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Integrin Alpha2 and *Integrin Beta3* Polymorphisms are Risk Factors for Infertility in Iraqi Infertile Females under *In vitro* Fertilization Program

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Abstract

The current study was designed to investigate the impact of the Single Nucleotide Polymorphism (SNP), rs5918 (T>C), of integrin beta 3 genes (ITGB3) and rs1801106 (G>A) of integrin alpha 2 genes (ITGB3) in peripheral blood samples of Iraqi female; 71 infertile females under IVF protocol (divided into two subgroups, 29 success implantation and 42 failure implantation) and 50 fertile females as control. From March 2021 to August 2021, blood samples were taken from the patients and control groups at Roh al-Hayat center in Baghdad, Iraq. The genotyping of SNPs was carried out by using high-resolution melting real-time polymerase chain reaction (HRM real-time PCR) of the purified genomic DNA obtained from blood samples. The findings demonstrated that fertile females had significantly greater levels of the TT and GG genotypes of rs5918 at ITGB3 and rs1801106 at ITGA2, respectively, than infertile females. that shows the GG genotype functions as a protective factor, while the TC genotype of rs5918 at ITGB3 and AA genotype of rs1801106 at ITGA2 act as risk factors. In conclusion, these SNPs in the ITGA2 and ITGB3 genes may have an influence on the predisposition of a certain group to infertility.

Keywords: HRM, RT-PCR, implantation, ITGA2 gene, ITGB3 Gene, IVF.

Introduction

Fertility is the ability of an individual to conceive and have children, whereas infertility is a disorder that inhibits a person's ability to conceive and bear children. Sterility is an irreversible condition, whereas infertility is not. Infertility is currently defined as the biological incapacity of a person to conceive or the failure to develop a clinical pregnancy following a period of 12 months during which frequent, regular, unprotected sexual activity was engaged in without the use of contraception[1,2]. On the other hand, it has been observed that couples with delayed pregnancies would experience a 50% drop-in infertility rate after two years of exposure to an unprotected sexual intercourse period [3]. According to estimates from the WHO, 186 million people or 48 million couples worldwide are infertile[4]. In the last thirty years, Iraq exposed to several instability crises aggravation many health problems one of these problems was the fertility status of many people in the country[5]. In a local previous study, it was shown that the expression compact of specific gene with its receptor could increase susceptibility or risk of failure implantation and infertility [6,7].

Infertility in women has three major root causes, including defects in ovulation, transport, and implantation, according to the Centers for Disease Control[8]. In vitro fertilization and embryo transfer

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(IVF-ET) technology is an important choice for infertile couples. Following decades of research, laboratory embryo culture systems and ovulation induction protocols have been progressively developed, leading to improved quantity and quality of embryos. The implantation rate continues to be between 25 and 40 percent, which limits the success of IVF-ET[9]. In order to achieve pregnancy, the infertile couple must go through a synchronized series of actions throughout the implantation process [10]. There are many mediators under the control of ovarian hormones that are crucial during endometrial receptivity. Some of those mediators include cytokines, growth factors, and cellular adhesion molecules (CAM), amongst others[11]. One of the most important of these adhesion molecules is the integrin, Integrin are a family of transmembrane heterodimeric glycoproteins that facilitate cell-extracellular matrix adhesion[12]**.** The integrin family consists of 18 alpha and 8 beta subunits, which combine to generate 24 different heterodimers. Each integrin heterodimer consists of a large extracellular domain region, two single-pass transmembrane helices (one in each subunit), and short cytoplasmic tails[13]. The expression of integrins in the endometrium provides an overview for the assessment of endometrial receptivity[14]. They are also expressed in the endometrium during the time of implantation[15]. During implantation, integrins play a role in the attachment of the cells to the extracellular membrane (ECM) and initiate signaling transduction from the embryo to the ECM to initiate the translation of genes involved in the implantation process [16]. The genetic variance effect on endometrial receptivity, to IVF -implantation improving, or susceptibility risk of failure implantation and could use as screening methods [17]. Integrin alpha 2, which is found on chromosome 5q23–31 and has 30 exons, encodes the alpha subunit of the transmembrane receptor for collagen and related proteins[18]. Integrin, generally, may have a fundamental role in the incidence of some diseases, as its role in the pathogenesis of sickle cell anemia[19]. Polymorphisms of *ITGA2* are the main causes and risk for cardiovascular diseases, myocardial infarction and type 2 diabetes mellitus[20,21]. Integrin beta 3 is a subunit of both the GpIIIb/ IIIa receptor (integrin α I I b β 3) Integrin Subunit Beta 3 (*ITGB3*) encodes for the beta subunit (IIIa) of the glycoprotein GPIIb/IIIa (aIIbb3 integrin)[22]. This glycoprotein has a key role in platelet aggregation, clot retraction, and stabilization of thrombi by binding fibrinogen and von Willebrand factor[23]. The implication of inherited and acquired thrombophilia in IVF-ET failure has been proposed, probably by impairing the initial vascularization process occurring at implantation[24]. The low expression of β3 integrin in females with unexplained infertility supports the possible role of these molecules in the endometrial receptivity during implantation[25]. According to Yousif and Almohaidi's study, there is a high correlation between the polymorphisms rs4642 and rs4634, which are located on *ITGB3* in exon 10, and IVF implantation failure in Iraqi females[26]. Therefore, the aim of this study is to show how rs18011o6 on Integrin alpha2 and rs5918 on Integrin beta3 affect implantation rates.

