International Journal of Molecular Biology and Biochemistry

ISSN Print: 2664-6501
ISSN Online: 2664-651X
Impact Factor: RJIF 5.4
IJMBB 2023; 5(2): 43-47
www.biologyjournals.net
Received: 23-07-2023
Accepted: 09-10-2023

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The variation of the androgen receptor single nucleotide polymorphism (rs6152) gene in different stages of endometriosis disease in Iraqi women

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DOI: https://doi.org/10.33545/26646501.2023.v5.i2a.50

Abstract
Gene (AR) is a nuclear receptor belonging to a family of super-nuclear futures. This study aims to screen the diagnostic and clinical of the (AR) gene single nucleotide polymorphism (rs6152) at various stages of endometriosis.
Methods: 120 serums of Iraqi women suffering from endometriosis were taken and it was distinguished into (I, II, III, and IV) stages according to the "American Society of Reproductive Medicine" (ASRM) guidelines. Iraqi healthy women numbering 50 were taken as a control group. The statistical software (SPSS. 2017) Version (25.0) were used to analyze the data.
Results: The (AR) gene single nucleotide polymorphism (rs6152) has three genotypes which are (AA, GA, and GG), 72% of the group in the control carry the (GA) genotype copy of the (AR) gene. The wild-type (GG) genotype was found in (85) patient cohort with a genotype frequency of (70.8%), while the mutant genotype (AA) was detected in 17 (14.2%), and (GA) was noticed in 18 patients with a genotype frequency (15.0%).
Conclusion: The augmentation of frequency of the (GG) genotype of this SNP in different stages of endometriosis can be considered promising signs to identify and diagnose patients with endometriosis.
Abbreviations: AR = androgen receptor, kDa = kilodaltons, DNA = Deoxyribonucleic acid, SNP= Single nucleotide polymorphism, bp = base pair.

Keywords: Endometriosis, androgen receptor, (rs 6152), stages

Introduction
Endometriosis is a benign gynecological disease that depends on estrogen (Ying G, *et al*. 2021) [28], it has been regarded as the second most prevalent cause of surgery in per menopause females after leiomyomata (Uterine fibroids), which shows an incidence of (about 70 - 80%) in women (Outi U, *et al*. 2021) [18].
AR (Androgen Receptor) is known also, as the Nuclear Receptor subfamily (3), group (C), member (4) ((NR3C4)). AR gene found on (Xq11–q12) of the (X) chromosome, encodes a nuclear receptor that belongs to the nuclear receptor's superfamily (Crawford ED, *et al*. 2018, Alessandra B, *et al*. 2022) [7, 1]. (AR) is a nuclear and steroid receptor that is triggered via Hormones that are androgenic such as testosterone and dihydrotestosterone (Tiziana S, *et al*. 2022, Eviania L, *et al*. 2022) [27, 12].
AR consists of eight exons separated by introns of various lengths (0.7–2.6 kb) that code for a (110-kDa) protein with (919) AAs (amino acids) (Alice Z, *et al*. 2019, Sujun W, *et al*. 2022) [2, 24]. The androgen receptors have two predominant isoforms AR-A (87 kDa) and AR-B (110 kDa) (Chao H *et al*. 2020) [5], together three main functional domains are included the N-terminal domain encodes via exon (1), DNA binding domain encodes via exon (2 and 3), and finally, the C-terminal ligand - binding domain encodes via exon (4–8) (Elizabeth VW, *et al*. 2022) [9]. AR is a nuclear and steroid receptor triggered via Hormones that are androgenic such as testosterone and dihydrotestosterone (Tiziana S, *et al*. 2022) [27]. AR is activated by phosphorylation with dimerization and then transported to the nucleus via the cytoplasm (Rachel AD, *et al*. 2016) [20]. Within the cytoplasm, the inactive AR is joining to heat-shock proteins such as HSP-40, HSP-70, and HSP-90 (Crona DJ, *et al*. 2017) [8].

