Prevalence and functional significance of 25-hydroxyvitamin D deficiency and vitamin D receptor gene polymorphisms in Asian Indians[1–4]

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ABSTRACT

Background: Recent studies show a wide prevalence of hypovitaminosis D in Asian Indians.

Objective: The objective was to assess the functional significance of 25-hydroxyvitamin D [25(OH)D] deficiency, vitamin D receptor (*VDR*) gene, and parathyroid hormone (*PTH*) gene polymorphisms in relation to bone mineral density (BMD) in urban Asian Indians.

Design: Serum total calcium, inorganic phosphorus, alkaline phosphatase, 25(OH)D, intact PTH, and BMD at lumbar spine, proximal femur, and forearm were measured in 105 adult subjects. The genotyping related to *VDR* (*Bsm*I, *Fok*I, and *Taq*I) and *PTH* (*Bst*BI and *Dra*II) gene single-nucleotide polymorphisms was carried out by polymerase chain reaction–restriction fragment length polymorphism analysis.

Results: The mean serum 25(OH)D concentration in the whole cohort was 9.8 ± 6.0 ng/mL, which was inversely related with serum intact PTH values ($P = 0.042$). Ninety-nine (94.3%) of the 105 subjects had vitamin D deficiency with 25(OH)D concentrations < 20 ng/mL. The age- and body mass index (BMI)–adjusted BMD value at the hip was higher in subjects with serum 25(OH)D values > 9.0 ng/mL than in those with values ≤ 9.0 ng/mL (0.893 ± 0.114 compared with 0.839 ± 0.112 g/cm^2, respectively; $P = 0.001$). The mean forearm and spine BMD values in subjects with TT (*VDR*, *Taq*I) or bb (*PTH*, *Bst*BI) genotypes were significantly higher than the values in subjects with Tt genotype and BB or Bb genotype, respectively.

Conclusion: Functionally significant 25(OH)D deficiency affecting BMD at the hip region is prevalent in urban Asian Indians. However, variation in BMD at the spine and forearm is related to *VDR* and *PTH* gene polymorphisms rather than to vitamin D status, at least in this hypovitaminotic D population. *Am J Clin Nutr* 2006;83:1411–9.

KEY WORDS Hypovitaminosis D, *VDR*, *PTH*, polymorphism, Asian Indians

INTRODUCTION

Serum 25-hydroxyvitamin D [25(OH)D] concentrations, body mass index (BMI; in kg/m^2), sex, age, and genetic factors are the important determinants of bone mineral density (BMD; 1–4). Studies conducted as early as the 1960s by Ahuja (5) and Gupta et al (6) reported up to 44% prevalence of osteoporosis and early occurrence of related fractures in Asian Indians. We reported wide prevalence of vitamin D deficiency and its significance in terms of secondary hyperparathyroidism in apparently healthy subjects residing in Delhi (7). Subnormal serum 25(OH)D concentrations could be related to poor sunshine exposure and skin pigmentation. BMD is higher in Indian patients with sporadic idiopathic hypoparathyroidism, which indicates the beneficial effect of low concentrations of serum parathyroid hormone (PTH) on BMD in these patients (8). Recent reports show a wide presence of hypovitaminosis D from other parts of northern India as well as southern India (9, 10). Only 2 studies were done in which the relation between BMD and serum 25(OH)D was assessed in Indians (11, 12). Arya et al (11) observed a correlation between serum 25(OH)D concentrations and BMD in a cohort of 92 healthy subjects from northern India. However, in healthy north Indian paramilitary forces, no correlation between serum 25(OH)D and BMD could be observed (12).

Among genetic factors that influence BMD, vitamin D receptor gene (*VDR*) is the most widely studied (13, 14). Morrison et al (15) first reported an association between BMD and *VDR* gene *Bsm*I single-nucleotide polymorphism (SNP). Subsequently, 3 other *VDR* gene SNPs (*Apa*I, *Taq*I, and *Fok*I) were associated with BMD changes in several ethnic groups (16–18). A few studies also assessed *PTH* gene SNPs in relation to BMD (19, 20). No study to date has assessed the relation between BMD and *VDR* and *PTH* gene SNPs in Asian Indians.

We assessed the relation between serum 25(OH)D concentrations and BMD in a cohort of 105 apparently healthy Asian Indian subjects after adjustment of the variation related to their age, sex, and BMI. We also analyzed the relation between BMD and various genotypes related to *VDR* gene polymorphisms and 2 *PTH* gene SNPs, ie, *Bst*BI and *Dra*II.

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[2] Presented at the 35th annual meeting of the Endocrine Society of India, New Delhi, India, November 2005.

[3] Supported by the All India Institute of Medical Sciences and by grant no. NI-696 from the Vulimiri Ramalingaswami Foundation (to RG and NG).

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Received January 11, 2006.

Accepted for publication February 28, 2006.

SUBJECTS AND METHODS

Subjects

Study subjects included 105 healthy persons (51 men and 54 women; $\bar{x}\pm$ SD age: 43.3 ± 9.7 y; BMI: 24.6 ± 3.6). The BMD was measured in all subjects at the All India Institute of Medical Sciences under the Indian Council of Medical Research task force study to establish normative data of BMD in Asian Indians. These subjects were recruited after randomization of a middle-income population staying in a government accommodation located at a central part of New Delhi (latitude 28.35 °N and longitude 77.12 °E; Meteorology Department, Delhi, India). Most of the male subjects included in the current study worked indoors as office staff, and the women were housewives. The study subjects were recruited after a careful history, physical examination, and normal baseline investigations, which included hemogram and renal and liver function tests. Subjects taking drugs that can affect bone mineral metabolism, such as glucocorticoids, antitubercular therapy, antiepileptics, levothyroxine, and hormone replacement therapy, and those with diabetes mellitus were not enrolled in the current study.

