Two novel missense mutations in exon 9 of TPO gene in Polycystic Ovary Syndrome patients with hypothyroidism

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Key words: missense mutations, TPO gene, Polycystic Ovary, hypothyroidism.

Abstract
This study was reflected on the relationship between the polycystic ovary syndrome (PCOS) and the genetic alternations in TPO gene. Fifty infertile Iraqi women with PCOS and 20 healthy women were included in this study. Blood samples were collected from the Infertility center of Al-Yarmok Teaching Hospital in Baghdad, during the period from November, 2010 to May, 2011. The age of infertile and fertile women was ranged from 16 to 45 years. The results of hormonal assay were as follows: There is significant (P ≤0.05) decrease in E2 and FSH levels in PCOS women and fertile women, There is significant (P ≤0.05) increase in LH levels in PCOS women and fertile women. There is no significant differences in Testosterone levels and the ratio of LH/FSH was ≥1.5. The molecular study was focused on the 18% of PCOS women with hypothyroidism. By sequencing for 27 samples; two novel different mutations were identified in the reading frame of the TPO gene in transcript variant of exon 9: c.1471delC (deletion C in codon 460) and c.1481delC (deletion C in codon 464). The percentage of mutations c.1481delC and c.1471delC recorded 55% and 44% of PCOS with hypothyroidism; respectively.

Introduction
Polycystic ovary syndrome (PCOS) is the commonest endocrine disorders affecting between 4% to 8% of women with reproductive age [1]. Patients with PCOS often present with clinical symptoms of hyperandrogenism and chronic anovulation [2]. PCOS patients predisposes a higher risk of developing the metabolic syndrome consisting of type 2 diabetes mellitus, dyslipidemia, hypertension, cardiovascular disease and a higher risk of endometrial and ovarian cancer [3]. Several hypothesis have been explain the pathogenesis of PCOS [4]:

An alteration in Gonadotropin - releasing hormone (GnRH) secretion leads to increase Luteinizing hormone (LH) secretion. An alteration in insulin secretion result in hyperinsulinemia and insulin resistance and Defect in androgen synthesis lead to increase in ovarian androgen production.
Typical ovarian ultrasonographic features suggested that consisted of 10-12 discrete follicles of less than 10 mm in diameter [5]. Biochemically, reveal elevated LH, low or normal FSH and the LH:FSH ratio can be increased to more than 2.5 [6]. There have been no genome wide screens of PCOS, and all reported genetic studies have used candidate gene approach in which genes are selected for analysis based on pathophysiology. Acceptable candidate gene for PCOS include those encoding proteins involved in steroid hormone biosynthesis, gonadotropins secretion or action, obesity and energy regulation, and insulin action [7].

The prevalence of hypothyroidism in women with reproductive-age defined as an abnormalities elevated thyroid stimulating hormone (TSH), hypothyroidism is associated with a broad spectrum of reproductive disorders ranging from abnormal sexual development to menstrual irregularities and infertility [8]. There is an association between disturbed thyroid function and ovarian function, infertility, and early pregnancy loss. These diseases are also described to often affected women with PCOS, therefore the relation of thyroid function and PCOS needs to be further investigated [9]. The aim of this study is to detect the hormonal disturbance and their relationship between PCOS and the genetic alternations in some segment of TPO gene.

**Materials and Methods**

**Subjects**

This study was included fifty infertile Iraqi women with PCOS and twenty healthy fertile women. The subjects were aged between 25 to 49 years. Patients and healthy were selected from women who attend the Infertility Center of Al-Yarmouk Teaching Hospital under license from Genetic Engineering and biotechnology Institute ethics committee-University of Baghdad, Al-Yarmouk hospital. All subjects were from the capital Baghdad. This study extended from November, 2010 to May, 2011. The data were collected together with the subject’s gynecological history and all their social, medical, and reproductive data according to a questionnaire forma. Venous blood sample 5 ml was collected from each woman of both PCOS and healthy control. Each blood sample was divided into two tubes, EDTA tubes for Molecular studies and clean dry plastic tube to obtain serum from clotted blood.

