri Lanka where Hb difference of 1 g/dl was observed in NTDT and TDT patients) [31]. Using these as the expected values, we have taken 65% as the lower proportion of rejection and 80% as the higher proportion for acceptance, alpha error of 5% and power of the study as 90%. The calculated minimum sample size was 75. The patients had to complete at least 3 months of therapy to be eligible for analysis considering the fact that the median time to response in hydroxyurea therapy is 3 months [28]. Accordingly, we decided to evaluate all patients with inclusion criteria during the study period ($n = 133$). However, 110 patients were finally evaluated after drop out of 18 patients who consumed the drug for less than 3 months, and 5 patients who became positive for HBsAg, HCV and HIV.

Since this study was on TDT (transfusion-dependent thalassaemia), another parameter which can be utilized for sample size calculation is the decrease in transfusion requirement. The study of Bordbar et al. [32] described that, based on the estimated decrease in transfusion requirement of about 0.2 mL/kg/day after hydroxyurea consumption, standard deviation = 0.3, $\alpha = 0.05$, and power at 95%, sample size was calculated as 60 patients.

So, by whatever method we calculate the sample size, we always get either 75 or 60 samples. The enrolled transfusion-dependent HbE-β thalassaemia patients in this study were 110, which was much more than either 75 or 60 samples.

Controls

Control group was selected to detect the prevalence of the six SNPs (at the BCL11A gene, the HBS1L-MYB intergenic region, the HBBP1 gene and − 158 position of HBG2 gene) and α-globin gene mutations for 3.7 and 4.2 deletions and triplications in the general population. So, 100 age- and sex-matched healthy individuals taken randomly from the general population acted as controls.

Assessment of response

Hydroxyurea response for this study was evaluated based on increase in Hb level (> 1.0 g/dl), increase in HbF levels (greater than the median increase of %HbF), reduction in transfusion requirements (> 50%) and the toxicity of hydroxyurea. Response to hydroxyurea treatment was considered significant if patients were able to alleviate the clinical symptoms (such as lethargy, fatigue, loss of energy, loss of appetite and complains of physical unwellness), could maintain their normal daily life activities, and to assure adequate growth in children [29].

Hydroxyurea treatment and toxicity

The dose of hydroxyurea was determined based on the body weight of the individual patients. It was started at a dose of 10 mg/kg of body weight/day, and for the patients who did not respond after 3 months, the dose was escalated as tolerated to 15 mg/kg body weight/day and finally to 20 mg/kg body weight/day. This is as per with the previous studies of hydroxyurea therapy in patients with β-thalassaemia [28]. Haematological toxicity was monitored on a regular monthly basis by observing the neutrophil counts and the platelet counts, which if observed to fall below $2500 \times 10^3/\mu l$ and $100 \times 10^3/\mu l$, respectively, the drug was stopped. The drug was again restarted at the previous lower dose when the blood counts resolved. Analysis was done, on a regular monthly basis for the first 3 months and then three-monthly, on automated analyser for liver function test (SGOT/SGPT, serum bilirubin) and renal function test (serum urea, creatinine). Patients who showed active liver disease (hepatitis B or hepatitis C infection) were excluded from the study. Hepatic toxicity was defined as an increase in liver enzymes (SGOT/SGPT) greater than thrice the upper limit of normal or total serum bilirubin concentration more than 3 mg%; renal toxicity was defined as decrease in creatinine clearance (CrCl < 60 ml/min). According to the protocol, dose adjustment was done in them as per creatinine clearance (CrCl) as given in "Recommendations of NHS, UK: Dosage Adjustment for Cytotoxics in Renal Impairment: January 2009". Moreover, alopecia, rash, skin hyperpigmentation and headache were reported as drug-related toxicity.

Haematological analysis

Haematological analysis was performed on Sysmex (KX- 21) automated cell counter for all the patients on a monthly basis. HPLC analysis was also done on a monthly basis by Bio-Rad, USA, Variant II. The values of %HbA0, %HbA2 and %HbF were estimated by HPLC. About 1 ml of blood was collected from each individual in EDTA vacutainer tubes (Becton-Dickinson, USA) and was tested on the day of collection, or they can be stored at 4 °C for up to 6 days prior to test. Standard protocol was followed to perform the test.

Molecular analysis

About 1 ml blood was collected from each individual in EDTA vacutainers (Becton-Dickinson, USA). DNA was isolated using QIAamp DNA Kit from Qiagen. DNA from each subject was tested for β-mutation using ARMS-PCR

(amplification refractory mutation system). This method is used to detect known β-thalassaemia mutations. Mutation analysis for five common β-thalassaemia genes prevalent in this region and responsible for > 90% of total mutations was performed in all the 110 patients. These include IVS1–5 (G → C), IVS1–1 (G → T), CD15 (G → A), CD30 (G → C), CD41–42 (-CTTT). ARMS-PCR was done by the method as described in Colah et al. [33], with slight modifications. After PCR, the samples were run in a 2% low melting agarose gel at 100 V for 45–60 min. After electrophoresis, the gel was stained with ethidium bromide and observed under UV light. Our selection of single nucleotide polymorphisms (SNPs) to be studied was based on well characterized SNPs [23–25, 34, 35] with a proven association with variable HbF levels in β-thalassaemia, sickle cell anaemia and heterozygotes for β-thalassaemia or HbE. Here, the 110 patients of study population were screened, and the analyses were compared to the 100 controls, for the six SNPs; at the BCL11A gene which occupies discrete sites within the β-globin gene cluster, the HBS1L-MYB intergenic region located outside the β-globin gene cluster, the HBBP1 gene (a pseudogene within the β-globin gene cluster) and − 158 position of HBG2 gene (gamma globin gene, Gϒ). The presence of rs7482144 at − 158 (C → T) of the HBG2 locus (XmnI polymorphism) was detected for each subject using RFLP-PCR (restriction fragment length polymorphism) following the method as described in Said et al. [35], modifications. For BCL11A locus, AvaII with slight restriction enzyme was used for rs7606173 SNP, AatII restriction enzyme for rs6706648 SNP; for the intergenic locus of HBS1L-MYB, RsaI restriction enzyme was used for rs11759553 SNP, HinfI restriction enzyme for rs4895440 SNP; for HBBP1 locus, Nsi1 restriction enzyme was used for rs2071348 SNP. These were also detected, for each subject, using RFLP-PCR following the method as described in Fanis et al. [25] and Roy et al. [23], with necessary modifications for each SNP. α-Globin gene deletion (3.7 and 4.2) and triplication (3.7 and 4.2) were detected for each subject by Gap PCR. Triplication was done by optimizing the method described by Wang et al. [36]. Deletion was done by optimizing the method described by Liu et al. [37].

