Research Paper

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SPECTRUM OF BETA THALASSEMIA MUTATIONS DETECTION IN SOUTH KARNATAKA POPULATION BY USING ARMS-PCR

Biochemistry

KEYWORDS :

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ABSTRACT

Beta-thalassemia is a highly prevalent autosomal recessive genetic disorder characterized by the reduced or absent expression of the beta globin gene, leading to an imbalance of alpha and beta globin chains in haemoglobin. Out of 300 different mutations causing beta-thalassemia, IVS1-1, IVS1-5, Codon 41/42, Codon 8/9 are most common in South Karnataka regions, the study was done to analyze the chromosomal background and to detect mutations of beta thalassemia by using ARMS-PCR assay. A total number of 30 diagnosed beta thalassemia cases were taken and DNA samples were used for amplification with different pair of allele specific primers having four pair of test primers and one pair of control primers. The obtained PCR products were electrophorized under optimal conditions. The present study suggests that the most commonly mutation was found to be IVSI-5. These data are useful in future molecular screening of the population for implementing a thalassemia prevention and control program. ARMS-PCR technique were found to be a valuable tool for analysis of beta-thalassemia mutations due to accurate, cost effective, simple and rapid screening.

INTRODUCTION

Beta-Thalassemia is a common autosomal recessive inherited single gene blood disorder in humans caused by mutations in the beta-globin (HBB) gene on the short arm of chromosome number-11. It is highly prevalent in the Mediterranean region, the Asian-African continents in general[1]. The HBB gene encodes the beta-globin chains of haemoglobin, an oxygen carrying protein composed of two alpha and two beta chain subunits found within red blood cells. This gene has three exons and two introns involved in beta-thalassemic pathogenesis. Mutations in the HBB gene lead to an altered beta-globin chain resulting in structural change of the protein conformation of haemoglobin. In beta-thalassemia, point mutations in the beta-globin structural gene are largely responsible for either decreased or no beta-globin synthesis. Previous studies have demonstrated the presence of population-specific mutations, with different frequencies probably caused by conservation of gene pools, selection, and genetic drift. Information provided on the distribution and the frequency of beta thalassemia mutations is useful to establish a program for carrier screening, genetic counselling, prenatal diagnosis, and for physicians to establish specific therapeutic approaches for patients with beta thalassemia.

Previous studies may not be sufficient to describe the complete spectrum of HBB gene mutations in this country. Very little data about HBB gene mutations in the population of south India are available so far. Therefore, the purpose of this study was to determine the spectrum of beta-thalassemia mutation in the population of the south Karnataka region.

MATERIALS AND METHODS

Patient samples

Thirty Beta-thalassemia patients from the Mysore surrounding region, who regularly attend in the department of Transfusion medicine and department of Paediatrics in the JSS Hospital Mysore, were included in the present study. All the thalassemia Patients were confirmed with red blood indices and haemoglobin variants separation by Cation exchange HPLC (BioRad-D10).

Table: 1. Sequence of primers and the concentration used in ARMS-PCR*

Beta thalassemia Mutation	Sequence	Primer Concentration (nM)	Product Size(bp)
IVSI-5 (G-C)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG[a]	80	319
IVSI-1 (G-T)	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA[a]	100	315
Cd 8/9 (+G)	CCT TGC CCC ACA CGG CAG TAA CGG CAC ACC[a]	20	250
Cd 41/42 (–TTCT)	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT[a]	20	476
Common Primers			
Control A	CAA TGT ATC ATG CCT CTT TGC ACC[a]	40	861
Control B	GAG TCA AGG CTG AGA GAT GCA GGA[a]	35	-
Common -E	TGA AGT CCA ACT CCT AAG CCA GTG	10	-

*Primers selected as per reference 4.

Blood samples were collected just before the transfusion, with the informed written consent of all parents of beta-thalassemic individuals following the institutional ethical guidelines. Thirty control cases of age and sex matched healthy individuals samples were also collected. 3mL Blood was collected in EDTA tubes and was stored at -80°C until DNA extraction.

