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Research article

Studies on molecular spectrum of beta thalassemia among residents of Chennai

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Abstract: Beta thalassemia is caused by a mutation in the human beta globin gene. More than 400 causative mutations have been characterized in the Hemoglobin Subunit Beta (HBB) gene. These causative mutations are present in the beta globin gene or the regulatory region. Though more than 400 causative mutations of HBB region have been described, rare and novel mutations are being reported in studies indicating the need for characterization of mutations in all regions and information regarding the same should be made available for successful implementation of prenatal diagnosis. The study aims to characterize the spectrum of beta thalassemia mutations in beta thalassemia heterozygous among residents of Chennai. A total of 5,207 cases were screened for beta thalassemia heterozygous by HPLC method. 387 beta thalassemia heterozygous identified by HPLC method were subjected to molecular DNA analysis by ARMS PCR technique and DNA Sanger sequencing for the characterization of causative beta thalassemia mutations. In the present study molecular characterization of beta thalassemia mutations revealed 30 different mutations with a high prevalence of IVS 1-5 (G-C) mutation, five new rare mutations viz., IVS II-1 (G>T), CD 37 TGG-TGA, IVS II 781 (C-G), CD114 CTG-CCG and Poly A (A-G) were diagnosed and reported first in India. One novel beta thalassemia mutation HBB.c319DelC was detected in the study. The diagnostic outcome of detecting the causative mutations for beta thalassemia imposes strong resources for developing easy and cheaper methods for prenatal diagnosis which will reduce the burden of disease.

Keywords: Beta Thalassemia; HPLC; Chennai; ARMS PCR; DNA sequencing

1. Introduction

Beta thalassemia is characterized by reduced synthesis of beta globin chains. This results in accumulation of α-globin chains, creating an imbalance between globin chain ratios. The severity of disease varies depends on the type of beta gene allele (β^o, β^+, β^{+++}). The relative interaction of β-globin chains with α-globin chains will mitigate the excess α-globin, thereby causing variable levels of severity of disease. The excess or unpaired free α-globin chains precipitates in the erythroblast cells causing hemolysis of red blood cells. Therefore, greater the free form of α-chains greater the severity of anemia and disease condition [1–3]. The causative mutations are present in the beta globin gene or the regulatory region [4].

The average carrier frequency for beta thalassemia in Africa is 13.3%. Beta thalassemia prevalence is high in Egypt in the range of 2.6%–4%. Every year in Egypt, it is anticipated that 1000 children with B-thalassaemia would be born[5]. Though, carrier frequency of beta thalassemia has been reported to be 10%, national prevention programs and prenatal diagnosis have reduced the rates to 4.0%–5.0% in recent studies [6].

Characterization of beta thalassemia mutation has a significant impact on the prevention of disease as diagnosis of silent atypical beta thalassemia carriers relies on molecular studies. Similarly, prenatal diagnosis of fetus is one of the future prospective of prevention program. This can be achieved by a large pool of data regarding the spectrum of mutations in all geographical regions. However, very few studies and reports are available about the causative beta thalassemia mutations in the state of Tamil Nadu. But due to the constant migration of people, the frequency of these disorders is increasing. Chennai is the fourth largest metropolitan city with increasing immigrant population from other parts of Tamil Nadu, other states and from other countries. The frequency of beta thalassemia ranges between 1 to 17% with an overall carrier frequency of 3%–4% in India, amounting to 30–40 million carriers [7]. A higher prevalence rate of 5%–17 % have been reported in some ethnic groups like Kuthis, Lohanas, Punjabis, Sindhis, few Muslims groups and in few tribal populations [8]. However, this varying frequency of beta thalassemia in different regions of India might be attributed to the unequal distribution of beta thalassemia gene in the Indian subcontinent [9]. Further, the beta thalassemia trait/heterozygous is observed in people without any ethnic relation to the disorder, due to population migration and intermarriage between different ethnic groups [10,11]. Thereby, the frequency and prevalence of hemoglobin disorders should be interpreted with caution due to heterogeneity of mutations. However, carrier frequency of thalassemia and hemoglobin variants is highly variable among different countries, with short geographical distances and even within small population groups [12]. Therefore, micro-mapping studies is required in thalassemia prevalent countries like India to identify the true burden of disease. On the other hand, immigration of people has made frequency of hemoglobin disorders worldwide for example in United Kingdom and America the frequency is very low but beta thalassemia is identified in immigrant population groups [13]. Similarly, there is continuous migration of different ethnic population to different regions of India. According to the 2001 census of India, migrants to Chennai city from parts of Tamil Nadu constitute 74.5% and migration from other parts of India is

23.8% and remaining 1.71% from other countries. The overwhelming reason for migration was job, carrier, business, education, marriages and transfer of jobs. Most of the migrants are younger people belonging to all genders. Chennai is the fourth largest metropolis, with emergent services in healthcare, employment and education is also the probable reason for migration. The impact of migration of hemoglobinopathy carriers from endemic areas to new regions along with widespread consanguineous marriage in the South Indian population has put forth the real need for screening.

The population of India as of mid-2019 is 1.39 billion [14]. According to 2011 census of India, the total population of Chennai metropolitan region is 8.65 million. The urban group comprises of < 30% of the population living in 200 towns and cities, while most people more than 70% live in rural areas in more than 550,000 villages. There is considerable ethnic, linguistic, religious cultural and genetic diversity [7]. The prevalence of beta thalassemia carriers in India is 5.3%–17.0% than the average 3%–4% estimated rate [15]. The clinical spectrum of the disorders varies from asymptomatic conditions to serious disorders like thalassemia major that require regular blood transfusions and extensive medical care [16]. Beta thalassemia homozygous causes severe hemolytic anemia requiring lifelong blood transfusions. Approximately 100,000 children are born annually with beta thalassemia syndrome. It has been estimated that around 7,500 to 12,000 beta thalassemia major affected children will be born annually in India of which only 5%–10% of affected children receive regular blood transfusion, follow-ups, iron chelation, quality of life is affected very much, where majority of people do not receive much considerable care. India spends nearly Rs.1000 crores per annum on the treatment of thalassemia patients [17]. Complications of iron overload, anemia, ineffective erythropoiesis, side effects of iron-chelating drugs may lead to mortality of the child [15]. Thus, the birth of a thalassemic child causes considerable physical and economic strain, not only on the affected child and its family, but also on the community and nation at large. Beta thalassemia heterozygotes have shown different degrees of ineffective erythropoiesis, leading to varying levels of iron absorption. The level of iron absorption depends on the degree of ineffective erythropoiesis [1]. However, it is evident that impact of anemia is caused either individually or in coexistence with other conditions including nutritional deficiency, parasitic infections, chronic infections, poor absorption of nutrients etc. The inherited or acquired disorders of hemoglobin; hemoglobinopathies and thalassemia also impose a role in the prevalence of anemia [18]. Though it is stated that beta thalassemia carriers has a selective advantage over iron balance, coexisting iron deficiency anemia condition and coexisting iron overload is not uncommon in India [19]. However, studies related to iron deficiency anemia among beta thalassemia condition has not been analyzed in South India and there is paucity of information regarding the iron status in these individuals. Despite, it is important to analyze the clinical diagnostic findings and iron status of beta thalassemia carriers before making a treatment decision. Hence, it is proposed to evaluate iron status among beta thalassemia carriers as an attempt to accomplish the future needs for proper diagnosis and management of disease.

Awareness program of thalassemia, prevention of birth of thalassemia major child by population screening, premarital screening, prenatal screening, and prenatal diagnosis before 11 weeks of gestation will control the affected thalassemia child birth [15]. For prenatal diagnosis to offer vast information regarding the causative mutations even in areas with small geographical distances is required. Therefore, it is proposed to investigate the spectrum of beta thalassemia mutations in beta thalassemia heterozygous among residents of Chennai.

2. Material and methods

In the present study, a total of 5,207 cases were analyzed. The study was conducted from the year 2014–2018. The study was conducted among patients referred by clinicians who have family suspicion of hemoglobinopathy, severe anemia cases and antenatal cases for screening of hemoglobinopathy. The samples were collected from 32 collection centers of Hitech Diagnostic Centre in various parts of Chennai. The study was approved by the Institutional Human Ethics Committee (IHEC), Hitech Diagnostic Centre, Chennai. Written consent was taken from all the patients for using their leftover samples for research purposes. Details regarding history of blood transfusion, relevant family and clinical history, epidemiological data pertaining to age, gender, ethnic origin, community,native language, and clinical history were collected.

Table 1. Internal control and common primers.

Internal control	Common primers
Internal control 1 (InC1)	5' CAA TGT ATC ATG CCT CTT TGC ACC3'
Internal control 1 (InC2)	5' GAG TCA AGG CTG AGA GAT GCA GGA3'
Common Primer (C1)	5' ACC TCA CCC TGT GGA GCC AC 3'
Common Primer (C2)	5' CCC CTT CCT ATG ACA TGA ACT TAA 3'

Quantitative enumeration of differently formed elements of blood and hemoglobin in whole blood by automated cell counter by sysmex XN-2000, sysmex corporation Kobe, Japan were done. Iron studies analysis is done by determination of iron level, total Iron binding capacity and ferritin measurement. Quantitative estimation of Iron and TIBC is done in automated equipment using Roche Cobas 8000. Ferritin levels were measured by ELISA method.

