Original Article

Effects of *Fok-I* polymorphism in vitamin D receptor gene on serum 25-hydroxyvitamin D, bone-specific alkaline phosphatase and calcaneal quantitative ultrasound parameters in young adults

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Several genes have been implicated as genetic determinants of osteoporosis. Vitamin D receptor (VDR) is an intracellular hormone receptor that specifically binds to the biologically active form of vitamin D, 1-alpha, 25-dihydroxyvitamin D_3 [1, 25$(OH)_2$D], and mediates its effects. One of the most frequently studied single nucleotide polymorphisms is the restriction fragment length polymorphism (RFLP) *Fok-I* (rs2228570). The presence of a *Fok-I* site, designated f, allows protein translation to initiate from the first ATG. An allele lacking the site (ATG>ACG: designated F), initiates from a second ATG site. In the present study, we explored the effect of the VDR *Fok-I* genotype on associations among serum bone-specific alkaline phosphatase (ALP), 25-hydroxyvitamin D_3 [25(OH)D], 1, 25$(OH)_2$D, and the dietary nutrient intake in healthy young Japanese subjects (n=193). Dietary nutrient intakes were calculated based on 3-day food records before the day of blood examinations. Quantitative ultrasound (QUS) parameters at the right calcaneus (heel bone) were measured. The allele frequencies were 0.622 for the F allele and 0.378 for the f allele in all subjects. Grouped by the VDR genotype, a significant positive correlation between the levels of serum bone-specific ALP and 25(OH)D was observed in the FF-type (p=0.005), but not in the ff-type. In addition, there was a significant positive correlation between the level of serum 25(OH)D and osteo-sono assessment index (OSI) in the FF-type (p=0.008), but not in the ff-type. These results suggest that the level of circulating 25(OH)D is an important factor when assessing the VDR *Fok-I* polymorphism to prevent osteoporosis.

Key Words: vitamin D receptor *Fok-I* polymorphism, bone-specific alkaline phosphatase, 25-hydroxyvitamin D_3, 1-alpha, 25- dihydroxyvitamin D_3, quantitative ultrasound

INTRODUCTION

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength, predisposing elderly people to an increased risk of fracture.[1] Osteoporosis results from complex interactions between genetic and environmental factors. Several genes have been implicated as genetic determinants of osteoporosis.[2-4] If genetic markers could be identified that indicate the risk of osteoporosis, they would be useful for its prevention and early, effective treatment.

The vitamin D receptor (VDR) is an intracellular hormone receptor that specifically binds to the biologically active form of vitamin D, 1-alpha, 25-dihydroxyvitamin D_3 [1, 25$(OH)_2$D] or calcitriol and interacts with specific nucleotide sequences (response elements) of target genes to produce a variety of biologic effects. The *VDR* gene is located on chromosome 12q12-q14, and common allelic variants in the gene that encode the VDR have been associated with the bone mineral density (BMD).[5-11] The most frequently studied single nucleotide polymorphisms (SNPs) are the restriction fragment length

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polymorphisms (RFLPs) *Bsm-I* (dbSNP:rs1544410) and *Fok-I* (rs2228570), as defined by the endonucleases *Bsm-I* and *Fok-I*, respectively.

Morrison et al first demonstrated a significant correlation between a *Bsm-I* RFLP in the *VDR* gene and both the concentration of osteocalcin in serum and BMD in Australian women.[5] *Bsm-I* is intronic and located at the 3' end of the gene.

The *Fok-I* RFLP, located in the coding region of the VDR gene, results in the production of a VDR protein that is three amino acids longer. The presence of a *Fok-I* site, designated as f, allows protein translation to initiate from the first ATG. An allele lacking the site (ATG>ACG: designated as F), initiates from a second ATG site. Arai et al demonstrated that the BMD of the lumbar spine was 12.0% greater for FF (ATG>ACG) homozygotes than for ff (ATG) homozygotes in 110 healthy premenopausal Japanese women.[7] They also suggested that the extent of vitamin D-dependent transcriptional activation of a reporter construct was approximately 1.7-fold greater for the variant of the *Fok-I* (F-type) than for the f-type.[7] Furthermore, it was reported that the stiffness index of the calcaneal bone mass measured with a quantitative ultrasound (QUS) bone densitometer was significantly higher in the FF type (p<0.05 versus ff type) in 126 premenopausal Japanese women aged 27.2±10.1 (mean±standard deviation) years old.[9]

