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Prevention of β-Thalassemia Major and Eβ-Thalassemia by Prenatal Diagnosis in Eastern India

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ABSTRACT Prenatal diagnosis is one of the most effe ctive and direct approaches for prevention of β-thalassemia and other Hemoglobinopathies. In this paper prenatal diagnosis was offered to 21 couples 'at risk' from populations of Eastern India with a view to assess the possibility of establishing a comprehensive prenatal diagnosis service. The basis of this consideration was the high prevalence and heterogeneity of β-thalassemia and Hemoglobin E disease (HbE) in Eastern India with predominance of 7 common mutations for β-thalassemia and codon 26 for Hemoglobin E disease. It was also observed that the most common mutation for β-thalassemia was IVS1 nt5 (G->C) followed by codon 26 (G->A) for HbE disease and both these mutations were found to interact very often to produce Eβ-thalassemia. DNA analysis for parents and prenatal samples was carried out using *in vitro* amplification by polymerase chain reaction (PCR) based amplification refractory mutation system (ARMS) and restriction fragment length polymorphism (RFLP). Prenatal diagnosis was offered in the second trimester of pregnancy following amniocentesis. In this study 100% diagnosis could be done for prenatal samples using these above techniques. Analysis of prenatal samples showed 36.4% affected and 63.6% unaffected pregnancies. Only 13.6% unaffected pregnancies were found to be normal while 50% were identified as carriers. The couples having affected pregnancies were counselled to terminate the pregnancies. Postnatal follow up confirmed the unaffected pregnancies. Poor awareness about the prevalence and adverse effects of this disease in the population of Eastern India was also observed in this study as 20 (95.2%) out of 21 couples 'at risk' came for prenatal diagnosis only after they had one or more affected child.

INTRODUCTION

β-thalassemia is one of the commonest autosomal recessive genetic disease in the Indian population which poses a major public health problem. The estimated number of β-thalassemia carriers in India is about 29.7 million and the births of β-thalassemia patients are 7000 per year (Verma et al. 1997). In Eastern India the occurrence of β-thalassemia and HbE (Haemoglobin E disease) mutation is fairly high in the normal population (Ajmani et al. 1978; Roychowdhary et al. 1994; De et al. 1997a, b). In homozygous form it results in severe anemia that requires regular blood transfusions from infancy for survival. As it is inherited in an autosomal recessive manner, couples who are both heterozygous for β-thalassemia gene carry a 25% risk of having a child with β-thalassemia major (homozygous for β-thalassemia gene). The main aim of genetic counselling for thalassemia is to reduce the frequency of births of these thalassemia major patients in the population. Prenatal diagnosis is one of the most effective and direct approach for the couples "at risk." for prevention of this life threatening disease as there is no satisfactory treatment available for these affected patients. Affected pregnancies can be diagnosed by molecular methods and decision for termination of pregnancy can be made before it is too late

Over the last 20 years more than 200 different varieties of molecular defects causing β-thalassemia were characterized affecting RNA transcription, processing or translation of the β-globin gene which are distributed in different parts of the world (Huisman et al.1998). The occurrence of these point mutations was found population specific and some population appear to have a characteristic set and frequency of β-thalassemia mutations. In our previous study the common mutations of β-thalassemia in Eastern India population has already been described (De et al. 1997b; Das et al. 2000). DNA analysis of the Eastern India population showed that the common mutations were IVS 1 nt5 (G->C), Frameshift (FS) Codon 41/42 (-CTTT), Codon 30 (G->C), Codon 15 (G->A), FS Codon 8/9 (+G), 619

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base pair (bp) deletion for β - thalassemias codon 6 for Haemoglobin S disease and Codon 26 (G->A) for Haemoglobin E disease (Hb E). Of them the most predominant mutation IVS 1 nt5 (G->C) was again often found to interact with Codon 26 to produce Eβ-thalassemia. This is an essential prerequisite to determine the feasibility of early prenatal diagnosis for β-thalassemia and haemoglobin E disease. (De et al. 1997b; Kazazian and Bohem 1988; Ng et al. 1994; Varawalla et al.1991)

