CLINICAL STUDY

Molecular defects of the *CYP21* gene in Spanish girls with isolated precocious pubarche

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Abstract

Objective: To determine the frequency of mutant alleles in the *CYP21* gene in Spanish girls presenting with precocious pubarche (PP) and to assess the relationships between genotype and endocrine-metabolic variables.

Design: Fifty-three unrelated girls with a history of PP (14 prepubertal, 8 pubertal and 31 postmenarcheal) and 35 controls were studied.

Methods: Genomic DNA was extracted from peripheral blood leukocytes. After selection against the pseudogen, an allele-specific PCR was used to identify 14 known mutations in the *CYP21* gene. The mutations studied were Pro30Leu, splice intron 2, Ile172Asn, Cluster E₆, Gly192Ser, Ins T, GT-CT, Gln318-stop, Arg357Trp, Trp406-stop, Pro453Ser, Arg483Pro, Arg483 frameshift and Val281Leu. A standard 2-h oral glucose tolerance test was performed in all PP girls. Ovarian 17-hydroxyprogesterone (17-OHP) responses to gonadotrophin-releasing hormone-agonist stimulation was assessed in postmenarcheal PP girls.

Results: Thirteen PP girls and eight control girls were heterozygous for one of the mutations studied. The frequency of the carrier status was 25% and 23% in the PP and control groups respectively. Severe mutations were found in 33% of the carrier girls. Serum 17-OHP responses to ACTH stimulation were similar in carriers and non-carriers (351 ± 65 vs 334 ± 22 ng/dl). The presence of ovarian hyperandrogenism and/or hyperinsulinism was also not related to the carrier status.

Conclusion: The incidence of molecular defects in the *CYP21* gene in the present study was comparable in the PP and control groups. We found no relationship between the presence of carrier status and endocrine-metabolic abnormalities. Prospective studies of larger cohorts of PP girls are needed to ascertain the long-term clinical relevance of *CYP21* heterozygosity.

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Introduction

Precocious pubarche (PP) is defined as the appearance of pubic hair before the age of 8 years in girls and 9 years in boys (1). Most cases of PP are due to an early and isolated maturation of the zona reticularis of the adrenal gland (precocious adrenarche). Defective steroidogenesis indicative of non-classical adrenal hyperplasia (NCAH) due to 21-hydroxylase deficiency is present in 7% of Spanish children with PP (2, 3); however, the incidence of this enzymatic defect in other populations has been reported to be higher (1, 4–6).

PP in girls is often preceded by a low weight at birth (7) and is associated with a constellation of postnatal endocrine-metabolic abnormalities including hyperinsulinaemia and dyslipidaemia of prepubertal onset, and functional ovarian hyperandrogenism (FOH) and

ovulatory dysfunction at adolescence (8–11). Genetic/environmental factors acting in a complex multifactorial manner have been suggested to underlie this sequence (12–14).

Previous studies in other populations have shown a high incidence of heterozygosity for *CYP21* gene mutations in girls with PP and/or FOH (15–17). The aim of the present study was to determine the frequency of mutant alleles in the *CYP21* gene in Spanish girls presenting with PP and to assess the relationships between genotype and endocrine-metabolic variables.

Study population and methods

Study population

A total of 53 unrelated girls with a history of isolated PP (age range, 6–18 years) and 35 control girls (age

range, 8–15 years) were studied. Controls were selected from short-normal children (heights in the 10–25 centile range) and children seen by other paediatric subspecialties in Barcelona Hospital. Fourteen PP girls were prepubertal, eight pubertal and thirty-one postmenarcheal (18).

