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Molecular aspects of the common types of β-thalassemia mutations among Sudanese patients: a cross sectional study

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Abstract. – OBJECTIVE: The study sought to identify the most common types of mutations in beta-thalassemia Sudanese patients in Khartoum State, as well as their relationship to anemia severity.

PATIENTS AND METHODS: From July 2017 to July 2021, a cross-sectional study was conducted on 100 samples from known beta-thalassemia patients attending public health hospitals in Khartoum State, and fifty samples were taken from apparently healthy controls. Using a PCR test, blood samples were analyzed to detect the most common types of mutations.

RESULTS: For the five mutations included in the study (IVS-I-110(G-A), IVS-I-1(G-A), IVS-I-6(T-C), IVS-I-5(G-C), and β_87(C-G)), 25% of the patients were positive and 75% were negative, while the entire control group was negative for all five mutations. Positive results were found in only three of the five mutations tested; the most common was IVS-I-110, which was found in 14 (56%) of the subjects, followed by IVS-I-6 (T) in 7 (28%), and IVS-I-1 in only 4 (16%) of the subjects. IVS-I-5 and β_87 mutations were not found in the study subjects. The Hb electrophoresis pattern revealed an increase in HbA2 (6.455±0.1318%), a decrease in HbF (1.865±0.1668%), and a decrease in HbA (78.50±0.2858%). The mean serum iron level was 82.99±3.063 ug/dL, which was considered normal.

CONCLUSIONS: According to the findings, the IVS-1-110 mutation is the most common among Sudanese beta-thalassemia patients. Each type of positive mutation caused mild to moderate anemia.

Key Words:
β-Thalassemia, Common mutations, Sudan, Molecular aspects.

Introduction

Thalassemia is a common genetic blood disease that is a major health issue in different parts of the world. On a global scale, gene frequencies for thalassemia range from 1% to more than 80% in malaria-endemic areas[1]. Approximately 1.5% of the world's population is a carrier of beta thalassemia, with about 60,000 symptomatic individuals born each year, the vast majority of whom reside in developing countries[2]. In Sudan, the prevalence of beta-thalassemia has been reported to be between 1 and 10%. Sudan is a multifaceted country with a diverse population made up of people from various ethnic groups. This reflects different levels of interbreeding and social interaction. However, a local database table remains unfinished[3]. As a result, detecting or identifying the most prevalent mutations causing thalassemia in Sudan may fill a gap in our local data store. To avoid beta-thalassemia, it is necessary to understand the molecular spectrum that occurs in vulnerable members of society, as the spectrum of mutations varies significantly across geographical regions, with only a few common globin mutations causing beta-thalassemia in each population[3]. The beta-thalassemia is an autosomal recessive disease. Affected parents are compelling heterozygotes with a single copy of a disease-causing beta-globin gene mutation. Each child of heterozygous parents has a 25% chance of being impacted, a 50% chance of being an asymptomatic carrier, and a 25% chance of being unaffected and not a carrier at conception. In each pregnancy, the proband's parents have a

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one-in-four (25%) chance of having another affected child[4]. The goal of this study is to find out which types of beta-thalassemia mutations are most common in Sudanese patients.

Patients and Methods

Design of the Study

This is a cross-sectional study of the most common mutations among beta-thalassemia patients admitted to Khartoum State's public health hospitals. The research was carried out between July 2017 and July 2021. A total of 100 samples were collected from patients who had already been diagnosed with beta-thalassemia, with 50 normal individual samples serving as controls.

Inclusion Criteria

Patients with beta-thalassemia, confirmed by CBC and hemoglobin electrophoresis, of all ages and sexes.

Exclusion Criteria

Patients in which other hemoglobin variants coexisted; blood transfused patients for less than 3 weeks, patients on iron therapy for the previous month, and patients with other hematological malignancies were excluded.

Data Collection

Data were collected by a designed questionnaire and laboratory investigations, coded and listed in a master sheet, and then computerized, including demographics and history of each participant.

Ethical Considerations

The local authorities in the study area approved the study, and the protocol was reviewed by the University of Gezira Ethics Committee (No. MGAS/0050632). The purpose of the study was explained to everyone who took part in it. All participants provided written, informed consent.

Sample Collection Technique

An antiseptic technique was used to collect 3 ml of venous blood, whole EDTA, and serum samples from controls and patients diagnosed with beta-thalassemia.

