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Research paper

In terms of the PCR-RFLP technique, genetic screening of Ala575Val inactivating mutation in patients with amenorrhea

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ABSTRACT

When mutation is inactivated in the follicle-stimulating hormone receptor (FSHR) gene of patients with amenorrhea, the receptor's functionality is abolished by completely blocking it, altering the ligand–receptor complex or altering the essential hormone signal transduction. This study aims to detect the frequency and pattern of chromosomal abnormalities and the presence of inactivating mutations (Ala575Val) at position 1540 of the FSHR gene in Iraqi women diagnosed with secondary amenorrhea (SA) and primary amenorrhea (PA). This cross-sectional study was carried out between February and August 2022. Depending on the initial diagnosis of amenorrhea, women were split into two groups: PA and SA. The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was done for those women with normal karyotyping and hypergonadotropic hypogonadism [follicle stimulating hormone (FSH) › 20 mIU/ml] to determine the genetic cause, such as inactivating mutation in Exon 10E. The FSH of blood in study patients with PA was 72.28 ± 23.60, luteinizing hormone (LH) was 20.60 ± 13.55, and BMI was 24.012 ± 4.06. In SA, the FSH, LH, and BMI values were 69.821 ± 35.95, 16.788 ± 14.12, and 26.12 ± 4.37, respectively. Also, highly significant differences were observed compared with healthy control subjects (*P*-value ‹ 0.005). The high matching between the results obtained by PCR-RFLP and those obtained by Sanger sequencing techniques used in this study confirmed no detected mutation in Ala575Val at position 1540 in all patients with PA and SA. The study concluded that in the PCR study, Ala575Val encoding genes are highly detected, while in PCR-RFLP, no action of MscI restriction enzyme in position 1540 (region of Ala575Val genotype) has emerged. This gives the impression that women with amenorrhea in the Iraqi population might not have any inactivating mutations in the FSHR gene.

Keywords: FSH receptor, mutation, karyotyping, amenorrhea

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INTRODUCTION

Amenorrhea is a medical term for the absence of a menstrual period for one month or more. It is one of the most common gynecological problems that adolescent or young adult girls face. It is considered natural in a few instances, such as during pregnancy, lactation, and menopause. It is also common once the uterus or ovaries have been surgically removed[1]. There are two types of amenorrhea: secondary amenorrhea (SA) and primary amenorrhea (PA). Amongst girls who have not developed secondary sexual characteristics, those who have not reached menarche by age 13 are said to have PA, and among girls who have developed secondary sexual characteristics, those who have not reached menarche by age 15 are said to have SA[2].

Amenorrhea is the sixth leading cause of female infertility, according to the World Health Organization, which estimates that the infertility rate of the world's population is 15%[3]. About 2–5% of women of childbearing age have amenorrhea, either PA or SA[4]. According to statistics from the Iraqi Ministry of Health for 2017 and 2018, problems with menarche and PA affect 9.68% and 17.78% of women, respectively[5].

The follicle-stimulating hormone receptor (FSHR) gene is about 190 kb long and is situated on chromosome 2p21–p16[6]. The human FSHR is a receptor coupled with G protein and has a long extracellular domain (ECD), seven transmembrane domains (7TMD) (three short intracellular loops and three extra loops), and one intracellular tail[7]. The FSHR gene has 10 exons and 9 introns. The ECD of the receptor is encoded by the first nine exons, whereas exon 10 encodes the C-terminal end of the ECD, the whole of 7TMD, and FSHR's intracellular domain. Although exon 10 is essential for signal transduction, ligand binding does not need it[8].

Inactivating mutations Ala575Val (rs386833511) found on Exon 10E at position 1540 of the FSHR can cause amenorrhea due to ovarian stimulation failure. Inactivating mutations affect the formation of the ligand–receptor complex or the signal transduction of the follicle-stimulating hormone (FSH), reducing the receptor function to a complete block. Premature ovarian failure, PA, and SA are all caused by FSHR-inactivating mutations[9].

Exon 10E of the FSHR gene in women with PA or SA, normal karyotypes, and high hypergonadotropic hypogonadism (FSH › 20 mIU/ml) has certain inactivating mutations, which our study aims to identify to facilitate the diagnosis of amenorrhea.

