The Impact of Follicle Stimulating Hormone Receptor (Rs6166) Gene Polymorphism on Infertility and Response to FSH Treatment in Iraqi Infertile Females

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Abstract
background: infertility is a disorder of the reproductive system described by the failure to obtain a clinical pregnancy after twelve months or more of regular unprotected sexual intercourse. Aim: to investigate the effect of polymorphism on response to FSH. Material and Methods: 260 women were taken and divided into three infertile groups according to response (poor, moderate, and high) responder groups, and one healthy group that considered as control group. Hormonal analysis that involved (FSH, LH, AMH, TSH, prolactin, and E2), as well as vaginal ultrasonography for measurement of antral follicle count, also genetic analysis for calculation of allele frequency and odd ratio, the ultrasonography was repeated after six days of giving recombinant FSH. Results: minor allele frequency were highest in poor responder group, and the odd ratio was more than two for (TT) genotype. Conclusion: the FSHR polymorphisms (rs6166) considered as one of the genetic factors responsible for fluctuation in response to FSH in Iraqi infertile women.
Keywords: FSH, infertility, polymorphism, response, allele frequency

1. Introduction

Follicle-stimulating hormone (FSHR) receptor, which is receptor that similar to rhodopsin, that corresponded to superfamily of receptors of G-protein-coupled, which composed from the intracellular moiety also extracellular domain which is a big leucine-rich repeat that passes seven times over the cellular membrane [1].
The gene of FSHR composed from ten exons, 1 to exon nine of which exons encode the extracellular domain while exon ten is encode the little portion of intracellular parts, transmembrane and extracellular domain. When follicle-stimulating hormone (FSH) binds to FSHR cause fast initiation of many cellular cascades, frequently protein kinase A, (cAMP) adenylyl cyclase, arrestin, and (mitogen-activated the protein kinase) Ras-MAPK, pathways of signal transduction. RAS-MAPK and Adenylyl cyclase activated by activation of adaptor proteins and heterotrimeric Gs protein, which resulting in recruiting the adenylyl cyclase, (phospholipase C), (GEFs) factors of guanine nucleotide exchange, subsequently [2,3].
There are many transcription factors, such as (CREB), cAMP regulatory element binding protein, and (ERK) extracellular signal regulated kinase, which lead to the last fertility effects of FSHR activated such as follicular development and maturation as well as lead to development of ovaries through orientation the promoter region belongs to some genes with induction and repression of these genes [4].
Infertility affects globally about 8–12% of couples in their age of reproduction. Females and males contribute unequally to overall cases of infertility [5].
FSHR associated medications like ovarian stimulation drugs, gonadotropins, and natural or synthetic derived follicle-stimulating medicines used for female's infertility [6].
These drugs used for stimulation of many follicles, that require to collect, maturate, and develop eggs with different usage protocols and dosages, these differences occur due to various patients that received these tratements through therapy protocols for infertility [7].
FSH, and similar therapies that specific ligands to bind FSHR, begin signal transduction sequences in cells (females granulosa cells), that cause follicles growth of ovaries, and the ovulation through expression changes in significant genes, so inducing differentiation with proliferation of females oocyte, drug efficacy and, safety ,side effects, treatment preferences, and tolerance studied by different physicians with patients demographic characters indicated weak to an elevated response to FSHR , sometimes excessive life-threatening response occur in some patients [8–10]. Women have impaired ovarian reserve or poor responders, those females who display two or all of three criteria: a) Abnormal ovarian reserve tests, (b) Poor ovarian response

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beyond ovarian stimulations previously, and (c) Advanced maternal age. Women with age 40 or over also retrieval of oocytes, these 3 are considered threshold values to distinguish of the poor ovarian responders. European Society for Human Reproduction and Embryology (ESHRE) included the anti-Müllerian hormone (AMH), and antral follicle count (AFC) [11].

