Original Article

Variations in the β-Globin genes of Sickle Cell Anaemia Patients in Zaria, Northwestern, Nigeria

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ABSTRACT

Context: Sickle Cell Anaemia (SCA) is a genetic disorder with a life-long disability, which is of public health importance. The diversity in its clinico-pathologic and laboratory presentations may be due to the interplay between additional genetic differences and environmental factors. The genetic factors may be within the β-globin gene itself, the β-globin gene cluster or elsewhere in the genome. **Aim:** To characterize the β-globin gene for variations associated with the Sickle Cell mutation. **Settings and Design:** A cross-sectional descriptive study involving 51 adult SCA patients attending Sickle Cell Clinic of Haematology Department Ahmadu Bello University (ABUTH) Zaria, Kaduna State, Nigeria. **Methods and Material:** The buccal swab specimens were collected and β-globin gene DNA sequencing was done. The sequences obtained were compared with a Genbank Reference β-globin gene (NC_000011.9) using Basic Local Alignment Search Tool (BLAST), and variations noted. Data generated were analyzed using SPSS Version 20.0. **Statistical analysis used:** Data generated was summarized by using charts, means±2SD, and 95% confidence intervals. **Results:** There were 40 (78.43%) females and 11 (21.57%) males. The mean age of the participants was 25.35 ± 7.67 years, 95% CI (23.20, 27.51). The classic sickle cell mutation A T was present in all participants. The mean number of β-Globin gene variations was 8.61±11.30, 95% CI (5.43, 11.78). The number of Substitutions were 122 (27.79%), insertions 184 (41.91%), and deletions 133 (30.30%). These occurred in various combinations. The mean number of substitutions, insertions, and deletions were 2.39±3.23, 3.61±7.66, and 2.60±2.46 with 95% CIs of (1.48, 3.30), (1.45, 5.76), and (1.92, 3.30) respectively. **Conclusions:** There are β-globin gene variations in SCA patients in Zaria, and locally relevant genetic database of the SCA population will be the cornerstone in understanding genotype-phenotype interactions in this disorder.

Key Messages: There are β-globin gene variations in SCA patients in Zaria. Generating locally relevant genetic databases of the SCA population will be the cornerstone in understanding genotype-phenotypeinteractions in this condition.

KEYWORDS: *β-globin gene, sca, variations*

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INTRODUCTION

Sickle Cell Anaemia (SCA) is a public health problem deserving national and international support.[1] It is a genetic disease that links biochemistry, pathology, natural selection, population genetics, gene expression, genomics,[2] and medical anthropology.[3,4] The genetic disorder has been shown to be a mutation in the DNA coding for the amino acid in the sixth position of the β-globin chain, which leads to a substitution of thymine for adenine.[5] This substitution is responsible for the myriad of features associated with this condition. However, these features may be attenuated or aggravated by additional mutations on the β-globin gene or elsewhere.[6]

The β-globin gene is located in the 11p15.5 region of Chromosome 11 constituting 1.6kb of the β-globin

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gene cluster.[7] Several disorders of the β-globin chain of haemoglobin leading to different disease phenotypes have been described.[8] Given the varying clinical severity of this Mendelian single-gene disease, it is obvious that other factors are responsible for these protean presentations. Such factors include environmental and allelic differences within the β-globin gene cluster and other unlinked loci. It has, therefore, been postulated that genetic analyses among sickle cell patients may eventually lead to therapeutic improvements.[2]

Several compound heterozygote forms of Sickle Cell Disease (SCD) have been described but these do not follow the pattern of increased severity with each additional mutation. Common forms include: Haemoglobin SC (HbSC), HbSD, and HbSE. Uncommon β-globin gene variants that have been found in association with HbS include: HbD Los Angeles, HbD Ibadan, Hb D Bushman, Hb City of Hope, and Hb C-Harlem to mention but a few.[4] A study in Thailand revealed 25 different beta globin mutations, with two Hb Variants identified for the first time in this population. These were Hb Tacoma and Hb Tende.[9]

