Appendix
Variable severity of β-thalassemia patients of Eastern India: effect of α-thalassemia and XmnI polymorphism

Abstract Sixty-four thalassemia and E-β thalassemia patients were studied for factors that modulate the severity of the disease; i.e., mutation of β-globin gene, presence of α-deletion, and presence of an XmnI site at the -158 position of the Gy gene. Presence of α-deletion and/or homozygosity for the XmnI site was in general associated with less-severe disease. About 12% of the patients harbored single α-gene deletion, and the gene frequency of the XmnI polymorphism in these patients is 0.48.

Key words β-Thalassemia • Severity • XmnI polymorphism • α-Thalassemia • Eastern India

Introduction

The frequency of β-thalassemia in Eastern India is quite high, and almost one-third of the mutant β-globin alleles involve HbE (codon 26 G→A). Heterozygotes of the HbE are clinically normal but in conjunction with other β-globin mutations HbE gives rise to E-β thalassemia, which constitutes a high percentage of thalassemic diseases in Eastern India [1]. Both β-thalassemia and E-β thalassemia are characterized by widely varying clinical symptoms. Even with the same β-globin mutations, Hb levels may vary between 3 and 13 g/dl, and the number of transfusions may range from once every fortnight to none at all.

There are several reasons for this type of anomalous phenotype. Co-inheritance of α-thalassemia with β-thalassemia or E-β thalassemia produces a milder phenotype [2, 3] as the β/α ratio moves closer to unity. The inheritance of a mild β-mutation and co-inheritance of genetic changes leading to hereditary persistence of fetal Hb (HbF) also reduce the severity of the disease. These changes might involve a large deletion of the β-cluster or certain point mutations in the promoter regions of Gy and Ay genes [4]. Of these, the presence of one polymorphic XmnI restriction site at -158 position of the Gy gene has been correlated with increased production of HbF in adults under hematopoietic stress [5].

We have studied 58 E-β thalassemia and 6 β-thalassemia patients for the β-globin gene mutations, the presence of the 3.7-kb and 4.2-kb deletions of the α-gene cluster, and the polymorphism at -158 position of the Gy globin gene. The patients were divided into three groups according to the severity of their disease. Group A consists of patients who undergo transfusion regularly. Patients of group B receive transfusions only occasionally, when Hb levels fall following infections. Group C patients do not receive transfusions. Attempts have been made to correlate the studied parameters with the status of the patients.
Patients and methods

Patients

Sixty-four patients, 58 with E-β thalassemia and 6 with β-thalassemia, attending the Hematology Clinic of Kothari Medical Centre, Calcutta and having the diagnosis of β-thalassemia or Hb E-β thalassemia were included in the study. The diagnosis was established by complete hemogram and routine Hb electrophoresis [6]. Before starting transfusions, the patients were observed for at least 3 months, and regular transfusion therapy was started if patients showed decreasing Hb levels and performance status along with increasing hepatosplenomegaly. Transfusions were carried out according to the guidelines of Thalassemia International Federation (TIF), along with regular chelation therapy with desferrioxamine and deferoxprone in group A patients. Transfusions started early in these thalassemia patients, the range being 6 months to 13 years, the mean value being 2.6 years. Of this group of 27 patients, 8 had their spleen removed.

Group B patients were followed regularly in the clinic by recording Hb levels, liver and spleen size, etc. Transfusions were given only when Hb levels fell following infections. In this group, transfusions started at ages 3–18 years, the mean value being 7.6 years. Of 8 patients in this group, 4 had their spleens removed.

Group C patients did not receive transfusions and the ages at which they were diagnosed ranged from 1 to 35 years, the mean value being 15 years. Of this group, 9 patients had their spleens removed. Although the storage iron levels were followed regularly, iron chelation therapy was not prescribed regularly in the last two groups.

The patients were from Calcutta and different districts of West Bengal. Their ages ranged between 4 and 43 years with a median age around 14–15 years. The patients are being treated by the same group of clinicians, following the same set of guidelines, so that their transfusion status is truly a reflection of the severity of their disease. Informed consent of the patients and their parents was obtained before the study.