Materials and Methods

Subjects

This study included one hundred and seventy (170) Iraqi women aged between (18 to 40) years. It was divided into (120) patients suffering from endometriosis (patient group) and 50 who took the control group. Endometriosis patients were classified according to the American Society for Reproductive Medicine (ASRM) guidelines into four stages (I, II, III, and IV). The blood samples were taken from both groups during the ovulation phase of the menstrual cycle.

Genotypic data

2 ml of the whole blood sample was transferred into an anticoagulant vacuum blood collection tube (EDTA–K3 tube glass 2ml), and at (−20 °C) was stored until analysis for genotyping (DNA) of the androgen receptor (AR) gene was extracted according to the protocol of the (ABIOpure, USA) kit. To determine the Purity and concentration of isolated (DNA) and detect the quality of samples for final applications, the Quantus Fluorometer was used. The concentration range of (DNA) is between (20 to 35 ng/µl).

AR (G/A) single nucleotide polymorphism (rs6152) of the AR gene, was genotyped through allele-specific multiplex Polymerase Chain Reaction (PCR) from Thermo Fisher Scientific, USA according to the American Society for Microbiology (ASM) protocol. Utilizing pre-designed specific primers supplied through (Macrogen Company, Korea) as described in below:

rs6152-F: 5`-TGTAAAACGACGGCCAGTGATGAGGAACAGCAACCTTC-3` and rs6152-R: 5`-CAGGAAACAGCTATGACCTTGTAGAGAGACAGGGTAGAC-3`.

The PCR component reaction in this study was performed in a total volume of (25 µl). Amount of (12.5 µl) of PCR master mix (Promega, USA), (2 µl) of DNA sample, (1 µl) of each forward, and (1 µl) of reverse primers and (8.5 µl) Nuclease Free Water (DNase, RNase free). The master mix contained (DNA polymerase, pyro phosphatase, pyrophosphate, cofactor - magnesium chloride ($MgCl_2$), dNTPs, buffer, detection dye, also stabilizer). The following conditions were used in the PCR program as shown in Table.

Table 1: PCR program

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	63	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

Following PCR amplification, 1.5% the agarose gel electrophoresis (Thermo, USA) was relied on to verify the existence of amplification. The bands were visualized using gel imaging system. The PCR products were transmitted for Sanger sequencing, utilizing the **(**ABI 3730*xl*), automated (DNA) sequencer by (Macrogen Corporation, Korea).

Statistical Analysis

The data in this study was interpreted using a software program (SPSS. 2017 version 25.0). The frequency, percentage, and mean were used to analyze the obtained data. To evaluate the difference in the mean level of numeric data between more than (2) variables ANOVA test was used. The chi-square test is also used to test the association between qualitative variables and to evaluate the studied biomarkers' sensitivity and specificity, the ROC test was used. P value regarded as significant if ($p \leq 0.05$).

Results and Discussion

Results

The (AR) gene single nucleotide polymorphism (rs6152) has three genotypes which are (AA, GA, and GG), 72% of the group in the control carry the (GA) genotype copy of the (AR) gene. The wild-type (GG) genotype was found in (85) patient cohort with a genotype frequency of (70.8%), while the mutant genotype (AA) was detected in 17 (14.2%), and (GA) was noticed in 18 patients with a genotype frequency (15.0%). In stage (I) of the disease 76.7% carry the (GG) genotype, all stage (II) patients carry the (GG) genotype, 56.7% of patients in stage (III) had (GG) genotype copy and 50% of patients in stage (IV) had (GG) genotype, a statistically significant association between carrying (GG) genotype copy and an endometriosis cohort, *p*-value (< 0.00), Table (2).

