Research article

Molecular characterization and mutational analysis of beta-globin gene among Kodagu population of Karnataka, India

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ABSTRACT

Introduction and Aim: Beta-thalassemia is a hereditary blood disorder caused by genotypic alterations in the HBB gene. Over 400 mutations have been identified, which are known to be responsible for this disease. In this study, we investigated the mutations for the globin gene prevalent among adult beta-thalassemia trait positive individuals and their distribution within the Kodagu community of Karnataka, India.

Materials and Methods: This study included 100 individuals (81 Females and 19 males) in the age group of 18-28 years previously tested as carriers for beta-thalassemia trait. Blood collected from each individual was subjected to genomic DNA extraction and PCR amplification of a 610 bp fragment of the beta-globin gene. The PCR product was purified and sequenced. The gene sequences were subjected to mutation analyses and characterization using bioinformatics tools.

Results: The study identified 13 known and 27 novel mutations for this study population. Among the known mutations observed 2 were of type synonymous (HBB:c.9T>C and HBB:c.18T>G), 5 missense (HBB:c.146T>G; HBB:c.149C>T; HBB:c.151A>T; HBB:c.203T>G; HBB:c.269G >A) and 6 intronic (HBB:c.92+2T >G; HBB:c.92+5G>C; HBB:c.315+16G>C; HBB:c.315+74T>G and HBB:c.315+81C>T). HBB:c.-6G>C at the 5'UTR region and IVS-II-16 G>C, an intronic variant, was observed to be the most common mutation showing highest frequency of 82.1%. Several novel mutation types were identified in Exon 2 (3 missense; 2 synonymous), Intron I and Intron II.

Conclusion: This study is the first of its kind for the Kodagu population, which identified several previously known and novel mutations in the HBB gene within this population. Since, our study subjects were carriers of very mild or silent beta-thalassemia trait, the mutations observed particularly the novel mutations warrant further investigation for a better understanding and management of β-thalassemia in this population.

Keywords: Beta-thalassemia; beta -globin gene; HBB; Kodagu population; DNA sequencing.

INTRODUCTION

Haemoglobin disorders present a significant health problem, among which thalassemias and allied disorders are a major concern and an increasing health burden globally (1). β-thalassemia an inherited blood disorder, has been associated with the defective synthesis of the β-globin chain, resulting in lowered haemoglobin levels, decreased red blood cell production, and severe hemolytic anaemia (1, 2). β-thalassemia has been further characterized as minor, intermedia and major based on the severity of the disease. While individuals with β-thalassemia minor usually are asymptomatic and exhibit no sign of the condition, individuals with the major form of the disease exhibit severe anaemia, which could be serious and life-threatening if left untreated. In contrast, β-thalassemia intermedia are highly variable, with severity falling between the two extremes of the major and minor forms. Individuals with β-thalassemia containing insufficient functional haemoglobin in their blood may develop severe anaemia (due to low production of red blood cells) or microcytic anaemia (due to abnormal production of microcytic red blood cells). Fatigue, weakness, shortness of breath, dizziness, and headaches are common symptoms of β-thalassemia anaemia.

In adult humans, hemoglobin A, is a hetero-tetramer composed of two α-globin and two β-globin polypeptides, each attached to a central heme ring. The haemoglobin (HBB) gene, located on the short arm of chromosome 11, encodes the β-globin polypeptide, which comprises three exons and two introns. Most β- thalassemia cases are caused by genetic abnormality events such as point mutations, large deletions, and small insertion/deletions of oligonucleotides within the HBB gene (3). The molecular basis of these mutations has been studied, and experimental evidence shows that some mutations can lead to altered transcription of the β-globin gene, causing an increase/decrease of the active protein function (4).

