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Role of the mutations $Trp^8 \Rightarrow Arg$ and $Ile^{15} \Rightarrow Thr$ of the human luteinizing hormone β-subunit in women with polycystic ovary syndrome

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Objective: To evaluate the clinical significance of LH in the form of a mutant β-subunit in women with polycystic ovary syndrome (PCOS).

Design: Prospective, controlled study.

Setting: University hospital.

Patient(s): Thirty healthy women and 30 women with PCOS.

Intervention(s): Clinical, ultrasonographic, and hormonal findings were used to define PCOS. Nucleotide mutations within codons 8 and 15 in the LH β-subunit gene ($Trp^8 \Rightarrow Arg$ and $Ile^{15} \Rightarrow Thr$) were analyzed with the use of polymerase chain reaction and subsequent restriction fragment length polymorphism.

Main Outcome Measure(s): Serum levels of gonadotropins, androgens, E_2, and prolactin were determined, and the results of restriction fragment length polymorphism were analyzed.

Result(s): Five women in the control group and one woman in the PCOS group were found to be affected by the LHβ gene mutations. No difference was observed in serum androgen and E_2 levels between the affected women and 25 healthy women who were homozygous for the wild-type LH. However, women whose serum LH levels were $\leq$5.1 mIU/mL had a higher risk of having mutant LH.

Conclusion(s): The frequency of LH mutations in women with PCOS is similar to that in healthy women. The presence of the variant does not cause any significant change in serum levels of androgens and E_2. (Fertil Steril® 1999;71:425–30. ©1999 by American Society for Reproductive Medicine.)

Key Words: LH, β-subunit, variant LH, mutation, PCOS

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Luteinizing hormone is a member of the glycoprotein hormone family that includes hCG, FSH, and TSH. These hormones are all α/β heterodimers with a common α-subunit and a unique β-subunit. The β-subunit confers biologic specificity. In women, LH promotes ovulation and luteinization of the ovarian follicle and enhances steroid production in the ovaries.

More sensitive immunoassay systems have revealed the existence of microheterogeneity of human LH in a large population (1, 2). Analysis of the structure of the LHβ gene in women with immunologically anomalous LH revealed two nucleotide substitutions in codons 8 (TGG $\rightarrow$ CGG) and 15 (ATC $\rightarrow$ ACC) that cause amino acid replacements from Trp^8 to Arg and from Ile^{15} to Thr (3). The same mutations also were reported from Finland, which suggests that this variant LH represents a universal polymorphism (4). Worldwide carrier frequency of this common genetic variant recently has been analyzed with two immunofluorometric assays using monoclonal antibodies and is reported to vary from 7% in U.S. Hispanics to 42% in Lapps of northern Finland (5). It was found to have higher in vitro bioactivity and a shorter half-life than the wild-type LH (4).

Abnormal secretion of LH may cause anovulation and luteal insufficiency, leading to menstrual irregularities, polycystic ovary syndrome (PCOS), and recurrent miscarriage. Although boys with the mutated genotype have been reported to be shorter and to have smaller

testicular volumes, the clinical significance of the variant LH remains unclear in women (3, 4, 6, 7). The role of this LH variant in women with PCOS also is unclear (8, 9).

The purpose of this study was to evaluate the clinical significance of the LHβ gene mutations $Trp^8 \Rightarrow Arg^8$ and $Ile^{15} \Rightarrow Thr^{15}$, both of which have ethnic and geographic differences, in women with PCOS.

MATERIALS AND METHODS

Subjects

The presence of the mutations $Trp^8 \Rightarrow Arg^8$ and $Ile^{15} \Rightarrow Thr^{15}$ of the human LH β-subunit was screened for in 30 women with PCOS and 30 healthy women (controls). Each woman underwent a screening history and physical examination. Before entering the study, all the women had taken no hormonal preparations for $\geq$3 months. Weight and height were obtained to calculate a body mass index ([BMI] wt [kg]/ht^2 [m^2]). A BMI of $\geq$25 was classified as obese, one of $<$25 as nonobese. Oligomenorrhea was defined as an intermenstrual interval of $>$35 days, and amenorrhea as the absence of menstrual periods for $\geq$6 months. Hirsutism was assessed by the protocol used by Ferriman and Gallwey, and a woman with a score of $\geq$8 was considered clinically hirsute (10, 11).

