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Study the polymorphism in UCSNP43, 44 to exon 3 of Calpain-10 gene of Polycystic Ovary Syndrome women in Thi Qar Province

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Abstract

The correlation between polycystic ovary syndrome (PCOS) and type-2 diabetes mellitus (T2DM) was reported regarding T2DM genes, which contribute in the pathogenesis of PCOS. The current study was aimed to investigate the association of CAPN-10 gene UCSNP-43, UCSNP-44 polymorphism with PCOS.

Thirty women with PCOS and 20 healthy, which are matched in their age, were selected to test the anthropometric and biochemical profile of our samples. Nucleic acid of samples was extracted and genotype analysis was done.

The results of patients-hormonal analysis were indicated that the level of follicle stimulating hormone was low and the levels of other hormones were high in comparison to that of healthy women. Biomass (BMI) and lipid profiles of PCOS patients were higher than from these in healthy women. Haplotypes of sequenced samples were determined for each gene fragment. The same three haplotypes of SNPs-43 were identified in both PCOS and controls samples. On the other hand, high diversity of haplotypes was found from SNPs-44. The meta-analysis with fixed and random effects odds ratio (ORs) on the basis of haplotypes frequencies were presented.

Keywords: Polycystic ovarian syndrome, Gene polymorphism, Type 2 diabetes mellitus, haplotype Calpain-10

دراسة تعدد الاشكال UCSNP43,44 للأكسون 3 للجين Calpain-10 في النساء المصابات بمتلازمة تكيس المبايض في محافظة ذي قار

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الخلاصة

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ان الهدف من البحث الحالية هو دراسة ارتباط تعدد الاشكال لجين CAPN 10(UCSNP-43، UCSNP-44) مع متلازمة تكيس المبايض.
الطريقة: شملت هذه الدراسة 30 امرأة تعاني من متلازمة تكيس المبايض و 20 امرأة في حالة صحية جيدة .
وقد تم عزل الحمض النووي الريبوزي منقوص الأكسجين (DNA)، والتحليل الوراثي لجميع السكان الدراسة باستخدام PCR-SNP.
النتائج: أظهرت نتائج هذه الدراسة انخفاض مستوى هرمون الولد للجريبات وارتفاع مستوى هرمون اللوتيني وارتفاع مستوى هرمون البرولاكتين، وارتفاع مستوى هرمون التستوستيرون (P <0.01) بين مجموعة مرضى متلازمة تكيس المبايض والنساء السليمات , واختلافات كبيرة في مؤشر كتلة الجسم وفي والدهون التي وجدت مستويات عالية من الكولسترول، والدهون الثلاثية في مرضى متلازمة تكيس المبايض مقارنة مع النساء السليمات ، تم تحديد النسخ المتنوعة لكل جزء الجينات من مرضى متلازمة تكيس المبايض والنساء السليمات . ولم تلاحظ أي فروق معنوية بين النساء المصابات بتكيس المبايض والنساء السليمات في الجزء SNPs-43 للجين , اما الجزء SNPs-44 لنفس الجين فقد لوحظ فروق معنوية وتنوع كبير في النسخ المتعددة بين النساء المصابات بتكيس المبايض والنساء السليمات .

Introduction

Polycystic ovary syndrome (PCOS) is a common cause of an ovulatory infertility and hirsutism resulting from a heterogeneous endocrine disorder of premenopausal women (Franks, 2008). This syndrome affects nearly (6-10%) of reproductive age women making it one of the most common endocrine disorders in this age group (Spritzer, 2003). PCOS patients also has metabolic characteristics that include prominent defects in both insulin action and β-cell function, which is characterized by insulin resistance (IR) (Ehrmann *et al* ., 1999), which is considered the main cause of pathogenesis of PCOS (Giallauria *et al* ., 2008). Hyperinsulinemia in PCOS patients leads to occur many disorders such as; hyperandrogenemia by stimulating ovarian androgen production, stimulate adrenal steroidogenesis by enhancing sensitivity to adrenocorticotrophic hormone (ACTH) that lead to menstrual disturbances, development of ovarian cysts, hirsutism and other related disorders (De Leo *et al*., 2000).

