The \((TAAA)n\) microsatellite polymorphism in the \(SHBG\) gene influences serum SHBG levels in women with polycystic ovary syndrome

Polonca Ferk, Natasa Teran and Ksenija Gersak\(^{1,2}\)

\(^{1}\)Department of Obstetrics and Gynaecology, Division of Medical Genetics, University Medical Centre, Ljubljana, Slovenia

\(^{2}\)To whom correspondence should be addressed at: University Medical Centre, Department of Obstetrics and Gynaecology, Slajmerjeva 3, SI-1000 Ljubljana, Slovenia. E-mail: ksenija.gersak@mf.uni-lj.si

BACKGROUND: Hyperandrogenaemia is a common feature of polycystic ovary syndrome (PCOS). The sex hormone-binding globulin (\(SHBG\)) gene was proposed as being a PCOS candidate gene. A possible influence of the microsatellite polymorphism \((TAAA)n\) in the \(SHBG\) gene on serum SHBG levels in PCOS patients was investigated.

METHODS: One hundred and twenty-three PCOS patients and 110 age-matched controls were included in the study. Peripheral blood samples were obtained. Genotyping of the \((TAAA)n\) polymorphism in the \(SHBG\) gene was performed. Serum LH, FSH, SHBG and total testosterone concentrations were determined.

RESULTS: \(SHBG\) alleles with 6–11 \(TAAA\) repeats were found. None of the \(SHBG\) alleles or genotypes were present at a significantly more frequent rate in PCOS patients compared with controls. Serum \(SHBG\) levels were significantly lower \((P<0.001)\) in PCOS patients compared with controls and were found to be strongly influenced by the \((TAAA)n\) \(SHBG\) polymorphism, in both the PCOS (55.3\%) and control (33.1\%) groups of patients.

CONCLUSIONS: The \((TAAA)n\) \(SHBG\) gene polymorphism might be an important predictor for serum \(SHBG\) levels and, consequently, for hyperandrogenaemic clinical presentation of PCOS.

Key words: hyperandrogenism/polycystic ovary syndrome/serum \(SHBG\) levels/(\(TAAA\))\(_n\) \(SHBG\) microsatellite polymorphism

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age, with hyperandrogenaemia and/or hyperandrogenism as main characteristics (Legro \textit{et al.}, 1998). Numerous studies on the aetiology of PCOS have been performed but the aetiological background remains unclear. On the basis of extreme phenotypic heterogeneity and because of familial clustering, PCOS is supposed to be a multifactorial and oligogenic disorder (Franks \textit{et al.}, 2001; Strauss III, 2003).

Among PCOS candidate genes, the sex hormone-binding globulin (\(SHBG\)) gene (17p13–p12) was proposed (Urbanek \textit{et al.}, 1999; Xita \textit{et al.}, 2003; Cousin \textit{et al.}, 2004). Human SHBG is a specific, 373-amino-acid plasma transport glycoprotein for sex steroid hormones, thus regulating access of sex steroid hormones to target cells (Cousin \textit{et al.}, 2004). It has been reported that serum SHBG levels are influenced by many factors, i.e. gender, age and hormonal, metabolic, genetic and nutritional factors (Hilpert \textit{et al.}, 2001; Xita \textit{et al.}, 2003; Cousin \textit{et al.}, 2004). Moreover, the essential conclusion from a previous wide HERITAGE Family Study was that serum SHBG levels are probably mainly dependent on genetic factors (Ukkola \textit{et al.}, 2002). In PCOS patients, decreased serum SHBG levels have been reported (Xita \textit{et al.}, 2003; Cousin \textit{et al.}, 2004).

Recently, the presence of a pentanucleotide microsatellite polymorphism \((TAAA)n\) in the promoter of the \(SHBG\) gene has been identified and its influence on \(SHBG\) transcriptional activity \textit{in vitro} has been described (Hogeveen \textit{et al.}, 2001). Two association studies have been performed on Greek PCOS (Xita \textit{et al.}, 2003) and French hirsute (Cousin \textit{et al.}, 2004) patients reporting that serum \(SHBG\) levels could be at least partly determined by the \(SHBG\) polymorphic variants. Noteworthy, Cousin \textit{et al.} (2004) reported a strong linkage disequilibrium between eight \(TAAA\) repeats of the microsatellite and a variant \((v)\) allele D327N in exon eight of the \(SHBG\) gene. The \(v\) allele introduces an additional consensus site for N-glycosylation leading to an increase in human SHBG half-life (Cousin \textit{et al.}, 1998), possibly resulting in higher serum \(SHBG\) levels in \(v\) allele carriers.

