Original Article

Association of Fibroblast Growth Factor-1 Promoter Polymorphism and its Serum Concentrations with Repeated Implantation Failure after *In vitro* Fertilisation: A Cross-sectional Study

Afshin Kharamani, Farhad Mashayekhi, Zivar Salehi

Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

Abstract

Background: Fibroblast growth factors (FGFs) play a key role in embryo implantation and support endometrial trophoblastic interaction. **Aim:** The aim of the study was to evaluate the association between FGF-1 (rs34011) gene variety and its serum concentration with repeated implantation failure (RIF). **Setting and Design:** The design of the study was a cross-sectional study. **Materials and Methods:** Four hundred infertile women with a history of RIF and 400 healthy women undergoing the first *in vitro* fertilisation-embryo transfer attempt with successful delivery (controls) were enrolled in the study. Genomic DNA was extracted from peripheral blood leucocytes and genotyped by Tetra-Primer Amplification Refractory Mutation System-Polymerase Chain Reaction. Serum FGF-1 concentration was evaluated with enzyme-linked immunosorbent assay. **Statistical Analysis Used:** The ANOVA test was used to analyse the difference between the means of the groups. **Results:** In RIF group, the genotype frequencies of the GG, GA and AA were 59%, 33.5% and 7.5%, respectively, whereas in controls were 72.5%, 24% and 3.5%, respectively. The G and A allele frequencies in the RIF group were 75.75% and 24.25%, while in controls were 84.5% and 15.5%, respectively ($P < 0.0001$). We have also shown that serum FGF-1 concentration in RIF and control groups was 17 ± 3.55 and 23.62 ± 4.91 pg/mL, respectively ($P = 0.008$). We have also shown that AA genotype is significantly associated with decreased serum FGF-1 concentration in RIF (AA, GA and GG serum levels were 9.55 ± 2.65, 14 ± 3.35 and 22.55 ± 7.26 pg/mL, and in controls were 12.22 ± 2.27, 18.44 ± 5.98 and 26.66 ± 8.29 pg/mL, respectively). **Conclusion:** The current study suggests that a significant association between FGF-1 (rs34011) promoter polymorphism and its serum concentration with RIF. The study also suggests that AA genotype is linked to lower FGF-1 serum levels and may play a risk factor for RIF.

Keywords: *Embryo implantation, embryo transfer, fibroblast growth factor 1, genetic polymorphism, polymerase chain reaction*

Introduction

Women using assisted reproductive technology may have repeated implantation failure (RIF). Despite the transfer of high-quality embryos, implantation failure is a very typical occurrence. Several factors have been identified as causes of implantation failure. Recurrent implantation failure (RIF) is a condition, in which a woman has had three or more unsuccessful attempts

Address for correspondence: Prof. Farhad Mashayekhi, Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran.
E-mail: mashayekhi@guilan.ac.ir, umistbiology20@gmail.com

Received: 18-04-2024 **Revised:** 09-05-2024
Accepted: 20-05-2024 **Published:** 28-06-2024

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/jhrs.jhrs_68_24

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How to cite this article: Kharamani A, Mashayekhi F, Salehi Z. Association of fibroblast growth factor-1 promoter polymorphism and its serum concentrations with repeated implantation failure after *in vitro* fertilisation: A cross-sectional study. J Hum Reprod Sci 2024;17:121-7.

at *in vitro* fertilisation (IVF) using embryos of high quality.[1]

Numerous reasons can contribute to implant failure. Many genes including fibroblast growth factors (FGFs) have been shown to play an important role in embryo implantation. Mutation in FGF receptor (FGFR) has been shown to result in defects at embryo implantation.[2] Previous studies have demonstrated that embryos with a mutation in FGFR1 are lost before or at gastrulation due to incomplete the epithelial-to-mesenchymal transition that is necessary for mesoderm development.[3] Progesterone (P4) and 17-estradiol (E2) affect endometrial tissue proliferation and differentiation to create an environment for the early development of the embryo. Stimulating the Epidermal growth factor receptor (EGF-R) system is one way that steroids may exert their mitogenic effects on the oviduct and uterus.[4] Some factors promote the expression of decidual FGFs. FGFs affect embryo implantation and support the interaction of endometrium and trophoblast.[5] It has been demonstrated that patients with RIF have lower levels of FGF-1 (also known as an acidic FGF) expression in their endometrium, which is especially essential for implantation in the luteal phase.[6] It was demonstrated that endometrial cell proliferation can be induced by FGF signalling, which is important for epithelial-stromal interactions.[7] FGFR1 is required during gastrulation for morphogenetic movements through the primitive streak.[8] Maintenance of trophoblast lineage is also controlled by FGFs.[9]

