

Promoter and 3'-Untranslated-Region Haplotypes in the Vitamin D Receptor Gene Predispose to Osteoporotic Fracture: The Rotterdam Study

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Polymorphisms of the vitamin D receptor gene (*VDR*) have been shown to be associated with several complex diseases, including osteoporosis, but the mechanisms are unknown and study results have been inconsistent. We therefore determined sequence variation across the major relevant parts of *VDR*, including construction of linkage disequilibrium blocks and identification of haplotype alleles. We analyzed 15 haplotype-tagging SNPs in relation to 937 clinical fractures recorded in 6,148 elderly whites over a follow-up period of 7.4 years. Haplotype alleles of the 5' 1a/1e, 1b promoter region and of the 3' untranslated region (UTR) were strongly associated with increased fracture risk. For the 16% of subjects who had risk genotypes at both regions, their risk increased 48% for clinical fractures ($P = .0002$), independent of age, sex, height, weight, and bone mineral density. The population-attributable risk varied between 1% and 12% for each block and was 4% for the combined *VDR* risk genotypes. Functional analysis of the variants demonstrated 53% lower expression of a reporter construct with the 1e/1a promoter risk haplotype ($P = 5 \times 10^{-7}$) in two cell lines and 15% lower mRNA level of *VDR* expression constructs carrying 3'-UTR risk haplotype 1 in five cell lines ($P = 2 \times 10^{-6}$). In a further analysis, we showed 30% increased mRNA decay in an osteoblast cell line for the construct carrying the 3'-UTR risk haplotype ($P = .02$). This comprehensive candidate-gene analysis demonstrates that the risk allele of multiple *VDR* polymorphisms results in lower *VDR* mRNA levels. This could impact the vitamin D signaling efficiency and might contribute to the increased fracture risk we observed for these risk haplotype alleles.

Introduction

The vitamin D receptor (*VDR* [MIM 601769]) (12q13) is a steroid receptor acting as a transcription factor responding to the biologically active form of the secosteroid vitamin D hormone. The vitamin D endocrine system is pleiotropic and plays an important role in skeletal metabolism, including intestinal calcium absorption and regulation of osteoblast differentiation, but it has also been shown to modulate immune response, insulin secretion, the renin/angiotension system, and growth of cancer cells (Haussler et al. 1998).

Rare, deleterious mutations in the *VDR* gene cause the well-known 1,25-dihydroxyvitamin D-resistant rickets (rickets type II), a rare monogenetic disease characterized by osteomalacia, alopecia, and increased 1,25-(OH)₂D₃ levels. Some isolated, more common *VDR*

SNPs have previously been associated with several complex diseases and traits, such as osteoporosis (Morrison et al. 1994; Young et al. 1996; Uitterlinden et al. 2001; Fang et al. 2003). However, the relationship between disease and haplotype alleles across *VDR* has not been systematically analyzed, and their effects on *VDR* function are poorly understood.

The interpretation of polymorphic variations in *VDR* is severely hindered by the fact that, until now, only a few polymorphisms in this large gene have been studied and that most of these are anonymous (nonfunctional) polymorphisms. To explain the associations observed with complex diseases, they should be in linkage disequilibrium (LD) with truly functional polymorphisms. Haplotype-based methods offer a powerful approach to studying the association of genetic variation with complex disease (Gabriel et al. 2002). Following a recent study of *VDR* polymorphisms (Nejentsev et al. 2004), we here present a detailed description of the genomic organization of the *VDR* gene region, the identification of 62 polymorphisms across relevant areas of the gene, and an analysis of LD and haplotype diversity of *VDR* variations in different ethnic groups. We use this information in a large-scale association analysis of haplo-

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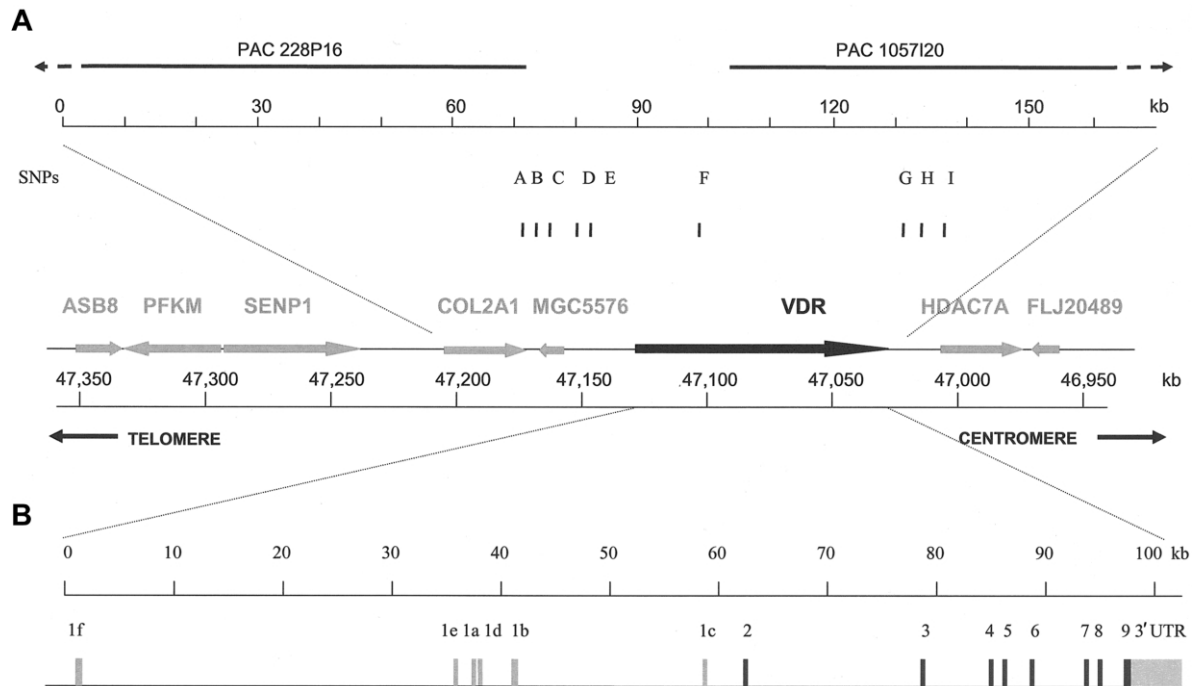


Figure 1 Genomic structure and LD map of the human *VDR* gene. *a*, Physical organization of the 12q12 area containing *VDR*, mostly based on the Celera database (kb 47032–47145 at chromosome 12q12). The arrows for each gene indicate the transcription direction, and distances are in kb. *b*, Genomic structure of the human *VDR* gene. Black bars indicate the coding exons of *VDR*, and the gray bars indicate 5' exons and the 3' UTR. *c*, Sequenced areas and positions of the 62 variations. Gray bars in the 3'-UTR indicate destabilizing elements (DE 1, 2, and 3 [Durrin et al. 1999]). The sequence-variation numbers refer to those given in table 3. *d*, Haplotype map of *VDR* in whites, Asians, and African Americans, based on SNPs with an MAF $\geq 5\%$ in each of the different ethnic populations. Common haplotype alleles in each block with a frequency $>3\%$ are presented below the blocks. SNPs and alleles in red indicate htSNPs. Fracture-risk haplotype alleles are underlined. The correspondence is shown for whites to haplotypes in block 5 of the *BsmI*-*ApaI*-*TaqI* haplotype alleles we defined elsewhere (Uitterlinden et al. 1996).

type-tagging SNPs (htSNPs) in relation to osteoporosis in a group of 6,148 white elderly men and women. We analyzed functionality of relevant polymorphisms in the promoter and 3' UTR to help understand the underlying mechanism of the association we observed.

Material and Methods

Subjects

We sequenced genomic DNA (see supplementary material and methods in online-only appendix A) from 15 young white individuals, including five homozygotes for each major *VDR* 3'-UTR haplotype—that is, 11, 22, and 33, as defined in a previous study (Uitterlinden et al. 1996) on the basis of *BsmI*, *ApaI*, and *TaqI* RFLPs. We studied LD in 234 random white blood-bank donor sam-

ples and in DNA from 107 Asian and 58 African individuals (from the Coriell Institute: 90 Chinese Han [group HD100], 9 Chinese [HD02], 8 Japanese [HD07], 47 African Americans [HD04 and HD50], and 9 Africans from south of the Sahara [HD12]). The association of *VDR* genotype with fracture risk was analyzed in the Rotterdam Study with 7,983 subjects (Hofman et al. 1991); DNA was collected from 6,580 of them, and 6,148 DNA samples were available for which genotyping of all SNPs was successful. Genomic DNA was isolated from blood in accordance with standard procedures.

