A Vitamin D Receptor Gene Polymorphism in the Translation Initiation Codon: Effect on Protein Activity and Relation to Bone Mineral Density in Japanese Women

HIDEKAZU ARAI,1 KEN-ICHI MIYAMOTO,1 YUTAKA TAKETANI,1 HIRONORI YAMAMOTO,1 YUCA IEMORI,1 KYOKO MORITA,1 TAKEHARU TONAI,2 TAKEHIKO NISHISHO,2 SHIGENOBU MORI,3 and EIJI TAKEDA 1

ABSTRACT

The effect of a T-C transition polymorphism at the translation initiation codon of the human vitamin D receptor (VDR) gene on the biological function of the encoded protein was investigated. Of 239 Japanese women volunteers subjected to genotype analysis for this polymorphism, 32 (13%) were genotype MM (the M allele is ATG at the putative translation start site), 75 (31%) were genotype mm (the m allele is ACG at the putative translation start site), and 132 (55%) were genotype Mm. The bone mineral density (BMD) in the lumbar spine (L2–L4) was determined for 110 healthy premenopausal women from the volunteers and was shown to be 12.0% greater (p < 0.05) for mm homozygotes than for MM homozygotes. Synthesis of the proteins by the M and m alleles from the cloned cDNAs in vitro and in transfected COS-7 cells revealed them to have a size of 50 and 49.5 kD, respectively, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This size difference is consistent with initiation of translation of the M allele-encoded protein from an ATG codon located at nucleotides +110 to +12 in the conventional open reading frame. The extent of vitamin D–dependent transcriptional activation of a reporter construct under the control of a vitamin D response element in transfected HeLa cells was ~1.7-fold greater for the m type VDR than for the M type protein. These results suggest that the polymorphism at the translation start site of the VDR gene may modulate BMD in premenopausal Japanese women. (J Bone Miner Res 1997;12:915–921)

INTRODUCTION

THE RISK OF OSTEOPOROTIC FRACTURE depends on the peak bone mass achieved in early adulthood as well as on the rate of bone loss later in life.1–3 The known determinants of peak bone mass are body mass index, calcium intake, exercise, and genetic factors,1–3 with the latter accounting for ~75–80% of the variability in these parameters.4 Common allelic variants in the gene that encodes the vitamin D receptor (VDR) have been associated with bone mineral density (BMD).5–10 Morrison et al.5,6 demonstrated a significant relation between a BsmI restriction fragment length polymorphism (RFLP) in the VDR gene and both the concentration of osteocalcin in serum and BMD in Australian women of English-Irish descent. However, the association between RFLPs at the VDR gene locus and BMD remains controversial.11–13

We previously analyzed the VDR cDNA sequence in three Japanese patients with vitamin D–dependent rickets type II.14 A T-C transition was present in the putative initiation codon of the three patients and one normal control, but not in that of two other control subjects. We have

1Department of Clinical Nutrition, School of Medicine, Tokushima University, Tokushima City, Japan.
2Department of Orthopedic Surgery and Institute for Clinical Research, Zentsuji National Hospital, Zentsuji, Japan.
3Department of Orthopedic Surgery, Asada General Hospital, Marugame, Japan.
now determined the allele frequencies for this polymorphism in Japanese women and investigated both its relation to BMD and the relative biological activities of the encoded proteins.

**MATERIALS AND METHODS**

**Subjects**

Allele frequencies for the VDR polymorphism were determined among 239 unrelated Japanese female volunteers (age range, 24–70 years) who resided in Kagawa prefecture, Japan. The effect of the VDR genotype on BMD was investigated in 110 normal, healthy, premenopausal women (age range, 24–45 years) selected from the volunteers. Subjects with a history of bone disease, illness, bilateral oophorectomy, or use of drugs that could affect bone turnover or BMD were excluded from this part of the study.

