Molecular and hematological spectrum of α-thalassemia in Saudi patients

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ABSTRACT

α-thalassemia (α-thal) is a genetic disorder characterized by a decreased synthesis of α-globin chains. A deletion mutation most often causes it in one or more α-globin chains. No comprehensive characterization studies have been conducted on α-thal patients in the Saudi population. Therefore, this research aims to identify the spectrum of genetic mutations responsible for α-thal in our region. Individuals with microcytic, hypochromic red blood cells and normal hemoglobin (Hb) A2 were enrolled. Sixty samples of individuals suspected of α-thal were selected for further genetic analysis. Multiplex ligation-dependent probe amplification assay was used to detect deletion mutations in α-globin genes. Among all samples tested, the $-\alpha^{3.7}$ deletion mutation was detected in 57 (95%) cases, whereas no mutation was detected in the remaining 3 (5%). In addition, 9 (15%) individuals were heterozygous for $-\alpha^{3.7}$, while $-\alpha^{3.7}$ homozygosity was found in 85% of the analyzed cases. The hematological characteristics of $\alpha^{3.7}$ subjects were significantly lower than the control group in the mean of Hb, hematocrit, mean corpuscular volume, mean corpuscular Hb, and mean corpuscular Hb concentration ($p<0.001$). These results highlight the importance of α-thal diagnosis and investigation in Saudi Arabia's pre-marital screening program for microcytic hypochromic individuals. Thus, it contributes to reducing the spread of genetic diseases.

Introduction

Thalassaemia is a worldwide public health issue. The global prevalence of thalassemia is significant, with up to 20% of the population harboring one or more α-thalassemia (α-thal) mutations and around 1.5% possessing one or more β-thalassemia mutations.[1,2] α-thal is the most prevalent hereditary hemoglobin (Hb) synthesis condition globally, particularly among Southeast Asian populations, the Mediterranean, India, and the Middle East.[3,4] In Saudi Arabia, a region where α-thal is highly prevalent, studies have shown that the incidence in some areas can reach as high as 25%.[5,6]

A normal individual typically has two pairs of α-globin genes, with two genes located on each chromosome (αα/αα).[7] α-thal syndrome is marked by diminished or missing synthesis of α-globin chains resulting from mutations or deletions in the HBA_1 and HBA_2 genes on chromosome 16. In α-thal, a deletion may eliminate one or both genes on a chromosome. The deletion mutations are the most common variant causing α-thal, which causes the deletion of one or more α-globin

chains. Therefore, it can be classified according to the number of missing or inactive α-globin genes. α^+-thal (one copy deleted or inactivated) and α^0-thal (both copies deleted or inactivated).[8,9] Depending on the number of impaired α-globin genes, the clinical symptoms of α-thal may vary from moderate anemia to severe, life-threatening disorders such as hydrops fetalis.[9,10] α-thal is defined as a silent carrier inherited as heterozygous of α^+-thal. On the other hand, α-thal is defined as an α-thal trait inherited by homozygous of α^+-thal or compound heterozygosity of α^0-thal. HbH disease is caused by compound heterozygosity for α^0/α^+-thal.

Furthermore, Hb Bart hydrops fetalis results from homozygosity for α^0-thal, characterized by significant tissue hypoxia. In the absence of medical intervention, it is incompatible with life and results in intrauterine or early neonatal mortality.[11,12] Molecular analysis is essential for accurate diagnosis, as the combination of deletion and non-deletion forms of α-thal and a combination of α^0/α^+-thal will increase the incidence of severe genetic disorders.[9]

Individuals who are heterozygous for α^+-thal (-α/αα) have low or no hematological changes, while those homozygous for α^+-thal (-α/-α) and heterozygous for α^0-thal (—/αα) show considerable microcytosis and hypochromia. The existence of a single functioning α-gene (—/-α) leads to persistent, moderate to severe hemolytic anemia, jaundice, and hepatosplenomegaly, a condition referred to as HbH.[13] Homozygosity for α^0-thal (—/—) results in Hb Bart's hydrops fetalis syndrome. Most thalassemia alleles are caused by deletions at the α-globin gene locus. Approximately 40 deletion mutations causing α-thal have been reported worldwide, such as $-\alpha^{3.7}$, $-\alpha^{4.2}$, -SEA, -FIL, -MED, $-^{20.5}$, and -THAI.[1,14-16] Furthermore, the two most common types of α^+-thal are $-\alpha^{3.7}$ and $-\alpha^{4.2}$.[12,17]

