Incidence of Classical 21-Hydroxylase Deficiency and Distribution of CYP21A2 Mutations in Estonia

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Congenital adrenal hyperplasia · 21-Hydroxylase deficiency · Classical 21-OHD, incidence · CYP21A2 genotype

Abstract
Aims: To determine the incidence of classical 21-hydroxylase deficiency (21-OHD) in Estonia from 1978 to 2004, and describe their phenotype and genotype. Methods: All Estonian endocrinologists informed us about their patients with 21-OHD. The diagnosis was confirmed in 20 patients, who were all screened for 8 common mutations of the CYP21A2 gene. Results: The 27-year period incidence was 1:25,500. The incidence from 1992 was 1:16,100, which more accurately reflects the real situation in Estonia. The salt-wasting form (SW) was diagnosed in 14 (7 males) and the simple virilizing form in 6 patients (1 male). The median age at diagnosis of the SW form was 30 days in males and 2 days in females. The investigation of 34 unrelated alleles showed that a common deletion/conversion was the most frequent mutation in our group (7/34). Six other mutations were present: p.Ile172Asn (5/34), 8-bp deletion (3/34), intron-2 splice mutation (3/34), p.Arg356Trp (3/34), p.Gln318X (3/34) and a small conversion (2/34). Mutations in 8 alleles remained uncertain. Conclusions: The incidence of classical 21-OHD in Estonia in 1992–2004 was 1:16,100. The genotype of our patients is similar to those from other Caucasian populations. The relatively late age at diagnosis and the skewed female:male ratio supports the need for newborn screening for 21-OHD.

Introduction
Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders resulting from the deficiency of one of the five enzymes required for the synthesis of cortisol in the adrenal cortex. More than 90\% of the cases are caused by a 21-hydroxylase deficiency (21-OHD) \cite{1}. 21-OHD is divided clinically as a classical form of CAH, which encompasses the salt-wasting (SW) and simple virilizing (SV) forms, and a non-classical form. The incidence of the classical form of CAH ranges from 1:9,800 in Sweden \cite{2} to 1:23,000 in New Zealand \cite{3}. However, there are populations with a very high incidence of 21-OHD, such as the Alaskan Yupik Eskimos with 1:282 \cite{4}.

The majority of affected females with the SW form are born with ambiguous external genitalia and therefore are diagnosed soon after birth. In newborn males with the SW form, the clinical picture is less clear and therefore the diagnosis is often made during the first 2–3 weeks of life with symptoms of adrenal crises. Females with the SV
form may also have ambiguity of external genitalia, but are more likely to manifest with androgenization of external genitalia and are usually diagnosed during the first 2 years of life. Males with the SV form may not be diagnosed until presenting with precocious pseudopuberty.

The disease shows autosomal recessive inheritance and is caused by mutations in the CYP21A2 gene located on chromosome 6 (6p21.3). The majority of these mutations are derived from the neighbouring pseudogene CYP21A1P sequence, via gene conversion events. According to the Human Genome Mutations Database, there are more than 100 known CAH-causing mutations, though 8–10 of them are responsible for 80–90% of all the cases of CAH [5, 6]. Most of the studies have shown a good correlation between phenotype and genotype [5–7] with few exceptions [8, 9]. In particular, variations have been seen in patients with the intron-2 splice mutation (g.655A>C>G) (usually characteristic for the SW form) and with p.Ile172Asn (mostly found in patients with the SV form) mutations.

The aims of this study were to determine the overall incidence of classical forms of 21-OHD in Estonia and to describe their phenotype and genotype.

**Patients and Methods**

**Patients**

All members of the Estonian Endocrine Society were asked by E-mail (if no response, then by phone) to inform us about their patients with CAH. Clinical picture, time of diagnosis and elevated serum 17-OHP levels confirmed the diagnosis of classical forms of CAH in 20 patients. Age at diagnosis, clinical picture and maximum 17-OHP values at diagnosis (n = 14) or later (n = 6) were collected from the notes. A patient was classified as of Estonian origin if both parents and all the grandparents considered themselves Estonian. All the other patients were studied as a group of non-Estonians, the majority of them of Slavic origin. The number of live births per year from 1978 to 2004 were taken from the Statistics Estonia website.

