Non classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency in families from a Greek island with a closed society

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Summary

In young members of a large family from a Greek island with a closed society, clinical and hormonal symptoms of 21-OH deficiency (CAH) were present. To discriminate those affected from those unaffected, we measured the basal and ACTH stimulated 30 values of 17-hydroxyprogesterone (17-0HP) progesterone (P) and cortisol (F) in combination with HLA-phenotypes in 25 out of 40 members of this family. The indices of the $Gutai_{30\text{-}min}$ assessment (17-0HP+P response to ACTH testing at 30 min), GF (F response at 30 min) and the ratio $GF_{30}/Guai_{30}$ named the Marina index were evaluated. The Marina index showed a very statistically significant difference among the three groups ($p < 0.001$). HLA phenotypes of the members of groups A and B showed a powerful association with B_{14}, DR_1, B_7, and B_{35} phenotypes that were related with 21-OH/CAH. In conclusion, in our study population, a high incidence of a clinically asymptomatic form of 21-OHdef was found only after the ACTH stimulation test. The Marina index seems to be of high diagnostic value in classifying disease severity.

Key words: Congenital adrenal hyperplasia, Greek island.

Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OH) is the most common enzymatic disorder in the metabolism of steroids in the adrenals [1]. The deficiency is inherited under the autosome recessive pattern and is located on the short arm of chromosome 6, between the histocompatibility antigens (HLA), B-cell and drug resistant phenotypes (DR) [2]. More specifically, the genetic disorder is located in the gene CYP21B which controls the function of cytochrome P450 C21 [3]. Due to that enzymatic deficiency a smaller quantity of cortisol is produced (final product), while an unacceptable quantity of androgens (precursor substances) are found in the patient's blood. The negative feedback effect of cortisol/adrenocorticotropin (F/ACTH) results in stimulation of the pituitary gland and increased release of ACTH, leading to stimulation of the adrenals and adrenal hyperplasia, which is responsible for the clinical manifestations of CAH [4, 5].

The severity and onset of clinical symptoms depend on the percentage of 21-hydroxylase deficiency enzyme (21-OH CAH) [6]. When expressed with its most severe type (classic CAH), the symptoms are masculinization of the female external genitalia during intrauterine life or loss of sodium chloride in the neonate leading to hydration and finally death, supposing that the pregnancy is not diagnosed early (prenatal diagnosis not only by measurement of 17-hydroxyprogesterone (17-OHP) in the amniotic fluid but also by detecting genetic changes of the gene CYP21B and given the appropriate corticosteroid treatment [7].

When expressed with its mild types, the symptoms due to blood androgen excess appear in puberty, therefore these forms are called "late onset" (late onset CAH, LO-CAH or non-classic types, NC-CAH). The symptoms are amenorrhea or oligomenorrhea, hirsutism and acne, oily skin, possibly a mild enlargement of the clitoris or masculine body image, short stature in girls, short stature and possibly sperm disturbances in boys, in the severe form of NC-CAH, due to hyperandrogenemia, which lead to penile enlargement but small testes, because the androgens are adrenal in origin. The outcome in both sexes is common; hyperandrogenemia, infertility and short adult height [8].

Each CAH form is linked to different human leucocyte antigen (HLA) phenotypes [9]. Based on literature data Bw_{47} phenotype is linked to the severe form and B_{14}, DR_1, and B_{35} phenotypes to the mild forms [7, 10]. Lately there have been reported cases of a non-symptomatic form of the 21-OH CAH (cryptic), in which despite their abnormal genotype and pathological response in the ACTH

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stimulation test, the patients appear with normal phenotype (regular menstruation and no clinical symptoms of hyperandrogenemia) [11].

The frequency of the classic type is between 1/5,000 and 1/25,000 among living infants in North America and Europe while that of the non-classic type is 0.2% among the white population, especially high in Eskenazi Jews and in some mediterranean countries (Spain, Italy). The frequency of the heterozygous types of 21-OH CAH is estimated to be 1/60 in the general Caucasian population and extremely high in Ashkenazi Jews (about 1/3) [1, 6, 7]. In our country several studies have been done in the Greek population [12-15]. Our aim is to contribute to the map making of the disease in the Hellenic area. Therefore we have launched two research programs approved and funded by the Ministry of Science and Technology and the Prefecture of Piraeus, respectively, to study the syndrome in the families of an island in the Saronic Gulf (with a closed society), in which young people with severe symptoms of hyperandrogenemia have been detected. The target of this study was to examine the diagnostic value of hormonal parameters and to associate the results of these parameters with HLA phenotypes, in order to discriminate between unaffected/affected people and to apply the appropriate medical treatment, especially to young people as well as prenatal care for young couples.

