SALAHADDIN UNIVERSITY-ERBIL

Association of Vascular endothelial growth factor gene 2578 C/A polymorphism with Recurrent Spontaneous Abortion risk at Erbil province

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Supervisor certificate

This **Graduation Research project** has been written under my supervision and has been submitted for the award of the degree of **B.SC** in Biology (Biomedical) with my approval as supervisor.

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Abstract:

Overview: Recurrent spontaneous abortion (RSA), one of the most common complications of pregnancy, refers to the occurrence of at least two consecutive unexplained pregnancy losses before the 20th week of gestation**.**

Objective: The aim of this research is to investigate the relationship between VEGF 2578 C/A polymorphism and RSA in Erbil province.

Materials and Method: The study involved 60 IRAQI subjects, who were divided into two groups; patients and controls. DNA have been extracted and ARMS-PCR has been done to amplify the target sequence then visualized through agarose gel electrophoresis.

Result: Result shows that CA and AA genotypes are protective factors for RSA with OR= 0.6000 and 0.3600 respectively, and they are not significant for both genotypes as P value = 0.4231 and 0.1573 for each gene respectively. Regarding allele frequencies, A allele shown to be protective factor for RSA in which OR=0.5848 with P value=0.1441 in which its not significant.

Conclusion: We conclude that 2578 VEGF C/A polymorphism doesn't cause RSA in Erbil population in spite of that it's a protective factor for RSA. Also there is a great variation between different populations so we suggest that to study the relationship of this gene in other cities with more number of patients to ensure the results that we got from this study.

Keywords: Recurrent Spontaneous Abortion, Polymorphism, gene.

1.Introduction

Recurrent spontaneous abortion (RSA), defined as three or more consecutive pregnancy losses before the 20th weeks of gestation, is a significant reproductive problem. Around 1–3% of couples trying to conceive experience RSA. The symptoms may include light spotting or brownish discharge, to heavy bleeding and bright-red blood or clots. The bleeding may come and go over several days (Carrington et al., 2005). There are many factors cause RSA such as uterine pathologies, endocrine dysfunctions, autoimmune diseases, acquired and inherited thrombophilia, nutritional factors, problems with implantation, anatomical and chromosomal abnormalities, genetic, placental anomalies, infection, smoking and alcohol consumption, use of contraceptive drug, exposure to environmental factors such as heavy metal, environment pollution, and radiation (Rai and Regan, 2006, Daya and Stephenson, 1996).

Chromosomal abnormalities in the conceptus are usually the characteristic findings in cases of spontaneous abortions occurring due to problems with the pregnancy itself. Chromosomal abnormalities occur in about 50 % of all products of conception from first trimester miscarriages, 5 % of late pregnancy losses and 0.5 % of livebirths. Abortions due to trisomy have a high risk of repeating followed by monosomy and polyploidy. Biochemical or clinical abortion occurs in 30-40 % of women who achieve pregnancy after in vitro fertilization (IVF) and embryo transfer (ET). Structural rearrangements are found in 3 % of cytogenetically abnormal conceptions (Boué et al., 1975).

Evidence for genetic contributions to obstetric disorders comes from candidate gene-based association studies, in which investigators have evaluated particular polymorphic variants of genes selected primarily because of their potential roles in pregnancy (Daher et al., 2006, Ciarmela et al., 2010). Most of the studied polymorphisms are considered functional, because they have been proven to influence the expression of mediators defining different phenotypes.

Cytokines, hormones, and angiogenic mediators play critical roles in reproductive events. Although they are necessary for normal pregnancy development, depending on the conditions such as quantity, stage of gestation, and locality of expression, these factors may affect trophoblast–endometrial interaction leading to RSA or other pregnancy complication (Choudhury and Knapp,

2001, Saini et al., 2011). One of the most important genes that has role in RSA is Vascular endothelial growth factor (VEGF), is a specific mitogen and survival factor for endothelial cells, and also a crucial promoter of angiogenesis in physiological and pathological conditions (Ferrara et al., 2003) (Bautch, 2012) (Byrne et al., 2005). During early gestation, VEGF is essential for the maturation of oocytes, the proliferation of trophoblasts, the implantation and development of the embryo, the angiogenesis of the placenta, and the growth of maternal and fetal blood vessels in the uterus (Jelkmann, 2001) (Su et al., 2011). The aim of this research is to investigate the relationship between VEGF 2578 C/A polymorphism and RSA in Erbil province.

2. Materials and Methods

2.1. Participants

The study involved 60 IRAQI subjects, who were divided into two groups; patients and controls. Patient Group: Included 30 patients recruited from Maternity and Zheen international hospitals. Patients were included in this group after being diagnosed with RSA according to the following criteria: (1) independent case-control or cohort studies; (2) inclusion of both RSA cases and non-RSA controls; (3) examination of the association between VEGF genetic polymorphisms and RSA risk; (4) inclusion of adequate data to calculate the effect size of allele or genotype frequencies; and (5) genotype distribution in healthy controls conforming to Hardy-Weinberg equilibrium(HWE). Control Group: Included 30 apparently healthy individuals whose age and sex were matched with those in the patients group. Venous blood samples (5ml) were collected and blood was assembled in tubes containing EDTA and was stored in freezer until DNA extraction.