The pre-marital screening program has been introduced in Saudi Arabia since 2001. It became mandatory by 2004 to test for various genetic disorders that may be passed from parents to their offspring.[18] The pre-marital screening process undergoes tests that help diagnose hemoglobinopathies, including peripheral blood smear, complete blood count (CBC), and high-performance liquid chromatography (HPLC). The diagnosis of α-thal has been minimal and cannot be diagnosed with a simple biochemical test. It is usually followed based on the results of CBC parameters indicating the presence of hypochromic microcytic red cells accompanied by normal HbA_2 to rule out β-thalassemia and normal iron levels to exclude iron deficiency anemia. However, this is inaccurate when determining α-thal populations' frequency.[19]

A wide range of molecular techniques has been used to identify mutations and deletions within the α-globin gene cluster, including reverse dot-blot hybridization (RDB), multiplex ligation-dependent probe amplification (MLPA), gap-polymerase chain reaction (PCR), and allele-specific PCR.[20] Gap-PCR is a common technique for detecting known deletions in the α-globin gene. Deletions are detected by specific primers designed to identify a known deletion mutation. On the other hand, MLPA is a technique used to detect α-thal resulting from rearrangements, mainly any deletions involving the α-globin gene. Moreover, MLPA is a comparatively recent approach that has been applied to the multiplex detection of any deletions or duplications in α-genes.[21-24]

α-thal is a significant health issue since its carrier status cannot be identified *via* conventional screening techniques, in contrast to β-thalassaemia. In Saudi Arabia, the prevalence of the α-thal trait was higher than the β-thalassemia carrier, which is 6.3% *versus* 0.4%.[6] The comprehensive impact of thalassemia on economic and healthcare systems remains unknown; however, it is recognized as escalating, not only in regions with high prevalence where patient survival and longevity are improving but also in areas experiencing increased prevalence due to immigration and demographic shifts.[3]

Despite the high prevalence of Hb disorders in Saudi Arabia, more current knowledge needs to be gained about the spectrum of thalassemia among the Saudi population. This knowledge gap is particularly evident in the case of α-thal. Therefore, this study is crucial as it aims to investigate the spectrum of α-thal among a sample of the Saudi population and their hematologic features among individuals who undergo a pre-marital screening program in the western province of Saudi Arabia.

Materials and Methods

Subjects

The initial analysis of hematological parameters was done on individuals attending the pre-marital screening in the Jeddah regional laboratory from February 2021 to March 2023. Following the assessment based on hematological parameters, 309 samples were diagnosed with microcytic [mean corpuscular volume (MCV) <80 fL] hypochromic [mean corpuscular hemoglobin (MCH) <27 pg /mL] anemia. After the second assessment based on Hb electrophoresis and iron studies, out of the 309 samples, 60 were subjected to an α-thal study. Individuals with a history of treatment with medications that may affect blood counts during the previous three weeks, such as hydroxyurea, were excluded. In addition, blood samples were taken from 30 healthy individuals as a control group. Medication history and written informed consent were obtained from all participants at the time of the study. The ethical approval was obtained from the Ministry of Health (No. 1322). The study followed the Helsinki Declaration rules and was approved by the Research Bioethics Committee of the Center of Excellence in Genomic Medicine Research.

Laboratory analysis

All individuals undergoing pre-marital screening were analyzed for hematological indices (CBC) using XN100 and Sysmex (Sysmex KX-100, Tokyo, Japan). HPLC was performed routinely for all individuals undergoing pre-marital screening using (VARINAT II, Bio-Rad, California, USA). Further serum iron and ferritin levels were assessed using Cobas 6000 c501. The ethylenediamine tetraacetic acid (EDTA) samples of individuals suspected of α-thal were selected for further genetic analysis at King Fahad Medical Research Center. MLPA assay was used to detect deletion mutations in α-globin genes.