Several types of Sickle haemoglobin variants with a second mutation on the β globin gene have been described and these have varying presentations.[10-18] They also cause diagnostic difficulties with routine diagnostic facilities.[10] HbS-Oman for example, which has two mutations on the same β-globin chain, has been described as a dominant Sickle cell syndrome.[18]

The molecular structure of the beta globin gene has a bearing on the primary, secondary, tertiary, and quaternary properties of the globin chains. The polypeptide chains interact with four haem moieties in haemoglobin. Any disorder at the genetic level may eventually distort the physicochemical properties of the molecule, the most significant being the effective carriage of oxygen from the lungs to the tissues.[19]

The other groups of abnormal haemoglobins, which may cause clinical disorders are unstable haemoglobins, haemoglobins with high or low oxygen affinity, and the methaemoglobins.[20]

The underlying pathophysiologic mechanisms differ in various forms of haemoglobin variants. While some have been elucidated, others remain putative. For instance Hb C-Harlem, which has been found in several black families, is less stable than HbA and HbS, undergoes sickling when deoxygenated and gels at higher concentrations than HbS.[17,21] On the other hand HbS-Travis has a mean gelling concentration similar to HbS, an increased oxygen affinity and exhibits instability.[16] Haemoglobin S-Antilles[22] and HbS-Oman[18] are considerably less soluble than HbS with the former also causing severe chronic haemolytic anaemia.

There is no genetic database of SCA patients in Nigeria. This study is, therefore, to characterize the β-globin gene for variations associated with the Sickle Cell mutation. This will serve as an initial step in acquiring locally relevant genetic databases.

Subjects and Methods

This was a cross-sectional study where buccal swabs for β-globin gene sequencing were obtained from 51 consenting adult SCA patients, who attended the Sickle Cell Clinic of the Department of Haematology Ahmadu Bello University Teaching Hospital Zaria Nigeria. All subjects were recruited based on alkaline haemoglobin electrophoretic pattern suggestive of HbSS and positive solubility tests. The Subjects rinsed their mouths with sodium chloride solution at least 1 hour after eating or drinking. Sterile cotton swab was inserted into the wide open mouth. This was rubbed vigorously on both cheeks at least 10 times. The samples were coded at the site of collection.

Deoxyribonucleic acid (DNA) was extracted as follows: 300µl of Cell Lysis Solution was dispensed into a 1.5ml microcentrifuge tube and sterile razor blade was used to cut the buccal swab. This was incubated at 65°C for 15 minutes. Then 1.5µl of Puregene Proteinase K was added and mixed by inverting 25 times. This was then incubated at 55°C for 1 hour. The Collection brush head was removed from the cell lysis solution. Then 100µl of Protein Precipitation Solution was added and vortexed vigorously for 20 seconds at high speed and then incubated on ice for 5 minutes. This was centrifuged for 3 minutes at 14,500*g*. This precipitated the proteins into a tight pellet. Then 300µl of Isopropanol and 0.5µl of Glycogen Solution were pipetted into a clean 1.5ml microcentrifuge tube. The supernatant wascarefully added. This was mixed by inverting 50 times, and was centrifuged for 5 minutes at 14,500*g*. The supernatant was discarded carefully and the tube drained on a clean piece of absorbent paper. Care was taken that the pellet remained in the tube. The pellet was air-dried for 15 minutes. Then 20µl of DNA hydration Solution was added and vortexed for 5 minutes at medium speed to mix. This was incubated at 65°C for 1 hour to dissolve the DNA. The above was incubated at room temperature overnight with gentle shaking using an agitator while ensuring the tube cap was tightly closed. The samples were then centrifuged briefly and transferred to Storage Tubes. The determination of DNA purity was done using the ratio:

$$\frac{A260}{A280} = 1.7\text{-}1.9$$

Two rounds of PCR were performed as follows: dH2O -17µl, Primer 1-0.5µl, Primer 2-0.5µl, Template -2µl [Table 1] and [Table 2]. PCR conditions were as follows: Pre-Denaturation-5min at 94°C, Denaturation- 0.5 to 1min at 94°C, Annealing- 1min at 50°C (approximately 5°C below primer Tm), Extension- 1min at 72°C, Final extension- 5min at 72°C. At the end of the second round PCR amplicons were then run on 1% agarose gel, visualized, cutout, and cleaned [Figure 6].

