Hormonal evaluation and mutation screening for steroid 21-hydroxylase deficiency in patients with unilateral and bilateral adrenal incidentalomas

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Abstract

Objective: The aims of the present study were (a) to examine the occurrence of 21-hydroxylase gene (CYP21) mutations in patients with unilateral and bilateral adrenal incidentalomas and (b) to correlate the results of mutation screening with hormonal parameters of 21-hydroxylase deficiency.

Design: The frequency of the eight commonly occurring CYP21 mutations in blood DNA samples of 19 patients with bilateral, as well as in blood and tumoral tissue DNA samples of 31 patients with unilateral adrenal incidentalomas, was determined. In all patients, hormonal evaluation for 21-hydroxylase deficiency was performed using measurements of basal and ACTH-stimulated plasma 17-hydroxyprogesterone (17-OHP) concentrations.

Methods: Blood and tumoral DNA samples were analyzed by allele-specific PCR for the detection of the eight commonly occurring CYP21 mutations (deletion/large gene conversion, intron 2 splicing, Ile172Asn, exon 6 cluster, Val281Leu, Leu307insT, Gln318Stop and Arg356Trp mutations). Plasma 17-OHP concentrations were measured by radioimmunounassay.

Results: Of the 19 patients with bilateral adrenal incidentalomas, one patient had homozygous (Val281Leu) and three patients had heterozygous germline CYP21 mutations (Val281Leu in two cases and Arg356Trp in one case). Heterozygous germline CYP21 mutations were also detected in five of the 31 patients with unilateral adrenal incidentalomas (Ile172Asn in three cases and Val281Leu in two cases). Mutation screening of tumoral DNA in unilateral incidentalomas showed the presence of corresponding germline mutations but no additional somatic mutations were found. ACTH-stimulated plasma 17-OHP concentrations were above 1500 ng/dl in all patients with bilateral incidentalomas who had homozygous and heterozygous CYP21 mutations, but heterozygous carriers with unilateral incidentalomas had highly variable ACTH-stimulated plasma 17-OHP levels (between 111 and 1705 ng/dl).

Conclusions: These results suggest a similar frequency of germline CYP21 mutations in patients with bilateral and unilateral adrenal incidentalomas (21.1% and 16.1% respectively). Therefore, it cannot be ruled out that, in at least some patients, CYP21 mutations may play a role in the pathomechanism of bilateral and unilateral adrenal incidentalomas. However, the lack of clear association of CYP21 mutations with increased ACTH-stimulated plasma 17-OHP response, especially in patients with unilateral incidentalomas, suggests that the effect of CYP21 mutations on adrenocortical tumor formation may also involve mechanism(s) independent of ACTH-induced changes in 17-OHP secretion.

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considered as being clinically non-functioning, although slight abnormalities in the function of the hypothalamic–pituitary–adrenal axis, perhaps suggesting an occult secretion of corticosteroids, have also been documented (14–16). Perhaps more interestingly, an exaggerated plasma 17-hydroxyprogesterone (17-OHP) response after adrenocorticotropic hormone (21-hydroxylation defect) has been documented in several series of studies, including a large number of patients with incidental adrenal tumors (10, 16–22). The latter observations led to the proposal that undiagnosed mild forms of congenital 21-hydroxylase deficiency or a heterozygous state for this disorder, which results in decreased cortisol secretion and consequently increased ACTH secretion, may be a predisposing factor for the development of incidental adrenocortical tumors. Consistent with this concept, one study showed that adrenocortical adenomas may be present in 82% of homozygous patients as well as in 45% of heterozygous carriers for congenital 21-hydroxylase deficiency (23).

