The Polymorphism in the Caudal-Related Homeodomain Protein Cdx-2 Binding Element in the Human Vitamin D Receptor Gene

HIDEKAZU ARAI, KEN-ICHI MIYAMOTO, MICHIKO YOSHIDA, HIRONORI YAMAMOTO, YUTAKA TAKETANI, KYOKO MORITA, MEGUMI KUBOTA, SHIGEKO YOSHIDA, MIKIKO IKEDA, FUMIKO WATABE, YASUHIRO KANEMASA, and EIJI TAKEDA

ABSTRACT

The major physiological activity of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is the regulation of calcium absorption in the small intestine, and the level of vitamin D receptor (VDR) is an important factor in this regulation. In a previous study, we indicated that the caudal-related homeodomain Cdx-2 played an important role in the intestine-specific transcription of the human VDR gene. In this study, the polymorphism was identified in the core sequence 5'-ATAAAAAACTTAT-3' in the Cdx-2 binding site in the VDR gene promoter. In 261 Japanese women with genotyped VDR polymorphisms, 48 were genotype Cdx-A (adenine at -3731 nucleotides [nt] relative to the transcription start site of human VDR gene 5'-ATAAAAAACTTAT), 82 were genotype Cdx-G (guanine at -3731 nt, 5'-GTAAAAACTTAT-3'), and 131 were genotype Cdx-A/G (heterozygote). In postmenopausal Japanese women, the bone mineral density (BMD) in the lumbar spine (L2–L4) with the Cdx-G homozygote was 12% lower than that with the Cdx-A homozygote (p < 0.05). In electrophoretic gel mobility shift assay (EMSA), the oligonucleotide with Cdx-G allele markedly decreased the binding to Cdx-2 compared with that in the Cdx-A allele. The transcriptional activity of the VDR promoter with Cdx-G allele was decreased to 70% of the Cdx-A allele. In addition, in the herpes simplex virus thymidine kinase promoter, the Cdx-2 binding element with the G allele showed significantly lower transcriptional activity than that of the A allele. Thus, the polymorphism in the Cdx-2 binding site of the VDR gene (Cdx-polymorphism) would affect the expression of VDR in the small intestine. In addition, this polymorphism may modulate BMD in postmenopausal Japanese women. (J Bone Miner Res 2001;16:1256–1264)

Key words: vitamin D receptor, gene promoter, caudal-related homeodomain protein, intestine-specific transcription, calcium absorption

INTRODUCTION

GENETIC FACTORS play a major role in determining bone mass, which is an important predictor of osteoporotic fracture risk.¹⁻³ Although the genetic basis of osteoporosis is not understood completely, polymorphism at the vitamin D receptor (VDR) locus has been implicated as a genetic marker for bone mineral density (BMD).⁴⁻⁷ VDR is most abundant in the small intestine and plays a key role in intestinal calcium absorption and cell differen-
tation. In humans and rodents after weaning, there is an onset of active calcium transport responsiveness to 1,25-
dihydroxyvitamin D \(_3\) \([1,25(OH)_2D_3]\) as well as a marked increase in intestinal VDR messenger RNA (mRNA) and serum 1,25(OH)2D_3. In contrast, VDR levels and calcium transport activity in the intestine decrease with advancing age. An interesting hypothesis involves the effect of aging or menopause on the intestinal VDR content as a contributing factor in the development of osteoporosis. In a previous study, we analyzed the function of the human VDR (hVDR) promoter in the intestinal cells and identified the intestinal-specific cis element of the hVDR gene promoter that interacts with a caudal-related homeodomain transcription factor Cdx-2. Cdx-2 interacts specifically with functional enhancer elements in the VDR gene in the small intestine and regulates its gene expression. In the present study, we found polymorphism in the Cdx-2 binding site of the hVDR gene promoter (Cdx-polymorphism). The relationship between the function of the promoter and BMD in post- and premenopausal Japanese women was investigated.

MATERIALS AND METHODS

Subjects

The subjects were 261 unrelated Japanese volunteers (females; range, 19–71 years) for analysis of frequencies of VDR polymorphism, residing in Okayama prefecture, Japan. Healthy, normal premenopausal and postmenopausal women volunteers were enrolled in the study of the effect of genetics on BMD and gave informed consent. Subjects with a history of bone disease, illness, bilateral oophorectomy, or drug use that could affect bone turnover and BMD were excluded from this study.