Statistical analysis

Data analyses were performed using Statistical Package for Social Sciences, (SPSS Inc., Chicago, IL) software version 21.0 and Medcalc version 11.0 (http://www.spss.com/downloads) (http://www.medcalc.be). The significance level for all statistical tests was set at a probability level of < 0.05. Results were expressed as mean ± SD. Correlation coefficients between all the variables pre- and post-therapy were calculated. Paired *t* test was used to compare means between the pre- and post-therapy for each of the continuous variables.

Results

Clinical parameters and phenotype of the patients

The total study population consisted of 48 males and 62 females. The baseline parameters such as mean age, height, weight, age of first blood transfusion, transfusion requirement per year, serum ferritin level, iron chelation therapy, baseline Hb, baseline HbF and MCV were evaluated for all the 110 patients, which are given in Table 1. 5.45% ($n = 6$) of the study population were splenectomized. Hence, 104 patients were evaluated for spleen size. All the 104 patients showed splenomegaly. There were no statistically significant differences ($p > 0.05$) in the values of the above parameters for the responders and the non-responders. Only the six splenectomized patients were all responders. Also, the patients with smaller spleen size showed response than the patients with larger spleen size who were non-responders, but the difference was not statistically significant ($p > 0.05$). The above-mentioned parameters for the responders and the non-responders have been tabulated in Table 1.

Response to hydroxyurea treatment

After 6 months therapy (based on mean increment of Hb > 1.0 g/dl according to previous study [38] and extrapolation from study from Sri Lanka where it is observed that 1 g/dl Hb increment may change the phenotype from TDT to NTDT [31]), 42 patients were found to be responders which is 38.18% of the total study population ($n = 110$). The responders showed an increase in Hb from a mean baseline value (baseline pre-transfusion Hb) of 6.45 g/dl (± 0.70) to 7.78 g/dl (± 0.72) post-hydroxyurea therapy (showing an increase of 1.33 g/dl). An increase in HbF level was observed among all the 110 patients on hydroxyurea treatment, though the range of increase varied to a great extent. The mean increase in HbF post-hydroxyurea therapy was 18.9% (± 6.53) for all patients. Based on median increase in HbF (14.72%) post-therapy, on an average after 8 months, 78 patients were found to be responders who showed an increase in HbF above the median value. The responders were 70.90% of the total study population ($n = 110$). The range of HbF increase was 14.72–34.85% for the responders, with a maximum increase of 34.85% post-therapy. This increase in HbF was observed post-hydroxyurea treatment after 8 months and was maintained until the study completion (28 months). On comparing the means of HbF of the responders pre- and post-therapy by performing paired *t* test, there was a significant increase in HbF ($p < 0.001$). The remaining 32 patients (29.09%) were considered non-responders, as their increase in HbF was below the median value (14.72%). The range of HbF increase for the non-responders was 2.03–13%. All those (42

Table 1 Various parameters compared between responders and non-responders

Parameters	Pre-treatment value for responders ($n = 78$)	Pre-treatment value for non-responders ($n = 32$)	p value
Age (mean) (years)	15.6 (± 11.5)	14.9 (± 10.7)	$p = 0.52$
Sex (male/female)	35/43	13/19	$p = 0.38$
Height (cm)	130 (± 22.8)	132 (± 22.4)	$p = 0.29$
Weight (kg)	29.9 (± 11.9)	31.4 (± 12.1)	$p = 0.82$
Mean age of first blood transfusion (years)	5.8 (± 8.48)	5.3 (± 8.2)	$p = 0.77$
Mean transfusion requirement (PRBC units/year)	32.49 (± 10.02)	34.21 (± 10.43)	$p = 0.43$
Serum ferritin (ng/ml)	1512.02 (±783.83)	1558.34 (±696.22)	$p = 0.67$
Splenectomy	6	0	$p < 0.0001$*
Spleen size (cm)	6.35 (±2.2)	10.1 (±1.01)	$p = 0.10$
Iron chelation therapy (deferoxamine)	75.41%	66.23%	$p = 0.51$
Dose of hydroxyurea (mg/kg/day)	11.26 ± 2	15.06 ± 2	$p = 0.73$
Hemoglobin (g/dl)	6.45 (± 0.70)	6.12 (± 0.78)	$p = 0.86$
HbF (%)	8.49% (±1.10)	7.98 (± 1.56)	$p = 0.32$
MCV (fl)	72.6 (± 1.2)	70.8 (± 1.7)	$p = 0.55$

Values represent the mean ± standard deviation for each group.

