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Association of the VEGF-1154G/A polymorphism with recurrent implantation failure in infertile Iranian women

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Abstract

Introduction: Most IVF embryos fail to implant before clinical diagnosis. Successful implantation of an embryo relies on the endometrium forming a connection with the embryo, thereby enabling blood vessel formation and a suitable environment for the embryo to develop. Vascular Endothelial Growth Factor (VEGF) plays a vital role in angiogenesis. The role of VEGF polymorphisms, including VEGF1154 G/A (rs1570360), has been shown in recurrent implantation failure (RIF) in various ethnic groups.

Materials and Methods: For this research, 148 healthy women with a previous successful pregnancy served as the control group, while the case group consisted of 75 women with Recurrent Implantation Failure (RIF) who had not achieved a pregnancy after transferring 10 embryos during three or more IVF cycles. Genomic DNA was extracted from peripheral blood cells. PCR was performed, and amplified DNA fragments were sequenced using the Sanger method.

Results: After analyzing with SHEsis application, while the control group exhibited Hardy- Weinberg equilibrium (HWE), a deviation was observed in the case group. However, the chi- square test did not reveal a statistically significant difference between the groups.

Conclusion: There was no significant association between this SNP and RIF in our case-control study. The presence of Hardy-Weinberg disequilibrium in the case group, despite the lack of significant difference from the control group, warrants further exploration of potential contributing factors and their implications for the study of this locus in relation to recurrent implantation failure.

Keywords: Recurrent implantation failure, VEGF 1154G/A polymorphism, Endometrium

1. Introduction

Successful angiogenesis is one of the most important factors in embryo implantation and healthy pregnancy progression. Any disruptions in angiogenesis can result in recurrent implantation failure (RIF) or pregnancy complications [1]. For a successful implantation and pregnancy, the developing embryo (blastocyst) needs to be embedded in the endometrium [2]. Several factors influence implantation, including: leukemia inhibitory factor (LIF), epidermal growth factor

(EGF), and cyclooxygenase-2 (Cox-2) [3]. Fibroblast growth factor 2 (FGF2) plays a vital role in the development of the first blood vessels of embryos [4]. Also, hyaluronidase-2 (HYAL2), which is released from the human embryo (Kong et al., 2021) and breaks down a large sugar molecule called hyaluronan, plays an important role in reducing edema in the implantation site, allowing the embryo to embed itself more securely in the uterine lining [5]. The likelihood of embryo implantation failure increases with delayed implantation timing. A study conducted by Wilcox in 1999 found that embryos implanted later than days 8 to 10 after ovulation have a higher risk of implantation failure than those implanted at an earlier stage [6]. A negative beta human chorionic gonadotropin (HCG) test, or a positive beta HCG test that do not progress beyond the visualization of fetus heartbeat via ultrasound, is considered as implantation failure. Recurrent implantation failure (RIF) refers to the failure of embryo implantation prior to clinical pregnancy diagnosis, following at least three fresh or frozen cycles of assisted reproduction technology (ART) and transfer of at least four good-quality embryos in a woman under 40 years old [7]. Several reasons cause abortion or implantation failure, which depends on embryo quality (morphology) and the condition of the endometrium [8]. Several factors contribute to implantation failure, for example, immune-related conditions like anti-phospholipid antibody syndrome, anti-thyroid peroxidase antibodies, infections, external influences, and unexplained causes [9]. Vascular endothelial growth factor (VEGF) plays a central role in angiogenesis [10]. *VEGF* is expressed by different types of cells, including those in the ovaries that support egg development, such as theca lutein cells and granulosa cells [11]. Within the endometrial tissue, *VEGF* expression significantly increases during the secretory phase of the menstrual cycle, particularly around the "window of implantation," meaning the period when embryo implantation is most likely to occur, with the highest expression seen in the mid-luteal phase; this upregulation of *VEGF* is crucial for facilitating blood vessel formation at the implantation site, allowing the embryo to properly embed in the uterine lining. Additionally, *VEGF* is expressed around day 22 of pregnancy by cytotrophoblast villous cells in the human placenta [12]. This gene is on chromosome 6p21.3 and has a length of 16279bp and eight exons [3]. It has six isoforms and consists VEGF183, VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 [1]. *VEGF* is believed to increase vascular permeability and promote the proliferation of endothelial cells for successful implantation of the embryo [2]. *VEGF*-1154 G/A, which is situated in the gene promoter, controls the expression of endometrial *VEGF*. The *VEGF*-1154 G/A polymorphism could potentially contribute to recurrent implantation failure. In 2012, the relationship between polymorphism of *VEGF*-1154 GA and recurrent pregnancy loss (RPL) was reported by Vidyadhari M [12]. *VEGF* helps the process of embryo implantation by increasing the growth of blood vessels in the embryo, improving the conditions of the blood vessels in the endometrium, and facilitating proper communication between the developing embryo and the endometrium [13].

