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Association of Single Nucleotide Polymorphisms in Vascular Endothelial Growth Factor with Recurrent Pregnancy Loss in Erbil Province: A Molecular Investigation

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ABSTRACT

Recurrent pregnancy loss (RPL) is defined as two or more failed pregnancies before 20 weeks of gestation. Genetic polymorphisms, especially alterations in the vascular endothelial growth factor (VEGF) gene, may increase the risk of pregnancy loss. This study aimed to investigate the connection between changes in the VEGF-A gene (-460C/T, -1190A/G, and -1154A/G) and RPL in women from the Erbil Province. The study included 60 women with a history of RPL and 30 healthy women with no history of miscarriage. Genomic DNA was isolated from whole blood samples. The VEGF-A promoter region was amplified using Touchdown PCR, and the resulting PCR products were analyzed by agarose gel electrophoresis and then processed for Sanger sequencing. Statistical analysis was performed using Yates-corrected χ^2 tests or Fisher's exact tests for small sample sizes. The results demonstrated that the -460 TT genotype was markedly more prevalent in RPL patients than in controls, increasing the risk by 3.3 times. The -460T allele significantly increased the probability of RPL (OR = 3.3, p = 0.0025, 95% CI: 1.49–7.29). For the -1190A/G polymorphism, the homozygous GG genotype was more prevalent in RPL cases, and the -1190G allele was associated with an increased risk (OR = 2.64, p = 0.0138, 95% CI: 1.26–5.52), identifying it as the risk allele for this SNP. In contrast, the -1154A/G polymorphism showed no significant differences in either genotype or allele frequencies between RPL cases and controls (p > 0.05). These findings suggest that some genotypic alterations, notably -460 TT and -1190 GG, may act as genetic risk factors for RPL.

1.Introduction

Abortion is a voluntary medical procedure to terminate a pregnancy, usually conducted using medication or surgical methods, whereas miscarriage refers to the involuntary and spontaneous loss of pregnancy, predominantly occurring in the first stages (Costescu et al., 2016). Recurrent pregnancy loss (RPL), characterized by two or more consecutive clinically confirmed spontaneous abortions prior to 20 weeks of gestation, leading to embryonic or fetal death, is a prevalent infertility-related condition affecting 1–5% of birthing individuals (Li et al., 2022). RPL is categorized into two types: primary and secondary. Primary recurrent pregnancy loss occur in women who have never given birth to a live child, whereas secondary recurrent pregnancy loss refers to women who have previously delivered a living child (Turesheva et al., 2023). Recognized causes of RPL consist of parental chromosomal irregularities, uterine issues, inherited thrombophilias, hormonal disorders, immune system factors, infections, and nutritional and environmental influences. However, most cases of RPL still have no identifiable cause and are considered idiopathic (Li et al., 2013).

One of the crucial factors involved in these processes is vascular endothelial growth factor (VEGF), which controls the differentiation, migration, and proliferation of endothelial cells and is essential for angiogenesis during placental development. Polymorphisms in the VEGF gene have been associated with an increased risk of the RPL (Şamlı et al., 2012, Guo et al., 2021). VEGF is a homo-dimeric glycoprotein that interacts with heparin-binding receptors on endothelial cells to regulate vascular permeability and angiogenesis (Almawi et al., 2016). The human VEGF family has five growth factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF), each of which works differently with the VEGFR cellular receptors (VEGFR-1, VEGFR-2, and VEGFR-3) (Failla et al., 2024). VEGF-A interacts with VEGFR2, also known as KDR or Flk-1, as well as VEGFR1; VEGF-C and VEGF-D associate with both VEGFR2 and VEGFR3 (Flt4), while PLGF and VEGF-B selectively attach to VEGFR1. Additionally, some neuropilins (NRPs), which are non-tyrosine kinase receptors, bind specific VEGF isoforms (Holmes and Zachary, 2005). Previous studies have reported associations between VEGF gene polymorphisms particularly in the promoter, 5'-untranslated, and 3'-untranslated regions and RPL (Aggarwal et al., 2011, Mrozikiewicz et al., 2023). Variations in the VEGF promoter are particularly significant, as they may affect gene expression and disrupt placental angiogenesis. The -1154G/A polymorphism, defined by the substitution of a guanine (G) allele with an adenine (A) allele at position -1154, has been extensively studied, with the A allele frequently reported to increase susceptibility to RPL (Vidyadhari et al., 2019). Similarly, the -460C/T polymorphism has been linked to an increased risk of miscarriage, especially in those with the CT and TT genotypes (Yalcintepe et al., 2014). Furthermore, there is limited research on the -1190A/G (rs13207351) polymorphism in the VEGFA gene in relation to RPL. This variation has predominantly been studied for its clinical relevance in head and neck malignancies and diabetic retinopathy (Yang et al., 2011, Dimitrakopoulos et al., 2021). This study aims to investigate the genotype and allelic distribution of SNPs associated with RPL and determine whether there is any correlation between RPL and the VEGF-A -460C/T, -1190A/G, and -1154A/G polymorphisms. These SNPs were selected because they are located in the VEGF promoter region and may influence gene expression related to angiogenesis. Additionally, the study examines demographic factors, including maternal age, family relationships, the number of healthy births, blood types, and past miscarriages, to better understand the genetic factors contributing to RPL.

