Saudi Journal of Biological Sciences 27 (2020) 1265–1270

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

New mutations of locus control region in Saudi sickle patients

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ARTICLE INFO

Article history:
Received 9 December 2019
Revised 16 January 2020
Accepted 22 January 2020
Available online 31 January 2020

ABSTRACT

Sickle cell anemia (SCA) is a common hematological disease affecting humans. Detection of a single base pair mutation in β-globin gene is an important diagnostic tool for SCA. 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.

Methodology: Blood samples from 69 unrelated sickle cell disease patients were obtained from the KKUH, Riyadh between 2017–2019. In this study, the DNA was extracted and PCR was performed. Additional PCR amplifications reactions covering the LCR were performed by using another different set of primers. Seven specific primer pairs were used to amplify seven regions in the locus control region (LCR) of β globin family. The generated fragments were sequenced to identify the possible alterations in this region.

Results: The results gained from sequencing experiments revealed a wide range of genomic alterations.

A total of 69 gene alterations have been recognized in the locus control region;-
The first fragment LCR-HS1 shows 20 alterations;
The second fragment LCR-HS2 revealed six changes;
The third fragment LCR-HS3 shows many changes;
The fifth LCR-HS5 region revealed four changes;
The sixth fragment LCR-HS6 revealed eight changes;
The seventh LCR-HS7 fragment demonstrates ten changes.

Conclusion: It 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.

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 anaemia 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 thalessemia 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. SCD is featured by many signs such as: inflammation, stroke, chronic haemolysis, and acute chest pain. Abnormal RBC shape causing them to lose their flexibility and turn into the form of solid sickles (Lionnetet al., 2012).

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 had these genetic blood diseases in the Saudi Arabia, of which 30% in the Eastern Region (Al-Naseri, 2009). El-Hazmi and others demonsated two

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.01.028
1319-562X/

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 2 μl 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).

Table 1
The primers that were designed and used in this study to multiply the LCR.

Primer	Primer seq 5′ – 3′	Product size (b.p)
LCR-1F	CCTGCAAGTTATCTGGTCAC	445
LCR-1R	CTTAGGGGCTTATTTTATTTTGT	
LCR-2F	CAGGGCAGATGGCAAAAA	460
LCR-2R	CTGACCCCGTATGTGAGCA	
LCR-3F	ATGGGGCAATGAAGCAAAGGAA	595
LCR-3R	ACCCATACATAGGAAGCCCATAGC	
LCR-5F	GCAAACACAGCAAACACAACGAC	442
LCR-5R	ATGGCAGAGGCAGAGGACAGGTTG	
LCR-6F	TTCCCAAAACCTAATAAGTAAC	520
LCR-6R	CCTCAGCCCTCCCTCTAA	
LCR-7F	TGCCCTGGCCCACAAGTATC	539
LCR-7R	TCAGGGGAAAGGTGGTATCTCTAA	

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

Table 3 presents a summary of hematological and biochemical measurements from SCD patients who have been followed for more than 4 years.

3.2. Relationship between genetic changes identified in beta globin and clinical symptoms in patients

The clinical symptoms of SCD patients vary from mild symptoms to severe complications that completely change their lifestyle and may even lead to disability from normal life. The changes identified above were correlated with the most frequent biochemical indicators among the surveyed patients to determine the relationship between these changes and the severity of those symptoms.

WBC count:

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 2
Hematological and biochemical measurements from SCA patients.

	WBC	Hgb	Plt	Bilirubin	LDH
Mean	92	11	368	48	446
SD	23	5	166	39	206

Table 3
Distrubution of LCR changes per base per sample.

LCR-HBB/F1 start at 668 end at 1110 LCR–HS1	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.	7	9.1%
	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 4397 end at 4991 LCR–HS2	1	4575 G > A het.	77	100.0%
	2	4631 G > A het.	77	100.0%
	3	4673 G > A het.	77	100.0%
	4	4950–4951 ->C homo ins.	2	2.6%
	5	4455–4456 –>TC homo ins.	1	1.3%
	6	4562 G > A het.	4	5.2%
LCR-HBB/F3	1	8874 G > A het.	7	9.1%
start at 8757	2	8899to 8902 TATA>—— homo del.	1	1.3%
end at 9219	3	8900to 8902 TA>—— homo del.	1	1.3%
LCR–HS3	4	8906–8910 – > CA homo ins.	1	1.3%
	5	8912 G > A het.	5	6.5%
	6	8914 A > G homo.	42	54.5%
	7	8915to 8920 TATA>—— homo del.	42	54.5%
	8	8921to 8922 –> AT homo ins.	10	13.0%
	9	8947 A > T het.	22	28.6%
	10	8948 A > T het.	77	100.0%
	11	8957 G > A het.	77	100.0%

