

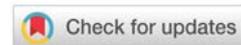
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***Corresponding author:** Sengul Tural, Associate Professor, Department of Medical Biology, Medical Faculty, University of Ondokuz Mayıs, Samsun, Turkey, Tel: 03623121919/2846; E-mail: stural@omu.edu.tr

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Research Article

Association between $LH\beta R$ gene variant and infertility

Elvan Yilmaz¹, Ayse Ozdemir², Mesut Onal², Davut Guven², Sengul Tural^{1*} and Idris Kocak²

¹Department of Medical Biology, Medical Faculty, University of Ondokuz Mayıs, Turkey

²Department of Gynecology and Obstetrics, Medical Faculty, University of Ondokuz Mayıs, Turkey

Abstract

Assisted reproductive techniques have been developed for infertility related problems and to offer more treatment options with increased infertility rates. The aim of the study was to investigate the effect of *Luteinizing hormone β receptor (LH β R)* gene variants on outcome of In Vitro Fertilization (IVF). This study included 69 cases (29 cases with failed of IVF treatment in IVF Center of Ondokuz Mayıs University Hospital by comparing with 30 healthy pregnant cases). DNA isolated from all the cases and *LH β* gene was analyzed by next generation DNA sequencing method. The statistical analysis was performed by SPSS and Chi-Square analysis. There was no statistically significant difference between patients and control groups for *LH β* gene exon 3 mutations of rs1056917 and rs149579838 ($p > 0.05$). When we investigate clinical findings according to the genotype, we found that, GG genotype of rs149579838 ($p = 0.04$, $\chi^2 = 6.381$) and AG genotype of rs1056917 ($p = 0.03$, $\chi^2 = 6.75$) was statistically significant for primary infertile cases. Our results suggest that women who have GG genotype for rs149579838 and AG genotype for rs1056917 are under risk for primary infertility. Large scaled, prospective and randomized trails are needed to demonstrate the relationship between *LH β* gene exon 3 mutations and infertility. These results should be confirmed by further and larger researches groups.

Introduction

Infertility is a condition in which women cannot conceive despite regular sexual intercourse during a year without contraception [1]. The incidence and frequency of infertility varies between societies. A healthy couple normally has 25% chance of conceiving in each ovulation cycle per month. This likelihood of pregnancy is assumed to be 57% for three months, 72% for six months, 85% for twelve months, and 93% for twenty four months. After age 35, quality oocyte production starts decreasing. Assisted Reproductive Techniques (ART) have been developed for infertility related problems and to offer more treatment options with increased infertility rates. Factors such as the age of the patient, etiology, and duration of infertility should always be considered. Infertility treatment should be beneficial both for the couple and the team that will implement it, when economically more advantageous, effective, and less deleterious protocols are developed. Despite ART progression, pregnancy rates per ART cycles initiated are still around 30% [2]. Follicle-Stimulating Hormone (FSH) and Luteinizing hormones are members of the glycoprotein hormone family that regulate gonadal function and menstrual cycle. The effects of FSH and LH are exerted

by their receptors. It is thought that the genetic diversity of receptors is an important predictor of ovarian response and ART treatment outcomes [3-6]. LH, with its receptor located on the cell surface, is important for the theca function, follicle maturation, and ovulation. The LH hormone has beta unit (LH β) and alpha unit (LH α). It is the beta unit that differs LH from the FSH and HCG hormones. The bioactivity of LH may vary with the genetic polymorphism of LH β 2 [7-10]. Liao, et al., have shown that polymorphism of LH β 1052A can cause infertility [7]. A recent systematic review and meta-analysis data summarize the clinical evidence regarding the impact of polymorphisms of gonadotrophins and their receptors on the outcome of controlled ovarian stimulation [11]. Additionally, LH receptor SNPs (LHCGR, rs2293275 and LHCGR, rs12470652) were reported to affect controlled ovarian stimulation and ART [12-14]. Our aim in this study is to investigate the effect of exon 3 variation in the *LH β* gene variants on outcome of IVF.

Materials and methods

Patients

Between September 2015 and June 2016 dates 59 patients were included the study (the study group of 29 had failed In



Vitro Fertilization (IVF) treatment in Ondokuz Mayıs University Faculty of Medicine, IVF Center (Samsun, Turkey) and the control group of 30 had adequate quality ovum. Informed consent in accordance with the study protocol was approved by the ethics committee of the Ondokuz Mayıs University Faculty of Medicine (OMU KAEK 2015/185). DNA material was isolated from all patients and *LHβ* gene was analyzed by next generation DNA sequencing method. Clinical finding of patients are seen in Table 1.

Table 1: Patients Clinical Findings.

