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MOLECULAR ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISM (SNPs) FOR β- GLOBIN GENE IN TRIBES OF MADHYA-PRADESH, INDIA

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ABSTRACT Hemoglobinopathies are associated with the inherited diseases characterized by structural or biochemical defects in haemoglobin chain. HBB (Human β-globin) gene relates naturally occurring genetic variation in humans. The purpose of the study is to analyze exon specific HBB gene mutations and to observe diversity in all 3 exons of HBB gene by Single Nucleotide Polymorphism (SNP) marker, among the three communities of M.P. ie. Raj-Gond, Baiga Kunbi. Total 520 samples are amplified with exon specific primers of HBB gene, followed by Polymerase Chain Reaction (PCR). Sequence analysis of 97.71% good quality sequences was performed followed by SNP-BLAST online tool and then analyzed for notified SNP against database at NCBI. Molecular marker based genetic diversity was observed and phylo-genetically analyzed by dendogram.

Total 191 sites in HBB1 and HBB2 region were detected as SNP variant and no polymorphic sites were observed in HBB3 region. The SNP variant site detected in the study are 3383 (T→C, rs: 63750898), 3466 (G→C, rs: 33960103) in HBB1 region, 3835 (G→C, rs: 63750898), 3893 (T→G, rs: 7480526) and 3900 (C→T, rs: 7946748) in HBB2 region. The BLAST-X analysis identified the presence of CDS from average ~ 127 positions. The SNP study was followed by phylogentic analysis for genetic diversity/distance/variation. Protein structure study can be carried out which allows visualization of the locations of mutation on the 3D structure.

KEYWORDS : HBB, phylogeny, SNP

INTRODUCTION

Natural selection sorts out and favours useful mutations while eliminate deleterious ones (Timothy Shanahan, 2004). In fact mutation and selection have a significant contribution in present day human diversity. Genetic disorders are good examples of such selection process. SNPs represent single-nucleotide polymorphism which usually occurs in non-coding regions more frequently than in coding regions or in general, where natural selection is functional for most favourable genetic adaptation (Barreiro L.B and Laval G., 2008). Beta thalassemia is caused due to mutations in the HBB gene on chromosome 11 (Campbell, James S., 2009) that inherited in an autosomal recessive fashion and changes in its DNA sequence causes reduced rate of synthesis of one of the globin chains that leads to form an abnormal hemoglobin and causes anaemia (Thein S.L, 2008). β-thalassemia is a subset of the β-hemoglobinopathies characterized by a hereditary anemia with a wide phenotypic spectrum (Weatherall DJ, Clegg JB. 2001). In India, the carrier frequency of β-thalassemia spans 0.3% to 15%, depending on the ethnic subgroup. On the African continent, the β-thalassemia carrier frequency varies from 1% to 13% (Weatherall DJ, Clegg JB et.al, 2001). In Europe, the percentage of the population carrying a significant variant associated with β-thalassemia ranges from 0.12% in Finland to 15% in Cyprus (Modell B, Darlison M, Birgens H, et.al, 2007). During the next 20 years, it is predicted that more than 900,000 children will be born with clinically significant β-thalassemia or abnormal hemoglobin (Vichinsky EP., 2005).

Present study – With a unique and almost pure gene pool, tribes have long been studied for different markers. The present study includes the variation analysis due to mutations in the tribes of Madhya Pradesh where the disease like malaria is a great threat to the ethnic diversities. This study is genetic screening of HBB gene in the tribes and castes of high risk malarial regions of M.P. using autosomal molecular markers.

Methodology – Despite of variety of mutation detection methods, β-thalassemia mutations may still go undetected, especially when detection strategies identify only a specific subset of the various β-globin variants. Direct DNA sequencing may enable more comprehensive detection of known and unknown β-globin gene mutations (Patrinos GP, Giardine B, Riemer C, et.al, 2004). In the present study, the process of sample collection was done in accordance with the ICMR guidelines. About 2 ml of blood sample was collected from healthy and patients of the disease with their written consent. Samples were processed for serological and molecular analysis. A part of the molecular analysis was performed at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad and Madhya Pradesh Council of Science and Technology (MPCST) Bhopal. DNA was isolated following Sambrook et.al (1989) method of lytic buffers. The isolated DNA washed twice with 70% ethanol, air dried and dissolved in TE buffer (10mM Tris HCl, pH 7.5, 1mM EDTA, pH 8).

Alternatively, DNA was isolated by an advance FTA classic card technique of DNA isolation and best suitable for population genetic study (Chaudhary R, Thangaraj K, et.al 2013). The preparation of DNA was performed using the Whatman FTA classic cards with optimization.

