0021-972X/99/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 84, No. 5
Printed in U.S.A.

Genotyping Is a Valuable Diagnostic Complement to Neonatal Screening for Congenital Adrenal Hyperplasia due to Steroid 21-Hydroxylase Deficiency*

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ABSTRACT

To evaluate genotyping as a diagnostic complement to neonatal screening for congenital adrenal hyperplasia, 91 children who had been diagnosed with this condition between 1986 and 1997 were analyzed for mutations in the steroid 21-hydroxylase gene. Screening levels of 17-hydroxyprogesterone were compared in patients representing different genotypes. Genotyping was performed using allele-specific PCR, the patients were divided into four groups according to the severity of their mutations, and screening results were compared between these groups as well as with 141 values representing false positive samples. The screening levels of 17-hydroxyprogesterone were significantly different in the five groups of samples. Values above 500 nmol/L were clearly associated with the most severe genotypes, whereas conclusions concerning disease severity could not be drawn from individual samples representing lower levels. For example, values around 150–200 nmol/L could be seen in children with all degrees of disease severity and could also constitute false positive samples. We conclude that genotyping is a valuable diagnostic tool and a good complement to neonatal screening, especially in confirming or discarding the diagnosis in cases with slightly elevated 17-hydroxyprogesterone levels. An additional benefit is that it provides information on disease severity, which reduces the risk of overtreatment of mildly affected children. (*J Clin Endocrinol Metab* **84:** 1505–1509, 1999)

CONGENITAL adrenal hyperplasia (CAH) is a group of inherited disorders caused by a deficiency in one of the enzymes necessary for the synthesis of cortisol in the adrenal cortex. More than 95% of all cases of CAH are due to 21-hydroxylase deficiency (21-OHD) (1, 2). This enzyme deficiency results in a reduced ability to synthesize cortisol and aldosterone. Reduced levels of cortisol lead to increased secretion of ACTH from the pituitary, which causes hyperplasia of the adrenals and an increase in steroid precursors that are shunted into the androgen-synthesizing pathway resulting in oversecretion of androgens.

The syndrome comprises a very wide spectrum of severity (1). The most severe form, with complete absence of 21-hydroxylase function, leads to acute circulatory collapse, usually during the first weeks of life. This salt-wasting (SW) crisis is due to a severe lack of mineralocorticoid, which causes loss of sodium in the kidney. Children with the SW form of the disease are exposed to excessive androgen levels during embryogenesis. The girls present with virilization of the external genitalia, *i.e.* clitoromegaly, fusion of the labia majora, and a common urethral and vaginal opening. A slightly less severe form of 21-OHD, with prenatal virilization but without life-threatening salt loss, is usually referred to as the simple virilizing (SV) form of CAH. Finally, milder forms of 21-OHD are associated with slightly elevated androgen levels, which are not sufficient to cause prenatal genital malformations. These forms of the disease may present as precocious pseudopuberty or growth acceleration in childhood or may cause menstrual disturbances, hirsutism, and infertility in adult women. They are often referred to as nonclassical (NC) CAH.

CAH due to 21-OHD is inherited as an autosomal recessive trait, and the molecular genetics of the disorder has been studied extensively. The 21-hydroxylase gene (*CYP21*) is located on the short arm of chromosome 6, between the human leukocyte antigen class I and class II gene clusters, in tandem with a highly homologous pseudogene (*CYP21P*) (3, 4). This genomic structure predisposes to misalignment during meiosis, which may result in recombination events between the *CYP21* and *CYP21P* genes. This can lead to deletion of *CYP21* as well as to an exchange of sequences between the two genes (5, 6). Deletion of *CYP21* together with nine smaller, pseudogene-derived sequence aberrations are responsible for around 95% of all affected Scandinavian *CYP21* alleles (7) and are also responsible for the majority of CAH patients in other ethnic groups (8–11). The remaining 5% of cases are due to rare, mostly population-specific mutations. In Scandinavia, we have identified 12 such rare mutations to date. Five are nonsense, splice, or frameshift mutations and have therefore not been assayed functionally *in vitro* (12–14). Seven are missense mutations that have displayed variable

Received September 1, 1998. Revision received January 13, 1999. Accepted January 25, 1999.