A Dye Terminator sequencing reaction was set up as follows: dH2O- 9.5µl, DNA template 0.5µl, Primer 2.0µl, and DTCS Quick start master mix 8.0µl [Table 3]. The sequencing reaction was set up in the PCR machine using the following Thermal cycling program: 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. This reaction was cleaned and loaded onto a Beckman Coulter Ceq 2000XL sequencing machine. The sequences were trimmed based on Quality Scores before assembly and were analyzed using Basic Local Alignment Search Tool (BLAST) by comparing with a Reference β globin gene sequence available at http://www.ncbi.nlm.nih.gov/gene/3043.

http://www.ncbi.nlm.nih.gov/nuccore/NC_000011.9?report=genbankandfrom=5246696andto=5248301andstrand=true.

Results

Out of a total number of 51 patients studied, there were 40 (78.43%) females and 11 (21.57%) males with a mean age of 25.35 ± 7.67 years, 95% CI (23.20, 27.51). The Hausa ethnic group constituted 33 (64.71%) of the participants, Yoruba 5 (9.80%), Others 13 (25.49%) [Figure 1].

The classic sickle cell mutation A T change was present in all participants. There were 184 (41.91%) insertions, 133 (30.30%) deletions, and 122 (27.79%) substitutions on the β globin gene of the participants. The mean number of variations on the β globin gene was 8.61±11.29, 95% CI (5.43, 11.78). The mean number of substitutions, deletions, and insertions in the participants were 2.39±3.23, 2.60±2.46, and 3.61±7.66 with 95% CIs of (1.48, 3.30), (1.92, 3.30), and (1.45, 5.76) respectively.

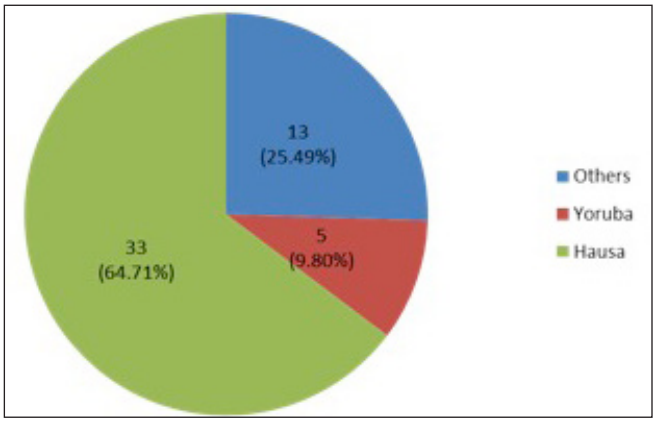

Figure 1: Ethnic Groups of Participants

Type of Substitution: Guanine was substituted by Cytosine (G>C) 53 times (43.44%) followed by the A>C 21 (17.21%), T>C 18 (14.75%), and A>G 9 (7.38%) [Figure 2].

Types of Deletions: Adenine was the most frequently deleted base 61 (45.86%) followed by Thymine 33 (24.81%), Cytosine 21 (15.79%), and Guanine 18 (13.53%) [Figure 3].

