Association study of calpain-10 Gene Polymorphism in Patients with Polycystic Ovarian Syndrome

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Abstract
Polycystic Ovarian syndrome (PCOS) is a common endocrine disorder with a global prevalence of 5-10% among women of reproductive age. PCOS is characterized by chronic anovulation, polycystic ovaries, hyperandrogenism, hirsutism, overweight, insulin resistance, and infertility. It is well-known that PCOS is a complex trait similar to type-2 diabetes in which both genetic and environmental factors play a crucial role in the pathogenesis of the disease. A couple of type-2 diabetes susceptibility genes including those for insulin secretion and action such as Calpain-10 have shown considerable contribution to genetic predisposition to PCOS. Since Calpain-10 gene seems to be a strong candidate gene for PCOS, we aimed to investigate the role of Calpain-10 gene polymorphism UCSNP-44 (T/C) located in a transcription enhancer element of the gene in disease susceptibility. We carried out a cross-sectional case-control study. Using simple random sampling, ninety healthy women were selected. All ninety patients fulfilled the 2003 Rotterdam criteria of PCOS. The subjects were genotyped for Calpain-10 gene polymorphism UCSNP-44 (T/C) using PCR-RFLP. Differences in genotype distributions between case and control subjects were examined via the chi-square test. Although PCOS showed a high prevalence among women in Bandar Abbas, Calpain-10 gene polymorphism UCSNP-44 (T/C) UCSNP44 did not influence the susceptibility to PCOS.

Keywords: Polymorphism; Calpain-10 Gene; Polycystic Ovarian Syndrome

Introduction
Polycystic ovarian syndrome (PCOS) was firstl reported as Stein & Levental syndrome in 1935 (1). PCOS, as a complex disease with both multiple genetic components and environment factors with various clinical manifestations and genetic heterogeneity, is a common endocrine disorder among women of reproductive age, affecting 5–10% of the population (2). The genetic basis of PCOS as a complex trait is not well known (3). PCOS is a heterogeneous endocrine disorder characterized by chronic anovulation, hyperandrogenism, overweight, insulin resistance and increased risk of diabetes mellitus type II (4). Oligomenorrhea or menstrual disorder is the primary main symptom of PCOS observed in 85-90% of the cases and 30-40% of the women with amenorrhea will develop PCOS in the future (5). Hirsutism is one the main symptoms of PCOS occurring in 17-83% of the women with PCOS (5, 6). Furthermore, infertility is another common symptom of PCOS with a prevalence of 35-94% (5-7).

PCOS is usually found in the young age and is a common complex phenotype with an undetermined definition (8). Although the clinical presentations of PCOS are detected in the young age, its establishment might occur atchildhood, even during the fetal life (9).
Of note, beginning of clinical signs before puberty is due to the increased LH concentration, leading to hyperandrogenism and ovulation disorders (10). These patients suffer from infertility in the reproductive age, an increase risk of cardiovascular and thrombophlebitis disorders (11-13), breast, ovary, uterus (14) and endometrial cancer with delayed menopause (15-18) and metabolic abnormalities such as dyslipidemia in the future (19-22).

The genetic basis of PCOS was reported by Cooper and his collagenous in 1968 (23). Although the etiology of PCOS is unknown yet, genetic factors play the leading role in its pathogenesis. In the recent decades, a number of studies were performed on the genetic basis and human reproductive hormones as well as chronic inflammation (24). Prevalence rates of PCOS as high as 5-6 times more in first degree relatives (25) and the higher prevalence in dizygotic twines compared with monozygotic twins have confirmed the genetic contribution to the disease (26). So far, 37 genes have been found as candidate genes including those related to sexual hormones, regulatory, insulin resistance, diabetes type II, cardiovascular disorder, insulin, and insulin receptor calpain-10 (27). All these studies suggest CAPN10 gene to be a strong candidate for both T2DM and PCOS. However, it remains unclear whether and which DNA variation in CAPN10 gene truly affects the disease risk. Human CAPN10 gene consists of 15 exons and maps to chromosome 2q37.3. CAPN10 gene plays a fundamental role in proinsulin processing, insulin secretion, and action. Strong evidence-based results suggest that UCSNP-44 may contribute to T2DM and PCOS susceptibility.

Considering the high prevalence of PCOS in Iran and the small number of studies evaluating candidate genes and PCOS association, the present study was designed to investigate the association between Captain-10 gene polymorphism UCSNP-44 (T/C) and PCOS in Bandar Abbas.

