

VEGFR2 Gene Variation is Associated with Ovarian Hyper Stimulation Syndrome

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ABSTRACT This study aimed to evaluate the effects of the Vascular Endothelial Growth Factor Receptor (VEGFR2) gene variant on the cases of high responses to ovarian stimulation and suffering from Ovarian Hyper Stimulation Syndrome (OHSS). This study included 57 cases (30 controls and 27 patients). Patients suffered from OHSS and controls were healthy pregnant women who had delivered children. DNA was isolated from 2 ml venous blood sample. Polymerase Chain Reaction (PCR) was followed by next generation DNA sequencing methods performed for VEGFR2 gene exon 7. As a result of the study, the VEGFR2 gene rs2305948 variation was statistically significant in the patient group of women who have the high number ovum ($p=0.006, \chi^2=7.560$). This variant rs2305948 may be a genetic marker for detecting women with poor and high responders before ovulation stimulation.

INTRODUCTION

Today, assisted reproductive technology (ART) has become a well-established and highly efficient therapy for infertility. Previous studies have evaluated genetic polymorphisms in ovarian stimulation to explain individuals' variability in response to ovulation induction. Controlled ovarian stimulation (COS) is crucial for optimising in ART and retrieving greater numbers of high-quality oocytes. Therefore, COH plays a principal role in achieving a high ART success rate and a better outcome in terms of oocyte and embryo quality (Roque et al. 2018; Lalethambika et al. 2016).

Pharmacogenetic studies have revealed a series of genetic markers involved in COS response. While applying assisted reproductive techniques, even if the same dosage of gonadotropins is used, it may result in differences oocyte numbers and quality (Dan et al. 2015). These results negatively affect the treatment and

inadequate response to gonadotropins necessitates the recurrence of IVF. In some cases, an exaggerated response to exogenous gonadotropin administration may cause Ovarian Hyperstimulation Syndrome (OHSS) that can be a life-threatening complication (Altmäe et al. 2011). Even if the relevant hormone or intracellular messenger molecule is sufficiently present, it may not interact with the receptor due to a genetic defect in the structure of the receptor. This structural problem may cause high response or poor response to ovarian stimulation. Hence, the effect of the drug varies between individuals and the same dose of medication may cause differences in oocyte number and quality.

VEGF has an important role in angiogenesis but also plays an important role in embryonic development, wound healing as well as female reproductive system and configuration, formation and bone growth. Increased vascular permeability associated with VEGF has been associated with OHSS. The VEGF and its receptors also seem to play a key role in this physiological process of OHSS (Hanevik et al. 2012; Nouri et al. 2014; O'Brien et al. 2014). Additionally it is stated that, follicle progression can be stimulated by VEGFR2 variants and have actions on follicular development through signal transduction pathways (Abadal-Majeh et al. 2019).

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The VEGF gene encodes one of the two receptors. This receptor, also known as kinase insert domain receptor (KDR/VEGF2), is a type III receptor tyrosine kinase. It functions as the main mediator of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis and sprouting (<https://www.ncbi.nlm.nih.gov/gene/3791>).

Aim of the Study

Based on these findings, in this study, the researchers aimed to evaluate the effects of the VEGF gene variant on the patients with a high ovarian response and suffering from OHSS during assisted reproductive techniques.

MATERIAL AND METHODS

Patients

This study included 57 cases (30 controls and 27 patients) with a high number of ovum recruited from the IVF Centre at the Ondokuz Mayıs University (Samsun, Turkey) between September 2015 and June 2016. Informed consent in accordance with the study protocol was approved by the ethics committee of Ondokuz Mayıs University Medical Faculty (KAEEK 2015: 157). A clinical evaluation was performed for all patients. The clinical diagnosis of OHSS was made as described by Fabregues et al. in 1998 (Fabregues et al. 1998). The controls were selected by excluding the diagnosis of OHSS.

DNA Extraction

DNA was isolated from 2 ml venous blood according to kit procedure (NucleoSpin, Macherey-Nagel, Germany) and stored at -20 °C until further analysis. DNA concentration and purity were evaluated using a Jenway Genowa Nano Drop1000 Spectrophotometer (Stone Staffs, UK). All individuals signed a written consent form after being informed about the details of the study.

