New mutations of locus control region in Saudi sickle patients

Faris Q. Alenzi * 

College of Appl. Med. Sci, Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia

1. Introduction

Sickle cell disease (SCD) is a chronic hereditary disease not only in the developing countries but also in the developed world. There are more than 500,000 newly diagnosed infants, and about 200,000 people with sickle cell anemia per year, according to the World Health Organization (Weatherall and Clegg, 2001; Williams and Weatherall, 2012; WHO report, 2006).

Genetic blood diseases including SCD and thalassemia are caused by molecular changes in specific blood proteins called globins. Haemoglobin (Hb) is made up of four chains of globin, each of which carries the heme group, which is associated with oxygen (Schechter, 2008). SCD is one of the most common and aggressive red blood cell (RBC) disorders, and is caused by abnormal haemoglobin known as HbS (Ashley-Koch et al., 2000). SCD results from a substitution of adenine (A) to thymine (T) in 6th codon of β-globin.

In Saudi Arabia, SCD was first identified in the eastern region in 1960 by Lehman (1963), which leads to many subsequent studies at the regional and national level to determine the clinical and genetic background of SCD. In Saudi Arabia, SCD is a relatively common hereditary disorder, ranging from 0.4 to 8% while SCD carriers ranged from 2% to 27%. These estimates do not include neonatal screening, which may increase the real frequency of the disease (Jastaniah, 2011).

There are more than three million people with these genetic blood diseases in the Saudi Arabia, of which 30% in the Eastern Region (Al-Naseri, 2009). El-Hazmi and others demonstrated two
different types of sickle cell anaemia, the average type dominated in the eastern region, while the severe type observed in most patients in the south western region (El-Hazmi, 1979; 1983; 1985; El-Hazmi et al., 1990; 1991; 1992, El-Hazmi and Warsy, 1996, El-Hazmi and Warsy, 1999, Al-Qurashi et al.2008).

The aim was to study the molecular survey of locus control regions (LCR) in Saudi patients with sickle cell anemia, and to identify the genetic variables and their clinical manifestations.

2. Materials and methods

2.1. Patients

The study was conducted on 69 of sickle cell anemia patients selected randomly from the attending the blood diseases clinic at King Khalid University Hospital (KKUH, Riyadh, KSA) from different regions of Saudi Arabia between June2017–June2019. Hematological and biochemical measurements and history of each patient were investigated. The study protocol respected the most recent Declaration of Helsinki, written informed consent and Research Ethics Committee approval were obtained from all cases.

2.2. Sample

10 ml of venous blood was withdrawn from each patient and distributed to two tubes (each containing 5 ml) of ethylenediamine tetra acetic acid (EDTA).

2.3. Extraction of DNA

DNA was extracted using Qiagen DNA extraction kit, according to the approved protocol by GentraPuregene Handbook 09/2007.

2.4. Primers & PCR

Primers were designed, requested and obtained through the Oligo ordering online. PCR primers were used (see Table 1.). Thirty-five cycles of PCR, with denaturation at 94o C for 30 s, annealing at 60o C for 1 min, and extension at 72o C for 1 min, were performed on a programmed-temperature system (Hybaid OmniGene; Midwest Scientific, Missouri, United States). After PCR amplification, 10 µl of the PCR products were mixed with 1 of the PCR products were mixed with DNA loading buffer and electrophoresed on a 2 percent agarose gel containing 0.2 µg/ml ethidium bromide in 0.5X TBE buffer. A DNA ladder was also run in parallel. The amplified gel was visualized and photographed under UV light (Bio-Rad Gel Doc 2000 Imaging System).

2.5. Statistical analysis

The data obtained was subjected to a statistical analysis using Window Excel and SPSS v17 statistical tools. ANOVAs tests for multiple comparisons and significant analysis (p < 0.05) were carried out.

3. Results

The study was conducted on 69 patients with sickle cell anemia from the outpatient clinic of blood diseases at the KKUH from different regions of Saudi Arabia.

In this study, locus control region of HBB was identified and propagated using the primers designed in Table 1. The analysis of the nucleotide sequencing for the entire LCR segment except the LCR4 was determined for study samples. These changes were widely varied between substitution at different nitrogen bases. Changes were found either homo or asymmetric. The number of changes also was identified in the control zones, was 69. The first zone of the control areas of the LCR-HBB / F1 gene begins at the number 668 of chromosome 11 and ends at 1110 and is called the LCR-HS1 region. Twenty changes have been detected in this area. The highest percentage in this region, are shown by all sample at 100%, while the lowest rate was 1.3% in twelve changes in the region in one patient only. The changes are shown in Table 2.

