



Evaluation of basal hormone levels and androgen receptor gene mutations in individuals with recurrent abortion

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ARTICLE INFO

Original paper

Article history:

Received: August 11, 2021

Accepted: November 13, 2021

Published: December 01, 2021

Keywords:

AR Gene Mutation; Hormonal Disorders; Pregnancy; Recurrent Miscarriage

ABSTRACT

A recurrent miscarriage is at least two consecutive miscarriages in the first trimester of pregnancy. Due to the dependence of pregnancy on endocrine changes in the menstrual cycle, its disorders can also affect the outcome of pregnancy. In addition to hormonal disorders, genetic changes are essential factors in recurrent miscarriage. The development and maturation of ovulation depend on the molecular signaling pathways that respond to androgens. Hundreds of mutations leading to resistance to androgen receptor (AR) gene function have been recorded, including the 5'UTR polymorphic region. Therefore, considering the role of androgen receptors and hormonal changes in recurrent miscarriage, this study was performed to investigate the relationship between hormonal changes and AR gene mutations in patients with recurrent miscarriage. In this regard, a case-control study was performed on 150 patients with miscarriage referred to the infertility center. Hysterosalpingography, parental karyotype, vaginal ultrasound, antiphospholipid antibody measurement, anticardiolipin antibody, history and physical examination were performed to evaluate the possible causes of recurrent miscarriage. Hormone levels of LH, FSH, TSH, and Prolactin were measured and compared in two groups with known and unknown causes. Blood samples were also taken from patients, and after DNA extraction, the PCR method was used to determine AR gene mutations. The mean age was 30.2 ± 7.1 years, the mean number of abortions was 2.6 ± 1.2 , and the mean duration of marriage was 6.1 ± 2.1 years. The mean of hormones in the two groups with known and unknown causes was compared, that TSH was significantly lower in the group with unknown cause ($P = 0.031$) and prolactin was higher in recurrent miscarriage patients with polycystic ovaries ($P = 0.048$). Regarding genetic evaluation, in the 5'UTR region of the androgen receptor gene, deletion of T nucleotide was observed in the +25 position, but no significant difference was found between the two groups. Generally, the findings of this study showed that thyroid dysfunction and hyperprolactinemia should be considered as an endocrine disorder in people with recurrent miscarriage, and genetic evaluation showed that the AR gene mutation was not associated with recurrent miscarriage.

DOI: <http://dx.doi.org/10.14715/cmb/2021.67.4.30>

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Introduction

Recurrent abortion (recurrent miscarriage) means two or three consecutive miscarriages in the first trimester of pregnancy (1). Although only 1% of couples have a recurrent abortion, it is a very frustrating experience for the patient and physician due to the uncertainty of the treatment and its etiology. There is a known cause only in less than 50% of recurrent miscarriages (2). In the study conducted by Diejomaoh *et al.* (3), the leading causes of abortion were uterine abnormalities (2.2%), chromosomal (2.2%), antiphospholipid antibodies (3.33%), polycystic ovary (PCO), infections, and other various causes (2.21%), and 6.35% had unknown reasons. Because normal pregnancies

depend on endocrine changes in the menstrual cycle, endocrine changes are likely to affect the outcome of pregnancy. These changes include luteal phase insufficiency, thyroid disease, hyperprolactinemia, and diminished ovarian reserve (DOR). Elevated LH causes premature aging of oocytes and lack of coordination in endometrial maturation (4).

Also, most patients with high LH have PCO characteristics and are obese patients with increased androgen levels. Although this is highly controversial, the increase in androgens may adversely affect the endometrium involved in recurrent miscarriages (5). The association between hypothyroidism and hyperprolactinemia with recurrent miscarriage is still significant. Recently, attempts have been made to link

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ovarian reserve markers (day three FSH and day three estradiol) to recurrent miscarriage (6).