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* This work was supported by the Swedish Medical Research Council (Projects 12198 and 4792); the Fredrik and Ingrid Thuring, Harald and Greta Jeansson, Novo Nordisk, Magnus Bergvall, and Sven Jerring Foundations; and the Ronald McDonald Pediatric Fund.

degrees of impaired enzyme function after expression in cultured cells (15–17).

We have found clear relationships between clinical disease severity and the *CYP21* mutations (7). In short, one group of mutations completely inactivates the gene and is referred to as Null mutations. Homozygosity or compound heterozygosity for any of these mutations is associated with the most severe form of CAH, with salt loss and prenatal virilization of external genitalia in females. The I2 splice mutation can be regarded as slightly less severe. It is associated with severe prenatal virilization in females, but a small number of children with this mutation have escaped salt loss. The $Ile^{172}Asn$ mutation is associated with the simple virilizing phenotype, although around 10% of cases with $Ile^{172}Asn$ develop salt-wasting symptoms, and the degree of virilization can vary. Finally, the $Val^{281}Leu$ mutation has in our experience not been found in any patient with virilized genitalia. Instead, it is associated with mild, late-onset symptoms. In conclusion, we find genotyping of *CYP21* mutations to be very useful for the prediction of clinical outcome in CAH patients. Similar experiences were reported previously (11), whereas others have concluded that the phenotype is not always concordant with the genotype (10).

Neonatal screening for CAH started on a national basis in Sweden in 1986 (18). Elevated levels of 17-hydroxyprogesterone (17-OHP) are used as an indicator of the disease (19, 20). By the end of 1997, more than 1.3 million Swedish children had been screened. The prevalence of CAH among these subjects was 1 in 9800, and 85% of the patients had the SW form of the disease (21). No children with other causes of CAH, such as 3β-hydroxysteroid or 11β-hydroxylase deficiency, were diagnosed during these years.

There are several obvious advantages of neonatal screening for CAH, such as earlier diagnosis, avoidance of salt crises, and an earlier correct gender assignment (22, 21). At the same time, however, it has raised a few novel questions, such as how to interpret a neonatal 17-OHP screening value in terms of disease severity. In particular, we have seen a tendency for overtreatment of mildly affected cases that have been diagnosed neonatally through screening. This is a risk when treatment is initiated before symptoms have had time to develop and, thus, the disease severity is not clinically assessed. On the other hand, it is one of the benefits of screening that salt loss and its potentially harmful effects on central nervous system development can be avoided. To evaluate genotyping as a diagnostic complement to the neonatal screening for CAH, we have compared *CYP21* genotypes and neonatal screening values of 17-OHP for 91 Swedish patients diagnosed with CAH between the years 1986 and 1997. Comparisons were also made to screening values of 17-OHP for 141 false positive cases.

Subjects and Methods

Neonatal screening for CAH

17-OHP was analyzed in filter paper blood spots collected on day 3, 4, or 5 after birth using RIA (1986–1990) or fluoroimmunoassay (1991–1997) (Delphia, Wallac, Finland) (19, 18). The cut-off limit for a positive test has gradually been lowered from 200 nmol/L in 1986 to 150 nmol/L in 1988, and, since 1991, it has been defined as 75 nmol/L plasma (assuming a hematocrit of 50%) for full-term infants. For infants born before the 37th week of gestation, a cut-off limit of 200 nmol/L after ether extraction was used. Recall was on the eighth day of life (median) (21). A second filter paper sample was requested for all cases with a positive screening value. The second sample was usually analyzed about 1 week after the first screening sample. Children in whom 17-OHP levels had decreased convincingly in the second sample (>20%) and/or in whom subsequent clinical examination excluded CAH were defined as false positive cases.

CYP21 genotyping

CYP21 mutation analysis was carried out using allele-specific PCR from genomic DNA prepared from venous blood samples (13). This detects the 95% of alleles that carry any of the common pseudogene-derived mutations. Additional rare alleles were characterized by direct DNA sequencing (12). Genotypes were divided into four groups depending on the mildest mutation: Null, I2 splice, $Ile^{172}Asn$, and $Val^{281}Leu$.