Although association studies of *Fok-I* polymorphisms in the VDR gene with BMD have been performed, their results are not in agreement and still remain controversial.[12,13] Ferrari et al reported that *Fok-I* VDR gene polymorphisms were not significantly correlated with BMD in 177 healthy European-Caucasian premenopausal females.[12] In addition, it was demonstrated that none of the individual VDR gene polymorphisms showed a consistent association with the baseline BMD or BMD change and the effect of the VDR genotype on the bone mass was negligible in Japanese women, as a part of the Japanese population-based osteoporosis (JPOS) study.[13]

Osteoporosis results from complex interactions between genetic and environmental factors. To reduce the risk of osteoporosis, it seemed to be important to maximize peak bone mass during youth. Nutrition is one of the most important environmental factors in the prevention of osteoporosis, so nutritional education targeted at accelerating and maintaining bone health is indispensable for young people. In the present study, we aimed to provide further insight into the effect of the *Fok-I* polymorphism of the *VDR* gene on the vitamin D status in young adults.

METHODS

The study protocol was approved by the Institutional Review Board of Japan Women's University, and the study was carried out according to the guidelines of the Declaration of Helsinki. Informed written consent was obtained from all study subjects.

Young subjects living in Tokyo, Japan, were recruited from November to March.[14] Participants were excluded if they had metabolic disease. This study population consisted of 97 healthy Japanese males and 96 females. All subjects were unrelated volunteers and aged 22.1±1.8 (mean±standard deviation) years old, with a height of 164.9±8.9 cm, weight of 57.2±9.2 kg, and body mass index (BMI) of 21.0±2.3 kg/m^2.

Dietary nutrient intakes were measured based on 3-day food records taken up to the day before blood examinations. Trained personnel reviewed the food records, and the nutrient content was determined with the use of Eiyo-Kun software (Kenpaku-sha, Japan).

Fasting blood samples were obtained and serum was kept frozen at -80℃ until measurement. Calcium was measured employing the o-cresol-phthalein complexion color development method,[15] and inorganic phosphorus was determined using the method of *p*-methylaminophenol reduction.[16] A bone formation marker, serum bone-specific alkaline phosphatase (ALP), was determined by enzyme immunoassay (Mitsubishi Kagaku Bio Clinical Laboratories Inc., Tokyo, Japan).

Serum 25(OH)D was measured using a radioimmunoassay (25-hydroxyvitamin D ^{125}I RIA Kit, DiaSorin, Stillwater, MN, USA).[17] The serum 1, 25$(OH)_2$D level was assayed using 1, 25$(OH)_2$D - RIA methods (Mitsubishi Kagaku Bio-Clinical Labs, Inc., Japan).[18-19]

Quantitative ultrasound (QUS) measurements at the calcaneus were performed using the AOS-100 machine (Hitachi Aloka Medical, Ltd.), which consisted of two transducers that were faced with silicone rubber coupling pads. The AOS-100 measured the speed of sound (SOS) as an index of bone density and the transmission index (TI) as an index of bone structure. The osteo-sono assessment index (OSI) was calculated using the following formula: $OSI = TI \times SOS^2$. The results given in T-scores represent the standard deviation of the subjects' OSI compared with normal values in a young adult reference population. The results given in Z-scores represent the standard deviation of the subjects' OSI compared with normal values for the age and gender.

All subjects were genotyped for *Fok-I* polymorphism in the *VDR* gene. DNA was extracted from whole blood (QIAamp DNA Blood Kit, Qiagen), and the polymerase chain reaction (PCR)-RFLP was performed for the VDR *Fok-I*. A 265-bp segment of the *VDR* gene including polymorphism sites was amplified by PCR.[6] All subjects were classified as either an FF homozygote, an Ff heterozygote, or an ff homozygote according to the digestion pattern.