Women with regular menstrual cycles (intervals between 21 and 35 days) were used as control subjects. Healthy women were not hirsute or infertile, had normal-appearing ovaries on ultrasound examination, and had normal serum androgen levels (testosterone $\leq$81 ng/dL, free testosterone $\leq$3.17 pg/mL, androstenedione $\leq$3.08 ng/mL, and DHEAS $\leq$430 μg/dL in women $<$20 years of age, $\leq$380 μg/dL in women 20–29 years of age, and $\leq$270 μg/dL in women 30–39 years of age). Baseline serum levels of FSH, LH, E_2, prolactin, and 17-hydroxyprogesterone (17-OHP) also were determined.

Polycystic ovary syndrome was defined as the presence of [1] bilateral polycystic ovaries on ultrasound examination, [2] oligomenorrhea or amenorrhea and/or chronic anovulation, and [3] hyperandrogenemia (high serum testosterone and/or androstenedione and/or free testosterone levels). All the women were euthyroid (serum TSH level, 0.35–5.5 μIU/mL). Their serum prolactin levels were $<$40 ng/mL. Since mild hyperprolactinemia (prolactin level, $<$40 ng/mL) has been described in women with PCOS, women with mild hyperprolactinemia were recruited into the study unless their history, physical examination, or computed tomographic examination showed any other reason for the hyperprolactinemia (12). Their serum testosterone levels were $<$200 ng/dL, and their DHEAS levels were $<$700 μg/dL (13).

In the PCOS group, if the basal serum 17-OHP level was $>$2 ng/mL, an ACTH stimulation test was done to exclude patients with late-onset congenital adrenal hyperplasia (14). Serum 17-OHP levels were determined before and 30 minutes after an IV injection of 0.25 mg of synthetic ACTH (Synacthen; Ciba, Basel, Switzerland). Women who had an increase of $>$3.3 ng/mL in the serum 17-OHP level after ACTH stimulation were excluded from the study. A serum cortisol level of $>$5 μg/dL after an overnight dexamethasone suppression test also was an exclusion criterion (15). Baseline serum levels of FSH, LH, and E_2 were determined. Women were excluded from the study if an adnexal mass was noted on pelvic sonography.

All hormonal investigations and ultrasound examinations were performed in the early follicular phase (days 3–5) of spontaneous bleeding or withdrawal bleeding induced with medroxyprogesterone acetate. All ultrasound examinations were performed by one operator (K.E.), either transabdominally or transvaginally (3.5-MHz and 5-MHz sector probes, respectively; Kretz, Zipf, Austria). Polycystic-appearing ovaries were defined sonographically as the presence of multiple ($>$10), small (2–8 mm in diameter) follicles in the periphery (in one plane) and increased stromal echogenicity as described by Adams et al. (16).

The study was approved by the Institutional Review Board at Istanbul University, and written informed consent was obtained from each subject.

Hormonal Assays

Blood samples were obtained through venipuncture and centrifuged within 2 hours after withdrawal. Serum was stored at $-$20°C; it was assayed for FSH, LH, E_2, and prolactin with chemiluminescent enzyme immunoassay kits provided by Chiron Diagnostics Corporation (East Walpole, MA) and for testosterone, free testosterone, androstenedione, DHEAS, and 17-OHP with commercially available RIA kits provided by Diagnostic Products Corporation (Coat-A-Count; Los Angeles, CA).

The intra-assay and total coefficients of variation for the low, middle, and high serum values were 2.8%, 1.7%, and 1.2% and 4.6%, 4.5%, and 4.7% for FSH; 4.7%, 4.5%, and 4.4% and 6.3%, 5.2%, and 5% for LH; 14.8%, 5.6%, and 6% and 18.4%, 9.4%, and 8.9% for E_2; and 2.5%, 2.8%, and 3.8% and 3.6%, 4%, and 4.5% for prolactin, respectively. The intra-assay coefficients of variation for different serum values were between 5% and 12% for testosterone, 3.2% and 4.3% for free testosterone, 3.7% and 9.4% for androstenedione, 6% and 9.8% for DHEAS, and 3.5% and 6.7% for 17-OHP. The interassay coefficients of variation were between 7.3% and 11% for testosterone, 3.4% and 5.5% for free testosterone, 4.9% and 14.8% for androstenedione, 7.3% and 9.5% for DHEAS, and 8.5% and 11% for 17-OHP.