Quantification of hemoglobin fractions and hemoglobin variant analysis was done by D-10 hemoglobin testing system (Bio-Rad Laboratories CA, USA) using HbA2/F/A1c dual program kit. Among 5207 cases, 387 beta thalassemia heterozygous identified by HPLC method were subjected to molecular DNA analysis. Initial screening of common beta thalassemia mutations was done by ARMS PCR technique. Samples that showed negative for these mutations were subjected to DNA Sanger sequencing to identify the causative beta thalassemia mutation. Genomic DNA was extracted using the Qiagen blood DNA extraction kit. DNA mutation analysis was carried out based on Amplification Refractory Mutation System (ARMS). The primer sequence for the analysis was shown in Table 1 and 2 [20].

PCR was performed in veriti thermal cycler, applied biosystems. Emerald Amp GT PCR (Takara Clon Tech) was used as a master mix. PCR procedure was done as per good laboratory practice. Two vials for the detection of mutation one tube for normal N and other mutant M were taken. 12.5 µl of emerald master mix was added to 1.5ml micro centrifuge vials. 5.0ul of dH_2O molecular grade was added to both the N and M vials. 1.0 µl of internal control primer 1, internal control primer 2 was added to normal and mutant vials. 1.0 µl of C1 or C2 primer was added to normal and mutated vials appropriately depending on the mutation to be he normal vial.1.0 µl of mutant primer was added to the mutant vial. 5.0 µl of genomic DNA was added to both the vials. The PCR program for the

amplification was carried out. Denaturation at 94°C for 5minutes, annealing at 63°C for 1minute. 25 cycles of extension at 72°C for 1.30 minutes, again denaturation at 94°C for 1 minute followed by 1 cycle annealing at 63°C for 1.0 minute was done. Final extension at 72°C for 3 minutes was performed. After PCR, the PCR products were electrophoresed on 1.5% agarose gel at 100V for 45 minutes. The gel is visualized under UV light. The interpretation was done based on the mutation specific band observed; the samples can be identified as normal, heterozygous or homozygous samples.

Table 2. Beta Thalassemia primers.

Sequence Name	Sequence 5' – 3'	Second Primer	Fragment Size /bp
IVS 1-5 (G-C)	N - CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	C1	285
	M- CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	C1	285
IVS 1-1 (G-T)	N- GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG	C2	454
	M- TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	C1	281
CD 41/42 (-CTTT)	N- GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	C1	443
	M- GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	C1	439
CD 8/9 (+G)	N-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	C1	214
	M-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	C1	215
CD 15 (G-A)	N-TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG	C2	500
	M-TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA	C2	500
Cap site +1 (A-C)	N-ATA TGT CAG GGC AGA GCC ATC TAT TGG TTA	C2	582
	M-ATA TGT CAG GGC AGA GCC ATC TAT TGG TTC	C2	582
CD 30 (G-C)	N- 5' TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC	C1	280
	M- 5'TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG	C1	280
CD 30 (G-A)	N- 5' TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC	C1	280
	M- 5'TAA ACC TGT CTT GTA ACC TTG ATA CCT ACT	C1	280
IVS 1-1 (G-A)	N- TTA AAC CTG TCT TGT AAC CTT GAT ACC CAC	C1	281
	M- TTA AAC CTG TCT TGT AAC CTT GAT ACC GAT	C1	281
CD 5 (-CT)	N- ACA GGG CAG TAA CGG CAG ACT TCT CCG CAG	C1	204
	M- ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA	C1	202

DNA Sequencing: Direct DNA sequencing is the method of choice to identify rare and novel mutation. In the present study, mutations which are not detected using ARMS PCR protocol were subjected to DNA sequencing. Human beta globin gene was sequenced to identify the causative mutations. DNA Sequence analysis of the complete HBB region was performed. Three sets of overlapping primers were designed to amplify the complete DNA sequence. Hence, three PCR wereperformed for each sample. Primer designing was done using online primer 3 software. Three sets of overlapping primers encompassing the complete HBB region were performed done.

First amplicon:
HBB For1 primer TGGAGCCACACCCTAGGGTT Tm – 62.5°C

HBB Rev1 primer	TGCAATCATTCGTCTGTTTCCCAT	Tm - 62°C
Second amplicon:		
HBB For2 primer	GAGTCTATGGGACGCTTGATG	Tm – 61.2°C
HBB Rev2 primer	GCTATTGCCTTAACCCAGAAATT	Tm – 61.2°C
Third amplicon:		
HBB For3 primer	ACTTTCCCTAATCTCTTTCTTTCAG	Tm – 60.9°C
HBB Rev3 primer	TTATGTTTTAAATGCACTGACCTCC	Tm – 60.9°C

The target region was amplified using HBB primer by PCR from the isolated DNA. Three tubes were processed for each sample. Three overlapping primers of the HBB gene were processed 12 µl of qiagen master mix, 2 µl of template DNA, 2 µl of forward and reverse primer was added and the final volume of PCR mix was made to 16 µl. PCR mix was added to 96 well plate and amplified in thermal cycler with initial denaturationat 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, 58°C annealing for 1 minute and extension at 72°C for 1 minute followed by final extension for 5 minutes. A single discrete PCR amplicon band of 600 bp was observed when resolved on 1.5% agarose gel. The PCR product was purified to remove contaminants. Cycle Sequencing: The purified PCR amplicon was sequenced using either forward or reverse primers. Sequencing was done by using BDT v3.1 cycle sequencing kit. Sequencing was done using ABI 3500 genetic analyzer. Data analysis was done using the online tool NCBI Blast and the mutations were identified [21]. Short nucleotide variations (SNVs) and single nucleotide polymorphism (SNPs) has been identified using sequence viewed BLAST. Genomic DNA sequences are used as input query sequence and variations identified from Human Genome Variation Society (HGVS). Reference SNP (rs) accession number from cited variants and clinical variations has been noted for each variation in the DNA sequence with pathological allele. The data were tabulated and statistically analyzed using statistical package for social sciences (SPSS Version 11.5).

3. Results

The screening and identification of beta thalassemia relies on hemoglobin fractions and RBC indices. Table 3 shows the gender wise distribution of mean hemoglobin fractions and RBC indices among beta thalassemia heterozygous. Statistical tool - Students t test is used to compare the mean differences between male and female genders. HbA2 the diagnostic marker was around 5.3%, similar in both male and female cases and the results are not statistically significant. The mild elevation of HbF among females was noted as the screening cases include pregnant cases. RBC count, hemoglobin and hematocrit levels are elevated in male than female cases and the results are statistically significant ($p<0.01$). Female beta thalassemia trait shows lower hemoglobin level of 9.84 g/dl compared to 11.52 g/dl in males.

Table 4 shows the distribution of beta thalassemia mutations. Molecular DNA analysis of beta globin gene was done in two phases. In the first phase all the 387 beta thalassemia heterozygous cases was subjected to ARMS PCR protocol to identify the common mutations. Eight types of beta thalassemia mutations and deletions were identified using ARMS PCR protocol which encompasses 336

cases of about 86.8%. In the second phase, remaining 51 cases which are not identified by ARMS PCR were subjected to DNA Sanger sequencing technology to identity the rare and novel mutations. Among 387 beta thalassemia heterozygous, 30 different types of mutations were detected of which 7 mutations and 1 deletion were identified by ARMS PCR and 22 mutations have been identified using DNA sequencing method. 4 cases about 1.0% of cases remained uncharacterized without any causative gene mutation. Among 387 beta thalassemia heterozygous investigated for mutation analysis, 70.8% cases showed IVS 1-5 G-C mutations, 619 bp deletion encounters about 23 cases accounting to about 5.94%, 17 (4.40%) cases of CD15 G-A mutations were observed. PCR gel documentation is shown in Figure 1. Three mutations CD30 G-C, IVS 1-130 G-A and CD41/42 were identified in 21 cases, each mutation comprising about 7 (1.80%) cases. Poly A (T-C) mutation was found in 6 cases (1.60%). Similarly, 15 cases covered another 3 mutation with equal frequency of 1.30% each of which comprises, CD121 GAA-TAA, IVS 1-1 G-T and Codon 16(-C). Mutations CD 8/9, IVS 1-130 G-C and -88 (C-T) mutations cover up to 1.5% of the total beta thalassemia heterozygous study group and each mutation has a frequency of 0.5%. Fifteen different mutations have been identified in 15 cases encompassing one case each with a frequency of 0.26%. Of the 15 cases, each case was identified with one mutation, one novel mutation HBB: c319 Del C was identified in the study. This novel mutation was characterized by DNA sequencing of the HBB gene. One case was identified with HbD homozygous hemoglobin variant, however the HPLC chromatogram and hematological variables showed features of beta thalassemia heterozygous. One case showed Poly A (A-G) in homozygous state, HPLC data showed elevated HbA2 of 10.2% which is high, compared to HbA2 of other beta thalassemia mutations. The sequence analysis data for each genomic DNA query sequence with their causative mutation / SNP was downloaded for all the 22 different types of mutations and shown in Figure 2 for few mutations. The diagnosed beta thalassemia mutations were classified into twelve types based on the classification, the identified mutations were categorized. It is of interest to note that all types of mutations have been observed in the present study.

Table 3. Gender wise distribution of hematological and biochemical analytes among beta thalassaemia heterozygous (n=387).

