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THE ASSOCIATION OF *HHEX* GENE POLYMORPHISM WITH THE INCIDENCE OF POLYCYSTIC OVARY SYNDROME (PCOS) IN IRAQ.

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ABSTRACT

This study was conducted to evaluate whether polymorphisms of *HHEX* gene (rs1111875) responsible for insulin secretion are associated with PCOS and PCOS with type2 diabetes mellitus (T2DM) in Iraqi women. Sixty PCOS and PCOS plus T2DM patients and 30 apparently healthy individuals were used in this study. Blood samples were obtained for DNA analysis and hormonal measurements. Genotyping of the *HHEX* gene was carried out by using PCR-RFLP method. There were no significant differences in allele frequency of the g.92703125A>G mutation (rs1111875) between the two groups of Iraqi patients women and controls. The AG heterozygous genotype percentage was significantly higher ($p<0.01$) in PCOS than control (56.66 *versus* 39.28%), and increased PCOS risk (OR= 1.328 , χ^2 =6.277). Significant association were found among AG and GG genotypes with LH and testosterone levels , in addition to F.B.S and LH : FSH ratio. An association was found between two genotypes of g.92703125A>G mutation (rs1111875) with the occurrence of both diabetic PCOS and PCOS in the Iraqi women population.

KEYWORDS: *HHEX* gene, PCOS, T2DM, PCR-RFLP.

INTRODUCTION

PCOS is one of the most common hormonal endocrine disorders estimated to affect 4-12% of women throughout the world in reproductive age (Garad *et al*.,2011; Bargiota and Diamanti-Kandarakis,2012). PCOS is one of the most common causes of hyperandrogenism (Polycystic Ovary Syndrome, 2013), hyperinsulinemia that cause insulin resistance, which is

also a prime indicator for woman have PCOS (Thomson *et al*., 2011) and chronic oligo-anovulation (Rotterdam consensus, revised 2004).

It was noted that there are genetic and environmental factors contribute to this hormonal imbalance, and these factors contribute to PCOS when combined with ovarian dysfunction, hypothalamic pituitary abnormalities, and obesity (Garad *et al*., 2011). Insulin resistance is a notable characteristic of PCOS(Nabag *et al*.,2014), although it's not considered as a parameter for diagnosis.

About 50–70% of women with PCOS also develop insulin resistance (Galluzzo *et al*.,2008). Insulin resistance and its accompanying hyperinsulinaemia may play a key role in the etiology of PCOS(Norman *et al*.,2007). If the patients are not treated, they will likely develop type 2 diabetes (T2DM) in the future.

T2DM is a disease characterized by impaired insulin sensitivity. Now, the hematopoietically-expressed homeobox (*HHEX*) gene and its SNP rs1111875 was identified as a promising candidate for type 2 diabetes (Kifagi *et al*.,2011). *HHEX* has recently been implicated in pancreas development and the regulation of insulin secretion (Ragvina *et al*.,2010). Studies found that risk allele has been associated with decreased pancreatic β-cell function(Rosengren *et al*.,2012). The current study conducted to investigate whether g.92703125A>G mutation (rs1111875) polymorphisms of *HHEX* gene associate with incidence of PCOS in Iraqi patients.

MATERIALS AND METHODS

This study was carried out in the Institute of Genetic Engineering & Biotechnology for post Graduate Studies - University of Baghdad during the period from May to October 2013. The study was approved by the Ethical Committee of the Institute of Genetic Engineering & Biotechnology – Baghdad University, and written consent was obtained from patients to participate in the study. Sixty women were studied comprised of 30 apparently healthy women served as control group, 30 women whom diagnosed as having PCOS and 30 diabetic PCOS. The patients groups had history of oligomenorrhea and evidence of hyperandrogenism (on clinical examination or by documented elevated testosterone levels). Women with any other cause of oligomenorrhea and hyperandrogenism were excluded. To ensure that phenotype was definitely PCOS, the women who had PCOS on ultrasonography was only enrolled. Blood sampling were collected during the follicular phase(day3or4)

were divided into two portions: First portion: EDTA tubes for DNA isolation (Molecular genetic studies). Second portion: the serum which subjected to measure LH, FSH, testosterone hormones, fasting blood sugar (FBS) and BMI. The hormonal and biochemical traits were given in Table 1 for patients with PCOS, PCOS+T2DM and controls.

