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Two single-nucleotide polymorphisms in the human vitamin D receptor promoter change protein–DNA complex formation and are associated with height and vitamin D status in adolescent girls

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Numerous association studies have dealt with single-nucleotide polymorphisms (SNPs) in coding and intronic regions of the human vitamin D receptor (hVDR) gene. We have hypothesized that phenotypic traits may also be associated with variations in VDR expression due to the presence of SNPs in promoter regions. In this work, we have studied two SNPs located 1521 bp (G/C) and 1012 bp (A/G) upstream of the transcriptional start site of the main human VDR gene promoter. One base-change in any of the two variant sites led to a dramatic change in protein–DNA complex formation using nuclear extracts from HEK293, Caco-2 and COS-7 cells. Genetic analysis of 185 healthy adolescent girls evidenced two major haplotypes: 1521G/1012A and 1521C/1012G and three main genotypes: homozygous for 1521G/1012A (21.1%), homozygous for 1521C/1012G (17.3%) and heterozygous 1521CG/1012GA (57.3%). On the basis of transfection data, promoter activity was nearly 2-fold higher with the 1521G/1012A haplotype, when compared with the 1521C/1012G haplotype. Clinical and biological association study in the adolescent cohort showed that girls with a CC/GG genotype had (i) lower circulating levels of 25-dihydroxyvitamin D, with no detectable consequence on calcium metabolism, (ii) lower serum IGF-1 levels and (iii) smaller height from 11 years of age up to adult height.

INTRODUCTION

Numerous studies have analyzed possible associations between several single-nucleotide polymorphisms (SNPs) of the human vitamin D receptor (hVDR) gene and diseases or phenotypic traits involving known vitamin D functions like bone mineralization, calcium metabolism, cell differentiation or regulation of the immune system (1). Several studies, including ours, have shown associations with body growth or final height (2–10). To date, most of the analyzed polymorphisms concern coding and intronic regions of the VDR gene, have poorly understood effects on VDR function, and are not unequivocally associated with diseases or traits. We, and others, have hypothesized that SNPs may be also located in promoter regions of the VDR, influence VDR expression, and therefore, be more tightly associated with phenotypic traits or diseases. The first of such SNPs has been found in a caudal-related homeodomain protein Cdx-2 binding element located 3731 bp upstream of exon 1a, which follow the so-called 1a-promoter of the hVDR gene (11,12) and shown to be associated with bone mineral density (12) and susceptibility to fracture in the elderly (13).

Another SNPs has been recently identified at −1012 site (G/A variation) of the 1a-promoter of the hVDR, and over-representation of the A allele was found in patients with malignant melanoma (14) and psoriasis (15). Yet the functionality of this latter site and possible impact of variants in healthy populations have not been evaluated.

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Keeping in line with the hypothesis that promoter SNPs may influence VDR expression and be associated with quantitative traits in healthy populations, we have screened 2 kb upstream of the transcriptional start site of the 1a-promoter of the VDR gene and identified two SNPs, one being the −1012 (G/A) and the other being localized at −1521 (G/C). We have shown their functionality, with dramatical changes in protein–DNA complex formation and transcriptional activity, in cell types that are classical vitamin D targets. This led us to test the influence of these human VDR gene promoter (hVDRp) SNPs on vitamin D status, height and bone mineralization in a cohort of healthy adolescent girls followed during a 4-year survey.

RESULTS

Two SNPs were found in the hVDRp

PCR amplification of the VDR promoter (2 kb) located upstream of the exon 1a showed the presence of two polymorphisms (Fig. 1), 1012 and 1521 bp upstream of the reported transcriptional start site of the promoter (16). The distribution of these variants in the studied cohort showed high homozygous frequencies at site 1521, (CC: 18.9%; GG: 21.1%) and 1012 (AA: 23.2%; GG: 18.7%). Two major haplotypes were found in the 370 studied chromosomes, 1521G/1012A (50.5% of the studied chromosomes) and 1521C/1012G (47.1%). Overall, three main genotypes were represented in our cohort (177 of 185 girls), homozygous for 1521G/1012A (21.1%), homozygous for 1521C/1012G (17.3%) and heterozygous 1521CG/1012GA (57.3%). Eight remaining individuals had a 1521CG/1012GG ($n = 2$, 1.1%), 1521CG/1012AA ($n = 3$, 1.6%), 1521CC/1012AG ($n = 2$, 1.1%) or a homozygous 1521CC/1012AA ($n = 1$, 0.5%) hVDRp genotype. The two promoter SNPs were independent of the *Bsm*I SNP located in intron 8 of the VDR gene and that has been previously determined in this cohort, with a null r^2 value between pairs of biallelic markers.

Nuclear complexes bound to the −1521 and −1012 regions depend upon polymorphism

Binding of transcription factors, or protein complexes, was investigated in the promoter region surrounding the two polymorphisms (14 bp upstream and downstream to the SNPs). As shown by Electrophoretic mobility shift assay (EMSA) using nuclear extracts of HEK293 kidney cells, oligonucleotides containing either G or C polymorphisms in the 1521 region bound a similar slow-migrating complex and different faster-migrating complexes (Fig. 2A). Results differed in the 1012 region, where 1012A and −1012G each bound a major complex with different migration properties. Similar binding patterns were found with Caco-2 (Fig. 2B) and COS-7 nuclear extracts (Fig. 2C).