**Mutation Analysis**

Blood samples of all 20 patients were screened for 8 common mutations using a panel of PCR ARMS tests. DNA was extracted from blood samples using the Puregene kit (Gentra Systems Inc.) and tested for 6 common CYP21A2 point mutations [p.Pro30Leu, g.655A>C>G (intron-2 splice site), p.Ile172Asn, p.Val281Leu, p.Gln318X, and p.Arg356Trp] using a series of ARMS PCR assays [10]. A further 2 common mutations, an 8 base pair (bp) deletion in exon 3 of CYP21A2 (g.707_714delGAGAGTAC) and a large deletion resulting in the formation of a ‘chimeric’ sequence – a fusion of 5’ pseudogene and 3’ functional gene (5’ CYP21A1P: 3’ CYP21A2) sequence were screened for by using two ARMS tests designed in-house at the National Genetics Reference Laboratory in Manchester, UK. Briefly, because the 5’ pseudogene sequence of the ‘chimeric’ sequence includes the exon 3 8-bp deletion, the method is based on the detection of the 8-bp deletion by ARMS primers either in the context of the pseudogene sequence (chimeric) or in isolation in the functional CYP21A2 gene (8-bp deletion). Two primary PCRs are performed in tandem using either a forward primer common to both the functional and pseudogene sequences (to detect the chimeric) (5’-GTTGCTGAACC-AGAGG-3’), or a primer annealing to only the functional gene (to detect 8-bp del) (5’-CAGGGTGCTTTAATTCATA-3’). In both cases, the reverse primer binds only the 3’ functional CYP21A2 sequence and is designed to span the sequence in exon 6, which in the pseudogene contains a cluster of three point mutations (5’-CCTCACGCTGTACATC-3’). Both secondary PCRs are identical involving ARMS primers to detect either the wild-type sequence in exon 3 (5’-AAAAAAAAAAGCTTTGCAGAGCAGRGACC-3’) or the 8-bp deletion (5’-CCGGTTTCCAGAGCAGRGACC-3’). R indicates an A/G: a ‘wobble’ position in the primers. The common CYP21A2 forward primer for the secondary ARMS reactions was 5’-TCAGTTCCCCACCCCTCAGC-3’ and the internal control reverse primer used to give an amplifier with the common primer of higher molecular weight to the ARMS products was 5’-CTACACAGAACCCTGTGTG-3’. 20-μl PCR reactions were carried out in Replinase buffer (1 M Tris–HCl, pH 9.0, 400 mM ammonium sulphate, 30 mM magnesium chloride) using Platinum Taq (Invitrogen) for the ARMS reactions. Reaction conditions for both primary and secondary PCRs were 94°C, for 15 min, followed by either 20 cycles (primary reaction) or 30 cycles (secondary reaction) of 96°C for 12 s, 60°C for 1 min, 72°C for 1 min, followed by a final extension of 72°C for 5 min. Thus a mutant band detected for the ‘chimeric’ reaction but not 8-bp reaction indicates a chimeric sequence, whereas a mutant band in both reactions indicates 8-bp deletion in functional CYP21A2 sequence. The allele frequency has been calculated from all 17 unrelated patients equalling 34 unrelated alleles. Due to insufficient DNA quality and/or quantity in the 7 unrelated patients, we could not perform the Southern blot analysis to determine whether the second allele has the same mutation as on the first allele (homozygosity) or if it carries a deletion/conversion (hemizygosity) which is not detected by our test.

**Genotype Groups**

All patients were divided into three different genotype groups proposed by Wedell et al. [11]. Group 0 contained mutations with complete loss of enzyme activity (deletions, conversions, deletion of 8 bp in exon 3, p.Gln318X, p.Arg356Trp). Group A contained patients who are carrying the intron-2 splice mutation (g.655A>C>G), which has been shown to result in low but measurable enzyme activity. Group B contained the p.Ile172Asn mutation, resulting in about 2% of normal enzymatic activity.