Materials and Methods

This study was carried out between March 2021 to April 2022 to determine the association of some biomarkers and ITGB3 polymorphisms with implantation failure in females attending General Hospitals in Baghdad, Iraq.

Subjects

The female participants in the research ranged in age from 20 to 43 and were all in reproductive health; those without endocrine disorders, polycystic ovary syndrome, or male infertility were eliminated ; 71 infertile females under IVF protocol (divided into two subgroups , 29 success implantation and 42 failure implantation after follow up all these cases to determine the results of implantation after blastocysts implantation process within IVF program)and 50 fertile females as control were involved in the study, each fertile female have, at least, one previous birth.

Blood sampling

Venous blood samples (3ml) were collected from each female. Each blood was drawn in an EDTA tube and stored at -18°C until DNA extraction for ITGB3 gene variations determination.

Genetic Analysis

Each Participant’s peripheral whole blood samples were used to extract genomic DNA using the EasyPure®Genomic DNA Kit (TransGen, biotech. EE101-01, China). A Rotor gene Q Real-time PCR System (QIAGEN) was used to perform qPCR-

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HRM, followed by an HRM analysis with 0.2 °C scaling from 55 to 95 °C. The qPCR-HRM was utilized on duplicate synthetic controls to detect allelic differences, and the integrated program's HRM Tool was used to create normalized melting curves (NMC) and differential curves (DC) (Rotor gene 4.4). Primers required for qPCR-HRM were displayed in Table 1, And components of the reaction were shown in Table 2.

Table 1. The primer pairs used to amplify exon 10 at *ITGB3* gene.

Gene	Forward primer (5´ to 3´)	Reverse primer (5´ to 3´)	Length (bp)	Ref.
ITGB3	CTCTTTGGGCTCCTGTCTTACAG	GCAGATTCTCCTTCAGGTCACA	68	27
ITGA2	TGAGTGACCTAAAGAAAGAGGAAGGA	CAAATGCAAGTTAAATTACCAGTACTAAAGCA	117	27

Table 2. The HRM SNPs experiment uses quantitative real-time PCR components.

Component	Volume 20 µl
2xTransStart® Tip Green qPCR Super Mix	10 µl
Forward primer	1 µl
Revers primer	1 µl
Nuclease free water	4µl
DNA	4 µl

According to the thermal profile, the cycling protocol was programmed for the following optimized cycles, as given in Table 3.

Table 3. The thermal profile of HRM genotyping.

Step	Temperature (°C)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	**5**	
Annealing	60	15	**40**
Extension	72	20	
HRM	55 °C -95 °C	0.2sec for 1 degree	

Statistical analysis

Statistical analysis of data was performed using SPSS (Statistical Package for the Social Sciences - version 26). One-way ANOVA and the least significant difference (LSD) test were performed to assess significant differences among means. $P< 0.05$ was considered statistically significant. SNP statistical analysis and the Odds ratios along with the confidence intervals were calculated by using the WINPEPI computer programs for epidemiologists-version 11.65. Allele frequencies of genes were calculated by direct gene counting methods, while a departure from Hardy-Weinberg (H-W) equilibrium was estimated using the H-W calculator for two alleles, which is available online at https://gene-calc.pl/hardy-weinberg-page.