The criteria used for the diagnosis PCOS subjects [10]:

- Oligo and/or anovulation.
- Clinical and/or biochemical features of hyperandrogenism.
- The presence of polycystic ovary morphology.

**Hormonal assay**

Hormonal analysis was performed by using Addendum-Mini VIDAS apparatus (VIDAS)12 mode l0, 1992, BioMerieux Company, France, through an enzyme linked fluorescent assay (ELFA) technique.

**Genomic DNA isolation**

The genomic DNA isolated from the whole fresh blood collected using Wizard genomic DNA purification kits (Promega, USA). The isolation of DNA was based on steps process provided with the kit.

**TPO mutations detection**

Most mutations in TPO gene occurs in exons 8 and 9 [11], therefore the specific primer was designed to amplify specific region in exon 9.

**Primer design**

Primers were designed depends on nucleotide sequence of TPO gene had been done by using Primer 3 programme and NCBI blast. Primer was supplied by Cinna Gen-Iran- Company as a lyophilized product. Lyophilized primer was dissolved in a free DNase/RNase water to give a final concentration of (100 pmol/μl) (as stock solution).The sequences of this primers were:


**PCR Program:**

PCR was carried in Veriti™ thermal cycle (Applied Biosystem) using the standard cycle procedure was a 5-minute denaturation at 95 °C for one cycle, then 35 cycles of 45 seconds of denaturation at 95 °C, 45 seconds of annealing between 63°C, 60 seconds extension at 72 °C and 7 min for final extension at 72 °C. PCR products were then analyzed by sequencing.
PCR products sequencing
The PCR products 27 samples of the exon 9TPO gene primer was sending to Source BioScience Company (Nottingham, UK) for sequencing. Sequence analysis was performed by direct sequence of the PCR products, using 373A automated DNA Sequencer (Applied Biosystem).

Statistical analysis
The statistical analysis system –SAS [12] was used to the effect of difference factors in traits in this study. Least significant difference (LSD) test was used to the significant compare between means, analysis were performed probability values less than 0.05 were considered statically significant.

Results and Discussion
Hormonal profile
The results obtained from hormonal analysis revealed that the E2 and FSH have a significant lower levels (34.89±2.39pg/ml ; 6.99 ± 0.41 µIU/ml respectively ) than healthy control group (54.07 ± 7.02 pg/ml; 13.56 ±3.79 µIU/ml respectively ) in PCOS women. Other parameters such as LH and LH/FSH ratio showed no significant levels. On the other hand, testosterone levels showed elevated level (1.43±0.29) ng /ml than control group (0.60 ±0.13) ng/ml in PCOS women with no significant different, Table (1).

The current results agreed with Chang and Katiz [13] who showed the E2 hormone level in PCOS women may be low to normal. The increase in serum AMH level in PCOS women resulted from an increased production of this hormone per follicle [14]; this amount led to an inhibits of aromatase activity therefore the follicle did not produce a sufficient amount of E2 hormone [15].

The elevated of testosterone was in agreement with the study of Carmina [16] who explained the LH hypersecretion which was in positive correlation with the elevated serum of 17-hydroxyprogesterone, androstenedione and testosterone. There were additional causes of hyperandrogenism [17] as:

- An increased synthesis of testosterone precursors due to a dysregulation of theca cell androgen production.
- Hyperinsulinemia, which has been proposed as the primary event leading to hyperandrogenism.
- An increased serine phosphorylation of the insulin receptor, resulting in an activation of both ovarian and adrenal P450c17α enzymes and promoting androgen synthesis.
- Genomic variants in genes related to the regulation of androgen biosynthesis and function.

The highlevel of LH which was noticed in this study was explained by MecCartney [18] who found that the PCOS women as exhibiting an accelerated frequency and / or higher abundance of LH pulses, augmentation of LH secretory burst mass, a more disorder in LH secretion. One study reported that 75% of PCOS women have an elevated LH level, because of the elevated insulin levels that cause the abnormalities in hypothalamic-pituitary-ovarian axis that lead to PCOS [19].