BMD was measured in all the subjects by using dual-energy X-ray absorptiometry (QDR 4500 Acclaim Series; Hologic Inc, Waltham, MA) at the lumbar spine (L1–L4 anteroposterior), left proximal femur, and left forearm regions. The CV of total BMD at various regions studied was 1%. The BMD values were also analyzed separately for neck, trochanter, intertrochanter, and Ward's triangle; the femur; and ultradistal, middle, and proximal one-third of the forearm. The BMD of the subjects was recorded in terms of absolute mineral content (in g/cm^2) at various sites. The *t* and *z* score analyses were not used in the current study because BMD data for a large population are yet not available for Asian Indians. Dietary calcium intake was assessed in all the subjects by a trained dietitian using a standard food-frequency questionnaire and published data on the mineral composition of Indian food (7, 21). Dietary products, including dairy products and food fats, are not fortified with vitamin D in India, and fish is a rare diet item.

A blood sample was drawn from the study subjects after an overnight fast without venostasis. The serum was separated in a refrigerated centrifuge at 2500 × *g* for 5 min at 4 °C and stored at −20 °C in multiple aliquots to measure concentrations of serum total calcium, inorganic phosphorus, alkaline phosphatase, intact PTH (iPTH), and 25(OH)D. Serum total calcium, inorganic phosphorus, and alkaline phosphatase were measured in duplicate with the use of commercial kits (Roche, Mannheim, Germany) on a semiautomated analyzer (Hitachi Photometer 4020; Boehringer, Mannheim, Germany). Intraassay and interassay CVs for serum calcium, phosphorus, and alkaline phosphatase assays were 3.6%, 3.8%, and 3.5% and 4.8%, 5.2%, and 4.1%, respectively, in our laboratory. Serum iPTH was measured with the use of an immunoradiometric assay (Diasorin, Stillwater, MN; normal range: 13–54 pg/mL; intraassay and interassay CVs: 4% and 8%, respectively). Serum 25(OH)D concentrations were estimated by using a radioimmunoassay (Diasorin; normal range: 9–37.6 ng/mL). Subjects were classified as vitamin D–deficient, –insufficient, and –sufficient on the basis of serum 25(OH)D concentrations of <20 ng/mL, 20 to 32 ng/mL, and >32 ng/mL, respectively, according to the recent consensus (22–24).

In 94 of the 105 subjects, *VDR* and *PTH* gene SNPs were analyzed by using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. Genomic DNA was extracted from the peripheral leukocytes in 10 mL venous blood and collected in a Falcon tube containing 400 μL 3% EDTA by the standard phenol-chloroform extraction procedure (25, 26).

Written informed consent was obtained from all study subjects. The Institutional Ethics Committee of the All India Institute of Medical Sciences approved the study protocol, including BMD assessment, biochemical estimations, and *VDR* and *PTH* gene SNP analysis in the study subjects.

Genotyping for the *VDR* SNPs (*Bsm*I, *Fok*I, and *Taq*I)

The *VDR* genotype at *Bsm*I, *Taq*I, and *FokI* SNP sites were assessed by using PCR-RFLP analysis as described below. We used an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA) for PCR amplification. The polyacrylamide gel electrophoresis (PAGE)–purified primers (Bio Basic Inc, East Markham, Canada), DNA *Taq* polymerase, deoxynucleoside triphosphates, and various restriction endonucleases (Fermantas Inc, Hanover, MD) used in the study were commercially procured.

BsmI polymorphism

The forward (5′-caaccaagactacaagtaccgcgtcagtg-3′) and reverse (5′-aaccagcgggaagaggtcaaggg-3′) primers used to genotype the *Bsm*I SNP were based on the report of Vandevyver et al (27). The conditions for PCR amplification were as follows: 200 ng genomic DNA, 100 pmol of each primer, 200 μmol each deoxynucleoside triphosphate/L, 2.5 mmol $MgCl_2$/L, 5 mmol Tris-HCl/L (pH, 8.0), 10 mmol NaCl/L, 10 μmol EDTA/L, 0.5 mmol dithiothreitol/L, 5% glycerol, 0.1% TritonX-100, and 1.25 U *Taq* DNA polymerase were constituted in a 25-μL reaction. The initial denaturation was performed at 94 °C for 3 min, followed by 35 cycles at 94 °C for 20 s, 62 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 6 min. The size of the amplicon produced after PCR was 822 base pairs (bp). After amplification, a 5-μL aliquot of PCR product was incubated at 37 °C with 4 U *Bsm*I restriction enzyme for 12 h. The digested products were electrophoresed on ethidium-stained 1.2% agarose gel in 0.5% Tris-borate/EDTA buffer for 1 h and visualized by using a ultraviolet transilluminator (**Figure 1**A).

The SNP leading to A→G substitution in intron 8 of the *VDR* gene leads to the generation of a *Bsm*I restriction site. Homozygous subjects with alleles containing nucleotide A at this position showed an intact 822-bp band and were designated as having BB *Bsm* genotype (GeneBank accession no. AC004466.1). Homozygous subjects with alleles containing G at this position showed 2 bands of 650 bp and 172 bp and were designated as bb. Subjects with heterozygote status at this position showed all 3 bands—ie, 822, 650, and 172 bp—and were designated as Bb (27).

