Cdx-2 Polymorphism in the Promoter Region of the Human Vitamin D Receptor Gene Determines Susceptibility to Fracture in the Elderly

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ABSTRACT

A Cdx-2 binding site polymorphism (G to A) in the promoter region of the human vitamin D receptor gene was reported. In an ecological study in eight ethnic groups and an association study in 2848 elderly whites, we found the A-allele to be associated with decreased fracture risk. Our findings expand previous similar findings in a Japanese study to whites and show a relationship with fracture risk of this functional polymorphism.

Introduction: A single nucleotide polymorphism (SNP) within a binding site of the intestinal-specific transcription factor Cdx-2 in the promoter region of the human vitamin D receptor (VDR) gene was previously reported. It was found to modulate the transcription of the hVDR gene and to be associated with decreased bone mineral density in a small group of postmenopausal Japanese women. In this study, we investigated the relationship between the VDR Cdx-2 genotype and risk of fracture.

Methods: We first determined the location of this SNP in the VDR gene by sequencing analysis, and we developed an allele-specific multiplex polymerase chain reaction test to determine the Cdx-2 genotype. We then performed an ecological study in eight ethnic groups and an association analysis in a large epidemiological cohort of 2848 Dutch white men and women, ≥55 years old.

Results and Conclusions: The location of the G to A substitution was found in the promoter region of exon 1e (1e-G→1739A) of the VDR gene. By comparing the frequency of the A-allele in eight different ethnic groups, we observed a negative correlation between prevalence of the A-allele and published hip fracture incidence rates in these ethnic groups (p = 0.006 for men and p = 0.02 for women), suggesting a protective effect of this allele on fracture risk. Subsequently, in the association study, the A-allele (population frequency 19%) was observed to have a protective effect on occurrence of osteoporotic fractures, especially for nonvertebral fracture in women (relative risk of AA versus GG genotype is 0.2; 95% CI, 0.05–0.8). This effect remained after adjustment for age, weight, and bone mineral density. We conclude that the A-allele of the VDR Cdx-2 polymorphism is present in whites, albeit at low frequency, and show a protective effect of this allele on risk of fracture.

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Key words: osteoporosis, ethnic groups, genetics, allele-specific multiplex polymerase chain reaction

INTRODUCTION

Polymorphisms of the vitamin D receptor (VDR) gene have been found to be associated with many clinical endpoints, such as osteoporotic fracture,1 osteoarthritis,2 diabetes,3 breast cancer,4 prostate cancer,5 and low bone mineral density (BMD).6 Most of these studies involved the analysis of polymorphisms that are located at the 3′ end of the VDR gene, such as the BsmI, ApaI, and TaqI restriction fragment length polymorphisms (RFLPs) or poly (A) repeat. However, these polymorphisms are not likely to be functional by themselves, either because they are in an intron or they do not change the sequence of amino acids, while the potential functional effect of poly (A) repeat polymorphism is still unclear. Another commonly studied polymorphism in the VDR gene is the FokI RFLP, which

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**MATERIALS AND METHODS**

**Subjects**

*Panel of ethnic group:* We genotyped a panel of DNA from 88 subjects of different ethnicities. The panel was obtained from the Coriell Institute (Camden, NJ, USA). It consists of DNA from 10 blacks (HD04), 9 Africans from south of the Sahara (HD12), 10 Chinese (HD02), 10 Japanese (HD07), 10 Southeast Asians (excluding Chinese and Japanese, HD13), 10 Northern Europeans (HD01), 9 Indo Pakistanis (HD03), 10 Middle Easterns (HD05), and 10 Mexicans (HD08). We grouped these 88 subjects into three major human race groups defined as African (19 subjects, HD04 and HD 12), Mongoloid (30 subjects, HD02, HD07, and HD13), and white (39 subjects, HD01, HD03, HD05, and HD08).

Data on the incidence rates of hip fracture in different ethnic groups were collected from published studies and are presented in Table 1. To enable comparisons among different published studies, we standardized the age-adjusted incidence of hip fracture in men and women aged \( \geq 55 \) years according to the U.S. population distribution in 1990. The direct standardization method was used; every age-specific rate was multiplied by the corresponding U.S. population in the same age category to get the number of hip fractures for each age category. The sum of fractures was then divided by the total U.S. population to get age-adjusted incidence of hip fracture. For each ethnic group, we computed the average incidence rates, which were weighted by the size of the study population, to compare incidence rates of hip fracture among these eight ethnic groups.