Isolation of DNA

Genomic DNA was isolated using either a modified phenol/chloroform extraction procedure or a Smallest genomic DNA isolation kit (Sumitomo KK, Osaka, Japan). The DNA samples isolated using the phenol/chloroform extraction procedure were dialyzed against Tris-EDTA buffer for 1 h before polymerase chain reaction (PCR) analysis to remove excess salts and residual phenol. With both extraction methods, the samples were analyzed multiple times to ensure the validity of the genotype.

DNA sequence analysis and detected polymorphism

The primers designed to detect the polymorphism at the hVD-SIF1 by PCR were synthesized as follows: 5’ sense primer, 5’-TAGAAAACATTGTAGAACATC-3’ (−3798 to −3777 nucleotide [nt] relative to the transcription start site of the hVDR gene); and 3’ antisense primer, 5’-AGC-TGTAGCAATTGAAAAGCTAC-3’ (−3662 to −3640 nt relative to the transcription start site). The PCR amplification reactions were performed at 95°C for 3 minutes in a single cycle, followed by 30 cycles at 95°C for 90 s, 59°C for 90 s, and 72°C for 2 minutes. The PCR products were purified using GenElute Agarose Spin Columns (Sigma, St. Louis, MO, USA). DNA sequencing used the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Tokyo, Japan) and was detected on an ABI PRISMTM 377 DNA Sequencer (Perkin Elmer).

Bone mineral densitometry

BMD, expressed as an areal density in grams per square centimeter, of the 124 normal women was measured in the lumbar spine (L2–L4) using dual-energy X-ray absorptiometry (DXA; Hologic QDR-1500; Hologic, Inc., Waltham, MA, USA).

The hVDR gene promoter and reporter vector construction

The isolation of the hVDR gene was performed as described previously. Briefly, the hVDR promoter region was subcloned into the luciferase reporter vector pGL3 (Promega, Madison, WI, USA), and its sequences were determined as described previously. These clones were termed pVDR-4000 and pVDR-4000MT. Site-directed mutagenesis in the 5’-flanking region of the hVDR gene was performed as described previously. The β-galactosidase expression vector pCMV-β (Clontech, Palo Alto, CA, USA) was used as an internal control. Each plasmid was purified using a Qiagen plasmid purification kit (Qiagen GmbH, Hilden, Germany).

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (HTB-37; Rockville, MD, USA), and COS-7 cells were obtained from Riken Cell Bank (Tokyo, Japan). The Caco-2 and COS-7 cells were cultured at 37°C under 5% CO2 in Dulbecco’s modified Eagle’s medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml of streptomycin. The Caco-2 medium also contained 1% nonessential amino acids (Gibco-BRL).

Transfection and luciferase assays

Cells were transfected using TransIT-LT1 lipofection reagent (Pan Vera Corp., Madison, WI, USA) with 0.3 μg of the luciferase reporter plasmids and 0.2 μg of pCMV-β per 5 × 10⁵ cells. In the Cdx-2 studies, 0.25 μg of the mouse Cdx-2 expression vector pReCMV-Cdx-2 was used for cotransfection as described previously, with 0.25 μg of the luciferase reporter plasmids and 0.1 μg of pCMV-β. In mock cotransfections, pReCMV-Cdx-2 was replaced with pReCMV. Cells were harvested 24–36 h later in cell lysis buffer, and the lysate was assayed for luciferase activity, β-galactosidase activity and protein concentrations. Luciferase activity was normalized to the activity of β-galactosidase.

Electrophoretic mobility shift assays

EMSAs were carried out using standard methods. A double-stranded nucleotide DNA fragment was synthesized by annealing two oligonucleotides. This fragment was la-
beled with \((\gamma^{33}\text{P})\)adenosine triphosphate (ATP; 110 TBq/mmol; ICN, Costa Mesa, CA, USA) using T4 polynucleotide kinase (Takara, Shiga, Japan). Extracts were incubated with the radiolabeled probe in binding buffer (10 mM HEPES [pH 7.9], 50 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.7 mM phenylmethylsulfonyl fluoride [PMSF], 2.0 mg/ml aprochinin, 2.0 mg/ml pepstatin, and 2.0 mg/ml leupeptin) in a final volume of 25 ml for 30 minutes at room temperature. The reaction mixture was then subjected to electrophoresis on a 4% polyacrylamide gel with 1/3 TAE (40 mM Tris-HCl, 40 mM acetic acid, and 1 mM EDTA) as an electrode buffer at a constant current of 30 mA for 1.5 h. The gel was dried and analyzed with a Fujix Bio-imaging analyzer BAS-1500 (Fuji Film, Tokyo, Japan).

