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ORIGINAL ARTICLE **OPEN ACCESS**

Study of Proteomic Diversity for Sickle Cell Disease in Tribe and Non-Tribe Population of Kumaun Region Of Uttarakhand

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ABSTRACT

The present study is an analysis of the prevalence of sickle cell disease (SCD) in Kumaun, region of Uttarakhand the investigations address the prevention of disease and some new information on frequencies of alleles among various tribal and non-tribal populations of Kumaun region. Blood samples were collected from twenty nine diseased individuals; hematological analysis such as peripheral cell count and red blood cell indices (RBC, Hb%, HCT, MCV, MCH, and MCHC) performed using standard procedures, SDS-PAGE was performed and based on banding pattern the UPGMA cluster separated in two major groups A and B. The present study revealed that the percentage of sickle cell disease is more in Tharu community of Sitarganj area than other tribal and non tribal communities in Kumaun region.

Key words: *Sickle cell disease, Haematological analysis, Tribal, Non-tribal, Kumaun region.*

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INTRODUCTION

Sickle cell disease (SCD) is diagnosed mainly through hematological studies and clinical manifestations. Sickle cell disease is a condition of anemia in which red blood cells contort into a sickle shape. The sickle-shaped RBCs stick to the walls of blood vessel, causing blockage that slows down or stops the blood flow [1] The lack of oxygen causes sudden and severe pain called pain crises [2].

Sickle cell disease (SCD) is one such blood disorder caused by the abnormal hemoglobin that damages and deforms red blood cells. When the abnormal red cells break it causes anemia and obstruct blood vessels leading to severe pain and results into multi-organ ischemic damage. In SCD severities varies from mild to asymptomatic state and severe symptoms and require hospitalization [3]. Variant hemoglobin is derived from gene abnormalities affecting the α-globin (HBA1 or HBA2) or β-globin (HBB) genes (exons) [4]. More than a thousand hemoglobin variants have been identified related to changes in the globin chains [5]. Qualitative changes correspond to amino acid substitutions resulting in hemoglobinopathies. Various methods for hemoglobinopathy in newborn screening and adult testing are being employed and most of such programmes use high performance liquid chromatography [6].

Sickle cell anemia is caused by a single code letter change in the DNA. An abnormal hemoglobin in which valine is replaced by glutamic acid causing the hemoglobin to become less soluble under decreasing oxygen concentrations and to polymerize into crystals that distort the red blood cell shape.

MATERIAL AND METHODS

The study was conducted at Earthworm Biotechnology and Microbial Metagenomics Laboratory, Department of Zoology, D.S.B. College, Nainital, Uttarakhand India; samples were collected from District Hospitals of Kumaun region, Uttarakhand, India and the families with the history of the disease were further investigated.

Survey and sample collection: This study was carried out in four families (Total Twenty-nine members of the family). Base line survey was conducted based on questionnaire survey for sickle cell disease patients in Kumaun region of Uttarakhand. Name, age and gender information recorded for further hematological examinations and for pedigree analysis of each family. Blood samples were collected from patients having no blood transfusion history using EDTA as anticoagulant by disposable syringes.

Family pedigree chart analysis: The family pedigree charts drawn with the help of family discussions i.e. in person interactions and counseling. All the four families were grouped based on inheritance of SCD from parents to progeny and all the diseased individuals have been highlighted in the chart. Family tree was prepared to understand the current status.

DIAGNOSIS AND TESTS:

Blood Smear and Hematological analysis: The blood smears were prepared and observed under the microscope for observation the shape of cells. SCD is a common life threatening genetic disorder that can be managed if diagnosed early by neonatal and prenatal screening. Hemoglobin A, F, C and S for selected samples quantified using conventional Hb electrophoresis on the semi-automated scan system.

PROTEOMIC ANALYSIS OF RBC

200µl of fresh blood collected from patients and normal individuals randomly, using EDTA as anticoagulant and immediately transferred to ice boxes. A proteomics approach (SDS PAGE) was used to observe protein profiles and diversity associated with SCD.

Isolation of total buffer soluble protein: RBC membrane proteins extracted according to [7] and [8] with minor modifications, Blood samples of selected individuals were transferred into the autoclaved centrifuge tubes. Red blood cells (RBCs) were washed twice with two volumes of ice cold saline solution (1 mM Tris, pH 8.0, 200 mM NaCl) and centrifuged at 2500 rpm for 5 min. at 4°C. The RBCs were lysed in 300µl of cold buffer containing 1mM Tris, pH 7.5, 1mM phenyl-methyl-sulphonyl fluoride (PMSF).