**Study population**

The study population sample of white elderly was derived from the Rotterdam Study (the source population), a single center prospective population-based cohort study including 7983 individuals, with 3105 men (38.9%) and 4878 women (61.1%), to analyze determinants and prognosis of chronic and disabling diseases in the elderly. The baseline measurements were performed between 1990 and 1993. The third follow-up examination phase took place from 1997 to 1999; the mean follow-up period was 6.6 years (range, 5.3–10.2 years). Baseline measurements of BMD were available for 5931 independently living subjects from the...
study. For the current study, 1453 of these were excluded based on age (>80 years), use of a walking aid, diabetes mellitus, or use of estrogen, thyroid hormone, or cytostatic drug therapy. From the 4478 remaining subjects, a random sample of 2848 subjects (the study population), with 1131 men (39.7%) and 1717 women (60.3%), was drawn at baseline, comprised of independently living participants ages 55–80 years. All subjects have records of incident nonvertebral fracture, while 1915 subjects (67.2% of study population) survived and had follow-up radiograph records to assess incident vertebral fracture. Data on dietary intake was available for 2536 subjects (90.0% of study population).

**Measurements**

Information about medical history, dietary habits, age at menopause, and smoking was obtained with a computerized questionnaire during a home interview. Intakes of calcium and total energy were calculated by food frequency questionnaire (based on all food and drinks consumed in 1 month) with the use of Dutch food composition tables. Dietary calcium intake was adjusted for total energy intake. Anthropometric measurements of participants were obtained at the research center. Body mass index (BMI) was calculated as weight (kg) divided by the height squared (m²). BMD (g/cm²) was determined by DXA (DPX-L densitometer; Lunar Corp., Madison, WI, USA) at the femoral neck and lumbar spine (vertebral L2–L4) as described before. All vertebral fractures were confirmed by radiographs of the spine from the fourth thoracic to the fifth lumbar vertebrae. Body mass index (BMI) was calculated as weight (kg) divided by the height squared (m²). BMD (g/cm²) was determined by DXA (DPX-L densitometer; Lunar Corp., Madison, WI, USA) at the femoral neck and lumbar spine (vertebral L2–L4) as described before. All vertebral fractures were confirmed by radiographs of the spine from the fourth thoracic to the fifth lumbar vertebrae. Body mass index (BMI) was calculated as weight (kg) divided by the height squared (m²). BMD (g/cm²) was determined by DXA (DPX-L densitometer; Lunar Corp., Madison, WI, USA) at the femoral neck and lumbar spine (vertebral L2–L4) as described before. All vertebral fractures were confirmed by radiographs of the spine from the fourth thoracic to the fifth lumbar vertebrae.

**Definition of fracture**

Nonvertebral fractures (including hip, wrist, and other fractures, but excluding head, foot, hand, and pathological fracture) were recorded by general practitioners (GPs) who covered 80% of the population. Research physicians confirmed follow-up information by checking GP’s patient records and collected the data of the remaining 20% of the population. Discharge reports and letters from medical specialists were additionally used to verify the hospitalized nonvertebral fracture patients. All fractures were coded events for a separate session. A medical expert in the field reviewed all coded events for a final classification. The incidence of nonvertebral fracture was defined as all new cases occurring during the follow-up period.

To assess presence of vertebral fracture, lateral radiographs of the spine from the fourth thoracic to the fifth lumbar vertebrae were obtained and analyzed morphometrically by the McCloskey-Kanis method as described previously. All vertebral fractures were confirmed through visual interpretation by an expert in the field. When a vertebra was determined to be normal at baseline and any of the three vertebral heights (anterior, central or posterior) showed a minimum decrease of at least 4.6 mm and 15% in absolute height of the later film, it was considered an incident vertebral fracture. The incidence of vertebral fracture was defined as all new cases occurring during the follow-up period.

