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ORIGINAL ARTICLE

Osteoporosis and its Association with Vitamin D Receptor, Oestrogen α Receptor, Parathyroid Receptor and Collagen Type I *alpha* Receptor Gene Polymorphisms with Bone Mineral Density: A Pilot Study from South Indian Postmenopausal Women of Tamil Nadu

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Abstract

The involvement of many putative genetic factors makes osteoporosis a complex disease. With increasing longevity of the Indian population, it's now being realized that, as within the West, osteoporotic fractures are also a significant explanation for morbidity and mortality in postmenopausal women. Studies have suggested that the genetic component liable for bone mass could be linked to single nucleotide polymorphisms. Therefore, this study is aimed to research the role of seven gene polymorphisms previously associated with bone phenotype in a cohort of postmenopausal South Indian women from Tamil Nadu. The subjects for the study ($n=300$) included 100 osteoporotic women (age 59.3 ± 9.26), 100 osteopenic women (age 55.6 ± 8.17) and 100 non-osteoporotic women as controls (age 55.4 ± 8.85).Genetic polymorphisms were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism. Case–control genetic association analysis of *Bsm*I of the VDR and *BstB*I of the PTH gene showed a significant allelic association with low bone mineral density amongst the osteoporotic postmenopausal women. The association of BMD with the VDR gene polymorphisms revealed that the average BMD in the *Bsm*I polymorphism with the recessive genotype GG in osteoporotic women was significantly reduced compared with the average BMD in osteoporotic women with AA and AG genotypes. In the *BstB*I polymorphism, the BMD in the osteoporotic subjects were significantly lower in the AA group than in the GA and GG groups. These results provide evidence for an independent association between BMD and rs1544410 in *VDR* and rs6254 in PTH and may contribute in being a possible genetic marker for predicting the disease susceptibility in the population tested.

Keywords Vitamin D receptor · Bone mineral density · Osteoporosis · Polymorphism · Postmenopausal women

Extended author information available on the last page of the article

Introduction

Osteoporosis, now considered a global health disorder, is rapidly introducing challenges in socio-medical research areas. The focus of this study is to perceive a more accurate genetic controlling mechanism for this malady. Clearly having a strong genetic component, osteoporosis is characterized by low bone mineral density (BMD), the deterioration of bone tissue and an increased risk of fracture (Kanis et al. 1994). The regulatory genes for these phenotypes have not been accurately defined, but genetic linkage studies have identified several quantitative trait loci for the regulation of BMD (Devoto et al. 1998). An increased incidence of fractures and a reduced BMD has been associated with polymorphisms of a number of classical genes, e.g. COLIA1, VDR, ESR1, PTH, LRP5 and OPG (Ferrari 2008).Without reference to the already prevailing role of a particular gene for bone metabolism, recent studies here sought association strategies with genome wide approaches in this area. Yet, the result of the final variant remains inconclusive. This highlights the importance of experimentally proven association studies of single nucleotide polymorphisms (SNP). Contradictory results were often obtained when analysis of polymorphisms in candidate genes were tested in independent cohorts. Thus for more accurate results in this area, replication and meta-analysis appears to be specific tools (Agueda et al. 2010).

After menopause, oestrogen deficiency results in decreased activity of vitamin D, the enzyme 1-α hydroxylase which converts, 25-OH vitamin D to 1, (OH)2 vitamin D, has a regulating effect on bone calcium homeostasis (Valdivielso and Fernandez 2006). Oestrogen also mediates calciotropic reactions in the body through the vitamin D receptor (VDR) and modulates the transcription of target genes such as calcium-binding proteins and osteocalcin, which help in calcium uptake or bone formation. The vitamin D receptor is expressed within the bone marrow on cells that consist of accessory and stromal cells. (Amiri et al. 2021) The VDR gene has been highlighted as one of the prominent genes for the genetic control of bone mass. Studies have revealed that several allelic variants of the gene encoding VDR can occur in coding or non-coding parts of the gene and lead to changes in the protein or alter its level and pattern of expression (Pike and Christakos 2017). SNPs recognized by appropriate restriction endonucleases, such as *Apa*I (rs7975232), *Taq*I (rs731236) and *Bsm*I (rs1544410), have been found to be associated with BMD in many studies. From a meta-analysis performed by Thakkinstian-co inferred that the A allele of the VDR *Bsm*I polymorphism followed a recessive model where the AA genotype was associated with a lower BMD (Thakkinstian et al. 2004a). In an investigation conducted by Uitterlinden et al. (2006) with 26,242 members from all over Europe were incorporated and genotyped utilizing different methods which were cross-validated between the various individuals from the consortium. However, the agreement on this relationship is not universal (Marozik et al. 2018). The varied views in studies addressing genetic risks may be attributed to genetic heterogeneity, population diversity and gene-environment interactions (Marozik et al. 2018; Bhanushali et al. 2009).