To complete hemolysis, samples were placed on ice for 10 minutes and then kept at -80°C for 15 minutes. Cellular debris was pelleted by centrifugation at 10,000 rpm for 15 minutes at 4°C and the hemolysate was stored at -80°C. Protein concentration of the hemolysate was measured using the Bradford method [9]. 15µg/well protein samples were separated on 15% SDS- PAGE gel [22]. The banding patterns observed with the gel documentation system [7, 8 and 10, 11].

DATA ANALYSIS:

Data was statistically analyzed to obtain significant and non-significant values with the help of Microsoft excel and Jacquard's coefficients among the samples by using NTSYS-pc (version 2.11W; Exeter Biological Software). All values were taken in triplicate for statistical analysis.

RESULTS

The baseline survey was conducted with the help of Doctors of district hospitals of Haldwani, Khatima and Rudrapur of Uttarakhand to understand the SCD registry and patient details. A total of four families based on the secondary data were followed and all the family members of related families surveyed for the detailed investigations the major findings are summarized in the table.1. A total 29 subjects were investigated and number of patients with Hb-AS: 0 (0%); Hb-SS: 3 (10.344%), Thalassemis Major: 1 (3.448%) and Thalassemis Minor: 5 (17.241%) rest 68.967% subjects were not found with any problems and conditions related to the haemoglobinopathies.

Table 1: Distribution of patients having haemoglobinopathies in different caste groups from major surveyed districts of Uttarakhand.

District	**Caste groups**	**Total No. of Samples of family**	**No. of Patients with Hb-AS**	**No. of Patients with Hb-SS**	**No. of Patients with** Thalassemis Major	**No. of Patients with** Thalassemis Minor
Pithoragarh	Hindu (T*)	03	-	-	-	01
Almora	Hindu (OBC**)	05	-	-	01	-
Udham Singh Nagar	Muslim (OBC)	12	-	03	-	-
Nainital	Hindu (OBC)	09	-	-	-	04
Total number of subjects:		**29**	**-**	**03**	**01**	**05**
Percentage			**0**	**10.344**	**3.448**	**17.241**

*T= Tribes, **OBC= Other Backward Class

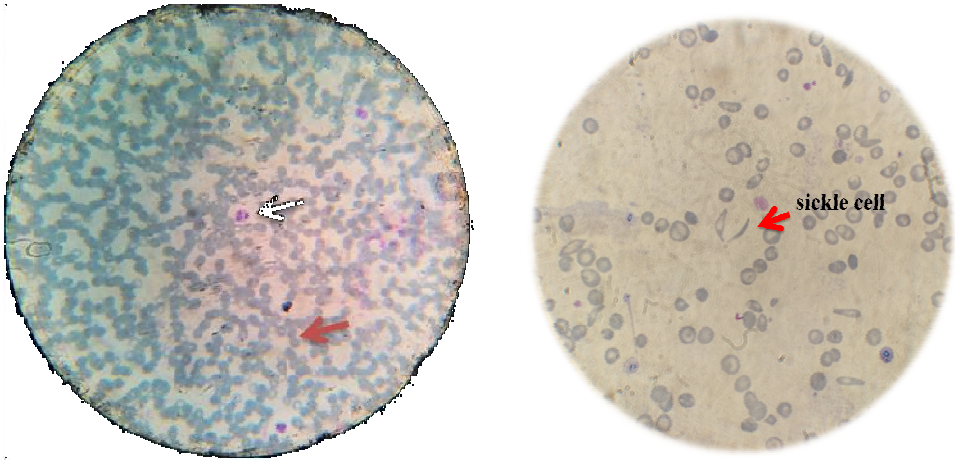

Figure 1: Images showing the blood cells. (A): White arrow shows white blood cells (WBC) and red arrow is showing the red blood cells (RBC) (Images are 20X magnification) in normal individuals (B) Sickle cell in blood smear 40X Magnification.