In a study by Trout *et al.* (7), performed on 57 women with recurrent abortion (36 with unexplained cause and 21 with known cause), women with unknown cause miscarriage had higher levels of FSH and estradiol than women who had a specific reason. Therefore, diminished ovarian reserve (DOR) may be involved in recurrent abortion and should be considered one of the causes in the follow-up of recurrent miscarriages.

Other causes of recurrent miscarriage include genetic changes in individuals. So far, the role of mutations in genes such as alpha and beta estrogen receptors and androgen receptors has been investigated in recurrent abortion (5). The androgen receptor gene (AR) is expressed in endometrial tissues and organs inside the pelvis and is responsible for transmitting cellular messages and proper hormonal function in endometrial tissue. The androgen receptor gene is located on the X chromosome at position Xq11-12 (8). This gene has eight exons and a relatively long 5'UTR. The final differentiation of ootids depends on the androgen receptor. AR appears to be involved in the final stages of egg differentiation (9). Thus, mutations in the AR gene are likely to lead to the formation of abnormally shaped eggs. So far, more than 500 mutations in the AR gene have been identified in people with androgen hypersensitivity syndrome (10). Also, different point mutations in the promoter region of the AR gene have been considered in the study of various diseases. The 5'UTR region of the AR gene also has polymorphic regions. Considering the role of 5'UTR in translation control, androgen receptor gene expression can be affected by mutations in this region (9).

Because knowing the cases of abortions related to hormonal disorders can make the mother as subject to endocrine treatment to prevent future abortions (11). Therefore, we aimed to evaluate the level of basal hormone and the association of mutation in the 5'UTR region (-23 to +214) of the androgen receptor gene in patients with recurrent miscarriages.

Materials and methods

Demographic, clinical, and hormone evaluations

The present case-control study was performed on 150 women referred to the infertility center with

recurrent abortion. After the initial visit and tests, patients were divided into two groups based on the presence or absence of a reason for abortion. The first group was 64 patients with recurrent abortion with a specific cause, and the second group was 86 patients with recurrent abortion without a specific cause. On average, patients were referred for treatment 3 to 6 months after abortion and were included in the study. Recurrent miscarriage was defined as at least two spontaneous terminations of gestation under 20 weeks. Also, not all subjects in both groups had a history of normal pregnancy.

Hysterosalpingography was performed on days 9-11 of the menstrual cycle to evaluate the structural status of the uterus. Also, parent karyotype was done to assess chromosomal abnormalities by blood culture and microscopic examination. Human anti-phospholipid and human anti-cardiolipin antibody ELISA Kit (MyBioSource, USA) measured anti-phospholipid antibodies and anti-cardiolipin antibodies as an immunological disorder.

Hormonal evaluation on the third day of the menstrual cycle was performed by Radioimmunoassay by RIA Assay Kit (IBL, USA) to assess the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and prolactin (PRL).

The questionnaire also includes: age, duration of the marriage, number of pregnancies, age of pregnancies per abortion, uterine abnormality, chromosomal abnormalities, polycystic ovaries, infection, family relationship, anticardiolipin antibody, antiphospholipid antibody, and hormone levels of TSH, LH, FSH, and prolactin were completed on the third day of each patient's menstrual cycle.

The positive familial relationship was defined as grade 3 familial dependence and polycystic ovary based on ten or more cysts with a diameter of 2-8 mm on one surface and an increase in ovarian stromal density on ultrasound third day of the menstrual cycle (12). Infection was considered as chorioamnionitis pregnancy due to fever and uterine sensitivity to touch and available laboratory evidence (13).

Anatomical abnormalities were also defined as abnormal uterine structural abnormalities (14). A recurrent abortion that has at least one of the causes of recurrent miscarriage, including anatomical

abnormalities, chromosomal abnormalities, polycystic ovaries, familial relationship, immunological disorders, was defined as recurrent abortion with a specific cause. Normal levels of experiments were compared as: FSH: 2.5-13 nmol/lit; LH: 1.4-13 nmol/lit; TSH: 0.3-5.1 nmol/lit; PRL: 3.2-25.3 nmol/lit; ACA<10 mIU/l; and APL<15 mIU/l.

