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Randomized genetic polymorphism analysis of sickle cell disease patients in a tertiary care teaching hospital Bhubaneswar

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Abstract
Sickle cell disease or HbSS genotype is associated with the most severe, most frequent pain and shortest life expectancy. However, the genetic modifiers play a very important role which result in extremely diverse clinical severity. This study is intended to analyse the hereditary polymorphism in 25 patients diagnosed as Sickle cell disease (SCD) presenting in a tertiary care hospital of Eastern India. The subjects were diagnosed as SCD based on clinical examination, hematological investigation, hemoglobin electrophoresis, parental screening by Hemoglobin electrophoresis and molecular diagnosis. Blood samples from 25 patients of diagnosed SCD were analysed. The whole DNA isolation was pursued and gene specific PCR amplification were performed. The amplified products were purified and sequencing was done. From the sequence information, the phylogenetic examination and sequence connected study was performed. The outcomes demonstrated a specific single band in 25 DNA amplified PCR item after agarose gel electrophoresis. The phylogenetic examination uncovered that some of the sequence were highly diverse and some of them also found in a single monophyletic clades. The sequence information linked with the goblin chain can be utilized in various fundamental and applied research in sickle cell anemia and furthermore in other hemoglobinopathies.

Keywords: SCD, randomized genetic polymorphism, PCR

Introduction
Sickle cell disease (SCD) is one of the best characterized human monogenic disorders. The development of molecular biology allowed the identification of several genomic polymorphisms responsible for its clinical diversity. Research on the first genetic modulators of SCD, such as coinheritance of a-thalassemia and haplotypes in the b-globin gene cluster, have been followed by studies associating single nucleotide polymorphisms (SNPs) with variable risks for stroke, leg ulceration, pulmonary hypertension, priapism and osteonecrosis, with differences in the response to hydroxyurea, and with variability in the management of pain [1]. SCD has vital implications for public health as both overall rate and commonness are high, which strengthens it as a huge social issue in numerous nations [2, 3]. The clinical assorted variety of SCD incorporates hemolytic and vaso-occlusive episode (VOE), stroke, acute chest syndrome, pulmonary hypertension, numerous organ dysfunctions and other different complexity [4].

As for the hereditary markers, SCA patients can likewise be carrier of at least one gene determinants, for example, the 3.7 Kb deletion of α-globin chain in α-thalassemia (−α3.7Kb-thal). The heterozygous (−α/αα) or homozygous (−α/−α) - α3.7Kb-thal genotype in SCA people is related with a decrease in HbS conentration, which brings down hemoglobin polymerization and cell damage and improves hemolysis profile [5, 6]. Thus, this affiliation advances changes in hematological and biochemical parameters of SCA [5-7]. The hematologic and laboratory changes in sickle cell anemia with coinherited ∝ thalassemia include: higher hemoglobin concentration, higher HbA2, lower reticulocyte count, lower MCV, lower bilirubin level, lower LDH, fewer dense and irreversibly sickled cells, increased erythrocyte lifespan; with minimal change in HbF concentration. The extent of these changes is related to the number of deleted ∝-globin genes. Similarly, in βS globin gene haplotypes are comprised of polymorphisms in the βS globin gene group, which are related with explicit degrees of fetal hemoglobin (HbF), adding to phenotypic decent variety in SCA patients [8-10].

The degree of amelioration depends on the severity of the β thalassemia alleles and the number of functional α globin genes. Taking these fact in to account in this study, we investigated the randomized DNA sequence polymorphism in patients from eastern India. This data will help to understand the genetic variation in SCD and enable the researcher throw light on the development of molecular marker for early diagnosis and phenotypic variation of SCD.

Material and Methods

The study included the diagnosed sickle cell anemia patients in different regional hospital of eastern India. A total of 25 patients were taken for this study. The blood samples were collected and following techniques were used.

