Background and objectives: This study aimed to characterize the spectrum of β-thalassemia mutations and haplotypes of sickle cell anemia in Beja tribes and other minor groups living in Port Sudan, Sudan.

Methods: This descriptive cross-sectional study was carried out from March 2011 to July 2013. Overall, 209 anemic patients were screened for hemoglobinopathy by capillary electrophoresis. The subjects were genotyped for β-thalassemia mutation by amplified refractory mutation system and for sickle cell haplotype by restriction-fragment length polymorphism.

Results: Of the 209 patients, 29 (13.87%) showed the typical -88(C→T) β-thalassemia mutation and 27 (12.91%) had sickle cell anemia, of whom 15 (55.6%) were heterozygous AS and 12 (44.4%) were homozygous SS. Based on results of the restriction-fragment length polymorphism; all subjects were with Benin haplotype (Benin/Benin).

Conclusion: Based on the results of this study, it is recommended to perform a potential carrier screening for the -88(C→T) mutation and sickle cell Benin haplotype by DNA analysis.

KEYWORDS: Genotyping, Hemoglobinopathies, Thalassemia, Sickle cell disease, Port Sudan.

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INTRODUCTION

Hemoglobinopathies may result from mutation of the α- and/or β-globin gene (1). The gene cluster responsible for the production of hemoglobin chains is hemoglobin β (HBB), which is located on the short (p) arm of chromosome 11. More than 250 mutations in the HBB gene have been found to cause β-thalassemia. B-thalassemia mutations are characterized according to the extent of defect in the formation of β-globin chains and reduced production of β-globin chains. Hence, mutation analysis of the β-globin gene is useful for predicting clinical phenotype of β-thalassemia, pre-symptomatic diagnosis of at-risk family members and prenatal diagnosis as well as prevention and control programs (2). Formerly, the distribution of thalassemia had been mainly limited to the Mediterranean area, the Middle East and Southern Asia, also known as the ‘thalassemia belt’. However, the recent migration of people has spread the thalassemia genes throughout the world. Given the demographic population shifts, thalassemia is at present considered as a global health problem.

Sickle cell anemia is caused by an HBB mutation that produces an abnormal form of the hemoglobin β-chain called hemoglobin S or (HbS). HbS results from substitution of glutamic acid with valine at position 6 in the β-chain. Abnormal HbS chains stick together and form long, rigid molecules that bend red blood cells into a sickle (crescent) shape, which can lead to shortage of red blood cells. The sickle-shaped cells can also block small blood vessels, thus causing pain and organ damage (3). Sickle cell disease (SCD) is believed to be the most frequent inherited blood disorder, affecting an estimated 100 million people worldwide, particularly the black people and those of the Mediterranean race. In Africa, SCD is the most prevalent genetic disease with a high mortality rate at age of one to five years. In the United States, sickle cell anemia is the most frequent autosomal recessive gene disorder that affects approximately one in every 375 persons of African ancestry (4). This disease was discovered early in Sudan, and the peak occurrence of SCD is among the Western Sudan population. It is believed that the sickle cell gene has been brought to Sudan by immigrants from the West African tribes, especially from Howsa, Wolof and Bargo (4).

Rates of sickle cell anemia and sickle trait vary in different areas of Sudan. The highest rates were observed in the Western and Eastern Sudan where one in every 123 children are affected. High consanguinity rates and malaria endemicity are strongly related to the distribution of the sickle cell gene in Sudan (5). In this study, we aimed to characterize the spectrum of β-thalassemia mutations and haplotypes of sickle cell anemia in Beja tribes and other minor groups living in Port Sudan, Sudan.

MATERIALS AND METHODS

This cross-sectional descriptive study was carried out from March 2011 to July 2013. The study included 209 patients who were selected from 600 patients referred to hematology laboratories in three major hospitals of Sudan for complete blood count (CBC). The subjects were selected according to the results of CBC and peripheral blood smear. Patients with laboratory features of anemia in the peripheral blood count were included and those with no laboratory evidence of anemia were excluded from the study. After obtaining verbal consent from all subjects, 5 ml of venous blood were taken and collected in potassium-EDTA tubes. Half of each blood sample was used for screening tests and the rest was stored at -20 °C until DNA extraction.

