Prenatal Diagnosis of HbE- β -Thalassemia: Experience of a Center in Western India

Roshan Colah¹ · Anita Nadkarni¹ · Ajit Gorakshakar¹ · Pratibha Sawant¹ · Khushnooma Italia¹ · Dipti Upadhye¹ · Harshali Gaikwad¹ · Kanjaksha Ghosh²

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Abstract The clinical presentation of HbE- β -thalassemia is extremely variable, however, many cases are severe and transfusion dependent. We offered prenatal diagnosis to 108 couples, 20 of whom came prospectively. CVS was done in 93 cases (9.5–13 weeks of gestation) while amniocentesis/cordocentesis was done for 15 cases in the second trimester. Diagnosis was done by reverse dot blot hybridization, ARMS, DNA sequencing and in a few cases by HPLC analysis of fetal blood. The genetic combinations in the couples at-risk were the following: HbE trait/ β -thal trait-95, HbE-thal/HbE trait-5, HbE homozygous/ β -thal trait-3, HbE-thal/ β -thal trait-3, HbE Lepore/ β -thal trait-1, HbE trait/HbD^{Punjab} trait-1. IVS1-5(G>C) was the

commonest β -thalassemia mutation followed by codon15(G>A), codon30(G>C), codons41/42(-CTTT), the 619 bp deletion and codon8/9(+G) in the β -thalassemic parent. However, several rare mutations seen in India like -90(C>T), -88(C>T), codon15(-T), IVS1-129(A>C), IVS1-130(G>C), IVSII-1(G>A), IVSII-837(C>T) and IVSII 848(C>A) were also encountered. Twenty-one fetuses were affected (HbE- β -thal-20, β -thal major-1) and all the couples opted for termination of the pregnancies. Couples with affected children wish to undergo prenatal testing for HbE- β -thal in subsequent pregnancies. More regional centers are needed for these services, particularly in West Bengal and the North-East where HbE is very common.

✉ Roshan Colah
colahrb@gmail.com

Anita Nadkarni
anitahnadkarni@yahoo.com

Ajit Gorakshakar
ajit5678@yahoo.com

Pratibha Sawant
pratibhamsawant@rediffmail.com

Khushnooma Italia
kyitalia4@gmail.com

Dipti Upadhye
dipti.upadhye@gmail.com

Harshali Gaikwad
harshali.gaikwad121@gmail.com

Kanjaksha Ghosh
kanjakshaghosh@yahoo.com

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Introduction

HbE- β -thalassemia is a common and clinically diverse condition seen most commonly in Southeast Asia, especially in Thailand, Cambodia, Laos and China as well as in South Asia in Sri Lanka, Bangladesh and parts of India [1–3]. HbE- β -thalassemia results from co-inheritance of a β -thalassemia allele from one parent and the structural variant Hemoglobin E which is also synthesized at a slightly reduced rate from the other. The disease has a very variable clinical presentation from being mildly symptomatic in around 15–20% of cases to resulting in severe anemia and a transfusion dependent disorder in others [4, 5].

The management of HbE- β -thalassemia patients depends on their ability to adapt to steady-state hemoglobin concentrations with a check on adequate physical growth

¹ National Institute of Immunohaematology, 13th Floor, N. M. S. Bldg, KEM Hospital Campus, Parel, Mumbai 400012, India

² Surat Raktadan Kendra, Surat, India

and absence of symptoms of anemia or an increase in spleen size. For those who are severely anemic and cannot maintain a steady state hemoglobin level, a regular blood transfusion regimen is set along with adequate iron chelation therapy [6, 7]. In India, the annual cost of management of these patients is high and estimated to be around US\$ 2400/- to 3500/- due to which very few patients receive optimal management while many of them die early as a consequence of severe anemia, unavailability of sufficient safe blood for transfusion and iron overload [8, 9].

The carrier frequencies of HbE are very high in some population groups particularly in the north-eastern states (3 to >50%) and in West Bengal in the east (3–4%) while the overall prevalence of β -thalassemia carriers in the country is 3–4% [10]. Thus, there are a large number of patients with severe HbE- β -thalassemia in India [11]. Control programs have been undertaken to reduce the burden of the disease by population screening, genetic counseling and prenatal diagnosis. We describe our experience with prenatal diagnosis for HbE- β -thalassemia in western India.

