THYROID PEROXIDASE (TPO) GENE MUTATION IN HYPOTHYROID FEMALES AND ITS ASSOCIATION WITH MENSTRUAL DISTURBANCES AND ABORTION IN THE POPULATION OF WEST BENGAL

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ABSTRACT
Thyroid Peroxidase (TPO) is the key enzyme in the biosynthesis of thyroid hormones. Mutations in TPO gene are associated with hypothyroidism. The objective of present study is to characterize the common mutations in TPO that affect the menstrual problems as well as pregnancy loss. In our study 200 hypothyroid patients (case) and their corresponding sex and age matched 200 normal individuals (control) were screened depending on their clinical manifestations. We screened a number of mutations in the exonic and intronic regions of the TPO gene in the patient population. A significant number of hypothyroid females having TPO gene mutations showed menstrual irregularity and spontaneous abortion. Irregular menstrual cycle was noticed in 78% of hypothyroid female patients. The clinical investigation also revealed the association of spontaneous abortion in 28% of the hypothyroid female. The results of present study underline a possible correlation between hypothyroidism and menstrual problems and/or pregnancy disorder.

Key words: Thyroid peroxidase, hypothyroidism, mutation, menstrual cycle, abortion.

INTRODUCTION
Thyroid Peroxidase (TPO) is the key enzyme in the biosynthesis of thyroid hormones. TPO is a membrane-bound glycoprotein (102 kDa), found as a dimer (Baker et al., 1994). Each monomer consists of 933 amino acid residues (Banga et al., 1990). TPO is located on the apical membrane surface of thyroid follicular cells and is responsible for the iodination and coupling of specific tyrosine residues in the thyroglobulin to form thyroxine (T4) and 3, 3’, 5-tri-iodothyronine (T3). The human TPO gene is located on chromosome 2p25 spanning 150 Kb of genomic DNA and contains 17 exons (Kimura et al., 1989). The genetic impairment of the TPO gene is the cause of thyroid dyshormonogenesis characterized by iodide organification defects (Bikker, 1997). Hypothyroidism is frequently responsible for irregularities of a woman’s menstrual cycle. Hypothyroidism produces menorrhagia (heavy periods), more frequent and longer periods, and dysmenorrhea (painful menstruation) (Koutras et al., 1997).

The relation of thyroid problems to miscarriage is an important issue that has attracted

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the interest of many investigators. Most studies have shown a significant positive association between the presence of thyroid auto antibodies and miscarriage rate. There are three possible explanations for the assumed association of thyroid autoimmunity with miscarriage or spontaneous abortion: 1) pregnancy loss is an epiphenomenon and not a direct effect of the thyroid auto antibodies, the presence of thyroid auto antibodies reflecting a generalized activation of the immune system. 2) delayed conception from the presence of thyroid auto antibodies; hence, when women with thyroid autoimmunity become pregnant, face a higher risk of miscarriage because of older age and 3) the pregnancy loss is secondary to a subtle deficiency in thyroid hormone concentrations or a lower capacity of the thyroid to adequately adapt to the demands of pregnancy (Lejeune et al. 1993). The present investigation is aimed to screen the mutations in the TPO gene leading to hypothyroidism and its association with menstrual disturbances and abortion in the population of West Bengal.

MATERIALS AND METHODS

Collection of study samples

200 hypothyroid patients (case) and their corresponding sex and age matched 200 normal individuals (control) were screened depending on their clinical manifestations, detailed familial history from the Institute of Post Graduate Medical Education & Research (IPGME & R), Kolkata. Peripheral blood samples were collected on the basis of prior consent given by patients/normal individuals, families and parents on behalf of minor children. The highest biomedical ethics had been enforced in this study. To the extent possible, complete pedigree samples were obtained.

Patients (Case): Subjects presenting over a period of 1 year to Endocrine OPD with hypothyroidism. Personal history includes the duration of disease, Age, Sex, Drug History, Menarche, Menstrual cycle abnormality, Abortions, Thyroid swelling, Lethargy, Weight gain/Weight loss, Cold intolerance and Constipation.