Oestrogen and its receptors play a key role in controlling skeletal growth and maintaining bone mass. Oestrogen therapy has been shown to prevent bone mineral loss, affirming the above statement. Many physiological processes, such as reproduction, the cardiovascular system and bone integrity, are influenced by oestrogen. The lipophilic nature of oestrogen enables it to diffuse through the plasma membrane and bind with its ERα1 receptors located in the nucleus and cytoplasm, thereby forming the oestrogen/ER complex. The binding of this complex to oestrogen response element sequences in the promoter regions of oestrogen-responsive genes triggers the removal of co-regulatory proteins (co-activators or co-repressors) from the promoter and alters gene expression regulation (Salanti 2005; Jensen and Jacobsen 1962). ER-α and ER-β are the two main isoforms of ER and are encoded by two separate genes. Chromosome 6q25.1 carries the ER-α1 gene, which has 140 kb of DNA comprising eight exons, encoding a protein of 595 amino acids with a molecular weight of approximately 66 kDa. Generally, it is observed that similar to the promoter, the first intron of a gene usually contains a larger number of regulatory sequences than the other introns. Either an increased or decreased risk of various diseases is associated with several of the SNPs identified in ER-α (Nilsson et al. 2001; Yoshidome et al. 2000). The *Pvu*II (rs2234693) and *Xba*I (rs9340799) site polymorphisms, both located in the first intron, are the best characterized SNPs of ER-α. Phenomena such as the timing of the onset of menopause, low BMD and vertebrate fracture risk in postmenopausal women, which are essentially oestrogen-dependent traits, was found to be associated with the *Pvu*II (rs2234693) and *Xba*I (rs9340799) SNPs. Modification of ER-α gene expression by altering the binding of transcription factors could be the underlying functional mechanisms of the *Pvu*II and *Xba*I polymorphisms (Gennari et al. 2005; Hill et al. 1989). The inconsistent results obtained in earlier studies associated with these polymorphisms relating to BMD may be due to their small sample size and their differences in terms of age, menopausal status (Yaich et al. 1992) and the ethnic background of the study populations involved. Further information on the association between ER-α1 polymorphisms and their impact on postmenopausal bone loss, including their influence as bone remodelling markers, are needed for proper confirmatory assessment (Weel et al. 1999).

Apart from vitamin D and oestrogen, the parathyroid hormone (PTH) secreted by the parathyroid gland has a controlling effect on the calcium homeostasis and bone remodelling. The PTH is an 84 amino-acid polypeptide hormone and the gene encoding it lies on the 11th chromosome. The extracellular calcium levels are maintained by PTH by three mechanisms; (i) enhanced gastrointestinal calcium absorption (ii) renal reabsorption of calcium and phosphate and (iii) osteoclastic bone resorption. The extracellular calcium (Sai et al. 2011) and other humoural factors including 1,25(OH)2D3 (Dawson-Hughes et al. 1991) regulate the synthesis and secretion of PTH from the parathyroid glands. The ageing process triggers off changes in the regulating factors thus affecting the secretion of PTH which in turn modulates the bone metabolism. Studies report that with ageing, the serum levels of PTH increases playing a vibrant role in the pathogenesis of involutional osteoporosis (Silver et al. 1985). This may be an evidence for the fact that some variations arising in the PTH gene has an effect on BMD and bone metabolism. To study the associations between BMD and PTH gene, we utilized restriction fragment length

polymorphisms (RFLPs) in the second intron of the PTH-*BstB*I gene polymorphism (rs6254) reported by Mullersman et al. (1992) to study the associations between BMD and this polymorphism of the PTH gene.

Another important candidate gene is the COLIA1gene receptor. The Type I collagen is a strong and plausible candidate gene for the regulation of BMD, since it constitutes the major protein constituent of the bone matrix. A possible association of allelic variants within the collagen 1 alpha 1 (COLIA1) gene with postmenopausal osteoporosis (PMO) has been reported by various studies (Mullersman et al. 1992; Jin et al. 2011). A novel G to T Sp1 polymorphism (rs1800012) was found to be within the binding site for transcription factor Sp1 in the first intron of COL1A1 which was associated with low BMD and an increased risk of fractures. The Sp1 polymorphism in COL1A1 is a functional genetic variant influencing the onset of osteoporosis by changing the bone quality and bone mass (Jin et al. 2011).