CBC was carried out using an automatic hematology analyzer (Sysmex KX 21N, Japan model 2007) and the peripheral blood smear. Electrophoresis was done using a fully automated capillary electrophoresis system (Cebia 2, Model 2007). DNA extraction was carried out using Jena Bioscience kits (Germany). The extracted DNA was checked with 1.5% agarose gel electrophoresis and stored at -70 °C until molecular testing.

Amplification-refractory mutation system (ARMS) was used to detect the -88(C→T) and -29(A→G) mutations, which are common in Afro-Americans (6). PCR reaction mixture (50 µl) contained 10X PCR buffer, 200 µM dNTPs, 1.5 mM MgCl₂ and 0.5 U of Taq DNA polymerase. Various primers were added to the reaction mixture [60 nM for -88(C→T) and 30 nM for -29(A→G)]. The common F primer was used as reverse primer for both forward primers (Table 1). The reaction was performed in two tubes for each patient.
(normal and mutant). The tubes were placed in a convergent thermal cycler (GmbH & Co. KG, SN: CT-TCL-205, Germany). The amplification conditions were as follows: initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 45 sec, annealing at 65 °C for 45 sec, extension at 72 °C for 1.5 min, and final extension at 72 °C for 7 min. The PCR products were separated on 1.5% agarose gel in 1X Tris-borate-EDTA buffer. The gel was stained with ethidium bromide and visualized under a UV illuminator.

**Restriction fragment length polymorphism (RFLP) for detecting sickle cell mutation**

Amplification of the HBB gene was carried by a standard PCR consisting of the following components: 5 μL of 10X PCR buffer, 1 μL (10 pm/μL) of forward primer, 1 μL (10 pm/μL) of reverse primer, 0.25 μL (25 mM) of dNTP mix, 1.5 mM (25 mM) MgCl2, 1 μL (100 ng) of DNA, 0.25 μl (5 U/μL) of Taq polymerase, and 40 μL of H2O. PCR cycling conditions consisted the followings: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 45 s, and final extension at 72 °C for 7 min.

PCR amplification was performed using the four pair of primers designed based on the published data for human β-globin chain sequence of known haplotypes (Cameroon, Benin, Senegal, Arab-Indian and Bantu) to amplify different DNA fragments of the β-globin S haplotypes.

Table 2 and 3 represent the restriction enzymes and primers used for the detection of different haplotypes.

For restriction enzyme analysis, 10 µl of the PCR product was mixed with 0.2µl (10 U) of restriction enzyme, 2.5µl of 10X buffer, and 12µl of H2O. The mixture was incubated at 37 ºC for 5-15 minutes, followed by 20 minutes of incubation at 80 °C for enzyme inactivation.

**Table 1: B-thalassemia primer sequences used in this study**

<table>
<thead>
<tr>
<th>Primers (5'-3')</th>
<th>Used with</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>African-American mutation primer</td>
<td>-88 (C-T) TCA CTT AGA CCT CAC CCT GTG GAG CCT CAT</td>
<td>F</td>
</tr>
<tr>
<td>Control primers</td>
<td>-29 (A-G) CAG GGA GGG CAG GAG CCA GGG CTG GGT ATG</td>
<td>F</td>
</tr>
<tr>
<td>Common F</td>
<td>CAA TAG GCA GAG AGA GTC AGT GCC TAT CA</td>
<td>861</td>
</tr>
</tbody>
</table>

**Table 2: Restriction enzymes used for detection of different sickle cell anemia haplotypes**

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>β Hind II/Xmn1</th>
<th>Hind III</th>
<th>HindI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senegal</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benin</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Cameroon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indian/Arab</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Bantu</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 3: Restriction enzymes and primers used for RFLP analysis of patients with sickle cell anemia**