**Genotyping**

Genomic DNA was isolated from peripheral venous blood samples according to standard proteinase K digestion and phenol-chloroform extraction. The position of the Cdx-2 (also denoted 1e-G–1739A) polymorphism is based on our sequence analysis of the 1e promoter region of the VDR gene (unpublished data). Two sets of primers were designed for the ASM-PCR test:

- **G-For:** 5′-AGGATAGAAAAATAGAAAAACATT-3′
- **G-Rev:** 5′-AACCCATAAAGAAAAATAGTTTTTTAC-3′
- **A-For:** 5′-TCCCTGAGTAACACTAGTTCAACAA-3′
- **A-Rev:** 5′-ACGGTTAAGTTCAGAAAAGATTTTAC-3′

A schematic representation of the method and localization of the allele-specific primer sets is shown in Fig. 1A. G-Rev and A-For are allele-specific primers. The primer **A-Rev:** 5′-AACCCATAAAGAAAAATAGTTTTTTAC-3′
- **A-For:** 5′-TCCCTGAGTAACACTAGTTCAACAA-3′
- **A-Rev:** 5′-ACGGTTAAGTTCAGAAAAGATTTTAC-3′

A schematic representation of the method and localization of the allele-specific primer sets is shown in Fig. 1A. G-Rev and A-For are allele-specific primers. The primer
A-For is designed from 5’ to 3’ of the sense strand (+ strand), and the last base is “A” at the site of the polymorphism. The primer G-Rev is from 5’ to 3’ of the antisense strand (– strand), and stops at “C” (the complement base of “G”) at the polymorphic site. These four primers generate three PCR fragments: primer set G-For and G-Rev specifically amplifies the G allele with a size of 110 bp, A-For and A-Rev specifically amplify the A-allele with a size of 235 bp, and the out-primer pair (G-For and A-Rev) amplifies the internal control PCR fragment with a size of 297 bp. A schematic representation of the gel electrophoresis pattern is shown in Fig. 1B. To verify the first result of genotype calls, a random set of 5% of the samples were genotyped again.

PCR and gel electrophoresis

The PCR amplification was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) with MicroAmp Optical 96-well Reaction Plate (Applied Biosystems) and in a PTC-225 DNA Engine Tetrad (MJ Research) with polypropylene 96- and 384-well thin wall microplates. Ten microliters of the PCR reaction system consisted of 1.0 μl 10× PCR buffer (1× buffer = 10 mM Tris-Cl, pH 8.3; 50 mM KCl; 1.25 mM MgCl2), 1.0 μl 10× dNTPs (0.2 mM), 0.4 pmol G-For, 0.6 pmol G-Rev, 0.6 pmol A-For, 0.4 pmol A-Rev, 0.5 U Super Taq (HT Biotechnology), and 10 ng genomic DNA. PCR was performed with an initial denaturation at 96 °C for 5 minutes, followed by 28 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 45 s. The final extension was at 72 °C for 5 minutes. PCR products were size-separated on a 2.5% agarose gel at 125 V for 1 h. The 100-bp DNA ladder of GIBCO BRL was used to determine the size of fragments.

Sequencing

We sequenced the entire region of 6.5 kb before the exon 1a of VDR gene in DNA from 15 young female whites as a part of our sequence analysis of the VDR gene (unpublished data). To sequence the region around the Cdx-2 polymorphism, the 297-bp PCR product, which was amplified with the out-primer set of G-For and A-Rev, was purified by using Quantum Prep PCR Kleen Spin Columns (Bio-Rad). We performed direct sequence analysis of PCR products by cycle sequencing using an ABI PRISM big dye kit from Applied Biosystems. The sequencing reaction (20 μl) included 4 μl of BigDye, 4 μl of 5× buffer (400 mM Tris-Cl pH 9.0, 10 mM MgCl2), 10 mM of G-For or A-Rev, 4 μl of Terminator Ready Reaction Mixture (Applied Biosystems), and 11 μl of PCR product. The sequencing reaction was performed in GeneAmp PCR system 9700 (Applied Biosystems) with the following 25 cycles: 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 minutes. The sequencing product was purified by using Micro Bio-Spin chromatography columns (Bio-Rad). Forward and reverse sequences of PCR products were produced with an ABI PRISM 310 or 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis

For the ecological study, Spearman’s correlation test was used to test for correlation between VDR Cdx-2 genotype distribution and the eight ethnic groups ranked by gender-specific hip fracture incidence. To compare the differences of the major anthropometric characteristics and clinical endpoints between the source population and the study population for the epidemiological study, we performed independent sample t-tests for continuous variables and the Pearson χ² analysis for categorical variables. Hardy-Weinberg equilibrium was tested for the Cdx-2 genotype by a χ² goodness of fit test. We grouped subjects by their genotype for the Cdx-2 polymorphism as GG, GA, and AA. The GG genotype was defined as reference group because it was the most frequent genotype in our study group. Differences in anthropometric characteristics by genotype were evaluated by ANOVA for continuous variables and Pearson χ² p value for categorical variables. Three possible genetic models were allowed to explain differences between groups, that is, an allele dose effect, a dominant effect, or a recessive effect. Allele dose was defined as the number of copies of a certain allele in the genotype, and genotype was treated as a continuous variable. For the A-allele, the genotype is expressed as a numeric variable with 0 = GG, 1 = GA, and 2 = AA. In case of a consistent trend reflected as an allele dose effect, a linear regression analysis was performed, and a “trend” p value was calculated to quantify the association. In case of a recessive or dominant effect of the test allele, a 2 × 2 χ² test or an independent sample t-test was performed to test for differences between two genotype groups. For recessive effects, homozygous subjects for the test allele (e.g., AA) were compared with the combined group of heterozygous carriers (i.e., GA) and noncarriers (i.e., GG). For dominant alleles, we compared test allele carriers (e.g., GA and AA) versus noncarriers (e.g., GG).

We first analyzed risk of any fracture, which includes vertebral and nonvertebral fracture, by the Cdx-2 genotype. This analysis was limited to the group for which data on vertebral fracture was available (n = 1915). We then stratified the analysis by type of fracture for vertebral versus nonvertebral fracture. For analysis of nonvertebral fracture, the complete study sample (n = 2848) could be included. Eighty-four individuals who had both vertebral and nonvertebral fracture during the follow-up time were included in all analyses. Differences in the nonvertebral fracture frequency by genotype were compared using Pearson χ² tests in general and a linear regression model for the allele-dose effect. Relative risk (RR) and 95% CI were calculated for the relationship between the Cdx-2 genotype and fracture using Cox regression models. The same models were used to estimate the RR adjusted for potential confounders, such as age, gender, BMI, and BMD.

To analyze effects of potential confounders and modification by dietary calcium intake, we also stratified by gender, percentiles, and particular cut-off level (less or more 600 mg/day) of dietary calcium intake. Differences in BMD by genotype were adjusted for age and BMI by a general linear model. All statistical analyses (except Hardy-Weinberg equilibrium) were carried out with the SPSS software package (version 9.0).
RESULTS

1e promoter region

We determined the sequence of the promoter region of 6.5 kb in front of the VDR gene exon 1a using a sequence walking strategy. We used reference sequence information from Miyamoto et al.,(32) Yamamoto et al.,(11) NCBI (http://www.ncbi.nlm.nih.gov/), and the Celera database. We found exon 1e (accession number of the NCBI genomic database is AH006427(33)) was only 2 kb in front of exon 1a (see Fig. 1A). The G to A substitution in the Cdx-2 binding site is located 1739 bp in front of exon 1e. Therefore, the Cdx-2 polymorphism is referred to as 1e-G1739A.

Genotyping

The ASM-PCR method to determine Cdx-2 genotype was applied to genotype 88 subjects from different ethnicities and 2848 men and women from the Rotterdam Study. Figure 2 shows a representative gel separation pattern of ASM-PCR genotype analysis for 95 samples and 1 negative control (no genomic DNA). To confirm the genotype result, we re-genotyped 5% random samples from those subjects and found no discrepancy. In addition, 15 random DNA samples were directly sequenced after PCR to evaluate the Cdx-2 genotype. Both genotype results were completely identical. No other polymorphism was found in this 297-bp region around Cdx-2 polymorphism in the 1e promoter region of the VDR gene according to the sequencing result of these 15 random samples. The genotype distribution in the sample from the Rotterdam Study obeyed Hardy-Weinberg equilibrium ($p = 0.17$). The frequency of the $G$ and $A$-allele in the large Dutch white population is 81% (4614/5696) and 19% (1082/5696), respectively. Allele frequencies were not different in men and women ($p = 0.93$), did not vary by age in men ($p = 0.42$) or in women ($p = 0.17$), and did not vary in the different subsets drawn from the Rotterdam Study ($p = 0.86$).