2. Materials and Methods

2.1. Sample Collection

Peripheral blood samples were collected from 90 participants at Zheen Hospital (Exo-gene lab) in Erbil City between July 2024 and October 2024. The participants were aged between 20 and 45 years. Patients and controls were given a particular questionnaire form, which had certain areas to fill in about the patient (name, consanguinity, address, age, blood group, number of miscarriages, genetic illnesses, and primary or secondary recurrent pregnancy loss). Furthermore, the questionnaire data were augmented with information obtained from hospital medical records, aided by supporting staff, and through direct patient interviews. The blood samples were stored in 2-ml EDTA tubes at -20°C to prevent degradation and denaturation.

2.2. DNA Extraction

Genomic DNA was isolated from 200 µL of whole blood utilizing the PureLink® DNA Isolation Kit (Invitrogen, ThermoFisher Scientific, USA), according to the manufacturer's instructions. The purity and quality of the extracted DNA were measured with a NanoDrop™ 1000 spectrophotometer, and the results were recorded in nanograms per microliter (ng/µl). The absorbance ratio (260/280), which shows DNA purity, was between 1.7 and 1.8.

2.3. Touchdown PCR (TD-PCR)

As part of the amplification process, a primer pair was designed for a specific promoter region in the VEGF-A gene. PCR amplification was performed using the Touchdown PCR (TD-PCR) method. The optimal temperature for the primers was 62°C, as shown in (Table 1). TD-PCR was used to detect the VEGF-A gene SNP using an Alpha-cycler 96 well. A 30 µl volume of PCR reaction made up of 1.5 µl of each forward and reverse primers, 10 µl of template DNA, and 17 µl of Taq 2x Master Mix Red with 1.5 mM $MgCl_2$ (Ampliqon, Denmark). The target gene was amplified with the TD-PCR method; thermal cycling settings are reported in (Table 2).

Table 1. Primer sequences for the promoter region of the VEGF-A gene with corresponding nucleotide sequence, melting temperature (Tm), and primer length (nt).

Product Name	Primer	Oligonucleotide Sequence (5′-3′)	Tm (°C)	Primer length(nt)
VEGF-A	Forward primer	5'-GAGCAGCGTCTTCGAGAGTG -3'	62.4	20
	Reverse primer	5'-TCAAATTCCAGCACCGAGCG -3'	62.8	20

Note: nt: nucleotides

Table 2. The TD-PCR cycle's steps for VEGF-A.

Stage	Step	Temperature (°C)	Time	No. of cycle
Initial step	Initial denaturation	95	5 min	1 cycle
Stage 1	Denaturation	94	30 sec	10 cycles
	Annealing	62	30 sec	
	Extension	72	30 sec	
Stage 2	Denaturation	94	30 sec	32 cycles

	Annealing	57	30 sec	
	Extension	72	30 sec	
Final step	Final extension	72	5 min	1 cycle
Hold	Hold	4	∞	

2.4. Gel Electrophoresis

A 2% agarose gel was prepared by dissolving agarose powder in 200 mL of 1× Tris-Borate-EDTA (TBE) buffer through heating, then cooling the solution to approximately 50°C. One microliter of DNA-safe stain (Sinaclon, Iran) was added for visualization. The gel was poured into a tray with a comb to create wells and allowed to solidify at room temperature for 15–20 minutes. After the gel set, 10 µL of each PCR product was put into wells, and 10 µL of the 100 bp DNA ladder (Bio-Rad, USA) was added to the first lane to serve as a molecular size marker. Electrophoresis was conducted at 200 volts for 20 minutes, allowing the negatively charged DNA fragments to migrate toward the positive electrode (anode). DNA bands were visualized under UV illumination using a transilluminator (InGenius3, Syngene, UK).