Genetic Analysis

Total genomic DNA isolated from the whole fresh blood collected in EDTA anticoagulant tubes using genomic DNA purification kit (Geneaid, Biotech Ltd.) as protocol supplied. *HHEX* gene was amplified by polymerase chain reaction (PCR), using forward primer: 5′-catcataacttctcactcccttcc-3, reverse primer: 5-' gctgcttatggaaactgcattact-3'. A total volume of 20 µl containing genomic DNA 100 *ng* was used as template in the reaction mixture, 10 µmol of each primer added to prepared Green *Accu Power ProFi Taq* PCR pre mix (Bioneer, Korea). Cycling program was denaturation at 94 ºC for 5 minutes, 30 cycles with 94 ºC for 1minute, 60 ºC for 1minute, 72 ºC for 1minute, and 72 ºC for 10 min. PCR product (161 bp) digested with *Xba I* (Biobasic, USA) for 4 hours at 37ºC to determine the genotypes of SNP rs1111875 A/G polymorphism at the intron 3 in the *HHEX* gene. Digested DNA fragments were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV trans-illuminator Vilberlourmat (Japan). Single 161 bp band indicates homozygosity for the GG genotype. Two fragments, 111 bp and 50 bp bands, indicates homozygosity for the AA genotype. Three fragments, 161, 111, and 50 bp bands, indicates heterozygosity for the AG genotype (Figure 1).

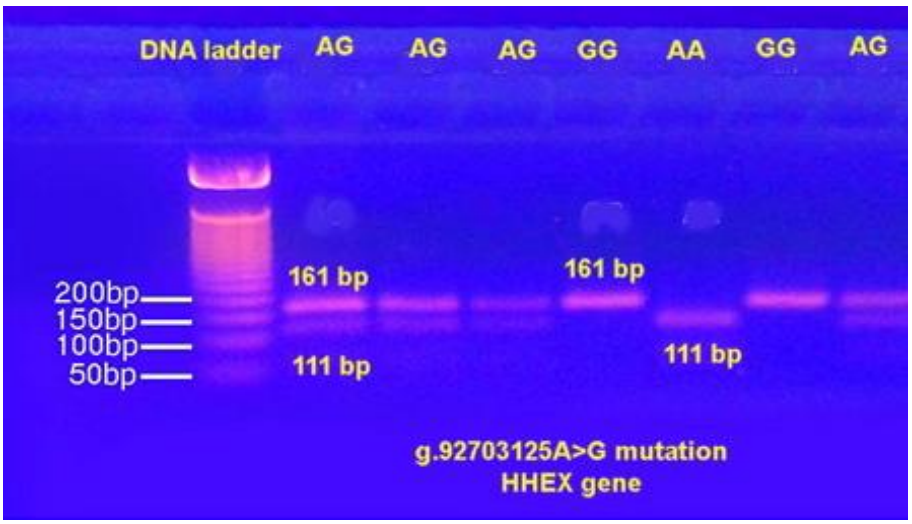

Figure1. PCR-RFLP analysis of the polymorphism of g.92703125A>G mutation inUTR region of *HHEX* gene. Agarose gel (2%) electrophoresis after *RsaI* digestion of PCR product.

Statistical Analysis

The Statistical Analysis System(SAS) (2012) was used to analyze the effects of different parameters studied. Least significant difference –LSD and Duncan (1955) multiple range test was used to analyze the difference among study groups for each parameter. Chi-square was used to exam the significance of genotype , alleles frequency in the study groups. Odd ratio was used to confirm the risk.

