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IMPACT OF THE TRANSCRIPTION FACTOR 7-LIKE2 (TCF7L2) GENE POLYMORPHISMS ON THE RISK OF POLYCYSTIC OVARY SYNDROME IN IRAQI PATIENTS

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ABSTRACT

The relationship of TCF7L2 gene polymorphisms (rs7903146) with the incidence of PCOS and diabetic PCOS in Iraqi patients (women) was evaluated in this study. Sixty PCOS and diabetic PCOS patients and 30 apparently healthy individuals were used in this study. Blood samples were obtained for DNA analysis and hormonal measurements. Genotyping of the TCF7L2 gene was carried out by using PCR-RFLP method, then the positive samples were subjected to sequencing analysis. Significant differences were observed in allele and genotype frequencies of TCF7L2(rs7903146) polymorphisms among study groups. There was significantly elevation of T allele in diabetic PCOS Compared with control (19% *versus* 7%) and increase risk (OR 1.325%, χ^2 4.793). Both CT and TT genotypes were significantly increased in patients compared with control and increased risk (OR 1.188, χ^2 4.829 ; OR 1.424, $\chi^2$4.922, respectively) and PCOS risk with CT genotype (OR 1.035 , χ^2 7.081). An association was found among CT, TT genotypes and hormonal, biochemical traits in patients. An association was found among Two genotypes (CT and TT) with the occurrence of both diabetic PCOS and PCOS in the Iraqi patients women.

KEYWORDS: TCF7L2, PCOS, RFLP, Diabetes mellitus Type2.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathy of women within the reproductive age that affect 6-8% of women in the world (Shayya and Chang, 2010). The major clinical features of PCOS are menstrual cycle disturbance, hyperandrogenism and polycystic ovaries. These symptoms are accompanied by obesity and infertility. PCOS is responsible for about 75% of anovulatory infertility (Seli and Duleba, 2002). Therefore, women with PCOS are at increased risk for diabetes mellitus (Azziz *et al.*, 2009). Women with PCOS have a high incidence of insulin resistance (Legro *et al.*, 1999), which accompanied by compensatory hyperinsulinemia and therefore presents an increased risk for type 2 diabetes (Ehrmann *et al.*,1999). Insulin resistance affects approximately 65-80% of women with PCOS and appears to play an important pathogenic role in the hyperandrogenism of both obese and lean women with PCOS (Nestler, 1997).

In PCOS, the insulin receptor defect adversely affects the insulin-mediated glucose transport into the muscles (Dunaif, 1997; Ciaraldi *et al.*, 1992). As a result, the glucose in blood remains elevated for a longer time and further stimulates pancreatic beta cells, which in order to improve muscular glucose uptake increase their secretion of insulin in a compensatory manner (Dunaif, 1997). This compensatory hyperinsulinemia of PCOS directly stimulates testosterone production by ovarian thecal cells, promoting the hyperandrogenic state (Nestler *et al.*, 1998), that is responsible for hirsutism, acne, alopecia, higher waist to hip ratio and the detrimental effects on follicular growth, leading to the anovulatory state, menstrual disturbances, in addition to the microcystic appearance of the ovaries that characterize this syndrome (Dunaif, 1997 ; Nestler, 1997 ; Nestler *et al.*, 1998).

Numerous risk variants have been identified as candidates for conferring susceptibility to polycystic ovary syndrome. Transcription factor 7-like 2 (TCF7L2) belong to a family of TCF/lymphoid enhancer factor (LEF) transcription factors and is a key component of the Wnt signaling pathway involved in the regulation of pancreatic beta-cell proliferation, differentiation and insulin secretion (Prunier *et al.*,2004).

The gene encoding TCF7L2 spans a 215,863 bases region on chromosome 10q25.3. It was first identified as a diabetes risk conferring gene in 2006 (Grant *et al.*, 2006). The most significant genetic association with diabetes was detected for two intronic single nucleotide

polymorphisms (SNPs),rs7903146 (intron 3) and rs12255372 (intron 4) located 50 kb from each other (Grant *et al.*,2006 ; Zeggini *et al.*, 2007 ; Tabara *et al.*, 2009 ; Savic *et al.*,2011).

The aim of our study was to investigate the potential effect of the rs7903146 (C/T) polymorphism in intron 3 of the TCF7L2 gene on clinical phenotype of polycystic ovary syndrome.