Values are the mean±standard deviation (SD), and Spearman rank correlation coefficients were calculated to analyze the relation between two parameters. Body and serum biochemical parameters and dietary nutrition intakes were compared among genotypic categories using ANOVA. Chi-square tests were conducted to examine whether the genotype frequencies were in Hardy-Weinberg equilibrium. Significance was accepted at p<0.05. Analysis was conducted using IBM SPSS Statistics (version 20, IBM Corporation, Somers, NY, USA).

RESULTS

In all subjects (n=193), the mean (±SD) dietary energy intake, calcium intake (mg/day), and vitamin D intake (μg/day) of the subjects were 2069±555 (kcal/day),

Table 1. Genotypes and allele frequencies

	Genotype			Allele (%)	
Fok- I	FF	Ff	ff	F	f
n	74	92	27	240	146
%	38	48	14	62.2	37.8

Table 2. Body and serum biochemical parameters and dietary nutrient intakes

	Fok- I polymorphism			*p*-values
	FF (n=74)	Ff (n=92)	ff (n=27)	
Body parameters				
Height (cm)	165.2±9.4	164.5±8.9	165.8±7.6	N.S.
Weight (kg)	57.7±9.3	56.5±9.7	58.3±7.1	N.S.
BMI (kg/m^2)	21.1±2.2	20.8±2.4	21.2±2.0	N.S.
Serum biochemical parameters				
Calcium (mg/dL)	9.7±0.5	9.8±0.4	9.7±0.4	N.S.
Phosphorus (mg/dL)	3.6±0.5	3.6±0.5	3.6±0.5	N.S.
Bone-specific ALP (U/l)	28.1±9.1	25.4±6.0	28.6±8.6	N.S.
25(OH)D (ng/mL)	19.8±6.8	19.7±6.5	22.2±8.2	N.S.
1,25(OH)$_2$D (pg/mL)	52±20	49±15	50±17	N.S.
Dietary nutrient intakes				
Calcium (mg/day)	549±218	558±216	571±265	N.S.
Vitamin D (μg/day)	5.4±4.1	5.7±5.1	7.6±5.1	N.S.

Each value represents the mean±SD.
BMI: body mass index; ALP: alkaline phosphatase; 25(OH)D: 25-hydroxyvitamin D_3; 1,25(OH)$_2$D: 1-alpha, 25- dihydroxyvitamin D_3; N.S.: not significant.

Table 3. Association between serum 25(OH)D and serum bone-specific alkaline phosphatase (ALP), 1,25(OH)$_2$D or dietary vitamin D intakes

	All (n=193)		*Fok- I* polymorphism					
			FF (n=74)		Ff (n=92)		ff (n=27)	
	r	*p*-values	*r*	*p*-values	*r*	*p*-values	*r*	*p*-values
Serum biochemical parameters								
Bone-specific ALP (U/l)	0.306	<0.001	0.323	0.005	0.298	0.004	0.345	0.078
1,25(OH)$_2$D (pg/mL)	0.083	0.253	0.152	0.197	-0.015	0.888	0.171	0.395
Dietary nutrient intakes								
Vitamin D (μg/day)	0.249	<0.001	0.371	0.001	0.196	0.061	0.147	0.465

ALP: alkaline phosphatase; 1,25(OH)$_2$D: 1-alpha, 25- dihydroxyvitamin D_3.

551±222 (mg/day), and 6.4±10.5 (μg/day), respectively.

The mean (±SD) levels of serum calcium and phosphorus were 9.7±0.4 and 3.6±0.5 mg/dL, respectively. The levels of serum bone-specific ALP, 25(OH)D, and 1, 25(OH)$_2$D were 26.9±7.8 U/l, 20.1±6.9 ng/mL, and 50±17 pg/mL, respectively.

To compare the influence on the QUS parameters, we measured the SOS, TI, OSI, T-score, and Z-score at the right calcaneus. In the subjects (n=175), the mean (±SD) levels of SOS, TI, and OSI and the T- and Z-scores were 1578±27 m/s, 1.17±0.11, 2.919±0.355, 104.3±11.3%, and 102.2±11.0%, respectively.