SNO	Parameters	Male=157		Female=230		t	Significance
		Mean	SD	Mean	SD		
1	HbF (%)	0.97	0.91	1.25	1.13	−2.56	<0.05
2	HbA0 (%)	81.41	2.41	81.66	2.39	−1.02	NS
3	HbA2 (%)	5.37	0.68	5.28	0.74	1.25	NS
4	RBC×10^6 cumm	6.04	1.11	5.17	0.81	8.87	<0.001
5	Hb (g/dl)	11.52	1.95	9.84	1.34	9.40	<0.001
6	HCT (%)	39.02	6.81	33.55	4.68	8.76	<0.001
7	MCV (fl)	65.77	9.39	65.35	6.68	0.94	NS
8	MCH (pg)	19.38	2.75	19.17	1.72	0.70	NS
9	MCHC (g/dl)	29.54	1.83	29.36	1.95	0.95	NS
10	RDW CV (%)	18.46	2.77	18.12	3.18	1.11	NS

Note: p<0.01 Significant at 1% level; p<0.05 significant at 5% level; NS- Not significant at 5% level.

Table 4. Distribution of beta thalassemia mutations among beta thalassaemia heterozygous (n=387).

SNO	Name of mutation (Common Names)	Mutation Region	Allele Type	rs number	HGVS nomenclature	n	%
1	IVS 1-5 G-C	Intron 1	β0	rs33915217	HBB:c.92+5G>C	274	70.8
2	619 bp del		β0		NG_000007.3:g.71609_72227 Del619	23	5.94
3	CD 15 G-A	Exon 1	β0	rs63750783	HBB:c.47G>A	17	4.4
4	CD 30 G-C	Intron 1	β0	rs33960103	HBB:c.92G>C	7	1.8
5	IVS 1-130 G-A	Intron 1	β0	rs33943001	HBB:c.93-1G>A	7	1.8
6	CD41/42	Exon 2	β0	rs281864900	HBB:c.126_129delCTTT	7	1.8
7	POLY A (T>C)	3'UTR,Poly A	β++	rs33978907	HBB:c.110T>C	6	1.6
8	CD121GAA-TAA	Exon 3	Dominant	rs33946267	HBB:c.364G>T	5	1.3
9	IVS 1-1 G-T	Intron 1	β0	rs33971440	HBB:c.92+1G>T	5	1.3
10	CODON 16 (-C)	Exon 1	β0	rs35662066	HBB:c.51delC	5	1.3
11	IVS II-1 G-A	Intron 2	β0	rs33945777	HBB:c.315+1G>A	3	0.8
12	CD 8/9	Exon 1	β0	rs35699606	HBB:c.27_28insG	2	0.5
13	IVS 1-130 G-C	Intron 1	β0	rs33943001	HBB:c.93-1G>C	2	0.5
14	IVS II-837 T-G	Intron 2	Unclear	rs35703285	HBB:c.316-14T>G	3	0.8
15	-88(C-T)	Promoter Mutation	β++	rs33944208	HBB:c.-138C>T	2	0.5
16	IVS II-1 G-T	Intron 2	β0	rs33945777	HBB:c.315+1G>T	1	0.26
17	CD 30 G-A	Intron 1	β0	rs33960103	HBB:c.92G>A	1	0.26
18	CD 37 TGG-TGA	Exon 2	β0	rs33974936	HBB:c.114G>A	1	0.26
19	CD39 C-T	Intiation Codon	β0	rs11549407	HBB:c.118C>T	1	0.26
20	CD106 CTG-CCG	Exon 3	Dominant	rs33941844	HBB:c.320T>C	1	0.26
21	CD114 CTG-CCG/Hb Durham	Exon 3	Dominant	rs36015961	HBB:c.344T>C	1	0.26
22	HBB:.C319 Del c		Novel	rs63750596	Novel	1	0.26
23	INI CD ATG>ACG	Initiation Codon	β0	rs33941849	HBB:c.2T>C	1	0.26
24	IVS II - 654 C>T	Intron 2	β0 / β+	rs34451549	HBB:c.316-197C>T	1	0.26
25	IVS II - 781 C>G	Intron 2	β+	rs193922560	HBB:c.316-70C>G	1	0.26
26	-223 (T-C) / -28 (A>G)	Promoter Mutation	Unclear /β+	rs139703273/rs33931746	HBB:c.-273T>C/HBB:C-78A>G	1	0.26
27	-28 (A-G)	Promoter Mutation	β+	rs33931746	HBB:c.-78A>G	1	0.26
28	-90 C-T	Promoter Mutation	β+	rs34999973	HBB:c.-140C>T	1	0.26
29	POLY A (A-G) HOMO AATAAA - AATGAA	3'UTR, Poly A	β++	rs63751128	HBB:c.*111A>G	1	0.26
30	CD121 GAA - CAA(HB D Punjab Homozygous)	Exon 3	B chain variant	rs33946267	HBB:c.364G>C GC HOMO	1	0.26
31	Uncharacterized			--	NA	4	1
	Total					387	100

Footnote of Table 4: Type of Beta globin gene defect and its frequency.

SNO	Type	Frequency/%
1	Promoter mutations - Transcriptions	1.28
2	Splice junction -m RNA Processing	6.46
3	Consensus Splice Site Mutations	70.8
4	Cryptic Splice site- mRNA Processing	1.06
5	RNA cleavage- Poly(A) signal (RNA Processing)	1.86
6	Initiation Codon - Translation	0.52
7	Nonsense codon -Translation	5.96
8	Frame Shift-Translation	3.6
9	Missense codon- protein structure	0.78
10	Splice Donor variant	0.26
11	Intron Variant	0.26
12	Deletions	6.2
13	Uncharacterised	1
14	Total	100%

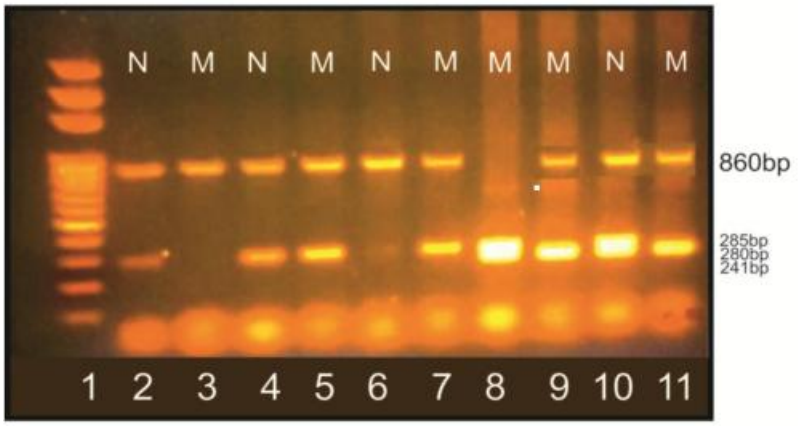

Figure 1. Detection of IVS 1-5 (G-C) mutation, 619 bp deletion and CD 30 (G-A) by ARMS PCR method.

Lane 1: 100 bp DNA ladder.860 bp fragment is an internal control. Lane 2 & 3: Control for IVS 1-5 (G-C) mutation marked by presence of 285 bp amplified product by normal specific primer in lane 2 and absence of amplified product by mutant specific primer in lane 3. Lane 4 & 5: Heterozygous IVS 1-5 (G-C) mutation marked by presence of amplified product of 285 bp by both the primers; normal specific primer in lane 4 and mutation specific primer in Lane 5. Lane 6 & 7: Homozygous IVS 1-5 (G-C) mutation marked by presence of amplified product of 285 bp by mutation specific primer in Lane 7 and absence of amplified product by normal specific primer in lane 6. Lane 8: Heterozygous -619bp deletion marked by the absence of internal control of 861 bp and presence of amplified product of 241 bp. Lane 9 : Homozygous -619bp deletion marked by the presence of both internal control of 861 bp and amplified product of 241 bp. Lane 10&11: Heterozygous CD 30 (G-A) mutation marked by presence of

amplified product of 280 bp by both the primers; normal specific primer in lane 10 and mutation specific primer in Lane 11.

Table 5. Mean hemoglobin fractions of beta thalassaemia heterozygous among various type of mutations (n=387).

SNO	Name of mutation (Common Names)	Mean Hemoglobin fractions		
		Hb F (%)	HbA0 (%)	HbA2 (%)
1	IVS 1-5 (G-C)	1.03	81.72	5.18
2	619 bp del	1.14	81.33	5.92
3	CD 15 (G-A)	1.61	80.93	5.84
4	CD 30 (G-C)	2.39	80.41	5.39
5	IVS 1-130 (G-A)	1.50	78.99	5.51
6	CD41/42	1.72	81.11	5.85
7	POLY A (T>C)	1.10	82.62	4.30
8	CD121GAA-TAA	1.66	79.52	5.72
9	IVS 1-1 (G-T)	1.86	81.06	5.34
10	CODON 16 (-C)	0.54	82.08	5.56
11	IVS II-1 (G-A)	0.93	81.47	6.13
12	CD 8/9	2.10	80.80	6.05
13	IVS 1-130 (G-C)	3.20	79.40	5.75
14	IVS II-837 (T-G)	1.86	79.86	5.56
15	-88(C-T)	1.30	82.15	5.20
16	IVS II-1 (G-T)	5.60	80.80	4.70
17	CD 30 (G-A)	0.00	79.90	5.20
18	CD 37 TGG-TGT	0.80	83.60	4.20
19	CD39 (C-T)	0.90	83.30	5.20
20	CD106 CTG-CCG	0.80	83.00	5.30
21	CD114 CTG-CCG/Hb Durham	1.10	81.30	4.40
22	HBB:.C319 Del c	0.00	86.70	4.60
23	INI CD ATG/ACG	3.10	78.90	8.00
24	IVS II - 654 (C-T)	1.50	81.20	6.00
25	IVS II - 781 (C-G)	0.00	80.30	6.00
26	-223 (T-C) / -28 (A>G)	1.10	82.80	6.10
27	-28 (A-G)	0.80	81.70	6.30
28	- 90 (C-T)	0.80	81.80	5.10
29	POLY A (A-G) HOMO	1.80	78.70	10.20
30	HB D	0.90	82.10	5.60
31	Uncharacterized	0.80	84.15	4.58
	F value	2.419	1.147	5.757
	Significance	P<0.01	NS	P<0.01

Note: p<0.01 Significant at 1% level; p<0.05 significant at 5% level; NS- Not significant at 5% level.

