21-hydroxylase deficiency: an exemplary model of the contribution of molecular biology in the understanding and management of the disease

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INTRODUCTION

The biosynthesis of cortisol, a hormone necessary for survival, occurs in the adrenal glands under the stimulus of adrenocorticotropic hormone (ACTH). The biosynthesis of all adrenal steroids is regulated by a negative feedback loop, but among all steroid hormones produced by the adrenals, cortisol is the only one to exert a significant feedback control on ACTH secretion. Thus, when cortisol secretion is insufficient, whatever the cause, the feedback loop opens and ACTH rises. There are five enzymes necessary for the biosynthesis of cortisol from cholesterol (fig. 1). A defect in any of these enzymes results in congenital adrenal hyperplasia (CAH). These disorders are so named because the adrenal glands are hyperplastic at birth due to unrestrained ACTH stimulation already in fetal life. However, deficiency in steroid 21-hydroxylase is by far the most frequent cause of CAH (≥90%).

The disease has two major consequences, a state of cortisol deficiency and an hyperproduction of adrenal androgens due to ACTH hyperstimulation (fig. 2). Steroid 21-hydroxylase deficiency (21-OHD), a monogenic autosomal recessive disorder, is one of the commonest metabolic disorders [19, 21, 26]. 21-OHD has a wide spectrum of clinical variants (fig. 3), which are not different diseases, but represent points of a spectrum of disease.

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Congenital adrenal hyperplasia (CAH) is a family of autosomal recessive disorders caused by mutations in genes encoding the enzymes involved in one of the various steps of adrenal steroid synthesis. Steroid 21-hydroxylase deficiency (21-OHD) is responsible for over 95% of the 5 forms of CAH, and results due to enzymatic defect owing to mutation in the CYP21 gene. The disease has two major clinical presentations. The “classical” form is severe, and divided into a salt wasting (SW) and simple virilizing (SV) subgroups. In both, affected female fetuses undergo virilization of the external genitalia.

Deficit en 21-hydroxylase : l’intérêt de la biologie moléculaire dans la compréhension de la maladie et la prise en charge des patients

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L’hyperplasie surrénale congénitale(CAH) correspond à une famille de maladies autosomales récessives ayant pour origine une mutation de gènes codant des enzymes participant à l’une des diverses étapes de la synthèse des stéroïdes surrénaux. Le déficit en stéroïde 21-hydroxylase (21-OHD) est responsable de plus de 95 p. 100 des cinq formes de CAH et résulte d’un déficit enzymatique par mutation du gène CYP21. Il existe deux présentations cliniques principales. La forme « classique » est sévère avec deux sous groupes de « salt wasting » (SW) et virilisants (SV). Dans les deux, le foetus féminin présente une virilisation des organes génitaux externes avec ambiguïté sexuelle à la naissance. Chez les deux sexes, il existe un risque vital dans la forme SW de CAH en cas de crise surénalienne. Le diagnostic anténatal est facilité par le dosage de 17-hydroxyprogésterone (17-OHP) dans le liquide amniotique ou la forme SW de CAH en cas de crise surénalienne. Le diagnostic anténatal est facilité par le dosage de 17-hydroxyprogésterone (17-OHP) dans le liquide amniotique ou après la naissance dans le sang périphérique. Une confirmation par analyse génétique est conseillée. La deuxième forme de 21-OHD, appelée « non classique » est moins sévère et le début des symptômes non spécifiques d’hyperandrogénie survient plus tard après la naissance, souvent au cours de la puberté. Un test à l’ACTH avec mesure du 17-OHP à 60 minuties permet le diagnostic. Dans les deux formes, une mutation du gène CYP21, que l’on peut identifier par des techniques de biologie moléculaire, est responsable. Il existe une bonne corrélation génotype-phénotype en raison du ni-veau variable de l’activité enzymatique résiduelle du 21-hydroxylase. Les formes SW, SV et NC sont associées à des mutations distinctes ou à l’association de mutations. Actuellement, il est possible grâce aux tests hormonaux et moléculaires de prédire la forme clinique après un dépistage postnatal et pour définir les valeurs seuils post-ACTH de 17-OHP distinguant les sujets atteints d’une forme non classique, les sujets hétérozygote et les sujets normaux.

Mots-clés : Hyperplasie congénitale des surrénales (HCS), déficit en 21-hydroxylase, ambiguïté sexuelle, perte de sel, conseil génétique, diagnostic anténatal, traitement anténatal.
severity directly related to the degree of enzymatic compromise conferred by a given genetic defect. Clinical forms of 21-OHD are divided into broad groups: “classic” (ex-congenital) and “non classical” (NC) (formerly termed late onset or cryptic) forms. The classical forms are subdivided into salt wasting (SW) type, and simple virilizing (SV) type according to the occurrence or not of a clinical salt loss in early infancy. In both types, affected female fetuses undergo virilization of the external genitalia prenatally and present at birth with sexual ambiguity (from simple clitoromegaly to complete male phenotype, 5 Prader stages) (fig. 4). Classical disease occurs in approximately 1/15,000 births, while the NC form occurs in approximately 1% of the general population, many of them being undiagnosed.