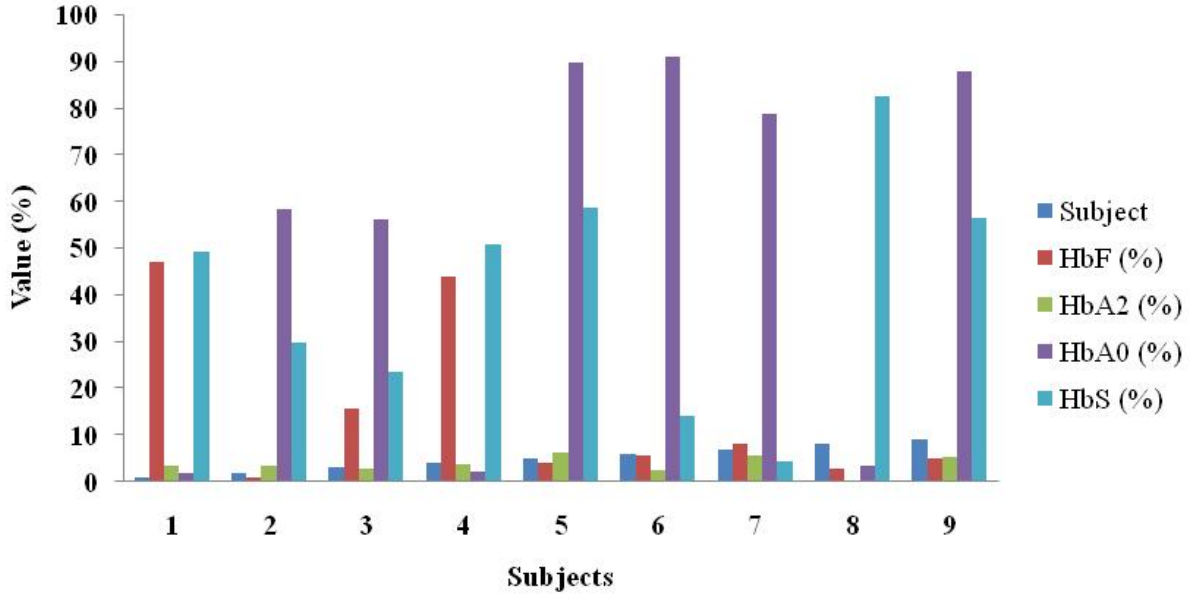

Figure 2: Distribution of Hb cases of nine subjects from Nainital and almora District.

Table 2: Most important features of HbS hemoglobinopathies [24]

Disease	Hemoglobin variants	Features
Sickle cell trait	Hb AS	Hb A > HbS; HbA: 50%-60%; Hb S: 30%-40% No apparent illness
Sickle cell disease	Hb SS	HbS (majority), Hb A_2: <3.5%, HbF (high), no HbA Severe disease with chronic hemolytic anemia
Sickle cell-β°- thalassemia	Hb S β°	HbS (majority), Hb A_2: >3.5%, Hb F (high), no HbA Low mean corpuscular volume and low man corpuscular hemoglobin; severe disease
Sickle cell- $β^+$- thalassemia	Hb $Sβ^+$/++	HbS (majority), Hb A_2: >3.5%, Hb F (high), Hb A: 5%-40% Variable mild-to-moderate sickle cell disease
Hemoglobin SC disease	Hb SC	Hb S: 50%, Hb C: 50% Moderate sickling disease but chronic chemolytic anemia may be present
Hemoglobin S/hereditary persistence of fetal hemoglobin		Hb S: 60%, Hb A_2: <3.5%, Hb F: 30%-40% Behaves as sickle cell trait

Table 3: Hematological examination of 09 patients from four families

Subject	HbF (%)	HbA2 (%)	HbA0 (%)	HbS (%)
1	47.10	3.50	01.80	49.10
2	01.00	3.50	58.22	29.90
3	15.60	2.90	56.25	23.40
4	43.80	3.80	02.10	50.85
5	04.00	6.20	89.80	58.49
6	05.50	2.60	90.80	14.00
7	08.11	5.50	78.80	04.50
8	02.80	3,60	03.40	82.40
9	05.00	5.20	87.80	56.49

FAMILY PEDIGREE CHART

Family pedigree charts (Figure.3) prepared to study the family history of SCD on the basis of questionnaire survey. Total four families were studied for SCD history after information collected from major district hospitals. Diseased persons were examined for SCD confirmation by SDS-PAGE and hematological analysis (Table 3).