Table 5 shows the mean hemoglobin fractions of 387 beta thalassemia heterozygous with various HBB mutations. In both β+ and β^o thalassemia, HbF levels are elevated. In the present study one way analysis of variance (ANOVA) statistical tool was used. Hemoglobin F value ranged from 0 to 5.6%. IVS II-1 (G-T) a β^o thalassemia mutation showed elevated HbF of 5.60% compared to all HBB mutations in the present study. Overall, there is significant association between HbF levels and the type of mutation. However significant changes or differences in HbF levels are associated between IVS 1-5 (G-C) mutation, CD 30 (G-A) and CD 30 (G-C) mutation. The diagnostic marker HbA2 of more than 4.0% is considered as beta thalassemia heterozygous. HbA2 levels have a significant association between the types of mutation.

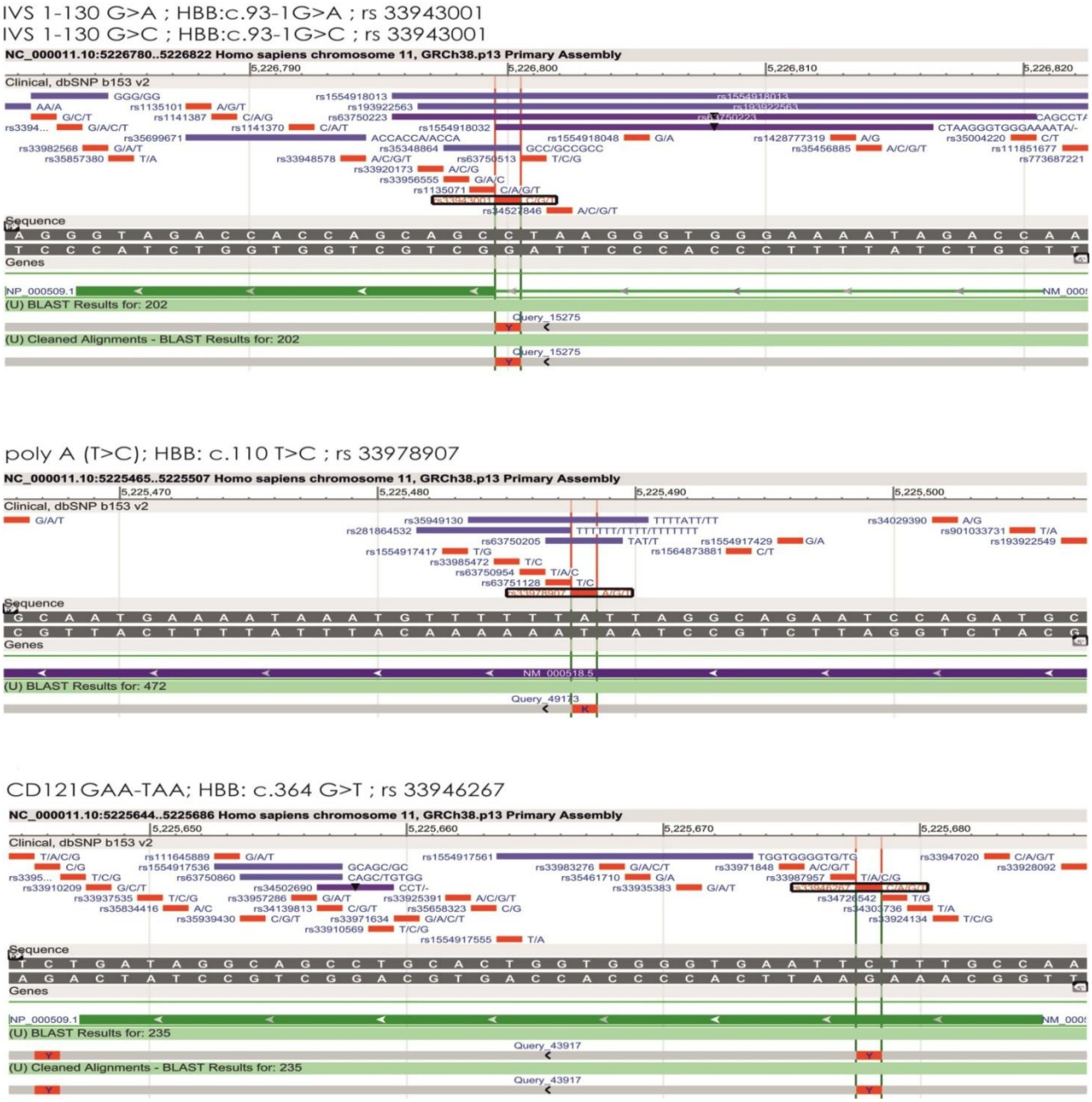

Figure 2. DNA sequence results of HBB Gene Mutation.

Table 6. Mean RBC Indices of beta thalassaemia heterozygous among various type of mutations (n=387).

SNO	Name of mutation (Common Names)	Mean RBC Indices RBC×10^6 cumm	Hb (g/dl)	HCT %	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (CV %)
1	IVS 1-5 (G-C)	5.55	10.59	36.07	65.44	19.20	29.40	18.06
2	619 bp del	5.60	10.48	34.49	62.26	18.97	30.03	17.99
3	CD 15 (G-A)	5.56	10.52	35.02	63.45	19.06	30.11	18.54
4	CD 30 (G-C)	5.81	10.90	37.54	65.44	18.87	28.93	18.67
5	IVS 1-130 (G-A)	5.83	10.83	38.04	65.34	18.54	28.44	19.51
6	CD41/42	6.13	11.24	38.10	62.17	18.32	29.48	18.10
7	POLY A (T>C)	5.08	10.42	35.70	70.10	20.03	28.65	15.88
8	CD121GAA-TAA	4.27	8.28	28.82	75.32	22.20	29.44	21.86
9	IVS 1-1 (G-T)	5.76	10.26	33.82	59.14	17.92	30.36	18.88
10	CODON 16 (-C)	6.04	11.46	39.14	65.10	18.98	29.22	18.18
11	IVS II-1 (G-A)	6.61	11.33	40.57	61.87	17.20	27.87	18.97
12	CD 8/9	5.25	10.30	34.00	64.65	19.60	30.40	19.10
13	IVS 1-130 (G-C)	5.25	9.40	30.55	58.25	17.90	30.75	18.45
14	IVS II-837 (T-G)	5.40	9.66	32.26	59.83	17.90	29.96	17.33
15	-88(C-T)	4.66	9.90	33.35	71.50	21.20	29.75	16.40
16	IVS II-1 (G-T)	4.45	9.40	28.90	64.90	21.10	32.50	25.20
17	CD 30 (G-A)	5.77	10.70	35.30	61.20	18.50	30.30	16.70
18	CD 37 TGG-TGT	5.84	12.00	41.00	70.20	20.50	29.30	14.50
19	CD39 (C-T)	4.77	9.20	29.90	62.70	19.30	30.80	15.60
20	CD106 CTG-CCG	5.10	9.80	32.40	63.50	19.20	30.20	16.70
21	CD114 CTG-CCG	2.47	4.80	16.20	65.60	19.40	29.60	31.60
22	HBB:.C319 Del c	3.49	7.90	30.70	88.00	22.60	25.70	27.90
23	INI CD ATG/ACG	3.49	6.40	22.30	64.50	18.30	28.40	29.60
24	IVS II - 654 (C-T)	5.58	11.50	38.60	69.20	20.60	29.80	14.70
25	IVS II - 781 (C-G)	5.03	8.80	36.50	72.60	17.50	24.10	20.50
26	-223 (T-C)/-284 (A>G)	4.41	10.10	36.90	83.70	22.90	27.40	14.10
27	-28 (A-G)	6.43	13.00	46.40	72.20	20.20	28.00	20.10
28	- 90 (C-T)	5.26	9.40	32.80	62.40	17.90	28.70	22.20
29	POLY A (A-G) HOMO	5.15	7.70	25.60	49.70	15.00	30.10	22.40
30	HB D	6.38	13.80	46.70	73.20	21.60	29.60	16.10
31	Uncharacterized	4.35	9.83	33.08	90.98	26.05	29.28	19.50
	F value	1.595	1.042	1.509	4.872	4.196	0.824	1.103
	Significance	NS	NS	p<0.05	p<0.01	p<0.01	NS	NS

Note: p<0.01 Significant at 1% level; p<0.05 significant at 5% level; NS- Not significant at 5% level.