*p values of < 0.01 are statistically significant and have been indicated by asterisks

patients) who showed an increase in Hb (responders) also showed an increase in HbF above the median value. Also, all the 78 responders showed a reduction in transfusion requirements after an average 8 months post-hydroxyurea therapy and maintained this until the study completion (28 months). The mean reduction in transfusion of the responders from the time of recruitment (before treatment) to post-hydroxyurea-therapy was 74.26% (± 8.32). For the 32 non-responders, the mean reduction in transfusion was either 32.83% (± 6.50) or there was no reduction at all. The maximum reduction in transfusion was 98.43% (requiring only one transfusion per year) post-therapy. On comparing the means of reduction in transfusions with increase in HbF levels pre- and post-therapy by performing paired t test, there was a significant correlation ($p < 0.001$). However, reduction in transfusions failed to show any significant correlation with increase in Hb levels. Serum ferritin levels were also measured pre- and post-hydroxyurea therapy. Baseline and post-therapy serum ferritin levels in the patients were 1512.41 (± 683.83) ng/ml and 1402.28 (± 596.22) ng/ml, respectively. The serum ferritin levels decreased in the responders post-therapy, but by performing paired t test, the decrease was not significant ($p > 0.05$). Due to regular transfusions, the patients showed severe iron overload (transfusional iron), which was measured on a regular basis by serum ferritin level. Hence, the patients underwent regular iron chelation therapy with deferoxamine. Reduction in iron overload was observed in the responder patients with reduction in transfusion requirements. But, there was no statistically significant difference ($p > 0.05$) in iron chelation therapy for the responders and the non-responders.

Hydroxyurea dose and toxicity

None of the 110 patients had haematological toxicity at the initial fixed low dose of 10 mg/kg/day. After 3 months follow-up of hydroxyurea treatment, the dose was increased to 15 mg/kg/day in 48 patients who did not show any response. Among them, 16 patients responded at the dose of 15 mg/kg/day. For the remaining 32 patients who did not respond even at 15 mg/kg/day, dose was increased to 20 mg/kg/day. Increase of the dose did result in a fall of neutrophil counts (below $2500 \times 10^3/\mu l$) and platelet counts (below $100 \times 10^3/\mu l$) in these 32 patients, in whom the drug as stopped. They were restarted with the lower dose of 15 mg/kg/day when the blood counts resolved. They were again re-challenged with the higher dose of 20 mg/kg/day after 1 month among whom 29 patients developed cytopenia and had to be continued with the lower dose of 15 mg/kg/day (who remained non-responders); the remaining 3 could tolerate the higher dose and were continued with 20 mg/kg/day. However, even after increasing the dose, these three patients remained non-responders. This study suggests that the optimal dose would be 10–15 mg/kg/day for the responder patients with a mean cumulative dose of 11.26 ± 2 mg/kg/day. Adverse effects included increase in SGOT/SGPT (thrice the upper limit of normal) in four patients. Decrease in creatinine clearance (CrCl < 60 ml/min) occurred in two patients. Dose was modified in them as per creatinine clearance (CrCl) as given in "Recommendations of NHS, UK: Dosage Adjustment for Cytotoxics in Renal Impairment: January 2009". No patient had to be put off from hydroxyurea therapy at any point of the entire study period (28 months). None of the patients presented with related adverse events, such as alopecia, rash, skin hyperpigmentation or headache.

Mutation and polymorphism analysis

The common β-gene mutations [IVS1–5 (G → C), IVS1–1 (G → T), CD15 (G → A), CD30 (G → C), CD41/42 (-CTTT)] were screened among all the patients. No significant correlation was observed between response to hydroxyurea and any type of β-mutations.

The statistical significance of association of each of the six SNPs rs7482144, rs7606173, rs6706648, rs11759553, rs4895440 and rs2071348, within the study population, with Hb increase and HbF increase for the responders are summarized in Table 2. For the 100 controls screened for the six SNPs, their prevalence within the population is also tabulated in Table 2.

The presence of rs7482144 at − 158 (C → T) of the HBG2 locus (XmnI polymorphism) showed the strongest association ($p < 0.0001$) with increase in HbF levels and Hb levels. The mean increase in HbF for the responders having XmnI homozygous was 33.24% and those having XmnI heterozygous was 30.57%. Prevalence of XmnI among the responders (in terms of HbF increase) was 23.08% ($n = 18$) homozygous and 66.67% ($n = 52$) heterozygous, which was more than the prevalence of XmnI within the normal population (18%), as compared to 15.62% prevalence within the non-responders ($n = 5$). Homozygous XmnI was absent within the non-responders and in the normal population. The effect of rs11759553 (intergenic region of *HBS1L-MYB*) on increase in HbF level was significant, and a p value of 0.0014 was obtained within the responders, with a prevalence of 25.18%, as compared to 26% prevalence within the normal population, whereas the effect of rs11759553 (intergenic region of *HBS1L-MYB*) on increase in Hb level was not significant ($p > 0.05$). The mean increase in HbF for the responders with rs11759553 (intergenic region of *HBS1L-MYB*) was 21.32%. rs2071348 (*HBBP1* gene) also showed significant association with HbF increase among the responders ($p = 0.002$), showing a prevalence of 52.09%, as compared to 45% prevalence within the normal population. The mean increase in HbF for the responders with rs2071348 (*HBBP1* gene) was 16.57%. But, rs2071348 (*HBBP1* gene) did not have a significant effect ($p > 0.05$) on the increase in Hb levels. On the other hand, rs7606173 and rs6706648 (on the *BCL11A* gene) and rs4895440 (intergenic region of *HBS1L-MYB*) failed to show association with HbF increase and Hb increase among the responders in a significant way. Their prevalence within the responders was 82.23%, 73.04% and 90%, respectively, while it was 89%, 94% and 98%, respectively, within the control group. These are tabulated in Table 2.