All subjects were genotyped for VDR *Fok-I* polymorphism (rs2228570). The distribution of VDR *Fok-I* polymorphism did not deviate from the Hardy-Weinberg expectations (p>0.05). As shown in Table 1, the allele frequencies were 0.622 for the F allele and 0.378 for the f allele. Seventy-four subjects were FF homozygote, 92 were heterozygous (Ff type), and 27 were ff homozygote. In male subjects (n=97), 36 showed the FF homozygote, 44 were heterozygous (Ff type), and 17 showed the ff homozygote. In female subjects (n=96), 38 showed the FF homozygote, 48 heterozygous (Ff type), and 10 showed the ff homozygote.

There was no significant difference among these genotype groups in terms of the height, weight, BMI, serum calcium, phosphorus, bone-specific ALP, 25(OH)D, 1, 25(OH)$_2$D, dietary calcium, or vitamin D intake, as shown in Table 2.

The concentration of serum 25(OH)D was significantly correlated with bone-specific ALP in the total subjects (r=0.306, p<0.001) (Table 3). Grouped by the *Fok-I* genotype, there were significant positive correlations between the serum 25(OH)D and bone-specific ALP in the FF homozygote (p=0.005) and Ff heterozygote (p=0.004), but not in the ff homozygote (p=0.078) (Table 3). There was no significant correlation between the level of serum 25(OH)D and serum 1, 25(OH)$_2$D.

As shown in Table 3, the serum 25(OH)D concentration was significantly correlated with the dietary vitamin D intake in the total subjects (p<0.001). Grouped by the *Fok-I* genotype, there were significant positive correlations between the serum 25(OH)D and vitamin D intake in the FF homozygote (p=0.001), but not in the ff

Table 4. Quantitative ultrasound (QUS) parameters

	Fok- I polymorphism			*p*-values
	FF (n=68)	Ff (n=86)	ff (n=21)	
SOS	1579±25	1576±26	1585±34	N.S.
TI	1.16±0.10	1.17±0.12	1.18±0.11	N.S.
OSI	2.92±0.325	2.92±0.371	2.97±0.393	N.S.
T-score	102±10.2	101.9±10.8	103.3±14.1	N.S.
Z-score	104±10.5	104.0±11.4	106±14.0	N.S.

Each value represents the mean±SD.
SOS: speed of sound; TI: transmission index; OSI: osteo-sono assessment index; N.S.: not significant
T score: compares the OSI in a similar manner using a young adult reference population.
Z score: compares the OSI to an age-matched reference population.

Table 5. Association between serum 25(OH)D and quantitative ultrasound (QUS) parameters

	All (n=175)		*Fok- I* polymorphism					
			FF (n=68)		Ff (n=86)		ff (n=21)	
	r	*p*-values	*r*	*p*-values	*r*	*p*-values	*r*	*p*-values
SOS	0.259	0.001	0.347	0.004	0.205	0.058	0.263	0.250
TI	0.127	0.094	0.300	0.013	0.037	0.737	0.062	0.790
OSI	0.163	0.031	0.318	0.008	0.076	0.489	0.086	0.712
T score	0.074	0.332	0.292	0.016	0.048	0.658	-0.057	0.807
Z score	0.115	0.130	0.255	0.036	0.018	0.873	-0.160	0.488

SOS: speed of sound; TI: transmission index; OSI: osteo-sono assessment index.
T score: compares the OSI in a similar manner using a young adult reference population.
Z score: compares the OSI to an age-matched reference population.

homozygote (p=0.465) (Table 3).

In order to confirm the influence of *Fok-I* polymorphism in the *VDR* gene on the bone mass, one hundred seventy five participants were analyzed to examine the association between 25(OH)D and QUS parameters. There was no significant difference among the genotype groups in the SOS, TI, OSI, T-score, and Z-score of the heel measured by a QUS bone densitometer (Table 4). As shown in Table 5, the concentration of serum 25(OH)D was significantly correlated with SOS and OSI in the 175 subjects (p=0.001 and p=0.031, respectively). Also, there was a significant positive correlation between the level of serum 25(OH)D and QUS parameters such as SOS (p=0.004), TI (p=0.013), OSI (p=0.008), T-score (p=0.016), and Z-score (p=0.036) in the FF-type, but not in the ff-type (Table 5).