**Isolation of DNA**

Genomic DNA was isolated from blood either by a modified phenol-chloroform extraction procedure\(^{115}\) or with a Smitest genomic DNA isolation kit (Sumitomo KK, Osaka, Japan). DNA samples isolated by the phenol-chloroform extraction procedure were dialyzed against Tris-EDTA buffer for 1 h to remove excess salts and residual phenol before polymerase chain reaction (PCR) analysis. With both extraction methods, the samples were analyzed multiple times to ensure the accuracy of the genotype.

**Bone mineral densitometry**

The BMD, expressed as an areal density in grams per square centimeter, of the 110 normal women was measured in the lumbar spine (L2–L4) by dual-energy X-ray absorptiometry (model QDR-1500, Hologic, Waltham, MA, U.S.A.) with coefficients of variation in our laboratory of 0.8%.

**Single-strand conformation polymorphism**

The primers designed to detect the polymorphism at the first ATG start codon by PCR single-strand conformation polymorphism (SSCP) analysis are shown in Fig. 1A.\(^{114,115}\) PCR was performed in a 10-\(\mu\)l reaction mixture containing 0.5 \(\mu\)g of genomic DNA, 25 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM 2-mercaptoethanol, 1 \(\mu\)g of gelatin, 0.5% NP-40, 0.5% Tween-20, 10 \(\mu\)M of each deoxynucleotide triphosphate, 10 pmol of each primer (primers 2a and 2b) (Fig. 1A), 0.1 \(\mu\)Ci of [\(\gamma\)-\(^{32}\)P]dCTP, and 1.25 U of Taq DNA polymerase (Toyobo, Tokyo, Japan). The amplification protocol consisted of 35 cycles of 94°C for 45 s (denaturation), 57°C for 45 s (annealing), and 72°C for 3 minutes (extension). The PCR products were diluted 1:10 with a solution containing 98% (w/v) formamide, 10 mM EDTA (pH 8.0), 0.05% bromphenol blue, and 0.05% xylene cyanol, and the resulting mixture was heated at 95°C for 3 minutes, cooled rapidly to 4°C, and maintained at the latter temperature for 5 minutes. A 5-\(\mu\)l portion of the mixture was then subjected to electrophoresis on a 6% polyacrylamide gel containing 1× TBE (90 mM Tris-borate and 2 mM EDTA); electrophoresis was performed at 30 W for 2 h in 1× TBE at room temperature.\(^{115}\) Each gel was dried on a vacuum gel dryer and exposed to X-ray film for 12–24 h with an intensifying screen. Bands were excised from the gel and rehydrated in 100 \(\mu\)l of water.\(^{115}\)

**DNA sequencing**

PCR products in the rehydrated gel pieces (100 \(\mu\)l) were purified by isopropanol precipitation with 200 \(\mu\)l of 4 M ammonium acetate and 400 \(\mu\)l of isopropanol. The precipitate was washed twice with 200 \(\mu\)l of 70% (v/v) ethanol and resuspended in 20 \(\mu\)l of water. DNA sequencing was performed by the dideoxy chain termination method with a T7 sequencing kit (Pharmacia, Uppsala, Sweden).

**In vitro expression of VDR proteins**

For this purpose, the oligonucleotide primers were synthesized as follows: 5′-CGGAATTCCGGGATGGAGG CATGG (ATG sense primer) or 5′-CGGAATTCCGGGAGG CCAATGGGCAATGG (ACG sense primer), and 5′-CGGA ATTCTCTATGTCAGGAGATCTCAT (antisense primer).\(^{117}\) All primers contained the recognition sequence for
EcoRI. The PCR products were digested with EcoRI and subcloned into the pSG-5 vector, which contains a promoter for SV40 and T7 RNA polymerase and encodes a 3’ poly(A) tail (Stratagene, La Jolla, CA, U.S.A.). The constructed expression vectors were termed pSG-ATG and pSG-ACG. In vitro translation of the human VDR cRNAs derived from these plasmids (pSG-ATG and pSG-ACG) was performed with the Single Tube Protein System 2 (Novagen, Madison, WI, U.S.A.), which is designated for efficient in vitro synthesis of proteins from supercoiled or linear DNA templates containing a bacteriophage T7 RNA polymerase promoter.(18)