2.5. Sanger Sequencing

Seventy-seven PCR products (52 patients and 25 controls) were sequenced in one direction from the forward strand using an Iranian 3130 genetic analyzer (Applied Biosystems, Hitachi High-Technologies, Tokyo, Japan) from the Immunogene Center in Erbil, Kurdistan, Iraq. FinchTV and Mutation Surveyor (SoftGenetics, State College, PA, USA) were used to analyze sequencing chromatograms, identify DNA sequence changes, and compare them to the wild-type VEGF-A reference sequence (NCBI RefSeq NG_008732.1).

2.6. Statistical Analysis

Statistical analyses were conducted using GraphPad version 8.0.2 (San Diego, California, USA). Group comparisons were conducted using the nonparametric Mann-Whitney test when appropriate, such as for analyzing participants' ages. VEGF-A genotype and allele frequency differences between RPL cases and controls were evaluated using 2×2 contingency tables with Fisher's exact testing for small sample numbers or Yates-corrected χ^2 tests. Odds ratios (ORs), which quantify the degree of correlation between allele frequencies and RPL, and 95% CIs were calculated using logistic regression analysis using the control group as a reference. All *P*-values were two-tailed, and $p < 0.05$ was considered statistically significant

3. Results

3.1. Demographical Study:

In this study, 90 women were recruited, 60 with history of RPL; their median age was 31.5 years (range: 20–42). The control group consisted of 30 fertile women with a median age of 28.5 years (range: 20–45). The median age did not significantly differ between the two groups ($p = 0.4$). Pedigree analysis revealed that 22 women across both groups were in consanguineous marriages: 16 in the RPL group (26.7%) and 6 in the control group (20%). However, this difference was not statistically significant ($p = 0.48$). Regarding the blood group distribution, there was a statistically significant difference between the RPL patients and controls ($p = 0.0012$). All patients were tested and confirmed negative for toxoplasmosis infection, as summarized in (Table 3).

Table 3. Baseline and demographic characteristics of the patients and controls.

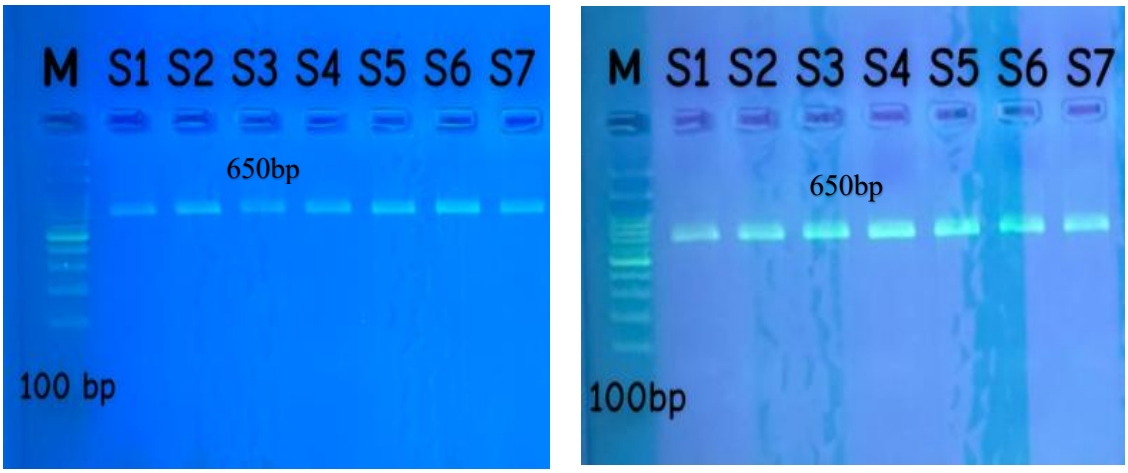
Variable	Number of Controls (%)	Number of Patients (%)	*p*-value
Total number of samples	30	60	
Age, median (range)	28.5 (20 – 45)	31.5 (20 – 42)	0.4
20-29	17 (56.7%)	20 (33.3%)	
30-39	9 (30%)	36 (60 %)	
40-45	4 (13.3%)	4 (6.67%)	
Consanguinity			0.48
NO	24 (80%)	44 (73.3%)	
YES	6 (20%)	16 (26.7%)	
Toxoplasma infection status			
Negative	30 (100%)	60 (100%)	
Positive	0	0	
Blood group			0.0012
O+	13 (43.33%)	23 (38.33%)	
A+	10 (33.33%)	14 (23.33%)	
B+	1 (3.33%)	17 (28.33%)	
O-	5 (16.67%)	0	
AB-	1 (3.33%)	2 (3.33%)	
AB+	0	3 (5%)	
A-	0	1 (1.67%)	
Normal Birth, median	2 (1 – 7)	0	0.0001
Primary or Secondary RPL			
Primary		45 (75%)	
Secondary		15 (25%)	
Number of miscarriages			
2 times		18 (30%)	
3 times		24 (40%)	
4 times		7 (11.67%)	
5 times		5 (8.33%)	
6 times		2 (3.33%)	
7 times		1 (1.67%)	
8 times		2 (3.33%)	
9 times		1 (1.67%)	