In India, β-thalassemia poses a significant health burden, and according to global estimates, 10% of thalassemic patients born every year are from India (5). β-thalassemia is heterogeneous at the molecular level, and several mutations leading to β-thal disease in the Indian population have been identified (6,7). Further, epidemiological surveys of β-thalassemia among the Indian population have estimated the carrier frequency rate of β-thal to vary between 1.0 to 17.0% with a mean prevalence of about 3.3% (5, 7-9). Heterozygous β-thalassemia, also known as β-thalassemia trait (BTT) is considered the most common autosomal recessive single gene disorder, a trait that is passed on from parents to their offspring (2). Hence, a routine premarital screening program is needed to estimate the true disease burden and prevent high-risk marriages. This study was conducted on young adults in the Kodagu community residing in the Kodagu district of Karnataka State, India. The Kodagu population are an ethnic group, wherein consanguineous marriages occur, thus being a high-risk community for genetic problems. In our previous study, we randomly screened individuals within the Kodagu community for the prevalence of β-thalassemia trait (BTT), which has been reported (10). In this study, DNA sequencing and analysis of the HBB gene of individuals positive for BTT was undertaken to assess the mutation patterns and their distribution in this population and associate it to their BTT phenotype, if any.

MATERIALS AND METHODS

Study population

For this study, a total of 100 individuals (81 Females and 19 males) in the age group of 18-28 years previously tested as carriers for β -thalassemia trait (BTT) by NESTROFT test (10) were selected for further sequencing and analysis of their HBB gene. Among these individuals, 60 confirmed positive for BTT had an MCH <27 pg, MCV<80 fl, and a HbA2 >3.5 %, while the remaining 40 showed MCH <27 pg, MCV<80 fl and borderline-normal HbA2 values (3-3.4%). Blood samples from each individual were collected separately for DNA extraction. Ethical consent was obtained from the Mangalore University Institutional Human Ethics Committee (No.MU-IHEC-2016-3 dt.18/04/2017) to carry out genetic testing of the blood samples. After obtaining prior informed consent, blood samples were collected from each study participant.

DNA isolation

Genomic DNA was extracted from 200 µl EDTA blood sample using a standard QIAmp DNA Blood Mini-kit (Qiagen, Germany) following the manufacturer's instructions. The concentration and purity of the extracted DNA were checked spectrophotometrically by measuring optical density at 260 and 280 nm wavelengths in Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA extracted was stored at –20°C until further use.

Polymerase chain reaction (PCR) amplification

Following DNA extraction, a 610 bp segment of the β-globin gene was PCR amplified using the primers: Fwd: 5'-ACCTCACCCTGTGGAGCCAC-3' and Rev: 5'-TCATTCGTCTGTTTCCCATTC-3'. This region comprised a section of the upstream sequence, 5'UTR, exon 1, intron-I, exon 2, and a part of intron-II. PCR was performed in a programmable thermocycler (Thermo Fisher, USA) having an initial delay at 95°C for 30 sec., followed by 35 cycles of denaturation of 95°C for 5 min, annealing at 60°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel. The gene products were excised, purified, and eluted using the QIAquick PCR Purification Kit. The purified PCR product was sequenced using a Big Dye terminator kit in a 3130xL genetic analyzer (Thermo fisher, USA) according to the manufacturer's instructions. Sequence alignment of the HBB gene sequences was performed using the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast) search against the Human chromosome 11 reference sequence (accession no. NC_000011.10) harboring the HBB gene. Multiple sequence alignment was performed using the Multalin program (http://multalin.toulouse.inra.fr/ multalin/). The mutations in the gene sequences were predicted using the novo SNP tool (http://www.molgen.ua.ac.be/bioinfo/novosnp/). To investigate pathogenicity of the mutations identified, the databases dbSNP (11) and HbVar (12) were used.

RESULTS

In this study 100 individuals, 60 were positive for the β-thalassemia trait (HBA2 >3.5) at the phenotypic level, and 40 individuals with slightly reduced HbA2 (3-3.4%) indicative of carriers of very mild or silent β-thalassemia trait were selected and subjected to sequencing and analysis of individual β-globin gene. A 610 bp corresponding to the HBB gene was amplified, sequenced, and looked for variations within the fragment (Fig.1).