In the past decade, the number of association investigations performed to find the genes responsible for osteoporosis has increased drastically. Various researchers studied the enormous numbers of SNPs within a set of genes in which majority are known to be associated with bone physiology or probably linked to other bone diseases. It has been accounted that genetic factors may be liable for the variations in the beginning of menopause and menarche (Mirinezhad et al. 2021). The impact of gene variants may likewise be affected by different variants within a same gene or by other gene-gene interactions just as interactions with the environment and epigenetic impacts like DNA methylation (Grant et al. 1996; Friso et al. 2002). The absence of similar studies on the genetic association with BMD from Tamil Nadu has been the driving force in conducting the present study. Considering the importance of these polymorphisms described above and the absence of similar studies on their allelic associations with BMD, this study aimed to investigate the genetic contributions of the commonly studied polymorphisms of *Apa*I, *Bsm*I and *Taq*I in VDR, *Pvu*II and *Xba*I in ER α1, *BstB*I in PTH and Sp1 in COLIA1 to the risk of osteoporosis in postmenopausal women of Tamil Nadu, south India.

Materials and Methods

Sample Collection

This study included three hundred women in the postmenopausal age group for whom the approval of the ethical committee and the consent of the participants were duly obtained. The women were divided into three groups of 100 each based on their BMD (*T*-score), as shown in Table 1. Exclusion criteria included endocrine disorders (such as hyperthyroidism and hypo- and hyperparathyroidism), chronic disorders of the liver and kidney and other skeletal diseases (Paget's disease, osteogenesis imperfecta and rheumatoid arthritis). Subjects using medications that are known to affect bone density and metabolism (such as calcium supplements, corticosteroids, anticonvulsants, hormone replacement therapy and heparin) or those with an unusual gynaecological history such as bilateral oophorectomy, irregular cycles or premature menopause before the age of 40 were excluded. The stratified

Table 1 Study subjects divided into the following groups

Study groups	*T*-score	Number of study subjects
Osteoporosis	− 2.5 and lower	100
Osteopenia (low bone mass)	− 1 and − 2.5	100
Normal bone mass (control)	− 1.0 and above	100

T-scores are based on statistical measurements called standard deviations (SD) that reflect the difference between the bone density and the average bone density in the reference population

random sampling method was used in this study. The selection of the study group being restricted only to postmenopausal women, all of whom were to be without any of the above mentioned major risk factors posed a limiting factor. Thus, reducing the sample size to achieve 80% statistical power. Hong and Park (2012), suggested that the dominant model required the smallest sample size (90 cases and 90 controls) to achieve 80% power when compared to other genetic models, under the following assumptions of 5% disease prevalence, 5% MAF, complete LD ($D' = 1$), 1:1 case-to-control ratio and 5% type I error rate (α) and odds ratios of heterozygotes at 2/ and odds ratios of rare homozygotes at 3.

BMD Measurements

A basic physical examination and the medical history of each subject were collected by interview with a structured questionnaire. BMD was estimated in all of the subjects using an ultrasound bone densitometer (model CM-200, Japan), which measures the speed of sound (SOS) at the calcaneus. BMD (*T*-score) values were calculated and classified as osteoporosis, osteopenia or normal individuals according to the World Health Organization recommendations (Genant et al. 1999).

BMI Measurement

Height and weight of all of the subjects were noted, and their body mass index (BMI) was calculated by weight (kg)/height (m^2).

Estimation of Osteocalcin in the Serum Samples

Blood samples from all of the study subjects were collected under aseptic conditions. The serum samples were separated and stored at − 20 °C. Osteocalcin was estimated by enzyme-linked immunosorbent assay (ELISA) using a human osteocalcin/bone gla protein (OT/BGP) ELISA kit (Bioassay Technology Laboratory, China), which is a solid phase enzyme immunoassay. The assay uses a monoclonal antibody directed against distinct epitopes of human osteocalcin. The amount

of substrate turnover was determined colorimetrically by measuring the absorbance at 450 nm (reference filter 630 nm), which is proportional to the osteocalcin concentration.