The developed classifications for infertility focused on specific features also women's age that provided a better nuanced picture of ovarian response if poor as a guide to physicians for patient management, the latter is called as patient-oriented strategies, encompassing individualized, oocyte number criteria [12,13].

FSH import the induction action by joining to (FSHR), the dose of FSH efficiency that given for patient mostly connected to the success of controlled ovarian hyper stimulation [10]. FSHR gene located at chromosome region (2p21) that consists of nine introns and ten exons [14].

The gene of Follicle stimulating hormone receptor is the most investigated genetic factor that mostly associated with COS, the most common and very well-investigated SNPs (single nucleotide polymorphism) in FSHR Asn680Ser (rs6166), and Thr307Ala (rs6165), both of polymorphisms found in exon number 10 of FSHR gene, with (Thr307Ala) in the hormone-binding area (extracellular domain of protein) also (Asn680Ser) of the intracellular domain [15].

2. Materials and Methods

Two hundreds sixty (260) women were involved in current study (210) of them undergoing ovarian stimulation by giving rFSH on second day of menstrual cycle,the present study was carried out from October, 2021 to July, 2022. The study was approved by the Scientific and Ethical Committee in Karbala University/college of Pharmacy. All subjects that included in study after a written consent were signed. Subjects that included in the study were classified into two groups: The first group called Control group that include 50 apparently healthy women. The second group called Patient group that include 210 infertile women that distributed into three groups according to treatment response [16]

Poor responder group

Include 70 infertile women which suffering from failure in response adequately for ovarian stimulation treatment and have AFC < 5 and /or AMH < 0.5.

Moderate responder group

Include 61 infertile women which have AFC 5-12 and/or AMH >2.

High responder

Include 79 infertile women which have AFC >12 and/or AMH > 5, the high responders were characterized by an excessive response for ovarian stimulation.

Exclusion criteria: endometriosis, polycystic ovary syndrome, and age of more than 34 years.

Five milliliters (5ml) of venous blood sample were withdrawn from all participant at the second day of menstrual cycle, two milliliters were placed in EDTA tube in order to undergoing DNA extraction, the rest were of blood placed in plain tube and serum was aspirated by centrifugation of blood in centrifuge at 3000 rpm for ten minutes and used for measurement of (basal E2, FSH, Prolactin LH, AMH, TSH) , then two milliliters of venous blood drawn from patient groups that included in study in order to measure serum levels of E2.

The SNPs (rs6166) that selected in study was selected according to (national center for Biotechnology Information) NCBI, clinical var reported high susceptibility to modulating ovarian response also affecting FSHR function.

DNA extraction procedure carried out in 4 steps including (lyses, binding, washing and elution). purity and concentration and of DNA were determined through the using of (NanoDrop)Nano-spectrophotometer.

(PCR) Polymerase chain reactions, were accomplished by using thermocycler, that depend on the amplification of specific region of genome. concentration of target sequence rises from single molecule to many million molecules. Allele specific type of PCR was used which also called as an (amplification refractory mutation system) ARMS-PCR. The main compone

nts consist of : DNA target which called (DNA template), the primers two types (forward and reverse), (*Taq polymerase*) mean *Thermus aquaticus* DNA polymerase enzyme, also need (dNTPs) deoxy nucleotide tri- phosphates, and buffer solution (Darawi *et al.*, 2013). A specific primer uesd to amplify FSHR gene rs6166, primers were designed according to https://www.ncbi.nlm.nih.gov/ websites and by using software (primer-blast).