The most important biological effects of the FGF are associated with angiogenesis, cellular differentiation and proliferation. Therefore, it is suggested that FGF plays an important role in decidua formation that leads to the successful establishment of pregnancy.[4] FGFs are also important in embryo development. Neugebauer *et al*. suggested that FGF signalling controls the length and function of cilia in diverse epithelia during embryo development.[10] Moreover, FGF plays a key role in neural crest cell migration and development.[11] It has been shown that the addition of FGFs to blastocyst increased the number of trophoblastic cells. Furthermore, FGF signalling induces cell division in the pre-implantation embryo.[12] It was suggested that suppression of FGF signalling is the primary cause of DNA (cytosine-5)-methyltransferase 3 beta (Dnmt3b) suppression in IVF embryos at the blastocyst-to-post-implantation transition. Dnmt3b is inhibited throughout *in vitro* development by repression of FGF signalling.[13] Due to the important role of FGF in embryo development, our goal was to evaluate the relationship between FGF-1 promoter (rs34011) polymorphism and its serum concentrations with RIF.

Materials and Methods

After obtaining written consent from all participants, infertile 400 women with a history of RIF (n = 400) after IVF (RIF group) and 400 women without RIF undergoing the first IVF transfer attempt with positive pregnancy (controls, n = 400) were enrolled in this study. They had undergone infertility examinations. The serum level of human chorionic gonadotropin was measured, and the amount of <0.5 IU/L was regarded as to be negative for pregnancy, as instructed by the manufacturer. After multiple IVF treatment cycles, serial negative pregnancy tests are considered RIF. Follicle-stimulating hormone levels of <12 mIU/mL and Anti-Müllerian hormone (AMH) levels >1.15 ng/mL were used to assess the functional ovarian reserve of the women in this study, all of whom were under the age of 40 years. Patients who (i) received at least four good-quality blastocysts in at least three cycles but had not yet become pregnant and (ii) were not pregnant during this frozen embryo transfer (ET) cycle were eligible for the RIF. The controls were the women who gave live birth after IVF.

This cross-sectional study was approved by the Graduate Education Council and Review Board of the university under the number 149974 and was performed in accordance with the Helsinki Declaration of 1975, as revised in 2013. Informed consent was taken from all adult research participants.

Genotyping

The sizes of samples were calculated using OpenEoi software (www.OpenEpi.com), and the power was set at 89.6%. Blood samples from RIF (n = 400) and controls (n = 400) women were collected and centrifuged at 1600 g for 10 min, and the serum was collected and kept at −70°C.

Gpp solution kit (Gen Pajoohan, Iran) was used for the extraction of genomic DNA from the blood according to the manufacturer's instructions. Using a spectrophotometer at 260 and 280 nm, DNA purity and concentration were determined. DNA samples were kept in buffer (0.1M Tris-Hcl, 0.05M EDTA pH 8.5, 1.25% SDS) at −20°C until used. Tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) was used for the genotyping process. The design of the primer for FGF-1 was carried out using oligo primer analysis software (version 7.54, Molecular Biology Insights Inc., Cascade, CO, USA).

The sequences of primers were as follows:

F inner (G allele): 5′-CTGGGATTACAGGCGTAAGCA ACCG-3′, R inner (A allele): 5′-AGCTCC

CAGGCCGGTCGT-3′, F outer: 5′-AGAGACCAGGA AACTAACGTGTGAACCGC-3′ and R outer: 5′-CCT GTAGCCCAAAATACGTTAATGATGAGGATG-3′.