Materials and Methods

Couples at risk of having a HbE- β -thalassemia child were referred to us for prenatal diagnosis while a few couples who had a previous child affected with HbE- β -thalassemia approached us directly. Depending on the gestational age of the fetus, chorionic villus sampling (10.5–13 weeks), amniocentesis (15–16 weeks) or cordocentesis (18–19 weeks) was planned. The diagnosis was done by DNA analysis in majority of the cases while fetal blood analysis was done in a few couples who came late. A total of 108 couples at-risk underwent prenatal diagnosis. After an informed consent, 5 ml blood of the couple and the affected child if available was collected in EDTA vacutainers for red cell indices, hemoglobin electrophoresis by HPLC on the Variant Hemoglobin Testing System (Bio Rad Laboratories, Hercules, USA) and DNA analysis. After confirming the genotypes of the parents, chorionic villus sampling was done in 93 pregnant women between 10.5 and 13 weeks of gestation. The villus samples were cleaned under a dissecting microscope and the DNA was isolated. Characterization of the β -thalassemia mutations and confirmation of the HbE mutation were done by reverse dot blot hybridization (RDB) [12] and amplification refractory mutation system (ARMS) [13]. The uncharacterized mutations were identified by automated DNA sequencing on the ABI prism 310 sequencer (Applied Biosystems, Foster City, CA, USA). Confirmation of the HbD^{Punjab} genotypes was done by PCR and restriction enzyme digestion using Eco RI [13]. DNA was processed for Hb Lepore genotypes by polymerase chain reaction

(PCR) [14] and then sequenced using the automated ABI Prism 310 sequencer. Amniocentesis was done for 5 women. Thirty to 35 ml of amniotic fluid was aspirated and the cells spun down and used for DNA analysis. Maternal contamination in the fetal DNA was ruled out by variable number of tandem repeat (VNTR) analysis.

Fetal blood sampling by cordocentesis was done for 10 women who came at around 18 weeks of gestation. The fetal blood sample was checked for maternal contamination by fetal cell staining using the Kleihauer-Betke slide method or by flow cytometry [15] after which the blood was analysed by HPLC on the Variant Hemoglobin Testing System [16]. After prenatal diagnosis, the continuing pregnancies were followed up and the infants were tested at birth or later.

Results

Prenatal diagnosis was done for couples at-risk of having a child with HbE- β -thalassemia, β -thalassemia major, HbLepore- β -thalassemia and HbD^{Punjab}E disease.

The origins of the couples coming for prenatal diagnosis from different states of India are shown in Table 1. Majority of them were from West Bengal but few belonged to other states while 8 couples came from Bangladesh. Most of the couples were Hindus—78 but there were also 30 Muslim couples. The 8 couples who came from Bangladesh were all Muslims. These 108 couples referred for prenatal diagnosis had different genetic combinations and based on this they were offered the option of prenatal diagnosis after counselling them. The different genetic combinations in these couples at-risk are given in Table 2. Couples where both the partners had HbE trait were advised against prenatal diagnosis as they were at-risk of having a HbE homozygous child, a condition which is very

Table 1 Distribution of the couples at-risk

	Number
Couples from different states	
West Bengal	79
Maharashtra	7
Orissa	3
Bihar	3
Gujarat	3
Uttar Pradesh	2
Assam	1
Karnataka	1
Goa	1
Other countries	
Bangladesh	8

Table 2 Genetic combinations in the couples at-risk

Genetic combinations in couples at-risk	Number of couples
HbE trait/ β thalassemia trait	95
HbE trait/HbE- β thalassemia	5
HbE homozygous/ β thalassemia trait	3
HbE- β thalassemia/ β thalassemia trait	3
HbELepore/ β thalassemia trait	1
HbE trait/HbD trait	1

Table 3 β -thalassemia mutations in the 98 thalassemic parents where molecular diagnosis was done

Mutations	Number of individuals
IVS 1-5(G>C)	65
Codon 15(G>A)	7
Codon 30(G>C)	6
619 bp deletion	4
Codons 41/42(–CTTT)	4
Codon 8/9(+G)	2
–90(C>T)	1
–88(C>T)	1
codon 15(–T)	1
IVS 1-129(A>C)	1
IVS 1-130(G>C)	1
IVS II-1(G>A)	1
IVS II-837(T>C)	2
IVS II-848(C>A)	2

mild. Hence those couples were not included in this study. Of the 108 couples at-risk, 20 couples came prospectively in the first pregnancy whereas 6 couples came twice for prenatal diagnosis and were included twice in the total count. There was one couple with a triplet pregnancy.

Most of the β -thalassemia mutations and the HbE mutation were characterised by RDB hybridization. The β -thalassemia mutation, IVS 1-5 (G>C) was the most common mutation found in 65 individuals. The other β -thalassemia mutations found in the 98 couples at risk where prenatal diagnosis was done by molecular analysis are given in Table 3. In the 10 couples who came after 18 weeks gestation, HPLC analysis of the fetal blood samples was done.

