Investigating pathogenic SNPs in androgen receptor with direct influence on polycystic ovary syndrome (PCOS) in women

Rehab S. Ramadhan[1] , Rebah N. Algafari[2*] and Aziz Latif Jarallah[1]

Abstract

Background: Polycystic ovary syndrome (PCOS) became one of the main reasons for infertility in women. It has an obvious effect on phenotype represented by hirsutism, increased body mass index, obesity, and acne, while biochemical tests show adverse hormonal imbalance with hyperandrogenism as testosterone levels increases. From molecular level point of view, pathogenic SNPs may change CAG repeats number along androgen receptor (AR) resulting in altered function of the gene causing different affinity to androgen hormones.

Methods: Recruiting 150 patients diagnosed with PCOS for the study, genomic DNA was extracted and amplified using specifically designed exon 1 PCR primers employing gene walking technique. The resulting amplicons were sequenced and thoroughly analyzed for polymorphism and CAG repeats number.

Results: Data obtained from recruiting 150 patients diagnosed with PCOS showed that sequences X:67545209–67545742; X:67545503–67545739 of exon 1 harbored 7 SNPs altered secondary structure of the resulting protein and forced toward the use of CAA as synonymous codon instead of the normal CAGs stretches. This led to produced alternative mRNA that eventually changed nonsense-mediated mRNA decay mechanism.

Conclusion: Probability of PCOS in women with polymorphic AR gene is higher than others, especially women with high number of CAG stretches. The new finding and highlight of this study is that alternative codon usage (CAAs) to produce the same amino acid (Gln) and compensate the reduced number of CAG repeats number may be attributed to epigenetic mechanism to mitigate the adverse effect of such change and maintain a normal function of AR gene. This finding was not previously reported in former studies.

Keywords: PCOS, Hormonal imbalance, CAG repeats, Androgen receptor polymorphism

Background

Polycystic ovary syndrome (PCOS) is a diverse condition found relatively 6–20% in females at reproductive age causing an endocrine disorder [1–3]. However, multiple diagnostic features can be found for PCOS accompanied by more comorbidities fall in hyperandrogenism, hirsutism, acne, amenorrhea or oligomenorrhea, insulin resistance, obesity, imbalanced hormonal system

affecting reproductive ability include luteinizing hormone (LH), hepatic lipid deposition, hypertension, an increased risk of miscarriage, and shape of polycystic ovarian on ultrasound [3–7].

In women, androgens come from ovaries and adrenal glands comprises 40–50% of the body testosterone with vital role in controlling fertility among them. Studies showed that tumors and PCOS are the main affecters in increasing their level without age relevance [8].

Hyperandrogenism is the distinct feature of PCOS. Therefore, polymorphisms of androgen activation genes are considered to be involved in the development of

*Correspondence: rebahalgafari@gmail.com
[2] Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq
Full list of author information is available at the end of the article

PCOS [9]. The N-terminal domain of AR encoded by exon 1 found to harbors variable expanses of CAG repeat that code for polyglutamine tracts (poly-Gln) regions [10–14].

The N-terminal transactivation domain of AR encoded by exon 1 harbors variable expanses of CAG repeat that code for polyglutamine tracts (poly-Gln) regions. CAG repeats vary normally from 8 to 35 and demonstrate constant offspring lineage [15]. In vitro studies results demonstrated that shorter poly-Gln in AR, the higher ability to activate reporter genes with elements responding to androgen [16].

The AR protein comprises four functional domains that begin with N-terminal domain (NTD) encoded by exon 1 which is related to AR transactivation activity with polyglutamine stretches encoded by CAG repeat segments ranging from 11 to 36 and polyglycine stretches encoded by GCC triplet segments, DNA-binding domain (DBD), the resilient joint region, and the ligand-binding domain (LBD) [17]. Previous studies found inverse correlation between CAG repeat number and AR activity, where shorter CAG repeats increase AR activity and is associated with hirsutism and ovarian hyperandrogenism, while longer CAG repeats lower AR activity causing hypoandrogenicity and male infertility [18].

This study was designed and conducted to identify the effective pathogenic SNPs within exon 1 with possible involvement to cause PCOS, role of CAG stretches number in AR activity as first study conducted involving Iraqi women.

Methods
Study approval
This research was reviewed and approved by Al-Nahrain University, Biotechnology Research Center IRB, Scientific Committee and Ethical Committee under reference No. 175.