Genetic Evaluations

After giving knowledge and obtaining consent, 4 ml of peripheral blood was collected from the subjects in tubes containing EDTA. The salting-out method was performed for DNA extraction (15). The extracted DNA was stored at -20°C until analysis. AR mutation in the -23 to +214 regions of the AR gene was performed by polymerase chain reaction (PCR). In this study, to investigate the presence of mutations in the -23 to +214 regions of the AR gene, the required primers were designed by Primer3 software, and the blast assigned the primers at the NCBI site to find regions with the homologous genome in the genome. The sequences of primers were:

Forward: 5'-GTTGCATTTGCTCTCCACCTCCC-3'

Revers: 5'-TCACCGAAGAGGAAAGGGCAGCTC-3'

Amplification of region -23 to +214 in AR gene was performed by PCR technique and using the Master Cycler Pro (Eppendorf, Germany), at a final volume of 25µl containing the template DNA, 20 pmol forward and reverse primers, 1.5mM magnesium chloride, 200 µM dNTP, and 1 unit Taq polymerase (Sigma-Aldrich, USA).

Amplification was selected in 30 cycles. The temperature of 64°C for 1 minute was considered the optimum temperature for the activity of the primers and 72°C for 1 minute for the extension. Initial denaturation was performed for 5 minutes at 94°C, and the final extension was performed for 5 minutes at 72°C. The obtained DNA fragments were then separated by 2% agarose gel and electrophoresis. The obtained fragment was 262 bp. The sequences of some samples were sent to the MacroGen Company to confirm the results by the Sanger method.

Statistical Analysis

Data were analyzed by SPSS software version 19. The X² and Fisher tests were used for qualitative data, and a t-test compared the mean in two independent

groups. In this test, P <0.05 showed a significant difference between the two groups.

Results and discussion

Demographic, clinical, and hormone results

One hundred fifty patients with recurrent abortion, including 64 (42.66%) cases with a known cause and 86 (57.34%) cases of unknown cause who had been referred to the infertility center, were included in the study. The frequency and percentage of abortion-related causes were shown in Table 1. Seven people were under 20 years old (4.66%), 104 people were 20-34 years old (69.34%), 39 people were 35 years old, and more aged (26%). The mean age of the study group was 30.2 ± 7.1, the mean duration of marriage was 6.1 ± 2.1 years, and the mean number of abortions was 2.6 ± 1.2.

Table 1. Frequency distribution and percentage of causes related to recurrent abortion in the subjects

Causes Associated with Abortion	Having Number (Percent)	Not Having Number (Percent)	Total Number (Percent)
Uterine anomaly	16 (10.67%)	134 (89.33%)	150 (100%)
Chromosomal abnormalities	13 (8.67%)	137 (91.33%)	150 (100%)
Polycystic ovaries	26 (17.33%)	124 (82.67%)	150 (100%)
Infection	5 (3.33%)	145 (96.67%)	150 (100%)
Family relationship	18 (12%)	132 (88%)	150 (100%)
Immunological disorder	17 (11.33%)	139 (92.67%)	150 (100%)

Table 2 shows the levels of hormones in the first group (64 recurrent abortion patients with a specific cause) and the second group (86 recurrent abortion patients without a specific cause). The results showed that there was a significant difference between the two groups in terms of TSH hormone (P = 0.004).

Table 2. Comparison of hormone levels in the first and the second groups

Hormone	First Group (n=64)	Second Group (n=86)	P-value
FSH	6.2 ± 3.4	6.8 ± 5.4	0.597
LH	7.9 ± 4.8	7.3 ± 3.5	0.434
TSH	2.4 ± 1.6	1.4 ± 1.1	0.004
PRL	16.9 ± 12.1	15.7 ± 11.8	0.522

The relative frequency distribution of abnormal status of hormones, LH, TSH, FSH, and PRL was evaluated separately for different causes of abortion. Prolactin levels were significantly higher in patients with polycystic ovaries (P = 0.048), and also in

patients with immunological disorders, TSH level was significantly lower ($P = 0.031$) (Table 3).