Of the 110 fetuses tested which included one triplet pregnancy, 30 were found to be normal. Thirty-four fetuses were heterozygous for HbE, 24 heterozygous for β -thalassemia, 1 heterozygous for Hb Lepore and 1 fetus was heterozygous for HbD. There were 20 fetuses who were compound heterozygous for HbE- β -thalassemia and 1 fetus was homozygous for β -thalassemia (Table 4). All the 21 couples who had affected fetuses opted for termination of

Table 4 - Diagnosis of the fetuses

Diagnosis	Number of fetuses
Normal	30
HbE trait	34
β thalassemia trait	24
Hb Lepore trait	1
HbD Punjab trait	1
HbE- β thalassemia	20
B thalassemia major	1

these pregnancies. In the couple with the triplet pregnancy 2 of the fetuses were normal and 1 had HbE trait. The results of prenatal testing in the couples who came twice are illustrated in Table 5. In 3 of the 6 couples (Nos 1–3) who came twice for prenatal diagnosis, the first time their fetuses were affected with HbE- β thalassemia and they had opted for termination of these pregnancies. One Muslim family already had one normal child and the second child had β -thalassemia trait after the first prenatal diagnosis but they decided to have a third child and came back for prenatal testing (No. 4). Another couple also had a child with β -thalassemia trait after their first prenatal diagnosis but came again soon in the next pregnancy (No 5). The last couple who came twice from Bihar already had 2 children with HbE- β -thalassemia. During the first prenatal diagnosis the fetus was diagnosed to be normal but she unfortunately had an abortion and thus came back in the subsequent pregnancy (No 6).

Discussion

HbE- β -thalassemia is not uncommon in India. Recent studies from West Bengal have shown that the prevalence of β -thalassemia carriers varies from 3.7 to 10.3% and Hb E carriers from 3.02 to 4.0% [10, 17, 18]. In the north eastern region, there is an extremely high prevalence of HbE being 30 to >50% in some tribal groups while the frequency of β -thalassemia carriers varies from 1.5 to 3.48% [3, 10]. In both these regions around 1% of anemic individuals referred for hemoglobin analysis by HPLC had HbE- β -thalassemia.

Around 15% of HbE- β -thalassemia patients in Southeast Asia are clinically mild and do not require regular blood transfusions [4]. However, it is difficult to predict whether the affected fetus will have a mild or severe clinical presentation as there are no genetic markers for the same. This results in a dilemma while offering genetic counselling to couples at-risk. Our recent study on HbE- β -thalassemia patients showed that 37.2% of patients had a mild phenotype [19] which was higher than that reported in Southeast

Table 5 Outcome of prenatal diagnosis in the 6 couples who came twice for prenatal testing

Serial no.	Genetic combinations in the couples	Previous children	1st Prenatal diagnosis	2nd prenatal diagnosis
1	HbE trait/ β thalassemia trait	HbE- β thalassemia-1	HbE- β thalassemia	HbE trait
2	HbE homozygous/ β thalassemia trait	Nil	HbE- β thalassemia	HbE- β thalassemia
3	HbE trait/ β thalassemia trait	HbE- β thalassemia-1	HbE- β thalassemia	Normal
4	HbE trait/ β thalassemia trait	Normal-1	β thalassemia trait	β thalassemia trait
5	HbE trait/ β thalassemia trait	Nil	β thalassemia trait	HbE trait
6	HbE trait/ β thalassemia trait	HbE- β thalassemia-2	Normal	Normal

Asia. Also, there are many severe patients who do not take regular blood transfusions due to various social and economic reasons and commonly present with severe ineffective erythropoiesis, peripheral hemolysis, iron overload and hypercoagulability leading to organ damage particularly in low and middle income countries including India [20]. Chakrabarti et al. [9] showed that in West Bengal, patients who received optimum management through unrestricted funding from corporate houses as a part of corporate social responsibility had a significantly better quality of life than those in routine care. However, this is possible only for around 15% of patients and majority of patients with β -thalassemia major or HbE- β -thalassemia are poorly transfused and not chelated.

Recent reports have shown that HbE- β -thalassemia is also seen in other states of the country in different ethnic groups both due to migration of people in search of a better livelihood and due to inter-marriages between communities which was not common earlier. Thus, certain population groups in West Bengal, Maharashtra, Uttar Pradesh, Bihar, Chhattisgarh, Jharkhand and Orissa have an overlapping distribution of both the β -thalassemia gene and the HbE gene [3, 19, 21] resulting in a high number of HbE- β -thalassemia cases.

As the HbE- β -thalassemia patients show variable clinical severity, many milder cases reach adulthood and knowingly or unknowingly get married to another β -thalassemia trait or HbE trait individual. Such couples are at a higher risk of having a child with β -thalassemia major, HbE- β -thalassemia or HbE disease in every pregnancy. Individuals with HbE disease present with mild clinical symptoms and hence couples where both the partners have HbE trait are not advised to undergo prenatal diagnosis [22]. We had 5 couples where one of the partners had HbE trait and the other had HbE- β -thalassemia but none of their fetuses had HbE disease.