Genotyping

We genotyped 47 SNPs (table 1) in three ethnic groups and 14 htSNPs and *FokI*, *BsmI*, *ApaI*, and *TaqI* RFLPs in 6,148 white subjects from the Rotterdam Study, with the use of high-throughput TaqMan allelic discrimination assays. The assay mixes (including unlabeled PCR primers, FAM and VIC dye-labeled TaqMan MGB probes) of three Assays-on-Demand and 36 Assays-by-Design were designed and provided by Applied Biosys-

Table 1

Allele Frequencies of 47 SNPs in Different Ethnic Groups

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 2**Oligonucleotides for EMSA**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

tems (ABI). The reaction system contained 1–5 ng of dried genomic DNA, 2.5 μ l of TaqMan Universal PCR Master Mix, 2 \times No AmpErase UNG, 0.125 μ l 40 \times Assay Mix or 0.0625 μ l 80 \times Assay Mix and was adjusted with Milli-Q H₂O for a total volume of 5 μ l. The genotyping results were analyzed using independent end-point readings by two operators of the ABI Prism 7900HT, and a random 5% of samples were independently repeated to confirm genotyping results. The disagreement rate varied from 0.3% to 1.2% for five htSNPs, whereas the genotype results for all other htSNPs were completely consistent.

Sequence Analysis, LD, and Haplotype Analyses

Human and mouse *VDR* genomic sequences (>105 kb from the Celera database) were analyzed using the Vista program (Vista Tools Web site) to visualize the pairwise percentage identity as calculated for every 100 bp. We used the TFSEARCH program (TFSEARCH: Searching Transcription Factor Binding Sites Web site) from the TRANSFAC databases developed by GBF-Braunschweig and the MetInspector from Genomatix software GmbH to search for potential transcription-factor binding sites (TFBSs) around promoter polymorphisms.

We first analyzed LD among all 62 polymorphisms determined on the basis of 15 sequenced white samples, to select 37 SNPs; we also included an additional 9 SNPs (A to I in fig. 1A and table 1) that flank *VDR* from the Celera database and E8-G+284A (a *BsmI* RFLP) (Morrison et al. 1992). (For polymorphism nomenclature, the first element of the polymorphism ID is the designation of exon [coding exons begin with capital “E”] or 3'-UTR [“U”]; in most IDs, this is followed by a hyphen and the major nucleotide allele [for whites]; then, the nucleotide location is designated as plus [+] or minus [–], relative to the first or last base of the nearest exon, or is shown without a sign if it is in the exon; and the last element of the polymorphism ID is the minor nucleotide allele [for whites].) Selection criteria for *VDR* SNPs in the LD analysis is based on whether the SNP (1) has a minor-allele frequency (MAF) >10% (3/30 alleles), as determined by our sequence analysis; (2) is in a potential promoter TFBS or destabilizing element in the 3' UTR; (3) is in a highly conserved region; or (4) is a tagging SNP (tagSNP) (on the basis of data from the 15 sequenced subjects).

We then genotyped 47 SNPs in whites, Han Chinese, and African Americans, to calculate allele frequencies

(table 1). We determined the race-specific SNPs, whose MAF is >3% in any of the ethnic study populations, and identified 42 SNPs for whites, 33 for Asians, and 41 for African Americans. We constructed haplotype structure by use of the PHASE program and then used the PHASE outcome to calculate the pairwise standardized disequilibrium coefficient (D') with Haploxt, to estimate the linkage magnitude between two SNPs, and depicted the graphic overviews of LD by use of the GOLD program (GOLD Web site). We identified haplotype blocks and calculated haplotype frequencies in each block by use of the HaploBlockFinder program (HaploBlockFinder Web site). Selection of the htSNPs in each ethnic group was based on the condition that (1) the minimal combination of htSNPs in each haplotype block represents $\geq 95\%$ of the haplotypes; (2) there is potential functionality in the 3' UTR or promoter; or (3) the SNP is unlinked to any block—for example, E2-C4T (the *FokI* RFLP).

Epidemiological Analysis

Genotype distribution was tested for Hardy-Weinberg equilibrium. Anthropometric measurements, BMI (weight in kg/height in m²), bone mineral density (BMD) (g/cm²; determined by dual energy x-ray absorptiometry [Lunar DPX-L densitometer]) at the femoral neck and lumbar spine (L2–L4), and other variables were measured as described elsewhere (Burger et al. 1994; Schuit et al. 2004). BMD measurements were available for 5,027 subjects (82% of the cohort with genotypes). Sex-specific *T* scores were calculated from the femoral neck BMD by use of the NHANES reference population (Looker et al. 1998) of white males and females aged 20–29 years. For assessment of the incidence of fracture, follow-up ended before January 1, 2002 (mean follow-up period 7.4 \pm 3.3 years). We defined “clinical fracture” to include all fractures confirmed by general practitioners or at hospitals, as described elsewhere (Fang et al. 2003; Schuit et al. 2004), and we excluded head, foot, hand, pathological, postprocedural, skull, and face fractures. The presence of vertebral fracture was analyzed as described elsewhere (McCloskey et al. 1993; Van der Klift et al. 2002).

We applied the Pearson χ^2 test to estimate differences in fracture frequency by genotype. We calculated the relative risk (RR) and 95% CI by logistic regression models and calculated hazard ratio (HR) for incidence of clinical, wrist, and hip fractures by Cox regression model. Both the logistic and the Cox regression models

Table 3**Sequence Variations of *VDR* Determined by Sequence Analysis of 30 White Chromosomes**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