Normal (Control): Age and sex-matched subjects with no goitre, no clinical evidence of hypothyroidism and normal levels of serum T3, free T4, TSH, anti-TPO antibody.

Anti-TPO antibody Assay

An ELISA was employed for the quantitative measurement of human TPO antibodies in serum. TPO antibody values of > 60 IU/mL were considered as positive (Maskari and Alnaqdy, 2006).

TSH Assays

Quantitative sandwich immunoassay kit (Siemens, India) was used to assay serum TSH level.

T3/FT4 Assay

Serum T3 or FT4 concentrations were determined by radioimmunoassay (RIA). $^{125}$I-labeled thyroid hormone (either T$_3$ or T$_4$) competes with thyroid hormone in the serum sample and for antibody sites on the tube, in the presence of blocking agents for thyroid hormone binding proteins (Burtis and Ashwood, 1994).

Urinary Iodine Assay

The iodine in the urine was measured by a modification of the traditional colorimetric method of Sandell and Kolthoff (1937).

Genomic DNA isolation from patients

Peripheral blood samples were collected from the case and control individuals. Genomic DNA was isolated from the blood leucocytes by using QIAamp Blood Kit (QIAGEN, Hilden, Germany).

Polymerase Chain Reaction

The human TPO gene (Exon 8 to Exon 14) was amplified by PCR. PCR was performed in a thermocycler (Applied Biosystems, Model No. 9902) using specific primers for each of the exons. The reaction mixture (25 µl) contained 40-100 ng of genomic DNA, 1.5 mM MgCl$_2$, 100 µM of each dNTP, 0.4 µM of each primer, and 0.5 unit of Taq DNA polymerase (Applied Biosystems). Denaturation at 95°C for 30 seconds, annealing at 55-60°C for 30 seconds, and extension at 72°C for
30 seconds x 44 cycles were performed.

**Agarose gel electrophoresis & Gel Extraction**

The PCR product was analyzed in 2% agarose gel electrophoresis (Fig. 1) for verifying the size of the PCR product. PCR fragments were purified from agarose gel using Gel Extraction kit (Genei Bangalore).

![Figure 1. PCR amplification product of Exon10 in 2% Agarose gel electrophoresis.](image)

**DNA sequencing**

The PCR products were sequenced by using Big Dye Terminator kit v3.1 (Applied Biosystems) in ABI Prism 377 DNA Sequencer (PE Applied Biosystems).

**Alignment of DNA sequences**

Sequences Alignment between sequences of Case and Control individuals was performed to find the best-matching piecewise (local) or global alignments of two query sequences using clustalW programme.

**RESULTS**

**Sex ratio of human samples**

Among the hypothyroid patients (case) 14.5% male, 85.5% female and the sex ratio (male: female) was 1:5.9 (in world population the ratio is 1:6). Among the normal (control) individuals the ratio (male: female) was 1:5.

**TSH level**

The patient population exhibited significant increase in the level of serum TSH. In control population average serum TSH level was $2.54 \pm 0.92 \, \mu\text{IU/ml}$. But in hypothyroid patient population average serum TSH level was $35.64 \pm 5.62$. Values are expressed as Mean ± SEM (Fig. 2A). P-Value < 0.05 is considered to be statistically significant.

**Anti-TPO antibody Result**

In the present investigation both cases and controls were assayed for serum anti TPO Antibody. We found 44 patients were anti TPO antibody positive (Fig. 2B).

![Figure 2. TSH Level (A) and Anti-TPO antibody Result (B).](image)
Urinary Iodine

In our study sample we observed 93% case and 95% control individuals were within the level of 10–20 µg/dL UIE which was in the range of optimal iodine nutrition (10–20 µg/dL) (WHO, UNICEF and ICCIDD, 2001).

Clinical Results

A significant number of hypothyroid females having elevated TSH and lower FT4 showed menorrhagia (heavy periods) and dysmenorrhea (painful menstruation). Irregular menstrual cycle was noticed in 78% of hypothyroid female patients. Spontaneous abortion in 28% of the hypothyroid female was also found (Table I and Fig. 3).