DNA Extraction and Determination of the VDR, ER α1, PTH and COLIA1 Genotypes

Approximately 3 mL of peripheral blood samples were collected from the subjects and stored in EDTA-coated vacutainers. Genomic DNA was extracted from whole blood samples by the non-enzymatic salting-out method (Suguna et al. 2014), which employs salting-out of cellular proteins by a saturated NaCl solution. Gradient PCR (Table 2) using common primers were performed in a thermocycler (Eppendorf). PCR was performed using 0.1 µg of extracted DNA in 50 µl of buffer solution 1× PCR buffer, 1.5 mM $MgCl_2$, 100 µM dNTP mix, 1 U of *Taq* DNA polymerase and 100 nM of each primer. Following PCR, the products were digested with specific restriction enzymes (Thermo Fisher Scientific, Finland). The digested products were analysed for the presence or absence of recognition sites by ethidium bromide staining of the fragments separated through a 3% agarose gel and photographed. The sizes of the bands were estimated using a 100 bp ladder. The primers, the PCR conditions and the genotype patterns are given in Table 2. The PCR-RFLP results were cross checked by randomly performing PCR-RFLP in triplicates in a set of representative samples and the results were confirmed.

Sequencing of the VDR, ER α1, PTH and COLIA1 Genes

The PCR product for each gene obtained was subjected to DNA sequencing with Sanger's sequencing method. The DNA sequencing was done to check and confirm whether the amplified product was the respective gene sequence. The obtained sequence was then subjected to BLASTN analysis to study the homology sequence of the amplified product.

Statistical Analysis

All analyses were carried out using the statistical package GraphPad Prism version 8. The association of genotype with BMD and serum osteocalcin was evaluated by analysis of variance (one-way ANOVA). Chi-square analysis was performed to determine the genotypic and allelic frequencies and to verify Hardy–Weinberg equilibrium (HWE). Osteoporosis risk was analysed based on the genotypes between women with osteoporosis and a normal bone mass (age-related control) by using the odds ratio (OR) and 95% confidence intervals (95% CI). CI was calculated for the dominant model using a 2×2 contingency table. Haploview4.2 (http://www.broadinstitute.org/haploview) (Barrett et al. 2005) was used to carry out linkage disequilibrium analysis (LD) and haplotype analysis. The LD values were calculated with D' and displayed with confidence bounds. A p value < 0.05 was selected to define statistical significance. Multiple linear regression analyses were performed to examine the interaction effects on BMD variations amongst the SNPs.

Table 2 PCR protocols and primer sequences for the amplification of different loci of the SNPs

Gene	Loci	Primers	Size (bp)	Allele	PCR protocol	Genotype pattern
Vitamin D receptor gene (Mitra et al. 2006a)	*Apa*I	5′-CAG AGC ATG GAC AGG GAG CAA G-3′ 5′-GCA ACT CCT CAT GGC TGA GGT CTC A-3′	740	T/G	94 °C for 1 min, 72 °C for 1 min, 72 °C for 1 min for 30 cycles	TT = 740 bp TG = 740 bp, 530 bp,210 bp GG = 530 bp, 210 bp
	*Taq*I			T/C		TT = 495 bp,245 bp TC = 495 bp, 290 bp,245 bp,205 bp CC = 290 bp, 245 bp, 205 bp
	*Bsm*I	5′-GGG AGA CGT AGC AAA AGG-3′ 5′-AGA GGT CAA GGG TCA CTG-3′	360	A/G	94 °C for 1 min, 62.4 °C for 1 min, 72 °C for1 min for 30 cycles	AA = 360 bp AG = 360 bp, 191 bp, 169 bp GG = 191 bp, 169 bp
Estrogen receptor type I alpha (Fa et al. 2009)	*Pvu*II	5′ATCCAGGGTTATGTGGCA ATGAC3′ 5′ACCCTGGCGTCGATTATC TGA3′	527	T/C	94 °C for 1 min, 59.4 °C for 1 min, 72 °C for 1 min for 30 cycles	TT = 527 bp TC = 527 bp,427 bp,100 bp CC = 427 bp,100 bp
	*Xba*I			A/G		AA = 527 bp AG = 527 bp,382 bp,145 bp GG = 382 bp,145 bp
Parathyroid gene receptor (Mullersman et al. 1992)	*BstB*I	5′-CATTCTGTGTACTATAGT TTG-3′ 5′-GAGCTTTGAATTAGCA-3′	600	G/A	95 °C for 30 s, 53.4 °C for 45 s, 72 °C for 1 min 30 s for 30 cycles	GG = 387 bp, 213 bp GA = 600 bp, 387 bp, 213 bp AA = 600 bp
Collagen type I alpha 1—Sp1 polymorphism (Grant et al. 1996)	*Msc*I	5′-TAACTTCTGGACTATTTG CGGACTTTTTGG-3′ 5′-GTCCAGCCCTCATCCTGG CC-3′	264	G/T	94 °C for 1 min, 66.8 °C for 1 min, 72 °C for 1 min for 35 cycles	GG = 264 bp GT = 264 bp, 246 bp