PCR Amplification and DNA Sequencing

The exon 7 region of the VEGFR gene was amplified with the primers F/R (F:5'-TTTCCAA-GACCATAGCTTACCAT-32 ; R:5'-CAGCAT-

CAGCATAAGAACTTGTA-32) by using GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, USA). The PCR reaction mixture (25 µL) containing 12.5 µL of One Taq 1X Master mix with Standart Buffer (New England Biolabs (Beijing) Ltd., China), 0.5 µL of each primers (BGI Tech Solutions Co. Ltd. (BGI-Tech), China) (0.2 µM), 2 µL of template DNA (1 ng/µL) and 9.5 µL of sterile deionised water. PCR conditions were as follows, that is, initial denaturation step at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60-62 °C for 30 seconds, 71 °C for 35 seconds, and a final extension of 7 minutes at 72 °C. PCR products of each sample were detected by using a 2.0 percent agarose gel and purified by using a GeneJET Gel Extraction Kit (Thermo Scientific, USA). Sequencing was performed at the Novogene Bioinformatics Technology Co. Ltd with an Illumina MiSeq platform according to protocols described by previous studies (Hanevik et al. 2012; Zou et al. 2016). Following the manufacturer's recommendations, a sequencing library was generated by using Nextera XT DNA Library Prep Kit for Illumina (New England Biolabs (Beijing) Ltd., China) and index codes were added (Zou et al. 2016). The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyser 2100 system. The last step, the library was sequenced on an Illumina MiSeq platform. Bioinformatics analyses. The sequences were analysed with the QIIME (Hanevik et al. 2010).

Ovulation Induction Protocol

Stimulation was performed according to the disease-free antagonist protocol. The dosage is adjusted according to ovarian response. When the serum estrogen (E2) concentration was 40pg/ml on the 3rd day of menstruation without any visible cystic structure under ultrasound examination, induction was started at a daily dose of 225 IU of FSH. Ovarian response was followed up by transvaginal ultrasonography and E2 levels. Exaggerated ovarian defined when the number of oocytes from individuals exceeded 20.

Statistical Analysis

SPSS statistical program was performed for correlation analysis. Comparing allele and gen-

otype frequencies between patient and control groups calculated by Chi-square analysis. Probability ratios (OR) and P values were also calculated. Genotype analyses were performed using the OpenEpi program (Dean et al. 2010 Version 2.3.1). The obtained results were evaluated in order to determine whether there is a relationship between polymorphic regions and infertility occurrence.

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RESULTS

The present study included 57 participants and mean age of the patients was 29.59 ± 4.948 years. Minimum age of the patients was 24 years, and maximum age of the patients was 43 years. Mean of the marriage duration of the patients was 6.48 years. Mean oocyte and embryo numbers of the patients was 9.92 ± 4.481 and 18.37 ± 5.534 , respectively. Hormonal laboratory findings revealed that means of FSH (mIU/ml), LH (IU/L) and

E2 (ng/ml) were 6.0259 ± 1.41760 , 6.4374 ± 3.76936 , 38.5252 ± 18.14024 , respectively (Table 1).

VEGFR2 Gene Next Generation DNA Sequencing Analysis Results

VEGFR gene rs2305948 mutant locus was found to be CC homozygote in 25 (50.0%) and CT heterozygote in 2 (28.6%) of the 27 patients studied. In the control group (n=30) CC homozygotes was detected at 25 (50.0%) and 5 (71.4%) CT heterozygotes. When genotype and allele frequencies of patients and controls were compared, there was no statistically significant relationship in terms of rs2305948 region ($p > 0.05$). When compared to TT +CT versus CC or TT versus CC + CT from genotypes, no statistical relation was found ($p > 0.05$) (Table 2).