Searching database for transcription factor-binding sites (Transcription Element Search System, TESS, using Transfac database) failed to identify transcription factors docking to the 1012G, 1521G or 1521C sites. In contrast, 1012A presented the sequence of a consensus GATA binding site.

Major complexes bound to 1521C and 1012G are related

Competition studies in EMSA using HEK293 nuclear extracts showed cross-competition between the 1521C and 1521G oligonucleotides for the slow-migrating complexes, thus confirming the similarity of these complexes, and no cross-competition for the faster-migrating complexes, which had distinct migration properties (Fig. 3A and B). No cross-competition for nuclear complexes docking was observed between the 1012G and 1012A oligonucleotides, further supporting that single nucleotide variations dramatically switch the nuclear protein complex bound to the 1012 site (Fig. 3C and D).

Remarkably, the faster-migrating complex bound to 1521C and the main complex bound to 1012G had comparable migrating properties and the two oligonucleotides were able to compete with each other for nuclear binding (Fig. 3A and C). This suggests that 1521C and 1012G sites (which are mostly found on the same allele) bind the same or closely related nuclear complexes. Similar results were found using the Caco-2 and COS-7 nuclear extracts (data not shown).

The hVDRp activity depends upon haplotype variation

Transfection experiments were performed in HEK293 and in COS-7 cells to test the effects on hVDRp activity of the two major haplotypes found in our cohort (1521C/1012G and 1521G/1012A). A recurrent and significant 1.9-fold higher hVDRp activity ($P < 0.0001$ in unpaired Student's t-test) was found with the promoter containing the 1521G/1012A allele (Fig. 4).

Genotypes of the hVDRp are associated with variations in skeletal growth

Genotype and phenotype associations were analyzed in the 177 adolescent and young adult females bearing one of the three most frequent VDR promoter genotypes (Tables 1 and 2), as the other genotypes included only three individuals or less. At the beginning of the study, the three genotypes did not differ as regards mean chronological age, body weight and body mass index. Age at menarche established at the end of the survey did not depend on the genotype (Table 1). Girls with a GG/AA genotype tended to have more advanced bone maturation and a higher bone mineral density, when adjusted for age and pubertal stage. But these differences did not reach significance. In contrast, height, expressed as standard deviations (SD) on French reference growth curves, was significantly associated with the hVDRp genotype, with a 0.7 SD lower height in girls with a CC/GG genotype versus girls with a GG/AA genotype ($P = 0.006$). Moreover, girls with a small size (≤ -1 SD) or a low bone mass (≤ -1 SD) were more frequently found in the CC/GG population (20%) than in the GG/AA population (3 and 6%). Interestingly, girls with the CC/GG genotype also had significantly lower mean levels of circulating total IGF-1.

At the beginning of the survey, no significant genotype association was observed with either mean serum calcium, urinary calcium excretion, serum PTH, serum osteocalcin, serum 1.25-$(OH)_2$D or serum alkaline phosphatase activity

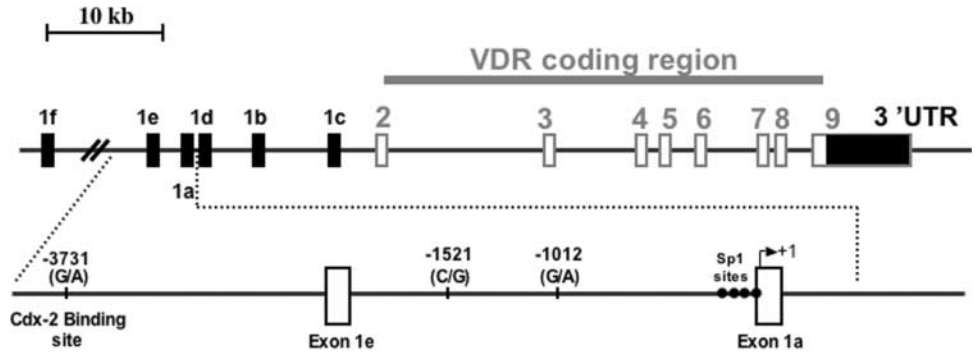

Figure 1. Two SNPs are present in the main promoter of the hVDR gene. PCR amplification was performed in the hVDRp, between exons 1a and 1e, using genomic DNAs extracted from human blood samples. The two polymorphisms are 1521 and 1012 bp, respectively, from the transcription start site of the Sp1-driven promoter described by Miyamoto *et al.* (16).