DNA isolation from the blood sample of sickle cell patient

Total genomic DNA was extracted from the blood sample of sickle cell patient by the help of HiPurA™ Blood Genomic DNA Mini Purification Kit. The blood samples were collected in EDTA-tube in labelled sterile containers. All samples were transferred to our laboratory and stored at -20°C.The frozen whole blood samples were thawed and 200 µl of the lysis solution (C1) was added to the sample. To obtain the homogeneous mixture the entire sample was vortexed thoroughly for few seconds. 20 µl of the reconstituted Proteinase K solution (20mg/ml) was added to the mixture. Then the mixture was incubated at 55^0c for 10 minutes followed by addition of 200 µl OF ethanol (96-100%) to the lysate obtained from above step. The mixture was mixed thoroughly by gentle pipetting. Subsequently to obtain RNA free genomic DNA, 20 µl of RNase A solution (20mg/ml) was added. The obtained lysate was transferred into the spin column provided by the kit followed by centrifugation at 10,000 rpm for 1 minute. The flow through was discarded and the column was placed in same 2.0 ml collection tube. Then 500 µl of diluted prewash solution was added to the column and centrifuged at 10000 rpm for 1 minute. The flow through liquid was discarded and the same collection tube was reused with the column. In washing steps, 500 µl of diluted wash solution was added to the column and centrifuged at (13000-16000 rpm) for 3 minutes to dry the column. The flow through liquid was discarded and the empty column was spinned for another 1 minute at the same speed. The collection tube was discarded which contained the flow through liquid and the column was placed in a new uncapped 2.0ml collection tube. From the column, elution of DNA was carried out by 100 µl of Elution Buffer (ET), which was added directly onto the column without spilling to the sides and incubated for 1 minute at room temperature. Then it was centrifuged at 10,000 rpm for 1 minute. This step was repeated again with another 100 µl of Elution Buffer (ET) for high yield of DNA. The elute was transferred to a fresh capped 2.0 ml collection tube for longer DNA storage.

Quantification of DNA

Quantification of DNA was estimated with the help of measurement of how much UV radiation absorbed by nucleic acid bases using UV- VIS Spectrophotometer Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA).The spectrophotometer calibration was done by using 2000 µl of tris acetate (TE) in a cuvette (quartz) at 260 nm & 280 nm. With 1995µl of TE, 5 µl of purified DNA sample was added mixed properly and O.D (absorbance) was taken at both 260nm and 280nm. O.D at 260nm was multiplied with the dilution factor and 50 to estimate the concentration of DNA sample [Concentration of DNA (µg/ml) = O.D at 260 x Dilution factor x 50]. The purity of the DNA was estimated by the ratio between the reading of spectrophotometer at 260 nm and 280 nm (O.D 260 nm/ O.D 280 nm). The ratio between 1.8 and 2.0 was taken for further downstream analysis, below or above 1.8 and 2.0 respectively were taken for further purification process. Intactness of genomic DNA, presence of contaminant like RNA and protein was determined with the help of 0.8% agarose gel electrophoresis. An aliquot of 2µl of DNA sample was taken with 50 ng of molecular weight marker (Lambda) to the agarose gel (0.8%) electrophoresis for about 2 hour. Staining of gel was done by using ETBR (ethidium bromide) 0.5µg/ml, visualized under UV Trans-illuminator machine and the photo was taken using a Gel-Doc system (Bio Rad, USA). Then according to the requirement (25ng to 50ng) the DNA was diluted for further work with 1X TE buffer.

PCR reaction

The sickle cell based DNA primer was designed using Primer3 software. Sickle cell amplification was carried out in a final volume of 25 µl PCR master mix containing 25 ng of template DNA, 2 µl of 1x PCR buffer, 2 µl of dntp mix (200µm), 1.5µl of $Mgcl_2$, 1 µl of each forward and reverse primers (10µM/L) and 0.17 µl of *Taq polymerase*(0.5U/µl). The PCR was programmed with following temperature profile: 94^0C for 5 min followed by 30 cycles of 94^0C for 1 min, 59.3^0C for 45 sec and 72^0C for 1 min, with a final extension of 10 min at 72^0C. The amplified products were separated on 1.5% agarose gel, staining of gel was done by using ETBR (ethidium bromide) 0.5µg/ml, visualized under UV Trans-illuminator machine and the photo was taken using a Gel- Doc system (Bio Rad, USA).

Sequencing and phylogenetic analysis

The purified PCR amplification products were sequenced using the BigDye Terminatorv3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, FosterCity, CA, USA). The sequence assembly and editing was performed with Sequencher 5.1 software and phylogenetic tree is constructed by using MEGA 6 software [11].