Table 1: Primers for 1st Round PCR

Name	Sequence	Tm (˚C)
beta 1 for	5'-TTC ACT AGC AAC CTC AAA CAG ACA CC-3'	58.9
beta Seq 2	5'-TAA TGT ACT AGG CAG ACT GTG TAA AG-3'	54.1

Table 2: Primers for 2nd Round PCR

Name	Sequence	Tm (˚C)
beta for	5'-ACA TTT GCT TCT GAC ACA ACT GTG T-3'	57.4
beta rev	5'-TTA GGG AAC AAA GGA ACC AAT AGA A-3'	57.3

Table 3: Primer for Sequencing PCR

Name	Sequence	Tm (˚C)
beta 1 for	5'-TTC ACT AGC AAC CTC AAA CAG ACA CC-3'	58.9

Table 4: Frequency of insertion of multiple nucleotide bases

Multiple Nucleotide Bases Inserted	Frequency	Percentage
AAG	1	3.85%
AT	4	15.38%
CA	3	11.54%
CCTCC	1	3.85%
CG	1	3.85%
CT	3	11.54%
GA	2	7.69%
GC	1	3.85%
GG	2	7.69%
GGAC	1	3.85%
GGC	1	3.85%
GT	1	3.85%
TC	2	7.69%
TG	2	7.69%
TT	1	3.85%
Total	26	100.00%