In 1980 after the first DNA polymorphisms had been detected in the β-globin gene cluster, prenatal diagnosis of β-thalassemia were carried out by using linked markers. Diagnosis by this method was indirect and has a theoretical error rate of about one in 300 to 500 due to meiotic recombination between a site used in tracking the β-thalassemia mutation and the mutation itself (Kazazian and Bohem 1988). At present almost 100% of the mutations can be detected using PCR-ARMS if supplemented by other methods such as Slot blot hybridization using allele specific oligonucleotide probes (ASO) (Ng et al. 1994), Reverse Dot Blot or RDB (Cai et al.1994) and Restriction Fragment length Polymorphism or RFLP (Varawalla et al.1992). Since DNA analysis did not depend on the gene expression, it could be performed by using DNA from any nucleated cell regardless of whether that cell synthesizes globin or not. Therefore prenatal diagnosis can be done successfully using amniotic fluid cells obtained at 15 –17 weeks of gestation.

MATERIALS AND METHODS

A total of 21 couples were offered prenatal diagnosis from amniotic fluid cell DNA. Of them, 19 couples who were diagnosed as carriers (each carrying one mutated allele) were thus at a 25% risk of having a child with β-thalassemia major. Among the 2 other families, mother of one family (family 2) was diagnosed with Eβ-thalassemia and in the other family the father was found with homozygous E state (family 10). So for these two couples the chances of having a thalassemic baby was 50%. Each couple received counselling on the risks of having a child with β-thalassemia major and Eβ-thalassemia and risk of the procedure of amniocentesis used for prenatal sampling. On the whole 22 pregnancies were tested as one family (family 14) came twice for prenatal diagnosis. (Table1)

For preliminary diagnosis i) estimation of haemoglobin and screening of red cell indices with an automated cell counter (Sysmex K1000, Japan), ii) hemoglobin (Hb) electrophoresis at pH 8.6 for detection of hemoglobin variants (Chandra et al. 1987) iii) fetal Hb estimation by alkali denaturation (Dacie and Lewis 1994), modified after Betke et al 1959 (Betke et al. 1959) and iv) HbA_2 estimation by gel elution technique (Adhikary et al. 1987) were carried out in all cases before prenatal samples were obtained. Thus the couples at risk of having a thalassemic baby were identified. The genetic mutations for all of them were also determined beforehand for each couple. The couples with an affected child, he or she was also considered for study especially when the mutations for the parents of that particular family could not be characterized. DNA was prepared from whole blood samples collected in the EDTA (ethylenediaminetetra-acetic acid) using Qiagen blood extraction mini kit (Cat No 51104). 15-20 ml of amniotic fluid was subjected to centrifugation and the cells were collected in 200-400µl of amniotic fluid while the rest of the amniotic fluid was discarded. DNA was extracted using the same kit following the same procedure instructed for whole blood.

Genotype Analysis: All samples were subjected to in vitro amplification by polymerase chain reaction (PCR) based amplification refractory mutation system (ARMS) technique using genomic DNA (Newton et al. 1989). Screening for the presence of 8 common β-thalassemia mutations of this region (De et al. 1997a) was carried out. These were: IVS1nt5 (G->C), frameshift (FS) Codon 41-42 (-CTTT), Codon 30 (G->C), Codon 15 (G->A), FS Codon 8/9 (+G), 619 bp deletion, HbS mutation at Codon 6 and Hb E mutation at Codon 26 (G->A). The mutations could be identified in 39 of the 42 individuals in 21 couples. The individuals whose β-mutations remained uncharacterised, their DNA was subjected for identification of three other mutations, IVS 1 nt1 (G->T), cap+1 (A->C) and –88 (C->T) which were reported to be present in Indian populations (Weatherall et al. 1995). When these mutations were found absent, genomic DNA was subjected to identify the polymorphic sites on the β-globin gene using PCR technique followed by restriction enzyme digestion (RFLP) (Old et al.1990; Varawalla et al. 1992; Thakur et al. 2000). For each polymorphic enzyme site, the

corresponding two primers were used in the PCR reaction. 5 different sites were tried whenever necessary. These were HindIII Aγ, HindIII Gγ, HincII 3'Ψβ, HincII 5'Ψβ and HinfI5'β. The digested products were visualized on a 3% agarose gel. The presence and absence of each site were identified as +/+ and -/- respectively. Genotype analysis of individuals was carried out from PCR-ARMS results for known mutations. For those with unspecified mutations, DNA samples were subjected to PCR followed by restriction enzyme digestion of the PCR product. Restriction Fragment length Polymorphism (RFLP) analysis was also used for confirmation of detection of mutations by ARMS technique. The affected pregnancies diagnosed with homozygous state of IVS 1 nt5 (G->C) were confirmed in a separate PCR-ARMS using normal primer instead of the mutant primer for this particular mutation. The genotypes of all the individuals were correlated with their respective Hb electrophoresis pattern and hematological phenotypes.

