Adrenal 21-hydroxylase gene mutations in Slovenian hyperandrogenic women: evaluation of corticotrophin stimulation and HLA polymorphisms in screening for carrier status

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Abstract

Objective: To study the incidence of 21-hydroxylase deficiency in Slovenian hyperandrogenic women, at the gene level. Previous endocrine studies indicated large differences in the incidence of 21-hydroxylase deficiency in hyperandrogenic women. The predictive values of the 17-hydroxyprogesterone (17-OHP) response to ACTH stimulation and of HLA typing in screening for carrier status were re-evaluated.

Design: Molecular analysis of CYP21 gene, ACTH stimulation and human leukocyte antigen (HLA) typing were performed in 83 consecutive Slovenian hyperandrogenic women.

Measurements: Cortisol and 17-OHP concentrations were measured at baseline and 60 min after ACTH stimulation. Basal adrenal androgen concentrations were also measured.

Results: None of 83 hyperandrogenic patients was affected with non-classical 21-hydroxylase deficiency, but 12 of 81 patients (14.8%) had high concentrations of 17-OHP after stimulation, indicative of carrier status. The increase in 17-OHP concentrations could be explained by a carrier status for CYP21 gene mutations in only three of 12 patients (25%), whereas seven of 69 patients (10.1%) with normal concentrations of 17-OHP after stimulation were found to be carriers of CYP21 gene mutations, indicating low positive predictive values of ACTH stimulation as a screening test for carriers of 21-hydroxylase deficiency. In total, 11 carriers were identified among 83 patients: seven CYP21 gene deletions/conversions, two Gln318Stop and one Val281Leu mutation and one gene conversion extending from exon 4 to exon 7 were found. The association between Val281Leu mutation and HLA-B14 antigen was confirmed in this Slovenian population.

Conclusions: Basal or ACTH-stimulated 17-OHP concentrations are not a good indicator of the carrier status for 21-hydroxylase deficiency among Slovenian hyperandrogenic patients. Reliable screening for carriers of 21-hydroxylase deficiency is possible only by molecular analysis of the CYP21 gene.

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Introduction

Non-tumoral hyperandrogenism is one of the most common endocrinological disorders in women. It is manifested by one or more of the following clinical signs and symptoms: oligo/amenorrhoea, decreased fertility, hirsutism, acne resistant to treatment or androgenic alopecia. Increased plasma concentrations of one or more adrenal or ovarian androgens are often found (1). There is no specific marker on the basis of which hyperandrogenism could be distinguished from the polycystic ovary syndrome (PCOS), especially as polycystic ovaries are also detected by ultrasound in healthy women without clinical signs of hyperandrogenism (2). Heterogeneity of clinical symptoms and biochemical features in hyperandrogenism complicates the search for its possible genetic causes (3). Mutations in the 21-hydroxylase gene, the 11β-hydroxylase gene, the 3β-hydroxysteroid dehydrogenase gene and the insulin receptor gene have been found to be associated with hyperandrogenism (4).

Non-classical congenital adrenal hyperplasia (NC-CAH) caused by 21-hydroxylase deficiency is one of the most common genetic defects. Its incidence is 1/1000 in white populations, in general but is much higher in some selected populations: 1/27 in Askhenazi Jews, 1/53 in Spaniards, 1/63 in Croats and 1/330 in Italians (5, 6). The incidence of NC-CAH in hyperandrogenic women has been reported to be 1.2–20% (7–9). This variability may be due to a different frequency of NC-CAH in studied populations of different ethnic origin or differences in selection criteria, but may also reflect...
21-Hydroxylase gene mutations in Slovenian hyperandrogenic women

Study participants and methods

Study participants

The study population consisted of 83 Slovenian female hyperandrogenic patients, aged 17–32 years (median 24 years), referred to the outpatient endocrinological unit in Ljubljana. They were complaining of one or more of the following clinical symptoms: oligo/amenorrhoea (12 of the patients were oligomenorrhoic, the others eumenorrhoic), hirsutism, acne resistant to treatment, or androgenic alopecia. The presence of hirsutism was assessed by the examining endocrinologist, but was not formally scored. The presence of polycystic ovaries as determined by ultrasound was not a selection criterion. Androgen-secreting tumours and thyroid dysfunction were excluded by clinical and hormonal findings.

