

ASSOCIATION BETWEEN AR GENE CAG REPEAT POLYMORPHISM AND SOME BIOCHEMICAL PARAMETERS WITH POLYCYSTIC OVARY SYNDROME IN IRAQI WOMEN

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ABSTRACT : CAG repeat in androgen receptor gene is one of very important genetic research to polycystic ovary syndrome (PCOS). Several studies have been presented to approve the association between AR gene (CAG) repeat polymorphism and PCOS patients without any reliable results. The aim of the present study was to estimate whether there is an alteration or relation in the AR gene (CAG) repeat length between patients with PCOS and healthy control in Iraqi women. Sixty Iraqi women with PCOS and thirty healthy women as a control were recruited in our research. Assays of biochemical parameters serum follicle-stimulating-hormone (FSH), serum luteinizing-hormone (LH), serum prolactin-hormone (PRL) and serum testosterone-hormone (T) were carried out, also BMI, age and hyper androgenism signs were recorded. PCR based assays were used to genotype of AR gene (CAG) repeat alleles. We detected that the CAG biallelic mean were comparable in PCOS women and controls (20.77 ± 0.65 vs. 20.87 ± 0.80 , $P = 0.626$). While when CAG biallelic repeats were divided into two groups; (short (CAG) repeat ≤ 20 and long (CAG) repeat > 20), the (CAG) repeat biallelic mean displayed more frequent in PCOS than in controls (60% vs 30%, $P < 0.0024$). In patients group, short (CAG) repeat was higher frequency 36/60 (60%) than long (CAG) repeat 24/60 (40%). Also, in patients serum testosterone (T) were positively correlated with the (CAG) repeat length polymorphism ($r = 0.26$, $P = 0.001$), while the clinical signs of hyperandrogenism with patients were showed a higher effect in short (CAG) repeat ≤ 20 alleles than long (CAG) repeat > 20 alleles as in hirsutism (30/43), acne (25/33) alopecia (27/38) and oily skin (18/31). Distribution of AR gene (CAG) repeats varied between patients with PCOS and controls, our finding results support the principle that there is a relation between AR gene (CAG) repeat polymorphism and PCOS in Iraqi women and (CAG) repeat polymorphism share in hyper androgenism in women with PCOS.

Key words : Polycystic ovary syndrome, PCOS, androgen receptor gene, polymorphism, CAG repeats, hyperandrogenism.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is known as the most prevalent reproductive age endocrine disease in women (Lizneva *et al*, 2016). It is a heterogeneous disease of unclear and unknown etiology, but it is widely regarded as having powerful proof that complicated relationships between genetic origins, environmental and behavioral variables contribute to this syndrome (Moghadam *et al*, 2018; Pasquali *et al*, 2016). PCOS affects up to 10% of women of reproductive age when using the diagnostic criteria of the National Institutes of Health (NIH) and up to 18% when PCOS is detected as the Rotterdam criteria (Mohammad *et al*, 2017).

PCOS is described as hyperandrogenism (evidence of excessive male hormone or androgenic effects)

typically associated with an ovulation symptoms (amenorrhoea, oligomenorrhoea and irregular cycles) associated with clinical and/or biochemical signs of androgenic increase (hirsutism, acne, alopecia) (Azziz *et al*, 2016). Diagnosis is based on the fulfillment of two of three values: uneven menstruation, high serum androgens (hyperandrogenism) such as testosterone or clinical signs, and an excessive number of preantral follicles in ultrasound ovaries (Rotterdam Consensus, 2004). The syndrome is the most mutual reason and source of ovulatory infertility (Kuang *et al*, 2015). PCOS is linked to obesity, insulin resistance and dyslipidemia, all of which are cardiovascular disease risk causes (Liu *et al*, 2019). 55-75% of PCOS patients had a large proportion of LH to FSH that clarifies enhanced concentrations of LH and low concentrations of FSH, the ratio of LH / FSH is usually

