Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 20/10/2024;
Date Revised: 05/01/2025;
Available Online: 22/03/2025;
Date Updated: 31/08/2025

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How to Cite:
Saqer LS, Hassan FM, Sharif FA (2025). α-globin gene 20.5kb deletion and triplication mutations among Palestinian patients with microcytic hypochromic anemia. Adv. Life Sci. 12(2): 340-345.

Keywords:
α-thalassemia; $-\alpha^{20.5}$ deletion; $\alpha\alpha\alpha^{anti3.7}$ triplication; Multiplex-PCR; Gaza Strip

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α-globin gene 20.5kb deletion and triplication mutations among Palestinian patients with microcytic hypochromic anemia

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Abstract

Background: α^0-thalassemias results from double gene deletions of the α-genes on the same chromosome as in $(-\alpha)^{20.5}$. In contrast to deletion, α-globin genes triplication ($\alpha\alpha\alpha^{anti3.7}$) is generated by homologous recombination during the process of crossover. This study was conducted to detect $(-\alpha)^{20.5}$ and $\alpha\alpha\alpha^{anti3.7}$ mutations in patients with microcytic hypochromic anemia in Gaza Strip-Palestine.

Methods: 200 subjects with microcytic hypochromic anemia with an age range of 18 to 48 years, were recruited from hematological departments of the main hospitals in Gaza Strip. The study subjects were those who underwent premarital β-thalassemia carrier screening, and their results were negative. Iron deficiency was excluded through measurement of serum iron and total iron binding capacity (TIBC). Complete blood count was done automatically. Molecular detection of $(-\alpha)^{20.5}$ and $\alpha\alpha\alpha^{anti3.7}$ mutations were performed by Multiplex-PCR.

Results: Sixty-six (33%) of the study participants proved to have α-thalassemia. The frequency of $(-\alpha)^{20.5}$ and $\alpha\alpha\alpha^{anti3.7}$ mutations were: 13.25% and 5.5%, respectively. Four genotypes were detected: $-\alpha^{20.5}/\alpha\alpha$ *accounted* for 26% (52/200), $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$ for 2.5% (5/200), $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$ for 4% (8/200) and $\alpha\alpha\alpha^{anti3.7}/-\alpha^{20.5}$ for 0.5% (1/200). The comparison of hematological parameters between $-\alpha^{20.5}/\alpha\alpha$ and $\alpha\alpha\alpha^{anti3.7}$ mutation carriers ($\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$ and $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$) revealed, though not statistically significant, higher levels in carriers of $\alpha\alpha\alpha^{anti3.7}$ mutation. The $-\alpha^{20.5}/\alpha\alpha\alpha^{anti3.7}$ genotype was observed in one case.

Conclusion: The rate of $-\alpha^{20.5}$ and $\alpha\alpha\alpha^{anti3.7}$ in the Palestinian population is high as compared to neighboring countries. Conducting further molecular testing to detect additional α-thalassemia mutations is needed in order to obtain a clearer picture of the genetic nature of this disease.

Introduction

α^0-thalassemia deletion is caused by complete or partial deletion of both α-genes in cis thus eliminating the dictated α-chain synthesis. Homozygotes for such deletions have the Hb Bart's Hydrops Fetalis Syndrome. There are about 20 α^0 thalassemia deletions that tend to be frequent in various populations, four of which are more commonly found: $--^{Med}$ (Mediterranean) and $(-\alpha)^{20.5}$ deletions in Mediterranean populations, and $--^{SEA}$ (South Asia) and $--^{FIL}$ (Filipino) in Southeast Asia. The breakpoints of the common α^0 thalassemia deletions are caused by non-homologous recombination [1]. $-(\alpha)^{20.5}$ deletion, an α^0-deletion commonly found in the Mediterranean and Central Asian populations. This deletion is reported to span 20.5 kb on the α-globin gene cluster, the 5' and 3' breakpoints are localized within an *Alu* region between the *HBZ* and *HBZP1* genes and the *HBA1* gene, respectively [2].