Table 3. The hormone levels in recurrent abortion patients with a specific cause

Abortion Causes	FSH (mean \pm SD)	LH (mean \pm SD)	TSH (mean \pm SD)	PRL (mean \pm SD)
Uterine anomaly				
Having	6.7 \pm 4.8	7.3 \pm 4.9	2.5 \pm 1.3	15.4 \pm 4.3
Not Having	6.6 \pm 4.3	7.9 \pm 3.9	1.6 \pm 1.2	16.5 \pm 12.2
Chromosomal abnormalities				
Having	5.3 \pm 3.6	8.6 \pm 7.2	1.8 \pm 1.1	18.3 \pm 15.5
Not Having	6.7 \pm 4.3	7.3 \pm 3.3	1.7 \pm 1.2	17.2 \pm 9.9
Polycystic ovaries				
Having	6.2 \pm 3.3	9.4 \pm 5.1	1.8 \pm 1.2	18.9 \pm 12.7*
Not Having	6.6 \pm 3.5	7.7 \pm 4.7	2.1 \pm 1.2	16.1 \pm 11.2
Infection				
Having	6.1 \pm 4.3	3.4 \pm 1.3	2.6 \pm 1.5	15.2 \pm 10.8
Not Having	6.3 \pm 4.1	7.4 \pm 4.2	1.9 \pm 1.0	16.3 \pm 12.2
Family relationship				
Having	6.4 \pm 3.4	8.9 \pm 4.8	2.1 \pm 1.3	13.9 \pm 4.7
Not Having	6.6 \pm 4.4	7.5 \pm 4.1	1.9 \pm 1.2	16.3 \pm 12.1
Immunological disorder				
Having	6.3 \pm 3.9	5.8 \pm 4.4	2.9 \pm 2.1*	13.1 \pm 6.2
Not Having	6.7 \pm 1.1	7.6 \pm 5.3	1.4 \pm 1.2	16.6 \pm 12.9

*: $P < 0.05$

Genetic evaluation results

After PCR, the results were evaluated with 2% agarose gel to investigate the presence of mutations in the -23 to +214 regions of the AR gene (Figure 1). The results showed that deletion of T nucleotide was observed in the +25 position in the study region. This mutation did not alter the AR gene and protein expression, indicating that there was no association between the occurrences of a mutation in the promoter region of -23 to +214 in the AR gene of women with recurrent miscarriage. In order to investigate the significant differences between the genotypes in the two groups, statistical studies were performed by SPSS software version 19 using a t-test. The value of obtained P was equal to 0.544. According to the P-value, it can be concluded that there was no correlation between AR gene mutations in the promoter region -23 to +214 with recurrent miscarriage in the study population. In the present study, hormonal tests including, FSH, LH, TSH, and PRL on the third day of menstruation were measured and compared in two groups of recurrent abortion

with a specific cause and without a specific cause (11).

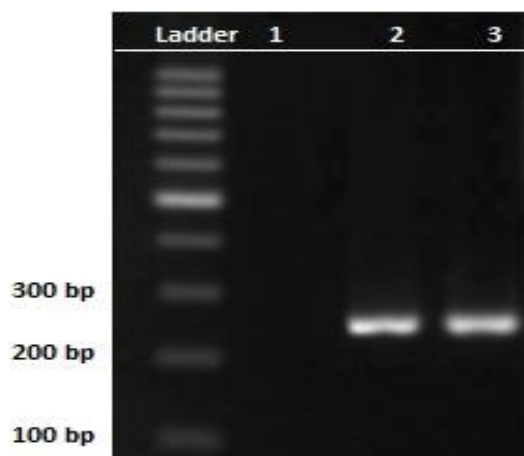


Figure 1. A 2% agarose gel sample to test for mutations in regions -23 to +214 of the AR gene identifies the amplified 262 bp fragment of the promoter region in the AR gene in two groups. Well No. 1: Negative control; Well No. 2: Sample of a person with recurrent miscarriage for a specific reason; Well No. 3: Sample of a person with recurrent miscarriage for no apparent reason