Hb Electrophoresis (Capillary Electrophoris)

Capillary electrophoresis (CE) is a technique for conducting hemoglobin electrophoresis in narrow capillaries filled with buffer. As a result, electro-osmotic flow occurs, and the differently charged Hb fractions are separated. These fractions are detected directly from cathode to anode at an absorbance wavelength of 415 nm, which is optimal for hemoglobin: Hb C, A 2, E, S, D, F, A, Bart's, J, and H.

Molecular Analysis

Polymerase chain reaction (PCR)

The primers described by Lamia's et al[5] were used for PCR. The primers' nucleotide substitution was focused on the nucleotide positions of IVSI-6 and IVSI-110. Primer nucleotide sequences, primer combinations for each reaction, and the length of the amplification product are all listed in Table I.

Table I. PCR primers sequence used for β-globin gene mutation.

Mutation name	Primer pair	Fragment, size(bp)
Normal IVS1-6	F:5'AGTTGGTGGTGAGGCCCTGGGCAGGTTGGT '3 R:5'CCCCTTCCTATGACATGAACTTAA'3	449
Mutant IVS1-6	F:5'TCTCCTTAAACCTGTCTTGTAACCTTCATG '3 R:5'ACCTCACCCTGTGGAGCCAC '3	286
Normal IVS1-110	F:5'ACCAGCAGCCTAAGGGTGGGAAAATACACC '3 R:5'ACCTCACCCTGTGGAGCCAC '3	419
Mutant IVS1-110	F:5'ACCAGCAGCCTAAGGGTGGGAAAATAGAGT '3 R:5'ACCTCACCCTGTGGAGCCAC '3	419
Normal IVS1-5	F:5 CTCCTTAAACCTGTCTTGTAACCTTGTTAC'3 R:5ACCTCACCCTGTGGAGCCAC'3	285
Mutant IVS1-5	F:5'CTCCTTAAACCTGTCTTGTAACCTTGTTAG'3 R:5'ACCTCACCCTGTGGAGCCAC'3	285
Mutant IVSI-1	F:5 TTAAACCTGTCTTGTAACCTTGATACGAATC'3 R:5'TTAAACCTGTCTTGTAACCTTGATACGAACC'3	218
Mutant -87	F:5CTCACCCTGTGGAGCCACACGCTAGGGTTGGCCAATCTAC'3 R:5GTAGATTGGCCAACCCTAGCGTGTGGCTCCACAGGGTGAG'3	289
Control	F: 5'-GAGTCAAGGCTGAGAGATGCA GGA-'3 R: 5'-CAATGTATCATGCCT CTTTGC ACC-'3	861

According to the allele-specific variant detected, twelve samples were chosen from 150 to confirm the single variant. Each variant received four samples (IVS I-1, IVS I-6, and IVS I-101) in addition to three controls (a total of 15 samples were sent for sequencing). Following DNA extraction, DNA primers for the target region (covering the three target single variants) were designed using Primer 3Plus[6] (Waltham, MA, USA). Previous samples (DNA extracted product) and designed primers were sent to BIONEER laboratories in Korea for oligonucleotide synthesis, region amplification, and Sanger DNA sequencing (for the forward or positive strand).

Statistical Analysis

The collected data were checked and analyzed using the SPSS Statistical Package for Social Science Software Version 23 (IBM Corp., Armonk, NY, USA). Microsoft Office Excel 2007 and Windows version 23 were used. The significance level was set at $p<0.05$. For gene sequencing, bioinformatics analyses were performed using the Chromatogram Explorer program version 5.0.2.3 (Arges, Romania), the Basic Local Alignment Search Tool (Megablast) program (Bethesda, MA, USA), and the CLC genomics workbench program version 20.0.3 (Redwood City, CA, USA).

Results

Demographic Data Analysis

This case-control study was carried out in Sudan among beta-thalassemia patients seen at public health hospitals in Khartoum State between July 2017 and July 2021. The study included 100 beta-thalassemia patients and 50 control samples. Adults (>18 yrs.) made up 64% of the 100 beta-thalassemic patients, followed by children (36%), while adults (>18 yrs.) made up 36% of the 50 controls, as shown in Table I. The male to female ratio was 41% and 59%, respectively, while the frequency in the control group was 50% for each (Table II).

Hemoglobin Electrophoresis Results

Samples were subjected to capillary electrophoresis testing; the mean of HbA was decreased by 78.50±0.2858%, while HbF and HbA2 were increased compared with the control group; all parameters were highly significant, as shown in Table II.