Results

Baseline Characteristics

The anthropometric measurements of the study group comprising 300 postmenopausal women between 45 and 80 years of age are tabulated in Table 3. Assessment of the tabulation highlighted that variations in serum osteocalcin levels and the *T*-score values for the BMD were found to be statistically significant ($p < 0.00001$). Significant variations were also observed with the age of the postmenopausal women ($p = 0.001$). However, there were no significant variations in BMI ($p = 0.602$) amongst the 3 groups. The genotype frequencies of the *Apa*I of VDR and *Pvu*II of ER-α1 and COLIA1 Sp1 gene polymorphisms in all of the groups were in Hardy–Weinberg equilibrium. The *Taq*I and *Bsm*I of the VDR gene polymorphism, the *Xba*I of ER α1 and the *BstB*I of the PTH receptor gene polymorphisms were not in Hardy–Weinberg equilibrium (Table 4). Our small sample size might be the reason for the disequilibrium. LD analysis was performed for *Apa*I, *Taq*I and *Bsm*I of VDR ($D' = 0.129$, $R^2 = 0.01$) and the *Pvu*II and *Xba*I of the ER-α1 ($D' = 0.064$, $R^2 = 0.004$) polymorphisms using the data of the controls and cases. None of the haplotypes showed an ambiguous significant difference between the cases and controls since their *p* values were not less than 0.05. None of the haplotypes had any significant difference between the cases and controls even when these data were subjected to 999-time permutation evaluation. All of these SNPs indicated strong evidence of recombination.

Osteoporosis risk was calculated using odds ratios (Table 5). Comparisons were conducted of the distribution of all of the SNP genotypes and alleles in osteoporotic women *vs* control women under the dominant model in postmenopausal women. VDR *Apa*I TT vs TG + GG genotypes, VDR *Bsm*I polymorphisms AA vs AG + GG genotypes and ER-α1*Pvu*II polymorphisms TT vs TC + CC genotypes were found to have a protective effect against osteoporosis ($p = 0.02$, OR = 0.4, 95% CI = 0.2–0.8) ($p = 0.02$*, OR = 0.4, 95% CI = 0.2–0.8) ($p = 0.01$*, OR = 0.3, 95% CI = 0.1–0.7), respectively).

Table 3 Anthropometric dimensions of the study groups

Variables	Osteoporotic $n = 100$	Osteopenic $n = 100$	Control $n = 100$	*p* value
BMD (g/cm^2)	− 2.79 ± 1.69	− 1.45 ± 0.841	0.06 ± 0.91	< 0.00001*
BMI (kg/m^2)	25.5 ± 6.06	26.01 ± 6.60	25.15 ± 5.31	0.602
Age (in years)	59.3 ± 9.26	55.6 ± 8.17	55.4 ± 8.85	0.001*
Osteocalcin (ng/ml)	31.6 ± 9.8	25.9 ± 7.5	15.1 ± 7.8	< 0.00001*

BMD bone mineral density, *BMI* body mass index

*$p < 0.05$ statistically significant for ANOVA. Values are expressed as Mean ± SEM

Table 4 Results of single marker association tests

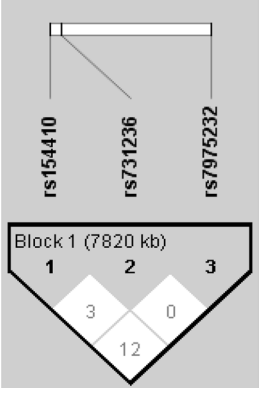
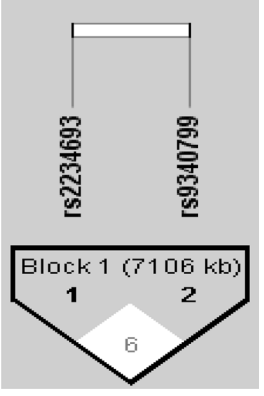
Gene	Chromosome position	LD plot	SNP ID	Genic position	Alleles (major/minor)	Major allele frequency	Minor allele frequency	HWE P
Vitamin D receptor	Chromosome 12q13.11		rs7975232	Intron 8	T/G	0.66	0.35	0.06
			rs731236	Exon 9	T/C	0.49	0.51	0.03*
			rs1544410	Intron 8	A/G	0.53	0.48	0.001*

Table 4 (continued)

Gene	Chromosome position	LD plot	SNP ID	Genic position	Alleles (major/minor)	Major allele frequency	Minor allele frequency	HWE P
Oestrogen α receptor	Chromosome 6q25.1		rs2234693	Intron 1	T/C	0.56	0.44	0.67
			rs9340799	Intron 1	A/G	0.59	0.42	0.001*
Parathyroid receptor	Chromosome 11p15.3	–	rs6254	Intron 2	G/A	0.50	0.50	0.00001*
Collagen type I alpha 1 receptor	Chromosome 17q21	–	rs1800012	Intron 1	G/T	0.91	0.09	0.09