Since XmnI polymorphism showed the strongest association with increase in HbF levels and Hb levels, it was analysed whether the presence of XmnI polymorphism had any association with the two most prevalent β-globin mutations (IVS 1–5 and CD15). It was found that XmnI had a negative

Table 2 Statistical significance of association of the six SNPs with Hb increase and HbF increase, and their prevalence

SNP	Candidate gene	Restriction enzyme	p value for responders (based on increase in HbF) ($n = 78$)	p value for responders (based on increase in Hb) ($n = 42$)	Prevalence within the responders (based on increase in HbF) ($n = 78$)	Prevalence within the general population ($n = 100$)
rs7482144	HBG2	XmnI (homozygous and heterozygous)	$p < 0.0001$*	$p < 0.0001$*	60%	18%
rs7606173	BCL11A	AvaII	$p = 0.082$	$p > 0.05$	82.23%	89%
rs6706648	BCL11A	AatII	$p = 0.0667$	$p > 0.05$	73.04%	94%
rs11759553	Intergenic region of HBS1L-MYB	RsaI	$p = 0.0014$*	$p > 0.05$	25.18%	26%
rs4895440	Intergenic region of HBS1L-MYB	HinfI	$p = 0.12$	$p > 0.05$	90%	98%
rs2071348	HBBP1	NsiI	$p = 0.002$*	$p > 0.05$	52.09%	45%

*p values of < 0.01 are statistically significant and have been indicated by asterisks

association with IVS 1–5 (i.e. XmnI was more prevalent in those without IVS 1–5), but the strength of association was weak (Pearson's $R = 0.227$, $P = 0.037$). On further analysis, it was also found that there was no significant association between XmnI polymorphism and CD-15 mutation in β-globin chain.

On screening α-globin gene mutations for 3.7 and 4.2 deletions and triplications, only one patient among the study population had alpha-alpha (anti-3.7) triplication and was a non-responder. Out of the 110 patients, 27 showed α-globin gene deletions. Among them, 19 patients had single deletion (− α/αα) (13 with 3.7 deletion and 6 with 4.2 deletion). Deletion of two α-globin genes (either − /αα or − α/− α) were found in eight patients (five with deletion of both 3.7 and 4.2 α-globin genes; and three were homozygous for α 3.7 deletion). All the 27 patients who had α-gene deletions showed even better response to hydroxyurea therapy among the responders and showed XmnI polymorphism (homozygous and heterozygous) and a significant increase ($p < 0.001$) in HbF (mean = 29.02%) and Hb (mean = 1.22 g/dl). The five patients who had deletion of two α-globin genes (both 3.7 and 4.2) and the three patients who were homozygous for α 3.7 deletion were the best responders among all. These eight patients were associated with XmnI homozygous polymorphism along with rs11759553 (intergenic region of HBS1L-MYB; restriction enzyme RsaI) and rs2071348 (HBBP1 gene; restriction enzyme Nsi1). Also, these eight patients showed the maximum increment in Hb (mean = 2.01 g/dl) and HbF (mean = 34.20%) which was highly significant ($p < 0.0001$), and their transfusion requirements decreased by 98.43% (requiring only one transfusion per year) which was also highly significant ($p < 0.0001$). These are tabulated in Table 3. They also showed the earliest response at 4 months. For the responders with XmnI heterozygous polymorphism, response time was a bit longer (average 6 months) but was less than the responders with SNPs at HBBP1 (rs2071348) and intregenic region of HBS1L-MYB (rs11759553), and for the responders having no alpha deletion, whose response time was much longer, 8 to 10 months (average 9 months). The prevalence of α-triplication within the control group ($n = 100$) was 23%. α 3.7 deletion and 4.2 deletion was 1% and 0%, respectively, within these controls.

Discussion

Being the commonest symptomatic haemoglobinopathy in many Asian countries, haemoglobinE/β-thalassaemia is a major health burden especially to the developing countries [39]. There is a great diversity in presentation of this disorder, the reasons for which are not completely understood. Hence, management is challenging and should be individualized. There is an emerging understanding of the genetic factors including β-thalassaemia mutations, alpha (α) thalassaemia mutations and a number of polymorphisms (SNPs) that influence the clinical course and severity of anaemia in HbE-β thalassaemia [40]. The main therapy at present for the patients is blood transfusion and iron chelation. Another curative treatment is bone marrow or stem cell transplantation, but the cost involved is prohibitive and also a lot of skilled expertise is required.

In this study, treatment with hydroxyurea was found to be effective in terms of increase in HbF levels, increase in Hb levels and reduction in transfusion requirements for most of the patients. Many of the patients also reported physical well-being while on hydroxyurea. There have been previous studies reporting clinical and hematologic improvement with hydroxyurea in patients with β-thalassaemia intermedia [40, 41]. Previous studies have shown rise in mean Hb level by 3–4 g/dl in children with thalassaemia intermedia and thalassaemia major after beginning treatment with hydroxyurea [42]. But, in this study, the increment of Hb was not as high as reported by them. Also, the increase was not always found to be reflected among the patients who otherwise showed significant

Table 3 Association of XmnI polymorphism, α-globin gene mutations, and decrease in transfusion with increase in HbF and Hb

	Increase in HbF% (mean)	Increase in Hb (mean) (g/dl)
XmnI homozygous ($n = 18$)	33.24	1.63
	$p < 0.001$	*$p < 0.001$*
XmnI heterozygous ($n = 52$)	30.57	0.91
	$p < 0.001$	$p > 0.05$
α-Deletion ($n = 27$)	29.02	1.22
	$p < 0.001$	*$p < 0.001$*
XmnI homozygous + α-double-deletion ($n = 8$)	34.20	2.01
	$p < 0.0001$	*$p < 0.0001$*
XmnI homozygous + α-single-deletion ($n = 10$)	33.76	1.89
	$p < 0.001$	*$p < 0.001$*
Decrease in transfusion (mean)	*$p < 0.001$*	$p > 0.05$

*Statistically significant results (p value < 0.05) are italicized

response for all of the above mentioned criteria. Thus, reduction in transfusion requirement with hydroxyurea therapy may not always reflect increase in Hb levels; also, the increase in Hb with hydroxyurea therapy was not always proportional to the increase in HbF. Similar results have been shown in previous studies on β-thalassaemia [28].

