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Effects of *FSHR* Gene Variants on Ovarian Response

Cayci Gulhan [1], Ayse Z. Ozdemir[2], Mesut Onal[2], [*]Sengul Tural[1], Davut Guven[2] and Idris Kocak[2]

[1]Ondokuz Mayis University, Medical Faculty, Department of Medical Biology, Samsun, Turkey
[2]Ondokuz Mayis University, Medical Faculty, Departmentof Gynecology and Obstetrics, Samsun, Turkey

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ABSTRACT The study aimed to evaluate the effects of Follicle Stimulating Hormone Receptor (*FSHR)* gene rs747317735 and rs6111 variants on ovarian response. The researchers evaluated 57 cases (27of them were poor ovarian responders, 30 cases have normal pregnancy). DNA was isolated from all the cases. The Polymerase Chain Reaction and the next generation DNA sequencing methods were performed for *FSHR* gene exon 10 analyses. As a result of DNA sequence, the researchers detected a statistically significant difference between patients and control groups for *FSHR* gene exon 10 rs6166 variant (p=0.033, χ^2=6.834). The C allele frequency was higher in the patient group (p=0.008, χ^2=2.897). It was detected that the patients have C allele or CC genotype of *FSHR* gene rs6166 variant were poor responders for application. It was also found that the frequency of patients have TT genotype was higher in the primary infertile group. These results should be confirmed by other and larger researcher groups.

INTRODUCTION

Infertility is a condition of not conceiving despite regular and unprotected sexual intercourse for a time longer than one year (Zegers-Hochschild et al. 2009). Of all healthy couples in the community, 10-15 percent suffer from infertility problems and more than 80 million couples in the world are infertile (Rosenbluth and Van Voorhis 2011). There have been significant developments leading to improved outcomes for most infertile couples in assisted reproductive techniques over the past 25 years (Motteram et al. 2015). During assisted reproductive technology (ART), multiple follicle development with controlled ovarian hyperstimulation (COH) resulting in high number of oocytes and embryos has been aimed at to increase the ART success. In ART, in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) is performed with oocytes obtained with gonadotropins. Despite the same dose of gonadotropins administered during stimulation with gonadotropins of ovaries, oocyte formation can occur in different numbers and quality (Flageole et al. 2019). This can negatively affect the treatment process of patients. In some cases it is seen that oocyte formation with insufficient numbers and poor quality leads to repetition of ART cycles (Livshytis et al. 2009; Palaban et al. 2014; Laleethambika et al. 2016).

Follicle stimulating hormone (FSH) is secreted from the pituitary gland to provide follicle development. Measuring only the FSH levels does not provide an adequate assessment of oocyte quality. The follicle stimulating hormone receptor (FSHR) plays an important role in this process (Flageole et al. 2019; Catteau et al. 2019). If there is enough hormone production while there is a problem in the receptor, hormone-receptor interaction will not take place and oocyte development will be negatively affected. Previous studies have analysed genes responsible for the production of FSH and FSHR, suggesting that some of the mutations, variations and polymorphisms of these genes may cause impairment in the ovarian response to gonadotropins (Livshytis et al. 2009; Palaban et al. 2014; Adolfo et al. 2017).

Objectives

In this study, the researchers aimed to investigate *FSHR* gene differences in individuals

[*]*Address for correspondence:*
Dr. Sengul Tural
Associate Professor
Ondokuz Mayis University,
Medical Faculty Department of Medical Biology,
Samsun,Turkey
Telephone: 03623121919/2846
E-mail: stural@omu.edu.tr

of poor ovarian response during assisted reproductive techniques.

MATERIAL AND METHODS

Patients

In this study the researchers evaluated 57 cases (27 poor responder) during ART treatment in Ondokuz Mayis University Faculty of Medicine IVF Center between September 2015 and June 2016 compared to 30 women who had a healthy pregnancy. Informed con-sent in accordance with the study proto-col was approved by the ethics committee of the Ondokuz Mayis University Faculty of Medicine. The con-trol group was selected by excluding the diagnosis of infertility. DNA was isolated from all the cases and the FSHR gene was analysed using the next generation DNA sequencing method. The study protocol was approved by the ethics commitee of Ondokuz Mayis University Faculty of Medicine (OMU KAEK 2015/185). Written informed was obtained from all the participants.

DNA Extraction

DNA was extracted from 2 ml venous blood according to kit procedure (NucleoSpin Blood DNA Isolation Kit) and stored at -20 °C until further analysis. DNA concentration and purity were evaluated using a JenwayGenowa Nano Drop 1000 Spectrophotometer (JenwayGenova Nano, UK). All individuals signed a written consent form after being informed about the details of the study.

