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Distribution of blood groups with β-thalassemia mutations in Iraqi patients, 2024

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Abstract: - β-thalassemia the furthermost widespread hereditary hematological condition, is brought on by decreased or missing ß globin chain production, which results in hemolysis and impairs erythropoiesis. It affects millions of people worldwide, including those living in developed and developing nations. Aim of research is study the distribution and relationship of blood types with β-thalassemia, in addition we aim to investigate the relationship, affinity and genetic predisposition between blood types and β-thalassemia. The retrospective study was done in Iraq which including 200 patients of β-thalassemia were examined for blood types test and molecular analysis for determine the various mutations. Our results showed that β- thalassemia in female (51%) more than male (49%) and blood groups was showed statistically high significant with gender, in female predominant blood group was O +Ve followed by B +Ve while in male was A +Ve followed by O +Ve. In addition to age of patients appeared significant differences with β-thalassemia the high rate was in aged group betwe en (18-25 years). Different mutations were detected in this study: IVS 2.1[G>A] (30%) was predominant mutation in patients with blood group O +Ve (30%) followed by 25 % in A +Ve, IVS 1.110 [G>A] (17%), IVS 1.6 [T>C] was 12%, IVS 1.5 [G>C] 8%, in addition to other mutations. In our research may be found some relation between blood group and β-thalassemia, which appeared high percentage in female and in young people in patients with blood group O +Ve.

Keywords: β- thalassemia, Blood groups, Gender, Age, β-Th Mutations.

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Introduction

Thalassemia is defines as the unusual generation or reduction in the rate of development of normal α- or β-globin subunits of hemoglobin (Hb). Chromosome 11 contains the genes that produce β-globin, whereas chromosome 16 has the genes that produce α-globin (1).

Beta-thalassemia is described by the absence or reduction in the rate of production of the β-globin chain (2). It was the first time defined by Cooley and Lee in 1925 (3). A quantitative disorder of β-globin production, β-thalassemia (βT) is defined by either diminished (β +) or absent (β 0) synthesis of normal hemoglobin A β-globin chains. Despite the fact that β-thalassemia was shown to be a historical illness (4).

Phenotypically there are three forms of ß-thalassemia. In mild to moderate microcytic anemia, ß-thalassemia minor is a heterozygous state characterized by a 50% reduction in ß-globin protein synthesis. Most affected people don't have any symptoms. Mild to moderate anemia is a symptom of ß-thalassemia intermedia (5). In most circumstances, blood transfusions are not necessary. The most severe kind, ß-thalassemia major, results in poor growth, delayed sexual maturation, skeletal deformities throughout infancy, and hemolytic anemia (6). βThalassemia is frequent in 60 nations, but it is most common in South Asia, particularly India (7) and Pakistan (8). An estimated 60,000 persons worldwide, mostly in the developing world, are predicted to have thalassemia annually. Every year, anywhere in the world (9). Iraq currently has an estimated total of 13390 registered cases of thalassemia, resulting in a prevalence of 3.4/10000. (10). According to a study conducted in Bagdad, 78.8% of the patients reported having related parents, meaning that roughly 66.0% of the patients were under the age of fifteen. Of all hemoglobinopathies, thalassemia accounted for 75% of cases in Iraq. In Basra province, the greatest prevalence was

noted, where 67% of all cases of β-thalassemia major were found thalassemia kinds (11).

In Najaf, made study that revealed that the most common allele in BT was the (Cod-88) mutation, accounting for 21% of the thalassemic samples analyzed in this urban area. Following closely behind were Cod 15 and Cod 8, with prevalence rates of 18% and 12%, respectively. (12). Blood groups are hereditary and are determined by the presence of surface antigens in red blood cells, playing a crucial role in transfusions. The ABO blood group system was discovered by Karl Landsteiner in 1900, marking a significant milestone in the history of blood transfusion. This was followed by the discovery of the Rh (D) antigen. (13)

Blood groups Rh (D) and ABO are distributed differently in different populations and races. In numerous genetic studies for trustworthy geographic information and in the blood transfusion process with related diseases, blood group is one of the significant and relatively well-known parameters that will ultimately aid in lowering the morbidity and mortality rate. Additionally necessary for efficient blood bank inventory management is an understanding of blood groupings (14, 15).

