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β^s-GLOBIN HAPLOTYPES IN PATIENTS WITH SICKLE CELL DISEASE IN GHANA

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Abstract

We studied β^s-globin haplotypes for the first time in 53 patients with SCD attending the Sickle Cell Clinic at the Korle-Bu Teaching Hospital, Accra, Ghana, and 30 control subjects of healthy blood donors from the year 2009 to 2012. We determined the haplotypes by PCR and RFLP, and the clinical and hematological variables by standard procedures. A total of 9 different haplotypes were defined for the population. These were: Ben/Atp, (Atp:Atypical), Ben/Ben, Ban/Ben, Atp/Atp, Cam/Ben, Ban/Atp, Sen/Sen, Sen/Atp and Cam/Atp. A total of 55 chromosomes (39.8%) were characterized as BEN haplotype, 23 chromosomes (16.7%) as BAN haplotype, 5 chromosomes (3.6%) as CAM haplotype, and 3 chromosomes (2.2%) as SEN haplotype. No Arab Indian haplotype was seen in the population. Fifty-two (52) chromosomes (37.7%) were characterized as Atypical (Atp) haplotype. The Ben and Atp haplotypes were the most prevalent among patients with SCD (51.2%), and HbCC in the control group (27.3%). However, in HbAA subjects, the Ban and Atp haplotypes were most prevalent (p=0.0001). None of the HbSS patients had a Sen haplotype even in the heterozygote. Sen was a rare haplotype in all subjects (p=0.0001) while Cam haplotype was also rare in the SCD population (p=0.0001). The respective allele frequencies for HbS, HbC, and HbA were determined to be 0.53, 0.25 and 0.22. The Linkage Disequilibrium (LD) of an HbS allele on a Ben haplotype was 0.32, while the HbA allele was in linkage with the Ban haplotype (LD=0.21). The hematological indices affirmed that, an HbS allele is necessary for severe disease phenotype.

Introduction

Sickle cell disease (SCD) is a co-dominant genetic disorder (Hunter, 2005; Ramirez and Frei-Jones, 2017; Steinberg and Adewoye, 2006) defined by the HbS variant of β-globin gene (Bolke and Scherer, 2012). The common forms are HbSS, HbSC, and HbSβ°/β^+ (Ababio and Quaye, 2016). These variants are characterized respectively by E6V ($\beta^{6\ GAG\rightarrow GTG}$), E6K ($\beta^{6\ GAG\rightarrow AAG}$), and $\beta^{\circ/+}$ mutations. The homozygous HbSS variant is the most commonly inherited SCD disorder in the world, Creary et al. (2007) referred to as sickle cell anemia (SCA). SCA manifests as a chronic hemolytic disease with episodic vaso-occlusive complication that results in pain and frequent hospitalizations (Steinberg, 1998). The World Health Organization (WHO) estimates that up to 25 million people Aliyu et al. (2008) have sickle cell disease. Sub-Saharan Africa accounts for 60% of this population while India and other parts of the world with African ancestry including the Middle East, have 33.3% and 13.3% respectively. In West Africa, Ghana has the second highest prevalence of SCD and it accounts for 2% of all Ghanaian newborns annually Aliyu et al. (2008). This trend is attributed largely to natural selection by *Plasmodium* species since it affords protection.