Key words: Congenital adrenal hyperplasia (CAH), 21-hydroxylase deficiency, sexual ambiguity, salt losing, genetic counseling, prenatal diagnosis, prenatal treatment.

THE MOLECULAR GENETIC BASIS OF THE DISEASE

It has been extensively studied [19, 24, 26]. 21-hydroxylation is mediated by cytochrome P450c21. Duplicated 21-OH genes, an active gene (CYP21) and a pseudo-gene (CYP21P) are located on chromosome 6p21.3, within the major human histocompatibility complex (HLA), about 30kb apart, adjacent to and alternating with the C4B and C4A genes encoding the fourth component of serum complement. A highly nucleotide homology between the duplicated region encompassing the 3 genes (CYP21, C4 and X) indicates frequent crossover events, making genetic distinction of the loci difficult.
Restriction length fragment polymorphism has first permitted to identify large lesions, either a complete deletion of C4B and CYP21 (12%) (a product of unequal crossing-over during meiosis) or gene conversion events (9%) (resulting in the transfer to CYP21B of mutations normally present in the pseudogene) [20]. However, it soon appeared that in 75-78% of the cases the enzyme defect was due to single mutations irrespective of the association with deletions (3%) duplication (20%) of C4A and CYP21 [20]. Twelve of the fifteen or so of the most common point mutations of the CYP21 gene are normally present in CYP21B, and can be identified directly on the CYP21 gene specifically by PCR.

In Lyon’s studies, as well as in that of other groups [15, 25, 26], frequency and type of CYP21 mutations in classic and NC forms differ.

Since a first report in 1986, Morel and associates have studied more than 2000 unrelated chromosomes from patients with classic forms (SW or SV) (70%) and NC patients (30%). To date, the genotyping strategy, deduced from previous studies, includes 3 steps in cascade: PCR-restricting enzyme digestions (6 frequent mutations), sequencing of 3 exons (7th, 8th and 10th), and sequencing of the remaining gene. Allele assignment is always done by family studies (whenever possible). Southern studies are done when a large lesion is suspected and in cases where genotypes do not match phenotypes. With this strategy, over 98% of 21-OHD alleles are identified [14].

In Lyon’s studies, as well as in that of other groups [15, 25, 26], frequency and type of CYP21 mutations in classic and NC forms differ. Studies of the in vitro activity of P450c21 of mutated genes transfected in COS cells has shown that there was various degree of enzyme deficiency, and permitted to conclude that there are good correlation’s between genotypes and phenotypes. Patients carrying mutations which destroy all P450c 21 ac-
activity (complete absence of the CYP21B gene, gene conversion, 8bp deletion or specific point mutations) have SW classic forms of 21-OHD, whether homozygous or heterozygous for any of these lesions. The most frequent mutation is the intron 2 splice site mutation (30.3%), followed by gene deletions (20.3%), the I172N mutation (19.7%) and large gene conversions (7.1%). So far, over 100 private rare mutations have been identified in the world [26], not including the 60 described in Lyon [14]. Overall, they represent likely less than 3% of CAH patients.

The positive predictive value for null mutations is a SW phenotype.

A change of isoleucine to asparagine at codon 172 (I172N) is the most common cause of the SV form of 21-OHD (in homozygous or heterozygous form with a null mutation).

Finally “mild” point mutations V282L, P453S and P30L are established causes of partial enzyme deficiency, and result in NC forms of the disease. Among 562 NC alleles studied in Lyon [23], 64% carried a mild mutation: V281L (55.5%), P30L (4.1%), P453S (4.1%), promoter (0.7%). Nevertheless, as 35% of NC alleles carried severe mutations (compound heterozygotes), more than 50% of NC patients present a risk to have a female virilized newborn. In brief, the presence of mild mutations on one allele will determine the clinical expression of the disease: compound heterozygote (severe/NC mutations) will have a NC forms of 21-OHD, but are at risk of having a child with classic 21-OHD if the partner also has one allele carrying a severe mutation (whether he (she) is a simple heterozygote, a compound heterozygote or affected with classic CAH) (fig. 5).