MATERIALS AND METHODS

This study was conducted in Genetic Engineering & Biotechnology Institute (GEBI) - University of Baghdad during the period from May to October 2013. This study was approved by the Local Ethical Committee of the GEBI Institute, and written consent was obtained from patients to participate in the study. Sixty women were studied(30 PCOS , 30 diabetic PCOS) comprised with 30 healthy women served as the control. The patient 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 samples were collected during the follicular phase (Day3 and 4) which divided into two portions: first, EDTA tubes for DNA isolation and the second was used for obtain the serum to measure LH, FSH, testosterone hormones, in addition to fasting blood sugar (FBS) (Table 1).

Genetic analysis

Total genomic DNA isolated from the whole fresh blood collected in EDTA anticoagulant tubes using genomic DNA purification kits (Geneaid Biotech Ltd.) as protocol supplied. TCF7L2 gene fragment (Figure 1) was amplified by polymerase chain reaction (PCR), using forward primer : 5′AATTAGAGAGCTAAGCACTTTTTAGGTA-3′ , reverse primer: 5′-CAAGCTTCTCAGTCACACAGG-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 TaqPCR premix (BioNeer, Korea) according to the manufacturer's instructions. Cycling parameters were: 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 products (176-bp) digested with RsaI (BioNeer, Daejeon, Korea) for 4 hours at 37ºC. Digested DNA fragments were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV

trans-illuminator Vilberlourmat(Japan). Single 176-bp band indicates the TT genotype. Two fragments149-bp and 27-bp bands, indicates the CC genotype. Three fragments, 176, 149, and 27-bp bands, indicates heterozygosity for the CT genotype (Figure 2).

Homo sapiens transcription factor 7-like 2 (T-cell specific, HMG-box) (TCF7L2), RefSeqGene on chromosome 10

NCBI Reference Sequence: NG_012631.1

>gi|255522815:53313-53488 Homo sapiens transcription factor 7-like 2 (T-cell specific, HMG-box) (TCF7L2), RefSeqGene on chromosome 10

AATTAGAGAGCTAAGCACTTTTTAGATACTATATAATTTAATTGCCGTATGA
GGCACCCTTAGTTTTCAGACGAGAAACCACAGTTACAGGGAAGGCAAGTAA
CTTAGTCAATGTCAGATAACTAGGAAAAGGTTAGAGGGGCCCTGGACACAG
GCCTGTGTGACTGAGAAGCTTG

Figure 1. Reference sequence (NG_012631.1) of TCF7L2 gene fragment used in this study.(ncbi.nlm.nih.gov)

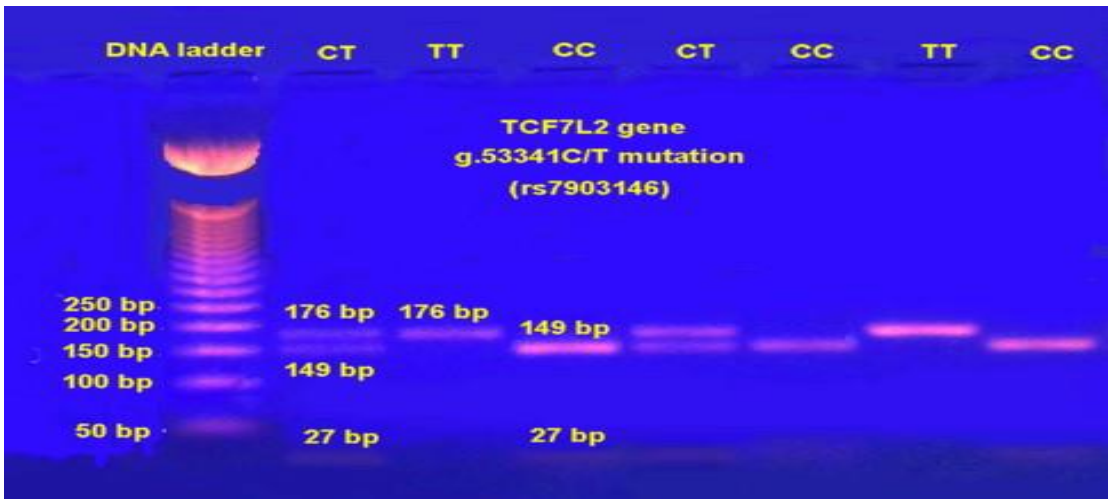

Figure 2. Restriction fragment length polymorphism analysis of the C/T polymorphism of intron 3 in the TCF7L2 gene. Agarose gel (2%) electrophoresis after RsaI digestion of the PCR. Lane 1: 50 bp DNA ladder, Lane 7: PCR product (176-bp); Lane 2 and 5: CT genotype (176-bp, 149-bp and 27-bp); Lane 4,6 and 8: CC genotype (149-bp and 27-bp), Lane 3: TT genotype (176-bp)

Statistical analysis

The Statistical Analysis System- SAS (2012) was used to test the effects of different factors in study parameters. Least significant difference –LSD and Duncan (1955) multiple

range test was used to examine the significance difference between the means of hormonal and another study traits and its association to genotypes. Chi-square was used to test the significance of genotype and alleles frequency in the three major groups. Odd ratio was used to confirm the risk factor.