FokI polymorphism

The forward (5′-agctggccctggcactgactctgctct-3′) and reverse (5′-atggaaacaccttgcttcttctccctc-3′) primers used to genotype *Fok*I were based on the report of Gross et al (28) and resulted in a 265-bp product. The constituents used for PCR were essentially

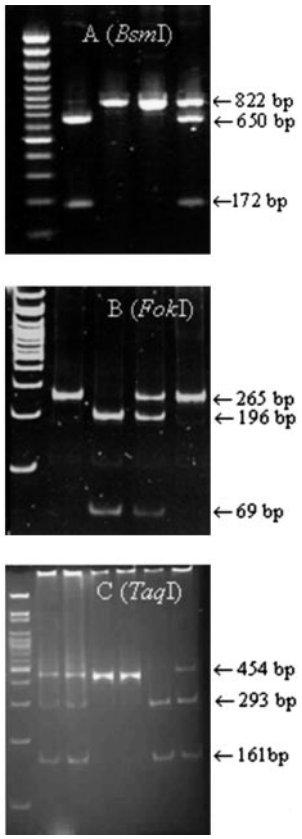

FIGURE 1. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of 3 *VDR* single-nucleotide polymorphisms (SNPs). (A) *Bsm*I, 1.2% agarose gel electrophoresis after digestion of 5 μL PCR products with 4 U *Bsm*I restriction enzyme at 37 °C for 12 h. Homozygous (BB) subjects showing an undigested 822-base pair (bp) product (lanes 3 and 4); heterozygous (Bb) subject showing 822-, 650-, and 172-bp fragments (lane 5); homozygous (bb) subject showing 650- and 172-bp fragments (lane 2). (B) *Fok*I, a 10% PAGE showing RFLP obtained after digestion of 4 μL PCR product with 4 U *Fok*I restriction enzyme at 55 °C for 6 h. Homozygous (FF) subjects showing an undigested 265-bp product (lanes 2 and 5); heterozygous (Ff) subject showing 265-, 196-, and 69-bp fragments (lane 4); homozygous (ff) subject showing 196- and 69-bp fragments (lane 3). (C) *Taq*I, a 10% PAGE showing RFLP obtained after 1-h digestion of 20 μL PCR product with 10 U *Taq*I restriction enzyme at 65 °C. Homozygous (TT) subjects showing an undigested 454-bp product (lanes 4 and 5); heterozygous (Tt) subjects showing 454-, 293-, and 161-bp fragments (lanes 2, 3, and 7); homozygous (tt) subject showing 293- and 161-bp fragments (lane 6). A 100-bp DNA ladder was run in lane 1 in all the gels.

the same as that described for the *Bsm*I genotyping. The conditions for PCR amplification were as follows: initial denaturation at 94 °C for 10 min followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min (29). A 4-μL amplified PCR product was digested with 4 U *Fok*I restriction enzyme at 55 °C for 6 h. The digested products were resolved by using 10% PAGE.

The SNP leading to the T→C substitution at the second nucleotide of exon 2 of the *VDR* gene resulted in the generation of a *Fok*I restriction site. Homozygous subjects with alleles containing nucleotide T at this position showed an intact 265-bp band and were labeled as FF. Homozygous subjects with alleles containing nucleotide C at this position showed 2 bands of 196 bp and 69 bp and were designated as ff. Subjects with heterozygote status at this position showed bands of 265, 196, and 69 bp and were designated as Ff (Figure 1B).

Taq*I polymorphism*

The forward (5′-cagagcatggacagggagc-3′) and reverse (5′-aggagaggcagcggtactg-3′) primers used to genotype the TaqI SNP were based on the report of Chang et al (30). The conditions for PCR (25 μL reaction mix) amplification were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The size of the amplicon produced after PCR was 454 bp. A 20-μL aliquot of PCR product was digested with 10 U *Taq*I restriction enzyme at 65 °C for 1 h. The digested products were resolved by 10% PAGE for *Taq*I restriction analysis.

The SNP leading to the T→C substitution at nucleotide position +1171 in exon 9 of the *VDR* gene will lead to generation of a *Taq*I restriction site. Subjects with alleles containing nucleotide T at this position showed an intact 454 bp and were designated as TT. Subjects with alleles containing nucleotide C at this position showed 2 bands of 293 bp and 161 bp and were designated as tt. Subjects with heterozygous status at this position showed all 3 bands—ie, 454, 293, and 161 bp—and were designated as Tt (Figure 1C).

PTH gene polymorphisms (*Bst*BI and *Dra*II)

The primers used for studying the *PTH* gene *Bst*BI and *Dra*II SNPs were based on the report of Sunthornthepvarakul et al (31). The conditions for PCR amplifications and restriction analysis were essentially the same as described in detail in our earlier report (23). The SNP leading to the G→A substitution in intron 2 of the *PTH* gene results in loss of the restriction site for endonuclease *Bst*BI. Homozygous subjects with alleles containing nucleotide G at this position showed 383- and 226-bp fragments and were designated as BB. Homozygous subjects with alleles containing nucleotide A at this position showed a 609-bp fragment and were designated as bb. Subjects with heterozygous status at this position showed 3 bands of 609, 383, and 226 bp and were designated as Bb.

The SNP leading to the C→A substitution at codon 52 (CGA) in exon 3 of the *PTH* gene leads to loss of restriction site for endonuclease *Dra*II. Homozygous subjects with alleles containing nucleotide C at this position showed 434-bp and 175-bp fragments and were designated as DD. Homozygous subjects with alleles containing nucleotide A at this position showed a 609-bp fragment and were designated as dd. Subjects with heterozygous status at this position showed 3 bands of 609, 434, and 175 bp and were designated as Dd.