The diagnosis of β- thalassemia can be performed using DNA testing, Hb- electrophoresis and complete blood counts (CBC). Amniocentesis, fetal blood collection, chorionic villus sampling, fetal cell analysis in maternal blood are examples of prenatal diagnostic procedures (16).

The objective will be to study the distribution and relationship of blood types with β-thalassemia, in addition we aim to investigate the relationship, affinity and genetic predisposition between blood types and β-thalassemia.

Materials & Methods

1. Subject study

Retrospective study was established in Molecular biology unit in Central Public Health Laboratory (CPHL), Ministry of Health, and Iraq/Baghdad. In 2023, 200 specimens were enrolled with β-thalassemia. The specimen was collected from thalassemia centers that present in Iraq`s cities. The exclusion criteria were patients with hemophilia, sickle cell anemia and iron deficiency. Criteria in this study undergo to work ethics in CPHL that include patient consent to receive information. All methods were performed by relevant guidelines and standard operating procedures (SOPs).

2. DNA Isolation:

The process of DNA extraction was doing by isolation nucleic acid kit manufacturing by VIENNA Lab Company, in this procedure we Use fresh or frozen blood (EDTA). Before we start DNA isolation, we should allow Lysis Solution and GENXTRACT Resin to reach room temperature (kit components). Pipette 100 µl blood samples into a 1.5 ml tube. After that, add 1 milliliter of Lysis solution, stir by repeatedly inverting, and let stand at room temperature for 15 minutes. Subsequently, centrifuge at 3,000 rpm for 5 minutes (or approximately 1,000 xg). Take out and dispose of the top (1 ml) of the supernatant, Leave pellet undisturbed. Then add 1 milliliter Lysis Solution, and mix by upsetting numerous times, Pellet should be completely resuspended, later Centrifuge at 12,000 rpm for 5 min, or (approximately 12,000 x g). Take out and dispose of the supernatant, leaving behind about 50 µl of a soft, visible pellet. Because GENXTRACT Resin sediments easily we should thoroughly swirl the bottle to resuspended it. Before taking out another aliquot, immediately repeat the resuspension each time. After that to the pellet, add 200 µl GENxTRACT Resin for 10 seconds close the tube and vortex. Then, Incubate at 56°C for 20 min. For ten seconds, vortex. Next, incubate at 98°C for 10 minutes. Vortex for ten seconds, then Centrifuge at 12,000 rpm for five minutes. Remain Cool on ice. Lastly, the supernatant needs to be frozen at -20°C or refrigerated (2-8°C; up to one week) in a new tube (17).

3. β – Thalassemia testing - Polymerase Chain Reaction (PCR) test

In PCR amplification which performed by β-Globin Strip Assay MED kit (VIENNA LAB Diagnostic GmbH) DNA isolation from EDTA blood samples. The amplification test was done by prepare reaction mix for amplification the target to detect β-thalassemia genes this process was performed by place reaction tubes inside, then star up the subsequent thermocycling program:

Pre-PCR at 94ºC for 2 min then the instrument will start thermocycling protocol for 35 cycles of denaturation stage at 94ºC for 15 seconds, after that the heat graduate to 58ºC at 30 seconds at annealing of primers stage and extension at 72ºC programmed for 45 seconds, final extension for 3 min at 72ºC. This program was followed according to the kit's instructions for optimum detection of the target (17).

4. Post-PCR (Hybridization β- Thalassemia)

In this step we used shaking water bath (at 45°C) (± 0.5°C). Allow Test strips, DNAT, Conjugate Solution, Wash Solution B and Color Developer to reach room temperature after Pre-warm Hybridization Buffer and Wash Solution A to 45°C. Prepare Typing Tray(s). Using clean tweezers, remove one Test strip for each sample. (Use gloves only when handling test strips, and use a pencil to Label Tests trips outside of the marker lines). Each lane to be used in the Typing Trays received 10 µl of DNAT (blue solution) in the lower corner. Next, 10 µl of amplification product was added to the corresponding drop of DNAT. Using pipette, thoroughly mix (The solution will remain blue). Wait five minutes at room temperature. After that filled each lane with 1 ml of Hybridization Buffer that had been pre-heated to 45°C. Shake the tray gently. The blue color will vanish.