Ecological study of the Cdx-2 polymorphism

The frequencies of the Cdx-2 alleles and the genotype distribution in different ethnic groups are presented in Table 2. Genotype distribution and allele frequency differed substantially among ethnic groups. When we combined individual Coriell panel groups into the three major races, we found the $A$-allele to have highest frequency in Africans (74%; 95% CI, 60–88%), intermediate in Asians (43%; 95% CI, 39–47%), and lowest in whites (19%; 95% CI, 18–20%). We observed an inverse relation between Cdx-2 A-allele frequency and the incidence rate of hip fractures, both for men and women (Spearman’s correlation test $r = -0.87$ $p = 0.006$ for men; and $r = -0.79$ $p = 0.02$ for women). In Fig. 3, we plotted the age-adjusted incidence rates of hip fracture in women of ≥50 years of age against the frequencies of the VDR Cdx-2 A-allele in the eight ethnic groups. We excluded HD12 (Africans from south of the Sahara), because no hip fracture incidence data are available for this group.
We then analyzed the relation between fracture risk and the VDR Cdx-2 polymorphism in a large white study population of Dutch men and women. We first compared baseline characteristics, including age, gender, height, weight, BMI, age of menopause, dietary calcium intake, and current smoking, between the source population and our study population. The subjects of our study group were 4 years younger, 0.6 cm shorter, and had 0.2 kg/m² lower BMI on average than the source population. In our study population, we observed that all fracture risk increased by increasing age (the linear regression p < 0.001 for all kinds of fractures), and the incidences of all fractures in women were significantly higher than in men (p < 0.001 for all). Baseline characteristics of men and women in the study population were not found to be significantly different when stratified by VDR Cdx-2 genotype (data not shown). The genotype distribution was not significantly different in men and women as well as in 5-year age categories.

We compared by Cdx-2 genotype the incidence of any fracture (including vertebral and nonvertebral fracture) and then separately by vertebral fracture and by nonvertebral fracture during the 6.6-year follow-up period (Table 3). We found subjects carrying the A-allele to have fewer fractures, with evidence for an allele dose effect. The association was borderline significant for vertebral fracture (p = 0.04) and any fractures (p = 0.06), while a similar trend was found for nonvertebral fracture (p = 0.12). When stratifying the same analysis by gender, the protective effect of the A-allele was similar in women and men. Because of the higher incidence of nonvertebral fracture in women (13.6%) than in men (6.2%) and the low frequency of the Cdx-2 A-allele in our white population, we further investigated the effect of Cdx-2 genotype on nonvertebral fracture (including hip fracture) in women separately (Table 4). Compared with the whole study population, we observed a similar protective effect of the Cdx-2 genotype in the women, which was borderline significant (p = 0.05). When RRs were calculated, we found presence of the Cdx-2 A-allele to be associated with reduced risk for fracture with evidence for an allele dose effect. The RR estimate did not essentially alter

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**TABLE 2. THE VDR 1E PROMOTER CDX-2 POLYMORPHISM IN DIFFERENT ETHNIC GROUPS**