Across sub-Saharan Africa and regions where SCD exists, differences in disease severity have been established (Kato et al., 2018). These are attributed partially to cis (Nagel et al., 1985, 1987) and trans-acting (Boyer et al., 1984; Dover et al., 1987, 1992) genetic factors within the β-globin gene cluster haplotypes and linked to the βs allele (Dover et al., 1992; Labie and Elion, 1999; Nagel and Steinberg, 2001; Zago et al., 2001, 2000). There are five predominant haplotypes in the β-gene cluster that affects the phenotype in SCD. These are the Senegal (Sen), Arab-India (Car), Benin (Ben), Cameroun (Cam), and Bantu (Ban) types in order of increasing clinical severity. Each haplotype is associated with a set of hematological and genetic factors that define the clinical phenotype (Akinsheye et al., 2011; Nagel et al., 1991, 1987; Schroeder et al., 1989). For instance the Senegal and Arab-Indian haplotypes have the highest HbF levels (>5), higher packed cell volume (PCV) and mildest clinical presentation (Akinsheye et al., 2011; Powars, 1991; Schroeder et al., 1989). In contrast, the Bantu haplotype is characterized by very low HbF (<5) (Liu et al., 2009; Nagel et al., 1987), low PCV and a severe clinical course (Antonarakis et al., 1982; Nagel and Labie, 1989). The Benin haplotype is intermediate. In spite of these distinctions, there is still considerable variation in the clinical course of subjects within each haplotype group. This indicates that other elements including environmental interactions could affect a clinical sub-phenotype.

In Ghana, SCD has a prevalence of 2% (Acquaye and Oldham,

1973; Konotey-Ahulu, 1974) with SCA being 50% of such cases. Sickle cell trait is found in approximately 13-15% of the population (Kreuels et al., 2009; Steinberg et al., 2008). SCA is associated with significant childhood and adult morbidity and mortality cause in Ghana (Commey and Dekyem, 1995; Konotey-Ahulu, 1974) which has led to considerable work on the epidemiology of the disease, its manifestation and clinical variables in the country (Acquaye and Oldham, 1973; Ankra-Badu, 1992; Commey and Dekyem, 1995; Konotey-Ahulu, 1974). However, the data linking these variables with genetic factors that specify differences in the disease presentation are unavailable (Ohene-Frempong et al., 1998). Here, we present a first report on the haplotypic clusters associated with the β^s globin gene that specify variations in the clinical subphenotypes of SCD in the country, using restriction fragment length polymorphism (RFLP).

Materials and Methods

Study subjects

Patients were recruited from the sickle cell clinic, Ghana Institute of Clinical Genetics (GICG) of the Korle-Bu, Teaching Hospital, Accra. A total of 83 subjects were studied; made up of 58 patients with sickle cell disease and 25 apparently healthy subjects who served as controls. The patients were Ghanaians with sickle cell disease who were attending the outpatient clinic and were in a steady state. Subjects with sickle cell disease above 13 years were included irrespective of their genotype. A subject in steady state had neither acute painful crisis nor any alterations brought on by therapy. The control subjects were individuals who qualified medically to donate blood at the National Blood Donation Centre, Korle-Bu Teaching Hospital, Accra. Subjects who declined to respond to the questionnaire were excluded as well as patients with palpable liver disease or known glucose-6-phosphate dehydrogenase deficiency (G-6-PD). Subjects were recruited consecutively at the outpatient unit after the University of Ghana Medical School ethical approval (MS-Et/M.10-P.3.4/2009-10) and informed consent. At enrolment, the age (years) was recorded, while measurements for Body Mass Index: (Weight (Kg); Height (meters), Systolic Blood Pressure (SBP, mmHg), and Diastolic Blood Pressure (DBP, mmHg) were taken. Information on any disease from the folders of the patients were noted and subjects who did not qualify by the inclusion criteria were not enrolled. Subjects were weighed in light clothing and without shoes. Weight was measured with a heavy-duty Seca 770 floor scale (Hamburg, Germany) to the nearest 0.1kg. Height was measured (to the nearest millimeter) using a height rod with the subject standing erect with heels together. Blood pressure (BP) was also measured to the nearest 2mmHg in the non - dominant arm of seated individuals on two occasions with a mercury sphygmomanometer and a cuff of appropriate size. Patients with SCD were included after confirmation of sickle cell disease by cellulose acetate electrophoresis. The electrophoresis were done at the Center for Clinical Genetics when the patients first visited for care.

Blood Sample collection and processing

Four millimeters (4 ml) blood was drawn from an antecubital vein by means of a plastic syringe and dispensed into EDTA tubes.

