The *ITGA2B* gene Polymorphism Associated with Glanzmannthrombasthenia in Sample of Iraqi Patients

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Abstract

Case control study was used ,with healthy individual control(n=20)and Glanzmannthrombasthenia patients(n=15). The diagnosis was depending on the clinical , hematological parameters and molecular analysis.All patients were severe bleeding symptoms with normal Hemoglobin , and plateles count ,and prolonged bleeding time.

It was successfully identified three SNP in *ITGA2B* gene the first SNP c.2653 T>G (rs5911)was presented with three genotypes (TT, TG and GG).The genotype frequencies of TT in control group (60.0 *vs*. 90.0%) show significant difference ($p \leq 0.05$) compared withGT patients . It was also noticed the frequency of mutant allele (*G*) revealed a significant difference ($p \leq 0.01$) in GT patients compared with controls group (33.3 *vs*. 7.5%; OR = 6.17; EF = 0.44; 95% C.I. = 1.55 - 24.53)respectively .

The c.641T>C (rs137852911) was given with three genotypes (TT, TC and CC) . The frequencies of these genotypes show non-significant difference ($p \leq 0.05$ between control and GT patient ,while the mutant allele (*C*) show significant difference ($p \leq 0.01$) in GT patientscompared with controls group (10 vs. 7.5%; OR = 1.37 ; EF = 0.11; 95% C.I. = 0.26 – 7.14).

The c.6438G>A showed three genotypes (GG, GA and AA) with two alleles (*G* and *A*).

The frequencies of these genotypes GG (60 vs. 95 %) , GA (33.3vs. 5.0%) and the third genotype (AA) 26.6 vs. 0%show significant difference ($p \leq 0.05$) between control and GT patient .It was also found the mutant allele (*A*)revealeda significant difference ($p \leq 0.01$) in GT patients compared with controls group (33.3 vs. 2.5%; OR = 11.87 ; EF = 0. 35 ; 95% C.I. = 1.41 – 99.80)respectively

Keywords:- *Glanzmannthrombasthenia, genotypes, revealed, bleeding time, frequencies*

Introduction

Glanzmannthrombasthenia (GT) is a rare, inherited disorder of platelet function characterized by absence of platelet aggregation ,and prolonged mucocutaneous bleeding tendency, caused by qualitative or quantitative defects of the platelet membrane integrin αIIbβ3[1, 2].

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The molecular basis of GT is linked to qualitative and / or quantitative abnormalities of αIIbβ3 integrin that between binding of the adhesive proteins lead to aggregating platelets and clot formation at position of wound [1]. patients have been separate into three groups:type I where platelets absence αIIbβ3 (have <5% of the normal platelet content), type II with residual αIIbβ3 (5–15%) that may be functional or not, while in type III with αIIbβ3 fails to function despite platelets possess upto 100% of normal levels [3].

Many Heterozygous or Homozygous mutations found in *ITGA2B*gene and these mutations are led to splice defects and stop codons truncate or lead to the loss

mRNA synthesis[3].

It was reported that GT appear in many ethnic populations such as Iranians,Indians, Iraqi Jews, Jordanian Arabs , and Palestinians in high frequency[4].

As a mention above this study was aimed to investigate the *ITGA2B* gene polymorphism in a sample of Glanzmannthrombasthenia Iraqi patients.

Materials and Methods

Case control study was used ,with healthy individual control(n=20)and Glanzmannthrombasthenia patients(n=15) were recruited at the Division of pediatric teaching hospital in Baghdad and Karama Teaching Hospital in wassit. Both laboratory data and clinical of every patient were gathered from their clinical records which included gender, age, age at determination, period of beginning , bleeding manifestations, and consanguinity of parents.Samples of patients and the control group were collected in EDTA, tubes, and hematology tests (Hb and plateles count)were performed by Coulter automated analyzer and the bleeding time examination was done.