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>Coriell code</th>
<th>Number of subjects</th>
<th>Genotype frequency (no.)</th>
<th>Allele frequency (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>GA</td>
</tr>
<tr>
<td>African</td>
<td></td>
<td>19</td>
<td>0.05 (1)</td>
<td>0.42 (8)</td>
</tr>
<tr>
<td>South Sahara</td>
<td>HD12</td>
<td>9</td>
<td>0</td>
<td>0.44 (4)</td>
</tr>
<tr>
<td>Black</td>
<td>HD04</td>
<td>10</td>
<td>0.10 (1)</td>
<td>0.40 (4)</td>
</tr>
<tr>
<td>Mongoloid</td>
<td></td>
<td>291</td>
<td>0.32 (92)</td>
<td>0.50 (147)</td>
</tr>
<tr>
<td>Southeast Asian</td>
<td>HD13</td>
<td>10</td>
<td>0.10 (1)</td>
<td>0.80 (8)</td>
</tr>
<tr>
<td>Chinese</td>
<td>HD02</td>
<td>10</td>
<td>0.30 (3)</td>
<td>0.50 (5)</td>
</tr>
<tr>
<td>Japanese</td>
<td>HD07</td>
<td>10</td>
<td>0.60 (6)</td>
<td>0.30 (3)</td>
</tr>
<tr>
<td>Japanese (Arai)</td>
<td></td>
<td>261</td>
<td>0.31 (82)</td>
<td>0.50 (131)</td>
</tr>
<tr>
<td>Japanese (total)</td>
<td></td>
<td>271</td>
<td>0.32 (88)</td>
<td>0.49 (134)</td>
</tr>
<tr>
<td>White</td>
<td></td>
<td>2887</td>
<td>0.67 (1921)</td>
<td>0.29 (848)</td>
</tr>
<tr>
<td>Mexican</td>
<td>HD08</td>
<td>10</td>
<td>0.60 (6)</td>
<td>0.30 (3)</td>
</tr>
<tr>
<td>Indo Pakistani</td>
<td>HD03</td>
<td>9</td>
<td>0.67 (6)</td>
<td>0.22 (2)</td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>HD05</td>
<td>10</td>
<td>0.60 (6)</td>
<td>0.40 (4)</td>
</tr>
<tr>
<td>Northern European</td>
<td>HD01</td>
<td>10</td>
<td>0.90 (9)</td>
<td>0.10 (1)</td>
</tr>
<tr>
<td>Dutch (current study)</td>
<td>2848</td>
<td>0.67 (1894)</td>
<td>0.29 (838)</td>
<td>0.04 (116)</td>
</tr>
<tr>
<td>Northern European (total)</td>
<td>2858</td>
<td>0.67 (1903)</td>
<td>0.29 (839)</td>
<td>0.04 (116)</td>
</tr>
</tbody>
</table>

* 95% CI of A allele frequency.
† Excluding Chinese and Japanese.
after adjustment for age, weight, and femoral neck BMD. We did not observe the calcium intake to modify the relation between VDR Cdx-2 genotype and fracture (data not shown).

**Association of Cdx-2 genotype with BMD**

We also analyzed the effect of Cdx-2 genotype on BMD but could not find an association of Cdx-2 genotype with femoral neck BMD or lumbar spine BMD. Our data showed that BMD at femoral neck and lumbar spine were correlated to calcium intake and age \((p < 0.001\) for both by linear regression analysis). In general, individuals with a high calcium intake had higher BMD than individuals with a low calcium intake. The dietary calcium intake was not different by Cdx-2 genotype \((p/\overline{H}11005 0.57, \text{which was adjusted for age and gender})\. We then went on to analyze the influence of dietary calcium intake on the relation between Cdx-2 genotype and BMD.

In quartiles of dietary calcium intake, no difference in BMD was observed by VDR Cdx-2 genotype. There were only 119 subjects (4% of the study population) with a calcium intake less than 600 mg/day. In this group, a trend could be observed toward the A-allele having increased BMD, but probably because we did not have sufficient subjects in the group, this failed to reach significance \((p = 0.47\) for femoral neck BMD and \(p = 0.56\) for lumbar spine BMD). In the group with high calcium intake (>600 mg/day), we did not see differences in BMD among VDR Cdx-2 genotype groups.