In this study, all the 110 patients showed an increase in HbF level, which has also been observed in a previous study where HbF was induced by hydroxyurea in β-thalassaemia/HbE patients [43]. But, the range of increase varied to a great extent, in this study.

Hydroxyurea is the most common pharmacological agent used in the HbF synthesis reactivation in patients with different haemoglobinopathies [44]. Haematological response to hydroxyurea varies among patients; in some, increase in HbF level can lead to reduced regular blood transfusion dependency, while others do not respond to hydroxyurea therapy. Therefore, it is important to identify possible genetic markers that correlate with drug response [44].

We did not find any β-globin gene mutation to show any pattern influencing the response among the patients in this study. In previous studies also, response to hydroxyurea in β-thalassaemia major and intermedia was not linked to the β-chain mutations [45, 46].

Point mutations at the promoter of the ϒ-globin gene and deletions within the β-globin gene cluster can result in increased levels of HbF [47]. It is known that the coinheritance of genetic factors like C → T polymorphism at − 158 base pair upstream Gγ gene (*XmnI* polymorphism) may affect the HbF production in patients with β-thalassaemia major, thalassaemia intermedia and HbE/β-thalassaemia [48–50]. This study also corroborates with the previous studies for the positive association between the Gϒ-globin (*HBG2*) gene promoter (*XmnI* polymorphism) on chromosome 11 and increase in HbF levels. An interesting observation of this study was that there is a very strong positive correlation between all the patients in the Xmn1 homozygous condition with increment in HbF levels and Hb levels and decrease in transfusion requirements. However, few studies did not find a significant correlation of response to hydroxyurea with XmnI polymorphism [51, 52].

HbF levels are also affected by genetic factors outside the β-globin gene cluster. This study showed that significant increase in levels of HbF among the responders was also primarily influenced by SNPs on rs1175955 on chromosome 6 (intergenic region of *HBS1L-MYB*) ($p = 0.0014$) and on rs2071348 (on the *HBBP1* gene) ($p = 0.002$). However, rs7606173 and rs6706648 (on the *BCL11A* gene) and rs4895440 (intergenic region of *HBS1L-MYB*) failed to show association with HbF increase among the responders ($p > 0.05$). But, previous studies have shown that SNPs located in the *BCL11A* gene and the *HBS1L-MYB* intergenic region act as a major ameliorating factor by modulating the HbF levels [37]. Roy et al. reported a significant association of HbF levels with *HBBP1* gene and with intergenic region of *HBS1L-MYB*, but failed to show significant association with *BCL11A* gene [23], which was in concordance with the results of this study. This suggests that the *BCL11A*, *HBS1L-MYB* and *HBBP1* loci have a minor effect on HbF level compared to the *Xmn*I in HbE-β thalassaemia patients. Also, an interesting study of genetic modifiers in response to hydroxyurea, reported by Karimi et al. [44], evaluated response to hydroxyurea with SNPs in *XmnI-HBG2*, *BCL11A*, *SARIA* and *OR51B2* gene in Iranian patients. In contrast to our study where we got a significant positive correlation with XmnI-HBG2 (rs7482144), they [44] failed to show any significant association with seven SNPs on *XmnI-HBG2* gene (rs11886868, rs112899491y, rs34427034, rs35321913y, rs086298, rs115249933, rs368698783, rs7937237, rs10742622). They got a significant association with only rs10837814 of OR51B2 gene among all the SNPs that they evaluated. Our study was in concordance with this study [44] where both failed to show significant association with *BCL11A* gene. However, the study of Karimi et al. [44] was on β-thalassaemia intermedia patients, and this study is on HbE-β thalassaemia patients.

Another interesting observation of this study was that all the patients having deletion of α-globin gene (− α/αα) (either 3.7 or 4.2) were responders and showed even better response among the responders in terms of their increase in HbF levels, Hb levels, the time required for the induction in HbF and a highly significant reduction in transfusion requirements, as compared to the non-deletional patients. The response was better for the patients having two α-globin gene deletions than single deletion. Out of the 14 patients with XmnI homozygous condition, 6 of them also had alpha double-deletion, who were the best responders among all showing a 98.43% reduction in transfusion (requiring only one transfusion per year). So it is evident that both alpha deletion and XmnI polymorphism are individually a determining factor in response to hydroxyurea, and presence of both gives the best result, though patients having both these criteria are quite rare (5.45% found in this study). In this study, only one patient had alpha-alpha (anti-3.7) triplication and was a non-responder. Previous studies have shown similar results for α-globin gene deletion and triplication in β-thalassaemia intermedia [53]. However, few studies have not found any significant association of response to hydroxyurea with α-globin gene deletions [54].

In this study, it was found that XmnI polymorphism did not have any significant association with the two most prevalent β-globin chain mutations (IVS 1–5 and CD-15). Chinelato et al. has similarly reported in their study on β-thalassaemia patients that there was no statistical significance between the presence of the *Xmn*I polymorphism and β-thalassaemia mutations [48].

In this study, there was reduction in the serum ferritin level for the responder patients post-therapy (due to decrease in

transfusion requirements), but the reduction was not statistically significant ($p > 0.05$). A longer follow-up could result in more reduction in the ferritin levels with sustained decrease in transfusion requirements. These observations are in line with those made in previous studies by Italia et al. in HbEβ-thalassaemia and homozygous β-thalassaemia patients [55, 56] where they observed a significant decrease in serum ferritin in responders but not in non-responders.