RESULTS

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 1. 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 / m2; 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 1: Fasting blood sugar, body mass index and some hormonal traits in women suffer from PCOS and PCOS with T2DM (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 (Mean ± SE) within each group. *refer to a significant at level ($p < 0.05$).

The results of LH, FSH, LH/FSH, testosterone, insulin and FBS are present in table 2. The results in Table 2 observed significant differences in these parameters within the different genotypes among study groups . Based on CT mutant heterozygous genotype, the levels of LH hormone was significantly ($p < 0.05$) increased in PCOS+T2DM and PCOS patient groups in comparison with control(7.36 and 6.86 *versus* 2.24 mIU\ml, respectively). The levels of FSH hormone was decreased in patient groups within

mutant genotypes but statistically no significant in comparison with control. The values of LH:FSH ratio were significantly increased ($p<0.05$) higher in PCOS and in PCOS+T2DM patients than control(1.32 and 1.10 *versus* 0.278, respectively) within CT mutant heterozygous genotype.

Testosterone hormone levels was significantly ($p < 0.05$) elevated in PCOS and PCOS + T2DM groups within CT mutant heterozygous genotype compared with control (0.831 and 0.822 *versus* 0.304 ng\ml, respectively) and was significantly ($p < 0.05$) higher in PCOS patients than control (1.00 *versus* 0.184 ng/ml, respectively) within TT mutant homozygous genotype.

Table 2: The relationship of TCF7L2 gene polymorphism with hormonal concentrations in study groups (Mean ± SE).

Parameters	Groups	Genotype			*LSD value*
		CC	CT	TT	
LH (m IU/ ml)	Control	2.20 ± 0.15 ^{Ba}	2.24 ± 0.98 ^{Ba}	2.20 ± 0.00 ^{Aa}	2.027
	PCOS	6.14 ± 0.70 ^{Aa}	6.86 ± 1.23 ^{Aa}	1.50 ± 0.00 ^{Aa}	6.139
	Diabetic PCOS	4.06 ± 0.89^{Aab}	7.36 ± 1.92 ^{Aa}	2.53 ± 0.74 ^{Ab}	4.626 *
	LSD value	3.215 *	4.870 *	2.807	0.00
FSH (m IU / ml)	Control	5.92 ± 0.31 ^{Aa}	7.16 ± 1.97 ^{Aa}	5.30 ± 0.00 ^{Aa}	4.144
	PCOS	5.09 ± 0.27 ^{Ba}	5.10 ± 0.32 ^{Aa}	3.40 ± 0.00 ^{Aa}	2.001
	Diabetic PCOS	4.25 ± 0.53 ^{Ca}	7.01 ± 1.63 ^{Aa}	5.10 ± 1.01 ^{Aa}	3.567
	LSD value	1.216 *	3.840	6.479	0.00
LH : FSH	Control	0.384 ± 0.03 ^{Ba}	0.278 ± 0.07^{Ba}	0.410 ± 0.00^{Aa}	0.283
	PCOS	1.20 ± 0.11 ^{Aa}	1.32 ± 0.20 ^{Aa}	0.440 ± 0.00^{Aa}	0.996
	Diabetic PCOS	0.975 ± 0.12^{Aa}	1.10 ± 0.20 ^{Aa}	0.567 ± 0.20^{Aa}	0.561
	LSD value	0.254 *	0.624 *	0.894	0.00
Testosterone (ng / ml)	Control	0.184 ± 0.03^{Ba}	0.304 ± 0.11^{Ba}	0.184 ± 0.03^{Ba}	0.281
	PCOS	0.627 ± 0.06^{Aa}	0.831 ± 0.19^{Aa}	1.00 ± 0.00 ^{Aa}	0.766
	Diabetic PCOS	0.620 ± 0.08 ^{Bab}	0.822 ± 0.17^{Aa}	0.282 ± 0.14^{Bb}	0.432 *
	LSD value	0.598 *	0.580	0.629 *	0.00
Insulin (µIU\ml)	Control	20.64 ± 0.58 ^{Bb}	25.80 ± 1.15 ^{Aa}	25.00 ± 0.00 ^{Aa}	4.089 *
	PCOS	30.17 ± 3.32 ^{Aa}	27.55 ± 5.17 ^{Aa}	30.00 ± 0.00 ^{Aa}	27.477
	Diabetic PCOS	32.13 ± 2.73 ^{Aa}	29.01 ± 8.14 ^{Aa}	19.70 ± 5.14 ^{Aa}	17.124
	LSD value	6.658 *	19.880	21.922	0.00
FBS (mmol / l)	Control	4.48 ± 0.08 ^{Bab}	5.10 ± 0.21 ^{Ba}	4.20 ± 0.00 ^{Bb}	0.754 *
	PCOS	4.86 ± 0.13 ^{Ba}	4.85 ± 0.27 ^{Ba}	5.70 ± 0.00 ^{Ba}	1.248
	Diabetic PCOS	10.90 ± 1.24 ^{Aa}	12.22 ± 2.41^{Aa}	13.15 ± 2.76^{Aa}	6.372
	LSD value	1.701 *	4.746 *	9.022 *	0.00

