ORIGINAL ARTICLE

The Spectrum of β-Globin Gene Mutations in Thalassemia Patients of South-Western Maharashtra: A Cross Sectional Study

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Abstract:

Background: -thalassemia is a heterogeneous group of inherited hematological disorder. Though the importance of mutations in the beta-globin gene causing -thalassemia have been reported worldwide, no data are available from rural population of South-Western Maharashtra. *Objective:* In the present study we aimed to characterize the mutations in ß-globin gene from ß-thalassemia patients from rural areas of South-Western Maharashtra. *Material and Methods:* The patients were analyzed for the ß-globin gene mutations included IVS I-1 (G-T), IVS I-5 (G-C), cd 71/72 (+A), cd 41/42 (-TTCT), codon (cd) 8/9 (+G), cd 17 (A-T), cd 95 (+A), cd 43 (-C), cd 41 (-C), cd 35 (C-A), cd 26 (G-T), cd 19 (A-G), cd 15 (-T), cd 27/28 (+C) and cd 14/15 (+G) with the help of Multiplexed Amplification Refractory Mutation System-Polymerase Chain Reaction (MARMS-PCR). *Results:* Out of the common mutations studied the cd 71/72 (21.54%), cd 19 (13.7 %). cd 41/42 (9.68%) and cd 41 (9.6%) showed high prevalence followed by cd17 (7.56 %). 7.27% patients showed IVSI-5 mutations, 6.26 % showed IVSI-1 mutations. Cd 15 mutations were present in 8.69 % patients and only 5.39 % subjects showed cd 8/9 mutations. This study provides the pattern of ß-thalassemia mutations from rural areas of Maharashtra in India. *Conclusion:* This study provides the pattern of ß-thalassemia mutations from rural population which will open a new avenue for implementation of molecular diagnostics for prenatal diagnosis and prevention of blood disorder by proper counseling in rural areas.

Keywords: Beta globin, -thalassemia, Hemoglobin, gene mutation, MARMS-PCR

Introduction:

Thalassemia is one of the major hemoglobino-pathy among the population all around the world mainly caused by deficient synthesis or absence of one or more of the polypeptide chains of human hemoglobin [1]. Thalassemia has been recognized by the World Health Organization (WHO) as an important genetically inherited disorder principally impacting on the population of low income countries [2]. In developing countries like India there is alarming rate of increase in thalassemia due to lack of knowledge and proper health care. About 10 % of total world thalassemia patients belong to Indian subcontinent [3]. The thalassemia syndromes are a group of inherited anemias characterized by deficient synthesis human hemoglobin as a consequence of large number of different genetic lesions in either a or ß-globin gene clusters [4]. Mainly there are two main types of thalassemia; -thalassemia caused due to mutation in -globin gene which led to decreased amount of a-globin chain synthesis whereas in ß-thalassemia there is reduced or absence of ß-globin chain synthesis. ß-thalassemia is one of the most common disorders in the world, involving a diverse group of defects in hemoglobin synthesis, all of which result from reduced output of ß-globin chains [1, 4]. ß-thalassemia is very heterogenous at molecular level with approximately more than 200 different disease causing mutations reported so far involving single nucleotide substitutions,

insertions or deletions of single nucleotides or small nucleotides frameshifts within the ß-globin gene [1, 5-6]. There are certain mutations responsible for more than 90 % of thalassemia cases and about 10 % mutations which remain unknown or considered as very rare ones [7].

 -thalassemia is the most common single gene disorder in Indian subcontinent. Some of the earlier reports have shown different mutations in Indians amongst which the common mutations included frameshift 8/9 (+G), nonsence cd 15 (TGG-TAG), frameshift 41/42 (-TCTT), frameshift16 (-C), IVSI -nt5 (G-C) accounting for over 90 % of mutations in ß-thalassemia patients [8-11]. The distribution of these mutations in various parts of India has been reported by number of investigations [12-17]. Commonly thalassemic persons are anemic and initially diagnosed clinically by considering hematological parameters like Hemoglobin (Hb gm %), Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH). Several molecular diagnostic processes have been evolved for the accurate diagnosis of thalassemia like, Amplification Refractory Mutation System (ARMS), Polymerase Chain Reaction (PCR), multiplex PCR etc. Multiplex-PCR protocols using ARMS (MARMS-PCR) has been widely used for identification of ß-thalassemia mutations [8, 18-20].