3.1. Clinical and blood data in the research sample

As shown in the Table 4, there was a significant difference between those individuals with LCR # F1, LCR # F1_10 mutation and who don’t (i.e., those 2 mutations can cause increase of the white blood cell count).

Hemoglobin level:

As shown in Table 5, there was no statistical difference between those with mutations, compared to the normal control group.
### Table 3
Distribution of LCR changes per base per sample.

| LCR-HBB/F1 start at 668 end at 1110 | Region/Fragment | Base change | N.Samples | Percent % |
|-------------------------------------|-----------------|-------------|-----------|-----------|
| 1                                   | 749–750 -/t homo ins. | 1           | 1.3%      |
| 2                                   | 751–752 -/c homo ins. | 7           | 9.1%      |
| 3                                   | 752–753 -/c homo ins. | 1           | 1.3%      |
| 4                                   | 752–753 -/t homo ins. | 1           | 1.3%      |
| 5                                   | 753–756 -/ct homo ins. | 1           | 1.3%      |
| 6                                   | 754–657 -/tt homo ins. | 2           | 2.6%      |
| 7                                   | 757–759 -/c homo ins. | 1           | 1.3%      |
| 8                                   | 765–767 -/t homo ins. | 2           | 2.6%      |
| 9                                   | 765–767 -/c homo ins. | 1           | 1.3%      |
| 10                                  | 805–807 -/c homo ins. | 1           | 1.3%      |
| 11                                  | 805–807 -/t homo ins. | 1           | 1.3%      |
| 12                                  | 704 ->T homo ins. | 1           | 1.3%      |
| 13                                  | 710 A→ homo del. | 13           | 16.9%     |
| 14                                  | 718–720 ->T homo ins. | 1           | 1.3%      |
| 15                                  | 789 G > A het. | 2           | 2.6%      |
| 16                                  | 797 G > A het. | 1           | 1.3%      |
| 17                                  | 817–819 ->t homo ins. | 1           | 1.3%      |
| 18                                  | 907 G > A het. | 1           | 1.3%      |
| 19                                  | 963 A > G homo. | 49           | 63.6%     |
| 20                                  | 1032 G > A het. | 77           | 100.0%    |

| LCR-HBB/F2 start at 4937 end at 4991 | Region/Fragment | Base change | N.Samples | Percent % |
|-------------------------------------|-----------------|-------------|-----------|-----------|
| 1                                   | 4575 G > A het. | 77           | 100.0%    |

| LCR-HBB/F3 start at 8757 end at 9219 | Region/Fragment | Base change | N.Samples | Percent % |
|-------------------------------------|-----------------|-------------|-----------|-----------|
| 1                                   | 8874 G > A het. | 7           | 9.1%      |

| LCR-HBB/F4 start at 8757 end at 9219 | Region/Fragment | Base change | N.Samples | Percent % |
|-------------------------------------|-----------------|-------------|-----------|-----------|
| 12                                  | 8960 T > A het. | 18           | 23.4%     |
| 13                                  | 8966G > T homo. | 76           | 98.7%     |
| 14                                  | 8969 A > T het. | 7            | 9.1%      |
| 15                                  | 8977 T > A het. | 18           | 23.4%     |
| 16                                  | 8994 A > G homo. | 77           | 100.0%    |
| 17                                  | 8997 T > G homo. | 69           | 89.6%     |
| 18                                  | 8997 T > G het. | 8            | 10.4%     |
| 19                                  | 9009C > A het. | 25           | 32.5%     |
| 20                                  | 9012 G > A het. | 77           | 100.0%    |
| 21                                  | 9021 G > A het. | 77           | 100.0%    |

| LCR-HBB/F5 start at 34,273 end at 34,816 | Region/Fragment | Base change | N.Samples | Percent % |
|-----------------------------------------|-----------------|-------------|-----------|-----------|
| 12                                     | 34,662C > A het. | 45           | 58.4%     |
| 13                                     | 34,663C > A het. | 45           | 58.4%     |
| 14                                     | 34,687C > T het. | 15           | 19.5%     |