### DISCUSSION

**Location of Cdx-2 polymorphism and genotyping method**

From our analysis of the physical map of the region in front of exon 1a and 1e of the VDR gene, we found that exon 1e was only about 2 kb in front of exon 1a. The Cdx-2 polymorphism is therefore positioned in a promoter region in front of exon 1e and not of exon 1a. Yamamoto et al.\(^{11}\) determined that the region around the Cdx-2 polymorphism is important for expression of the VDR in intestinal cell lines. However, the constructs used in that study contained either only exon 1a or both 1e and 1a. The effect of the promoter of exon 1e on the expression of the VDR gene was not analyzed separately in that study. Crofts et al.\(^{33}\) analyzed the tissue-specific mRNA expression of multiple promoters of the VDR gene and found exon 1a expressed in all tissues or cell lines in that study. Transcripts containing exon 1f and 1e were found to be expressed in kidney and two tumor cells, but exon 1e was not analyzed separately. Cdx-2 protein is an intestine-specific transcription factor that could regulate the expression of VDR in the same tissue and influence calcium homeostasis consequently. Therefore, to confirm whether 1e is a tissue-specifically expressed

### TABLE 3. RISK OF FRACTURE ACCORDING TO THE VDR CDX-2 GENOTYPE IN MEN AND WOMEN

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Case/total (%)</th>
<th>Any fracture</th>
<th>Vertebral fracture</th>
<th>Nonvertebral fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crude</td>
<td>Adjusted(^{\dagger})</td>
<td>Crude</td>
</tr>
<tr>
<td>GG</td>
<td>381/1915 (19.9)</td>
<td>268/1270 (21.1)</td>
<td>103/567 (18.2)</td>
<td>70/383 (8.4)</td>
</tr>
<tr>
<td>GA</td>
<td>267/1270 (21.1)</td>
<td>128/1270 (10.1)</td>
<td>96/567 (17.2)</td>
<td>70/383 (8.4)</td>
</tr>
<tr>
<td>AA</td>
<td>104/567 (18.2)</td>
<td>8/567 (1.4)</td>
<td>5/567 (0.9)</td>
<td>5/567 (0.9)</td>
</tr>
</tbody>
</table>

\(^{\dagger}\) RR was adjusted for age, gender, weight, and femoral neck BMD.

### TABLE 4. RR OF NONVERTEBRAL FRACTURE IN 1717 WOMEN ACCORDING TO THE VDR CDX-2 GENOTYPE

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Case/total (%)</th>
<th>Crude RR (95% CI)</th>
<th>Adjusted RR (95% CI)(^{\star})</th>
<th>Adjusted RR (95% CI)(^{\dagger})</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>139/1139 (12.2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GA</td>
<td>57/505 (11.3)</td>
<td>1.0 (0.7–1.3)</td>
<td>1.0 (0.7–1.3)</td>
<td>1.0 (0.7–1.3)</td>
</tr>
<tr>
<td>AA</td>
<td>2/73 (2.7)</td>
<td>0.2 (0.06–0.9)</td>
<td>0.2 (0.05–0.8)</td>
<td>0.2 (0.05–0.8)</td>
</tr>
<tr>
<td>Per copy of A allele</td>
<td>0.8 (0.6–1.0)</td>
<td>0.8 (0.6–1.0)</td>
<td>0.7 (0.5–1.0)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{\star}\) RR was adjusted for age and weight.

\(^{\dagger}\) RR was adjusted for age, gender, weight, and femoral neck BMD.
The frequency of the A-allele in this Dutch white population (19%) was much lower than that reported previously for the Japanese population (43%). Our analysis of the ethnic panel also suggests large differences in A-allele frequency of this polymorphism, ranging from 19% in North European whites to 74% in African subjects. The A-allele frequency in the small sample of Japanese subjects used for the ecological study (25%) differed from that found in the relatively large study (261 subjects) by Arai et al. (43%), which can be because of differences in power between these two samples. The previously reported hip fracture incidence rates appeared to be highest in subjects of northern European extraction and lowest in those of Asian and African origin. By comparing incidence rates with the frequency of the A-allele among these ethnic groups, we observed an apparent inverse relationship between the A-allele frequency and hip fracture rate (Fig. 3). However, results from ecological studies have to be interpreted with caution because we cannot rule out alternative explanations for the observed relation (“ecological fallacy”). Yet, the protective effect on fracture of the Cdx-2 A-allele suggested by this comparison is in line with the results from our epidemiological study in the white population of elderly subjects, especially for nonvertebral fracture in elderly women (Table 4). In the previous Japanese study, Arai et al. reported a significant association between A-allele and an increased BMD in postmenopausal women, and this would predict the A-allele to be associated with a decreased risk of fracture as observed in our current study. Nevertheless, separate epidemiological studies of the relation between the VDR Cdx-2 polymorphism and fracture risk have to be performed in the different ethnic groups to determine if the protective effect of the A-allele on fracture risk is true and consistent.