Results and Discussion

This study examined *ITGB3* and *ITGA2* polymorphism in fertile and infertile females who are under IVF protocol divided between the success of implantation and failure of it.

rs5918 polymorphism at *ITGB3*

Integrin beta 3 rs5918 polymorphism was genotyped in DNA samples from all study groups, and detection was made by HRM real-time PCR. Fig. 1 displays the thermocycler's output from the HRM analysis process for the three genotypes of rs5918. Fig. 2 shows a genomic region of rs5918 polymorphism.

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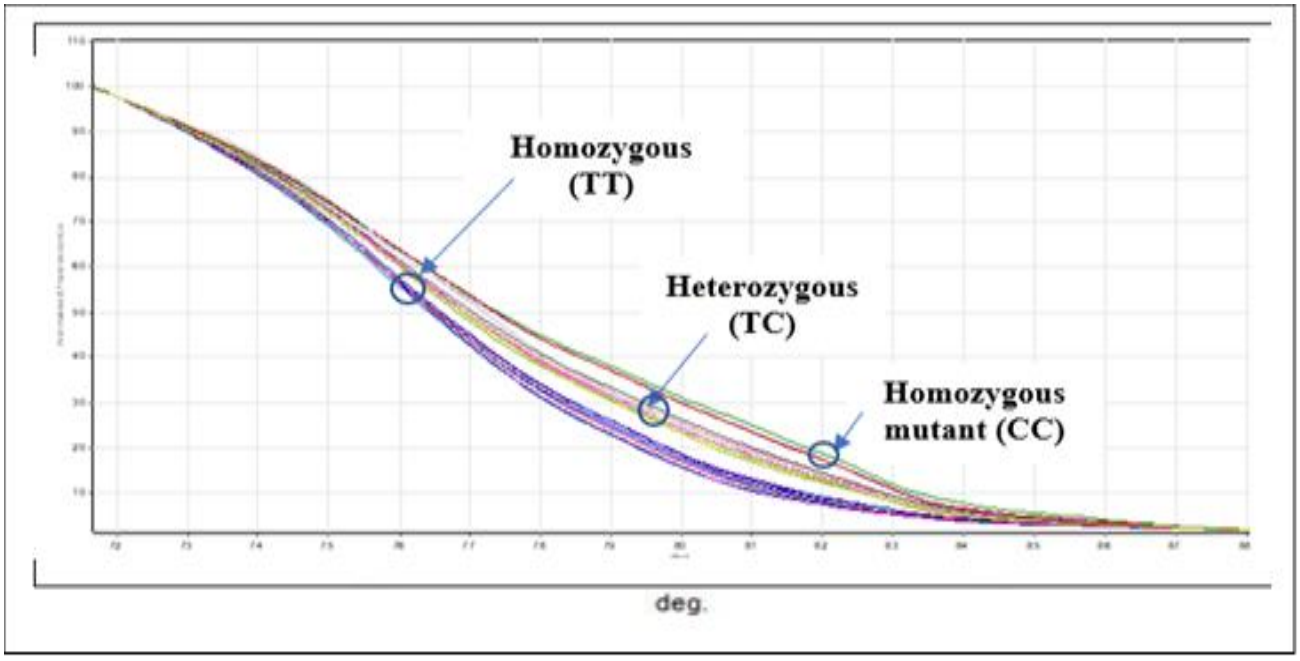