It was observed that all the six splenectomised patients were responders, though splenectomised patients in this study were rare, only 5.45%. The patients with an initial smaller spleen size at the time of recruitment showed better response than the patients with larger spleen size who were non-responders, though the difference of baseline spleen size in both the groups was not statistically significant. Thus, baseline spleen size and splenectomy could be considered as a factor to predict response of hydroxyurea, but further studies on this is required. Previous studies have also documented a similar trend in spleen size and hydroxyurea therapy [57, 58].

Earliest response to therapy, in this study, was noted at 4 months for patients having both XmnI homozygous polymorphism and alpha globin gene double-deletion. But, response time was much longer (8–10 months, average 9 months) for the other responders. Most of the studies have reported long-term hydroxyurea therapy [59] which is in concordance with this study. However, Dixit et al. reported response within 1 month of starting therapy in majority of the thalassaemia intermedia patients. They claimed that response to hydroxyurea could be predicted by short trial (3 months) of therapy [46]. But, from this study, we cannot say that short trial could be predictive for hydroxyurea therapy and we would prefer longer periods of follow-up to see the effectiveness of hydroxyurea.

Decrease in transfusions with hydroxyurea therapy, in this study, though, did not correlate with increase in Hb levels, and it correlated with increase in HbF levels. This has also been observed in a previous study in β-thalassaemia/HbE patients [58].

Also, this study suggests that higher dose of hydroxyurea is ineffective and the optimal dose would be 10–15 mg/kg/day for the responder patients with a mean cumulative dose of 11.26 ± 2 mg/kg/day. All patients could tolerate a fixed low-dose treatment with hydroxyurea without any major side effects. This observation was in concordance with previous studies where mostly lower dose of 10–15 mg/kg per day was used initially [59]. Mussalam et al. [28] also observed myelotoxicity especially at doses > 20 mg/kg per day, which could be reversed upon dose reduction. Very few studies on dose escalation up to 20 mg/kg body weight/day could be found on literature review [53, 60–62]. However, a 2009 report suggested that a dose increase to 30 mg/kg per day in a small group of non-responsive patients did not provide any additional benefit [60]. Also, Ghasemi et al. [63] showed that a slow dose escalation up to 35 mg/kg body weight/day can be well tolerated without serious side effects.

Finally, we conclude that hydroxyurea treatment is effective in transfusion-dependent HbE-β thalassaemia patients. It can be a cost-effective and safe alternative method for blood transfusion and chelation therapy, particularly in the developing countries. The response is best in patients having both XmnI polymorphism and alpha deletion, while *BCL11A*, *HBS1L-MYB* and *HBBP1* loci have a minor effect on HbF level as compared to the *XmnI* polymorphism. So, in future, molecular analysis of individual patients can help clinicians to provide more personalized patient care according to the genetic background of each patient, which can lead to better targeted therapy.

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Compliance with ethical standards

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed written consent was obtained from all individual participants included in the study and parents of paediatric participants.

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References

1. Modell B, Darlison M (2008) Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ 86:480–487
2. Weatherall DJ, Clegg JB (2001) Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ 79: 704–712
3. Kiran SS, Aithal S, Belagavi CS (2016) Hemoglobin E hemoglobinopathy in an adult from Assam with unusual presentation: a diagnostic dilemma. J Lab Physicians 8(2):116–119
4. Mohanty D, Colah RB, Gorakshakar AC, Patel RZ, Master DC, Mahanta J, Sharma SK, Chaudhari U, Ghosh M, Das S, Britt RP, Singh S, Ross C, Jagannathan L, Kaul R, Shukla DK, Muthuswamy V (2013) Prevalence of β-thalassemia and other haemoglobinopathies in six cities in India: a multicentre study. J Community Genet 4:33–42
5. Deka R, Reddy AP, Mukherjee BN, Das BM, Banerjee S, Roy M, Dey B, Malhotra KC, Walter H (1988) Hemoglobin E distribution