DNA Extraction

DNA extraction was carried out by using HiMedia DNA extraction and purification kit according to manufacturer's protocol. The concentration quantified using a quartz microcuvette. The DNA yield was checked with 0.8% agarose gel electrophoresis. The extracted and purified DNA is collected into a 1.5ml RNA free mini centrifuge tubes and are stored at -20°C until fur-

ther analysis

Amplification refractory mutation system

ARMS is a PCR based method, which uses allele-specific priming. In this method, an oligonucleotide primer with a triple end complementary to the sequence of a specific mutation, coupled with a common primer is used in one PCR reaction. In parallel, a corresponding normal primer coupled with a common primer is used in another PCR reaction[3].

Primers were selected according to the ARMS protocol for beta-thalassemia mutations commonly found in south Karnataka region. Four mutation primers selected are IVS1-5, IVS1-1, Codon 41/42 and Codon 8/9 (Varawalla NY et, al)[4]. The primers for DNA amplification were synthesized by SIGMA-Aldrich Laboratories USA. Primer sequences of the selected mutations, concentration of primer used for the reaction and amplified PCR product size are shown in Table 1. These primers were paired with common forward primer-E, to obtain ARMS products of specific molecular weights. An internal control to amplify 861bp fragment along the HBB-gene was amplified in every PCR reaction with internal control primers A and B separately.

ARMS protocol

ARMS-PCR amplification was carried out in 30µl reactions mixture containing 200µM dinucleotide triphosphates, 10X PCR buffer (200 mM Tris-HCI, 1.5 mM MgCI, pH 8.4), Different concentration of ARMS primers[5], 1.5 Units Taq DNA polymerase. To this 4µL Genomic DNA is added.

Eppendorf Master Cycler Gradient was used for amplification. The program set up of the PCR was as follows with the initial denaturation at 95°C for 5 minutes, followed by 30cycles of denaturation 94^0C for 45 seconds, annealing 61^0C for 1 minute and extension for 1 minute at 72°C. A final extension of 5 minutes at 72°C was allowed and the reaction was maintained at 4^0C until analysis by gel electrophoresis.

Optimization of the ARMS

Optimization for specific amplification of the mutations was carried out by manipuanlation of different $MgCI_2$ concentrations, primer concentration, anneaing temperatures and Taq polymerase concentrations.

The amplified products were electrophoresed along with 1Kbp Marker on 1.5% agarose gel containing Ethidium-Bromide dye, at 100 V for 1 hour. DNA bands were visualized under UV- illuminator and then documented using GENE-sys gel documentation unit.

RESULTS

A sample size of 60 in which 30 thalassemic cases were taken and about 30 healthy controls. Four sets of primers for different mutations were taken and was subjected to PCR with a pair of internal controls. The obtained PCR products showed that about 53.3% among thalassemia children had IVSI-5 mutation and about 10% revealed Cd 41/42 mutation followed by 10% of IVSI-1 mutation and minimum of about 6.6% of Cd 8/9, this study also showed about 20% of cases are unknown mutations. Using the ARMS, DNA from 30 normal individuals was amplified for each of the four common beta-mutations evaluated to check for false positive results. In each of the normal DNA samples, only the 861 bp internal control was amplified after ARMS-PCR and no beta-thalassemia mutant bands were observed. Details of study results are shown in table 2.

Table 2: Various mutations detected in present study

Mutation	Number of cases	Percentage
IVSI-5 (G-C)	16	53.3
IVSI-1 (G-T)	3	10
Cd 8/9 (+G)	2	6.6
Cd 41/42 (_TTCT)	3	10
Unknown	6	20

DISCUSSION

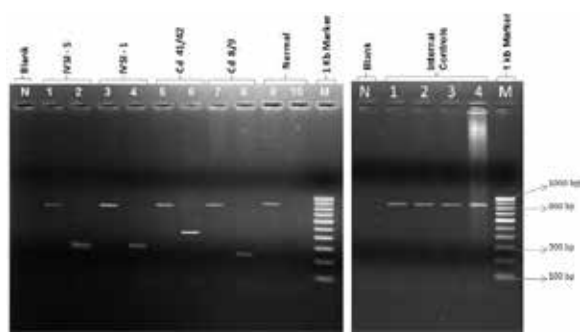
Beta-thalassemia is a highly prevalent autosomal recessive disorder characterized by the reduced or absent expression of the beta-globin gene, leading to an imbalance of alpha and beta globin chains. So far, over 300 beta-thalassemia alleles have been characterized in or around the beta globin region[6]. Normally beta-thalassemia trait in India is 3.3% with 1–2 per 1,000 couples being at risk of having an affected offspring each year, leading to a high societal burden[7]. As the ethnic composition of the Indian population is heterogeneous, each region of the country has its own distinct set and frequency of beta-thalassemia mutations.