**Statistical analysis**

Analysis of variance (ANOVA) was performed using the Statview statistical package (Abacus Concepts, Inc., Berkeley, CA, USA) on a Power Macintosh G3. Fisher’s protected least significant difference (PLSD) test was used to assess the relationship between the genotype and BMD. Significant levels quoted are for the initial \(f\) test on null hypothesis (no difference between the means) of the overall effect. Student’s \(t\)-test was used for pairwise comparisons.

**RESULTS**

**Polymorphism on the Cdx-2 binding region of the hVDR gene**

In 261 DNA specimens, direct sequence analysis was performed by using two primers positioned in the promoter (Fig. 1A). This analysis shows that the polymorphism appeared in the Cdx-2 binding region (Fig. 1A).

In 261 women with genotyped VDR polymorphisms, 48 were genotype Cdx-A (the \(A\) allele is 5'-ATAAAAAACTTAT-3' at -3731 to -3720 base pairs [bp] relative to the transcription start site of the hVDR promoter), 82 were genotype Cdx-G (the \(G\) allele is 5'-GTAAAAACTTAT-3' in the binding element), and 131 were genotype Cdx-A/G (heterozygote; Fig. 1B). The allelic frequency of the Cdx-A allele was 0.43. The frequencies of the Cdx-G allele were 0.57. The genotypic distribution did not differ in each age group.

**Characteristics of the subject population**

The findings for each parameter in pre- and postmenopausal groups are presented in Tables 1 and 2. There were no significant differences by Cdx genotype groups for age, weight, body lean, body mass index (BMI), age at menopause, serum calcium, and daily calcium intake.

**BMD in relation to VDR genotype**

A statistically significant intergroup difference in BMD of the postmenopausal group but not the premenopausal group was found at the lumbar spine among the different genotypes (Figs. 2A and 2B). In postmenopausal women, the subjects in the Cdx-A group had a significantly higher BMD (0.945 ± 0.025 g/cm²) than subjects in either the Cdx-G or the Cdx-A/G groups (0.723 ± 0.017 g/cm² and 0.755 ± 0.026 g/cm²), respectively. When compared with Cdx-A/A homozygotes, Cdx-G/G and Cdx-A/G genotype had lower BMD at the lumbar spine by 23% (\(p < 0.05\)) and 20% (\(p < 0.05\)), respectively.

Age-adjusted BMD in postmenopausal women was 0.2 SD lower at the lumbar spine (L2–L4) in Cdx-G homozygotes than in Cdx-A homozygotes (Z = 0.38 Cdx-A/A vs. Z = 0.10 Cdx-G/G; \(p < 0.05\)). Heterozygotes Cdx-A/G had intermediate BMD at the lumbar spine, 0.1 SD greater than the Cdx-G/G subject (Figs. 3A and 3B).

**Analysis of EMSA in the Cdx-A and Cdx-G genotype**

In a previous study, we found that the hVD-SIF1 sequence 5'-ATAAAAACTTAT-3' is the target sequence of FIG. 1. Sequence determination on the polymorphism site in the promoter of the hVDR gene. The primer for amplification of the Cdx-2 binding element was designed as described in the Material and Methods section. DNA was extracted from 261 volunteers and the Cdx-2 binding site was amplified using PCR and then analyzed by direct sequence analysis. (A) Represents the genotype in Cdx-A and Cdx-G. Sequence data showed that the Cdx-A allele is 5'-ATAAAAAACTTAT-3' at -3731 to -3720 base pairs [bp] relative to the transcription start site of the hVDR promoter, 82 were genotype Cdx-G (the G allele is 5'-GTAAAAACTTAT-3' in the binding element), and 131 were genotype Cdx-A/G (heterozygote; Fig. 1B). The allelic frequency of the Cdx-A allele was 0.43. The frequencies of the Cdx-G allele were 0.57. The genotypic distribution did not differ in each age group.

