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Frequency of Methyletetrahydrofolate Reductase Enzyme Mutation among Sudanese Patients with Sickle Cell Anemia in Khartoum State

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Abstract

Background: Sickle cell disease is an autosomal recessive and chronic hemolytic anemia whose clinical manifestations arise from the tendency of the hemoglobin (HbS or sickle hemoglobin) to polymerize and deform red blood cells into the characteristic sickle shape. The homozygous state (HbSS or sickle cell anemia) is the most common form of sickle cell disease,
Methylenetetrahydrofolate reductase (MTHFR) has a major impact on the regulation of the folic acid pathway due to the conversion of 5, 10-methylenetetrahydrofolate (methylene-THF) to 5-methyl-THF.
Objective: This study aimed to determine the frequency of the mutation of MTHFR in patients with sickle cell anaemia and to measure the prevalence of MTHFR mutation among the study population .
Methods: A total of 125 patients less than 17 years with sickle cell anaemia were examined for the mutation in the (MTHFR) gene.In this study we used Chelex method to extract of DNA and used Gel Electrophoresis to explain the band of homozygous or heterozygous mutation in MTHFR in locus A1298C.
Result: This study found that the frequency of mutation in MTHFR in A1298C was 19% in SCD patient (homozygous was 11.4%, while heterozygous was 7.6 %). significant relationship between mutation in MTHFR and SCD patient (P=001).
Conclusion: This study revealed that the three is high frequency of mutation of MTHFR enzyme among Sudanese patients with SCA (19%), 11.85% had heterozygous allele and 7.8% had homozygous allele.

Keywords: Sickle cell disease, Methylenetetrahydrofolate reductase enzyme (MTHFR), single nucleotide polymorphisms (SNP), DNA extraction, PCR.

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Introduction:

Sickle cell disease is an autosomal recessive and chronic hemolytic anemia whose clinical manifestations arise from the tendency of the hemoglobin (HbS or sickle hemoglobin) to polymerize and distort red blood cells into the characteristic sickle shape. The homozygous state (HbSS or sickle cell anemia) is the most common form of sickle cell disease, and the heterogonous stat (Hb AS) is refer as sickle cell trait.This property is due to a point mutation in single nucleotide change in the ß-globin gene leading to substitution of valine for glutamic acid at position 6 of the -globin chain (ß 6glu→val or ß s).[1]

persons who are affected with sickle cell anemia have two copies of this variant (Hb SS), and the primary hemoglobin present in their red blood cells is sickle hemoglobin. Individuals affected with other types of sickle cell disease are compound heterozygotes. They possess one copy of the Hb S variant plus one copy of another β-globin gene variant, such as Hb C or Hb β-thalassemia. These individuals produce a mixture of variant hemoglobin's. Carrier individuals have one copy of the sickle variant and one copy of the normal β-globin gene (Hb AS), producing a mixture of sickle hemoglobin and normal hemoglobin. The carrier state for sickle cell disease is often referred to as "sickle cell trait." Although individuals with sickle cell trait do not express sickle cell disease, one study found that sickle cell trait may be a risk factor for sudden death during physical training. [2] In addition, individuals with sickle cell trait are protected from malaria infection. [1] The high frequency of the Hb S variant is believed to be a result of this protective effect.

The distribution of sickle cell anaemia provides evidence for origin of the mutation in several locations within Africa (the Senegal, Benin and Bantu haplotypes) and Asia (the Arab–Indian haplotype), The Sudanese haplotype is camerion haplotype. [3]

Inheritances:

Sickle-cell conditions are inherited as autosomal recessive pattern .The types of haemoglobin a person makes in the red blood cells depend on what haemoglobin genes are inherited from his parents. If one parent has sickle-cell anaemia (SS) and the other has sickle-cell trait (AS), there is a 50% chance of a child's having sickle-cell disease (SS) and a 50% chance of a child's having sickle-cell trait (AS). When both parents have sickle-cell trait (AS), a child has a 25% chance (1 of 4) of sickle-cell disease (SS). [3]