The basic purpose of the present study was to investigate whether the \((TAAA)n\) microsatellite polymorphism in the \(SHBG\) gene is associated with the presence of PCOS and with serum \(SHBG\) levels in PCOS patients.
Materials and methods

Subjects
One hundred and twenty-three patients fulfilling the criteria for PCOS (Homburg, 2002; The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004) were enrolled in the study group. They were included consecutively between the years 2002 and 2005 during their treatment at the Department of Obstetrics and Gynaecology, University Medical Centre Ljubljana, Slovenia. All patients had menstrual cycle abnormalities (amenorrhoea/oligomenorrhoea) and polycystic ovaries (PCO) on ultrasound. The morphological characterization of PCO was made according to the international consensus criteria (Balen et al., 2003): 12 or more follicles measuring 2–9 mm in diameter and/or increased ovarian volume (>10 cm^3). Hyperandrogenism was assessed by the presence of hirsutism (Ferriman–Gallwey index score of at least eight) (Balcn et al., 2005) and/or by serum free testosterone (TF) levels, calculated from serum total testosterone (TT) and SHBG levels (Ly and Handelsman, 2005), by serum dehydroepiandrosterone sulphate (DHEA-S) and by serum androstendione (A) levels. Other possible causes of hyperandrogenism (late onset congenital adrenal hyperplasia, Cushing’s syndrome, androgen-secreting tumours) were previously excluded by endocrinologists. The control group consisted of 110 age-matched healthy volunteers with proven fertility (seen in our clinic for normal pregnancy), no menstrual cycle irregularities, with no clinical or biochemical hyperandrogenism and without PCO. They also had no history of endocrinological or autoimmune disorders and no surgery to the pelvic region. All women were of European (Slovenian) origin and were not genetically related.

The study protocol was approved by the National Medical Ethics Committee of the Republic of Slovenia (No. 97/05/01). Informed written consent was obtained from all women enrolled in the study.

Hormonal assays
Serum LH, FSH, SHBG, TT, DHEA-S and A levels were measured. Serum LH, FSH and SHBG concentrations were determined by chemiluminescent immunometric assays using LH-Immuli, FSH-Immuli and SHBG-Immuli, respectively (Diagnostic Products Corporation, Los Angeles, CA, USA). Serum TT, DHEA-S and A levels were measured using commercial radioimmunoassay kits (TT: DiaSorin, Saluggia, Italy; A: Diagnostic Systems Laboratories Inc., Costa Mesa, CA, USA; A: Diagnostic Systems Laboratories Inc., Webster, TX, USA). The intra-assay and inter-assay coefficients of variation were between 1.6 and 13.0%. Serum samples were obtained in the early follicular phase of the menstrual cycle or randomly in amennorhoeic patients.

Genotype analysis
For all patients and all controls, genomic DNA was isolated from peripheral blood leukocytes using the commercial FlexiGene kit (Qiagen GmbH, Hilden, Germany), following protocols recommended by manufacturer.

Genotyping for the (TAAAA)n SHBG polymorphism was performed by classical PCR using hot-start AmpliTaq Gold™ DNA polymerase (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) and the following primers: forward 5'-GAA GCC ACC TTT GCA CTA CCT-3', reverse 5'-CCA AAG GCC ATT CAG GCA AAA-3' and with TaqMan® MGB probes VIC®-labelled: 5'-CTA GGA GAA GAC TCT TC-3', 6-FAM®-labelled: 5'-CTA GGA GAA AAC TCT TC-3'). The Taqman Universal PCR protocol was applied: initial denaturation step (10 min, 95°C) was followed by 40 cycles of (i) denaturation (15 s at 92°C) and (ii) annealing + extension (1 min, 60°C). Allelic discrimination analysis was done using ABI Prism sequence detection software.

