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Is there an Association Between-2549 Insertion/Deletion Polymorphisms in the Promotor Region of the Gene Encoding for VEGFA as a Risk Factor and the Idiopathic Recurrent Spontaneous Miscarriage in a Sample of Jordanian Women?

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Abstract

Background: At least 50% of the cases of recurrent spontaneous miscarriage is aetiologically idiopathic. Recently various genetic polymorphisms have been proposed as susceptibility risk factors for pregnancy loss.

Objective: The aim of the present case control-study is to establish the association between the functional -2549 I/D polymorphisms in the promoter region of the vascular endothelial growth factor A (VEGFA) gene and idiopathic recurrent spontaneous miscarriage (IRSM) in a sample of Jordanian women.

Subjects and Methods: 328 subjects were recruited, 103 and 98 women with primary and secondary IRSM, respectively, 127 normal women were selected as a control group. Genomic DNA was isolated from a blood sample withdrawn from each participant, then, -2549 I/D polymorphisms of the VEGFA gene were genotyped by Polymerase Chain Reaction (PCR).

Results: The obtained results revealed that ID polymorphism and D allele of VEGFA -2549 I/D polymorphisms have the highest frequencies in both primary and secondary IRSM patients, no significant difference between the three groups regarding polymorphisms and allele frequencies, patients with DD+ID genetic models have positive association with high risk of IRSM versus II model, and patients with D allele are more liable to have IRSM than those having I allele, no significant difference in the association of VEGFA -2549 I/D polymorphisms with IRSM in the three genetic models of the primary and secondary IRSM patients.

Conclusion: patients with ID genetic model of -2549 I/D polymorphisms in the VEGFA gene's promoter region and D allele have a higher risk for IRSM.

Keywords: Polymorphisms; Miscarriage; VEGFA; Idiopathic; Insertion/Deletion; Recurrent spontaneous miscarriage are aetiologically idiopathic; PCR

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Introduction

IRSM, the frequent obstetric complication, can be explained as a loss of three or more successive pregnancy prior to the 20th weeks of gestation. Up to 50% of IRSM patients have unknown underlying etiology and it affects 1-5% of the women who are seeking bear children [1]. Different factors like endocrine dysfunctions, autoimmune diseases, and uterine pathologies in addition to environmental and nutritional factors have been demonstrated for their direct and/or indirect effects on miscarriage [2]. Placental circulation in addition to fetal vasculature should be sufficient for maintaining normal pregnancy which is requiring angiogenesis. Such a process is dependent upon multiple factors, including VEGFA [3].

VEGFA, the potent angiogenic factor, is secreted by both fetal and maternal trophoblastic cells for enhancing vascular permeability, hematopoiesis, endothelial cell proliferation and survival [4]. VEGF has receptors (VEGFR1/Flt-1, VEGFR2/KDR/Flk-1) [5], its functions

are related to human reproduction, including placental organization and fetal angiogenesis, in addition to pre-decidualization and gametogenesis [6]. The rate of expression of VEGFA/VEGFR was the highest in the maternal side of the placenta [7], which is different under conditions of normal pregnancy versus the complicated one. This is because of the abnormalities of angiogenesis during implantation and placenta formation early in pregnancy which may lead to IRSM [3].

The possible role of VEGF has enticed the interest of researchers and clinicians. Thus far, several single nucleotide polymorphisms (SNPs) based on nucleotides substitutions and those dependent on insertion (I) or deletion (D) or both of one or more nucleotides (indels) have been described in the VEGF gene. Several SNPs are identified to be associated with the susceptibility to several cancers, metabolic or vascular disorders [8]. Although several SNPs were discussed in IRSM and other reproductive disorders, the interests in insertion/deletion (I/D) polymorphisms researches become more prominent, due to their participation in genetic and phenotypic divergence and diversity [9,10].