Participants
Inclusion criteria
All participants were of 14–40 years old attending fertility clinic of Kamal Al-Samarie Hospital in Baghdad from period May 2020 to August 2021. A total of 150 PCOS patients and 100 controls with matching age group were recruited for this study after they gave their written and verbal consent. The diagnosis of PCOS came depending on Rotterdam criteria [5] when at least two symptoms were identified such as signs of hyperandrogenism, oligo- or anovulation, or polycystic ovaries. Control women had a normal menstrual cycle (26–35 days), normal range of fertility hormones, and normal morphology of ovaries.

Control women visited the Fertility Clinic due to unrelated PCOS issue or male factors-related infertility.

Exclusion criteria
Women suffering from ovary tumors, ovary cancer, subjected to ovarian cyst excision surgery, and who were injected with fertility hormones for IVF super-ovulation program were excluded from this study. Research steps are illustrated in Fig. 1.

Clinical, hormonal, and body criteria measurement
The anthropometric variables of all participants, including their age, body height and weight, and menstrual cycles, were recorded. The levels of fertility hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestradiol (E2), progesterone (P), total testosterone (TT), prolactin, and anti-Müllerian hormone (AMH) were assayed in the clinical laboratory of the hospital by enzyme-linked immunosorbent assay (ELISA) and chemiluminescence immunoassay (CLIA). Hormone levels were recorded in stage of follicular phase at day 3 and 8 after period.

Molecular assay for exon 1 analysis
DNA extraction
Blood lymphocytes were isolated and purified by Lymphoprep (Nycomed Pharma, Oslo, Norway) and stored saline at − 20 °C. DNA was extracted from these cells using Genaid Total DNA extraction kit, a product of Macrogen Company, Korea. DNA purity and concentration were measured using Techne NanoDrop system (England), and samples were kept under − 20 °C until further processing.