PCR Amplification and Next Generation DNA Sequencing

The exon 10 region of the *FSHR* gene was amplified with the primers (F): 5-TTTGTGGTCATCTGTGGCTGC-3(R): 5-CAAAGGCAAGGACTGAATTATCATT-3 by using the Applied Biosystems Gene Amp 9700 PCR System thermal cycler. The PCR was conducted in triplicate for each sample of the action mixture (25ìL) containing 50-100 ng of template DNA, 0,2-0,4 ìM of each primers (Fermentas), 1-2 mM 1xPCR Buffer (Mg^{+2} containing) 0,625U Taqpolimeraz (ThermoScientific). PCR conditions included initial denaturing step at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds denaturation, 60-62 °C for 30 seconds binding, 71 °C for 35 seconds elongation and a final extension of 7 minutes at 72 °C. Subsequently, PCR products of each sample were detected by using a 2.0 percent agarose gel and purified by using a Gene JET Gel Extraction Kit (ThermoScientific).

Sequencing was performed at the Ondokuz Mayis University Blacksea Advanced Technology Center by IlluminaMiSeq platform according to protocols described by previous studies (Caporaso et al. 2012). Following the manufacturer's recommendations, a sequencing library was generated by using the Nextera XT DNA Library Prep Kit for IlluminaMiseq (New EnglandBiolabs) and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (ThermoScientific, USA) and Agilent Bioanalyser 2100 system. The last step, the library was sequenced on an IlluminaMiseq platform. Bio informatics analyses sequences were analysed with the QIIME (Caporaso et al. 2010) software package.

Ovulation Induction Protocol

Stimulation was performed according to the disease-fix antagonist protocol. The dosage is adjusted according to the ovarian response. When the serum estrogen (e2) concentration was 40 pg/ml in the third day of menstruation and cystic structure was not seen on ultrasound examination, induction was started at a daily dose of 225 IU of FSH. Ovarian response was followed by transvaginal ultrasonography and e2 levels. Follicle growth was monitored by transvaginal ultrasound and 10,000 units human chorionic gonadotrophin (HCG) injection was administered when at least two or three follicles of e"18 mm were seen. Oocyte retrieval was performed 34 to 36 hours afterwards under transvaginal ultrasound guidance using a 17-gauge needle. After a single embryo was transferred the remaining good quality embryos were preserved frozen for the next transfers.

Determination of oocyte quality was based on the concept of oocyte quality that is normally associated with the mature stage, in coinci-

dence with the emission of polar body I and arrest at metaphase II (Coticchio et al. 2004).

Statistical Analysis

A correlation analysis between clinical findings and genotypes was performed using the SPPS statistical program. Chi square, probability ratios (OR) and P values were also calculated to compare allele and genotype frequencies between patient and control groups. 57 genotype analyses were performed using the SPSS program (Dean et al. 2013 Version 3.0.1). Pearson Chi square was used when the sample number was above 25, and Yates Chi square was used when it was below 25. Fisher exact or Mid-P exact was applied when the number of samples was below 5.

The obtained results were evaluated in order to determine whether there is a relationship between polymorphic regions and infertility occurrence.

RESULTS

The present study included 57 participants and mean age of the patients was 36.07±5.86 years. The minimum age of the patients was 24 and maximum age of the patients was 45 years. Mean of the marriage duration of the patients was 4.75±4.33 years. Mean oocyte and embriyo numbers of the patients was 2.55±1.15 and 1.40±1.11, respectively. Hormonal laboratory findings revealed that means of FSH (mIU/ml), LH (IU/L) and E2 (ng/ml) were 10.96 ± 5.86, 7.34 ± 5.23 and 0.54 ± 0.30, respectively (Table 1).

There was a statistically significant difference in frequencies of *FSHR* C/T alleles with patients having a higher representation of T alleles and lower representation of C alleles when compared to control group (p=0.008). Results of the *FSHR* gene variants study revealed 7 (58.3%) CC homozygous, 7 (28%) CT heterozygous, and 13 (65%) TT homozygous in the patient group, while 5 (42.7%) CC homozygous, 18 (72%) CT heterozygous and 7 (75%) TT homozygous were in the control group. When compared between trials and controls, FSHR exon 10 region rs747317735 variant sequence yielded significant difference with regards to CC phenotype (p=0,033, χ^2=6,834). When genotypes CC+CT versus TT were compared, a statistically significant association was observed (p=0.024).

When genotype and frequencies of allele were compared, there was no statistically significant relation in terms of rs6166 region (p=0,353) between the study and the control groups (Table 2).