DISCUSSION

This study investigated the contribution of SNPs in the human VDR associated with bone metabolism. The genotype frequencies in the FF homozygote, Ff heterozygote, and ff homozygote of the VDR *Fok-I* polymorphisms were 38.3, 47.7, and 14.0%, respectively. The allele frequencies were 0.622 for the F allele and 0.378 for the f allele (Table 1). Morita et al investigated VDR *Fok-I* polymorphisms in a large-scale cohort study (JPOS).[13] They reported that the genotype frequencies of the FF, Ff, and ff types were 38.7% (n=554), 47.7% (n=684), and 13.6% (n=195), respectively, and the allele frequencies were 0.625 for the F allele and 0.375 for the f allele,[13] and these data were similar to those in the present study. Harris et al. demonstrated that distribution of *Fok-I* genotypes differed significantly by race (p<0.001): 65% of African American versus 37% of Caucasian were FF homozygotes and 4% of African American versus 18% of Caucasian were ff homozygotes in 154 premenopausal American women (72 African American and 82 Caucasian) aged 20-40 years old.[20]

Many studies examining the role of polymorphisms in VDR gene *Fok-I* polymorphisms for BMD determination have generated conflicting results. The multiple factors contributing to the pathogenesis of osteoporosis include genetic and environmental factors. In the present study, there were no significant differences among these genotype groups in terms of the serum bone-specific ALP, 25(OH)D, 1, 25$(OH)_2$D, SOS, TI, OSI, T-score, and Z-score by QUS measurements (Tables 2 and 4). Interestingly, the serum bone-specific ALP activity was significantly correlated with serum 25(OH)D levels in the total subjects (p<0.001) (Table 3). We examined the effect of the *Fok-I* genotype on the association with the serum 25(OH)D, and demonstrated that there were significant positive correlations between serum bone-specific ALP and 25(OH)D concentrations in the FF type and Ff type (Table 3). These results suggest that an adequate level of serum 25(OH)D may be important for people with the FF type or Ff type.

Bone-specific ALP is an enzyme that plays a role in bone mineralization. The present immunoassay method can only detect serum bone-specific ALP activity in total ALP, and provides an assessment of the skeletal status. The levels of total ALP are higher in children than in adults, because bone-specific ALP activity is elevated as a result of the promoted bone formation. The physiological roles of ALP are not well understood, but strong evidence is provided by the rare genetic disease hypophosphatasia. Hypophosphatasia is an inherited disorder characterized by a defect in skeletal mineralization caused by tissue-nonspecific ALP (liver/bone/kidney; TNSALP) deficiency. Previously, we discovered

a more significant correlation between SNPs in the TNSALP gene (rs3200254) associated with the BMD among 501 postmenopausal women.[4] These results indicated that bone-specific ALP was an important determinant of the bone mass .

Dietary vitamin D absorbed from the gastrointestinal tract and vitamin D synthesized in the epidermis in response to ultraviolet radiation undergo conversion to 25(OH)D in the liver, with the subsequent conversion of 25(OH)D to 1, $25(OH)_2D$ via complex metabolic pathways in the kidney. Since there were limitations of dietary vitamin D intake for vitamin D status without knowledge of sunlight exposure, we determined the concentrations of serum 25(OH)D in all subjects. Serum level of 25(OH)D is a good reflection of cumulative effects of exposure to sunlight and dietary intake of vitamin D, and is therefore used to evaluate vitamin D status.[21] We examined that the difference in dietary vitamin D intake influenced the serum biochemical parameters, especially the level of serum 25(OH)D. Actually, dietary vitamin D intake was significantly correlated with the concentration of serum 25(OH)D in the total subjects ($p<0.001$) (Table 3). According to the Japanese Dietary Reference Intakes (DRIs) by the Ministry of Health, Labour, and Welfare, an adequate intake (AI) of vitamin D is estimated to be 5.5 (μg/day) for young adults aged 18-29 years.[22] The AI of the vitamin D intake in Japanese DRIs was calculated to maintain a normal serum PTH level, and a higher concentration of serum 25(OH)D (>50 nmol/L) is recommended to prevent osteoporosis.[22] The total vitamin D intake was 6.4±10.5 (μg/day) in the present study, and this level of intake is similar to the average in young adults aged 20-29 years (6.0 μg/day) based on a national nutrition survey in Japan.[23]