The occurrence of disease-causing 21-hydroxylase gene (CYP21) mutations in peripheral blood and/or tumoral tissue samples of patients with different types of adrenal tumors has been examined in two recent studies (24, 25). Beuschlein et al. (24) investigated the mutational spectrum and mRNA expression of the CYP21 gene in six aldosterone-producing adenomas, seven cortisol-producing adenomas, four adrenal carcinomas and two adrenocortical incidentalomas. They found that neither of the two adrenocortical incidentalomas had homozygous or heterozygous CYP21 mutations, although the mRNA contents of the two tumors were markedly lower than those measured in aldosterone-producing adenomas. In the study of Kjellman et al. (25), 27 patients with sporadic adrenocortical tumors including six patients with hormonally silent benign tumors were screened and the results showed the absence of the commonly occurring CYP21 mutations in these patients. However, the results of the two studies do not entirely contradict the previously proposed role of CYP21 mutations in the development of hormonally inactive adrenal tumors, because an ACTH-stimulation test had not been performed and, therefore, it is not known whether or not the few patients studied could have an exaggerated 17-OHP response. In addition, the occurrence of CYP21 mutations has not been previously examined in patients with bilateral adrenal incidentalomas. We therefore performed the present studies with two principal aims: (a) to examine the occurrence of CYP21 mutations in patients with unilateral and bilateral adrenal incidentalomas and (b) to correlate the results of mutation screening with hormonal evidence of 21-hydroxylase deficiency using measurements of basal and ACTH-stimulated plasma 17-OHP concentrations in the same patients with unilateral and bilateral adrenal tumors.

**Patients and methods**

**Patients**

The patients were evaluated for adrenal incidentalomas at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1995 and 2000. Nineteen patients (14 females and five males, mean age 54 years) had bilateral adrenal tumors which were discovered during abdominal ultrasound or CT performed for unrelated reasons. None of the patients had clinical evidence of hormonal overproduction. In all patients, a detailed endocrine evaluation was performed, which excluded primary aldosteronism, Cushing’s syndrome, hyperandrogenism and pheochromocytoma. In all these patients, CT scans showed homogenous adrenal lesions whose size remained unchanged during follow-up lasting 12–60 months. None of the patients underwent adrenal surgery.

The study also included 31 patients (27 females and four males, mean age 51 years) who were operated on for unilateral adrenal incidentalomas between 1995 and 2000. In these patients, hormonal evaluation performed before unilateral adrenalectomy failed to prove hormonal overproduction disorders. Although surgery was performed in all cases because of some suspicion of malignancy because of a tumor diameter larger than 4 cm or an increase in tumor size during patient follow-up, none of the tumors proved to be malignant and in all cases histological examination showed benign adrenocortical adenomas.

**Endocrine evaluation**

In all patients, hormonal evaluation included measurements of plasma cortisol, 17-OHP, androstenedione, dehydroepiandrosterone and testosterone at 0800 h under resting conditions, as well as plasma cortisol measurements after overnight low-dose dexamethasone administration. Plasma renin activity and aldosterone were also determined under resting conditions and in an upright posture after furosemide administration.

All patients underwent an ACTH stimulation test using 2 mg synthetic ACTH1–24 (Cortrosyn Depot; Organon, Oss, The Netherlands). An i.m. injection was given at 1400 h, and blood was drawn the next morning between 0800 and 0900 h for measurements of plasma cortisol and 17-OHP. In patients with unilateral adrenal incidentalomas, the test was performed both before and after adrenal surgery. In a previous study, the plasma 17-OHP concentrations (mean±S.D.) after this ACTH stimulation test in 78 patients with unilateral adrenal incidentalomas and in 60 normal subjects were 192.6±180.1 ng/dl (58.3±54.5 nmol/l) and 601.5±274.3 ng/dl (18.2±8.3 nmol/l) respectively (16). None of the patients from this previous study was included in the present study.
Plasma cortisol, 17-OHP, androstenedione, dehydroepiandrosterone, aldosterone, and testosterone concentrations were measured with previously published radioimmunoassay methods, using highly specific antisera (26, 27). The intra-assay coefficient of variation for these assays was between 6% and 10%, and the interassay coefficient of variation was between 5% and 9%. Plasma renin activity was determined using a commercially available kit (Rianen; DuPont, Boston, MA, USA).

**Mutation analysis of the CYP21 gene**

Written informed consent for mutation analysis was obtained from all patients. The use of adrenal tumor samples for molecular biological studies was approved by the local Ethical Committee of Semmelweis University.

Genomic DNA was extracted from peripheral blood leukocytes. Samples of adrenal tumor tissues were collected at adrenalectomy, frozen in liquid nitrogen, and stored at −80°C until analyzed. The tissues were homogenized and used for DNA extraction. DNA was extracted from peripheral blood leukocytes and from adrenal tumor homogenates using the DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim Corp., Indianapolis, IN, USA).