**Results**

**Incidence and Clinical Picture at Diagnosis**

Altogether, 20 patients with classical forms of CAH were identified in Estonia. This makes the incidence of classical 21-OHD in Estonia over the 27-year period (1978–2004) about 1 in 25,500 live births. However, the
incidence over the last 13-year period (1992–2004), after Estonia regained independence in 1991, was 1:16,100 per live birth. 14 patients (70%) were suffering from the SW form (7 males and 7 females) and 6 patients from the SV form (1 male, 5 females). 13 patients (65%) were Estonians, and the remaining 7 patients (35%) non-Estonians. This corresponds to the average proportion of nationalities in Estonia. When we looked at the distribution of clinical forms separately, we found the SW form more common in Estonians (12 out of 14) and the SV form in non-Estonians (5 out of 6). However, the number of patients was too small for a comparative analysis of different subgroups. There were two families with more than 1 affected sibling. In the first family (non-Estonian), both a brother and sister were affected by the SV forms, and in the second family (Estonian) a brother and 2 sisters were affected by the SW forms (table 1).

The median age at diagnosis of the SW form was 30 days in males and 2 days in females. One boy with the SW form was diagnosed at the age of 1 year: during the first year of life he had several long-lasting and severe infections, some of them with hypoglycaemic episodes indicative of an addisonian crisis. Four females and all 7 males with the SW form presented with signs of adrenal crisis (hyperkalaemia and hyponatraemia). All females with SW had clitoromegaly (table 1).
Mutation Analysis

Seven different CYP21A2 mutations were found in 34 alleles of all 17 unrelated patients (table 1). The most frequent mutation was a deletion/conversion (chimeric) occurring in 7 alleles, 4 of them in unrelated Estonians. p.Ile172Asn was the most common point mutation occurring in 5 alleles, 4 of them in unrelated non-Estonian patients. The intron-2 splice-mutation (g.655A>C), 8-bp deletion (g.707_714delGAGACTAC), p.Arg356Trp and p.Gln318X occurred each in 3 alleles. Two mutations were small conversions involving 4 sequential point mutations (p.Ile172Asn, p.Gln318X, p.Val281Leu, p.Arg356Trp) in the 3′ end of the gene. In the 7 unrelated patients we were not able to distinguish whether the second allele has the same mutation as on the first allele (homozygosity) or if it carries a deletion/conversion (hemizygosity) which is not detected by our test (table 2). Patient 20, who was heterozygous for p.Gln318X, had some other mutation not included in the panel on her second allele. The mutational spectrum of 34 unrelated alleles is given in table 2.

There were 10 patients in group 0, 6 in group B and 3 in group A. We were unable to determine the genotype group of patient 20 (table 1). As the patient has not been fully characterized, one cannot exclude that she is heterozygous for a milder mutation.

Discussion

This is the first study describing the incidence, phenotype and genotype of classical forms of CAH in Estonia. All patients with a diagnosis of the classical form of CAH agreed to participate in the study. Thus, the incidence, calculated on the number of clinically diagnosed cases, reflects the real situation in the country. Brosnan et al. [12] showed no significant difference in the incidence based either on clinically diagnosed cases (1:17,396), or in the results of the screening programme (1:15,974) in three states of the USA. The biggest survey from the 6.5 million newborn infants screened in 13 countries gave an overall incidence of the classical form of CAH of 1 in 15,000 live births [13, 14], which is higher than the 1:25,500 we found over the 27-year period (1978–2004), but very similar to the 1:16,100 found in the last 13 years. One of the reasons for the discrepancy between these two periods may be the fact that after regaining our independence in 1991, about 200,000 people emigrated from Estonia in the following years and therefore some patients with CAH might also have left. According to the data from the Statistics Estonia Death Register, there have been no lethal cases from adrenal disorders since 1997, but computerized data from previous years are not available. Thus, it is possible that there might have been some lethal cases of the SW form in previous years. Third, and not least important, is the improvement of general knowledge of the disease and our diagnostic standards, especially after serum 17-OHP assays became routinely available in Estonia in 1989. The skewed female: male ratio in the SV group (5 females and 1 male) indicates that there could be some undiagnosed male cases of the SV form in Estonia, particularly from the late 1970s and 1980s when the SV form was diagnosed in 3 girls, but not in any boys. Therefore, it is likely that the incidence of CAH over the last 13 years (1:16,100) reflects more accurately the real situation in Estonia. This is slightly smaller than the figure from Sweden, where the incidence of CAH based on clinically diagnosed cases was 1:11,500 [2].