DNA mapping

DNA was extracted from the buffy cost of blood with EDTA anticoagulant by the usual method [7]. The status of XmnI polymorphism at -158 position of the Gy gene was determined by amplification of a 641-bp fragment around the site by polymerase chain reaction (PCR). The fragment was purified by precipitation with ammonium acetate and isopropanol at room temperature, digested with XmnI overnight at 37°C, and analyzed by electrophoresis on 3.0% agarose gel with staining by ethidium bromide. Digestion produced bands of 223 bp and 418 bp when both chromosomes had the site, 641 bp, 223 bp, and 418 bp, when only one chromosome had the site, whereas absence of the site in both the chromosomes showed only the original 641-bp band (Fig. 1). 3.7-kb and 4.2-kb deletions of α-gene cluster were determined by PCR [8]. Briefly, appropriate primers were used to amplify relevant fragments. In the case of a 3.7-kb deletion, normal and mutant primers amplified 1.8-kb fragments. Hence two different reactions had to be set up per sample to amplify the respective fragments. In the case of a 4.2-kb deletion, normal primers amplified a 581-bp fragment and mutant primers amplified a 2.1-kb band. These fragments were identified by electrophoresis on 1.5% agarose gel (Fig. 2).

Alpha triplication was studied similarly using PCR [9]. Here normal and mutant primers amplified 1.8-kb bands, so once again two different reaction sets were used. The different β-globin gene mutations were determined by the PCR-based method of Amplification Refractory Mutation System (ARMS) as described earlier [1].

Fig. 1 XmnI digestion of 641-bp polymerase chain reaction (PCR) product. Lane 1, +/-; lane 2, +/+; lane 3, molecular weight marker (50-bp ladder); lane 4, +/-; lane 5, -/-

Fig. 2 Detection of 3.7-kb α-globin gene deletion by PCR. Lane 1, λ/HindIII marker; 2,4, mutant allele-specific primer; 3,5, normal allele-specific primer

fragments. In the case of a 4.2-kb deletion, normal primers amplified a 581-bp fragment and mutant primers amplified a 2.1-kb band. These fragments were identified by electrophoresis on 1.5% agarose gel (Fig. 2).

Alpha triplication was studied similarly using PCR [9]. Here normal and mutant primers amplified 1.8-kb bands, so once again two different reaction sets were used. The different β-globin gene mutations were determined by the PCR-based method of Amplification Refractory Mutation System (ARMS) as described earlier [1].
The patients were divided into three groups A, B, and C, according to their transfusion protocol. There were 27 patients in group A, all being E-P thalassemia patients. None had an α-gene deletion, and 1 had triplication of α-gene. Of the patients, 21 had IVS I-nth 5 (G→C) mutations with HbE, 1 had 41/42 (-CTTT), 1 cap+1 (A→C), 1 -88(C→T), 1 IVS I-130 (G→C), and 1 remained uncharacterized. None of the patients in this group was homozygous for the XmnI (+/+). genotype, 7 were homozygous for the XmnI (-/-) genotype, and 20 were heterozygous (+/-).

There were 8 patients in group B who were on infrequent transfusions. Of these, 6 had the 3.7-kb α-gene deletion, 6 were heterozygous for the XmnI (+/-) genotype, and 2 had the XmnI (-/-) genotype. All were E-β thalassemia patients, with 5 of them having the IVS I-nth 5 (G→C) mutation, 1 codon 15 (G→A) mutation, 1 -88 (C→T) mutation, and 1 41/42 (-CTTT) mutation along with the HbE mutation.

There were 29 patients in group C of non-transfused patients. Of these, 5 were β-thalassemia homozygotes, 3 had IVS I-nth 5 (G→C) mutation, and 2 (brothers) the codon 30 (G→C) mutation. One patient was a compound heterozygote for the IVS I-nth 5 (G→C) and cap+1 (A→C) mutation. The other 23 were E-β thalassemia patients, with 18 having the IVS I-nth 5 (G→C) mutation, 2 having the codon 30 (G→C) mutation, 1 the codon 15 (G→A), and 1 codon 8/9 (+G) along with HbE mutation. The β-mutation in 1 patient remained uncharacterized.