The hormonal assay showed that the FSH was significantly lower in PCOS compared with healthy group, this result was in agreement with the finding by Begawy [20].The reduction levels of FSH can be explained by:

- High levels of inhibin that have been found in the PCOS women which lead to FSH reduction [21].
- Overexpression of Follistatin leading to the increase of ovarian androgen production [7].
- In PCOS the estrone level increases due to conversion of androstenedione in adipose tissue which additionally stimulates LH and inhibits FSH [22].

The results obtained revealed that there was LH/FSH >1.5 in PCOS women (1.493±0.168) compared with healthy group (0.991 ± 0.008), these results were in agreement with Arroyo et al [23] who demonstrated that >75% of PCOS women with dysregulation in gonadotropic function and explained that the normal pulsatile secretion of LH was increased by an increased frequency and amplitude of pulses, while that of
Table (1): Mean endocrine-metabolic values (± SE) of polycystic ovary syndrome and healthy women.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Endocrine-metabolic values</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCOS</td>
<td>Healthy control</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>34.89 ± 2.39</td>
<td>54.07 ± 7.02</td>
</tr>
<tr>
<td>T (ng/ml)</td>
<td>1.43 ± 0.29</td>
<td>0.60 ± 0.13</td>
</tr>
<tr>
<td>LH (μIU/ml)</td>
<td>10.04 ± 0.98</td>
<td>13.51 ± 3.88</td>
</tr>
<tr>
<td>FSH (μIU/ml)</td>
<td>6.99 ± 0.41</td>
<td>13.56 ± 3.79</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.49 ± 0.168</td>
<td>0.99 ± 0.008</td>
</tr>
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</table>


TSH, T4 and T3 levels:
Data listed in Table (2) showed that there were no significant differences in TSH, T4 and T3 levels in 82% of PCOS women, but the levels of this hormones was recorded significantly increase in 18% of PCOS with hyperthyroidism. This study focused on this ratio to determine the genetic alternations of TPO gene.

The relationship between PCOS and thyroid disorders such as hyperthyroidism has been studied well. With few exceptions, similar effect of subclinical hypothyroidism on various clinical and metabolic parameters has not received much attention in patients with PCOS[24]. Hypothyroidism aggravation PCOS by decreasing sex hormone binding globulin (SHBG) concentration, increasing conversion of androstenedione to testosterone hormone and aromatization of estradiol that lead to hyper androgenism which a whole mark of PCOS[25].

Table (2): Levels of TSH, T4 and T3 polycystic ovary syndrome women with hypothyroidism

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Mean ± SE</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCOS with hypothyroidism</td>
<td>Healthy control</td>
</tr>
<tr>
<td>TSH µIU/ml</td>
<td>19.22 ± 3.51</td>
<td>2.15±0.50</td>
</tr>
<tr>
<td>T3 n. mol/L</td>
<td>7.15 ± 1.58</td>
<td>1.97±0.16</td>
</tr>
<tr>
<td>T4 n. mol/L</td>
<td>42.54 ± 4.87</td>
<td>96.25±5.13</td>
</tr>
</tbody>
</table>

* (P ≤ 0.05), ns: non-significant. TSH: Thyroid-Stimulating Hormone, T3: Triiodothyronine, T4: Thyroxin, SE: Standard error, PCOS: Polycystic Ovary Syndrome, LSD: Least Significant Differences.

Molecular identification of TPO
The molecular part of this study was focused on the analysis of extracted DNA for PCOS with hypothyroidism patients by using specific primer PCR amplification. The genomic DNA which was extracted from blood of polycystic ovary syndrome showed a high concentration of 6μg/ml.

Polymerase chain reaction (PCR) analysis
The present study used PCR technique to detect region of the TPO gene(exon 9). The PCR results revealed that identical bands related to the TPO exon9, PCR amplified region showed a molecular weight of 307bp.