PCR was done under the following conditions: initial denaturation at 96ºC for 6 min, 35 cycles of denaturation at 96°C for 40 s and annealing at 57°C for 42 s, with a final step at 73°C for 5 min to allow a complete PCR fragment extension. Two bands of 447 bp and 151 bp were seen for the GG genotype, three bands of 447, 338 and 151 bp for GA and two bands of 447 and 338 bp for AA [Figure 1]. The amplicons were electrophoresed with a 100 bp DNA molecular marker (SMOBIO Tech., Inc., Taiwan) on a 1.5% agarose gel. Twenty per cent of the samples were re-genotyped by another laboratory member to increase the quality of genotyping and its validity and no difference in the results was found.

Serum fibroblast growth factor-1 levels by enzyme-linked immunosorbent assay

To separate the serum, the whole blood samples were centrifuged at room temperature. Then, the serum samples were kept at −80°C for further analysis. For measurement of FGF-1 serum concentration, enzyme-linked immunosorbent assay Kit (ab219636) (Abcam, UK) was used according to the manufacturer's instructions.

Statistical analysis

Based on the minor allele frequency of the studied Single-nucleotide polymorphism (SNP), to calculate the required sample size, OpenEpi software (www.OpenEpi.com) was used. The Chi-square test was used to calculate Hardy–Weinberg equilibrium (HWE) to compare the expected and observed genotype frequencies between the two groups. AA, GA and GG genotypes were considered for rs34011. Chi-squared test was used for the calculation of the statistical significance of differences between RIF and control groups. To define the strength of the link between rs34011 genetic polymorphism and RIF, odds ratio (OR) and 95% confidence interval (CI) were used. For statistical analysis, MedCalc statistical software (Version 17.9.7, Mariakerke, Belgium) was used.

The receiver operating characteristic (ROC) curve was used to assess the sensitivity, specificity and area under the curve (AUC). Statistical results with $P < 0.05$ were considered significant.

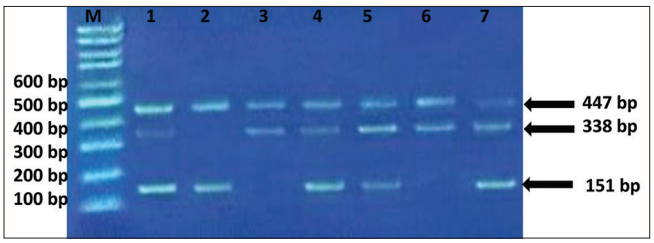

Figure 1: Detection of fibroblast growth factor (FGF)-1 polymorphism by tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR). T-ARMS-PCR for FGF-1 polymorphism detection two bands of 447 and 151 base pairs were produced by the GG genotype (lane 2), three by the GA genotype (lanes 1, 4, 5, and 7), and two by the AA genotype (lanes 3 and 6). The molecular weight marker is shown in the left part of the gel

Results

Hardy–Weinberg equilibrium

All of the samples were successfully genotyped. The observed genotype frequencies in RIF and controls were in the HWE (χ^2 test = 3.10, P = 0.07; χ^2 test = 2.80, P = 0.09, respectively). Thus, no population stratification and no sampling bias have been seen. For repeat analysis, at least 10% of the samples were chosen at random, resulting in 100% agreement.

Genotyping

Genotyping for rs34011 promoter polymorphism of FGF-1 was performed in 400 RIF cases and 400 controls. Genotyping of rs34011 has been done by the T-ARMS-PCR method [Figure 1]. All information about allele and genotype frequencies for RIF and control groups is presented in Table 1. There were significant variations in genotypes and alleles distribution were seen between the two groups (χ^2 = 17.64, P = 0.0001; χ^2 = 19.23, P = 0.0001, respectively).