| LCR-HBB/F6 start at 61,441 end at 61,961 | Region/Fragment | Base change | N.Samples | Percent % |
|-----------------------------------------|-----------------|-------------|-----------|-----------|
| 12                                     | 61,495 to 61,496 -/C homo ins. | 1          | 1.3%      |
| Region/Fragment | Base change | N.Samples | Percent % |
|-----------------|------------|-----------|-----------|
| LCR-HBB/F1      |            |           |           |
| start at 668    |            |           |           |
| end at 1110     |            |           |           |
| LCR-HS1         |            |           |           |
| Region/Fragment | Base change | N.Samples | Percent % |
| LCR-HBB/F7      |            |           |           |
| start at 63,586 |            |           |           |
| end at 64,134   |            |           |           |
| 3 flanking      |            |           |           |
| sequences       |            |           |           |
| (enhancer       |            |           |           |
| 1 64,081 A > T homo. | 6 | 7.8% |
| 2 64,081 A > t het. | 4 | 5.2% |
| 3 63,925C > A homo. | 1 | 1.3% |
| 4 63,925C > A het. | 3 | 3.9% |
| 5 63918–63919 ->C homo ins | 77 | 100.0% |
| 6 63,923 G > A het. | 1 | 1.3% |
| 7 63,843 G > C hom | 1 | 1.3% |
| 8 63,843 G > C het. | 3 | 3.9% |
| 9 63,818 G > A het. | 1 | 1.3% |
| 10 63,826 G > A het. | 1 | 1.3% |

Table 4
Relationship between WBC count and mutation.

| Part/Variable | WBC |          |          |
|---------------|-----|----------|----------|
|               | Mean | Median   | Standard Deviation |
| LCR#F1_2      | 10.89 | 11.05   | 4.21     |
|               | 15.65 | 15.20   | 3.44     |
| LCR#F1_10     | 10.66 | 11.05   | 3.54     |
|               | 18.01 | 16.50   | 6.12     |
| LCR#F1_13     | 11.58 | 11.33   | 4.41     |
|               | 11.06 | 11.33   | 3.90     |
| LCR#F3_9      | 11.52 | 11.19   | 4.64     |
|               | 10.84 | 11.33   | 3.55     |

Table 5
Relationship between Hb level and mutation.

| Part/Variable | HB or Hgb |          |          |
|---------------|-----------|----------|----------|
|               | Mean      | Median   | Standard Deviation |
| LCR#F1_2      | 91.1      | 91.7     | 20.9      |
|               | 97.5      | 102.0    | 15.7      |
| LCR#F1_10     | 91.0      | 91.7     | 20.8      |
|               | 98.1      | 106.0    | 15.9      |
| LCR#F1_13     | 92.4      | 91.7     | 19.0      |
|               | 88.0      | 91.7     | 27.2      |
| LCR#F3_9      | 92.2      | 91.7     | 22.3      |
|               | 90.2      | 91.7     | 15.4      |

Table 6
Relationship between Platelets count and mutation.

| Part/Variable | Plt |          |          |
|---------------|-----|----------|----------|
|               | Mean | Median   | Standard Deviation |
| LCR#F1_2      | 368  | 362      | 172       |
|               | 368  | 333      | 103       |
| LCR#F1_10     | 378  | 366      | 174       |
|               | 318  | 323      | 104       |
| LCR#F1_13     | 377  | 364      | 169       |
|               | 301  | 315      | 131       |
| LCR#F3_9      | 389  | 379      | 174       |
|               | 299  | 298      | 115       |
Platelets Count:

As shown in Table 6, there was a significant difference between those with mutations in LCR # F3_9, compared to the normal control group (i.e., this mutation may cause a decrease in platelets counts).

Level of Lactate Dehydrogenase (LDH):

As shown in Table 7, there was no statistical difference between those with mutations, compared to the normal control group.

Bilirubin level:

As shown in Table 8, there was a significant difference between those with mutations in LCR # F1_13, compared to normal individuals (i.e, this mutation can increase bilirubin level).

Using Mann-Whitney test to determine whether there is a relationship between the parameters including: WBC, HB, PLT, LDH, Bilirubin and those individuals with mutations, Table 9 showed significant effect of some mutations on those individuals examined Hb, Platelets, and Bilirubin.

4. Discussion

Analysis of the local control regions (LCR) showed many differences and changes on SCD patients. These changes widely varied among the substitutions within different nitrogen bases. Changes found were either homo or asymmetric. The number of changes identified from SCD patients in local control areas was 69.