A nucleotide sequence analysis of all the HBB gene fragments generated in this study revealed the sequences to be highly similar (>95% identity) with no significant phenotypic association between those with elevated or reduced HBA2 levels. However, a comparative analysis of individual sequences to the reference HBB gene sequence (NC_000011.10) using BLAST identified several SNPs (single nucleotide polymorphisms) within the amplified gene sequence.

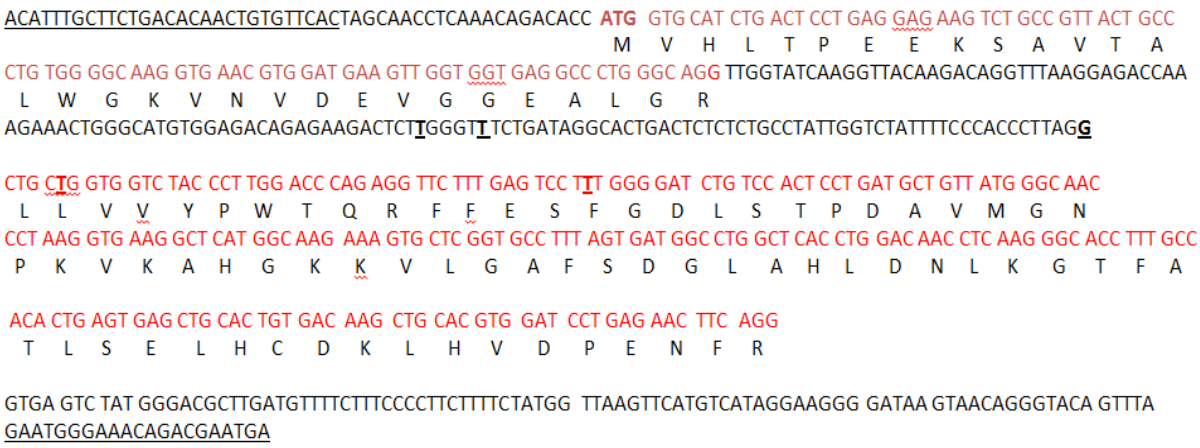

Fig. 1 : Representation of the nucleotide sequence generated by the amplification of 610 bp of the HBB gene. The forward and reverse primers are underlined. Nucleotides marked red are the exon regions and the single letter codes are their deduced amino acids.

These changes were matched to mutations documented in the Human Hemoglobin Variants and Thalassemia mutations (HbVar) database (https://globin.bx.psu.edu/hbvar/ accessed February 05, 2021) which lists more than 940 mutations for the β-globin gene, characterized from different populations worldwide. In total, 13 known and 27 novel mutations were identified, the results shown in Tables 1 and 2, respectively.

Among the known mutation types two were synonymous mutations (HBB:c.9T>C His>His; HBB:c.18T>G Pro>Pro) within the Exon-1 region and 5 missense mutations with Exon-2 (Table1). Among the synonymous mutations the frequency of HBB: c.9T>C was observed to be the highest (77%). The missense mutations occurring in the Kodagu population belonged to HBB variant Hb Okaloosa (rs33952850), Hb Las Palmas (rs33960931), Hb Zurich-Langstrasse (rs63750336), Hb Manukau (rs33918343) and Hb Créteil (rs33917628). In addition to a mutation in the coding regions, 5 intronic variants (2 in Intron I and 3 in intron II) were also observed in the HBB gene sequenced. The most frequent mutation was HBB:c.315+16G>C [IVS-II-16 G>C], accounting for 82.1% prevalence in the Kodagu population.

This study also reports the occurrence of several novel HBB mutations within the Kodagu population, the list of which is presented in Table 2. Based on mutation types, these novel mutations could be categorized as occurring in Exon 2 (3 missense; 2 synonymous), Intron I and Intron II (Table2). Nucleotide substitutions within three variants (HBB: c.192 T->G; HBB: c.196 A->G and HBB: c.199_200 AA->GG) resulted in missense mutation within the exonic-2 region of the HBB gene.