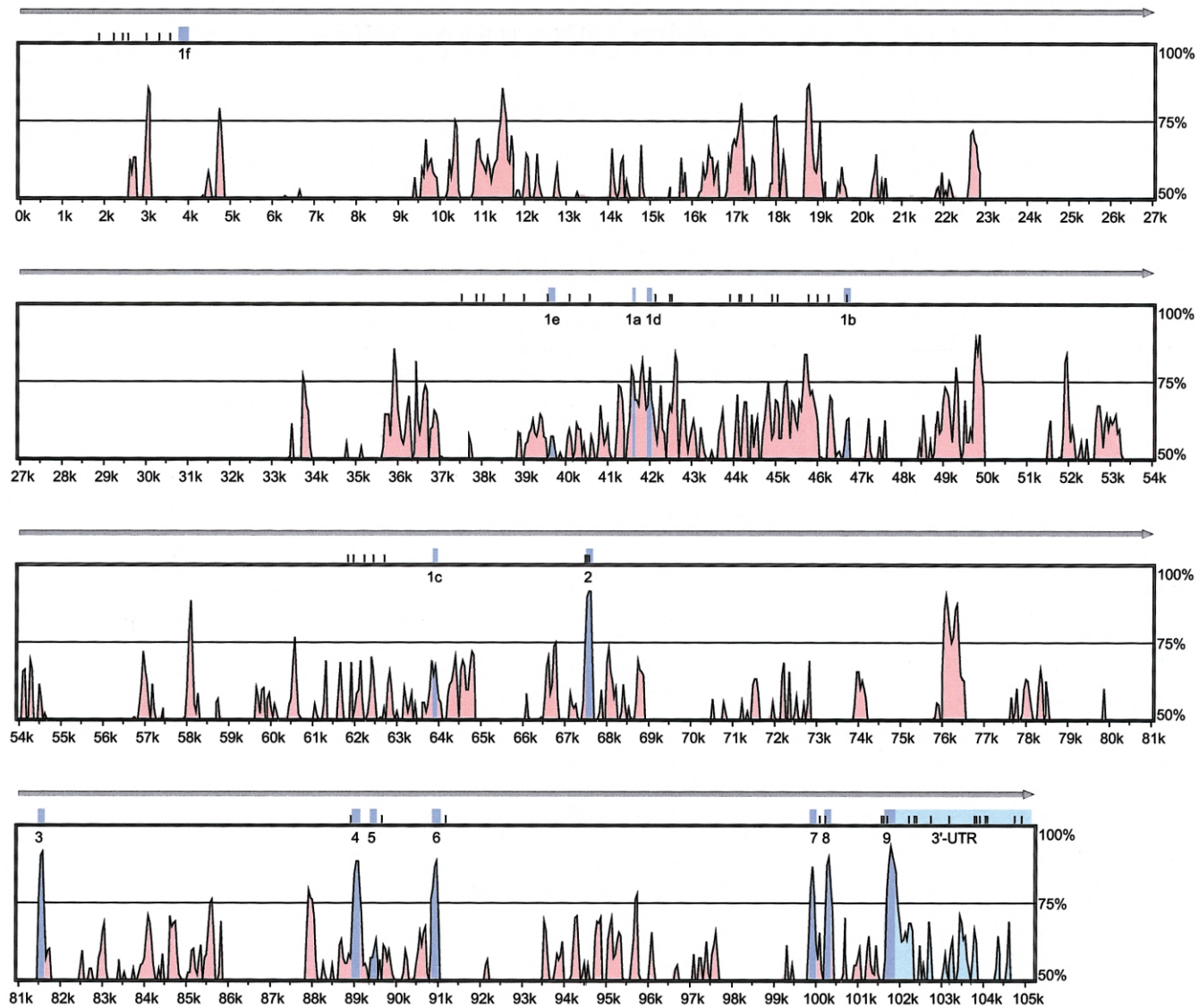
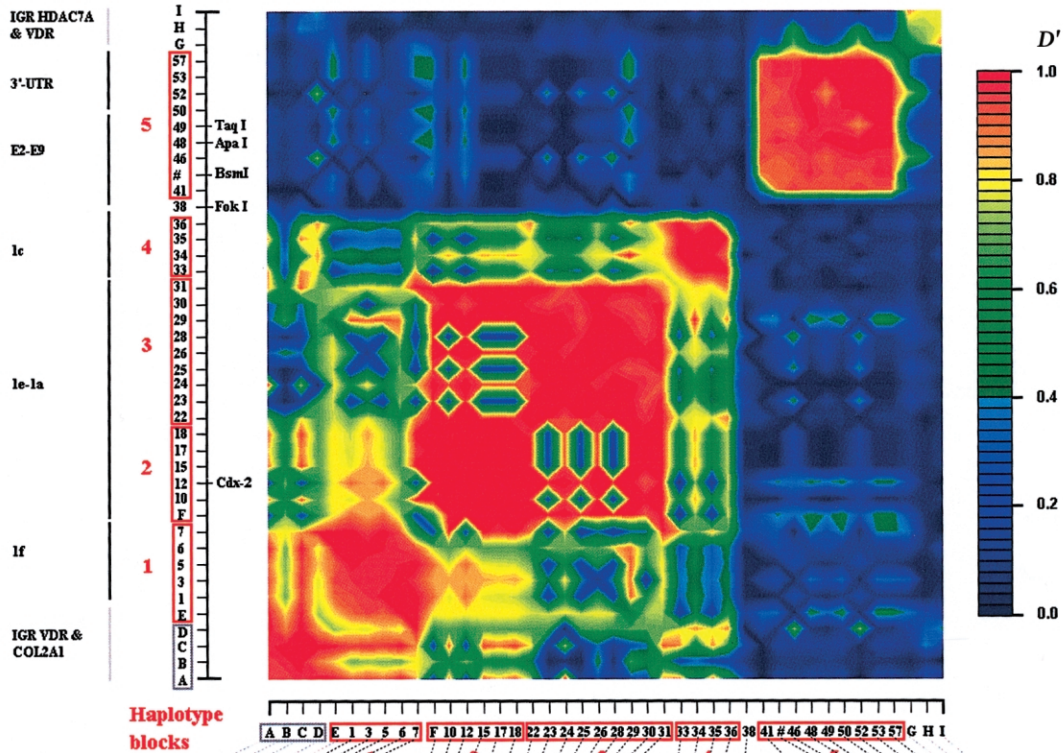
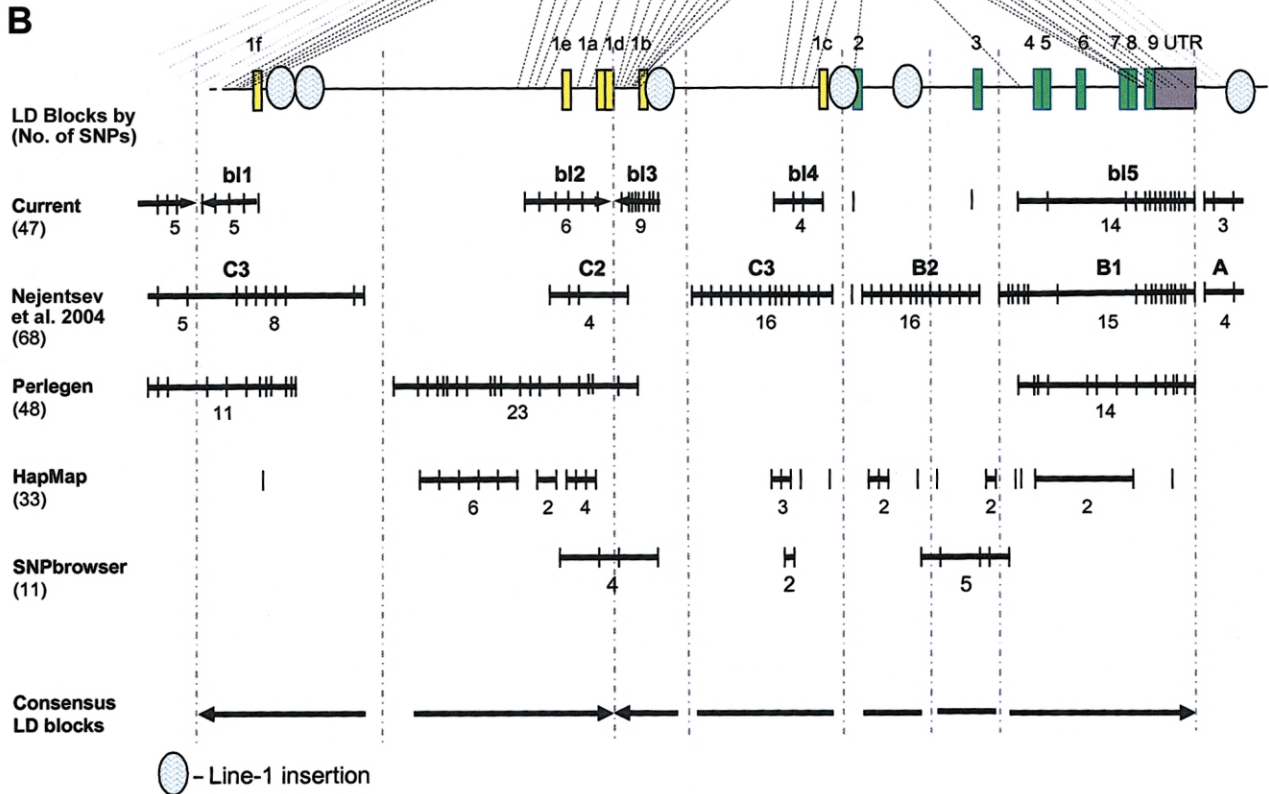


Figure 2 Conservation of the human and mouse genomic *VDR* gene sequence. The Y-axis is the homology rate between human and mouse; the X-axis is the physical distance on the human *VDR* gene. All exons are indicated in purple, the 3' UTR in light green, and the conserved noncoding region in red. The small black bars on the top of each frame indicate the polymorphisms we observed by resequencing, and the gray arrow on top indicates the transcription direction of *VDR*.

A



B



were adjusted for potential confounders, such as age, sex, height, weight, BMD, and bone loss. The population-attributable risk (PAR) was calculated for genetic and other markers for clinical fracture risk. All statistical analyses were done using SPSS (version 11.0). The PAR was calculated as $P_e \times (HR - 1) / [P_e \times (HR - 1) + 1]$, where P_e is the proportion of the study population that is exposed to the risk factor for fracture and where the HR is for the risk factor.

Functional Experiments

Details on the electrophoretic gel mobility shift assay (EMSA) and the transactivation assays for promoter polymorphisms as well as the mRNA levels and stability measurements for the 3'-UTR polymorphisms are provided in online-only appendix A. The oligonucleotides used for EMSA are provided in table 2.