CT genotype frequency was found to be statistically significant in individuals with more than 20 oocyte counts in the study group ($p = 0.006$) (Table 3). In addition, no significant relationship was found between the number of patients who obtained oocytes over 15 in the patients group and the presence of CT genotype frequency ($p=0.189$). Muta-

Table 1: Clinical findings of patients

	Patients group (N=27)		
	Mean $\pm$ SD	Median (Min-Max)	Range
Age	29.59 $\pm$ 4.948	29.00 (24-43)	19
Marriage time (year)	6.48 $\pm$ 4.061	6.00 (2-17)	15
Hemogram	12.9625 $\pm$ 1.36535	12.9500 (9.30-15.10)	5.80
FSH (mIU/ml)	6.0259 $\pm$ 1.41760	6.10000 (4.00-9.20)	5.20
LH (IU/L)	6.4374 $\pm$ 3.76936	6.2000 (0.70-19.56)	18.86
E2 (ng/ml)	38.5252 $\pm$ 18.14024	36.9800 (3.70-99.00)	95.30
Oocyte number	18.37 $\pm$ 5.534	17.00 (13-32)	19
Embryo number	9.92 $\pm$ 4.481	10.00 (2-19)	17

Table 2: Distribution genotype and allele frequencies of VEGFR gene rs2305948 polymorphism between patients and controls

SNP	Genotype / allele	Patients (n=27) (%)	Controls (n=30) (%)	χ^2	P value	OR (%95 CI)
VEGFR rs2305948	CC	25(50.0)	25 (50.0)	1.131	0.288	
	CT	2(28.6)	5 (71.4)			
	TT	0	0			
	CC+CT:TT	27: 0	30: 0	1.131	0.28	2.5 (0.4428-14.11)
	CC: TC+TT	25: 2	25: 5			
	C	52	55			
	T	2	5	0.057	0.305	2.364 (0.4392-12.72)

*Statistically significant results are written in bold.

Table 3: Genotype frequencies according to the oocyte-embryo numbers and age of patients

	Total n (%)27	VEGFR rs2305948			P value	χ^2
		CC	CT	TT		
<i>Oocyte Number</i>						
<20	21 (77.8)	21 (84)	0(0.0)	0 (0.0)	*0.006	7.560
>20	6 (22.2)	4 (16.0)	2 (100)	0 (0.0)		
<i>Embryo Number</i>						
<15	12 (44.4)	12 (48.0)	0(0.0)	0 (0.0)	0.189	1.728
>15	15 (55.6)	13 (52.0)	2 (100)	0 (0.0)		
<i>Age</i>						
20-30	16 (59.3)	14 (56.0)	11 (0.0)	0 (0.0)	0.223	1.485
30-40	11 (40.7)	11 (44.0)	0(0.0)	0 (0.0)		

*Statistically significant results are written in bold.

tion-dependent genotype variation was not statistically significant with patient age ($p = 0.223$).

DISCUSSION

In humans, VEGF has a biological effect by binding to the VEGF1 and VEGF2 receptors. Vascular endothelial cells are also responsible for vascular permeability of VEGF (Ferrara 2004). A number of studies have examined the relationship between peripheral blood concentration of VEGF and OHSS risk. In these studies while positive associations were shown, contradictory results were found as well (Enskog et al. 2001; Pau et al. 2006).

VEGFR2 gene has a polymorphic region rs2305948 (C > T) located in exon 7 and causing amino acid change in codon 297 (valine > isoleucine). The site of amino acid substitution is in the ligand binding domain of the extracellular domain of the receptor. In vitro studies indicate that this polymorphic region affects the binding of VEGFR and VEGFR-2 kinase insert domain receptor (KDR). Clinically, it has been found that rs2305948 polymorphic region is associated with coronary artery disease, intracerebral haemorrhage and stroke (Wang et al. 2007; Zhang et al. 2009). The rs2305948 T and rs10020464-rs7692791 haplotypes have been associated with neovascular age-related macular degeneration (Fang et al. 2009). It was detected there is a polymorphism in the promoter region of VEGF that affects the transcriptional activity of VEGFR (Kariyazono et al. 2004; Wang et al. 2007). However, there are

also polymorphisms that affect the coding region of the VEGF gene and affect the binding activity to VEGF 2 of VEGF (Lledo et al. 2014).