Table 1: Known HBB mutations observed in Kodagu population

HGVS nomenclature	Amino acid substitutions	Mutation Type	Name	Mutation position	dbSNP ID	Frequency (%)
HBB:c.-6G>C	-	NA	ODISHA	5'UTR	-	78 (82.1)
HBB:c.9T>C	His>His	Synonymous	NA	Exon1	-	77 (81.1
HBB:c.18T>G	Pro>Pro	Synonymous	NA	Exon1	-	2 (2.1)
HBB:c.146T>G	Leu>Arg	Missense	Hb Okaloosa	Exon2	rs33952850	1 (1.1)
HBB:c.149C>T	Ser>Phe	Missense	Hb Las Palmas	Exon2	rs33960931	1 (1.1)
HBB:c.151A>T	Thr>Ser	Missense	Hb Zurich-Langstrasse	Exon2	rs63750336	1 (1.1)
HBB:c.203T>G	Val>Gly	Missense	Hb Manukau	Exon2	rs33918343	1 (1.1)
HBB:c.269G >A	Ser>Asn	Missense	Hb Créteil	Exon2	rs33917628	1 (1.1)
HBB:c.92+2T >G	-	Splice Donor variant	IVS-I-2 T>G	Intron 1	rs33956879	1 (1.1)
HBB:c.92+5G >C	-	Intron variant	IVS-I-5 G>C	Intron 1	rs33915217	3 (3.2)
HBB:c.315+16G >C	-	Intron variant	IVS-II-16 G>C	Intron II	rs10768683	78 (82.1)
HBB:c.315+74T>G	-	Intron variant	IVS-II-74 T>G	Intron II	rs7480526	8 (8.4)
HBB:c.315+81C>T	-	Intron variant	IVS-II-81 C>T	Intron II	rs7946748	5 (5.3)

Table 2: Novel HBB mutations observed in Kodagu population

Nucleotide change	Amino acid substitutions	Mutation type	Mutation position	Frequency (%)
HBB: c.192 T->G	His->Gln	Missense	Exon2	1(1.1)
HBB: c.195 C->G	Gly->Gly	Synonymous	Exon2	2 (2.1)
HBB: c.196 A->G	Lys->Glu	Missense	Exon2	2 (2.1)
HBB: c.199_200 AA->GG	Lys->Gly	Missense	Exon2	2 (2.1)
HBB:c.306 G>A	Glu->Glu	Synonymous	Exon2	1(1.1)
IVS-I-73 T>G	-	Intron variant	Intron I	1(1.1)
IVS-I-78 T>G	-	Intron variant	Intron I	1(1.1)
IVS-II-13 G>A	-	Intron variant	Intron II	1(1.1)
IVS-II-14 A>G	-	Intron variant	Intron II	2 (2.1)
IVS-II-15 C>A	-	Intron variant	Intron II	2 (2.1)
IVS-II-16_17 InsG	-	Intron variant	Intron II	14 (14.7)
IVS-II-16_17 Ins C	-	Intron variant	Intron II	18 (19.0)
IVS-II-17 G>T	-	Intron variant	Intron II	17 (17.9)
IVS-II-19 del T	-	Intron variant	Intron II	16 (16.8)
IVS-II -49 T>G	-	Intron variant	Intron II	1(1.1)
IVS-II-51_52 Ins G	-	Intron variant	Intron II	1(1.1)
IVS-II-61 C>G	-	Intron variant	Intron II	1(1.1)
IVS-II-68 A>G	-	Intron variant	Intron II	35 (36.8)
IVS-II-68 A>T	-	Intron variant	Intron II	4 (4.2)
IVS-II-68, del A	-	Intron variant	Intron II	18 (19.0)
IVS-II-68_69 InsA	-	Intron variant	Intron II	1(1.1)
IVS-II-69 del G	-	Intron variant	Intron II	46 (48.4)
IVS-II-70 del G	-	Intron variant	Intron II	24 (25.3)
IVS-II-72 A>G	-	Intron variant	Intron II	22 (23.2)
IVS-II-77 G>A	-	Intron variant	Intron II	2 (2.1)
IVS-II-78 T>A	-	Intron variant	Intron II	18 (19.0)
IVS-II-79 A>T	-	Intron variant	Intron II	19 (20.0)