Statistical analysis

The data are presented as means ± SDs. Statistical analysis was performed by using SPSS statistical software (version 10.0; SPSS Inc, Chicago, IL). Student's *t* test was used to assess the significance of differences in mean values of age, BMI, daily intake of dietary calcium, and concentrations of serum total calcium, inorganic phosphorus, alkaline phosphatase, and iPTH in the 2 groups of subjects with the use of a serum 25(OH)D concentration cutoff of 9.0 ng/mL (the lowest "normal" value of the kit used for the estimation of 25(OH)D; group 1, ≤9.0 ng/mL; group 2, >9.0 ng/mL). For assessing the difference in the mean BMD values between these 2 groups of subjects, age- and BMI-adjusted BMD values were used. However, for analyzing the forearm BMD, adjustment for sex was also done. The Pearson

TABLE 1
Clinical characteristics, biochemical indicators, and bone mineral density (BMD) in subjects with different serum 25-hydroxyvitamin D [25(OH)D] concentrations[1]

	25(OH)D ≤ 9 ng/mL (n = 64)	25(OH)D > 9 ng/mL (n = 41)	P[2]
Age (y)	42.9 ± 9.4[3]	44 ± 10.2	0.54
M:F	27:37	24:17	0.44
BMI (kg/m^2)	25.1 ± 3.8	23.9 ± 3.1	0.10
Serum calcium (mg/dL)	9.4 ± 0.7	9.6 ± 0.4	0.095
Serum inorganic phosphorus (mg/dL)	4.1 ± 0.6	4.1 ± 0.5	0.99
Serum alkaline phosphatase (IU/L)	228 ± 68	217 ± 64	0.41
Serum iPTH (pg/mL)	53.1 ± 31.3	39.3 ± 17.5	0.012
Serum 25(OH)D (ng/mL)	6.3 ± 1.2	15.3 ± 6.3	< 0.001
Daily intake of dietary calcium (mg)	1277 ± 433	1275 ± 465	0.99
BMD			
L1–L4 AP spine (g/cm^2)	0.930 ± 0.132	0.954 ± 0.119	0.12
Femoral neck (g/cm^2)	0.727 ± 0.109	0.779 ± 0.105	0.002
Trochanter (g/cm^2)	0.609 ± 0.084	0.666 ± 0.101	< 0.001
Intertrochanter (g/cm^2)	1.006 ± 0.141	1.072 ± 0.142	0.003
Total hip (g/cm^2)	0.839 ± 0.112	0.893 ± 0.114	0.001
Ward's triangle (g/cm^2)	0.583 ± 0.141	0.592 ± 0.296	0.59
Ultradistal forearm (g/cm^2)	0.391 ± 0.064	0.402 ± 0.062	0.36
Mid forearm (g/cm^2)	0.544 ± 0.058	0.565 ± 0.063	0.119
Proximal forearm (g/cm^2)	0.632 ± 0.064	0.652 ± 0.075	0.37
Total forearm (g/cm^2)	0.525 ± 0.058	0.541 ± 0.062	0.20

[1] iPTH, intact parathyroid hormone; AP, anteroposterior.
[2] P for BMDs are age- and BMI-adjusted for the spine and hip and age-, BMI-, and sex-adjusted for the forearm.
[3] $\bar{x}$ ± SD (all such values).

correlation test was used to assess the relation among various indicators and age- and BMI-adjusted BMD values. The difference in the mean value of the various biochemical and BMD indicators in subjects with 3 different genotype groups related to *VDR* and *PTH* gene polymorphisms was analyzed by using an analysis of variance (ANOVA) followed by Scheffe's test for posthoc analysis if the ANOVA was significant. A P value < 0.05 was considered significant. Genotype distribution of the 3 *VDR* SNPs and 2 *PTH* gene SNPs in the study population was assessed for Hardy-Weinberg equilibrium by using the chi-square test. The linkage disequilibrium among alleles of the 3 *VDR* SNPs was analyzed by using the software program SHEsis (http://202.120.7.14/analysis/myAnalysis.php; 32). Fisher's exact test was used to assess the significance of difference in the frequency of various haplotypes related to the 3 *VDR* gene SNPs in subjects with different BMD values in the SHEsis program.

RESULTS

The BMI values among the study subjects showed normal distribution with no skewing at either end. The mean daily intake of dietary calcium of the group was 1277 ± 444 mg, which was normal according to the Indian normative data published by the Indian Council of Medical Research (33). The dietary vitamin D content was not estimated for the study subjects because no published data are available for vitamin D content of the dietary items consumed in India.

The mean serum 25(OH)D concentrations observed in the current cohort of 105 study subjects was 9.8 ± 6.0 ng/mL; this value did not differ significantly between men and women in the current study (10.8 ± 6.8 and 8.8 ± 4.9 ng/mL, respectively; P = 0.09). A significant inverse relation was observed between serum 25(OH)D and iPTH values (r = −0.20, P = 0.042). Of the subjects studied, 94.3% had serum 25(OH) concentrations suggestive of vitamin D deficiency (<20 ng/mL) and 4.8% had vitamin D insufficiency (20–32 ng/mL). Only 1 subject had sufficient vitamin D nutritional status (>32 ng/mL).