Isolation of DNA from the EDTA blood of patient samples and control groups using DNA Kit from Promega. regions of *ITGA2B* gene were amplified by PCR using primers . Which was designed using the program PRIMER3 .Genomic DNA was used as the template for 25 PCR

reaction system containing 12 µM of Master Mix, 10 µM each of oligonucleotide primers, 8.5µM Nuclease Free Water and 2 µM Taq DNA Polymerase . Thermocycling conditions of primers F5`-TATGTCTGAGTCTTGGAGCCC-3` and R 5`-CAGAGAGCCTGCTCACTACGA-3`,andprimers F5`-GTTGCTTTGGGTACAAGAATG-3` and R 5`-CTCCCACCAAGTCCTAATAATC-3`were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation , annealing , and extension at 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s Respectively. A final extension step (72 °C, 7 min) was performed at the end. other primers F 5`-CTTCCAGGTGATGAGACCCG-3`and5-R5`-TCTGGAATGGCGGTGTTACC-3` have same thermocycling conditions but have different annealing temperature (65 °C).

Using ABI3730XL, a Sanger sequencing analysis was performed on the PCR product by Macrogen Corporation - Korea, and the results were analyzed using a genious program.

All data was analyzed statistically by using SPSS software (Statistical Package for Social Sciences) version 20 and p-values were set at 0.001 . Data are presented as Mean ± SD (standard deviation). The results were analyzed using analysis of variance (ANOVA) .

Depending on Hardy-Weinberg equilibrium, the difference between the expected and observed frequencies of the genotype was determined as well with respect to the expected and observed alleles at the control and patient groups.Alleles and genotypes of *ITGA2B* genes were presented as percentage frequencies, and significant differences between their distributions in GT patients and controls were assessed by two-tailed Fisher's exact probability (P). In addition, odd ratio (OR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between *ITGA2B* and *ITGB3* alleles and genotypes with the disease , these estimations were calculated by using the WINPEPI computer programs for epidemiologists,which is available free online at http://www.brixtonhealth.com.