	Patients Clinical Findings	
	Mean±SS	Median (Min-Maks)
Age(year)	33.86±5.443	34.00(22-47)
Marriage (year)	8.1379±2.49887	7.0000(2-24)
FSH level	7.7597±2.49887	8.0000(3.60-12.40)
LH level	7.2314±3.49679	6.6800(3.50-19.56)
E2 level	39.9724±16.50274	37.0000(15-99)
Oocyte	10.82±7.394	10.50(1-31)
Embryo	5.86±4.751	4.00(1-19)
Frozen embryo	3.13±1.642	3.00(1-6)

DNA extraction

DNA was extracted from 2ml venous blood according to kit procedure (Nucleo Spin Blood DNA Isolation Kit 74951.50, Germany) and stored at -20°C until further analysis. DNA concentration and purity were evaluated using a Jenway Genowa Nano Drop 1000 Spectrophotometer (Jenway Genova Nano, UK). All individuals were assigned a written consent form after being informed about the details of the study.

PCR amplification and DNA sequencing

The Exon 3 region of the *LHβ* gene was amplified with the primers (F):5'-AGTCTGAGACCTGTGGGGTCAGCTT-3';(R):5'-GGAGGATCCGGGTGTCAGGG CTCCA -3' by using Applied Biosystems Gene Amp 9700 PCR System thermal cycler. The PCR was conducted in triplicate for each sample of the reaction mixture (25μL) containing 50-100ng of template DNA, 0,2-0,4μM of each primers (Fermentas), 1-2mM 1xPCR Buffer (Mg^{+2} containing), 0,625U Taq polimeraz (Thermo Scientific). PCR conditions were as follows: initial denaturing step at 94°C for 3minutes, followed by 35 cycles of 94°C for 30seconds denaturation, $60-62^{\circ}\text{C}$ for 30seconds binding, 71°C for 35seconds elongation and a final extension of 7minutes at 72°C . Subsequently, PCR products of each sample were detected by using a 2.0% agarose gel and purified by using a GeneJET Gel Extraction Kit (Thermo Scientific).

Sequencing was performed at the Novogene Bioinformatics TechnologCo.,Ltd. with an Illumina MiSeq platform according to protocols described by previous studies following the manufacturer's recommendations, a sequencing library was generated by using Nextera XT DNA Library Prep Kit for Illumina Miseq (New England Biolabs) and index codes were added. The library quality was assessed on the Qubit@2.0Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system. The last step, the library was sequenced on an Illumina Miseq

platform (Bioinformatic analyses). The sequences were analyzed with the QIIME 11softwarepackage.

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 40pg/ml on the 3rd day of menstruation and cystic structure was not seen on ultrasound examination, induction was started at a daily dose of 225IU of FSH. Ovarian response was followed up by transvaginal ultrasonography and E2 levels. Follicle growth was monitored by transvaginal ultrasound and 10000units Human Chorionic Gonadotrophin (HCG) injection was administered when at least two or three follicles of $\geq 18\text{mm}$ were seen. Oocyte retrieval was performed 34-36hours afterwards under transvaginal ultrasound guiding using a 17-gauge needle. After a single embryo transfer the remaining good quality embryos were frozen for next transfers. It was accepted that exaggerated ovarian response was obtained in individuals with an oocyte number more than 20.

Statistical analysis

A correlation analysis between clinical findings and genotypes was performed using the SPSS statistical program. Chi square, probability ratios (OR) and *P* values were also calculated to compare allele and genotype frequencies between patient and control groups. 59 genotype analyzes were performed using the SPSS program (Dean, et al., Version 2.3.1). 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. $P < 0.05$ was regarded as statistically significant. The obtained results were evaluated in order to determine whether there is a relationship between polymorphic regions and infertility occurrence.

Results

Results of the *LHβ* gene rs1056917 region revealed 6(60%) AA homozygous, 16(51.6%) GT heterozygous, and 7(38.9%) GG homozygous in the study group, while 4(40%) AA homozygous, 15(48.4%) GA heterozygous and 11(61.1%) GG homozygous in the control group. Results of the *LHβ* gene rs149579838 region study revealed 21(47.7%) GG homozygous, 2(100%) CG heterozygous and 6(46.2%) CC homozygous in the study group, while 23(52.3%) GG homozygous, 7(53.8%) CC heterozygous in the control group. The *LHβ* gene exon 3 region did not show a statistically significant difference between both groups by next generation DNA sequencing of rs1056917 and rs149579838 regions ($p > 0.05$) (Table 2). When the genotype distributions of infertile women were examined, rs149579838 variant region GG genotype ($p = 0.04$, $\chi^2 = 6.381$) and rs1056917 mutant region AG genotype ($p = 0.03$, $\chi^2 = 6.75$) were statistically significant in primary infertility patients (Table 3).

Discussion

In recent years, with the help of assisted reproductive techniques, the fertility rates of infertile couples have increased



Table 2: Genotype Distribution and frequencies.