Place Tests trips into the appropriate lanes with the marked side up and lines visible. Fully submerge test strips, 30 minutes were spent incubating at 45°C on the water bath`s shaking platform or thermo shaker. Set moderate shaking frequency to avoid dropping. Keep the shelter of the water bath or thermo shaker closed to avoid differences in temperature. Removed hybridization solutions by micropipette tips. Started wash steps by added 1 ml Wash Solution A (pre-warmed to 45°C). Rinse briefly (10 sec). Eliminate liquids by micropipette tip, then added 1 ml Wash Solution A (45°C). Incubate for fifteen minutes at 45°C on the trembling platform of the water bath or thermo shaker. Remove liquids by micropipette tips (Repeat this step another time).

For Color development steps 1 ml Conjugate Solution was added. (Aspirate liquids) Incubate for 15 minutes at room temperature on a rocker. A 1 ml Wash Solution Buffer was added. Rinse briefly ten seconds, liquids were aspirated out. 1 ml of Wash Solution Buffer was then added. Use an orbital shaker or rocker to

incubate for five minutes at room temperature. By aspirating out the liquids, remove them (do this step again). 1 ml of Color Developer was added. Incubate on a rocker for 15 minutes at room temperature in the dark. The test strips should be wash several times with distilled water and let strips dry in the dark on permeable paper (17).

Analytical Statistics:

The SAS-Statistical Analysis System (2018) software was utilized to measure the effect of the separate categories on the study parameters. In this study, the chi-square test was utilized to compare percentages (0.05 and 0.01 probability) statistically significant (18).

Results

The study was carried out in Iraq population among beta-thalassemia patients, EDTA-blood samples was sent from different thalassemia centers between January 2023 to December 2023 which included 200 patients with beta-thalassemia. In study group the ratio of male to female was 49 % to 51 %, the blood groups showed statistically high significant with gender as shown in table (1), which appeared high percentage in male for blood group A+ (43.8%) followed by group O+ about (27.5%) but in female blood group O+ gave high percentage compared with male, that followed by B+ about (27.18%).

Table (1): Distribution of blood groups in β-thalassemia patients depending to gender

Blood group	Male No. (%)	Female No. (%)
A+	43 (43.88%)	12 (11.65%)
A-	0 (0.00%)	2 (1.94%)
B+	17 (17.35%)	28 (27.18%)
B-	2 (2.04%)	4 (3.88%)
AB+	5 (5.10%)	4 (3.88%)
AB-	0 (0.00%)	0 (0.00%)
O+	27 (27.55%)	49 (47.57%)
O-	4 (4.08%)	3 (2.94%)
Total	98	102
P-value	0.0001 **	0.0001 **
** (P≤0.01).		

The data of study compromised the age groups in male and female showed significant differences with blood group distribution which divided into (18-25), (27-35), (36- 40) this age group appeared not significant differences with blood groups as shown in Table (2) & (3). Blood group A+ appeared high percentage at age 18-2 about (6.63%) in male and followed by O+ (5.07%), while Blood group O+ gave maximum (14.04%) in female, then blood group B+ recorded about (6.48%), we found that the high peaks of distribution for ABO blood group appeared in age group (18-25 years).

Table (2): Distribution of blood groups in β-thalassemia patients depending on age in male

Blood group/Male	Age (18-25)	Percentage%	(26-30)	Percentage %	(31-35)	Percentage %	(36-40)	Percentage %
A+	17	6.63%	14	4.48%	7	1.12%	3	0.18%
A-	0	0	0	0	0	0	0	0
B+	6	2.34%	5	1.6%	4	0.64%	2	0.12%
B-	0	0	2	0.64%	0	0	0	0
AB+	2	0.78	2	0.64%	1	0.16%	0	0
AB-	0	0	0	0	0	0	0	0
O+	13	5.07%	8	2.56%	4	0.64%	1	0.06%
O-	1	0.39	1	0.32%	0	0	0	0
Total	39		32		16		6	
Chi-square	--	11.74 **	--	8.924 **	--	4.067 *	--	1.026 NS
P-value	--	0.0001	--	0.0001	--	0.0352	--	0.285
* (P≤0.05), ** (P≤0.01).								