**RESULTS**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cdx-A</th>
<th>Cdx-A/G</th>
<th>Cdx-G</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>48</td>
<td>131</td>
<td>82</td>
<td>261</td>
</tr>
<tr>
<td>(%)</td>
<td>18.4</td>
<td>50.2</td>
<td>31.4</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Because the polymorphism is located at this core sequence (the 5’-terminal site), we investigated the effect of the polymorphism on the binding to Cdx-2 (Fig. 4). EMSAs were performed using oligonucleotides containing specific mutations of this sequence (MT; Cdx-G) as competitors of the wild-type sequence (WT; Cdx-A). As previously reported, the formation of this complex was markedly inhibited in the presence of the Cdx-2 antibody (cdx2).(18) The oligonucleotide of Cdx-A (WT) completely inhibited the binding of Cdx-2, did not affect the binding to that in the WT (Fig. 4).

Similarly, other oligonucleotides, which mutated the core sequence of the binding of Cdx-2, did not affect the binding activity. As previously reported, the formation of this complex was markedly inhibited in the presence of the Cdx-2 antibody (cdx2).(18)

**Effect of Cdx-polymorphism in the hVDR gene promoter**

In a previous study,(18) we found that the DNA fragment containing the hVD-SIF1 binding site increased transcription when placed upstream of the TK promoter.

Here, we constructed the vector containing the Cdx-A and Cdx-G genotype on the Cdx-2 binding element. As shown in Fig. 5, the cotransfection of Cdx-2 expression vector and the TK promoter fused the Cdx-2 binding site and was stimulated about 3.4-fold of the control. In contrast, the cotransfection of Cdx-2 expression vector and the TK promoter fused the Cdx-G (Cdx-G/VD100) significantly increased the luciferase activity compared with the control. Thus, the transactivation function of Cdx-2 in the mutated vector (Cdx-G) was significantly lower (about 30% less) than that of the Cdx-A.

Moreover, we investigated the transcriptional activity in Caco-2 cells by using the native hVDR promoter (Fig. 7). We constructed two clones, pVDR-4000 and pVDR-4000MT, which contained allele A and allele G in the Cdx-2 binding site, respectively. As shown in Fig. 7, the transcriptional activity of the hVDR gene promoter with genotype Cdx-G was significantly lower (about 15% less) than that of the Cdx-A.
DISCUSSION

BMD is a predictor of fracture risk and in elderly women may be determined by both peak bone density and postmenopausal loss.\(^1\–^3\) There has been substantial interest in the association of polymorphic VDR alleles and the risk of osteoporosis.\(^4\–^7\) In a previous study, we reported that the hVDR gene has two potential translation initiation sites (FokI polymorphism).\(^20\) The polymorphic variant of hVDR (missing the FokI restriction enzyme site), which lacks only the first three amino acids, interacted more efficiently with transcription factor IIB (TFIIB) and also possessed elevated transcriptional activity compared with the full-length receptor (first ATG).\(^24\) In premenopausal Japanese women, BMD at an L2–L4 was 10.7% lower in 16 women homozygous for the first ATG compared with 30 women homozygous for the absence of the first ATG.\(^20\) However, in the postmenopausal women, we did not find any significant changes in BMD on FokI genotype (data not shown). Moreover, recent studies indicate that the effect of the FokI polymorphism on BMD could prove to be less than originally thought, as was found in the case of the BsmI polymorphism.\(^25\–^30\) It is more probable that confounding environmental and regional factors such as high calcium diet may be masking the relationship between the FokI genotype and BMD.\(^31\–^33\)

VDR levels and calcium transport activity in the intestine decrease with advancing age.\(^15\–^17\) An interesting hypothesis involves the effect of aging or menopause on the intestinal VDR content as a contributing factor in the development of osteoporosis.\(^15\) In addition, the regulation of VDR gene expression has been well studied regarding intestinal calcium absorption in neonatal development.\(^16\) VDR controls the expression of calbindin-D9k, which is important to intestinal calcium transport, because changes in the amount of VDR mRNA are known to be associated with intestinal calcium transport.\(^15\) In a previous study, using an intestinal cell line that expresses Cdx-2 as a model system, we showed that the Cdx-2 binding sites (hVD-SIF1) were a key determinant for the expression of the VDR gene.