The majority of the evidence supports the finding that most women with PCOS have both insulin resistance and compensatory hyperinsulinemia. Therefore, several candidate genes involving signaling pathways (insulin secretion and action) the insulin gene (INS), the insulin receptor gene (INSR), and calpain-10 gene (CAPN10) are examined for PCOS. (Wiltgen *et al*., 2007), Calpains are calcium-dependent intracellular nonlysosomal proteases that are capable of hydrolyzing specific substrates involved in calcium-regulated signaling pathways Calpain-10 is an atypical member of the calpain family and is expressed at the mRNA and protein levels by several tissue types including pancreatic β islet cells; liver; skeletal muscle; and adipocytes (Sreenan *et al*., 2001), The gene encoding calpain-10 (CAPN10) consists of 15 exons and is located on chromosome 2 q37.3. It was shown to be related to proinsulin processing, insulin secretion and insulin resistance, CAPN10 variants are known to influence cholesterol levels blood pressure values, and insulin resistance phenotypes in the Spanish population (Horikawa *et al*., 2000).

The aim of our study was to investigate the role of CAPN10 in PCOS patients from Iraq. This hypothesis with an association study were tested using a population-based series of patients with PCOS for the frequency of two intronic polymorphisms within CAPN10 (USCSNP-43, UCSNP-44). Our results indicate that CAPN10 gene may play a role in PCOS susceptibility in humans.

Material and Methods

Thirty consecutive Polycystic Ovary Syndrome women from bent – Al Huda hospital and general population from June, to August, 2016.

The age are extended from 20 to 45 years and were identified using the 2006-Androgen Excess Society (AES) criteria: 1. hyperandrogenism, clinical or biochemical and either; 2. oligo-anovulation or 3. polycystic ovarian morphology. All subjects underwent transabdominal ultra sound in the follicular phase to evaluate ovarian morphology and any lesions in the pelvic area.

Sampling: Two milliliters of peripheral blood was collected in EDTA for DNA isolation, and 5 ml of blood in plain vial for serum preparation from all the patients and controls along with clinical data, personal history and family history.

Biochemical and hormonal findings: Serum preparation was done immediately using centrifuge, and stored in 20 C until processing of biochemical parameters (triglycerides (TG) > 1.7mmol/L (>150mg/dl), high density lipoprotein (HDL) < 1.3 mmol/L (<50 mg/dl)) , hormones (LH , FSH , prolactin , testosterone) measured by miniVidas method in both patients and controls and waist circumference (WC) >88 cm.

Extraction of DNA and Genotype Analysis: Genomic DNA was isolated from the peripheral blood of samples according to the Genomic DNA Mini Kit (Blood) , geneaid, Thailand , The extracted DNA was estimated by 0.8% agarose gel electrophoresis in 1X TAE (40 mM Tris acetate; 2 mM EDTA, pH 8.3), and quantified using a Nano drop (BioDrop µLITE, Biodrop, UK).The DNA was stored at -200 C until handled. Genotyping for the CAPN-10 UCSNP-43, UCSNP-44 polymorphism was achieved by polymerase chain reaction (PCR) with primers was designed using the NCBI primer BLAST online software (GenBank Acc. No. NG_028911.1). The designed oligonucleotide primer pair is; UCSNP-43, forward primer: 5'-CGGGTGGTGCTTATATCACG -3'; reverse primer: 5-' GGCACACATGTTCTCTGTGG -3' UCSNP-44 forward primer: 5'- CCTTGCAGGGAGACTCTGTT-3' reverse primer: 5'-TGCCCACACACAGAAACTCT- 3' synthesized from Bioneer (Korea), The PCR reaction was completed using AccuPower PCR premix (Cat # K-2012, Bioneer - Korea). The optimum annealing temperatures were determined empirically in our extracted DNA template using gradient PCR (ver. Mastercycler-nexus, Eppendorf, 22331Hamburg). The amplification was begin by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing temp for UCSNP-43 . 60.5 for 30 seconds and for UCSNP-44 . 59.5 extension at 72 C for 30 seconds, final extention at 72 C for 5 minutes, and elongation at 72°C for 30 sec, and was concluded with a final extension at 72°C for 5 min. UCSNP-43 PCR Product size 512 bp and 787 bp for UCSNP-44 , then this product, After performing PCR thermocycling, PCR products were verified by electrophoresis on a 1.5% .