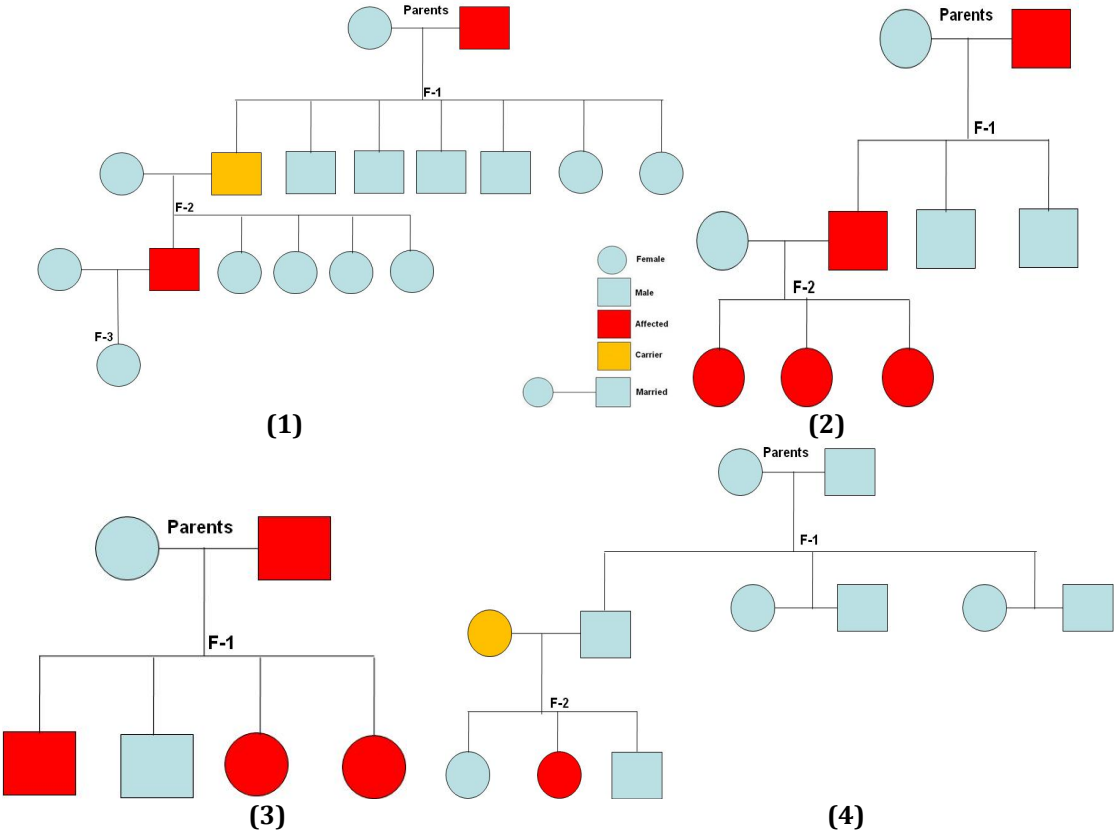

Figure 3: Family pedigree chart for SCA analysis ancestor to their progeny.

The present study revealed that there is serious lack of awareness including premarital SCD genetic counseling to reduce the cases. The SCD is not only limited to tribes it has been observed across the populations especially where congenial marriage are common in practice. In case of tribes the population is very small and live in very small pockets of Uttarakhand. Therefore it's very important to conserve this important human genetic pool, it can be achieved by better health management and counseling. Many studies reported that SCD was predominantly related to tribes and communities where congenial marriages are common [12 to 13] but during the present investigation we observed the occurrence of SCD in those families where congenial marriages are prohibited which indicates that the disease vulnerability is all across the population which needs immediate attention.

PROTEOMIC ANALYSIS

The increasing monarchy of investigative proteomics has added a sole dimension to the study of the complex path physiology involved in SCD. The proteomic study based on 1D SDS-PAGE for SCD identification was able to distinguish between carrier and diseased)