Note: All patients tested negative for Toxoplasma infection; therefore, statistical analysis for this variable was not applicable.

3.2. Agarose Gel Electrophoresis

Figures 1a and 1b present the results of gel electrophoresis for the PCR products of the amplified VEGF-A gene at the promoter region. These figures show discrete bands that correspond to the expected PCR product size 650 bp, as established by comparison

with the 100 bp DNA ladder. The clarity and intensity of the bands indicate a high yield and specificity of the PCR process.



A) B)

Figure 1. Agarose gel electrophoresis of the PCR-amplified VEGF-A promoter region. A) Patient group: lane 1 represents the 100 bp DNA ladder, while lanes 2–8 display PCR products from patient samples. B) Control group: lane 1 represents the 100 bp DNA ladder, while lanes 2–8 display PCR products from control samples. A 100 bp DNA ladder was used as a molecular size marker in all analyses.

3.3. Genetic Association of VEGF-A Gene Polymorphisms with RPL

Table 5 presents the genotype frequencies of VEGF -460 C/T (rs833061), -1190 A/G (rs13207351), and -1154 A/G (rs1570360) in both the control and RPL patient groups. These polymorphisms are found in the promoter region of the VEGF-A gene, as indicated in (Table 4). In this study, the genotypic frequencies of the three variants among RPL patients and control women are summarized in (Table 5). The genotype distribution for -460 C/T among RPL patients were 44.23% for C/C, 21.15% for C/T, and 34.62% for T/T, compared to 72% for C/C, 16% for C/T, and 12% for T/T in the control group. The -460T allele raised the probability of RPL 3.3 times (OR = 3.3, 95% CI: 1.49–7.29, p = 0.0025). Furthermore, for the -1190 A/G polymorphism, the genotype frequencies were as follows: 44.23% of cases and 68% of controls were homozygous AA; 15.38% of cases and 12% of controls were heterozygous AG; and 40.38% of cases and 20% of controls were homozygous GG. The mutant homozygous genotype GG of the -1190 A/G polymorphism was significantly more prevalent in RPL patients than in controls. The relative risk associated with the -1190G allele was 2.64, with a 95% confidence range of 1.26 to 5.52. In addition to the, -1154A/G polymorphisms the results show that the women with RPL had genotype frequencies of 42.3% for A/A, 7.69% for A/G, and 50% for G/G, whereas control women demonstrated frequencies of 56% for A/A, 12% for A/G, and 32% for G/G. There was no significant difference observed in the genotypic and allelic frequencies between patients and controls (p > 0.05). In Table 6, the allele frequencies of the three polymorphisms are presented.

Table 4. Three SNPs in the VEGF-A promoter region identified from the gene's RefSeq in the NCBI database.

Strand	Sequence
SNP -460 C/T	GTGTGGGGTTGAGGGC/TGTTGGAGCGGGG
SNP -1190 A/G	GGCCAGGCTTCACTGA/GGCGTCCGCAGAG
SNP -1154 A/G	CGAGCCGCGTGTGGAA/GGGGCTGAGGCTC

Table 5. Genotype frequencies of VEGF-A gene polymorphisms (-460C/T, -1190A/G, and -1154A/G) in women with RPL compared to healthy controls.

Genotypes	RPL (n=52)	Controls (n=25)	Total (n=77)	*p*-value
-460 C/T T/T C/T C/C	 18 (34.6) 11 (21.15) 23 (44.23)	 4 (12) 3 (16) 18 (72)	 22 (28.57) 14 (18.18) 41 (53.24)	 TT vs CC:0.078 CT vs. CC:0.24 CC vs. CT,TT:0.02*
-1190 A/G G/G A/G A/A	 21 (40.38) 8 (15.38) 23 (44.23)	 5 (20) 3 (12) 17 (68)	 26 (33.7) 11 (14.2) 40 (51.9)	 GG vs. AA:0.05 AG vs. AA:0.57 AA vs. AG,GG:0.05
-1154 A/G G/G A/G A/A	 26 (50) 4 (7.69) 22 (42.3)	 8 (32) 3 (12) 14 (56)	 34 (44.15) 7 (90.9) 36 (46.75)	 GG vs. AA:0.26 AG vs. AA:0.82 AA vs. AG,GG:0.3

Note: Data are presented as n (%). RPL recurrent pregnancy loss.