about (1:1) whereas the percentage of PCOS patients is greater than (2:1) (Zainulabdeen *et al*, 2014). An ovulation and hyper androgenism are mainly a result of disordered gonadotropin secretion, Enlarged LH stimulates the development of ovarian testosterone (T) while comparative FSH deficiency results in the arrest of ovarian folliculogenesis and may also lead to constitutive rises in ovarian and adrenal androgen secretion. Increased T concentrations of hypothalamus feedback contribute to disordered gonadotropin release (Hayes *et al*, 2015). Under pathological situations abnormal ovarian and adrenal steroid synthesis leads to hyper androgenism, increasing androgen leads to overactivation of the androgen receptor (AR), the human AR gene is situated on the X-chromosome and consists of eight exons and seven introns (Rajender *et al*, 2013).

The AR gene is a member of the ligand-activated transcription factor family that regulates many biological developments and is encoded by a gene that is situated at Xq11-q12 (Shukla *et al*, 2016). The AR gene comprises four functional domains: the N-terminal transactivation domain (NTD), the DNA-binding domain (DBD), the hinge region and the Ligand-binding domain (LBD). The N-terminal transactivation domain is encoded by exon one with a highly polymorphic (CAG) replicating region (Crona *et al*, 2017). The AR gene includes CAG repeats that impact the receptor function, where fewer repeats lead to enhanced sensitivity of the receptor to androgens circulating and more repeats lead to reduced sensitivity of the receptor (Francomano *et al*, 2013). The (CAG) repeat varies between (10-35) and microsatellite region (CAG)_n repeat codes a poly-glutamine area and influences the transactivation function of the AR, an *in vitro* research showed a reverse connection between the amount of repeats and the activity of AR, AR alleles with a lesser number of (CAG) repeat showed higher activity while the increase in the number of (CAG) repeats gradually decreased activity (Tirabassi *et al*, 2015).

The aim of the current study was to estimate whether there is an alteration or relationship in the AR gene (CAG) repeat length between patients with PCOS and healthy control in Iraqi women, also evaluated the influence of (CAG) repeat on different biochemical parameters (FSH) (LH) (PRL) (T) also age, body mass index (BMI) and as well as on clinical androgen sign (hyperandrogenism) in PCOS patients. Our study is the first kind to investigate the association between Androgen receptor gene (CAG) repeat polymorphism and PCOS women in Iraqi population.

MATERIALS AND METHODS

The study consisted of (90) Iraqi women divided into two groups (60 PCOS patients and 30 healthy control). Their age range was between (18-36) years, the samples were collected from Kamal Al-Samarrai Hospital and Baghdad teaching hospital. The medical history was taken from all patients, the diagnosis of PCOS was prepared conferring to the Rotterdam criteria, which are based on presence of two of three factors including irregular menses (oligo-or anovulation), elevated serum androgens (hyperandrogenism) such as testosterone or clinical sign such as hirsutism and a great number of preantral follicles appearing in ovaries on ultrasound. Healthy women diagnosed with steady cycle and no signs of hyperandrogenism.

The collection of blood specimens

Blood samples were collected by taking 6 ml of venous blood from each patient and healthy women, during (2nd - 5th) day of the menstrual cycle and two ml of whole blood were employed in tube that containing EDTA (Ethylene Diamine Tetracetic Acid) for DNA extraction and the other (four ml) of whole blood were put in non-heparinized tube; serum was separated from this blood by centrifuging for 5 minutes at 3000 round per minutes (rpm), for additional hormone tests. Patients with a hormonal therapy, adrenal hyperplasia and Cushing's syndrome were excluded from the study.