Materials and Methods

Patients

The subjects in our study belong to a numerous family of 40 living members formed by nine smaller families (3 generations) (Figure 1). The target person who induced us to create this study belongs to the central family, the originating family, and then the study expands to the rest of the families in which the two branches of the smaller families meet. This young woman (26 years old) presented with severe hyperandrogenemia, abnormal menstruation and infertility. She was treated with oral contraceptives as was thought to have polycystic ovarian syndrome (PCOS). After three-months of prednisolone treatment, she had regular menstruation, became pregnant, and finally after a hormonal controlled pregnancy, gave birth to a healthy female infant with normal external genitalia.

Study design

The methodology in the diagnosis of our patient population in relation to 21-OHdef and the differential diagnosis from other endocrinological disorders consisted of: a) detailed personal and family history, b) clinical examination, c) complete evaluation of hormonal levels before and after an ACTH stimulation test, and d) evaluation of the HLA phenotypes genetically linked to the disease. Patients with Cushing syndrome, adrenal or ovarian virilizing tumors, hyperprolactinemia or thyroid dysfunction were excluded from the study. None of the patients received hormonal therapy for at least 12 weeks before testing. All patients underwent an acute adrenal stimulation test to rule out or confirm 21-hydroxylase deficiency, as described below. The study was approved and funded by the Ministry of Science and Technology and the Prefecture of Piraeus and the Institutional Review Board of Athens University.

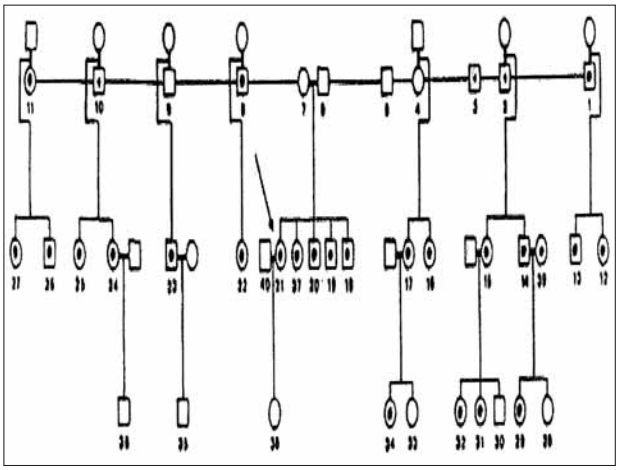

Figure 1. — Family tree of population with 21-OH deficiency. The target-patient is marked with → and the patients that were stimulated with ACTH are marked as: male: dotted square; female: dotted circle.

We present the results of the hormonal evaluation, applied to the 25 younger members of the families. We evaluated the basal levels of 17-OHP, progesterone (P) and cortisol (F) and response to the ACTH stimulation test by measuring the levels of 17-OHP, P and F, 30 min after the ACTH stimulation test ($GF_{30} = F_{30}\text{-}F_{0}/_{30}$, $Gutai_{30} = 17\text{-}OHP_{30}\text{-}17\text{-}OHP_{0}+P_{30}\text{-}P_{0}/_{30}$). Thereby we measured and evaluated the speed of cortisol production (GF, 30 min) and the ratio of these two parameters, an index suggested for the first time, called Marina (named for the target member in our study): $GF_{30}/Gutai_{30}$ expressing the ratio of the final product to the precursor substances.

ACTH stimulation test

All patients were informed about and accepted the diagnostic procedure. After overnight fasting, 0.25 mg of ACTH (1-24) (Synacthen, Ciba-Geigy, Basel, Switzerland) were injected as an intravenous bolus with the patient in the supine position, in the morning, between 08:00 and 10:00 a.m. No dexamethasone was administered before testing. The test was performed in the early follicular phase (days 3-7 of the menstrual cycle) if the person was a woman, while in cases with secondary amenorrhea, menstrual bleeding was produced after progesterone administration for ten days. Blood samples were obtained before (0 min) and 60 min following ACTH administration. Serum was separated, aliquoted and stored at -20°C until assay.

Hormonal assays

Hormone measurement of F was performed by polarization fluoroimmunoassay using commercially available kits (Medgenix, Fleurus, Belgium; TDX Abbott Laboratories, IL, USA). Sensitivities, intra- and interassay coefficient of variation (CV %) were found to be 12.41 nmol/l, 5.1 and 7.0 for F; 17-OHP measurements were performed with RIA kits provided by Diagnostic Systems Laboratories (Webster, TX USA), with CV 6.3%. Progesterone measurements were performed by fluoroimmunoassay and the CV was found to be 0. 25 nmol/l, 5.0 and 7. 0.