![FIG. 2. Distribution of genotypes and spinal BMD (L2–L4). Genotype was determined by A or G in the position of −3731 nt relative to the transcription start site. Cdx-A are the homozygotes that represent the ATAAAAACTTAT; Cdx-G are the homozygotes that represent the GTAAAAACTTAT; Cdx-A/G are heterozygotes. Numbers represent the population who has the specified genotype. BMD, expressed as an areal density in grams per square centimeter, was measured in the lumbar spine (L2–L4); *p < 0.05. (A) Premenopausal women; (B) postmenopausal women.](image)

![FIG. 3. The effect of Cdx genotype on BMD. Base line age-adjusted BMD (Z score ± SEM) by genotype at the lumber spine. *p < 0.01. (A) Premenopausal women; (B) postmenopausal women.](image)
in the small intestine. In the present study, we identified the polymorphism in the Cdx-2 binding site of the hVDR gene. Among the three groups, there are no significant changes in daily calcium intake, body weight, age, BMI, or plasma calcium levels. The BMD in the lumbar spine (L2–L4) with the Cdx-G homozygote was 12% lower than that with the Cdx-A homozygote ($p < 0.05$).

The polymorphism (A/G) is located at the 5’-terminal of the core sequence (5’-ATAAAAACTTAT-3’) for the Cdx-2 binding. Comparison of the caudal-related element sequences indicates that the position of the polymorphism is important to the Cdx-2 binding. EMSA and transfection study, the polymorphism of genotype Cdx-G clearly decreased Cdx-2 binding activity.

The promoter function in the Cdx-G genotype was 70% of the Cdx-A genotype. The functional study suggests that intestinal VDR content of the Cdx-G genotype may be lower than those of the Cdx-A genotype. The observation that individuals with the Cdx-A genotype are low bone densities implies an important role for the intestinal VDR in determining bone density in postmenopausal women. This association is only seen in postmenopausal women but not premenopausal women. For this reason, the effect of estrogen may be masking the relationship between the Cdx genotype and BMD in premenopausal women.

In postmenopausal women, estrogen deficiency may affect the levels of VDR expression. Menopause and estrogen deficiency are associated with apparent intestinal resistance to vitamin D, which can be reversed by estrogen replacement. A recent study reported that estrogen up-regulates VDR expression in the duodenal mucosa and concurrently increases the responsiveness to endogenous 1,25(OH)2 D3. Modulation of intestinal VDR activity by estrogen and subsequent influence on intestinal calcium absorption could be one of the major protective mechanisms of estrogen against osteoporosis.

In this study, the estrogen deficiency may decrease intestinal VDR levels in postmenopausal women. The Cdx polymorphism may influence intestinal VDR levels only in the estrogen deficiency. Although we did not determine whether VDR genotype influenced intestinal calcium transport activity, the levels of intestinal VDR may be important to determine BMD in postmenopausal women.
In our subjects, we also analyzed the association of BMD and BsmI or FokI VDR polymorphisms. Cdx polymorphism was independent on FokI and BsmI VDR polymorphism. In the premenopausal subjects, we found a significant correlation between FokI and BMD as reported previously. FokI VDR polymorphism may be more highly associated with BMD of the lumber spine in premenopausal women. With advancing age, the relation between the FokI genotype and BMD may not be apparent. In contrast, the Cdx polymorphism is associated with BMD of postmenopausal women, suggesting that this polymorphism may account for bone loss with aging.

ACKNOWLEDGMENTS

This work was supported by grant 11557202 (K.M.) from the Ministry of Education, Science, Sports and Culture of Japan. E.T. also was supported by grants from the Setsuro Fujii Memorial Foundation.

REFERENCES

to the lactase-phlorizin hydrolase and sucrase-isomaltase promoters are functionally related oligomeric molecules. FEBS Lett 342:297–301.


Address reprint requests to:
Hidekazu Arai, Ph.D
Department of Clinical Nutrition
School of Medicine
University of Tokushima
Kuramoto-cho 3, Tokushima City
Tokushima 770-8503, Japan

Received in original form April 20, 2000; in revised form October 18, 2000; accepted February 27, 2001.