Cellulose acetate electrophoresis

The cellulose acetate strips were immersed in Tris-buffer (Ohene-Frempong et al., 1998) for five minutes, blotted evenly between two sheets of Whatman no. 3 filter paper to remove excess moisture, and mounted horizontally in the electrophoretic tank. The strips were allowed to equilibrate in the closed unit for 10 minutes. The test samples and controls (i.e. lysates) were applied as 1–1.5cm strips and placed 0.5 cm on the cathode side of the mid-point of the strip (Figure 1). Several specimens were run simultaneously. Optimal separation occurred in 45 minutes at 20 to 25 volts/cm with a current of 0.3 – 0.5 mA/cm. The strips were removed after a 45-minute period of electrophoresis and cut midway between the areas of greatest concentration. Hemoglobin bands were then developed in 5% Ponseau-S stain and de-stained with 5% Tricarboxylic acid.

Full blood count

The automated SWELAB analyzer was used for complete blood count after a control sample had been used to standardize the analyzer. The analysis involved first mixing the blood on a roller and placing it on a rack in the Swelab^{TM} AlfaLyse analyzer (Stockholm, Sweden) for automated sampling and analysis.

DNA extraction and storage

Genomic DNA was extracted using QIAmp DNA kit (Qiagen Inc., Valencia, CA) and quantified with the Fisher Thermo Scientific Nanodrop 2000/2000C, serial number 8273, USA. All DNA isolates were reconstituted in 50 µl of Qiagen buffer and stored at -20°C until Hemoglobin β genotyping and β-locus haplotyping.

Detection of SCD by RFLP on the hemoglobin beta (HBB) gene

The primer set F:5'-AGGAGCAGGGAGGGCAGGA-3'; R:5' -CCAAGGGTAGACCACCAGC-3', was used to generate a primary amplicon of length 358 bp. This amplicon was then digested with Mnl I [5'CCTC(N)7'3;3'GGAG(N)'5'] which permitted the discrimination between HbAA, HbSS/CC/SC, HbSC/AC, HbAE and HbEEs genotypes. A second digest with Dde I (C/TNAG) enabled an unambiguous discrimination between HbSS, HbCC, HbSC, HbAS and HbAC. Each digest was performed at 37°C for 2.5 hours by the manufacturer's protocol and resolved on 3% agarose gels.

The PCR reaction mix consisted of 1 μl DNA, 0.2 mM of each dNTP, 2 μM of each primer set, 1 unit of DNA Hotstart polymerase and a buffer containing 2.5 mM $MgCl_2$ in a total volume of 20 μl.

The PCR cycling conditions for the amplification of the HBB gene were 15 minutes incubation at 94°C, followed by 45

amplification cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec and elongation at 72°C for 60 sec and a final elongation at 72°C for 10 minutes. The reaction was performed on a DNA thermal cycler TC-5000 with the lid heated to 105°C at the start of the cycling. At the end of the amplification, fractions of the PCR products were visualized by electrophoresis on a 1.5% agarose gel and documented with a gel imager.

β-Globin locus haplotyping by RFLP analysis

The five classical RFLP sites used to determine sickle cell β-globin gene haplotypes are shown in Figure 1 (Enevold et al., 2005; Hanchard et al., 2007). These were used to define the haplotypes for the study population. Table 1 is the full list of the primers and amplicon sizes generated from the PCR, specific to each restriction enzyme site that was used for the restriction digests in defining the haplotypes. Each reaction mix consisted of 1µl DNA, 0.2 mM dNTP, 2 µM each of a primer set, 2.5mM $MgCl_2$, 0.5 units Hotstart DNA polymerase, 4 µl of PCR coral load buffer 1X and PCR water added to a total volume of 20 µl. The PCR conditions for amplification were initial denaturation at 94°C for 15 min, followed by 45 cycles of 1 min denaturation at 94°C, 30 sec annealing at 56°C and 30 sec elongation at 72°C for 1 minute, and then final elongation at 72°C for 10 minutes.