The results showed that the incidences of GG, GA and AA genotypes in the RIF group were 59%, 33.5% and 7.5%, and in controls were 72.5%, 24% and 3.5%. To analyse the polymorphic sites, four genetic models (codominant, dominant, recessive and overdominant) were used [Table 1]. The AA genotype was shown to be related to RIF when compared to that of the GG genotype in a codominant model (OR = 2.63, 95% CI = 1.36–5.08, P = 0.003). In a recessive model (AA vs. GG + GA), we showed that the AA genotype is connected to an increased risk of RIF (OR = 2.23, 95% CI = 1.16–4.28, P = 0.001). The G and A alleles incidence in RIF and controls were 75.75%, 24.25% and 84.5%, 15.5%, respectively. We also showed that the A allele is linked with a higher risk of RIF [OR = 1.74, 95% CI = 1.35–2.24, $P < 0.0001$, Table 1].

Serum fibroblast growth factor-1 concentration in repeated implantation failure and control groups using enzyme-linked immunosorbent assay

We also showed that the levels of serum FGF-1 in RIF (17 ± 3.55 pg/mL) were significantly lower than in the control group (23.62 ± 4.91 pg/mL) [P = 0.008, Figure 2].

We also find that AA is significantly related to lower serum FGF-1 concentration in RIF (AA, GA and GG

Table 1: Genotypes frequencies of fibroblast growth factor-1 promoter polymorphism in repeated implantation failure and control groups

Genetic model	Genotype	RIF, *n* (%)	Controls, *n* (%)	OR (95% CI)	*P*
Codominant	G/G	236 (59)	290 (72.5)	1.00	
	G/A	134 (33.5)	96 (24)	1.71 (1.25–2.34)	0.0007
	A/A	30 (7.5)	14 (3.5)	2.63 (1.36–5.08)	0.003
Dominant	G/G	236 (59)	290 (72.5)	1.00	
	G/A + A/A	164 (41)	110 (27.5)	1.83 (1.36–2.46)	0.0001
Recessive	G/G + G/A	370 (92.5)	386 (96.5)	1.00	
	A/A	30 (7.5)	14 (3.5)	2.23 (1.16–4.28)	0.001
Overdominant	G/G + A/A	266 (66.5)	304 (76)	1.00	
	G/A	134 (33.5)	96 (24)	1.59 (1.17–2.17)	0.003
Alleles	G	606 (75.75)	676 (84.5)	1.00	
	A	194 (24.25)	124 (15.5)	1.74 (1.35–2.24)	<0.0001

RIF=Repeated implantation failure, OR=Odds ratio, CI=Confidence interval

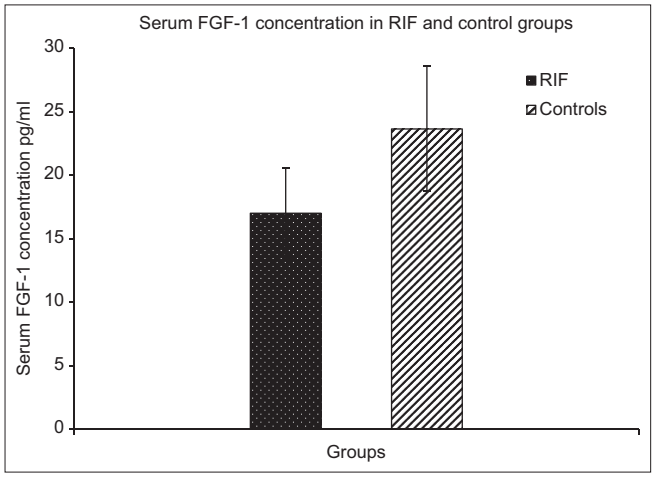

Figure 2: Serum levels of fibroblast growth factor (FGF)-1 in the repeated implantation failure (RIF) patients and the control group. The RIF had significantly lower serum FGF-1 levels (17.55 ± 3.55 pg/mL) than the control group (23.62 ± 4.91 pg/mL). The patients with RIF had a significantly lower serum FGF-1 level than the control group ($P = 0.008$). FGF-1 = Fibroblast growth factor, RIF = Repeated implantation failure

serum concentrations were 9.55 ± 2.65, 14 ± 3.35 and 22.55 ± 7.26 pg/mL, and in controls were 12.22 ± 2.27, 18.44 ± 5.98 and 26.66 ± 8.29 pg/mL, respectively) [Figure 3a and b].