Table (1) Primers sequences of FSHR rs6166

Primers		Sequence (5'⟶3')	Product Size (bp)
Primers sequences of FSHR rs6166	Reverse	CTGCTATGAAATGCAAGCCCAAATTTAT	-
	Allele C F1	TTAGAGGGACAAGTATGTAAGTGGAACCAT	134
	Allele T F2	TTAGAGGGACAAGTATGTAAGTGGAACCAC	134

3. Statistical Analysis

By using Statistical Package for Social Science (SPSS 25 IBM, Armonk, USA), one-sample Kolmogorov-Smirnov test used to know how the values are distributed. The data were presented as the mean ±SD, the differences in means of the variables between control and patient groups (poor, moderate, and high responder) were analyzed by using one-way analysis of variance (ANOVA). P value of less than 0.05 was considered

as statistically significant. The results of genotyping expressed as frequency and percentage by using SPSS, allele frequency were obtained by using Hardy-Weinberg equilibrium online calculator for all genotypes in the study. Odds ratio (OR) and confidence interval 95% (CI-95) were used to examine the association of these genotypes on the study clinical and biochemical markers also on the development of infertility.

4. Results

The results of clinical and demographic characteristics were shown in table (2), the mean ± SD result of Age, BMI, and age of menarche, for control and patient groups (Poor, Moderate, and high responder infertile women) revealed non-significant statistical difference between the two groups (p>0.05).

Table (2): Socio-demographic data of the control group and patient groups (Poor, Moderate, and high responder infertile women).

Mean ± SD	Control	Poor responder	Moderate responder	High responder
Number	50	70	61	79
Age(year)	25.78 ±2.66	26.27 ±2.95	25.39±3.09	26.42 ±3.16
Body mass index (Kg/m2)	24.62 ±0.74	24.13±3.05	23.96±2.38	24.26. ±2.68
Age of menarche (Year)	12.76±1.3	13.19 ±1.49	12.95 ±1.37	13.13 ±1.1

The mean ±SD of serum FSH levels for control and patient groups (Poor, Moderate, and high responder infertile women) were 6.27 ± (1.43) mIU/mL, 9.64 ± (0.45) mIU/mL, 6.64± (1.48) mIU/mL, 5.67 ± (0.26) mIU/mL respectively.

There were insignificant statistical differences (P > 0.05) in means of serum levels of FSH for control and moderate groups, while there was very highly significant increase (P < 0.001) in mean serum levels of FSH for poor responder group as compared with control group.

The results of LH levels for control and patient groups (Poor, Moderate, and high responder infertile women) were 5.85 ± (0.72) mIU/mL, 8.09± (1.14) mIU/mL, 5.48± (1.52) mIU/mL, 7.42. ±(0.66) mIU/mL respectively. ANOVA showed that there were no statistical differences (P > 0.05) in mean serum levels of LH between moderate responder group and control group, in contrast there were high significant increases (P < 0.001) in mean of LH in high and poor responder groups when compared to moderate responder and control groups, there were very high significant decrease (P < 0.001) in mean of LH in high responder group in comparison with poor responder groups.

The results of E2 before and after treatment, Size of Graafian follicle, Number of Graafian follicle levels for patient groups (Poor, Moderate, and high responder infertile women) listed in Table (3). ANOVA revealed very high significant increases (P < 0.001) in the mean of E2 after treatment with FSH, Size of Graafian follicle, and Number of Graafian follicle for moderate and high responder groups when compared with poor responder group, also there were very high statistical significant increases (P < 0.001) for high responder group in comparison with moderate responder group.

Table (3): The mean ±SD of E2 before and after treatment, Size of Graafian follicle, Number of Graafian follicle in patient groups (Poor, Moderate, and high responder infertile women).

		Poor responder	Moderate responder	High responder
Number		70	61	79
E2 (pg/mL)	Before treatment	38.96±6.52	33.22±5.62	30.23±5.24
	After treatment	80.26±4.67	311.02±34.61	704.78±138.85
Size of Graafian follicle (mm)		9.76±2.23	18.31±1.16	21.91 ±0.9
Number of Graafian follicle		1.04±0.2	2.07±0.25	3.05±0.22

The heterozygous genotype (CT) of FSHR gene was more abundant than (CC) and (TT) genotypes in control group with a frequency of (46,40,14%) respectively with major allele frequency of 63(63%) and minor allele frequency of 37(37%) figure (1).