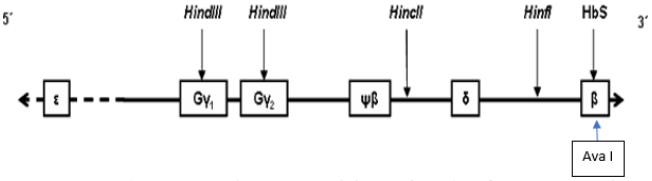

Figure 1. The approximate positions for the five RFLP sites are indicated (Hanchard et al., 2007)

Haplotype assignment

Each haplotype was assigned following restriction digest of PCR products generated with primers as shown in Table 1. Hind III, Hinc II, Ava II, and Hinf I- restriction enzymes were used for the digestion according to the manufacturers instructions. Respective haplotype assignment for the digests were: Ben (HincII/5'ε, HincII/ε, HindIII/Gγ, HindIII/Aγ, Hinc/$\psi\beta$, HinfI-, AvaII/β: (- - - - - - +), Ban (- - + - - - +; - - + - - + +), Cam (- - + + + + +), Sen (- - + - + + +), Atypical (Atp): (- - - - - - -, - - - - - + +, - - - - - + - +) where a () or (+) represents the absence or presence of a restriction site on the amplicon.

Results and Discussion

The 58 patients with SCD consisted of 33 HbSS and 25 HbSC individuals, while the 25 control subjects consisted of 11 HbCC and 14 HbAA individuals Figure 1.

The clinical variables are presented in Table 2. Significant differences in the clinical variables between patients with SCD and controls were observed for BMI, hemoglobin, rbc count, RDW, hematocrit, platelet count, total count for white blood cells and the differentials (lymphocytes and granulocytes). Specifically, patients with SCA were leaner, with reductions in all red blood cell indices while the indices for white blood cells (total count and differentials) were elevated. Patients with SC phenotype had apparently similar findings as the controls in terms of clinical indices. An elevation in RDW with normal MCV is characteristic of SCA patients. The hematological indices seem to indicate that an HbS allele is necessary for severe disease phenotype. When the hematological profiles were stratified by haplotypes, the subject numbers were too small for any meaningful conclusions to be deduced.

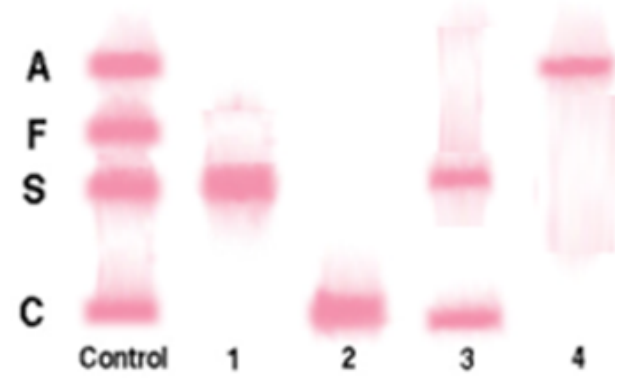

Figure 2. Cellulose acetate electrophoretogram

β-Haplotypes by RFLP: The β-globin haplotypes and allele frequencies are presented in Table 3. A total of 9 different haplotypes were defined for the population (Table 3; Figure 3.0 to Figure 3.5).