Result

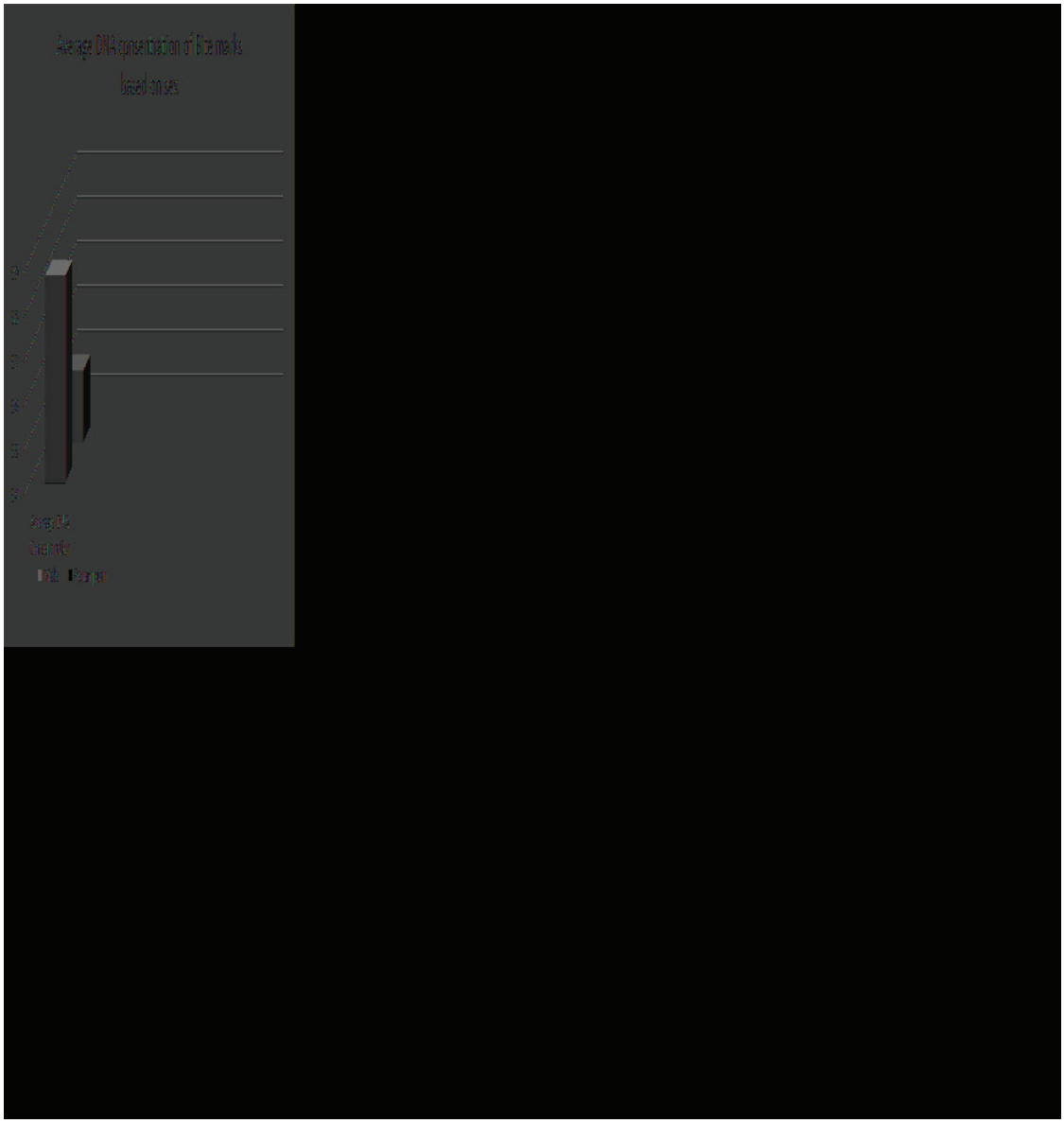
GT was diagnosed depend on of Clinical signs and data of each patient . The results of the hemoglobin test and platelet count were within the normal range.Where were the results of an examination HB mean 11.7±1.7 of patients and mean 12±1 of control group . While the average platelet count test values were mean 217.1±9.2 of patients and mean 296±9.9 of control group .The platelet count and the hemoglobin parameters and indexes measured in the current work were not significantly different ($p<0.001$) between Glanzmannpatients , and control groups.

Whereas, there was a significant difference between the values of bleeding time result (mean13.9±3.2) for patients compared to the control group (mean 4.7±1.5).

The results showed that after ethidium bromide staining, a main band (length 980 bp) appeared on the gel, which represented the amplification of the restricted

region from 44375568 to 44376560 in the *ITGA2B* gene containing exons 23,24 and 25with intros between these exons . The other result of the amplification of the DNA fragments by the primers is the region between 44384919 and 44385928 with a band of 1000 bp on the gel and the exons 3,4,5,6 and 7 .The DNA fragment between 44383196 and 44384189 was amplified by PCR and primer set. The length of the product DNA fragment was 990 bp, including exons 11 and 12 figure(1)

Sequencing analysis can detect a total of 3 different mutations in the *ITGA2B* gene.c. 2653 T> G mutation is the replacement of nucleotide *T* allele in position chr17:44375697 (GRCh38.p12) in exon 24 with *G allele* , which causes the amino acid isoleucine to become a serine substitution at position 874 of the GPIIbfigure (2).c.641T>C mutation at position chr17: 44385193 (GRCh38.p12) revealed that the mutation changed T>C in exon 6 and caused the amino acid leucine to become proline at position 214 in the polypeptide chain figure (3). c.6438G>A mutation of chr17:44383668 (GRCh38. p12) of exon 12 was detected by sequencing analysis, and caused the amino acid at GPIIb 345 to replace methionine with isoleucinefigure (4) .