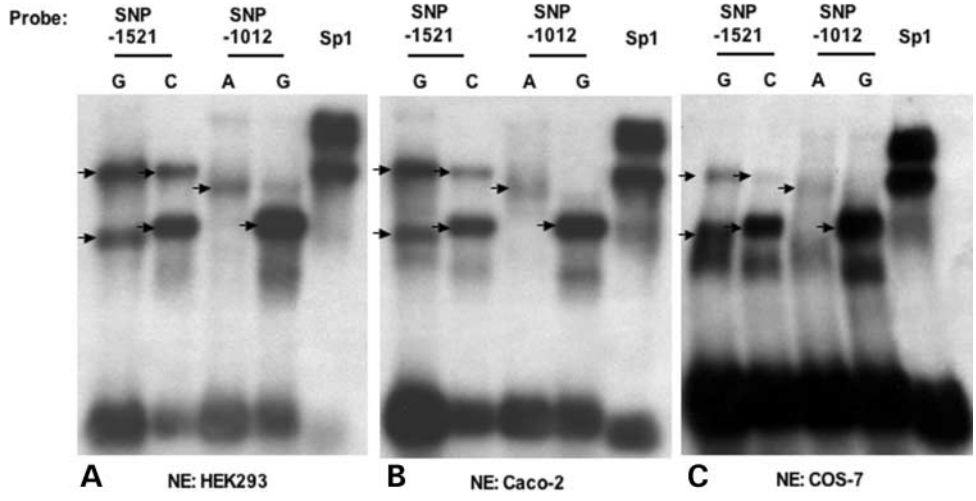

Figure 2. EMSA analysis of nuclear extract complexes bound to polymorphism sites. Gel shift assays were performed with labeled oligonucleotides representing 1521G, 1521C, 1012A, 1012G SNPs using nuclear extracts of HEK293 (**A**), Caco-2 (**B**) and COS-7 (**C**). Experiments were also performed using a canonical Sp1 site as a control for the nuclear extract integrity.

(Table 2). But girls with the CC/GG genotype had significantly lower mean circulating levels of 25-(OH)D, the storage form of vitamin D ($P = 0.006$). Furthermore, 20% of the CC/GG girls had 25-(OH)D levels below the threshold of vitamin D deficiency (10 ng/ml), whereas they were only 3% in the GG/AA group.

Multiple regression analysis (Table 3) confirmed the significant contribution of the VDR promoter genotype to height, serum IGF-1 levels and serum 25-(OH)D levels at the beginning of the survey. In contrast, none of the tested clinical and biological variables, besides 25-(OH)D levels (18 ± 1.6, 19 ± 0.9 and 23 ± 2.0 ng/ml in BB, Bb and bb girls, respectively, $P < 0.05$), was associated with the *Bsm*I genotype (Table 3).

Analyzing age-dependent height in the whole cohort showed significantly shorter size in the CC/GG group at all ages, from 11 years up to the end of puberty (Fig. 5), with no genotype dependent changes in growth velocity during the 4-year survey (1.6 ± 0.4, 1.8 ± 0.2 and 1.7 ± 0.3 cm/yr in the CC/GG, CG/GA and GG/AA groups, respectively). In addition, the observation that 90 girls had reached the age of 18 or more at the end of the survey allowed a preliminary analysis of adult size in this cohort (Table 4). Height in female young adults with a CC/GG genotype was 164.2 ± 1.5 cm, >3 cm shorter ($P = 0.05$) than in the heterozygous (167.2 ± 0.8 cm) and GG/AA groups (167.6 ± 1.3 cm).

DISCUSSION

Promoter regions are potential candidates for the presence of functional SNPs affecting gene expression in human population (17). We have focused on the main human VDR promoter (so-called 1a-promoter), identified two functional SNPs and found significant associations between these SNPs and a major human trait, namely height.

The two polymorphisms are located 1521 bp (G/C) and 1012 bp (A/G) upstream of the transcriptional start site of the promoter (16). These two SNPs are among the 245 polymorphisms recently identified on the human VDR gene (18). However, their functionality has not been studied so far. This study shows that the two SNPs are functional: (i) one

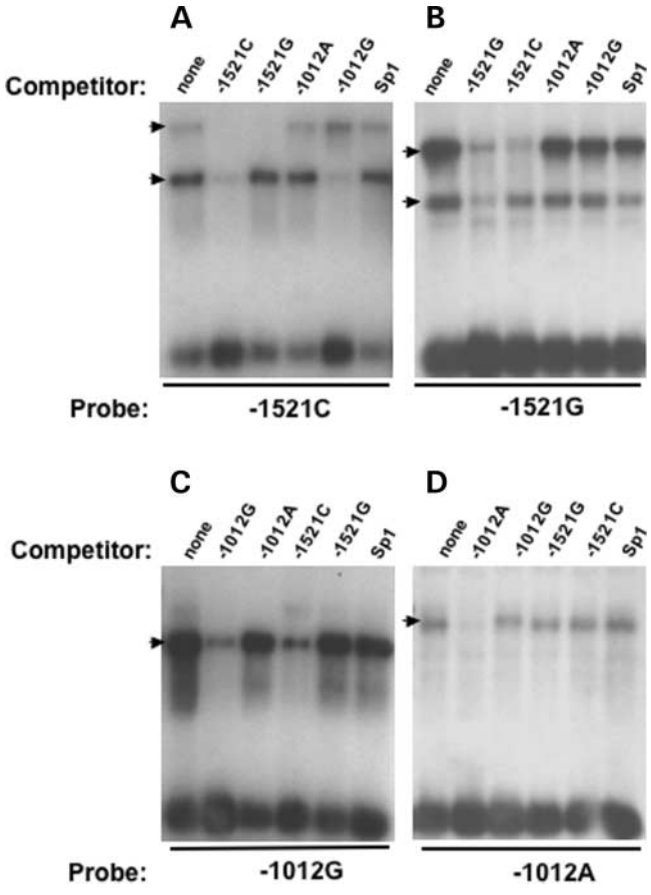

Figure 3. Cross-competition analysis between SNP sites in EMSA experiments. Gel shift assays were performed with labeled oligonucleotides representing 1521C (**A**), 1521G (**B**), 1012G (**C**) and 1012A (**D**) SNP sites using HEK293 nuclear extracts. Competition experiments were performed as indicated using a 100-fold excess of unlabeled oligonucleotide. Sp1 site was used as a non-specific competitor.