Statistical analysis
Chi-square test was used to compare SHBG allele and genotype frequencies between the study and the control group of patients. Student’s t-test was used to compare mean values of BMI, age and the analysed hormones between the two different groups of women, whereas ANOVA was performed when comparing mean values of serum SHBG levels between the six different SHBG genotypes. Pearson’s correlations for assessing linear relations and Spearman’s correlations for assessing non-linear relations were applied. Multiple linear regression analysis with serum SHBG as a dependent variable and multivariate logistic regression analysis with PCOS status as an outcome were performed. All statistical analyses were done using SPSS for Windows (version 12.0; SPSS, IL, USA). A P-value of <0.05 was considered statistically significant.

Results
Clinical and biochemical characteristics of our PCOS and control patients
In PCOS patients, mean BMI and mean serum TT, TF, A and LH levels were significantly higher (P < 0.001 for all the parameters), whereas mean serum SHBG levels were significantly lower compared with control patients (P < 0.001); serum FSH and DHEA-S levels did not differ significantly between the two groups of patients (P = 0.385 and P = 0.656, respectively) (Table 1). All our control women were lean (18 kg/m^2 ≤ BMI ≤ 25 kg/m^2), whereas 13% of PCOS patients were overweight (BMI > 25 kg/m^2); the remaining patients were lean.

In PCOS patients, BMI showed a significant positive correlation with serum TT levels (R = 0.276, P = 0.002). Serum SHBG levels were not significantly correlated with BMI in either the PCOS or the control group of patients. However, a trend to lower serum SHBG levels in overweight PCOS women was observed (Figure 1), but the number of overweight PCOS patients was too low for relevant statistical analysis.
SHBG genotyping

(TAAA)n, SHBG polymorphism

SHBG alleles with 6, 7, 8, 9, 10 and 11 TAAA repeats were found. The allele distribution did not differ significantly between the study and the control group of patients (P = 0.169; due to its low frequency, the allele with 11 repeats was excluded from the statistical analysis), although an excess of nine-repeat-alleles in PCOS patients and six-repeat-alleles in controls was evident (Figure 2). The following (TAAA)n SHBG genotypes were observed: 6/6, 6/7, 6/8, 6/9, 6/10, 6/11, 7/7, 7/8, 7/9, 7/10, 7/11, 8/8, 8/9, 8/10, 8/11, 9/9, 9/10, 9/11 and 10/10; some of which were present only in controls (Figure 3).

Regarding the polymorphism, the study and the control group of patients were both in Hardy–Weinberg equilibrium.

Logistic regression analysis using parameter SHBGp [referring to the biallelic mean repeat number of both of the two (TAAA)n SHBG alleles in pair] as an independent variable, BMI and age as covariates and PCOS status (PCOS/control) as a dependent variable revealed that the genetic parameter does not significantly predict the development of PCOS (P = 0.326).

D327N SHBG polymorphism

The allele frequencies for the D327N SHBG polymorphism in PCOS and control patients were: 93.0 versus 90.4% for the wild-type (W) allele and 7.0 versus 9.6% for the v allele, respectively. The genotype frequencies in PCOS and control patients were as follows: 86.0 versus 82.6% for the W/W (G/G) genotype, 14.0 versus 15.6% for the W/v (G/A) genotype and 0.0 versus 1.8% for the v/v (A/A) genotype. Between the two groups of patients, differences in the allele as well as in the genotype distributions were of no statistical significance.

Regarding the polymorphism, both the study and the control groups of patients were in Hardy–Weinberg equilibrium.

Relationship between the D327N and (TAAA)n SHBG polymorphisms

The v D327N SHBG allele was strongly segregated with eight-TAAA-repeat SHBG allele: 93% of PCOS patients and all (100%) control women bearing the v D327N allele had at least one allele with eight TAAA repeats (χ² test: P < 0.001 for both groups of patients); this observation demonstrates a significant linkage disequilibrium between the D327 N and (TAAA)n SHBG polymorphisms. None of the PCOS patients and only two control women [one with 8/8 (TAAA)n SHBG genotype and the other with 8/9 (TAAA)n SHBG genotype] had the homozygous v/v D327 N genotype.