Human VEGF, the highly polymorphic, cell-specific mitogenic gene especially in the promoter, 5'-and 3'-untranslated regions, located on chromosome 6p21.3 has consisted of seven introns and eight exons, spanning approximately 14 kb [11]. More than twenty-five different polymorphisms have been identified in the gene encoding for VEGFA [12] which were suggested to influence the levels of its expression.

The most common genetic association with IRSM that was investigated for 20 polymorphisms are -1154 G/A, +936 C/T, -2578 C/A, and -634 G/C SNPs, the obtained results from these studies are inconsistent [13-15]. In the VEGFA gene, a functional I/D polymorphism is located at position -2549 in the promoter region [16]. The deletion of an 18 base pair (bp) long sequence (D allele) results in a 1.95-fold elevated transcriptional activity compared to the allele containing the insertion (I allele) [17].

VEGF gene -2549 I/D polymorphisms may be related to other diseases. In a recent study, it had been shown that uterine leiomyoma was linked to VEGF gene -2549 I/D polymorphisms [18], while, another study concluded that raised susceptibility to diabetic nephropathy in north Indian population has an association with DD genotype and D allele in I/D polymorphism at -2549 position of VEGF gene [19]. Previous literature had evaluated the effect of VEGF gene polymorphisms on IRSM and revealed novel data [15,20-24]. Our study made a comparison with the previous meta-analysis and to the best of authors, knowledge; it is the first study to be conducted in Jordan.

The aim of our study is to explore whether there is an association between the functional -2549 I/D polymorphism in the promoter region of the VEGFA gene and IRSM in a sample of Jordanian women.

Methodology

Subjects

Two hundred and one women who had experienced at least two successive spontaneous abortions and stillbirths were recruited from out-patient clinics attendants of the Obstetrical Clinic, Obstetrics and Gynecology Department, Al-Karak governmental Hospital. One hundred and twenty-seven women who experienced at least two live births and no abortion and who have no history of infertility were included in the study as a control group. Written informed consent was obtained from each participant in the study. The study was approved by the Medical Ethics, Committee of the Faculty of Medicine, Mutah University.

Exclusion criteria

Women with chromosomal anomalies in either partner, endocrine or metabolic disorders, anti-phospholipid syndrome, autoimmune disease or other systemic diseases, previous arterial or venous thrombosis, or structural uterine anomalies detected by ultrasonography and/or hysteroscopy were excluded from the study.

The subjects enrolled in the study were subdivided into three groups:

- Group I: primary IRSM (n=103) with a history of two or more pregnancy losses but no live birth.
- Group II: secondary IRSM (n=98) experienced three or more pregnancy losses after one live birth.
- Group III: control (n=127) with at least two live births with no abortion nor a history of infertility.

The cases with the history of recurrent pregnancy loss (primary and secondary) were subjected to ultrasound and hysterosalpingography for the detection of anatomic abnormalities of the genital tract such as the woman with a septate uterus were excluded from the study.

-2549 Insertion/Deletion polymorphisms analysis

Five ml of blood was withdrawn from each participant in the study (IRSM patients and the healthy controls) for DNA extraction using the method adopted by Sambrook j, et al. (1989) [25]. The analysis of 18 bp I/D polymorphisms of -2549 in the promotor region of VEGFA gene was determined by PCR in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) using a pair of primers (sense-5′-CCTGGAGCGTTTTGGTTAAA-3′ and antisense-5′-ATATAGGAAGCAGCTTGGAA-3′) [16]. The reaction total volume was 50 µl containing 100 ng DNA template, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 200 mM each of dATP, dCTP, dGTP and dTTP (Integrated DNA Technologies/USA), 2.5 mM $MgCl_2$, 0.5 mM of each primer, and 1 U Taq DNA Polymerase (iNtRON Biotechnology/Korea). The reaction started with an initial denaturation for 2 min at 95°C, then, the samples were subjected to 30 cycles of three file program in the thermal cycler (45 sec at 95°C, 30 sec at 55°C) and 1 min at 72°C, followed by a final extension at 72°C for 5 min. The 18 bp I/D polymorphism PCR products were electrophoresed on 2% agarose gel, stained and visualized under UV light. The 100 bp DNA ladder was indicated to the left side of gel, two fragments were revealed two bands 234 bp and 18 bp (not seen) for I allele, and one band 216 bp for D allele.

