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Clinical and molecular studies related to bone metabolism in patients with congenital adrenal hyperplasia

Abstract: Patients with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency need glucocorticoid (GC) therapy, which alters bone mineral metabolism. We analyze clinical and biochemical parameters and different polymorphisms of candidate genes associated with bone mineral density (BMD) in CAH patients. The CAH patients treated with GC and healthy controls were studied. Anthropometric parameters, biochemical markers of bone turnover, and BMD were evaluated. Polymerase chain reaction technique was used to genotype different candidate genes. The 192-192 genotype frequency (*IGF-I*) was lower in poorly controlled patients than that from controls. In CAH patients, FF genotype (vitamin D receptor, *VDR*) correlated with lower lumbar spine BMD and there was a significant association between the 0-0 genotype (*IGF-I*) and high values of β-CrossLaps and a low total BMD. This study contributes to understanding of the association of genetic determinants of BMD with the variable response to GC treatment in CAH patients and demonstrates the usefulness of these genetic polymorphisms.

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Introduction

Patients with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) need a life-long glucocorticoid (GC) replacement therapy (1). Treatment of CAH patients is still a matter of review, especially in terms of obtaining an optimal bone mass and adult height according to genetic potential (2). An adequate control of CAH results from a sufficient GC replacement dose to normalize androgen precursor secretion and to ensure normal skeletal growth and maturation (1). Besides, it is important to consider different predictor variables, such as CAH genotypes and their correlation with the different clinical forms, mean parental height, body mass index (BMI), between others (2, 3).

Bone loss induced by long-term GC is the most prevalent mode of secondary osteopenia (4). GC affects bones directly by altering osteoblasts and osteoclasts metabolism and indirectly, through alterations in the growth hormone axis (5). Long-term GC therapy can lead to a modification in bone mineral density (BMD). However, the effects of GC on bone mass in CAH patients are a matter of controversy, as BMD may be increased (1), normal (6), or reduced (7). Studies show contradictory results in terms of concentrations of bone markers (8, 9), but their correlation with components of the GH-IGF-I axis has still not been sufficiently analyzed.

Different polymorphisms of candidate genes related to bone metabolism, such as vitamin D receptor (VDR),

estrogen receptor (ERα), type I collagen (COLIA1), insulin-like growth factor-I (IGF-I), and glucocorticoid receptor (GCR), have been associated with variations in bone homeostasis and in therapeutic response in several bone diseases (10). Therefore, we hypothesized that CAH patients receiving GC treatment might show different impact on bone metabolism according to their genotypes. In this sense, a biallelic polymorphism of GCR gene has been associated with increased GC sensitivity (11), while no link to BMD of CAH patients treated with GC has been demonstrated (12).

Previous studies suggest a significant role for IGF-I in determining BMD. The polymorphic cytosine-adenine $(CA)_n$ repeat upstream of the transcription start site of the human *IGF-I* gene has been found to be associated with serum levels of IGF-I, BMD, and fracture risk in various ethnic groups (13). Cetinkaya and Kara (14) did not find changes in BMD in CAH patients after GC treatment, but suggest that VDR polymorphisms should be studied among other factors. With regard to ER and COLIA1 genotypes, no associations with the GC response of CAH patients have been reported yet. Based on these considerations, the aims of the present work were to analyze the following in CAH patients: (a) clinical and biochemical parameters as possible indicators of osteopenia development; (b) polymorphisms of candidate genes associated with BMD; (c) possible associations of the polymorphisms with bone turnover markers, BMD, and the IGF system.

Subjects and methods

Subjects

The CAH patients (n=67), 58% females (F) and 42% males (M) at mean chronological age of 13.8 years old presented 21-OHD and were treated with GC and/or mineralocorticoid when they were salt wasting. Based on 17-hydroxyprogesterone (17-OHP), δ4-androstenedione (δ4-A), dehydroepiandrosterone-sulfate (DHEA-S) and testosterone serum levels, patients were classified according to the presence (WC, well controlled) or absence (PC, poorly controlled) of an adequate clinical, biochemical, and auxological status. Patients and controls (n=119, 75% females and 25% males, age range 1.6–30 years old) came from the Hospital de Niños Santísima Trinidad, Córdoba, Argentina. The protocol was approved by both the Bioethical Committees from the Hospital and the Facultad de Ciencias Médicas, UNC, Argentina. Written informed consent was obtained from patients and healthy individuals or their parents or guardians. The World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects was taken into account.