RESULTS

A total of 21 couples who were at a risk for a child with β-thalassemia major or Eβ-thalassemia, opted for prenatal testing. The age group for father and mother ranged from 26 to 46 years and 20 to 38 years respectively. Blood samples for all the individuals were subjected to Hb electrophoresis to identify whether they were β-thalassemia carriers (minor) or HbE heterozygote (carriers).Table 1 show details of the haematological parameters, DNA analysis results and other relevant information about these 21 families. In some patients (offspring of some couples) the levels of HbF, HbA_2 or HbE were found to be lower than expected due to repeated blood transfusions. In one family (family 2), the mother was found to be with Eβ-thalassemia and in another family the father was with homozygous Hb-E disease (family 10) identified by Hb electrophoresis and then confirmed by DNA analysis. As expected, the most common mutation was IVS 1 nt5 (G->C) followed by Codon 26. (See Table 2) In 42 individuals (21 couples), 25 alleles (56.8%) were identified with IVS 1nt5 (G->C), 13 (29.6%) were with Codon 26 (G->A), 2 (4.5%) were with Codon 8/9 (+G) mutations, 1 with 619 bp deletion (2.3%) and 3 (6.8%) were with unspecified mutation. In 2 individuals who were thalassemia patients, one was compound heterozygous for Codon 26 (G->A) / 619bp del (in mother of family 2) and the other person was found homozygous for codon26 (G->A) mutation (in father of family 10). Direct identification method using PCR-ARMS resulted in an identification of 93.2% alleles while, the rest 6.8% were diagnosed through an indirect approach using RFLP method. A success rate of 100% was achieved for prenatal diagnosis, when the direct method was backed up by RFLP analysis.

Most of the couples who came for prenatal testing opted for it at mother's 2nd pregnancies (63.6%). Only one couple where the mother was educated and was aware that she was an Eβ thalassemia patient came for prenatal testing for their 1st baby. All others came after they had either at least one affected child or had experienced repeated abortions or deaths of children at a very young age. One family (family 14) came twice for prenatal testing ie. for both their 6th and the 7th pregnancies (Table 1).

Analysis of the prenatal samples resulted in the identification of 8 (36.4%) affected pregnancies of which 5 cases were with β-thalassemia in homozygous state (β-thalassemia major) and 3 cases were with Eβ-thalassemia. 6 pregnancies were found with β-thalassemia carriers, 5 other pregnancies were with HbE heterozygote (total carriers 50%) and 3 (13.6%) cases were found to be normal pregnancies indicating a total of 14 (63.6%) unaffected pregnancies. (Table 4) One prenatal sample of a couple (family 21) in whom mother's mutation was unknown, showed absence of father's mutation (HbE) and was therefore predicted to have either β-thalassemia minor or to be normal. The absence of HbE mutation for this foetus was again confirmed indirectly by identifying the polymorphic sites of DNA in mother, father, one affected child and amniotic fluid cell and studying their linkage analysis for transmission of mutation.(Table 5) It was not possible for us to diagnose whether the foetus was β-thalassemia minor or normal for this pregnancy since we could not study another polymorphic restriction site (HincII 5'Ψ B) due to insufficient specimen. β-carrier status was also confirmed in case of family 14 for their 2nd pregnancy which was diagnosed with IVS 1nt5 (G->C) mutation.The two other families (family 6 and family 16) where one of the parents was with unspecified mutation were subjected to RFLP analysis using at least 2

Table 1: Haematological picture and DNA analysis results in different members of 21 families