Molecular biology of Sickle cell anemia:

Sickle-cell gene mutation probably arose spontaneously in different geographic areas, as suggested by restriction endonuclease analysis. These variants are known as Cameroon, Senegal, Benin, Bantu and Saudi-Asian. Their clinical importance springs from the fact that some of them are associated with higher HbF levels, e.g., Senegal and Saudi-Asian variants, and tend to have milder disease.In people heterozygous for HbS (carriers of sickling haemoglobin), the polymerisation problems are minor, because the normal allele is able to produce over 50% of the haemoglobin. In people homozygous for HbS, the

presence of long-chain polymers of HbS distort the shape of the red blood cell from a smooth doughnut-like shape to ragged and full of spikes, making it fragile and susceptible to breaking within capillaries. Carriers have symptoms only if they are deprived of oxygen (for example, while climbing a mountain) or while severely dehydrated. Under normal circumstances, these painful crises occur about 0.8 times per year per patient. The sickle-cell disease occurs when the seventh amino acid (if the initial methionine is counted), glutamic acid, is replaced by valine to change its structure and function.

The gene defect is a known mutation of a single nucleotide (single-nucleotide polymorphism - SNP) (A to T) of the β-globin gene, which results in glutamic acid being substituted by valine at position 6. Haemoglobin S with this mutation is referred to as HbS, as opposed to the normal adult HbA. The genetic disorder is due to the mutation of a single nucleotide, from a GAG to GTG codon mutation, becoming a GUG codon by transcription. This is normally a benign mutation, causing no apparent effects on the secondary, tertiary, or quaternary structure of hemoglobin in conditions of normal oxygen concentration. What it does allow for, under conditions of low oxygen concentration, is the polymerization of the HbS itself. The deoxy form of hemoglobin exposes a hydrophobic patch on the protein between the E and F helices. The hydrophobic residues of the valine at position 6 of the beta chain in hemoglobin are able to associate with the hydrophobic patch, causing hemoglobin S molecules to aggregate and form fibrous precipitates.

The allele responsible for sickle-cell anaemia is autosomal recessive and can be found on the short arm of chromosome 11. A person that receives the defective gene from both father and mother develops the disease; a person that receives one defective and one healthy allele remains healthy, but can pass on the disease and is known as a carrier. If two parents who are carriers have a child, there is a 1in 4 chance of their child developing the disease and a 1in 2 chance of their child's being just a carrier. Since the gene is incompletely recessive, carriers can produce a few sickled red blood cells, not enough to cause symptoms, but enough to give resistance to malaria. Because of this, heterozygotes have a higher fitness than either of the homozygotes. This is known as heterozygote advantage.

Due to the adaptive advantage of the heterozygote, the disease is still prevalent, especially among people with recent ancestry in malaria-stricken areas, such as Africa, the Mediterranean, India and the Middle East [4]. Malaria was historically endemic to southern Europe, but it was declared eradicated in the mid-20th century, with the exception of rare sporadic cases. [5]

Methylenetetrahydrofolate reductase(MTHFR)

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that in humans is encoded by the MTHFR gene. [6] also has a main impact on the regulation of the folic acid pathway due to the conversion of 5, 10-methylenetetrahydrofolate (methylene-THF) to 5-methyl-THF. Two common

nonsynonymous coding region polymorphisms (677C>T and 1298A>C) in the MTHFR gene were shown to confer reduced enzyme activity in vitro assays leading to a reduced pool of methyl-THF and the MTHFR variant 677C>T was related with an increased risk of hyperhomocysteinemia, particularly in folate-deficient states [7]. With regard to the MTHFR 677C>T polymorphism, results from in vitro] assays showed a reduction in enzyme activity to 65% for the heterozygous and to 30% for the homozygous state of the 677T variant[8]. For the 1298A>C polymorphism, enzyme activity in vitro is diminished in homozygous variants and, to a lesser extent, in heterozygotes compared with those homozygous for the wild-type allele. [9] ,Genetic variation in this gene influences susceptibility to occlusive vascular disease, neural tube defects, colon cancer and acute leukemia, and mutations in this gene are associated with methylenetetrahydrofolate reductase deficiency. [10]