The percentage of cross-reactivity of the FSH assay with LH and hCG, of the LH assay with hCG, β-hCG and FSH, and of the E_2 assay with estriol, estrone, estrone sulfate, and DHEAS was $<$1%.

Specific Amplification of the LHβ Gene

The nuclear DNA fractions were obtained from peripheral blood lymphocytes and used directly as template DNA for the polymerase chain reaction (PCR) as described previously (3, 17). In brief, the LHβ genes of all the women were amplified with F1 (5′-GAAGCAGTGTCCTTGTCCCA-3′) and R1 (5′-GAAGAGGAGGCCTGAGAGTT-3′) primers (MWG Biotech, Ebersberg, Germany), which span exon 2, intron 2, and exon 3 of the LHβ gene. R1 has specific nucleotide mismatches to β-hCG.

Total genomic DNA was subjected to 35 cycles of denaturation (95°C for 1 minute), annealing (62°C for 2 minutes), and extension (72°C for 2 minutes) using a thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) in a 100-μL reaction mixture containing 0.025 U/mL of Taq polymerase (MBI Fermentas Inc., New York, NY), 10 μL of PCR buffer (500 mmol/L of KCl, 100 mmol/L of tris(hydroxymethyl)aminomethane chloride, and 0.8% Nonidet P40; MBI Fermentas Inc.), 1.5 mmol/L of $MgCl_2$, 200 μmol/L of deoxynucleoside triphosphate (50 μmol/L each of deoxyadenosine triphosphate, 2-deoxyguanosine-5′-triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate), and 10 pmol/L of primers. This method already has been shown to amplify only the LHβ gene without contaminating the PCR product with the hCGβ genes (3). The PCR products were electrophoresed in 3% agarose gel to confirm the presence of the specific product of 660 base pairs (bp).

Restriction Fragment Length Polymorphism

Women with PCOS and healthy controls underwent restriction fragment length polymorphism analysis to screen for the mutations in codons 8 and 15 using restriction enzymes NcoI and FokI (MBI Fermentas Inc.), respectively. Because the mutations at Trp^8 to Arg (CCACGG) and at Ile^{15} to Thr (NNNNNNNNNNNNCACCC) destroyed the recognition sites of NcoI (CCATGG) and FokI (NNNNNNNNNNNNNCATCC), respectively, LHβ gene fragments, amplified by PCR using primers of F1 and R1, were digested along with these restriction enzymes. Ten units of the enzyme was added to the PCR reaction mixture and incubated overnight at 56°C for FokI digestion and at 37°C for NcoI digestion. An aliquot of the mixture then was examined by 20% polyacrylamide gel electrophoresis.

NcoI digestion in normal LH homozygous individuals results in three bands of 475 bp, 100 bp, and 85 bp (3). In the presence of the mutation at codon 8, the enzyme recognition site is lost so that in homozygotes, there are two bands of 475 bp and 185 bp, whereas in heterozygotes, there are four bands of 475 bp, 185 bp, 100 bp, and 85 bp (3).

With FokI digestion of the PCR product in homozygous normal individuals, the following band pattern occurs: 390 bp, 176 bp, 51 bp, and 43 bp. In the presence of the mutation at codon 15 that destroys the restriction site, fragments of 433 bp, 176 bp, and 51 bp are seen in homozygotes and fragments of 433 bp, 390 bp, 176 bp, 51 bp, and 43 bp are seen in mutant heterozygotes (3).

Statistical Analysis

Data are expressed as means ± SD. Normal distribution of the age, BMI, and hormonal data of the two groups was tested with the Kolmogorov-Smirnov test. Because these parameters were distributed normally, Student's *t*-test was used to compare the PCOS and control groups. The χ^2 test was used to compare the proportion of women who had the LH variant in the two groups.