Although several international studies on the genetic and molecular aspects of thalassemia disorder have been successfully carried out, unfortunately no molecular diagnostics has yet been regularly implemented in routine use for the identification of beta globin gene mutations from this disorder in developing countries including India. Very few studies from different regions of India have reported the ß-globin gene mutations of ß-thalassemia but no reports are available from rural areas of western Maharashtra population. Therefore, in the present study we have aimed to characterize the mutations in ß-thalassemia patients mostly from rural areas of the districts including Satara, Sangli and Kolhapur of Western Maharashtra region. As outcome of conducting genetic analysis at molecular levels in the rural areas will be helpful to us to understand the nature of mutations in the thalassemia disorder so that genetic counseling will be given to carrier families and specific treatments would be planned to the affected patients. The findings presented in this paper will be useful for implementing the molecular diagnostics for prenatal diagnosis of genetic blood disorder like thalassemia in a rural hospital.

Material and Methods:

Subjects:

The MARMS-PCR protocol was analyzed on genomic DNA obtained from blood of patients routinely visiting Department of Pediatrics, Krishna Institute of Medical Sciences, Karad for blood transfusion. 2.0 milliliter (ml) of peripheral blood samples were collected from fifty individuals clinically diagnosed as thalassemia major and equal number of normal age matched controls after receiving their informed consent. All subjects were more than one year of age and attended the hospital on routine basis.

Hematological Analysis:

Hematological parameters including Hemoglobin (Hb), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Packed cell volume (PCV) were analyzed immediately after collection of whole blood, using an automated hematology analyzer (Tulip Diagnostics).

Genomic DNA isolation from human blood:

Two milliliter (ml) of whole blood from patients and normal age matched controls was collected in

sterile purple top vacutainer after receiving informed consent. Genomic DNA extraction was carried out from the peripheral blood sample using Purelink genomic DNA extraction and purification Kit (Invitrogen, Life technologies) following the manufacturer's instructions. The quantitative and qualitative analysis of genomic DNA was carried out by Spectrophotometer and agarose gel electrophoresis

Multiplexed Amplification Refractory Mutation System-Polymerase Chain Reaction (MARMS-PCR)Assay:

The ß-thalassemia mutations commonly found in the Indian population were included in this study. The mutation analysis was carried out using Multiplexed ARMS-PCR. The PCR was first optimized to obtain all possible amplicons. PCR amplification was carried out in 25 μL reaction mixture volume containing 10X PCR assay buffer containing 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl2, 50 mM of KCl, 200 μM each dNTP, 1U of Taq DNA polymerase (Merk Millipore) and 0.1nmole of each primer listed in table1 thereafter 500ng of purified DNA template was added to the reaction mixture. The PCR reaction for amplification was carried out in a Master Cycler gradient PCR machine (Eppendorf). The PCR amplification programme was performed as per the following sequence, initial denaturation was done at 95ºC for 10 minutes, followed by 30 cycles of denaturation at 95ºC for 45 seconds, annealing at 62ºC for 45 sec, and extension at 72ºC for 1 minute following final extension at 72ºC for 10 minutes. Thereafter the amplification products were separated by 2.0% agarose gel electropho-resis in 1X TAE buffer. The gel was stained with ethidium bromide (10mg/ml) and visualized under UV Transilluminator and photographed in gel documentation system (Bio-Rad Laboratories).

The primers were used for detection of common ß-thalassemia mutations including IVS I-1, IVS I-5, cd 71/72, cd 41/42, cd 8/9, cd 17, cd 95, cd 43, cd 41, cd 35, cd 26,cd 19, cd 15, cd 27/28 and cd 14/15 as described by [20] (Table-1). Two different pairs of control primers were also used as internal control for the PCR. DNA for all mutations as described earlier were amplified in three separate reactions. In the first two reactions first six mutations were amplified together with common primer whereas in other third reaction two different forward primers were used along with reverse mutation primers.

Statistical Analysis:

Statistical analysis of quantitative variables was performed using One-Way ANOVA and two tailed student t-test for comparing means. All statistical calculations were performed using SPSS software version 10 for Windows.