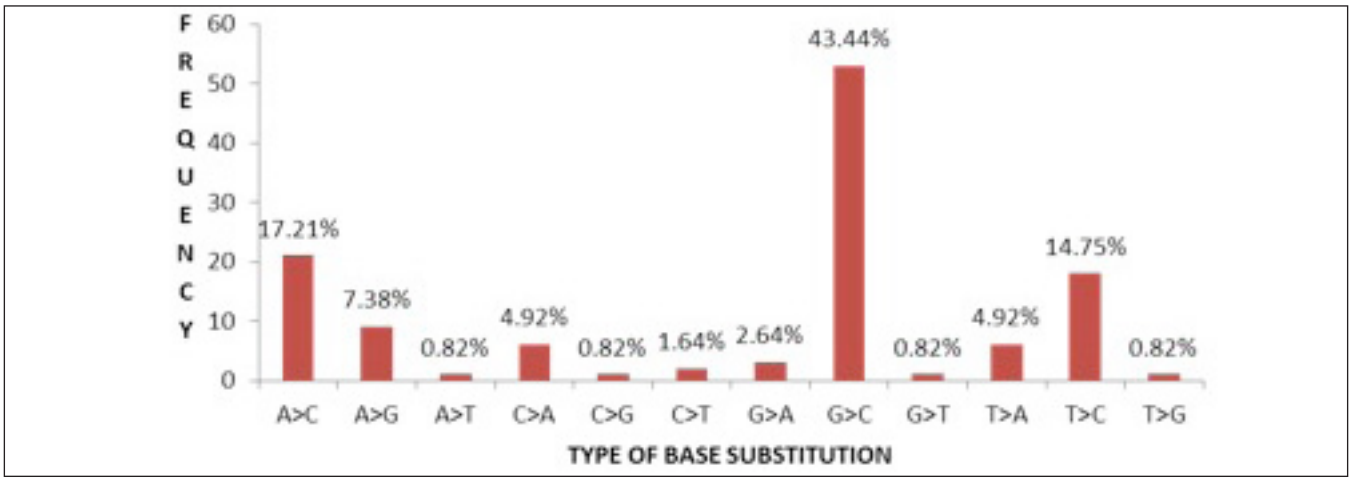

Figure 2: Type of base Substitution

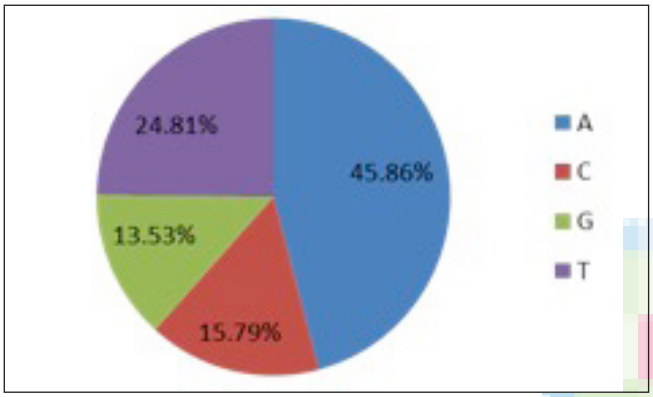

Figure 3: Distribution of type of base deletion

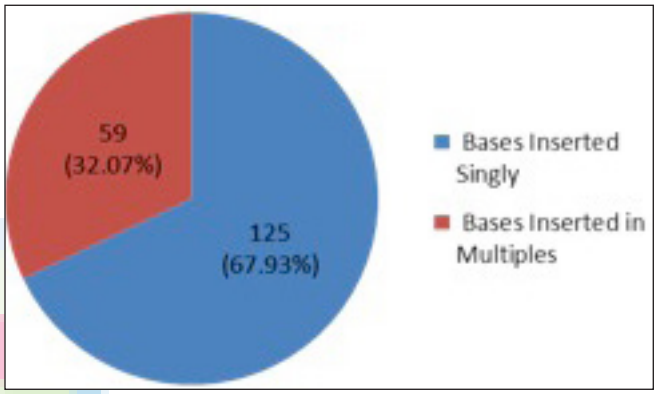

Figure 4: Single versus multiple base insertions

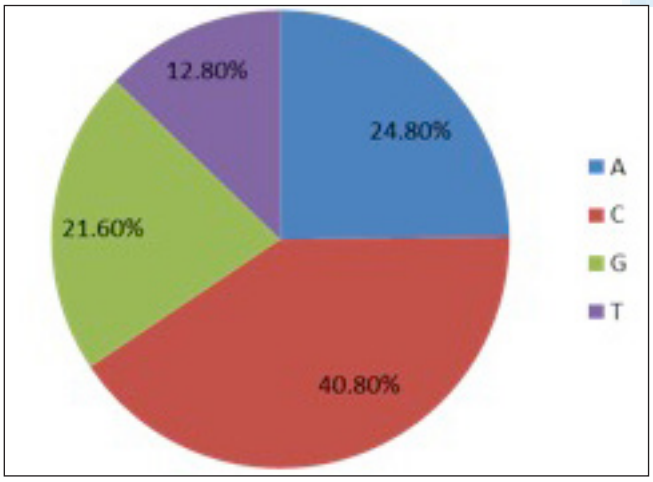

Figure 5: Type of singly inserted bases

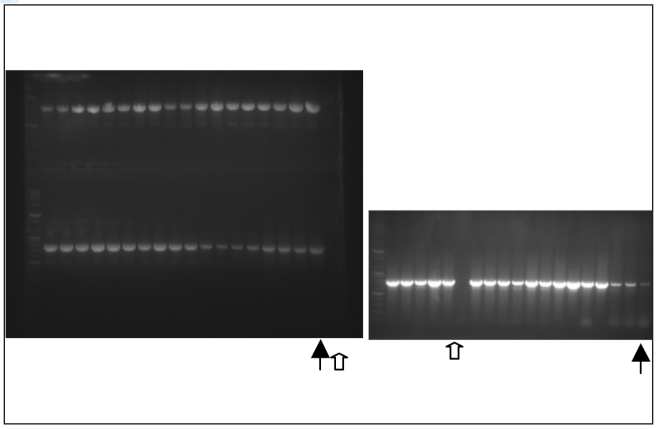

Figure 6: Photomicrographs of 1% Agarose gel electrophoresis showing about 800 bp of PCR products. A 100 bp Plus DNA ladder was used. Negative control lane Positive control lane

Singly Versus Multiply Inserted Nucleotide Bases: One hundred and twenty five bases were inserted singly (67.93%) while 59 (32.07%) were inserted in groups of two or more [Figure 4].

Distribution of singly inserted nucleotide bases: Cytosine was inserted most frequently 51 (40.80%). This was followed by Adenine 31 (24.80%), Guanine 27 (21.60%), and Thymine 16 (12.80%) [Figure 5].