Influence of the (TAAA)n SHBG polymorphism on serum SHBG levels

A significant negative correlation was found between serum SHBG levels and SHBGp [referring to the shorter of the two (TAAA)n SHBG alleles; ρ = −0.787, P < 0.001] as well as between serum SHBG levels and SHBGl (referring to the longer of the two (TAAA)n SHBG alleles; ρ = −0.372,

Table I. Means ± SD of age, BMI and hormones in PCOS and control patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24.4 ± 4.4</td>
<td>25.3 ± 3.8</td>
<td>0.287</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>44.4 ± 19.1</td>
<td>61.0 ± 14.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TT (nmol/l)</td>
<td>12.0 ± 0.4</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>TF (nmol/l)</td>
<td>3.0 ± 1.3</td>
<td>2.4 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>10.1 ± 6.5</td>
<td>4.6 ± 1.0</td>
<td>0.385</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>22.3 ± 3.1</td>
<td>21.2 ± 1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>355.6 ± 235.4</td>
<td>333.1 ± 209.7</td>
<td>0.656</td>
</tr>
<tr>
<td>A (ng/dl)</td>
<td>250.1 ± 110.3</td>
<td>165.2 ± 65.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1. Serum SHBG levels in relation to BMI in PCOS and control group.

Figure 2. Distribution of the (TAAA)n SHBG allele frequencies (%).

Figure 3. Distribution of the (TAAA)n SHBG genotype frequencies in PCOS and control patients.
serum SHBG levels as a dependent variable and BMI as a possible covariate was performed, for both the PCOS and the control group of patients. The results show that in both the PCOS and the control group of patients, serum SHBG levels were significantly influenced by the genetic parameter, SHBGp (P < 0.001 in both, the PCOS patients and the controls), whereas BMI was significant for the dependent variable only in controls (P = 0.024), but not in PCOS patients (P = 0.331). The respective linear regression models explained 55.3% (for PCOS patients) and 33.1% (for controls) of the variability (adjusted R² values) in serum SHBG levels.

The difference in serum SHBG levels was not statistically significant between PCOS and control women having 6/6 genotype (P = 0.756), whereas the difference between the two groups of patients reached statistical significance with the other most frequent (TAAAA)ₙ SHBG genotypes (6/8, 6/9, 8/8, 8/9 and 9/9) (P = 0.011, P = 0.035, P < 0.001, P = 0.001 and P < 0.001, respectively), with serum SHBG levels being significantly lower in PCOS women. Within groups, PCOS patients and controls, the serum SHBG levels were found to be significantly different between the six (6/6, 6/8, 6/9, 8/8, 8/9 and 9/9) genotypes (P < 0.001 within both groups) (Figure 4).

Influence of the D327N SHBG polymorphism on serum SHBG levels

Within both the study and the control group of patients, serum SHBG levels differed according to the D327N SHBG genotype (P = 0.011 for PCOS patients and P = 0.025 for controls) (Figure 5). A significant decline in serum SHBG levels was observed in heterozygous W/v individuals in comparison with homozygous W/W individuals; in controls, the only two v/v homozygotes had even lower serum SHBG levels than heterozygotes.

Discussion

The basic purpose of the present study was to investigate a possible association of the (TAAAA)ₙ repeat polymorphism in the SHBG gene with the development of PCOS as well as with serum SHBG levels in Slovene patients.

The (TAAAA)ₙ SHBG polymorphism could be one of the factors representing a genetic link to the developmental origin hypothesis for PCOS (Abbott et al., 2002, 2005; Dumesic et al., 2005), by which individuals with genetically determined low SHBG levels may be exposed to high free androgen levels during fetal life, programming their future PCOS hyperandrogenic characteristics. Since longer SHBG alleles were found to have lower transcriptional activity in vitro (Hogeveen et al., 2001), leading to lower serum SHBG levels and consequently to higher levels of bioavailable androgens (Xita et al., 2003; Cousin et al., 2004), an excess of longer SHBG alleles in PCOS patients was expected. However, no association between the (TAAAA)ₙ SHBG genotypes and PCOS was observed and the allelic variants were similarly distributed in our study and control patients, although a trend to higher frequency of nine-repeat-alleles and to lower frequency of six-repeat-alleles in PCOS group was evident. To evaluate this trend with statistical (non)significance, many more patients should be tested; however, the observation is confirmatory to a significantly higher frequency of longer (TAAAA)ₙ SHBG alleles (with more than eight repeats) previously found in 185 Greek PCOS patients (Xita et al., 2003).