Figure 1. The result output of HRM for the three genotypes in rs5918

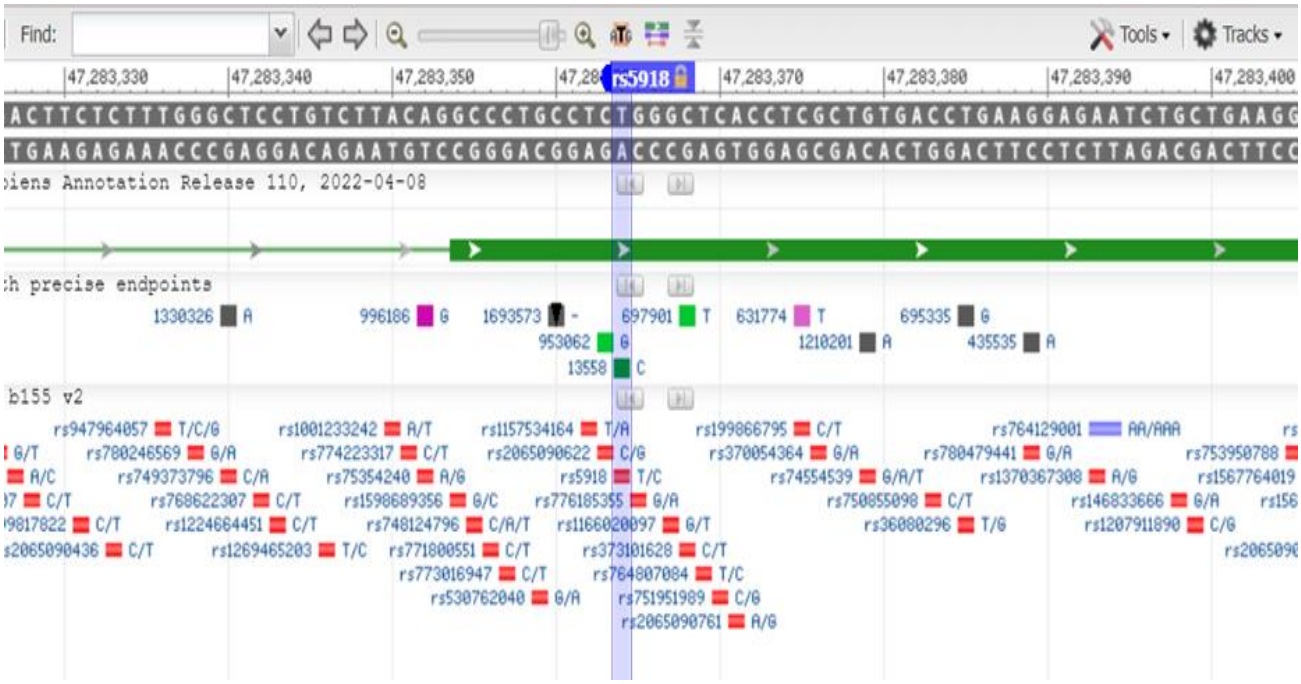

Figure 2. Variation viewer for genomic region of rs5918 polymorphism

The rs5918 of *ITGB3* gene (176T>C; located on chr17:47283364 was presented with three genotypes (TT, TC and CC) that were corresponding to two alleles (T and C). This SNP led to the substitution of leucine (Leu) to Proline (Pro). Therefore, the polymorphism has commonly been described as Leu33Pro [28]. Due to this substitution, the IIb-3 complex with very high affinity may be able inhibit platelet-fibrin interactions, and potentially be more effective as antithrombotic agents [29].

The genotyping results of rs5918 polymorphism was presented in Table 4 which demonstrates rates meaningful and significant differences in genotype distribution and allele frequency of rs5918 T > C polymorphism, among infertile and fertile groups. The odds ratio was >1 for both TC and CC genotypes, this indicates that the two genotypes act

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as risk factors for disease. While the wild genotype (TT) represents the protective factor. The observed TT genotype was high in control compared to other genotypes (TC, CC genotypes). This supports the protective efficacy of the TT genotype, as mentioned in Table 4. The frequency of the T allele is 77 in infertile and 22 in fertile; the frequency of the T allele as a whole is 99 from 240 alleles; as a result, minor allele frequency (MAF) = 99/240 = 0.41 (41%). This variation is regarded as a common variant by the HapMap project[30]. Minor allele frequency for this variant was 0.007, 0.13 and 0.128 for Asian, European-ancestry and African respectively, while it was absent in the Chinese study[31].The comparison between the success and failure cases shown in Table 5, recorded that the odds ratio for the wild type (TT) was (2.10), which gives an indication that it acts as a risk factor for the disease, in any way, there are no significant differences for the genotype and allele between success and failure groups. Even the preventive effects of genotype CC and allele C were evident, with corresponding risk factors of 0.63 and 0.69. As a result, from this finding could deduced that Iraqi women who had variant C at this site typically respond well to IVF treatment.

Table 4. Genotype and allele frequencies of *ITGB3* gene rs5918 polymorphism between infertile groups and control group.