The mean age of the subjects was 30.2 ± 7.1 years and the mean number of abortions was 2.6 ± 1.2 . In the study of Nardo *et al.* (16), the mean age of the study group was 32 years and the average number of abortions was 4. In the study of Clifford *et al.* (17), the mean age was 32.9 years and the mean number of abortions was 4. In the study of Rai *et al.* (12), the mean age was 33 years and in the study of Bussen *et al.* (18), the mean age of the study group was 33.2 years and the mean number of abortions was 3.9. According to the average age in the present study, its lower age may be due to the lower age of marriage in the study area.

In the present study, the percentage of uterine anomalies was 10.67%, chromosomal abnormalities were 8.67%, polycystic ovaries (PCO) were 17.33%, infection was 3.33%, the family relationship was 12%, and immunological factors were 11%. In a study by Diejomaoh *et al.* (3), the significant causes of abortion included uterine abnormalities (2.2%), chromosomal abnormalities (2.2%), antiphospholipid antibodies (33.3%), PCO, infections, and miscellaneous causes (21%), and 6.35% had unknown reasons. In the study by Clifford *et al.* (17), 56% of patients had PCO and antiphospholipid antibodies were found in 14% of women. Chromosomal abnormalities were present in

3.6% of patients. In Balasch study (19), 50% of patients had a miscarriage with a specific cause. In the study of Rai *et al.* (12), PCO was present in 7.40% of cases and in the study of Lidell *et al.* (20), PCO was present in 36% of cases. In the study by Christiansen *et al.* (21), 10% had uterine structural disorders, 10% had endocrine disorders, and 15% had antiphospholipid antibodies. According to the mentioned studies, finding the cause and recognizing the existing reasons related to abortion in people with recurrent abortion has a great help in eliminating the causes and thus improving the prognosis of pregnancy. In the present study, the mean of hormones in people with recurrent miscarriage is in the normal range. Compared with hormones performed between the two groups of recurrent miscarriage with known and unknown causes, only TSH was lower in the unspecified group.

In the study by Christiansen *et al.* (21), 2% of women with a miscarriage in the second trimester were hypothyroid. Therefore, it was suggested that thyroid function be evaluated in all women with abortion. In the study of Bussen *et al.* (18), in two groups, recurrent miscarriage was performed compared to women with male or tubal factor infertility. Prolactin and dehydroepiandrosterone (DHEA) in the recurrent abortion group were higher than the control group (despite being within the normal range), and TSH was similar in the two groups. They linked the findings to the cause of recurrent miscarriage and believed that knowing them would help endocrine therapy.

In the study by Gurbuz *et al.* (22), FSH, estradiol, LH, prolactin, and DHEAS in recurrent abortions without a specific cause were higher than in the group with a particular reason, and TSH and testosterone were similar in the two groups. In conclusion, endocrine disorders of the follicular phase have not been implicated in recurrent miscarriage.

In the study by Nardo *et al.* (16), no association was found between PCO and increased LH secretion, high testosterone, and BMI with the prognosis of pregnancy in women with unexplained recurrent miscarriage. In the study by Trout *et al.* (7), women with recurrent miscarriage without a known cause had a higher prevalence of elevated serum FSH and estradiol on the third day compared with the control group (recurrent miscarriage with a specific reason).

Therefore, the involvement of ovarian reserve in recurrent abortion is possible, and it is considered one of the necessary follow-ups in cases of recurrent abortion. Tulppala *et al.* (23) found that the presence of PCO could not predict miscarriage, but PCO and hyperandrogenism could be associated with recurrent miscarriage.

