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Original Article

Correlation of Estrogen Receptor Alpha Serum Level with Gene Polymorphism and Its Effect on Women with Unexplained Infertility, Basra, Iraq

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Abstract

Infertility of unknown etiology is considered a significant medical and health problem. This study focused on the role of the estrogen receptor alpha (*ESRα*) gene polymorphism, *PvuII* (rs2234693), and its effect on the amount of *ESRα* in the blood of women who cannot get pregnant for unknown reasons. A total of 184 females were evaluated, including 102 with unexplained infertility (UI) and 82 age-matched control females (with at least one living child and no history of infertility). Blood samples were collected, genomic DNA was isolated from blood samples, and the genotyping of the *ESRα* gene was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *ESRα* expression levels were assessed by the ELISA. The study revealed that the mean serum level of *ESRα* was significantly higher in the case group than in the control group ($P<0.05$). Furthermore, the genotypes (TT, TC, and CC) and alleles (T and C) significantly influenced the plasma level of *ESRα* in the study population. Moreover, the presence of the C allele was considered a risk factor, and the polymorphism had a significant effect on *ESRα* expression level in women with UI.

Keywords: Estrogen receptor alpha, Female infertility, Gene polymorphism, Genotyping, RFLP

1. Introduction

Successful embryo implantation is influenced by impaired endometrial receptivity, which might lead to infertility in women (1). Several factors, including hormones, receptors, adhesion molecules, growth factors, and cytokines, mediate the mother's embryo-fetal interaction, making it easier to accept the blastocyst and implant it (2).

The release of ovarian steroids controls uterine receptivity throughout the menstrual cycle. During the preovulatory period, estrogen stimulates the growth of the endometrium, while progesterone alters the endometrium secretory structure (3). Ligand-specific intracellular receptors mediate estrogen and progesterone effects in stromal and epithelial endometrial cells (4, 5). Estrogen receptor alpha (*ESRα*) is up-regulated in reaction to estrogen during the proliferative phase and down-regulated in response to progesterone during the implantation (6). In most mammalian species, ESR loss has been reported at the moment of implantation (7). A crucial step in forming endometrial receptivity is when the ESR reduction coincides with endometrial gene expression in the middle of the luteal phase (8). The absence of ESR during implantation has been hypothesized to affect the expression of proteins that control endometrial receptivity (9).

Central and peripheral nervous systems are affected

by estrogen activity, which is mediated by ESRs. The genes for ESRs α (*ESR*α, *ESR*1) and β (*ESR*β, *ESR*2) are essential in regulating the physiological resistance to estrogen. *ESRα* gene is present on chromosome 6q25. It consists of eight exons which are divided into seven intronic regions with a total size of 140 kb (10).

ESRα codes a protein of 595 amino acids, and the most widely studied polymorphism of *ESRα* involves *PvuII* (rs2234693). The *PvuII* (T397C) polymorphism occurs due to T/C transition in the first intron, controlled ovarian hyperstimulation (COH), and *in vitro* fertilization (IVF) pregnancy outcomes (11, 12).

Undiagnosed/unexplained infertility (UI), also known as idiopathic infertility, is when couples cannot conceive for unknown reasons (13). The diagnosis of UI occurs when normal ovulatory function (including basal body temperature, cervical mucus changes, serum luteinizing hormone surge, or mid-luteal progesterone), tubal patency (hysterosalpingogram and/or laparoscopy), and standard semen analysis are confirmed (14).

It was assumed that changes to molecular and cellular indicators might be the cause of UI. This study investigated the *ESRα* gene polymorphisms and their effect on its serum levels in females with UI, compared to average fertile, healthy females. It also investigated *ESRα* gene polymorphisms in females with UI to determine whether potential allelic variants may contribute to infertility and assess whether *ESRα* genotypes will induce changes in circulating levels of *ESRα* and affect the success of pregnancy.

