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Abstract

Objective: Congenital adrenal hyperplasia (CAH) shows a range of severity which is explained in part by the different mutations of the CYP21 gene. To better understand the incomplete concordance between genotype and phenotype in CAH the role of the sensitizing N363S polymorphism of the glucocorticoid receptor (GR) was examined in CAH patients.

Design: CAH patients were screened for N363S. Laboratory findings and clinical characteristics of carriers and non-carriers were analyzed retrospectively.

Methods: The CYP21 gene of 200 CAH patients was analyzed by allele-specific PCR. The GR gene was tested for N363S by PCR followed by restriction fragment length polymorphism. Antropometric data (height, weight), degree of intrauterine virilization, hormone concentrations (17-OH-progesterone, dehydroepiandrosterone (DHEA), aldosterone, testosterone, plasma renin activity), substitution doses and clinical course were analyzed.

Results: The carrier frequency of N363S in CAH patients was equivalent to that of the general Hungarian population (6% vs 7.8%). Interestingly, none of the non-classical CAH (NC-CAH) patients were carriers of the polymorphism. Carrier girls had milder genital virilization than mutation-matched non-carrier controls. There was no significant difference between the carriers and non-carriers in either the substitution doses, the hormonal, or the auxiological parameters.

Conclusions: The association of sensitizing the GR variant with impaired cortisol production in CAH might be compensatory in mild NC-CAH and may prevent severe intrauterine virilization in classical form. Although the exact role of N363S in extrauterine life should be further investigated, the consideration of certain genetic polymorphisms of CAH patients may lead to better, individualized therapeutic regimes.

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Introduction

The most common form of congenital adrenal hyperplasia (CAH) is caused by the mutation of the 21-hydroxylase gene (1). Since the residual in vitro enzyme activity of the mutated 21-hydroxylase varies between 50% and zero (2), a broad spectrum of CAH phenotypes exist. Classically, three forms of CAH have been described: the salt-wasting (SW) type, being the most severe, life-threatening form, the simple virilizer (SV) and the least severe non-classical form (NC-CAH). However, our current understanding suggests that CAH is better described as a continuum of disorders (3) rather than three distinct forms. Several explanations have been suggested for this continuity. First, the residual enzymatic activity of compound heterozygotes, carrying mutations that differ in severity on the two alleles, is often different from that of classical homozygous mutations. Although the phenotype is determined predominantly by the less severely mutated allele (5), other genetic and environmental factors have a certain potential to modify the phenotype, for example androgen sensitivity, glucocorticoid sensitivity or 11β-hydroxysteroid dehydrogenase (HSD) activity (6). Additionally, there are a number of reports of genotype–phenotype non-concordance among siblings (7, 8).

Several polymorphisms of the glucocorticoid receptor (GR) gene have been described. Individuals carrying the N363S polymorphism of GR have been reported to respond with a larger suppression of serum cortisol in the dexamethasone (DEX) test, corresponding to higher in vivo glucocorticoid sensitivity of the hypothalamic–pituitary–adrenal (HPA) axis (9). Carriers of N363S polymorphism have been reported to be more prone to obesity (10); the relationship to other pathologic conditions such as diabetes, coronary heart disease or hypertension is more controversial (11–13).

We hypothesize that in states of insufficient cortisol production the individual variations of glucocorticoid sensitivity can modify the severity of the disease.
Therefore, our aim was to investigate the effect of the N363S polymorphism of GR on the phenotype of CAH patients.

**Methods**

**Patients**

**Genetic study of the population.** Two hundred pediatric CAH patients were involved. The CAH diagnosis was confirmed by both hormonal and genetic testing in each case. The sex ratio was 72 boys (46 SW, 16 SV and 10 NC-CAH) and 128 girls (66 SW, 39 SV and 23 NC-CAH). The carrier frequency of N363S polymorphism in Hungary was determined as being 7.8% in a previous study involving 102 random healthy adults (14).

**Phenotype analysis.** Twenty N363S non-carrier CAH patients were matched, as far as possible, to gender and the CYP21 allele class of the carriers (Table 1). The mutations were classified by the degree of enzymatic compromise according to the approach of Wedell et al. (15). CAH patients were classified as SW if signs of mineralocorticoid deficiency (i.e. dehydration, hyponatremia, and hyperkalemia) developed in the first months of life. The SV form was defined by the signs of precocious pubarche. NC-CAH was defined by signs of androgen excess presenting after the age of physiological pubarche.

