The (TAAAA)$_n$ microsatellite polymorphism in the SHBG gene influences serum SHBG levels in women with polycystic ovary syndrome

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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 (TAAAA)$_n$ 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 (TAAAA)$_n$ polymorphism in the SHBG gene was performed. Serum LH, FSH, SHBG and total testosterone concentrations were determined.

RESULTS: SHBG alleles with 6–11 TAAAA 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 (TAAAA)$_n$ SHBG polymorphism, in both the PCOS (55.3%) and control (33.1%) groups of patients. CONCLUSIONS: The (TAAAA)$_n$ 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/(TAAAA)$_n$ 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 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 et al., 2001; Strauss III, 2003).

Among PCOS candidate genes, the sex hormone-binding globulin (SHBG) gene (17p13–p12) was proposed (Urbanek et al., 1999; Xita et al., 2003; Cousin 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 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 et al., 2001; Xita et al., 2003; Cousin 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 et al., 2002). In PCOS patients, decreased serum SHBG levels have been reported (Xita et al., 2003; Cousin et al., 2004).

Recently, the presence of a pentanucleotide microsatellite polymorphism (TAAAA)$_n$ in the promoter of the SHBG gene has been identified and its influence on SHBG transcriptional activity in vitro has been described (Hogeveen et al., 2001). Two association studies have been performed on Greek PCOS (Xita et al., 2003) and French hirsute (Cousin et al., 2004) patients reporting that serum SHBG levels could be at least partly determined by the SHBG polymorphic variants. Noteworthy, Cousin et al. (2004) reported a strong linkage disequilibrium between eight TAAAA 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 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 (TAAAA)$_n$ 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 cm³). Hyperandrogenism was assessed by the presence of hirsutism (Ferriman–Gallwey index score of at least eight) (Balen 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 androstenedione (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-Immulite, FSH-Immulite and SHBG-Immulite, respectively (Diagnostic Products Corporation, Los Angeles, CA, USA). Serum TT, DHEA-S and A levels were measured using commercial radioimmunoassay kits (TT: DiaSorin, Salugia, Italy; DHEA-S: ICN Biomedicals 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 amenorrhoeic 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 CTC GAG CAG CAG CAG CAG TGA-3'; reverse 5'-AGA AAT CAC CCA CTC CCT GA-3'. The reaction conditions were as follows: initial denaturation step (10 min at 95°C) was followed by 30 cycles of (i) 45 s of denaturation at 94°C, (ii) 45 s of the annealing step at 63°C and (iii) 1 min of extension at 72°C; the final extension lasted 7 min at 72°C. To separate PCR products of different sizes, electrophoresis on Spreadex™ gels (Elchrom Scientific, Cham, Switzerland) at 55°C was performed, according to manufacturer’s recommendations (Spreadex™ EL400 Mini, 141 min). The number of TAAAA repeats was calculated from the size of PCR products (in relation to M3 marker; Elchrom Scientific) and our own internal standard. The latter was prepared from six different PCR fragments, 133, 138, 143, 148, 153 and 158 bp in size, which, after direct sequencing, were identified as having 6–11 TAAAA repeats, respectively.

The D327N polymorphism (G > A) in exon 8 of the SHBG gene was genotyped by real-time PCR using TaqMan® technology (ABI Prism™ 7000 Sequence Detection System v1.1; Applied Biosystems, Weiterstadt, Germany). The reaction mixture (25 µl) consisted of 70 ng of genomic DNA, 1× TaqMan® Universal PCR Master Mix, No AmpErase® UNG (including AmpliTaq Gold® DNA polymerase) and 1× TaqMan® SNP Genotyping Assay (with unlabelled primers F: 5'-AAT GCC ACC TTT GCA CTA CTT-3', R: 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/m² ≤ BMI ≤ 25 kg/m²), whereas 13% of PCOS patients were overweight (BMI > 25 kg/m²); 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**

*SHBG* alleles with 6, 7, 8, 9, 10 and 11 TAAAA 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 (TAAAA)_n SHBG genotypes were observed: 6/6, 6/7, 6/8, 6/9, 6/10, 6/11, 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 (TAAAA)_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 (TAAAA)_n SHBG polymorphisms**

The v D327N SHBG allele was strongly segregated with eight-TAAAA-repeat SHBG allele: 93% of PCOS patients and all (100%) control women bearing the v D327N allele had at least one allele with eight TAAAA repeats (*χ^2* test: *P* < 0.001 for both groups of patients); this observation demonstrates a significant linkage disequilibrium between the D327 N and (TAAAA)_n SHBG polymorphisms. None of the PCOS patients and only two control women [one with 8/8 (TAAAA)_n SHBG genotype and the other with 8/9 (TAAA)_n SHBG genotype] had the homozygous v/v D327 N genotype.

**Influence of the (TAAAA)_n SHBG polymorphism on serum SHBG levels**

A significant negative correlation was found between serum SHBG levels and SHBGs [referring to the shorter of the two (TAAAA)_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 (TAAAA)_n SHBG alleles; *ρ* = −0.372).

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**Table 1.** Means ± SD of age, BMI and hormones in PCOS and control patients

<table>
<thead>
<tr>
<th>Variable (nmol/l)</th>
<th>PCOS</th>
<th>Controls</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHBGg</td>
<td>44.4 ± 19.1</td>
<td>61.0 ± 14.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TT</td>
<td>3.0 ± 1.3</td>
<td>1.2 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TF</td>
<td>39.8 ± 18.3</td>
<td>12.8 ± 5.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH</td>
<td>10.1 ± 6.5</td>
<td>2.4 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FSH</td>
<td>4.8 ± 2.4</td>
<td>4.6 ± 1.0</td>
<td>0.385</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>22.3 ± 3.1</td>
<td>21.2 ± 1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>DHEA-S (mg/dl)</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>

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

Figure 2. Distribution of the (TAAAA)_n SHBG allele frequencies in PCOS and control patients.

Figure 3. Distribution of the (TAAAA)_n SHBG genotype frequencies in PCOS and control patients.
Accordingly, multiple linear regression analysis with a parameter $SHBG_p$ as a predictor, 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, $SHBG_p$ ($P < 0.001$ 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 $R^2$ 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) $SHBG$ 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 heterogeneous 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 evidenced 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)₉ SHBG alleles were observed not only in PCOS patients but also in controls.

In order to provide additional information about the (TAAAA)₉ 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)₉ SHBG alleles in comparison with other women with short (TAAAA)₉ SHBG alleles who do not develop PCOS.

Interestingly, mean serum SHBG levels were the lowest in 8/8 (TAAAA)₉ SHBG PCOS women. Since the mutant D327N variant in exon 8, which delays human SHBG half-life, and a variant with eight TAAAA 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 patient. However, the observed lowering effect of the v 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)₉ SHBG polymorphism seems to be an important predictor for serum SHBG levels and consequently for hyperandrogenenaemic 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.

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References


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