PCR amplification and characterization of CAG repeats
CAG repeats were characterized by the number of units in the AR exon1 depending on previously published technique [19]. Four primers were designed depending on NCBI sequence with accession number NG_009014.2 to amplify the entire exon1 using genome walking technique with the following sequence (5′–3′): AR-1-Forward GTGCTGGACACGACAACAAC, AR-1-Reverse CTCATTCGGACACACTGGCT with product size 307 bp, AR-2-Forward TAGGGCTGGGAAGGGTCTAC, AR-2-Reverse GCTGTTGCTGAAGGAGTTGC with product size 573 bp, AR-3-Forward GCAACTCCTTCAGCAACAGC, AR-3-Reverse GCCTTCTAGCCCTTTGGTGT with product size 394 bp, and AR-4-Forward GGTGAGCAGAGTGCCCTATC, AR-4-Reverse GTTGTTGTCGTGTCCAGCAC with product size 556 bp. Genomic DNA with concentration of 100 ng was amplified by PCR using the following protocol: denaturation
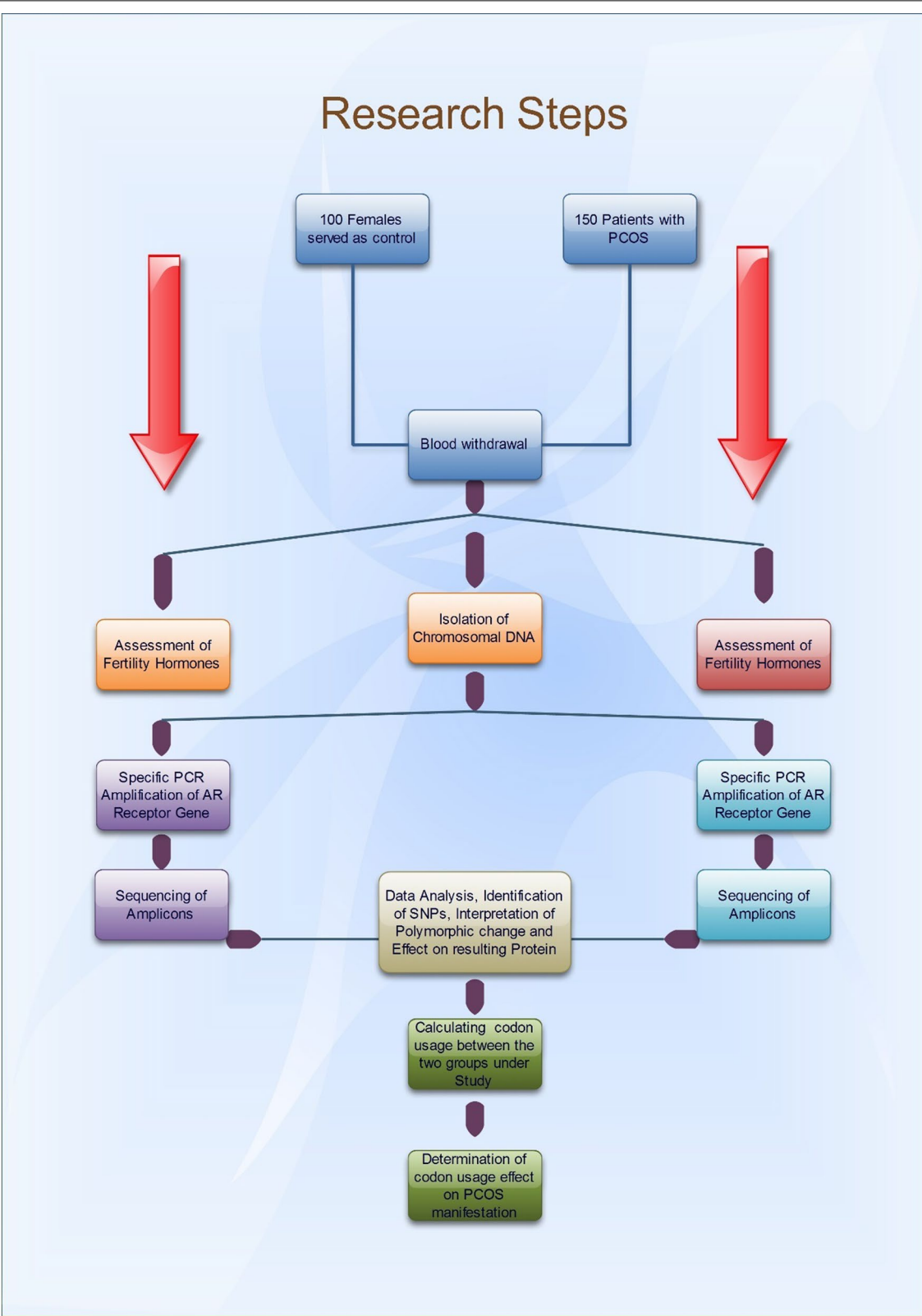
Fig. 1 Flowchart showing research steps, participants, and main goal of this work

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cycle at 94 °C for 5 min. followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and final extension cycle at 72 °C for 10 min. Successful amplification was confirmed by electrophoresis at 10 v/cm field strength for 90 min., and resulting amplicons were sequenced by Macrogen company, Korea using Sanger method.

Software and data analysis

Data analysis was performed using BlastRef Sequence, BlastX, and Blastp available tools at https://www.ncbi.nlm.nih.gov, BLAT, genome alignment, TBLASTN at http://asia.ensembl.org., Phyre 2 at http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index, and DNASp6 as a standalone software. All data were statistically analyzed by SPSS software (version 12; SPSS Inc., Chicago, IL, USA).

Table 1 Hormonal and anthropometric data of PCOS patients and the control

Clinical parameter	Control ($n = 100$)	PCOS ($n = 150$)	*p* Value
Age, year	28.79 ± 2.94	28.63 ± 3.36	> 0.05
FSH (IU/L)	6.70 ± 1.22	$3.87 \pm 1.41*$	< 0.05
LH (IU/L)	5.17 ± 1.66	$8.65 \pm 4.64*$	< 0.05
Basal PRL (ng/ml)	3.7 ± 1.53	$17.71 \pm 6.08*$	< 0.05
E2 (pg/ml)	35.36 ± 10.98	38.76 ± 17.17	< 0.05
P (ng/ml)	0.59 ± 0.18	$0.76 \pm 0.46*$	< 0.05
TT (ng/dl)	23.34 ± 7.49	$40.54 \pm 17.45*$	< 0.05
AMH (ng/ml)	4.36 ± 2.49	$9.80 \pm 4.94*$	< 0.05

Values are given by mean $\pm$ standard deviation. *$p < 0.05$ related to control group