Molecular Analysis

According to the PCR results, 25% of the patients were positive and 75% were negative for the five mutations included in the study (βIVS-I-110 (G→A), βIVS-I-1(G→A), βIVS-I-6(T→C), βIVS-I-5(G→C) and β_87(C-G)), while all patients in the control group were negative for all five mutations, as illustrated in Figure 1. Positive results were found in only three of the five mutations tested; the most common and frequent one detected was IVS-I-110, which was positive in 14 (56%) of the subjects, followed by IVS-I-6 (T→C), which was positive in 7 (28%), and the least common mutation, IVS-I-1, which was positive in only four (16%) of the subjects, as shown in Figure 2. Males and females had 8 (32%) and 17 (68%) mutations, respectively, while children had 9 (36%), and adults had 16 (64%) (Table III). The three mutations found in this study caused mild to moderate anemia; the frequency of mild to moderate mutations was 72% and 28%, respectively (Table IV). Table V and VI show a significant correlation between the mean hemoglobin concentration for mutated and non-mutated individuals and controls, with a p-value of 0.000 for mild and moderate anemia. The results obtained from the sequencing of beta-thalassemia samples clearly confirmed that all samples possessed the three mutations detected by PCR. The mean of the Hb pattern in each type of mutation is illustrated in Figure 3, while Figures 4, 5, and 6 show the correlation of hemoglobin A2, F, and A, respectively, in different degrees of severity of beta-thalassemia and control.

Discussion

Sudan is a diverse nation with a complex population made up of people from various ethnic groups, which reflects the various levels of intermarriage and social interaction. Prevention of thalassemia necessitates an understanding of the molecular mutations and genotypes found in the beta-thalassemia population. This study used 100 samples for hemoglobin quantification and qualification, red cell indices, and genotyping. The study included 100 beta-thalassemic patients and 50 control samples. Adults (>18 years) made up 64% of the patients, while children (under 18 years) made up 36% of the patients. The male-to-female ratio was 41% male to 59% female, respectively. Hb electrophoresis results show that the mean of HbF was 1.865±0.1668%, which was

Table II. Age and gender distribution in the study group and controls.

Age group	Study subjects		Control	
	No	Frequency	No	Frequency
Adults (>18 Yrs.)	64	64%	33	66%
Children (< 18Yrs.)	36	36%	17	34%
Total	100	100%	50	100%
Gender group				
Male	41	41%	25	50%
Female	59	59%	25	50%
Total	100	100%	50	100%

Table III. Hb electrophoresis pattern mean values in the study group.

Hb electrophoresis parameters	HbA2 %	HbF%	HbA%
Study subjects	6.45 ± 0.1318	1.865 ± 0.1668	78.50 ± 0.2858
Controls	1.962 ± 0.06821	0.6958 ± 0.0250	297.05 ± 0.1129
p-value	0.0001	0.0001	0.0001
Significance level	*p*-value is significant if it is	< 0.05	

high, and HbA2 is 6.455±0.1318%, which is also significant, which agrees with previous study[7] that show high HbA2 > 3.5%.

Out of 100 patients, 25% were positive and 75% were negative for the five mutations included in the study (IVS-I-110(G-A), IVS-I-1(G-A), IVS-I-6(T-C), IVS-I-5(G-C), and β_87(C-G)), while all patients in the control group were negative for all five mutations. Positive mutations were found in 25% of patients, with the most common being the IVS-I-110(GA) mutation (intronic cryptic splice site mutation) found in 14 (56%), followed by the IVS-I-6 (TC) mutation (consensus splicing) seen in 7 (28%), and the IVS-I-1 mutation found in 4 (16%), while all control groups were negative for all five mutations. All three mutations detected in this study were the most commonly reported in 22 Arab countries, particularly Egypt, and were of Mediterranean and eastern Mediterranean origin[8]. However, IVSI-5, an Asian Indian origin mutation, and β_87, of Mediterranean origin mutation, were not found in all subjects in this study.