Genotype	Study groups		Odds ratio	CI 95%	Fisher exact probability	Attributable fraction	Prevented fraction
	Infertile	Fertile					
TT	11(15%)	34(68%)	0.09	0.04 to 0.21	0.0[7.2E-9]		91.2%
TC	41(59%)	10(20%)	5.66	2.44 to 13.4	0.0[2.2E-5]	82.3%	
CC	18(26%)	6(12%)	2.54	0.94 to 7.48	0.053	60.6%	
Total	70	50					
Alleles distribution							
T	63(45%)	78(78%)	0. 23	0.13 to 0.41	0.0[2.5E-7]		76.9%
C	77(55%)	22(22%)	4.33	2.43 to 7.80	0.0[2.5E-7]	76.9%	

Table 5. Genotype and allele frequencies of *ITGB3* gene rs5918 polymorphism between success and failure groups.

Genotype	Study groups		Odds ratio	CI 95%	Fisher exact probability	Attributable fraction	Prevented fraction
	Failure	Success					
TT	8(20%)	3(10%)	2.10	0.51 to 10.5	0.261	52.4%	
TC	24(59%)	17(59%)	1.00	0.37 to 2.65	0.903	———	0.3%
CC	9(22%)	9(31%)	0.63	0.21 to 1.90	0.343	———	37.5%
Total	41	29					
Alleles distribution							
T	40(49%)	23(40%)	1.45	0.73 to 2.89	0.267	31.0%	———
C	42(51%)	35(60%)	0.69	0.35 to 1.37	0.267	———	31.0%

In addition, the studied population is constant according to Hardy-Weinberg Equilibrium as shown in Table 6. Whereas among control genotypes, the frequencies of genotypes and alleles were not consistent according to Hardy–Weinberg Equilibrium.

Table 6. Expected Frequencies of rs5918 at *ITGB3* Using Hardy-Weinberg Equilibrium for the expected frequencies of genotypes.

Groups		TT	TC	CC	X^2	P-value
Success genotypes	Observed	3	17	9	1.466	0.480 C
	Expected	4.56	13.88	10.56		
Failure genotypes	Observed	8	24	9	1.204	0.754 C
	Expected	9.76	20.49	10.76		
Control genotypes	Observed	34	10	6	8.704	1.012 NC
	Expected	30.42	17.16	2.42		
Total observed		45	51	24		

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Distribution consistent with Hardy Weinberg's law at the level of significance: 0.05 if P-value > 0.05 and X^2 < 3.84, distribution does not consistent with Hardy Weinberg's law at the level of significance:0.05 if P-value < 0.05 and X^2 > 3.84.

rs5918 polymorphism is associated with heightened platelet aggregability by enhancing the binding of *ITGB3* receptor to fibrinogen[32]. The presence of the *ITGB3* variants has been associated with an increased risk of arterial thrombosis[33]. The implication of inherited and acquired thrombophilia in IVF failure has been proposed, probably by impairing the initial vascularization process occurring at implantation[34]. It has been shown that there was a correlation between rs5918 T>C polymorphism and recurrent prognosis losses (RPL) risk in a sample of Iranian subjects in which this polymorphism was mainly observed among RPL cases[35]. Some of the performed studies have shown a significant association of rs5918 and rs1800790 variants with RPL through augmentation of immunological responses and coagulation activity and thereby placental dysfunction[36,37]. In contrast, Al-Astal and her collogue could not find any association between the risk of idiopathic recurrent abortion and rs5918 T > C polymorphisms either alone or in combination with other thrombophilia variants among cases with either primary or secondary RPL[38]. Also current results goes with a previous Iraqi study emphasize that gene polymorphism could effect on gene expression that related with endometrial and causes failure of implantation under IVF program for Iraqi Arab females [39]. Additionally, these SNPs require additional research on various ethnic groups within the Iraqi population with a large sample size.

rs18011o6 polymorphism at *ITGA2* gene

DNA samples from all study groups were genotyped for the *ITGA2* rs18011o6 polymorphism, which was then detected by HRM real-time PCR. The analysis of the three genotypes HRM results is shown in Fig. 3. The rs1801106 polymorphism's genomic region is depicted in Fig. 4.