In poor responder group there were 21 homozygous patients (30%), 32 heterozygous (45.7%), and 17(24.3%) homozygote mutant type with an allele frequency of 74(53%) for (C) allele and 66(47%) for (T) allele, while in moderate responder group (39.3%) had homozygote wild type, (44.3%) heterozygous and (16.4%) with homozygous mutated genotype, the allele frequency was (61%) for major allele (C) and (39%) for minor allele (T).

Among 79 high responder patients, there were 36 heterozygous (CT) genotypes (45.6%),30 (CC) genotypes (38%) and 13 (TT) genotypes (16.5%) for the SNP rs6166 gene, so minor allele frequency was 62(39%)for allele T, and major allele frequency was 96 (61%).

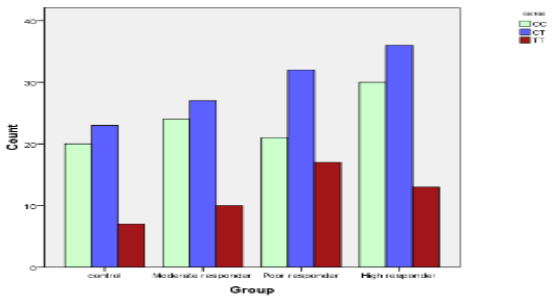

Figure (1): Genotype distribution in (rs6166) SNP among control and patient groups (poor, moderate and high responder).

As shown in table (4) Odds ratio (OR), P-value and Confidence interval (CI) for study groups were summarized , in poor responder group when compared with control group the odd ratio (OR) of CT genotype is 1.32, CI 95% is 0.58-2.98 and (P =0.49) as compared to wild (reference) which represented by CC genotype, while the TT genotype of this SNP has the odd ratio of 2.31, CI 95% is 0.79-6.75and (P =0.04) as compared to wild genotype(reference), the (OR), CI 95% and P value of CT genotype for moderate responder group when compared with control group were 0.97, 0.43-2.2 and (0.95) respectively as compared to wild (reference) genotype that represented by CC genotype, while the TT genotype of this SNP has the odd ratio of 1.63, CI 95% is 0.52-5.05and (P =0.39) as compared to (CC) wild genotype that considered as (reference).

The high responder group as compared with control group show odd ratio (OR) of CT genotype equal to 1.04, CI 95% is 0.48-2.25 and (P =0.91) as compared to wild (reference) which represented by CC genotype, as well as the TT genotype of this C>T polymorphism has the odd ratio of 1.48, CI 95% is 0.51-4.3 and (P =0.46) as compared to wild genotype(reference).

Table (4): Odds ratio (OR), P-value and Confidence interval (CI) of the FSHR (rs6166) C/T genotypes in (poor, moderate, and high responder) groups.

	Genotype	OR	CI 95%	P value
Poor responder versus control group	CC(Reference)	---	---	---
	CT	1.32	0.58-2.98	0.49
	TT	2.31	0.79-6.75	0.04
moderate responder versus control group	CC(Reference)	---	---	---
	CT	0.97	0.43-2.2	0.95
	TT	1.63	0.52-5.05	0.39
High responder versus control group	CC(Reference)	---	---	---
	CT	1.04	0.48-2.25	0.91
	TT	1.48	0.51-4.3	0.46

OR (odd ratio), CI 95% (confidence interval)

5. Discussion

Because the important FSH roles in females follicular growth, also ovarian steroidogenesis, the mutations in gene of FSHR might affect the ability for reproduction [17]. In current study investigation focused on the association between (rs6166) FSHR polymorphism and (clinical, and hormonal parameters) for study groups.