The most common lesion in classic 21-OHD is the A->G substitution 13bp before the end of intron 2 resulting in aberrant splicing of pre-mRNA, but for yet unexplained reasons, it does not always correlate with clinical expression. It should also be stressed that genetic diagnosis is more complicated for 21-OHD than for many other monogenic disorders due to the high variability of the locus. This includes coexistence of mutations within an allele or the presence of more than one CYP21/C4 repeat unit on the same chromosome. With some exceptions, a careful study resolved most apparent discrepancy between genotype and phenotype i.e. gene conversions associated with NC, V281L always associated with other mutations in SW, or a CYP21 duplication [16].

The present consensus is that in general, a good genotype-phenotype relationship is seen in patients with either the severest or the mildest mutations. Some exceptions are observed with the Intron 2 mutation, or the P30L mutation of intermediate severity.

**CORRELATION GENOTYPE/PHENOTYPE AND HETEROZYGOTE DETECTION PROVIDE A NEW LOOK ON GENETIC COUNSELLING FOR PRENATAL DIAGNOSIS AND TREATMENT**

Genetic counseling

In a family with a previously affected child, complete genetic analysis is made in the index case, and the lesions identified in the parents (in order to exclude a de novo mutation). A clinico-genetic correlation should be made, that is that both parents should also have hormonal studies, i.e. measurement of basal or ACTH stimulated 17-hydroxyprogesterone (17-OHP) levels, in order to
eliminate a NC form of the disease (whether cryptic or undiagnosed).

Genetic counseling is more difficult when the index case is a parent or a relative, or even when there is no index case. There is an increasing demand for genetic counseling from CAH patients or relatives of a CAH patient, when they desire children. The genetic status of the partner is unknown, but the couple want to know what is the risk of having a CAH child, and what would be the clinical expression in case of CAH. Heterozygote detection is not a goal for prenatal diagnosis per se, but has to be performed at this occasion.

Although neonatal screening is now available, as it is feasible by a simple technique (17-OHP measurement on dried blood samples), such studies do not allow the detection of NC forms of 21-OHD and all the more so that of heterozygotes in a general population. On the other hand, it is not yet conceivable to use molecular genetic studies for population screening of 21-OHD heterozygotes. However, adequate hormonal studies can now allow the detection of 21-OHD heterozygotes. Indeed, heterozygote subjects show subtle anomalies after ACTH stimulation. In the past, it has been shown that, as a group, heterozygote subjects exhibited post-ACTH levels of 17-OHP significantly higher than in normal controls. Unfortunately, because of a large overlap between the 2 groups, the test is not accurate on an individual basis. In our experience ≥75% of the obligate heterozygotes tested (parents of CAH affected children) did not have post-ACTH levels of 17-OHP above normal (Forest, unpublished). This is why a more sophisticated hormonal testing was developed.

DETECTION OF HETEROZYGOTES

Post-ACTH levels of 21-deoxycortisol have been shown for many years to be elevated in 21-hydroxylase deficiency, and proposed for making the detection of heterozygotes demonstrated by HLA typing [13]. This compound is the product of the 11-hydroxylation of 17-OHP. It is produced in minute amounts in normal subjects because 17-OHP is not a good substrate for 11-hydroxylation. It is obviously quite elevated in patients presenting with classic 21-deficiency because of the very marked rise in 17-OHP levels. The only indication of measuring 21-deoxycortisol is to detect heterozygotes. We have developed a very specific assay (specific chromatography before radioimmunoassay with in-house antibodies). With this assay, after a simple short ACTH test, 21-deoxycortisol rises significantly in heterozygotes, with a minimal overlap with the control subjects (fig. 6). The test appears discriminative in 94% of the individuals tested as controlled by further molecular analysis of the subjects (Morel and Tardy, unpublished data).
Our strategy for detection of heterozygote is thus currently straightforward. The study is indicated only when one person of a given couple is known to be heterozygote for a severe mutation of CYP21 (or eventually at risk for it). Search for heterozygote will be made in the partner. The first step is to give the subject a short ACTH test and measure 17-OHP (for control of adequate ACTH stimulation) and 21-deoxycortisol, 60 and 90 min later. If the subject is predicted to be heterozygote, a molecular genetic studies of her (his) CYP21B genes is made (and in the nuclear family as well). According to the gene lesions found, one can now predict whether the offspring is at risk for a classic or NC form of CAH.

**Prenatal Diagnosis of 21-Hydroxylase Deficiency**

At first, it was made after amniocentesis, using HLA typing and/or steroid analysis although interruption of treatment was not advisable at that time. HLA typing should no longer be used [20]. Prenatal diagnosis was next performed on chorionic villus sampling (CVS) and molecular studies of the 21-hydroxylase genes performed. This permits an earlier diagnosis, with improved safety. Correlation genotype/phenotype and heterozygote detection provides a new look on genetic counseling for prenatal diagnosis. Therefore, genotyping must be made with segregation of mutations in families, before genetic counseling, particularly in case of a demand of prenatal diagnosis and treatment. Indeed, one can now predict not only the risk for a couple to have an affected child, but also what could be the clinical form (classic or NC) of the disease. Genotypes of deletion, gene conversion or severe mutations motivate prenatal diagnosis (and eventually treatment of affected female fetuses), while genotypes of less severe mutations (whether homozygous or heterozygous) do not (fig. 7).