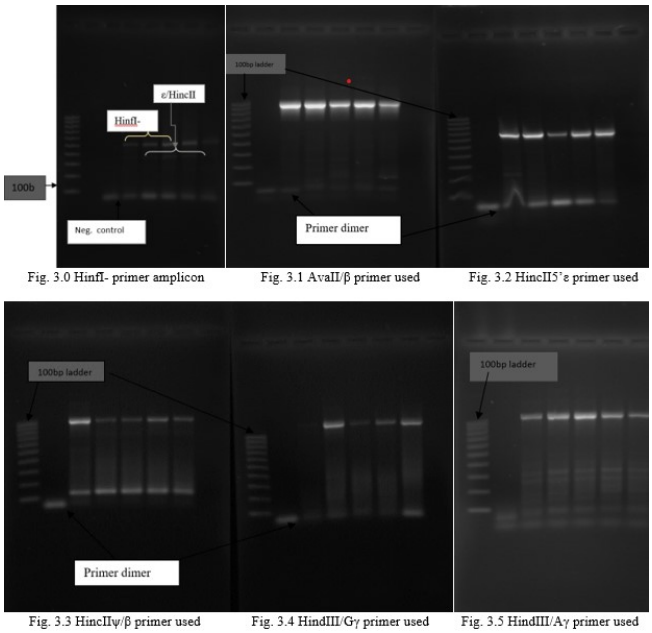

Fig. 3.0 HinfI- primer amplicon | Fig. 3.1 AvaII/β primer used | Fig. 3.2 HincII5'ε primer used

Fig. 3.3 HincIIψ/β primer used | Fig. 3.4 HindIII/Gγ primer used | Fig. 3.5 HindIII/Aγ primer used

Figure 3. β-globin haplotypes and allele frequencies

These were: Ben/Atp, (Atp:Atypical), Ben/Ben, Ban/Ben,

Table 1. Primers for genotyping of Beta-globin locus haplotypes by RFLP

Haplotype	Primer sequence	Fragment (bp)	Reference
Hinf I-	F:5'-TAAAGAGAAATAGGAACTTGACTG-3' R:5'-TCCAAGGGTAGACCACCAGC-3'	989	(Enevold et al., 2005)
Hinf I+	F:5'-TAAAGAGAAATAGGAACTTGACTC-3' R:5'-TCCAAGGGTAGACCACCAGC-3	828	(Currat et al., 2002)
ε/Hinc II	F:5'-AAGGCTTTAGTCCCACTGT-3' R:5'-ACCCTCTTCATCATCTTCCA-3'	336	(Hanchard et al., 2007)
Hinc II/5'ε	F:5'-CCTTCCCAGTGAGAAGTATAAGCAG-3' R:5'-AGTCATTGGTCAAGGCTGACCTGTG-3'	710	(Modiano et al., 2001)
Hind III/Gγ	F:5'-CAATTGAAACATTTGGGCTGGAGTAG-3' R:5'-CCTCTTTAGGCATGCGTCAACACTT-3'	1202	(Modiano et al., 2001)
Hind III/Aγ	F:5'-CAATTGAAACATTTGGGCTGGAGTAG-3' R:5'-TTTCTTAGGCATCCACAAGGGCTGT-3'	1042	(Modiano et al., 2001)
Hinc II/$\psi\beta$	F:5'-GATGAGGGAACAGAAGTTGAGATAG-3' R:5'-GTTCTCTCTTTTCTTGCAGGATTGC-3'	996	(Modiano et al., 2001)
Ava II/β	F:5'-TTGGGGATCTGTCCACTCCTGAT-3' R:5'-CCAGCCTTATCCCAACCATAAAATAA-3'	963	(Modiano et al., 2001)