RESULTS

Both allele and genotype frequencies of g.92703125A>G mutation in *HHEX* gene are presented in Table 1. No significant differences were noted among study groups as related with the frequency of both A and G alleles. Also, there were no significant differences in AA genotype frequency among all study groups. The frequency of AG genotype was significantly (p<0.05) higher in diabetic PCOS patients compared with control subjects (48.3 *versus* 39.3 %, respectively; *OR*=0.088; X^2=4.055, p<0.05). In addition, the frequency of AG genotype was significantly (p<0.05) higher in PCOS than diabetic PCOS patients (56.7 *versus* 48.3%, respectively; *OR*=0.529; X^2=3.988, p<0.05). Highly significant (p<0.01) increase in AG genotype frequency in PCOS patients when compared with apparently healthy subjects (56.7 *versus* 39.3%, respectively; *OR*=1.328; X^2=6.277, p<0.01). These results indicate that AG genotype represent a risk factor for PCOS incidence in Iraqi women with PCOS. The frequency of GG genotype was significantly (p<0.05) lower in PCOS patients compared with apparently healthy subjects (26.6 *versus* 39.3%, respectively; *OR*=1.082; X^2=5.026, p<0.05). In the same trend, GG genotype frequency was significantly lower in PCOS compared with diabetic PCOS patients (26.6 *versus* 34.5%,respectively; *OR*=0.641; X^2=3.981, p<0.05).

The results of genotype frequencies in the study groups for FBS, BMI and hormonal traits are presented in Table 2. No significant differences in FBS values were observed among genotype frequencies within each group.

Table 1. The allele and genotype frequencies of g.92703125A>G mutation in *HHEX* gene in the present study groups.

Groups		Alleles, n (%)		Genotypes, n (%)		
		A	G	AA	AG	GG
G1:Control		23 (41.1%)	33 (58.9%)	6 (21.4%)	11 (39.3%)	11 (39.3%)
G2: PCOS		27 (45%)	33 (55%)	5 (16.7%)	17 (56.7%)	8 (26.6%)
G3:Diabetic PCOS		24 (41.4%)	34 (58.6%)	5 (17.2%)	14 (48.3%)	10 (34.5%)
Comparisons						
G1 *versus* G2	*OR*	0.028	0.028	0.428	1.328	1.082
	X^2	1.027	1.027	0.869	6.277 **	5.026 *
G1 *versus* G3	*OR*	0.0072	0.0072	0.327	0.088	0.225
	X^2	0.0037	0.0037	0.569	4.055 *	0.866
G2 *versus* G3	*OR*	0.026	0.026	0.088	0.529	0.641
	X^2	1.019	1.019	0.259	3.988 *	3.981 *

*OR= odd ratio; χ^2=chi square. *,**means a significant difference at 0.05 and 0.01 .*

FBS values in all genotypes were significantly (p<0.05) higher in diabetic PCOS than other groups (9.6, 13.4 and 11 mmol / l for AA, AG and GG, respectively), whereas no significant differences were noted between apparently healthy subjects and PCOS patients as related with fasting blood sugar values.

As related with body mass index results, both PCOS and diabetic PCOS patients had significantly (p<0.05) higher BMI than apparently healthy subjects (34.2 and 36.5 *versus* 27.8 kg / m^2, respectively).

There were no significant differences among genotypes within each group in testosterone concentrations. In subjects with AA genotype, testosterone concentrations in diabetic PCOS were significantly (p<0.05) higher than those in PCOS and control groups (0.65 *versus* 0.54 and 0.18 *ng*/ml, respectively). Moreover, in subjects with AG genotype, testosterone concentrations in PCOS and diabetic PCOS patients were significantly (p<0.05) higher than apparently healthy subjects (0.71 and 0.77 *versus* 0.20 ng / ml, respectively). In subjects with GG genotype, testosterone concentrations were significantly (p<0.05) higher in PCOS than diabetic PCOS patients and control subjects (0.85 *versus* 0.34 and 0.20, respectively).

LH concentrations were significantly (p<0.05) higher in PCOS patients with AA genotype compared with subjects with AG and GG genotypes (9.7 *versus* 6.4 and 3.8 m IU/ml, respectively). Also, in subjects with AA genotype, LH concentrations were significantly (p<0.05) higher in PCOS patients than in diabetic PCOS and apparently healthy subjects (9.7 *versus* 5 and 2.1 m IU/ml, respectively). Moreover, in subjects with AG genotype, LH

concentrations were significantly (p<0.05) higher in PCOS and diabetic PCOS patients than in apparently healthy subjects (6.4 and 5.5 *versus* 2.3 m IU/ml, respectively).