Genetics:

The enzyme is coded by the gene with the symbol MTHFR on chromosome 1 location p36.3 in humans.[4] There is DNA sequence variants (genetic polymorphisms) associated with this gene. In 2000 a report brought the number of polymorphisms up to 24.[11]Two of the most investigated are C677T (rs1801133) and A1298C (rs1801131) single nucleotide polymorphisms (SNP).

C677T SNP (Ala222Val)

The MTHFR nucleotide at position 677 in the gene has two possibilities: C (cytosine) or T (thymine). C at position 677 (leading to an alanine at amino acid 222) is the normal allele. The 677T allele (leading to a valine substitution at amino acid 222) encodes a thermolabile enzyme with reduced activity.

Individual with two copies of 677C (677CC) have the "normal" or "wild type" genotype. 677TT individuals (homozygous) are said to have mild MTHFR deficiency. 677CT individuals (heterozygous) are almost the same as normal individuals because the normal MTHFR can make up for the thermolabile MTHFR. About ten percent of the North American population are T-homozygous for this polymorphism. There is ethnic variability in the frequency of the T allele frequency in Mediterranean/Hispanics > Caucasians > Africans/African-Americans).

The degree of enzyme thermolability (assessed as residual activity after heat inactivation) is much greater in 677TT individuals (18-22%) compared with 677CT (56%) and 677CC (66-67%). [12]

Individuals of 677TT are predisposed to mild hyperhomocysteinemia (high blood homocysteine levels), because they have less active MTHFR available to produce 5-methyltetrahydrofolate (which is used to decrease homocysteine). Low dietary intake of the vitamin folic acid can also cause mild hyperhomocysteinemia.

Low folate intake affects individuals with the 677TT genotype to a greater extent than those with the 677CC/CT genotypes. 677TT (but not 677CC/CT) individuals with lower plasma folate levels are at risk for elevated plasma homocysteine levels.[13] In studies of human

recombinant MTHFR, the protein encoded by 677T loses its FAD cofactor three times faster than the wild-type protein , [14] 5-Methyl-THF slows the rate of FAD release in both the wild-type and mutant enzymes, although it is to a much greater extent in the mutant enzyme,[15].677TT individuals are at a decreased risk for certain leukemias, [16]and colon cancer.

Mutations in the MTHFR gene could be one of the factors leading to increased risk of developing schizophrenia [17]. Schizophrenic patients having the risk allele (T\T) show more deficiencies in executive function tasks.

A1298C SNP (Glu429Ala):

At nucleotide 1298 of the MTHFR, there are two possibilities: A or C. 1298A (leading to a Glu at amino acid 429) is the most common while 1298C (leading to an Ala substitution at amino acid 429) is less common. 1298AA is the "normal" homozygous, 1298AC the heterozygous, and 1298CC the homozygous for the "variant". In studies of human recombinant MTHFR, the protein encoded by 1298C cannot be distinguished from 1298A in terms of activity, thermolability, FAD release, or the protective effect of 5-methyl-THF. [18] The C mutation does not appear to affect the MTHFR protein. It does not result in thermolabile MTHFR and does not appear to affect homocysteine levels.

Compound Heterozygotes:

Mutations at 677 and 1298 are both in the same gene, MTHFR. They are at different locations in the same gene. Some studies have shown that the MTHFR protein in people with the genotype 677CT 1298AC does its job a bit less well than the normal MTHFR. [2]

Severe MTHFR deficiency:

Severe MTHFR deficiency is rare (about 50 cases worldwide) and caused by mutations resulting in 0-20% residual enzyme activity.[11] Patients exhibit developmental delay, motor and gait dysfunction, seizures, and neurological impairment and have extremely high levels of homocysteine in their plasma and urine as well as low to normal plasma methionine levels[19].