Screening of mutations

In the present investigation we observed a number of nucleotide changes or mutations in different exons of TPO gene in the patient population. Only few are given in Table II & III.

DISCUSSION

Earlier studies reported that exons 8-10 encode the catalytic center of the TPO protein (hemebinding region) which is crucial for the enzymatic activity (Bikker, 1997). Exon 10 plays an important role in the structure of TPO enzyme. Thus,

Table I. Clinical spectrum of some selected hypothyroid female patients with irregular menstrual cycle and abortion cases.

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Female Patient sample ID</th>
<th>Age (years)</th>
<th>FT4 (ng/dl) (normal range = 5.10-14.10)</th>
<th>TSH (mcU/ml) (normal range = 0.27-4.20)</th>
<th>Anti TPO Ab (IU/ml) (&gt;60 = +ve)</th>
<th>Abortion</th>
<th>Menstrual cycle duration</th>
<th>Status of Menstrual cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>18</td>
<td>0.82</td>
<td>15.90</td>
<td>56.60</td>
<td>nil</td>
<td>Irregular (7-8 days)</td>
<td>Menorrhagia &amp; dysmenorrhea</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>38</td>
<td>3.10</td>
<td>34.23</td>
<td>608.90</td>
<td>spontaneous</td>
<td>Irregular (7-9 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>26</td>
<td>1.49</td>
<td>37.21</td>
<td>416.40</td>
<td>spontaneous</td>
<td>Irregular (5-6 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>36</td>
<td>1.08</td>
<td>10.50</td>
<td>54.00</td>
<td>nil</td>
<td>Irregular (1-2 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>5</td>
<td>109</td>
<td>29</td>
<td>1.50</td>
<td>35.22</td>
<td>104.40</td>
<td>spontaneous</td>
<td>Irregular (6 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>6</td>
<td>111</td>
<td>36</td>
<td>1.82</td>
<td>35.75</td>
<td>19.50</td>
<td>spontaneous</td>
<td>Irregular (8-10 days)</td>
<td>menorrhagia &amp; dysmenorrhea</td>
</tr>
<tr>
<td>7</td>
<td>113</td>
<td>33</td>
<td>2.30</td>
<td>87.46</td>
<td>57.30</td>
<td>nil</td>
<td>Irregular (1-2 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>8</td>
<td>120</td>
<td>28</td>
<td>2.12</td>
<td>8.23</td>
<td>226.00</td>
<td>spontaneous</td>
<td>Irregular (9 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>9</td>
<td>144</td>
<td>45</td>
<td>1.50</td>
<td>14.70</td>
<td>416.40</td>
<td>spontaneous</td>
<td>Irregular (10-15 days)</td>
<td>menorrhagia &amp; dysmenorrhea</td>
</tr>
<tr>
<td>10</td>
<td>147</td>
<td>21</td>
<td>3.12</td>
<td>10.40</td>
<td>335.70</td>
<td>spontaneous</td>
<td>Irregular (1-2 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>11</td>
<td>148</td>
<td>18</td>
<td>0.28</td>
<td>14.00</td>
<td>73.70</td>
<td>nil</td>
<td>Irregular (7-8 days)</td>
<td>menorrhagia</td>
</tr>
<tr>
<td>12</td>
<td>153</td>
<td>32</td>
<td>1.56</td>
<td>25.49</td>
<td>250.30</td>
<td>spontaneous</td>
<td>Irregular (1-2 days)</td>
<td>menorrhagia</td>
</tr>
</tbody>
</table>
Figure 3. Percentage of patients with spontaneous abortion (A) and irregular menstrual cycle (B).