Statistical analysis

The Kruskal-Wallis test, paired *t* test, Mann-Whitney U ranking sum test, and the SPSS computer program (SPSS, Inc., Chicago IL) were used in the statistical analysis.

Results

A total of 114 children were diagnosed with CAH in Sweden during 1986–1997. *CYP21* mutation analysis was performed for 107 of these children. Only 2 patients had the $Pro^{30}Leu$ mutation, 1 had the unique $Gly^{291}Ser$ mutation, and 2 had combinations of more than 1 partially inactivating mutation in the same allele ($Ile^{172}Asn+Pro^{453}Ser$ and I2 splice+$Ile^{172}Asn$). These patients were not included in the statistical analyses. Four infants were excluded because of prematurity (<37 weeks gestation), and 2 patients had their screening samples taken on day 1 after birth and therefore were not included. Five girls who had been diagnosed prenatally and treated with dexamethasone *in utero* until term were also excluded.

The genotypes of the remaining 91 full-term infants who were diagnosed with CAH are summarized in Table 1. The Null genotype group consisted of 22 infants. The I2 splice group comprised 26, and the $Ile^{172}Asn$ group consisted of 30 infants. Thirteen infants belonged to the mildest genotype group, represented by the $Val^{281}Leu$ mutation. A second sample was obtained before steroid treatment was initiated for 19 infants in the $Ile^{172}Asn$ group and 6 in the $Val^{281}Leu$ group. The second sample was obtained at about 8–12 days of age.

Screening values for all full-term infants born between 1991–1997 with false positive screening tests were used for comparison (n = 141). A second sample was obtained from 126 of these children. Genotyping was performed in 13 of the false positive subjects. No *CYP21* mutations were found in any case.

The 17-OHP screening values for the CAH children ranged from 41–1371 nmol/L plasma. Screening values of 17-OHP were significantly different among the three genotype groups, Null, I2 splice, and $Ile^{172}Asn+Val^{281}Leu$ ($P < 0.05$; Fig. 1). The two mildest groups did not differ from each other, but were different from the false positive group. Therefore, 17-OHP values representing the second sample, from the recall filter papers, were compared among the $Ile^{172}Asn$, $Val^{281}Leu$, and false positive groups (Fig. 2). The groups

TABLE 1. *CYP21* genotypes of 91 Swedish full-term infants diagnosed with CAH between 1986 and 1997

Genotype group	N1	N2	Genotypes included in the group	n
Null	22		Null[a]/Null[a]	22
I2 splice	26		I2 splice/Null[a]	17
			I2 splice/I2 splice	6
			I2 splice/Null[a] or I2 splice/I2 splice	3
$Ile^{172}Asn$	30	19	$Ile^{172}Asn$/Null[a]	7
			$Ile^{172}Asn$/I2 splice	14
			$Ile^{172}Asn/Ile^{172}Asn$	9
$Val^{281}Leu$	13	6	$Val^{281}Leu$/Null[a]	5
			$Val^{281}Leu$/I2 splice	5
			$Val^{281}Leu/Ile^{172}Asn$	2
			$Val^{281}Leu$/Null[a] or $Val^{281}Leu/Val^{281}Leu$	1

[a] The different completely inactivated alleles that were included in the Null group were: *CYP21* deletion, Gln^{318}stop, Arg^{356}Trp, Arg^{356}Pro, I1 splice, Arg^{483}GGtoC, cluster E6+Val^{281}Leu (two mutations in the same allele), $Val^{281}Leu+Leu^{307}$insT, $Leu^{307}insT+Gln^{318}$stop, Gln^{318}stop+Arg^{356}Trp, and $Ile^{172}Asn$+Cluster E6+$Val^{281}Leu+Leu^{307}insT+Gln^{318}stop+Arg^{356}$Trp (six mutations in the same allele). N1 denotes the number of subjects from whom a first (days 3–5) screening sample was available. N2 denotes the number of subjects from whom a second (days 8–12) screening sample was available.