Two silent point mutations, HBB: c.195 C>G and HBB:c.306 G>A, where the codon change resulted in encoding the same amino acid, glycine, and glutamine respectively were identified. At position IVS-II-16_17 of the HBB gene an insertion of either guanine or cytosine was observed in 14.7 and 19.0% of the studied population respectively. In 17.9% of the individuals, a substitution of guanine to thymine (IVS-II-17 G>T) was observed at the same nucleotide position. Similar substitutions were also observed at nucleotide position 68 of intron-II where arginine was replaced by either guanine (35 individuals) or thymine (4 individuals). Deletion of arginine and insertion of an additional arginine at this 68-nucleotide position was also observed (Table 2). The nucleotide guanine (G) was deleted at positions 69 and 70 of intron-II in 48.4 and 25.3% of the Kodagu subjects (Table 2). Transversion mutations adenine to thymine (IVS-II-78, T>A) and thymine to adenine (IVS-II-79, A>T) was observed in 19 and 20% of the subjects respectively at the intron-II region of the HBB gene (Table 2).

DISCUSSION

In recent years, the measurement of blood HbA2 levels has been considered a reliable diagnostic marker for the routine identification of people who are carriers of the β-thalassemia trait (BTT). A level of HbA2>3.5% is considered a sign of BTT (13). However, recent findings report that this is not always the case as elevated/reduced levels of HbA2 could be modulated by several other genetic factors (3,14). Hence, DNA analysis is needed to identify and characterize mutations that cause β-thalassaemia. Further, it was found that β-globin gene mutations vary among different ethnic groups; and in each group, only a few mutations are responsible for the disease (15,16). In this study, 13 known and 27 novel mutations were identified in the β-globin gene of the Kodagu population screened. Among the known mutations, the highest frequency (82.1%) was observed at the 5'UTR region wherein the nucleotide guanine was substituted by cytosine (HBB: c.-6G>C). This SNP mutation was recorded earlier in about 20% of the population of Odisha State in India [6]. This assumes significance as mutations in or close to the 5' untranslated region is known to down-regulate transcription, which could result in mild b^+-thalassemia (17). The HBB gene consists of three coding regions (Exon1-3), and approximately half of the known β-thalassemia mutations are reported for these regions, which interfere with translation and could produce unstable globin chains of varying lengths or premature termination of translation (18). Two synonymous variations (HBB:c.9T>C His>His and HBB:c.18T>G Pro>Pro) within Exon 1 were found in this study, although they didn't seem to affect thalassemia. In Exon-2, five missense mutations were identified in this population (Table1). The variant Hb Okaloosa was reported from six members of a

Caucasian family, the USA, and is considered a relatively unstable variant with low oxygen affinity (19). Hb Las Palmas, first identified in Spain, is an unstable variant in which a C>G substitution occurs in Exon2 codon 149, replacing the serine residue with phenylalanine in the β-globin chain (20). Hb Zurich-Langstrasse HBB c.151A>T is a missense variant that results in a conservative amino acid change (Thr51Ser) in the globin domain. This mutation has been reported in a hematologically asymptomatic individual and is considered possibly benign with no impact on protein function (https://globin.bx.psu.edu/hbvar/hbvar.html). One of the participants in this study had the Haemoglobin Manukau (67 Val—>Gly) mutation, which had previously been reported in two brothers with symptoms of non-spherocytic haemolytic anaemia mitigated by coexisting α-thalassaemia, who became transfusion dependent by 6 months of age (21). Additional structural changes for this loci has been also reported, valine mutated to alanine (Hb Sydney (22) and valine to glutamine (Hb M-Milwaukee-I, (23). Hemoglobin Creteil (β: Ser89Asn) discovered in one of the participants in this study, is a variant haemoglobin with high oxygen affinity and very low allosteric function. Experimental evidence shows that serine mutation to asparagine causes severe disordering of the β-chain COOH-terminal tetrapeptide, destabilizing the quaternary structure of deoxy-hemoglobin A (24).