*p value < 0.05 statistically significant

Table 5 Odds ratio analysis amongst cases and control

Genotype contrast	Polymorphism	Dominant model	Odds ratio	95% CI	*p* value
Osteoporotic vs controls	*Apa*I	TT vs TG + GG	0.4	0.2–0.8	0.02*
	*Taq*I	TT vs TC + CC	0.6	0–1.3	0.2
	*Bsm*I	AA vs AG + GG	0.3	0.1–0.7	0.01*
	*Pvu*II	TT vs TC + CC	0.2	0.1–0.5	0.0009*
	*Xba*I	AA vs AG + GG	0.6	0.3–1.1	0.1
	*BstB*I	GG vs GA + AA	0.7	0.3–1.4	0.3

CI confidence interval

*p value < 0.05 statistically significant

Association Between VDR Genotypes with BMD

The distribution of the genotype frequencies of women in the normal, osteopenic and osteoporotic groups with respect to their BMD with VDR gene *Apa*I, *Taq*I and *Bsm*I polymorphisms is illustrated in Table 6. There was no relationship found between the *Apa*I and *Taq*I polymorphisms and the BMD values ($p > 0.05$). Concerning the *Bsm*I polymorphism, the osteoporotic women with the recessive "GG" genotypes were found to have a significantly lower BMD ($p < 0.05$), than the osteoporotic women with "AA" and "AG" genotypes.

Association Between ERα1 Genotypes with BMD

In all of the postmenopausal women, the ERα1 *Pvu*II genotypes did not show any association with BMD (controls TT = 0.6 ± 3.6, TC = 0.1 ± 0.9, CC = 0.1 ± 0.9; $p = 0.5$, osteopenic TT = − 1.5 ± 0.8, TC = − 1.3 ± 0.8, CC = − 1.4 ± 0.8; $p = 0.6$, osteoporotic TT = − 2.9 ± 1.5, TC = − 2.5 ± 2.0, CC = − 3.1 ± 0.7; $p = 0.2$). In contrast, in the ER-α1 *Xba*I polymorphism, the genotypes of osteopenic postmenopausal women (AA = − 1.1 ± 1.1, AG = − 1.6 ± 0.6, GG = − 1.5 ± 0.5; $p = 0.03$*) showed a significant association with BMD (Table 6). The osteoporotic and control groups (osteoporotic AA = − 2.9 ± 1.3, AG = − 2.5 ± 2.1, GG = − 2.6 ± 1.7; $p = 0.5$, control AA = − 0.04 ± 0.8, AG = 0.1 ± 1.0, GG = 0.02 ± 0.6; $p = 0.6$) showed that there was no association of the *Xba*I polymorphism with BMD. Although the *Xba*I polymorphism of the ER-α gene showed an association ($p = 0.03$) with BMD in the postmenopausal osteopenic group, its association with BMD seems to be negligible and this must be further addressed.

Association Between *BstB*I Genotypes with BMD

Table 6 gives the association of *BstB*I genotypes with osteoporosis, osteopenia and the normal bone mass for *BstBI*. The *T*-scores of BMD in the osteoporotic subjects were significantly lower in the osteoporotic women with AA group than in the osteoporotic women with GA and GG (AA = − 3.3 ± 0.7, GA = − 2.1 ± 2.3, GG = − 2.9 ± 1.4; $p = 0.01$*). In contrast, the *BstB*I genotypes between the

Table 6 The relationship between the RFLPs and the bone mineral density for *Apa*I, *Taq*I, *Bsm*I, *Pvu*II, *Xba*I and *BstBI* amongst the study groups using one-way (ANOVA)