Genomic DNA extraction, amplification and analysis of (CAG) repeat

DNA was extracted from the all (PCOS cases and controls) blood samples by taking 2ml whole blood that placed in EDTA tubes for molecular studies using DNA purification kit (QIAamp, Germany). (CAG) repeat were genotyped using Polymerase chain reaction (PCR)-based assay of Genomic DNA, PCR amplification were detecting in a total volume of 25µl involved (3µl genomic DNA, 5.5µl Nuclease-Free water, 12.5µl Q5 High-Fidelity 2X Master Mix and 2µl of each primer). The AR gene (CAG) repeat regions were amplified using the forward primer (5' -CCTGTTGAACTCTTCTGAG-3') and the reverse primer (5' -TCTGGGACGCAACCTCTCTC-3') (Peng *et al*, 2014). PCR condition were: firstly initiation denaturation at 92°C for (2 minutes) followed by 35 cycle program with denaturation at 94°C for (40 seconds), annealing at 61.5°C for (40 seconds), elongation at 72°C for (40 seconds), and final elongation at 72°C for (5 minutes). PCR products were scanned by electrophoresed on 1.5% agarose gel in 1X TAE buffer. In the electrophoresis of gel procedure every subject was showed DNA band between (497-516 bp) (Fig. 1)

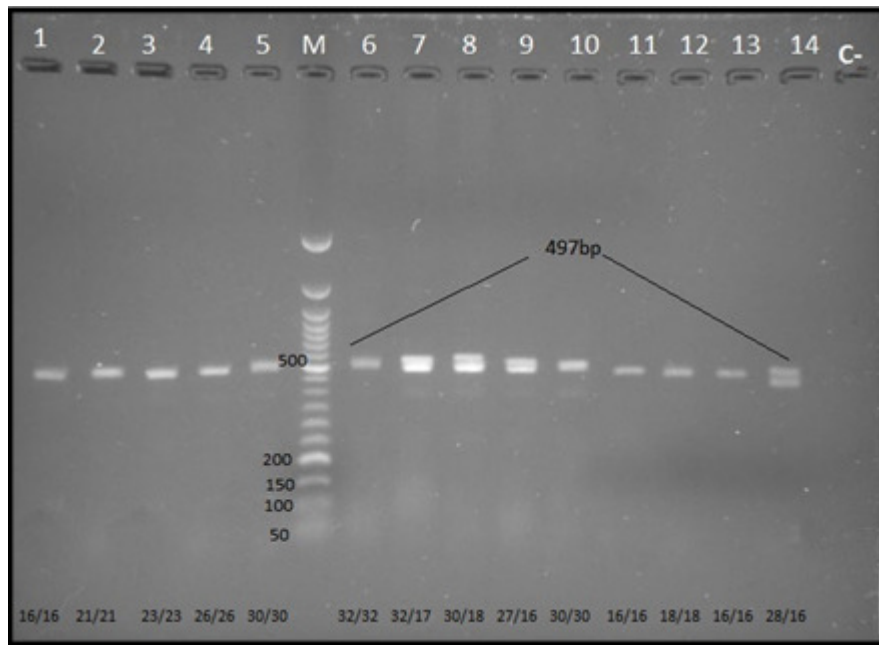


Fig. 1 : Identification of AR-gene (CAG) repeat length genotype between (497-517bp) from gel electrophoresis of PCR products, individual AR-gene genotypes are termed as number of CAG repeats for total AR-alleles, -C: as negative sample, M: ladder as marker.

and captured by gel documentation system to the observed specific bands. Samples that showed double band were purified and separated using Gel DNA Recovery Kit from (Zymo Research, USA) when another agarose gel electrophoresis was performed to separate the two heterozygous bands, getting single band individually once cutting and purifying. Finally, the (CAG) repeat numbers of homozygous bands and single band of heterozygous bands were calculated by sequencing process and analysis of Sequences was made by Basic Local Alignment Search Tool (BLAST) software and Molecular Evolutionary Genetics Analysis (MEGA) software.

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used in the research parameters to identify the impact of differential variables. (T-Test) was used to significant compare between means. Correlations coefficient -r among variables were done. Chi-square test was used in this research to significant compare between proportion (0.05 and 0.01 probability).

RESULTS

Clinical and hormonal analysis of PCOS patients and controls

The clinical and hormonal traits of women with PCOS and without PCOS as (controls) were termed in Table 1. In PCOS patients there were highly significant different as predictable in LH, LH/FSH, T, PRL and BMI ($P > 0.01$) also age ($P > 0.05$) than control. While FSH level was significantly lower than control. The women with PCOS

were had a higher percentage of hirsutism (43/60) 71.60%, Acne (33/60) 55%, Alopecia (38/60) 63% and Oily skin (31/60) 51.60% than controls as were showed in Table 2.