Fig. (1): PCR products of TPO gene/exon 9 on 2% agarose gel at 70 voltages for one hour.
Lane 1: DNA ladder
Lane 2,3,4,5,6,7,8,9 and 10: PCR products of the exon 9 from PCOS women with hypothyroidism.
mRNA-\textit{TPO} sequences

All the nucleotide sequences of mRNA-\textit{TPO} sequence (bases 1 to 3145) of \textit{Homo sapenis} (Human) were downloaded from Gene bank (http://www.ncbi.nih.gov/nuccore/NM_000547.4), as shown in figure (2).

<table>
<thead>
<tr>
<th>Codons</th>
<th>Alternating codons</th>
<th>Alternating codons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons</td>
<td>Alternating exons</td>
<td>Alternating exons</td>
</tr>
<tr>
<td>Variations</td>
<td>S' UTR</td>
<td>S' UTR</td>
</tr>
<tr>
<td></td>
<td>Non-synonymous coding</td>
<td>Splice site SNP</td>
</tr>
<tr>
<td>Other features</td>
<td>UTR</td>
<td></td>
</tr>
</tbody>
</table>

1381 GACGCCTGTGTACCAGAGGCGGCCAACACGCTGCACAGATCATCACCCT
1290 GGACGCCGTGTAACCCAGAGGCGGCACAGTGCACAGATCATCACCCT

\textit{KM} Y * R

1441 GAGGGGATACATCCCCAGGGATAGGGGACAGGCTGGCTGCAGGTAAGGCCTCA
1350 GAGGGGATACATCCCCAGGGATAGGGGACAGGCTGGCTGCAGGTAAGGCCTCA

\textit{MR} R Y \textit{YM}

1501 TGAAGGCTAGCTACCCAGCGCAGCCCTCTGCAACGTCTCTCAACAGCCGCTTT
1410 TGAAGGCTAGCTACCCAGCGCAGCCCTCTGCAACGTCTCTCAACAGCCGCTTT

R Y R * Y

1561 CCGCTTCAGCCATGCCAGATTCCACGCTGGCTGAGGGCTTGAAGAGGCCAGCTCCAGGA
1470 CCGCTTCAGCCATGCCAGATTCCACGCTGGCTGAGGGCTTGAAGAGGCCAGCTCCAGGA

Fig. (2): Nucleotides sequence of the healthy human cDNA-TPO gene, exon 9 sequence are uppercase letters, the amino acids sequence were showed under the nucleotides sequence. Y: C or T, R: G or A, M: A or C, KM: G or T or A or C, YR: C or Tor G or A and *: single nucleotide polymorphism

\textbf{Exon 9 (\textit{TPO} gene) alterations:}

The exon 9 of the \textit{TPO} gene was screened by sequencing from nine PCOS with hypothyroidism. All results 27 samples were directly compared with human reference mRNA-\textit{TPO} sequence (http: NCBI Reference Sequence: NM_000547.4) by software program (Chromas Pro,version:1.5) that available in web site (http://www.technelysium.com.au/chromas.html). Data present in figure (3) showed that there were two alternations in exon 9. The study have detected by sequencing two novel mutations: one of These mutations was deletion in one nucleotide: (c.1471delC) deletion Cytosine in 1471 base sequence leading to change the amino acid in position 460 (CCC) that coding for proline, another one was (c. 1481delC) deletion Cytosine in 1481 base sequence leading to change the amino acid in position 464(CAG) that coding for phenylalanine. Figure (4) showed the results of alignment of control group cDNA – TPO

>gi|253735815|ref|NM_000547.5| Homo sapiens thyroid peroxidase (TPO), transcript variant 1, mRNA, Length=3152
GENE ID: 7173 TPO | thyroid peroxidase [Homo sapiens] (Over 100 PubMed links)
Score = 361 bits (400), Expect = 1e-97
Identities = 212/218 (97%), Gaps = 2/218 (1%)
Strand=Plus/Plus

Query 33  CTGGAGCCAGGCCCTTCTCAGCAGTACGTTGGGTCCCTATGAAAGCTATGACTCCACCGCC  90
Sbjct 1463 CTGGAGCCAGGCCCTTCTCAGCAGTACGTTGGGTCCCTATGAAAGCTATGACTCCACCGCC  1522

Fig.(3): Alignment of patients cDNA – TPO gene/exon 9 with the reference sequence (http: NCBI Reference Sequence: NM_000547.4) by software program.