Family members of patients who had CYP21 gene mutations or increased 17-OHP after an ACTH test were asked to participate in the study. Eleven family members of four patients agreed to take part in HLA typing and molecular analysis, but were not hormonally tested.

Two control groups were analysed for point mutations in the CYP21 gene. The first control group of 18 young women aged 20–29 years (median 21 years) served also to provide information on the normal basal and stimulated 17-OHP and cortisol concentrations in the healthy Slovenian population. All were eumenorrhoic, without clinically evident signs of hirsutism or acne, and none was receiving hormonal medication. The second control group consisted of 38 DNA samples randomly chosen from Slovenian female blood donors previously typed for HLA (16).

The study design was approved by the Slovene Ethical Committee for Research in Medicine. Written informed consent was obtained from all participants in the study.

ACTH testing and hormonal analysis

The ACTH test was performed with 0.25 mg ACTH i.m. (Synacthen; Ciba–Geigy, Basel, Switzerland) between 0800 and 0900 h. Healthy controls and eumenorrhoic hyperandrogenic women were tested in the early follicular phase. Blood samples were obtained at 0 min and 60 min after the administration of ACTH and were assayed for 17-OHP and cortisol. Normal upper basal and stimulated concentrations of 17-OHP were determined as mean+2S.D. of those in healthy controls and were less than 4.1 nmol/l for basal 17-OHP and less than 9.8 nmol/l for stimulated 17-OHP. In patients, basal blood samples were also assayed for free testosterone (normal value <7.7 pmol/l), androstenedione (normal value <11.6 nmol/l) and dehydroepiandrosterone sulphate (DHEAS; normal value <8.8 mmol/l). The following RIA kits were used: free testosterone, Diagnostic Products Corporation (DPC; Los Angeles, CA, USA); androstenedione, Immunotech International (Marseille, France); DHEAS, Diagnostic System Laboratories (Webster, TX, USA); 17-OHP, DPC. A fluoroimmunoassay kit from Wallac (Turku, Finland) was used to determine cortisol concentration. The mean intra- and interassay coefficients of variation (CV) were less than 6%.

HLA typing

Standard microlymphocytotoxicity testing (17) was used to determine class I HLA phenotypes in 77 female hyperandrogenic patients. A local set of serologically defined polyclonal alloantisera and polyclonal alloantisera from Biotest (Dreieich, Germany) were used. HLA-DRB1 alleles were analysed by oligonucleotide hybridisation of PCR-amplified second exons of the HLA-DRB1 gene (18) or by sequence-specific PCR


**Molecular analysis**

DNA was isolated from peripheral blood leucocytes (20). Ten micrograms genomic DNA were digested with TaqI restriction endonuclease. Fragments were separated on 0.7% agarose gels and blotted on the Hybond N+ membrane (21). Two cDNA probes, CYP21 probe (22) and C4 probe (23), were labelled with 32P-dCTP and used to determine CYP21 and C4 gene deletions, duplications and large gene conversions in 64 hyperandrogenic patients. Both probes were obtained through the courtesy of M C Carroll (Harvard University, Boston, MA, USA). Because only TaqI digests were analysed, gene conversions involving the TaqI restriction site were not distinguishable from deletions of the CYP21 gene. Deletions/conversions of CYP21 were identified by decreased intensity of the 3.7 kb TaqI fragment compared with that of the 3.2 kb TaqI fragment (24).