Table (3): Distribution of blood groups in β-thalassemia patients depending on age in female

Blood group/Female	Age (18-25)	Percentage%	(26-30)	Percentage%	(31-35)	Percentage%	(36-40)	Percentage%
A+	9	4.86%	6	2.04%	4	0.6%	1	0.05%
A-	2	1.08%	1	0.34%	0	0	0	0
B+	12	6.48%	8	2.72%	4	0.6%	2	0.1%
B-	3	1.62%	1	0.34%	0	0	0	0
AB+	2	1.08%	2	0.68%	0	0	0	0
AB-	0	0	0	0	0	0	0	0
O+	26	14.04%	12	4.08%	7	1.05%	2	0.1%
O-	0	0	4	1.36%	0	0	0	0
Total	54		34		15		5	
Chi-square	--	14.702 **	--	7.984 **	--	4.266 *	--	0.891 NS
P-value	--	0.0001	--	0.0085	--	0.0376	--	0.175
* (P≤0.05), ** (P≤0.01).								

In current study, Beta thalassemia patients' diagnosis according to Hb electrophoresis and determined HbF and HbA2 value after that all specimens undergo to molecular diagnosis by polymerase chain reaction (PCR) techniques to detection the variants of mutation detected by DNA amplified to the target of beta globin proteins by using β-Globin Strip Assay kit could show in Table (4).

According to their frequency, there are 17 different mutations which gave significant differences with ABO blood groups with Rh+ factor that showed:

IVS 2.1[G>A] was high percentage about (30%) in blood type O+, then followed by A+ (25%), then B+ about (16%) this mutation was appeared more frequency compared with other mutations. while was IVS 1.1 [G>A] in blood group A+ and O+ the same percentage about (3%), IVS 1.110 [G>A] was (10%) in A+ and (7%) in O+, IVS 1.130 [G>C] was high ate in O+ about (6%), IVS 1.5 [G>C] was (5%) in O+ and about (3%) in B+, IVS 1.6 [T>C] was (7%) in O+ and (5%) in A+ followed by (4%) in B+, IVS 2.745 [C>G] was (3%) in B+ and (2%) in A+ & O+, IVS 2.848 [C>A] was (1%) in both B+& O+, Codon 8/9[+G] was (2%) in B+ followed by (1%) in A+ & O+, Codon 44[-C] was maximum in O+ about (5%) and (2%) in A+, Codon 39 [C>T] was (4%) in O+ then (2%) in A+, Codon 8 [-AA] was high rate in B+ about (4%) and (2%) in A+, Codon 5 [-CT] was (3%) in B+ and (1%) in both A+ & O+, Codon 27 [G>T] was (1%) in O+, Codon 6 [G>A] was (1%) in both A+ & AB+ , -101 [C>T] was (1%) in A+, B+, AB+,O+, -87 [C>G] was (2%) in O+.

Table (4): Distribution of blood groups with β-thalassemia mutations

β-thalassemia Mutations	**Blood group**							
	A+	A-	B+	B-	AB+	AB-	O+	O-
IVS 2.1[G>A]	25	0	16	2	3	0	30	3
IVS 1.1 [G>A]	3	0	2	0	0	0	3	0
IVS 1.110 [G>A]	10	0	5	0	2	0	7	0
IVS 1.130 [G>C]	0	0	1	0	0	0	6	1
IVS 1.5 [G>C]	2	0	3	2	1	0	5	1
IVS 1.6 [T>C]	5	0	4	0	1	0	7	1
IVS 2.745 [C>G]	2	0	3	1	1	0	2	0
IVS 2.848 [C>A]	0	0	1	0	0	0	1	0
Codon 8/9[+G]	1	1	2	0	0	0	1	0
Codon 44[-C]	2	1	1	1	0	0	5	1
Codon 39 [C>T]	2	0	1	0	0	0	4	0
Codon 8 [-AA]	2	0	4	0	0	0	0	1
Codon 5 [-CT]	1	0	3	0	0	0	1	0
Codon 15[TGG>TGA]	0	0	0	0	0	0	0	0
Codon 27 [G>T]	0	0	0	0	0	0	1	0
Codon 6 [G>A]	1	0	0	0	1	0	0	0
-101 [C>T]	1	0	1	0	1	0	1	0

-87 [C>G]	0	0	0	0	0	0	2	0
-30 [T>A]	0	0	0	0	0	0	0	0
Total	57	2	47	6	10	0	76	8
P-value	0.0001 **	0.271 NS	0.0001 **	0.208 NS	0.096 NS	NS	0.0001 **	0.062 NS
Confidence Interval (C.I)	8.41-22.54	0.79-1.65	6.95-15.02	0.82-1.76	0.88-1.81	-	11.69-27.52	0.85-1.78
** ($P \leq 0.01$).								