Human leukocyte antigen (HLA) typing

HLA A and B phenotypes were determined on peripheral blood leukocytes using the standard National Institutes of Health (NIH) two-stage microtoxicity test [16]. HLA DR typing was performed using the PCR-SSP (sequence specific primer) technique [17, 18].

Table 1. — *Classification of the study population and levels of hormonal parameters (x ± SEM) in the three groups.*

Group	No.	Basal levels of 17-OHP (ng/ml)	Gutai 30 min test (ng/dl/min) (17-OHP30-17-OHP0)+(P30-P0) 30 min	GF30 F30-F0 30 min	Marina index GF30' Gutai30'
1	3	> 100 (105-172)	107 ± 25	76.7 ± 18.5*	0.85 ± 0.3*
2	16	≤ 3 (0.7-3)	21.6 ± 3.5	601.5 ± 42.2*	35.8 ± 4.2*
3	6	< 3 (0.5-2.8)	4.8 ± 0.9	385.0 ± 29.6*	96.8 ± 19.5*

* = p < 0.0001: One-way ANOVA analysis.

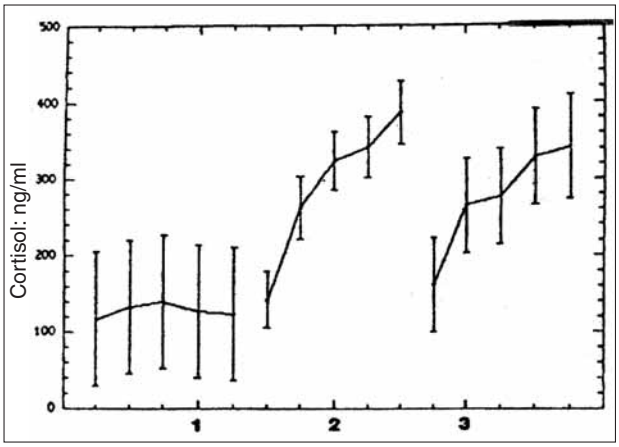

Figure 2. — Levels of cortisol (ng/ml) after the ACTH stimulation test at 0, 15, 30, 45, and 60 min of the groups in our study.

Statistical analysis

One-way ANOVA analysis was used for the statistical analysis of the hormonal parameters; a p value less than or equal to 0.05 was considered significant.

Results

By using as a criterion the basic values of 17-0HP (3 ng/ml) and the criteria of Gutai, 30 min (7 ng/dl/min) result, the subjects of our study were divided in three groups with numerous important differences concerning clinical manifestations of 21-OH CAH and also the hormonal parameters (Table 1, Figure 2).

I. Three members of the central family, the target person and two of her brothers, with an obvious clinical picture of the 21-OH CAH (the woman with secondary amenorhea, short stature, muscular body image, hirsutism, acne, infertility, mild enlargement of the clitoris but not masculinized external genitalia and the brothers with short stature, acne and sperm disturbances), resulted in very high basic levels of 17-0HP (105-172 ng/ml) and of P (4-8.8 ng/ml), and a very fast production of the same hormones after the ACTH stimulation test ($Gutai_{30}$ = 76-156 ng/dl/min). The speed of cortisol production was statistically significantly smaller compared to the other two groups, a fact that can be seen in the very low Marina ratio (Table 1).

II. Sixteen subjects from both branches of the other families, mainly without obvious clinical manifestations of 21-OH CAH and with very low basic values of 17-0HP < 3 ng/ml, resulted to have a pathological response to the ACTH stimulation test ($Gutai_{30}$ = 8.40-62 ng/dl/min), and the highest speed of cortisol production. As a result the Marina index was significantly improved in comparison to group I (Table 1).

III. Six other subjects with the same low levels of 17-OHP < 3 ng/ml, and with a normal clinical picture, resulted to have a normal response to the ACTH stimulation test ($Gutai_{30}$ = 2-7 ng/dl/min) and a moderate production of cortisol, and as a result, the highest Marina index compared to the other two groups (Table 1).

Identification of the HLA phenotypes which are genetically linked to 21-OH CAH, has shown a very frequent appearance of B_{14}, DR_1 B_7, and B_{35} phenotypes with localization of the previously reported phenotypes to the central family and to the middle of the right branch where the members of group B mainly belong (Figure 1).