Molecular analysis

Multiplex ligation-dependent probe amplification

DNA from EDTA blood samples was isolated from leukocytes using a DNA Micro kit from QIAGEN (Hilden, Germany) following the kit's protocol. DNA concentration was determined by NanoDrop spectrophotometry (Thermo Scientific, Waltham, MA, USA). According to the manufacturer's instructions, the assay was performed using the SALSA MLPA kit, EK5-FAM MRC-Holland. At least three normal control

samples were used for each group of patient samples. The MLPA testing was performed as described by White *et al*. and Schouten *et al*.[22,25] Approximately 200 ng of genomic DNA in a final volume of 5-8 µL was heated for 5 min at 98°C and cooled to 25°C for 1 min. 1.5 mL of the probe mix and 1.5 mL SALSA hybridization buffer (MRC-Holland, DL, Amsterdam, Netherlands) were added to each sample, followed by heat denaturation at 95°C 1 min sand hybridization at 60°C 16-20 h. Ligation was performed by adding 32 µL of ligase-65 master mix (3 µL of ligase buffer A, 3 µL of ligase buffer B, 1 µL of ligase-65 enzyme, and 25 µL of dH2O) to the specimen DNA, which was incubated at 54°C for 15 min, followed by 95°C for 5 min. Then, the reaction was stopped by cooling to 20°C. PCR amplification was performed using 10 µL of PCR master mix (0.5 µL of SALSA polymerase, 2 µL of SALSA primer mix, 7.5 µL of dH2O) and a 40 µL ligated product. PCR conditions were as follows: 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a 20-min final extension at 72°C and a 15°C hold. A size standard [0.2 µL (LIZ) GS 500] was added to each sample. PCR products were detected and quantified by capillary electrophoresis in an ABI Prism 3500 instrument (Applied BioSystems, Waltham, MA, USA). The MRC-Coffalyser software (MRC-Holland, DL, Amsterdam, Netherlands) was used as an analysis tool for normalizing MLPA data. The expected results of allele copy numbers 0, 1, 2, 3, and 4 (normal) corresponded to probe ratios of 0, 0.25, 0.5, 0.75, or 1.0, respectively. The MLPA results for each sample were imported and analyzed using Coffalyse software.

Whole-exome sequencing

Whole-exome sequencing (WES) enables the simultaneous analysis of multiple variants, which was performed on the samples with the negative result by the MLPA test. The quality of the DNA sample was measured by fluorescence-based quantification of double-stranded DNA (dsDNA) method using Qubit dsDNA High Sensitivity Assay Kit (Cat. no. Q32854) (Qubit dsDNA High Sensitivity Assay Kit) with a quantification range 0.2-100 ng. DNA sequencing was performed on a Nextseq machine (Illumina, San Diego, CA, USA), and the analytical process was carried out by using the Illumina Pipeline software program through stages of sequencing, quality control, mapping reads to the reference genome (alignment), filtering, and trimming, sequence coverage reporting, and variant calling.

Table 1. Summary of hematological analysis of suspected α-thalassemia subjects.

Parameter	Mean±SD (range) n=60
WBC (×10³/µL)	5.9±1.8
RBC (×10⁶/µL)	5.7±0.4
Hb (g/dL)	12.06±1.1
Hct (%)	39.8±3.8
MCV (fL)	68.9±3.1
MCH (pg)	20.8±0.9
MCHC (g/dL)	30.28± 0.4
RDW	14.9±1.08
PLT (×103/µL)	305±64.43
HbA (%)	97.18±0.52
HbA_2 (%)	2.5±0.3

SD, standard deviation; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Hct, hematocrit test; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count.

Statistical analysis

Analysis of variance was used to determine the correlation between genotype and Hb level. The Mann-Whitney test and *t*-test were used to compare the scores of means. Moreover, categorical variables were compared using the Chi-squared test. Statistical analysis was carried out using SPSS version 19. A p-value less than 0.05 was considered statistically significant.

Results

Study population

The frequency of α-thal among our cohort of 309 samples was 19.4%. Their hematological data is summarized below in Table 1. The female-to-male sex ratio was estimated at (14:6), and the median age was 28.5.

Multiplex ligation-dependent probe amplification analysis for the detection of deletional forms of α-thalassemia

A deletional mutation was detected in 57 (95%) out of 60 samples suspected with α-thal, while 3 (5%) were negative for deletion mutations. All 57 positive subjects with α-globin deletion showed an $-\alpha^{3.7}$ allele deletion mutation. Out of (57) $-\alpha^{3.7}$ positive cases, 9(16%) individuals were heterozygous $-\alpha^{3.7}$, while $-\alpha^{3.7}$ homozygosity was found in 48(84 %) of positive cases (Figure 1).

The whole-exome sequencing analysis for the detection of non-deletional variants

The remaining samples that showed no deletion mutations in the α-globin genes were subjected to further investigations using WES. No significant variants related to anemia or α-thal were detected. The five most predicted variants associated with disease symptoms are summarized in (Table 2).