This study also reports the prevalence of several novel mutations among the Kodagu population, not reported earlier (Table 2). These were all seen in Exon-2 region of the β-globin gene and were either of synonymous or missense mutation types. Further research is needed to understand how these unknown mutations affect β-globin chain and are linked to the β- thalassemia trait.

In addition to mutation in the coding regions, intronic variants were also observed in the HBB gene sequenced. Among the known mutations occurring at the intronic regions, the most frequent mutation was HBB:c.315+16G >C [IVS-II-16 G>C], accounting for 70.5% of the population of Kodagu district. An earlier study also reported similar results that found IVS-II-16 G>C to be the most frequent mutation among the Odisha population in India (6). Another intron variant common among Odisha and Kodagu populations was IVS-II-81C>T (rs7946748). Among the intron variants, the second highest mutation observed was IVS-II-74 T>G occurring in 8.4% of the population. In the HbVar database, this mutation (rs7480526) has been listed as a frequent SNP of the HBB gene, associated with a normal clinical presentation. The mutations IVS-II-16 G>C and IVS-II-74 T>G have been previously reported in the Karnataka population with frequencies of 36% and 22%, respectively (25). A splice donor variant IVS-1-2 T>G (rs33956879) was identified in one individual. The occurrence of this mutation has been considered pathogenic as the T->G change in the GT dinucleotide, which is required for normal splicing, completely prevents this process and no normal mRNA is produced.

The intron variant IVS-I-5 G>C has been reported as a frequent mutation in Asian Indian, UAE, and East Asian populations (26) and was observed to occur in 3.2% of the Kodagu population tested (Table1). An earlier study reported the prevalence of this mutation to be 45.6% in the Karnataka population (8). According to experimental evidence, the G>C mutation at this position of the IVS-I greatly reduces the splicing efficiency of the normal 5' splicing site, resulting in aberrant β-globin chain formation (18).

In this study, the molecular characterization and mutational analyses of the HBB gene based on DNA sequencing were undertaken for individuals exhibiting elevated or borderline normal BTT. In this study, we identified a wide spectrum of known and novel mutations within the Kodagu population. Among the known mutations the most frequent were HBB: c.-6G>C (82.1%), HBB: c.9T>C (81.1%), IVS-II-16 G>C (75.8%). The majority of the novel variants identified were within intronic (IVS-I and IVS-II) regions, which is significant because mutations in introns have been shown to have a significant effect on -globin gene expression, RNA splicing, and mRNA stability (27). The intronic variant exhibiting highest frequency was IVS-II-69 del G (48.4%) followed by IVS-II-68 A>G (36.8%), IVS-II-70 del G (25.3%), IVS-II-72 A>G (23.2%). Similarly, five novel mutations presented in this study were in the Exon 2 region of the HBB gene. Although prevalent in low frequencies, the implications of these exonic mutations need to be understood, as previous studies have indicated that the severity of β-thalassemia disease is associated with mutations in exons within the HBB gene. The novel variants observed in this study require further investigation to better understand their influence on the proper production and functioning of the β-globin protein.

CONCLUSION

To our knowledge, this study is the first of its kind to be undertaken in the Indian Kodagu population. Although we observed the occurrence of different Hb variants in this population, it was difficult to conclude, as the study was based on the partial amplification and sequencing of the HBB gene. However, the study hints at the presence of novel mutations among the population that tested positive for the β-thalassemia trait. This study emphasizes the importance of complete mutation profiling of the β -globin gene to investigate and validate the clinical consequences of the mutations in this population. This might prove to be a useful tool for better understanding the disease and aiding in implementing strategies such as carrier screening, premarital genetic counseling, and

preventive and treatment measures for better β-thalassemia management in this population.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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