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Growth, calcium status and vitamin D receptor (VDR) promoter genotype in European children with normal or low calcium intake☆

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ABSTRACT

Calcium homeostasis and bone metabolism require the vitamin D receptor (VDR) to function properly, as evidenced in patients and transgenic mice with VDR mutations. We have shown that (A/G) polymorphism at the −1012 locus of the VDR promoter (rs4516035) is frequent in European populations, may influence VDR expression, is associated with height in French adolescent girls, and is associated with their lumbar spine mineral density in case of insufficient milk intake. Here, an association study was performed in a cohort of Moldovan children and adolescents, living at latitude similar to the first cohort but receiving a cereal-based diet with very low milk/dairy product intakes. Children and adolescents in this cohort had similar 25-(OH) D levels, but a short stature and low serum calcium levels, compared to the first cohort. Their height remained associated with the A-1012G VDRp genotype. In addition, their serum calcium levels were associated with VDRp polymorphism, excepted when their 25-(OH) D levels were low (below 33 nmol/L). In conclusion, the −1012 VDRp genotype appears to be associated with height in European children whatever their calcium/dairy product intakes, and may modulate their calcium homeostasis in conditions of low calcium/milk intakes when vitamin D status is sufficient.

1. Introduction

Calcium homeostasis strongly depends on vitamin D status, production of 1,25-dihydroxyvitamin D3, the active form of vitamin D, and on the expression of the vitamin D receptor (VDR), a nuclear receptor and a transcription factor known to regulate the expression of genes important for calcium balance and bone metabolism. The human vitamin D receptor (VDR) gene is 65 kb in length from its main promoter [1] to the PolyA signal [2]. Numerous single nucleotide polymorphisms (SNPs) have been described so far (more than 300) all along the VDR gene [3,4]. Several of these SNPs have been found to be associated with various human traits involved in growth, bone metabolism or calcium homeostasis. However, only few have been shown to be functional in vitro, mainly, the FokI SNP that produces a length variation of the VDR protein, and polymorphisms in the 3′-UTR that change VDR mRNA decay [4]. Promoter regions are also good candidates to explain polymorphism-induced functional effects, through changes in transcription activity, effects that can be caused by variations in transcription factor binding and/or effect. To date, several functional polymorphism sites have been identified on the VDR gene promoter. The first has been localized on the binding site of Cdx-2, a transcription factor specific of the intestine mucosa known to regulate VDR expression [5,6]. Later, two other functional SNPs have been localized in the more proximal region of the VDR promoter, and found to produce important variations in the VDR promoter activity in cell culture concomitantly with changes in the binding of transcription factor complexes [4,7–8]. Hypotheses that changes in VDR expression associated with these polymorphisms might have some impacts in vitamin D-dependent functions have been supported by the recent findings of associations with human traits in various domain such as growth [8], bone density [9], fracture risk [4] or cancer risk [7,10].

2. Subjects and methods

- 204 apparently healthy children and adolescents were seen at the end of the winter while staying as full-time boarders in a single Moldovan boarding school (latitude: 47°N). They stayed at this boarding school during the academic year, but lived in a rural family environment in the summer. Only children with known chronic disease and/or body measures below or above 2 SDs were excluded from the study (118 boys and 86 girls aged

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Table 1
Characteristics of the two cohorts.

	Moldovan Cohort	Moldovan Cohort	French Cohort
n =	118 boys	86 girls	138 girls
Age (years)	11.2 ± 1.7	11.5 ± 1.8	13.2 ± 1.4
Prepubertal (%)	45	38	17
Height (cm)	140 ± 10	143 ± 11	157 ± 9
Weight (kg)	34.6 ± 7.1	35.7 ± 9.2	46.9 ± 9.0
Height (SDs)	−0.694 ± 0.827	−0.577 ± 0.936	0.200 ± 0.991
BMI (kg/m^2)	17.4 ± 1.9	17.2 ± 2.5	18.9 ± 2.3
BMI (SDs)	−0.07 ± 1.00	−0.41 ± 1.07	−0.133 ± 0.879
Serum calcium (mmol/L)	2.12 ± 0.31	2.18 ± 0.30	2.50 ± 0.08
Corrected serum calcium (mmol/L)*	2.21 ± 0.32	2.24 ± 0.34	–
Serum Proteins (g/L)	68 ± 11	70 ± 10	–
Serum phosphate (mmol/L)	1.35 ± 0.38	1.34 ± 0.37	1.36 ± 0.16
Serum magnesium (mmol/L)	0.71 ± 0.14	0.69 ± 0.13	0.79 ± 0.09
Serum 25-hydroxyvitamin D (nmol/L)	45.6 ± 15.0	40.3 ± 14.8	46.8 ± 21.0**
Average calcium intake (mg/day)	493	493	865 ± 251

Values are given as mean ± SD.
* Serum calcium values were corrected for serum proteins.
** Samples in this cohort were collected all year around. When restricted to samples collected between January and April, values were 41.3 ± 19.6 nmol/L (*n* = 104).

Table 2
VDR −1012 G/A polymorphism and variables in the Moldovan cohort (cohort I).