Diagnostic value of fibroblast growth factor-1 serum concentration

To evaluate the diagnostic value of the FGF-1 serum concentration, ROC analysis was performed [Figure 4]. Differential expression of FGF-1 between RIF and control groups showed a sensitivity of 64.70%, specificity of 55.39% and AUC value of 0.86 (95% CI: 0.76–0.96).

Discussion

RIF describes when embryos of morphologically good quality are repeatedly transferred into a normal uterus without success. Even though there has been a lot of research done on the main causes of RIF, we still do not know much about this condition. Polymorphism in many genes has been shown to be important in pregnancy outcome.[14,15]

To identify potential risk factors, we have examined FGF-1 gene variation and serum levels in RIF patients. This is the first project studying the relationship between FGF-1 (rs34011) polymorphism and its serum concentrations with RIF after IVF. FGFs have been shown to play a vital role in embryo implantation. Decreased FGF-1 expression has been shown in the endometrium of patients with RIF, which is mainly necessary for implantation in the luteal phase.[16] It has been shown that FGF signalling is important for the maturation and regeneration of endometrium following menstruation.[17] Changes in the expression of FGF-1 in infertile women have been documented, which shows that FGF-1 is an important maternal factor affecting implantation. It has been demonstrated that FGFs and their receptors are expressed in the endometrium and embryo.[18] It was suggested that during early embryo development, the endometrium produces FGFs, which may play a role in embryo development and implantation.[19] Mashayekhi *et al*. have shown that FGF signaling is an essential factor in brain development and proliferation of neural cell proliferation.[20] It has been suggested that Alzheimer's disease (AD) patients had higher levels of FGF-1 in their serum and cerebrospinal fluid than healthy controls.[21] It has been shown that elevated FGF serum concentration is related to a higher risk of major adverse cardiovascular events, and cardiovascular or all-cause mortality in patients with coronary artery disease (CAD), even after adjustment for renal function. Blood FGF levels may provide important predictive information in CAD patients.[22]

Phenotypic differences between individuals, such as disease risk and drug response, may be caused by

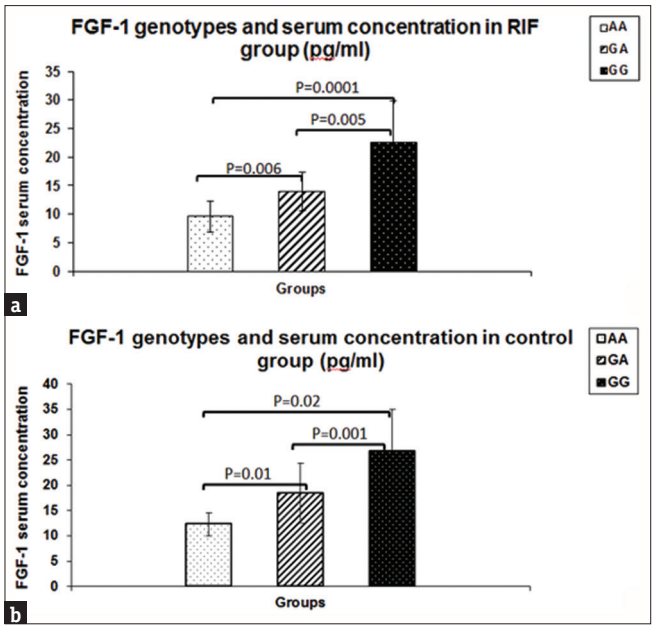

Figure 3: Serum levels of fibroblast growth factor (FGF)-1 and genotypes were found to be correlated in repeated implantation failure (RIF) patients (a) and the control group (b). In RIF, the AA genotype is associated with a lower serum concentration of FGF-1 (AA, GA, and GG serum levels were 9.55 ± 2.65, 14 ± 3.35 and 22.55 ± 7.26 pg/mL, respectively, whereas controls' serum levels were 12.22 ± 2.27, 18.44 ± 5.98 and 26.66 ± 8.29 pg/mL). FGF-1 = Fibroblast growth factor, RIF = Repeated implantation failure