Discussion

β-thalassemia is a hereditary disorder that results in severe anemia due to defective erythropoiesis, there are limited therapeutic options. The grade of inequity between the alpha globin and non-alpha globin chains is correlated with the severity of the disease (19). Patients with thalassemia disease need to blood transfusion continuously to avoid anemia (there are not enough healthy red blood cells in the body).

In this study the dominant blood group in male differed from female, in male blood group A+ gave high rate compared with female while blood group O+ in female showed high percentage compared with male after that other blood groups various according to their percentage, people with blood groups AB (-Ve) (0%) and A (– Ve) (1.9%) are less probable to get the disease than other blood group that gave some prediction about which types of blood groups that can carried β-thalassemia gene more than others, this result was agreed with other study that showed the less group was AB (–Ve) with 0 % while A (-Ve) 1% (6).

Our findings appeared significant differences in age for male and female with blood groups at age (18-25, 26-30 and 31-35), the all-age groups gave high rate in A +Ve 6.63%, 4.48 %, 1.12 % in male respectively, whereas in female was O +Ve 14.04 %, 4.08 %, 1.05 % respectively. A (-Ve) gave 0 % in male but in female gave 1.42 % at youth age. O (-Ve) showed high percentage in female about 1.36 % compared to male was 0.7 %. In male at age (26-30), B -Ve was 0.64 % and other groups was 0 % while in female at age (18-30) was 1.96% in addition to AB +Ve appeared approximately the equal rates in male and female while AB (-Ve) was 0 % in both genders. The Rh (-) phenotype in β-thalassemia patients showed decrease in percentage compared with Rh (+) phenotype which indicates that Rh (-) is resistant to thalassemia. In age group (36-40) appeared not significant differences in both male and female with blood groups. As a results of our research, we can find the thalassemia gene occur more in youth age and in Blood groups with Rh+ factor.

The various mutation types that showed in our research so as in our molecular work from Iraq regarding BT. Mutations in this study detection by use B-Globin Strip Assay. Our findings that appeared the predominant mutation IVS 2.1 [G>A] was 38.3%, followed by IVS 1.110 [G>A] 11.6 %, IVS 1.6 [T>C] 8.7 %, IVS 1.5 [G>C] 6.8 %, Codon 44[-C] 5.3%, IVS 2.745 [C>G] 4.3 %, IVS 1.1 [G>A] and IVS 1.130 [G>C] were 3.8 %, Codon 39 [C>T] and Codon 8 [-AA] was 3.4 %, Codon 8/9[+G] and Codon 5 [-CT] were 2.4 %,-101 [C>T] 1.9 %, IVS 2.848 [C>A], Codon 6 [G>A] and -87 [C>G] were 0.9 % and finally Codon 27 [G>T] 0.4 % as showed in figure (1) which agreed with local study in 2008 the following mutation frequencies were reported by individuals registered in thalassemia centers of Baghdad, Ninawa and Al-Muthanna governorates : IVS 2.1 (G>A) 21.53%, IVS 1.110 (G>A) 19.23%, IVS 1.6 (T>C) 10.76%, cod 44 (-C) 9.23%, cod 5 (-CT) 8.46%, cod 39 (C>T) 7.69%, cod 8 (-AA) 6.15%, IVS 2.745 (C>G) 5.38%, IVS 1.5 (G>C) 3.84% and codon 8/9 (+G) 0.76%. (20). In 2013, study in Baghdad city showed six of mutations from 17 which were IVS-I-110 (G>A), IVS-II-1 (G>A), IVS-I-5 (G>C), codons 8/9 (+G), IVS-I-I (G>A) and codon 44 (-C), established 78.0% of the mutations categorized and new mutations which were reported for first period in Iraq by sequencing included IVS-I, 25 bp deletion, IVS-II-848 (C>A), -28 (A>C), IVS-I-130 (G>C), IVS-I-128 (T>G) and codons 41/42 (-TTCT) (21). Other study in 2009 in Ninawa governorate gave same mutation but in different rates and predominant mutation in this study was IVS 1.110 (G>A) 27.08%, followed by IVS 1.6 (T>C) 14.5%, cod 8 (-AA) 12.5%, cod 39 (C>T) 12.5%, IVS 2.1 (G>A) 12.5%, cod 44 (-C) 4.16%, IVS 1.5(G>C) 2.08% and Cod 5(-CT) 2.08% (22).