Table 6. Allelic frequencies of VEGF-A gene polymorphisms (-460C/T, -1190A/G, and -1154A/G) in women with RPL compared to healthy controls.

Alleles	RPL (n=104)	Controls (n=50)	OR	95%CI	*p*-value
-460 C/T C T	 57(54.8) 47(45.1)	 40(80) 10(20)	 0.3 3.3	 (0.14–0.67) –7.29)1.49(	 0.0025 **
-1190 A/G A G	 54 (51.92) 50 (48.08)	 37(74) 13(26)	 0.38 2.64	 (0.18–0.80) (1.26–5.52)	 0.0138 *
-1154 A/G A G	 48(46.1) 56(53.8)	 31 (62) 19(38)	 0.53 1.9	 (0.26–1.05) (0.96–3.17)	 0.0851 NS

Note: Data are presented as n (%). RPL recurrent pregnancy loss; OR odds ratio; CI confidence interval; NS = not statistically significant. T allele is the risk allele for -460C/T SNP; G allele is the risk allele for -1190A/G SNP.

3.4. Chromatogram Results of VEGF-A Gene Polymorphisms in RPL

The PCR products of SNPs with different genotypes were selected and verified by direct Sanger sequencing of -460C/T (rs833061), -1190A/G (rs13207351), and -1154A/G (rs1570360). The sequences were analyzed using Mutation Surveyor and FinchTV. Analysis of three different PCR

product samples confirmed the presence of the -460C/T (rs833061) polymorphism. The results revealed a polymorphism in the promoter region at position -460 base pairs (bp), where cytosine (C) is substituted by thymine (T) (Figure 2). In this figure, "Y" is a degenerate code representing the heterozygous genotype CT. For the -1190A/G (rs13207351) and -1154A/G (rs1570360) polymorphisms, adenine (A) is substituted by guanine (G) (Figures 3 and 4). "R" is a degenerate code representing the heterozygous genotype AG.