Table 6 shows the mean RBC indices of beta thalassemia heterozygous among various types of mutations. RBC count levels range from 4.27×10^6–6.61×10^6cumm among beta thalassemia mutations. In the present investigation, one way analysis of variance (ANOVA) statistical tool was used. The rule 3 of hematology to assess RBC count i.e. RBC multiplied by 3 will be equal to hemoglobin, however in all beta thalassemia cases RBC count was relatively high with that of hemoglobin levels among all beta thalassemia mutations indicating microcytic hypochromic anemia. Hemoglobin levels range from 4.80–13.80 g/dl. HbD structural variant showed normal hemoglobin of 13.8 g/dl. CD114 CTG-CCG mutation showed the lower hemoglobin level of 4.80 g/dl. The diagnostic criteria of beta thalassemia heterozygous rely on microcytosis, hypochromia and HbA2 values. Hence all beta thalassemia heterozygous cases irrespective of the causative mutation showed reduced MCV and MCH. RDW levels vary ranging from 14.10%–31.60%. Among RBC indices HCT, MCV and MCH shows significant association between the type of mutation, other parameters RBC count, hemoglobin, MCHC and RDW showed non-significant results ($p>0.05$).

Table 7. Mean concentration of biochemical variables among beta thalassemia heterozygous in various type of mutations (n=387).

SNO	NAME OF MUTATION (Common Names)	Mean Biochemical Variables		
		IRON (µg/dl)	TIBC (ug/dl)	Ferritin (ng/ml)
1	IVS 1-5 G-C	77.67	338.76	133.79
2	619 bp del	91.86	322.00	194.68
3	CD 15 G-A	77.62	359.71	131.88
4	CD 30 G-C	69.75	344.40	166.95
5	IVS 1-130 G-A	92.41	409.25	125.15
6	CD41/42	91.00	316.50	60.28
7	POLY A (T>C)	77.97	331.33	69.31
8	CD121GAA-TAA	35.55	156.00	395.65
9	IVS 1-1 G-T	37.28	366.00	57.22
10	CODON 16 (-C)	81.72	407.50	33.90
11	IVS II-1 G-A	68.05	372.00	64.15
12	CD 8/9	22.90	363.00	6.24
13	IVS 1-130 G-C	54.85	334.50	52.25
14	IVS II-837 T-G	94.32	297.00	152.35
15	-88(C-T)	95.92	291.00	168.47
16	IVS II-1 G-T	47.50	312.00	18.48
17	CD 30 G-A	--	--	19.17
18	CD 37 TGG-TGT	78.29	399.00	23.08
19	CD39 C-T	65.30	292.00	29.02
20	CD106 CTG-CCG	--	--	18.74
21	CD114 CTG-CCG/Hb Durham	68.80	323.00	169.10
22	HBB:.C319 Del c	138.80	290.00	129.80
23	INI CD ATG/ACG	--	--	297.20

Continued on next page

SNO	NAME OF MUTATION (Common Names)	Mean Biochemical Variables		
		IRON (µg/dl)	TIBC (ug/dl)	Ferritin (ng/ml)
24	IVS II - 654 C-T	--	--	146.00
25	IVS II - 781 C-G	155.64	329.00	72.61
26	-223 T-C / -28 (A>G)	90.98	372.00	19.43
27	-28 (A-G)	53.89	310.00	106.60
28	- 90 C-T	--	--	46.63
29	POLY A (A-G) HOMO	62.07	226.00	260.60
30	HB D	32.70	214.00	104.00
31	Uncharacterized	77.93	303.25	210.48
	F value	0.722	1.102	0.476
	Significance	NS	NS	NS

Note: $p<0.01$ Significant at 1% level; $p<0.05$ significant at 5% level;NS- Not significant at 5% level.

Table 7 shows the mean concentration of biochemical variables among beta thalassemia trait in various types of mutations. In the present investigation, one way analysis of variance (ANOVA) statistical tool was used. Among the 32 types of mutation, iron, TIBC results were not available for few samples due to non-availability of samples. All beta thalassemia trait cases were processed for ferritin measurements. Iron and TIBC have diurnal variation. However,body iron status is checked by Iron, TIBC and ferritin levels. Iron levels vary from 22.90 to155.64 ug/dl. The mean ferritin in CD 8/9 mutation is as low as 6.24 ng/ml to 395.65 ng/ml in CD121 GAA-TAA mutation. One case showed two mutations -223 T>C and -28 (A>G), however the patient presented as carrier without past history of blood transfusion, the ferritn measurement in this case showed reduced level of 19.43 ng/ml indicating coexisting anemia condition. But Poly A(A-G) homozygous mutation with elevated HbA2 level showed Ferritin of 260.40 ng/ml. Hence, it is evident from the data that there is no significant association between the type of mutation and biochemical iron markers ($p>0.05$). Thereby, screening beta thalassemia carriers for iron markers irrespective of the causative mutation is recommended.

Table 8. Distribution of HbA2 fractions in beta thalassaemia heterozygous (n=387).

SNO	HB A2	n	%
1	4.0–4.50	40	10.34
2	4.51–5.0	107	27.65
3	5.01–6.0	194	50.13
4	More than 6.0	46	11.89
	Total	387	100.0

Table 8 shows about the distribution of HbA2 fractions among beta thalassemia heterozygous. Among 387 molecular tested beta thalassemia heterozygous samples, more than 50% (194 cases) showed HbA2 levels in the range of 5.0%–6.0%. 107 (27.65%) cases showed HBA2 in the range of 4.51%–5.0%. In remaining cases, HbA2 levels were in the range of 4.0%–4.5% and more than 6.0%. The diagnostic reliability of HbA2 measurement in diagnosing beta thalassemia heterozygous is variable. Hemoglobin variant HbD is identified by molecular analysis, however HPLC data showed HbA2 of

5.60%. Similarly, 4 cases uncharacterized for beta thalassemia mutations had HbA2 in the range of 4.0%–6.0%. Hence HbA2 measurement with molecular diagnosis is imperative to diagnose BTT.

Table 9. Religion wise distribution of beta thalassaemia heterozygous (n=387).

No	Religion	Beta thalassaemia heterozygous	
		n	%
1	Hindu	237	61.24
2	Muslim	62	16.02
3	Jain	49	12.66
4	Christian	26	6.72
5	Sindhi	7	1.81
6	Sikh	3	0.78
7	Marvadi	2	0.52
8	Sowrashtra	1	0.26
	Total	387	100

Table 9 shows the religion wise distribution of beta thalassemia. Among 387 beta thalassemia heterozygous cases, 61.24% covers Hindu religion, followed by 16.02% Muslims, 12.66% Jains, 6.72 % Christians, 1.81% Sindhis, 0.78% Sikh, 0.52% Marvadi and 0.26% Sowrashtra. Hindu population showed a higher distribution as the city of Chennai has higher proportion of Hinduresidents. The frequency distribution of BTT among various religions is in accordance with the relative population religion of Chennai [22].

Table 10. Characterization of beta thalassemia heterozygous residing in chennai based on their origin place (n=387).

Origin	n	%
Tamil Nadu	286	73.90
Chennai	151	52.80
Rest of Tamil Nadu (Immigrants)	135	47.20
Rest of INDIA (Immigrants)	101	26.10
Total	387	100.00

Table 10 shows the characterization of beta thalassemia heterozygous residing in Chennai based on their origin place. Among 387 beta thalassemia heterozygous, 286 (73.90%) cases showed origin place from Tamil Nadu and remaining 101 (26.1%) were from rest of India. Among 286 cases with origin of Tamil Nadu, 151 (52.8%) cases have Chennai as their origin place and 47.2% from rest of Tamil Nadu. This table explains the native and immigrant population from other cities of Chennai and other states of India.

4. Discussion

The population of India 2019 is around 1.36 billion. The current metropolitan area population of Chennai city is 9.1 million. The prevalence of beta thalassemia heterozygous in India is 5.3%–17.0%

than the average 3%–4% estimated rate. It has been estimated that around 7500 to 12,000 beta thalassemia major affected children will be born annually in India of which only 5%–10% of affected children receive regular blood transfusion, follow-ups, iron chelation to avoid iron overload and intact care [15]. An important health concern in India is the prevention of beta thalassemia through genetic counselling and prenatal diagnosis [23,24]. With this increasing population growth and increasing frequency of affected children, screening for inherited disorders of hemoglobinopathies and thalassemia in each state is of important needful factor. Thalassemia being an endemic disease, carrier frequency is highly variable, only micro-mapping studies will reveal the exact incidence of the disease. The incidence of beta thalassemia inTamil Nadu was reported as 4.0% [25]. However, very few studies and reports are available about the causative beta thalassemia mutations. But due to the constant migration of people, the frequency of these disorders is increasing. Chennai is the fourth largest metropolitan city with increasing immigrant population from other parts of Tamil Nadu, other states and from other countries. However, in the present study frequency of beta thalassemia mutations has been evaluated among residents of Chennai population.