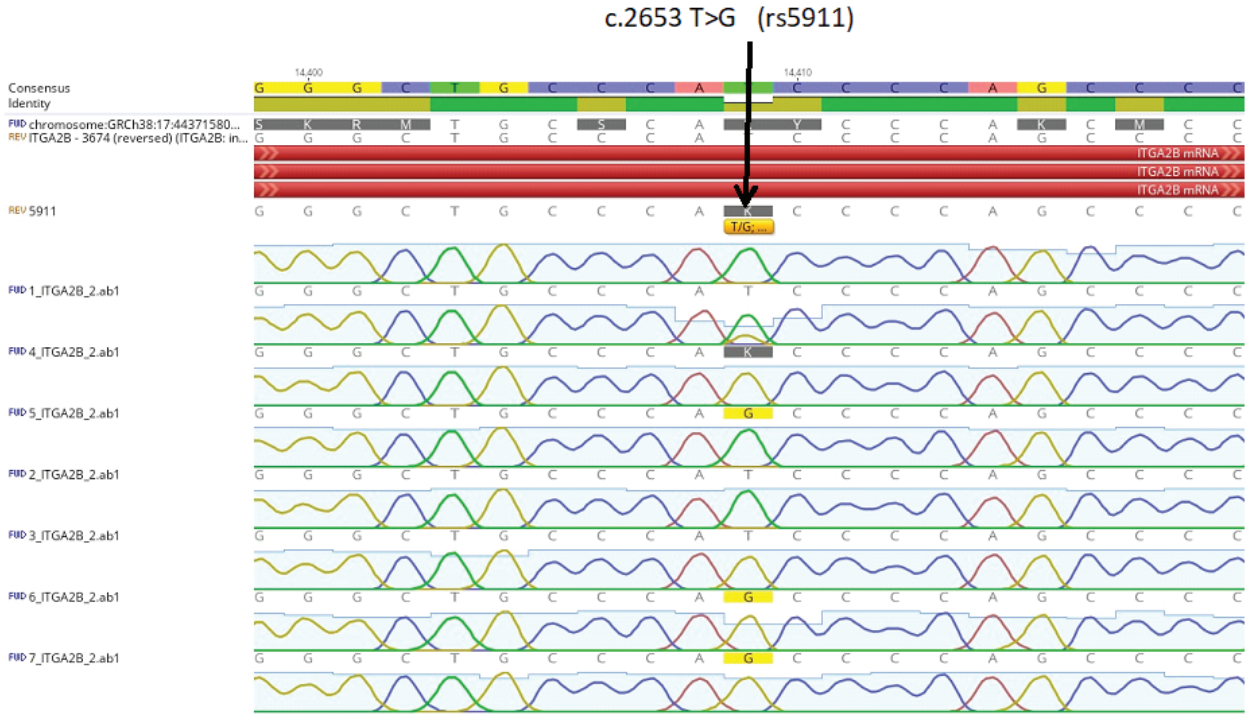


Figure 2 Nucleotide sequencing analysis of *ITGA2B* gene. Results for nucleotides are shown for a the GT patient. The nucleotide substitution T>G at position 2653 in exon 24 and the corresponding amino acid change from Ile874 (ATC) to Ser874 (AGC) are indicated