base-change in any of the two variant sites led to a dramatic switch in the nature of the complex bound to this part of the VDR promoter and (ii) it resulted in significant change in hVDRp activity. The two polymorphism sites, in EMSA studies, bound complexes present in different cell types such as enterocytes (Caco-2 cells), human tubular kidney cells (HEK293) and African green monkey kidney cells (COS-7 cells). Therefore, we can speculate that the impact of these polymorphisms on VDR expression will affect major target tissues for vitamin D, including intestine and kidney. As −1521C and −1012G sites are likely binding the same complex and have resembling sequence, we drew a consensus sequence ACC/AA/TTGCTT/AT, where underlined positions correspond to polymorphism positions at −1521 ad −1012 sites. No transcription factor-binding element has been found, using each individual sequence or the consensus sequence, during extensive TESS searches using the Transfac database. In contrast, similar TESS searches support the hypothesis that the −1012A binding area is a GATA site. Further EMSA and competition experiments confirmed the ability of the 1012A site to bind GATA factors (19).

On the basis of transfection data using the two hVDRp main haplotypes, hVDR promoter activity in renal HEK293 cells and COS-7 cells was nearly 2-fold higher with the 1521G/1012A haplotype, which contains the GATA site, when compared with the 1521C/1012G haplotype, which contains two docking sites to the same unknown transcription factor complex. It is thus possible that the VDR content is reduced in cells (and individuals) bearing a CC/GG genotype.

Genotyping the present cohort of healthy French Caucasian girls evidenced SNPs frequencies similar to those described for the −1012 (14,18) and the −1521 (18) sites in Britain populations. Both genotypes were linked, very likely due to their close location (509 bp apart), and there was a high frequency of homozygous haplotypes, 21.1% 1521GG/1012AA (GG/AA) and 17.3% 1521CC/1012GG (CC/GG), allowing association studies. These two polymorphisms localize to the haplotype block C covering the intron 2–exon 1f regulatory region of the VDR gene described by Nejentsev *et al*. (18). They are in linkage disequilibrium with the Cdx-2 polymorphism, which is also in haplotype block C, but not with other studied polymorphisms, such as *Bsm*I *Apa*I and *Taq*I in haplotype block B or *Fok*I located in the breaking spot separating blocks B and C (18).

Keeping in line with a putative lower hVDR expression in cells with a CC/GG genotype, we have searched for an association between hVDRp genotypes and markers of vitamin D status and vitamin D responsiveness. Girls with a CC/GG genotype had lower circulating levels of 25-(OH)D and tended to have higher, although not significant, circulating levels of 1,25-dihydroxyvitamin D. Similarly, low normal 25-(OH)D and elevated 1,25-$(OH)_2$D levels have been observed in children with genetically altered VDR function and severe vitamin D resistance (20). These findings are thought to result from increased 1,25-$(OH)_2$D production and decreased 1,25-$(OH)_2$D catabolism, both being VDR dependent (21). Yet, the absence of association between VDRp genotypes and either serum calcium, serum PTH or urinary calcium excretion suggests that vitamin D resistance in the CC/GG cohort was moderate and not sufficient to alter calcium metabolism.

Of particular interest, height in CC/GG girls was found to be markedly smaller during the 4-year survey that spanned the pubertal growth spurt up to adult height. Earlier studies have considered association between height and VDR polymorphisms located in coding and intronic regions of the VDR gene, namely *Bsm*I, *Apa*I, *Taq*I and *Fok*I. *Fok*I site influences the length and activity of the VDR protein (21). The other polymorphism sites have no known functional activity, but they are tightly linked to other VDR gene polymorphism sites, which may influence VDR mRNA stability or VDR transcriptional activity (19). Associations between height and VDR genotypes at these polymorphism sites have been reported in pre-pubertal and pubertal children (3–5,8,9), adult men (5) and adult women (3,10). However, other studies have found no such associations in young or adult males (4,6,8,10) or females (6,7,22 and present study). Our findings are the first shown with variants located in the main regulatory region of the VDR gene, at polymorphic sites important for the regulation of the promoter activity. These findings suggest that the hVDRp genotype may contribute to