The demographic and hormonal data of the women with mutant LH were compared with those of the healthy women who were homozygous for the wild-type LH with the use of the Mann-Whitney *U* test. Receiver operating characteristic analysis of the serum LH values of the women was performed to examine the optimal sensitivity and specificity of serum LH for the detection of mutant LH.

Diagnostic sensitivity and specificity were calculated as follows: the number of women with PCOS with a positive test result was divided by the total number of women with PCOS, and the number of control women with a negative test result was divided by the total number of control women. The receiver operating characteristic curve was constructed by plotting the sensitivity against the false-positive rate (1-specificity) for each serum LH value. The LH value with the optimal sensitivity and specificity was chosen as the cutoff value. Based on this cutoff point, the odds ratio was calculated. $P<.05$ was considered statistically significant.

RESULTS

Women with PCOS and controls were similar in age but had different BMIs. The mean (±SD) age of the women with PCOS was 25.53 ± 4.57 years (range, 18–34 years) and that of the healthy women was 27.3 ± 4.8 years (range, 16–36 years). The mean (±SD) BMI was 30.67 ± 7.43 kg/m^2 in the PCOS group and 24.2 ± 4.41 kg/m^2 in the control group. Of the women with PCOS, 26 (86%) were hirsute, 23 (77%) were obese, and 14 (47%) were infertile. One patient had acanthosis nigricans, and 4 had mild hyperprolactinemia.

The hormonal profiles of the women in both groups are shown in Table 1. The women with PCOS had higher serum levels of LH, testosterone, free testosterone, 17-OHP, and DHEAS, and lower levels of FSH. The serum E_2, prolactin, and androstenedione concentrations of the two groups were similar ($P>.05$).

Restriction fragment length polymorphism analysis used to screen for the mutations in codons 8 and 15 using the restriction enzymes NcoI and FokI revealed one (3%) affected woman in the PCOS group and five (17%) in the control group (Fig. 1). The difference between the groups with respect to the frequency of this polymorphism was not statistically significant ($P>.05$). Except for one woman in

TABLE 1

Demographic and hormonal data for women with PCOS and healthy women.

Variable	Study group: PCOS	Study group: Control	*P* value
Age (y)	25.5 ± 4.6	27.3 ± 4.8	NS
BMI (kg/m^2)	30.7 ± 7.4	24.2 ± 4.4	<.001
FSH level (mIU/mL)	5.7 ± 1.7	7.2 ± 2.8	.01
LH level (mIU/mL)	12.2 ± 6.9	7.1 ± 3.7	.001
LH/FSH ratio	2.1 ± 1.0	1.0 ± 0.5	<.001
E_2 level (pg/mL)	62.4 ± 43.2	59.1 ± 37.7	NS
Prolactin level (ng/mL)	15.3 ± 9.1	13.4 ± 8.6	NS
Testosterone level (ng/mL)	72.6 ± 31.6	35.7 ± 20.7	<.001
Free testosterone level (pg/mL)	3.5 ± 1.7	1.5 ± 0.7	<.001
Androstenedione level (ng/mL)	1.7 ± 1.1	1.6 ± 0.7	NS
DHEAS level (μg/dL)	261.4 ± 102.5	185.8 ± 91.7	.004
17-OHP level (μg/L)	1.3 ± 1.0	0.7 ± 0.5	.004

Note: All values are means ± SD. BMI = body mass index; NS = not significant; 17-OHP = 17-hydroxyprogesterone; PCOS = polycystic ovary syndrome.

TABLE 2

Demographic and hormonal data for women with mutant LH and healthy women homozygous for the wild-type LH.