In Arab countries, the IVS-I-110 (G>A) mutation is the most common in Lebanon, Syria,

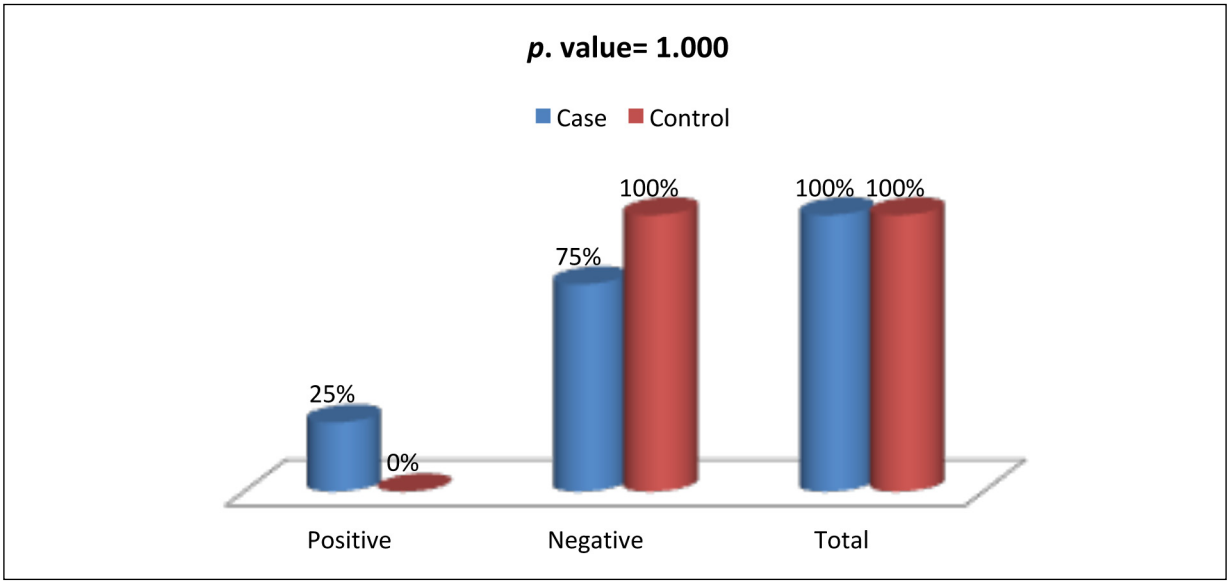

Figure 1. Positive and negative PCR frequency between the study groups.