Of these 29 patients, 5 carried the 3.7-kb deletion of the α-gene and 1 carried the 4.2-kb deletion, bringing the incidence of α-thalassemia to 20.7% in this group. Of the 3 IVS I-nth 5 (G→C) homozygotes, 2 carried the 3.7-kb deletion and 1 carried the 4.2-kb deletion.

The genotype of the XmnI polymorphism in group C was homozygous (+/+ in 6 patients and heterozygous (+/-) in the others. Of the (+/+ ) patients 4 were β-thalassemia homozygotes, 2 for IVS I-nth 5 (G→C), and 2 for codon 30 (G→C). None was -/- at the XmnI locus in this group.

Discussion

The disease β-thalassemia is well known for its widely variable phenotypes in patients of apparently identical genotypes. With the same pair of β-globin mutations, some might be almost asymptomatic while others require frequent transfusions. Attempts have been made for some time to understand the molecular basis of these differences. Co-inheritance of α-thalassemia is one of the factors that ameliorates the severity of β-thalassemia. Such co-inheritance of α-thalassemia with β-thalassemia and sickle cell disease has also been noticed in India. An α-thalassemia gene frequency of 0.32 has been found in tribal sickle cell patients of South India, and in Orissa it was estimated at 0.29 [10, 11]. In the non-tribal population of the Punjab, Maharashtra, and Gujarat area, two studies reported an α-thalassemia gene frequency of approximately 0.1 [12, 13]. No data on the E-β or β-thalassemia patients of caste population of Eastern India are available to date. In this study, we found that 8 of 64 patients, i.e., 12.5% of the patients, harbor 3.7-kb or 4.2-kb deletions of the α-gene cluster. Interestingly, all were in the non-transfused or infrequently transfused group of patients.

Persistence of Hbf production in adult life is another factor that greatly influences the severity of β-thalassemia. Several loci, in and outside the β-globin gene cluster, have been implicated in higher production of Hbf. Environmental factors are also involved, and the whole story is by no means complete. Of the various loci involved, the XmnI restriction site at position -158 of Gγ gene has been strongly correlated with Hbf production. We studied this in our patients and found that homozygosity of this site (+/+ ) is strongly correlated with a mild phenotype and its absence (-/-) with a severe phenotype. Of the 128 chromosomes studied, 62 harbored the restriction site and 66 did not; hence the ratio of + allele to - allele is 0.94 in these patients. It has not been possible to measure the Hbf values in all patients, but a small study has been performed on 18 patients. The percentage of Hbf, as obtained by high-
Table 1  Mutation of the β-globulin gene, presence of an α-deletion, XmnI polymorphism at position -158 of the Gγ gene, and fetal hemoglobin (HbF) levels in transfused (group A), infrequently transfused (group B), and non-transfused (group C) thalassemia and B-β thalassemia patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Mutation</th>
<th>α-Globin</th>
<th>XmnI</th>
<th>HbF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21</td>
<td>IVS I nt5/E</td>
<td>1 ααααα</td>
<td>17 ++/-</td>
<td>0.24±0.15 g/dl</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>41/42/E</td>
<td>N</td>
<td>1 +/-</td>
<td>(n=9)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-88/E</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>IVS I nt130</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Cap+1/E</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Uncharacterized/E</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>IVS I nt5/E</td>
<td>13.7, 4 N</td>
<td>1 -/-</td>
<td>0.85±0.57 g/dl</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>41/42/E</td>
<td>N</td>
<td>-/-</td>
<td>(n=4) P&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-88/E</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Co15/E</td>
<td>3.7</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>IVS I nt5/E</td>
<td>23.7, 15 N</td>
<td>1 +/+</td>
<td>2.67±1.15 g/dl</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Co30/E</td>
<td>N</td>
<td>1 +/+</td>
<td>(n=5) P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Co15/E</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Co8/9/E</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Uncharacterized/E</td>
<td>3.7</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>IVS I nt5/IVS Int5</td>
<td>23.7, 14.2</td>
<td>2 +/+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Co 30/Co 30</td>
<td>N</td>
<td>2 +/+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>IVS I nt5/Cap+1</td>
<td>N</td>
<td>+/-</td>
<td></td>
</tr>
</tbody>
</table>

N, Neither 3.7-kb nor 4.2-kb deletion of α-globin gene detected.