in ten endogamous population groups of Assam, India. Hum Hered 38:261–266
6. Fuchareon S, Weatherall DJ (2012) The hemoglobin E thalassemias. Cold Spring Harb Perspect Med 2:a011734
7. Orkin SH, Kazazian HH, Antonarakis SE, Ostrer H, Goff SC, Sexton JP (1982) Abnormal RNA processing due to the exon mutation of β^{E}-globin gene. Nature 300:768–769
8. Weatherall DJ, Clegg JB: The thalassaemia syndromes. 3rd. Blackwell Scientific; 2001
9. Hardison RC (2012) Evolution of hemoglobin and its genes. Cold Spring Harb Perspect Med 2:a011627
10. Oneal PA, Gantt NM, Schwartz JD, Bhanu NV, Lee YT, Moroney JW, Reed CH, Schechter AN, Luban NLC, Miller JL (2006) Fetal hemoglobin silencing in humans. Blood 108:2081–2086
11. Stamatoyannopoulos G, Nienhuis AW (2001) Molecular basis of blood diseases, ed 3. Saunders, Philadelphia, PA
12. Wood WG (1993) Increased HbF in adult life. Baillieres Clin Haematol 6(1):177–213
13. Baliga BS, Pace BS, Chen HH, Shah AK, Yang YM (2000) Mechanism for fetal hemoglobin induction by hydroxyurea in sickle cell erythroid progenitors. Am J Hematol 65(3):227–233
14. Karimi M (2009) Hydroxyurea in the management of thalassemia intermedia. Hemoglobin 33:S177–S182
15. Akinsheye I, Alsultan A, Solovieff N, Ngo D, Baldwin CT, Sebastiani P, Chui DHK, Steinberg MH (2011) Fetal hemoglobin in sickle cell anemia. Blood 118(1):19–27
16. Pule GD, Mowla S, Novitzky N, Wiysonge CS, Wonkam A (2015) A systematic review of known mechanisms of hydroxyurea-induced foetal haemoglobin for treatment of sickle cell disease. Expert Rev Haematol 8:669–679
17. Stamatoyannopoulos G, Grosveld F (2001) The molecular basis of blood diseases, 3rd edn. W. B. Saunders, Philadelphia, pp 135–182
18. Sankaran VG, Orkin SH (2013) The switch from foetal to adult haemoglobin. Cold Spring Harb Perspect Med 3(1):a011643
19. Weatherall DJ (1998) Pathophysiology of thalassaemia. Baillieres Clin Haematol 11:127–146
20. Weatherall DJ (2001) Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat. Rev. Genet. 2: 245–255
21. Thein SL, Menzel S, Peng X, Best S, Jiang J, Close J, Silver N, Gerovasilli A, Ping C, Yamaguchi M, Wahlberg K, Ulug P, Spector TD, Garner C, Matsuda F, Farrall M, Lathrop M (2007) Itergenic variations of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal haemoglobin levels in adult. ProcNatlAcadSci USA 104(27):11346–11351
22. Menzel S, Garner C, Gut I, Matsuda F, Yamaguchi M, Heath S, Foglio M, Zelenika D, Boland A, Rooks H, Best S, Spector TD, Farrall M, Lathrop M, Thein SL (2007) A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat. Genet. 39(10):1197–1199
23. Roy P, Bhattacharyya G, Mandal A, Dasgupta UB, Banerjee D, Chandra S, Das M (2012) Influence of BCL11A, HBS1L-MYB, HBBP1 single nucleotide polymorphisms and the HBG2 XmnI polymorphism on HbF levels. Hemoglobin 36(6):592–599
24. Nuinoon M, Makarasara W, Mushiroda T, Setianingsih I, Wahidiyat PA, Sripichai O, Kumasaka N, Takahashi A, Svasti S, Munkongdee T, Mahasirimongkol S, Peerapittayamongkol C, Viprakasit V, Kamatani N, Winichagoon P, Kubo M, Nakamura Y, Fucharoen S (2010) A genome-wide association identified the common genetic variants influence disease severity in β°-thalassemia / hemoglobin E. Hum. Genet. 127(3):303–314
25. Fanis P, Kousiappa I, Phylactides M, Kleanthous M (2014) Genotyping of BCL11A and HBS1L-MYB SNPs associated with fetal haemoglobin levels: a SNaPshotminisequencing approach. BMC Genomics 15(108):1471–2164
26. Badens C, Joly P, Agouti I, Thuret I, Gonnet K, Fattoum S, Francina A, Simeoni MC, Loundou A, Pissard S (2011) Variants in genetic modifiers of β-thalassemia can help to predict the major or intermedia type of the disease. Haematologica 96(11):1712–1714
27. Rosnah B, Rosline H, Zaidah AW, Noor Haslina MN, Marini R, Shafini MY, Nurul Ain FA: Detection of common deletional Alpha-Thalassaemia spectrum by molecular technique in Kelantan, Northeastern Malaysia. Isrn Haematology. 2012; article ID 462969: 3 pgs
28. Musallam KM, Taher AT, Cappellini MD, Sankaran VG (2013) CME article. Clinical experience with fetal hemoglobin induction therapy in patients with β thalassemia. Blood 121(12):2199–2212
29. Olivieri NF, Muraca GM, O'Donnell A, Premawardhena A, Fisher C, Weatherall DJ (2008) Studies in haemoglobin E beta-thalassaemia. Br. J. Haematol. 141(3):388–397
30. Dixit A, Chatterjee TC, Mishra P, Choudhry DR, Mahapatra M, Tyagi S, et al: Hydroxyurea in thalassemia intermedia—a promising therapy. Ann Hematol. 2005 Jul 19 [cited 2016 Dec 25]; 84(7): 441–6]
31. Kalantri SA, Ray R, Chattopadhyay A, Bhattacharjee S, Biswas A, Bhattacharyya M (2018) Efficacy of decitabine as hemoglobin F inducer in HbE/β-thalassemia. Ann. Hematol. 97:1689–1694. https://doi.org/10.1007/s00277-018-3357-y
32. Bordbar MR, Silavizadeh S, Haghpanah S, Kamfiroozi R, Bardestani M, Karimi M (2014) Hydroxyurea treatment in transfusion-dependent β-thalassemia patients. Iran Red Crescent Med J 16(6):e18028
33. Colah R, Mohanty D (2008) Laboratory manual for screening diagnosis and molecular analysis of haemoglobinopathies and red cell enzymopathies. Bhilani Publishing House, Mumbai India, pp 98–101
34. Lettre G, Sankaran VG, Bezerra MA et al (2008) DNA polymorphisms at the BCL11A, HBS1L-MYB and β-globin gene associate with fetal haemoglobin levels and pain crises in sickle cell disease. Proc. Natl. Acad. Sci. U. S. A. 105(33):11869–11874
35. Said F, Abdel-Salam A (2015) XmnI polymorphism: relation to β-thalassaemia phenotype and genotype in Egyptian children. The Egyptian Journal of Medical Human Genetics 16:123–127
36. Wang W, Ma ESK, Chan AYY, Prior J, Erber WN, Chan LC, Chui DHK, Chong SS (2003) Single-tube multiplex-PCR screen for anti-3.7 and anti-4.2 α-globin gene triplications. Clin. Chem. 49(10): 1679–1682
37. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB (2000) Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br. J. Haematol. 108:295–299
38. Chowdhury PK, Jena R, Chowdhury D (2016) Red cell indices as predictors of response to hydroxyurea therapy in HbE/beta thalassaemia patients. Blood 128:4838
39. Weatherall DJ (2005) The challenges of thalassaemia for the developing countries. Ann. N. Y. Acad. Sci. 1054:11–17
40. Olivieri NF, Pakbaz Z, Vichinsky E (2011) HbE/beta-thalassaemia: a common & clinibordbarcally diverse disorder. Indian J Med Res 134:522–531
41. Saxon BR, Rees D, Olivieri NF (1998) Regression of extramedullary haemopoiesis and augmentation of fetal haemoglobin concentration during hydroxyurea therapy in β thalassaemia. Br. J. Haematol. 101:416–419
42. Bradai M, Abad MT, Pissard S, Lamraouni F, Skopinski L, de Montalembert M (2003) Hydroxyurea can eliminate transfusion requirements in children with severe β-thalassaemia. Blood 102: 1529–1530
43. Watanapokasin Y, Chuncharunee S, Sanmund D, Kongnium W, Winichagoon P, Rodgers GP, Fucharoen S (2005) In vivo and in vitro studies of fetalhemoglobin induction by hydroxyurea in