We investigated the bone mass status using the QUS method. QUS is a non-invasive technique for measuring the acoustic properties of bone. Although Dual-energy X-ray absorptiometry (DXA) remains the gold standard technique for measuring the BMD, QUS is an attractive alternative method of bone assessment because it is radiation-free, low-cost, simple, and is portable. In recent years, QUS has been applied in the prediction of osteoporotic fractures, in monitoring therapies, and in the investigation of secondary osteoporosis. Several studies showed the positive correlation between calcaneal QUS and BMD of the spine or the proximal femur assessed by DXA.[24-26] As shown in Table 5, there was a significant positive correlation between the level of serum 25(OH)D and QUS parameters such as the SOS, TI, or OSI or T- or Z -scores in the FF homozygote, not in the ff homozygote. The observation that higher 25(OH)D levels are associated with a higher BMD in adults of all ethnicities is well documented.[27-28]

Vitamin D functions in the body through both an endocrine (regulation of calcium absorption) and autocrine (facilitation of gene expression) mechanism. The autocrine mechanism is able to function normally as long as adequate serum levels of 25(OH)D are maintained, on which its function is completely dependent. Lower levels of serum 25(OH)D are associated with a greater risk of osteoporosis and many chronic diseases based on large, prospective, community-based studies.[29] Most recently, it was demonstrated that a common variation (rs7968585) within the *VDR* gene modifies associations of low 25(OH)D concentrations with major clinical outcomes of incident hip fracture, myocardial infarction, cancer, and mortality among cardiovascular Health Study participants (n=1514).[30]

The present study suggests that the serum 25(OH)D concentration is one of the important factors when assessing VDR *Fok-I* polymorphism, and our findings indicate the gene-nutritional factor-related interactions associated with the osteoporosis risk. As there are limitations of this association study due to the small sample size, as well as a lack of extensive functional studies, further analysis of bone metabolism including the vitamin D status is necessary for the prevention and treatment of osteoporosis.

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AUTHOR DISCLOSURES

There are no competing interests for all authors. We hereby declare that the work reported herein was conducted by and originated entirely from us.

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Original Article

Effects of *Fok-I* polymorphism in vitamin D receptor gene on serum 25-hydroxyvitamin D, bone-specific alkaline phosphatase and calcaneal quantitative ultrasound parameters in young adults

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维生素 D 受体基因 *Fok-I* 多态性对青年血清 25-羟维生素 D 和骨特异性碱性磷酸酶以及跟骨定量超声参数的影响

一些基因已经被证实为骨质疏松症的基因决定性因素。维生素 D 受体（VDR）是一种细胞内的激素受体，它可以特异性地与活性状态的维生素 D，1-α, 25-二羟基维生素 D_3 [1,25(OH)2D] 结合，并且调节它的作用。研究最多的单核苷酸多态性是限制性片段长度多态性（RFLP）*Fok-I*（rs2228570）。在 *Fok-I* 上一个位点，被定为 f，使蛋白质转录从第一个 ATG 开始。一个等位基因缺失位点 ATG>ACG：定为 F，转录是从第二个 ATG 开始。本研究探究了健康日本青年人群中（n=193）VDR *Fok-I* 基因型对血清中骨特异性碱性磷酸酶（ALP）、25-羟基维生素 D_3[25(OH)D]、1,25$(OH)_2$D 以及饮食中营养物的摄入之间的影响。饮食中营养素的摄入是根据验血前 3 天的饮食记录计算的。定量超声（QUS）参数通过右侧跟骨测得。在整个样本中，F 等位基因频率为 0.622，f 等位基因频率为 0.378。根据 VCR 基因型分组，在 FF 基因型组发现血清中骨特异性 ALP 的水平与 25(OH)D 有显著正相关（p=0.005），但未在 ff 基因型组中发现。同时在 FF 基因型组中发现血清中 25(OH)D 水平与骨超声评价指数（OSI）显著正相关（p=0.008），但未在 ff 基因型组发现。这些结果表明，当评估 VDR *Fok-I* 多态性时血液中 25(OH)D 的水平是预防骨质疏松的一个重要因素。

关键词：维生素 D 受体 *Fok-I* 基因多态性、骨特异性碱性磷酸酶、25-羟基维生素 D_3、1-α, 25-二羟基维生素 D_3、定量超声