PATIENTS AND METHODS

Selection of Study Patients

Seventy two women participated in this cross-sectional study carried out from February 2022 to August 2022. The first group consisted of 42 patients (25 women with PA and 17 women with SA) who suffered from amenorrhea with hypergonadotropic hypogonadism (high level of FSH › 20 mIU/ml). The second group represented 30 healthy control women with regular menstruation cycles.

Women with amenorrhea, either PA or SA, were referred to one of the following institutions: Department of Obstetrics and Gynaecology in Ramadi Teaching Hospital for Child and Maternity and Ramadi and Private Clinics in Al-Anbar Governorate and Baghdad city. The initial diagnosis of amenorrhea was made by an expert clinician based on their medical history and hormonal profile, ultrasonography results, and physical examination.

Inclusion criteria for PA were women who did not have secondary sexual characteristics or women who were more than 16 years of age with average growth and development of secondary sexual characteristics (pubic and axillary hair and breast development). The inclusion criteria for SA were women with no menstrual period for three to six months. The exclusion criteria were women with abnormal karyotype, FSH level less than 20 mIU/ml, abnormal hormonal status such as autoimmune thyroid disorders, and imbalance of the prolactin hormone in addition to infections and exposure to radiation.

Ethics Statement

In accordance with the Declaration of Helsinki, this study received approval from the University of Anbar's Medical Ethics Committee on February 13, 2022, in Ramadi, Iraq (approval number 8). All study participants provided their verbal or written consent.

COLLECTION OF SAMPLES

Blood Sample for the Serological Study

Two millilitres of peripheral blood samples was collected using a sterile disposable syringe, which was drawn in a 6 ml gel and clot activator tube. After allowing them to clot in a water bath (37°C), the sera were separated by centrifugation for 5 minutes at 1500 rpm. FSH and luteinizing hormone (LH) analyses were done by Tosoh Bioscience (AIA-1800, Japan) by taking 100 µl of serum.

Blood Sample for the Chromosomal Study

Two millilitres of peripheral blood samples was taken from women with PA and SA using a sterile disposable syringe and injected into a lithium heparin tube. The blood was promptly cultured and analyzed for chromosomal abnormalities.

Blood Sample for the Molecular Study

For patients with normal karyotyping, another blood aspirate for 2 ml of blood into EDTA tubes has been taken for FSH receptor gene mutation and polymorphism.

Serology Part of the Study

TOSOH Automated Immunoassay System (TOSOH Bioscience, Japan) has been used to evaluate all participants' submissions for hormonal studies, including FSH and LH in human serum. The normal range for FSH in women during the follicular phase is 4.5–11.0 mIU/ml. In LH, the normal range is 1.7–13.3 mIU/ml.

Cytogenetic Study

Karyotyping

Lymphocyte cultures were done according to the standard procedure[10,11,12]. A volume of 1500 µl of peripheral blood samples was aseptically transferred into sterile culture tubes with 4 ml of RPMI-1640 medium solution (HiMedia, India). In each centrifuged conical tube, 1 ml of fetal bovine serum (Biowest, European), 200 µl of Phytohemagglutinin (Biowest, European), and antibiotics (60 µl of ampicillin and 60 µl of streptomycin) were added.

The culture medium was kept at –20°C until further analysis. The culture tubes were appropriately marked before being incubated in an incubator for 72 hours. After 71 hours, each culture tube received 100 µl of the anti-metaphase drug colchicine (Biowest, European) to arrest the cells. The cell suspensions underwent 15 minutes of 1500 rpm centrifugation after 30 minutes of incubation. After discarding the supernatant, the pellet was treated with a gentle drop-by-drop application of hypotonic solution (0.075 M KCl). The centrifuged tubes were then incubated for 20 min at 37°C. Once more, the tubes were carefully centrifuged at 1500 rpm for 15 minutes. Then, the supernatant was removed, and to the pellet was added 7 ml of fixation solution that was freshly prepared (3:1; methanol to glacial acetic acid) and mixed thoroughly. The tubes were washed with freshly prepared fixative solution repeatedly 3–4 times. The supernatant was then removed after the completion of centrifugation, and till a clear pellet was obtained, the centrifugation was repeated. The pellet was stored with 2 ml of fixation solution at –20°C.