In apparently healthy subjects, FSH concentrations were significantly (p<0.05) in those with AG and GG genotypes compared with AA genotype (7 and 6.3 *versus* 4.9 m IU/ml, respectively). FSH concentrations were significantly (p<0.05) higher in PCOS patients with AA genotype compared with PCOS patients with GG genotype (6 *versus* 4.3 m IU/ml, respectively). In subjects with AA genotype, FSH concentrations were significantly (p<0.05) less in diabetic PCOS patients compared with apparently healthy subjects and PCOS patients (4.1 *versus* 4.9 and 6 m IU/ml, respectively).

LH:FSH ratio was significantly (p<0.05) higher in PCOS patients with AA genotype compared with AG and GG genotypes (1.58 versus 1.26 and 0.90, respectively). In subjects with AA and AG genotypes, LH:FSH ratio was significantly (p<0.05) higher in both PCOS and diabetic PCOS patients compared with apparently healthy subjects (1.58 and 1.27 versus 0.43 for AA genotype and 1.26 and 1 versus 0.33 for AG genotype, respectively).

Table 2.The relationship of *HHEX* gene polymorphism with hormonal concentrations in study groups (Mean ± SE).

Parameters	Groups	Genotype			*LSD value*
		AA	AG	GG	
FBS (mmol / l)	Control	4.7 ± 0.16 ^{Ba}	4.8 ± 0.15 ^{Ba}	4.3 ± 0.12 ^{Ba}	0.438
	PCOS	4.7 ± 0.14 ^{Ba}	4.8 ± 0.20 ^{Ba}	5.1 ± 0.24 ^{Ba}	0.894
	Diabetic PCOS	9.6 ± 1.57 ^{Aa}	13.4 ± 1.81 ^{Aa}	11.0 ± 1.47 ^{Aa}	5.783
	LSD value	1.834 *	3.501 *	5.162 *	
BMI (kg / m^2)	Control	27.8±2.65 ^{Ba}	30.4 ± 1.37 ^{Aa}	28.4 ± 1.51 ^{Aa}	6.275
	PCOS	34.2 ±3.71 ^{Aa}	32.2± 1.31 ^{Aa}	34.8 ± 8.01 ^{Aa}	12.55
	Diabetic PCOS	36.5 ±6.28 ^{Aa}	31.6 ± 1.63 ^{Aa}	35.8 ± 2.33 ^{Aa}	9.115
	LSD value	5.271 *	9.843	19.693	
Testosterone (ng / ml)	Control	0.18±0.04 ^{Ba}	0.20 ± 0.05 ^{Ba}	0.20 ± 0.04 ^{Ba}	0.143
	PCOS	0.54±0.07 ^{ABa}	0.71 ± 0.09 ^{Aa}	0.85 ± 0.25 ^{Aa}	0.502
	Diabetic PCOS	0.65±0.08 ^{Aa}	0.77 ± 0.13 ^{Aa}	0.39 ± 0.09 ^{Ba}	0.439
	LSD value	0.412 *	0.455 *	0.4092 *	
LH (m IU/ ml)	Control	2.1 ± 0.39 ^{Ba}	2.3 ± 0.41 ^{Ba}	2.2 ± 0.20 ^{Aa}	1.147
	PCOS	9.7 ± 0.99 ^{Aa}	6.4 ± 0.84 ^{Aab}	3.8 ± 0.67 ^{Ab}	3.392 *
	Diabetic PCOS	5.0 ± 0.53 ^{Ba}	5.5 ± 1.20 ^{Aa}	4.0 ± 1.66 ^{Aa}	4.612
	LSD value	3.339 *	3.617 *	2.792	
FSH (m IU / ml)	Control	4.9 ± 0.69 ^{Ab}	7.0 ± 0.77 ^{Aa}	6.3 ± 0.42 ^{Aa}	1.419 *
	PCOS	6.0 ± 0.29 ^{Aa}	5.1 ± 0.25 ^{Aab}	4.3 ± 0.39 ^{Ab}	1.255 *
	Diabetic PCOS	4.1 ± 0.59 ^{Ba}	5.7 ± 1.60 ^{Aa}	5.0 ± 0.70 ^{Aa}	3.502
	LSD value	1.135 *	3.539	5.522	
LH : FSH	Control	0.43±0.08 ^{Ba}	0.33 ± 0.04 ^{Ba}	0.36 ± 0.04 ^{Aa}	0.148
	PCOS	1.58±0.18 ^{Aa}	1.26 ±0.14 ^{Aab}	0.90 ±0.15 ^{Ab}	0.566 *
	Diabetic PCOS	1.27±0.14 ^{Aa}	1.00 ± 0.12 ^{Aa}	0.80 ± 0.21 ^{Aa}	0.482
	LSD value	0.367 *	0.619 *	0.764	