Severity was correlated with low values of HbF. Using Student's t-test, the value of HbF in group A was significantly different from that in group C (non-transfused patients). Those with the ++ genotype had consistently high HbF levels. However the reverse was not true, and some patients with the -/- genotype had a HbF level between 0.75 and 1.0 g/dl.

Apart from obvious academic interest, these studies are also important for genetic counseling. Co-inheritance of α-thalassemia and homozygosity (+/+ of the XmnI site at position -158 of the Gγ gene on a background of the mild IVS1-nt 5 (G→C) mutation makes a patient almost asymptomatic, and these factors should be taken into account while counseling. It should also be mentioned that there are several patients in this study who have not co-inherited an α-globin gene deletion or (+/+ status of the XmnI polymorphism, but still do not need transfusions. They might harbor non-deletional mutations of α-globin gene or other factors that influence the severity of E-β thalassemia.

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Detection of a Rare Splice Acceptor Site Mutation (IVS I nt 130 G→C) of the β Globin Gene in 3 Patients of Eastern India

To the Editor: Twenty-nine mutations of the β globin gene have been reported so far to occur in Thalassemia patients of India. Only 5 or 6 mutations seem to occur at an appreciable frequency, the most common one being IVS I nt 5 (G→C) [1]. Others occur at different frequencies at different regions of the country, reflecting the composite nature of Indian population. A few cases have remained uncharacterized in all studies, and the frequency of uncharacterized mutations is highest (~9%) in Bengal [1], indicating presence of hitherto unidentified mutations present in population of this region. We report here the presence of a rare splice acceptor site mutation IVS I nt 130 (G→C) in three patients of West Bengal. This mutation, previously reported in Japan and Turkey, has not been detected in Indian population so far.

The first subject is a 10-year-old Bengalee HbE β-thalassemia patient with hematological features of Hb 8.5 g/dL, PCV 28.5, MCV 72.4, MCH 21.7, MCHC 29.9, HbF 4%, and HbE + β 19.2%. The boy is regularly transfused. His mother is a carrier of HbE mutation. None of the β globin mutations commonly occurring in Indian population could be detected in the boy's or his father's DNA. So a 499-base pair fragment, from the 1st nucleotide of the 19th codon to the 72nd nucleotide of the second intron was amplified by polymerase chain reaction (PCR) and subjected to conformation-sensitive gel electrophoresis analysis (CSGE) [2]. Heteroduplex formation was indicated in both the boy's and his father's DNA. This fragment did not carry any sequence polymorphism in the father's case but still showed heteroduplex formation. Upon sequencing, using thermal cycle sequencing protocol, a G→C substitution was found in the 130th position of the first intron (Fig. 1). This change in splice acceptor site produces a β^+ mutation which has not been reported in India so far.

This mutation abolishes a CviII restriction site in the DNA and can be detected by the failure of CviII to digest the 0.5-kb fragment at the mutation site, so that instead of 88,201- and 211-bp fragments, 211- and 289-bp fragments are obtained. The exact nucleotide change can be confirmed by sequencing.

Two other patients also harbored this mutation along with the IVS I nt 5 (G→C) mutation.

Of the three chromosomes harboring the IVS I nt 130 (G→C) mutation, 2 were subjected to haplotype analysis of the β globin gene cluster by PCR-RFLP, using the sites HindII Iα, HindIII Gry, HindIII Aγ, HindIII S'β, HindIII 3'β, and HindII B [3]. One chromosome was associated with the pattern + + + + + +, which is very common in Indian population, both in normal and β thalassemia chromosomes. The other chromosome carried the haplotype + − + − + − +, which we found also with chromosomes carrying the Co 30 (G→C) mutation [3]. This haplotype has been reported to be associated with the Co 30 (G→C) mutation in Tunisian people [4].

Though the two chromosomes bear different 3' haplotypes, the only β globin site studied has the same status (+) in both, and the difference in haplotypes might be explained as a result of recombination. This mutation has also been reported in Turkey and Japan at a very low frequency, but to the best of our knowledge the haplotypes associated with the chromosomes have not been published. So it is not possible to decide whether this mutation originated here or whether its presence in India is a result of the Muslim invasion of India in the middle ages. Incidentally this is also the first application of CSGE in the detection of β globin gene mutation.