Slide preparation

The slide was made using the protocol laid down by Al-Mustansiriyah University, Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), which involves removing the cell culture from the freezer. The cell culture mixture was well mixed by a vortex mixer, and cell suspension (3–4 drops) was dropped uniformly from an acceptable distance (30–50 cm) at a 45 degree angle onto oil-free slides, which were then allowed to dry at room temperature before being labeled.

The slides were stained with Giemsa stain, practical grade (HiMedia, India). Giemsa stain powder weighing 2 g and 100 ml of absolute methanol were combined to make the solution. The solution was stirred for 3 hours using a hotplate magnetic stirrer model L-81 with a magnetic bar at room temperature. The solution was filtered using a 0.45 µm Millipore sterile syringe filter membrane and stored in a tightly closed dark bottle. One millilitre of the stock solution is mixed with 4 ml of Sorenson's buffer for staining immediately[13].

Karyotype analysis

A fluorescence microscope (Euromex, Arnhem, Netherlands) and the MetaClass Karyotyping apparatus (Microptics S.L., Barcelona, Spain) were used to determine the chromosomal status.

Standard cytogenetic protocols examined chromosome preparations and metaphases using peripheral blood cultures. G-banding was used for cytogenetic analysis. All participants had 20 metaphases examined. In some cases, the analysis was expanded to 50 metaphases, particularly when mosaicism and abnormalities were suspected. The International System of Human Cytogenetic Nomenclature was used to report chromosomal abnormalities[14].

Molecular Part of the Study

Nucleic acid extraction

A SaMag blood DNA extraction kit was employed with a SaMag-12 automatic nucleic acid extraction system (Sacace biotechnologies, Italy) for extraction of genomic DNA from 400 µl of human whole blood, according to the Al-Ouqaili and Al-Qaysi protocols[15,16]. The principle of blood DNA extraction consisted of steps of lysis, binding, washing, and elution.

Nucleic acid concentration and purity

Quantus™ Fluorometer with QuantiFluor® dsDNA System (Promega, USA) was used to detect the extracted DNA concentration to determine the sample's quality for subsequent applications. Ninety-nine microlitres of diluted Quantus Flour Dye was mixed with 1 µl of DNA after 5 minutes of incubation at room temperature. DNA concentrations were then measured as previously reported[17]. DNA purity was also calculated using an OD260/OD280 UV spectrophotometer (Unico/USA).

Polymerase chain reaction amplification

The requested primers were purchased from Alpha DNA Company, Canada, for use in conventional polymerase chain reaction (PCR) for detecting FSH receptors' genes. The primers were dissolved and lyophilized in nuclease-free water to make a stock solution to reach a final concentration of 1000 pmol/l. The specific primers of inactivating mutation Ala575Val were F: 5′ CCTTGTGCTCAATGTCCTGG 3′; and R: 5′ GCTTTGGACACAGTGATGAG 3′.

A PCR thermal cycler (Esco, USA) was used to amplify DNA fragments. The template DNA and primers were added to an AccuPower® PCR PreMix tube, which was requested from Bioneer, Korea. PCR was carried out in a 20 µl mixture containing 1 U Top DNA polymerase, 250 µM dNTP (dATP, dCTP, dGTP, and dTTP), 1X reaction buffer with 1.5 mM $MgCl_2$, amplification primers (2 µl, each), target DNA (5 µl), and nuclease-free water (11 µl).

The inactivating mutation (Ala575Val) PCR comprised initial denaturation for 5 min at 95°C, denaturation for 45 sec at 95°C, annealing for 50 sec at 56°C, extension for 1 min at 72°C, and final extension for 5 min at 72°C.

Then, 5 µl of the PCR product was added to each 1.5% agarose gel well. The electrodes were attached to the power supply at 50 voltages for 5 min and then 100 voltages for 60 min. DNA bands were visualized using an ultraviolet transilluminator (Vilber format, USA). The PCR product has a size of 219 bp (Ala575Val).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

RFLP Analysis of the Ala575Val (rs386833511) Mutation

The restriction fragment was performed using the MscI restriction enzyme (BioLabs-USA). MscI digestion occurred at 37°C for 3 hours, and inactivation occurred at 80°C for 20 min and was visualized on 1.5% agarose gel stained with ethidium bromide in 1X TBE Buffer solution at a voltage of 100 volts for 55 min.