Anthropometric parameters

Standard deviation scores (SDS) of stature and weight and parental height were determined and were used to determine the BMI, which is expressed as weight (kg)/square of height (m^2).

Bone mineral density

Lumbar spine (L1–L4) and femoral neck BMD were measured using dual-energy X-ray absorptiometry (DXA) Hologic QDR 4500 W densitometer (Hologic, Inc., Bedford, MA, USA). DXA scans were following a strict protocol and internal controls. BMD values are expressed in g/cm^2 and in *Z*-score from a data bank of national values obtained in normal children of the same age range (15).

Biochemical assays

Testosterone, DHEA-S levels, and biochemical markers of bone turnover were analyzed by ECLIA (Roche Diagnostics, Mannheim, Germany). Serum IGF-I and IGFBP-3 were determined by immunoradiometric assays (Diagnostic Systems Lab. Inc., TX, USA), 17-OHP, and δ4-A by RIA (DPC).

Genotyping

DNA was extracted from whole blood using a standard red cell lysis and proteinase K digestion technique and specific primers were used for the polymerase chain reaction (PCR) to amplify gene fragments of *VDR* (*Bsm I* and *Fok I* sites), *ERa* (*Pvu II* site), COLIA1 (site *Bal I*), and polymorphic CA_n repeat of *IGF-I* and GCR (*Bcl I* site), as described previously (16). All PCR amplifications were carried out in a Hybaid thermal cycler (Omnigene, Hampton Hill, Middlesex, UK). Genotypes for *BsmI* polymorphisms were termed BB, Bb and bb; those for *Fok I* were termed FF, Ff, ff; genotypes for *COLIA1* were termed SS, Ss, ss; and those for *PvuII* were termed PP, Pp, and pp. Uppercases letters represent absence and lowercase letters represent presence of restriction sites. Genotypes of *IGF-I* polymorphisms were: 192-192, 0-0, and heterozygous genotypes (17). The *Bcl I* polymorphism of the GCR gene was amplified and PCR products were digested with 7.5 Units Bcl I enzyme (New England Biolabs GmbH, Frankfurt am Main, Germany) at 50°C for 120 min, and fragments were analyzed on 2% agarose (418 bp fragment). The presence of a cleavage site for the Bcl I enzyme produced two smaller fragments (267 and 151 bp). Homozygous *Bcl I* genotype was CC genotype, whereas the heterozygous one was GC genotype. In the homozygous wild-type genotype, the 418 bp fragment remained undigested (GG genotype) (18).

Statistical analysis

All the analyses were performed using the statistical software R 4.14.2 (R-project, Boston, MA, USA) for Windows. Genotype frequencies for all loci were tested against Hardy-Weinberg ratios by the χ^2-test. Anthropometric data, biochemical variables, and associations

between genotypes at each genetic locus and BMD values or other variables were analyzed using Pearson's χ^2-test and the Wilcoxon or Kruskal-Wallis test, as indicated in the next section. Differences were considered statistically significant at $p<0.05$.

Results

Anthropometric and biochemical variables of the whole group of CAH patients are shown in Table 1. Age, height, and BMI were similar in both WC and PC groups. No differences were found in the concentration of IGF-I and IGFBP3 between WC and PC patients and in the values of bone turnover markers such as osteocalcin and β-CrossLaps. Dose range of GC was from 7.10 to 26.7 mg/m^2/day in the WC group and from 7.10 to 30 mg/m^2/day in the PC group.

Frequencies of polymorphic variants of *VDR*, *ERα*, *COLIAI*, *IGF-I*, and *GCR* genes were also evaluated. Genotype distributions were in agreement with those expected according to the Hardy-Weinberg equilibrium in all loci analyzed. The distributions of the different genotypes were similar between the control group and CAH patients. No differences in the distribution of the polymorphic sites Fok I and Bsm I (*VDR*), Pvu II and Xba 1 (*ERα*), Bal 1 (*COLIAI*), and Bcl I for the *GCR* gene as well as in the length of the CA repeat from *IGF-1* gene, were found (Table 2). Interestingly, the frequency of different genotypes related to the *IGF-I* gene was similar between the healthy control group and the WC group, but it was significantly different between the PC and the healthy control group (p=0.030; Table 3). The frequency of PC patients with the genotype 192-192 was lower than that from the control group (48% vs. 70%), while the percentage of homozygosity for the absence of 192 bp of CA repeat was more than double in PC patients than that of the healthy controls (33% vs. 14%). Another important finding was that the FF genotype appeared to be correlated with lower lumbar spine BMD, but not with total BMD, in the whole population of CAH patients (Table 4). We have observed that the *Z*-score BMD from the ff genotype was not different compared to those from the other genotypes, while the *Z*-score BMD from FF genotype was significantly different compared to that from the Ff genotype (post hoc analysis of Kruskal-Wallis test). Therefore, the allele F is apparently responsible for the differences in lumbar spine BMD observed between patients carrying FF and patients with Ff genotype.