2. Materials and Methods

2.1. Sampling

A case-control study was conducted from November 2021 to March 2022. The patient population comprised 102 females with primary and secondary UI who attended the reproductive clinic at Basra Maternity and Child Hospital, Basra, Iraq, and received a gynecological diagnosis. They were between 20 and 50 years. Each participant filled out a thorough questionnaire form. The Control group included 82 women attending the family planning or healthcare facility who seemed to be in good health, were not pregnant, and were matched in age to the research group.

2.2. Molecular Study

A 3 ml sample of fresh venous blood was taken from every participant, 2 ml of which was in EDTA tubes for total DNA extraction by the whole blood Wizard® Genomic DNA Purification Kit (Promega, USA). The DNA isolation was based on the kit manufacturer's instructions.

2.3. Serological Study

The remaining 1 ml of fresh blood was centrifuged in a gel tube for serological study, and the obtained serum was kept frozen at -20°C until detecting the serum levels of *ESRα* by special ELISA kits supplied by Bioassay Technology Laboratory (BT, CHINA, SHANGHAI Cat. No E7871Hu). All samples were measured according to the manufacturer's instructions in the kit.

2.4. *ESRα* Genotype Determination

The *PVUII* c454-397T>C (NCBI ID: rs2234693) SNP polymorphism of *ESRα* was identified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (by design primers), forward CCACCATGCTCAGTCTCTA and revers ACCACCATGCTCAGTCTCTA (Accession number NG_008493. 2). New England Biolabs (NEB, USA) digested PCR products with the restriction endonucleases *PVUII* Catalogue number R0151S. The digested DNA was then run on 1.5% agarose gel and sent for sequencing.

2.5. Sequencing

To corroborate the findings of the RFLP genotype, six samples were taken from PCR products in a volume of 50µl and forwarded to Macrogen (Korea) for sequence analysis and phylogenetic tree using the Neighbor-Joining method with p-distance value via 1,000 replicates of bootstrap.

2.6. Statistical Analysis

Statistical Package for the Social Sciences (SPSS, version 20) program was used for statistical analysis.

Data were evaluated using the Chi-squared, t-student, and Pearson correlation tests. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the Study Population

A total of 184 females were evaluated in this study, including 102 patients with UI and 82 fertile females as the control group. The age group of the studied females was between 20-50 years, and the average age was 31.59±6.79. The distribution of cases and control groups in terms of age, as well as the type and duration of infertility, is presented in table 1.

Table1. Characteristics of study populationbased on age, type of infertility, and duration of infertility

		Cases (102)		Control (82)		*P*-value
		N0	%	No	%	
Age groups	20-29	54	52. 9	50	61. 0	0. 478
	30-39	27	26. 5	20	24. 4	
	40-49	21	20. 6	12	14. 6	
Type of infertility	Primary	77	75. 5			
	secondary	25	24. 5			
Duration of infertility	<10	16	15. 7			
	>10	86	84. 3			

For comparison, the studied subjects were divided into three age groups of 20-29, 30-39, and 40-49 years. The majority of females with UI were 20-29 years. There was no significant difference in terms of age between the cases and the control group (*P*>0.05). Most patients (n=77, 75.5%) had primary infertility with more than 10 years (84.3%).

The mean serum level of *ESRα* was significantly higher in the case group (1352.6±660.5), compared to the control one (748.6±408.5). As illustrated in table 2, the *P*-value was <0.05.

Table 2. Differences in mean serum level ESR alphain the study population

Test	Cases group, No. =102, Mean±SD	Control group, No. =82, Mean±SD	*P*-value
ESRalpha	1352. 6± 660. 5	748. 6± 408. 5	0. 001

The association of serum levels of *ESRα* with the type and duration of infertility is shown in tables 3 and 4, which demonstrate no significant differences in the quantity of *ESRα* in primary or secondary infertility or the duration of infertility in the study groups (P>0.05).