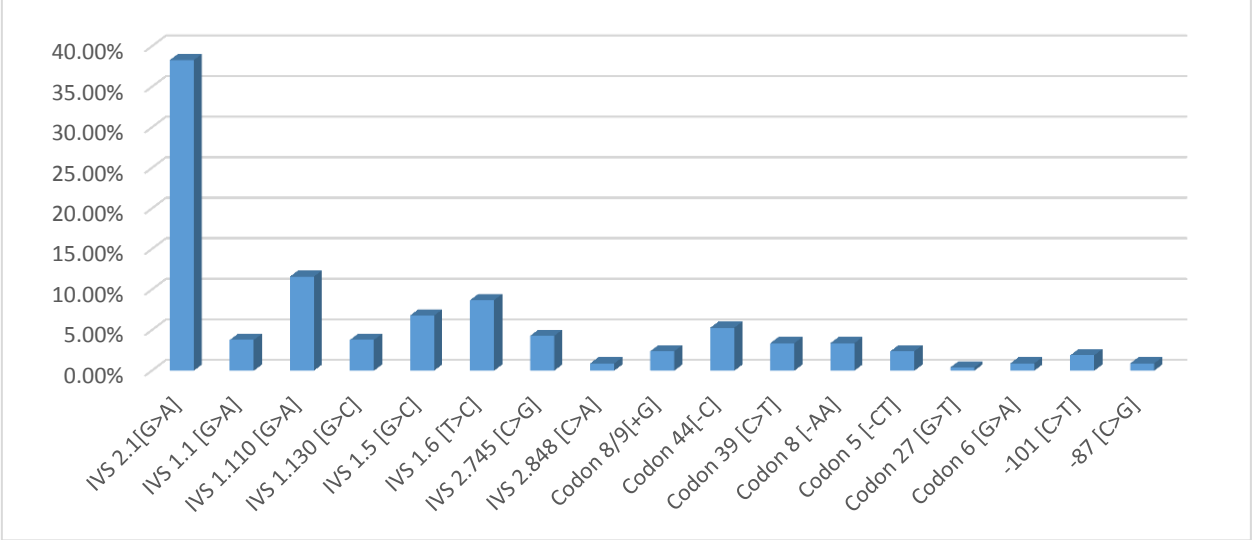

Figure (1): Distribution of β-thalassemia mutations in Iraqi patients

The study in North of Iraq appeared various rates in β-thalassemia mutations, the most frequent were IVS-II-I (G>A) (47.2%), followed by IVS-I-6 (T>C) (23.3%) and IVS-I-110 (G>A) (5%) (23). In 2016 about 4.6% of people are carriers of the ß-thalassemia gene in Basrah, Southern Iraq. This study gave various results the most common β- thalassemia mutations are found in codon-15(G-A) and IVS 1nt-5(G-C), which are present in 31 (37.3%) and 18(21.7%) of patients correspondingly, codon 8/9 is present in 14(16.9%) and codon 30 is present in 8 (9.6%) patients (24). In Al-Najaf province the results presented that the highest incidence of (Cod-88) mutation about 21 % and followed by Cod 15 and Cod 8 which were 18% and 12%, individually. The lowest rates were for Cod 8\9, Cod IVS and Cod 30 gave 5 %, 3 % and 2 % (25).

There are eleven alleles which causing thalassemia in Iraq with their mutation frequencies, we can note in Basrah and Najaf shared in some mutations causing ß-thalassemia like codon-15(G-A), codon 8/9 and codon 30, and in Baghdad and North of Iraq shared with some mutation like IVS-I-6 (T>C) (23.3%) and IVS-I-110 (G>A), that mean some frequencies can be distribution in some provinces of Iraq and other frequencies in other governates, like some mutation may be found in north and middle of Iraq but not found in South. In Turkey`s studies appeared the same mutation that gave in our study especially IVS-I-110 (G>A), IVS 1.6 [T>C], Codon 44[-C], Codon 39 [C>T] and Codon 8 [-AA] (26).