DNA sequencing and sequencing Analysis Each single samples' pattern for the amplified UCSNP-43 (512bp), UCSNP-44 (787bp) fragment was purified and sequenced from both ends (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. The reference sequences of UCSNP-43 ,UCSNP-44 (GenBank acc. No. NG_028911.1) were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov). The sequencing results of the PCR products of different SSCP patterns were edited, aligned, and equated with their reference sequences using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA).

Data and statistics

Body mass index = weight/height2 (kg/m2) and Statistical analysis was done using "minitab " statistical software (MSS), USA. One-way ANOVA with post t test was achieved using "minitab" software. A p-value of < 0.01 was considered statistically significant. Signeficant , odds ratio (OR), and 95% confidence interval (CI) were done to assess the relationship between the groups.

Results

PCOS patients versus Control subjects

1- Biochemical Study :

Significant differences were found between PCOS patients and healthy women in this study criteria there were significantly higher testosterone level , prolactin level and higher LH level, lower FSH level (P<0.01) were observed in PCOS patients in comparison with the healthy women ,and the lipid profile and BMI of PCOS patients, were higher Significantly than of healthy women .Table(1)

Table (1) Comparison of demographic parameters between PCOS and healthy groups

Parameters	Groups	N.	Mean ± StDev	Significant
FSH	Patient	30	4.732 ± 1.793	0.008
	Healthy	20	6.078 ± 1.483	
LH	Patient	30	16.018 ± 4.889	0.000
	Healthy	20	4.973 ±1.134	
Prolactin	Patient	30	21.778 ± 10.253	0.000
	Healthy	20	12.350 ± 2.751	
Testosterone	Patient	30	0.95 ± 0.49	0.000
	Healthy	20	0.38 ± 0.18	
Cholesterol	Patient	30	181.31 ± 31.59	0.007
	Healthy	20	145.69 ± 12.11	
T.G.	Patient	30	142.86 ± 32.02	0.009
	Healthy	20	121.47 ± 24.63	
BMI (kg/m2)	Patient	30	85.22 ± 5.46	0.000
	Healthy	20	51.85 ± 2.82	

p≤ 0.01

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2- Genetic Study :

The single nucleotide polymorphism (SNP) analysis was made to determine genotypes of the UCSNP43,44 at the Exon 3 in Calpain 10 gene was amplified by PCR and the products was 512 -bp for UCSNP43 and 787 for UCSNP44 (Figure 1 , 2) ,and the haplotype of gene fragment were determined in PCOS patients and healthy women.

We found that the same three haplotypes of SNPs-43 were known in both PCOS and healthy women samples, but there was high diversity of haplotypes from SNPs-44. The results of meta-analysis with fixed and random effects ORs on the basis of haplotypes frequencies are showed in table (2) .

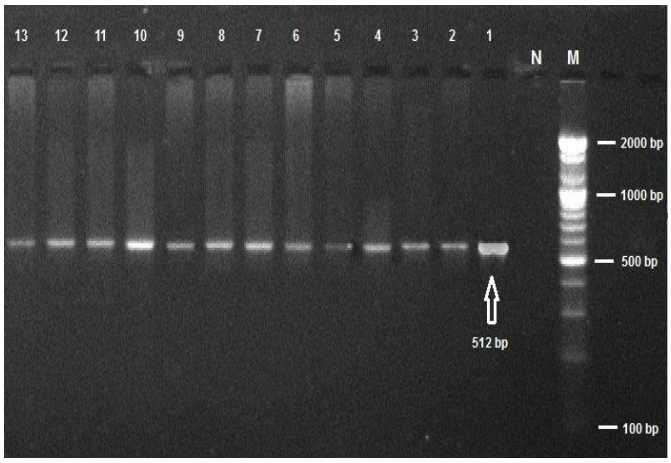

Figure (1) Size product of UCSNP43 in exon 3 of CAP N 10 gene Agarose gel (1%) electrophoresis. (M) DNA ladder