When the researchers looked at the clinical characteristics and *FSHR* genotype distributions of the patients, it was also found that individuals with TT genotype for rs747317735 have higher frequency in the primary infertile group (p = 0.046) (Table 3).

DISCUSSION

FSH is used as a basic hormone in gamete maturation and in the treatment of hypogonadotropic hypogonadism and infertility in both sexes. Drug dosage applied in assisted reproductive techniques and number and quality of oocytes obtained are important factors affecting success in the treatment. For this reason, many studies have been conducted in order to solve this problem. In particular, pharmacoge-

Table 1: Clinical findings of patients

	Patients group (N=27)		
	Mean ± SD	*Median (Min-Max)*	*Range*
Age	36.07 ± 5.86	29.00 (24-45)	21
Marriage time (year)	4.75 ± 4.33	3.5 (1-20)	20
Hemogram	13.10 ± 1.10	12.90 (11.2-15.90)	4.70
FSH (mIU/ml)	10.96 ± 5.86	10.00 (4.70-37)	32.30
LH (IU/L)	7.34 ± 5.23	6.68 (2.90-32)	29.10
E2 (ng/ml)	44.80 ± 24.67	41 (4.47-122)	117.53
Oocyte number	2.55 ± 1.15	3 (1-4)	3
Embryo number	1.40 ± 1.11	1 (0-4)	4

Table 2: The distribution of *FSHR* gene genotypes in study and control groups

SNP	*Genotype / Allele*	*Patient*	*Controls*	χ^2	*P value*	*OR (%95 CI)*
		(n=27) (%)	*(n=30) (%)*			
FSHR	CC	7(58.3)	5 (41.7)			
rs747317735	CT	7(28.0)	18 (72.0)	6.834	**0.033***	
	T T	13(65.0)	7 (35.0)			
	CC+CT:TT	14 : 13	23: 7	3.842	**0.024***	0.327(0.1055-1.019)
	CC:TC+TT	7: 720	5: 25	0.733	0.39	1.75 (0.482-6.354)
	C	21(%)	38(%)			
	T	33(%)	32(%)	2.897	0.08	0.535 (0.260-1.102)
FSHR	GG	26(49.1)	27 (50.9)			
rs6166	AG	1(25.0)	3 (75.0)	0.863	0.353	
	AA	0	0			
	GG+AG: AA	27: 0	30 : 0			
	GG : AG+AA	26: 1	27: 3	0.864	0.35	2.889 (0.282-29.58)
	G	53(%)	57(%)			
	A	1(%)	3(%)		0.70	2.789 (0.2814-27.65)

*Statistically significant results are written in bold.

Table 3: The distribution of *FSHR* gene genotypes according to the clinical findings

	Total n(%) 27	*FSHR rs747317735*			*P value*	*FSHR rs6166*			*P value*
Infertiliy type		*CC*	*CT*	*TT*		*GG*	*AG*	*AA*	
Primary	25 (92.6)	5 (71.4)	7(100.0)	13(100.0)	0.046*	23 (92.0)	2 (8.0)	0	0.923
Seconday	2 (7.4)	2 (28.6)	0 (0)	0 (0)	6.171	1(100.0)	0 (0)	0	0.087

*Statistically significant results are written in bold.

netic studies have been carried out to determine the variability in response to the genetic differences among the individuals, variants investigated in exon 10 as G919A (Ala307Thr), G2039A (Ser680Asn), G29A mutations and C566T (Ala189Val) inactivation mutations, which occur in the exon 10 regions of the receptor gene of the FSH hormone and is required for normal reproductive function in women, affect the number and quality of oocytes in the treatment of female fertility. Similar and different results have been presented in the literature. As a result of this study, in terms of TT genotype in the study and control groups when compared with rs747317735 variant region of exon 10 region of FSHR gene, there was a statistically significant difference (p=0, 033, χ^2=6,834). With regard to FSHR gene rs747317735 region (C> T) variations, the T allele frequency was higher in the patient group than in the control group (p = 0.008, χ^2 = 2.897). According to the results of this study, in the process of assisted reproductive treatment, in terms of FSHR gene rs6166 region, it was found that individuals with T allele and TT genotype could not generate a sufficient number of quality oocytes. When the researchers looked at other studies, for instance, in the study of Wunsch et al. (2005), mutational studies conducted in the coding part of FSHR revealed two common changes in 307 and 680 positions of exon 10. In women receiving reproductive assistance, studies have shown that changes in exon 10 can affect receptor proteins and sensitivity to FSH induction (Wunsch et al. 2005). In their study Wunsch et al. (2007) showed that patients with-29 A/A homozygous were found to have lower amount of FSH than the other patients.