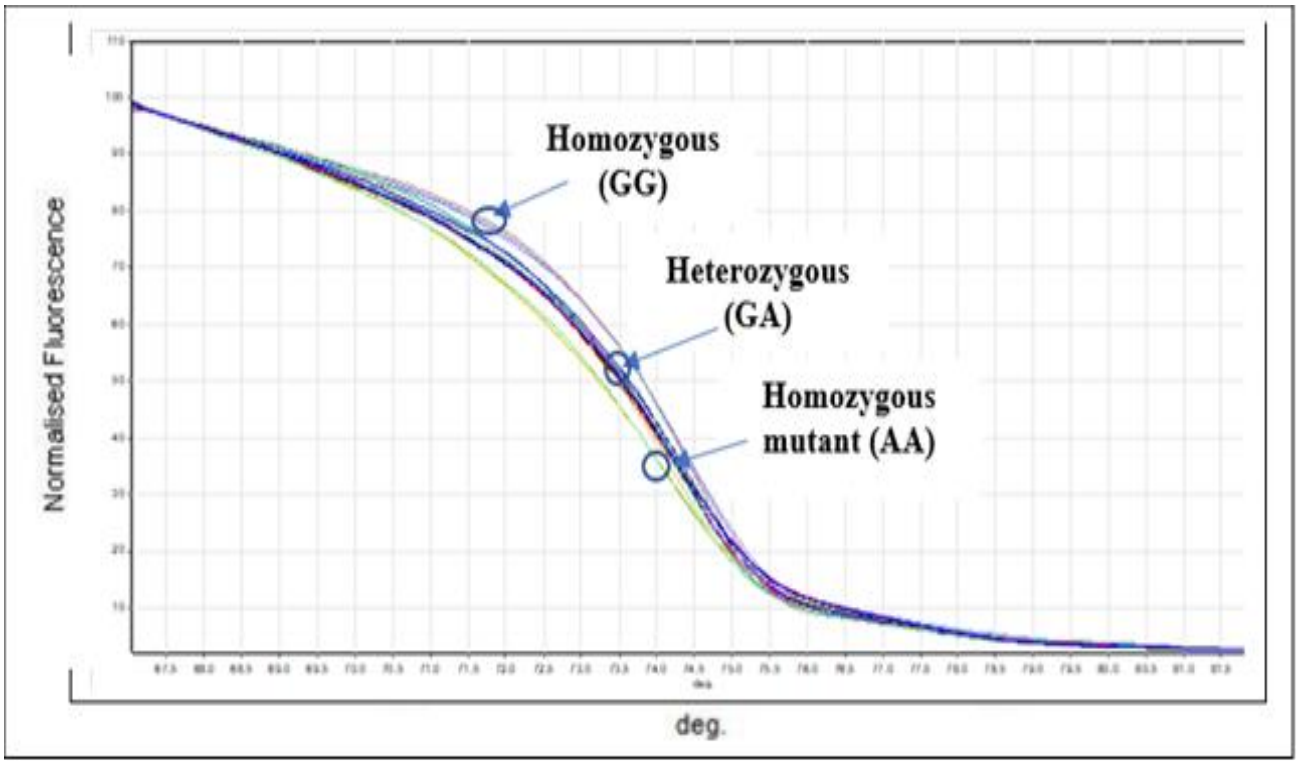

Figure 3. The outcome of HRM for the three rs1801106 genotypes

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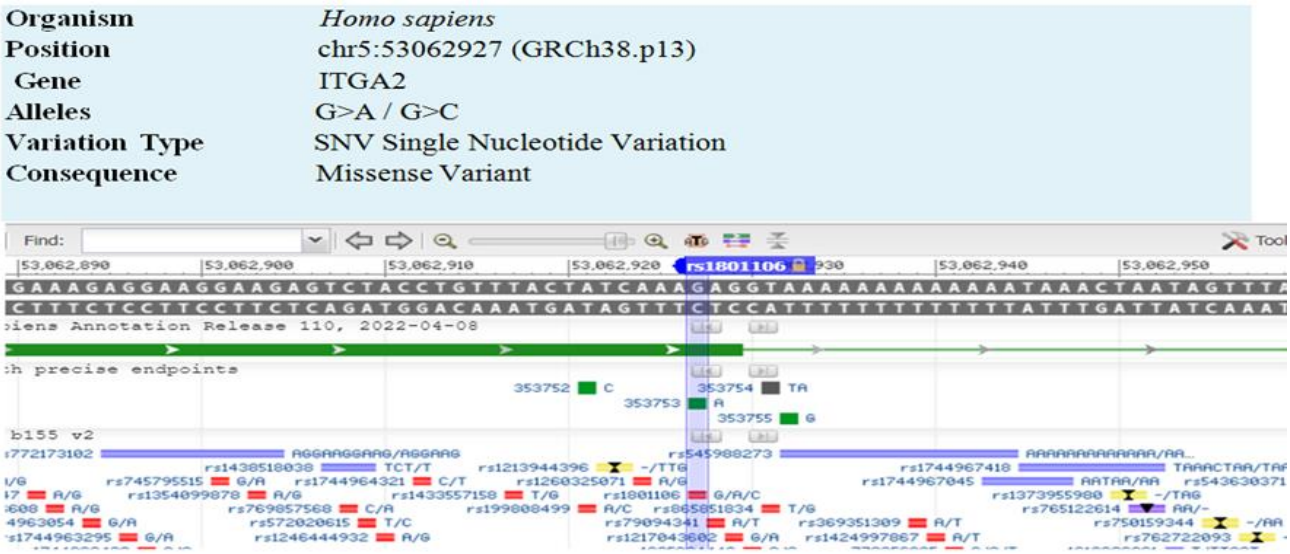