Statistical analysis

Data were analyzed by IBM SPSS version 20. Pearson's χ^2 and ANOVA tests were used to assess differences between the studied groups. Allele and genotype frequencies were determined by allele counting, Odds ratio (OR) and 95% confidence interval (CI) were also determined. Concordance of the polymorphisms frequencies with Hardy-Weinberg equilibrium was evaluated using χ^2.

Results

The study of VEGFA -2459 I/D polymorphisms among the different groups (Table 1) revealed that I/D polymorphism is the highest in frequency, while, II gene is the lowest one and there is no statistically significant difference among the three groups (χ^2=0.625, p=0.960). The frequency of D allele was the highest in patients with secondary IRSM (61.1%) and the least among the controls (56.3%),

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Table 1: Polymorphisms, frequency of the VEGFA -2459 I/D in the IRSM groups (primary and secondary) of women and the controls.

Group/polymorphism	DD (no.%)	ID (no.%)	II (no.%)	χ^2	P value
Control	46 (36.22%)	51 (40.15%)	30 (23.63%)	0.625	0.960
Primary IRSM	36 (34.95%)	44 (42.72%)	23 (23.33%)	-	-
Secondary IRSM	39 (39.80%)	42 (42.86%)	17 (17.34%)	-	-
Total IRSM	75 (37.31%)	86 (42.79%)	40 (19.90%)	-	-

Table 2: Alleles frequency of -2549 I/D polymorphisms in IRSM groups (primary and secondary) and the controls.

Group/allele	D (no.%)	I (no.%)	χ^2	P value
Control	142 (55.90%)	112 (44.10%)	0.341	0.529
Primary IRSM	116 (56.31%)	90 (43.69%)	-	-
Secondary IRSM	120 (61.22%)	76 (38.78%)	-	-
Total IRSM	236 (58.70%)	166 (41.30%)	-	-

while, I allele frequency was higher in the control group (43.7%) than in patients with primary IRSM (43.6%) and secondary IRSM (38.9%) and no statistically significant difference among the three groups (χ^2=0.341,p=529) (Table 2).

The obtained results revealed an OR of 1.28 with 95% CI=0.94 to 1.85) suggesting that the patients with dominant and co-dominant genetic models (DD+ID) had positively associated with the risk of IRSM compared to the recessive genetic model (II). Another comparison between dominant gene (DD) on one side with recessive and co-dominant genes (ID+II) on the other side, showing an OR of 1.24 with 95% CI=0.88 to 1.93 which is still indicating that IRSM is more likely to occur in the patients with the dominant gene. Patients with DD polymorphism had more liability (Odds ratio=1.34 with 95% CI=0.99 to 1.97) to have IRSM than those with the recessive gene (II). In contrast, Patients with DD and II polymorphisms are less likely to have IRSM than patients with ID polymorphism (Odds ratio=0.94 with 95% CI=0.98 to 1.75and Odds ratio=0.96 with 95% CI=0.83 to 1.44, respectively). The assessment of D and I alleles revealed that patients with D allele are more liable to have IRSM than patients with I allele (Odds ratio=1.33, 95 % CI=0.94 to 1.97) (Table 3).

Additionally, the results revealed that there is no statistically significant difference in the association of VEGFA -2549 I/D polymorphisms with IRSM under dominant, recessive and co-dominant genetic models of the primary and secondary IRSM patients, while, the assessment of D and I alleles revealed that IRSM is more likely to occur in patients with D allele more than patients with I allele (Odds ratio=1.33, 95% CI=0.94 to 1.97) (Table 4).