In another study, TNP at 29th position was found to be associated with the transcriptional activity of the FSH receptor gene and AA genotype related to poor treatment response (Achrekar et al. 2009).

In another study conducted in 2010, 29 AA genotype prevalence in individuals with primary and secondary amenorrhea was found to be higher when compared with the control group. Primary amenorrhea patients with AA genotype at the 29th position was found to have higher serum FSH levels (Achrekar et al. 2010).

Investigations in different populations have shown that baseline FSH levels are higher in individuals with Ser/Ser variants at position 680 (Sudo et al. 2002; Falconer et al. 2005; Greb et al. 2005; Simoni et al. 2008). Mutations in the FSHR gene sequence can cause effective functional changes in the amino acids in the FSHR protein. FSHR gene mutations may be effective in receptor expression at the cell surface, FSH binding capacity, and FSH signal transduction.

In 1995, Aittomaki et al. found a mutation that caused ovarian failure due to resistant ovarian syndrome in the 7th exon of the FSHR gene and affected women are considered infertile with no management other than the ovulation induction treatment (Ghadami et al. 2008).

The activator mutation of the *FSHR* is defined in the extracellular ligand binding portion of the protein. C566T transition at exon 7 causes Val to replace the Ala at the 189th residue (Tapanainen et al. 1998). The phenotype of women with Ala189Val mutation is considered to be more important than women who express partially inactive forms of FSHR (Rannikko et al. 2002).

In homozygous females in terms of Val 189, follicular maturation is inhibited and granulosa cells undergo apoptosis. Ala189Val mutation affects signal transduction after FSH binding due to structural change of FSHR. The mutation alters the cell membrane targeting of FSHR and the secondary messenger activity of the receptor (Loutradis et al. 2008). In a study conducted in 2014, 96 cases treated with assisted reproductive techniques were studied and the FSHR gene was examined in the cumulus cell specimens, of these patients Eight percent were found to have deletions and splices in the exon and intron of the FSHR gene. It has been noted that alternative splice variants in the FSHR gene may cause differences among individuals (Karakaya et al. 2014).

In a study conducted on Asian women, Palaban et al. investigated polymorphism in the FSHR gene Ser680Asn (N680S) and found that the frequency of the SS genotype in women who could not obtain sufficient oocytes was higher (OR 1.61, p = 0.08) (Palaban et al. 2014). In a recent study, Flageolle et al. reported a compound heterozygous for two *FSHR* variants, I160T, a known pathologic variant, and N558H which has never been previously reported (Flageolla et al. 2019). In a previous study the *FMR1* gene was analysed in primary ovarian insufficiency patients in Turkish population and 6.6 percent mutation frequency was detected (Tural S et al. 2015). Simoni et al. investigated the polymorphic regions rs6166 (c.2039AOG, p.N680S), rs6165 (c.919AOG, p.T307A), and rs1394205 (c. K29GOA) of FSH and reported that FSHR gene variants affect serum FSH levels and gonadal response in both sexes (Simoni and Casarini 2014).

Similarly, in this study, there was a statistically significant difference in TT genotype when compared with the rs747317735 variant region of FSHR gene exon 10 in study and control groups ($p = 0.033$, $\chi^2 = 6.834$). The T allele frequency, in terms of the rs747317735 region variations (C> T), was higher in the patient group than in the control group ($p = 0.008$, $\chi^2 = 2.897$). According to the results of this study, in the process of adjuvant therapy, it was found that there were not enough quality oocytes in individuals with T allele and TT genotype in rs747317735 region of FSHR gene. It is known that serine →asparagine amino acid translocation occurs in the 680 position of FSHR gene exon 10 and this conformational change occurring in the receptor causes changes in the hormone-receptor binding activity (Wunsch et al. 2007).

CONCLUSION

The researchers detected that the patients with C allele or CC genotype of *FSHR* gene rs6166 variant were poor responders for application. It was also found that frequency of patients with TT genotype were higher in the primary infertile group. In conclusion, the inability to obtain adequate quality oocytes during the ovulation induction depends on the *FSH* receptor mutations that occur in some of the infertile patients. These results should be confirmed by other and larger research groups.

RECOMMENDATIONS

There is a need for more comprehensive, prospective, randomised studies to clarify the relationship of oocyte quality and genetic variations in the exon 10 region of the *FSH* receptor gene.

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ABBREVIATIONS

FSHR: Follicle Stimulating Hormone Receptor
ART: Assisted reproductive technology
COH: Multiple follicle development with controlled ovarian hyperstimulation
IVF: In vitro fertilization
ICSI: Intracytoplasmic sperm injection
PCR: Polymerase Chain Reaction

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