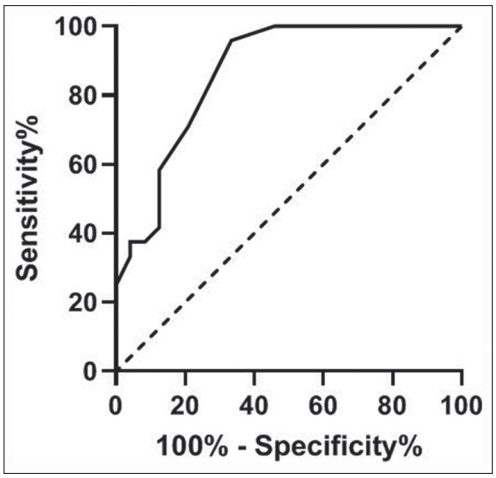

Figure 4: Receiver operating characteristic (ROC) analysis. To evaluate the diagnostic value of the fibroblast growth factor (FGF)-1 serum concentration, ROC analysis was performed. Differential expression of FGF-1 between repeated implantation failure and control groups showed a sensitivity of 64.70%, specificity of 55.39% and area under the curve value of 0.86 (95% confidence interval: 0.76–0.96)

genetic polymorphisms. The identification of the variants that modulate gene expression and/or protein function is to be made easier by defining genetic polymorphisms. This is the first study about the association of FGF-1 gene polymorphism and its serum concentration with embryo implantation failure. However, the association of FGF-1 gene polymorphism with other diseases has been documented. It was suggested that the FGF-1 gene is associated with AD.[23] Rafiqdoost *et al*. suggested that FGF-1 polymorphism is linked to a lower risk of non-syndromic cleft lip with or without a cleft palate, according to some studies.[24] Bian *et al*. showed that there is no relationship between the FGF-1-1385 genetic variation and AD risk in China.[25] It has been suggested that variants in FGF are linked to susceptibility to Parkinson's Disease.[26] Marwa *et al*. suggested that the pathogenesis of pre-eclampsia in Tunisian women may be influenced by the genetic variants of FGF-1.[27] An association between the FGF promoter polymorphism and the possibility of developing adenomyosis and endometriosis in north Chinese women has been proposed. These gynaecological diseases appear to be less likely to affect people who carry the FGF gene's G allele.[28] It has been suggested that there is an association of FGF-1 rs250108 with oestrogen receptor (ER)-negative compared to ER-positive breast cancer.[29] It has also been shown that genetic variation in FGF-1 appears to be related to survival after diagnosis of breast cancer.[30]

This study found a strong correlation between serum concentration and FGF-1 gene polymorphism with RIF following IVF. We have also demonstrated a correlation between IVF-ET failure and the AA genotype, which is linked to a lower serum concentration of FGF-1. We also showed that FGF-1 serum concentration decreases in patients with RIF compared to controls. As FGFs affect embryo implantation and support the interaction of endometrium and trophoblast and early embryo development, decreased serum FGF-1 in the patients with RIF in our study may be important in the result of IVF-ET. When interpreting the results, it is important to consider this study's limitations. First, there has been a relatively small sample size and the results should be interpreted with caution. Second, because only a population in the north of Iran was included in this study, it may be difficult to apply these findings to other populations. Third, since this project only examined one FGF-1 SNP, it is impossible to rule out the possibility that other genetic variants influence IVF-ET outcomes. Finally, the outcome of IVF-ET is influenced by a variety of factors, both individually and collectively. As a result, additional factors ought to be included in our subsequent research.

Conclusion

The current study findings suggest that serum FGF-1 serum concentration and promoter variation are related to RIF in the studied population. It is also suggested that the AA genotype is linked to the decreased serum FGF-1 concentration and may be regarded as a risk

factor for RIF. However, to obtain a final conclusion and confirm the results, larger studies with more samples are needed.

Authors' contributions

AK: Methodology and experimental studies, Investigation, literature search, data acquisition. FM: Supervision, Conceptualization, data analysis, manuscript preparation, manuscript editing and manuscript review. ZS: Methodology, Software, clinical studies, data acquisition, statistical analysis.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Data availability statement

The data are available with the corresponding author and willing to share it on request.

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