<table>
<thead>
<tr>
<th>Restriction enzymes</th>
<th>Primers (5'-3')</th>
<th>Product size, bp</th>
<th>Presence of site, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xmn1</td>
<td>F: 5' AACTGTTCATTATAGGATT</td>
<td>655</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>R: AGGAGCATTATGATAACCTCAGAC</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>1.Xmn1's 5 to γγ'</td>
<td>F: AGT GCT GCA AGA AGA ACT ACC</td>
<td>328</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>R: CTC TG C ATG GGC AGT GAG CTC</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>2. HindI's 5 to βγ</td>
<td>F: AGT AGA GGC TTT ATT TGG AGG</td>
<td>638</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>R: GTT AAG GTG GTT GAT GAT AAC</td>
<td>302</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Of the 209 samples subjected to capillary electrophoresis, 29 (13.87%) had β-thalassemia. The typical -88 (C→T) β-thalassemia mutation was found in all subjects, but the -29 (A→G) transition was not observed at all (Figure 1).

The -88 (C→T) β-thalassemia mutation was most prevalent among the Hadandawa (37.9%), Bani Amer (34.5%) and Abderahmanab (10.3%) tribes, respectively. The frequency of the mutation was 3.4% in the rest of the tribes (Table 4).

The results obtained from sequencing of β-thalassemia samples also confirmed that all samples had the -88 (C→T) mutation (Figure 2).

Sequencing of β' and β genes
DNA purification and standard sequencing were performed for both genes by Macrogen Co. (Seoul, Korea). Sequence chromatogram was viewed by the FinchTV viewer application (http://www.geospiza.com/finchtv.shtml), and then nucleotide sequences of the genes were searched for similarity using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) (7).

Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignments using the phylogeny software (http://www.phylogen.fr/simple.phylogeny.cgi) (8,9).

Table 4- Distribution of the -88 (C→T) β-thalassemia mutation among different tribes in Port Sudan

<table>
<thead>
<tr>
<th>Tribe</th>
<th>Number (Male:Female)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hadandawa</td>
<td>11 (5:6)</td>
<td>37.9</td>
</tr>
<tr>
<td>Bani Amer</td>
<td>10 (8:2)</td>
<td>34.5</td>
</tr>
<tr>
<td>Abderahmanab</td>
<td>3 (1:2)</td>
<td>10.3</td>
</tr>
<tr>
<td>Armada</td>
<td>1 (1:0)</td>
<td>3.4</td>
</tr>
<tr>
<td>Hawa</td>
<td>1 (1:0)</td>
<td>3.4</td>
</tr>
<tr>
<td>Nuba</td>
<td>1 (1:0)</td>
<td>3.4</td>
</tr>
<tr>
<td>Rubatab</td>
<td>1 (1:0)</td>
<td>3.4</td>
</tr>
<tr>
<td>Danagla</td>
<td>1 (1:0)</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td>29 (19:10)</td>
<td>100</td>
</tr>
</tbody>
</table>

The results obtained from sequencing of β-thalassemia samples also confirmed that all samples had the -88 (C→T) mutation (Figure 2).

Figure 2- The -88 (C→T) mutation in four β-thalassemia samples compared to the reference sequence. Additional three base pair substitutions can be seen in the upper alignment.
Based on the phylogenetic tree, the tribal groups acquired the mutation independently. Moreover, the mutation seems to have appeared in Bani Amer and Nuba long before Hadandawa and Danagla (Figure 3).

Among the 27 (12.91%) samples positive for presence of HbS, 15 (55.6%) were heterozygous AS and 12 (44.4%) were homozygous SS. RFLP indicated that all these samples were of Benin haplotype (Figures 4 and 5).