To screen for point mutations, exons 1–3 and exons 3–8 of the CYP21 gene were selectively amplified with two sets of primers (25) in all patients and controls. Dot blot hybridisation analysis of the PCR product with 3'DIG-ddUTP end-labelled oligonucleotides was used to screen for the presence of normal or mutated sequences at the CYP21 gene: Pro30Leu (exon 1), A/C656G (-13 intron 2), Ile172Asn (exon 4), Ile-Val-Glu-Met236–239Asn-Glu-Glu-Lys (exon 6), Val281Leu (exon 7), ins5107 (exon 7), Gln118Stop (exon 8) and Arg56Trp (exon 8). Oligonucleotide sequences were essentially as previously described (25). Allele-specific PCR was used to analyse for an 8 bp deletion in exon 3 (26).

For sequence analysis, the CYP21 gene and its proximal promoter region were first specifically amplified in two large fragments, the first extending from -264 to the proximal promoter region to the CYP21-specific site in exon 3 (986 bp, forward primer 5'-AGCTGACTCTGGATGCAGGA-3', reverse primer 5'-AGCAGGGAGTAGTCTCCCAAG -3') and the second comprising exons 3 to 10 (2095 bp, forward primer 5'-GTG-CTCTGGAGACTACCTC-3', reverse primer 5'-ACTTGTTTFACAGGGAAGGAG-3'). Amplified fragments were purified on QIAquick PCR purification columns (Qiagen, Hilden, Germany) and sequenced by α-35S-dATP incorporation with the forward and reverse primers shown in Table 1, using an AmpliCycle sequencing kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ, USA).

**Statistical analysis**

Hormonal data are expressed as means±s.d. Student’s t-test was used for statistical analysis of hormonal data. χ2 and Fisher’s exact test were used to compare HLA phenotype frequencies between patients and controls. Statistical significance was assigned a value of P<0.05.

**Results**

**17-OHP responses to ACTH stimulation**

The individual values of basal and stimulated 17-OHP concentrations in Slovenian hyperandrogenic patients are shown in Fig. 1. All patients were subjected to an ACTH test, but 17-OHP was determined only in 81 patients, among whom 20 (24.7%) demonstrated basal 17-OHP values greater than 4.1 nmol/l. At 60 min, increased 17-OHP concentrations (range 9.8–21.5 nmol/l) were found in 12 of 81 patients (14.8%). No patient demonstrated stimulated 17-OHP greater than 36.6 nmol/l that would be consistent with 21-hydroxylase deficient NC-CAH (7). Basal 17-OHP concentrations were significantly greater in hyperandrogenic women than in healthy controls (3.3±0.2 compared with 2.5±0.8 nmol/l; P=0.006). However, stimulated 17-OHP concentrations were similar in both groups (7.0±3.1 compared with 6.6±2.0 nmol/l). Basal cortisol (397±152 nmol/l and 396±116 nmol/l) and stimulated cortisol concentrations (727±147 nmol/l and 716±72 nmol/l) were not significantly different between the groups.
CYP21 gene mutations and HLA associations

Eleven heterozygous carriers of CYP21 mutations were identified among 83 hyperandrogenic patients analysed (13.3%). Basal and stimulated 17-OHP concentrations, HLA haplotypes and specificity of mutations in hyperandrogenic carriers of CYP21 gene mutations are shown in Table 2.

CYP21 gene deletion/conversion carriers were identified among seven of 64 hyperandrogenic patients (10.9%), of whom two had high and four had normal stimulated 17-OHP values. In one patient, 17-OHP was not determined. CYP21 gene deletions/conversions were not associated with HLA-B47 typical for CAH in Northern European populations. HLA-B40, but not HLA-DR1, was present in two patients with CYP21 gene deletion/conversion.

Three heterozygous carriers of CYP21 point mutations and one gene conversion encompassing four exons were identified among 83 hyperandrogenic patients (4.8%). Point mutations identified were Val281Leu in one and Glu318Stop in two patients. Similar to the findings described by Weddel et al. (27), in one patient, four consecutive mutations were found – Ile172Asn,

Table 2 17-OHP concentrations at baseline and 60 min after ACTH stimulation, and HLA haplotypes, in female hyperandrogenic carriers of CYP21 gene mutations.