In all patients, PP was attributed to pronounced adrenarche, as suggested by elevated androstenedione and/or dehydroepiandrosterone sulphate (DHEAS) levels at PP diagnosis (1, 19) and by exclusion of NCAH by means of an adrenocorticotrophin (ACTH) test (20, 21). None of the subjects presented evidence for thyroid dysfunction or hyperprolactinaemia, or had a family or personal history of diabetes mellitus. Fifteen (48%) of the postmenarcheal PP girls had FOH, defined as a combination of at least three of the following: amenorrhea or oligomenorrhea (duration of menstrual cycles ≥ 45 days); hirsutism (Ferriman and Gallwey score ≥ 8) (22); elevated serum androstenedione, total testosterone and/or free androgen index (testosterone $\times 100/\text{sex hormone-binding globulin (SHBG)}$, an index of free testosterone (23)); 17-hydroxyprogesterone (17-OHP) hyper-response (≥ 160 ng/dl) to gonadotrophin-releasing hormone agonist (GnRHa; leuprolide acetate (500 μg s.c.); Procrin; Abbott, Madrid, Spain) (10).

The clinical characteristics of the study population are described in Table 1.

Methods

Genomic DNA was extracted from peripheral blood leukocytes. After selection against the pseudogen, an allele-specific PCR was used to identify 14 known mutations of the CYP21 gene, according to the protocol of Wedell & Luthman (24, 25). The mutations studied were Pro30Leu, splice intron 2, Ile172Asn, Cluster E₆, Gly192Ser, Ins T, GT-CT, Gln318-stop, Arg357Trp, Trp406-stop, Pro453Ser, Arg483Pro, Arg483 frameshift and Val281Leu.

After 3 days of a high carbohydrate diet (300 g/day) and an overnight fast, a standard 2-h oral glucose tolerance test (oGTT) was performed in all PP girls. Oral glucose (1.75 g/kg body weight; maximum 75 g) was given at 0800 h, and blood was sampled at 0, 30, 60 and 120 min for measurement of glucose and immunoreactive insulin, as described (26). For calculation of mean serum insulin (MSI) during the oGTT,

the area under the insulin curve was calculated according to the trapezoidal rule. MSI levels ≥ 54 IU/l per min in prepubertal girls and ≥ 84 IU/l per min in pubertal and postmenarcheal girls were considered abnormal (8, 9). All girls had normal glucose tolerance, according to the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus criteria (27).

Postmenarcheal girls were studied either in the follicular phase of the menstrual cycle (days 3–8) or after 2 months of amenorrhea.

Hormone assays

Serum glucose was measured by the glucose oxidase method. Immunoreactive insulin was assayed by IMX (Abbott Diagnostics, Santa Clara, CA, USA). The mean intra- and interassay coefficients of variation were 4.7% and 7.2% respectively. Serum 17-OHP, testosterone, SHBG and DHEAS levels were assayed as previously described (3, 6). Serum samples were stored at -20°C until assay.

Statistical analyses and ethics

Anthropometric data and hormonal results are expressed as means $\pm$ S.E.M. unless stated otherwise. Comparisons were made by two-sided *t*-test. *P* values < 0.05 were considered statistically significant. Conditional regression analysis was performed to analyse the relationships between genotype and the different endocrine-metabolic variables.

The study was approved by the Institutional Review Board of Barcelona Hospital. Informed consent was obtained from parents and/or girls, as well as assent from minors.

Results

Thirteen PP girls and eight control girls were heterozygous for one of the mutations studied (Table 2). The frequency of the carrier status was 25% and 23% in the PP and control groups respectively. Severe mutations such as Gln318stop and intron 2 (655 A or C to G) which abolish or severely impair enzyme activity were found in heterozygous form in 33% of the carrier girls; conversely, mild mutations (Val218Leu, Pro453Ser and Pro30Leu) accounted for

Table 1 Clinical characteristics of the study population. Values are means $\pm$ S.E.M.