Atp/Atp, Cam/Ben, Ban/Atp, Sen/Sen, Sen/Atp and Cam/Atp. A total of 55 chromosomes (39.8%) were characterized as BEN haplotype, 23 chromosomes (16.7%) as BAN haplotype, 5 chromosomes (3.6%) as CAM haplotype, and 3 chromosomes (2.2%) as SEN haplotype. No Arab Indian haplotype was seen in the population. 52 chromosomes (37.7%) were characterized as Atypical (Atp) haplotype. Among SCD patients, the Ben and Atp haplotypes were the most prevalent, as was the HbCC subjects in the control group (Table 3). However, in HbAA subjects, the Ban and Atp haplotypes were the most prevalent (p=0.0001). None of the HbSS patients had a Sen haplotype even in the heterozygote. Sen was a rare haplotype in the population (p=0.0001) while Cam haplotype was also rare in the SCD population (p=0.0001). The respective allele frequencies for HbS, HbC, and HbA were determined to be 0.53, 0.25 and 0.22. Based on expectation maximization using these frequencies and haplotype frequencies, the Linkage Disequilibrium (LD) of an HbS allele on a Ben haplotype was 0.32. The HbS allele appears to be in linkage with the Ben haplotype, while the HbA allele was in linkage with the Ban haplotype (LD=0.21). HbSC and HbCC subjects had similar prevalence of Ben, Atp and Ban haplotypes and appear unlinked to a haplotype. There was considerable heterogeneity in the population as seen in the number of atypical haplotypes (p=0.001) which ranked second after the Ben haplotype.

We have determined for the first time, the haplotype genetic background in Ghanaian patients with the β^s allele. Our results show that the β^s allele in Ghana is predominantly associated with the Benin and Atypical haplotypes in the homozygous β^{ss} or heterozygote β^{sc} respectively. Interestingly this haplotype was relatively high in control subjects with homozygous β^c allele. In central West Africa, the reported geographical distribution for this haplotype covered Nigeria, Benin, Togo and Cote DIvoire. Our study affirms the haplotypic pattern (Antonarakis et al., 1984; Lee et al., 2002; Swensen et al., 2010) in Ghana as being consistent with the pattern observed in the region. Considering that the Bantu haplotype was on an ancestral βA genetic background, with the Cameroon and Senegal haplotypes being rare in the population, it appears there has been very little admixture in the population with an environmental factor driving and sustaining the selection of the haplotypes. The atypical haplotype appeared to be largely on a Ben and to a smaller extent Bantu haplotype background. The five typical haplotype backgrounds seen in Africa on the β^s allele: Bantu, Cameroon, Benin, Senegal and Arab Indian haplotypes are known to have occurred independently from recombinational events and gene conversions. In population genetics, the process of natural selection is such that if the mutation of an allele is more recent and rapid (hard selective sweep), then, the haplotype background which is more ancestral is retained. On the other hand a softer and slower genetic change will result in the accumulation of variant haplotypes within the ancestral background (Gueye Tall et al., 2017; Swensen et al., 2010). β^s and β^c have been implicated to confer protection against malaria in the heterozygous (Hermisson and Pennings, 2017; Pennings and Hermisson, 2006). This fitness advantage has enabled the selection of both alleles in malaria-endemic environments. Recently it was shown that the protective mechanism of the alleles has convergence on a reduced cytoadherence of infected RBCs in the vasculature. The findings in the present study appear to suggest a genetic sweep (Hermisson and Pennings, 2017; Verra et al., 2009) within the population driven by strong environmental factors leading to the transfer of the mutant β^s allele unto the benign Benin haplotype, which is being selected for survival, together with an ancestral β^c allele in the population. These haplotypes confer variable disease severity to patients, with the severest being the Bantu haplotype and the least the Ben haplotype within the central West African region (Esoh and Wonkam, 2021; Nagel and Stein-