Finally, in the CAH population there was a significant association between the 0-0 genotype (*IGF-I*) and the high values of β-CrossLaps and a possible association between

Table 1 Anthropometric and biochemical variables of well-controlled and poorly controlled CAH patients in response to GC treatment.

	WC, n=32	PC, n=34	p-Value
Age	15.45±1.75	12.37±1.34	0.1850
Height (SDS)	−0.52±0.23	−0.12±0.36	0.5117
BMI	21.48±0.77	22.13±1.04	0.9387
17-OHP, ng/mL	2.98±0.51	71.37±15.16	<0.0001
δ4-A, ng/mL	1.06±0.25	7.88±2.19	<0.0001
DHEA-S, ug/dL	44.35±17.47	73.27±16.01	<0.0046
Testosterone, ng/dL	93.61±27.27	179.41±34.97	<0.0086
IGF-I (SDS)	0.17±0.19	0.55±0.19	0.1740
IGFBP3, ng/mL	4744±221	5416±235	0.0664
Osteocalcin, ng/mL	61.76±9.01	68.36±8.23	0.2795
β-CrossLaps, ng/mL	1.14±0.15	1.25±0.14	0.4806
GC dose, mg/m^2/day	16.01±0.97	17.18±1.15	0.5446

Data are expressed as means±SE. The Wilcoxon test was used. Statistically significant at $p<0.05$.

WC, well controlled; PC, poorly controlled (based on levels of 17-OHP); 17-OHP, 17-hydroxyprogesterone; δ4-A, androstenedione; DHEA-S, dehydroepiandrosterone sulfate; SDS, standard deviation score.

Table 2 Frequency of different polymorphic sites from the VDR, ER, COLIA-I, GCR, and IGF-I genes in CAH and healthy control groups.

Genotype	Control	CAH	χ^2	p-Value
Bsm-I				
Bb	31 (33)	32 (21)		
Bb	51 (55)	53 (35)	0.33	0.849
BB	19 (20)	15 (10)		
Fok- I				
Ff	9 (10)	12 (8)		
Ff	55 (60)	53 (35)	0.41	0.813
FF	36 (40)	35 (23)		
Pvu-II				
Pp	38 (44)	40 (26)		
Pp	44 (51)	46 (30)	0.73	0.695
PP	19 (22)	14 (9)		
Bal-I				
Ss	4 (4)	2 (1)		
Ss	41 (40)	34 (21)	0.42	0.832
SS	55(53)	64 (39)		
Bcl-I				
CC	46 (55)	45 (29)		
GC	42 (50)	41 (26)	0.09	0.955
GG	13 (15)	14 (9)		
IGF-I				
192-192	70 (83)	55 (36)		
192-0	15 (18)	21 (14)	4.78	0.092
0-0	14 (17)	24 (16)		

The frequency of genotypes is expressed as percentage of the total population. The number within parentheses represents the number of cases. The Pearson's χ^2-test was used for frequency analysis. Statistically significant at $p<0.05$.

Table 3 Frequency of IGF-I genotypes in CAH and healthy control groups.

Genotype	Healthy control	WC patients	PC patients	χ^2	p-Value
IGF-I					
192-192	71 (84)	61 (20)	50 (17)	7.00[a]	0.030
192-0	15 (18)	24 (8)	18 (6)		
0-0	14 (17)	15 (5)	32 (11)		

The frequency of genotypes is expressed as percentage of the total population. The number within parentheses represents the number of cases. The Pearson's χ^2-test was used for frequency analysis.
[a]PC patients vs. healthy control group. Statistically significant at $p<0.05$.

the 0-0 genotype and the low total BMD expressed in g/cm^2 (p=0.05) (Table 5).

Discussion

In the present study we analyzed clinical and biochemical parameters and different polymorphisms of candidate genes associated with BMD as possible indicators of the development of osteopenia in CAH patients treated with GC and also evaluated their links with markers of bone turnover, BMD, and IGF system.

Table 4 Relationship between Fok-I genotypes (VDR gene) and lumbar spine and total BMD in CAH patients.