Table II. Mutation in TPO gene coding region of hypothyroid females who showed spontaneous abortion and irregular menstrual cycle

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Exon</th>
<th>Codon position</th>
<th>Codon change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>144</td>
<td>10</td>
<td>603</td>
<td>CTG&gt;CGG</td>
<td>Leu&gt;Arg</td>
</tr>
<tr>
<td>147</td>
<td>10</td>
<td>620</td>
<td>GAC&gt;AAC</td>
<td>Asp&gt;Asn</td>
</tr>
<tr>
<td>148</td>
<td>10</td>
<td>620</td>
<td>GAC&gt;AAC</td>
<td>Asp&gt;Asn</td>
</tr>
<tr>
<td>153</td>
<td>10</td>
<td>626</td>
<td>TAC&gt;TTC</td>
<td>Tyr&gt;Phe</td>
</tr>
<tr>
<td>109</td>
<td>10</td>
<td>641</td>
<td>GAA&gt;AAA</td>
<td>Glu&gt;Lys</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
<td>646</td>
<td>AGG&gt;GGA</td>
<td>Arg&gt;Gly</td>
</tr>
</tbody>
</table>

Figure 4A. Nucleotide changes in normal and patient (Sample- 144, Exon 10, Codon -603, CTG>CGG)
Association of TPO mutation with menstrual problems and spontaneous abortion

Figure 4B. Nucleotide changes in normal and patient (Sample-109, Exon-10, Codon-641, GAA>AAA)

Table III. Intronic nucleotide polymorphism(s) in Thyroid Peroxidase (TPO) gene in hypothyroid female patients

<table>
<thead>
<tr>
<th>Sample Id</th>
<th>Intron</th>
<th>Gene Position</th>
<th>Nucleotide Change</th>
<th>Database Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>10</td>
<td>79644</td>
<td>G&gt;A</td>
<td>1497831</td>
</tr>
<tr>
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<td>79644</td>
<td>G&gt;A</td>
<td>1497831</td>
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<td>10</td>
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<td>G&gt;A</td>
<td>1497831</td>
</tr>
<tr>
<td>144</td>
<td>10</td>
<td>79644</td>
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<td>153</td>
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<td>79644</td>
<td>G&gt;A</td>
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<td>147</td>
<td>10</td>
<td>79644</td>
<td>G&gt;A</td>
<td>1497831</td>
</tr>
</tbody>
</table>

Figure 5. G>A Nucleotide changes in normal and patient
mutations in these regions are expected to have major effects on TPO activity resulting in severe hypothyroidism. In many cases altered amino acids have opposite properties. For example in case of Leu 603 Arg, non polar, hydrophobic leucine (Leu) amino acid is replaced by polar, hydrophilic, positive charged Arginine (Arg) amino acid. In Glu 641 Lys, negative charged amino acid is replaced by positive charged amino acid. Therefore, mutations in this amino acid may cause change in the activity of TPO enzyme which ultimately may reduce the functional efficacy of the enzyme. In the present investigation, we observed a number of nucleotide changes in the intronic region of the TPO gene in the patient population. Though this is not clear whether the effect of these intronic mutations in the function of TPO gene, it may be hypothesized that the intronic mutations may have some regulatory role in the modulation of protein behavior. A mutation (usually a base substitution) within an intron may result in an alternative splice site that competes with the normal splice sites during RNA processing. It is important to note that urinary iodine levels of both cases and controls are in normal range. Therefore, urinary iodine levels indicate that iodine deficiency is currently not a public health problem in our study population.

Irregular menstrual cycle was noticed in 78% of hypothyroid female patients. The clinical investigation also observed the association of spontaneous abortion in 28% of the hypothyroid female. There are several convincing data which suggest linkages between menstruation and thyroid disease (Koutras et al. 1997). Hypothyroidism is associated with menorrhagia, dysmenorrheal, infertility, premenstrual syndrome, polymenorrhea and menorrhagia (Joshi et al. 1993). TSH which is markedly increased in hypothyroidism has a small follicle stimulating hormone (FSH) and luteinizing hormone (LH) like effects. On the other hand, thyroid auto antibodies or anti TPO antibodies are associated with pregnancy loss (Poppe et al. 2003; Bussen and Steck, 1995). Hence, women with thyroid autoimmunity when become pregnant carry a higher risk of miscarriage (Lejeune et al. 1993). The results of present study underline a possible correlation between hypothyroidism and menstrual problems and/or pregnancy disorder.

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