	VDR genotype			*P* values	
	G/G	G/A	A/A	G/G vs. A/A	G/G vs. G/A
Frequency (*n* =)	21.1% (43)	44.1% (90)	34.8% (71)		
Age (years)	11.4 ± 0.3	11.5 ± 0.2	11.3 ± 0.2	0.8670	0.8365
Height (SDs, WHO standard)	−0.95 ± 0.14	−0.72 ± 0.10	−0.57 ± 0.10	**0.0351**	0.1769
BMI (SDs)	−0.16 ± 0.12	−0.23 ± 0.12	−0.13 ± 0.12	0.8990	0.6353
Serum calcium (mmol/L)*	2.13 ± 0.05	2.24 ± 0.04	2.27 ± 0.04	**0.0336**	**0.0544**
Serum proteins (g/L)	70.2 ± 1.6	68.2 ± 1.1	69.1 ± 1.3	0.5819	0.3085
Serum phosphate (mmol/L)	1.42 ± 0.07	1.32 ± 0.04	1.37 ± 0.04	0.4664	0.1494
Serum magnesium (mmol/L)	0.68 ± 0.02	0.72 ± 0.02	0.72 ± 0.02	0.1618	0.1489
Serum 25-hydroxyvitamin D (nmol/L)	42.1 ± 1.9	43.5 ± 1.7	45.4 ± 1.9	0.2811	0.6432

Values are given as mean ± SE.
Significant values ($p < 0.05$) are in bold.
* Serum calcium were adjusted to serum protein levels.

7–16 years). They took all their meals in the boarding school and had mostly a cereal-based diet with little access to meat, milk and dairy products. Their intakes of total protein (average: 75 g/day including 24 g/day animal protein), energy (2842 kcal/day), and calcium (493 mg/day including 69 mg/day as milk and dairy products), were evaluated, based on week menus given to all boarders

- 138 healthy Caucasian adolescent girls living in France (latitude 49°N) were part of a previously reported cohort [11] restricted to girls aged 10–16 years to enable comparisons with the Moldovan cohort. Their mean protein, energy and calcium intakes calculated on a one-week food recall were 71 g/day (including 48 g/day of animal protein), 1989 kcal/day and 865 mg/day, respectively.
- Weight and height were recorded as absolute values and as SDs values based on World Health Organization standard curves [12].

Blood samples were taken with consents of parents and kids and with the agreement of the local ethical committees. Biochemical analyses included serum calcium, phosphates, and 25-hydroxyvitamin D (25-(OH) D), All serum 25-(OH) vitamin D were assayed in our laboratory using in-house competitive protein binding assays that detected equally the vitamin D3 and D2 forms, and with continuous external quality assessment of the 25-(OH) vitamin D assays [13]. The values of the 20 DEQAS controls assayed simultaneously (range: 9–79 nmol/L) were −0.11 ± 0.72 SD units (mean ± SD) from the all laboratories means [13]. The cut-off value of the serum vitamin D level for the vitamin D status study was 33 nmol/L. It corresponds to the threshold of the lower vitamin D tertile.

- Polymorphism testing: Genomic DNA was prepared from blood, using commercial extraction kits (Qiagen, Courtaboeuf, France). Polymorphism rs4516035 located −1012 in the promoter of the human vitamin D receptor (hVDRp) was assayed using fluorescent hybridization probe melting curves in real time PCR. Specific PCR and hybridization primers (Tib MolBiol, Berlin, Germany) and PCR conditions were described in Esterle et al. [9].

3. Results

Full-time boarders children and adolescents (mean age: 11.4 years) had short stature and low calcium and magnesium levels (Table 1). Their 25-(OH) D levels, measured during winter–spring (January–April), were not different from those found in the French adolescent girls measured between February and May (cohort II). The two populations had a high prevalence of low 25-(OH) D values. Indeed, levels below 30 nmol/L were found in 15% and 28%, and levels below 40 nmol/L were found in 45% and 46% of cohorts I and II, respectively.

Genotyping the two cohorts for the polymorphism located −1012 of the promoter region of the VDR gene [8], showed similar distribution, with 21% and 17% genotype G/G, 44% and 52% genotype A/G and 35% and 31% genotype AA in cohorts I and II, respectively.

Analysis of growth parameters showed a significant association between height, expressed as SDs, and −1012 VDRp genotype in the two cohorts, with the G/G children and adolescents being 0.4–0.6 SDs shorter than their A/A counterparts (Tables 2 and 3).

Analysis of biochemical parameters showed a significant association between serum calcium levels and VDRp genotype in cohort I (Table 2). A similar trend was not significant in French adolescent girls (Table 3). Of interest, when considering possible interactions with the vitamin D status, the VDRp/serum calcium association was

Table 3
VDR −1012 G/A polymorphism and variables in the French adolescent girls (cohort II).