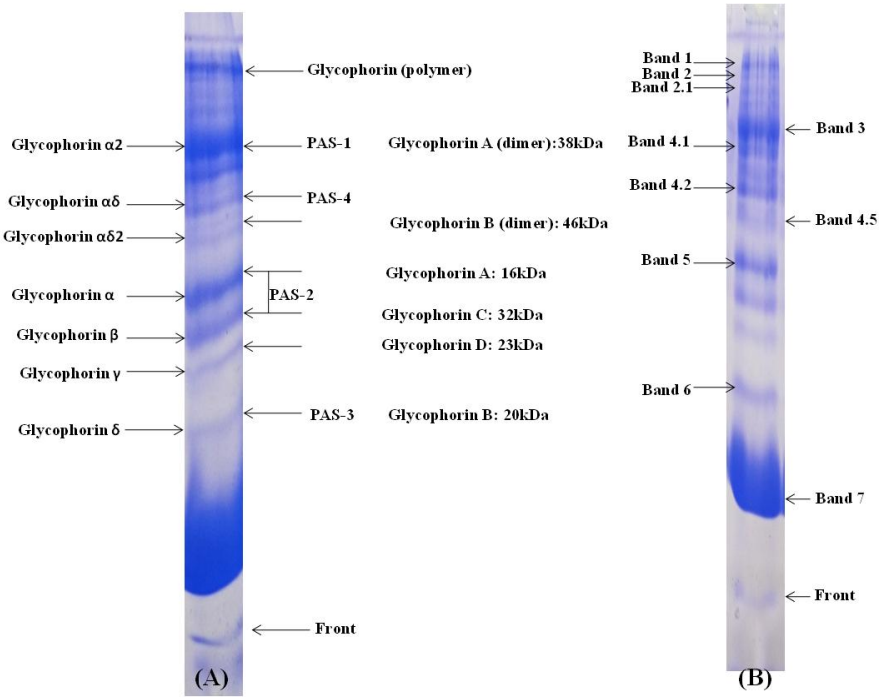

Figure 4: SDS-PAGE gel electrophoresis of human red blood cell protein. Healthy human RBC protein stained with Coomassie brilliant blue [14]. A; RBC proteins and glycoproteins have detected on SDS gels. B; showing the typical human red blood cell proteins separated by SDS-PAGE, which were similarities as shown by [15].

Samples of SCD diseased showed ~30kDa, ~20kDa and ~18kDa bands (Figure 5). It is clearly indicated in Figure 5 of UPGMA cluster constructed with the help of NYSYSpc software.

Two major clusters were generated and one cluster has 17 individuals and cluster two have 07. Cluster one was further divided in five sub-clusters while cluster two divided in three sub-clusters (Figure 6).

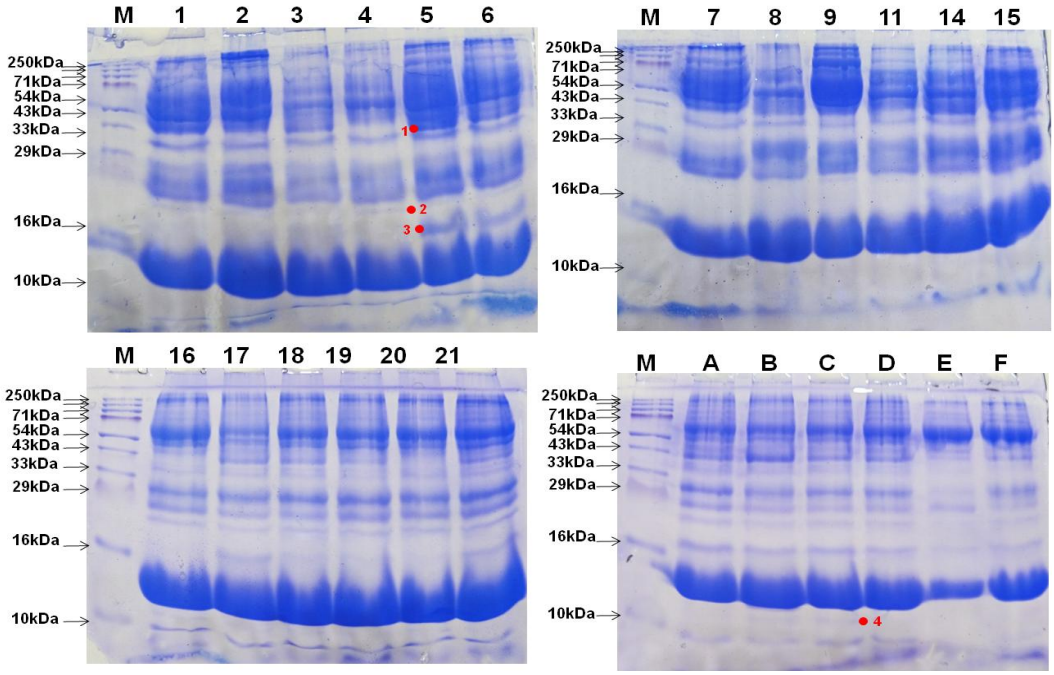

Figure 5: SDS-PAGE gel analysis of collected blood sample for identification of proteomic diversity from 4 families. The sample numbers 1 to 9 family (one), 10 to 15 family (Two), 16 to 21 family (Three) and A to F family (Four). Sample 4, 16, 20 and E were not appeared because all mentioned samples were SCD positive. Red dots indicate unique bands.