2.2. DNA Analysis

The genetic analysis was performed at Zheen International Hospital in Molecular Genetics department. Five milliliters of peripheral blood were collected from both RSA patients and control. Blood samples were kept, in anticoagulant tubes at 4°C, (Add Prep Genomic DNA extraction kit) was used to isolate genomic DNA from peripheral leukocyte. The isolation was made according to the manufacturer protocol.

Tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) is a

simple and rapid method with a high level of accuracy for the detection of SNPs (Hashemi et al., 2012b, Hashemi et al., 2012a, Hashemi et al., 2011). This system was used for genotyping of VEGF 2578 C/A polymorphisms. Genotyping of VEGF 2578 C/A was performed using two outer primers (FO and RO) and two inner allele-specific primers (FI and RI) for the SNP as its shown in Table I.

Table 1: Primer sequences for the identification of VEGF 2578 C/A polymorphisms

Gene	Polymorphism	Primer sequences 5′-3′	PCR product size (bp)	Detection methods
VEGF	2578 C/A	FO:GCCAGCCCTTTTCCTCATAAGGGCCTTA	406	Tetra-ARMS-PCR
		RO: ACATCTTCCCTAAGTGCTCCCAAAGGCC		
		FI: GCCAGCTGTAGGCCAGACCCTGGTAA	205	
		RI:CCAGTCAGTCTGATTATCCACCCAGACCG	256	

Amplification was done with an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of 45 seconds at 94 °C, 1 minute at 63 °C, and 45 seconds at 72 °C, with a final step at 72 °C for 10 minutes. PCR products were verified on 2.0% agarose gel contained 5 μg/mL ethidium bromide and photographs was taken to confirm genotyping quality, all polymorphisms in random samples were genotyped.

2.3. Statistical analysis

Statistical analysis was done using Graph Pad Prism 9 statistical software. Two sample t-test was used to compare the recurrent spontaneous abortion group and healthy control. Genotype and allele frequencies of cases and controls were analyzed using the Chi-square ($\chi 2$) test and both genotype and allelic odds ratio (ORs) and 95% confidence interval (CI) were calculated to determine the Association of VEGF 2578 C/A polymorphisms with Recurrent spontaneous abortion. A p-value of less than 5% ($p<0.05$) was set to be statistically significant.

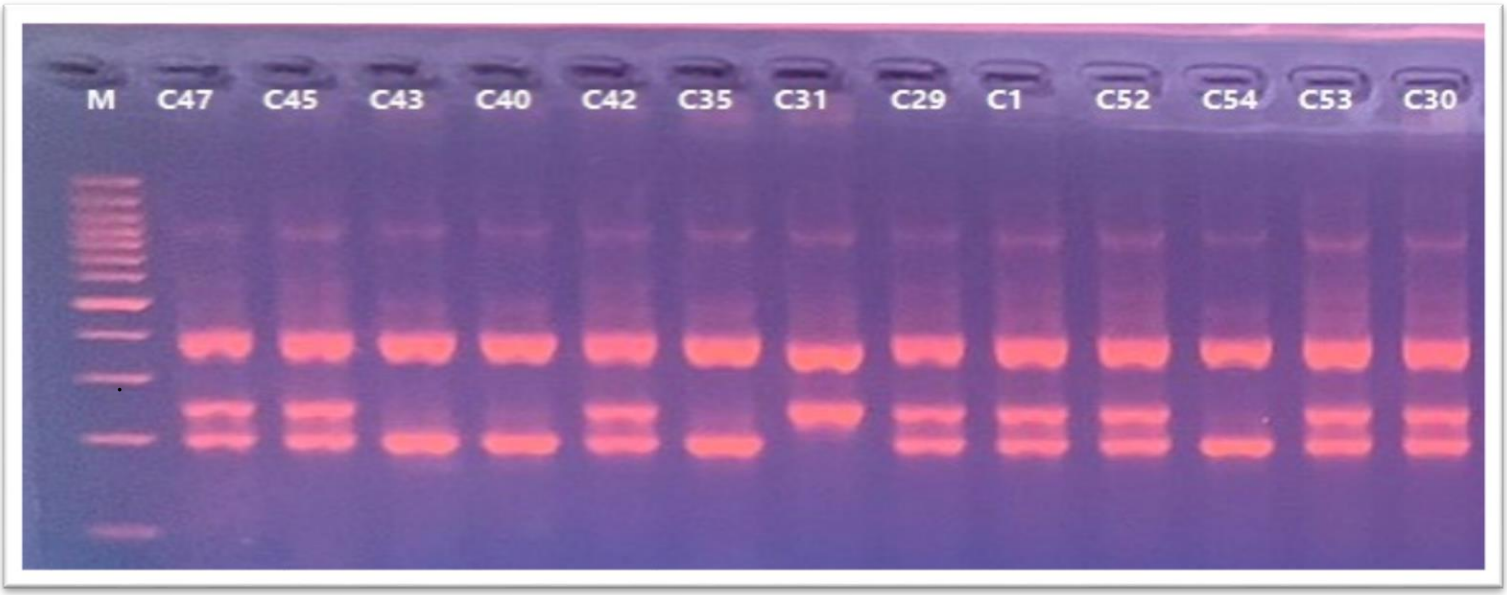
3. Results:

The samples composed of 30 RSA patients and 30 healthy individuals. Table 2 displays the association of genotype and allele frequencies of 2578 C/A polymorphism with recurrent spontaneous abortion polymorphism. Our result shows that CA and AA genotypes are protective factors for RSA with OR= 0.6000 and 0.3600 respectively, and they are not significant for both genotypes as P value = 0.4231 and 0.1573 for each gene respectively. Regarding allele frequencies, A allele shown to be protective factor for RSA in which OR=0.5848 with P value=0.1441 in which its not significant.

Table 2: association of RSA with carriage of alleles/genotypes of 2578 VEGF gene polymorphism.

Polymorphism	RSA (N=30)		Control (N=30)		OR	95% CI	P value
	No	%	No	%			
CC	10	33	6	20	1	-	-
CA	14	47	14	47	0.6000	0.1867 to 1.961	0.4231
AA	6	20	10	33	0.3600	0.08322 to 1.639	0.1573
C	34	57	26	43	0.5848	0.2925 to 1.220	0.1441
A	26	43	34	57			

Figure 1: 2578 VEGF C/A polymorphism electrophoresis design of the tetra amplification refractory system-polymerase chain reaction (T-ARMS-PCR). Lanes 1-13 are samples; M is a DNA marker.



4.Discussion and conclusion:

RSA is known as a multifactorial disease, which is associated with polymorphisms of some genes (Robertson et al., 2006). VEGF belongs to a multifunctional family of growth factors, and is especially important in endothelial cells (Yancopoulos et al., 2000). It is responsible for the proliferation, migration and differentiation of endothelial cells (Bikfalvi, 2004). VEGF, in both developmental stages and in adults, is necessary for vasculogenesis and angiogenesis (Shalaby et al., 1995).

Also VEGF is especially important for a successful pregnancy. Studies carried out on VEGF knock-out mice, showed that VEGF plays an important role in the early stages of pregnancy during the process of angiogenesis (Rowe et al., 2003). Its responsible for increased vascular permeability and the proliferation of endothelial cells for the successful implantation of the embryo (Rowe et al., 2003). Any dysfunction or reduced expression of the gene and its products may lead to failure of implantation.

Recent studies have shown that 30 single nucleotide polymorphisms (SNPs) of the gene, especially VEGF-1154A, VEGF-2578A and VEGF-936T, are related to lowered expression of VEGF and are responsible for the reduced production of the protein (Papazoglou et al., 2005).

Extensive body of evidence suggest that VEGF (2578C/A) cannot be a risk factor for RSA (Masoumi Moghaddam et al., 2012) which its appropriate with the research that we did, we found that 2578 VEGF C/A polymorphism relation with RSA is not a risk factor while it's a protective factor for RSA, in which CA and AA genotypes are protective factors for RSA with (OR= 0.6000, P value = 0.4231) and (OR=0.3600, pvalue0 0.1573) for each gene respectively. Regarding allele frequencies, A allele shown to be protective factor for RSA in which OR=0.5848 with P value=0.1441 in which its not significant.

However, no statistically significant association was observed between −2578C/A (rs699947) and RSA risk. One possible reason for this pattern of results could be that rs1570360, rs3025039, rs2010963, and rs3025020 polymorphisms were more impactful than other SNPs on VEGF gene expression and protein production, thereby possibly explaining inter-individual differences in disease incidences of RSA. Furthermore, in the subgroup analysis by geographic position, significantly increased RSA risk was observed in non-Asian populations for rs1570360 polymorphism and Asian populations for rs3025039 polymorphism. A possible reason for geographic variation could be that great disparities in common SNPs in the VEGF gene that influence the risk of RSA are mostly due to genetic drift and natural selection (Xiao et al., 2011).

While another study revealed a statistically significant difference higher frequency of homozygous mutant genotype (AA) of VEGF C2578A gen among cases compared to control, manifested by very high odds ratio [OR= 2.18, P=<0.001]. Also, there is a statistically significant difference lower frequency of normal wild genotype (CC) in VEGF C2578A gene among cases compared to controls [OR= 0.22, P=<0.001] (El-baz et al., 2014). However, in our study, there was no significant difference in the VEGF genotype between the two groups.

Finally, we conclude that, 2578 VEGF C/A polymorphism doesn't cause RSA in Erbil population in spite of that it's a protective factor for RSA. Also there is a great variation between different populations so we suggest that to study the relationship of this gene in other cities with more number of patients to ensure the results that we got from this study.

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