Table 3. Mean serum levels of ESR alpha in patients only

Parameters	Primary infertility group, No. =77, Mean±SD	Secondary infertility group, No. =25, Mean± SD	*P*-value
ESR alpha	1328.2±732. 8	1427.6±356. 8	0. 516

-No. : The number of patients, SD: Standard Division

Table 4. Differences in serum ESR alphalevels in a group of patients based on infertility duration

Parameters	<10 years	>10 years	*P*-value
ESR alpha	1446. 4±328.8	1335.1±328.8	0. 539

The results of examining the relationship between the age of the patients and the average serum levels of *ESRα* using statistical analysis (Pearson's correlation) showed no significant correlation between the age of the study population and the mean serum levels of *ESRα* (Table 5, Figure 1).

Table 5. Correlation of serum level of (ER1) with the age of the study population

Parameters	Pearson correlation coefficient (R)	*P*-value
ESRα	0. 092	**0. 214**

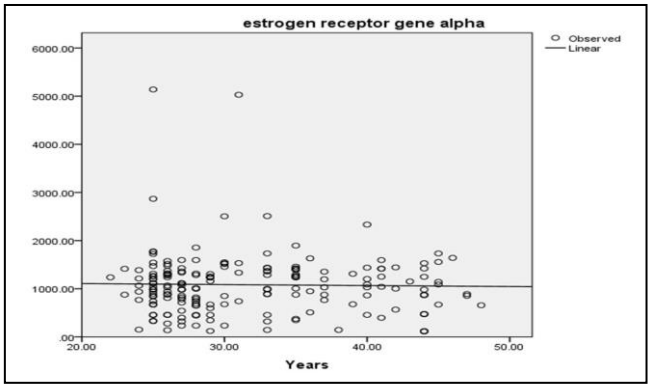

Figure 1. Scatter plot of positive linear correlation between ESRαand the age of the study population

3.2. *ESRα* Genotypes

The gene segment that contains the single nucleotide polymorphism within the *ESRα* gene region was amplified from the extracted DNA for each sample of patients with UI and the healthy controls. The PCR was performed under optimum conditions, and the PCR product underwent electrophoresis on 1.5% agarose gel. The results showed a single clear band with a molecular size of 530 bp (Figure 2).

3.3. Genotyping of *ESRα*

The PCR product of the samples was digested using the restriction enzyme (*PvuII*) and then run on 2% agarose gel. The wild-type (WT) genotype (TT) would have two bands (334 and 196 bp).

The hetero genotype (TC) was confirmed once three bands (530 bp, 334 bp, and 196 bp) were shown in the gel. Finally, the mutant genotype (CC) was detected once the gel expressed only one band of (530 bp) (Figure 3).

The PCR-RFLP bands of the SNP rs2234693 T/C of the *ESRα* underwent genetic analysis, and the results revealed three genotypes (TT, TC, and CC) in the patient and control samples. The findings revealed a difference in genotype repetition between the control group and the patients. As a homogeneous genotype, CC showed a higher frequency among patients, compared to the control group (7.8%, 0.0%, respectively), with a statistically significant difference (*P*=0.002). The odd ratio of (OR=0.436) means that the presence of the C allele is considered a risk factor.

Comparing the heterozygous TC genotype of *PvuII* to the homozygous TT (*P*=0.02) and the uncommon homozygous CC (*P*=0.002) genotype of cases and the controls showed that the heterozygous TC genotype was more prevalent in the infertile group and was substantially related to the risk of infertility (Tables 6 and 7).

3.4. *Sequencing and* Phylogenic Tree of *ESRα* Gene

The findings showed that three genotypes (homozygous wild type TT, heterozygous TC, and homozygous mutant CC) were used. To corroborate the findings of the RFLP genotype, PCR was carried out in a volume of 50 µl and forwarded to Macrogen (Korea) for sequence analysis (Figures 4, 5, and 6).

Based on the genes of the *ESRα*, a molecular had been created. The phylogenetic trees of specimens of homo sapience based on the sequence of the *ESRα* gene are shown in figures 7 and 8.