β-thalassemia /haemoglobin E patients. Exp Hematol 33:1486–1492
44. Karimi M, Zarei T, Haghpanah S, Moghadam M, Ebrahimi A, Rezaei N, Heidari G, Vazin A, Khavari M, Miri HR (2017) Relationship between some single-nucleotide polymorphism and response to hydroxyurea therapy in Iranian patients with β-thalassemia intermedia. J. Pediatr. Hematol./Oncol. 39(4):e171–e176
45. Italia KY, Jijina FJ, Merchant R, Panjwani S, Nadkarni AH, Sawant PM, Nair SB, Ghosh K, Colah RB (2009) Response to hydroxyurea in β thalassemia major and intermedia: experience in western India. Clin. Chim. Acta 407(1–2):10–15
46. Dixit A, Chatterjee TC, Mishra P, Choudhry DR, Mahapatra M, Tyagi S, Kabra M, Saxena R, Choudhry VP (2005) Hydroxyurea in thalassemia intermedia—a promising therapy. Ann. Hematol. 84: 441–446
47. David J, Weatherall JBC (2001) The thalassaemia syndromes, 4th edn. Oxford, UK, Blackwell Scientific
48. Chinelato IS, Carrocini GCS, Bonini-Domingos CR (2011) *Xmn*I polymorphism frequency in heterozygote beta thalassemia subjects and its relation to fetal hemoglobin levels. Rev Bras Hematol Hemoter 33(6):483
49. Nguyen TK, Joly P, Bardel C, Moulsma M, Bonello-Palot N (2010) FrancinaA :the XmnI (G)gamma polymorphism influences hemoglobin F synthesis contrary to BCL11A and HBS1L-MYB SNPs in a cohort of 57 beta-thalassemia intermedia patients. Blood Cells Mol Dis 45(2):124–127
50. Olivieri NF, Pakbaz Z, Vichinsky E (2011) Hb E/beta-thalassaemia: a common & clinically diverse disorder. Indian J Med Res 134(4): 522–531
51. Ali N, Ayyub M, Khan SA, Ahmed S, Abbas K, Malik HS, Tashfeen S (2015) Frequency of Gγ-globin promoter −158 (C>T) XmnI polymorphism in patients with homozygous/compound heterozygous beta thalassaemia. HematolOncol Stem Cell Ther 8(1):10–15
52. Karimi M, Haghpanah S, Farhadi A, Yavarian M (2012) Genotype-phenotype relationship of patients with β-thalassemia taking hydroxyurea: a 13-year experience in Iran. Int J Hematol 95:51–56
53. Panighari I, Dixit A, Arora S, Kabra M, Mahapatra M, Choudhry VP, Saxena R (2005) Do alpha deletions influence hydroxyurea response in thalassaemia intermedia? Hematology 10:61–63
54. Yavarian M, Karimi M, Bakker E, Harteveld CL, Giordano PC (2004) Response to hydroxyurea treatment in Iranian transfusion dependent beta-thalassemia patients. Haematologica 89(10):1172–1178
55. Italia KY, Jijina FJ, Merchant R, Panjwani S, Nadkarni AH, Sawant PM (2009) Response to hydroxyurea in beta thalassemia major and intermedia: experience in western India. Clin. Chim. Acta 407(1–2):10–15
56. Italia KY, Jijina FF, Merchant R, Panjwani S, Nadkarni AH, Sawant PM, Nair SB, Ghosh K, Colah RB (2010) Effect of hydroxyurea on the transfusion requirements in patients with severe HbE-beta-thalassaemia: a genotypic and phenotypic study. J Clin Pathol 63(2): 147–150
57. Cario H, Wegener M, Debatin KM, Kohne E (2002) Treatment with hydroxyurea in thalassemia intermedia with paravertebral pseudotumors of extramedullary hematopoiesis. Ann. Hematol. 81:478–482
58. Watanapokasin Y, Chuncharunee S, Sanmund D, Kongnium W, Winichagoon P, Rodgers GP, Fucharoen S (2005) In vivo and in vitro studies of fetal hemoglobin induction by hydroxyurea in β-thalassemia /haemoglobin E patients. Exp Hematol 33:1486–1492
59. De Paula EV, Lima CSP, Arruda R, Alberto FL, Saad STO, Costa FF (2003) Long-term hydroxyurea therapy in beta-thalassaemia patients. Eur J Haematol 70:151–155
60. Sylvia ST, Elliott V, Sandra L, Nancy O, Nancy S, Frans KA (2008) Hydroxycarbamide-induced changes in E/beta thalassaemia red blood cells. Am J Hematol 83(11):842–845
61. Treatment with hydroxyurea – thalassaemia.com. Experimental drug therapy to increase fetal haemoglobin. Treating Thalassaemia. Northern California comprehensive thalassaemia center
62. Arruda VR, Lima CSP, Saad STO, Costa FF (1997) Successful use of hydroxyurea in β-thalassemia major. N Engl J Med 336:964–965
63. Ghasemi A, Keikhaei B, Ghodsi R (2014) Side effects of hydroxyurea in patients with thalassaemia major and thalassaemia intermedia and sickle cell anaemia. Iran J PedHematol Oncol 4(3):114–117