Family No.		*Hb g%*	*MCV fl*	*MCH pg*	*HbF %*	*HbA_2 %*	*HbE %*	*DNA Analysis (Type of Mutation & status of both alleles)*	*Diagnosis and Remarks*
1	Father (46)	11.7	70.4	22.1	0.8	5.0		F/S Codon 8/9(+G) / N	β-Thalassemia Carrier
	Mother (38)	7.1	84.5	23.4	0.6		19.6	Codon 26(G->A) / N	HbE Carrier
	Ist Child (12M)							F/S Codon 8/9 / Codon 26(G->A)	Transfusion dependent (300unit) Eβ-Thalassemia
	2nd pregnancy Amniotic fluid cells							Codon 26(G->A) / N	**HbE Carrier (Foetus not affected)**
	Cord blood collected during delivery							Codon 26(G->A) / N	**HbE Carrier**
2	Father (31)	12.2	65.4	20.2	0.6	4.4		IVS 1-5 (G->C) / N	β-Thalassemia Carrier
	Mother (28)	6.1	67.8	18.7	18.6		46.8	619 bp del/Codon 26 619bp del / N	Eβ- Thalassemia, 5 units of blood transfussion
	1st pregnancy Amniotic fluid cells								**β-Thalassemia Carrier (Foetus not affected)**
	Father (35)	13.0	68.4	20.9	0.4	4.5		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (22)	8.7	64.3	19.3	0.6	4.3		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (3M) 4 MTP done								Died of thalassemia
	6th pregnancy Amniotic fluid cells							IVS 1-5 (G ->C) / IVS 1-5 (G ->C)	**Homozygous β-Thal (Foetus affected)**
4	Father (38)	12.3	72.5	22.3	0.6	5.8		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (35)	11	77.3	25.0	0.7		21.0	Codon 26(G->A) / N	HbE Carrier
	Ist child (3y 7m M)	6.9	63.8	17.8	34		38.1	IVS 1-5 (G -> C) / Codon 26 (G->A)	Eβ-Thalassemia
	2nd pregnancy Amniotic fluid cells							Codon 26(G->A) / IVS 1-5 (G ->C)	**Eβ-Thalassemia (Foetus affected)**
5	Father (34)	13.6	95.3		0.6		22.6	Codon 26(G->A) / N	HbE Carrier
	Mother (29)	9.4	82.5		0.4	4.2		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (3y8mF)	5.1	81.1		30		47.4		Eβ- Thalassemia
	2nd pregnancy Amniotic fluid cells							Codon 26(G->A) / N	**HbE Carrier (Foetus not affected)**
	Baby on follow up :								
	1st follow up (7mF)	9.5	76.7	23.1	4.2		25.0	Codon 26(G->A) / N	**HbE Carrier**
	2nd follow up (1y3m)	11.7	75.7	25.4	1.0		24.3	Codon 26(G->A) / N	**HbE Carrier**
6	Father (37)	10.0	62.5	18.7	0.6	4.4		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (28)	9.4	67.1	21.2	0.4	5.7		Uncharacterized(?) / N	β-Thalassemia Carrier
	1st child (5F)	8.7	80.7	27.1	1.0	2.9		IVS 1-5 (G ->C) / ?	Homozygous β-Thal,

Table 1: Contd. .....