In our study the boys received their diagnosis at the median age of 30 days, which is later than the 21 days reported in Sweden before the introduction of a newborn screening programme [2]. However, in countries with

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Alleles found</th>
<th>Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del/conv&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>20.6</td>
</tr>
<tr>
<td>Small conversion</td>
<td>2</td>
<td>5.9</td>
</tr>
<tr>
<td>p.Ile172Asn</td>
<td>5</td>
<td>14.7</td>
</tr>
<tr>
<td>p.Arg356Trp</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>p.Gln318X</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>Intron-2 splice</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>8-bp deletion</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>Uncertain alleles</td>
<td>8</td>
<td>23.5</td>
</tr>
</tbody>
</table>

The frequency is calculated from all unrelated alleles (n = 34).

- Only a chimeric form of a deletion or a large gene conversion was detected.
- Homozygosity was not distinguished from hemizygosity due to a lack of parental samples. The uncertain allele may involve the same mutation or a deletion/conversion mutation not detected by our test.

Table 2. The mutational spectrum of CYP21A2 among the patients with a 21-OHD in Estonia
We were surprised to find differences in clinical forms and mutational spectrum between Estonians and non-Estonians. The Estonian population has been influenced by different waves of migration from Europe (Germany, Sweden, Denmark) and Russia. The comparison of mitochondrial DNA in Estonians has shown similarity with Western European, mostly Scandinavian, countries [17, 18] and also with the South-Western Russian population [Prof. A. Metspalu, pers. commun.]. The studies of other genetic diseases (cystic fibrosis, phenylketonuria) in Estonia have shown that mutation frequency in Estonian and Russian patients is quite similar [19, 20]. The spectrum of CYP21A2 gene defects in Russian patients was similar to those reported in other Caucasian populations [15]. The ratio between the SW and SV forms in non-Estonian unrelated patients (2:4) is very unusual and may be just due to the small number of patients in this group. It is important to underline that childhood mortality and morbidity in Estonia does not differ significantly between Estonians and non-Estonians. Therefore it is unlikely that we have missed the SW forms only in non-Estonian children. Thus, the differences in phenotype and genotype between Estonians and non-Estonians are most likely due to the sample size rather than true distributions.

The genotype-phenotype correlation in our study was good. In groups 0 and A, there was no phenotypic variance as expected. In group B, where the predicted residual enzyme activity is about 2%, genotype-phenotype correlation is variable [1, 7, 8]. Most commonly, group B is associated with the SV form of CAH [8, 11]. In our study there were 5 patients with the SV form and 1 with the SW form, which were classified in group B. This SW patient (No. 18 in table 1) had the p.Ile172Asn/8bp del genotype. The detection of a wild-type allele at position g.999, as well as the mutated allele (i.e. p.Ile172Asn), was indicative of a possible duplication of CYP21A2. The presence of the 8-bp deletion in trans (i.e. on the other chromosome) to the point mutation would normally destroy a primer binding site for the ARMS assay on the chromosome carrying the 8-bp deletion. This would not lead to the corresponding wild-type amplimer from the p.Ile172Asn ARMS test. Clinically she had clitoromegaly at birth and during the second week of life she developed adrenal crisis with hyponatraemia and hyperkalaemia. A similar mutation (p.Ile172Asn/8bp del) in SW patients has also been reported by Krone et al. [21]. They also described a marked divergence in group B, where 26% of the patients had the SW form.
Conclusion

This is the first study reporting the incidence of classical 21-OHD in Estonia. The mutational spectrum of the CYP21A2 gene is similar to those from other Caucasian populations. The relatively late age of diagnosis and the skewed female:male ratio supports the need for newborn screening for CAH in Estonia.

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