$\alpha^{3.7}$ triplication is an increase in α gene copies that occur on one of the chromosomes. $\alpha^{3.7}$ triplication is generated in a process called unequal crossover during the recombination of α1 and α2 genes, which creates the $\alpha\alpha\alpha^{anti3.7}$ triplicated allele [3]. The mutual $\alpha\alpha\alpha^{anti3.7}$ allele of this unequal crossover consequently contains three α- globin genes because of the addition of a hybrid α-globin gene consisting of the 5′ portion of *HBA1* and the 3′ portion of *HBA2*. Because a single-gene deletion and its corresponding triplication are mutual products of unequal crossover, equal occurrences of these alleles would be probable under the assumption of evolutionary neutrality for both alleles. In sperm, a high rate of mutual unequal crossover of the α-globin genes has been recognized, with equal occurrences of the $\alpha^{3.7}$ deletion and $\alpha\alpha\alpha^{anti3.7}$ triplication [4]. However, between these mutual alleles, the $\alpha^{3.7}$ chromosomes are more common than the $\alpha\alpha\alpha^{anti3.7}$ chromosomes, given a strong indication of positive environmental selection for the single-α-globin gene deletion in certain populations [5,6]. To our knowledge, this is the first study in the Gaza Strip investigating the *α-globin* gene mutations. In this study, multiplex PCR was used to identify the α-thalassemia 20.5 double deletion and $\alpha\alpha\alpha^{3.7}$ triplication mutations among Palestinian patients suffering from microcytic hypochromic anemia.

Methods

The current analytical cross-sectional study employed two hundred microcytic hypochromic anaemic patients with MCV <80fL and/or MCH <27 pg collected from three hospitals in Gaza Strip: Al-Shifa, Gaza-European and Nasser Medical complex. All participants underwent β-thalassemia carrier screening premarital, and their results were negative. To exclude iron deficiency anemia; serum iron and TIBC were measured. Complete blood count was performed by an automated cell counter (XT1800i, Sysmex, Japan). All data were analyzed by SPSS version 20 using Independent Samples t-test and ANOVA for comparison of the hematological data. P value < 0.05 was considered statistically significant. The experimental work was conducted at the Labs of Medical Science department at the University College of Science and Technology - Gaza Strip, Palestine.

Ethical Considerations

An authorization to carry out the study was obtained from the Palestinian Ministry of Health and the Helsinki committee. In addition, all the subjects involved in this study gave their oral consent to participate in the study.

Detection of the α thalassemia mutations

DNA was isolated from fresh EDTA whole blood cells by using Promega kit for human DNA isolation (Promega, USA). The quality and concentration of isolated DNA were determined by using Nanodrop spectrophotometer (IMPLEN-USA). One µl of the isolated DNA of each sample was used for this purpose. Multiplex-PCR was performed using the primers listed in Table 1.

Two micro-tubes were used: for reaction I $-(\alpha)^{20.5}$ multiplex PCR [7]: we used 15 µL master mix (Bioline), 2 µL deionized water, 1 µL DNA template, and 0.2 µL of each primer listed in Table 1 for α^o multiplex PCR (2 pmol) in one micro-tube (0.2 ml) and were mixed. In the second micro-tube used for reaction II- $\alpha^{anti3.7}$multiplex PCR [8], 7 µL master mix (Bioline), 2 µL deionized water, 1 µL DNA template, and 0.3 µL of forward primer (3 pmol) and 0.1 µL of each reverse primer in one micro-tube (0.2 ml) were mixed. Amplification of a large (2.5 kilobase) segment of the *LIS1* gene 3'UTR (the *LIS1* gene at 17p13.3) was included as internal control for monitoring the success amplification of reaction I. The sequence of primers used annealing temperature, and the PCR amplicons sizes are demonstrated in table1.

The thermal cycler (BECO-Germany) program was set as follows: initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 61.5 °C (reaction I) or 67.8 °C (reaction II) for 4 min, followed by an extension step at 72 °C for 4 min, and a final elongation at 72 °C for 5 min terminated the PCR reactions. A 1.5% agarose gel stained with ethidium bromide was used to detect the PCR products and visualized on a UV transilluminator. We estimated the PCR products sizes by comparison with 1kb ladder DNA run on the same gel.

Name	Sequence (5' to 3')	Product size	Annealing T°
Reaction I: -(α)$^{20.5}$ **multiplex PCR**			
LIS1	**LIS1(F)**: GTCGTCACTGGCAGCGTAGATC	2503	61.5 °C
	LIS1(R): GATTCCAGGTTGTAGACGGACTG		
-(α) $^{20.5}$	**20.5 (F)**: GCCCAACATCCGGAGTACATG	1007	
	3.7/20.5 (R): AAAGCACTCTAGGGTCCAGCG		
α2 gene	**α2/3.7(F)**: CCCCTCGCCAAGTCCACCC	1800	
	α2 (R): AGACCAGGAAGGGCCGGTG		
Reaction II: α $^{anti3.7}$ **multiplex PCR**			
Normal αα "α2 gene"	**3.7(F)**: AAGTCCACCCCTTCCTTCCTCACC	2217	67.8 °C
	3.7(R1): ATGAGAGAAATGTTCTGGCACCTGCACTTG		
Normal αα"α1 gene"	**3.7(F)**: AAGTCCACCCCTTCCTTCCTCACC	2213	
	3.7(R2): TCCATCCCCTCCTCCCGCCCCTGCCTTTTC		
α $^{anti3.7}$	**3.7(F)**: AAGTCCACCCCTTCCTTCCTCACC	2440	
	3.7(R1): ATGAGAGAAATGTTCTGGCACCTGCACTTG		