In present study, there were very high significant($p<0.001$) increase in the mean of serum FSH for poor responder group in comparison to the control group ,in contrast there were very high significant($p<0.001$) decrease was present between (moderate and high responder infertile women) when compared to poor responder group ,as the egg quality, and quantity, declined the pituitary gland increases, the level of FSH in order to maintain normal follicular development, hence the basal FSH level give an indicate, of ovarian response to ovarian stimulation treatment [18],these results were in line with Jaiswar S. reported that Basal serum FSH concentration were important in predicting ovarian reserve/response and infertile women with high serum FSH level are at high risk of development poor response to ovarian stimulation treatment [19].

In this study there were significant increase in mean of LH serum level in poor responder group and in high responder group as compared to control group. FSH and LH are crucial for follicles development in female. LH enhances the growth of large follicles as well as increases granulosa cell FSH activity by increasing androgen synthesis then promotes the recruitment of follicles, so when LH level was abnormal that lead to abnormal follicular development [20].These results were in agreement with finding of study [21].

Measurement of E2 level after ovarian stimulation treatment would be helpful to estimate follicle maturation and to predict the ovarian response. E2 is a steroid hormone, secreted by granulosa cells, of developing ovarian follicles. The low level of estradiol indicate a reduction in the ability of ovarian follicles to grow and produce estradiol in response to FSH (Huang et al., 2018), because the main functions of FSH is follicular development and stimulation of estradiol production which might be uncoupled and/or involve different downstream pathways of the FSH receptor

The results of present study that show the allele frequency were listed in table(3-4) and (3-5)for rs6166 and rs6165 revealed that heterozygous genotype of both SNPs were more abundant than homozygous mutant and wild genotypes in control and patient groups.These results were consistent with those of Unsal T.which show that the heterozygous genotype frequency of rs6166and rs6165 was higher than wild and homozygous genotypes in both control and infertile women groups [22],while in poor responder group TT genotype is higher than TT genotype in control ,moderate and high responder group in rs6166 ;yet these increment in TT genotype is much higher in rs6165 for poor responder group as compared with TT genotype in control, moderate and high responder groups, these observations, were consistent with, the results obtained by Rod A. who noticed that position, 307 of FSHR SNP(rs6165) was more representative, than position 680 of SNP(rs6166)[23] ,Tarek M. indicated that the mutant

genotype was 2.5 fold higher in poor responders group than in good responders group[24].

The expression of odd ratio and association between allele frequencies for rs6166 in (poor, moderate and high responder groups) and control group displayed in table (3-6) Odd ratio for CT genotype of rs6166 for moderate, high and poor responder groups reveal insignificant differences that mean no association between CT genotype in all groups with infertility occurrence.

The odd ratio for the TT genotype in the moderate and high responder groups have the same level of insignificancy. The odd ratio for TT genotype in the group of poor responder was greater than two, indicating a link between this genotype with occurrence of infertility, this significant differences mean that polymorphism occurrence (C>T) in rs6166 play a role in pathogenesis of infertility.

The odd ratio in poor responder group for mutant genotype (TT) that belong to rs6165 have more risk than rs6166 on the occurrence of infertility in Iraqi infertile women, these finding were in agreement with Maryam K. which reported that rs6165 was associated with increased risk of infertility [25].

This study revealed that the minor allele frequency for rs6166 and rs6165 were very high for T allele in poor responder group when compared with T allele in control, moderate and high responder groups as shown in tables(3-4) and(3-5),these finding suggested that the (T allele) of rs6165 and rs6166 was associated with possible risk of female infertility in Iraqi infertile women and these SNPs seem to exert pathogenic contribution to female infertility.

These results were in contrast to a study performed in the Indian population 2019 which states that rs6166 had no association with infertility pathogenesis [26], while results of current study were agree with several studies that show that the T allele of rs6166 was associated with increased risk to infertility, in several population as stated by Maryam and Tarek [21].

6. Conclusion

The allele frequency of mutant genotype was found high in poor responder group in comparison to (high responder, moderate responder, and control) groups, this lead to make a generalized conclusion that a mutant (TT) genotype polymorphism leads to a poor response for FSH receptor.

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