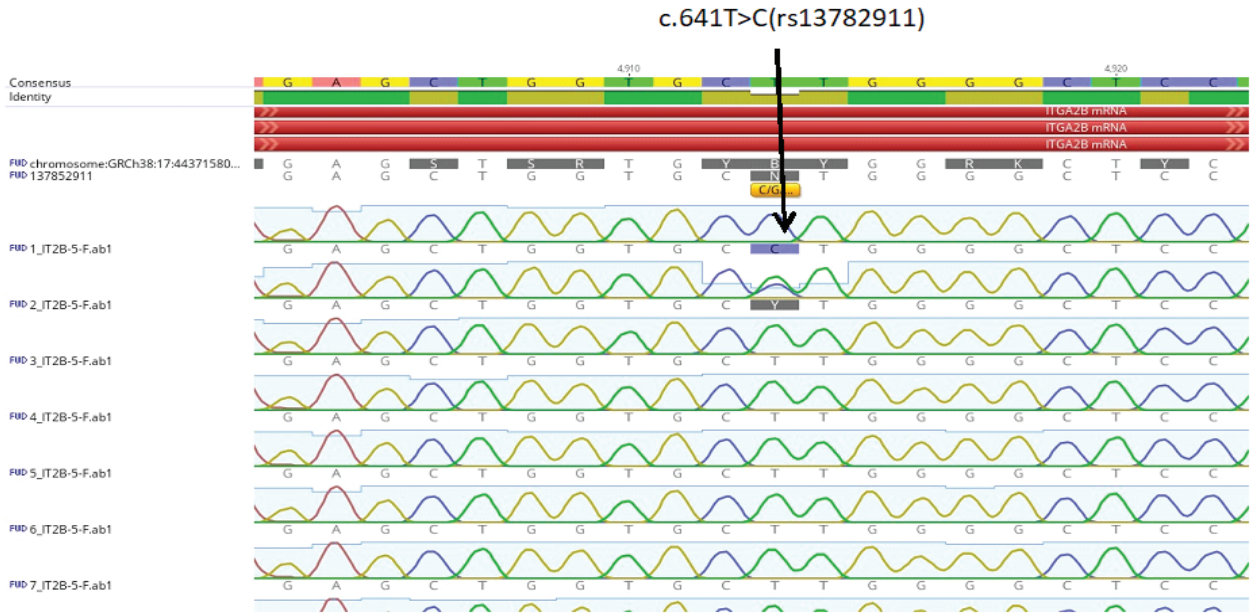


Figure 3. Nucleotide sequencing analysis of *ITGA2B* gene. Results for nucleotides are shown for a the GT patient. The nucleotide substitution T>C at position 673 in exon 6 and the corresponding amino acid change from Ieu 214 (CTT) to Pro 214 (CCT) are indicated.