Distribution of Multiply inserted nucleotide bases: AT were inserted most frequently 4 (15.38%). This was followed by CA and CT 3 (11.54%) each and CCTCC was 1 (3.85%) [Table 4].

Table 5: Overview of number of types of variations according to site

Variations	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Total
Substitutions	0	38	15	3	7	63
Deletions	0	26	14	13	1	54
Single Insertions	0	50	27	4	7	88
Multiple Insertions	0	18	3	0	2	23
Total	0	132	59	20	17	228

Discussion

The finding in this study of a higher number of insertions compared to substitutions and deletions, is at variance with the assertions that single base substitutions are the commonest type of mutations,[23] and that deletions are three times more common than insertions.[24] A possible explanation for this departure may be that whereas this study was restricted to part of the β-globin gene, the other studies are general inferences for the whole human genome. However, it is important to note that Africa is the most genetically diverse region in the world as evidenced by extensive genetic variations even between closely related African populations. The β-globin gene and other candidate genes have been postulated as possible targets for understanding these variations.[23] Ascribing any of the variations noted in this study to an ethnic group will be misleading due to the small sample size and sampling technique utilized.

Although the results of this study indicate the presence of additional genetic variations existing in association with the sickle cell mutation, it remains to be seen if they have any significant relationship with clinical and haematological factors. This is because some mutations may be 'Silent' or 'Neutral'.[25] This could be ascribed to the degenerate nature of the DNA code. Therefore, the same protein will be synthesized regardless of the type of change that occurs. Neutral mutations, therefore, do not confer any advantage or disadvantage.[26] Most mutations either have very small effects or are neutral.[25,27] Neutral deletions in the β-Globin gene cluster have been described.[28] Some additional mutations found in association with the sickle cell anaemia mutation in HbC-Harlem,[19] Hb Jamaica Plain,[14] HbS-South End,[10] and HbS-Oman[18] worsen the clinical picture. These differences may be due to varying degrees of Epistasis as earlier described. This is a phenomenon where many mutations interact with one another thus altering their individual effects. These interactions can be within a gene or among different genes. The ramification of this concept in our study participants is that genome wide association studies may shed more light on such possible interactions.[29,30]

Another interaction that will need to be assessed is the possibility of Suppressor mutations. These are mutations that occur in a second location so that the harmful or deleterious effects of another mutation are abolished or ameliorated. Suppressor mutations may be within the same gene (intragenic) or in an entirely different gene (extragenic).[31] Thus, Suppressor mutations in this context may be in the Untranslated Regions (5' or 3' UTR), in other parts of the β-Globin Gene Cluster or other genes entirely.

Additionally, since the DNA of the Participants was gotten from buccal cells there is a possibility that some of the mutations are Somatic rather than Germline. Somatic mutations only become important if they occur systemically and affect the survival of the individual.[27] The possibilities of confounders such as environmental factors and epistatic interactions will need to be accounted for in future studies. Also, future studies will need to explore gene expression profiles in SCA anaemia patients with a view to further elucidate the effects of these variations. Nevertheless, this study may be used to generate the hypothesis, that the number or presence of additional β-Globin gene variations in SCA patients does not necessarily alter the phenotype. This will need to be tested in larger, more controlled study designs especially prospective cohort models. These will then provide grounds for comparisons as it is difficult to find studies, local or foreign, with which sufficient and relevant comparisons can be made presently.

Majority of the types of variations documented in this study occur in the introns [Table 5]. This may be important as intronic alterations, especially deep intronic changes, may lead to intronic sequence exonisation.[32,33] Conversely, it has been suggested that substitutions in non-coding DNA most often do not have effects on gene expression.[34]

The findings from this study underscore the need for population-based Biobanks. The establishment of such data bases will serve as a tool for a comprehensive study of genetic and environmental determinants of SCA in particular and the other diseases in general. This will give context and consideration to the diverse ethnic groups in Nigeria.[35]

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Nil

Conficts of interest

There are no conflicts of interest.

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