Exon 9 control : 403230601

>gi|253735815|ref|NM_000547.5| Homo sapiens thyroid peroxidase (TPO), transcript variant 1, mRNA, Length=3152
GENE ID: 7173 TPO | thyroid peroxidase [Homo sapiens] (Over 100 PubMed links)
Score = 410 bits (454), Expect = 3e-112
Identities = 231/234 (99%), Gaps = 0/234 (0%)
Strand=Plus/Plus

Query 27  GGANCCCTGGGAGCCAGGCCCTTCTCAGCAGTACGTTGGGTCCCTATGAAAGCTATGACTCCACCGCC  86
Sbjct 1458 GGANCCCTGGGAGCCAGGCCCTTCTCAGCAGTACGTTGGGTCCCTATGAAAGCTATGACTCCACCGCC  1517

Fig.(4): Alignment of control group cDNA – TPO gene/exon 9 with the reference sequence (http: NCBI Reference Sequence: NM_000547.4) by software program.
Effect of mutations

The effect of mutation on TPO gene was represented by effecting on thyroid peroxidase synthesis. Table (3) showed that there was shift mutation in exon 9 of TPO gene leading to the impact on phenotype of the thyroid peroxidase. The deletion and insertion mutation of non-multiple of (3 bp) lead to frameshift mutation, these mutations resulted in a completely different translation and also may be cause stop codon which truncates the protein synthesis [26]. Missense mutation which a single codon is altered so that one amino acid in protein is replaced with a different amino acid, the severity of a missense mutation depends on the nature and location of the amino acid was substituted, a silent mutation is an alteration in the DNA sequence that has no effect on the operation of the cell, in other word this mutation do not alter the phenotype [27].

Table (3): Type of effect of mutations in thyroid peroxidase gene in Iraq women with polycystic ovary syndrome

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Change in amino acid</th>
<th>Wild type</th>
<th>Mutant type</th>
<th>Effect in translation</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1471delC*</td>
<td>Deletion C in 460 site</td>
<td>CCC</td>
<td>CC-</td>
<td>Frameshift</td>
<td>4</td>
</tr>
<tr>
<td>c.1481delC*</td>
<td>Deletion C in 464 site</td>
<td>CAG</td>
<td>-AG</td>
<td>Frameshift</td>
<td>5</td>
</tr>
</tbody>
</table>

C: Cytosine, G: Guanine, T: Thymine, * novel mutation

Thyroid peroxidase is the enzyme that is responsible for the synthesis thyroid hormone (T3 and T4) and catalyze both iodination and coupling of iodotyrosine in TG [28], this enzyme is a glycosylated membrane bound hemoprotein localization the apical membrane of the thyrocyte where it plays an essential role in thyroid hormone synthesis. The exon 7, 8 and 9 encoding the catalytic heme binding domain of the TPO enzyme [11].

Ohtaki [29] reported the TPO molecular abnormalities leading to the following situations: TPO unable to bind home, TPO cannot bind with thyroglobulin or iodide as substrate and abnormal TPO lead to wrong cellular localization.

Percentage of Mutations:
Sequencing of PCR production of exon 9 showed the mutation c.1481delC recorded 55% of PCOS with hypothyroidism women and 10% of all PCOS, while c.1471delC recorded 44% of PCOS with hypothyroidism women and 8% of all PCOS women.

Conclusions
PCOS can be considered as a complex, heterogeneous metabolic syndrome triggered by the interact effect of genetic and environmental factors. There was an association between PCOS and hypothyroidism because the thyroid hormone plays an important role in reproductive function. By sequencing, many mutations (substitution, deletion and insertion) have been reported in TPO gene leading to defect in TPO which play an essential role in thyroid hormone synthesis.

References