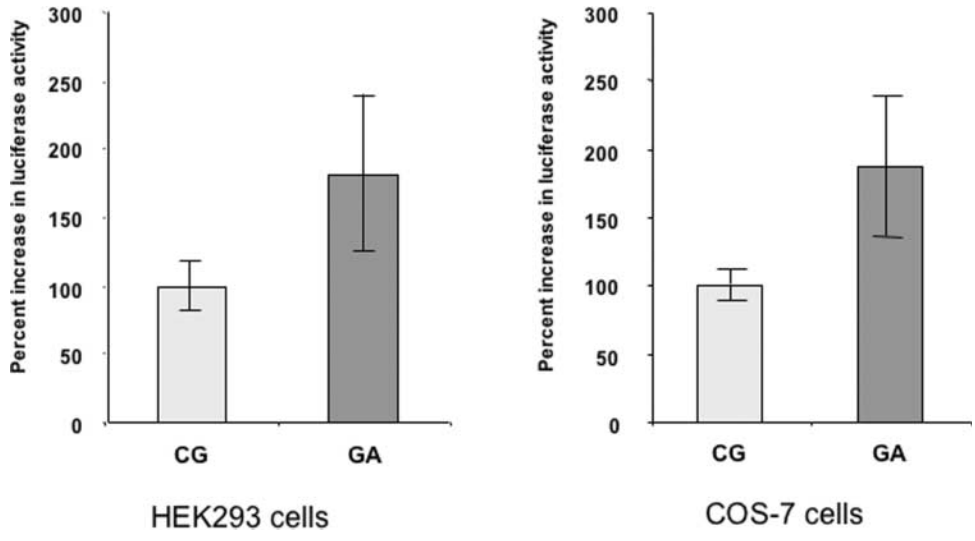

Figure 4. Promoter activity of the major hVDRp haplotypes.Transfection experiments were performed in HEK293 and COS-7 cells using the 2 kb hVDRp reporter vector containing the 1521G/1012A allele or the 1521C/1012G allele. Transfections were normalized by β-galactosidase activity that was produced by 50 ng of co-transfected pCMVbeta-gal vector. The values represent the mean of seven (HEK293 cells) and five (COS-7 cells) different experiments ($\pm$SD) done in triplicate. $P < 0.0001$ between haplotypes for each experiment in ANOVA testing.

growth, up to adult stature, via variations in the transactivation capacity of the hVDRp, in addition to other hVDR polymorphisms influencing the stability of hVDR mRNA or the length of the hVDR protein. Of interest, hVDRp genotypes were associated with height, but neither with body weight, body mass index, bone mineral content nor circulating markers of bone metabolism (osteocalcin and alkaline phosphatase activity). It appears thus that the putative hVDRp genotype effect on growth might specifically concern the growth plate cartilage function, rather than bone growth and mineralization. Along this line, associations between VDR genotype and bone density during growth have been observed in some studies (8,23,24), but not all studies (5,6,25), and have been attributed to variance in bone size rather than in bone mineralization (4,9).

Several global genome scanning for genetic determination of height loci have highlighted the chromosome 12 as a region of interest (26), and a peak of linkage with adult height has been observed at the chromosome marker D12S398 locus (27–29). Interestingly, VDR gene is located at chromosome 12q12–q14, and the peak of linkage is located only 4.5 Mb from the VDR gene, a spacing smaller than the average spacing between two markers in these studies. Therefore, the VDR gene could be one of the genetic determinants of adult height pointed out by genome scan studies.

Several evidences point out vitamin D as a likely contributor to growth: (i) its activity on cell proliferation in bone and growth plate cartilage (30,31), (ii) the growth delay observed in patients with vitamin D deficiency or pseudo-deficiency rickets and (iii) the growth enhancing effect of vitamin D during intrauterine and postnatal life in children (32). VDR is likely to be involved in this action, as suggested by phenotype and genotype association studies including the present one, and by the observation of a reduced growth in transgenic mice with an invalidated vitamin D receptor (33); however, how VDR controls growth remains to be elucidated. The observed differences in height do not seem related to differences in sexual maturation or bone differentiation, as age at menarche and bone age were not associated with hVDRp genotype. In contrast, the significant association with circulating levels of IGF-1 suggests that this major factor for skeletal growth mediates in part the observed association with height. There is no evidence as yet for a direct interaction between VDR and the IGF-1 gene. But several interactions between vitamin D and the IGF system have been reported: (i) 1,25-$(OH)_2$D increases IGF-1 circulating levels *in vivo* (34) and in bone cell cultures (35–37); (ii) it increases the expression of the IGF-Type 1 receptor in growth plate chondrocytes (38); (iii) it increases the expression of several IGF-binding proteins in osteoblasts and bone marrow stromal cell cultures, namely IGF-BP2 and IGF-BP3 (39), IGF-BP4 (37–40) and IGF-BP5 (41); (iv) a VDR responsive element has been identified on the promoter region of IGF-BP3 (42). Finally, it has been proposed that VDR genotype effects result from variations in VDR expression in the intestine and, subsequently, in the intestine ability to absorb calcium (43–46). Biological and clinical association studies gave no evidence for a lower calcium absorption in the CC/GG girls. But interactions of calcium (or dairy product) intake and VDR genotype on pubertal growth cannot be excluded.

In conclusion, we identified two functional SNPs located in the main VDR promoter. One base-change in any of the two variant sites led to a dramatic change in the nature of protein–DNA complex formation and to significant changes in the transactivation capacity of the VDR promoter. Results of the present association studies in female adolescents suggest that these two SNPs may be of great interest for the understanding of individual phenotypic variations in response to vitamin D.