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 values of FBS were significantly ($p<0.05$) higher in PCOS + T2DM patients within CT mutant heterozygous genotypes (12.22 mmol/l) than control (5.10 mmol/l). While in TT mutant homozygous genotype, FBS values were significantly($p<0.05$) higher in diabetic PCOS than control (13.15 *versus* 4.20 mmol/l).

Table 3 showed allele and genotype frequencies of g.53341C>T mutation (rs7903146) in this study. The frequency of C allele was significantly ($p < 0.01$) higher than T allele frequency. The frequency of T allele was 32.8% in PCOS+T2DM patients and 12.5% , 21.7% in both control and PCOS patients respectively. The distribution results showed that the frequency of CC normal homozygous genotype was significantly ($p < 0.01$) higher than mutant genotypes CT and TT respectively. The frequency of mutant CT heterozygous genotype in PCOS+T2DM was significantly ($p < 0.05$) higher than in control (31.03 *versus* 17.85%,respectively; OR=1.188 , χ^2=4.829 , $p < 0.05$). High significant ($p < 0.01$) increase in CT heterozygous genotype was observed in PCOS patients when compared with apparently healthy control (36.66 *versus*17.85%,respectively; OR= 1.035, χ^2=7.081). The frequency of mutant homozygous genotype TT was significantly ($p<0.05$) increased in PCOS+T2DM in comparison with apparently healthy control (17.2 *versus* 3.57%; OR=1.424, χ^2=4.922, $p<0.05$). These results indicate that CT genotype represent a risk factor for PCOS incidence in Iraqi women with both PCOS and PCOS+T2DM, while TT genotype represent a risk factor for PCOS incidence in Iraqi women with PCOS+T2DM.

Table 3: The allele and genotype frequencies of g.53341C>T mutation (rs7903146) in TCF7L2 gene in the present study groups.

Groups		Alleles, n (%)		Genotypes, n (%)		
		C	T	CC	CT	TT
G1:Control		49(87.5%)	7(12.5%)	22 (78.57%)	5 (17.85%)	1 (3.57%)
G2: PCOS		47(78.3%)	13(21.7%)	18 (60.0%)	11(36.66%)	1 (3.33%)
G3:Diabetic PCOS		39(67.2%)	19(32.8%)	15 (51.72%)	9 (31.03%)	5 (17.2%)
Comparisons						
G1 *versus* G2	*OR*	0.378	0.378	0.547	1.035	0.0224
	X^2	4.027 *	4.027 *	6.329 **	7.081 **	0.0277
G1 *versus* G3	*OR*	0.841	0.841	0.863	1.188	1.424
	X^2	7.255 **	7.255 **	6.052 **	4.829 *	4.922 *
G2 *versus* G3	*OR*	0.512	0.492	0.366	0.319	1.254
	X^2	4.682 *	4.682 *	4.178 *	1.044	4.509 *

*refer to a significant at level ($p < 0.05$). ** refer to a significant at level ($p < 0.01$).

DISCUSSION

It was noted that out of 180 PCOS women, 44.4 % overweight obese women and 55.6 % normal weight women (Mahmoud *et al.*, 2014). Recently, Nabag *et al.* (2014) found that Sudanese women with PCOS were obese with BMI>30, with high levels in Fasting blood glucose (FBG) that was encountered in 30% and 5% were diabetic whereas the elevation of fasting insulin levels in this study was not significant.