In the present study different types of mutations have been identified of which 8 mutations are detected using ARMS PCR and remaining 23 mutations were diagnosed by DNA sequence analysis. 274(70.8%) cases showed IVS 1-5 (G-C) mutation. The results are in accordance with previous studies. IVS1-5 (G-C) is the commonest mutation identified in the present study. IVS 1-5 (G-C) mutations were the most frequent mutation throughout the country [26,27]. Studies in South India also reported higher frequency of IVS 1-5 (G-C) mutation [28–30]. Sinha et al., has reported this mutation with a prevalence of 67.9% in south India, similarly east, west and northern regions also accounted for 54.7% frequency of IVS 1-5 (G-C) mutation [31]. In a study conducted by Varawalla et al., 48 out of 59 cases in TamilNadu showed IVS 1-5 (G-C) mutation. This mutation was the commonest among Indian subcontinent [32]. The reason behind this is stated as consanguineous marriage within the same ethnic background might be the reason behind the occurrence of one predominant mutation [24]. However, it is evident that IVS 1-5 (G-C) mutation is not only the predominant mutation but also oldest beta thalassemia mutation in Indian sub-continent irrespective of the frequency levels among each region [32] As the study is conducted in Chennai which constitutes the dravidian population with dravidian languages as native tongue this mutation was more pronounced in hindus with tamil as native language The reason behind IVS 1-5 (G-C) mutation as the predominant mutation in Chennai is that this mutation might be the ancestral mutation in the dravidian population as stated [31]. Furthermore, IVS 1-5 (G-C) mutation was detected in our neighboring countries either as a predominant mutation or with a lower frequency rate. In a study conducted in California from newborn samples, IVS 1-5 (G-C) mutation was reported in Asian Indians, Chinese, Taiwanese and South East Asians [33]. Our neighboring countries Pakistan, Bangladesh and Iran have a high prevalence of IVS 1-5 (G-C) mutation [34–36]. The mutation was also reported with lower frequency in Saudi Arabia, Syria and Iraq [37–39].

In Iran, southeast region IVS1-5 (G-C) was found to be a common mutation [40]. Gene deletion causing beta thalassemia either down regulate the beta globin gene or the regulatory Locus Control Region (LCR). Major gene deletions are less prevalent in beta thalassemia in contrast to α-thalassemia which involves large deletions [2]. Of the few deletions reported, 619 bp deletion has been reported high in Sindhi and Punjab populations of India [23]. In the present study, 23 out of 387 cases (5.94%) showed 619 bp deletion. 619 bp deletion is of Indian and Asian origin causing β^o thalassemia. In the present study, 619 bp deletion was noted as the second predominant mutation, however, only one case of 619 bp

deletion was reported in earlier studies in Tamil Nadu [23,24]. Haplotyping analysis of 619 bp deletion has revealed the origin is from Sindh, however, gene flow through migration of population has shown higher frequency in the present study [32]. In earlier studies, 619 bp deletion was reported with a frequency of 7.28% in India [41]. Higher frequency rates were reported in north, central, and western India, however south and eastern India has shown a lower frequency rate [31]. In a study conducted among multi-ethnic Indian population, 619 bp deletion was not present from the state of Tamil Nadu, Kerala and Andhra Pradesh whereas 0.8% was reported in the state of Karnataka [26]. Similar to the present study, 619 bp deletion was reported as the 2nd common mutation in western India [42]. The incidence of this mutation in other parts of India was 12.6% in Gujarat and 1.7% in Maharashtra [27]. Edison et al., in his beta thalassemia analysis in the Indian population reported frequency of 619 bp deletion to be 22.2% in Gujarat, Haryana 25%, MadhyaPradesh 16.9%, Maharashtra 13.48% and Rajasthan 41.17% [28]. The state of Odisha showed absence of 619 bp deletion [43]. In Uttar Pradesh frequency of 2.5% has been reported [44]. 619 bp deletion the prevalent mutation of sindh and punjabi population was reported with a frequency of 18.7% among punjabis in a study conducted in Chandigarh [45]. From the above distribution and frequency, it is apparent that gene flow and population migration have exhibited varying levels of frequency throughout India. 17 (4.4%) cases showed CD 15 (G-A) mutation. This mutation has an Asian Indian, Pakistan and Japanese origin. In a study by Varawalla et al., CD 15 (G-A) mutation was reported among Asian Indians with higher prevalence among people originated from northwest Pakistan and none has been noted in Tamil Nadu [23]. Edison et al., reported 16.18% in Andhra Pradesh, 13.56% in Karnataka, 2.25% in Maharashtra, 6.38% in the state of Orissa, whereas Kerala, Punjab and Rajasthan have not presented with this mutation. Frequency distribution in Tamil Nadu for CD 15 (G-A) mutation was found to be higher with a frequency rate of 13.21% and second predominant mutation [28]. However, in the present study this mutation was the third predominant mutation with a frequency of 4.40%. Similarly Sinha et al., has reported this mutation in all parts of India [31]. Bashyam et al., has documented this mutation as 9% in Andhra Pradesh [29]. In a study among multi-ethnic Indian population, CD 15 (G-A) mutation was absent in the state of Tamil Nadu and Kerala, might be due to very small sample size in this region whereas it has been reported in western, northern and eastern India [26]. CD 15 (G-A) was reported as the 2nd predominant mutation in India [41]. In Maharashtra natives from Bombay region, high prevalence of 18% CD 15 (G-A) mutation was reported [46]. Though CD15(G-A) was reported with high frequency in Maharashtra state, review of research papers in India showed that this mutation was found to be one of the common mutations of beta thalassemia in India due to heterogeneous population nature in India [27,42,44,47–49]. Codon 30 (G-C) was observed in 7 (1.80%) cases. The frequency of this mutation in the present study was in accordance with earlier studies [31]. Nadkarni et al., has reported 3.348% in the Indian population [41]. However,in few studies, this mutation was not reported in Tamil Nadu [23,26,28,50]. Similarly. Kerala population was not presented with CD 30 (G-C) mutation and low frequency of 0.85% was noted in Karnataka [28]. Western India, including Maharashtra and Gujarat states, has reported a frequency of 4.2% [27]. 1.7% frequency was reported in Uttar Pradesh [44]. CD 30 (G-C) mutation prevalence is almost similar in all parts of India. However, frequency of less than 10% was noted from other regions of India. IVS 1-130 (G-A) mutation shows frequency of 1.80% in the present study which is higher than earlier studies. This rare mutation was reported in low frequency in the Indian population [28,41,51]. Sinha et al., in his review on the spectrum of mutation among the Indian population reported the absence of this mutation throughout the

country [31]. CD 41/42 was reported as the second predominant mutation in Tamil Nadu [23,24]. CD41/42 a south eastern asian and Indian ethnic origin mutation is prevalent among the Indian population. Earlier studies reported a frequency of 4.411% [41]. However, mutation frequency ranging from 0.8% to 37.5% has been noted in studies of India [26]. Sinha et al., review recorded 2.3% frequency in southIndia, with overall Indian frequency of 6.1% [31]. Edison et al., has reported 2.36% in Tamil Nadu, 0.74, 0.169 and 4.48% frequency were reported among Andhra Pradesh, Karnataka and Kerala respectively. Varying frequency in other states up to 11.54% has been reported. It has been noted next to IVS 1-5 (G-C), CD 41/42 mutation is present in almost all regions of India [28]. CD 41/42 was reported as 7.38% in north eastern region of India [52]. Other regional frequencies showed South Western Maharashtra (2.38%), Gujarat (6.9%), Uttar Pradesh (12.4%), Punjab (9.8%), West Bengal (3.58%) [27,44,47,49]. However, in Thailand CD 41/42 was reported as the predominant mutation [53]. DNA sequence analysis revealed 6 (1.60%) cases of Poly A (T-C) mutation. The mean HbA2 of this mutation is 4.30% ranging from 4.2 to 4.6% which is lowest among 30 types of characterized mutations. Similar to the present study, hematological variables among Poly A (T-C) mutation was reported with low HbA2 levels [54]. This data reveals that low HbA2 in Poly A (T-C) mutation should be diagnosed with care. DNA sequencing helped to identify the mild β^{++} thalassemia. Accurate sensitive diagnosis by molecular techniques is of prime importance along with hematological parameters to avoid misdiagnosis of beta thalassemia heterozygous. The frequency of Poly A (T-C) in the present study was 1.6% in accordance with earlier studies [51]. Frequency of 8% has been reported in the state of Karnataka [55]. Sinha et al., studies showed 4.7% frequency among south India and 0.4% among east India [31]. In earlier studies, Poly A (T-C) mutation was absent in Tamil Nadu / Kerala, but the frequency in Karnataka state was 1.16% similar to present study [27]. South Indian states reported 2.94% in Andhra Pradesh, 14.41% in Karnataka, 10.85% in Tamil Nadu, 5.97% in Kerala, 2.25% in Maharashtra, 2.13% in Orissa, 0.76% in West Bengal [28]. From the earlier studies, it was found that Poly A (T-C) mutation is highly prevalent in the state of Karnataka. South Indian states have shown moderate prevalence compared to lower frequency in other parts of the country. Due to smaller geographical distance and higher rate of migrants of people around this Dravidian originated states, has led to the increased distribution of this mutation in the southern region. 5 (1.30%) cases show this dominantly inherited CD121 GAA-TAA mutation. Gorakshakar et al., reported this mutation in India for the first time [56]. Later Colah et al., has reported one homozygous CD 121 GAA-TAA mutation [57]. 0.267% frequency of CD 121 (G-T) mutation was reported in India [41]. 1.6% prevalence of this mutation has been reported in the state of Karnataka, with an overall frequency of 0.08% in India [26]. The frequency of the present study was in concurrencewith earlier studies. CD121 GAA-TAA is a rare mutation in India, review of earlier studies revealed theabsence of mutation in various geographical regions of India. IVS 1-1 (G-T) mutation was noted in1.3% cases. In earlier studies, frequency in the range of 0.8% to as high as 23.5% have been reported [23,26]. In Sinha et al., review stated this mutation was absent in south India, with an overall frequency of 6.2% in India [31]. This mutation was also absent in the state of Andhra Pradesh [29]. Similarly, Edison et al., also showed the absence of this mutation in Andhra Pradesh and Kerala followed by 3.39% and 0.47% in Karnataka and Tamil Nadu respectively, whereas, Rajasthan, Sindh migrants, Punjab and Haryana have high prevalence of this mutation [28]. A frequency of 4.933% was reported in the Indian population [41]. In the present study 5 (1.30%) cases showed Codon 16 (-C) as the causative mutation for beta thalassemia. The mutation is of Asian Indian origin. This mutation shows elevated ferritin of 395.65 ng/ml compared to other mutations.