Family No.		*Hb g%*	*MCV fl*	*MCH pg*	*HbF %*	*HbA_2 %*	*HbE %*	*DNA Analysis (Type of Mutation & status of both alleles)*	*Diagnosis and Remarks*
									50 units blood transfusion
	2nd pregnancy Amniotic fluid cells							IVS 1-5 (G->C) / ? RFLP done for Unknown	**Homozygous β-Thal (Foetus affected) ***
7	Father (35)	13.6	80.0	25.9	0.6		26.0	Codon 26(G->A) / N	HbE Carrier
	Mother (29)	9.9	72.1	22.9	1.0	4.1		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Ist Child (10M)								Died
	2nd pregnancy Amniotic fluid cells							The above mutations not detected (N / N)	**Normal foetus**
	Baby on follow up (7mF)	11.4	72.0	22.0	0.4	2.1		Same as above (N / N)	**Normal**
8	Father (35)	15.8	79.4	26.9	1.1		22.1	Codon 26(G->A) / N	HbE Carrier
	Mother (28)	11.3	65.3	20.6	1.3	4.8		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (11M)								Normal (not affected)
	2nd child (9F)	12.3	82.0	27.3	2.7		5.8	IVS 1-5 (G ->C) / Codon 26(G->A)	Eβ- Thalassemia 96 unit blood transfusion
	3nd pregnancy Amniotic fluid cells							Codon 26(G->A) /	**Eβ- Thalassemia (Foetus affected)**
9	Father (36)	12.8	69.1	22.1	0.6	5.3		IVS 1-5 (G ->C)	
	Mother (30)	10.6	62.5	19.1	1.0	5.4		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (11mM)							IVS 1-5 (G ->C) / N	β-Thalassemia Carrier Died (β-Thalassemia) 1 unit of blood transfusion
	2nd pregnancy Amniotic fluid cells							IVS 1-5 (G ->C) / IVS 1-5 (G ->C)	**Homozygous β-Thal (Foetus affected)**
10	Father (40)	13.5	69.3	20.8	0.9		91.3	Codon 26/Codon 26	Homozygous E disease
	Mother (34)	10.2	68.9	19.1	2.6	4.4		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (5M)								Transfussion dependent Eβ-Thalassemia
	Cousin of Father								Transfussion dependent Eβ-Thalassemia (Died)
	2nd pregnancy Amniotic fluid cells							Codon 26(G->A) / N	**HbE Carrier (Foetus not affected)**
11	Father (26)	12.0	69.8	21.5	0.6	4.6		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (22)	9.1	60.7	18.4	0.6	5.6		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (1 F)	6.9	62.6	20.2	4.3	3.4			4 units of blood transfusion, Died
	2nd pregnancy Amniotic fluid cells							IVS 1-5 (G ->C) / IVS 1-5 (G ->C)	**Homozygous β-Thal (Foetus affected)**

Table 1: Contd. .....

Family No.		*Hb g%*	*MCV fl*	*MCH pg*	*HbF %*	*HbA_2 %*	*HbE %*	*DNA Analysis (Type of Mutation & status of both alleles)*	*Diagnosis and Remarks*
12	Father (35)	13.5	67.2	20.6	0.7	4.1		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (30)	9.8	65.7	19.9	0.6	4.0		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (1y10m M)								Undiagnosed, Died
	2nd child (10m M)								β-Thalassemia , Died
	3rd child (6F)	7.5	64.3	18.1	5.0	4.0		IVS 1-5 (G ->C) /	β-Thalassemia, under
	4th pregnancy							IVS 1-5 (G ->C)	transfusion
	Amniotic fluid cells							The above mutation	**Normal foetus**
	Baby on follow up (7mF)	10.4	76.8	26.2	0.4	2.3		not observed **N / N**	**Normal**
13	Father (31)	14.2	81.1	26.3	0.4		22.0	Codon 26(G->A) / N	HbE Carrier
	Mother (25)	9.9	68.3	20.5	0.2	4.2		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (2y1m F)	7.9	67.7	19.5	41		39.1		Eβ- Thalassemia
	2nd pregnancy							Codon 26(G->A) / N	**HbE Carrier**
	Amniotic fluid cells								**(Foetus not affected)**
14	Father (47)	12.2	72.4	22.0	0.9	4.3		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (37)	11.8	85.3	27.0	1.3		28.6	Codon 26(G->A) / N	HbE Carrier
	1st child (7F)								Died, Transfusion dependent thalassemia
	2nd and 3rd pregnancy								MTP
	4th (6d F) and 5th child (16hrs M)								Premature delivery. Died
	6th pregnancy							IVS 1-5 (G ->C) / N	**β-Thalassemia Carrier**
	Amniotic fluid cells								**(Foetus not affected)**
	Baby (7mF) on follow up	9.1			1.4	4.7			**β-Thalassemia Carrier**
	7th pregnancy							IVS 1-5 (G ->C) / N	β-T**halassemia Carrier***
	Amniotic fluid cells								**(Foetus not affected)**
15	Father (26)	12.3	66.2	21.0	0.9	4.0		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (22)	10.0	68.5	21.3	0.8	4.4		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (2F)								Transfusion dependent Died
	2nd pregnancy							IVS 1-5 (G ->C) / N	**β-Thalassemia Carrier**
	Amniotic fluid cells								**(Foetus not affected)**
16	Father (26)	12.0	63.8	20.1	1.2	5.1		Uncharacterized (?) /N	β-Thal Carrier (unknown)*
	Mother (22)	9.1	63.5	20.9	1.0	4.3		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (2y6m M)	11.0	80.1	26.9	4.7	2.3		IVS 1-5 (G ->C) / ?	Transfusion dependent β-Thalassemia
	2nd pregnancy							IVS 1-5 (G ->C) / N	**β-Thalassemia Carrier**
	Amniotic fluid cells								**(Foetus not affected)**

Table 1: Contd. .....