Material and Methods

The study was approved by the ethical review committee and each subject read and signed the informed consent form. The present study was conducted in accordance with ethical standards of Helsinki Declaration. In the present cross sectional case-control study, 90 women aged 18-40 years who were referred to infertility clinic of Bandar Abbas from 2012 to 2013 were assessed. Sampling was performed by the simple consecutive method. The control subjects were lean healthy female volunteers and consecutive overweight patients attending the clinic for the treatment of overweight and obesity, lacking signs or symptoms of hyperandrogenism, menstrual dysfunction, and infertility. A diagnosis of PCOS was confirmed by two positive signs out of three Rotterdam diagnostic criteria (irregular menarche, hyperandrogenism, clinical signs such as acne, hirsutism, alopecia, and ovarian sonography displaying two cystic ovaries carrying more than 12 cysts with diameters of 2-9 mm).

The subjects had a minimal two-year history of infertility. Other infertility causes including male factor infertility, endometriosis, and tubal factor were ruled out in the patients. Women with a history of hormonal therapy during the past three months, any forms of malignancy such as congenital adrenal hyperplasia, nonclassic defect of 21-hydroxilase enzyme, couching syndrome and hypothyroidism were excluded from the study (28). A self-designed questionnaire including information on clinical signs (hirsutism, acne, alopecia, etc.), waist/hip ratio, age, age at menarche, menarche disorders with oligomenorrhea, blood hypertension, body mass index, diabetes type II, infertility type an hormonal level of LH/FSH, TSH and prolactin was administered. Oligomenorrhea was defined as menstrual periods occurring six times or less within one year. Physical examinations for hyperandrogenism including the evaluation for hirsutism, acne, and male pattern alopecia were performed. A diagnosis of hirsutism was made using the Ferriman–Gallwey score. Comedones with many papules were considered as severe acne, and a moderate to severe decline in the hair complex and a recess in the frontal hair line were considered as alopecia.

Blood samples were collected after 12 hours (overnight) fasting. Cells were used for DNA extraction and plasma for biochemical analysis. The latter was stored at –80°C if not analyzed immediately.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using a standard protocol. To 1ml of blood, an equal volume of ice-cold C1 buffer (4X) and 12ml of ice-cold sterile water were added to lyse the RBC cell membrane. Then, the nuclei were pelleted at 1500xg for 15 minutes at 4°C. The pellet was washed again with 1X C1 buffer. After that, 12ml of nuclear lysis buffer, 0.8ml of 10% SDS and 50µl of a 20µg/µl solution of proteinase-K were added and
the pellet was resuspended by brief vortexing. After incubation at 65°C for 3 hours, the proteinaceous material was precipitated with the addition of 4ml of 6M NaCl. After a 15-minute spin at 2500 rpm, the supernatant was transferred to another tube and 2 vol. of room temperature pure ethanol was added to precipitate the DNA. The precipitated DNA was then washed twice with 70% ethanol, air-dried and dissolved in 500µl TE buffer at 65°C for 2 hours in a dry bath. Appropriate dilutions in TE buffer were made to determine the absorbance at 260 nm and 280 nm. DNA quality was assessed using the 260/280-nm ratio. DNA with a ratio between 1.5-2.0 was considered to be of good quality. Then, 5-10µl of stock DNA was electrophoresed on 0.8 % agarose gel (Sigma, St. Louis, USA) and stained with ethidium bromide (Sigma, St. Louis, USA) to determine the integrity. The DNA quantification was based on 260-nm absorbance. The stock solution of the DNA was diluted to 50 ng/µl and used in further genotyping experiments.

**Genotyping**

Human CAPN10 consists of 15 exons and maps to chromosome 2q37.3. Each 25µl PCR solution contains 18.1µl water, 2µl buffer, 1.5µl MgCl2, 0.5μl dNTPs, 0.7 µl Taq enzyme, 0.7 µl primer F (5’-CATCCATAAGCTTCCACGCCTC-3’), and 0.7µl primer R (5’-CTCATCCTCAACAAAGTCAAGG-3’) (prepared from gene fanavarvan company). One µl extracted DNA was added to each tube and centrifuged and then transferred to a thermocycler device. PCR products were loaded on 2% gel-agarose media and observed using a UV transilluminator. In the SNP genotyping process, 15µl of the solution was used as the reaction volume and accordingly, 1.5µl buffer, 1µl Hha1 enzyme to digest the products, and the rest of the PCR product were mixed in the Ependorph tube. This compound was kept at 37°C for two hours and then electrophoresed on 2.5% gel agarose (containing 0.5 µl ethidium bromide) for one hour (voltage: 95 V) and observed under ultraviolet light. The number and size of amplicons were compared with DNA ladder where the size of amplicons were 60 bp and 75 bp, respectively. An independent observer read and confirmed all the genotypes and discrepancies, if any, were resolved by repeated PCR-RFLP.