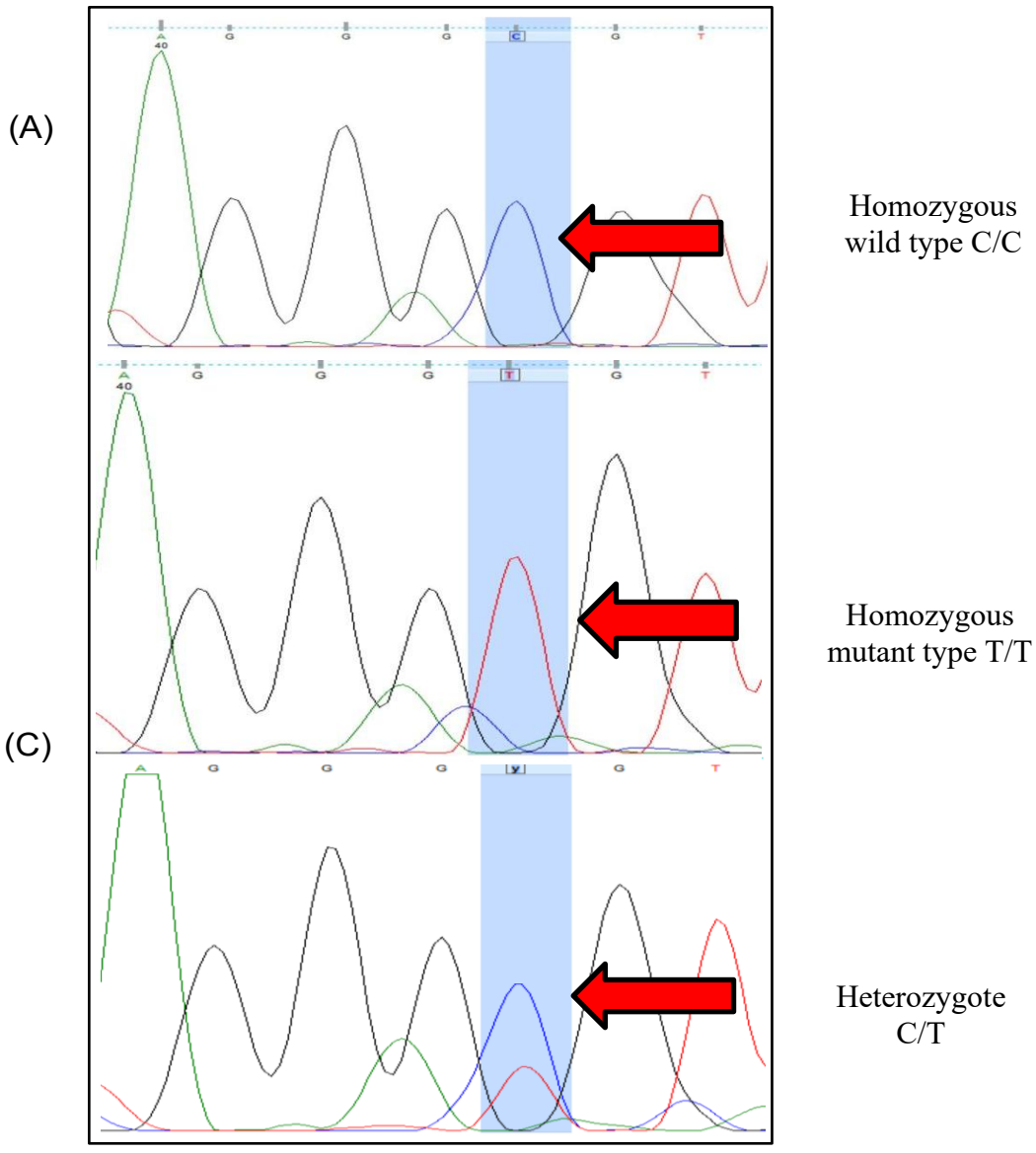


Figure 2. DNA sequencing chromatograms illustrating the -460C/T (rs833061) polymorphism in three PCR product samples. (A) Homozygous wild-type genotype CC, indicated by a single arrow-marked peak. (B) Homozygous mutant genotype TT, shown as a single peak with an arrow. (C) The heterozygous genotype CT was represented by two peaks with an arrow; represented Y