Hematological parameters analysis

The hematological features of α-thal subjects were compared with the mean of 30 control samples. The red blood cells (RBC) counts in the α-thal samples were lower than in the control group ($p<0.01$). In addition, the red cell distribution width (RDW) value was significantly lower among the α-thal subjects than in the control group ($p<0.001$). However, comparing other hematological parameters such as Hb, hematocrit test (Hct), MCV, MCH, and mean corpuscular Hb concentration (MCHC) between the α-thal and control samples showed a significant decrease in the α-thal group ($p<0.001$). The HbA level was significantly lower in the α-thal group compared to the control group at ($p<0.05$) (Table 3). There was no significant difference among $-\alpha^{3.7}$ homozygous and $-\alpha^{3.7}$ heterozygous in the mean of RBC, Hct, Hb, MCV, MCH, MCHC, RDW, platelets count, HbA and HbA_2 level. White blood cell count was significantly lower in $-\alpha^{3.7}$ homozygous cases than in $-\alpha^{3.7}$ heterozygous subjects ($p<0.05$) (Table 4).

Table 2. Variants of unknown significance identified by whole-exome sequencing.

Candidate gene	Position in transcript	Mutations (%) Zygosity	Consequence
SPTA1	NM_003126.2	Heterozygous	Splice region variant, intron variant
MTR	NM_000254.2	Homozygous	missense variant
BRCA1	NM_007294.3	Heterozygous	missense variant
STAT1	NM_007315.3	Heterozygous	intron variant
PLEKHM1	NM_014798.2	Heterozygous	3_prime_UTR_variant

Table 3. Hematological characteristics of the α-thal and control group.

Parameter	α-thal group (n=60) Mean±SD	Control (n=30) Mean±SD	p
WBC ($\times 10^9$/L)	5.85±1.88	6.4±1.7	0.19
RBC ($\times 10^{12}$/L)	5.78±0.4	5.4±0.4	0.005
Hb (g/dL)	12.08±1.18	15.3±0.8	<0.001
Hematocrit (%)	39.9±3.9	46.8±2.1	<0.001
MCV (fL)	69.0±3.2	87.3±4.8	<0.001
MCH (pg)	20.89±1.01	28.03±2.7	<0.001
MCHC (g/dL)	30.26±0.48	32.7±0.63	<0.001
RDW (%)	14.9±1.11	12.7±1.3	<0.001
Platelets (×109/L)	308.8±65.8	288.2±95.6	0.022
HbA (%)	97.2±0.51	97.4±0.61	0.025
HbA_2 (%)	2.5±0.31	2.5±0.61	0.024

α-thal, α-thalassemia; SD, standard deviation; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