All the 5 cases had HbA2 in the range of 5.01 to 6.0%. Though the mutation ethnicity is of Asian Indian origin the frequency of this mutation is less than 1.0% in all parts of India except central India which had a frequency of 1.5% [31]. Varied levels of frequency in different states with an overall frequency of 2.1% in India have been reported [26]. CD 16 (-C) mutations were noted in Gujarat, Maharashtra, Sindhi migrants, Punjab and Haryana states [58]. CD 16 (-C) mutation was absent in Tamil Nadu as reported in earlier studies [23,24,28,30]. Though the frequency of CD 16 (-C) mutation was nil in Tamil Nadu it has been reported in southern states and certain western Indian region. IVS II-1(G-A) mutation was present in 3 (0.8%) cases with elevated HbA2 of 6.13%, mean RBC count was relatively high 6.61×10^6 cumm when compared with other beta thalassemia mutations in the present study. Regional profile of beta thalassemia mutations in India showed the absence of IVS II-1 (G-A) mutation indicating its lower frequency [31]. In Colah et al., studies, 0.08% prevalence of IVS II-1 (G-A) was noted in the state of Karnataka, other regions showed nil frequency of this mutation [26]. One case of IVS II-1 (G-A) was reported in Gujarat [58]. IVS II-1 (G-A) was reported as a rare mutation in urban east India [43]. This mutation was reported with lower prevalence in earlier studies [41,51]. The frequency of IVS II-1 (G-A) mutation in the present study is in line with earlier reported frequency in southern states [28]. Haplotype analysis revealed the origin of mutation in Turkey and invasion or immigration to Punjab with the same haplotype has been stated and gene flow of beta thalassemia is well explained [32]. Codon 8/9 mutationis present in 2 cases 0.50% in present study. The two cases had severe anemia with mean ferritin as low as 6.24 ng/ml.

CD 8/9 is stated to be the one of the common beta thalassemia mutations among Sindhis [58]. Similarly,CD 8/9 has been reported with increased frequency among sindh population, whereas 619 bp deletion is the predominant mutation of sindh followed by CD8/9 mutation [23,58].

It has been noted that this CD 8/9 mutation is less frequent in south Indian population. Many studies are in accordance with the reported frequency of 0.85% among Karnataka and absence of this mutation among 3 south India states Andhra Pradesh, Keralaand Tamil Nadu [28]. 2 cases (0.5%) showed IVS 1-130 (G-C) mutation. This is a rare mutation observed with low frequency whereas in earlier studies higher frequency of 12.5% in Tamil Nadu was reported and low levels in Gujarat and Uttar Pradesh was noted [26]. Edison et al., reported this rare mutation in the state of Tamil Nadu while other states showed nil frequency [28]. Sinha et al., studies in India have not reported this mutation throughout India [31]. However this mutation was reported in our neighboring countries [59].IVS II 837 (T-G) is a rare mutation of Indian origin which is present in 0.5% cases. IVS II 837 (T-G) was reported among Asian Indians [23]. Nadkarni et al., has reported a 0.569% frequency of this mutation in India [41]. This rare mutation was reported in south Indian states of Andhra Pradesh and Karnataka [29,55]. In previous studies frequency of 7.63, 5.9 and 0.94% among Karnataka, Kerala and Tamil Nadu respectively were reported [28]. In other regions of India the frequency of IVS II 837 (T-G) mutation was absent. However, IVS II 837 (T-G) was the predominant mutation in Goa with a frequency of 53.8% followed by 4.0% in Karnataka and 0.3% in Maharashtra [26]. IVS II 837 (T-G) mutation was noted only in south India with a frequency of 3.0%. It can be stated that this mutation though rare is prevalent among south Indians and absent in other regions of India [31]. Thakur et al., reported this mutation in 2 cases from Karnataka state [60]. This rare mutation of Asian Indian origin is also reported in Saudi Arabia [61]. -88 (C-T) mutation has a lower frequency of 0.5% in the present study. Varawalla et al., has reported 1 case from Punjab [23]. Sinha et al., found this mutation only from north India around 2.5%, otherwise -88 (C-T) is absent in west, east, central and south India [31]. The

rare mutation was reported in 2 cases (0.16%) among Indian population [58]. Similarly, a very low frequency of 0.19% has been reported in India encompassing 4.17% in Haryana, 1.54% in MadhyaPradesh, 3.85% in Punjab, however, this mutation was not recorded in south Indian states [28]. So, it is evident that the mutation is prevalent in lower frequencies among north Indians, however, gene flow have made this mutation to be present among Chennai residents. Though not novel, IVS II-1 (G-T) mutation has been reported first in India in the present study. The mutation causes β^{o}thalassemia. This rare mutation has been identified in a 26 year female individual, Christian community of Chennai origin. In this case the HbF level was high 5.6%. It is of interest to note that two beta thalassemia heterozygous cases with this mutation reported earlier has exhibited higher HbF levels. The reason stated for this elevated HbF could be due to beta thalassemia unlinked to beta globin loci [62]. This mutation has been identified earlier in Surinam in a 13 year old Surinamese girl [62,63]. The Indo-Surinamese are the largest ethnic group residing in Suriname as the British sent many Indians to work in British colonies. After the independence of Surinam, many Indo-Surinamese immigrated to Netherlands. Thus, the immigrant population from India to Suriname and genetic drift might be the reason for the presence of IVS II–1 (G-T) mutation. However, IVS II- 1 (G-A) mutation which occurs in the same position by substitution of nucleotide A had a higher prevalence in India as reported in earlier studies [26,41]. However, epidemiological studies of beta thalassemia mutations, history, ethnic origin and haplotyping analysis will explain the presence of this mutation in India. In the present study, one (0.26%) case with CD 30 (G-A) mutation was present. This mutation is rare in the present study with low frequency. However,in earlier studies CD30 (G-A) was reported in Tamil Nadu, Andhra Pradesh and Gujarat region whereas Kerala and Karnataka showed nil frequency [28]. The average frequency of CD 30 (G-A) in India was 0.8% [26]. This mutation was absent in a large study covering geographical regions and states of India [31]. Low frequency of this mutation has been reported in various studies in India [27,41,51,58]. From the existing published data, mutation CD 30 (G-A) has a lower frequency and present sparsely in the Indian population. The ethnicity of CD 37 TGG-TGA mutation is Saudi Arabia. However, our neighboring country Pakistan has a lower frequency of CD 37 TGG-TGA mutation. In the present study, one case of CD 37 TGG-TGA was observed. This rare mutation to the best of knowledge and from review of literature, this is the first report in India. CD37 TGG-TGA though it is not a novel mutation; this rare mutation has been identified in the present study. A 23 years male individual presented with a beta thalassemia picture in HPLC. The countrywide distribution of CD 37 has been discussed. Boehm et al., reported the first novel CD 37 (G-A) mutation in Saudi Arabian family [64].This mutation was characterized in Saudi Arabians [38]. Vrettou et al., has noted this mutation among the population of Greece [65]. CD 37 (G-A) mutation has been reported in Jordan country of 6.3%frequency [66]. Christopoulos et al., has reported these mutations among Egyptians patients. [67]. This mutation was also reported in Brazil [68]. In India CD 39 (C>T) mutation is very rare and present in subminimal frequencies among the Indian population.

In the present study, only one case of CD 39 mutation has been noted. Several large volume and large sample sized studies also have not reported this mutation [27,28,31,41,51,58]. However, CD 39 (C-T) mutation was reported as 0.04% in the state of Karnataka, a reservoir of rare mutations [26]. Similarly, 12 rare mutations including CD 39 C>T mutation was reported in East India [43]. The origin of this mutation is Mediterranean and it has a high prevalence in Europe, Africa, Saudi Arabia and Asian Continents. In European countries, CD 39 (C-T) was reported as the second predominant mutation in a study in Greece and Romanian population [69,70]. In Algeria, CD39 was reported as the predominant

mutation in north east Algerian population [71]. In Brazil, CD 39 was reported as a common mutation [72]. In Saudi Arabia, the HBB gene spectrum reveals CD 39 mutation as one of the common and prevalent mutations [37,38,73]. Asian Countries including Iran and Syria, mutations were present in varying frequencies [74–76]. CD106 CTG-CCG mutation is a dominantly inherited beta thalassemia and present in 1 case. This mutation was first reported in 1972 [77]. Hb Southampton mutation at codon 106 is also called as Hb Casper has been reported in Argentinean boy with severe hemolytic anemia [78].