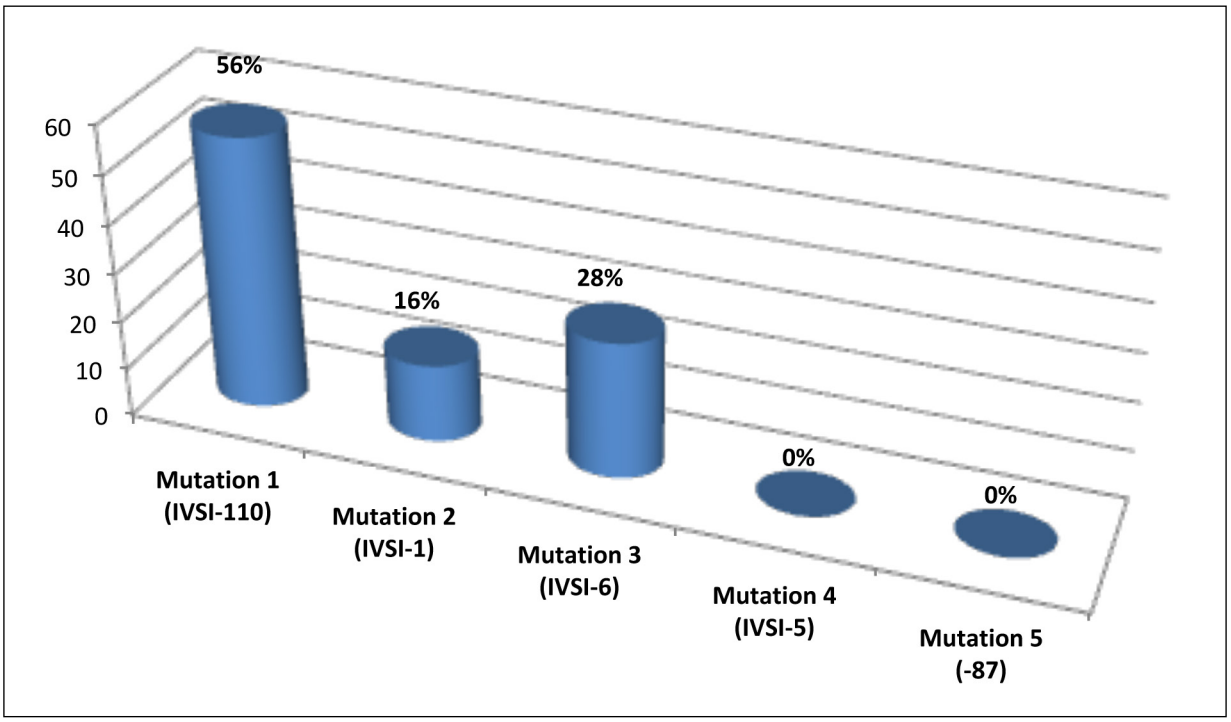

Figure 2. Frequency of different types of mutations among the study.

Jordan, Egypt, Tunisia, and Algeria; these findings are inconsistent with our study. The most frequently mutated allele detected by real-time PCR in 20 thalassemic patients in Egypt was IVS I-110, all but one was heterozygous for IVS I-110, and only one was homozygous[8,9].

The most common mutations among Lebanese thalassemic patients were IVSI-110 (29.87%), IVSI-6 (20.74%), IVSI-1 (14.07%), IVSII-1 (9.13%), Cd29 (9.13%), and Cd30 (3.95%)10. These mutations are also found in high numbers in Sudan. In Iran, statistical analysis revealed that IVS-II-I (G-A) (25.4%) was the most common mutation[11].

All of the mutations found in this study, IVSI-110, IVSI-6, and IVSI-1, were the most commonly reported in Arab countries. The associated β-thalassemia phenotypes of these mutations vary across Arab countries. The IVS I-110 mutation is a Mediterranean mutation that is common in Arab countries, with rates ranging from about

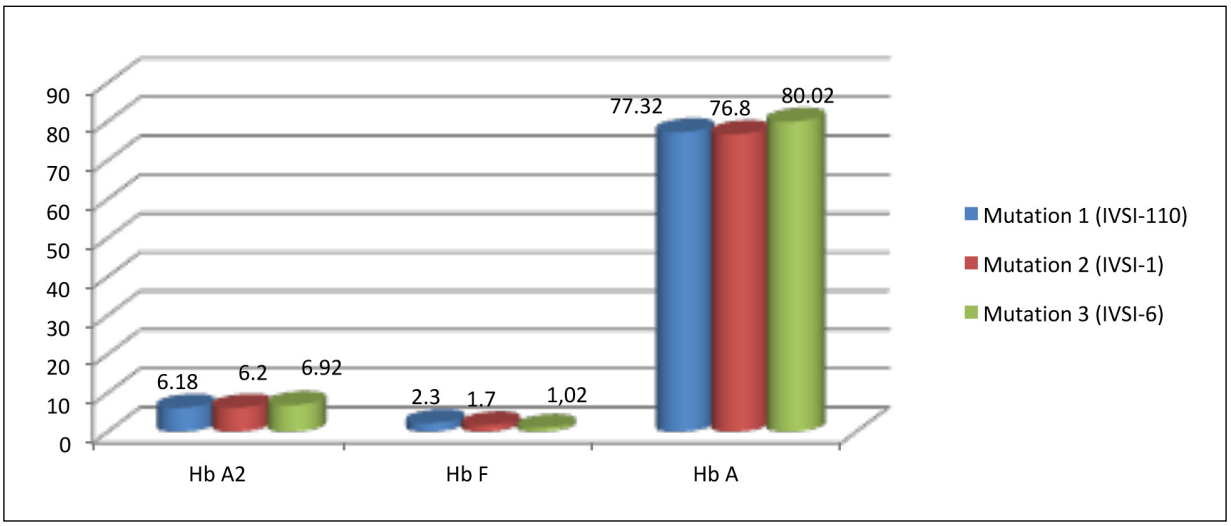

Figure 3. Mean of hemoglobin pattern in each type of mutations.

Table IV. Frequency of mutations according to gender and age.

Mutation Frequency	Males	Females	Adults	Children's	Total
No	8	17	16	9	25
%	32%	68%	64%	36%	100

Table V. Frequency and the degree of anemia in different types of mutations.