**Statistical Analysis**

To test for Hardy-Weinberg equilibrium, the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers was determined with the χ² test using the De-finetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Allele and genotype frequencies among study subjects were estimated through gene counting and analyzed by means of the χ² or Fisher's exact test and logistic regression to assess the independent effect of either allele or genotype on the presence of PCOS by the SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA) and EPIINFO version 6. Continuous data is expressed as mean ± SD. Baseline characteristics and demographic features were compared using the unpaired t- test for continuous data and the χ² test for categorical data from simple interactive statistical analysis (http://home.clara.net/sisa/twoby2.htm). A P value less than 0.05 was considered statistically significant.

**Results**

**Baseline characteristics of study groups**

In the present study, 180 PCOS patients and healthy women aged 18-40 year were assessed. Clinical, biochemical, and metabolic characteristics of the women are compared in Table 1. Obesity (waist circumference more than 85cm), hirsutism and irregular menarche (monthly cycles at intervals more than 35 days), LH/FSH >2, and BMI>25 (mean: 28.19) were significantly more in the PCOS group than the control group (P<0.05 for all). There was no significant association (P > 0.05 for all) between two groups for diabetes mellitus type II, hypertension (>140/90 mmHg) and hormonal levels (TSH > 5 and prolactin >29.2). The intra-assay and inter-assay coefficients of variation were less than 5% for all the biochemical measurements.

**Association analysis of the Caplain-10 gene polymorphism UCSNP-44 (T/C) with PCOS**

According to the findings of Table 2, in PCOS patients and control group, 95% of women had TT genotype and 2.2% had CC genotype. The frequency of the C allele in PCOS and control group was 3.3% and 2.2%, respectively. The frequency of the TC genotype in the PCOS and control group was 2.2% and 4.4%, respectively. None of the genotypes had a significant association between PCOS and the Caplain-10 gene polymorphism UCSNP-44 (T/C) (P = 0.99).

**Discussion**

PCOS is the most common endocrine disorder...
characterized by amenorrhea or oligomenorrhea, hyperandrogenism, hirsutism, obesity, insulin resistance, and increase risk of susceptibility to diabetes mellitus type II (4). Despite years of research and huge amounts of investment, the etiology of PCOS is still poorly understood. PCOS is a disorder that primarily affects 5-10% of the women of the reproductive age (7). Although the etiology of PCOS is complex and incompletely understood, genetic factors play a leading role in the pathogenesis of the disease. In recent decades, a couple of studies were carried out on PCOS pathogenesis in the context of reproductive, insulin receptors and chronic inflammation genes (29). It is most probably a heterogeneous disorder which results from the interaction of multiple genes. Nowadays, molecular genetic methods have been introduced to evaluate single genes in multifactorial disorders (23, 30). Hyperandrogenism, anovulation, and polycystic ovaries with a familial pattern confirm the genetic basis of PCOS. Insulin resistance is a essential key constituent contributing to PCOS pathogenesis. Insulin regulates metabolic homeostasis and contributes to ovarian steroidogenesis. Candidate

### Table 1: Clinical, biochemical, and metabolic characteristics of PCOS and control patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS women</th>
<th>Control women</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td>18-29 (78.9%)</td>
<td>18-29 (62.2%)</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>29-40 (21.1%)</td>
<td>29-40 (37.8%)</td>
<td></td>
</tr>
<tr>
<td>BMI&gt;25</td>
<td>57 (63.3%)</td>
<td>27 (30%)</td>
<td>0.00</td>
</tr>
<tr>
<td>Waist round&gt; 85</td>
<td>58 (64.4%)</td>
<td>37 (41.1%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>47 (52.2%)</td>
<td>24 (26.7%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Acne</td>
<td>10 (11.1%)</td>
<td>4 (4.4%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Galactorrhea</td>
<td>9 (4.4%)</td>
<td>8 (3.3%)</td>
<td>0.99</td>
</tr>
<tr>
<td>LH/FSH&gt;2 (MIU/ml)</td>
<td>16 (17.8%)</td>
<td>1 (1.1%)</td>
<td>0.00</td>
</tr>
<tr>
<td>TSH (MIU/ml)</td>
<td>2 (2.2%)</td>
<td>2 (2.2%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Prolactin (MIU/ml)</td>
<td>17 (18.9%)</td>
<td>14 (15.6%)</td>
<td>0.693</td>
</tr>
<tr>
<td>Diabetes Mellitus II</td>
<td>2 (2.2%)</td>
<td>1 (1.1%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1 (1.1%)</td>
<td>1 (1.1%)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

### Table 2: Frequency retribution of Calpain-10 among PCOS and control patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Control (%)</th>
<th>PCOS (%)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain-10</td>
<td>UCSNP44 (rs 2975760)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>95.5</td>
<td>95.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>4.4</td>
<td>2.2</td>
<td>0.99</td>
<td>1(0.242-4.128)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC+CC</td>
<td>4.4</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allelic Frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>97.7</td>
<td>96.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>2.2</td>
<td>3.3</td>
<td></td>
<td></td>
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</tbody>
</table>

Afrouz Khazanipou, Abdolazim Nejatizadeh, Seyed Mehdi Kalantar and Colleagues
gene analyses have dissected genes related to insulin secretion and action for their association with PCOS susceptibility. Moreover, insulin resistance and diabetes type II are more observed in PCOS families (31). Although a large number of genomic variants have been revealed to be associated with PCOS, no single candidate gene has emerged as a compelling biomarker so far.