Table 2: The Polymorphism of Androgen Receptor Gene (AR) (rs6152) in Studied Groups (*p-value > 0.05)

Patients		AR GENE (rs6152)			Total	p-value
		AA	GA	GG		
		17 (14.2%)	18 (15.0%)	85 (70.8%)	120 (100.0%)	< 0.00*
Stage of endometriosis	Stage 1 N (30)	7 (23.3%)	0 (0.0%)	23 (76.7%)	30 (100.0%)	
	Stage 2 N (30)	0 (0.0%)	0 (0.0%)	30 (100.0%)	30 (100.0%)	
	Stage 3 N (30)	5 (16.7%)	8 (26.7%)	17 (56.7%)	30 (100.0%)	
	Stage 4 N (30)	5 (16.7%)	10 (33.3%)	15 (50.0%)	30 (100.0%)	
Control N (50)		0 (0.0%)	36 (72.0%)	14 (28.0%)	50 (100.0%)	
Total		17 (10.0%)	54 (31.8%)	99 (58.2%)	170 (100.0%)	

Results of the amplification of (rs6152) of human samples and controls were fractionated on (1.5%) agarose gel electrophoresis stained with ethidium bromide (m: 100 bp) ladder marker. lanes (1-13c) resemble (752 bp) PCR products Figure (1).

Analysis of (rs6152) SNP of androgen receptor (AR) gene using Sanger sequencing. Single “G” peak indicative of a G homozygous allele. Single “A” peak indicative of an A homozygous allele. In addition, the presence of the “G” and “A” peaks is indicative of the G/A heterozygous allele Figure (2).