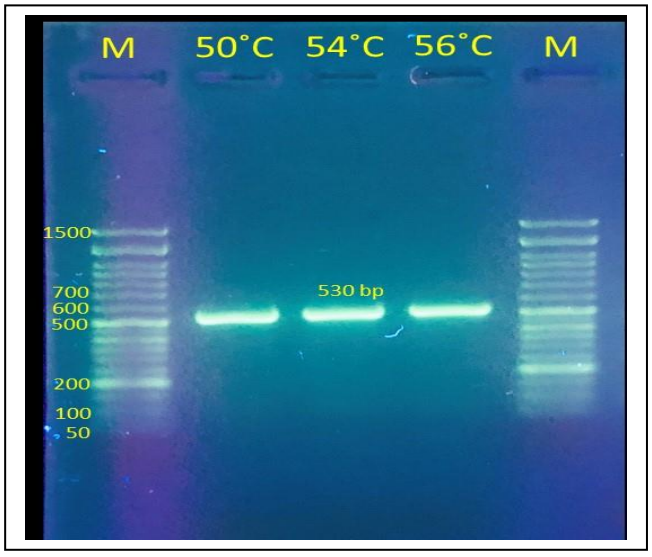

Figure 2. PCR products of the amplification of a partial region of gene ESR alphaof *Homo sapiens*
(530 bp)The gel was 1. 5%, and the DNA dye was Red Safe (Intron, Korea). V: 90, Time: 45 minutes. M: DNA ladder (50-1500)bp

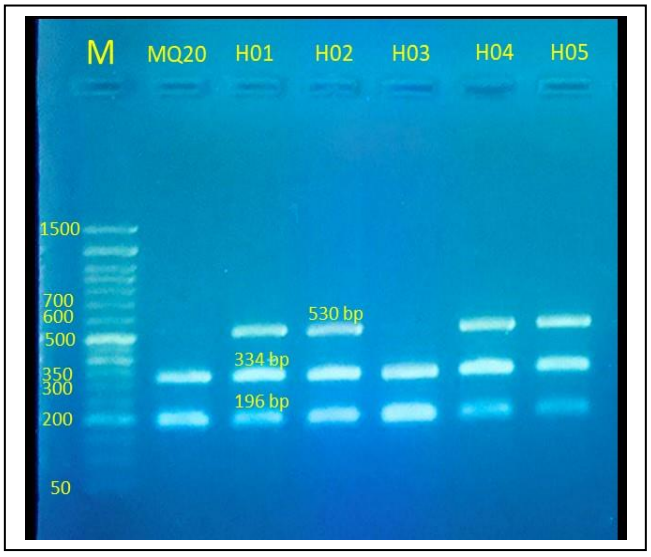

Figure 3. Gel electrophoresis of PCR-RFLP bands of SNP rs2234693 T/C of gene *ESR alpha*

Table 6. Genotype distribution of *PvuII* in *ESR alpha gene* among study groups

	Genotype	Cases (102)	Controls(82)	OR	95% CI	*P*-value
	TT	34 (33. 3)	44 (53. 7)	Ref	Ref	Ref
	TC	60 (58. 8)	38 (46. 3)	0. 489	0. 267-0. 896	0. 02
~	**CC**	8 (7. 8)	0 (0. 0)	0. 436	0. 339-0. 561	0. 002

Table 7. Association between the –*PvuII* in *ESR alpha gene* SNP and ER 1 alpha serum level among study groups

	Case No= 102	Control No=82	*P*-value
Alleles	**ER 1 serum concentration Mean ± SD**		
T	1365. 88± 673. 9	748. 57± 408. 5	**0. 001**
C	1445. 49±753. 97	672. 11± 368. 37	**0. 001**
Genotype	**ER 1 serum concentration Mean ± SD**		
TT	1166. 29± 331. 6	814. 6± 433. 54	**0. 001**
TC	1478. 98± 786. 37	672. 11± 368. 37	**0. 001**
Cc	1196. 46± 479. 33	0	