<table>
<thead>
<tr>
<th>Patient</th>
<th>17-OHP (nmol/l)</th>
<th>HLA haplotype</th>
<th>CYP21 mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td>E36</td>
<td>6.1</td>
<td>9.9</td>
<td>A2 A24 B62 Bx DR1 DR13</td>
</tr>
<tr>
<td>E46</td>
<td>6.8</td>
<td>9.6</td>
<td>A3 A24 B41 B40 DR11 DR13</td>
</tr>
<tr>
<td>E2</td>
<td>1.3</td>
<td>5.0</td>
<td>A2 A28 B51 B44 DR16 DR7</td>
</tr>
<tr>
<td>E12</td>
<td>5.0</td>
<td>8.9</td>
<td>A24 A26 B51 B56 DR3 DR11</td>
</tr>
<tr>
<td>E21</td>
<td>ND</td>
<td>ND</td>
<td>A2 A11 B38 B35 DR1 DR16</td>
</tr>
<tr>
<td>E33</td>
<td>1.9</td>
<td>4.1</td>
<td>A2 A24 B21 B40 DR11 DR14</td>
</tr>
<tr>
<td>E89</td>
<td>2.1</td>
<td>3.8</td>
<td>A3 A34 B44 B35 DR7 DR14</td>
</tr>
<tr>
<td>E83</td>
<td>3.6</td>
<td>12.8</td>
<td>A3 A32 B14 BX DR13 DR13</td>
</tr>
<tr>
<td>E54</td>
<td>5.0</td>
<td>6.5</td>
<td>A3 A26 B51 B35 DR4 DR8</td>
</tr>
<tr>
<td>E77</td>
<td>2.5</td>
<td>8.4</td>
<td>A1 A2 B8 B50 DR3 DR7</td>
</tr>
<tr>
<td>E72</td>
<td>2.9</td>
<td>7.0</td>
<td>A2 A23 B62 B50 DR1 DR3</td>
</tr>
</tbody>
</table>

ND, not done; conv, conversion; del, deletion; N/M, heterozygous carrier; Ex. exon. Polymorphisms and neutral mutations are not shown.
cluster of mutations in exon 6, Val^{281}Leu and insertion of T_{307} – and a gene conversion extending from exon 4 to exon 7 was suspected, because of the normal response to ACTH stimulation. The carriers of Glu^{318}-Stop mutation, a mutation frequently found in patients with classical 21-hydroxylase deficiency, had normal 17-OHP values after stimulation. Gln^{318}Stop mutation was associated with HLA-A2 and -B50 in both patients; in one, this association was confirmed by a family study (Fig. 2A).

The carrier of the Val^{281}Leu mutation on the HLA-B14 DR13 extended haplotype showed an elevated 17-OHP response to ACTH stimulation. To investigate further the association between HLA-B14 and Val^{281}Leu mutation in the Slovenian population, five additional DNA samples of HLA-B14 carriers were selected from the registry of the Tissue Typing Centre in Ljubljana and analysed for point mutations in the CYP21 gene. Three of them (60%) were found to be heterozygous carriers of the Val^{281}Leu mutation (Table 3).

No CYP21 point mutations were found in 56 healthy controls. However, a heterozygous Gln^{318}Stop mutation associated with the HLA-A2 B21 DR4 haplotype (Fig. 2B) was found in the mother and the sister of one hyperandrogenic patient without this mutation.

All mutations detected by oligonucleotide hybridisation were confirmed by sequencing. In addition, sequence analysis of the proximal promoter region, exons and exon–intron junctions of the CYP21 gene was used to screen those hyperandrogenic patients who had high 17-OHP values after stimulation for other mutations or polymorphisms that could explain their exaggerated response to the ACTH test. Sequencing analysis revealed 10 neutral polymorphic sites (mostly in exons 1, 7 and 10) in 11 patients, but no additional mutations were found. In the carrier of a gene conversion extending from exon 4 to exon 7, polymorphisms characteristic of the pseudogene were also present in a heterozygous form.