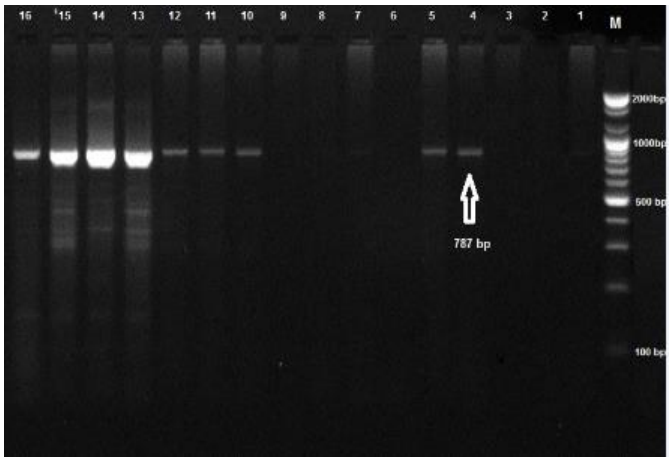

Figure (2) Size product of UCSNP44 in exon 3 of CAP N 10 gene Agarose gel (1%) electrophoresis. (M) DNA ladder

Table (2) haplotypes frequencies for UCSNP-43, UCSNP-44.

	Haplotypes	PCOS	Controls	*p*-value Odds ratio (95% CI)
SNPs-43	1	3	3	1.00 1.00 (0.041- 24.55)
	2	0	1	0.32 0.26 (0.01 - 8.52)
SNPs-44	3	1	0	0.32 3.86 (0.12- 126.74)
	4	0	1	0.32 0.259 (0.01 - 8.52)
	5	0	1	0.32 0.26 (0.01 - 8.52)
	6	0	1	0.32 0.26 (0.01 - 8.52)
	7	0	1	0.32 0.26 (0.01 - 8.52)
	8	1	0	0.32 3.86 (0.12 -126.74)
	9	1	0	0.32 3.86 (0.12 - 126.74)
	10	1	0	0.32 3.86 (0.12 - 126.74)
	11	1	0	0.32 3.86 (0.12 - 126.74)

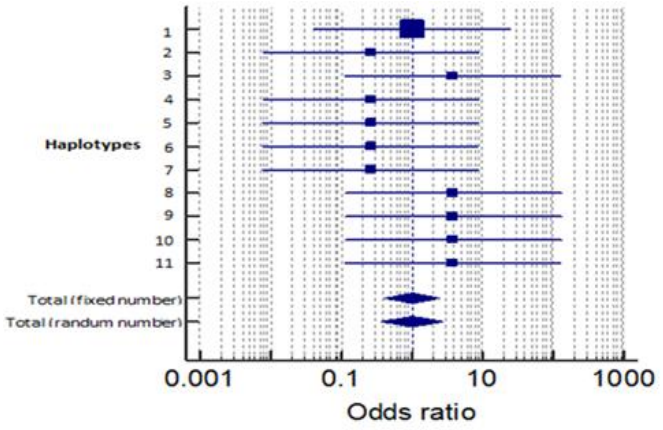

Figure (3) haplotypes frequencies for UCSNP-43 .UCSNP-44

Discussion

One of the most prevalent disorders in women at childbearing age is PCOS (Boyle *et al* ., 2012), which has a diversity of clinical and metabolic findings. There have been great discussions about whether it one disorder or multiple associated pathologic disorders (Legro *et al*., 2013). The current understanding is that

PCOS is not only a gynecological condition but a metabolic syndrome with associated disorders such as obesity, hormonal disturbance, insulin resistance and dyslipidemia (Teede, Deeks and moran ., 2010).