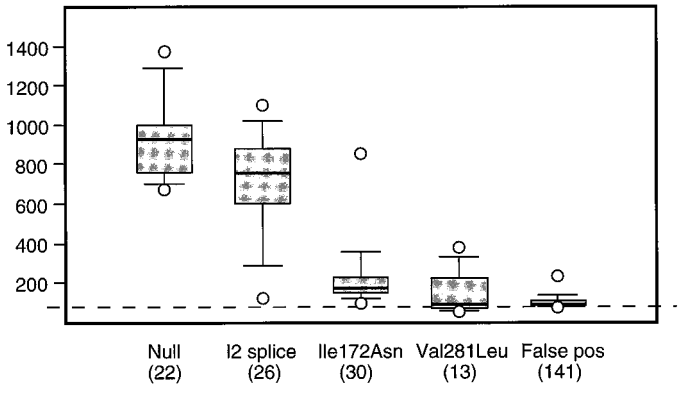

FIG. 1. Neonatal screening values of 17-OHP in relation to the *CYP21* genotype group. Screening values of false positive cases are also included. The box plots show median values together with the 10th, 25th, 75th, and 90th percentiles. In addition, maximum and minimum values in each group are indicated by *circles*. The cut-off limit for a positive test (75 nmol/L) is indicated by the *dashed line*. The four groups, Null, I2 splice, $Ile^{172}Asn+Val^{281}Leu$, and false positive, were significantly different from each other ($P < 0.05$, by Kruskal-Wallis test and Mann-Whitney U ranking sum test).

representing the $Ile^{172}Asn$ and $Val^{281}Leu$ mutations were then significantly different from each other ($P < 0.05$) as well as from the false positive group.

Five CAH girls were not included in the calculations because they had been treated prenatally with dexamethasone until term. Three of them belonged to the Null group and had screening values of 711, 985, and 750 nmol/L; two belonged to the I2 splice group and had values of 279 and 727 nmol/L. Thus, the prenatal treatment only marginally affects neonatal 17-OHP levels. The Scandinavian experience regarding the overall outcome of prenatal treatment has been reported previously (23).

As a rule, the full-term infants with false positive screening tests had only moderately elevated 17-OHP levels, in the same range as those in patients with genotypes associated with the SV or NC forms of the disease (Fig. 1). Among the children with false positive tests, there were 2 infants for whom the second sample showed an increased 17-OHP level compared to the first (97 to 154 and 104 to 175 nmol/L) and 4 who had unchanged levels in the second sample. In 3 of these 6 cases, including the 2 with increased values, *CYP21* mutations were excluded by genotyping. In an additional 7 infants, the 17-OHP value did not decrease convincingly. Thus, in 10.3% of the false positive cases (13 of 126), the second sample was not conclusive. A relatively large group of these infants (45%) was born in gestational week 37. This is in keeping with the fact that immature infants have higher levels of 17-OHP in the screening. The symptoms of these children are not known to the screening laboratory in any great detail. However, a large proportion of these children seem to have been under perinatal stress, *e.g.* they had respiratory distress, intracranial hemorrhage, sepsis, or cardiac malformation with cardiac failure.

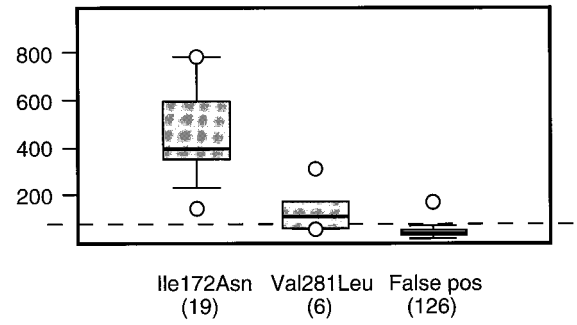

FIG. 2. Neonatal screening values of 17-OHP, second sample, in relation to *CYP21* genotype groups $Ile^{172}Asn$ and $Val^{281}Leu$. Screening values of false positive cases are also included. The box plots show median values together with the 10th, 25th, 75th, and 90th percentiles. In addition, maximum and minimum values in each group are indicated by *circles*. The cut-off limit for a positive test (75 nmol/L) is indicated by the *dashed line*. The three groups were significantly different from each other ($P < 0.05$, by Kruskal-Wallis test and Mann-Whitney U ranking sum test).