All 200 CAH patients were treated and followed up by a single center. The case histories were analyzed retrospectively.

**Methods**

There was no neonatal screening for CAH in Hungary during the study period. Ambiguous genitalia were scored according to Prader at diagnosis (16). Auxological data of patients were compared to national standards.

**Results**

**Frequency of N363S polymorphism of GR among Hungarian CAH patients**

First, the allele frequency of N363S polymorphism of GR was tested in the Hungarian CAH population. Twelve of the investigated two hundred CAH patients carried the N363S polymorphism (6%; 6 boys and 6 girls), compared to 8 of 102 controls (7.8%). Comparison using chi square test showed, there was no significant difference in the overall carrier frequency ($P = 0.54$).

The classification of CAH patients to classical or non-classical forms roughly characterizes the severity of the disease. If classified into subtypes, all N363S carriers had classical CAH: 8 of the 12 carriers were SW, four were SV and none of them had NC-CAH, while in the whole study population 17% had NC-CAH (33/200) and 83% had classical CAH (Fig. 1). It is noteworthy that the whole study population exhibits an accurate representation of the Middle European frequency of CAH subtypes (20) and it is intriguingly different from that of the carriers ($P = 0.1$).

The predicted phenotype regarding the CAH subtype based on CYP21 mutations was different from the clinical phenotype in three carriers (Table 2). Patient 1 was predicted to have SW CAH; however, there was no evidence of salt wasting. Patient 1 was diagnosed at a very early age (day 1). As a result, the pre-treatment period was very short, thus making it difficult to make a proper assessment of electrolyte homeostasis. Patient 6 is a compound heterozygote for the intron 2 which is

**Table 1** Non-carrier controls matched to the carriers for gender and severity of mutation.

<table>
<thead>
<tr>
<th>CYP21 alleles (%)</th>
<th>Carriers</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (%)</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>A (%)</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>B (%)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>C (%)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>No. of patients</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Boys</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Girls</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

The classification of mutations is as follows: 0: Del, Q318X, R356W, L307TIns, ClusterE6, conv; A: Intron 2; B: I172N; C: V281L.
known to be associated with variable phenotypes. Patient 11 is a compound heterozygote for a mild (V281L) and a severe (Del) mutation. This constellation has previously been reported as being associated with both classical and non-classical phenotypes (21).

Since the frequency of N363S is higher in classical than in non-classical CAH (7.2% vs 0), this suggests that the carrier status of N363S affects the clinical severity of CAH. As a result, we further scrutinized the pre-treatment clinical characteristics and the treatment follow-up data of the patients.

**Pre-treatment clinical characteristics of carriers differ from the matched non-carrier controls**

We could not compare the carriers to the non-carriers as a whole, because the two groups were different in a number of important parameters: sex ratio (carriers: 6 boys and 6 girls vs non-carriers: 66 boys and 122 girls), ratio of non-classical patients (carriers: 0/12, non-carriers: 33/188) and allele frequencies (carriers: 0 58%, A 32%, B 5%, C 5% vs non-carriers: 0 27%, A 47%, B 19%, C 7%). Therefore, a control group was matched to the carriers as detailed in the Methods (Table 1). Compared to the matched controls, the most important difference was that carrier girls tended to have less severely virilized genitalia at birth (Fig. 2). A majority of the carrier girls had Prader 2 genitalia (clitoromegalia with no or minimal labial fusion) while most control girls had Prader 3 or 4 genitalia (large clitoromegalia with marked labial fusion).

However, there was no significant difference between carriers and controls in terms of age at diagnosis (carriers 2 months (1 day–10 years); controls 1 month (2 days–5.5 years)) (Table 2). At diagnosis all 17-OHP levels were in the pathological range, while carriers had lower 17-OHP levels than controls (366 ± 283 ng/ml vs 822 ± 727 ng/ml, $P = 0.09$). The ratio of SW patients was almost identical in the carrier (8/12) and the non-carrier (15/20) group ($P = 0.46$). There was no demonstrable difference between the growth pattern or the required glucocorticoid dose of the carrier and the non-carrier group.