Al‑Mulhim *et al.* (2014) found that Saudi women with PCOS had higher LH, prolactin and testosterone levels but lower FSH Also, they found that the size of both ovaries was significantly greater in PCOS group and there were more follicles in the ovaries of the PCOS group.

In addition, Schmidt et al.(2011) found high levels in testosterone and lower FSH levels compared with controls in postmenopausal women with PCOS. However, Akbarzadeh *et al.* (2012) found a positive relationship between plasma testosterone and insulin levels with incidence of PCOS in women with normal BMI. Result of this study found that Iraqi women with PCOS,PCOS+T2DM had higher testosterone levels but lower FSH than controls , and these results agree with Schmidt *et al.* (2011) and Rotterdam ESHRE/ASRM(2004). LH and prolactin levels were significantly higher in patients groups (Rotterdam ESHRE/ASRMSponsored,2004).

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). Women with PCOS are generally overweight or obese (Al‑Mulhim *et al.* 2014) and this agree with the results of this study. Although PCOS women have a higher incidence of type 2 diabetes, but this study basically choose PCOS patient group suffered with type 2 diabetes, and high blood glucose levels (FBS) were found in both patients groups in this study, and this agree with Nabag et al. (2014). The hormonal profile of Iraqi women with PCOS was in accordance with the previous studies.

Buraczynska *et al.*(2014) found that the frequency of the T allele was significantly higher in patients with diabetic nephropathy *versus* non-diabetic renal disease. Similarly, Palizban *et al.* (2012) found that the frequency of the T allele of g.53341C>T polymorphism was significantly higher in diabetic patients (47.3%) compared with normal subjects (34.4%) in a Persian population. In addition, Cauchi et al. (2007) found that the

frequencies of the g.53341C>T alleles observed in the randomly selected healthy Brazilian volunteers were 63% for the C allele and 37% for T allele. The minor allele frequency (T allele) observed in other populations were in Northern Europeans (18-30%), other Caucasians (27-34%), Asians (3-28%), and Africans (28-35%).

In this study the percentage of the T allele frequency was a highest in the PCOS+T2DM patients among study groups. So, the result of current study that related with T allele frequency agreed with (Buraczynska *et al.*(2014) and (Palizban *et al.*(2012).The T allele frequency in current study ranged 7-19% which agree with Asian ethnic group listed in findings of Cauchi *et al.*(2007).

Case–control study of Shen, *et al.* (2014) on the relationships between TCF7L2 genetic polymorphisms and polycystic ovary syndrome risk, found evidence that TCF7L2 genetic polymorphisms may contribute to susceptibility to PCOS, especially for the g.53341C>T polymorphism among Caucasians and Asians. While, the study of Ben-Salem *et al.* (2014) found there was a weak or no contribution of TCF7L2 gene polymorphism to PCOS in Tunisian women.

Results of this study as related with allele and genotype frequency agree with results of Shen, et al. (2014) who found significant positive correlations between TCF7L2 genetic polymorphisms and an increased risk of PCOS among Caucasians and Asians but not among Africans; and diagree with the results of (Ben-Salem , *et al.* (2014) in Tunisian women with polycystic ovary syndrome. So a possible explanation for this incongruity could be differences in ethnic background, Since Iraqi women consider as Asians racial.

Results of the association of the TCF7L2 polymorphism with clinical and metabolic features in this study found a significant association between CT mutant heterozygous genotype and the high levels of LH, testosterone, F.B.S., LH\FSH in PCOS +T2DM patients group. The TT mutant homozygous genotype was significantly associated with the high levels of testosterone, in PCOS patients group. The CC normal homozygous genotype was found to be significantly associated with the increase levels of LH, insulin, F.B.S. in PCOS+T2DM patients group.

Ramos *et al.*(2013) found no differences between genotypes and haplotypes of TCF7L2 gene for clinical and metabolic variables in South Brazilian women with PCOS, while Franco

et al.(2011) found that carriers of the CT genotype for rs7903146 of TCF7L2 gene had significantly lower insulin levels 2 h after a 75-g glucose load than carriers of the CC genotype in Japanese-Brazilians . Amisi *et al.* (2013) found that body mass index was not statistically different between PCOS insulin resistance and control Congolese women. So, the result of present study agree with the results of Amisi *et al.*(2013) ,and disagree with the results of Ramos *et al.*(2013) and Franco *et al.*(2011).

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