Several genetic polymorphisms that may be effective in the development of OHSS risk have been investigated such as, *FSHR*, *LHB*, *ESR*, *AMHR*, *SHBG*, *CYP19*, *MTHFR*, *BMP15*, *GDF9*, *SOD2* and *p53* (Wang et al. 2007). In the present study, the frequency of CT genotype was found to be statistically significant in patients who were highly responsive to ovarian stimulation in terms of VEGFR2 gene exon 7 rs2305948 variant region ($p = 0.006$, $\chi^2 = 7.560$). Similarly, in another study with VEGF gene and OHSS patients, it suggested that rs2305945 G/T polymorphic region rs2305948, rs1870378, rs2305945 (C-T-G) haplotype is effective in the development of OHSS (O'Brien et al. 2014).

In Hanevik et al.'s study, they reported that the VEGF + 405CC genotype and OHSS were statistically related (OR 3.4, 95% CI 1.01-11.7) (Hanevik et al. 2012). There was no correlation between the other 5 SNP regions and the development of OHSS. The VEGF + 405C genotype was also associated with ovarian cancer (Steffensen et al. 2010). In another study, the VEGF + 405C genotype was identified as a risk factor for the development of endometriosis (Bhanoori et al. 2005). In addition to VEGF, the VEGFR gene has also been associated with some cancers, stroke, systemic lupus erythematosus, and recurrent abortions in previous studies (McClure et al. 1994; Nouri et al. 2014; Atalay et al. 2016; Taha et al. 2016; Yueniwati et al. 2016).

Kaczmarek et al. (2007) studied the effects of VEGF/VEGFR on OHSS development, based

on 116 patients with OHSS and 124 patients as the control group, the rs2071559 (VEGFR2-604), rs2305948 (VEGFR2-1192), rs1870377 (VEGFR2-1719), rs2010963 (VEGF-405), and rs111458691 (VEGFR1-519) gene regions were researched. They found that frequency of T allele VEGFR1-519 polymorphism was high in OHSS patients ($P=0.02$, OR: 3.62, CI: 1.16-11.27) and VEGFR1-519 and VEGF-405 polymorphic regions were significantly different between patients and control group ($p=0.02$, OR: 3.79 CI: 1.98-11.97 and $p=0.005$ OR: 0.29, CI: 0.17 – 0.50). In this study, it was shown that VEGF and its receptor play an important role in vascular permeability and in the development of OHSS and pathophysiology of OHSS (Kaczmarek et al. 2007). In Nouri et al.'s study they found that rs2305948 (VEGFR2-1192) gene region mutations did not correlate statistically with other parameters except for the number of oocytes obtained. They also found a significant association of the VEGF405 polymorphism (rs2010963) and the VEGFR1-519 polymorphism (rs111458691) with the occurrence of OHSS (Nouri et al. 2014). In a previous study, the *FMR1* gene was analysed in primary ovarian insufficiency patients in Turkish population and 6.6 percent mutation frequency detected (Tural et al. 2015). In a recent study, Abedal-Majed et al. hypothesised that follicular progression during puberty is affected by diet. They tested this hypothesis and reported that follicle progression can be stimulated by VEGF165 and inhibited by VEGF165b through diverse signal transduction pathways (Abedal-Majed et al. 2019).

CONCLUSION

The results of this study were in accordance with the literature. According to the results, it was determined that in ART procedures individuals with CT genotype for the *VEGFR2* gene rs2305948 region, develop much higher number of follicles and these individuals are also at risk for developing OHSS. This variant rs2305948 may be a genetic marker for detecting women who are poor and high responders before ovulation stimulation.

RECOMMENDATIONS

There is a need for more comprehensive prospective and randomised studies in order to clarify

the association of *VEGFR2* gene mutations with OHSS or exaggerated ovarian response.

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ABBREVIATIONS

ART: Assisted reproductive technology
COH: Multiple follicle development with controlled ovarian hyperstimulation
IVF: In vitro fertilisation
ICSI: Intracytoplasmic sperm injection
PCR: Polymerase Chain Reaction
VEGF: Vascular Endothelial Growth Factor

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