Variables	*Apa*I polymorphism								
	Controls			Osteopenic			Osteoporotic		
	TT	TG	GG	TT	TG	GG	TT	TG	GG
BMD	0.2 ± 52.8	− 0.05 ± 0.8	− 0.05 ± 0.5	− 1.5 ± 0.6	− 1.3 ± 0.9	− 1.2 ± 1.1	− 3.1 ± 1.3	− 2.5 ± 1.9	− 2.6 ± 1.6
p	0.3			0.4			0.2		
Variables	*Taq*I polymorphism								
	TT	TC	CC	TT	TC	CC	TT	TC	CC
BMD	− 0.02 ± 0.85	− 0.01 ± 0.64	0.27 ± 1.26	− 1.2 ± 1.1	− 1.5 ± 0.6	− 1.4 ± 0.8	− 3 ± 2.1	− 2.6 ± 1.5	− 2.8 ± 1.5
p	0.3			0.5			0.6		
Variables	*Bsm*I polymorphism								
	AA	AG	GG	AA	AG	GG	AA	AG	GG
BMD	− 0.05 ± 0.63	0.11 ± 1	0.02 ± 0.9	− 1.6 ± 0.3	− 1.3 ± 0.9	− 1.4 ± 0.9	− 1.9 ± 2.5	− 2.9 ± 1.4	− 3.2 ± 0.7
p	0.7			0.5			0.02*		
Variables	*Pvu*II polymorphism								
	TT	TC	CC	TT	TC	CC	TT	TC	CC
BMD	0.6 ± 3.6	0.1 ± 0.9	0.1 ± 0.9	− 1.5 ± 0.8	− 1.3 ± 0.8	− 1.4 ± 0.8	− 2.9 ± 1.5	− 2.5 ± 2.0	− 3.1 ± 0.7
p	0.5			0.6			0.2		
Variables	*Xba*I polymorphism								
	AA	AG	GG	AA	AG	GG	AA	AG	GG
BMD	− 0.04 ± 0.8	0.1 ± 1.0	0.02 ± 0.6	− 1.1 ± 1.1	− 1.6 ± 0.6	− 1.5 ± 0.5	− 2.9 ± 1.3	− 2.5 ± 2.1	− 2.6 ± 1.7
p	0.6			0.03*			0.5		

Table 6 (continued)

Variables	*BstBI* polymorphism								
	GG	GA	AA	GG	GA	AA	GG	GA	AA
BMD	0.2 ± 0.8	− 0.04 ± 0.8	0.06 ± 1.1	− 1.3 ± 0.9	− 1.5 ± 0.8	− 1.3 ± 0.7	− 2.9 ± 1.4	− 2.1 ± 2.3	− 3.3 ± 0.7
p	0.6			0.4			0.01*		

**p* value < 0.05 statistically significant-(one-way ANOVA); COLIA1-SpI polymorphism data is not shown since the TT genotype was not observed in the study population

osteopenic and control postmenopausal women did not have a significant association with BMD.

Association Between Collagen Type I alpha 1 (COLIA1) Sp1 Polymorphism Genotypes with BMD

The present study examined the role and relevance of the Sp1 polymorphism in the first intron of the COLIA1 gene (rs1800012) in PMO and their association with BMDs at the calcaneus, which has not been previously reported from Tamil Nadu, south India. Our study reports that the *Sp*1 polymorphism does not influence the risk of osteoporosis amongst control (GG = 0.07 ± 0.9, GT = 0.04 ± 0.9; $p = 0.8$), osteopenic (GG = − 1.43 ± 0.8, GT = − 1.48 ± 0.8; $p = 0.7$) and osteoporotic (GG = − 2.6 ± 2, GT = − 2.9 ± 1.2; $p = 0.3$) (data not included in Table 6) postmenopausal women of Tamil Nadu.

Gene to Gene Interaction Analysis

The results of multiple linear regression analyses amongst the study groups (included as supplementary file 2). The most promising gene–gene interactions were *Apa*I × *Bsm*I and *Xba*I × *BstB*I for BMD ($p = 0.013$* and $p = 0.008$**, respectively). The overall interactions at the intercept amongst the SNPs showed a significant association with BMD (Adj $R^2 = 0.1197$, $p = 0.0005$*) in the regression model used for the analysis.

Discussion

SNPs are rapidly becoming informative genetic markers for disease susceptibility since they are scattered throughout the genome and show a high degree of variability. This case–control study was undertaken to investigate: (i) the role of VDR, ER-α1, PTH and COLIA1genes in south Indian postmenopausal women of Tamil Nadu, and (ii) their associations with PMO, a multi-factorial disorder. This study aimed to determine the genetic variants amongst the genes and their contributing risk factors in this targeted population. A total of seven polymorphic variants were selected and evaluated for this study. These variants have been established as PMO risk factors. Our specific area of interest was to determine if their effect was similar or varied in comparison to other populations and thereby to ascertain their combined effect. The analysis in this regard was done with the cases and controls with well defined inclusion criteria.