Family No.		*Hb g%*	*MCV fl*	*MCH pg*	*HbF %*	*HbA_2 %*	*HbE %*	*DNA Analysis (Type of Mutation & status of both alleles)*	*Diagnosis and Remarks*
17	Father (38)	11.5	71.1	22.6	1.8	5.8		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (26)	12.5	75.7	24.5	1.0		26.0	Codon 26(G->A) / N	HbE Carrier
	1st child (8M)	8.7	77.6	24.7	4.2		15.6	IVS 1-5 (G ->C) / Codon 26(G->A)	Eβ-Thalassemia (40 units of blood transfusion)
	2nd pregnancy Amniotic fluid cells							Codon 26(G->A) / N	**HbE Carrier (Foetus not affected)**
18	Father (31)	12.0	65.6	20.1	1.0	4.2		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (29)	10.8	74.7	23.3	0.8		23.3	Codon 26(G->A) / N	HbE Carrier
	1st pregnancy								Abortion
	2nd pregnancy Amniotic fluid cells							IVS 1-5 (G ->C) / Codon 26(G->A)	**Eβ-Thalassemia (Foetus affected)**
19	Father (33)	12.8	70.5	33.4	0.4	4.4		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (29)	9.6	64.4	21.4	0.6	4.8		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	1st child (2y 6m F)	9.5	72.1	30.1	0.4	4.8		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	2nd child (11mF)	7.3	80.0	22.8	17.0	2.1		IVS 1-5 (G ->C) / IVS 1-5 (G ->C)	β-Thalassemia, Died 3 units blood transfussion
	3rd pregnancy Amniotic fluid cells							Above mutations not found, **N / N**	**Normal Foetus**
20	Father (37)	13.9	69.8	22.4	2.1	4.5		IVS 1-5 (G ->C) / N	β-Thalassemia Carrier
	Mother (25)	10.2	66.1	21.3	1.0	5.4		FS 8/9 (+G) / N	β-Thalassemia Carrier
	1st child (4F)	10.5	73.6	38.4	0.6	4.2			βTthalassemia Carrier
	2nd child (3F)	5.4	84.8	19	22	3.2			β-Thalassemia (48 units of blood transfusion)
	3rd pregnancy Amniotic fluid cells							IVS 1-5 (G ->C) / FS 8/9 (+G)	**Homozygous β-Thal (Foetus affected)**
21	Father (28)	14.0	84.4	26.7	0.9		20.7	Codon 26 (G->A) / N	HbE Carrier
	Mother (24)	7.8	63.9	18.8	0.6	5.3		Uncharacterized / N	β-Thalassemia Carrier
	1st child (6y 6mF)	5.2	72.6	22.2	5.4		21.6	Uncharacterized (?) / Codon 26 (G->A)	Eβ-Thalassemia (with Unknown β-mutation)*
	2nd pregnancy Amniotic fluid cells							Codon 26 not present	**(Foetus not affected)**

Figures in parenthesis- Age in Years, y-Year, m-Month, d-Days, M- Male, F- Female,
N - Normal allele, (?) - Uncharacterized, del – Deletion, thal- Thalassemia
* Cases which were further studied by RFLP

polymorphic sites. HindIII Aγ, HindIII Gγ sites were found to be useful for linkage analysis in these two families. The indirect evidence after RFLP study showed that the baby was not affected in one (family 16) but was found to be affected in the other ie family 6.(Table 5)

All the parents were advised to come back when the baby is more than 6 months old for follow up. In one case (family 1) the mutation found in the prenatal diagnosis was also confirmed in the cord blood cell DNA which was collected at the time of the birth of the baby. 4 couples came back for follow up (families 5, 7, 12 and 14) after their babies were born and were more than 6 months old. In all cases the diagnosis done at prenatal stage were found to be correct (Table 1). Those who had affected pregnancies were advised to terminate the pregnancy. Some couples (families 14 for 7th pregnancy, 19 and 21) are expected to turn up for follow up within a year and a half. There is no news from some other families. No foetal loss was reported so far following the procedure of amniocentesis for this purpose.