Region/Fragment	Base change	N.Samples	Percent %	
LCR-HBB/F4	12	8960 T > A het.	18	23.4%
start at 8757	13	8966G > T homo.	76	98.7%
end at 9219	14	8969 A > T het.	7	9.1%
LCR–HS3	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 G–promoter region	1	34,502 G > A het.	1	1.3%
	2	34,662C > A het.	45	58.4%
	3	34,663C > A het.	45	58.4%
	4	34,687C > T het.	15	19.5%

Region/Fragment	Base change	N.Samples	Percent %	
LCR-HBB/F6 start at 61,441 end at 61,961 (AT)x(T)y region	1	61,495 to 61,496 ->C homo ins.	1	1.3%
	2	61,522 to 6523 ->A homo ins.	1	1.3%
	3	61,558 to 6559 ->A homo ins.	2	2.6%
	4	61586 T > C homo.	2	2.6%
	5	61,586 - - > CA homo ins.	3	3.9%
	6	61590 T > C homo.	2	2.6%
	7	61,593C > T homo.	8	10.4%
	8	61,594 - - >AT homo ins.	9	11.7%

(*continued on next page*)

Table 3 (*continued*)

LCR-HBB/F1 start at 668 end at 1110 LCR–HS1	Region/Fragment	Base change	N.Samples	Percent %
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	Normal	10.89	11.05	4.21
	Mutated	15.65	15.20	3.44
LCR#F1_10	Normal	10.66	11.05	3.54
	Mutated	18.01	16.50	6.12
LCR#F1_13	Normal	11.58	11.33	4.41
	Mutated	10.06	11.33	3.90
LCR#F3_9	Normal	11.52	11.19	4.64
	Mutated	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	Normal	91.1	91.7	20.9
	Mutated	97.5	102.0	15.7
LCR#F1_10	Normal	91.0	91.7	20.8
	Mutated	98.1	106.0	15.9
LCR#F1_13	Normal	92.4	91.7	19.0
	Mutated	88.0	91.7	27.2
LCR#F3_9	Normal	92.2	91.7	22.3
	Mutated	90.2	91.7	15.4

Table 6
Relationship between Platelets count and mutation.

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

Table 7
Relationship between LDH level and mutation.

Part/Variable		LDH		
		Mean	Median	Standard Deviation
LCR#F1_2	Normal	550.6	539.3	502.5
	Mutated	426.2	453.0	121.9
LCR#F1_10	Normal	547.6	539.3	503.2
	Mutated	455.8	453.0	119.0
LCR#F1_13	Normal	560.6	539.3	516.5
	Mutated	434.1	497.0	228.7
LCR#F3_9	Normal	558.7	539.3	564.8
	Mutated	490.8	539.3	120.9

Table 8
Relationship between Bilirubin level and mutation.

Part/ Variable		Bilirubin		
		Mean	Median	Standard Deviation
LCR#F1_2	Normal	48.1	48.1	35.2
	Mutated	48.9	47.0	21.5
LCR#F1_10	Normal	47.5	48.1	34.2
	Mutated	55.1	47.0	34.9
LCR#F1_13	Normal	50.8	48.2	36.0
	Mutated	35.2	42.0	18.0
LCR#F3_9	Normal	49.2	48.0	37.2
	Mutated	45.6	47.6	25.1

Table 9
Relationship between Lab parameters and presence of mutation(s).

Part/Variable	Significance Level (P- Value) one tail				
	WBC	HB	PLT	LDH	Bilirubin
LCR#F1_2	0.001 *	0.100	0.437	0.065	0.337
LCR#F1_10	0.001 *	0.122	0.101	0.15	0.257
LCR#F1_13	0.194	0.5	0.093	0.06	0.048 *
LCR#F3_9	0.495	0.328	0.034 *	0.448	0.343

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 mutation 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

homozygous for A1 haplotype. Moreover, SPNs 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 Hb F 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 β-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).

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