In this study the couples at-risk were individually counselled about the possibility of having an affected child depending on their genotypes and the life-long consequences of the disease. This helped all the 21 couples where prenatal diagnosis indicated that the fetus would be affected to decide on the option of termination of these

pregnancies. Fortunately, all the 3 fetus in the triplet pregnancy were unaffected (normal-2, β thalassemia trait-1). Such pregnancies can cause difficulties if selective termination has to be done for one or two of the fetuses. Many couples who already had a child with HbE- β -thalassemia in the family wished to undergo prenatal diagnosis in the subsequent pregnancies to avoid this from recurring.

Heterozygous carriers are generally asymptomatic and it is necessary to confirm their genotypes, particularly those with borderline HbA₂ levels before offering prenatal diagnosis. One of the couples in our study had HbE/HbLepore in one parent and β -thalassemia trait in another parent. They were at-risk of having a HbE- β -thalassemia or a HbLepore- β -thalassemia child. With both these combinations, the child can present with a clinically severe form of transfusion dependent disease. Co-inheritance of HbE with HbLepore leads to a mild disorder but often gets misdiagnosed as HbE- β -thalassemia instead of HbE/HbLepore. In such cases, accurate DNA analysis of the couple and the affected child if available along with an extended family study should be undertaken before the interventional procedure for fetal sampling. Further, the results of DNA analysis should be correlated with the clinical, hematological and biochemical data as relying only on the findings of HPLC may at times lead to a misdiagnosis [23]. There have been eleven cases with HbE/HbLepore reported so far, three cases from a family in Bangladesh, two cases in Thailand, two cases in Spain and three cases from India [23–28].

One of our couples had HbE trait in one parent and HbD Punjab trait in the other. They were at-risk of having a child with HbD^{Punjab}E disease. However, the individuals with this genotype are usually mild [29] and prenatal diagnosis in such situations is discouraged. Nevertheless in this family prenatal diagnosis was done as the couple at-risk were insistent.

Prenatal diagnosis has been one of the best ways to reduce the burden of the disease in many Southeast Asian countries like Singapore, Cambodia and Thailand [30–32]. Of the many thalassemia control programs in Middle Eastern countries, the ones that significantly reduced the

birth of β -thalassemia children were in Iran, Bahrain, Iraqi Kurdistan and Turkey through prenatal diagnosis and therapeutic abortion [33]. Peng et al. 2013 reported a marked decline in the number of newborns with thalassemia major with the national program for screening pregnancies to control the spread of thalassemia in Taiwan [34]. In Thailand, in regions with a high frequency of both α and β -thalassemia, a combination of both hemoglobin analysis and DNA testing was done to confirm the carrier status as underdiagnoses of carriers hampered the effectiveness of the thalassemia prevention and control program [35].

In India, facilities for both first and second trimester prenatal diagnosis of hemoglobinopathies are available but there are only 12 to 15 centers in the country mainly in large metropolitan cities and there are very few obstetricians and sonologists who have adequate training for fetal sampling procedures [36, 37].

One of the major drawbacks is that pregnant women come late in antenatal clinics in public hospitals with only 15–20% registering in the 1st trimester due to which second trimester diagnosis also continues to be required [37–39]. However, when the diagnosis is done by HPLC analysis of fetal blood, the results have to be interpreted with caution if the parents have mild β^+ or β^{++} mutations (borderline HbA₂ or near normal indices) as there are chances of unusually higher HbA₀ levels in homozygous fetuses [40]. Such cases must be further confirmed by DNA analysis. At times, the father is not available during prenatal testing, and the affected child if available is investigated for mutation analysis. Other hurdles include non-cooperation of the husband for investigations and various social issues like stigmatization [37].

Each fetal tissue or fetal blood sample needs to be checked for maternal contamination for which VNTR or STR analysis is done using different highly polymorphic markers. None of our fetal samples had any maternal contamination. Even though strict quality control is followed during prenatal diagnosis, a small risk of fetal loss and misdiagnosis remains. We had one fetal loss following cordocentesis. There was no misdiagnosis in the babies who could be tested after birth to reconfirm the diagnosis. Prenatal diagnosis of HbE- β -thalassemia is important to reduce the burden of the disease and in a vast country like India many more centers would be needed so that couples would not need to travel long distances.

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Compliance with Ethical Standards

Conflicts of interest There is no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

Human and Animal Rights All procedures performed in this study involving human participants were in accordance with the ethical standards of the institution. and/or National Research Committee.

Informed Consent Informed consent was taken from all the couples who went for prenatal diagnosis.

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