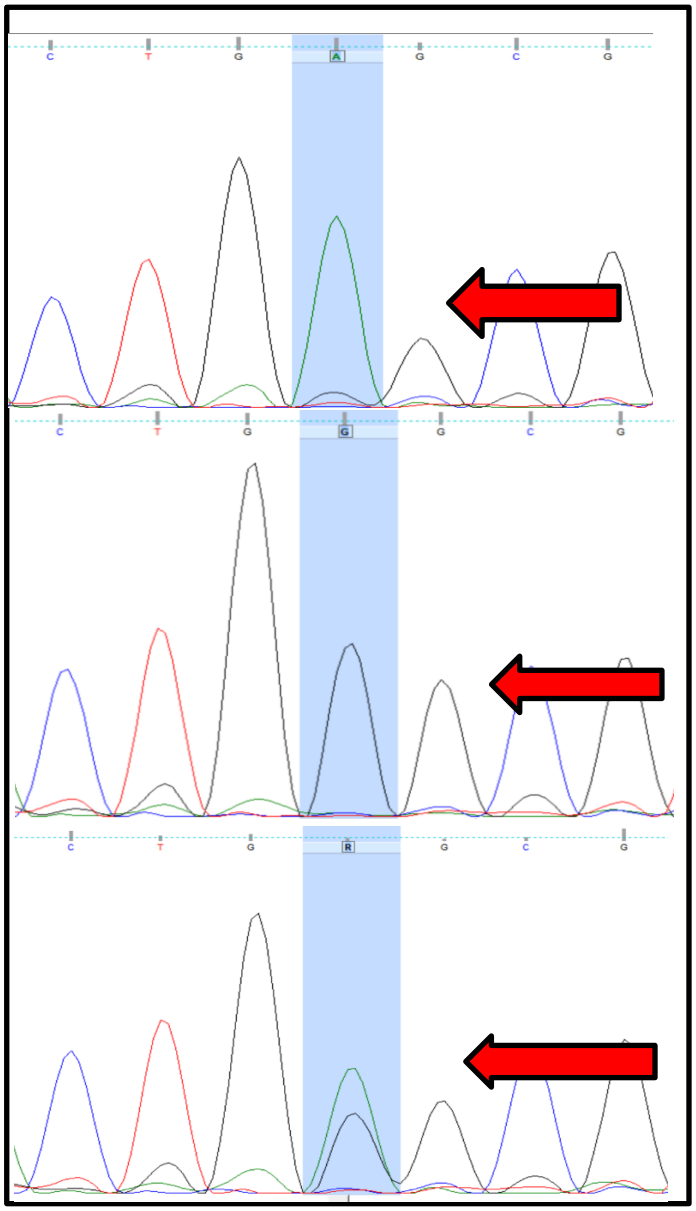


Figure 3. DNA sequencing chromatograms illustrating the -1190A/G (rs13207351) polymorphism in three PCR-amplified samples. (A) Wild-type homozygous genotype AA, indicated by a single arrow-marked peak. (B) Mutant homozygous genotype GG, shown as a single peak with an arrow. (C) The heterozygous genotype AG was represented by two peaks with an arrow; represented R

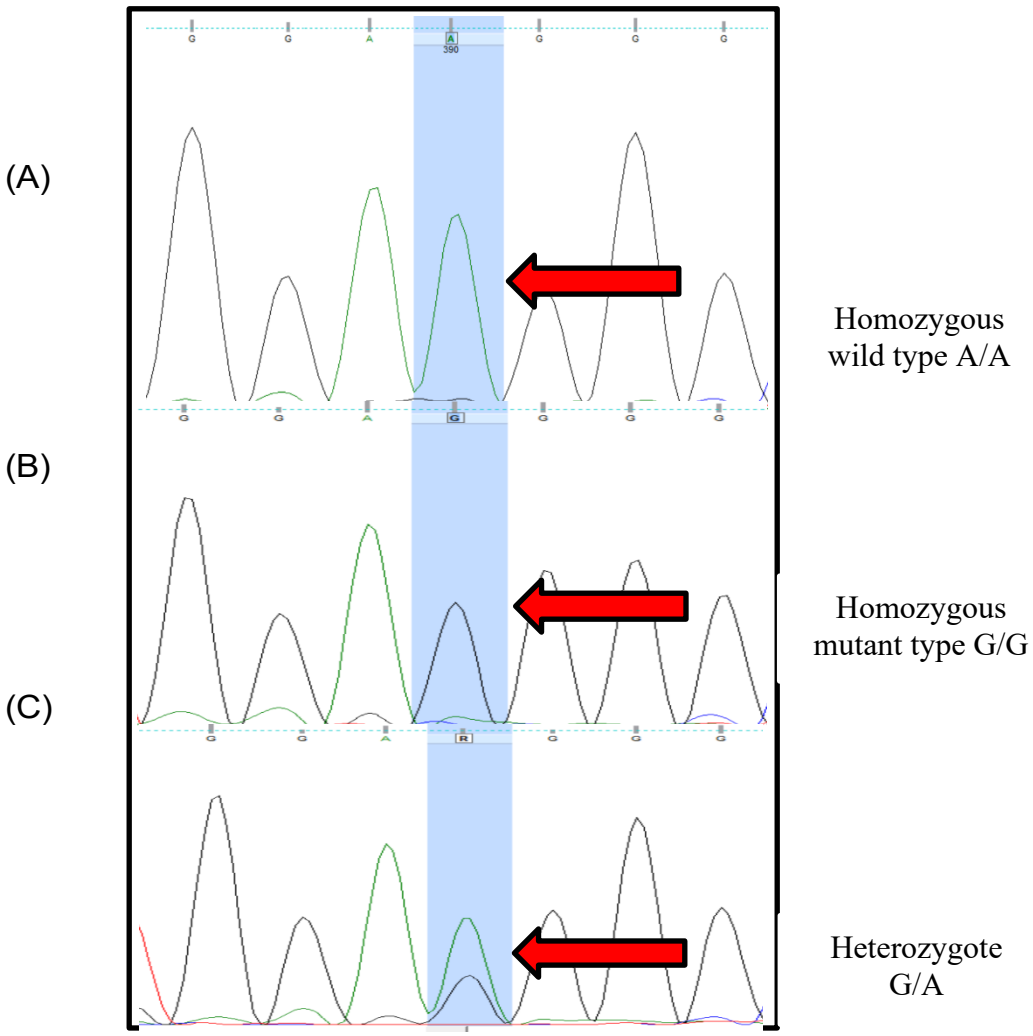


Figure 4. DNA sequencing chromatograms illustrating the -1154A/G (rs1570360) polymorphism in three PCR-amplified samples. (A) Wild-type homozygous genotype AA, indicated by a single arrow-marked peak. (B) Mutant homozygous genotype GG, shown as a single peak with an arrow. (C) The heterozygous genotype AG was represented by two peaks with an arrow; represented R