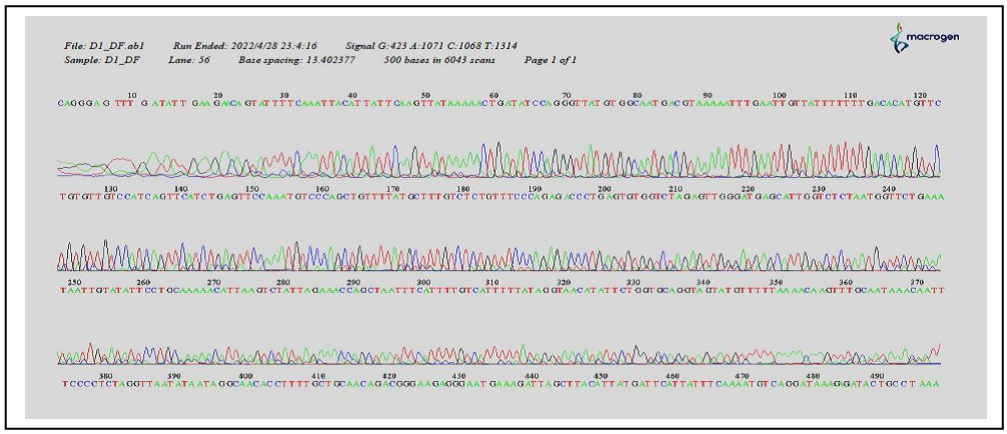

Figure 4. Sangar sequence method Analysis for Sample (1) ESR alpha Forward primer

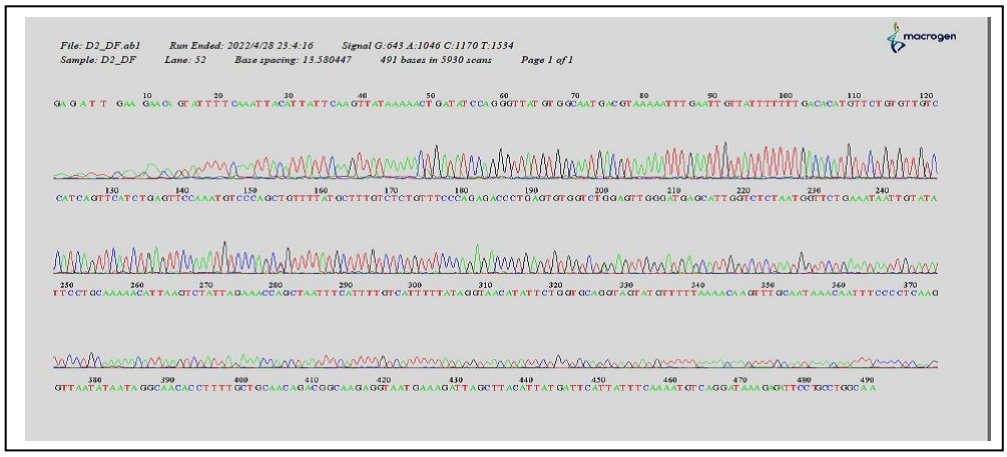

Figure 5. Sangar sequence method Analysis for Sample (2) *ESR alpha* Forward primer

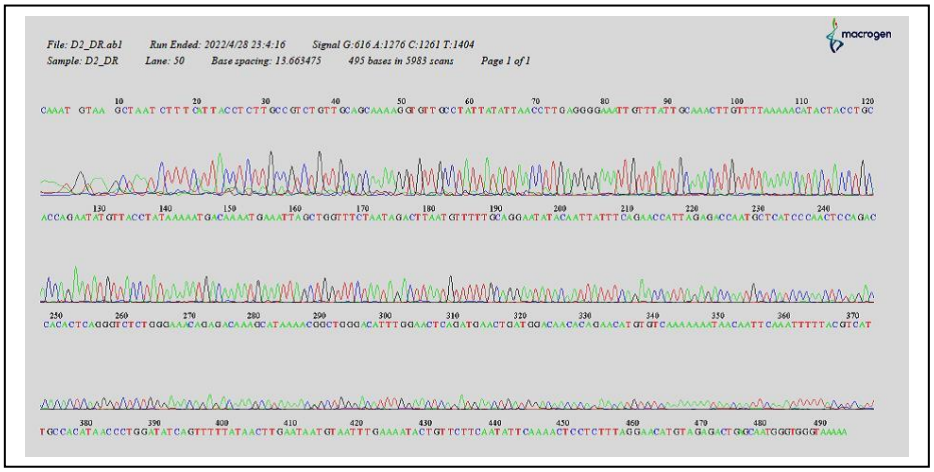

Figure 6. Sangar sequence method Analysis for Sample (2) ESR alpha Reverse primer