DNA amplification - Amplification for the exon specific DNA of HBB gene was done using a set of exon specific primers (Hung-Chang Shih, Tze-Kiong Er et.al, 2009). PCR amplification was carried out in 200µl with 40ng of DNA template. The reaction mixture was prepared in a 1.5 ml of tube containing, 10pmol of exon specific primer, 200µM dNTPs, 10X PCR buffer, 25mM MgCl2 and 1U *Taq* polymerase. The amplified product was electrophoresed (120V in 2% agarose) and visualized under UV. On obtaining a single band without dimmers, the PCR product was then sequenced. Direct sequencing of the amplified product was carried out by Big Dye Terminator reaction kit (Applied Biosystems, Foster City, CA). The sequencing conditions were: 45 cycles at 96C for 10 min, 55C for 15 sec., 72C for 15 sec. And 72C for 1 minutes (Hung Chang Shih, Tze Kiong Er, et.al 2009). On-screen alignment of chromatograms was done by Auto assembler (Mac software package, PE-Applied Bio-systems) and Applied Biosystems automated sequencers. Factura, a pre-filter or clean-up program is used for sequence analysis. FASTA format of these sequences was made and the obtained sequences were analyzed using population genetic software CLUSTALX (Jeanmougin et.al., 1998). The output file is then used in MEGA (Molecular Evolutionary Genetics Analysis) Version 5.2 cited by Tamura et.al 2007 to align and to find out variable sites. Power marker version 3.25 software, cited by Liu and Muse 2005, is used to calculate genetic distance and to prepare dendrogram by applying UPGMA (Unweighted Pair Group Method with Arithmetic mean).

RESULT AND DISCUSSION - The genetic distance and diversity was studied by using Nei's method. Figure indicates Gond and Bansod are closely related with a distance lesser than 0.2 among themselves.

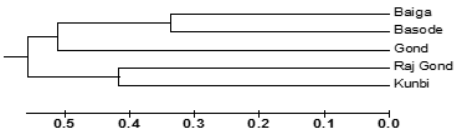

Figure 1: Dendogram showing genetic distance among the communities, based on serological marker

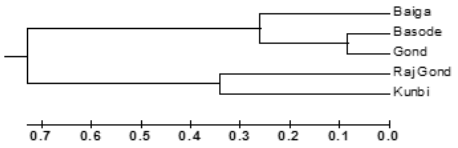

Figure 2: Dedogram showing genetic relationship among the communities, based on HBB SNP marker

The community Gond and Bansod clustered together and Baiga is externally linked to the cluster of these two. Raj-Gond and Kunbi were clustered in same group. Similar clustering pattern was observed in the dendogram based on serological ABO marker. The genetic distance values of the studied populations are compared with the other tribe such as Bhil of Jhabua district studied by Papiha et.al (1978) and Keer tribe of Sehore district a study by Bharti et.al (2007) of Madhya Pradesh.

Exon specific HBB gene Analysis

To detect SNP sites and variation, sequence analysis was performed by SNP-BLAST online tool for all three exonic regions of HBB gene (Chang JG, Chen PH, Lee SS, 1992). Sequences of exonic regions and one standard HBB gene sequence of Genbank (28302128) were aligned through multiple sequence alignment (MSA) by ClustalW software tool. Screening of the HBB gene mutation was focused on promoter and exon specific regions as it was reported to be more susceptible polymorphic site (Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, 2004). After analysing 97.71% good quality sequences, the detected SNP sites are at position 3383 (T→C, rs: 63750898), 3466 (G→C, rs: 33960103) in HBB1 region, at position 3835 (G→C, rs: 63750898), 3893 (T→G, rs: 7480526) and 3900 (C→T, rs: 7946748) in HBB2 region. Similar results were obtained by Wittwer CT, Reed GH, Gundry CN and Pryor RJ in 2003 by high resolution genotyping amplicon melting analysis method of mutation detection. As compare to Raj-Gond, Kunbi and Baiga community, Raj-Gond tribe shows highest SNP site (67.77%) at all reported mutations (rs: 63750898, 33960103, 7480526 and 7946748) at known five positions i.e. 3383, 3466, 3835, 3893 and 3900. Among all obtained SNPs sites, 3835 (G→C, rs: 63750898) is found more (46%), 3893 (T→G, rs: 7480526) is 42% and 3900 (C→T, rs: 7946748) is found to be least but with countable percent 29% in people of all three Raj-Gond, Baiga and Kunbi community. No clear cut polymorphic site was observed in SNP analysis of exon HBB3 region. The SNP study was followed by population diversity studies based on phylogenetic analysis. Diversities are compared by populations with one another and with the data available from the literature.

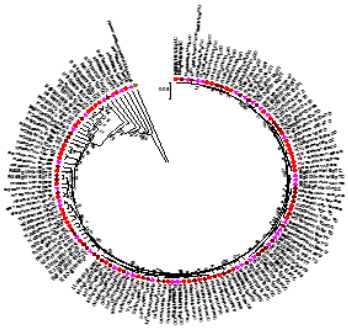

Figure 3: Dendogram of HBB Exon1. Code G10BtFHb1ABP0 indicates, Community, Region, Sex, Blood group, Rh factor (+/-) and mutation. Red circle: Kunbi, Pink triangle: Baiga, Brown diamond: Raj Gond and Black diamond: standard mRNA HBB sequence.