4. Discussion

Members of the VEGF family have roles in several biological processes, including oxidative stress, inflammation, angiogenesis, lymphopoiesis, and lipid metabolism (Kang et al., 2024). When comparing RPL to controls, significantly reduced vascular, stromal, and glandular expression patterns of endometrial VEGF were seen (Abu-Ghazaleh et al., 2023). The results of this study indicate that consanguinity may not be a substantial independent risk factor for RPL in this population, since the statistically significant $p = 0.48$ was observed between the RPL and control groups. Additionally, the age difference between the two groups was shown to be statistically insignificant ($p > 0.05$). VEGF-A is a highly polymorphic gene with several identified SNPs in the promoter regions, as reported by Ajaz et al. (2021). This study investigated the association of three VEGF-A SNPs (rs833061, rs13207351, and rs1570360) with RPL in the Erbil Governorate. The VEGF -460T>C polymorphism is associated with pre-

eclampsia, polycystic ovarian syndrome (PCOS), and recurrent spontaneous miscarriage (RSM), as well as other obstetric and gynecological disorders (Zeng et al., 2021).

This study indicated that the mutant homozygous TT genotype of the -460C/T polymorphism was significantly more frequent in RPL patients than in the control group, suggesting the T allele is a risk factor for RPL and increased the risk 3.3 times, and this agrees with what was discovered by Almawi et al. (2013), who similarly identified an elevated risk of RPL linked to the homozygous 460C/T genotype in their investigation. In contrast, Şamlı et al. (2012) reported that there was no significant difference in the C/T genotype frequency between the cases and the control groups. The study comprised just 30 control subjects and 38 patients with RPL. In addition, the -1190A>G polymorphism, found in the promoter area of the VEGF-A gene, is expected to affect transcription factor binding, as this region plays a key role in the control of gene expression, according to Buroker (2014) in his analysis of regulatory SNPs and transcription factor binding sites in VEGFA and their link to human disease. Yang et al. (2011) argue that Chinese individuals with type 2 diabetes mellitus who are homozygous for the minor allele of this SNP exhibit an elevated risk of developing diabetic retinopathy. The current study identified a notable correlation between the -1190A/G (rs13207351) polymorphism and RPL. The homozygous mutant genotype (GG) of the -1190A/G polymorphism was substantially more common in RPL patients than in controls. G allele compared to A allele, with a pooled odds ratio of 2.64 (95% confidence interval 1.26, 5.52; p = 0.01). There was limited data regarding the possible role of rs13207351 in RPL. Moreover, the genotypic and allelic frequencies of -1154A/G presented no significant connection between cases and controls, with a p-value above 0.05. And this agrees with Xing et al. (2011), who reported that there were no significant differences in the frequency of VEGF-1154G/A genotypes between RPL patients and healthy controls in the Chinese Han population. Similarly, Moradi et al. (2024) observed no significant variation in genotype distribution of the -1154A/G polymorphism between women with recurrent implantation failure (RIF) and controls. In contrast, Papazoglou et al. (2005) found that the A allele of the -1154A/G variant was significantly associated with an increased risk of RPL. These discrepancies may indicate variations in ethnic background, sample size, or study methodology, highlighting the necessity for larger, multi-center investigations to elucidate the potential role of the -1154A/G polymorphism in RPL susceptibility.

A limitation of this study is that serum VEGF-A levels were not measured, which could provide functional support for the genetic findings.

5. Conclusion

This study studied the correlation between three single nucleotide polymorphisms in the promoter region of the VEGF-A gene—namely, -460C>T (rs833061), -1190A>G (rs13207351), and -1154A>G (rs1570360) — with the incidence of recurrent pregnancy loss in women from Erbil city. The results indicated a notable correlation between RPL and the -460C>T and -1190A>G polymorphisms. Specifically, the homozygous TT genotype of -460C>T and the GG genotype of -1190A>G were more prevalent in RPL patients, indicating that these nucleotide alterations (C→T and A→G, both categorized as transitions) may increase susceptible to RPL. In contrast, no significant correlation was found between RPL and the -1154A>G polymorphism. which also involves a transition, indicating that this particular nucleotide change may not contribute to disease risk in this population. Future studies should integrate haplotype analysis to better assess the cumulative genetic impact of VEGF polymorphisms. In addition, further investigation into the functional role of the -

1190A>G allele is recommended to clarify its contribution to the etiology of RPL.

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