Table 2: Frequency of different mutations in the 21 couples tested

Mutations identified	*No of mutant alleles*	*No of normal alleles*	*% of mutation observed*
IVS 1nt5 (G-C)	25	25	56.80
Codon 26 (Hb E)	13	10	29.60
Codon 8/9 (+G)	2	2	4.50
619bp deletion	1	0	2.30
Uncharacterized	3	3	6.80
Total	44	40	100.00

Table 3: Time of approach for prenatal diagnosis in each of 22 pregnancies from 21 couples tested

Time at which couples came for prenatal diagnosis	*Total no. of prenatal diagnosis done at each pregnancy*	*% of prenatal diagnosis done at each pregnancy*
1st pregnancy	1	4.60
2nd pregnancy	14	63.60
3rd pregnancy	3	13.60
4th pregnancy	1	4.60
6th pregnancy	2	9.00
7th pregnancy	1	4.60
Total	22	100.00

DISCUSSION

Prenatal diagnosis is one of the most effective and direct approach for prevention of thalassemia disease. β-thalassemia and Eβ-thalassemia constitute two most common and serious health problems in Eastern part of India which claims many childhood deaths every year. In a country like India where 50% populations are below poverty line, prenatal diagnosis should be a necessary criterion during early pregnancy where parents are known to be thalassemia carriers. The expensive and long term intensive medical care can place a burden on the afflicted families and society as a whole (Balgir 2000; Choudhary et al.1998). A strategy for an effective thalassemia prevention programme must include in the first place the education and awareness in the population about the prevalence and adverse effects of the disease, cost of treatment and associated problems. It is also necessary to use the cost effective methods of carrier detection in the population (osmotic fragility test of the red cell, Hb electrophoresis, estimation of HbA_2 and other relevant hematological parameters). Antenatal patients should be offered thalassemia screening. All first degree relatives and spouses of new carriers detected should be screened and genetic counseling given to all who carry the thalassemia gene. Subsidized screening would be an added incentive especially for the poor

Table 4: Incidence of affected/unaffected pregnancies from the 21 couples tested

Type of Pregnancy Identified	*Total no. found in each case*	*% of each type of pregnancy recorded*	*Total % of Unaffected/ affected pregnancies*
Normal	3	13.63	63.65
β-Carrier	6	27.30	
E-Carrier	5	22.72	
Eβ-thalassemia	3	13.63	36.35
β-thalassemia major	5	22.72	
Total	22	100.00	100.00

Table 5: RELP Linkage analysis results in some selected families

Family No.		*Tye of Mutation & status of both alleles*	*RELP sites investigated*					*Remarks*
			Hind III	*Hind III*	*Hinc II*	*Hinc II*	*Hinf I*	
			GY	*AY*	*5'B*	*3'B*	*3"B*	
6	Father (BC)	IVS 1-5 (G ->C) /N	+/-	+/-				
	Mother (BC)	Uncharacterized /N	+/-	-/-				
	1st child (β thal)	IVS 1-5 (G ->C) / Uncharacterized	-/-	-/-				
	2nd pregnancy Amniotic fluid cells	IVS 1-5 (G ->C) /?	-/-	-/-				**Foetus Affected** IVS 1-5 (G ->C) / Uncharacterized
14	Father (BC)	IVS 1-5 (G ->C) /N					+/+	
	Mother (BC)	Codon 26(G->A)/ N					+/-	
	6th pregnancy Amniotic fluid cells	IVS 1-5 (G ->C) /N						
	Baby on follow up (BC)	IVS 1-5 (G ->C) /N					+/+	
	7th pregnancy Amniotic fluid cells	IVS 1-5 (G ->C) /N					+/+	**Foetus not affected** IVS 1-5 (G->C)/N
16	Father (BC)	Uncharacterized/N	+/-	+/-				
	Mother (BC)	IVS 1-5 (G ->C) /N	-/-	-/-				
	1st child (β-thal)	Uncharacterized/ IVS 1-5 (G ->C)	+/-	+/-				
	2nd pregnancy Amniotic fluid cells	IVS 1-5 (G ->C) /?	-/-	-/-				**Foetus not affected** IVS 1-5 (G->C)/N
21	Father (EC)	Codon 26 (G ->A)/N	+/-	-/-	+/-	+/-	+/+	
	Mother (BC)	Uncharacterized /N	+/-	-/-	-/-	+/-	+/-	
	1st child (β-thal)	Uncharacterized / Codon 26 (G ->A)	+/-	-/-	+/-	+/-	+/-	
	2nd pregnancy Amniotic fluid cells	Codon 26 (G ->A) absent Uncharacterized /N (?) or N / N (?)					+/+	**Foetus not affected** Insufficient specimen for further Investigation