The age pattern of the study included was between the age of 1 to 14 years who exhibited severe anaemia, pallor and spleenomegaly and underwent repeated blood transfusion for every 15-20 days depending upon the severity of the disorder. The haematological parameters of these cases had marked decrease in Haemoglobin, MCV and MCH.

The haemoglobin variants show decreased HbA and a relative increase of HbF and HbA_2.

Mutation spectrum of beta-thalassemia patients

The molecular screening protocol we followed using the PCR, which included four common mutations, consisting of 2 control primers and 4 test primers enabled us to identify the causative mutation in Chromosome 11.



Figur:1 Right- Ethidium-bromide stained agarose gel picture of PCR products for the beta thalassemia mutations study. N is negative control(blank), lane 1, 3, 5, 7 and 9 are amplified PCR product of internal control (861bp). lane 2 has the amplified product of the mutation IVSI-5 (319bp), lane 4 consists of the mutation IVSI-1 (315bp), lane 6 has the amplified product of the mutation Cd41/42 (476bp), lane 8 has the PCR product for the mutation Cd8/9 (250bp)., lane 10 consists of PCR product of control that is negative for mutation, lane M 1-kb DNA ladder. Left – PCR products of normal samples (861bp).

10X PCR buffer with about 1.5 unit of Taq polymerase with dNTPs were mixed with respective concentrations and the obtained PCR products were run in an electrophoretic chamber. Our study showed that the IVS-I-5 (G-C) is the most prevalent mutation among the beta-thalassemic South Karnataka population ranging up to 53.3% followed with a relative frequency of 10 % followed by Cd 41/42 and with a relative frequencies of IVSI-1 and Cd 8/9 with10% and 6.6% respectively. This analysis was done among the mutations chosen there was 20% cases with unknown mutations. These results will help the beta thalassemic patients of especially South Karnataka population for genetic

counselling which furthermore would help in gene therapy.

Similar studies were conducted in different regions of the India Edison et al, 2008 conducted a study in Tamil nadu and stated that 65.6% of cases were affected with the Poly A(T<C) allele mutant[8]. Likewise a study carried out by Garewal et al, 2005, showed the provinces of Maharashtra and Punjab had a higher prevalence of IVSI-5(G-C) accounts for 54.7% and codon 15 (G>A)[9]. A study done by Census Of India 2001a reported IVSI-1(G>T) and codon 12(G>A) as the fourth commonest regional mutation of the Western India with 7.6%.

Reich et al 2009 studied on the mutations spread in Dravidian states namely Andra Pradesh, Karnataka, Tamil Nadu and Kerala presenting that the most common mutation to be found was IVSI-5(G>C) with 67.9% and codon 15 as second most common followed by Poly-A site(T>C) allel[10].

Kulkarni et al conducted a study in whole of Karnataka population and reported that the commonly found mutants were as follows IVSI-5G >C (28%), IVSII-74 T >G (22%), codon 3 (T > C) (19%)[11]. S. Sinha et al, conducted study on beta-thalassaemia mutations in India at state and regional levels. The obtained data was that 52 mutations accounted for 97.5% of all beta-thalassaemia alleles in India, with IVSI-5(G-C) the most common mutation (54.7%)[12].

Since beta-globin gene mutations are responsible for beta-thalassemia disease, detection of these particular genetic defects is important, particularly for genetic counselling and prenatal diagnosis. Several PCR-based detections of these mutations have been collectively introduced. The ARMS-PCR employed to characterize the beta-globin mutation. We have clearly shown that the simple ARMS-PCR technique can accurately identify common beta-thalassemia mutations in south Karnataka beta-thalassemic patients. This could mean that the characterization of these mutations can be easily performed by only a single PCR step followed by agarose gel electrophoresis without any further steps being taken. However, the primers used in this study could detect only common beta-thalassemia mutations, leaving some cases unidentified. In those problematic individuals, the mutation detection was accomplished by direct nucleotide sequencing.

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