The genotypes of six children diagnosed with CAH between 1988 and 1997 despite negative screening values were also determined and compared to their neonatal 17-OHP screening values (Table 2). Four of these children with false negative screening tests had genotypes associated with the mildest form of CAH (mutation $Val^{281}Leu$). None of these had any symptoms from birth. One boy had the $Pro^{30}Leu$ mutation. He was diagnosed neonatally because his elder sister was known to be affected with CAH. The sister, who was born before the neonatal screening program was initiated, was detected due to growth acceleration, premature pubarche, and modest clitoral enlargement at 3.8 yr of age. The $Pro^{30}Leu$ mutation is known to be associated with nonclassical CAH, but it usually produces somewhat more symptoms, including slight clitoromegaly, compared to the $Val^{281}Leu$ mutation (24). One girl had the $Ile^{172}Asn$ mutation, which is associated with the simple virilizing form of CAH. When she was diagnosed at 2.5 yr of age, signs of prenatal virilization were found (clitoromegaly and moderate labial fusion). These malformations, which had been missed neonatally, were subsequently surgically corrected. Thus, the

TABLE 2. CAH patients with false negatives screening samples

Yr of birth	Sex	CYP21 genotype	17-OHP screening value (nmol/L)	Age at diagnosis (yr)
1985	F	$Ile^{172}Asn$/I2 splice	71	2.5
1987	F	$Val^{281}Leu/Ile^{172}Asn$	61	5.7
1988	M	$Val^{281}Leu$/I2 splice	55	Neonatal[a]
1989	F	$Val^{281}Leu$/Null	66	8.1
1991	F	$Val^{281}Leu/Ile^{172}Asn$	50	4.3
1991	M	$Pro^{30}Leu$/Null	41	Neonatal[a]

[a] No symptoms, diagnosed by more extensive laboratory measurements during the first weeks of life despite negative screening values due to elder sibling with nonclassic CAH.

severity of symptoms of the false negative children at diagnosis was in conformity with their genotypes.

Discussion

Neonatal screening for CAH has several obvious benefits, including earlier diagnosis, avoidance of salt crises, and an earlier correct gender assignment (22, 21). At the same time, however, because the diagnosis is made before the onset of overt clinical symptoms, it has raised a few novel questions, such as how to interpret a neonatal 17-OHP screening value in terms of disease severity. As shown in Fig. 1, a severely elevated screening value of 17-OHP was clearly associated with a genotype in which Null or I2 splice was the mildest mutation. In fact, apart from one child with an $Ile^{172}Asn$/Null genotype who had a screening value of 852 nmol/L, all children in the $Ile^{172}Asn$ and $Val^{281}Leu$ groups had values below 400 nmol/L. The girl with a level of 852 nmol/L was initially taken for a boy and started to show reduced serum sodium levels before treatment was initiated; she thus belonged to the most severely affected children seen in the $Ile^{172}Asn$ genotype group. For the above reasons, we believe that a screening value above 500 nmol/L can be regarded as evidence of the most severe forms of CAH. However, with regard to lower screening levels of 17-OHP, no safe conclusions about disease severity can be drawn, even though the genotypes were significantly different from each other when grouped. For example, a value around 150–200 nmol/L was seen in relation to all degrees of disease severity, from salt wasting to mild CAH (genotype groups I2 splice, $Ile^{172}Asn$, and $Val^{281}Leu$), and could even constitute a false positive test. For infants with moderately elevated 17-OHP levels, it is important that the diagnosis is also indicated by other laboratory tests or clinical signs before treatment is started to avoid excess use of steroids.

One consequence of introducing neonatal screening for CAH that must be considered is the fact that patients who would otherwise be diagnosed due to late-onset symptoms will now be identified from birth. It cannot be taken for granted that these patients will only benefit from being treated from this early age, before the onset of symptoms. In fact, we have seen a tendency for overtreatment of these mildly affected cases from birth because the degree of disease severity has not been clear. Verifying a mild genotype (*e.g.* the $Val^{281}Leu$ mutation) means that the risk of salt loss is extremely low. In addition, there have been observations indicating that early growth may not be increased in these patients until after 18 months of age (25). Thus, with careful clinical monitoring, including measurements of bone age, height, growth velocity, and steroid levels, glucocorticoid replacement therapy can be kept to a minimum in children in whom the mildest mutations are identified. In addition to these somatic examinations, however, it may also be important to consider possible influences of excess steroid hormones on behavior (26).