The N363S polymorphism has previously been reported to be associated with obesity in elderly patients (10). Glucocorticoid treated CAH patients are often obese as an adverse effect of the substitution therapy. In the present study the prevalence of marked obesity defined by a body mass index (BMI) above the 97th percentile for age was the same in the carrier and the control group (6/20 and 4/12). Interestingly, the average age at the onset of obesity was higher in the carrier group (median 7 years vs 3.5 years).

**Discussion**

The present study scrutinizes the N363S polymorphism of the GR as one of the genetic factors responsible for the variability of the genotype–phenotype correspondence in CAH in the pre-treatment period.

**Table 2** Some important clinical features of the N363S carrier CAH patients. Phenotype prediction was made by the method of Wilson et al. (20).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>CYP21 mutation</th>
<th>Predicted phenotype†</th>
<th>Gender</th>
<th>Age at diagnosis</th>
<th>17-OHP at diagnosis (ng/ml)</th>
<th>Prader</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SV P30L, conv/Del</td>
<td>SW</td>
<td>F</td>
<td>1 day</td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>SW Del/Del</td>
<td>SW</td>
<td>M</td>
<td>3 weeks</td>
<td>860</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>SW I172N, V281L, ClusterE6, L307T, Q318X, R356N/Del</td>
<td>SW</td>
<td>M</td>
<td>1 month</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>4*</td>
<td>SV Intron 2/I172N</td>
<td>SV</td>
<td>F</td>
<td>4 years</td>
<td>519</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>SW Intron 2/Intron 2</td>
<td>SW</td>
<td>M</td>
<td>3 weeks</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SV Intron 2/Q318X</td>
<td>SW</td>
<td>M</td>
<td>2 months</td>
<td>170</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>SW Del/Del</td>
<td>SW</td>
<td>F</td>
<td>3 months</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>SW Intron 2/Intron 2</td>
<td>SW</td>
<td>M</td>
<td>1.5 years</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>SW Intron 2/Intron 2</td>
<td>SW</td>
<td>M</td>
<td>2 months</td>
<td>470</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>SW Del/Del</td>
<td>SW</td>
<td>F</td>
<td>1 month</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>SV V281L/Del</td>
<td>NC</td>
<td>F</td>
<td>10 years</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>SW Intron 2, V281L, Q318X, R356W/L307Tins</td>
<td>SW</td>
<td>F</td>
<td>1.5 months</td>
<td>700</td>
<td>4</td>
</tr>
</tbody>
</table>

*The female patients with intron 2/I172N mutation discussed in the text. †Italics indicate that the actual phenotype is different from the predicted.*
In most cases the severity of CAH is consistent with the compromise in enzymatic activity conferred by each mutation (2). Most patients are compound heterozygotes and they are likely to exhibit their less severely impaired allele. In the past there have been several attempts to predict the severity of symptoms from the mutations of the CYP21 gene alone; the overall correct classification rate was between 79% and 88% (22, 23). However, these studies have concentrated on the presence or absence of salt-wasting as the dominant feature of the CAH phenotype, although the other symptoms (e.g. degree of genital virilization, bone maturation, age at pubarche) also constitute a continuum. Excellent distinction was found between the severe mutant and the wild genotypes, but, importantly, a high degree of overlap was found between moderate genotypes and those either more or less affected (21). Several explanations have been proposed for the incomplete correspondence between genotype and phenotype. The role of certain CYP21 genotypes has been investigated in detail (e.g. the leakiness of intron 2; the marginal enzymatic activity of 1172N (5)). This has provided an explanation for some, but not all, of the reported problematic cases. Hence, we have investigated the role of a genetic factor other than CYP21.

The N363S polymorphism of the GR has been shown to slightly increase the sensitivity of the GR both in vitro and in vivo. In vitro, the N363S polymorphism has been shown to be associated with increased glucocorticoid sensitivity, resulting in an increased transactivating capacity, while having no influence on the repressing capacity of the GR in COS-1 cells (24). Also, a greater sensitivity to DEX in the carriers’ lymphocytes in mitogen-induced cell proliferation assay has been reported. However, the exact molecular mechanism is unknown. In vivo, in the 0.25 mg DEX test, larger cortisol suppression and higher insulin response have been seen in N363S carriers (9). In some studies, carriers had higher BMI (11) and lower bone mineral density (9), while other studies failed to find any difference (10, 13, 25).