Ethics and biosafety:

The study protocol was approved by Institutional Ethics and Biosafety Committee of Krishna Institute of Medical Sciences. Informed consent was obtained before collecting the blood samples and the confidentiality of results was maintained.

Results:

Molecular analysis of ß-thalassemia mutations was carried out by the MARMS-PCR system to identify the mutations in ß-globin gene from ß-thalassemia patients. The multiplexed ARMS procedure was first standardized and then subsequently tested on test samples and normal controls. In first reaction mixture the primers for ß-thalassemia mutations IVSI-1, cd 71/72 and cd 8/9 were added whereas in second reaction the primers were added for ß-thalassemia mutations like IVSI-5, cd41/42 and cd17 along with common internal control primers which produced 493 bp fragment (Fig. 1A & B). In the third

Table 1: Primers used for detection of beta globin gene mutations by MARMS-PCR

β-thalassemia mutation	Primer sequence	Amplicon size (bp)
Group 1		
Control F	5'- CAACTTGCTCAAGCATACACTC - 3'	
Control R	5'- AATAATAGGCATAGTGACAAGTGC - 3'	493 bp
Primer F1	5'- TGAAGTCCAACTCCTAAGCCAGTG - 3'	
IVSI-1 R	5'- TTAAACCTGTCTTGTAACCTTGATACGAAA - 3'	315 bp
IVSI-5 R	5' – CTCCTTAAACCTGTCTTGTAACCTTGTTAG - 3'	319 bp
cd 8/9 R	5' – CCTTGCCCCACACGGCAGTAACGGCACACC - 3'	250 bp
cd 41/42 R	5'- GAGTGGACAGATCCCCAAAGGACTCAACCT - 3'	476 bp
cd 17 R	5'- CTCACCACCAACTTCATCCACGTTCAGCTA – 3'	275 bp
cd 71/72 R	5'- GGTTGTCCAGGTGAGCCAGGCCATCAGTT - 3'	569 bp
Group 2		
Control F	5'- TCCAACTCCTAAGCCAGTGC - 3'	
Contro R	5'- CGATCCTGAGACTTCCACACTG - 3'	804 bp
Primer F2	5'- TCCAACTCCTAAGCCAGTGC - 3'	
cd 95 R	5'- GGATCCACGTGCAGCTTTG - 3'	649 bp
cd 43 R	5'- TGGACAGATCCCCAAAGGACTA - 3'	497 bp
cd 35 R	5' – GAACCTCTGGGTCCAAGGT - 3'	475 bp
cd 26 R	5'- ACCTGCCCAGGGCCTA - 3'	310 bp
cd 19 R	5'- CACCAACTTCATCCACGCTC - 3'	292 bp
cd 14/15 R	5'- TTCACCTTGCCCCACCA - 3'	276 bp
Primer F3	5'- CGATCCTGAGACTTCCACACTG - 3'	
cd 41 R	5'- ACCCTTGGACCCAGAGGTTT - 3'	351 bp
cd 27/28 R	5'- TGGTGGTGAGGCCCCT - 3'	516 bp
cd 15 R	5'- ACCCTTGGACCCAGAGGTTT - 3'	558 bp

reaction mixture cd95, cd43, cd41, cd35, cd26, cd19, cd15, cd 27/28 and cd 14/15 primers along with common internal control primers which amplified 804 bp fragment were mixed in the reaction as an indicator of status of PCR reaction (Fig. 2A & B). We were able to get appropriate bands for all the mutations except cd 8/9 in first reaction and cd 41/42 in second reaction which led to nonspecific amplifications (data not shown). In third reaction many mutations did not show amplification of specific pcr fragment like cd95, cd 43, cd35, cd26 cd 27/28 and cd 14/15 but other

studied mutations show specific amplification. The normal controls did not show any other bands than internal control band in each reaction.