1- Biochemical Study

In the present study, higher BMI was, encountered, in PCOS, patients (table 1); Those PCOS women, usually have greater abdominal fat spreading (truncal abdominal fat distribution) and gluteofemoral deposition Women with PCOS usually have so-called central obesity (Visceral adiposity) or upper-body obesity, and therefore tend to have an increased waist-hip ratio and waist to thigh ratio (Yanira *et al* ., 2006). A likely explanation for the mechanism following the development of obesity in PCOS women is a combined effect of genetic factor in which certain SNP associated with obesity which leads to rising BMI in PCOS, supporting the phenotype thought of obesity is a consequence of polygenic mechanisms (Ewens *et al*., 2011). In the context to other factors like obesogenic environmental factors due to poor diet and reduced exercise where women with PCOS appear to have a significantly lower basal metabolic rate than do age- and BMI-controls (1446 kcal/day Vs 1841 kcal/day) (Barber *et al*., 2006).

Regardless of the weight factor, PCOS patients in the current research had higher levels of LH, while FSH was found to be low (table 1). A findings that was also reported by other researchers (Iwasa *et al*., 2009; Fakhoury *et al*., 2012; Saxena *et al*., 2012). The synthesis and secretion of FSH and LH are strongly dependent on the model of the GnRH stimulus, with fast frequencies boost LH and slower FSH synthesis and secretion The underlying cause of this pattern of gonadotropin secretion is linked to an accelerated GnRH pulse generator activity and heightened pituitary response to GnRH (Alnakash et al., 2007). Insulin also contributes to the excessive LH-secretion observed in women with PCOS by enhancement of GnRH pulsatile secretion or pituitary responsiveness to GnRH (Kovacs *et al*., 2002).

The data of this research showed PCOS patients have an elevated prolactin (table 1). A findings that was also reported by other researchers (Anna *et al* ., 1995 ; Gaitonde *et al* ., 2012) , In 7 % Spanish women which have hyperandrogonism have hyperprolactinemia, and half of those cases were associated with macroprolactin which pointed toward PCOS as the primary etiology of hyperandrogonism, it is ambiguous whether the relation is coincidental and independent or somehow related (Escobar-Morreale *et al* ., 2006)

That mean high prolactin levels are related with anovulation and may cause infertility, it is considered as the most incessant reason for anovulatory sterility, although spontaneous pregnancy may occur occasionally. The prevalence of hyperprolactinemia stays below 1% in normal population but may be elevated than 17% in reproductive disorders females, while PRL acts directly on the ovary which inhibits the hCG-induced follicle rupture and leads to suppression of ovulation.(Shibli-Rahhal,2011) , Hyperprolactinemia in females causes delayed puberty, hypogonadotropic hypogonadism primary or secondary amenorrhea, and galactorrhea, hyperprolactinemia in males may result in as a first signs of decreased libido or impotence, however also cause inefficient sperm production and infertility (Colao, 2004).

The data of this study showed PCOS patients have an elevated testosterone (table 1) several studies have indicated that PCOS usually produce excess androgen (Iwasa *et al*., 2007). Familial combination of biochemical abnormalities in relatives of PCOS patients based on the genetic traits (Legro *et al*., 1998b). The genetic susceptibility for a poor function of the aromatase enzyme amplifies the androgen elevation by a slow conversion of androgens to estrogens. The increase of androgen that resulting from decrease activity of Aromatase in follicles from patients with PCOS, and that the possible contribute to abnormal follicle development (Franks, Stark and Hardy, 2008).

As noticed in the results of lipid profile in this study, high levels of the cholesterol, triglyceride were observed in PCOS patients women when compared with healthy women (table 1) which was also found between obese PCOS patients women and obese healthy women control. Similar findings were reported in other studies (Villa, 2011; Cristian-Ioan, Nicolae and Dan, 2012).

The causes of dyslipidemia in PCOS patients women are again multifactorial. Insulin resistance appears to have essential role in stimulation of lipolysis and change the expression of lipoprotein lipase (LPL) and hepatic lipase Insulin resistance will increases hepatic gluconeogenesis and inhibiting glucose uptake and oxidation in skeletal muscle. The glucose in the liver converted to free fatty acids and cholesterol (Gateva and Kamenov, 2012).