In our study, carrier girls were less severely virilized than the matched controls. Although the difference failed to reach the usual level of statistical significance, as a result of the limited sample size, its biological and medical significance is immense. Even a small reduction in virilization notably improves the outcome of the feminizing genitoplasty which is a major component of the quality of life in adulthood. Its importance to patients is well illustrated by the fact that, although prenatal therapy often fails to completely prevent virilization, most mothers are ready to undergo the severe side-effects of prenatal dexamethasone therapy in order to prevent more severe prenatal virilization (1, 2).

Milder genital virilization is unlikely to be a result of the compound heterozygosity of CYP21 of the N363S carriers. Only one of the six carrier girls carried the intron 2 and/or the 1172N mutations (intron 2/1172N compound heterozygote); therefore, in the remaining cases the variable nature of these mutations did not contribute to this phenomenon. The mechanism of prenatal virilization in CAH is well known: the insufficient fetal adrenal gland is unable to produce enough cortisol to suppress the hypothalamus and the resulting elevated level of adrenocorticotropin (ACTH) stimulates excess androgen production, thereby causing prenatal virilization of female fetuses (1). However, the mechanism of virilization utilizes the same cortisol–ACTH–cortisol feedback loop as characterized by the DEX test. In terms of virilization, the early stages of pregnancy are very important, as known from studies of prenatal therapy (26). Recently it has been shown that in the first trimester a certain amount of maternal cortisol is able to cross the placenta (6). Theoretically, it is possible that this maternal cortisol is able to develop greater suppression of fetal ACTH production, thereby causing less early virilization in carrier fetuses. Later in pregnancy less maternal cortisol crosses the placenta and therefore this beneficial effect might decrease. In cases of partial compromise in enzymatic activity the small amount of fetal cortisol might act on the polymorph GR.

In our study, the presence of salt-wasting was able to be predicted accurately based on the mutation of CYP21. All actual deviations from the expected phenotype were explained either by the case histories or the mutation of the CYP21 gene. The N363S polymorphism seemed not to affect mineralocorticoid homeostasis, as was expected.

Obesity often complicates CAH therapy, although the exact cause of obesity is unknown (27). Reports of the association of N363S polymorphism with obesity are controversial (10, 11, 28). However, in our study the frequency of obesity was the same in the carrier and the non-carrier groups, but the onset of obesity was later in the carriers and seemed to be progressive during puberty. This suggests that N363S is associated with a
different pattern of obesity, developing with pubarche and being persistent through adult life, in contrast to the non-carriers who were obese from early childhood with no change at pubarche.

The carrier status of N363S might have direct implications for CAH therapy. In some cases it is more difficult to achieve good hormonal control than in others (2). Some patients might even need adrenalectomy to suppress adrenal androgen production (29). Combination therapies with glucocorticoid, mineralocorticoid, aromatase-inhibitor (testolactone), androgen receptor blocking drugs (flutamide) (30), 11β-HSD inhibitor (carbenoxolone) or growth hormone (31) have been tested. However, the exact role and indication of these drugs and approaches in the treatment of CAH is still a matter of debate. It should be considered that for carriers with greater glucocorticoid sensitivity lower doses of hydrocortisone therapy supplemented with antiandrogens or mineralocorticoids might be more appropriate to prevent obesity and minimize cardiovascular risk in adult life. However, further studies are needed to assess this possibility.

Our study is the first to raise the potential advantage of N363S polymorphism of GR in 21-hydroxylase deficiency. The N363S polymorphism ameliorates the disease outcome in CAH, prevents the early virilization of female fetuses and might modify the pattern of obesity. However, the allele frequency of this polymorphism is relatively low in the population and therefore our study might not be able to demonstrate any further differences because of the limited sample size. Investigation of genetic polymorphisms in CAH patients might lead us to a better understanding of phenotype–genotype non-concordances and may help us to develop individualized therapeutic regimens for our patients.

Acknowledgements

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References