Result

Gene specific PCR amplification

Out of total 25 samples collected from different hospitals, which were subjected for DNA isolation and purification, 24 successful PCR amplification could be achieved using a gene specific to sickle cell hemoglobinopathy. The primer sequence was 5'-GCTTACATTTGCTTCTGACACAAC-3' forward and 5'-CCCCTTCCTATGACATGAACTTAA-3' having annealing temperature 55°C and GC percentage more than 40 resulted in a specific band of ≈550 bp in all the 24 samples collected for analysis. No bands were found from the negative PCR reaction (Fig. 1).

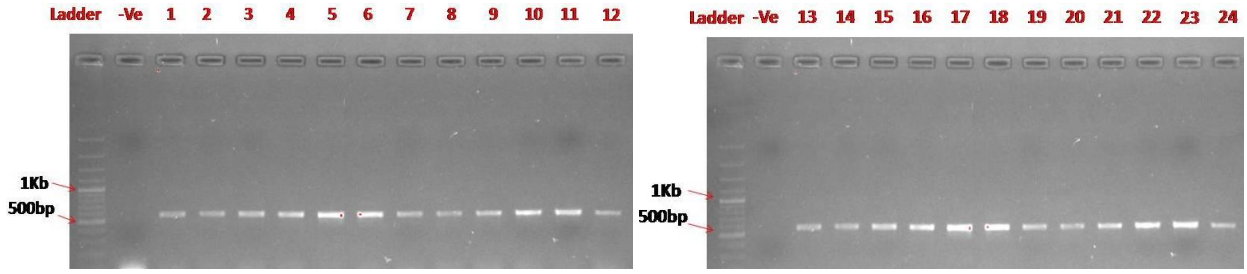

Fig 1: Agarose gel banding of a specific 550bp length DNA amplified after PCR using hemoglobinopathy gene specific primer. (The arrow indicates 500bp and 1Kb ladder length of the used 100 bp ladder plus. -Ve well in all four indicates no amplification and 1-24 well indicates the amplified PCR product at length ≈550 bp.

Sequencing and its analysis

All 45 PCR amplified products were purified and sequenced and the sequence information were analysed. Most of the sequence were found conserve. The sequence data are provided here in the list as in supplementary file S1. The sequence were named with Sum1 to Sum 45 and further analysis were done by blast X, where the maximum identity value, maximum score, and E- values were found. Blast X compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database. The maximum identity protein sequence with all above data were analysed which is listed in the table.1

Table 1: Maximum similarity results of homology search (Blast X) for each 45hemoglobinopathy linked sequence in the non-redundant protein sequence database of NCBI's GenBank.

S. No.	Acc. no.	Description	Max- score	Max -identity	E- value
1	XP_011830555.1	Hemoglobin subunit beta isoform X1 [*Mandrillus leucophaeus*]	90.62%	244	4e-79
2	AAQ24848.1	Hemoglobin beta chain [*Homo sapiens*]	100.00%	82.0	2e-17
3	AAQ24847.1	Hemoglobin beta chain [*Homo sapiens*	97.50%	80.9	7e-17
4	1Y4V_B	Chain B, Hemoglobin Beta Chain [*Homo sapiens*]	100.00%	84.0	8e-17
5	1Y5J_B	Chain B, Hemoglobin Beta Chain [*Homo sapiens*]	100.00%	84.0	8e-17
6	1Y31_B	Chain B, Hemoglobin Beta Chain [*Homo sapiens*]	100.00%	83.6	1e-16
7	2HHE_B	Chain B, Hemoglobin (deoxy) (beta Chain) [*Homo sapiens*]	100.00%	84.0	9e-17
8	1BUW_B	Chain B, Crystal Structure Of S-Nitroso-Nitrosyl Human Hemoglobin A [*Homo sapiens*]	100.00%	83.6	1e-16
9	XP_025213810.1	Hemoglobin subunit beta isoform X1 [*Theropithecus gelada*]	89.06%	237	2e-76
10	AAK20080.1	Mutant beta globin [*Homo sapiens*]	98.75%	167	1e-49
11	ACU56984.1	Beta-globin [*Homo sapiens*]	97.37%	157	2e-49
12	BAV25189.2	Beta-globin [*Homo sapiens*]	98.67%	156	5e-49
13	CAA26204.1	Beta-globin [Pan troglodytes]	85.71%	156	4e-45
14	ADV59922.1	Hemoglobin Beta Chain [*Homo sapiens*]	98.67%	156	2e-45
15	CAA26204.1	Beta-globin [Pan troglodytes]	86.81%	159	3e-46
16	AAY15222.1	Beta-globin [*Homo sapiens*]	100.00%	159	3e-46
17	AAT36651.1	Chain B, Hemoglobin Beta Chain [*Homo sapiens*]	100.00%	158	3e-46
18	1Y2Z_B	Chain B, Hemoglobin Beta Chain [*Homo sapiens*]	97.37%		4e-48
19	AAR96398.1	Hemoglobin Beta [*Homo sapiens*]	96%	151	1e-47
20	AAG46184.1	Mutant Beta [*Homo sapiens*]	98.68%	159	2e-46
21	BAV25189.2	Beta globin [*Homo sapiens*]	98.67%	156	5e-49
22	XP_011830555.1	PREDICTED: hemoglobin subunit beta isoform X1 [*Mandrills leucophaeus*]	90.62%	244	3e-79
23	XP_025213810.1	hemoglobin subunit beta isoform X1 [Theropithecus gelada]	88.28%	236	4e-76
24	711685A	hemoglobin [*Cebus albifrons*]	92.11%	149	4e-45