### Adrenal function and serum androgen concentrations in hyperandrogenic carriers of CYP21 gene mutations

In 27 of 82 hyperandrogenic patients (33%), one androgen (mostly free testosterone or DHEAS) was increased in serum, and in 20 of the 82 patients (24%), two androgens were increased. Androstenedione was increased only in two of the 82 (2.4%). To evaluate the influence of CYP21 gene mutations on adrenal function, 17-OHP, cortisol and adrenal androgen concentrations were compared between hyperandrogenic carriers of CYP21 gene mutations and patients without mutations (Table 4).

### Discussion

The present study represents the first systematic screening for 21-hydroxylase deficiency in Slovenian patients.
Table 4 17-OHP, cortisol and androgen concentrations in Slovenian female hyperandrogenic patients with and without CYP21 gene mutations. Values are expressed as mean ± S.D.

<table>
<thead>
<tr>
<th>Carriers of CYP21 mutations</th>
<th>Patients without CYP21 mutations</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP&lt;sub&gt;0&lt;/sub&gt; (nmol/l)</td>
<td>3.7 ± 1.9</td>
<td>3.2 ± 2.0</td>
</tr>
<tr>
<td>17-OHP&lt;sub&gt;60&lt;/sub&gt; (nmol/l)</td>
<td>7.6 ± 2.8</td>
<td>7.0 ± 3.1</td>
</tr>
<tr>
<td>17-OHP&lt;sub&gt;60&lt;/sub&gt; (nmol/l)</td>
<td>3.9 ± 2.3</td>
<td>3.8 ± 2.9</td>
</tr>
<tr>
<td>Cortisol (nmol/l)</td>
<td>308 ± 57</td>
<td>409 ± 157</td>
</tr>
<tr>
<td>Free testosterone (pmol/l)</td>
<td>11.5 ± 9.4</td>
<td>7.9 ± 4.0</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>8.0 ± 3.6</td>
<td>5.9 ± 2.9</td>
</tr>
<tr>
<td>DHEAS (nmol/l)</td>
<td>10.1 ± 4.1</td>
<td>8.1 ± 3.8</td>
</tr>
</tbody>
</table>

17-OHP, 17-OHP<sub>60</sub>, 17-OHP<sub>60</sub>, concentrations at baseline and 60 min after stimulation with ACTH, and the difference between these values.

hyperandrogenic women. ACTH stimulation, HLA typing and molecular analysis of the CYP21 gene region were used to define the carrier status for 21-hydroxylase deficiency.

High 17-OHP concentrations after stimulation, indicative of heterozygous carriers of 21-hydroxylase deficiency (10), were found in 12 of 81 hyperandrogenic patients (14.8%). As no patient demonstrated a stimulated 17-OHP concentration indicative of non-classical 21-hydroxylase deficiency, we cannot draw any conclusions as to the incidence of non-classical 21-hydroxylase deficiency in Slovenian hyperandrogenic women; it may be lower than or consistent with the reported 1–2% frequency in white hyperandrogenic women in general (7, 8). However, high frequencies of non-classical 21-hydroxylase deficiency that are found in some other selected populations (5) are practically excluded in the Slovenian population. Therefore routine screening for 21-hydroxylase deficiency is not recommended in Slovenian hyperandrogenic women.

Molecular analysis of the CYP21 gene revealed that 11 of 83 hyperandrogenic patients (13.3%) were heterozygous carriers of 21-hydroxylase deficiency. These results differ from those of Azziz & Owerbach (10), who found carriers of inherited defects in CYP21 in the majority of patients with an exaggerated 17-OHP response to ACTH stimulation. No point mutations were identified in 56 healthy Slovenian controls.