Results

Measurement of hormones

Data were recorded from 150 PCOS cases and 100 controls who were 14–40 years old. The endocrine and anthropometric parameters' comparison of two categories of participants is listed in Table 1. PCOS patients showed higher body mass index (BMI), prolactin (PRL), luteinizing hormone (LH), progesterone (P), oestradiol (E2), total testosterone (TT), and anti-Müllerian hormone (AMH) levels compared to controls. Follicle-stimulating hormone (FSH) level decreased significantly ($p < 0.05$) with no significant differences related to age.
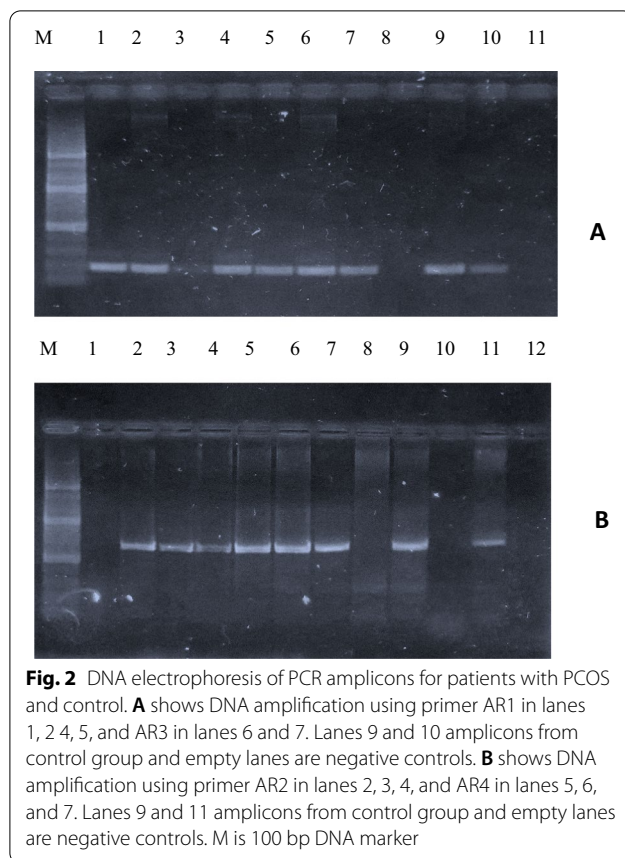
Clinical biochemical and anthropometric

In Table 2 biochemical and clinical characteristics were listed for 150 subjects diagnosed with PCOS who met Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group [5] characteristics. Multiple phenotypic and biochemical parameters were recorded and compared with control.

Molecular analysis of AR exon 1
Determination of amplicons sequence criteria

Amplicons sequences obtained from patients and control were blast using tool available at http://asia.ensembl.org/ to determine the exact location, length, and position of the sequences on exon 1. Results obtained showed that the sequence covered was X: 67545209 to 67546114 distributed as follows: X:67545209 to X:67545742; X:67545503 to X:67545739; X:67545763 to X:67546114; X:67545758 to X:67546115; and X:67545760 to X:67546114. Further analysis showed that the first two locations are vulnerable for DNA change since multiple transitions and transversion were found within the sequences causing significant effect on amino acid

Table 2 Clinical and biochemical presentation of women with PCOS and non-PCOS control groups

Criteria	PCOS	*Mann–Whitney confidence interval, p*	Control	Significance
Polycystic ovaries, %	150	NS	100	NS
Oligomenorrhea, %	64	0.002	7	0.001
Amenorrhea, %	9	0.041	2	NS
Hyperandrogenemia, %	57	< 0.001	Non	NS
Hirsutism and/or acne, %	57	0.017	10	NS
Hirsutism, %	28	NS	6	0.003
Acne, %	48	0.009	18	NS
BMI, kg/m^2	25.5 ± 6.2	< 0.001	24.4 ± 3.8	< 0.001
Waist, cm	73.8 ± 16.2	< 0.001	68.0 ± 11.6	$< .001$
Waist-to-hip ratio	0.80 ± 0.10	< 0.001	0.77 ± 0.07	$< .001$

NS non-significant

Fig. 2 DNA electrophoresis of PCR amplicons for patients with PCOS and control. **A** shows DNA amplification using primer AR1 in lanes 1, 2 4, 5, and AR3 in lanes 6 and 7. Lanes 9 and 10 amplicons from control group and empty lanes are negative controls. **B** shows DNA amplification using primer AR2 in lanes 2, 3, 4, and AR4 in lanes 5, 6, and 7. Lanes 9 and 11 amplicons from control group and empty lanes are negative controls. M is 100 bp DNA marker

sequences and protein configuration. Electrophoresis of PCR amplicons is given in Fig. 2.