Results

Genomic Structure of VDR and Homology Analysis

The physical organization of the human *VDR* gene region on chromosome 12q12-14 (kbp 46950–47350) (fig. 1a) and the *VDR* gene structure (fig. 1b) is based on PAC clones (PAC228P16 and PAC1057I20), data from two published articles (Miyamoto et al. 1997; Crofts et al. 1998), the Celera database, and our resequencing efforts. *VDR* encompasses at least 105 kb; the large 5' region of noncoding exons 1f–1c is 60 kb, with exon 1f located 35 kb upstream of exon 1e, whereas exon 1e is 2 kb upstream of exon 1a. The nearest genes are *COL2A1*, at a distance of 20 kb upstream (with a small gene-like structure, MGC5576, close to *COL2A1*), and *HDAC7A*, at a distance of 10 kb downstream.

The overall percentage of identity between the entire human and mouse *VDR* genomic sequences is 28.8% (fig. 2). Although coding exons were found in a highly conserved region (86.5%–92.6% identity), except for exon 5 (61.2% identity), we also observed regions of high homology in intronic areas—in total, 14 kb of such regions between exons 1f and 1e, 5 kb of regions between exons 1b and 1c, and a small 500-bp region between exons 2 and 3. Interestingly, there is a lack of homology of human exons 1b, 1e, and 1f with the mouse

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 4 LD maps of *VDR* in different ethnic groups. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

gene, whereas exons 1a, 1d, and 1c are well conserved. Overall, much of the lack of homology can be explained by the presence of *Alu* repeats in the human *VDR* gene. We detected 62 *Alu*-like sequences, mostly located in introns and between exons 1f and 1e, although one is located in the 3' UTR (data not shown).

Resequencing of the VDR Gene

We found 62 polymorphisms (fig. 1c and table 3), including 57 SNPs and 5 VNTR polymorphisms, of which 18 polymorphisms (including 15 SNPs) were not present in the Celera database (chromosome 12, position 47033000–47139000 in the National Center for Biotechnology Information [NCBI] Human Genome [build 35.1, April 2005 freeze]), the NCBI dbSNP (build 123), the Human Gene Mutation Database (HGMD), the HGBASE database, and/or other publications. Of 62 polymorphisms, 9 are located in highly conserved non-coding regions (identity rate >70%) in the 3' UTR and the promoter region. The average frequency of variation across 22 kb was 1/355 bp, with a range from 1/247 bp (13/3.2 kb) in the 3' UTR, to 1/315 (13/4.1 kb) in exons 2–9 and surrounding introns, to 1/408 (36/14.7 kb) across the promoter region. Fifty SNPs with an MAF $\geq 5\%$ in the 15 subjects were identified as “informative SNPs.”

Three informative polymorphisms were located in the so-called destabilizing elements (described elsewhere [Durrin et al. 1999]) in the 3' UTR of *VDR* (fig. 1c). Four informative SNPs were observed in coding exons 2–9: a previously identified C→T substitution (E2-C4T, a *FokI* RFLP), E2-C59T and E8-C2T, which are synonymous substitutions, and a previously identified synonymous SNP (E9-T32C, detected as a *TaqI* RFLP). In the promoter-region exons 1a–1f, we found 36 sequence variations, including 5 VNTRs and 31 SNPs. One SNP in exon 1b (1b-C25A) would change the pre-

Figure 3 LD structure of *VDR* in whites. *a*, Blocks with pairwise $D' > 0.8$ are numbered 1–5. The analyzed SNPs (table 1) include 39 *VDR* SNPs, 5 SNPs in the *COL2A1* and *VDR* intergenic region (“IGR *VDR* & *COL2A1*”), and 3 SNPs in the *VDR* and *HDAC7A* intergenic region (“IGR *HDAC7A* & *VDR*”). SNP IDs correspond to those in figure 1 and tables 1 and 5. The red boxes on the X- and Y-axes indicate the high-LD blocks used to define haplotype alleles. The physical organization of *VDR* is represented with vertical lines on the Y-axis (see also fig. 1). *b*, Aligned LD analyses from different sources and estimated consensus LD structure of *VDR*. The total number of SNPs analyzed in each study is indicated in parentheses. Thick lines indicate haplotype blocks, with the number of analyzed SNPs below the line and the name of the block above the line.

dicted amino acid sequence by replacing a threonine with a lysine (1b-Thr8Lys). TRANSFAC analysis of the 5' upstream regions of promoter exons 1a–1f indicated that 14 polymorphisms change the core recognition sequence of potential transcription factors (see table 3).

LD and Haplotype Analyses

In whites, we identified five blocks with high LD (numbered 1–5 in fig. 3a), which ranged in size from 2 to 17 kb. SNPs in such a block are in strong LD with the other SNPs inside the block but show very little LD with SNPs outside the block. Four blocks (blocks 1–4) were found in the promoter region, whereas the largest block (block 5) encompasses 17 kb and includes exons 4–9 and the 3' UTR. Blocks 2 and 3, encompassing the 1b–1e promoter region, could not be considered as one LD block in our analyses, even when $D' > 0.50$ is taken as the cutoff to define a block. There are also clear areas of very low (or absent) LD—that is, between exons 1e and 1f (blocks 1 and 2), between exons 2 and 3 (blocks 4 and 5), and at the end of *VDR* (3' of block 5). The E2-C4T SNP (the *FokI* RFLP) has no LD with any of the other SNPs and cannot be assigned to any of the blocks. The most distal 3' haplotype block (block 5) shows no LD with SNPs after *VDR*, and the most proximal 5' (block 1) shows only weak LD with more 5' SNPs near and within *COL2A1*. At least seven blocks could be identified when we compared different sources of *VDR* LD block structures (fig. 3b). It is difficult to define the exact boundaries of these low-LD areas because not all studies analyzed a high density of SNPs across *VDR*. Interestingly, we observed Line-1 repetitive elements to be localized between the boundaries of the LD blocks of *VDR* (fig. 3b).

The LD map of Han Chinese is similar to that of

whites (figs. 1d and 4a), but with only four haplotype blocks and with areas of low LD colocalizing with those in whites. For Asians, we observed a large haplotype block in the promoter region (from 1e-T–3743C to 1b-T–391C), which corresponds to the combined blocks 2 and 3 in whites. This larger promoter block was also observed in whites when we used SNPs with an MAF $\leq 5\%$ (data not shown). The LD map of African Americans is more fragmented and is substantially different from those of whites and Asians (figs. 1d and 4b). There are eight small haplotype blocks and seven unlinked SNPs. Again, the E2-C4T SNP (the *FokI* RFLP) was found to be independent of any other haplotype block. Some areas of low LD are colocalizing in all three ethnic groups, indicated in fig. 1d as A (separating exon 1f from the upstream region to *COL2A1*), C (separating exon 1c from exons 1e–1b), D (separating exon 2 from exons 3–9/the 3' UTR), and E (separating exon 4 to the 3'-UTR from the area downstream of *VDR*), whereas B (separating exon 1f from exons 1e–1b) is shared between whites and African Americans but is absent in Han Chinese. In African Americans, two additional areas of low LD (B1 and B2) can be distinguished.

We reconstructed haplotype alleles and analyzed diversity and frequencies across ethnic groups (fig. 1d; only haplotypes with MAF $> 3\%$ are shown). The number of haplotype alleles in each of the LD blocks increases from Han Chinese to whites and is largest in African Americans. Only for LD blocks relatively “conserved” across ethnic groups is comparison possible, and this shows substantial differences in haplotype allele frequencies. For example, in block 1 (around exon 1f), the most common allele in whites has a frequency of 44.6%, but this same allele has a frequency of 4.7% in Han Chinese and 10.3% in African Americans. Similarly, the most

Table 4
Characteristics of the Study Population

CHARACTERISTIC	VALUE FOR TOTAL COHORT	VALUE FOR COMBINED PROMOTER AND 3'-UTR <i>VDR</i> GENOTYPE ^a			P
		0	1	2 or 3	
No. (%) of individuals	6,148	1,586 (25.8)	3,560 (57.9)	1,002 (16.3)	...
No. (%) of females	3,649 (59.4)	932 (58.8)	2,102 (59.0)	612 (61.1)	.49
Age (years) ^b	69.5 ± 9.1	69.1 ± 9.1	69.5 ± 9.1	70.0 ± 9.4	.03
Height (cm) ^c	166.8 ± 9.5	167.0 ± 9.5	166.7 ± 9.4	166.6 ± 9.8	.16
Weight (kg) ^d	73.1 ± 12.0	73.3 ± 12.2	73.1 ± 12.0	72.9 ± 11.7	.55
Femoral neck (FN) BMD (g/cm ²) ^e	.867 ± .142	.872 ± .143	.864 ± .142	.868 ± .145	.40
Lumbar spine BMD (g/cm ²) ^e	1.090 ± .198	1.098 ± .197	1.089 ± .198	1.082 ± .199	.25
FN BMD change (10 ⁻³ g/cm ² /year) ^f	-2.1 ± 9.4	-1.6 ± 8.6	-1.9 ± 9.5	-3.2 ± 10.0	.02

^a The definition of the combined genotypes “0,” “1,” and “2 or 3” are given in table 6.