SNP	Genotype/ Allele	Patients (n=29) (%)	Controls (n=30) (%)	χ^2	P value	OR (%95 CI)
LHBR rs1056917	AA	6(60.0)	4(40.0)	1.305	0.52	
	GA	16(51.6)	15(48.4)			
	GG	7(38.9)	11(61.1)			
	GG+GA:AA	6:23	4:26	0.566	0.45	1.696(0.4249-6.766)
	GG:AG+AA	22:7	19:11	1.092	0.29	1.82(0.588-5.627)
	G	30	37	0.016	0.89	1.046(0.521-2.1)
A	28	33				
LHBR rs149579838	GG	21(47.7)	23(52.3)	2.152	0.34	
	CG	2(100.0)	0(0.0)			
	CC	6(46.2)	7(53.8)			
	GG+CG : CC	23:60	23:7			
	GG : CG+CC	21: 8	23:7	0.050	0.80	1.167(0.339-4.008)
	G	44	46	0.140	0.70	0.798 (0.246-2.588)
	C	14	14	0.010	0.91	0.956 (0.409-2.234)

Statistically significant results are written in bold.

Table 3: Genotype distribution according to clinical findings.

	LHBR rs 1056917			P value	LHBR rs 149579838			p χ^2 value
	AA	AG	GG		CC	GC	GG	
Embriyo count								
10<	4(66.7)	13(81.3)	7(100.0)	0,27	5(83.3)	2(100)	17(81)	
10>	2(33.3)	3(18.8)	0(0)	2.57	1(16.7)	0(0)	4(19.0)	0.79 0.46
Oocyte count								
10<	2(33.3)	7 (43.8)	5(71.4)	0.3 2.17	2(33.3)	2(100) 0(0)	10(47.6)	0.26 2.68
10>	4(66.7)	9(56.3)	2(28.6)		4(66.7)		11(52.4)	
Infertility type								
Primary	6(100)	16(100)	5(71.4)	0.03	6(100)	1(50.0)	20(95.2)	0.04 6.38
Secondary	0(0)	0(0)	2(28.6)	6,75	0(0)	1(50.0)	1(4.8)	

Statistically significant results are written in bold.

[7]. Gonadotropins are pituitary glycoprotein hormones playing an important role in the regulation of fertility. The FSH and LH hormones are pituitary hormones that control ovarian estrogen production. In particular, Njiyu, et al., emphasized that LH synthesis and LH receptor affinity is usually high during oocyte maturation and ovulation period [15].

Despite the limited data obtained in previous studies, it is found that LH and LH receptor genes have been associated with primary amenorrhea and anovulation [16]. LHB gene mutations alter structure and function of LH by activating or inactivating its bioactivity. This change may cause gynecological problems such as anovulation, amenorrhea, and polycystic ovary syndrome. Women with LHB gene mutations often have amenorrhea and infertility [4]. Additionally, studies showed that LHB G1502A gene polymorphism is associated with infertility and endometriosis [8,17-20]. In another study conducted by Piersma, et al., who investigated the LHB gene 291Ser variations and reported that there was no any increase in LHB susceptibility [21]. Maman, et al., examined the LHB expression patterns in human granulosa cells and observed correlation with oocyte function. As a result of this study, they argued that low and high LHB expression might be associated with low fertilization capacity [22]. Additional study reported that mutations in LHCGR gene exon 1 were associated with low oocyte quality in women receiving IVF treatment [23].

Papamentzelopoulou, et al., examined cumulus cells of women treated with assisted reproductive techniques and found a link between the LHR genetic splice variant expression and the ovarian response. In their study it was shown that the profile of LHR gene expression may be a biomarker for ovarian response during assistive reproductive techniques [24]. Davar, et al., again examined the LH gene in Iranian women and the GG, GA and AA genotypes were analyzed by polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) in their study and no correlation was found between LH gene and ovarian change and infertility [7]. They also found that the genotype and allele frequencies of LHB gene polymorphisms did not change in healthy women. In a study conducted in Chinese women in 2015, the LHB gene broad-spectrum rs13405728 polymorphic region was examined and no relation was found between follicle number and anti-mullerian hormone levels [7]. It is known that the LHB gene exon 3 G1502A mutation (Gly102Ser) alters the LH bioactivity by causing amino acid changes [25]. We think that this may change the binding capacity of the hormone to its receptor, which may in turn result in poor response to the drugs applied in the infertility treatment.

As a result of DNA sequence, we detected that while there was no statistically significant difference for FSHR gene rs1056917 and rs149579838 variants between cases and controls



($p > 0.05$). There was a statistically significantly difference when compared genetic distributions based on the clinical findings of the patients. GG genotype for the *LHβ* gene rs 149579838 region ($p = 0.04$, $\chi^2 = 6.381$) and the AG genotype for rs1056917 region ($p = 0.03$, $\chi^2 = 6.75$) were higher in the infertile group. These variants may cause infertility in women. Further and larger studies are needed in order to confirm these results.

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