Sequencing

The Sanger dideoxynucleoside sequencing technique using ABI3730XL by Macrogen Corporation (South Korea) was used to detect any polymorphisms in the samples of this study. The sequences were then run through the standard gene Basic Local Alignment Search Tool (BLAST) program, which is available online at the National Centre Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov), and sequences were aligned using the genius software.

Statistical Analysis

The statistical analysis was carried out using SPSS software version 22 (Armonk, NY, USA), and the studied groups were compared using an independent *t*-test. A *P*-value of 0.05 was considered the significant. Results were presented as mean ± standard deviation (SD) or the number of participants.

RESULTS

The age of patients with PA in our study (25, 34.7%) ranged from 14 to 25 years with a mean age of 18.2 ± 2.8 years. On the other hand, the age of patients with SA (17, 23.6%) ranged from 18 to 35 years with a mean age of 25.0 ± 5.9. The ages of women with regular periods ranged from 14 to 33 years, with a mean age of 22.5 ± 5.0 years.

FSH of blood in study patients with PA was 72.28 ± 23.60, LH was 20.595 ± 13.55 (mIU/ml), and body mass index (BMI) was 24.012 ± 4.06 (kg/m^2). In SA, the FSH, LH, and BMI values were 69.821 ± 35.95 (mIU/ml), 16.788 ± 14.12 (mIU/ml), and 26.117 ± 4.37 (kg/m^2), respectively. The statistical analysis of this study's female patients and healthy female subjects revealed statistically significant differences between the two groups (*P*-value ‹ 0.05). All details are shown in Tables 1 and 2.

Our study results revealed that there are highly significant differences observed in study patients with LH and FSH concentrations in both study patients and control healthy subjects, as represented in Table 2.

The Cytogenetic Part in Patients with Primary and Early SA

As a control, the chromosomal analysis of women with PA and SA was performed on 42 cases and 30 cases of women with regular menstruation cycles. The karyotyping results were typical in 33 cases (78.6% of all cases with amenorrhea). The representative karyotype for the normal ones is represented in Figure 1. However, chromosomal aberrations were present in seven (28.0%) patients with PA and two (11.8%) patients with SA.

In the present study, to examine the association between amenorrhea with inactivating mutation (Ala575Val) in the FSH receptor gene, patients with normal karyotype and hypergonadotropic hypogonadism (FSH › 20 mIU/ml) were selected. Eighteen patients with PA and 15 with SA were compared with 30 women with regular menstruation cycles who have proven fertile; they served as controls.

The PCR product of inactivating mutations in Exon 10E of FSHR gene (Ala575Val) transition in all diagnosed patients with PA and SA and control groups had a size of 219 bp (Figure 2). No any inactivation mutation occurred in our study in PCR–restriction fragment length polymorphism (PCR-RFLP) analysis. This is reflected by the normal action of the restriction enzyme MscI, which was used to digest the PCR product (219 bp) into two fragments having sizes of 119 bp and 100 bp, as shown in Figure 3.

Table 1. Parameters associated with PA and SA and controls.

Parameters	PA (*n* = 25)	SA (*n* = 17)	Controls (*n* = 30)
Age (years)	18.2 ± 2.77 (14–25)	25.0 ± 5.93 (14–33)	22.5 ± 5.04 (14–33)
BMI (kg/m^2)	24.012 ± 4.06	26.1176 ± 4.37[b]	23.493 ± 2.84[b]
FSH (mIU/ml)	72.28 ± 23.60[a]	69.821 ± 35.95[b]	5.879 ± 0.96[b]
LH (mIU/ml)	20.595 ± 13.55[a]	16.788 ± 14.12[b]	5.224 ± 1.855[a,b]

[a]Values significantly differ between control subjects and patients with PA (*P*-value ‹ 0.05; independent sample test).
[b]Values are significantly different between control subjects and patients with SA (*P*-value ‹ 0.05; independent sample test).
Abbreviations: BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; PA, primary amenorrhea; SA, secondary amenorrhea.

Table 2. Comparison of FSH and LH in women with PA and SA with healthy women in the control group.

Parameters	Study cases and control women	No.	Mean	Standard deviation	*P*-value significance (2-tailed)
FSH	PA cases	25	72.28	23.60	0.00
	Control women	30	5.879	0.96	
	SA cases	17	69.821	35.95	0.00
	Control women	30	5.879	0.96	
LH	PA cases	25	20.595	13.55	0.00
	Control women	30	5.224	1.86	
	SA cases	17	16.788	14.12	0.00
	Control women	30	5.224	1.86	

Abbreviations: PA, primary amenorrhea; SA, secondary amenorrhea.