Different capital letters refer to a significant difference among groups within each genotype. Different small letters refer to a significant difference among genotype within each group. *refer to a significant at level ($p < 0.05$).

The data of Fasting blood sugar (FBS), body mass index (BMI) and hormonal traits for control, PCOS and PCOS with T2DM groups in this study are presented in Table 3 F.B.S , BMI , LH, LH:FSH ratio, testosterone and insulin values were significantly ($p<0.05$) higher in diabetic PCOS patients compared with control subjects (11.6 *versus* 4.6 mmol/l ; 34.0 *versus* 29.1 kg / m^2; 4.9 *versus* 2.2 mIU / ml ; 0.96 *versus* 0.37 ; 0.64 *versus* 0.20 ng / ml ; 29.4 *versus* 21.7 µIU / ml, respectively). LH, LH:FSH ratio, testosterone and insulin values were significantly ($p<0.05$) higher in PCOS patients compared with control subjects (6.3 *versus* 2.2 mIU / ml ; 1.2 *versus* 0.37 ; 0.71 *versus* 0.20 ng / ml ; 29.2 *versus* 21.7 µIU / ml, respectively). LH: FSH ratio was significantly ($p<0.05$) higher in PCOS than diabetic PCOS

patients (1.2 *versus* 0.96, respectively). FSH levels were unaffected in both PCOS and diabetic PCOS patients.

Table 3. FBS,BMI and hormonal traits in study groups (Mean ± SE) .

Parameters	Groups			LSD *value*
	Control	PCOS	PCOS + T2DM	
F.B.S (mmol / l)	4.6 ± 0.08 [b]	4.9 ± 0.13 [b]	11.6 ± 1.06 [a]	1.710 *
BMI (Kg\m^2)	29.1 ± 0.97 [b]	33.2 ± 2.24 [ab]	34.0 ± 1.58 [a]	4.817 *
LH (mIU / ml)	2.2 ± 0.19 [b]	6.3 ± 0.62 [a]	4.9 ± 0.84 [a]	1.733 *
FSH (mIU / ml)	6.1 ± 0.41 [a]	5.0 ± 0.20 [a]	5 .3± 0.64 [a]	1.252
LH:FSH ratio	0.37 ± 0.03 [c]	1.2 ± 0.10 [a]	0.96 ± 0.09 [b]	0.237 *
Testosterone (ng / ml)	0.20 ± 0.03 [b]	0.71± 0.08 [a]	0.64 ± 0.08 [a]	0.187 *
Insulin (µIU / ml)	21.7± 0.63 [b]	29.2 ± 2.70 [a]	29.4 ± 2.94 [a]	6.606 *

Different small letters refer to a significant difference among study groups within each parameter. * indicate to a significant difference at 0.05 level.

DISCUSSION

The results of present study are in agreement with study of Haleem *et al*.(2014) who found that out of 110 married infertile women in Baghdad, 85.4% belong to age less than 35 years and of which 33.3 % with PCOS, 41.3 % had hormonal problems and 86.8 % had problems in ovulation function and study of Mahmoud *et al*.(2014) who found that in 180 PCOS women, there were 80 overweight obese women (44.4%).

Recently, Nabag *et al*. (2014) found that Sudanese women with PCOS were obese with BMI >30, with high levels in Fasting blood glucose. Al-Mulhim *et al*. (2014) found that Saudi women with PCOS had higher testosterone but lower FSH and significantly higher LH hormone level and no differences in the blood sugar level. Study of Schmidt *et al*. (2011) on reproductive hormone levels in postmenopausal women with PCOS and found high levels in testosterone and lower FSH than controls**.** Moreover, Akbarzadeh *et al*. (2012) found a positive relationship between plasma testosterone and insulin levels with incidence of PCOS in women with normal BMI.