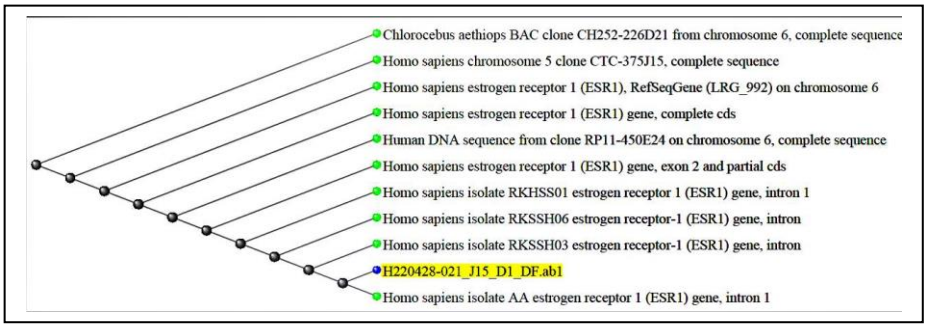

Figure 7. Phylogenetic tree of ESR alpha *gene*

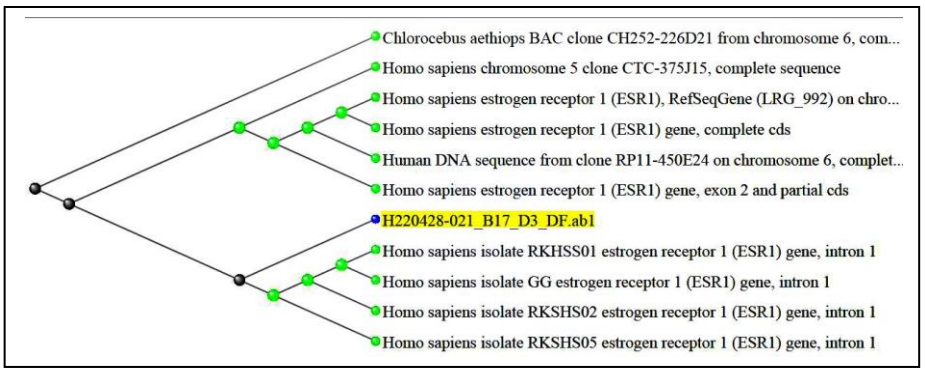

Figure 8. Phylogenetic tree of ESR alpha *gene*

4. Discussion

The objectives of diagnostic fertility are to determine the cause, provide a prognosis, and formulate a treatment strategy. In the current study, the age of the enrolled subjects ranged from 20 to less than 50 years, with a mean age of 31.5924±6.79216. Based on the findings, the majority of infertile women were 20 to 29 years. Previous research has also shown that the majority of infertile women (57.2%) were 20-30 years and 29±6 years, in descending order (12, 15). Regarding the two types of infertility, primary and