DNA samples obtained from peripheral blood and tumoral tissues were analyzed for deletion/large gene conversion of the CYP21 gene locus, as described (28). Mutation analysis of the CYP21 gene by allele-specific PCR, after selection against the CYP55 pseudogene was performed using the method of Wedell & Luthman (29). CYP21 was first selectively amplified by PCR using oligonucleotides CYP55, 5'-CCTGTTCCTTTGGGAGACTACTAT and CYP12, 5'-ACTGTTTTACAGGGGGGAGG for a 2080 bp fragment and CYP1, 5'-TTCAGGGGATTCAGAAGGC and CYP48, 5'-CAGAGGGAGTAGTCTCT for a 1129 bp fragment of the CYP21 gene. The PCR products containing the selectively amplified CYP21 fragments were further analyzed by allele-specific PCR using oligonucleotide sequences for the detection of intron 2 splicing, Ile172Asn, exon 6 cluster, Val281Leu, Leu307insT, Gln318Stop and Arg356Trp mutations. After an initial denaturation at 96°C for 3 min, 30 cycles at 96°C for 1 min, 56°C for 30 s and 72°C for 3 min were performed in a buffer containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl, 0.2 mM deoxynucleotide triphosphate, 1 U Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), 0.15 or 0.5 μM oligonucleotide primers (Invitrogen Life Technologies, Glasgow, UK) and 5% glycerol. The final extension was accomplished at 72°C for 10 min. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Statistical analysis**

The results are expressed as means±s.d. Statistical analysis included non-parametric tests (Mann–Whitney–Wilcoxon test, Spearman rank correlation). P < 0.05 was considered to be statistically significant.

**Results**

The clinical data of patients with bilateral and unilateral adrenal incidentalomas are shown in Table 1. The mean age was similar in the two groups of patients. There was a high female/male ratio in both groups of patients. The size of unilateral incidentalomas (31±50 mm) was larger as compared with the size of bilateral adrenal tumors (24±22 and 23±28 mm in the left and right incidentalomas respectively).

Basal plasma 17-OHP concentrations were similar in the two groups of patients with adrenal tumors (59±58 and 67±65 ng/dl in patients with unilateral and bilateral adrenal tumors respectively). After ACTH administration, plasma 17-OHP was substantially increased in both groups with higher levels attained in patients with bilateral (1770±1459 ng/dl) compared with those with unilateral adrenal incidentalomas (1287±731 ng/dl). However, this difference between the two groups was not statistically significant. The ACTH-stimulated plasma 17-OHP concentrations of individual patients indicated that 14 of the 19 patients with bilateral tumors and 19 of the 31 patients with unilateral adrenal tumors showed an exaggerated response to ACTH stimulation (>1000 ng/dl), which is usually considered as hormonal evidence for a decreased activity of the 21-hydroxylase enzyme. In contrast, after adrenal surgery none of the 31 patients with unilateral adrenal incidentalomas had an ACTH-stimulated plasma 17-OHP concentration above 1000 ng/dl. The difference in ACTH-stimulated plasma 17-OHP levels before (1287±731 ng/dl) and after (349±217 ng/dl) adrenal surgery was statistically significant (P < 0.05).

<table>
<thead>
<tr>
<th>Incidentoma</th>
<th>No. of patients</th>
<th>Female/male</th>
<th>Age (years)</th>
<th>Tumor size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Bilateral</td>
<td>19</td>
<td>14/5</td>
<td>54</td>
<td>42–67</td>
</tr>
<tr>
<td>Unilateral</td>
<td>31</td>
<td>27/4</td>
<td>51</td>
<td>28–76</td>
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**Table 1 Clinical data of patients with bilateral and unilateral adrenal incidentalomas.**
Mutation screening performed in peripheral blood DNA samples of 19 patients with bilateral adrenal incidentalomas revealed a Val281Leu mutation in a homozygous form in one patient. In addition, three patients had CYP21 mutations in heterozygous forms (Val281Leu in two cases and Arg356Trp in one case). Thus, homozygous or heterozygous mutations were detected in 21.1% of patients with bilateral adrenal tumors. In all these patients, ACTH-stimulated plasma 17-OHP levels were above 1500 ng/dl (Table 2).