To study the relation between the vitamin D nutritional status and BMD, the cohort of 105 subjects was split into 2 groups at a serum 25(OH)D cutoff of 9.0 ng/mL (group 1: ≤9.0 ng/mL; group 2: > 9.0 ng/mL). The mean value of serum 25(OH)D in the 64 subjects included in group 1 (6.3 ± 1.2 ng/mL) was significantly (P < 0.001) less than that in the 41 subjects in group 2 (15.3 ± 6.3 ng/mL). The mean serum iPTH was significantly higher in the group 1 than in the group 2 subjects (53.1 ± 31.3 and 39.3 ± 17.5 pg/mL, respectively; P = 0.012). The 2 groups were comparable in terms of mean age, male-to-female ratio, BMI, daily intake of dietary calcium, and concentrations of serum total calcium, phosphorus, and alkaline phosphatase (**Table 1**). The differences in the BMD values between the 2 groups of subjects with different serum 25(OH)D concentrations were measured after adjustment for age and BMI. This was considered necessary because the BMD values at hip, spine, and forearm showed significant positive correlation with BMI (hip: r = 0.38, P < 0.001; spine: r = 0.19, P = 0.06; forearm: r = 0.20, P = 0.043) and significant inverse correlation with age (hip: r = −0.30, P = 0.002; spine: r = −0.20, P = 0.047; forearm: r = −0.40, P < 0.001). However, to assess the similar difference in the forearm BMD, adjustment was also made for sex, because, in the current group, forearm BMD was significantly less in the female than in the male subjects (total forearm BMD: 0.563 ± 0.055 and 0.502 ± 0.048 g/cm^2, respectively; P < 0.001).

The mean BMD values of the total hip, including femoral neck, trochanter, and intertrochanter, were significantly (P = 0.001) lower in group 1 than in group 2 (Table 1). However, no

significant difference was observed in the mean BMD at the lumbar spine and total forearm between the 2 groups of subjects with significantly different serum 25(OH)D concentrations ($P = 0.12$ and 0.20, respectively). When the correlation analysis was performed with age- and BMI-adjusted BMD values on the one hand and various other indicators related to bone mineral homeostasis on the other, a significant positive correlation was observed between the total hip BMD value and serum 25(OH)D ($r = 0.35$, $P < 0.001$). The total hip BMD also showed a significant inverse correlation with serum iPTH concentrations ($r = -0.24$, $P = 0.014$). However, age- and BMI-adjusted BMD values at the lumbar spine and total forearm showed only a trend toward correlation with serum 25(OH)D concentrations ($r = 0.19$, $P = 0.052$ and $r = 0.18$, $P = 0.062$, respectively) and showed no correlation with serum iPTH values.

The genotype distribution for the *VDR* SNPs showed that the study subjects were in Hardy-Weinberg equilibrium for *Bsm*I and *Fok*I sites ($P = 0.31$ and 0.69, respectively). However, at the *Taq*I site, the study population was in Hardy-Weinberg disequilibrium ($P = 0.001$) because of the rarity of the homozygous mutant genotype (*Taq*I tt = 4). The study subjects were in Hardy-Weinberg equilibrium for both the *PTH* gene SNPs, ie, *Bst*BI and *Dra*II ($P = 0.31$ and 0.80, respectively). The linkage disequilibrium analysis by using the SHEsis program showed linkage of *Taq*I with *Bsm*I SNP with linkage D score of 0.607.

To assess the relation of BMD with *VDR* and *PTH* gene SNPs, subjects with 3 genotypes related to each of the *VDR* and *PTH* gene SNPs studied were compared (**Table 2** and **Table 3**). The mean BMD values of total forearm and ultradistal and proximal one-third of forearm were significantly higher in subjects with the TT genotype of the *Taq*I *VDR* gene polymorphism than in subjects with the Tt/tt genotypes (Table 2). The subjects with the TT genotype also had a higher mean lumbar spine BMD than subjects with the Tt genotype. The subjects with the TT genotype had 13–19% higher mean BMD values at various forearm sites and 11% higher BMD of the total lumbar spine than subjects with the Tt genotype. The mean BMD value of the total hip was not significantly different among the subjects with the 3 *Taq*I *VDR* genotypes (ANOVA, $P = 0.055$).

The mean BMD at the forearm and lumbar spine was significantly higher in subjects with the bb genotype of the *PTH* gene *Bst*BI polymorphism than the subjects with the BB/Bb genotypes (Table 3). No significant differences were observed in the mean BMD at any of the studied sites among subjects with the 3 genotypes related to *Bsm*I and *Fok*I SNPs of the *VDR* gene and the *Dra*II SNP of the *PTH* gene.

To assess the role of various haplotypes constructed by using the 3 *VDR* genetic loci studied [*Taq*1 (T/t), *Bsm*I (B/b), and *Fok*I (F/f)] in determining the BMD, we divided the study cohort into 2 groups at their median BMD cutoff. The frequency of haplotype tbF was significantly lower among subjects with lumbar spine BMD above the median value than the subjects with lumbar spine BMD below the median (4.04% compared with 15.24%, respectively, $P = 0.007$).

DISCUSSION

The present study confirms our earlier observation of a wide prevalence of vitamin D deficiency (94.3%) in Asian Indians residing in Delhi (7). Recently, other investigators have also reported a similar occurrence of vitamin D deficiency among pregnant women from another northern Indian city, Lucknow (latitude 26.55 °N and longitude 80.59 °E), and in postmenopausal women from Tirupati in southern India (latitude 13.40 °N and longitude 77.2 °E) (9, 10, 34). Although the subjects from Tirupati had daily exposure of near-perpendicular tropical sunshine for 4–6 h, hypovitaminosis D observed in them could be explained by their relatively dark skin complexion (10).