Table 1. Clinical data at the beginning of the survey

Genotype	CC/GG	CG/AG	GG/AA
n	32	106	39
Age (years)	14.9 ± 0.49	14.8 ± 0.28	14.6 ± 0.47
Calcium intake (mg/d)	896 ± 41	882 ± 25	891 ± 42
Age at menarche (years)[a]	13.3 ± 2.0	13.1 ± 0.1	13.2 ± 0.2
Bone maturation[b]	−0.72 ± 0.17	−0.62 ± 0.14	−0.38 ± 0.25
Weight (kg)	47.7 ± 1.8	49.9 ± 0.9	49.0 ± 1.3
Body mass index (kg/m^2)	19.2 ± 0.5	19.4 ± 0.2	18.9 ± 0.4
Height (SD)[c]	0.14 ± 0.20	0.71 ± 0.10*	0.84 ± 0.17*
Percent girls with height ≤ −1 SD	20%	7%	3%
Bone mineral density (Z score)[c]	0.04 ± 0.19	−0.05 ± 0.09	0.27 ± 0.16
Percent girls with Z score ≤ −1 SD	20%	16%	6%

Data are mean ± SE. Significant differences are noted. No significant differences were found between CG/AG and GG/AA.
[a]Age at menarche has been documented for all girls, at the beginning or during the survey.
[b]Bone maturation is the difference between bone age and chronological age. It could only be evaluated in girls under 16 years of age ($n = 15$, 55 and 23, respectively, in the three genotype groups).
[c]Mean absolute values for height and bone mineral density have not been calculated because of the wide age-range of the cohort.
*$P \leq 0.008$ when compared with CC/GG using ANOVA (Statview 5.0).

Table 2. Biological data at the beginning of the survey

Genotype	CC/GG (30)	CG/AG (103)	GG/AA (36)
Serum values			
Calcium (mg/dl)	9.88 ± 0.04	9.96 ± 0.03	9.92 ± 0.04
PTH (pg/ml)	27.5 ± 2.2	26.6 ± 1.1	24.1 ± 1.5
1,25-(OH)2D (pg/ml)[a]	78 ± 7	76 ± 4	71 ± 8
25-(OH)D (ng/ml)	17.7 ± 1.4	20.3 ± 0.9	23.0 ± 1.6***
Percent girls with 25-(OH)D ≤ 10 ng/ml	20%	9%	3%
Osteocalcin (ng/ml)	86 ± 9	91 ± 5	87 ± 8
Alkaline phosphatase activity (IU/l)[b]	327 ± 27	340 ± 17	291 ± 23
IGF-1 (ng/ml)	417 ± 25	467 ± 16*	508 ± 36**
IGF-1 (SD)	−0.30 ± 0.16	0.04 ± 0.92	0.17 ± 0.19*
Urinary values			
Calcium excretion (mg/100 ml G.F.)	0.045 ± 0.008	0.037 ± 0.003	0.038 ± 0.005

Data are mean ± SE. Significant differences are noted. No significant differences were found between CG/AG and GG/AA.
[a]The number of samples assayed in each genotype group was 10, 36 and 10, respectively.
[b]The number of samples assayed in each genotype group was 15, 54 and 23, respectively.
*$P < 0.05$; **$P = 0.03$; ***$P = 0.006$ when compared with CC/GG using ANOVA (Statview 5.0).

MATERIALS AND METHODS

Population cohort

The studied cohort included 185 unrelated healthy French Caucasian girls with no known alteration in calcium or bone metabolism (mean ± SD: 14.7 ± 2.8 years; range: 11–22 years). All girls had been enrolled for the 4-year survey of their bone mineral mass acquisition (47). Twenty-one girls were first seen before puberty, 57 during puberty and 107 after menarche. Chronological age, bone age, pubertal stage, height, weight, BMI, DXA lumbar bone mineral density, daily calcium intake and *Bsm*I polymorphism in the VDR gene had been recorded at the time of blood and urine sampling and after 2 and 4 years. Serum and urinary calcium and creatinine concentrations and serum alkaline phosphatase activities were determined using an automatic auto-analyzer. Serum IGF-1, osteocalcin and PTH were assayed using commercial kits (47). Serum 25-hydroxyvitamin D (25-(OH)D) was assayed using in-house competitive protein binding assays (48) with continuous external quality assessment of the 25-(OH)D assay (49). Serum 1,25-$(OH)_2$D was assayed using a commercial radioimmunoassay kit (Nichols Institute Diagnostics, Paris, France). Height and serum IGF-1 levels were evaluated both as absolute values and in comparison to reference growth curves for French children (50), and bone mineral density was evaluated in g/cm^2, and as Z score adjusted for age and pubertal stage using in-house reference curves. A local Ethics Committee on human subjects approved the genomic studies and the subjects or their parents had given written informed consent.