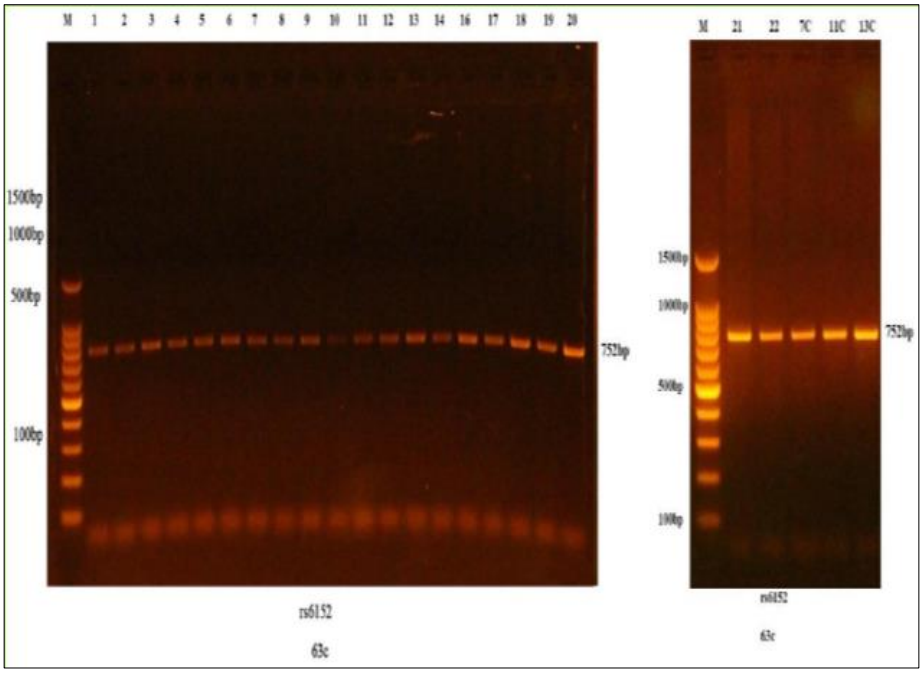

Fig 1: Agarose Gel Electrophoresis

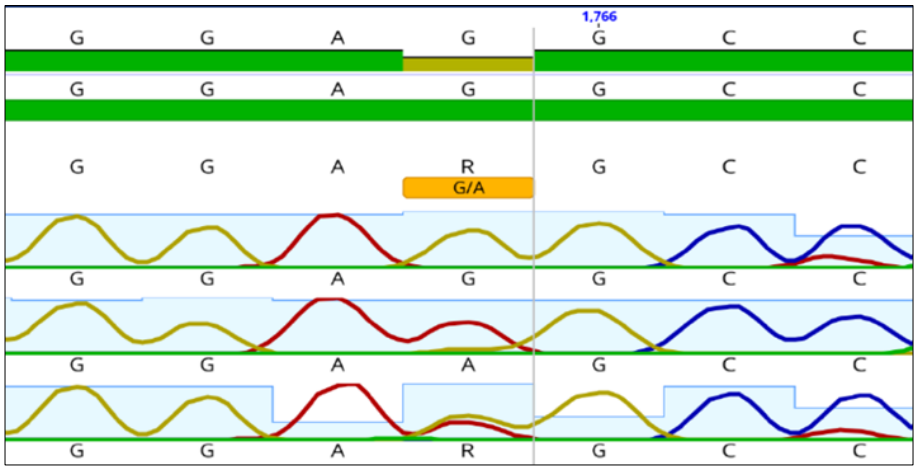

Fig 2: Data Analysis of (rs6152) SNP of androgen receptor (AR) gen

Discussion

In this study has been assayed (rs6152) or as known (G1733A) polymorphism of the androgen receptor (AR) gene in different stages of endometriosis patients. This SNP is located in chromosome (X): 67545785 (GRCh38.p13), (X):66765627 (GRCh37) (NCBI dbSNP), between exon (1) and (2) polymorphic trinuclotide repeats (The CAG and the GGC) that code for polyglutamine and polyglicine pathways (Ángela PD, *et al*. 2017, Shireen H F, *et al*. 2021) [3, 22].

The results of this study found a significant statistical association between carrying (GG) genotype copy and endometriosis patients when compared to healthy individuals. The outcome of this study is in line with Carneiro *et al* who observed abundance of androgens and androgen receptors (ARs) are identified in endometriotic lesions in endometriosis patients, indicating that endometriotic tissue is responding to both local as well as systemic androgens that have the ability to operate on endometriotic cells (Carneiro M M, *et al*. 2008) [4]. So is similar other research focused on this fact of the androgen receptors (ARs) in endometriosis (Hsin-S W, *et al*. 2012, Stefano A, *et al*. 2020) [13, 23].

Because of the reality of endometriosis, is a benign, chronic lesion that is responsive and depends on the estrogen hormone. (Liberia T, *et al*. 2022) [14], which is produced through its precursor the androgens (pro-estrogen) that have a significant impact on a female's normal physiology and uterine diseases (Luu-T V, *et al*. 2010, Raffaele N, *et al*. 2022) [15, 21].

On this basis, circulating levels of androgens (Androstenedione as well as testosterone) are extremely important because androgens act as a substrate for the aromatase (Cytochrome p450) enzyme which has elevated levels in eutopic and ectopic endometriosis (Yoshihiro JO, *et al*. 2014, Tea LR *et al*. 2020) [29, 26]. Leading to the biosynthesis of estrogen (or estradiol E2) (Evan R S, *et al*. 2001, Elodie C, *et al*. 2020) [11, 10]. Additionally, androgen modulates the influences of estradiol within the endometrial (Önder Ç, *et al*. 2017) [17].

Sherry *et al*. Upon assay reported that the androgen receptor (AR) gene single nucleotide polymorphism (rs 6152) and the risk of repeated spontaneous abortion (RSA) for Iraqi women, is associated with (GG) genotype of (rs 6152) SNP (Shireen H F, *et al*. 2021) [22]. This conclusion agrees with the findings for the same SNP (rs6152) in Mexican and

Greek women (Ángela P-D, *et al*. 2017) [3], and also the Iranian population (Tahere J, *et al*. 2013). The evidence of an association between AR (rs6512) and RSA according to the previous review supports the results of our study because endometriosis is linked to repeated spontaneous abortion depending on epidemiological data (Pietro S, *et al*. 2016, Chloe H, *et al*. 2020, Zhuang Y, *et al*. 2022) [19, 6, 30].

Conclusion

Our research is the first to explore the relationship between different stages of endometriosis and (rs6152 /G1733A) polymorphism of the androgen receptor (AR) gene, which is illustrated augmentation of frequency of (GG) genotype of this SNP, which may contribute to activating the androgen receptor (AR) transcription network and lead to elevated estradiol production and increasing the severity of endometriosis lesions. Thus, they can be considered promising signs to identify patients with endometriosis.

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