The main goal of this study was to examine the association between the *VEGF*-1154 G/A polymorphism and recurrent implantation failure in infertile women referred to the Royan Institute.

2. Materials and Methods

2.1 Study participants

The control group comprised 148 fertile women with no history of pregnancy complications and at least one live birth. The case group comprised 74 female patients who had experienced repeated implantation failure, as defined by established criteria. Definition the age range of the patients was 22-37 years. The patients had a normal karyotype and no major concerns during genetic counseling. Patients with a history of cancer and chemotherapy, systemic autoimmune disorders, inherited thrombophilia, hormonal diseases, uterine anatomic defects, and other known infertility-related conditions such as endometriosis or PCOs, which could negatively affect embryo implantation, were excluded from the study.
This study complied with the Code of Ethics of the World Medical Association's (Declaration of Helsinki) guidelines for research involving human subjects and was approved by the ethics board of the Royan Institute (Reference number 91000518). Informed consent was obtained from all participants after they were fully briefed.

2.2 Specimen collection and detection process

After collecting a sample of peripheral blood, genomic DNA was extracted using the salting out method. For designing primers of the genomic regions of interest, the Perl primer (version v1.1.21) software was used [Table 1].
PCR was performed, and the product was run on 1% agarose gel with ethidium bromide, and a 215 bp band was observed on the gel.
The optimum conditions for PCR were 30 cycles of denaturation at 94°c for 1 min, annealing at 65°c for 25 s, and extension at 72°c for 45 s and a final extension step of 72 °C for 2 min to terminate the process. PCR

products were collected and sent to Faza Pajooh Company (Tehran, Iran) for sequencing. This company determined the sequencing fragments using Sanger sequencing in an ABI 3730 XL capillary sequencer system. To confirm the authenticity of PCR products using the primers, the results of sequencing were examined using two databases: the Basic Alignment Local Search Tool (BLAST) and the UCSC Genome Browser.

2.3 Statistical analysis

To analyze the results, the SHEsis software was used. The Chi-square test was employed to compare the case and control results. A significance level of 0.05 was considered. The Hardy-Weinberg equilibrium (HWE) test was used to show that there was no selection bias, population stratification, or genotyping errors in the control group.

3. Results

In the control group, 100 out of 296 alleles were A (33.8%) and 196 out of 296 were G (66.2%). In contrast, in the patient group, 45 out of 150 alleles were A (30.0%) and 105 out of 150 were G (70.0%). Despite the disparity between the two groups, the observed difference did not attain statistical significance (P=0.389). Allele frequencies are shown in Table 2.

In the control group, the number of A/A genotypes was 19 out of 148 (12.8٪); the number of G/G genotypes was 67 out of 148 (45.3٪), and the number of G/A genotypes was 62 out of 148 (41.9٪). In the patient group, the number of A/A genotypes was 12 out of 74 (16.2٪), and the number of G/G genotypes was 42 out of 74 (56.8٪); the number of G/A genotypes was 20 out of 74 (27.0٪). No statistically significant difference was observed between the two groups (P=0.096) [Table 3].

The sequencing results were assessed using Finch TV software, as shown in Figure 3. Analysis using the HWE method indicated a state of equilibrium in the control group. The Hardy-Weinberg equilibrium was not observed in the patient group, with a statistically significant result (p-value=0.004). The chi-square test revealed no significant distinction in allele frequency or genotype between the control and patient groups. Thus, no significant association between this genetic variation and RIF was found in this case-control analysis.

Table1. Oligonucleotide sequence of *VEGF* primers

Primer	Primer sequence	TM(°C)	٪GC	Size bound (bp)
VEGF F	CAGGCTGTGAACCTTGGTG	60/00	57/89	215
VEGF R	CCGCTACCAGCCGACTTT	58/00	61/11	

Table 2. Alleles frequency for the rs1570360 *VEGF* gene polymorphism in the control and patient groups.

Alleles	Control n. (%)	Case n. (%)
A	100 (33.8)	45 (30.0)
G	196 (66.2)	105 (70.0)

Table 3. Genotypes frequency for rs1570360 of *VEGF* polymorphism in the patient and control groups.

Genotypes	Control n. (%)	Case n. (%)
A/A	19 (12.8)	12 (16.2)
G/G	67 (45.3)	42 (56.8)
G/A	62 (41.9)	20 (27.0)

4. Discussion

Implantation failure is one of the most common disappointing situations for couples trying to get pregnant using assisted reproductive techniques (ART) [14]. Genetic and epigenetic factors from both the embryo and endometrium may be involved in implantation failure. One of the main steps in embryo

implantation is embryo attachment to the endometrium and subsequent angiogenesis, which ensures adequate blood supply for embryo development. Disorders in angiogenesis can lead to recurrent implantation failure and pregnancy loss.