Effect of DNA change on amino acid sequence

In present study, DNA resulted from PCR amplification for all patients was sequenced to determine the effective change influencing the primary structure of the protein encoded by exon 1. We were able to find seven SNPs in sequences X:67545209–67545742 and X:67545503–67545739 with effect on amino acid sequence. These changes are listed in Table 3.

None of these SNPs were not detected in control or the consensus sequence. The *in silico* analysis of these SNPs demonstrated that SNPs at positions X:67545218, X:67545264 might be pathogenic, while the remaining SNPs were likely to be benign. This was determined by effect of these SNPs on resulting amino acid and later on protein secondary structure, folding, and disorder in further analysis using Phyre[2] software.

Figure 3 shows chromatograms of SNPs detected in PCOS patients during DNA sequencing.

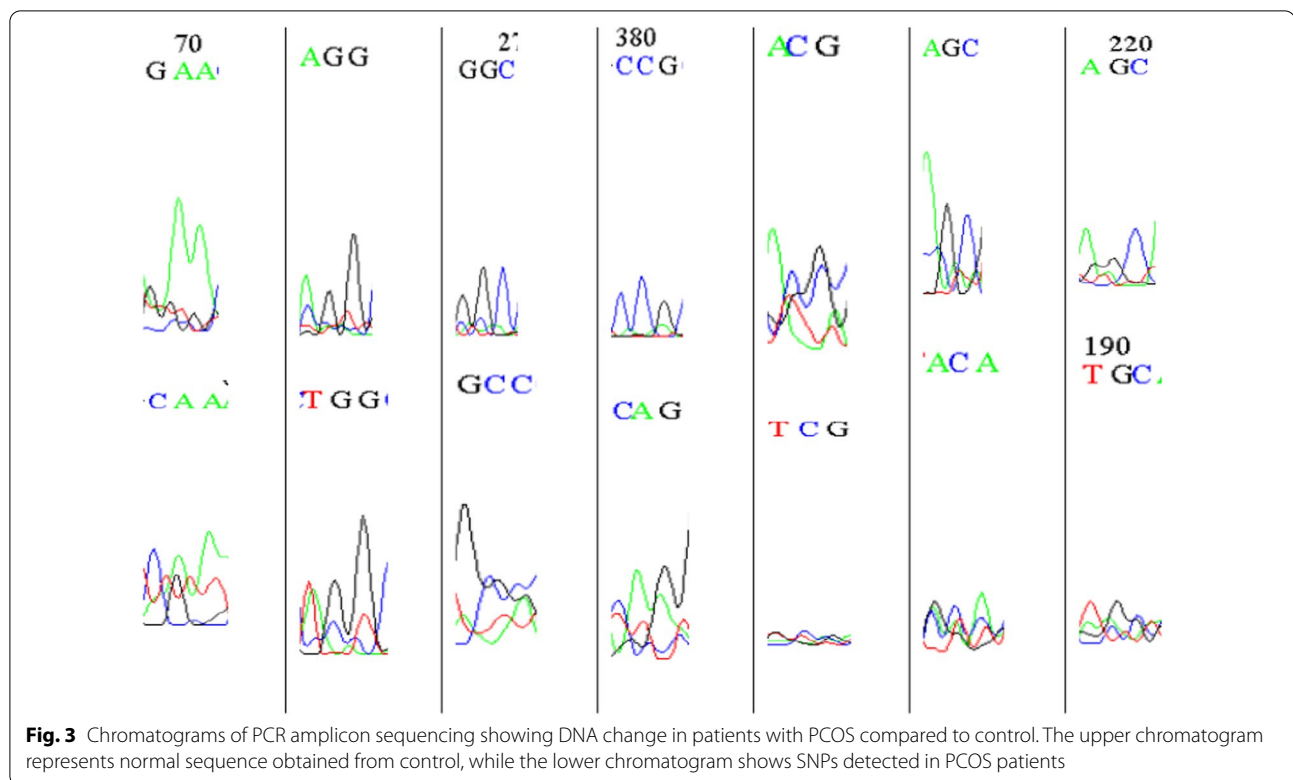**Effect of amino acid substitution and protein configuration**
Translation of DNA transcripts is a critical stage toward creating a protein with optimum function. Change in the genetic code assembled in mRNA may not cause a change in the final form of the protein due to redundancy or sustaining the same criteria of the amino acid. However, in our case, alteration in some amino acid sequence occurred in patients, especially these with different criteria altered the secondary form of the protein by changing in both alpha helix and beta strand. Applying DNA sequence obtained from PCOS patients to determine the secondary protein structure using Phyre[2] software showed significant change in the secondary structure compared to control as elaborated in Fig. 4.