Immunoblot analysis

Proteins synthesized by in vitro translation or total soluble extracts prepared from transfected COS-7 cells were mixed with sodium dodecyl sulfate (SDS) sample buffer, boiled for 2 minutes, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels.(14) The separated proteins were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes for immunoblot analysis as described previously.(14) The membranes were then incubated with Tris-buffered saline containing 5% (w/v) nonfat dried milk before exposure for 90 minutes to rat anti-VDR monoclonal antibodies (9A7g). (19) Immune complexes were detected with rabbit anti-rat IgG conjugated with horseradish peroxidase and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL, U.S.A.).

Construction of reporter plasmids

The plasmid pGCAT containing the rat 24-hydroxylase gene promoter (nucleotides [nt] −291 to +9, relative to the transcription start site (20)), which itself contains a vitamin D response element (VDRE) located between nt −226 and −238,(20) was digested with SalI and XbaI and the DNA insert subcloned into the pGL-2 basic vector (Promega, Madison, WI, U.S.A.) with XhoI and BamHI restriction sites.

Cell culture and transfection

COS-7 and HeLa cells were cultured at 37°C under 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (Sanko-Jyunyaku, Tokyo, Japan) containing 10% fetal bovine serum (Equitech-Bio, Inc., Ingram, TX, U.S.A.). Transfection of HeLa or COS-7 cells for transactivation experiments was performed by 5 μl of LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) with 0.5 μg of the VDRE-containing vector, 0.5 μg of human VDR expression vector (pSG-ATG or pSG-ACG), and 0.5 μg of the β-galactosidase expression vector pCMV-β (Clontech, Palo Alto, CA, U.S.A.), the latter as an internal standard to normalize transfection efficiency.(21) After transfection, the cells were incubated under standard conditions for 36 h and then exposed to 50 nM 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in DMEM without serum for 15 h. Cells were then harvested in cell lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 10% glycerol, and 1% Triton X-100) and the lysate assayed for luciferase activity,(21) β-galactosidase activity,(21) and protein concentration. (22)

Statistical analysis

Analysis of variance (ANOVA) was performed using the Statview statistical package (Abacus Concepts, Inc., Berkeley, CA, U.S.A.) on a Macintosh LC 630. Fisher’s protected least significant difference (PLSD) test was used to assess the relationship between genotype and BMD. Significant levels quoted are for the initial F-test on null hypothesis (no difference between the means) of the overall effect. The student’s t-test was used for pairwise comparisons.

RESULTS

Polymorphism in exon 2 of the human VDR gene

PCR-SSCP analysis of genomic DNA from 239 Japanese women was performed with two primers positioned in introns 1 and 2 of the VDR gene (Fig. 1A). Three migration profiles were apparent on electrophoresis (Fig. 1B), and bands containing the most radioactivity were eluted from SSCP gels (Fig. 1) and subjected to DNA sequencing. The M allele was shown to contain ATG, whereas the m allele contains ACG, at the putative translation start site. The T-C transition is indicated with an asterisk. In intron 1 and exon 2, sequences are shown in lower- and upper-case, respectively.
As characterized by three bands and corresponding to heterozygotes for the start site polymorphism. No significant difference was observed in the extent of expression of the two proteins, given the absence of a Kozak consensus sequence surrounding the putative initiation codon for Mp and Mp, we measured the relative efficiencies of transcription and translation of the two proteins both in vitro and in vivo. Immunoblot analysis revealed that the extent of expression of Mp was ~20% less than that of MP both in vitro (Fig. 6A) and in transfected HeLa cells (Fig. 6B). Thus, the increased transactivation observed with Mp relative to that apparent with MP does not seem to be attributable to increased expression of the former.