Type of mutation		Mild	Moderate	Severe	Total
Mutation 1 (IVS1-110)	Frequency	10	4	0	14
	Mean Hb g/dL	10.6	8.0	0	-
Mutation 2 (IVS1-6)	Frequency	5	2	0	7
	Mean Hb g/dL	10.54	7.85	0	-
Mutation 3 (IVS1-1)	Frequency	3	1	0	4
	Mean Hb g/dl	10.1	8.1	0	-
Total mutated frequency		18	7	0	25
		72%	28%	0	100%

Table VI. Mean hemoglobin g/dL concentration between mutated, none mutated and controls.

Type of mutation	Severe (< 7 g/dl)	Moderate (7 - 9.4 g/dl)	Mild (9.5 – 11.5 g/dl)	Normal
Mutation 1 (IVS1-110)	0	8.0	10.6	0
Mutation 2 (IVS1-6)	0	7.85	10.54	0
Mutation 3 (IVS1-1)	0	8.1	10.1	0
Negative patients	6.45	8.4	10.64	12.79
Controls	0	0	0	14.30
p-values	0.07	0.000		
Significance level	*p*-value is significant if it is < 0.05			

1% in Bahrain and the UAE to 48% in Egypt and 56% in the current study. The IVS I-110 G>A mutation is thought to have spread in the Eastern Mediterranean and North Africa during the Ottoman Empire, which ruled the area from the 16th to the early 20th centuries[11].

Furthermore, it is believed that IVS I-110 G>A was introduced to the Arab world through human migration; in our study, IVS I-110 (56%) was the most common mutation detected.

Although the IVS I-1 mutation is common in Arab countries, its frequency in the Czech Republic is the highest (36%), while in Arab countries it is 26% in Egypt and lower in other countries. This mutation was detected in (16%) in this study, which was the lowest mutation detected[11].

The IVS I-6 G>A mutation is a Mediterranean mutation of Portuguese origin that is found at low frequency in Arab countries except in the Palestinian territories, where it accounts for nearly half of all HBB gene mutations. The IVS I-6 mutation, which was discovered as the second mutation, was found to be localized in the Palestinian territories' mountain region, which reflects the isolation of the population of this region, which was inhabited by Samaritans[11]. The absence of IVSI-5, a mutation of Asian and Indian origin, and β_87, a mutation of Mediterranean origin, was due to some limitations of this study.

The severity of anemia in various types of mutations was classified as mild, moderate, or severe depending on Hb levels. The results showed that the majority of them presented with mild to moderate anemia, with the frequency of mild to moderate mutations being 72% and 28%, respectively, and severe anemia not being detected in this study when compared to a previous study[7] conducted in Port Sudan to screen for hemoglobinopathy. Hemoglobinopathy was found in 59 (28.22%) of the subjects, with 29 (49.15%) having beta-thalassemia trait and 1 (1.69%) having beta-thalassemia major. These discovered mutations have varying effects on the splicing process, depending on the nature and position of the mutation. Splicing consensus sequence mutations cause varying degrees of defective splicing, resulting in aberrant tran-

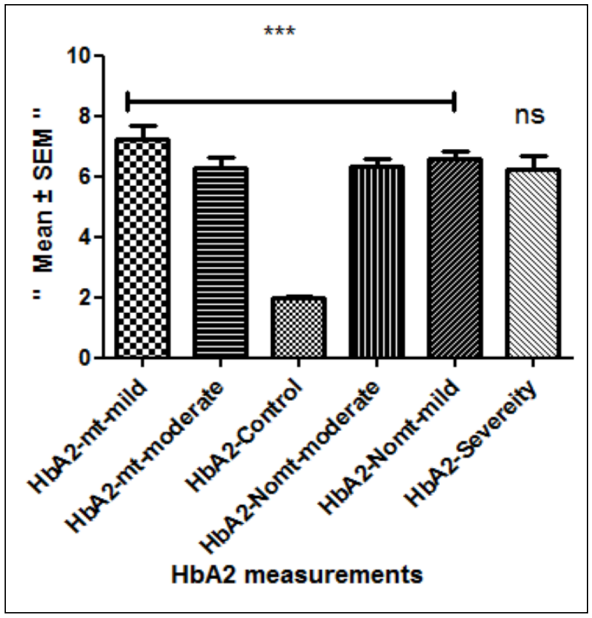

Figure 4. Correlation of hemoglobin A2 in different degree of severity of Beta-thalassemia and control. SEM= standard error mean, ns= non-significant, mt= mutated, nomt= non-mutated.

scripts and lower functional beta-globin levels, as well as milder forms of β-thalassemia. Other exon or intron sequence mutations may activate a cryptic splicing site, resulting in abnormal mRNA processing. Even in these cases, defective splicing occurs to varying degrees, resulting in mild to severe phenotypes. The substitution of C for T in the adjacent nucleotide, intron 1 position 6, on the other hand, has only a minor effect on normal RNA splicing[12].