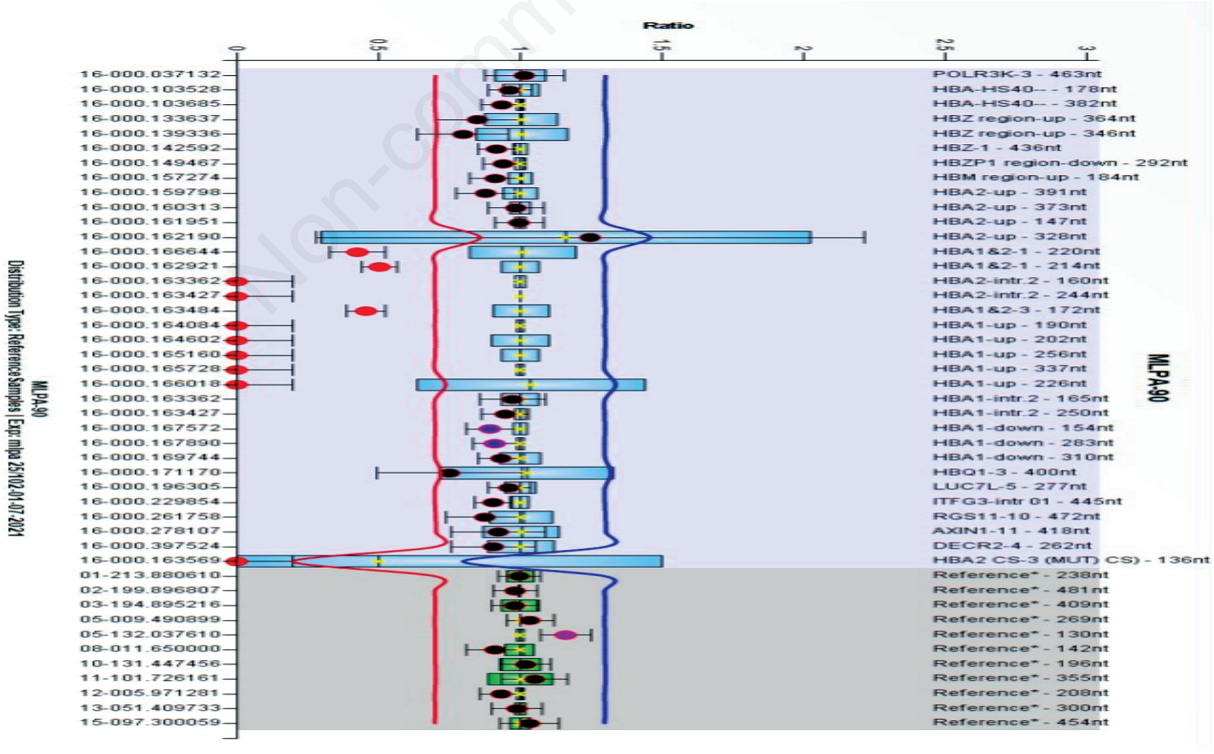

Figure 1. Determination of deleted region in *HbA* gene. In -$\alpha^{3.7}$ allele deletion, 3.7 kb deleted regions of the *HbA* gene, deletion removes the 3' portion of the HBA_2 and the 5' portion of the HBA_1 gene.

Table 4. Hematological characteristics of α- thalassemia subjects according to their genotype.

Parameter	Homozygous (n=48) Mean±SD	Heterozygous (n=9) Mean±SD	p
WBC (×10^9/L)	5.42±1.37	8.21±2.8	0.01
RBC (×10^{12}/L)	5.8±0.42	5.64±0.21	0.42
Hb (g/dL)	12.2±1.18	11.3±1.1	0.23
Hematocrit (%)	40.3±3.95	37.9±3.81	0.33
MCV (fL)	69.2±2.22	67.2±7.16	0.31
MCH (pg)	21.0±0.72	20.1±2.05	0.16
MCHC (g/dL)	30.3±0.5	29.9±0.2	0.25
RDW (%)	14.9±1.08	14.7±1.5	0.78
Platelets (×109/L)	305.5±71.7	326.3±6.65	0.55
HbA (%)	97.1±0.52	97.7±0.057	0.06
HbA_2 (%)	2.6±0.32	2.3±0.057	0.06

SD, standard deviation; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

Discussion

α-thal typically results from deletions involving the HbA_1 and HbA_2 genes. However, several non-deleted mutations in the *HbA* genes that cause α-thal have been identified worldwide.[26] Moreover, α-thal is a challenge for genetic counselors advising couples suspected of being at risk without knowing their genotype. There is limited published information on the molecular basis of α-thal in the Saudi population. This study investigated the spectrum of genetic mutations responsible for α-thal in our region. The molecular characteristics of α-thal have been studied in different Arab countries. Identifying the most common mutations in each population greatly facilitates molecular genetic testing. This is the first study in Jeddah to determine the spectrum of α-thal mutations and their hematologic features among individuals who visited pre-marital screening centers.

The complex molecular basis of α-thal has presented a challenge for molecular diagnostics. Previously, diagnostic tools such as quantitative real-time PCR, multiplex PCR, and RDB were frequently employed using PCR-based methodologies.[22] MLPA has recently become an effective method for mutation analysis of α-thal.[27] Colosimo *et al*. found MLPA an efficient technique for screening α-thal variants.[23] They highlighted the obstacles of RDB and proposed MLPA as a suitable alternative to the more time-consuming and labor-intensive techniques like Southern blot or Gap PCR. Another study reported that MLPA results showed undetected deletions in the RDB kit.[28]

The frequency of α-thal carriers in Arab countries ranged from 1% to 58%.[18] In the current study, a deletion mutation in the *HbA* gene was demonstrated in 95% (57) of suspected α-thal subjects. The $-\alpha^{3.7}$ deletion is a worldwide widespread mutation among all ethnic groups. The prevalence of the $-\alpha^{3.7}$ deletion mutation among the Saudi population is estimated to be 43.3% and 64%.[5,29-31] Moreover, its prevalence in surrounding countries is 58.3% in Oman,[32] 68.6% in the Emirates,[33] and 32.0% in Bahrain.[34] However, the most prevalent mutation reported in Kuwait is $polyA_1$, with a prevalence of 86.7%.[35] In this study, only the $-\alpha^{3.7}$ deletion mutation was identified. This result is consistent with previous studies in Saudi Arabia.[5,36] Moreover, a study conducted in Al-Ahsa and Qatif in the Eastern Province of Saudi Arabia reported that $-\alpha^{3.7}$ deletion mutation was found in 21.7% of transfusion-dependent β-thalassemia patients.[14] A high prevalence of $-\alpha^{3.7}$ mutations was observed in 43% of the studied population in Jordan.[37]