Figure 4. Variation viewer for genomic region of rs1801106 polymorphism

The genotyping results of rs1801106 polymorphism are displayed in Table 7, which indicates that there are significant differences in genotype distribution and allele frequency of rs1801106 polymorphism, among infertile and fertile groups. Both the GA and AA genotypes' odds ratios were more than 1, which suggests that the two genotypes serve as risk factors for illness. While the protective factor is represented by the wild genotype (GG), these results were identical to what current study obtained when comparing success and failure, but significant differences were absent in the two genotypes GA and AA, as shown in table 8. The observed genotype frequencies in both success and control were higher than those predicted by the Hardy-Weinberg Equilibrium as shown in table 9. These values were not consistent with H.W.E. so these results may suggest that evolutionary selection may be taking place at this locus in the Iraqi population.

Table 7. The genotype and allele frequencies of the *ITGA2* rs18011o6 polymorphism in comparison to the control group and infertile groups.

Genotype	Study groups		Odds ratio	CI 95%	Fisher exact probability	Attributable fraction	Prevented fraction
	Infertile	Fertile					
GG	26(37%)	34(68%)	0.28	0.13 to 0.60	0.001	———	72.2%
GA	21(30%)	10(20%)	1.71	0.73 to 4.19	0.225	41.7%	———
AA	23(33%)	6(12%)	3.59	1.36 to 10.3	0.007	72.1%	———
Total	70	50					
Alleles distribution							
G	73(52%)	78(78%)	0. 31	0.17 to 0.55	0.0[3.4E-5]	———	69.3%
A	67(48%)	22(22%)	3.25	1.83 to 5.86	0.0[3.4E-5]	69.3%	———

Table 8. Genotype and allele frequencies of *ITGA2* gene rs18011o6 polymorphism at between success and failure groups.

Genotype	Study groups		Odds ratio	CI 95%	Fisher exact probability	Attributable fraction	Prevented fraction
	Failure	Success					
GG	10(24%)	16(55%)	0.26	0.09 to 0.74	0.009	———	73.8%
GA	15(37%)	6(21%)	2.21	0.73 to 7.07	0.152	54.8%	———
AA	16(39%)	7(24%)	2.01	0.70 to 6.06	0.165	50.3%	———
Total	41	29					
Alleles distribution							
G	35(43%)	38(66%)	0.39	0.19 to 0.79	0.008	———	60.8%
A	47(51%)	20(60%)	2.55	1.27 to 5.16	0.008	60.8%	———

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Table 9. Expected Frequencies of rs1801106 at *ITGA2*Using Hardy-Weinberg Equilibrium for the expected frequencies of genotypes.

Groups		GG	GA	AA	X^2	P-value
Success genotypes	Observed	16	6	7	8.522	0.014 NC
	Expected	12.45	13.1	3.45		
Failure genotypes	Observed	10	15	16	2.609	0.721 C
	Expected	7.47	20.06	13.47		
Control genotypes	Observed	34	10	6	8.704	1.012 NC
	Expected	30.42	17.16	2.42		
Total observed		60	31	29		

Distribution consistent with Hardy Weinberg's law at the level of significance: 0.05 if P-value > 0.05 and X^2 < 3.84, distribution does not consistent with Hardy Weinberg's law at the level of significance:0.05 if P-value < 0.05 and X^2 > 3.84.