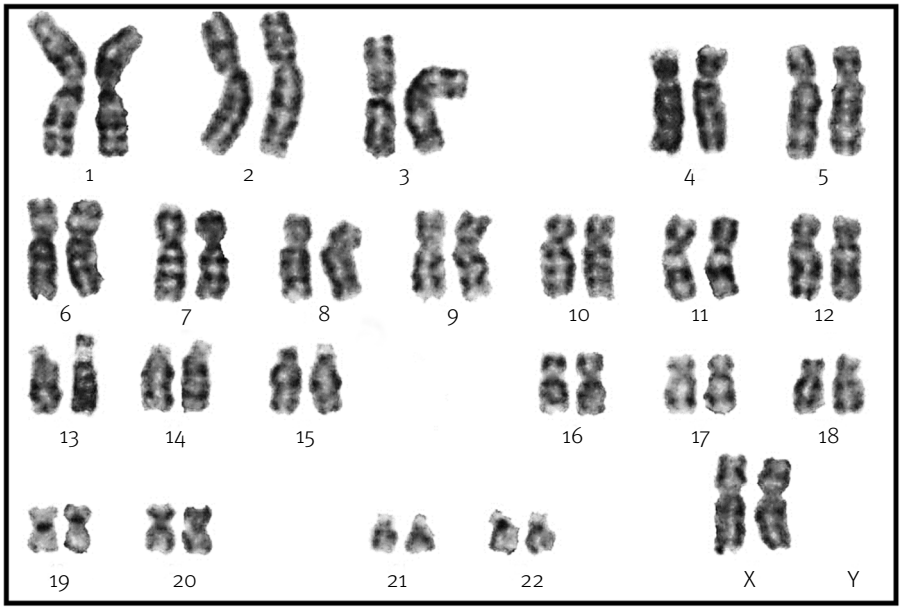

Figure 1. The normal representative karyotype (46, XX) for study patients with amenorrhea, either primary or secondary subjects.

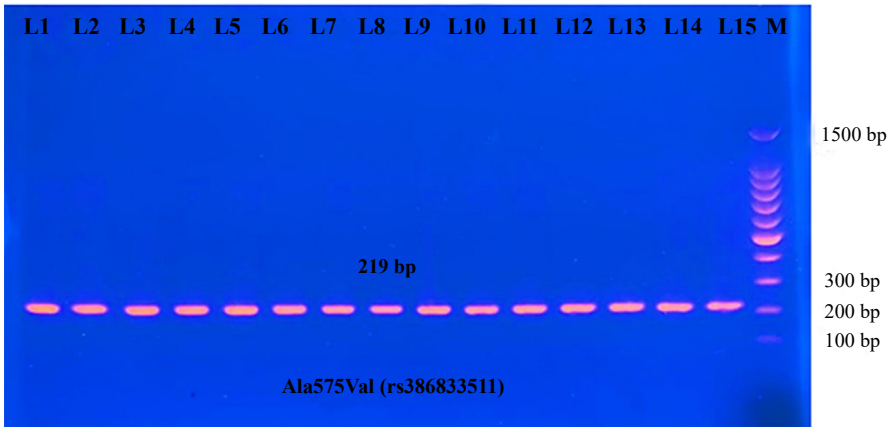

Figure 2. The PCR product of inactivating mutation in the Ala575Val (rs386833511) gene.

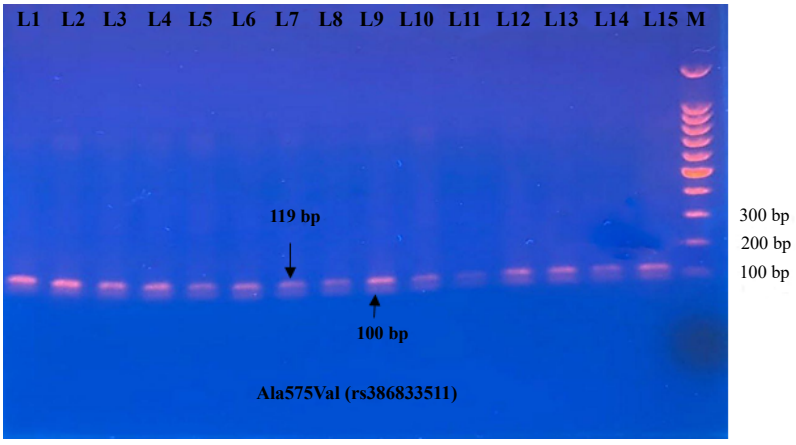

Figure 3. PCR-RFLP analysis of the inactivating mutation Ala575Val (rs386833511) was carried out using MscI restriction enzymes.