N-Normal allele, BC-b carrier, B-thal - b Thalassemia, EC- HbE carrier; (+) presence of site, (-) absence of site , ? –Not known

families to agree to screening. Thus identification of couples 'at risk" can be done prospectively before or after marriage. Prenatal diagnosis can be offered to these couples whenever necessary before the birth of an affected child.

Thalassemia prevention programmes have been actively pursued in many Meditarnean countries, including Sardinia, Cyprus and Greece (Angastiniotis 1990; Angastiniotis and Hadjiminas 1981; Cao et al. 1984; Hadji et al. 1987). The programme had notable success in eliminating live births with β-thalassemia major in these countries.

From the present study a high percentage of affected pregnancies (36.4%) were observed. 63.6% pregnancies were found to be unaffected. (Table 4) The high prevalence of β-thalassemia and Haemoglobin E disease in Eastern India is also responsible for the substantial increase in the percentage of affected pregnancies. Table 3 also shows that the highest percentage of counselling was carried out in 2nd pregnancies ie. all the couples except one (family 2) were unaware that they were carriers of β-thalassemia or HbE disease and came for counselling only after an affected child was born (Table 3). This indicates poor awareness about the disease among common people.

Among the individuals of the 21 couples tested, IVS1 nt5 (G->C) mutation was found to be most common (56.8%) followed by Hb E (29.6%) which has already been described as the most predominant mutations in Eastern India (De et al. 1997 b; Das et al. 2000). The percentage of unspecified mutations described by De et al. 1997b were recorded as 17.9% due to non-availability of some primers necessary for identification of 3 more mutations for that study population such as Codon 8/9 (+G), Codon 15 (G->A) and Codon 30 (G->C) which were also found to be common in Eastern India. At present only 6.8% mutations remained unspecified for

which prenatal diagnosis was carried out with the help of RFLP technique. Thus the success rate of prenatal diagnosis for this study was 100%. The use of RFLP for complementing the identification of the known mutations by PCR-ARMS as well as its importance in diagnosis of transmission of unknown mutations was also reported. (Kazazian and Boehm 1988; Weatherall et al. 1988; 1995; Old et al. 1990; Venkatesan et al. 1992; Varawalla et al. 1992; Ng et al. 1996; Thakur et al. 2000)

The high prevalence and heterogeneity of β-thalassemia and the occurrence of its 8 common mutations with the predominance of IVS 1nt5 (G->C) for β-thalassemia and Codon 26 (G->A) for Haemoglobin E disease in Eastern India indicate that prenatal diagnosis is very much possible in this population.The people in this region seems to be an ideal population for implementing direct detection of mutations by PCR-ARMS supplemented by RFLP linkage analysis towards establishing a comprehensive prenatal diagnosis service during the midtrimester of pregnancy following amniocentesis. The prenatal samples may also be obtained from other sources such as foetal blood cells (15 to 17 weeks) and chorionic villus sampling during 9 to 11 weeks. (Weatherall 1991) Previous studies (Weatherall et al. 1988, 1995) have also shown that it is possible to achieve a success rate close to 100% for prenatal diagnosis when the direct detection of mutation is backed up by RFLP analysis. Potential difficulties were reported as maternal tissue contamination, crossover and nonpaternity (Weatherall et al. 1988, 1995; Varawalla et al. 1992) In case no informative markers were available for a novel mutation in a particular family, direct DNA sequencing may be carried out. Therefore our current strategy for prenatal diagnosis is to screen the parents "at risk" and to identify the mutations present in them beforehand and then to check the presence or absence of these mutations in the amniotic fluid cells. The confirmation of the diagnosis should be carried out by another PCR-ARMS or RFLP linkage analysis whichever possible.

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