Methods:

Ethical approval and consent to participant:

Approval of This study was obtained from hematology department of medical laboratory science (MLS), Omdurman Islamic University, and ministry of health issued by the local ethical committee, Khartoum State, Sudan. Written consent was taken from each member of the study.

Study Location:

A case control study. With total samples were 125, which include 79 sample from patient with sickle cell anaemia and 46 as normal control.

The study was conducted in Khartoum State in different haemglobinopathy laboratory centres (Khartoum Teaching Hospital, Ministry of Health Biochemistry department, faculty of Medicine University of Khartoum, Haematology Department, National Health Laboratory, and Federal Ministry of Health).

Study populations:

The study population was children of less than 17 years diagnosed with sickle cell disease based on their Hb electrophoresis on cellulose acetate paper

at alkaline pH, who attend one of the laboratory centres either for their regular chick up or for diagnosis and evaluation.

Sample collections:

5ml EDTA blood was collected under sterile condition, store at 4ºC for 7 days till Buffy coat preparation, and haemolysed sample for Hb electrophoresis were also collected and store for DNA extraction.

DNA extractions by Chelex method:

DNA was extracted from all samples using th Chlex method according to manufactures instructions.

Polymerase Chain Reactions (PCR):

Extracted DNA was brought from-20 ºC, thawed and centrifuged again to pellet down any remaining Chelex and kept on ice cro-rack for processing , at the same time primers, dNTPs, and buffer were brought at room Temperature and kept on ice cro-rack for thawing and sterile PCR water was brought out and aliquoted on 1.5 ml tube and kept as above.

Master Mix preparation:

Samples and reagents were brought out from the freezer and kept on ice in a frozen cryo-rack during assembly procedure. A4 worksheet with PCR samples data were recorded for each samples to be tested. Master mix (MM) was prepared using forward and reversed primer ,the amount of each reagents were calculated to go into the MM in 1.5 µl sterile tube, according to the number of samples to be processed with an extra one more samples than actually being tested to compensate for retention of solution in pipette tips and tube. PCR reagents, except for samples DNA, were added in the order listed on the worksheet, adding water first and Taq polymerase last. The specified volume of MM was added into the each tube, all reagents were kept in a frozen-cryo-rack during mixing and returned to the freezer immediately after use, caps were closed tightly and the PCR tubes were moved to samples loading area.

In the samples preparation area specified volume of sample was loaded into an appropriately labeled PCR tube. To avoid contamination, the tips were always changed and the avoidance of touching the side tube and capped was recommended.

PCR amplification of MTHFR gene:

Genomic DNA was amplified with primer flanking Codon A1298C of MTHFR gene with flowing forward primers: 5’CAAGGAGGAGCT GCTGAAGA3’

And reverse primer: 5’CCACTCCAGCATCACT CACT3’

PCR optimizations:

Small fraction of sample was first subjected to PCR amplification, after successful amplification the rest of the samples were analyzed in batches.

DNA visualization:

Agarose gel by electrophoresis.,5 µl from each PCR product were mixed with 2 µl Brmoephenol blue dye and loaded into 1.5% Agarose gel dissolved in TBE (Tris-borate Ph 8.0 and 0.002 M EDTA Ph 8.0). The gel was stained with ethidium bromide (0.5mg/ml), run for 30 minutes at 60 volt/cm for conventional PCR.

Results:

Study subjects comprised 79 SCD patients (39males and 40 females; mean age 4 month -17 years) and 46 controls (28 males and 18 females; mean age 4 -14 years).The DNA were extracted to detect the incidence of the mutation in the (MTHFR) gene, among the diagnosed SCD patients and normal control in Khartoum State.