secondary, 75.5% of the patients were affected by primary infertility, and 24.5% had secondary infertility. This is in agreement with a previous study that diagnosed (78.7%) had primary infertility, and (21.3%) had secondary infertility (16). Liaqat, Hasnain (7) reported that (69.5%) suffered from primary infertility and (30.5%) from secondary infertility. However, Hsieh, Wang (8) reported a reverse finding in which infertility was primary in 14.3% and secondary in 85.7% of the cases. Estrogen, which has well-known roles in the uterus, ovaries, mammary glands, and hypothalamic-pituitary axis, is an essential component in female reproduction. The vital mediator for the function of estrogen is created by special receptors, which are *ESRα* and *ESRβ* genes. The expression levels of *ESRα* are maximum in the ovary and uterine endometrium, consistent with the fact that the female reproductive system is the primary target of estrogens. In this study, the mean serum levels of *ESRα* were significantly higher in females with UI, compared to the control fertile group (P<0.05). In cases of UI females, the main target of treatment is assisting reproduction techniques. Numerous factors have been linked to the success of such treatment. Despite all patients receiving the identical IVF follicular stimulation procedure, there were considerable differences in patients' follicular responses. The estrogen hormone is a significant element in determining the quality of an oocyte because it influences oocyte maturation and promotes optimal oocyte cytoplasm and oolemma maturation. A high-quality oocyte is necessary for maturation, fertilization, and post-embryonic development. All of the above facts of the effect of estrogen hormones depend on ESRs, so we can suggest that the female who is diagnosed with UI and found to have an average level of ESR can benefit from IVF (13).

This study evaluated one *ESRα* SNP (*PvuII*) in females with UI. This polymorphism is located in intron 1 (rs2234693). This study concentrated on this region for genetic investigation since this part affects the pregnancy rate after IVF (16). *It has also been suggested that PvuII polymorphism is linked to an increased risk of developing endometriosis,* COH, and the pregnancy outcomes of IVF (13). A significant difference was observed between patients and the control group in terms of TT+TC versus CC genotypes (P=0.05). Data showed that the level of CC homozygote genotype (mutant type) was significantly higher in patients, compared to the control group with OR=0.436, meaning that the presence of the C allele is considered a risk factor or a predisposing factor in combination with other issues for the development of the disease. Since the TT homozygote (wild) genotype was significantly higher in the control group than in the patients' group, this may hint that the presence of this genotype and the T allele has a protective effect against the development of the disease. The heterozygote genotype in the form of TC was significantly different between the cases (58.8%) and the control group (46.3%). It was associated with the risk of infertility, compared to the homozygous TT (P=0.02) and rare homozygous CC (P=0.002) genotype of cases and the control group with an OR ratio of 0.489. As a result, women in our research group with heterozygous TC genotypes were more likely to have infertility than those with CC or TT genotypes (12, 15).

In this study, there was a significant influence of the genotypes (TT, TC, CC) and alleles (T, C) on the plasma level of *ESRα* in the study population (P=0.0001). However, the *ESRα* gene polymorphism's impact on gene expression was investigated for the first time. Regarding the TT homozygote genotype and the T allele, as found in this study, this genotype is considered a protective factor because it was higher in the control group than in the patients. It was also associated with a significantly lower level of serum *ESRα* in the control group, which confirms the protective effect of this allele and the genotype. The results also showed that the mean serum level of *ESRα* was significantly higher in patients (P<0.05) with TC

genotype than in the control group. Therefore, as mentioned previously, the TC genotype is considered a risk factor for the development of infertility in females carrying it. Concerning the CC mutant homozygote genotype, it was detected in the patient group only and was associated with a high serum level of *ESRα* ($P<0.05$).

Finally, the results showed that the frequency of the high *ESRα* production allele (C-allele) was higher in patients, compared to the control group. This was statistically significant, and the mean serum level of *ESRα* was higher in cases ($P<0.05$).

Therefore, given how it influences the etiology and prognosis of other diseases, there is a possible favorable effect of gene polymorphism on infertility (17, 18). The present study showed the importance of *ESRα* gene polymorphisms (rs2234693) and *ESRα* serum level in UI patients in Basra, Iraq.

Authors' Contribution

Study concept and design: M. Q. T.
Acquisition of data: W. N. I.
Analysis and interpretation of data: M. S.
Drafting of the manuscript: M. Q. T.
Critical revision of the manuscript for important intellectual content: W. N. I.
Statistical analysis: M. S.
Administrative, technical, and material support: M. Q. T.

Ethics

The ethical approval was obtained from the Ethical Approval Committee in the College of Medicine, University of Basra, Barah, Iraq, and was offered acceptance and approval by the Research and Development Center, the Ministry of Health.

Conflict of Interest

The authors declare that they have no conflict of interest.

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