^b Age was adjusted for sex.

^c Height was adjusted for age and sex.

^d Weight was adjusted for age, sex, and height.

^e BMD (subset $n = 5,027$) was adjusted for age, sex, height, and weight.

^f BMD change in 7.4 years follow-up (subset $n = 2,391$) was adjusted for age, sex, and clinical fracture.

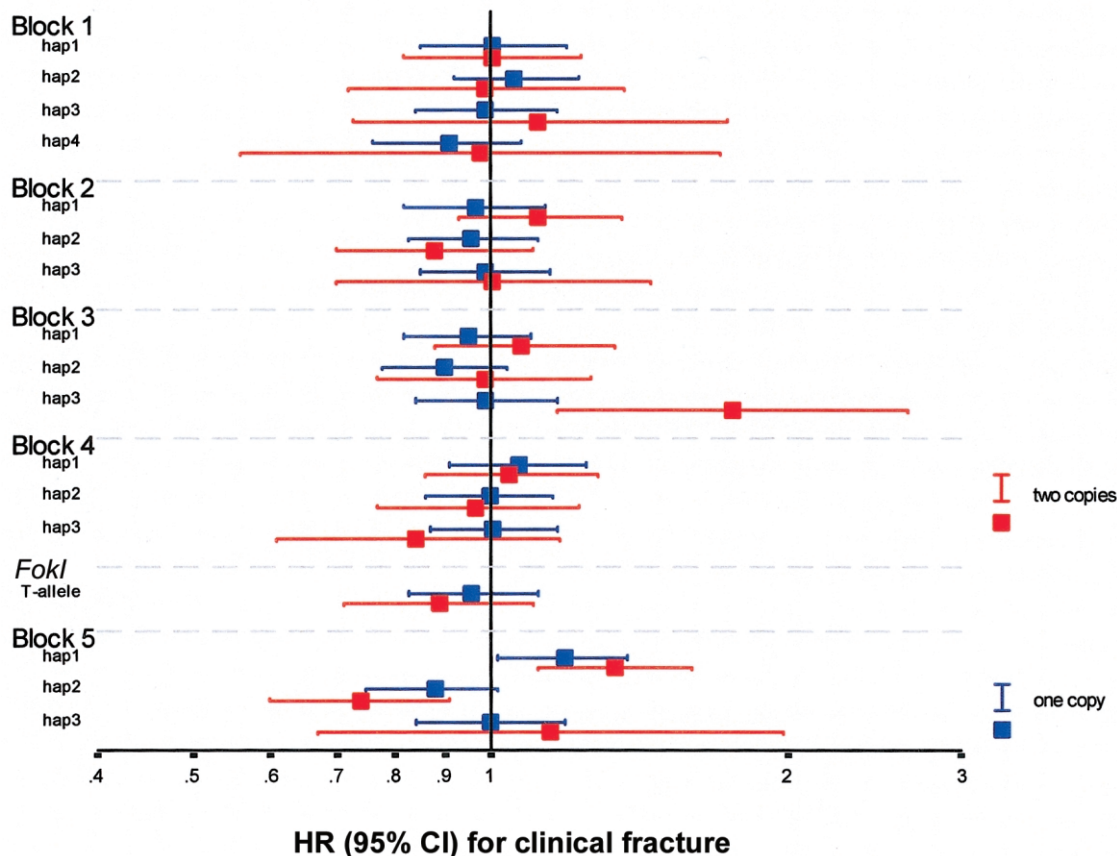


Figure 5 HR for clinical fracture, by VDR genotypes based on haplotype alleles in five haplotype blocks (1–5) and the *FokI* RFLP. The HR point estimates and the surrounding 95% CIs are represented with colored squares and lines. The HR for one copy of the test allele versus no copy is in blue; the HR for two copies of the allele versus no copy is in red. The logarithmic HR is plotted for the common haplotype alleles (frequency >3%) in all haplotype blocks (see fig. 1d for whites) and the *FokI* RFLP.

common allele in block 4 (around exon 1c) has frequency 40.2% in whites, 32.3% in Han Chinese, and 13.8% in African Americans.

Although, for whites, 15 tagSNPs (14 htSNPs and E2-C4T) are required to cover the common genetic diversity across *VDR*, the required number is only 10 for Han Chinese but increases to 28 for African Americans. When only the tagSNPs were used to genotype a population (6,148 subjects from the Rotterdam Study), the LD pattern obtained was similar to that obtained using the 34 original SNPs with an MAF >5% in the smaller sample of 234 blood-bank donors, which indicates that the tagSNPs effectively predict the other SNPs within the blocks.

We observed that the haplotypes constructed from the well-known *BsmI*, *ApaI*, and *TaqI* RFLPs, as described elsewhere by our group (Uitterlinden et al. 1996), could correctly predict all the common haplotypes at the 3' UTR in whites, defined using the nine SNPs with an MAF >5% (fig. 1d). For Han Chinese, such correspon-

dence is lower because of the U-D796T polymorphism, which has changed phase, whereas, for African Americans, the haplotype structure of this area is much different.

Study of Association with Osteoporosis

Baseline characteristics of 6,148 white elderly of the Rotterdam Study are presented in table 4. The 15 tag-SNPs were in Hardy-Weinberg equilibrium. Haplotype alleles in blocks 2, 3, and 5 had consistent—albeit not always significant—effects on clinical fractures (fig. 5), with similar effects in men and women and for vertebral, hip, and wrist fractures (data not shown).

Subjects homozygous for the block 2–hap1 allele, homozygous for the block 3–hap3 allele, or carrying the block 5–hap1 allele had an increased risk for clinical fracture of 15% ($P = .06$), 74% ($P = .002$), and 23% ($P = .004$), respectively (tables 5 and 6). We then investigated the combined effect of promoter and 3'-UTR risk

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 6 EMSA of 1a-A–1012G for GATA protein. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

genotypes on fracture risk and found that subjects carrying two or three *VDR* risk genotypes had a 48% increased risk for clinical fracture ($P = .0002$). Although age was borderline significantly different by combined *VDR* genotype, all associations with fracture were independent of age, sex, height, weight, and bone loss (data not shown). By our observations, BMD did not influence the association of individual or combined blocks with fracture risk.

The PAR of the *VDR* genetic markers (table 7) was 1% (frequency of subjects homozygous for the block 3–hap3 allele = 2%) and was 2% for the promoter region (frequency of subjects homozygous for the block 2–hap1 allele = 21%) but increased to 12% for the 3' region, including the 3' UTR (frequency of the block 5–hap1 allele carriers = 68%). This was higher than the 1% PAR of the best validated genetic marker for osteoporosis so far (*COL1A1* Sp1 [Grant et al. 1996; Uitterlinden et al. 1998a]; in our study population, the frequency of subjects homozygous for the Sp1 T allele was 3%) and was similar to the PARs for smoking and use of a walking aid.

Functionality Studies of Promoter Polymorphisms

Choosing from among 12 polymorphisms located at potential TFBSs in the *VDR* promoter region, we per-

formed EMSA for four common (MAF >20%) SNPs (1e-C–2090T, 1e-G1739A, 1a-G1521C, and 1a-A–1012G) in the promoter region of exons 1e and 1a, using Caco2 or HEK293 nuclear extracts (fig. 6). We identified a putative GATA-binding site for the A allele of the 1a-A–1012G SNP in the exon 1a promoter region (AGATAT in reverse orientation) and demonstrated that the G allele has markedly decreased binding to GATA compared with that of the A allele.

Reporter constructs containing the 2-kb 1a-promoter sequence with the two SNPs 1a-G–1521C and 1a-A–1012G in block 2 (fig. 7) showed that, in HEK293 cells, the normalized luciferase activity of the haplotype allele was decreased by 53% and 50%, compared with that of the hap 2/3 and hap 4/5 alleles, respectively ($P = 5 \times 10^{-7}$ and 8×10^{-7}). This indicates that the G allele of the htSNP 1a-A–1012G has a transcription rate twofold lower than that of the A allele. The same results were observed in COS-7 cells (data not shown).