Phylogenetic analysis of the 45 PCR amplified sequence

Phylogenetic analysis is done here, which estimate the evolutionary relationships of the nucleotide found in our study from the blood sample of the hemoglobinopathy patients. The MEGA 6 software is used here to get the test neighbor-joining tree from the 24 nucleotide sequence (Fig. 2)

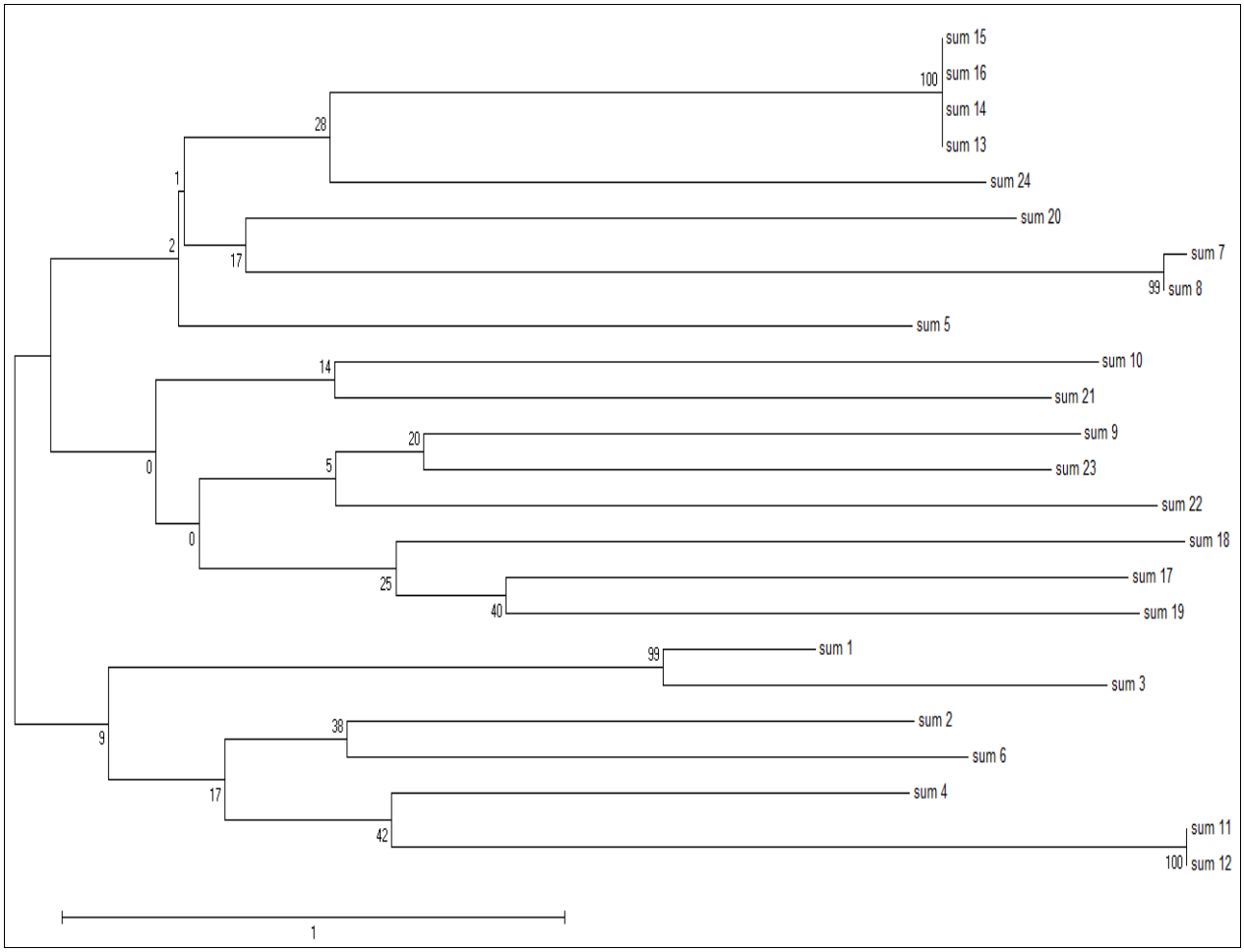

Fig 2: Phylogenetic tree of 24 sequence from hemoglobinopathy patient blood DNA. Phylogenetic analysis was performed by the neighbour joining method using MEGA 6.

Discussion

Although all patients with SCD have the same mutation, severity of their clinical manifestations are very different from one to another [12]. Some genetic modifiers are identified for SCD including alpha thalassemia [13-15], elevated HbF, and glucose 6 phosphate dehydrogenase (G6PD) deficiency. However these factors don't completely clarify the diversity in the clinical findings of patients with SCD [14, 15]. Today, the introduction of genome analysis due to the development in molecular marker has been helpful in identifying the genomic polymorphisms responsible for the clinical diversity in patients with SCD. Some DNA polymorphisms in the β-globin gene are associated with mild form of SCD [16] that we analyzed here in our study through the gene specific PCR amplification. It provided an unique DNA band in all our 45 DNA isolated from blood sample of different patients from eastern India hospital. After sequencing the data were also analyzed through the homology search (BlastX) for each 45 hemoglobinopathy linked sequence in the non-redundant protein sequence database of NCBI's GenBank (Table. 1).