The β-thalassemia phenotypes linked to these mutations exhibit variation among Arab nations. The Mediterranean mutation known as IVS I-110 is prevalent in Arab nations; rates of this mutation range from 1% in Bahrain and the United Arab Emirates to 48% in Egypt and 56% in the present study. When the Ottoman Empire existed, which dominated the area from the sixteenth to the early twentieth centuries, it is believed that the IVS I-110 G>A mutation spread throughout the Eastern Mediterranean and North Africa (27). This study was first study in Iraq that showed the distribution between ß-thalassemia mutations and blood groups with Rh factor, as shown in Table (4) the 2.1 IVS was the most frequent that appeared in this study and appeared in most of blood groups as following: A+ was (32%), B+ (20%), AB+ (4%) and O+ (38%) as we found this mutation was predominant with Rh (+)while in Rh (–)showed low percentage or absent as in O (-) about 4% and B (-) 2 % when A- and AB- were (0) as shown in figure (2).

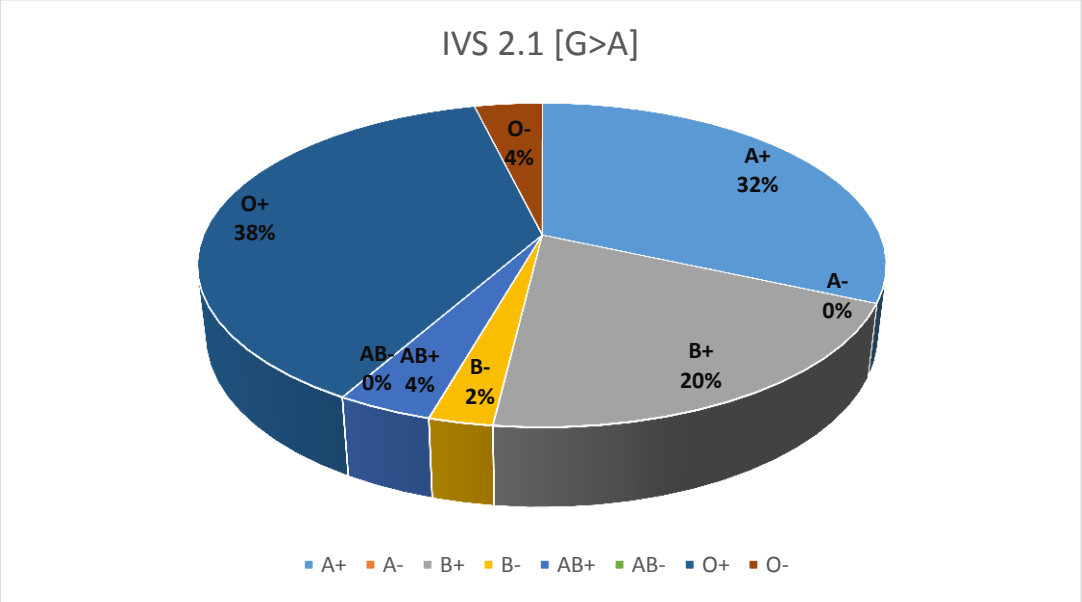

Figure (2): Distribution of predominant mutation IVS 2.1 [G>A] with blood groups in Iraq`s patients

when IVS 1.110 [G>A] was found in patients with blood group Rh+ was high rate in A+ followed by O+, B+ and AB+ but in blood groups with Rh- was absent, while in IVS 1.130 [G>C], IVS 1.5 [G>C], IVS 1.6 [T>C], Codon 44[-C], Codon 39 [C>T], were high rate in O+, in first mutation was appeared in B+ but other four mutations appeared in both A+ and B+. Finally, our research was showed high significant differences with distribution of mutations causing of β-thalassemia in Iraq establish in most in patients with blood groups Rh+ and type O+ gave high rates compared with other groups. Blood groups O with Rh + was predominant in this study which agreed with local study that recorded high percentage for O+ and followed B+ and A+ (28).

Conclusion

The research appeared that the blood groups in thalassemia patients different in male from female, in male predominate type is A+ while in female is O+. In polymerase chain reaction the IVS 2.1 [G>A] mutation is the utmost common among Iraqi β-thalassemia patients their have Blood groups with Rh+, high percentage for O+ followed by A+ and B+. IVS 1.110 [G>A] was second mutation followed by IVS 1.5 [G>C] and IVS 1.6 [T>C].

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