El-Tayeb et al (2008) reported interesting results that the prevalence of sickle cell anaemia and thalassemia in Al-Qassim region, to be 0.165% and 0.252% respectively. Also showed that the clinical manifestations of sickle cell disease vary widely among patients, where some patients with sickle cell anaemia have mild disease, while others suffer from a severe form and relatively high mortality at early age.

Our current results were obtained from 69 SCD patients at the KKUH from various regions of Saudi Arabia, exhibiting a wide spectrum of the disease from mild to severe stages. This was evidenced by the follow-up of several indicators in patients' records throughout the study period. The differences in SCD patients were related to the need for medical emergency assistance, as well as the need to stay at the hospital for several days to receive the necessary treatment.

A comprehensive molecular analysis of the nucleotide sequence for six LCR outside the region was performed here, results as shown below: (LCR–HS1: (HBB-LCR1), LCR–HS2: (HBB-LCR2), LCR–HS3:(HBB-LCR3), G-promoter region: (HBB-LCR5), (AT)x(T)y region: (HBB-LCR6); and 3_ flanking; sequences (enhancer): (HBB-LCR7).

The relationship between clinical symptoms and LCR mutations, showed a close relationship between an increase of WBCs and those changes: (751–752 & change: 805–807 -/c homo ins), in the LCR-HS1 region: (HBB-LCR1). Another relationship between a decrease of platelets and those changes: (8947 A > T het, in the LCR-HS3) segment: (HBB-LCR3). A third relationship was found between elevated Bilirubin level and (a deletion of 710 A - homo del in LCR-HS1) (HBB-LCR1).

Ngo et al. (2013) studied forty four Saudi HbS homozygous patients with A haplotype. They found that A11 cases were...
homozegous for A1 haplotype. Moreover, SNPs in BCL11A and HBS1L-Myb were associated with high HbF (Ngo et al., 2013). Tunisian group found four independent regions in LCR Hb beta globin namely: (the 5’ region of LCR-HS2 site, the intervening sequence region of two fetal G gamma and A gamma genes and 5’ of beta-globin gene. There was a close relationship between high HbF level and the 5’ region of LCR-HS2, which could improve our standing on disease severity (Moumni et al., 2016). Two additional studies agreed with the Tunisian’s results (Zago et al., 2001; Ben Mustapha et al., 2012).

We published a very recent paper studying the nucleotide sequences and the molecular survey of beta-globin gene in Saudi patients from 77 SCD patients. A total of 47 alterations have been recognized. SCD had a negative effect on many organs and outcomes (Alenzi and AlShaya, 2019).

In conclusion, it has been clear that this study has successfully identified LCR mutations for random Saudi patients with SCD. The above results should be taken further to set up management strategies to improve outcomes.

Acknowledgments

Special thanks to Dr. Dalal AlShaya (PNU), Prof. AbdulKareem Al-Momen (KSU) and Dr. Mai AL-Mohanna (KFSHRC) for advice and providing the samples. This project has been funded by a grant from the PSAU (grant no: 2019/03/10211).

References

Weatherall, D.J., Clegg, J.B., 2001. Inherited haemoglobin disorders: an increasing global health problem. Bull. World Health Organ. 79 (8), 704–712.

WHO report. (2005–2006-2011). Sickle-cell disease and other haemoglobin disorders. Fact sheet N°308.

Ashley-Koch, A., Yang, Q., Olney, R.S., 2000. Sickle hemoglobin (Hb S) allele and sickle cell disease: a huge review. Am. J. Hematol. 65 (2), 142–145.

Schechter, A.N., 2008. Hemoglobin research and the origins of molecular medicine. Blood 112 (10), 3927–3938.

Lionnet, F., Hammoudi, N., Stoianov, K.S., Avellino, V., Groteau, G., Girot, R., Haymann, J.P., 2012. Hemoglobin sickle cell disease complications: a clinical study of 179 cases. Haematologica 97 (8), 1136–1141.

Jastaniah, W., 2011. Epidemiology of sickle cell disease in Saudi Arabia. Ann. Saudi Med. 31 (3), 289–293.

El-Hazmi, M.A.F., 1979. Aspect of human hemoglobin and hemoglobin haemoglobinopathies in the arbian hemansula- studies at genetics and molecular level. KACST, 1982–1992.