Discussion

IRSM is a disorder affected by factors that are related to genetics and non-genetics. Certain studies have revealed an association between genetic variants as risk factors and IRSM [26-29]. VEGF is the main factor for vasculogenesis in both pathological and physiological conditions [30-32]; it is overexpressed in a variety of tissues, including the reproductive system of women, ischemic tissues, cancers and during cellular transformation [33], the enhancement of angiogenesis up-regulates the plasma level of VEGF [34].

The role of VEGF in both placental and fetal angiogenesis has been concluded from gene studies [35,36]. VEGF is necessary for the oocytes maturation, trophoblasts proliferation, embryo implantation, the placental angiogenesis, and the maternal and fetal blood vessels growth [13,37]. According to the above, it is reasonable that VEGF may be involved in the pathogenesis of IRSM in women.

Table 3: The association of VEGFA -2549 I/D polymorphisms with IRSM in patients versus the controls.

VEGFA polymorphism	OR	95% confidence interval	χ^2
DD+ID versus II	1.28	0.94 to 1.85	0.108
DD versus ID+II	1.24	0.88 to 1.93	0.087
DD versus II	1.34	0.99 to 1.97	0.126
DD versus ID	0.94	0.98 to 1.75	0.212
II versus ID	0.96	0.83 to 1.44	0.263
D versus I	1.31	0.98 to 1.94	0.171

Table 4: The association of VEGFA -2549 I/D polymorphisms with the primary IRSM patients versus the secondary IRSM patients.

VEGFA polymorphism	OR	95% confidence interval	χ2	P value
DD+ID versus II	0.69	0.72 to 1.47	0.407	0.523
DD versus ID+II	0.81	0.82 to 1.46	0.2	0.655
DD versus II	0.94	0.94 to 1.58	0.014	0.906
DD versus ID	0.87	0.79 to 1.73	0.393	0.531
II versus ID	0.71	0.91 to 1.44	0.299	0.584
D versus I	1.33	0.94 to 1.97	0.131	0.538

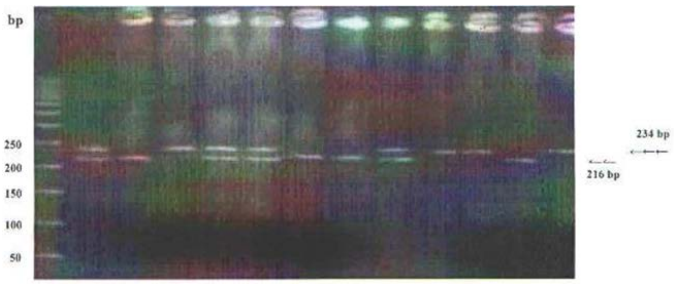

Figure 1: IRSM patients with the different VEGFA -2459 I/D polymorphisms. 2% agarose gel electrophoresis for PCR amplicons of VEGFA -2549 I/D polymorphisms, the dominant gene (DD) (234 bp), the recessive gene (II) (216 bp), the co-dominant gene (ID) (216, 234 bp), 100 bp DNA ladder is indicated to the left side of the gel.

Genetic polymorphisms may affect the production of VEGF, like I/D 18 base pair (bp) polymorphism at site -2549 of the VEGF gene which was demonstrated to be involved in the development of many diseases, especially those based on angiogenesis [38], including breast cancer, Alzheimer's disease and preeclampsia [39], [40-42]. The homozygous (DD) and heterozygous deletion (DI) genotypes have more transcriptional activity of the VEGF gene and therefore more production [16].

The obtained results in the present study revealed that the frequency of ID polymorphic form of VEGFA -2459 I/D genetic models among primary, secondary and total IRSM groups of women was higher than II and DD models, while, II polymorphism was the lowest one. The assessment of the frequency of D and I alleles showed that the D allele was the highest in frequency in secondary IRSM women and the least among the controls, I allele frequency was higher in the control group than in women with primary and secondary IRSM. There was no statistically significant difference between the three groups regarding the frequency of polymorphisms and alleles.

