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Genotype–phenotype correlation and report of novel mutations in β-globin gene in thalassemia patients☆

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ABSTRACT

Heterogeneity in thalassemia is due to various modifying factors viz. coinheritance of α-gene defects, abnormal hemoglobin, *XmnI* polymorphism, variation in repeat sequences present in LCR, and silencer region of the gene. The present work on populations from eastern regions of India was undertaken to study the genetic profile of heterogeneity in thalassemia patients. Mutation analysis in 126 index families revealed the presence of 3 novel mutations: CD2 (−A) in the 1st exon, −42 (C–G), and −223 (T–C) in the promoter region of β-globin gene. The modifying effect of coexisting α-gene defects, and abnormal Hb (HbS) was clearly observed in our study, however ameliorating effect of T allele of *XmnI* polymorphism was not found. Analysis of the regulatory regions (LCR) exhibited new combinations ($CA_{15}TA_5$ and $CA_{13}TA_8$) in HS1 region and one $(AT)_{10}T_3$ in $(AT)_xT_y$ silencer region. Thus disparate factors, when considered together, were able to explain several of the thalassemic phenotypes, otherwise not explained by the β globin mutations. However, there were still some cases in this group whose molecular origin could not be ascertained. Our findings confirm not only the extensive genotypic and clinical heterogeneity in β thalassemia but also the need to look for more modulators and modifiers to better understand the genotype–phenotype correlation in thalassemia.

1. Introduction

β-Thalassemia, an autosomal recessive disorder, shows considerable clinical heterogeneity, phenotype varying from life threatening severe anemia and transfusion dependency to near normal, asymptomatic condition. More than 300 mutations, both in and around beta globin gene [1,2] are attributed to this phenotypic spectrum. β-Thal has been shown to have considerable presence in the Indian subcontinent, especially in the western, northern and north-eastern regions. Based on the genotypic information from these regions, 5 major mutations have been identified that are believed to account for more than 80% of the patients. However, occurrence of β-thalassemia in the larger part of the land mass of India is only now being realized [3,4]. Therefore the present study has been undertaken to have an estimate of the mutation profile of the β-globin gene, its LCR and α globin gene defects and *XmnI* polymorphisms leading to β-thalassemia to enable us to define their modulatory role in the heterogeneity and diversity of the β-thal phenotypes in cases from the eastern region of India (Eastern Uttar Pradesh and adjoining states of Bihar, Jharkhand and Chhattisgarh). We report not only a mutation profile different from that presently attributed to the population in the Indian subcontinent but also a few novel mutations within β-globin gene and its promoter region. We also derive possible combinations of genotypes and their interaction to explain the genetic and phenotypic correlation among the studied subjects.

2. Materials and methods

The thalassemia subjects registered in Varanasi Regional Thalassemia Welfare Society and Pediatrics Department of the University Hospital were referred to the Centre for Genetic Disorders (CGD) of the University for DNA testing, which is a government-registered center for diagnosis of various genetic disorders. 3–4 ml of blood was taken in EDTA coated vials following a written informed consent taken from the guardian of the patients. Blood morphology was studied, and CBC count was taken using an automated counter, Hb-electrophoresis was performed for any abnormal hemoglobin followed by column chromatography for the estimation of HbA_2. DNA was isolated by modified salting out method [5]. Initial screening of mutations was done for 18 "common" mutations by ARMS test. In cases where either only one mutation or no mutation in beta globin gene was detected, complete β-globin gene sequencing as well as LCR analysis was done using ABI-3130 sequence analyzer (Invitrogen, USA). *Xmn*I polymorphism was checked by PCR-RFLP. Coinheritance of alpha gene defects was studied by Gap-PCR. The study was approved by Institutional Ethical Committee, BHU.