Epidemiological study in whites

In the population-based cohort study of white elderly subjects, we performed a large-scale association analysis to investigate the relationship between the Cdx-2 polymorphism and fracture risk. The genotype distribution followed the Hardy-Weinberg equilibrium, suggesting absence of selection bias. The associations between Cdx-2 genotype and fractures were observed and independent of age, gender, weight, and BMD. We found a trend of decreasing frequency of fracture by increased number of A-alleles, suggesting an allele dose effect. Because we did not have sufficient statistical power, in particular for the AA genotype group, the associations were borderline significant, which makes it difficult to distinguish an allele-dose effects from a recessive effects.

The association of the VDR Cdx-2 A-allele with reduced fracture risk is consistent with the results of functional studies of this polymorphism that were previously reported. In these studies, the A-allele was found to bind more efficiently the Cdx-2 protein and showed increased transcription level of the VDR gene. The Cdx-2 transcription factor plays an important role in intestine-specific gene transcription. As a transcription factor, Cdx-2 could mediate the transcription of the VDR gene through the special cis-element in the 1e promoter region of the VDR gene, thereby affecting the expression of the VDR in the intestine. Thus, the VDR content of intestinal cells of the GA and AA genotype may be higher than that of the GG genotype. The VDR is a transcription factor and regulates the transcription of other downstream genes in many tissues. Interestingly, by using VDR-knockout mice, the expression of two intestinal calcium channels, epithelial calcium channel (ECaC) and calcium transport protein type 1 (CaT1), was shown to be strongly vitamin D dependent. The VDR mediates the effect of vitamin D. It can therefore be hypothesized that VDR Cdx-2 A-allele carriers could have higher intestinal calcium absorption, because of the elevated expression of these intestinal calcium channel proteins. Increased calcium absorption in turn could increase the BMD, and might, thus, contribute to a decreased fracture risk.

To investigate the potential mechanism underlying the association between fracture and the VDR Cdx-2 genotype, we further analyzed the relationship between BMD and Cdx-2 genotype. However, in our study of Dutch white elderly, we could not observe VDR Cdx-2 genotype to be associated with BMD, which is in contrast with the results reported for the Japanese population. Arai et al. found the VDR Cdx-2 A-allele to be associated with increased BMD at the lumbar spine in a Japanese population of postmenopausal women. However, several environmental factors involved in bone metabolism could be different between the Japanese and the Dutch population, including dietary calcium intake and serum vitamin D level. For example, the dietary calcium intake of this Dutch study population is higher than that of the Japanese population (average intake of 1117 versus 600 mg/day). We therefore went on to analyze the influence of dietary Ca intake on the relationship between Cdx-2 genotype and BMD in our study population.
In our study group, increased dietary calcium intake was associated with increased BMD, but the association was independent of VDR Cdx-2 genotype. However, when we analyzed subjects with a dietary calcium intake of less than 600 mg/day (n = 119), which is the similar to that of the Japanese population, we observed a trend toward the A-allele being associated with increased BMD, but this failed to reach significance, probably because of low statistical power. Thus, to draw conclusions in this respect, it is necessary to analyze VDR Cdx-2 genotype in relation to BMD and fracture in a population with relatively low calcium intake, and for whites, in an even larger sample size than that of the current study population.

In conclusion, in this study, we introduced a simple and specific genotyping method for association analysis of the Cdx-2 polymorphism in the 1c promoter region of VDR gene. In an ecological study, we found a strong correlation between frequency of Cdx-2 A-allele and the incidence rates of hip fracture from different ethnic groups. In an epidemiological study, we demonstrated that the A-allele has a protective effect on the risk of fracture in white elderly, especially for women. The association seems not to be directly explained by differences in BMD. Our results prompt the further association analysis of this polymorphism in relation to fracture risk and environmental factors, particularly in Asian and African populations.

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