Table 1: The sequence of PCR primers used to detect -(α)$^{20.5}$ and α $^{anti3.7}$ by Multiplex–PCR. The table demonstrates the product size and annealing temperature for each reaction.

Results

The study population consisted of 200 participants: 106 were males and 94 females, the participant's age ranged from 18 to 48 with an average of 30.35±9.6 years. All of the participants were microcytic hypochromic anemic patients with MCV: 74.9fl ±4.4 and/or MCH 25.16 pg ±1.8. Iron deficiency was excluded as a cause of their anemia by measuring their serum iron and TIBC. All the subjects underwent premarital carrier screening for β-thalassemia and their results came out negative. Among the 200 participants, 66 (33%) proved to be carriers for α-thalassemia based on the molecular analysis. An assessment of the hematological parameters among the mutation carriers and non-carriers are summarized in Table 2. The comparison showed no significant difference (p> 0.05) in terms of MCV, hemoglobin, RBCs, and RDW, regardless of gender. But the difference in the mean MCHC reached significance in α- thalassemia mutation carriers when compared with non-carriers with p-values: 0.05 and 0.003 for females and males, respectively.

Hematological parameters	Group			
	Non-carriers (No.)		α- Thal. mutation carriers	
	Male (76)	Female (58)	Male (30)	Female (36)
Hb	12.3±1.6	11.76±1.03	12.9±1.1	11.66±1.1
p-value			0.12	0.64
MCV	74.86±4.79	75.27±4.6	74.27±4.07	74.99±4.04
p-value			0.55	0.76
MCH	25.17±2.02	25.13±1.95	25.7±1.5	24.75±1.48
p-value			0.19	0.31
RBCs	4.90±0.60	4.68±0.44	4.96±0.54	4.64±0.50
p-value			0.65	0.66
MCHC	33.62±1.55	33.41±1.85	34.57±1.13	32.66±1.7
p-value			**0.003**	**0.05**
RDW	13.83±1.52	13.83±1.3	13.7±1.2	14.02±1.1
p-value			0.73	0.48

Table 2: The comparison between α-thalassemia mutation carriers and non-carriers according to Hematological parameters.
Hb: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, RBCs: red blood cells, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width. *p-values from Student's *t*-test for independent samples.

Of the two hundred DNA samples analyzed, 33% have been observed to be carriers of α-gene defect. Based on the molecular investigation, the mutations of interest ($-\alpha^{20.5}$) deletion and triplicated ($\alpha\alpha\alpha^{anti3.7}$) were observed. The mutant alleles represented 18.75% of total α- *globin* alleles (mutant and normal alleles). The frequency of $-\alpha^{20.5}$ mutation was (13.25%) while $\alpha\alpha\alpha^{anti3.7}$ (5.5%) Table (3). The frequencies of the $-\alpha^{20.5}$ deletion was higher in female (7.25%) than in male (6.0%) subjects. The frequencies of $\alpha\alpha\alpha^{anti3.7}$ triplication, however, were the same in both genders (2.75%).

Among the α-thalassemia gene mutation carriers, four different α-globin genotypes were identified: $-\alpha^{20.5}/\alpha\alpha$ (26%), $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$ (2.5%), $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$ (4%) and $\alpha\alpha\alpha^{anti3.7}/-\alpha^{20.5}$(0.5%).

According to the results shown in table 4, the highest levels of RBCs and Hb were found in the $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$genotype [mean±SD:5.02±0.36 and 13.03±1.57], respectively. Whereas the lowest levels of MCV and MCH were seen in the -α$^{20.5}$/αα (MCV:74.16fl and MCH: 25.04pg).As for $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$genotype the level of MCV was slightly decreased but the mean of RBCs, Hb and MCH levels were higher than in $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$ genotype; however, when combined with $-\alpha^{20.5}$ deletion (seen in one case) the levels of MCV and MCH were increased while the Hb and RBCs decreased (Table 4).