Analysis of the human VDR promoter SNPs

Genomic DNA was prepared from blood, using commercial extraction kits (Qiagen, Courtaboeuf, France). The human VDR promoter was PCR-amplified with 50 ng of genomic DNA as template using 0.5 μM of 3′ forward primer 5′-GGA

Table 3. Multiple regression analysis of clinical and biological data at the beginning of the survey

	Height	Weight	BMD	Serum calcium	Urinary Ca/creat	Serum PTH	Serum 25-(OH)D	Serum ALP	Serum osteocalcin	Serum IGF1
Age	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.7105	0.2312	0.9647	**<0.0001**	**<0.0001**	0.0636
BMI	0.5145	—	**<0.0001**	**0.0229**	0.2345	0.7274	0.2540	0.1987	**0.0201**	**0.0068**
Ca intake	0.2946	0.9783	0.4115	0.4904	0.0516	0.9389	0.1700	0.1833	0.2707	0.0610
IGF-1	**0.0038**	**0.0003**	**<0.0001**	0.3539	0.1878	0.2201	0.9384	0.3115	0.2181	—
25-(OH)D	**0.0343**	**0.0209**	**0.0179**	0.1242	**0.0249**	0.0606	—	0.6958	0.2231	0.9384
PTH	**0.0106**	0.3344	0.9172	0.2721	**0.0007**	—	0.0606	0.0601	**0.0206**	0.2201
Month[a]	—	—	—	—	—	—	**0.0170**	—	—	—
*Bsm*I	0.2599	0.6900	0.9513	0.6520	0.5415	0.8733	0.0569	0.2160	0.3527	0.8451
VDRp[b]	**0.0086**	0.2048	0.4227	0.9980	0.4371	0.5481	**0.0307**	0.7294	0.4903	**0.007**
Adjusted r^2	0.521	0.463	0.637	0.163	0.127	0.012	0.124	0.198	0.6540	0.118

Multiple regression analysis was performed using Statview 5.0. All independent variables tested are listed.
[a]Month of blood collection was tested only for 25-(OH)D as this variable fluctuates with the season.
[b]Genotype CC/GG, CG/AG, GG/AA.
Values in bold letters have reached significance ($P \leq 0.05$).

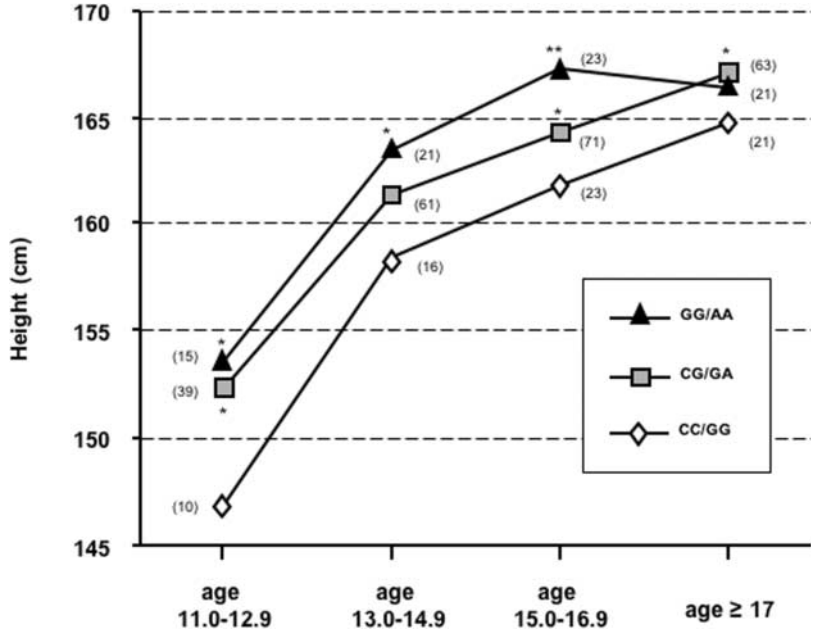

Figure 5. hVDRp genotype-associated growth curve during female puberty. The 177 girls seen at the onset of the survey have mostly been seen thrice during the 4-year survey. Their height has been plotted according to their VDRp genotype and age category with no individual reported twice in the same age category. Numbers in parentheses indicate the number of subjects for each point. Significant differences are noted, single asterisk indicates $P \leq 0.05$; double asterisks indicate $P = 0.001$ for mean GG/AA or CG/GA heights versus respective mean CC/GG heights in the same age categories, using ANOVA (Statview 5.0).

CTT GCA GAG AAT GTC CCA AG-3′ (−1609 to −1586 pb from the start site of the hVDRp) and 0.5 μM of 3′ reverse primer 5′-GAT AGG CAC CGC TCT ATC TGC-3′ (position −856 to −835 pb). The PCR amplifications were performed using either 2 units of Taq polymerase or 4 units of BioXAct short (Abcys S.A., Paris France), enzyme for difficult templates in the presence of 2.0 mM $MgCl_2$ and 1× buffer of the enzyme supplier. Thermal conditions required for the PCR reactions were 94°C for 3 min, followed by 35 cycles of 20 s of denaturation at 94°C, 20 s of annealing at 56°C and 1 min of elongation at 72°C, followed by a final step of 5 min of elongation at 72°C. PCR products were purified using High Pure PCR Product Purification Kit (Roche Diagnostics). Sequencing was carried out by the Sequencing Facility of the 'Institut Cochin' (Inserm U567, Paris, France). Statistical analysis (ANOVA, Student's *t*-test and multiple regression analysis) has been performed using the Staview 5.0 software.