In this study we used Chelex method to extract of DNA and used Gel Electrophoresis to explain the band of homozygous or heterozygous mutation in MTHFR in locus A1298C.

The incidence of mutation in MTHFR in A1298C was 19% in SCD patient (homozygous was 11.4%, while heterozygous was 7.6 %).

The significant relationship between mutation in MTHFR and SCD patient compared with control (P=000).

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Table 1: Shows mean and range of age /years and six among patients and controls:

	Patients	Controls
No (125)	79	46
Age in years mean, (range)	7.3(0.4 -17 years)	28.63(4-84)
Sex Male (45)	22(42.3%)	28(60.9%)
Female (59)	30(57.69%)	18(39.1%)

Table 2: Shows the mean and range of some hematological parameter among patient and control:

hematological parameter	Patients	Controls
Hb gm/dl	7.9(3.7-14.9)	12.8(9.9-16.0)
Hct% mean (range)	24.6 (13.5-44)	38.3%(29.7-49)
MCV/fl	85.8(8-102)	90.6(80-100)
MCHC gm/dl	32.7 (24-41)	32 (30-36)
WBC 10x3 cell/L	14.7(4.5-46.6	8(4-13)

Table 3: Shows the differentiation between Patients and Control:

Characteristics		Patients	Controls
Contgenousity:	**Yes**	62%	69.6%
	No	34%	31.4%
Hb variants			
	AA	00%	100%
	AS	31.6%	00%
	SS	68.4%	00%
MTHFR	**AA**	79.7%	97.8%
Homozygous		11.4%	0.0%
Heterozygous		7.6%	2.2.0%

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Table 4: Showed Main characteristics of the MTHFR (1298A>C) genetic variants

			Gene		
	Allele variant	Amino Acid change	Location	Position	NCBIdbSNP rs
Polymorphism	1298A>C	E429A	1p36.3	Exon 8	rs1801131

Table 5: showed the Comparison of allele and genotype frequency of 1298 normal/abnormal single nucleotide polymorphism of MTHFR in Sickle cell patients of males and females:

Gender	Male	Female	Total
No	39	40	79
Normal	34	29	63
Heterozygous	3	6	9
Homozygous	2	4	6
Normal	36	32	68
Abnormal	3	8	11

Table 6: Shows genotype frequency of the 1298 normal/abnormal single nucleotide polymorphism of MTHFR in Sickle cell patients and controls:

Genotype	Sickle cell disease(n=79)	Controls (n=46)
Normal	63(79.7%)	45(97.9%)
Heterozygous	9(11.4%)	0(00%)
Homozygous	6(7.6%)	1(2.1%)
P=000		

Table 7: shows allele frequency of the 1298 normal/abnormal single nucleotide polymorphism of MTHFR in Sickle cell patients and controls:

Allele	Sickle cell disease(n=79)	Controls (n=46)
Normal	135 (86.5%)	90 (97.8)
Abnormal	21(13.5%)	2 (2.2%)
	P=003	

Table 8: shows the relationship of Contgenousity marriage and MTHFR alleles:

		1298 genotype			Total
		AA allele	AC allele	CC allele	
Contgenousity	Yes	27	4	3	34
	No	16	5	3	24
Total		43	9	6	58
P=001					