Table 2. Clinical variables in the study population

	SS	SC	CC	AA	p-value
N	33	25	11	14	
BMI(kg/m^2)	20.7 ± 4.3*	24.9 ± 3.5	25.9 ± 2.4	25.6 ± 2.6	0.000
Age(years)	30.6 ± 13.7	29.8 ± 11.0	31.6 ± 5.1	33.1 ± 10.1	0.849
SBP(mmHg)	120.9 ± 25.2	118.2 ± 0.6	120.7 ± 7.1	117.1 ± 7.3	0.862
DBP(mmHg)	71.0 ± 15.7	73.4 ± 7.3	79.5 ± 11.1	76.4 ± 7.4	0.175
Rbc(10^{12}/L)	3.0 ± 0.7	4.2 ± 2.4	4.9 ± 0.3	4.8 ± 0.6	0.000
MCV(fL)	81.4 ± 9.2	77.0 ± 14.8	80.6 ± 3.3	81.0 ± 5.6	0.419
RDW(%)	21.3 ± 3.6	17.7 ± 13.3	15.4 ± 0.9	15.2 ± 0.8	0.036
Hct (L/L)	24.3 ± 4.9	32.1 ± 4.9	39.6 ± 3.4	38.4 ± 4.4	0.000
Plt(10^9/L)	559.4 ± 621.6	209.0 ± 91.0	214.5 ± 48.1	234.9 ± 74.6	0.004
MPV(fL)	7.9 ± 2.0	8.1 ± 1.1	8.0 ± 0.6	7.8 ± 0.6	0.925
Wbc(10^9/L)	14.2 ± 7.4	7.4 ± 3.6	5.0 ± 1.6	5.2 ± 1.3	0.000
Hb(g/dl)	8.6 ± 1.7	11.4 ± 1.6	13.8 ± 1.2	13.6 ± 1.7	0.000
MCH(pg)	28.7 ± 3.4	27.4 ± 2.3	28.2 ± 1.5	28.7 ± 1.9	0.286
MCHC(g/L)	35.3 ± 1.5	35.6 ± 2.0	35.0 ± 1.1	35.4 ± 1.0	0.747
Lym(109^9/L)	7.7 ± 2.2	3.2 ± 3.4	2.2 ± 0.6	2.2 ± 0.8	0.000
Gra (10^9/L)	7.2 ± 5.2	3.7 ± 4.1	2.3 ± 1.0	2.4 ± 0.8	0.000

P-values below 0.05 were said to be significant. ***Mean** ± **SD***

Table 3. Proportions of subjects with SCD and control subjects for the classically defined beta-globin gene haplotypes

Haplotype	HbSS(%)	HbSC(%)	HbCC(%)	HbAA(%)
Ben/Atp	17/30(56.7)	5/13(38.5)	3/11(27.3)	4/15(26.7)
Ben/Ben	6/30(20)	1/13(7.7)	1/11(9.1)	0/15(0)
Ban/Ben	3/30(10)	1/13(7.7)	2/11(18.2)	2/15(13.3)
Atp/Atp	2/30(6.7)	0/13	0/11	0/15
Cam/Ben	1/30(3.3)	0/13	1/11(9.1)	0/15
Ban/Atp	1/30(3.3)	5/13(38.5)	1/11(9.1)	8/15(53.3)
Sen/Sen	0/30	1/13(7.7)	0/11	0/15
Sen/Atp	0/30	0/13	0/11	1/15(6.7)
Cam/Atp	0/30	0/13	3/11(27.3)	0/15

Key: Benin was designated Ben, atypical as Atp, Bantu as Ban, Senegal as Sen, and Cameroun as Cam

berg, 2001; Weatherall et al., 2002) due to regulatory levels of expression of HbF associated with each haplotype. Since the βA allele appears to be linked to the Bantu haplotype, it appears that environmental forces selecting for a less risky haplotype with the greatest chance for survival, are driving the selection for the Ben haplotype on the β^s allele within the region. It is interesting that from the eastern to the western corridor of the West African coast, the haplotypes associated with the β^s allele get more benign regarding disease severity. It is unequivocally established that malaria disease is driving a balanced act of maintaining the β^s allele in areas of endemic Plasmodium falciparum infection as the heterozygous confers protection as opposed to the homozygous. In a recent study, it was suggested that one probable mechanism in operation for the maintenance of the different haplotypes geographically is through allelic exclusion.

Conclusion

In conclusion, the present study puts Ghana on the β^s allele haplotype map in Central West Africa, showing the Ben haplotype as the dominant genetic background in the country.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

Data Availability

Data is available at Synapse with an ID being syn29715327 and a running title as SCD haplotypes.

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