### DISCUSSION

Genetic effects on BMD and bone turnover have been related to allelic variation in the VDR gene in Caucasians. Genetic analysis of multiple RFLPs (BsmI, ApaI, TaqI) showed that the BbAAT genotype is relatively common (16.7%) in Caucasians but virtually nonexistent in Japanese (1.4%). The BMD of the lumbar spine associated with the common genotype BbAAT is 9.3% less than that associated with the bbaAT genotype in premenopausal Japanese women. Yamagata et al. also showed that BMD is affected by the VDR genotype in Japanese women, but with different allelic frequencies than in Caucasians. We detected only two (0.8%) BB homozygotes among our 239 volunteers, and the BMD of these two women did not differ significantly from that of the bb homozygotes (data not shown). In addition, no significant difference was observed in the mean

**FIG. 3.** Distribution of genotypes and spinal BMD (L2–L4). DNA was extracted from 110 normal healthy women and then analyzed by PCR-SSCP. Genotype was determined by the T-C transition. MM = homozygotes for the first ATG, Mm = heterozygotes for the first ACG, Mm = heterozygotes. Numbers in parentheses represent the population who have the specified genotype. p < 0.05, p < 0.01.

Profile Mm was characterized by three bands and corresponded to heterozygotes for the start site polymorphism.

**Relation between BMD and VDR genotype**

Of the 239 subjects, PCR-SSCP analysis revealed that 32 (13.4%) were genotype MM, 75 (31.4%) were genotype mm, and 132 (55.2%) were genotype Mm. The frequencies of the m and M alleles were 0.59 and 0.41, respectively. The distribution of genotypes did not differ with age (data not shown).

The BMD of the lumbar spine (L2–L4) in 110 healthy premenopausal women from the 239 volunteers was measured and compared among genotypes. Age (mean [SD] 32.1 [7] years), weight (48 [5.5] kg) and height (154 [4.3] cm) did not differ between the VDR genotypes. Differences in BMD after adjusting for age, weight, and height were assessed by analysis of covariance. The BMD of mm homozygotes (1.043 ± 0.025 g/cm²) was significantly greater (p < 0.05) than of either Mm heterozygotes (1.022 ± 0.017 g/cm²) or MM homozygotes (0.931 ± 0.026 g/cm²) (Fig. 3).

**Biological activities of the exon 2 variants of the human VDR**

The putative translation start site is highly conserved in mouse, rat, and human VDR cDNA, suggesting that the exon 2 polymorphism may affect the function of the human VDR protein. We considered two possibilities: (1) that an ATG codon located at nt +10 to +12 in the human VDR cDNA sequence reported previously serves as the initiation codon in the m allele, or (2) that translation is initiated by a non-ATG codon, ACG at nt +1 to +3, in the m allele, as previously shown for the Krox24 gene. To distinguish between these possibilities, we subjected plasmids encoding the two VDR variants to in vitro transcription and translation and analyzed the resulting proteins by immunoblotting with rat anti-VDR monoclonal antibodies. The ATG cDNA variant yielded one major protein of 50 kD, which was designated MP (Fig. 4A). The plasmid encoding the ACG variant gave rise to a 49.5 kD protein, designated mP. In addition, COS-7 cells were transfected with expression vectors encoding each of the VDR variants, and soluble cell extracts were subsequently subjected to immunoblot analysis with the antibodies. The ATG and ACG variants yielded proteins of 50 and 49.5 kD, respectively (Fig. 4B).

Next, the transactivation function of the VDR isoforms was investigated in HeLa cells transfected with the appropriate VDR expression vector and a luciferase reporter construct containing the rat 24-hydroxylase gene VDRE. Exposure of cells to 50 nM 1,25-(OH)₂D₃ increased luciferase activity by 19.5-, 11.2-, and 15.5-fold in those expressing mP, MP, or both mP and MP, respectively (Fig. 5). Similar results were obtained with COS-7 cells (data not shown).