Surrounding sequence analyses showed that the 1e-C–2090T and 1e-G–1739A SNPs are located at potential Cdx-2-binding sites (fig. 8a). EMSA confirmed that the G allele of 1e-G–1739A and the T allele of 1e-C–2090T have a relatively decreased binding to Cdx-2, compared with their allelic counterparts (fig. 8b). The risk allele of the GATA and Cdx-2 promoter SNPs are contained in block 2–hap1, which is the risk allele for fracture.

Functionality Studies of 3'-UTR Polymorphisms

We found that, 24 h after transfection of reporter constructs containing the complete 3.2-kb 3' UTR of hap1 or hap2, the neomycin-normalized *VDR* mRNA level

Table 5

Incidence of Fractures in 6,148 Men and Women from the Rotterdam Study, by *VDR* Genotype Defined by Haplotype Allele Status in Blocks 2, 3, and 5

INDIVIDUAL LD BLOCK AND MEASURED VALUE	GENOTYPE GROUPS ^a		P
	Nonhomozygous	Homozygous	
Block 2–hap1:			
No. of cases/total subjects (%)	695/4,844 (14.3)	211/1,304 (16.2)	.06
Crude HR (95% CI)	1	1.15 (.97–1.35)	.06
Block 3–hap3:			
No. of cases/total subjects (%)	876/6,019 (14.6)	30/129 (23.3)	.006
Crude HR (95% CI)	1	1.74 (1.22–2.50)	.002
	Noncarrier	Carrier	
Block 5–hap1:			
No. of cases/total subjects (%)	256/1,988 (12.9)	650/4,160 (15.6)	.004
Crude HR (95% CI)	1	1.23 (1.07–1.42)	.004

NOTE.—Of 6,148 subjects overall, the total number of cases was 906 (14.7%). The values in bold italics represent significant associations.

^a “Nonhomozygous” includes the genotype groups without the risk allele and the heterozygotes. “Homozygous” is the genotype group homozygous for the risk allele. “Noncarrier” is the genotype group without the risk allele, and “Carrier” includes the genotype groups heterozygous and homozygous for the risk allele.

Table 6**Incidence of Fractures in 6,148 Men and Women from the Rotterdam Study, by Combined VDR Genotype**

MEASURED VALUE AND FRACTURE TYPE	TOTAL	NO. OF RISK GENOTYPES IN BLOCKS 2, 3, AND 5			TREND <i>P</i>
		0 ^a	1 ^b	2 or 3 ^c	
No. of cases/total subjects (%):					
Clinical fracture	906/6,148 (14.7)	207/1,586 (13.1)	516/3,560 (14.5)	183/1,002 (18.3)	.001
Vertebral fracture ^d	335/3,055 (11.0)	78/814 (9.6)	186/1,748 (10.6)	71/493 (14.4)	.01
Hip fracture	261/6,148 (4.2)	61/1,586 (3.8)	144/3,560 (4.0)	56/1,002 (5.6)	.05
Wrist fracture	257/6,148 (4.2)	58/1,586 (3.7)	145/3,560 (4.1)	54/1,002 (5.4)	.04
Crude HR (95% CI):					
Clinical fracture	...	1	1.14 (.97–1.33)	1.48 (1.21–1.80)	.0002
Vertebral fracture	...	1	1.12 (.85–1.48)	1.59 (1.13–2.24)	.01
Hip fracture	...	1	1.06 (.79–1.43)	1.49 (1.04–2.14)	.04
Wrist fracture	...	1	1.13 (.83–1.53)	1.53 (1.06–2.21)	.03

NOTE.—The values in bold italics represent significant associations.

^a “0” indicates the nonhomozygous genotype groups for blocks 2 and 3 (where “nonhomozygous” includes the genotype groups without the risk allele and the heterozygotes) and the noncarrier genotype groups (i.e., the groups without the risk allele) for block 5.

^b “1” indicates either the homozygous genotype group (i.e., the group homozygous for the risk allele) for block 2 or 3 or the carrier genotype group (i.e., the group with the risk allele) for block 5.

^c “2 or 3” indicates two or three of the risk genotype groups—the homozygous genotype groups for blocks 2 and 3 and the carrier genotype group for block 5.

^d Vertebral fracture was diagnosed by x-ray.

of the hap1 transcript was 15% lower than that of the hap2 transcript when results of all tested cell lines were combined ($P = 2 \times 10^{-6}$; $n = 53$), and similar patterns were observed in individual cell lines (fig. 9a and 9b). We then investigated the stability of VDR mRNAs transcribed from hap1 and hap2 and observed that the decay rate of VDR mRNA for hap1 was 30% faster than that for hap2 in the MG63 cell line 24 h after inhibiting transcription ($P = .02$; $n = 9$) (fig. 9c).

Discussion

A major limitation so far of association studies using VDR polymorphisms in relation to complex-disease end points has been the small number of analyzed polymorphisms and, thus, the lack of knowledge about the influence of and relation between other polymorphisms in the gene. In addition, the lack of statistical power of most studies to detect the expected subtle effects as well as the misconceptions about how such small biological effects could be translated into risk of disease have led to a number of controversies in the field. In the current study, we identified 62 polymorphisms in potentially functional areas of VDR, characterized the LD structure, and identified htSNPs. We here combined several studies and databases (Nejentsev et al. 2004; Hinds et al. 2005; Applied Biosystems SNPbrowser Software Web site; HapMap Data Web site; Perlegen Genome Browser Web site) to conclude that there are seven haplotype blocks across the VDR gene in whites. We used 15 tagSNPs to represent the common haplotypes for five of these blocks in potentially functional areas of VDR.

In this study, we also constructed an LD map and identified htSNPs of VDR for three major ethnic populations. There are several further approaches to identify the (set of) responsible functional SNPs in the LD areas where we identified risk haplotype alleles. Since African Americans have, in general, smaller LD blocks, replication of the associations in African Americans could focus on a smaller region with concomitantly fewer SNPs to test functionally. Subsequently, the functionality test we describe here (see figs. 7 and 9) can be repeated, but with constructs of promoter and 3'-UTR variants that differ at only one position.

A large association study with the htSNPs in a population-based cohort (6,148 elderly subjects) identified haplotype alleles in three LD blocks that confer risk for fracture, and we present evidence of intragenic additive effects. These relationships were found in both men and women and for different types of fractures (including vertebral, hip, and wrist fractures) and were independent of age, height, weight, BMD, and BMD change. In our study population (which had a 15% prevalence of fracture among 6,148 subjects), we have 80% statistical power to detect a 25% increased fracture risk for a polymorphism and/or haplotype with a frequency >10%. We analyzed haplotypes instead of individual SNPs, which limited the number of tests. For blocks 2 and 5, we did not adjust the significant *P* value by the conservative Bonferroni correction for multiple comparisons because, in our previous studies (Uitterlinden et al. 2001; Fang et al. 2003), we observed two fracture-risk alleles in these blocks in a subset of our current study, and we confirmed the association in this study—

Table 7

PAR of Independent Risk Factors for Clinical Fracture in 6,148 Men and Women from the Rotterdam Study

Risk Factor	Frequency at Baseline (%)	HR (95% CI) ^a	PAR in % (95% CI) ^a
Age >75 years	32	2.3 (2.1–2.6)	29 (25–34)
T score <–2.5	16	2.7 (2.3–3.1)	21 (17–25)
Current smoking	23	1.3 (1.1–1.5)	6 (2–10)
Use of walking aid	12	1.3 (1.1–1.6)	3 (1–4)
Genetic markers:			
COL1A1 Sp1 T-allele homozygote	3	1.4 (1.0–1.9)	1 (0–3)
VDR risk genotype:			
Block 2–hap1	21	1.1 (.9–1.3)	2 (0–5)
Block 3–hap3	2	1.6 (1.2–2.4)	1 (0–3)
Block 5–hap1	68	1.2 (1.1–1.4)	12 (4–21)
Carrier of 2 or 3 VDR risk genotypes	16	1.3 (1.1–1.5)	4 (2–6)

^a All HRs and PARs were adjusted for age and sex.

but this time for the haplotype alleles encompassing the previously observed risk alleles. However, we used $P = .005$ (.05/11) on the basis of a Bonferroni correction for analyses in the other three blocks and the *FokI* RFLP; only the association between block 3–hap3 and fracture risk remains borderline significant ($P = .002$). Thus, we cannot reject the possibility that some of the associations we observed are false positives, even in this very large population.