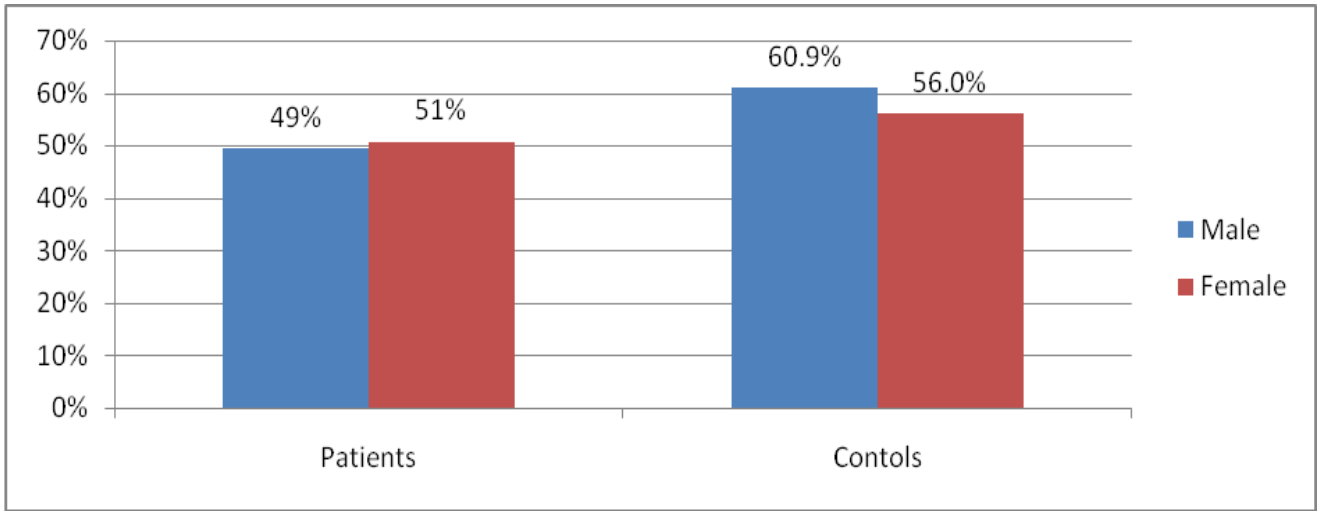

Figure 1: Shows Frequency of distribution of males and females

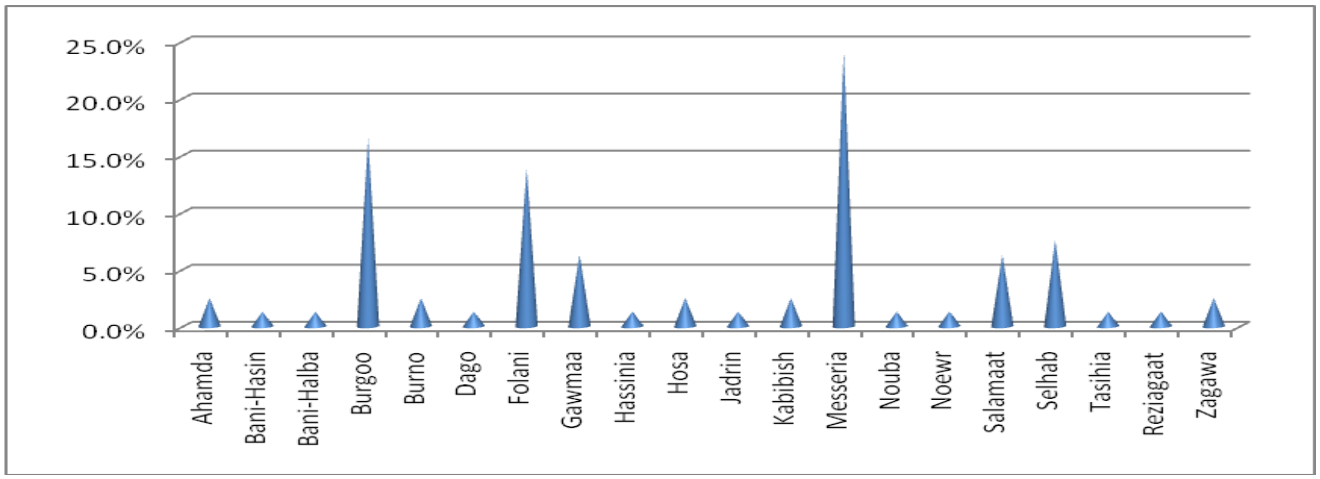

Figure 2: Shows Frequency of the different tribes with sickle cell disease:

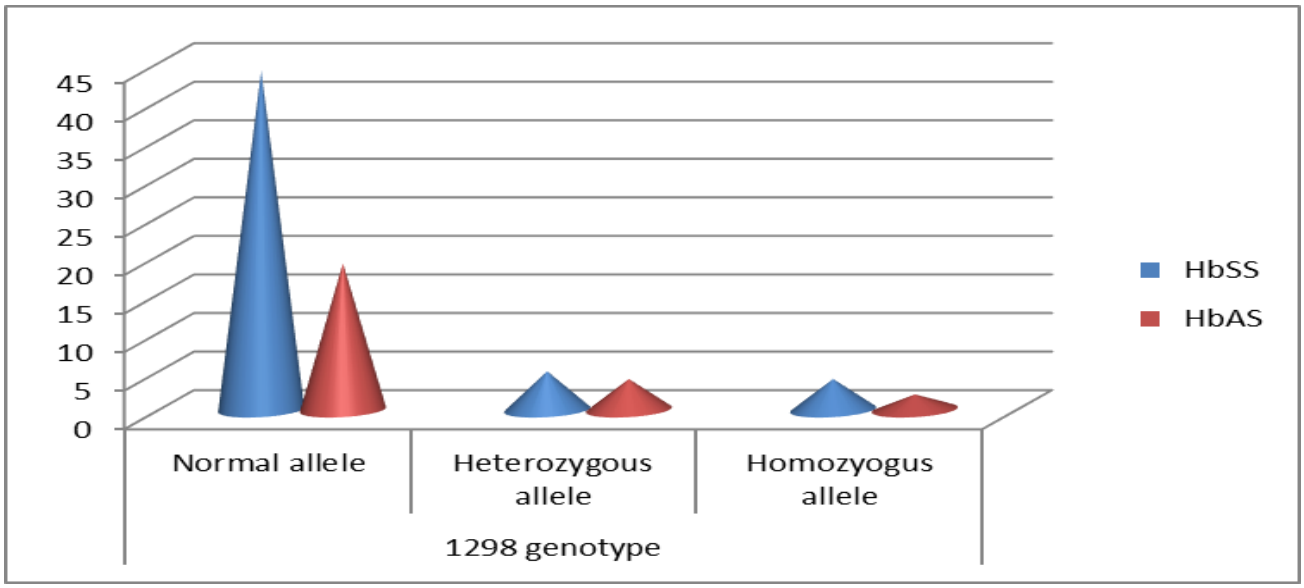

Figure 3: Shows Association of 1298 MTHFR genotype and Hb variants

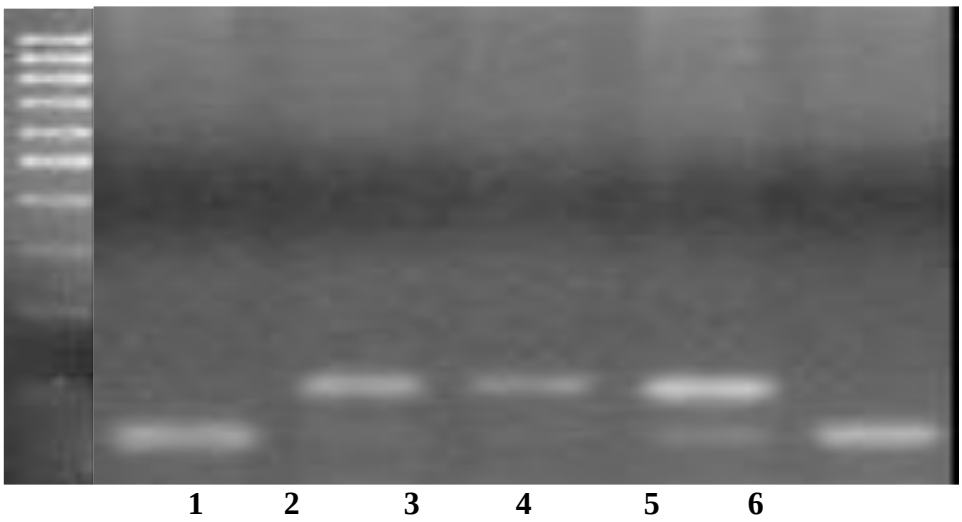

Figure 4: Agarose gel electrophoresis image showing the sizes of RFLP using *MboII* digestion, lane 1 represents the molecular weight marker 100 (bp), lane 2 and 6 is 72 bp (wild type), lane 3 and 4 were homozygous, lane 5 (heterozygous)

Discussion

In this study the populations were 46 normal as control and 79 patients with sickle cell disease to detect mutation in MTHFR gene. The study was carried out in Tropical Medicine Research Institute in National Health Laboratory in Khartoum State, from March 2011 to July 2011.

In this study we found that, frequency of MTHFR gene mutation among patients with sickle cell disease 19%, compare with normal control 2.1%

68.4% had haemoglobin SS and 31.6% had haemoglobin AS and the only one case of MTHFR gene mutation was from haemglobin AA and his origin was from Hausa tribe.

Female represent (50.6%) of the studied patient while male represent (39.3%) and their age range from 4 month to 17 years.

The frequency of homozygous mutation in MTHFR in female is 4% and male is 2%, while the frequency of heterozygous mutation in MTHFR in female is 6% and male 3%.

The significant relationship between SCD patient and the mutation in MTHFR was (P=000) as genotype.

The significant relationship between SCD patient and the mutation in MTHFR was (P=003) as allele.

We observed frequency of Contgenousity marriage in the patients group 62% and this could be a risk factor for genetic disease as sickle cell anaemia and mutation of MTHFR as shown in this study.