Fetal sexing is part of the prenatal diagnosis of 21-OHD, because the parents’ decision might be influenced by the sex of the fetus, and it is mandatory when prenatal treatment is considered. In the past karyotype was performed on cultured amniocytes (at around 19-20 WA). Later, karyotype or SRY were determined on CVS sample (that is at 11-13 WA). Recently, fetal sexing was proposed on the determination of SRY on cell free fetal DNA present in maternal blood as early as 6-11 WA [2].

**Prenatal Treatment of 21-Hydroxylase Deficiency**

Prenatal therapy has been proposed for preventing the in utero virilization of CAH females [3]. The rationale of the treatment was to provide sufficient glucocorticoid levels to the fetus to suppress the excessive adrenocorticotropic stimulation. Dexamethasone (Dex) was chosen because it crosses the placenta and has a long half-life. The effective Dex dose was determined as the lowest dose at which 17-OHP levels were normalized in the amniotic fluid [8], and appeared to be 20µg/kg maternal weight per day. The early Lyon’s protocol was as follows: 1) Indications: prenatal treatment was offered only to mothers at risk of having a fetus with classical form of the disease and who decided to continue pregnancy whether the fetus was affected or not. 2) Contraindications: mothers at risk for or (of having) diabetes, hypertension, obesity, insulin resistance, cardiovascular disease or any situations aggravated by glucocorticoids. 3. Treatment was started early (≥ 8th week of amenorrhea, WA), i.e. prior to any possible prenatal diagnosis, continued until term only in case of a CAH female fetus, and stopped in all other cases (fig. 8). 4) Treatment was not interrupted before knowing the results of prenatal diagnosis. 5) Maternal compliance was followed on maternal level of cortisol and DHA, while efficacy of adrenal fetal suppression was attested by low maternal estriol levels monthly after the 6th month of pregnancy. 6) The mothers were given low-salt diet, and asked to pay careful attention to their weight gain.

At first, prenatal diagnosis was made after amniocentesis, using HLA typing and/or steroid analysis although interruption of treatment was not advisable at that time. French Multicenter studies, have concluded that this treatment was efficient in preventing virilization in CAH female fetuses. Later, prenatal diagnosis in treated
mothers was performed on (CVS) by molecular studies of the CYP21. This permitted an earlier diagnosis, with improved safety.

In order to amass a sufficient sample size to generate statistically powerful information to assess the effect of prenatal treatment, data were also gathered from numerous European centres. Data for 253 pregnancies in 215 mothers were collected, and recently published in full [9]. The cumulated European experience shows that the benefits of Dex treatment clearly outweighs the risks and can help to allay anxiety and encourage future pregnancies in these families. From these studies and from the experience accumulated in the literature, in particular that of Dr New’s group in USA [22], it can be concluded that Dex treatment is efficacious in preventing virilization of external genitalia in CAH fetuses. Quality of results rely on early initiation of treatment and a divided (i.e. bid or tid) daily dose. It is also important to remember that successful prenatal therapy depends upon parental motivation and effective collaboration among specialized teams of pediatricians, gynecologists, biologists and geneticists.

The occurrence of variable complications has been reported only in few patients (e.g., excessive weight gain, severe striae, mood fluctuations and irritability, acne and edema), reversible after delivery. However, only an excessive weight gain has been proven in a study with a control group [17]. This suggests that the minimal effective dose should be used [1] and that unnecessary treatment should be discontinued as early as possible. Indeed, 7 out of 8 fetuses do not need prenatal therapy (male and unaffected females). This is why the early fetal sexing is a noticeable progress, since made before starting treatment. In our group male fetuses at risk for classic CAH are no longer treated (fig. 8).

Among an international cohort of over 800 new borns treated in utero [9, 12, 17, 18], no teratogenic effect was reported. The new-borns had normal birth weight and birth length as a group; normal growth characterized the infants treated in utero during the first year of life. Passage of developmental milestones was normal though several adverse events, both in treated mothers and infants. Some adverse events have been noted in

Figure 8: Flow chart of prenatal treatment in a couple at risk for a classic form of 21-hydroxylase deficiency [6].
the literature in a few treated infants. As it remains unknown whether these events are attributable to the treatment, the latter must still be regarded experimental, and its use should be centralized and meticulously monitored until more experience has been gained. Prenatal treatment has been controversial, on the ground of an followed-up studies are currently started.

**REFERENCES**