Selective amplification of different known mutations with selective oligonucleotide primers is shown in Fig. 1 & 2.
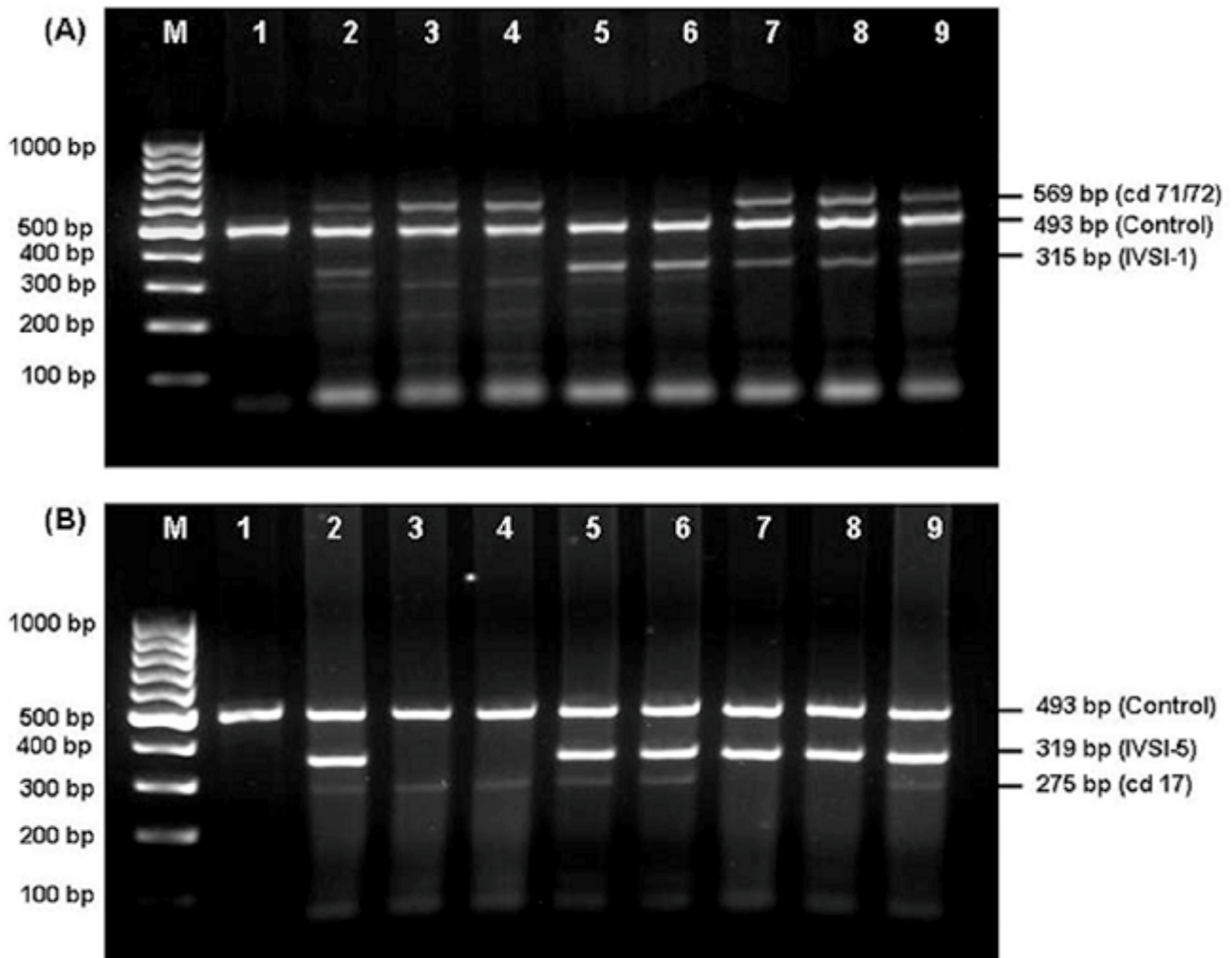

Fig. 1 A: Representative Ethidium Bromide Stained Agarose Gel Image showing Multiplexed ARMS-PCR for the -thalassemia Mutations using Group-1 Primers (IVSI-1, d 71/72)

Fig. 1 B: (IVSI-5, cd17) in Patients from Western Maharashtra. -thalassemia Patients indicated by the Presence or Absence of the Relevant Amplicons Band on Agarose Gels.