Associations of (CAG) repeat polymorphism in women with PCOS

The range of (CAG) repeat in PCOS patients was (8-34), while in healthy women (CAG) repeat numbers ranged from (10-36). The distribution of the number of allelic (CAG) repeats for PCOS patients and control is offered in Fig. 2.

The variance in the mean (CAG) repeats between PCOS patients (20.77 ± 0.65) $n=60$ alleles vs. control (20.87 ± 0.80) $n = 30$ alleles was not significant difference ($P=0.6267$). While when (CAG) alleles were classified in to short biallelic (≤ 20 repeats) and long biallelic (>20 repeats), according to median number of (CAG) repeats which was (20) in our population, a significant differences ($P < 0.01$) in the distribution of CAG total alleles (2n) and biallelic mean between PCOS patients and controls were found (Table 3). In women with PCOS, short biallelic was higher frequency 36/60 (60%) than long biallelic 24/60 (40%) while was opposing in controls when long biallelic was higher frequency than short biallelic.

Associations of CAG repeat polymorphism and androgen effect in PCOS patients

Our result displayed no significant differences in hormonal parameters in PCOS patients with short and long alleles (Table 4) excepting serum-Testosterone (T)

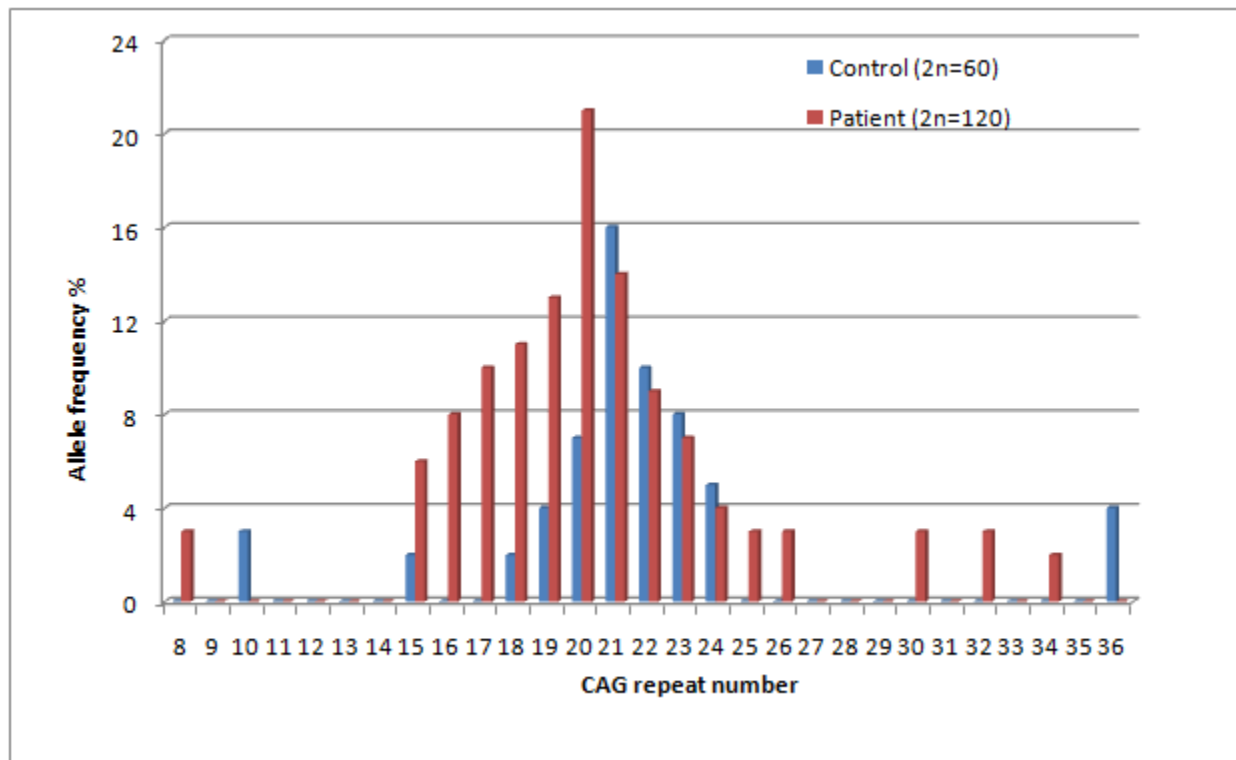


Fig. 2 : Distribution of the (CAG) repeats alleles in patients with (PCOS and controls).