El-Hazmi, M.A.F., 1983. Abnormal hemoglobins and allied disorders in the Middle East – Saudi Arabia. In: Distribution and evolution of hemoglobin and globin loci. Bowman JF, ed. 1983, pp. 239–249.

El-Hazmi, M.A.F., 1992. Haemoglobinopathies, thalassaemias and enzymopathies in Saudi Arabia. Saudi Med. J. 13 (6), 488–499.

El-Hazmi, M.A.F., 1985. Incidence and Frequency of Haemoglobinopathies and Thalassaemia in the North West Sector of Arabia. Saudi Med. J. 6 (2), 149, 162.

El-Hazmi, M.A.F., Warsy, A.S., 1999. Appraisal of sickle cell and thalassaemia genes in Saudi Arabia. Eastern Mediterranean Health J. 5 (6), 1147–1153.

El-Hazmi, M.A.F., Jabbar, F.A., Al-Faleh, F.Z., Al-Swailem, A.R., Warsy, A.S., 1991. Pattern for sickle cell, thalassaemia and glucose 6 phosphate dehydrogenase deficiency genes in northernwestern Saudi Arabia. Hum Genet Hered 41 (1), 26–34.

Williams, T.N., Weatherall, D.J., 2012. World distribution, population genetics, and health burden of the hemoglobinopathies. Cold Spring Harb Perspect Med 2 (9).

Al-Qurashi, M.M., El-Mouzan, M.I., Al-Herbish, A.S., Al-Salloum, A.A., Al-Omar, A.A., 2008. The prevalence of sickle cell disease in Saudi children and adolescents. A community-based survey. Saudi Med J 29, 1480–1483.

Al-Naseri, E. Awareness of mothers in nutrition their children affected with sickle-cell anemia and beta-thalassaemia in Jeddah. (Master Thesis). King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia, 2009.

El-Tayeb, E.N., Yambo, M., Abdur-Rahim, K., Gustavson, K.H. 2008. Prevalence of beta-thalassaemia and sickle cell traits in premarital screening in Al-Qasim Saudi Arabia. Genet Couns. 19 (2), 211–218.

El-Hazmi, M.A.F., Bahakim, H.M., Al-Swailem, A.M., Warsy, A.S., 1990. The features of sickle cell disease in Saudi children. J. Trop. Paedr. 36, 148–155.

El-Hazmi, M.A.F., Warsy, A.S., 1996. Hemoglobinopathies in Arab Countries. Genetic Disorders among Arab Populations. New York: Oxford University Press.

Ngo, D., Bae, H., Steinberg, M.H., Sebastiani, P., Soloviov, N., Baldwin, C.T., Melista, E., Salaya, S., Farrer, L.A., Al-Sulaiman, A.M., Al-Baghi, W.H., El-Baghi, M.H., Naserullah, Z., Akinsheye, I., Galagher, P., Luo, H.Y., Chai, D.H., Farell, J.J., Al-Ali, A.K., Alsultan, A., 2013. Fetal hemoglobin in sickle cell anemia: genetic studies of the Arab-Indian haplotype. Blood Cells Mol. Dis. 51 (1), 22–26.

Moumni, I., Ben Mustapha, M., Ben Mansour, I., Zorai, A., Douzi, K., Sassi, S., Chouaich, D., Mellouli, F., Bejaoui, M., Abbes, S., 2016. Fetal hemoglobin in Tunisian sickle cell disease patient: relationship with polymorphic sequences CEE to the β-globin gene. Indian J. Hematol. Blood Transfus. 32 (1), 114–119.

Zago, M.A., Silva Jr, W.A., Gualandro, S., Yokomizu, L.K., Araujo, A.G., Tavela, M.H., Gerard, N., Krishnamoorthy, R., Elion, J., 2001. Rearrangements of the beta-globin gene cluster in apparently typical betaS haplotypes. Haematologica 86 (2), 142–145.

Ben Mustapha, M., Moumni, I., Zorai, A., Douzi, K., Ghanem, A., Abbes, S., 2012. Microsatellite and single nucleotide polymorphisms in the β-globin locus control region-hypersensitive Site 2: SPECIFICITY of Tunisian βS chromosomes. Hemoglobin 36 (6), 533–544.

Alenzi, F.Q., AlShaya, D.S., 2019. Biochemical and Molecular analysis of the beta-globin gene on Saudi sickle cell anemia. Saudi J Biol Sci. 26 (7), 1377–1384.