Lane M. 100 bp DNA ladder (Invitrogen), Lane 2. Sample from normal control, Lanes 2-9 showing PCR product as an indication of presence of different mutations in different patients. Internal control of fragment size 493 bp has been included in each reaction to ensure the effective amplification. Arrows show location of each PCR product using indicated primers.
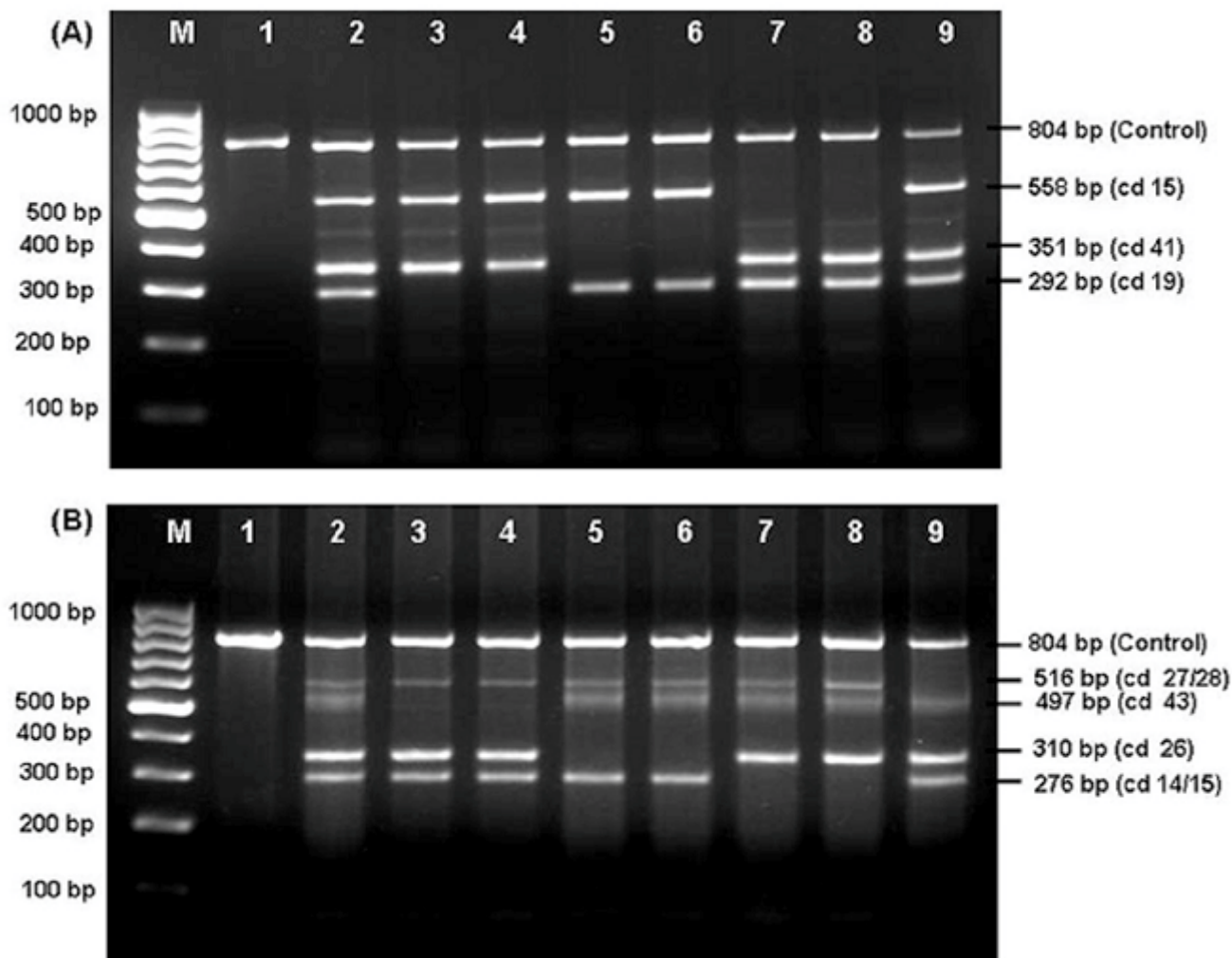
Fig. 2A: Representative Agarose Gel Image showing Amplification of -globin Gene Mutations by using Group-2 Primers (cd15, cd19, cd41)

Fig. 2B: cd14/15, cd26, cd27/28, cd43 in -thalassemia Patients indicated by the Presence or Absence of the Relevant Amplicons.