Study population	<i>n</i>	Age (years)	Post-ACTH 17-OHP (ng/dl)	MSI SDS	Post-GnRHa 17-OHP (ng/dl)
PP prepubertal	14	8.4 $\pm$ 0.3	236.1 $\pm$ 39.1	1.5 $\pm$ 0.4	—
PP pubertal	8	9.9 $\pm$ 0.2	256.8 $\pm$ 83.4	0.9 $\pm$ 0.8	—
PP postpubertal	31	13.7 $\pm$ 0.4	333.6 $\pm$ 29.2	2.1 $\pm$ 0.3	152.0 $\pm$ 60.7
Controls	35	13.5 $\pm$ 1.2	—	—	—

MSI SDS, mean serum insulin S.D. score.

Table 2 CYP21 gene mutations in PP and control girls.

Mutation	PP (n = 53)	Control (n = 35)
Val281Leu	3	4
Gln318-stop	4	2
Pro453Leu	3	1
Pro30Leu	3	0
Intron 2	0	1
Total	13 (25%)	8 (23%)

67% of the mutations detected in both groups. Serum 17-OHP responses to ACTH stimulation were similar in carriers and non-carriers (351 ± 65 vs 334 ± 22 ng/dl) (Fig. 1). Three heterozygous girls and three non-carrier girls had 17-OHP values post ACTH between 500 and 1000 ng/dl.

The presence of FOH and/or hyperinsulinaemia was also not related to the carrier status, as only four (27%) of the 15 postmenarcheal girls with FOH and two (8%) of the 24 PP girls with abnormal MSI levels were found to have mutations in the CYP21 gene.

Discussion

NCAH due to 21-hydroxylase deficiency is probably the most frequent autosomal recessive genetic disorder in humans (4). The incidence of NCAH among children presenting with PP based solely on biochemical assessment has been reported to vary from 0% to 40% (2, 3, 5, 6, 28–32). Molecular studies, however, have shown that the frequency in non-Jewish Caucasian populations is less than 10% (4).

The incidence of molecular defects of the CYP21 gene in the present study was comparable in the PP and control populations (25% vs 23% respectively). In a small population of PP boys ($n = 7$), heterozygosity for this molecular defect was also 28% (authors, unpublished observations).

Previous reports have shown higher (15) or similar frequencies (17, 33, 34) of heterozygosity in both PP

children and hyperandrogenic women. However, some of these studies included patients with NCAH (15), were performed in unselected groups of hirsute women (34), or included different racial groups (17). In our series, the frequency of heterozygosity in the control group was found to be unexpectedly higher than previously reported in other Caucasian populations (16, 17, 33, 34). The observed carrier frequency might be accounted for by a series of founder effects, genetic drift, or a high frequency of *de novo* mutations (35).

We found no relationship between the presence of a carrier status and the subsequent development of FOH at adolescence. Indeed, the presence of CYP21 mutations was equally prevalent among PP patients who developed FOH and those who did not. Heterozygosity for 21-hydroxylase deficiency has been suggested to increase the risks of developing clinical signs of androgen excess (16, 33, 34, 36); however, additional studies in obligate heterozygotic carriers have failed to show evidence of these associations (37). This phenotypic heterogeneity may reflect the effects of other multiple susceptibility genes or allelic variants, genetically based variations in androgen biosynthesis or sensitivity to androgens, epigenetic influences, and/or the role of environmental factors (17, 35, 38).

One-third of heterozygotes in both the PP and control populations were found to have severe CYP21 gene mutations that abolish or severely impair enzymatic activity. Although the frequency in this and other hyperandrogenic populations (27) has to be taken into account, the available data do not allow conclusions to be drawn with regard to the need for genetic counselling in this population at such an early age. In conclusion, the incidence of molecular defects in the CYP21 gene in the group of Spanish girls with PP was comparable with the control group. Prospective studies of larger cohorts of PP girls are needed to ascertain the long-term clinical relevance of CYP21 heterozygosity, before considering the need for genetic counselling in selected cases.

Figure 1 Baseline and post-ACTH 17-hydroxyprogesterone levels in PP girls with (right-hand pair of symbols) and without (left-hand pair of symbols) mutations. n.s., not significant. Values are means $\pm$ S.E.M.

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