	VDR genotype			*P* values	
	G/G	G/A	A/A	G/G vs. A/A	G/G vs. G/A
Frequency (*n* =)	18.8% (*n* = 26)	48.5% (*n* = 67)	32.6% (*n* = 45)	–	–
Age (years)	13.4 ± 0.3	13.0 ± 0.2	13.4 ± 0.2	0.9255	0.2321
Height (SDs, WHO standard)	−0.088 ± 0.194	0.081 ± 0.121[a]	0.545 ± 0.136	**0.0087**	0.4502
Serum calcium (mmol/L)	2.48 ± 0.02	2.51 ± 0.01	2.50 ± 0.01	0.2362	0.1216
Serum phosphate (mmol/L)	1.34 ± 0.02	1.36 ± 0.02	1.34 ± 0.02	0.4882	0.8831
Serum magnesium (mmol/L)	0.78 ± 0.02	0.79 ± 0.01	0.79 ± 0.01	0.7414	0.5548
Serum 25-hydroxyvitamin D (nmol/L)	38.9 ± 3.3	50.0 ± 2.3	46.7 ± 3.3	0.1263	**0.0217**

Values are given as mean ± SE.
Significant values ($p < 0.05$) are in bold.
[a] $p = 0.0138$ vs. A/A.

Table 4
VDR genotype and variables according to 25-(OH) D status in the Moldovan cohort (cohort I).

−1012 VDR genotype	25-(OH) D <33 nmol/L			25-(OH) D ≥33 nmol/L		
	G/G	G/A	A/A	G/G	G/A	A/A
n =	11	30	17	32	61	54
Age (years)	11.3 ± 0.6	11.7 ± 0.4	11.9 ± 0.4	11.4 ± 0.3	11.3 ± 0.2	11.2 ± 0.2
Adjusted serum calcium (mmol/L)	2.16 ± 0.09	2.18 ± 0.05	2.17 ± 0.06	2.12 ± 0.06[a]	2.28 ± 0.05	2.30 ± 0.04
Serum phosphate (mmol/L)	1.53 ± 0.15	1.31 ± 0.07	1.31 ± 0.08	1.39 ± 0.08	1.32 ± 0.05	1.39 ± 0.04
Serum magnesium (mmol/L)	0.69 ± 0.06	0.70 ± 0.03	0.82 ± 0.03	0.68 ± 0.03	0.73 ± 0.02	0.72 ± 0.02
Serum 25-(OH) D (nmol/L)	28.4 ± 4.6	28.3 ± 0.7	27.4 ± 1.0	46.8 ± 1.8	51.1 ± 1.9	51.0 ± 1.8
Height (SDs)	−0.86 ± 0.20	−0.54 ± 0.19	−0.58 ± 0.22	−0.98 ± 0.18[b]	−0.81 ± 0.11	−0.57 ± 0.12
BMI (SDs)	−0.27 ± 0.18	−0.67 ± 0.19	−0.28 ± 0.23	−0.21 ± 0.15	−0.36 ± 0.16	−0.82 ± 0.14

Values are given as mean ± SE
[a] $p = 0.0425$ vs. G/A and $p = 0.0233$ vs. A/A
[b] $p = 0.0470$ vs. A/A

found in Moldovan children with 25-(OH) D above 33 nmol/L, but not in those with 25-(OH) D levels in the lower tertile of the total cohort, below 33 nmol/L (Table 4).

4. Discussion

We and others have shown previously that the −1012 VDR polymorphism is functional as cells transfected with a −1012A VDR promoter show increased VDR transactivation activity compared to cells transfected with a −1012G VDR promoter [4,8]. This polymorphism is also of interest as it shows distribution differences among ethnic groups. Many Caucasians (32–35%) but very few Asians or Sub-Saharian Africans (less than 1%) bear a G/G genotype [4]. Previous reports suggest that this "Caucasian" genotype may not be as favorable as the A/A genotype, regarding height in adolescent girls [8] or bone mineralization in adolescent girls having low milk intakes [9]. The present work brings further support to the hypothesis that height during growth is in part controlled by VDR expression and may be hampered in children and adolescents bearing a G/G genotype. This effect does not appear to be linked to a likely influence of the VDRp genotype on the ability of the intestine to absorb calcium, as it was found in two Caucasian cohorts, irrespective of their calcium intakes.

Of interest, VDRp genotype was also found to be associated with serum calcium levels in the cohort of children and adolescents with very low milk/calcium intakes, the lower values being again found in children with the G/G genotype. Even though neither PTH nor 1,25-$(OH)_2$ D could be assayed, it is tempting to suggest that children with the lower VDR expression had more difficulties to absorb calcium and, thus, to maintain their serum calcium levels in the normal range. Our observation that the association between VDRp genotype and serum calcium levels was not found in 25-(OH) D deficient children (<33 nmol/L) suggests that a sufficient production of 1,25-$(OH)_2$ D3, the active form of vitamin, is required to reveal the VDRp genotype effects.

In conclusion, the −1012 VDRp polymorphism appears more and more to be functional and to play a significant role in the individual control of calcium and bone metabolism as regards calcium intakes and vitamin D status. All studies published so far suggest that Caucasians may have greater difficulties than other ethnic groups to achieve optimal calcium homeostasis and bone mineralization when their milk/calcium intakes are insufficient, because of their high prevalence of an unfavorable −1012 VDRp genotype.

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