Lane M. 100 bp DNA ladder, lanes 1- normal control, Lanes 2-9 showing PCR product as an indication of presence of different mutations in different patients. Internal control of fragment size 804 bp was included in each reaction to ensure the effective amplification. Arrows show location of each PCR product using indicated primers.

The major mutations identified were cd71/72 (21.54%), cd 19 (13.7%) cd 41/42 (9.68 %), cd 41 (9.60 %), cd15 (8.69 %), cd17 (7.56 %) followed by IVSI-5 (7.27%) and IVSI-1 (6.26%) and cd 8/9 (5.39%). The frequency comparison of ß-

thalassemia mutations in individuals incorporated in this study is shown in pie chart (Fig. 3). The mutations incorporated in this study including cd 95, cd 43, cd 35, cd 26, cd 27/28 and cd 14/15 did not show any signal. Amongst the total 15
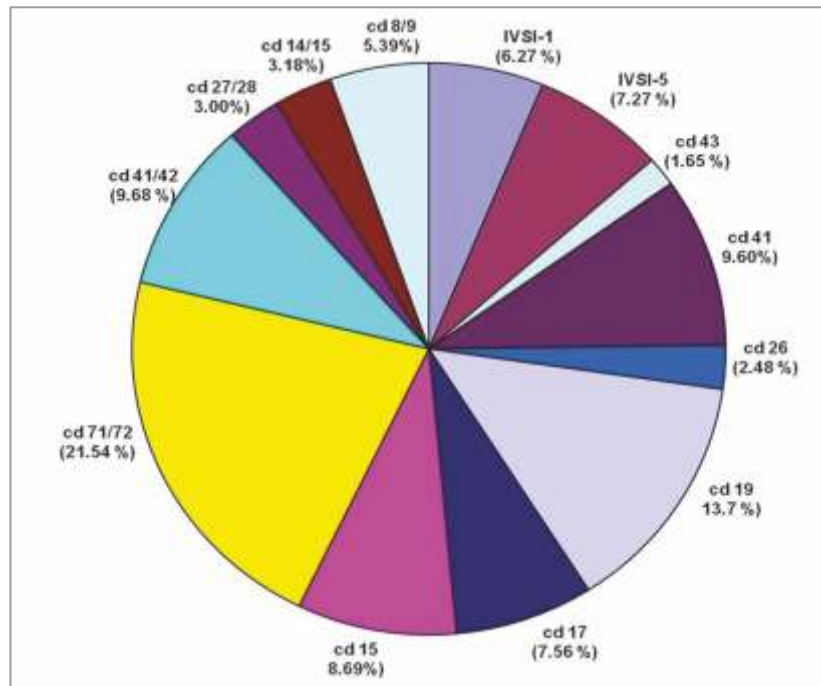
Fig. 3: Pie Chart showing Percent Frequency Distribution of Common Mutations in ß-globin Gene in ß-thalassemia Patients from Rural Areas of Western Maharashtra from India.

mutations studied the mutations including cd 71/72, cd 41/42 and cd 19 were the most common mutations constituting 44.92 % of all the mutations characterized. A 60 % ß-globin gene defect was identified in all 50 (100%) cases with Hb levels ranging from 2.9 to 6.9 g/dL. Significant differences were observed in most hematological parameters when compared to normal controls. All the mutations presented in this study when compared with hematological parameters showed closer similarity for the symptoms with the lowest red blood cell values with mean MCV of 79.51($\pm$4.43) fl, MCH of 25.05 ($\pm$1.89) pg and PCV 20.24 ($\pm$5.82) respectively (data not shown).