On the basis of the studies related to the intestinal calcium absorption, inverse relation with serum iPTH concentration, and protection from fracture risk, a recent consensus has suggested that a "normal" concentration of circulating serum 25(OH)D is at least 32 ng/mL (22–24). It is interesting that, in the current study and using the current consensus, 94.3% of the apparently healthy northern Indian subjects residing in Delhi were vitamin D deficient. To study the relation between the vitamin D nutritional status and BMD, we had stratified the whole cohort into 2 groups at a serum 25(OH)D cutoff of 9.0 ng/mL [the lowest reported normal value of the kit used for the estimation of 25(OH)D in this study]. The significantly lower serum 25(OH)D concentrations observed in the group 1 subjects than in the group 2 subjects in the current study were associated with a low BMD at the hip region. However, the effect of subnormal serum 25(OH)D concentrations on BMD at the lumbar spine region was not significant. This was further evident on correlation analysis in which serum iPTH showed a significant inverse correlation with BMD of the hip region but not with BMD of the spine and forearm regions. Thus, it seems that 25(OH)D deficiency coupled with consequently elevated serum PTH is most detrimental for the BMD at the hip region in urban Asian Indian subjects. The pattern of a significant relation between serum 25(OH)D concentrations and hip BMD but not with lumbar spine or forearm BMD observed in the current study is in accordance with the observation of Arya et al (11) in Indians. Although several investigators reported significantly lower hip BMDs in both premenopausal and postmenopausal white subjects with low serum 25(OH)D concentrations (1, 2, 35–37), changes observed for the lumbar spine and forearm BMDs have been variable (35–40). Besides, vitamin D supplementation was shown to have beneficial effect predominantly on the BMD at the hip rather than at the forearm (41, 42).

Differential effects of vitamin D deficiency on BMD at various sites possibly involve factors other than hypovitaminosis D. In this context, genetic variation related to *VDR* polymorphism was extensively studied in various populations (13, 14). Among the 3 *VDR* SNPs studied by us, subjects with *Taq*I-TT genotype had a significantly higher BMD at the forearm than did subjects with Tt and tt genotypes. In 1995, Spector et al (43) first reported an association of the TT genotype of *Taq*I polymorphism with significantly higher BMD. Recently, Nguyen et al (44) reported a lower risk of femoral fracture in postmenopausal women with the TT genotype. Liu et al (13) reviewed studies of *VDR* gene SNPs and variation in the BMD. Of the 4 *VDR* SNPs, variation in the genotype related to *Taq*I (TT homozygotes) and *Bsm*I (bb homozygotes) polymorphisms were reported to be associated with higher BMD (13). A significant linkage with 97% concordance between *Taq*I and *Bsm*I SNPs was shown in the white subjects (15). Because of the linkage between *Taq*I and *Bsm*I, it is difficult to segregate the individual effect of genotype related to these 2 sites on the BMD. In the current study, the lower D score observed between *Taq*I and *Bsm*I SNPs (0.607) could possibly

TABLE 2
Clinical characteristics, biochemical indicators, and bone mineral density (BMD) in subjects with different *VDR* genotype groups[1]