Fok-I	Lumbar spine BMD	Total BMD
Ff	−0.10±1.04	0.67±1.36
Ff	0.13±0.32	0.86±0.42
FF	−1.41±0.37[a]	−0.64±0.24

Data are expressed as the *Z*-score mean±SE for lumbar spine and total BMD. Statistical analysis: Kruskal-Wallis test.
[a]FF vs. Ff; statistically significant at $p<0.05$.

The CAH patients can reach an adult height consistent with their genetic potential. Published reports on adult height reached in CAH patients are highly variable and are influenced by different factors (19). The data on adult height SDS of our patients are comparable with those reported in the literature (20). The possible causes of short height in a patient subgroup have led to the analysis of influences of the GH-IGF-I axis. It has been suggested that DHEA-S may modulate the biological response of IGF-I (21, 22). However, comparable with most of the published data, our patients presented IGF-I values within the reference ranges (23).

Bone metabolism during follow up of CAH patients shows controversial results (1, 24, 25). Elevated androgens frequently found in CAH patients may have protective effects on bone integrity (26). Our results showed BMD values comparable to those of the normal population, without significant differences between groups. In agreement with other reports (24, 27), serum levels of bone turnover markers resulted normal, suggesting that the bone was probably metabolically stable at the moment of study.

As tissue sensitivity to GC is partially determined by genetic factors, different polymorphisms of the GCR have been investigated. The G allele has been associated with increased sensitivity to GC action (28, 29), but not in pediatric CAH patients. Our data did not show differences in the allelic frequencies in comparison with that from the healthy controls. The same was observed in the distribution of the ERα polymorphism.

Conclusions about *COLIA1* polymorphism in children are inconsistent (30–33). In a previous study involving small for gestational age (SGA) children, we reported that *COLIA1* polymorphic site seems to be associated with a low lumbar spine or femoral neck BMD (16). However, CAH patients presented an allele distribution comparable to that of the normal population, without significant differences in the levels of bone turnover markers or BMD.

Fok-1 polymorphism of the VDR gene has been previously related to differences in BMD (10, 34). In our study, FF

Table 5 Relationship between IGF-I genotypes with BMD and β-CrossLaps in CAH patients.

IGF-I	Lumbar spine BMD		Total BMD		β-CrossLaps
	g/cm^2	*Z*-score	g/cm^2	*Z*-score	
192-192	0.76±0.04	−0.73±0.39	1.00±0.06	0.34±0.57	1.02±0.13
192-0	0.85±0.07	0.41±0.44	1.10±0.04	0.86±0.47	1.24±0.26
0-0	0.73±0.11	−0.38±0.432	0.84± 0.09[a]	−0.21±0.45	1.57±0.18[b]

Data are expressed as means±SE. Statistical analysis: Kruskal-Wallis test.
[a]0-0 vs. 192-0, p=0.05.
[b]0-0 vs. 192-192, $p<0.01$.

genotype correlated with lower *Z*-score BMD from lumbar spine without significant differences considering *Z*-score BMD from total body. This is not surprising because it has been observed that osteoporosis is frequently detected in one or two sites of the skeleton as those certain regions are more susceptible than others to develop this disease (35, 36).

Accordingly, VDR gene has been reported to have multiple promoters that result in multiple tissue-specific transcripts. These allelic variants may be responsible for subtle differences in the VDR isoforms (37). These data might be of considerable interest and require further studies.

Polymorphisms near the promoter region in the IGF-I gene have been associated with changes in serum levels of IGF-I, body height, birth weight, and BMD. The presence of the 192 alleles has been linked to higher levels of IGF-I and BMD, along with a lower risk of fracture than other allelic variants (38, 39). Moreover, children born SGA with catch-up growth presenting the 192-192 bp genotype, had an adequate growth (16). The prevailing genotype of CAH patients under study was 192-192 bp which is consistent with our previous observations (16). Patients with inadequate control of CAH showed a lower percentage of the genotype 192-192 than controls. The molecular mechanism of association with more complex aspects of the CAH phenotype needs further investigation. Based on the finding of a significant association between the 0-0 genotype and the higher values of β-CrossLaps with a trend towards a lower total BMD than the healthy control group, we concluded that CAH patients lacking allele 192 showed a different response to GC treatment, along with higher bone resorption. Comparable results were shown previously for SGA children (16).

In conclusion, this study contributes towards better understanding of the genetic determinants as indicators of BMD in CAH patients and provides new information that can be helpful for future genetic association studies that analyze multiple polymorphisms as a whole.

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