Analysis of data obtained for secondary structure of protein in PCOS patients and control shows significant change represented by change in alpha helix length and change of from alpha helix form to beta strand with twice the ratio (A compared to B), while the obvious change in (D and C) was the change from alpha to beta strand in multiple locations. Parameters changed resulted from amino acids substitution (AAS) are listed in Table 4.

The prediction is 3 states including α-helix, β-strand, or coil. Each part of the protein is represented by a color according to the type of fold: α-helices (green), β-strands (blue arrows), and faint lines (coil). Confidence of prediction is proportionally inverse to disorder with high confidence (red) and low confidence (blue). Data obtained

Table 3 Location, genetic code change, and effect on amino acid sequence in patients with PCOS

Location of genetic change in the AR gene	Change in genetic code	Change in amino acid	Change of chemical criteria
X:67545218	GAA > CAA	E (Glu) > Q (Gln)	Acidic to polar
X:67545264	AGG > TGG	R (Arg) > W (Trp)	Basic to non-polar
X:57545509	GGC > GCC	G (Gly) > A (Ala)	Polar–polar
X:67545583	CCG > CAG	P (Pro) > Q (Gln)	Non-polar to polar
X:67545633	ACG > TCG	T (Thr) > S (Ser)	Polar–polar
X:67545672	AGC > ACA	S (Ser) > T (Thr)	Polar–polar
X:67545673	AGC > TGC	S(Ser) > C (Cys)	Non-polar to Non-polar

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Fig. 3 Chromatograms of PCR amplicon sequencing showing DNA change in patients with PCOS compared to control. The upper chromatogram represents normal sequence obtained from control, while the lower chromatogram shows SNPs detected in PCOS patients

with secondary structure were with 78–80% accuracy, and disorder prediction is on average 30%.

Analysis of CAG repeat length

Determination and estimation of the number of CAG repeats were performed during this study for patients with PCOS compared to control. We conclude that with higher number of CAG repeats, the AR becomes more sensitive to testosterone even with slight change in number of these stretches and might play a significant role in the syndrome. These stretches detected are shown in Fig. 5.

For further analysis, since the difference in CAG stretches is not significant between patients and control, we suspected the reason may be attributed to difference in codon usage in both PCOS patients. Results obtained are listed in Table 5.

The table explains the following results: two redundant codons were used for Gln; CAG that is of interest and the synonymous CAA. In patients with PCOS, both codons are almost equally used (observed value), and expected value should be constant, but RSCU results show decrease in CAG usage compared to control, meaning CAG sequence was less used in the genetic code for glutamine synthesis with deviation toward CAA codon to compensate this decrease.

Discussion

As reported by previous articles, androgens play critical roles in influencing the follicular development-associated downstream genes expression controlling stages of development of follicle through their regulatory function mediated by specific AR receptors [20, 21] and revealed that nucleotide alteration [22, 23] or altered expression of AR may enhance the progression of PCOS [24, 25].