	*Taq*I			*Fok*I			*Bsm*I		
	TT ($n = 12$)	Tt ($n = 78$)	tt ($n = 4$)	FF ($n = 53$)	Ff ($n = 33$)	ff ($n = 8$)	BB ($n = 14$)	Bb ($n = 53$)	bb ($n = 27$)
Age (y)	36.8 ± 4.7[2]	46.0 ± 10.5	43.3 ± 12.1	44.0 ± 9.7	45.5 ± 11.0	46.4 ± 12.7	43.0 ± 12.3	45.0 ± 9.5	
M:F	8:4	37:41	1:3	25:28	15:18	6:2	6:8	32:21	8:19
BMI (kg/m^2)	24.7 ± 2.9	24.8 ± 3.8	27.2 ± 7.5	25.0 ± 4.0	25.3 ± 3.8	22.4 ± 2.7	25.4 ± 6.0	24.8 ± 3.5	24.7 ± 3.4
Serum total calcium (mg/dL)	9.6 ± 0.4	9.5 ± 0.6	10.2 ± 0.4	9.6 ± 0.5	9.5 ± 0.8	9.6 ± 0.6	9.9 ± 0.6	9.5 ± 0.6	9.5 ± 0.5
Serum phosphorus (mg/dL)	3.9 ± 0.4	4.1 ± 0.6	4.2 ± 0.3	4.0 ± 0.5	4.0 ± 0.6	4.1 ± 0.3	4.1 ± 0.3	4.0 ± 0.6	4.1 ± 0.6
Alkaline phosphatase (IU/L)	225 ± 58	228 ± 66	229 ± 25	282 ± 452	240 ± 68	209 ± 42	224 ± 64	232 ± 61	221 ± 71
Serum iPTH (pg/mL)	50.9 ± 19.1	47.5 ± 32.5	41.7 ± 33.9	46.7 ± 35.7	49.2 ± 23.2	47.4 ± 29.6	46.3 ± 29.7	50 ± 34.3	44 ± 25
Serum 25(OH)D (ng/mL)	10.4 ± 6.1	9.9 ± 6.4	9.6 ± 5.3	9.4 ± 4.5	10.0 ± 6.5	13.3 ± 12.4	8.9 ± 4.4	10.9 ± 7.1	8.6 ± 5.0
BMD									
L1–L4 AP spine (g/cm^2)[3]	1.037 ± 0.125^a	0.910 ± 0.127^b	0.977 ± 0.097^{a,b}	0.929 ± 0.133	0.920 ± 0.133	0.969 ± 0.134	0.939 ± 0.096	0.940 ± 0.130	0.904 ± 0.152
Femoral neck (g/cm^2)	0.820 ± 0.087	0.731 ± 0.117	0.797 ± 0.050	0.745 ± 0.114	0.748 ± 0.118	0.724 ± 0.127	0.760 ± 0.099	0.751 ± 0.116	0.722 ± 0.123
Trochanter (g/cm^2)	0.679 ± 0.060	0.620 ± 0.104	0.615 ± 0.021	0.618 ± 0.097	0.647 ± 0.105	0.608 ± 0.085	0.628 ± 0.069	0.646 ± 0.100	0.588 ± 0.102
Intertrochanter (g/cm^2)	1.100 ± 0.100	1.015 ± 0.155	1.056 ± 0.069	1.014 ± 0.142	1.057 ± 0.169	0.989 ± 0.106	1.043 ± 0.122	1.046 ± 0.150	0.978 ± 0.154
Total hip (g/cm^2)	0.927 ± 0.078	0.838 ± 0.125	0.866 ± 0.047	0.844 ± 0.120	0.869 ± 0.126	0.819 ± 0.100	0.850 ± 0.093	0.865 ± 0.122	0.822 ± 0.129
Ward's triangle (g/cm^2)	0.669 ± 0.077	0.557 ± 0.231	0.639 ± 0.053	0.591 ± 0.135	0.543 ± 0.322	0.570 ± 0.151	0.621 ± 0.124	0.565 ± 0.264	0.565 ± 0.135
Ultradistal forearm (g/cm^2)[3]	0.471 ± 0.051^a	0.383 ± 0.058^b	0.375 ± 0.025^b	0.396 ± 0.064	0.384 ± 0.052	0.401 ± 0.086	0.377 ± 0.065	0.399 ± 0.056	0.387 ± 0.073
Mid forearm (g/cm^2)[3]	0.625 ± 0.050^a	0.544 ± 0.060^b	0.534 ± 0.030^b	0.557 ± 0.069	0.539 ± 0.049	0.575 ± 0.071	0.533 ± 0.069	0.559 ± 0.058	0.550 ± 0.072
Proximal forearm (g/cm^2)[3]	0.728 ± 0.059^a	0.633 ± 0.067^b	0.644 ± 0.064^b	0.652 ± 0.080	0.619 ± 0.057	0.681 ± 0.051	0.643 ± 0.067	0.651 ± 0.073	0.630 ± 0.076
Total forearm (g/cm^2)[3]	0.599 ± 0.058^a	0.517 ± 0.056^b	0.514 ± 0.025^b	0.532 ± 0.067	0.516 ± 0.051	0.550 ± 0.062	0.513 ± 0.061	0.533 ± 0.057	0.525 ± 0.073

[1] iPTH, intact parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; AP, anteroposterior.
[2] $\bar{x}$ ± SD (all such values).
[3] Within a genotype group, means in a row with different superscript letters are significantly different, $P < 0.05$ (ANOVA with Scheffe's test).

TABLE 3
Clinical characteristics, biochemical indicators, and bone mineral density (BMD) in subjects with different *PTH* genotype groups[1]

	*Bst*BI			*Dra*II		
	BB ($n = 54$)	Bb ($n = 30$)	bb ($n = 9$)	DD ($n = 51$)	Dd ($n = 34$)	dd ($n = 8$)
Age (y)	45.8 ± 10.3[2]	44.0 ± 10.4	39.9 ± 10.5	45.6 ± 11.3	44.6 ± 9.4	38.5 ± 6.0
M:F	22:32	18:12	6:3	29:22	14:20	3:5
BMI (kg/m^2)	24.6 ± 3.9	25.3 ± 4.0	24.3 ± 2.7	24.6 ± 3.2	25.1 ± 4.7	24.7 ± 4.1
Serum total calcium (mg/dL)	9.4 ± 0.7	9.7 ± 0.5	9.8 ± 0.2	9.6 ± 0.6	9.6 ± 0.6	9.3 ± 0.5
Serum inorganic phosphorus (mg/dL)	4.1 ± 0.6	4.0 ± 0.6	3.9 ± 0.2	4.0 ± 0.6	4.1 ± 0.4	4.0 ± 0.5
Serum alkaline phosphatase (IU/L)	223 ± 71	233 ± 48	222 ± 68	237 ± 64	216 ± 62	203 ± 55
Serum iPTH (pg/mL)	49.0 ± 34.8	42.5 ± 25.0	56.5 ± 24.8	44.5 ± 25.8	54.0 ± 38.9	39.6 ± 22.5
Serum 25(OH)D (ng/mL)	9.2 ± 5.2	10.1 ± 5.9	14.6 ± 11.4	10.2 ± 7.0	9.8 ± 5.4	9.9 ± 5.5
BMD (g/cm^2)						
L1–L4 AP spine (g/cm^2)[3]	0.932 ± 0.125[a]	0.880 ± 0.115[a]	1.066 ± 0.138[b]	0.931 ± 0.142	0.927 ± 0.124	0.914 ± 0.117
Femoral neck (g/cm^2)	0.755 ± 0.119	0.714 ± 0.115	0.781 ± 0.084	0.742 ± 0.104	0.745 ± 0.136	0.752 ± 0.113
Trochanter (g/cm^2)	0.626 ± 0.099	0.615 ± 0.106	0.666 ± 0.076	0.635 ± 0.095	0.616 ± 0.113	0.611 ± 0.053
Intertrochanter (g/cm^2)	1.041 ± 0.157	0.990 ± 0.148	1.057 ± 0.088	1.027 ± 0.135	1.022 ± 0.178	1.029 ± 0.124
Total hip (g/cm^2)	0.858 ± 0.124	0.819 ± 0.120	0.901 ± 0.084	0.857 ± 0.110	0.840 ± 0.145	0.848 ± 0.086
Ward's triangle (g/cm^2)	0.613 ± 0.143	0.493 ± 0.314	0.610 ± 0.096	0.549 ± 0.258	0.600 ± 0.162	0.616 ± 0.099
Ultradistal forearm (g/cm^2)	0.387 ± 0.067	0.390 ± 0.051	0.437 ± 0.056	0.397 ± 0.066	0.381 ± 0.056	0.409 ± 0.063
Mid forearm (g/cm^2)	0.545 ± 0.069	0.552 ± 0.051	0.597 ± 0.054	0.553 ± 0.068	0.545 ± 0.057	0.578 ± 0.060
Proximal forearm (g/cm^2)	0.637 ± 0.080	0.643 ± 0.055	0.694 ± 0.074	0.648 ± 0.082	0.633 ± 0.058	0.663 ± 0.064
Total forearm (g/cm^2)[3]	0.519 ± 0.067[a]	0.526 ± 0.047[a]	0.583 ± 0.057[b]	0.532 ± 0.069	0.517 ± 0.052	0.542 ± 0.060