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REFERENCES
Major Beta-Globin Gene Mutations in Eastern India and Their Associated Haplotypes

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Abstract

324 alleles of the β-globin gene from unrelated thalassaemia patients native to the eastern region of India (mainly from the state of West Bengal) were analysed for β-globin gene mutations by the amplification refractory mutation system (ARMS). The major mutations that were detected are IVS-1 pos 5 (G-C), codon 26 (G-A) and codon 30 (G-C) with frequencies of 0.45, 0.33 and 0.05, respectively. Haplotype analysis revealed a very strong linkage disequilibrium of IVS-1 pos 5 (G-C) with one particular haplotype. HbE was found to be associated with two major haplotypes. Codon 30 (G-C) was associated with a haplotype that is the same as that found in the African population. Haplotype associated with codon 8/9 (+G) was the same as that found in northwest India. These findings have implications for the use of molecular diagnosis for genetic counselling and prenatal diagnosis of β-thalassaemia in this region.

Introduction

In India, β-thalassaemia is the most common monogenic disorder. The average incidence of β-thalassaemia trait in India is 3.3% with 1–2 per 1,000 couples being at risk of having an affected offspring each year. The disease is characterised by its genetic heterogeneity at the molecular level, and more than 300 mutations of the β-globin gene have been characterised all over the world, though each population seems to harbour only a few of these mutations. As the ethnic composition of the Indian population is varied and complex [1], each region of the country has its own distinct set of mutations [2,3]. The eastern region of the country is very poorly characterised in this regard. The Bengalee population, the subject of our study, is an admixture of indigenous people with later invaders who came here from time to time and left their genetic imprints. Here the haemoglobin variant HbE is very common along with β-thalassaemia. HbE in conjunction with β-thalassaemia produces HbE-β-thalassaemia, a more common form of the disease in this region of the country. In order to characterise the molecular basis of both β-thalassaemia and HbE-β-thalassaemia within the population of eastern India, we have studied 324 alleles from unrelated patients attending the Haematology Department of the School of Tropical Medicine, Calcutta. Screening was
one using the polymerase chain reaction (PCR)-based method of the amplification refractory mutation system (ARMS) for the five β-thalassaemia mutations, namely IVS-1 pos 5 (G-C), codon 30 (G-C), codon 15 (G-A), codon 8/9 (+G) and a 619-bp deletion from the 3' end of the β-globin gene. HbE alleles were screened for the mutation codon 26 (G-A). The spectrum of the mutation pattern revealed from such a study should provide the basis for prenatal diagnosis and genetic counselling of affected individuals.

Subjects and Methods

The population sample consisted of 170 unrelated patients from the Haematology Department of the School of Tropical Medicine, Kolkata. The diagnosis of β-thalassaemia was established by clinical, hematological and biochemical examination at the School of Tropical Medicine. Of the total number of individuals studied, 141 were from the Hindu community of which 8 were Brahmins and 23 were from the Muslim community from the State of West Bengal. There were 3 individuals each from the adjacent states of Bihar and Orissa. The total number could be categorised as 49 homozygous β-thalassaemia, 107 HbE-β-thalassaemia and 2 HbS-β-thalassaemia cases. Ten individuals were carriers of the β-thalassaemia allele. Two cases of homozygous HbE were also studied. For haplotype analyses, DNA samples from the parents of the patients were also studied.

**DNA Isolation**

DNA isolation from the buffy coat cells of EDTA-anticoagulated blood samples was done as described [5].

**Detection of Mutations**

For the detection of the β-thalassaemia mutations, the PCR-based technique of the ARMS was used as described [2]. Five mutations, namely IVS-1 pos 5 (G-C), codon 30 (G-C), codon 8/9 (+G), codon 15 (G-A) and a 619-bp deletion from the 3' end of the β-globin gene were screened for. Detection of the HbE alleles by ARMS [6] was performed as described (fig. 1).

**Haplotype Analysis**

The PCR-based technique was used [7] (fig. 2). The six restriction sites on the β-globin gene cluster, namely Hind II e, Hind III GY, Hind III AY, Hind II 5' yf, Hind II 3' yf, Hind I 3' y were studied. Primers and PCR conditions were used as described [8]. Thirty-nine alleles of IVS-1 pos 3 (G-C), 44 alleles of HbE, 2 of codon 8/9 (+G) and 1 of codon 30 (G-C) were analysed for determining the haplotypes linked to the respective mutations. It was not possible to assign haplotypes in all cases due to heterozygosity for a particular RFLP and non-availability of family members.