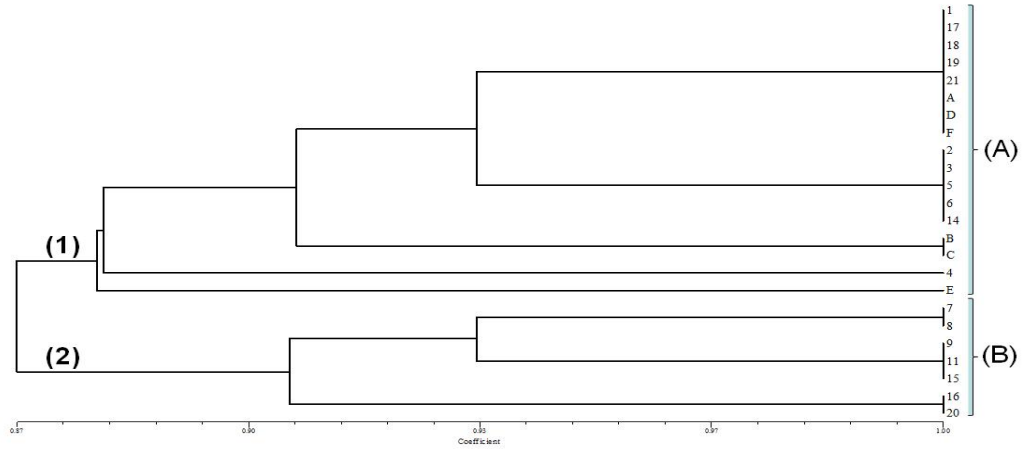

Figure 6: UPGMA clustering of SCD samples. Total 15 to 20 bands were monomorphic and 2 to 3 bands were polymorphic. Based on banding pattern samples were grouped in two major clusters.

DISCUSSION

Present investigation will be helpful for identification of SCD in tribe and non-tribe populations. The Indian Council of Medical Research and the National Rural Health Mission in different States are undertaking outreach programmes for better management and control of the disease [6]. Further there are challenges of clinical trials for studying the chronic SCD.

According to some researchers the surface of the human red blood cell is dominated by a small number of abundant blood group active proteins. The major proteins are the anion transport protein (band 3) which has AB (H) activity and Glycophorin A which has MN activity. Band 3 and Glycophorin A are of equal abundance in the normal red cell membrane (approximately $l0^6$ copies of each) and the two proteins may associate together as a complex [16 and 17]. The Band3 and Band 4 have different expression in SCA and healthy individuals similar kind of observation have been reported by many other researchers [18]. Glycophorin A was the most affected protein in sickle RBCs by the ERK1/2 pathway, which contained 12 unique phosphorylated peptides, suggesting that in addition to its effect on sickle RBC adhesion. Increased glycophorin A phosphorylation via the ERK1/2 pathway may also affect glycophorin A interactions with band 3, which could result in decreases in both anion transport by band 3 and band 3 trafficking [19, 20 and 21].

Table 4: Red blood cell membrane proteins [14]

Band	Molecular Mass (kDa)	Designation	Function
3	100	Band 3 (AE1)	Anion transporter
4.1	82	Band 4.1	Components of cytoskeleton
4.2	76	Band 4.2	
4.5	55	Band 4.5 (GLUT1)	Glucose transporter
5	43	Actin	Components of cytoskeleton
7	31	Stomatin	regulator of monovalent cation flux across the membrane [22]

In the present study it was observed that Band 3 and Band 4 have different expression in SCA and healthy one (Table 4). It is also proved that this study is helpful in preliminary screening of SCA which well substantiated by similar studies made the researchers [14]. It is also realized that the proteomic method of screen the disease will be cost effective to screen larger population. Stomatin is a 31 kDa integral membrane protein, named after the rare human haemolytic anaemia hereditary stomatocytosis. It regulates monovalent cation flux across the membrane [23].

CONCLUSION

The Findings and observations reveled that the degree of awareness at all level needs improvement to check the SCD especially the genetic counseling and health management practices to the affected individuals by healthcare providers and the public in general. Radom screening of SCD and other Hemoglobinopathies using cost effective methods are needed to achieve goal.

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CONFLICT OF INTEREST:
The author declares no conflicts of interest.

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