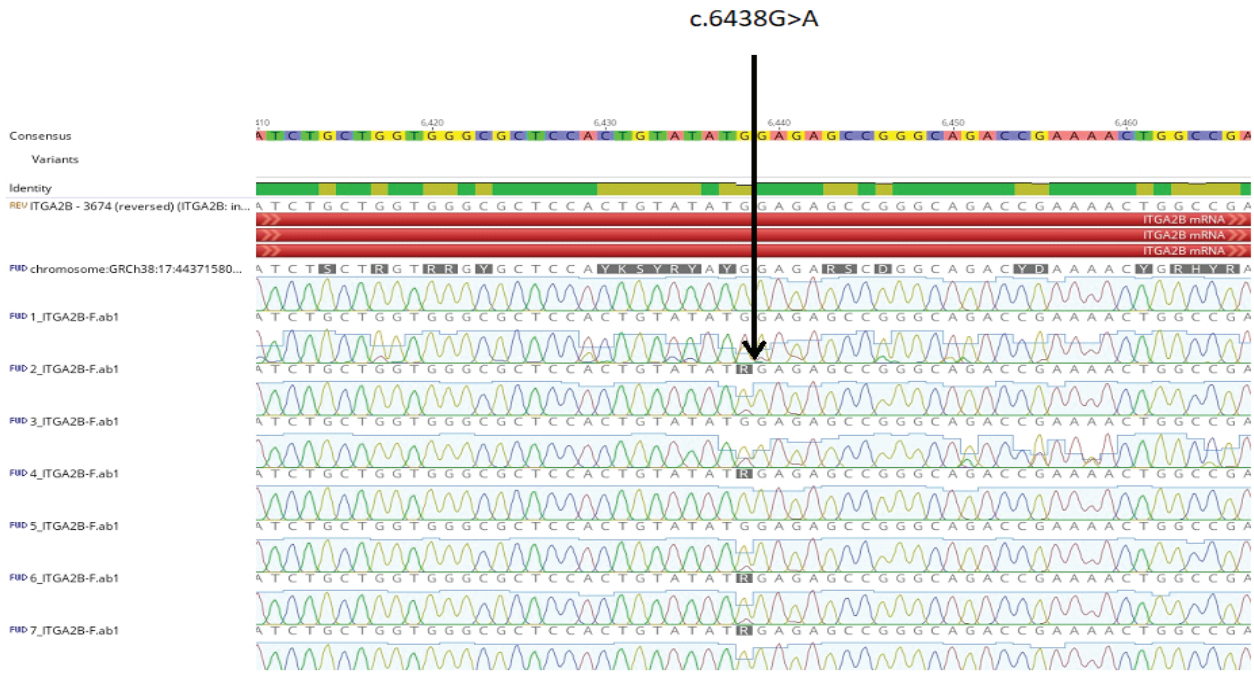


Figure 4. Nucleotide sequencing analysis of ITGA2B gene. Results for nucleotides are shown for a the GT patient. The nucleotide substitution G>A in exon 12 and the corresponding amino acid change from Met 345 (GGC) to Ile 345 (AGC) are indicated

The SNP (c.2653 T>G (rs5911) at position 2653in exon 24 was presented with two alleles (*T* and *G*) and three genotypes (TT, TG and GG). These genotypes showed deviation from HWE in GT patients, because there was a significant difference between the observed and expected genotype frequencies ($p \leq 0.01$),it was also noticed a deviation from HWE in controls treatment with significant difference ($p \leq 0.01$).

Comparing GT patients to controls revealed that genotype frequencies of TT (60.0*vs*. 90.0%) and TG (13.4*vs*. 5.0%),while the third genotype (GG) was observed with a frequency 26.6 *vs*.5.0% in patients and control group respectively,The genotype TT in control group was significant difference ($p \leq 0.05$) compared to GT patient , butTG and GG were show non-significant difference between healthy subject and GT patient .It was also noticed the frequencyof mutant allele (*G*) showa significant difference ($p \leq 0.01$) in GT patients compared to controls (33.3*vs*. 7.5%; OR = 6.17, EF = 0.44; 95% C.I. = 1.55 - 24.53) that mean (*G*) allele(OR = 6.17) is risk factor associated with GT patients and negatively associated with healthy subject, while in the control group the frequency of (*T*) alleleshow a significant difference ($p \leq 0.01$) compared to GT patients (92.5*vs*.66.6;Reciprocal of OR =6.17 , EF= 0.932 ; 95% C.I. = 0.04- 0.65) . In this case the (*T*) allele associated with healthy subject and considered as protective factor , but in GT patients group was negatively associated withReciprocal of OR =6.17(Table).

The SNP rs137852911 (c.641T>C) at position 9313 in exon 6 was presented with two alleles (*T* and *C*) and three genotypes (TT, TC and CC). These genotypes showed deviation from HWE in GT patients, because there was a significant difference between the observed and expected genotype frequencies ($p \leq 0.01$),it was also noticed the genotypesfrequencies (TT, TC and CC) show deviation from HWE in controlsgroup with significant difference ($p \leq 0.01$).

Comparing GT patients to controls revealed that genotype frequencies of TT (86.6vs. 90.0%) , TC (6.6vs. 5.0%) ,and The third genotype (CC) 6.6 vs.5.0% . These genotypes were non-significant difference ($p \leq 0.05$) compared between control and GT patient. it was also noticed the frequency of mutant allele (*C*) show a significant difference ($p \leq 0.01$) in GT patients compared with controls (10 vs. 7.5%; OR = 1.37 ; EF = 0.11;

95% C.I. = 0.26–7.14) , while in the control group the frequency of (*T*) allele show a significant difference ($p \leq 0.01$) compared to GT patients (90 vs.92.5;Reciprocal of OR = 1.73, EF0.71 ; 95% C.I. = 0.14- 3.8) . In this case the (*T*) allele associated with healthy subject and non-associated with GT patients(Reciprocal of OR = 1.73) and considered as protective factor (Table).