Studies on the relationship between genetic polymorphisms in the *VEGF* gene and recurrent spontaneous abortion (RSA) have been published prior to its relation with repeated implantation failure. Zhang *et al.* (2012), studied the results of the 8 previous reports on the relation of *VEGF* gene polymorphisms and the risk of RSA. They concluded that the *VEGF* gene polymorphisms, including 2578 C/A (rs699947) and 1154 G/A (rs1570360), were not significantly associated with the risk of RSA, whereas -634 G/C (rs2010963) under recessive model and -936 C/T (rs3025039) polymorphisms under codominant and dominant models have been associated with it [8]. Another meta-analysis in 2015 by Xu *et al.*, which included 10 case-control studies, confirmed -1154 G/A variations with RSA. In a study by Amin and his colleagues, the relationship between all the variants of VEGF, including VEGF-1154 AA genotypes and RSA was reported [15]. In the study conducted in 2010 by Lee et al., there was not significant relationship between *VEGF*1154 G/A polymorphism and RSA in Taiwanese women (Taiwanese Han) [7]. In their study, the patient group consisted of 115 patients (62 women with two implantation failures and 53 women with three or more) and the control group consisted of 170 cases .The frequency of 1154 G/A allelic polymorphism in various ethnic groups, was different (the frequency of G/A alleles: 0.5-0.6/0.4-0.5 in Whites, 0.2-0.8 in Asians).This difference can be related with the small number of studied samples or really the effect of ethnicity and relatively low influence of this polymorphism in the recurrent implantation failure. There was also no significant difference in the coagulation status. In the analysis of the *VEGF* gene haplotype, there is an imbalance of communication linkage disequilibrium (LD) between the promoter and untranslated3´ region (3´ UTR) of the *VEGF* gene [14].

The goal to increase the rate of embryo implantation in ART lead to extensive study on the effect of genetic variations on it. Therefore, the study of VEGF variations in relation to RSA was repeated in relation to failure of embryo implantation.

Goodman et al., in 2008, reported the frequency of the homozygous VEGF -1154AA genotype was significantly higher among women experiencing implantation failure compared with control women and alongside p53 codon 72 Pro/Pro, and PAI-1 4G/4G may be useful to identify women at risk for implantation failure after IVF-ET.

Oliveira et al., in 2013, studied VEGF SNP in 86 couples with RIF. They reported no association between VEGF -1154G/A polymorphisms and implantation or pregnancy rates after IVF/ICSI-embryo transfer in couples with RIF.

Vagnini et al in 2015 found an association between the VEGF-1154G/A polymorphism and RIF in Brazilian women [10].

A study by Shim et al., in 2018, indicated that the *VEGF* -2578AA genotype, -634G allele and -2578A/-1154A/-634G/936C haplotype may serve as genetic markers for susceptibility to RIF [3].

Azahara et al in 2020 investigated the *p53* and *VEGF*-1154 polymorphism in RIF patients and suggested that patients carrying variants in p53 and VEGF may be at increased risk of RIF.

Elmi *et al.* in 2023 in a case- control study consisting 60 Iranian RIF patient and 60 control concluded that VEGF polymorphism may influence embryo implantation, and the VEGF rs1570360 AA genotype may predispose individuals to recurrent implantation failure after IVF.

This study, carried out at the Royan Institute on 222 subjects with recurrent implantation failure (RIF) and controls, and similarly to Oliveira at al. in 2013, found no significant association between the VEGF-1154 G/A polymorphism and recurrent implantation failure. The discordant outcomes may be attributed to disparities in ethnic background between the population in this investigation and other research. In the case group, Hardy-Weinberg equilibrium is disrupted, whereas the control group remained in equilibrium. The uneven distribution in the case group implies that one genotype, specifically the G/A genotype, was less common than expected based on the equilibrium model in the control group, indicating a departure from the ideal conditions. The insignificance of this difference may be attributed to the relatively small number of patients in the case group, which could have masked a potentially meaningful association.

5. Conclusion

Our case-control study found no significant association between this single nucleotide polymorphism (SNP) and recurrent implantation failure (RIF). Hardy-Weinberg disequilibrium in the case group, coupled with the lack of a significant

difference from the control group, calls for further investigation into factors that may be responsible for the observed deviation and its implications for understanding the relationship between this locus and recurrent implantation failure. It is essential to take into account various genetic, geographic, and other factors that could potentially impact RIF.

Ethical Considerations

Compliance with ethical guidelines

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author's contributions

Conception and design of study: Hamideh Moradi, Zeinab Rokhsat talab, Hamid Gourabi

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Revising the manuscript critically for important intellectual content: Hamideh Moradi, Hamid Gourabi.

Conflict of interest

The authors have no conflict of interest

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