Discussion:

ß-globin gene is present on the short arm of chromosome 11 in a cluster with the other genes [21-22]. ß-thalassemia is very heterogenous at molecular level with approximately more than 200 different disease causing mutations within the ß-globin gene reported so far [1, 5-6]. With exception of a few deletions, majority of ß-thalassaemias are caused by point mutations within the globin gene or its immediate flanking sequences [23-24]. Thus the degree of globin chain imbalance is responsible for thalassaemia syndromes. Several molecular techniques for DNA analysis like PCR have played an important role in identification of such mutations [8, 18-20]. PCR strategy has come into practice for elucida-ting ß-thalassemia mutations involved in screening of the common mutations that predominate in the at-risk population. The ARMS-PCR technique is a popular technique to identify the known ß-thalassemia mutations since twenty years [8, 18]. In this study, we have successfully applied multiplexed ARMS-PCR system for the detection of fifteen ß-thalassemia mutations commonly found in Indian population

with the help of two groups of primers (Table-1). Amongst these fifteen mutations nine mutations have shown very high prevalence in the patients of ß-thalassemia and the remaining six mutations are probably infrequent to be seen among the limited sample size (n=50). Group 1 and 2 primers have been able to identify mutations in only 87.87% of ß-thalassemia samples. The nine most common mutations found in this study have been also identified as common by Bharadwaj *et al* [20] but differed from those reported by Thedaswad *et al* [25]. The most prevalent mutation identified in all studies is cd 71/72 whereas cd 8/9 mutation is the lowest among all heterozygous ß-thalassemia mutations in this study. Same results have been observed in other Indian study by Varawalla *et al* [8] but this is in contrast to the Mediterranean region where IVSI-110 (G-A), IVSI-6 (T-C) are the predominant ß-thalassaemia mutations [20]

From the distribution of most appropriate -thalassaemia mutations profiles widely studied nationally at regional level, it has been postulated that the majority of mutations originate from major states of Western India including Maharashtra and Gujarat, North region (Uttar Pradesh) and East region (West Bengal) [26]. The ten most common ß-thalassaemia mutations including IVSI-5, IVSI-1, cd 41/42, cd 8/9, cd 15, cd 30, cd 16, cd 15, cd 27/28 and IVSI-1 are identified in the northern region of Indian subcontinent [26]. North Indian population is genetically heterogeneous and accounts for 50.7% of all ß-thalassaemia mutations whereas the Western region deviates from the national pattern where somewhat high prevalence of the IVSI-1 (8.7%) and cd 15 (7.6%) is seen as the fourth common regional mutation. The Eastern region of India including the states like West Bengal, Bihar, Orissa, Jharkhand exhibit higher prevalence of IVS I-5 at 71.4%, with cd30 and cd

15 the second and third most common alleles, accounting for 5.8% and 5.4% of the total prevalance respectively, followed by cd 41/42 with a prevalence of 4.3%. [27]. Four states of Southern India like Andhra Pradesh, Karnataka, Tamil Nadu and Kerala have a predominantly a population with 67.9% prevalence of IVSI-5 mutations whereas other second most common mutation is cd 15 (8.8%) [28].

There are a very few reports available on thalassemia mutations from Maharashtra state, in contrast to these reports. Our results have shown a marginal difference with respect to ß- globin gene mutations. Thus the data on molecular genetic testing from the population of low income community of Western Maharashtra regions provide a specific ß-globin gene mutation profiles. This could be highly effective in helping to screen and prevent ß-thalassaemia trait with the use of more efficient and cost effective protocols. Thus, the outcomes derived from the basic data collected in the present study would provide a platform on which future health care planning for the prevention and treatment of ß-thalassaemia in rural Maharashtrian community and sub-populations can be undertaken. This study will further be extended to prenatal analysis by molecular means and genetic counseling at population level. Also a baseline database of these diagnosed patients and families will be established with an intension to identify the primary support services to be provided to the families carrying beta thalassemia disorder. There are only few centers, which offer prenatal diagnosis of -thalassemia in Maharashtra. Therefore considering this matter of great importance, we could establish prenatal diagnosis services at the rural hospital and offer prenatal diagnosis. Additional studies are required to fully assess the ß-globin gene polymorphism in the

South –Western Maharashtrian population and to estimate the population's level of ß-thalassaemia mutations.

Conclusion:

Significant prevalence of the mutations different from those in other ethnic populations was seen in this small institution-based study. To the best of our knowledge, these are the first cases of ß-thalassaemia using DNA testing methods reported in rural Maharashtra. This study provides the pattern of ß-thalassemia mutations from rural population which will open a new avenue for implementation of molecular diagnostics for prenatal diagnosis and prevention of blood disorder by proper counseling in rural areas of western Maharashtra.

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