PCOS exhibit phenotypical features like hirsutism, overweight and obesity, and biochemical changes like hormone imbalance in patients. In our investigation, patients with PCOS showed typical phenotypic and biochemical features for the syndrome as shown in Tables 1 and 2 with significant elevation in prolactin, LH, oligomenorrhea or amenorrhea, infertility, and high testosterone level which is considered as the main feature in this syndrome as hyperandrogenism. Obese normal women are fertile in general, but elevated BMI may relate to high risk of infertility. Obesity may result from ovulation, menstrual, and infertility disorders and increases the hormonal and clinical features of PCOS [26–28]. Thus, hormonal imbalance causing certain body illness symptoms can be associated with the pathogenesis of PCOS [29, 30]. Furthermore, in many cases, the estrogen dominance results from excess testosterone converted by the body to estrogen in PCOS patients. Such elevation
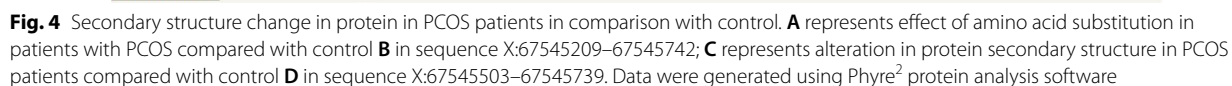
Fig. 4 Secondary structure change in protein in PCOS patients in comparison with control. **A** represents effect of amino acid substitution in patients with PCOS compared with control **B** in sequence X:67545209–67545742; **C** represents alteration in protein secondary structure in PCOS patients compared with control **D** in sequence X:67545503–67545739. Data were generated using Phyre^2 protein analysis software

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Table 4 Parameters related to secondary protein structure generated by Phyre^2 software

Parameter	Patients with PCOS Percentage	Control Percentage	t-test/p
Sequence	X:67545209–67545742		
Alpha helix	35	39	0.85
Beta strand	6	3	0.35
Disordered	30	32	NS
Sequence	X:67545503–67545739		
Alpha helix	45	50	0.72
Beta strand	5	7	0.28
Disordered	25	33	NS

NS non-significant, $p < 0.05$

may result in high fat accumulation and water retention leading to obesity [31].

Polymorphism and mutation of AR CAG repeat length were central point of research in the etiology of PCOS. Many researches have been published to relate genetic change and CAG repeat polymorphism in AR gene with PCOS, without concurrent results [9, 18]. However, in this study we used different approaches to analyze effect of polymorphism in developing PCOS depending on relative sensitivity of AR gene in regard to Gln production that has been encoded by redundant of triplets coding for it and their usage. In previously published report [19, 32], shorter CAG repeats are associated with higher sensitivity of AR, resulting in hyperandrogenism. However, such assumption was not completely confirmed, since such conclusion came from X chromosome inactivation in cases where no significant difference was found between PCOS patients and control [16], which is the same result we found in this study showing no significant difference in CAG repeats between both subjects participated in this study was found. Moreover, we found that the number of repeats in Iraqi women is higher than previously reported in both patients and control [33, 34] which might be an ethnic criterion for those women. In addition, multiple SNPs were identified along exon 1 which controls
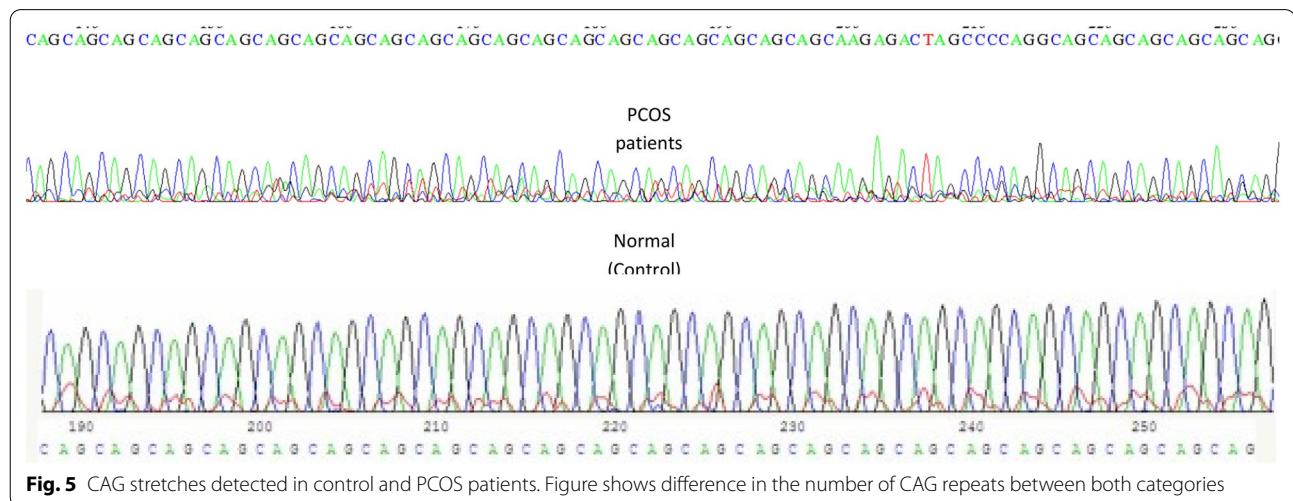
Fig. 5 CAG stretches detected in control and PCOS patients. Figure shows difference in the number of CAG repeats between both categories