**Figure 3-** Phylogenetic tree of the β-thalassemia mutation in four tribal groups

**Figure 4-** PCR amplification for the Hinf I reaction showing the typical 638 bp bands

**Figure 5-** PCR amplification for the Hinf I enzyme digestion

**Figure 6-** Sequencing alignment for Hinf I PCR products. The sample belonged to a patient from the Bani Amer tribe. 15 base pair substitution can be seen as compared to the reference sequence. Three recognition sites can be seen in the figure and the fourth one has been diminished due to a single bp substitution in the query strand (A→G).

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Sequencing was carried out for selected samples with the sickle cell hemoglobin. The sequences obtained for Hind III PCR products yielded no significant similarity when compared to the reference genomic strands on the BLAST. However, the presence of the cutting site (GANTC) was recognized by the Hinf I enzyme (Figures 4-6).

Figure 6. Sequencing alignment for Hinf I PCR products. The sample belonged to a patient from the Bani Amer tribe. 15 base pair substitution can be seen as compared to the reference sequence. Three recognition sites can be seen in the figure and the fourth one has been diminished due to a single bp substitution in the query strand (A→G).

DISCUSSION

Currently, little is known about the occurrence and manifestations of hemoglobinopathies in Eastern Sudan, namely the Red Sea State. Approximately 250 million people (4.5% of the world population) carry a potentially pathologic hemoglobinopathy gene. About 300,000 infants are born with major hemoglobinopathies every year. Owing to the rise in global immigration, hemoglobinopathies have also appeared in non-endemic areas. Many developing countries have provided a convenient genetic approach for controlling chronic childhood diseases and hemoglobinopathies, including thalassemia and SCD (10). According to Bain, the prevalence of HbS, HbC and β-thalassemia in Sudan is 1-17%, 0%, and 1-10% (overall prevalence: 4%), respectively (11). However, Elderdery et al. reported two cases with HbSC in Sudan (11, 12). They also reported the S gene as the most common variant among the Western tribes (12%) and the general Sudanese population (6.1%) (13). These results are in line with our findings. In another study on relatives of sickle cell patients in Western Sudan, 54% of the subjects were SCD (14). Another study by Elderdery et al. reported HbS as the most common abnormal Hb, which could be due to inclusion of a known population of patients with the disease (15). Using molecular analysis of the β-globin gene cluster haplotypes, four of the five typical β-S-globin haplotypes [Cameroon (35.0%), Benin (29.4%), Senegal (18.2%) and Bantu (2.8%)] were identified in a Sudanese population (16), and three atypical haplotypes were identified in 17 patients (14.6%). Limited data are available on the prevalence and manifestations of thalassemia in the Sudanese population (17). In Nigeria, the prevalence of homozygous SCD (Hb SS), Hb SC disease and sickle trait (HB AS) was reported to be 3.1%, 1.1% and 23.7%, respectively (18). The prevalence of sickle cell trait was significantly lower in our study, which can be attributed to the higher prevalence of the S gene in the West African populations (19). In a study by Elgari et al., the frequency distribution of sickle cell phenotypes including SS, AS, SC and SF based on hemoglobin electrophoresis was 36.2%, 61.2%, 1.3% and 1.3%, respectively (20). These findings are consistent with ours except for the absence of Hb SC in our study population. The worldwide prevalence of thalassemia carriers is more than SCD, but the high frequency of the sickle-cell gene in certain areas results in a high birth rate of homozygotes. Overall, SCD accounts for about 70% of total hemoglobin disorders (10). In our study, the sequencing of the mutant genes for both sickle cell anemia and β-thalassemia suggests that the genes were introduced to the target population through migration of several tribes of African descent into the area in the beginning of the last century rather than being arisen from malaria endemcity.

heterozygous carriers (Hb AS), 42% were normal (Hb AA), and 4% were diagnosed with...
CONCLUSION

The data obtained from this study clearly indicates that hemoglobinopathies are common among people of the Beja tribes, which requires establishment of centers for the diagnosis and control of the disease. The hemoglobin variants and thalassemia might have been brought about by the migration of many tribes of African descent to Sudan during the early years of the last century.

REFERENCES