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Cases were categorized as TM or TI on the basis of age of onset and transfusion requirement. Thalassemia major were the patients showing the symptoms within 3 years of age [6], and were transfusion dependent, while those with late onset and occasional transfusion demand were categorized as TI. Average age of presentation in TM and TI group was respectively, 1 and 12 yrs in the studied cohort.

3. Results and discussion

3.1. The mutation profile

Within a period of 7-years, a total of 126 cases were referred to the CGD for mutational profiling. Of these, 22 were obligate carriers (OC) where only parental mutational analysis was done because of the expiry of patients due to β-thalassemia during or before the study. Of the remaining 104, 78 were Thalassemia Major (TM), 17 were Thalassemia Intermedia (TI), and 5 were β Thalassemia Trait (BTT) while 3 had alpha thalassemia and 1 sickle cell disease. Among the TM, 2 were S-β and 8 E-β while 10 cases revealed only one mutant beta gene allele and 3 no mutant allele. The rest (55) were double mutants for the β-globin gene. A total of 7 α gene triplications were also observed in different TM samples. Two of the cases co-inherited α gene deletion. Among the TI cases the breakup was: 6 E-β (one with $\alpha\alpha\alpha^{3.7}$ and one with $\alpha\alpha/\alpha^{3.7}$), 2 S-β, 3 with both β alleles affected ($\alpha\alpha/\alpha^{3.7}$, $\alpha^{3.7}/\alpha^{3.7}$, $\alpha\alpha\alpha^{3.7}$), 5 with one β gene mutation of which one had a single α gene deletion and one with $\alpha\alpha\alpha^{3.7}$. One TI case did not reveal any mutation in the β globin gene. Table 1 illustrates the mutation profile in the families studied, revealing IVS1-5 to be the most abundant (51.5%) followed by CD30, CD16 and CD15 whose frequency was higher than CD8/9, CD41/42, the mutants reported to be the most prevalent after IVS 1–5, in most parts of India [3]. Of another common "Indian" mutation, IVS1-1 (G-T), only 5 alleles were found in individuals of 2 families of Sindhi community (migrants from Pakistan or northern India). In 10.4% (26/230) of the alleles no mutation was detected.

3.2. Novel mutations in β-globin gene

A novel single base deletion (−A) in codon 2 was recorded in the mother of a deceased thalassemic child (Fig. 1). As per records, the child presented clinical symptoms of thalassemia at an age of 6 months, with fever and pallor. The child only survived for 11 months with two transfusions occurring within survival period. IVS1-5 was the other mutation recorded in the father. The novel deletion would result in a stop codon after third position of the growing amino-acid chain, purportedly leading to β^0 condition from that allele.

Complete β globin gene sequencing revealed two additional variations in heterozygous condition: −42 (C–G), −223 (T–C) from promoter region in a TI and TM case, respectively (Fig. 1). The TI case was a female, presenting at 16 yrs of age with severe anemia. The blood picture showed marked anisopoikilocytosis, microcytosis, polychromias, tear drop and a number of nucleated red cells. Mutational analysis of this transfusion-independent patient revealed CD30 and −42 (C–G) changes. The patient also inherited a triplicated α-gene allele. In a study [7] −56 (G–C) mutation has been reported as a negative modulator of globin gene expression which may be comparable to the −42 variant seen in the present report. The other patient, a TM, presented at 6 months of age with hypochromic, microcytic anemia and a number of fragmented cells revealed a single mutation in the promoter region, −223 (T–C). He is on regular transfusion. Since the region between −100 to −300 is an important regulator of β-globin gene [8], we surmise a likely association of the observed mutation with the disorder. We have not found any other mutation in this individual, and it seems unlikely that a heterozygous mutation alone in the regulatory region should manifest in a TM phenotype. Therefore, additional genetic contributors need to be factored in to explain this phenotype. These 2 variants were not found in any of the 65 (130 chromosomes) normal individuals nor in any beta thal cases globally.