Our results revealed here that Sum [15, 16, 17, 18] sequence were fall under the monophyletic clade, which are very much similar with the protein sequence of beta-globin of *Pan troglodytes* and *Homo sapiens.* Similarly Sum [7, 8] and [11, 12] are also showed in two different monophyletic clades in different groups which revealed the protein sequence similarity with Chain B, Crystal Structure Of S-Nitroso-Nitrosyl Human Hemoglobin A *[Homo sapiens*] and beta globin gene of *Homo sapiens* respectively. Sum [10, 21] fall closely to each other, like [9, 22, 23] in the same groups which represents the protein sequence of hemoglobin subunit beta isoform X1. In other group will find Sum [18, 17, 19] which find similarity with the Chain B, Hemoglobin Beta Chain [*Homo sapiens*]. Sum 1 and 3 sequence is 99% similar with the protein sequence of Hemoglobin beta chain [*Homo sapiens* and *Mandrills leucophaeus*]. Similarly the sequence of Sum [2, 4, 6] are found closely associated with the protein sequence of Chain B, Hemoglobin Beta Chain [*Homo sapiens*]. All the above mentioned factors are nothing but the DNA label polymorphisms in the β-globin gene found associated with hemoglobinopathy condition that results in abnormal structure of globin chains of the haemoglobin molecule.

Conclusion

In the above study, the DNA analysis marks the molecular variety identified in patients with Sickle cell Disease. These genetic modifiers may contribute to the phenotypic variations in these patients in India as well as in other parts of the world where this disease is prevalent. The hereditary polymorphisms that are identified with the regulation elements of SCD empower the researcher to assess the assorted variety in hereditary level to discover the molecular kit for early diagnosis of the linked diseases. Further studies on hereditary polymorphisms are crucial to SCD and if

possible, to discover genetic modulators of disease severity that could guide prognosis to determine preventive measures, the best treatment for intense and incessant organ damage.

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