Carriers of CYP21 gene mutations could not be distinguished from other hyperandrogenic patients on the basis of their response to ACTH stimulation; in fact, the increase in 17-OHP concentrations after ACTH stimulation could be explained by a carrier state for CYP21 gene mutations only in three of 12 patients analysed (25%). In addition, CYP21 gene mutations were found in 7 of 69 hyperandrogenic patients (10.1%) with normal 17-OHP values after stimulation. The response to the ACTH test was normal also in the carrier of a gene conversion extending from exon 4 to exon 7. Our results indicate that 7 of 11 carriers (72.7%) would be missed if ACTH stimulation alone was used as a screening test, and that carriers of severe mutations characteristic of classical 21-hydroxylase deficiency alleles (five of seven deletions/conversions, two of two Gln<sup>118</sup>Stop mutations) would be at risk of being given inadequate genetic advice. CYP21 gene mutations that contributed to increased 17-OHP concentrations after ACTH stimulation were two gene deletions/conversions and one Val<sup>281</sup>Leu mutation. The Val<sup>281</sup>Leu mutation resulting in an enzyme with decreased activity (28) seems to compromise the adrenal function of the carrier to a greater extent than does the complete absence of the product of one allele.

Our study confirmed the association between Val<sup>281</sup>Leu mutation and HLA-B14, a marker characteristic of NC-CAH in several populations (15, 29) also in the Slovenian population. Further studies of HLA haplotypes in Slovenian patients with CAH and their families are needed to ascertain associations between HLA haplotypes and CYP21 gene mutations before HLA typing can be used to screen for carriers.

Heterozygous carriers of CYP21 mutations had higher concentrations of serum free testosterone, androstenedione and DHEAS than patients without CYP21 gene defects. However, the two groups did not differ in basal or ACTH-stimulated 17-OHP concentrations. This is in agreement with observations that heterozygosity for CYP21 mutations may result in mild abnormalities of androgen biosynthesis reflected by higher mean free testosterone (13, 30) or by higher mean DHEAS concentrations (10). Our results suggest that, in contrast with findings in patients with NC-CAH, among hyperandrogenic women, basal or ACTH-stimulated 17-OHP concentrations are not a good indicator of the carrier status for inherited defects of CYP21, as observed also by other investigators (13, 31, 32) who defined the carrier status by molecular analysis of CYP21 gene, the most accurate method to define carriers for inherited defects of CYP21 (10). Older studies reported that the majority of heterozygotes for 21-hydroxylase deficiency can be detected by measuring the 17-OHP concentration 60 min after ACTH stimulation; however, the diagnosis of 21-hydroxylase deficiency was based on HLA genotyping (33). Perhaps
measuring 21-deoxycortisol (34) and the ratio of 17-OHP/21-deoxycortisol (35), not done in our study, might improve prediction of heterozygous carriers of 21-hydroxylase deficiency by hormonal testing.

It remains unclear what was the cause of borderline elevations in ACTH-stimulated 17-OHP concentrations in nine of 12 hyperandrogenic patients (75%), in whom even extensive sequencing of the CYP21 gene and its proximal promoter region revealed no mutations. Falsely positive ACTH tests may be due to high 17-OHP concentrations reflecting adrenal and non-adrenal secretion of this steroid precursor (30). Factors other than a mild impairment of 21-hydroxylase activity may also contribute to biochemical findings in these patients (29). Studies of the 17α-hydroxylase/17–20-lyase gene showed that mutations in this gene do not contribute to increased stimulated 17-OHP concentrations in Slovenian hyperandrogenic women (37). Further studies may show which additional factors influence the phenotypic traits of 21-hydroxylase deficiency in heterozygous carriers. In conclusion, our results demonstrate the difficulty in choosing appropriate cut-offs in biochemical tests and the low sensitivity of ACTH stimulation for detection of carriers of 21-hydroxylase deficiency. Reliable detection of carriers is possible only by molecular analysis of the CYP21 gene.

Acknowledgements

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