Genetic screening in both peripheral blood and tumoral tissue DNA samples of 31 patients with unilateral adrenal incidentalomas failed to show CYP21 mutations in homozygous forms. However, five patients with heterozygous mutations were identified (16.1% of patients with unilateral tumors) which showed the presence of the same mutations in both peripheral blood and tumoral tissue DNA (Ile172Asn in three cases and Val281Leu in two cases). Interestingly, only two of the five patients had increased ACTH-stimulated plasma 17-OHP levels suggestive of a decreased activity of the 21-hydroxylase enzyme (Table 2).

Comparison of the results of genetic screening and hormonal data indicated a considerable degree of divergence between the mutational spectrum of the CYP21 gene and ACTH-stimulated plasma 17-OHP concentrations. As shown in Fig. 1, homozygous or heterozygous CYP21 gene mutations were frequently present in patients with bilateral adrenal incidentalomas who had ACTH-stimulated plasma 17-OHP levels above 1500 ng/dl, although these mutations were absent in a patient showing the highest plasma 17-OHP level after ACTH stimulation. The discrepancy between genetic and hormonal studies was even more apparent in patients with unilateral incidentalomas, as ACTH-stimulated plasma 17-OHP concentrations remained in the normal or low range in three of the five heterozygous carriers.

**Discussion**

In the present study, we performed hormonal evaluation and mutation screening for 21-hydroxylase deficiency in a large number of patients with unilateral and bilateral adrenal incidentalomas. A homozygous germline Val281Leu mutation was found in one of the 19 patients with bilateral adrenal tumors, this patient had a markedly increased plasma 17-OHP response to ACTH stimulation. Since normal basal 17-OHP together with a markedly increased ACTH-stimulated 17-OHP concentration predict a partial decrease of 21-hydroxylase enzyme activity, and homozygous Val281Leu mutation results in a partial inactivation of the enzyme (30), this patient appears to represent a clinically unrecognized case of the non-classical form of congenital 21-hydroxylase deficiency. In agreement with this finding, a few cases of congenital 21-hydroxylase deficiency presenting as bilateral adrenal masses in middle-aged or older subjects have already been reported (31–33).

In patients with bilateral adrenal incidentalomas, germline CYP21 mutations were also present in a heterozygous form. Of the 19 patients, two patients were identified as heterozygous carriers for a Val281Leu mutation and one patient was a heterozygous carrier for a Arg356Trp mutation. In all these three patients ACTH-stimulated plasma 17-OHP

| Table 2 Basal and ACTH-stimulated plasma 17-OHP concentrations in patients with homozygous and heterozygous CYP21 mutations. |
|---|---|---|---|---|---|
| Adrenal | Patient no. | Mutation | Adrenal tumor (mm) | Plasma 17-OHP (ng/dl) |
| incidentaloma | | | | Basal | ACTH-stimulated |
| Bilateral | 1 | Val281Leu homozygous | 43 (left) | 237 | 5012 |
| | 2 | Val281Leu heterozygous | 34 (right) | 125 | 2570 |
| | 3 | Val281Leu heterozygous | 30 (right) | 39 | 2118 |
| | 4 | Val281Leu heterozygous | 25 (left) | 19 | 1505 |
| Unilateral | 1 | Ile172Asn heterozygous | 20 (left) | 197 | 1142 |
| | 2 | Ile172Asn heterozygous | 40 (right) | 31 | 111 |
| | 3 | Ile172Asn heterozygous | 20 (left) | 106 | 717 |
| | 4 | Val281Leu heterozygous | 35 (right) | 36 | 247 |
| | 5 | Val281Leu heterozygous | 32 (right) | 49 | 1705 |

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concentrations were also well above the normal range (>1500 ng/dl), but these hormone levels were lower than that found in a patient with a homozygous Val281Leu mutation. An exaggerated 17-OHP response to ACTH stimulation has been reported in several earlier studies in heterozygous carriers of congenital 21-hydroxylase deficiency (34–36). However, it is difficult to explain why a heterozygous state results in a mild decrease of enzyme activity in the presence of a non-mutated allele.