The effect of the rs1801106 genotypes on platelet adhesion assay demonstrated that platelets from GG donors bound to decorin more strongly than those from GA+AA donors, yet the two groups bind to collagen I. Therefore, the present study suggests that rs1801106 affects the affinity of *ITGA2*B1 for decorin and that it probably functions as a crucial part of the binding site for decorin. These findings outline a new paradigm for alternative ligand binding and offer the first proof that *ITGA2* SNP rs1801106 has an impact on *ITGA2*B1 function[40]. Integrin alpha 2 is a major receptor for collagen mediating platelet adhesion and activation is found on platelet membranes[41]. On the other hand, *ITGA2* is present in human endometrial and trophoblast cells and may play an important role during the early stages of implantation and placentation [42]. All current finding goes with previous Iraqi finding [6,39] that Genes Polymorphism and expression could effect on IVF outcomes.

Conclusion

The TT genotype of rs5918 is considered a protective agent, while TC and CC genotypes act as etiological factors associated with infertility. Allele C is an etiological factor associated with implantation failure, while allele T is the protective agent. In addition, the GG genotype of rs1801106 in *ITGA2* was significantly higher in fertile females than infertile, which indicates that the GG genotype acts as a protective factor, while the AA genotype is an etiological factor. These findings could have a biological explanation that involves either change to implantation events or to the vascular supply throughout the early stages of pregnancy. Furthermore, any variation in the *ITGB3* gene could lead to changes either in thrombophilia which in turn affects the vascular supply in the primary intervillous space during the first days of gestation or in implantation events.

Acknowledgment

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- No animal studies are present in the manuscript.
- No potentially identified images or data are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at the College of Science for Women, the University of Baghdad,

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Baghdad, Iraq, and Roh al-Hayat Center in Baghdad, Iraq.

Authors' Contribution Statement

This study was carried out with the contribution of all authors. A. M. S. A. was the one who developed the idea of the project and fully supervised it and designed the primers, while the practical part and writing the theoretical part was the responsibility of R. A. Y.

Journal Declaration:

Second author A. M. S. A. is an editor for the journal but did not participate in the peer review process other than as an author. The authors declare no other conflict of interest.

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تعدد الطرز الوراثية لمورث *الإنتجرين ألفا 2* ومورث *الإنتجرين بيتا* 3 كعوامل خطر للعقم عند الإناث العراقيات العقيمات الخاضعات لبرنامج الاخصاب خارج الجسم

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الخلاصة

صُممت الدراسة الحالية للتحري عن تأثير تعدد الطرز أحادي النوكليوتيدات (SNP) ، للتتابع المرجعي 5918 ،(T> C) لمورث إنتيجرين بيتا 3 (ITGB3) وللتابع المرجعي 1801106 (G> A) إنتجرين ألفا 2 (ITGB3) في عينات دم اناث عراقيات. 71 أنثى مصابة بالعقم خاضعة لبروتوكول أطفال الأنابيب (مقسمة إلى مجموعتين فرعيتين ، 29 عملية زرع ناجحة و 42 عملية زرع فاشلة) و 50 أنثى خصبة كمجموعة سيطرة. تم الحصول على عينات الدم من المرضى ومجموعات السيطرة في المستشفيات العامة في بغداد ، العراق من مارس 2021 إلى أبريل 2021. تم إجراء التنميط الوراثي لتعدد الطرز باستخدام تحليل الذوبان عالي الدقة في الوقت الحقيقي (HRM في الوقت الحقيقي PCR) من الحمض النووي الرايبوزي منقوص الاوكسجين الجينومي المنقى المأخوذ من عينات الدم. أظهرت النتائج أن الطرز الوراثية TT و GG للتتابعات المرجعية 5918 في ITGB3 و 1801106 في ITGA2 على التوالي ، كانت أعلى بشكل ملحوظ في الإناث الخصبة من الاناث العقيمات. يشير ذلك إلى أن الطراز الوراثي GG يعمل كعامل وقائي ، بينما يعمل الطراز الوراثي TC للتتابع المرجعي 5918 في ITGB3 و AA من الطراز الوراثي للتتابع المرجعي 1801106 في ITGA2 كعوامل خطر. الاستنتاج ، ان لتعدد الطرز في جينات ITGA2 و ITGB3 تأثير على استعداد مجموعة من الاناث ضمن الدر اسة للإصابة بالعقم.

الكلمات المفتاحية: تحليل عالي الدقة ، الانغراس ، جين الانتجرين الفا 2 ، جين الانتجرين بيتا 3 ، الاخصاب خارج الجسم .