In El-Bahrain Al Jishi et al. (2005) described, for the first time, a 41-year-old married woman with a combination of Takayasu arteritis (TA) and primary antiphospholipid antibodies (aPL) syndrome who underwent carotid stenting. She had single nucleotide polymorphism (SNP) double homozygosity for methylenetetrahydrofolate reductase (MTHFR); C677T (T/T genotype) and A1298C (C/C genotype). Serum homocysteine was elevated and that might be attributed to C677T SNP. It was thought that MTHFR 1298C/C genotype would be an independent risk factor for ischemic stroke. In our study we agree with them in mutation of MTHFR A1298C, but we disagree with them in association between MTHFR and the disease.

The previous study in Saudia Arabia Fawaz et al (2004) found that there is role T/T (and C/T) at the MTHFR 677 (41.38%) in the patients with sickle cell anaemia this study agree with our study which we found mutation in the MTHFR in (19%) of patient with Sickle cell anaemia, but we disagree with it because in our study locus of MTHFR was A1298C.

The MTHFR 677TT genotype was detected in 1.8% of SCD patients in Brazil, in the patients with sickle cell anaemia this study agree with our study which we found mutation in the MTHFR in (19%) of patient with Sickle cell anaemia, but we disagree with it because in our study locus of MTHFR was A1298C.

The previous study in USA They studied the frequency of the thermolabile methylene tetrahydro-folate reductase (MTHFR) variant (C677T) in adult sickle cell patients with and without AVN. The frequency of the MTHFR mutation was 35.6% in patients with AVN and 12.9% in those without AVN (p=0.006). These

data suggest that the thermolabile MTHFR variant may be a contributing risk factor for AVN in some populations with sickle cell disease. We agree with it, because in our study there is mutation in the MTHFR in (19%) of patient with Sickle cell anaemia but we disagree with it because in our study locus of MTHFR was A1298C.

To our best knowledge this is the first study in Sudan to investigate MTHFR gene polymorphism in sickle cell disease.

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