Table 1 : Clinical and hormonal trait in the PCOS patients and controls.

Clinical and hormonal tests	Patient (mean $\pm$ SD)	Control (mean $\pm$ SD)	P-value
Age (years)	26.80 $\pm$ 0.61	24.43 $\pm$ 0.85	0.0258**
BMI (kg/m ²)	31.41 $\pm$ 0.73	25.74 $\pm$ 0.87	0.0001**
FSH (mIU/ml)	4.25 $\pm$ 0.16	5.60 $\pm$ 0.19	0.0001**
LH(mIU/ml)	8.22 $\pm$ 0.27	4.87 $\pm$ 0.18	0.0001**
LH/FSH	2.034 $\pm$ 0.08	0.863 $\pm$ 0.02	0.0001**
T (ng/ml)	1.182 $\pm$ 0.05	0.422 $\pm$ 0.02	0.0001**
PRL(ng/ml)	32.56 $\pm$ 1.31	14.11 $\pm$ 0.56	0.0001**

(BMI) body mass index, (FSH) follicle-stimulating-hormone, (LH) luteinizing-hormone, (T) testosterone- hormone, (PRL) prolactin-hormone, ** (P<0.01) (P<0.05) Significant.

Table 2 : Distribution of PCOS patients according to Symptoms.

Symptoms	PCOS (n=60)	Estimated percentage % for patients
Hirsutism	43	71.60%
Acne	33	55%
Alopecia	38	63%
Oily skin	31	51.60%

Table 3 : Distribution of CAG repeat of PCOS patients and controls conferring to qualitative groups of CAG repeats.

Repeat size	Total number of CAG repeat alleles ^a		P-value	CAG repeat biallelic mean ^b		P-value
	PCOS (2n = 120)	Control (2n = 60)		PCOS (n = 60)	Control (n = 30)	
≤ 20	70 (58.33 %)	17 (28.33 %)	0.0026**	36 (60 %)	9 (30 %)	0.0024**
> 20	50 (41.66 %)	43 (71.66 %)		24 (40 %)	21 (70 %)	

^a $\chi^2 = 9.788^b$, $X^2 = 9.806$ ** (P<0.01) Significant.

which was higher in long (CAG) repeat > 20 alleles (P< 0.01). Pearson correlation coefficient-r analysis were presented a positive correlation between the (CAG) repeat length and (T) level in women with PCOS (R = 0.27, P < 0.05). While the clinical signs of hyper androgenism of patients were showed a higher effect in short (CAG) repeat ≤ 20 alleles than long (CAG) repeat > 20 alleles such as hirsutism (30/43), acne (25/33) alopecia (27/38) and oily skin (18/31) as termed in Table 5.

DISCUSSION

PCOS is the most popular endocrine reproductive disorder-aged women with unknown etiology, characterized by hyper androgenism (increased androgen effect) and considered to be a key feature of PCOS disease (Lizneva *et al*, 2016; Azziz *et al*, 2016). Allowing to our finding results, patients with PCOS tended to have lower mean of CAG repeats than women without PCOS with no significant differences. In concurrent with previous study (Peng *et al*, 2014), while when (CAG) alleles were divided into two groups (long biallele and

Table 4 : Clinical and hormonal parameters in PCOS women with short and long (CAG) repeats.