Variable	Study group: No variant LH (n = 25)	Study group: Variant LH (n = 6)	*P* value
Age (y)	27.8 ± 4.3	23.7 ± 6.2	NS
BMI (kg/m^2)	24.5 ± 4.0	22.5 ± 5.8	NS
FSH level (mIU/mL)	7.6 ± 2.8	5.6 ± 1.5	NS
LH level (mIU/mL)	7.9 ± 3.5	4.7 ± 3.8	.03
LH/FSH ratio	1.1 ± 0.5	0.9 ± 0.6	NS
E_2 level (pg/mL)	54.1 ± 26.9	96.3 ± 69.9	NS
Prolactin level (ng/mL)	13.3 ± 7.5	15.9 ± 13.5	NS
Testosterone level (ng/mL)	35.8 ± 19.5	38.0 ± 27.0	NS
Free testosterone level (pg/mL)	1.4 ± 0.7	2.2 ± 1.1	NS
Androstenedione level (ng/mL)	1.6 ± 0.6	2.0 ± 0.7	NS
DHEAS level (μg/dL)	178.5 ± 89.4	256.4 ± 125.7	NS
17-OHP level (μg/L)	0.6 ± 0.4	1.1 ± 0.6	NS

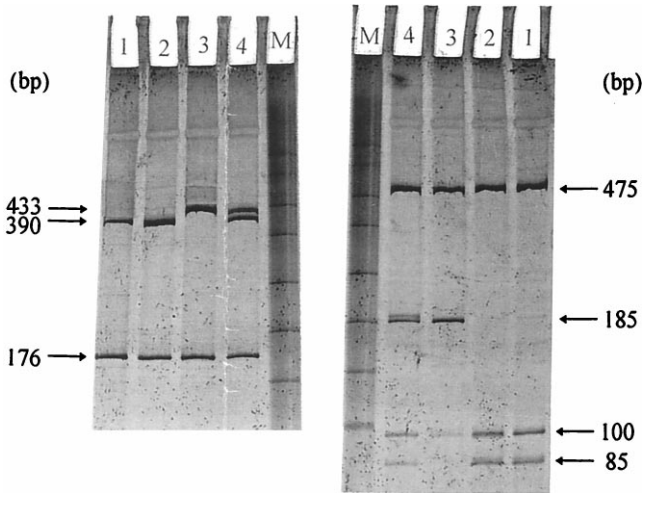
Note: All values are means ± SD. BMI = body mass index; NS = not significant; 17-OHP = 17-hydroxyprogesterone.

the control group, all the women with mutant LH were homozygous (83%). All the homozygotes were not obese (BMI <25 kg/m^2), and the heterozygote was obese.

When the hormone levels of the 6 women with variant LH were compared with those of the 25 healthy women who were homozygous for the wild-type LH, all but the LH level were similar (Table 2). The women with variant LH had lower serum LH levels than the healthy women. The optimal sensitivity and specificity was attained with a cutoff value of 5.1 mIU/mL. The women whose serum LH levels were ≤5.1 mIU/mL had a higher risk of having mutant LH (odds ratio 26 [95% confidence interval, 2.4–289]; $P = .004$; sensitivity 83%, specificity 84%).

FIGURE 1

Polyacrylamide gel electrophoresis (20%) of the PCR products after FokI (*left*) and NcoI (*right*) digestion. *Lanes 1* and *2* were homozygous for the wild-type LH. *Lane 3* was homozygous and *lane 4* was heterozygous for the variant LH. *Lane M*: molecular size markers; pUC mix (MBI Fermentas Inc., New York, NY). Sizes (base pairs [bp]) of DNA fragments are indicated on each side of the figure.



DISCUSSION

Investigation of the subjects with immunologically anomalous LH revealed that the variant form of LH is the result of two point mutations at codons 8 (Trp → Arg) and 15 (Ile → Thr) in the LHβ gene (3). Initial studies reported that this mutant LH is a common DNA polymorphism with a prevalence of 28% in a healthy Finnish population and about 18% in England (4, 8). Nilsson et al. (5) recently calculated the worldwide frequency of the variant LH with the use of two immunofluorometric assays (5). They also claimed that the LH variant is less common in Asian countries.

In our study, the frequency was 17% in healthy women and 3% in women with PCOS. Since the frequency in our control group was consistent with the frequencies reported from other Asian countries (i.e., Thailand, China, Japan), we believe that the frequency in the Turkish population is similar to our findings (5). However, we are aware that screening of a larger group, including men, is necessary to determine the actual frequency of the mutant LH in the Turkish population.