[1] iPTH, intact parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; AP, anteroposterior.
[2] $\bar{x}$ ± SD (all such values).
[3] Within a genotype group, means with different superscript letters are significantly different, $P < 0.05$ (ANOVA with Scheffe's test).

be due to the fact that the study population was not in Hardy-Weinberg equilibrium at the *Taq*I site as a result of the relative rarity of the tt genotype. The higher mean spine and forearm BMDs in subjects with the TT genotype related to the *Taq*I SNP but not among subjects with the bb genotype (*Bsm*I) or with the *Fok*I SNP indicated the predominant influence of the *Taq*I SNP in determining the variation of BMD in Asian Indian subjects. The importance of the t allele in *Taq*I SNP in determining BMD in Asian Indian subjects was further confirmed on haplotype analysis. The frequency of the tbf haplotype was significantly higher in subjects with a spine BMD value below the median than in subjects with a BMD value above the median (15.24% and 4.04%, respectively). Studies assessing the variation in the BMD with *PTH* gene SNPs are limited (19, 20). In the current study, subjects with the *Bst*BI-BB genotype showed a lower mean BMD at the forearm and spine than did subjects with the bb genotype. This finding of the current study is different from that reported in Chinese and Japanese populations (19, 20).

The effect of *VDR* polymorphisms on BMD was variable in several studies (13, 45–47). Such variability was postulated to be due to the masking of the *VDR* gene–related effect under different environmental conditions, including dietary calcium intake (13, 46). Kiel et al (46) observed the effect of *Bsm*I *VDR* SNP only in subjects with dietary calcium intakes > 800 mg/d. Similarly, Rubin et al (45) could relate *Taq*I SNP to BMD only when the daily intake of dietary calcium was >684 mg. However, the results of the recent studies have shown an association of *VDR* *Taq*I polymorphism with BMD even under calcium-deprived states in subjects with the tt genotype (47). In the current study, a significantly lower BMD at the forearm and lumbar spine was observed in subjects with the Tt genotype (*Taq*I SNP) even in the presence of vitamin D deficiency.

The mechanism related to differences in the VDR expression or stability in relation to *VDR* polymorphism was a subject of study to date (48–50). Crofts et al (48) in 1996 showed a higher *VDR* mRNA expression in the peripheral leukocytes of subjects with a T allele than in those of subjects with a t allele of *VDR* *Taq*I SNP. Subsequently, Verbeek et al (50) in 1997 showed 30% lower reverse transcriptase–PCR product was derived from the t allele mRNA obtained from the peripheral leukocytes than from that derived from the T allele. However, Yamagata et al (49) could not associate *VDR* mRNA expression with BMD. The role of other genes in linkage with various *VDR* SNPs cannot be excluded as the cause of the variation in the BMD associated with *VDR* polymorphisms.

To conclude, vitamin D deficiency is present in most of the Asian Indians living in Delhi. Such vitamin D deficiency when coupled with higher serum PTH concentrations has functional relevance, as indicated by the significantly low BMD, especially at the hip region. Moreover, the genetic factors also influence the BMD in the subjects, as reflected by a significantly lower forearm BMD in subjects with the Tt/tt genotype related to the *Taq*I SNP of the *VDR* gene and the BB/Bb genotype of the *Bst*BI SNP of the *PTH* gene.

This work received the AR Seth Award for the Best Research work of the year at the 35th annual meeting of the Endocrine Society of India, New Delhi, India, November 2005.

MRV performed the vitamin D receptor gene PCR-RFLP study, analyzed the data, and wrote the manuscript. RG designed the work; standardized the PCR-RFLP assay for the vitamin D receptor and parathyroid hormone

genes; conducted the biochemical analysis, including serum total calcium, phosphorus, and alkaline phosphatase; and supervised the study, including analysis of the data and writing and editing of the manuscript. NG performed the 25(OH)D and serum PTH assays. DR helped in the vitamin D receptor polymorphism analysis. NT helped organize the bone mineral data estimation. NK helped recruit volunteers for the study. None of the authors had a personal or financial conflict of interest.

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