3.3. Modifying effect of β-gene mutations, α-gene profile and XmnI Polymorphism

The variability in the phenotype with a similar genotype could readily be explained in the 14 cases of HbE and 4 HbS cases, where 6 E-β and 2 S-β belonged to TI group, and the rest (8 E-β and 2 S-β) belonged to TM group, owing to variable amounts of HbE and S in different individuals. In the other 2 compound heterozygous cases, one having a combination of CD8/9 with a milder mutation (Capsite +1) and $\alpha\alpha/\alpha^{3.7}$, and the other with αααα3.7 and −42 (C–G) (a milder mutation) along with CD30, had, we suppose, tilted the phenotype from TM to TI. The modulatory effect of alpha gene defects was clearly observed in 4 TI cases: 2 with alpha gene deletion and double mutant for β globin gene and 2 with single beta gene defect and αααα3.7.

However, the suggested ameliorating effect of *Xmn*I polymorphism at −158 (C–T) in γ globin gene in which TT genotype is associated with enhancement of fetal hemoglobin was not observed in the present cohort. The frequency of C and T allele was, respectively 0.82 and 0.18 with no significant difference in the level of HbF between the CC and CT groups. Although, incidence of T allele of −158 $^{G}\gamma$ gene polymorphism among TI cases was significantly higher than in TM, a correlation with high HbF could not be established. One TM patient (homozygous for IVS1-5) having the TT genotype had 92% fetal hemoglobin but equally high HbF was recorded in several patients with CC genotype. Apparently, factors other than the *XmnI* polymorphism in γ globin gene are involved in elevating the HbF level.

3.4. LCR analysis

Globin gene expression is coordinated by the Locus Control Region (LCR), nearly 60 kb upstream of ε gene. Various DNaseI hypersensitive sites (HS1, HS2, HS3, and HS4), containing repeat elements or other unique sequences, act as modifiers of the β-globin activity. In the β thal cases where mutation in one or none of the β-globin alleles was detected, we examined polymorphisms in the LCR (HS1, HS3, HS4), enhancer (3′ to β globin) and silencer region (5′ upstream of β globin). While no significant modifying effect was observed in HS3, HS4 and 3′ flanking regions of β globin gene (3FS), various combinations observed in HS1 region, characterized by a $(CA)_x(TA)_y$ repeat motif (Table 2) showed 2 novel combinations as compared with an earlier report from India [9]. These two combinations were: $(CA)_{15}(TA)_5$ in a TM

Table 1
Spectrum of mutations among various disease groups. The highlighted 3 mutations are novel to literature. OC = Obligate Carrier.

Mutations	TM (156 alleles)	TI (17 cases)	BTT (5 alleles)	OC (44 alleles)	Total
IVS1-5(G–C)	73	8	2	22	105 (52.7%)
CD16 (−C)	10	1	0	4	15 (7.5%)
CD30 (G–A)	9	4	0	3	15 (7.5%)
CD8/9(+G)	13	2	0	0	14 (7.0%)
HbE	7	6	0	1	14 (7.0%)
CD41/42(−TCTT)	9	1	0	2	12 (6.0%)
CD15 (G–A)	6	0	0	0	06 (3.0%)
IVS1-1(G–T)	4	0	1	0	05 (2.5%)
HbS	2	2	0	1	04 (2.0%)
619 bpΔ	2	0	1	0	03 (1.5%)
−88 (+T)	1	0	0	0	01 (0.5%)
Capsite +1 (A–C)	1	1	0	0	02 (0.1%)
CD5 (−CT)	1	1	0	0	02 (0.1%)
IVS1-1(G–A)	0	0	1	0	01 (0.5%)
CD2(−A)	0	0	0	1	01 (0.5%)
−42 (C–G)	0	1	0	0	01 (0.5%)
−223 (T–C)	1	0	0	0	01 (0.5%)
Total	139	27	5	34	206
Uncharacterized	17			8[a]	25 (10.4%)

[a] One of the family had both the parents with a triplicated alpha allele.