Only in Finland, Japan, and England was variant LH, which consists of two mutations (LH-M8/15), screened for

with the use of genetic methods (4, 7, 8). The frequencies reported from other countries were determined by calculating the ratio of LH values measured with two immunofluorometric assays, one of which uses two LH β-subunit–specific monoclonal antibodies and the other of which uses two different monoclonal antibodies that react with the β-subunit and the intact dimer, respectively (5).

Analysis of the effects of these mutations separately by site-directed mutagenesis showed that many properties of the mutant LH with only the mutation within codon 8 (LH-M8) are similar to those of LH-M8/15 (18). It also has been shown that calculating the ratio of LH values measured by the two immunofluorometric assays does not discriminate between LH-M8 and LH-M8/15 (18). Since our study is a genetic one and all the women with variant LH had both the mutations, we showed that despite the fact that its prevalence is different in Asian countries, the genetic structure of the variant LH is constant.

The role of LH in the pathogenesis of PCOS has been debated (19). Variant LH, as a result of its increased bioactivity, may cause hyperandrogenemia and PCOS. The existence of a more potent form of LH in patients with PCOS was suggested before the LH variant was defined, and it has been reported that many women with PCOS have an increased ratio of bioactive to immunoactive LH (20–23). Furui et al. (3) and Suganuma et al. (7), who also defined the LH variant, reported that two of five cases homozygous for the variant LH were diagnosed as PCOS. However, we observed a similar frequency of the LH variant in women with PCOS and healthy women.

Rajkhowa et al. (8) also screened 212 normal ovulatory women and 153 women with PCOS for the LH variant and reported that its prevalence was not higher in women with PCOS, although it was overrepresented in obese women with PCOS. In contrast, Huhtaniemi et al. (9) reported that variant LH occurs with normal frequency in nonobese patients with PCOS but is underrepresented in obese patients with PCOS. We could not analyze the relation between obesity in women with PCOS and the mutant LH because of the limited number of women in our study. However, in our study, healthy women, in spite of having a lower BMI than women with PCOS, had a higher rate of mutant LH (17% versus 3%), although the difference was not statistically significant (Table 1). We believe that obese patients with PCOS do not have a higher incidence of variant LH.

Another difference that we noted was the rate of homozygotes; 5 (83%) of our subjects were homozygous. However, only 1 (2%) of 64 mutant subjects were homozygous in the United Kingdom population in which Rajkhowa et al. (8) investigated the relation between PCOS and variant LH. In analyzing the pathogenic significance of variant LH in women with PCOS, this high rate of homozygotes is our most significant finding.

To analyze the relation between variant LH and the hormonal milieu, especially androgens and E_2, we compared the serum hormone levels of the 6 patients with mutant LH with those of the 25 controls who were homozygous for the wild-type LH. Subjects with variant LH had lower serum LH levels. The other hormone levels were similar in the two groups. The difference in serum LH levels between the groups probably was due to the inability of the antibodies used by our LH kit to recognize the LH variant. The finding that serum androgen levels were similar supports the idea that variant LH does not cause hyperandrogenemia and PCOS.

We also believed that serum LH levels might be helpful in screening women with mutant LH. We observed that a woman with a baseline serum LH level of ≤5.1 mIU/mL had a higher risk of having variant LH and should undergo genetic evaluation. This cutoff value for baseline serum LH may be useful in developing countries where kits that can detect variant LH are not yet available. Knowing the LH status of a woman will prevent misinterpretation of serum LH values in some clinical states, such as during ovulation induction therapy to detect a premature LH surge and while diagnosing conditions associated with high serum LH levels, such as PCOS and menopause.

In conclusion, [1] the prevalence of the variant LH in our Turkish population was approximately 17%, [2] the rate of homozygotes may be higher in our country than in other countries, [3] the prevalence of variant LH in women with PCOS is similar to that in the normal population, [4] this polymorphic LHβ allele is not overrepresented in obese women with PCOS, and [5] women with a baseline serum LH level of ≤5.1 mIU/mL have a higher risk of having variant LH and should undergo genetic evaluation.

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