The three genotype groups, Null, I2 splice, and $Ile^{172}Asn+Val^{281}Leu$, were already significantly different from each other in the first screening sample (days 3–5). The $Ile^{172}Asn$ and $Val^{281}Leu$ groups could clearly be separated by the second measurement, which was obtained around days 8–12. Thus, a more obvious increase in 17-OHP evolves over time in the moderate $Ile^{172}Asn$ group compared to the milder $Val^{281}Leu$ group. Increasing 17-OHP values with time was also seen in the screening program in Texas, where two screening samples were taken routinely. A relatively large proportion of patients affected by milder forms of CAH was identified with the second sample, which was obtained at 2 weeks of age (27).

We also compared 17-OHP levels after subdividing the patients according to the different genotypes within the four major groups, *i.e.* we compared individuals who were homozygous and hemizygous for the mutations. Hemizygosity (I2 splice/Null: median, 746 nmol/L; $Ile^{172}Asn$/Null: median, 222 nmol/L) was associated with higher screening values than homozygosity (I2 splice/I2 splice: median, 710 nmol/L; $Ile^{172}Asn/Ile^{172}Asn$, median, 152 nmol/L), indicating a gene dosage effect. These differences were not statistically significant, however.

The relatively large interindividual spread in screening values is not surprising, as it is well known that individual 17-OHP values are affected by factors such as concurrent illness and stress. One patient in the I2 splice/Null subgroup was delivered by elective cesarean section because he was in the breech position. This patient had the lowest 17-OHP screening value within his subgroup (186 nmol/L). This is not surprising because it has been reported that early corticosteriod levels are influenced by birth conditions (28).

The patients who had the $Pro^{30}Leu$ mutation, which is known to be associated with nonclassical CAH (24), were not included in our calculations because only two cases were found with genotypes including this mutation. These two patients were both males, both had a $Pro^{30}Leu$/Null genotype, and both were born in 1991. Their screening values were considerably different from each other; one had a value of 41 nmol/L and was thus a false negative case (Table 2), whereas the other one had a value of 419 nmol/L. We have previously found that some patients with $Pro^{30}Leu$ have gene conversions in their promoter, changing *CYP21* sequences into pseudogene-derived sequences, whereas others retain the *CYP21* promoter (unpublished). As the *CYP21P* promoter is known to be partially active compared to that of *CYP21* (29), patients with $Pro^{30}Leu$ in combination with the promoter mutation can be expected to be more severely affected than those with $Pro^{30}Leu$ alone. However, we sequenced the *CYP21* genes of the two boys who had $Pro^{30}Leu$ up to 800 bases upstream of translation initiation. Both patients had the *CYP21P* promoter in the *CYP21* allele that carried $Pro^{30}Leu$, *i.e.* the complete 5′-parts of *CYP21* were converted into *CYP21P* in both cases. Thus, we could not

find any explanation for the large difference in their screening values.

For 13 infants with positive screening samples who were later shown to be unaffected, *i.e.* in 10.3% of the false positive cases, the second screening sample was inconclusive. As *CYP21* genotyping identifies 95% of all alleles causing CAH, and the majority of the remaining 5% are Null mutations, mutation analysis is a reliable as well as rapid way of discarding or verifying the diagnosis of CAH in these cases. It is important to shorten the time of uncertainty as much as possible to minimize the negative psychological effects on the family of a false positive screening result (30).

In Sweden, genotyping is performed for most, if not all, children in whom positive screening samples of 17-OHP are found. We find this to be an ideal diagnostic complement, both in providing prognostic information on the degree of disease severity as well as in discriminating against false positive cases. However, as shown in Table 2, some patients with mild and even moderate forms of the disease will invariably escape diagnosis in the screening unless it is designed to produce unacceptable numbers of false positive results. Obviously, the well-being of these children will continue to be dependent on the perceptiveness of clinicians who are skilled at identifying symptoms and signs of androgen excess.

Acknowledgment

We thank Nils Larsson for advice on the statistical analyses.

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