The rate of mutation occurrence in females was higher than that in males: 36/66 women (54.54%) and 30/66 men (45.45%). In the heterozygous group: 26/30 men (86.67%) and 32/36 women (88.88%), whereas 4/30 men (13.33%) and 4/36 women (11.11%) were in the homozygous group.

However, the combination $-\alpha^{20.5}$ and $\alpha^{anti3.7}$ was observed in only one male. Regarding the $-\alpha^{20.5}$ mutation; we observed that MCH levels and RDW were almost equal in the $-\alpha^{20.5}/\alpha\alpha$ genotype when compared with normal genotype, however the mean of MCV levels and RBCs count were lower in $-\alpha^{20.5}/\alpha\alpha$ genotype ; but these differences did not reach statistical significance. In contrast all the hematological parameters, except MCH and RDW, were elevated in the $\alpha\alpha\alpha^{anti3.7}$ homozygotes as compared to the heterozygotes.

The highest levels of Hb, RBCs, MCV and MCH were observed in the $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$ and the $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$ genotypes.

Discussion

The most frequent mutation in our population $-\alpha^{20.5}$ was observed in 53 out of 200 patients and represented a frequency of 26.5%. This mutation is not often reported in Arab countries, the Middle East, and Asia. It was reported to be the less frequent deletion in the studies conducted by Mesbah-Amroun et al., [9] in Algeria (2008) with 6.5%, 8.9% in Saudi population [10] and 9% in the United Arab Emirates [11].

Gender	Allele Frequency (%)			Genotype (%)					Total (%)
	αα	$-\alpha^{20.5}$	$\alpha^{anti3.7}$	αα/αα	$-\alpha^{20.5}/\alpha\alpha$	$\alpha^{anti3.7}/\alpha\alpha$	$\alpha^{anti3.7}/\alpha^{anti3.7}$	$\alpha^{anti3.7}/-\alpha^{20.5}$	
Male	177(44.25)	24(6.0)	11(2.75)	76(38)	23(11.5)	2(1)	4(2)	1(0.5)	106(53)
Female	148(37)	29(7.25)	11(2.75)	58(29)	29(14.5)	3(1.5)	4(2)	-	94(47)
Total	325(81.25)	53(13.25)	22(5.5)	134(67)	52(26)	5(2.5)	8(4)	1(0.5)	200

Table 3: The distribution of α-Globin alleles and genotype distribution among gender: male and female

Genotype	Freq %	RBCs	Hb	MCV	MCH	MCHC	RDW
αα/αα	67	4.81±0.55	12.09±1.44	75.04±4.7	25.15±1.98	33.53±1.68	13.83±1.43
$-\alpha^{20.5}/\alpha\alpha$	26	4.75±0.55	12.09±1.37	74.16±3.94	25.04±1.51	33.49±1.64	13.93±1.24
$\alpha^{anti3.7}/\alpha\alpha$	2.5	4.88±0.66	12.24±1.37	76.68±6.29	25.14±1.27	32.94±2.47	14.4±1.12
$\alpha^{anti3.7}/\alpha^{anti3.7}$	4	5.02±0.36	13.03±1.57	76.29±2.59	25.89±1.84	33.95±2.17	13.29±0.80
$\alpha^{anti3.7}/-\alpha^{20.5}$	0.5	4.12	11.3	78	27.4	35.2	13.5

Table 4: The prevalence of α-globin genotypes among the study population and the main value hematological parameters of each genotype.

Population	Individuals studied	Genotype frequency of $\alpha\alpha\alpha^{anti3.7}$ (%)	References
Gaza Strip/Palestine	200	14(7%)	Present study
Jerusalem /Palestine	73	1(2.1%)	[29]
Lebanon	70	1(1.4%)	[15]
Jordan	286	2(1%)	[16]
Israel	232	13(5.5%)	[32]
Oman	634	3 (0.47%)	[30]
North Morocco	1658	2(0.06%)	[31]
Turkey	78	15 (9.6%)	[21]
Iran	6946	49(1.1%)	[33]
Cyprus	485	(1%)	[34]
Indian	1253	15 (1.1%)	[35]

Table 5: The comparison demonstrates the difference in the frequency of the $\alpha\alpha\alpha^{anti3.7}$ allele in our population and some countries.

This mutation was detected in Sulaimani region in Iraq by Amin et al[12] (9.8%) but was not detected in Erbil province [13] in contrast Al-Allawi et al [14] identified this mutation by 2.9% in Northeastern Iraq. On the other hand, this mutation was not observed in Lebanon, Jordan or Tunisia in the studies conducted by Farra et al.[15], Qaddoumi et al [16] and Zorai et al.[17] respectively.