Parameters	(CAG) repeats mean $\pm$ SD		P-value
	≤ 20 (n=36)	> 20 (n=24)	
Age (year)	26.25 $\pm$ 0.70	27.62 $\pm$ 1.08	0.271 NS
BMI (kg/m ²)	31.42 $\pm$ 0.89	31.40 $\pm$ 1.25	0.985 NS
FSH (mIU/ml)	4.19 $\pm$ 0.21	4.34 $\pm$ 0.25	0.655 NS
LH (mIU/ml)	8.17 $\pm$ 0.36	8.29 $\pm$ 0.43	0.835 NS
LH/FSH	2.05 $\pm$ 0.11	2.00 $\pm$ 0.12	0.767 NS
T (ng/ml)	0.913 $\pm$ 0.02	1.585 $\pm$ 0.07	0.0001**
PRL (ng/ml)	32.68 $\pm$ 1.62	32.38 $\pm$ 2.24	0.910 NS

BMI- body mass index, FSH- follicle-stimulating-hormone, LH- luteinizing-hormone, T- testosterone-hormone, PRL- prolactin-hormone, ** (P<0.01) Significant, NS: Non-Significant.

short biallele) in PCOS patients showed that shorter (CAG) repeats was higher frequency than longer (CAG) repeats and a significant differences were found. Similar to our finding (Shah *et al*, 2008; Xita *et al*, 2008 and Schüring *et al*, 2012). These results were opposing to previous studies (Skrigatic *et al*, 2012) detected that the mean (CAG) repeat length in PCOS patients was higher than control while no significant differences while (Yuan *et al*, 2015) showed that there was no significant differences in bi-allelic mean (short and long repeats). We compared the alteration between hyperandrogenic and non hyperandrogenic in the two groups of PCOS patients. So, we evaluated the effect of (CAG) repeats number on different clinical and hormonal parameters as well as on androgen signs in patients. In the association of the (CAG) repeats length and (T), there is no congruous conclusion. Jääskeläinen *et al* (2005) repeated no association between the mean (CAG) biallelic repeat and (T). Also, Liu *et al* (2008) found that there is no alteration in the mean (CAG) repeat of low and high total (T). Conversely, Skrigatic *et al* (2012) verified a positive connotation between the (CAG) biallelic repeat length and (T). In our group of patients (T) level were significantly higher in long (CAG) repeat length (P=0.0001). Pearson correlation coefficient-r analysis were showed a positive relationship between the (CAG) repeat length and (T) level in PCOS women (R=0.27, P<0.05). In other hand the signs of androgen influence (hirsutism, acne, alopecia and oily skin) were showed a highest percentage of effect in short (CAG) repeat < 20 alleles than long (CAG) repeat > 20 alleles as a reverse effect than (T). Similar to our results, Van Nieuwerburgh *et al* (2008) were showed in signs of androgen influence on patients. In spite of the statement that patients with shorter (CAG) repeats were had anormal level from (T) than PCOS patients with longer (CAG) repeats. PCOS patients with shorter (CAG) repeats were higher

Table 5 : Clinical signs of hyper androgenism in PCOS patients with short and long (CAG) repeats.

Androgen Sign	(CAG) repeats	
	≤ 20	> 20
Hirsutism	30 (69.76%)	13 (30.23%)
Acne	25 (75.75%)	8 (24.24%)
Alopecia	27 (71.05%)	11 (28.94%)
Oily skin	18 (58.06%)	13 (41.93%)

significant effect and had highest percentage of hyper androgenism (hirsutism, acne, alopecia and oily skin) compared with long (CAG) repeats, which were lowest percentage and without significant difference of hyper androgenism signs (hirsutism, acne, alopecia and oily skin). These results supplies the theory that patients with short (CAG) repeats raise polycystic ovarian syndrome even without high androgen levels and with signs of hyper androgenism, while patients with longer (CAG) repeats only increase PCOS when their androgen levels have increased without any other sign of hyper androgenism. The variance of our results and other studies can be clarified by difference in sample size, heterogeneous of PCOS patients, background and personal genetic history of patients.

CONCLUSION

At the conclusion our result finding there was a relationship between AR gene (CAG) repeat polymorphism and PCOS in Iraqi women. PCOS may consequence from either high androgen concentrations or increased androgen sensitivity induced by a more active androgen receptor with lower (CAG) repeat and PCOS hereditary androgen sensitivity changes may result PCOS.

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