Results of current study found that Iraqi women with PCOS and/or PCOS+T2DM had higher testosterone levels but lower FSH than controls , and this agree with what was reported by Rotterdam ESHRE/ASRM(2004) and Schmidt *et al*. (2011). LH was significantly higher in patients groups (Rotterdam ESHRE/ASRM, 2004).

Insulin was higher as expected in the patients groups compared with the control group. Women patients with PCOS usually have insulin resistance, which leads to hyperinsulinemia. The ovaries of women seem to be particularly sensitive to high blood levels of insulin and respond by overproducing androgens like testosterone(Akbarzadeh *et al*. 2012).

Since PCOS women have a higher incidence of type 2 diabetes, in this study basically choose PCOS patient group suffer from type 2 diabetes, and high blood glucose levels (FBS) were observed in the both patients groups in this study, and this agree with Nabag *et al*. (2014).

Xu *et al*.(2010) was reported that the G allele frequency in Han PCOS was higher than in other ethnic groups. Maggie *et al*.(2008) confirmed the associations of *HHEX* gene with risk for type 2 diabetes (OR= 1.2). Cai *et al*.(2011) reported a relationship between the *HHEX* gene polymorphism and type 2 diabetes in different ethnicities including Asian, Caucasian, Indian and African American. Odd ratio for type 2 diabetes of the rs1111875 polymorphism was 1.20 (95% CI: 1.16–1.24) in Asians, and 1.16 (95% CI:1.10–1.23) in Europeans and was demonstrated that the risk allele of *HHEX* polymorphisms (rs1111875 and rs7923837) is a risk factor for developing T2DM. Chang *et al*.(2014) was demonstrated that further meta-analysis pooling 20 studies in Han Chinese confirmed the association of *HHEX* gene among 10 genetic variants with T2DM.

The results of this study showed that the frequency of mutant G allele was significantly higher than that of the normal A allele in Iraqi patients women with both PCOS and diabetic PCOS (OR= 0.061) and agree with Xu *et al*.(2010) and this results might associated with ethnicity, because the Iraqi women an Asian racial (Chang *et al*.,2014),but disagree with Maggie *et al*.(2008) and Cai *et al*.(2011) because the odd ratio of Iraqi patients women with PCOS+T2DM in current study was lower than that of Maggie *et al*.(2008) and Cai *et al*.(2011) that might related to the sample size of patients women participated in this study compared with study of Maggie *et al*.(2008) and Cai *et al*.(2011).

The results in Table 2 related with genotype frequency revealed that the AG mutant heterozygous genotype was significantly increased in PCOS among the different genotypes. Based on Cai *et al*.(2011) and Qian *et al*.(2012) one plausible hypothesis was mediated that Iraqi patients women with PCOS whom carrying G risk allele of rs1111875 SNP might have the ability developing to T2DM in future.

Results of the association in table 2 revealed that the levels of LH, Testosterone hormones and LH:FSH ratio was significantly increased in patients groups within heterozygous AG mutant genotype. The levels of testosterone was significantly increased in PCOS within homozygous GG mutant genotype. The values of FBS was significantly increased in diabetic PCOS patients group within both mutant genotypes. Therefore, the results in the present study found an association of LH, LH:FSH ratio, testosterone and FBS values with the mutants rs1111875 genotypes of *HHEX* gene in Iraqi patients women with both PCOS and diabetic PCOS. Kim *et al*.(2012) found that *HHEX* gene was not associated with PCOS, and the SNP has no significant associations with other serum hormonal and metabolic markers. Result of the FBS in this study agree with the results of Kim *et al*.(2012). Despite vast divergence in allele distributions, subgroup analyses by ethnicity showed comparable risk estimates between Asians and Caucasians for three examined polymorphisms(Li *et.al*.,2012).

CONCLUSION

No significant differences were noted in the mutant G allele among the study groups. The AG genotype was significantly increased in PCOS patients. Significant association were found in AG genotype with high levels of LH, testosterone and LH:FSH ratio in both PCOS and diabetic PCOS. The GG genotype with the high levels of testosterone in PCOS patients and F.B.S. in diabetic PCOS patients.

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