Since PCOS is a heterogenous syndrome with a complex clinical picture and with multifactorial pathogenesis, it was more appropriate to investigate an association of the SHBG polymorphism with low serum SHBG levels as a specific feature of PCOS (also confirmed in our patients) rather than with the presence of the syndrome itself. In this respect, an important observation was that a great proportion of variability in serum SHBG levels could be explained by the SHBG polymorphism, similar to the findings in 303 French hirsute women (154 PCOS women among them) (Cousin et al., 2004). In addition, longer allele genotypes have been associated with lower serum SHBG levels in Greek PCOS patients (Xita et al., 2003).

As evidently suggested from the results obtained in our study, the (TAAAA)ₙ SHBG polymorphism might have a strong influence on serum SHBG levels and this effect is probably...
independent of the presence of PCOS; significantly lower serum SHBG levels in women with longer (TAAAA)_n SHBG alleles were observed not only in PCOS patients but also in controls.

In order to provide additional information about the (TAAAA)_n SHBG allele expression in vivo (Jänne et al., 1998; Hilpert et al., 2001; Hogeveen et al., 2001; Hogeveen et al., 2002), we further focused on serum SHBG levels in patients with the six most frequent genotypes (6/6, 6/8, 6/9, 8/8, 8/9 and 9/9). Serum SHBG levels were significantly lower in PCOS patients when compared with controls with the same genotype; accordingly, it could be speculated that additional factors might contribute to final determination of serum SHBG levels in PCOS patients. The exception was the 6/6 genotype where there was no significant difference in mean serum SHBG levels between our PCOS and control patients; in this respect, other influences, genetic and/or environmental, may be considered to play an important role in the development of PCOS in some women with short (TAAAA)_n SHBG alleles in comparison with other women with short (TAAAA)_n SHBG alleles who do not develop PCOS.

Interestingly, mean serum SHBG levels were the lowest in 8/8 (TAAAA)_n SHBG PCOS women. Since the mutant D327N variant in exon 8, which delays human SHBG half-life, and a variant with eight TAAA repeats in the promoter of the SHBG gene were described to be in a strong linkage disequilibrium (Cousin et al., 2004), effects of 8/8 genotypes on serum SHBG levels could be masked by the point mutation. Therefore, the relationship between the two SHBG polymorphisms was also tested in our PCOS and control population and our results confirmed linkage disequilibrium between the two polymorphisms, with similar frequencies as described before. Additionally, the variant and wild-type D327N allele frequencies were similar to that previously described (Cousin et al., 2004). Because of similar allele and genotype frequencies between the PCOS and the control group of patients, the D327N SHBG polymorphism probably do not contribute to differences in serum SHBG levels between the two groups of patients. However, the observed lowering effect of the variant D327N SHBG alleles on serum SHBG levels, in both the study and the control group, was contradictory to previous findings (Cousin et al., 1998, 2004).

In previous studies, particularly in a genome-wide linkage scan (Ukkola et al., 2002), numerous candidate genetic loci exhibited significant linkages for serum SHBG levels, suggesting that the latter are probably regulated by many genes rather than a single genetic marker. Therefore, interactions between different genetic factors should be considered and tested in future.

An additional finding from previous studies was that serum SHBG levels are also significantly influenced (negative correlation) by BMI, but the influence was mostly restricted to non-obese patients (Cousin et al., 2004). In contrast, our results did not show a significant correlation between serum SHBG levels and BMI, although a trend to lower serum SHBG levels in overweight PCOS patients could be observed; however, it could not be explained with statistical significance. Namely, only a minor proportion of our PCOS women presented with obesity. In this sense, our PCOS patients should be considered as a specific PCOS subgroup.

To summarize the key results from our study, the (TAAAA)_n SHBG polymorphism seems to be an important predictor for serum SHBG levels and consequently for hyperandrogenenaic clinical presentation in PCOS. However, since this genetic effect is evident in both the PCOS patients and the controls, although being very relevant for the presence of PCOS and much stronger in PCOS compared with control women, it is probably not exclusive to the PCOS phenotype. Accordingly, the SHBG gene may be considered as being a modifier gene for PCOS phenotype.

Acknowledgements

This work was supported by grants from the Ministry of Education, Science and Sport of Republic of Slovenia (No. J3-6429-0312). We are very grateful to our patients for their participation in this study. We would like to give special thanks to Mrs Paula Duff for revising the English text.

References


Submitted on December 13, 2005; resubmitted on October 19, 2006; accepted on October 26, 2006