Discussion

Our findings allowed us to make some comparisons of our diagnostic methodology with the international standards. Therefore, we suppose that the cutoff limit of basic values of 17-OHP > 3 ng/ml, or according to others the limit of basal values of 17-OHP > 2 ng/ml, to establish suspicion of the 21-OH CAH, especially in high-risk members of suspect families, should not always be fixed in order to perform a check of the differential diagnosis with the ACTH stimulation test. It is evident from our study, but as other researchers also propose [11, 12], there are clinically asymptomatic forms of 21-OH CAH which can be exposed only after the stimulation of the adrenals with ACTH [19, 20].

Accordingly, it is particularly important for the study of families with 21-OH CAH that all members of the families be examined according to the protocol of our study. On the other hand, very high basic values of 17-0HP > 8 ng/ml and a dramatic increase after an ACTH stimulation test have to be recognized as safe signs for the diagnosis of NC-CAH, as can also be seen from group I.

Also it should to be noted that the criteria of $Gutai_{30}$ > 6.5 ng/dl/min or > 7 ng/dl/min, which is internationally accepted as a clean-cut diagnosis of the disease, can clearly detect severe cases of NC-CAH and give a degree of enzymatic insufficiency with the calculation of the precursor substances, however cannot clearly differentiate asymptomatic forms from normal cases [21]. We also believe that since there is not a calculation of the speed of production of the final product of cortisol, we do not obtain an integrated picture of the enzymatic reserve of each person.

Moreover, in the histogram proposed by New *et al.* at the two axons where the basic values of 17-OHP and the values of $17\text{-}OHP_{60}$ min stand after the ACTH stimulation test, there is an important overlap between heterozygous carriers (asymptomatic forms) and normal persons [22]. For this reason we have tried to evaluate some other indices that can reflect the speed of cortisol production. We suppose that the GF index confirms the arrangement of clinically asymptomatic forms into a separate group based on the highest production of cortisol compared to the other groups. The good basic values of cortisol

together with the capability of production of sufficient quantities in cases of stress, is of particular importance for the normal clinical and biochemical profile of a person, even if there is a pathological genotype. In these cases there is no activation of the hypophyseal/adrenal axis, there is no permanent hyperfunction of the adrenals and the concentration of androgens in blood, and as a result there are no final clinical features of CAH presenting with various degrees of hyperandrogenemia. Accordingly, the ratio of cortisol/precursor substances, the Marina index, which for the first time is proposed because it includes the GF parameter, we assume that it is more indicative for the precise, clinical characterization of both healthy and affected persons, since it better approaches the enzymatic adequacy of the person and measures the person's ability to react to stress.

As a conclusion, we assume that a) the ratio of the clinically asymptomatic forms in the subjects of our study is big enough and testifies to our diagnostic methodology for their exposure and identification; b) the Marina index has an important diagnostic value for the categorization and the classification of patients since it seems to better approach the enzymatic adequacy of the person which is importantly relevant to his/her genetic defect; c) with the identification of HLA phenotypes that are genetically linked to the disease and the comparison with the hormonal data, the subjects of group I should genetically present the more severe form of NC-CAH, the genotype severe/mild; the persons of group II should belong to the milder forms of the 21-OHdef: mild/mild, severe/normal or mild/normal; and the persons of group III, the phenotypically normal, probably belong to other categories of enzyme defects and/or present PCOS or a very small number of these are heterozygous carriers, or they have a completely normal genotype. With the progress of molecular biology it has become possible to analyze the gene CYP21B which encodes the enzyme 21-hydroxylase [13, 23]. As a result we know today that the various forms of the disease are due to determinate genetic changes (mutations, deletions, changes to the clusters of bases, etc.) of the CYP21B gene which encodes the enzyme 21-hydroxylase. In this way, the results of hormonal evaluations, the stimulation of the adrenals with ACTH, and the results of the HLA phenotypes can be controlled and confirmed at the gene level. The accurate diagnosis of CAH can be confirmed by molecular gene analysis [24-28], but even today in clinical practice, molecular biology is not routinely available. The ACTH stimulation test remains the principal diagnostic tool. The reason for a quick diagnosis of this enzymatic disorder in clinical practice using the adrenal stimulation test is the simplicity of this test. Thus, we have presented an index, that based on the adrenal stimulation test, affords a quick approach and diagnosis of the severity of 21-OH CAH, especially in members of families highly suspicious for CAH and belonging to closed societies, which is easy to detect and with low-cost effectiveness that can be followed-up later with molecular gene analysis, if deemed necessary.

Finally, in our study population, a high incidence of a clinically asymptomatic form of 21-OH CAH was found, revealed only after the ACTH stimulation test. The proposed index Marina seems to be of high diagnostic value in classifying the disease severity.

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