High androgen levels additionally worsen the disturbances in the lipid metabolism; it may lead to

abnormalities in lipoprotein profile by working directly at the liver through the induction of hepatic lipase activity. This enzyme has a role in the catabolism of HDL particles decreases LPL activity in abdominal fat cells another reason are The obesity usually leads to a more atherogenic lipoprotein pattern suggesting reduced capacity for cholesterol removal from tissues with diminished antiatherogenic potential (Harmanci *et al*., 2012; Yasui *et al*., 2012).

2- Genetic Study

The genetic agents play the main role in the etiology of PCOS (Govind, Obhrai and Clayton, 1999). However, the heredity of PCOS remains unknown and recent studies found this disorder may be a complex of diseases which indicate that several genes with environmental factors to induce this phenotype. (Legro and Strauss, 2002; Diamanti- Kandarakis and Piperi, 2005). Different family studies proposed that PCOS has strong genetic basis (Urbanek, 2007) which found the first-degree relatives of PCOS patients have hyperandrogenemia and insulin resistance (Legro *et al*., 1998b).

Several candidate genes including those related to insulin resistance and androgen biosynthesis or action have been associated with the syndrome (Yesilada *et al*., 2006).

There are many hypotheses about the biological role of the expressed cysteine protease calpain-10 in the etiology of metabolic syndrome, obesity andT2DM which effects on proinsulin processing and on glucose-induced insulin secretion, action, and sensitivity (Sreenan ., 2001), Basing on familial aggregation of PCOS patients the guide of genetic material is heterogeneous and autosomal dominant and as previous studies association of CAPN10 and PCOS and appears an appropriate elect gene (Gonzalez *et al* ., 2002 ; Gonzalez *et al* ., 2003) produced promising, but also conflicting, data.However, many genes have been supposed but they have yet to be identify. (Amato ., 2004). This may be due to a few studies that are incapable clear of detecting a simple or moderate odds ratios (Ridderstrale, Parikh and Groop ., 2005). Furthermore, the different phenotype characters of the PCOS, including that expressed clinically during reproductive age in women, and in consequence, the absence of constant criteria of PCOS study samples which contribute to these paradoxical results (Diamanti-Kandarakis., 2005).

Our study investigated the association between the Caplain-10 gene haplotype UCSNP-43, 44 and PCOS for the first time in Iraqi population; however, there is a little number of reports on the role of calpain 10 in the pathogenesis of PCOS. Our results showed that the values of ORs from haplotypes frequencies confirm the association between SNPs-43 and PCOS (OR=0.26. p= 0.32) This results agree with (david *et al* ., 2002) , which reported PCOS patients relative to their unaffected family members to UCSNP-43 and, in the case of white subjects, relative to a set of data from unrelated controls from several Northern European populations, Both the white and African American PCOS subjects with the high-risk haplotype combination showed a 2-fold increase in risk for PCOS by calculating odds ratios for PCOS, Whereas, we revealed a high association (increase risk) between SNPs-44 and PCOS in some cases (OR=3.86, p=0.32) (fig 3.). Similar results was observed in (Vollmert *et al*., 2007), also Our findings were similar to the findings of (Gonzalez *et al* ., 2002 ; Talbott ., 2004) who they found that PCOS was associated with SNP-44 in Spanish women , In contrast, some other study have shown no significant association between PCOS and UCSNP-44 (Huang *et al* ., 2012) .

Bongardt *et al* (2007) detected an association between PCOS and the C allele of UCSNP-44, which was with the ins/del polymorphism and also associated with PCOS in Caucasians populations.

Yilmaz *et al* (2009) reported that allele distribution of Calpain 10 SNP 44 gene polymorphism was observed significantly different between the two groups. Calpain 10 SNP 44 TC genotype frequency was found to be increased in PCOS subjects compared to the control subjects. Furthermore, in an relationship study carried out among South Indian Women, Dasgupta *et al* (2012) showed a significant link between UCSNP-44 genotype CC and PCOS with highly significant odds ratio when compared to TC and TT.

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