According to our association analysis, the genetic effect of the VDR polymorphisms on fracture risk is modest (15%–48%), which corresponds to the modest difference (15%) in the VDR mRNA level by genotype in our functionality analysis. In an earlier study (Mann et al. 2001), the “Ss” (or GT) genotype of *COL1A1* Sp1 was found to have a 21% increased *COL1A1*:*COL1A2* protein ratio, as measured in osteoblasts, compared with that of the “SS” (or GG) genotype. Another functional study of a 3′-UTR SNP (+1073C/T in the oxidized LDL receptor gene [*OLR1*]) that is associated with increased risk for Alzheimer disease demonstrated that C-allele carriers had a 41% decreased *OLR1* mRNA level compared with that of TT homozygotes (Lambert et al. 2003). These examples indicate that genetic effects of polymorphisms on gene expression and clinical phenotypes are modest.

Elsewhere, we reported that haplotype 1 of the 3′-end variants, defined by only *BsmI*-*ApaI*-*TaqI* RFLPs, was associated with increased fracture risk in 1,004 postmenopausal women (Uitterlinden et al. 2001), which is a subgroup of the current study population. We here confirm this association between *BsmI*-*ApaI*-*TaqI* haplotype 1 and fracture risk in the complete study population ($P = .03$; data not shown). Yet, in our more detailed haplotype analysis based on more SNPs, we defined a subtype of the *BsmI*-*ApaI*-*TaqI* haplotype 1—that is, of block 5–hap1u (40.2%) (fig. 1d)—that shows

a stronger and more significant association with fracture risk. These subtle differences in the exact definition of the risk allele could contribute to heterogeneity in association results observed in different studies. Although we focused here on the more common haplotype alleles in the population, we cannot exclude the idea that (several) less-frequent risk alleles may contribute to the VDR genotype-dependent fracture risk. Further studies will be necessary to assess their contribution. We show that effects are modest (~20%–70% increased risk), as can be expected for common variants in relation to complex disease. The VDR risk haplotype alleles therefore have a modest influence on individual risk of fracture but make a substantial contribution at the population level (PAR = 4%–12%) in comparison with other genetic markers we previously identified in this population, such as *COL1A1* Sp1 (Uitterlinden et al. 1998b) and *ESR1* *PvuII* and *XbaI* polymorphisms (van Meurs et al. 2003). The most prominent genetic effect on fracture risk, according to the PAR analyses, is the block 5–hap1 risk allele. Many previous conflicting association studies of VDR and BMD as well as fracture usually analyzed (very) small study populations and used the *BsmI*, *ApaI*, or *TaqI* polymorphisms in this block but mostly analyzed them separately. We therefore suppose that the controversy can partly be explained by a lack of statistical power as a result of small sample size and failure to use haplotypes. In addition, population stratification, such as mixed ethnic groups with different allele frequencies; population-specific differences in some environmental factors, such as (dietary) calcium intake, (dietary) vitamin D intake, and sunlight exposure; and other characteristics of the study population pertinent to bone metabolism and fracture risk could result in heterogeneity of association observed across different study populations. On the basis of a meta-analysis of published data on the relationship between

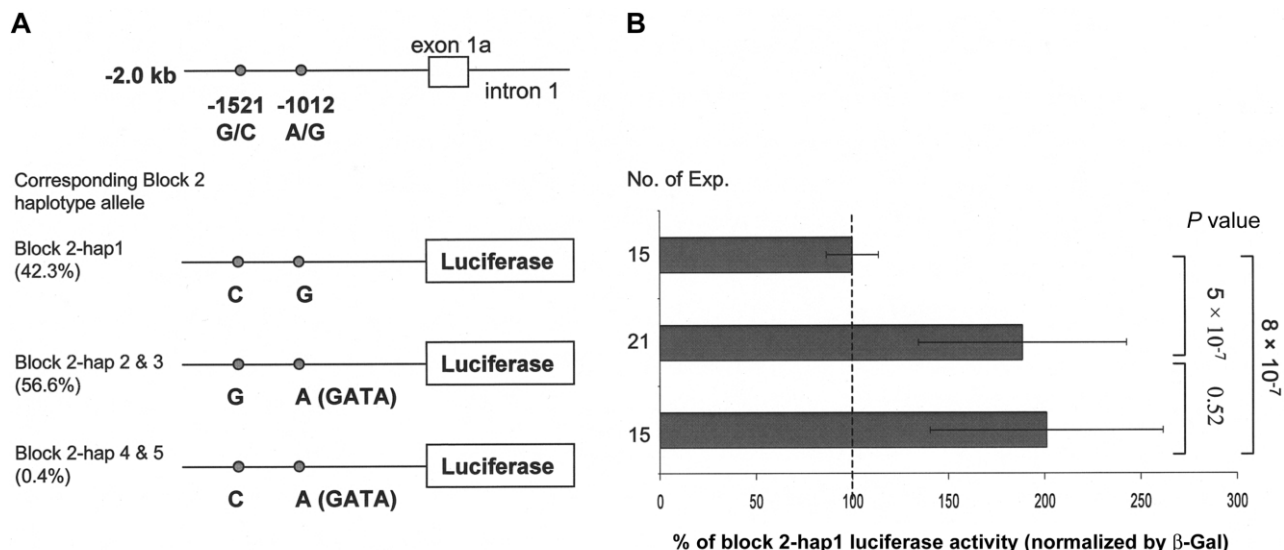


Figure 7 Relative luciferase activity in HEK293 cells of VDR exon 1a promoter sequences, including two SNPs. *a*, The three constructs containing the 2-kb 1a promoter sequence with SNPs 1a-G-1521C and 1a-A-1012G. *b*, β-galactosidase (beta-Gal)-normalized luciferase activity for the three constructs. The block 2-hap1 allele is set at 100% to be the reference group; P values were calculated by independent t test.

the VDR *BsmI* RFLP and fracture risk, we have some evidence that this could be an important factor (Y. Fang, F. Rivadeneira, J. Ioannidis, and A. Uitterlinden, unpublished data). We have data on serum vitamin D and on dietary calcium intake in a subset of our study population ($n = 1,312$ and $4,747$, respectively), but we do not have reliable dietary vitamin D intake data. We note that our study population has a very high dietary calcium intake (1,120 mg/d). For the VDR-fracture relationship, we repeated the analyses but stratified them by the median, tertiles, and quartiles of vitamin D level or dietary calcium intake. However, the association did not differ between these strata, and no interaction was observed (data not shown).

By analyzing functionality of the risk alleles in vitro, we demonstrate that the molecular mechanisms underlying these associations are likely to involve a lower expression of VDR mRNA. Some of the promoter polymorphisms result in altered transcription-factor binding for Cdx-2 and GATA. The previously reported Cdx-2 site at 1e-G-1739A (Yamamoto et al. 1999; Fang et

al. 2001) and the GATA site at 1a-A-1012G (Halsall et al. 2005) are encompassed in the block 2-hap1 risk allele. Our functionality experiment and previous ones (Arai et al. 2001) show that these two weak-binding alleles together result in decreased transcription activity of this VDR promoter. Further research is needed to establish, in more detail, in which cells and/or tissues this promoter part is influencing VDR expression. Although GATA is expressed in many tissues, Cdx-2 is expressed predominantly in the intestines. Thus, the hap1 allele might cause relatively lower VDR expression in target cells for vitamin D, including the intestines.