hVDR gene promoter and reporter vector construction

The human VDR promoter region was subcloned into the pGL2 luciferase reporter vector (Promega Corporation, Madison, WI, USA) from the commercially available BAC vector RP11-89H19 (Roswell Park Center Institute Human BAC Library via Invitrogen Life Technologies) containing a 58 kb fragment of human genomic DNA (GenBank accession

Table 4. Height and bone mineral density in the 90 young adult females of the cohort[a] (over 18 years)

Genotype	CC/GG	CG/AG	GG/AA
n	19	52	19
Height (cm)	164.2 ± 1.5	167.2 ± 0.8*	167.3 ± 1.3*
Bone mineral density (g/cm^2)	1.038 ± 0.030	1.008 ± 0.020	0.993 ± 0.030

Data are mean ± SE. Significant differences are noted. No significant differences were found between CG/AG and GG/AA.
[a]Girls considered were aged 18 years or more at the end of the survey.
*$P \leq 0.05$ when compared with CC/GG using ANOVA (Statview 5.0).

number AC121338) located between exon 1f and exon 3 of the hVDR gene.

The 2024 bp fragment of hVDRp was obtained by cutting the BAC RP11-89H19 vector with *Eag*I and *Bgl*II restriction enzymes. Then, the fragment was placed into pGL2basic vector in the *Bgl*II site, and a new *Eag*I site, generated by an oligonucleotide insertion in the *Nhe*I and *Hin*dIII sites: Sense oligonucleotide CTAGCTCGAGATCTAACTGCAGGTTATT CAAGTCGGCCGTA and Antisense oligonucleotide AGCTT ACGGCCGACTTGAATAACCTGCAGTTAGATCTCGAG.

Transformant *Escherichia coli* clones (strain DH5α) were screened using a ^{32}P-labeled oligonucleotide probe (5′-GCT AGC TTT CCC ACG ATG CTT TGG GCA AG-3′) and positive clones were confirmed by sequencing.

Site-directed mutagenesis at the SNP locations in the hVDRp was performed using the Gene Editor TM system (Promega Corporation). The mutated oligonucleotides used were 5′-AGG CGA ATA GCA AT**G** TCT TCC CTG GCT AA-3′ for −1012 SNP and 5′-GCT AGC TTT CCC AC**C** ATG CTT TGG GCA AG-3′ for −1521 SNP (the mutated base in underlined). Each site-directed mutant was confirmed by sequencing.

Cell culture

Human intestinal cells (Caco-2), human kidney cells (HEK293) and African green monkey kidney cells (COS-7) were obtained from the American Type Culture Collection (HTB-37, Rockville, MD, USA). All cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 mg/ml of streptomycin sulfate, 0.25 μg/ml amphotericin B).

Transfection and luciferase assays

For these transfection studies, HEK293 and COS-7 cells were plated in six well plates (2×10^5 cells/well) with 2 ml of medium for 24 h. Cells were transfected using 2 μl of fuGENE™ six transfection reagent (Roche Diagnostics, France) with 0.95 μg of the pGL2 vector containing a 2024 pb hVDR promoter fragment coupled to the firefly luciferase reporter gene. In each transfection, 50 ng of the β-galactosidase expression vector pCMVβ (Clontech, Palo Alto, CA, USA) was used to correct for transfection efficiency. The cells have been grown for an additional 48 h and harvested with 200 μl of 1× lysis buffer (Promega Corporation). Luciferase activities have been measured in 10 μl of cell extract with LG Berthold Lumat LB 9507 and corrected average β-galactosidase activity determined by a standard colorimetric procedure using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate.

Electrophoretic mobility shift assays

Nuclear proteins were extracted as described previously (51) in a 300 mM KCL solution. The following double-stranded oligonucleotide probes (SNP identified in bold highlighted) were generated: −1012 A sense: 5′-AGG CGA ATA GCA AT**A** TCT TCC CTG GCT AA-3′; −1012 A antisense: 5′-TTA GCC AGG GAA GA**T** ATT GCT ATT CGC CT-3′; −1012 G sense: 5′-AGG CGA ATA GCA AT**G** TCT TCC CTG GCT AA-3′; −1012 G antisense: 5′-TTA GCC AGG GAA GA**C** ATT GCT ATT CGC CT-3′; −1521 G sense: 5′-GCT AGC TTT CCC AC**G** ATG CTT TGG GCA AG-3′; −1521 G antisense: 5′-CTT GCC CAA AGC AT**C** GTG GGA AAG CTA GC-3′; −1521 C sense: 5′-GCT AGC TTT CCC AC**C** ATG CTT TGG GCA AG-3′; −1521 C antisense: 5′-CTT GCC CAA AGC AT**G** GTG GGA AAG CTA GC-3′.

The double-stranded synthetic oligonucleotides were [γ-^{32}P]-ATP end-labeled by T4 polynucleotide kinase (Invitrogen Life Technologies) and purified on a non-denaturing 10% polyacrylamide gel prior to gel shift studies. EMSA and competition experiments were described previously (52). Competition experiments were performed using an 100-fold of a non-radioactive probe.

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