Several mutations have been identified among Saudis, including -MED (2.6%), -FIL (6.4%), $-\alpha^{4.2}$ (3.8%), and $-\alpha^{20.5}$ (8.9%),[5,14,38] but these mutations were not detected in our subjects. This may be due to the low frequency of these mutations, the limited number of samples, or the focus of the study on a particular region. The results of the current study showed that the homozygosity for α-thal was high. About 80% of individuals suspected of having α-thal were $-\alpha^{3.7}$ homozygous, and 15% were $-\alpha^{3.7}$ heterozygous. Similar to our findings, a study conducted in the Jazan region reported that 57% of the studied population were $-\alpha^{3.7}$ homozygous.[36]

α-thal typically results from deletions involving the HbA_1 and HbA_2 genes. However, several non-deleted mutations in the *HbA* genes that cause α-thal have been identified worldwide.[26] Furthermore, a single point mutation in the *ATR* gene leads to an α-thal-like phenotype.[39] In this study, no significant variants related to anemia or α-thal were detected in the samples that showed no deletion mutations using the MLPA technique (results not shown). The condition of a negative sample could be the silent type of β-thalassemia or carry another unknown mutation. A non-deletion of $\alpha^{PA\text{-}1}$ (c.*94A>G) was more common among Kuwaitis (33.3%).[40] In addition, a compound heterozygous for $\alpha^{-3.7}/\alpha^{PA\text{-}1}$ was detected in 20.5% of the studied population in Kuwait City.[40] It has been reported that the polyA mutation is also thought to be shared among Saudis (41%).[31] Moreover, the polyA mutation was previously identified among Saudis in 2.6% of the studied population. However, it was not identified in our negative samples.[14]

Frequently, $-\alpha^{3.7}$ is associated with mild anemia and a slight decrease in Hb levels without clinical symptoms. Our results showed that not all thalassemia traits were related to mild hematological phenotypes. There was no correlation between MCV, MCH values, and the number of functional α-globin genes. A significantly lower WBC count was observed with decreasing α-genes (*t*-test, $p<0.05$). However, MCV and MCH are still poor predictors of the $-\alpha^{3.7}$ genotype. In contrast

to our study, Akhavan-Niaki *et al.* showed that MCV and MCH values were significantly lower in -$\alpha^{3.7}$ homozygous patients than in -$\alpha^{3.7}$ heterozygous patients.[41]

Corpuscular indices obtained from hematological counters may serve as valuable prediction indicators for the number of deleted α-genes. It has been reported that one α^0 allele should be suspected with MCV<70.80 fL and MCH<21.90 pg.[42] In this study, the analysis of differences in the hematological parameters between the α-thal and the control groups refers to a decrease in Hb, hematocrit, MCV, MCH, and MCH values in the α-thal group compared to the control group. Moreover, a significantly higher percentage of RDW was observed in the α-thal carrier compared to the control group. These findings were similar to those obtained from the study by Akhavan-Niaki *et al.* They reported that the MCV and MCH values were significantly decreased in α-thal carriers. This was useful for selecting suspected α-thal carriers based on hematological features.[41,42]

α-thal with $\alpha^{3.7}$ deletions usually has no clinical effect. However, the compound heterozygosity of this mutation with α^0-thal can lead to HbH disease. Health education, molecular screening, and genetic counseling are recommended to reduce the consequences of homozygosity resulting from inbreeding, especially when severe mutations in alleles predominate among affected families.[43]

The small number of patients in our cohort is one of the limitations of our study, and there certainly is a need for studies in larger populations.

Conclusions

The most common α-gene abnormality in our cohort is $\alpha^{3.7}$ deletions among microcytic hypochromic Saudis in Jeddah. Routine screening for common α-thal mutations should be considered among microcytic hypochromic in pre-marital screening programs in Saudi Arabia. α-thal is common in Saudi Arabia. Cultural traditions and the increased prevalence of consanguineous marriages make it crucial to investigate mutation types and identify α-thal carriers to improve prevention and therapy.

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