The c.6438G>A at position 6582 in exon 12 was presented with three genotypes (GG, GA and AA) ,and two alleles (*G* and *A*) .These genotypes showed deviation from HWE in GT patients, because there was a significant difference between the observed and expected genotype frequencies ($p \leq 0.01$),it was also noticed a deviation from HWE in controls treatment with significant difference ($p \leq 0.01$).

Comparing GT patients to controls revealed that genotype frequencies of GG (60 vs. 95 %) , GA (33.3vs. 5.0%) ,and The third genotype (AA) 26.6 vs.0% .The GA and AA genotypes were show non-significant difference between healthy subject and GT patient, while The genotype GA was significant difference ($p \leq 0.05$) compared between control and GT patient. it was also noticed the frequency of mutant allele (*A*) show a significant difference ($p \leq 0.01$) in GT patients compared to controls (33.3 vs. 2.5%; OR = 11.87 ; EF = 0. 35 ; 95% C.I. = 1.41 – 99.80) that mean (*A*) allele is risk factor associated with GT patients and negatively associated of healthy , while in the control group the frequency of (*G*) allele show a significant difference ($p \leq 0.01$) compared to GT patients (66.6 vs. 97.5; Reciprocalof OR = 11.87; EF0.98 ; 95% C.I. = 0.01-0.71) and positively associated of health and negatively associated of GT patients (Table).

Table : Genotype and allele frequencies and epidemiological parameters for Iraqi GT patients and controls group.

***ITGA2B* gene**	**GT patient (15)**			**Control (20)**			**Epidemiological parameters**				
SNP	**Observed N (%)**	**Expected N (%)**	**HWE *P* .value**	**Observed N (%)**	**Expected N (%)**	**HWE *P* .value**	**OR**	***p*.value**	**EF**	**PF**	**C.I 95%**
rs5911											
TT	9 (60)	6.6(44.4)	0.01	18(90)	17.11(85.5)	0.01	6	0.05	0.06	0.75	0.03 to 0.95
TG	2(13.4)	6.6(44.4)	0.01	1(5)	2.7(13.8)	0.01	2.92	0.5	0.12	0.08	0.26 to 33.24
GG	4(26.6)	1.6(11.1)	0.01	1(5)	0.11(0.5)?	0.01	6.91	0.1	0.06	0.2	0.73 to 65.57
T	20(66.6)		0.01	37(92.5)		0.01	6.17*	0.01	0.9	0.7	0.04 to 0.65
G	10(33.3)		0.01	3(7.5)		0.01	6.17	0.01	0.4	0.2	1.55 to 24.53
rs137852911			0.01			0.01					
TT	13(86.6)	12.1(81)	0.01	18(90)	17.1(85.5)	0.01	0.72	1	0.7	0.25	0.10 to 5.47
CT	1(6.6)	2.7(18)	0.01	1(5)	2.7(13.8)	0.01	1.36	1	0.14	0.01	0.08 to 21.70
CC	1(6.6)	0.15(1)	0.01	1(5)	0.11(0.5)?	0.01	1.36	1	0.14	0.01	0.08to 21.70
T	27(90)		0.01	37(92.5)		0.01	1.37*	1	0.7	0.25	0.14 to 3.80
C	3(10)		0.01	3(7.5)		0.01	1.37	1	0.1	0.02	0.26 to 7.14
c.6438G>A			0.01			0.01					
GG	9(60)	19(58.7)	0.01	19(95)	19(95)	0.01	12.67	0.02	0.01	0.2	0.01 to 0.71
GA	5(33.3)	1(35.7)	0.01	1(5)	0.9(4.8)	0.01	9.50	0.06	0.9	0.8	1.03 to 87.25
AA	1(26.6)	0(5.4)	0.01	0(0)	0.01(0.06)	0.01	4.24	0.42	0.09	0.07	0.18 to 102.51
G	23(66.6)		0.01	39(97.5)		0.01	11.87*	0.01	0.98	0.89	0.01 to 0.71
A	7(33.3)		0.01	1(2.5)		0.01	11.87	0.01	0.35	0.21	1.41 to 99.80