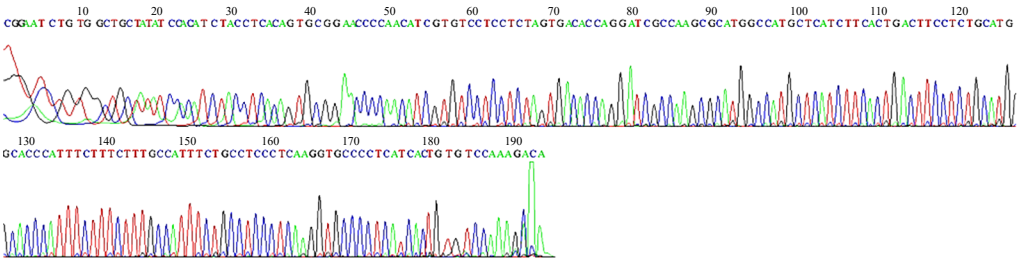

Figure 4. DNA Sanger sequencing of the PCR product (Ala575Val) indicating a wild-type sequence.

In the present study, 10 PCR products each from patients with PA and SA and the control group were submitted for DNA sequencing. Then the yielded sequences were analyzed by NCBI blast online. These results confirmed the data obtained by the PCR-RFLP for the Ala575Val (rs386833511) gene, which revealed no detected mutation in all patients with PA and SA, as shown in Figure 4.

DISCUSSION

The study of the Ala575Val inactivating mutation gene in patients with amenorrhea was the first gene study conducted in Iraq and the neighboring Arab countries. This inactivating mutation, predicting an alanine to valine substitution, is located in Exon 10E of the FSHR gene in the sixth helices of the transmembrane domain of FSHR of the receptor molecule[18].

Inactivating FSHR mutations have been known to cause altered cell surface expression, decreased binding capacity, and impaired signal transduction and receptor expression. This can lead to delayed puberty, hypogonadotropic hypogonadism, PA or SA, infertility, ovarian dysgenesis, predisposition to sex cord ovarian tumors, and impaired spermatogenesis in men[19]. Inactivating FSHR mutation was first described in Finnish women in 1996 by Aittomäki and associates[20].

In our study, we observed highly significant differences in the levels of LH and FSH between patients with amenorrhea and the healthy subjects. This appears to be normal in the hypergonadotropic hypogonadism clinical situation, where the levels of hormones like FSH are sharply increased. In this study, the patients had clinical characteristics; ovaries and uterus were small in size with an absence of follicles. Those subjects had a higher basal FSH level (FSH < 20 mIU/ml), demonstrating the loss of negative estrogen feedback[21].

This study's results revealed that there is no detection of any inactivation mutation in Exon 10E of FSHR gene (Ala575Val) transition in all study patients with either PA or SA in addition to the control groups. It is well known that the FSHR's inactivation is most likely due to the mutation at position 575. Our result was in agreement with those reported by Nishu and co-workers[22], who concluded that the absence of inactivating mutation was observed through the PCR-RFLP study on Exon 10A and Exon 10B. On the other hand, Achrekar et al.[23] concluded that women from the western Indian population diagnosed with PA have a novel homozygous mutation (Ala575Val) in the FSHR gene. In addition, Desai et al.[18] reported that an Ala575Val mutant was present in a subject with PA, which leads to poor secondary organ development. This demonstrated that the receptor's cell signaling activity was impaired, which caused the receptor to become inactive.

The study concluded that in the PCR study, Ala575Val-encoding genes are highly detected, while in PCR-RFLP, there was no action of MscI restriction enzyme in position 1540 (region of Ala575Val genotype). This provides an impression that no inactivating mutations in the FSHR gene are detected in the women suffering from amenorrhea in the Iraqi population.

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