To rule out the possibility that the significant difference in transactivation function between MP and mP is attributable to a difference in the extent of expression of the two proteins, given the absence of a Kozak consensus sequence surrounding the putative initiation codon for MP and mP, we measured the relative efficiencies of transcription and translation of the two proteins both in vitro and in vivo. Immunoblot analysis revealed that the extent of expression of Mp was ~20% less than that of MP both in vitro (Fig. 6A) and in transfected HeLa cells (Fig. 6B). Thus, the increased transactivation observed with Mp relative to that apparent with MP does not seem to be attributable to increased expression of the former.
BMD between the Bb heterozygotes and the bb homozygotes in our healthy Japanese subjects. In contrast, the frequencies of the \textit{m} and \textit{M} alleles of the VDR start site polymorphism were 0.59 and 0.41 in our healthy Japanese subjects, respectively, and the BMD in \textit{mm} homozygotes was significantly (12.0%) greater than in \textit{MM} homozygotes. Thus, the polymorphism at the translation initiation site is likely to be a more useful marker than the \textit{BsmI} RFLP for the assessment of BMD in Japanese women.

Analysis of the sequences of human, mouse, and rat VDR cDNAs revealed that the open reading frame is highly conserved and is initiated from the first ATG codon, whose
position is also conserved.\textsuperscript{17,25,26} We have now shown that two proteins, MP and mP, appear to be synthesized from different start sites of the human VDR gene. Whereas translation of MP is likely initiated from the first ATG codon, mP translation appears to be initiated at the second ATG codon in the same reading frame. Thus, mP should lack the three NH\textsubscript{2}-terminal amino acids of MP, which is consistent with the size difference (\textasciitilde0.5 kD) between the two proteins on SDS-PAGE but which requires confirmation by NH\textsubscript{2}-terminal microsequencing. The mP protein induced transactivation of a VDRE-containing promoter construct in HeLa and COS-7 cells to a greater extent than did MP. This difference in biological activity of the two VDR isoforms did not appear attributable to a difference in the extent of their expression. The reason for the difference in activity of the two proteins, for example, whether it is due to a difference in the ability to bind 1,25(OH)\textsubscript{2}D\textsubscript{3}, to associate with the retinoid X receptor, to bind to the VDRE, or to activate transcription per se, remains to be determined.

Morrison et al.\textsuperscript{5} demonstrated a relation between the VDR genotype and the concentration of osteocalcin in serum. Dawson-Hughes et al.\textsuperscript{27} showed that intestinal calcium absorption is reduced in women with the BB genotype. In the present study, we analyzed the transactivation function of the MP and mP isoforms of human VDR with the VDRE in the rat 24-hydroxylase gene promoter. Alignment of this VDRE with those of the osteocalcin, osteopontin, and calbindin-D\textsubscript{9K} genes reveals that they all consist of a direct repeat of two half-sites separated by a 3-bp spacer.\textsuperscript{28} Thus, the polymorphism at the translation start site of the VDR gene may affect the expression of genes containing such VDREs. If the M and m allele of the VDR differ functionally in vivo, this difference might also be reflected in a difference in the serum osteocalcin concentration and calcium absorption among subjects with different genotypes. The latter should also be manifest by the difference in size of the MP and mP proteins.

In summary, we have characterized a polymorphism at the translation initiation codon of the human VDR gene. This T-C transition appears to result in the synthesis of a smaller (by three amino acids) protein with increased biological activity and to be associated with an increased BMD in premenopausal Japanese women.

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REFERENCES


Address reprint requests to:
Ken-ichi Miyamoto, Ph.D.
Department of Clinical Nutrition
School of Medicine
Tokushima University
Karamoto-Cho 3, Tokushima City
Tokushima 770, Japan

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