EF: Etiological fraction , PF: Preventive Fraction , OR :Odds ratio , C.I. : Confidence interval

HWE :Hardy-Weinberg Equilibrium , * : Reciprocal of odds ratio

Desiccation

GT has been reported among Iraqi patients by several groups [5, 6], including a series of 12 patients from Iraqi-Jewish which was described in 1991(7), and other group from Iraqi-Jewish was described in 2001 (8).The average age of patients was 20 years in this study and this is consistent with Al-Rahaland Giath 2015, which stated that this disease affects children and young adults. Most of the cases were caused by the consanguineous marriage to the parents .Consanguineous marriage is a common behavior in Iraqi populations, also prevalent in some populations, such as Iranians, Saudi Arabia, and Southern Indians.[6] Consanguineous marriage between parents is an important risk factor for GT[5].

The normal level of Hb was expected in Glanzmann, because the defects of these diseases directly affect platelets, not directly affect the level, number and nature of red blood cells. However, in some samples, the level of hemoglobin has decreased, especially in some women, the common problem that troubles women with Granzman is menorrhagia[9]. The platelet count results of all patients and the control group were normal, and the difference was not statistically significant and this is result correspond with Sabhan*etal*.2017.

Regarding prolonging the bleeding time of patients (n = 15), statistical analysis showed a significant difference ($p \leq 0.01$) compared with the control group.Prolonged bleeding time suggests delayed primary hemostatic thrombosis due to poor platelet aggregation[5].

As a mention above my be Rs5911 T >G considered as missense mutation that causes the substitution of isoleucine at position 843 of the GPIIb heavy chain with a serine amino acid.Therefore, rs5911 may work by affecting post-fibrinogen binding events, including cytoskeletal reorganization and clot retraction, and in some way affect the stability of platelet/fibrinogen interaction[10],depending on this finding the (*G*) allele considered as risk factor associated with GT patients while the (*T*) allele associated with healthy subject and considered as protective factor.

The c.641T>C mutation (missense mutation) was detect at position chr17:44385193 (GRCh38.p12) that change T>C in exon 6, and lead to changing amino acid leucine to Proline in position 214 in polypeptide chain.

The c.641T>C mutation results in the creation of a proline-glycine-alanine-proline (PGAP) sequence at the amino-terminal end of the b-turn structure. The rigidity of the two proline residues linked by flexible glycine and alanine residues may create a twist in the secondary structure affecting the conformation of the b-turn. The inability of mutant GPIIb/ IIIa receptors to be recognized by conformation-dependent antibodies, to be activated into a high-affinity ligand binding conformation, and to adhere to immobilized fibrinogen suggests that this structural alteration affects ligand-binding and that the Leu214Pro mutation has either an indirect or direct effect on this site [11]. in this study , it was show the (*C*) allele considered as risk factor associated with GT patients while the (*T*) allele associated with healthy subject and considered as protective factor.

c.6438G>A mutation in exon 12 was lead to substitution amino acid Methionine to Isoleucine acid at position 345 of the GPIIb .The change in the amino acid Methionine to Isoleucine may lead to a change in the structure of the receptor and thus affect the function of the receptor.

In this result , it was found the (*A*) allele considered as risk factor associated with GT patients while the (*G*) allele associated with healthy subject and considered as protective factor.

Conflict of Interest: There is no conflict of interest among the authors.

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Ethical Clearance: This study is ethically approved by the Institutional ethical Committee.

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