Table 5 Codon usage in PCOS patients and control

Codon	PCOS patients			Control		
	Observed frequency	RSCU	Scaled chi^2	Observed frequency	RSCU	Scaled chi^2
Sequence: X:67545209–67545742						
CAG (Q)	4	1.44	0.658*	6	1.71	0.669
CAA (Q)	3	0.86	0.652*	1	0.29	0.233
Sequence: X:67545503–67545739						
CAG (Q)	3	1.37	0.625*	6	1.71	0.669
CAA (Q)	2	0.80	0.541*	1	0.29	0.233

The table was generated using DNASp6 with effective number of codons (ENC): 52.961, and codon bias index (CBI): 0.510

RSCU relative synonymous codon usage. $p = 0.05$

AR activity and harbor most of CAG repeats [16, 35]. These were 7 SNPs with in which two changed the physicochemical criteria of amino acids affecting the protein PI (isoelectric point), while the others affected CAG stretches toward reduction in number. However, they are extended to change the secondary structure which might affect the final form of the protein. Most of the effect was observed in X:67545503–67545739 sequence, while sequence X:67545209–67545742 was mostly affected by CAG repeats change.

The observation within this study is that, in the participants with PCOS and control, the CAG repeats were more than previously reported [16, 33]. Even with non-significant difference in their number in both participants' categories, it seems that an increased number of these stretches render the AR receptor more sensitive to any slight change. More confirmation came from codon usage which showed significant difference in CAG repeats usage between patients and control. Such reduction in CAG repeats usage forced the AR to use synonymous codon CAA for compensation. On molecular level, such shift codon usage can produce a variant mRNA which eventually altered nonsense-mediated mRNA decay (NMD) [36, 37].

With such possible explanation for higher sensitivity of AR receptor in women with PCOS, the use of androgen blocking drugs can be a promising medication to reduce the impact of this physiological problem on these women and may elevate their fertility level.

Conclusion

The AR exon 1 is considered to be highly vulnerable for genetic alteration. This alteration is featured by the presence of effective SNPs at specific location of the exon and change in CAG repeats number. With exon harboring high content of CAG repeats, it becomes more sensitive to any change in their number and compensates such decrease with synonymous codon. It seems that a switching in codon usage for Gln amino acid present in AR exon 1 from CAG to CAA with a mechanism may be related to upstream control elements that we recommend to be studied.

Abbreviations

AAS: Amino acids substitution; AMH: Anti-Müllerian hormone; AR: Androgen receptor; BMI: Body mass index; CLIA: Chemiluminescence immunoassay; DBD: DNA-binding domain; E2: Oestradiol; ELISA: Enzyme-linked immunosorbent assay; FSH: Follicle-stimulating hormone; LBD: Ligand-binding domain; LH: Luteinizing hormone; P: Progesterone; PCOS: Polycystic ovary syndrome; PRL: Prolactin; SNP: Single nucleotide polymorphism; TT: Total testosterone.

Author contributions
The first author RSR conducted planning the research, sample collection, and DNA isolation, the second author RNA performed PCR amplification, primer design, sequencing, data analysis, correspondence, and writing the paper, and third author ALJ is in charge of data curating. All authors read and approved the final manuscript.

Availability of data and materials
Data, samples, and genomic materials used during this study are available and stored at Biotechnology Research Center, Al-Nahrain University, and Al-Esraa University College.

Declarations

Ethics approval and consent to participate
Adult participants gave written consent to be a part of this work, while underage patients' parents (either one) agreed to let their daughter to be a part of this study which was given as a written consent. In both categories, obtaining consents to participate was done after clarifying the goal of the study and with no personal, details, and images of the patients to be published.

Consent for publication
Results of this research were approved by all authors and agreed for publication.

Competing interests
This research was conducted without conflict of interest among authors, funding agencies, or with any other research group in others institutes.

Author details
[1] Al-Esraa University College, Baghdad, Iraq. [2] Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq.

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