VEGFA variants-IRSM interrelationship was confirmed by the following studies, which illustrated the association of labor before 37th weeks of gestation and VEGF 936C/T polymorphisms in Greeks [43] and North Indians study which claimed that subjects with VEGF gene polymorphisms -1154G/A and +936C/T have higher risk of abortion [44], but Lee HH, et al. (2010) who studied Koreans and concluded that VEGF polymorphisms of in IRSM are inconclusive, therefore, further studies are needed to be done with more sample sizes and in different races or ethnicities [45]. Another Brazilian study involving -634, 936 VEGF polymorphisms suggested that no correlations were found in any of the investigated polymorphisms, even among co-dominant, dominant and alleles [46].

Our results suggested that -2549 I/D polymorphisms of the dominant (DD) and co-dominant (ID) models had positively associated with IRSM risk versus the recessive one (II). Also, the comparison between the dominant gene compared to the co-dominant and the recessive ones indicated that IRSM is more likely to occur in the patients with the dominant gene. In contrast, patients with dominant and recessive polymorphisms are less likely to have IRSM than patients with co-dominant polymorphism. Furthermore, it is shown from the results of the present study that patients with D allele are more likely to have IRSM compared to those having I allele, but, no significant difference in the association between the three genetic models and IRSM in primary and secondary patients.

Pereza n, et al (2015) studied -2549 I/D polymorphisms of the VEGFA gene in the promoter region in couples and found that the frequency of ID gene is the highest in both diseased and control patients [24]. This is consistent with the findings of this study, however, the comparison between DD and ID genetic models was less than the results in the present research. Additionally, Hashemi M, et al. (2018) found that the Or of ins/ins versus del/del and del/ins+ins/ins compared to del/del were 2.85 and 2.19, respectively, while, the allelic OR was higher than the values obtained in the present study [21]. However, this inconsistency in the results can be explained by the racial and ethnicity difference of patients.

VEGF -2549 polymorphisms were studied in patients with diabetic retinopathy, where ID genotype was also identified as a potential risk factor for the disease, whereas in other studies, DD polymorphism was detected as a risk factor for diabetic retinopathy, where ID genotype was also identified as a potential risk factor for the disease, whereas in other studies, DD polymorphism was detected as a risk factor for diabetic retinopathy [47,48].

VEGF gene polymorphisms in patients with diabetes mellitus were evaluated; the study identified a statistically significant association between DD and DD+ID genotypes as risk factors, on one hand, D allele on the other hand, and the development of diabetes mellitus and its neuropathic complication, while, the frequency of II genotype was more in the controls suggesting the role of this polymorphism and me allele which was detected to be more frequent than D allele in the controls in the prevention of diabetes mellitus pathogenesis. Those findings are proving the association between the existence of D allele and the increase in the risk of vascular complications of diabetes mellitus [49].

An additional study clarified the role of VEGF SNPs in metabolic syndrome pathogenesis, stating that the association between remains to be evaluated should be done using functional studies by enrolling larger homogenous populations in various racial and ethnic populations [9].

The limitations of this study are including, a relatively small number of IRSM patients. It should be carried out on a larger group of patients to provide the data for explaining the role of -2549 I/D VEGFA gene polymorphisms in the development of IRSM. Also, the correlation between the levels of VEGFA and the different polymorphisms was not included in the present study which is requiring further investigations in the future.

Conclusion

The results of the present study showed that women with ID model of the -2549 I/D polymorphisms in the promoter region of the gene encoding for VEGFA and those who carry D allele are at higher risk to have IRSM. More genetic studies in different ethnic populations for the VEGFA gene polymorphisms regarding larger loci for more SNPs are needed to shed the light on the association between the studied polymorphisms of the VEGFA gene including -2549 I/D genotypes and IRSM in the Jordanian women for the accurate diagnosis and proper management.

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