The results of Li *et al.* (24) study showed no association between recurrent miscarriage and hyperprolactinemia and abnormal thyroid function. But endothelial and endocrine disorders were present in a quarter of women with recurrent miscarriages for no apparent reason. In the present study, the levels of FSH, LH, TSH, and prolactin in various causes related to abortion were investigated, which in people with immunological causes, TSH levels were significantly lower ($P = 0.031$). Kutteh *et al.* (25) found that antithyroid antibodies, as an autoantibody, could indicate autoimmune activity and be associated with an increased risk of termination of pregnancy and postpartum thyroid disease. In this study, antithyroid antibodies were more common in women with recurrent miscarriages.

In the Lazaru study (26), thyroid peroxidase antibodies were present in 10% of women at 14 weeks of gestation, which was associated with increased pregnancy failure, increased prevalence of thyroid dysfunction, and increased risk of postpartum thyroiditis. Findings indicate the presence of hypothyroidism (including subclinical hypothyroidism) in about 2.5% of pregnancies, which is also known as a cause of infertility. Therefore, hypothyroidism is one of the possible causes of abortion, which is recommended to be considered as one of the evaluation tests in patients with recurrent abortion. In the study of the frequency distribution of different causes of abortion, according to the abnormal status of hormonal tests, hyperprolactinemia was observed in people with PCO ($P = 0.048$). In the Gu study (27), the findings indicated the effect of hypoprolactinemia and hyperprolactinemia on abnormal follicular maturation or luteal dysfunction to varying degrees. Prolactin levels have been linked to luteal function in early pregnancy. In the study of Ando *et al.* (28), hyperprolactinemia was one of the causes of recurrent miscarriage (with unknown cause). Patients with hyperprolactinemia disorders without corpus luteum dysfunction were treated with

bromocriptine, which has been shown to be effective in maintaining pregnancy.

Regarding genetic evaluation, a T nucleotide deletion at +25 position was observed in the 5'UTR region of the androgen receptor gene. This single nucleotide mutation did not alter the expression of the androgen receptor gene, and this indicates that there is no association between mutations in the promoter region -23 to +214 in the AR gene with recurrent miscarriage in the study population. Functional defects and mutations in the androgen receptor gene are associated with many diseases such as endometriosis and polycystic ovary syndrome (29). Today, the study of molecular issues of androgen receptors is considered one of the most critical fields related to genetic disease (30). In this study, mutations in the AR gene and their association with recurrent miscarriage were studied. This gene is located on the X chromosome and contains eight exons. The androgen receptor gene (AR) is expressed in endometrial tissues and pelvic organs and is responsible for transmitting cellular messages and proper hormonal function in endometriotic tissue (31). The androgen gene receptor is a transcription factor and plays a role in controlling the biological pathways by regulating the expression of genes involved in puberty and the development of reproductive organs. The promoter region of the AR gene is known as one of the polymorphic regions (32). The study of the relationship between genetic diseases and androgen receptors is a crucial issue contributing to effective therapies. The androgen receptor gene contains CAG repeats. Thus, point mutation or variation of CAG repetitive sequence in this gene is associated with the transmission of various diseases (33). According to studies, mutations and variation in CAG replicate sequence in the AR gene are associated with various diseases, including endometriosis (34-37). Since the mutation in promoter region -23 to +214 AR gene has not been considered with recurrent miscarriage, the present study was performed for the first time for this purpose. In this study, only one aspect of the influential factors was investigated. Therefore, it is necessary to investigate the role of other genetic, environmental, and even other polymorphisms of the AR gene in recurrent miscarriage.

The findings of this study showed that thyroid dysfunction and hyperprolactinemia should be

considered as endocrine disorders in people with recurrent miscarriages. The results of the genetic evaluation showed that the AR gene mutation was not associated with recurrent miscarriage.

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