The IVS1-6 T→ C mutation is generally associated with milder β-thalassemia, depending on the site and nature of the mutation. The IVS1-110 G to A mutation was the first in a β-thalassemia gene to be discovered. It is one of the most common types of β-thalassemia in the Mediterranean population and is frequently associated with mild β-thalassemia phenotypes[13]. This supported our findings that all of the mutations found in this study were located in intron 1 and between the intron 1 and exon 1 junction, and all of the patients had mild to moderate thalassemia.

Conclusions

The study concluded that the IVS-1-110 (GC) mutation is the most common among Sudanese thalassemia patients. The PCR results obtained showed that 25% of patients were positive and 75% were negative for five mutations; including within the study, positive results were detected in only three mutations out of five; the most common one detected was IVS-I-110, followed by IVS-I-6, and the most recent frequency mutation

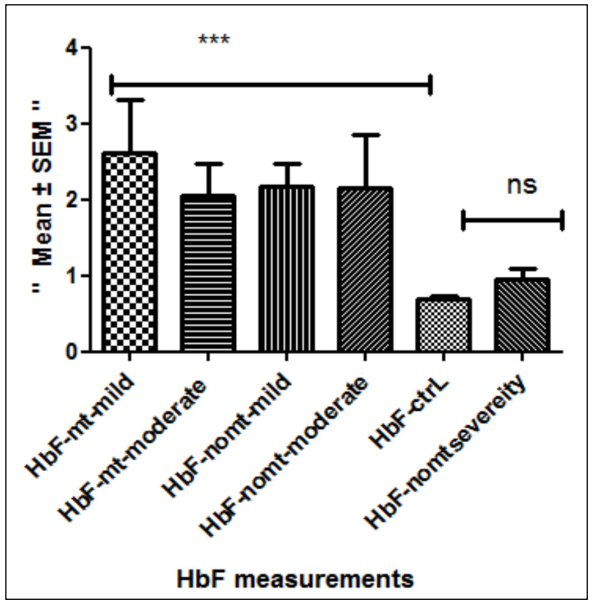

Figure 5. Correlation of hemoglobin F in different degree of severity of Beta-thalassemia and control. SEM= standard error mean, ns= non-significant, mt= mutated, nomt= non-mutated, ctrl= control.

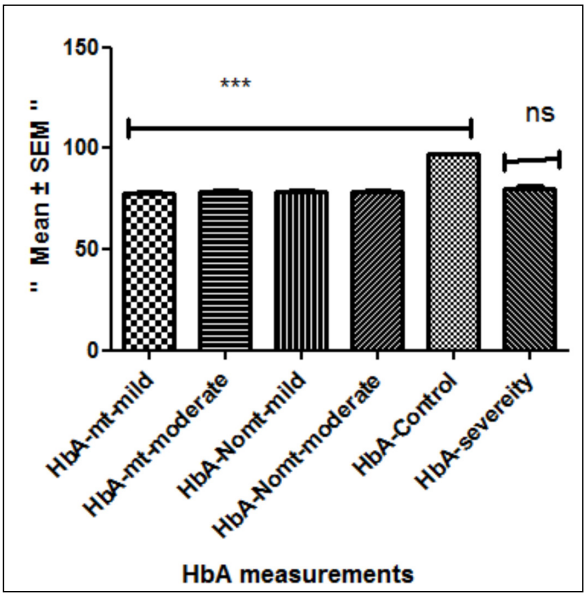

Figure 6. Correlation of hemoglobin A in different degree of severity of Beta-thalassemia and control. SEM= standard error mean, ns= non-significant, mt= mutated, nomt= non-mutated.

was IVS-I-1. The other two mutations not detected in this study were IVSI-5 and β_87, the absence of which was due to some study limitations.

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Conflict of Interest
Authors declared that there is no conflict of interest in this research.

Authors' Contributions
All authors worked together to complete this project. The final manuscript was read and approved by all authors.

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Ethics Approval
The local authorities in the study area approved the study, and the protocol was reviewed by the University of Gezira Ethics Committee (No. MGAS/0050632).

Informed Consent
All participants provided written, informed consent.

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