Variation reater than 5% was shown by sequences of 7 subjects comprised of 4 Baiga, 2 Kunbi and 1 Raj-Gond. The std. Ref. sequences of HBB gene (GI: 28302128) of Arab origin was externally linked to Indian clustered exon1 region of HBB gene with greater than 20% variation. 2-5% variation was observed among the 13.4% Kunbi, 5.7% Baiga and 9.2% Raj-Gond. Rest 67% sequences were observed to be closely related with less than 2% variation.

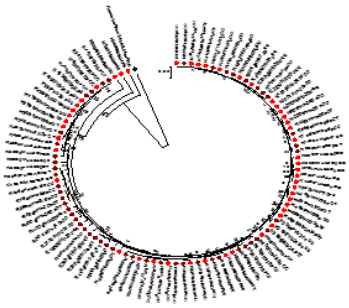

Figure 4: Dendogram of HBB Exon2. (Code G10BtFHb2ABP0 indicates, Community, Region, Sex, Blood group, Rh factor positive or negative and mutation). Red circle: Kunbi and Brown diamond: Raj Gond and Black diamond: standard mRNA HBB sequence.

Three types of SNP mutations were identified as 3835 G→C (rs: 63750898), 3893 T→G (rs: 7480526) and 3900 C→T (rs: 7946748). 46% allelomorphs of 3835 G→C type mutation were observed consist of 22% of Raj-Gond and 24% of Kunbi. 42% allelomorphs of 3893 T→G type mutation were observed consist of 18% Raj Gond and 24% of Kunbi. 29% allelomorphs of 3900 C→T type mutation were observed consist of 14% Raj-Gond and 15% of Kunbi. The frequencies of all three types of mutations were comparatively higher in Kunbi community. Family wise related sequences are shown to be clustered together, but still there is intermixing of groups of Raj-Gond with Kunbi communities. Exon2 region of HBB gene seems to be highly conserved among the Indian population with random distribution of three allels in both the communities.

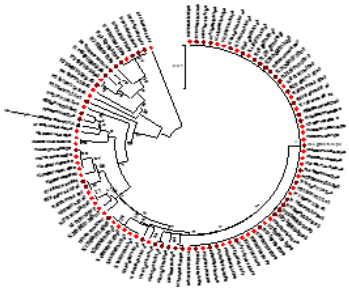

Figure 5: Dendogram of HBB Exon3. (Code G10BtFHb3ABP0 indicates, Community, Region, Sex, Blood group, Rh factor positive or negative and mutation). Red circle: Kunbi, and Brown diamond: Raj Gond and Black diamond: standard mRNA HBB sequence

The MSA indicates greater than 97% similarity among the sequences (red colour characters are conserved among the sequences). SNP-BLAST analysis of exon3 region of HBB gene has not shown any significant SNP in exon3 region of 100 sequences. Reference sequence of HBB gene (NCBI ref: 28302128) of Arab origin was also formed internal cluster indicating close relationship among the sequences by exon3. It can be predicted that the exon3 region of HBB gene is seem to be highly conserved among the Indian population and shows close similarity even with the Arab origin sequences.

Similar SNP studies based on HBB and G-6-PD molecular marker in the population of Asian sub continents were performed by Nguyen Thi Hue et.al (2009) and Hung Chang Shih et.al in 2009. Interestingly two subjects of Raj-Gond out of these four mutants had family malarial history with regular anti-malarial drug consumption. This may show that very rarely de novo mutations can arise that causing the more severe condition of chronic non spherocytic hemolytic anemia (Mason et.al 2007).

CONCLUSION -

SNP data of the present study concludes that the mean pair-wise distance is lower between Gond and Bansod (0.0833) as compared to Gond and Kunbi and highest between Gond and Baiga (0.7155). Thus, from Sero-genetic and exon specific DNA analysis, Gond appears to be

a close lineage with Bansod in due course of evolution. In terms of their geographical relation also Gond from Chicholi and Bansod from Betul are closer than Raj-Gond from Sagar. But due to variation in SNP analysis Kunbi comes out to entirely different from the Gond and Baiga. In conclusion, it can be said that the present work has provided the original data of the serological and exon specific molecular markers in the selected tribes/caste of Madhya Pradesh. This study has helped to genetically characterize the tribes and caste of Madhya Pradesh and also to complete the genetic map of Madhya Pradesh.

Such research studies would be helpful in future not only in revealing the phylogenetic relationship among the individuals in one tribe and those with the other but will also form the foundation to understand the genetic basis of complex diseases found in tribes. Study of HBB gene with all 3 exons with protein structure can be performed for more and complete analysis.

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