In 2013 this mutation was reported in a uruguayan woman [79]. To the best of my knowledge this mutation has not been reported in India. CD114 CTG-CCG mutation is a dominantly inherited beta thalassemia. In the present study, a 29-year female presented with severe anemia of hemoglobin 4.8 g/dl, HbA2 and HbF levels were 4.4 and 1.1%respectively. RDW-CV was high 31.6% due to coexistence of anemia. The patient showed microcytic hypochromic anemia along with malaria parasite was observed in smear which added interest to the research process. Beta thalassemia cases have selective advantage over malarial infection however it has been stated that dominantly inherited beta thalassemia does not have selective advantage over malaria and it is prevalent in malaria endemic population [2]. It is of interest to note that similar observation has been observed in the present study with features of beta thalassemia heterozygous with dominant inheritance and presence of malaria in the individual. In 1994, INT CD ATG/ACG mutation was identified in Durham as novel mutation [80]. Later it has been reported in Russian patients [81]. This rare mutation was first identified in India in the present study. In the present study 1 case, 0.26% of INT CD ATG/ACG mutation was observed and the frequency distribution was in accordance with earlier studies [28]. Though ethnicity of this mutation is Yugoslavian origin it is found in low frequency in India and it has been reported by many researchers. Shaji et al., has reported Int codon ATG >ACG mutation in his studies [51].

In earlier studies on beta thalassemia mutation analysis among Indian population from all regions revealed the presence of this mutation only in the state of Tamil Nadu with a frequency of 0.94%, other regions including south, east, west and north India does not present this mutation [28].

This is a rare mutation in India with a frequency of <1.0% noted. However, identification of these rare mutations is of great importance during prenatal diagnosis. IVS II 654 (C-T) mutation is observed in one case 0.26% in the present study. This is a rare mutation in India and has been reported in earlier studies [6,26,27,41,57]. However, in Tamil Nadu, this mutation was not reported in previous studies. In the present study 0.26% frequency of IVS II 781 (C-G) mutation was observed. This rare mutation was first reported in India in the present study. Henderson et al., reported this mutation for the first time in U.K. [82]. A 30 year female individual presented with -223 (T-C) and -28(A-G) HBB mutations. The patient presented with HbA2 of 6.1% with microcytic hypochromic anemia. Both beta thalassemia causative mutations are involved in the transcription process in the promoter region. -223 (T-C) of unknown phenotype and -28 (A-G) mutation with mild β+ phenotype together presented as a typical beta thalassemia carrier in HPLC analysis. The mutations are rarer in India. Compound heterozygous mutation presented as a heterozygous carrier is a rare condition. The patient has not undergone any blood transfusions and presented with mild anemia. Once again it is of interest to describe that -28 (A-G) promoter mutation is also of south Indian type with prevalence in southern parts of India. Regional state-wise characterization of beta thalassemia mutation by authors has revealed the presence of -28 A-G mutation among south Indian population [28,31]. Gorakshakar et al., reported this rare Chinese origin mutation -28 (A-G) first in India [56]. However, -223 (T-C) mutation was first reported in Uttar Pradesh [83]. To the best of my knowledge, this mutation was not reported in other states of India and

the present study has identified this mutation in Chennai, Tamil Nadu. Coexistence of two beta thalassemia mutations is a rare occurrence, however the patient presented with typical characteristics of beta thalassemia heterozygous. -90 (C-T) is also a rare mutation which is noted in the present study in one case (0.26%). This rare mutation was reported in India in the state of West Bengal and Kerala [28]. Sinha et al., has noted 0.8% frequency in east India [31]. This mutation was noted with 0.089% frequency in India [41]. A 29-year female presented with elevated HbA2 of 10.2% and 1.8% HbF with severe MCHC anemia. DNA sequencing analysis revealed Poly A (A-G)AATAAA>AATGAA mutation in homozygous state. However, the patient presented herself with features of β thalassemia carrier. Poly A (A-G) mutation affects the 3' UTR region leading to cleavage of mRNA transcripts. This unstable mRNA transcript leads to a less severe form of β^{++}thalassemia. Reduced mRNA cleavage and formation of unstable mRNA might be the reason behind mild beta thalassemia features even in homozygous state. Typical to these findings in the present study it has been reported earlier [84]. A 15-year-old male presented as beta thalassemia heterozygous with HbA2 of 5.6% HbF of 0.9%. RBC indices were moderate with mild microcytic hypochromic picture. Hemoglobin was 13.8 g/dl. DNA sequence analysis revealed CD121 GAA-CAA mutation a β chain variant HbD-Punjab in homozygous state. HPLC chromatogram and RBC indices were suggestive of beta thalassemia heterozygous. However molecular analysis showed homozygous HBB c.364 G>C: GC Homozygous. Molecular finding is of great value as HbA2 measurement alone cannot be used as a reliable marker in diagnosing the condition [85,86]. HbA2 has a measure of unreliability in beta thalassemia heterozygous has also been implicated in the present study. It has been stated that the reliability of HbA2 levels is a persistent problem when HbD is involved. Similarly, HbA2 will not serve as a confirmatory marker while differentiating HbD homozygous with that of coexisting HbD with beta thalassemia [86]. HBB c:319 Del C novel mutation was identified in a 24 year female with HbA2 of4.6%. Hemoglobin levels were low 7.9 g/dl. The patient belongs to a muslim family with ancestor's origin place from Tuticorin. Native language was urdu and from previous two generations residing in Chennai district. DNA sequencing analysis revealed a deletion in the third exon of the β- globin gene, at codon 106 thereby producing novel β-thalassemia mutation. Spectrum of mutations in the present study showed different types of mutation. Of the 30 types of mutations promoter mutations covered 4 types. It is to note that all the promoter mutations cause mild beta thalassemia. Most of the beta thalassemia inheritance is by autosomal recessive. However, three dominantly inherited beta thalassemia mutations which occur in exon 3 have been identified in the study. Two beta thalassemia mutations, one with homozygous Poly A (A-G) and Initiation codon mutation had a substantially high HbA2 levels than other causative mutations. IVS 1-5 (G-C) mutation is almost evenly distributed among residents of Chennai who had their origin place as Chennai or from other districts of Tamil Nadu or from other states indicating that mutation frequency and prevalence is distributed throughout India.

In earlier studies, molecular characterization of β thalassemia mutation was done by ARMS PCR to identify the common, known mutations. However due to development advancement in technology and cost-effective screening tools to identify rare and novel mutations are made available in the diagnostic platform has led to the identification of different causative mutations. A total of 30 different mutations have been reported in the study. Though IVS 1-5 (G-C) mutation covered 70.8% beta thalassemia mutation, five rare mutations and one novel mutation have been identified. In spite of modernized molecular techniques, 1% of the mutation in the present study remains uncharacterized. IVS1-5 (G-C), 619 bp and CD I5 (G-A) mutation covered 81.14% cases. There are extensive variations of βthalassemia

mutations in the population. Detailed discussion on each identified mutation has been furnished to enhance the prenatal diagnosis and diagnostic implications of finding a rare mutation to avoid the birth of thalassemia major. Earlier few studies have been done on western and northern Indian states about the prevalence of beta thalassemia mutations. Based on these studies, IVS 1-5 (G-C), 619 bp deletion, IVS 1-1 (G-T), CD 8/9 and CD 41/42 mutations were considered as the five common beta thalassemia mutations [23,58]. Later characterization of beta thalassemia mutations using ARMS PCR was done in many studies by screening these common mutations. However, due to heterogeneous variation and diversity of people in different regions with varying ethnicities, collection of new rare mutations have been addressed with varying prevalence using new molecular techniques. IVS 1-5 (G-C) which is considered as the oldest mutation is prevalent all over India. Other mutations are present invariable frequencies in different geographical areas. However, the people of India follow the practice of marrying members of the same caste with the same inner caste division. Along with this endogamy, consanguinity is common among south Indians. Personnel in India have no barriers to move to either part of the nation for their occupation or education, marriages, etc. with this setting and immigrant population, origin and ethnicity-based screening protocols will be a challenging issue. Hence characterization and beta thalassemia mutation using DNA sequence analysis will reveal either known mutations or rare novel mutation.

5. Conclusion

Molecular characterization of beta thalassemia mutations revealed 30 different mutations with a high prevalence of IVS 1-5 (G-C) mutation, five new rare mutations viz., IVS II-1 (G>T), CD 37 TGG-TGA, IVS II 781 (C-G), CD114 CTG-CCG and Poly A (A-G) were diagnosed and reported first in India. One novel beta thalassemia mutation HBB.c319DelC was detected in the study. The diagnostic outcome of detecting the causative mutations for beta thalassemia imposes strong resources for developing easy and cheaper methods for prenatal diagnosis which will reduce the burden of disease. Further, the importance of the molecular diagnostic work to be discussed with the physician as false- negative beta thalassemia heterozygous diagnosis has a 25% chance of getting a beta thalassemia major child when the other partner is BTC. This is very important in our country as consanguineous marriage rates are high. Genotype characterization of beta thalassemia mutations is very significant for disease management as well as proper accomplishment of prenatal diagnosis. Treatment of beta thalassemia is expensive and exerts a burden both financially and morally decreasing the quality of life of the entire family. Carrier screening should be made extensive by premarital screening, prenatal screening by CVS diagnosis and termination of pregnancy in diagnosed cases will avoid adverse conditions of newborn. Awareness of the disease and counseling of the affected family will reduce the burden. For a full-fledged effective prenatal diagnosis of fetuses, screening the immediate family members and siblings by genotype analysis will be effective and helpful to avoid transfusion dependent beta thalassemia homozygous disease.

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Conflict of interest

Authors declare no conflict of interest in this manuscript.

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