The 3' UTR of genes is known to be involved in regulation of gene expression, especially through regulation of mRNA stability. The *BsmI*, *ApaI*, and *TaqI* SNPs are anonymous, and block 5 does not include polymorphisms beyond the 3' UTR of VDR. Therefore, SNPs in the 3' UTR are the most likely candidates for the truly functional sequence variations that may explain the associations we observed. We identified differences in VDR mRNA expression level and stability between the hap1 and hap2 alleles, which differ at only five positions across the 3.2-kb 3' UTR. The fracture-risk allele hap1 causes 15% lower levels of mRNA expression than does hap2, in all tested cell lines. This is in line with the 30% faster decay of or lower stability of VDR mRNA we observed in MG63, an osteoblast cell line. This observation also corresponds to other studies performed in vivo and in vitro (Morrison et al. 1992, 1994; Carling et al. 1997; Ogunkolade et al.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 8 Two SNPs in the 1e promoter region of the human VDR gene, located at a Cdx-2 binding site. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

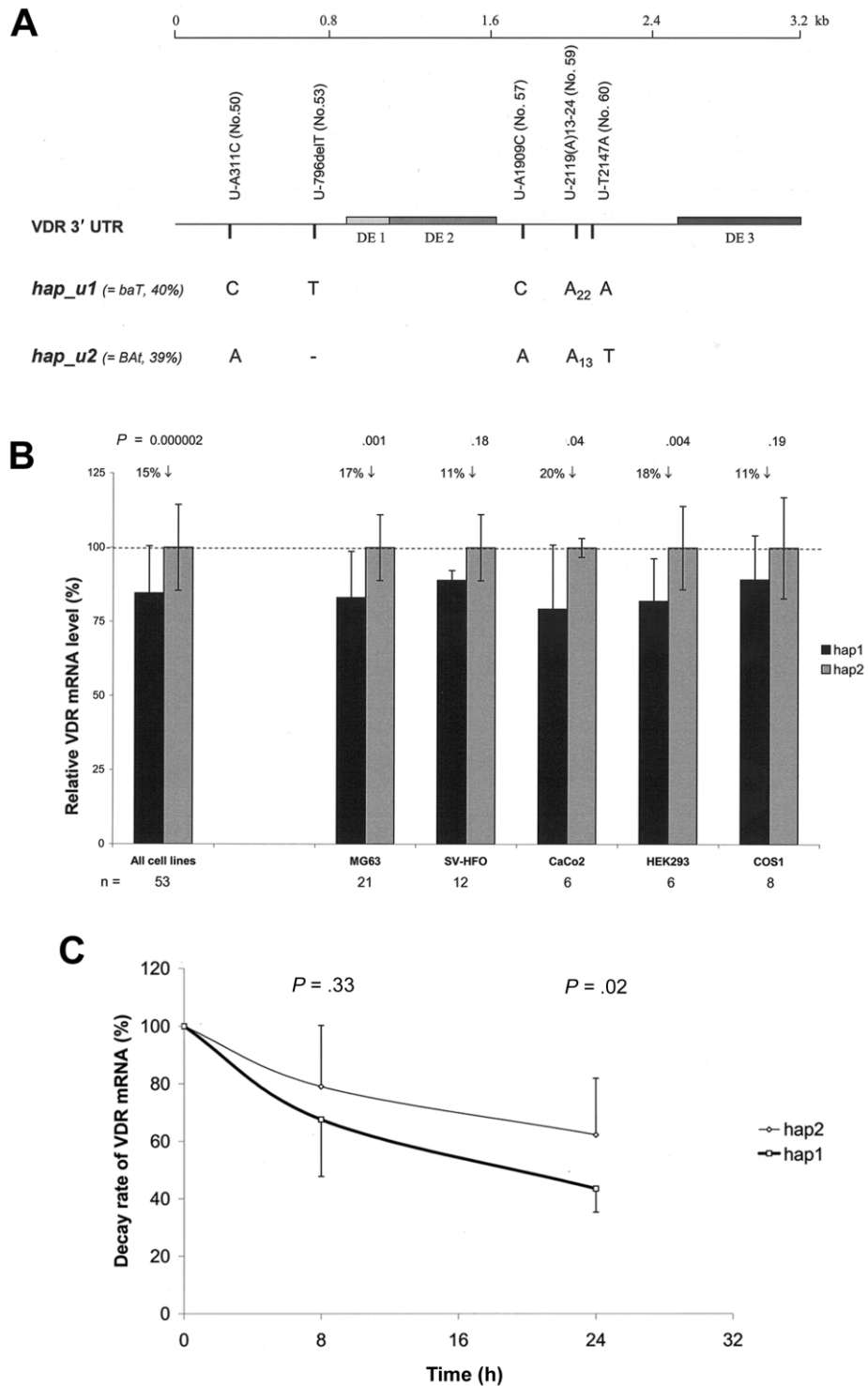


Figure 9 VDR mRNA expression level and stability analysis by 3'-UTR haplotypes in different cell lines. *a*, VDR 3'-UTR with sequence variations that distinguish hap1 (corresponding to block 5-hap1) from hap2 (corresponding to block 5-hap2). The SNP variation number (*in parentheses*) refers to those given in table 3. *b*, Neomycin-normalized VDR mRNA expression levels (mean \pm SD) for VDR 3'-UTR hap1 versus hap2. The level of hap2 is set at 100% to be the reference group; *P* values were calculated by independent *t* test; *n* = number of experiments for each cell line. *c*, Decay rate of VDR mRNA by hap1 versus hap2, determined in the MG63 cell line. Time point 0 h is defined as 100% mRNA level for both haplotypes; *P* values for each time point were calculated by independent *t* test.

2002). This is likely to also result in lower numbers of VDR protein being present in target cells for vitamin D, giving such target cells a decreased response to vitamin D.

VDR as a transcription factor influences the expression of downstream genes, such as *TRPV5*, *TRPV6*, and *Calbindin*, which are involved in calcium absorption and could therefore impact BMD and thus fracture risk. However, we found that the association between VDR genotype and fracture risk was independent of BMD, which suggests that other mechanisms (such as bone microarchitecture, bone quality, and bone strength) determine fracture risk. Alternatively, we can hypothesize that the bone tissue of the subjects carrying a risk allele may have a somewhat lower sensitivity to vitamin D, since the expression of VDR is lower. Therefore, the osteoblast activity could be lower, and the bone formation rate (in the bone-remodeling balance) could be decreased. The age-related expansion of the outer diameter of long bone is associated with a marked increment of bone strength. We have observed recently that the fracture-risk haplotype alleles were also associated with decreased bone size (Y. Fang, J. van Meurs, F. Rivadeneira, N. van Schoor, J. van Leeuwen, P. Lips, H. Pols, and A. Uitterlinden, unpublished data) in the same population. This genotype-related bone geometry difference reflects that the bone gain resulting in outer bone diameter expansion is smaller than the bone loss resulting in inner bone diameter expansion. This bone geometry difference leads to a decrease in bone strength for the risk-allele carriers, possibly increasing the fracture risk.

We demonstrate that polymorphisms within the promoter area and the 3'-UTR area of a gene have effects that can influence VDR gene function in certain cells and/or subjects. Thus, the 5'-promoter and 3'-UTR polymorphisms together can determine how much of a given VDR mRNA level will be expressed in a given target cell. The combined risk genotypes in the promoter region and 3' UTR represent a moderate genetic effect of the entire VDR gene on fracture risk. The vitamin D endocrine system has been implicated in several other complex diseases, including osteoarthritis, diabetes, and cancer. Whether our findings have relevance for these other diseases needs to be tested in separate association studies using the LD and haplotype information we provide here.

In conclusion, we systematically scanned sequence variations across VDR, identified LD structure and htSNPs for different ethnic populations, and demonstrated that polymorphisms in the 5' promoter region and the 3' UTR of VDR contribute to fracture risk in a large population. Functionality experiments in the 5' promoter region and the 3' UTR support and provide

a possible molecular explanation for the association with fracture risk that we observed.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Applied Biosystems SNPbrowser Software, http://events-na.appliedbiosystems.com/mk/get/snpb_landing?isource=fr_E_RD_www_allsnps_com_snpbrowser
 dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/> (for detected polymorphisms [accession numbers ss32465817–ss32465820 and ss32465633–ss32465690])
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/index.html> (for reference sequences [accession numbers BV210413–BV210452 and AY827085–AY827088])
 Genomatix, http://www.genomatix.de/cgi-bin/.matinspector_prof/mat_fam.pl
 GOLD, <http://www.sph.umich.edu/csg/abecasis/GOLD>
 HaploBlockFinder V0.7, <http://cgi.uc.edu/cgi-bin/kzhang/haploBlockFinder.cgi>
 Haploxt, <http://archimedes.well.ox.ac.uk/>
 HapMap Data, <http://www.hapmap.org/cgi-perl/gbrowse/gbrowse/hapmap>
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for VDR)
 Perlegen Genotype Browser, <http://genome.perlegen.com/browser/index.html>
 Phase, <https://depts.washington.edu/ventures/clickthru/ReleaseAgreement.php?raf=PHASEV2>
 TFSEARCH: Searching Transcription Factor Binding Sites, <http://www.cbrc.jp/research/db/TFSEARCH.html>
 Vista Tools, <http://www.gsd.lbl.gov/VISTA/index.shtml>

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