It has been shown that patients with bilateral adrenal incidentalomas have a significantly higher ACTH-stimulated plasma 17-OHP response compared with those with unilateral tumors, and that increased plasma 17-OHP levels suggestive of 21-hydroxylase deficiency are more frequent in patients with bilateral than in unilateral tumors (17). Our study also showed higher ACTH-stimulated plasma 17-OHP concentrations in patients with bilateral adrenal incidentalomas, although the difference between patients with bilateral and unilateral tumors was not statistically significant.

In our study, heterozygous germline CYP21 mutations were also detected in five of the 31 patients with unilateral adrenal incidentalomas (Ile172Asn in three cases and Val281Leu in two cases). However, the ACTH-stimulated plasma 17-OHP concentrations in three of the five patients failed to reflect the heterozygous carrier state (<1000 ng/dl). The discordance between the results of mutation screening and ACTH-stimulated plasma 17-OHP levels in these three patients with unilateral adrenal incidentalomas is not fully understood. It has been shown that not all heterozygous carriers show an exaggerated plasma 17-OHP response to ACTH stimulation (37), which indicates that the stimulation test commonly used to document a partial deficiency of 21-hydroxylase enzyme is not always a reliable tool to detect heterozygous carriers. Other studies also showed a remarkable clinical and hormonal heterogeneity of homozygous patients or heterozygous carriers (38, 39), suggesting that other genes or other effects involved in the metabolism of adrenal steroids may influence the clinical and/or hormonal abnormalities present in these patients. Alternatively, it cannot be ruled out that the effect of CYP21 mutations on adrenocortical tumor formation (23) may involve mechanism(s) independent of the ACTH-induced changes in 17-OHP secretion.

The CYP21 gene is a frequent target of mutations, and congenital 21-hydroxylase deficiency is the most frequent autosomal recessive disorder in the human. The prevalence of 21-hydroxylase deficiency in the Caucasian population has been estimated as one in 1000, but selective groups may have a higher prevalence (39–42). Based on plasma 17-OHP responses to ACTH stimulation, heterozygous carrier frequencies have been estimated as one in 16 for Caucasians (42). Molecular genetic screening of normal newborn infants in New Zealand showed that one in 20 were heterozygous for CYP21B mutations, which implies a disease frequency similar to that estimated in the Caucasian population (43). In our study, the detection of one homozygous and three heterozygous germline mutations in 19 patients with bilateral (21.1%), and five heterozygous germline mutations in 31 patients with unilateral incidentalomas (16.1%) suggests a higher frequency of mutations in both groups of adrenal tumors compared with that found in the general European population.

Finally, the results of the present study also indicate that increased ACTH-stimulated plasma 17-OHP levels can be detected in a considerable proportion of patients with unilateral and bilateral adrenal incidentalomas who do not have homozygous and heterozygous mutations of the CYP21 gene. The reason for increased 17-OHP levels in these patients remains unknown, but several possibilities can be considered. The mutation screening method used in our study
has been proved to detect homozygous normal, homozygous mutant or heterozygous genotypes for the eight commonly occurring mutations with the exception of heterozygous deletion (28, 29, 44), which could result in some underestimation of the occurrence of heterozygous carriers. However, it is unlikely that heterozygous deletions not revealed by our method significantly influenced the mutational spectrum detected in our patients. In our study, we also excluded the possibility that the exaggerated plasma 17-OHP response to ACTH in patients with no detectable germine CYP21 mutations was due to somatic mutations, since mutation screening in patients with unilateral adrenal incidentalomas revealed the same results in peripheral blood and tumoral DNA samples with no additional mutations in tumoral DNA. It may be relevant, however, that earlier studies showed down-regulation of 21-hydroxylase mRNA, if it occurs in the absence of 21-hydroxylase messenger ribonucleic acid expression in these tumors remains to be elucidated. Alternatively, it is also possible that mechanisms other than impairment of 21-hydroxylase activity should be considered, since decreased activity of the 11β-hydroxylase enzyme resulting in increased ACTH-stimulated plasma 17-OHP levels in patients with adrenal incidentalomas has already been documented (19).

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