Pouranfard et al. [18] reported the $-\alpha^{20.5}$ mutation in 0.6% in a cross-sectional study carried out in a province located southwest of Iran. Our results differ from those reported from other neighboring Mediterranean such as Greece (12.7%), Cyprus (8.3%) and Turkey (5.8%) [19-21]. The high frequency of this mutation in our population may be due to the frequent consanguineous marriage.

In our study, the $-\alpha^{20.5}$ deletion was found in fifty-two cases as $-\alpha^{20.5}/\alpha\alpha$ and one case was compound heterozygous with the $\alpha\alpha\alpha^{anti3.7}$ triplicated allele.*α-globin* gene triplication is, actually, an increase in α-genes that occurs on one of the chromosomes. α-gene triplication ($\alpha\alpha\alpha^{anti3.7}$) mechanism is an uneven crossover during the recombination of α1 and α2 gene [22-25]. If the crossover occurs between the homologous Z2 and Z1 boxes, also called a "rightward crossover", then $-\alpha^{3.7}$ single-gene deletion allele and the mutual $\alpha\alpha\alpha^{anti3.7}$ triplicated allele result.

There are various studies which displayed the frequency of the *α-globin* gene triplication in healthy subjects and thalassemia patients. The incidence of the *α-globin* gene triplication is diverse, and it is reliant on the prevalence of thalassemia disease in the studied countries [26]. There are two types of triplicated α-globin genes: $\alpha\alpha\alpha^{anti3.7}$ and $\alpha\alpha\alpha^{anti4.2}$ [27,28]. The $\alpha\alpha\alpha^{anti4.2}$ is usually detected in Asians whereas the $\alpha\alpha\alpha^{anti3.7}$ is predominant in Africans, Middle Eastern, and Mediterranean populations [26].In our study, we found heterozygous triplications $\alpha\alpha\alpha^{anti3.7}$ in 5 cases and homozygous triplication in 8 cases and one subject carried a $\alpha\alpha\alpha^{anti3.7}$ allele and the $-\alpha^{20.5}$allele (genotype: $\alpha\alpha\alpha^{anti3.7}/-\alpha^{20.5}$). The $\alpha\alpha\alpha^{anti3.7}$ allele accounted for 5.5% of the total α-globin alleles (22/400). All 14 carriers of the triplicated α-globin genes including the $\alpha\alpha\alpha^{anti3.7}/-\alpha^{20.5}$ case did not present any clinical manifestations except microcytic hypochromic anemia at the time of examination. The blood parameters including the RBCs, Hb, MCV, MCH, MCHC and RDW were measured and statistically analyzed through ANOVA.

No significant parameter difference was identified among the different genotypes in comparison with the non-carrier subjects ($p > 0.05$) (data not shown). We observed that the higher mean of Hb level and RBCs was in $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$ genotype among the three different genotypes. $\alpha\alpha\alpha^{anti3.7}/-\alpha^{20.5}$genotype has the lowest Hb level (11.3g/dl) and RBCs count beside the highest value of MCV and MCH among the 3 genotypes. It appears that the extra α-gene in the case of the $\alpha\alpha\alpha^{anti3.7}$ triplication mutation replaces the decrease caused by the deletion of the *α-globin* gene due to the $-\alpha^{20.5}$ mutation.

In our study fourteen cases were found to have $\alpha\alpha\alpha^{anti3.7}$triplication (29.33% of mutant chromosome:22/75) while this mutation was reported in 2.7 % of mutant chromosomes in a study conducted by Hamayel et al. in Jerusalem/Palestine[29]. Various studies identified the $\alpha\alpha\alpha^{anti3.7}$triplication: Lebanon

[15], Jordan [16], Oman [30], North Morocco [31], Turkey [21] and others (Table 5).

It should be noted that the frequency of the α-thalassemia carriers should be higher in Gaza Strip because in this study only microcytosis and hypochromia cases were included in this study, so the prevalence of the α-thalassemia carriers might be underestimated and only two mutations have been investigated in our study population.

Still, the $-\alpha^{20.5}$ deletion with a frequency of 13.25% and the $\alpha\alpha\alpha^{anti3.7}$ triplication with a frequency of 5.5% constitute important α-thalassemia mutations in the investigated population and should be considered when investigating microcytic hypochromic patients.

Author Contributions

Lamiaa Sobhi Saqer carried out the experiment and wrote the manuscript. FathElrahman Mahdi Hassan and Fadel Akram Sharif verified the analytical methods and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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