All the primers used were procured from Life Technologies, USA.

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DNA isolation from the buffy coat cells of EDTA-anticoagulated blood samples was done as described [5].

**Detection of Mutations**

For the detection of the β-thalassaemia mutations, the PCR-based technique of the ARMS was used as described [2]. Five mutations, namely IVS-1 pos 5 (G-C), codon 30 (G-C), codon 8/9 (+G), codon 15 (G-A) and a 619-bp deletion from the 3' end of the β-globin gene were screened for. Detection of the HbE alleles by ARMS [6] was performed as described (fig. 1).

**Haplotype Analysis**

The PCR-based technique was used [7] (fig. 2). The six restriction sites on the β-globin gene cluster, namely Hind II e, Hind III GY, Hind III AY, Hind II 5' yf, Hind II 3' yf, Hind I 3' y were studied. Primers and PCR conditions were used as described [8]. Thirty-nine alleles of IVS-1 pos 3 (G-C), 44 alleles of HbE, 2 of codon 8/9 (+G) and 1 of codon 30 (G-C) were analysed for determining the haplotypes linked to the respective mutations. It was not possible to assign haplotypes in all cases due to heterozygosity for a particular RFLP and non-availability of family members.

All the primers used were procured from Life Technologies, USA.
Table 1. Frequency of HbE β-thalassaemia mutations in the eastern Indian population

<table>
<thead>
<tr>
<th>Mutation Description</th>
<th>Number of Alleles</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 IVS-1 pos 5 (G-C)</td>
<td>149</td>
<td>0.4598</td>
</tr>
<tr>
<td>2 Codon 26 (G-A)</td>
<td>107</td>
<td>0.3302</td>
</tr>
<tr>
<td>3 Codon 30 (G-C)</td>
<td>17</td>
<td>0.0524</td>
</tr>
<tr>
<td>4 Codon 8/9 (+G)</td>
<td>3</td>
<td>0.0092</td>
</tr>
<tr>
<td>5 Codon 15 (G-A)</td>
<td>3</td>
<td>0.0092</td>
</tr>
<tr>
<td>6 619-bp deletion</td>
<td>3</td>
<td>0.0092</td>
</tr>
<tr>
<td>7 Uncharacterised</td>
<td>42</td>
<td>0.1296</td>
</tr>
<tr>
<td>Total</td>
<td>324</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2. Haplotypes associated with the various β-globin gene mutations

<table>
<thead>
<tr>
<th>Mutations Description</th>
<th>Number of Alleles</th>
<th>Haplotypes</th>
<th>Linkage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS1 pos 5 (G-C)</td>
<td>33</td>
<td>+--------+</td>
<td>86</td>
</tr>
<tr>
<td>IVS1 pos 5 (G-C)</td>
<td>1</td>
<td>+--------+</td>
<td>-</td>
</tr>
<tr>
<td>IVS1 pos 5 (G-C)</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbE-codon 26 (G-A)</td>
<td>31</td>
<td>+--------+</td>
<td>65</td>
</tr>
<tr>
<td>HbE-codon 26 (G-A)</td>
<td>8</td>
<td>-+------</td>
<td>23</td>
</tr>
<tr>
<td>HbE-codon 26 (G-A)</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Codon 30 (G-C)</td>
<td>1</td>
<td>+--------+</td>
<td>-</td>
</tr>
<tr>
<td>Codon 8/9 (+G)</td>
<td>2</td>
<td>+--------+</td>
<td>-</td>
</tr>
</tbody>
</table>

The polymorphic sites are in the following order: Hind II e, Hind III Gy, Hind III Ay, Hind II-5' vp, Hind II-3' vp and Hind I-p.