Given that PCOS is associated with at least 50% increased risk of developing type 2 diabetes mellitus (T2DM), genes linked to T2DM may possibly also play a role in the PCOS pathogenesis. CAPN10 gene plays a decisive role in proinsulin processing, insulin secretion, and action. The single nucleotide polymorphism (SNP) of the Caplain-10 gene polymorphism UCSNP-44 (T/C) was selected to evaluate its contribution to T2DM and PCOS susceptibility. Our study investigated the association between the Caplain-10 gene polymorphism UCSNP-44 (T/C) and PCOS syndrome for the first time in Iran; however, the findings demonstrated no association with susceptibility to PCOS.

Our findings were similar to the findings of Haddad et al. in 2002 in Mexican people (32). It seems that Calpain-10 gene may not influence susceptibility to diabetes type II and PCOS. Others studies have reported no association between PCOS and diabetes type II among white American and American-African women (33). In 2003, Gonzalez et al. evaluated four SNPs (SNP19, SNP43, SNP44, and SNP63) from calpain-10 gene. It was found that PCOS syndrome was associated with SNP-44 in Spanish women (22, 34). In 2002, Gonzalez et al. reported that although SNP-44 was related with the risk of diabetes type II development, it did not influence susceptibility to PCOS pathogenesis (34). In another study, SNP-43 from calpain-10 gene revealed an association with diabetes type II disorder, but allele C in SNP-44 was related with diabetes type II in PCOS women (35).

In contrast, some other studies have show no significant association between PCOS and UCSNP-44 (36). Bongardt et al. detected an association between PCOS and the C allele of UCSNP-44, which was in LD with the ins/del polymorphism and also associated with T2DM in Caucasians populations (37). Yilmaz et al. 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 (38). Furthermore, in an association study carried out among South Indian Women, Dasgupta et al. showed a significant association between UCSNP-44 genotype CC and PCOS with highly significant odds ratio when compared to TC and TT (39).

The present findings are consistent with the results of some studies, while some other reports suggest a strong association between Calpain-10 gene polymorphism UCSNP-44 (T/C) and PCOS syndrome. We believe that our results should be reproduced via genome wide scans and family-based tests together with large-scale case-control studies and gene-environment interactions to make conclusive comments on the genetics of PCOS. Such an approach will also curtail the effect of the ethnic and environmental discrepancies.

Our findings in conjunction with other reports imply that contribution of other gene interactions could have clinical implications. Such interactions may give rise to different relationships between genotypic variation and phenotypic variation in different environments.

Given the considerable frequency of the patients bearing a number of risk-conferring genotype combinations, genotyping of other gene polymorphisms along with positive family history of PCOS could help to identify individuals with a high risk of susceptibility to PCOS. The findings reported here add to the accumulating data from animal models and human studies; however, further studies need to be conducted to address the molecular basis for such effects.

References


بررسی میزان فراوانی پلی مورفیسم زن های کالپاین 10 در سندرم تخمدان پلی کیستیک

چکیده
سندرم تخمدان پلی کیستیک (PCOS)، شایع‌ترین اختلال اندوکرین در زنان سنین باروری است و دارای خصوصیات هیپرآندروژنیسم و عدم تخمک‌گذاری مزمن همراه با علائمی از قبیل: تخمدان‌های پلی کیستیک، هیرسوتیسم، چاقی، مقاومت به انسولین و ناباروری می‌باشد. این سندرم یک اختلال چندژنی با اتیولوژی نامشخص می‌باشد که بر روی علائم آن مستلزم وجود هر دو عوامل ژنتیکی و محیطی است. هدف از این مطالعه بررسی فراوانی پلی مورفیسم ژن کالپائین (CAPN) در زنان مبتلا به سندرم تخمدان پلی کیستیک در شهر بندرعباس می‌باشد. این مطالعه در مرکز تحقیقاتی درمانی ناباروری، دانشگاه علوم پزشکی یزد و مرکز تحقیقات پزشکی مولکولی دانشگاه علوم پزشکی هرمزگان، بندرعباس ایران انجام شد. پس از ثبت نسبي OR و تست 

واژگان کلیدی: پلی مورفیسم؛ سندرم تخمدان پلی کیستیک؛ زن کالپاین 10.