Results

Mutation Analysis

A total of 324 unrelated alleles were examined by ARMS, of which 107 were from HbE. Of the 217 β-thalassaemia alleles, 149 had the IVS-1 pos 5 (G-C) mutation, making it the most frequently occurring mutation in this region of India. Of these, 71 were HbE-β-thalassaemia and 24 homozygous β-thalassaemia cases. There were 17 alleles carrying codon 30 (G-C) mutation of which 4 were homozygotes. There were 3 cases each of codon 8/9 (+G), codon 15 (G-A) and a 619-bp deletion. All codon 15 (G-A) mutations were found in cases of HbE-β-thalassaemia. Out of the 3 alleles of codon 8/9 (+G) and a 619-bp deletion, 2 were found in homozygous β-thalassaemia cases. Forty-two cases remain uncharacterised. The frequency of the different mutations is listed in table 1.

Haplotype Analysis

A total of 2 β-globin haplotypes were identified among 44 HbE chromosomes. With the exception of 5 chromosomes to which no definite haplotype could be assigned, 31 had the haplotype +-------- (Hind II e, Hind III Gy, Hind III Ay, Hind II-5' vp, Hind II-3' vp, Hind I-3' β) and 8 had the haplotype -+-----.

Among the IVS-1 pos 5 (G-C) chromosomes no definite haplotype could be ascertained for 5 chromosomes. The rest of the IVS-1 pos 5 (G-C) alleles were linked to the +-------- haplotype with the exception in one case where the haplotype was +--------. The 2 codon 8/9 (+G) alleles studied were of the +-------- haplotype. The codon 30 (G-C) allele revealed a -+----- haplotype. The mutations associated with their respective haplotypes are listed in table 2.

Discussion

We have determined the various β-thalassaemia mutations in 324 alleles and linked some of the mutations to their respective haplotypes from the population sample of eastern India. This is the first report of linkage of RFLP markers with the globin gene mutations in this region of India.

From our study, the frequency of the HbE allele is 0.33. The incidence of HbE amongst the population studied is quite high and goes well with the data of previous workers who have also reported the high prevalence of HbE amongst the population of other regions of eastern India [9,10]. This mutation, apart from producing a variant haemoglobin, also produces a cryptic splice site so that the level of β-globin mRNA production is reduced and the gene behaves like a mild β-thalassaemia gene [11]. Among the β-thalassaemia mutations, IVS-1 pos 5 (G-C) is the most common mutation occurring at a frequency 0.45. This happens to be the most frequent mutation in many other parts of India as well, i.e. Punjab, Gujerat, Maharashtra and southern India [2, 8].

The mutations codon 15 (G-A), codon 8/9 (+G) and a 619 bp deletion occurred at low frequencies. These mutations are endemic in the northwestern regions of India and Pakistan and have probably infiltrated into the eastern region from there by the Muslim invaders. Codon 8/9 (+G) mutations were found only in the Muslim population in our study.
The only mutation other than IVS-1 pos 5 (G-C) present at an appreciable frequency is codon 30 (G-C) which was identified in 17 alleles. This mutation has been reported by Varawalla et al. [2] in the populations of Gujarat, Punjab and Sindh. It was originally reported to occur among Tunisians [12].

Two haplotypes could be identified with 39 HbE alleles studied (5 alleles could not be characterised). ___-___-___ was revealed to be the major haplotype and represented 31 HbE alleles (70%). The +---___ haplotype was present in 8 cases (18%). These 2 haplotypes have been previously been reported to be associated with the 6bE alleles, of the population of Laos, Thailand and Cambodia [13]. However, the ++-+++ haplotype, the major haplotype in the population studied, is the less prevalent haplotype there and the +---___- haplotype is more frequent. Historical facts reveal that there has been interaction between these two populations. However, isolated pockets of the Tibeto-Burman-speaking Bodo ethnic group residing in Assam [9] and Deshi population of the Ialsa district of West Bengal [10] show a high incidence of HbE. Keeping the ancestry of the above populations in mind, the origin of HbE in the eastern region of India remains an open question. It is very interesting to note that the HBE allele has spread in Southeast Asia to the eastern region of India and cannot be traced any further westwards on the continent.

The IVS-1 pos 5 (G-C) mutation was found to be strongly linked to the +---___ haplotype (82%). This linkage has also been reported for pockets of the Tibeto-Burman-speaking Bodo ethnic group residing in Assam [9] and Deshi population of the Ialsa district of West Bengal [10] show a high incidence of HbE.

References