Polymorphisms in the VDR Gene and BMD

Vitamin D assumes a significant part in the maintenance of Ca^{2+} signalling pathways and reduction in the levels of vitamin D could trigger a few diseases such as cardiovascular, diabetes and osteoporosis (Ghazizadeh et al. 2020). Gene expression

array examination has shown that, Vitamin D can direct up to 5% of the human genome and various observational investigations have recommended a relationship amongst factors like BMI, BMD and Vitamin D insufficiency (Sharifan et al. 2021). The data obtained for the SNP in the VDR gene of our study are unlikely to be considered a determining factor associated with BMD (Table 6). The association between the VDR gene and BMD was also largely inconsistent when compared to the significant associations reported amongst Korean, Thai and Caucasian postmenopausal women (Peacock 1995; Kurabayashi et al. 1999). In this study, genotype GG of the *Bsm*I VDR gene was correlated with a low BMD in the osteoporotic postmenopausal groups (Table 6). Similar results were reported by Mitra et al. (2006a) from women of a Maharashtrian ethnic origin. Another study by Farkhondeh et al. (2013) also reports that the GG genotype of the *Bsm*I VDR gene was significantly associated with BMD in the lumbar spine and could be a potential genetic marker of BMD in Iranian postmenopausal women vulnerable to the disease. In the LD analysis, all three SNPs showed very strong evidence of recombination amongst the groups. However, the linkage of *Apa*I, *Taq*I and *Bsm*I in the osteopenic and osteoporotic groups did not reach a strong level of expectancy. Haplotype analysis and further permutation tests revealed that the most frequent haplotype was ACT amongst the osteoporotic subjects and it was less common amongst the control subjects, whilst haplotype ATT was most frequent for osteopenic subjects (included in supplementary file 1). Though this study did not give the expected results of the linkage amongst the *Apa*I, *Taq*I and *Bsm*I variants, earlier studies show an evidence of strong linkage between the above mentioned three variants of the VDR gene located within the chromosomal regions 12q12. The study conducted in Belarusian and Lithuanian women highlighted the greatest degree of LD between *Apa*I and *Bsm*I, followed by *Bsm*I and *Taq*I and lastly between *Apa*I and *Taq*I (Marozik et al. 2018). A similar trend was reported in a Caucasian population (Uitterlinden et al. 2004) and a British population (Chen et al. 2009), and it agreed with the observations made in an Italian population (Thakkinstian et al. 2004b). Most likely, the strong LD coefficient may be explained by the location of these three SNPs at the 3′ end of the VDR gene, which are likely to have adaptive proximity.

Polymorphisms in the ER α1 Gene and BMD

Since the physiological functions of oestrogen are mediated through specific oestrogen receptors, genetic variations in these receptors will influence the risk of osteoporosis. No significant association between the genotypes of the *Pvu*II and *Xba*I polymorphisms and BMD was found in the postmenopausal women in our study. Similar observations were reported by Saoji et al. (2019) and Fa et al. (2009) in their study on postmenopausal and premenopausal women from northeast India and from Iranian Women, respectively. Subjects with AA and TT genotypes amongst the study groups of postmenopausal northern Indian women of Maharashtrian ethnic origin had higher average BMDs than those with CC and GG genotypes (Mitra et al. 2006b). The south Indian population of pre- and postmenopausal women from Andhra Pradesh also showed that individuals with CC genotypes had a significantly

lower average BMD than those with TT genotypes for *Pvu*II polymorphisms. However, for the *Xba*I polymorphism, the individuals with the AA genotype amongst the pre- and postmenopausal groups showed a comparatively higher BMD than those with the GG genotype (Jeedigunta et al. 2010). The results of the above mentioned studies do not confirm the results of the present study. The T allelic frequency was found to be higher in the controls ($T=0.6$) for *Pvu*II polymorphisms than in the osteoporotic postmenopausal women ($T=0.5$) (Table 1). However, these results for *Pvu*II are in agreement with those of Mitra et al. (2006b) but not for *Xba*I. The frequency of the AA allele in the *Xba*I polymorphism was found to be higher in the osteoporotic group ($A=0.7$). Studies on Danish and Korean postmenopausal women affirmed an association of A alleles with osteoporosis (Han et al. 1999a).This study revealed no significant LD between the *Pvu*II and *Xba*I polymorphisms, as seen in Table 4. In the LD analysis, both SNPs showed strong evidence of recombination amongst the groups. Haplotype analysis and further permutation tests revealed the following data: (i) amongst the subjects, the TA haplotype was the most frequent, whilst the CG haplotype was the least frequent. Some studies (Weel et al. 1999) reported haplotype CG to be associated with a decreased BMD in postmenopausal women whilst haplotype TA was associated with an increased BMD. Haplotype TG did not show any such association. A few researchers have indicated no association between the 2 haplotypes (Mitra et al. 2006b). The development and progression of various diseases, including osteoporosis, can be attributed to these genotypic variations. However, the underlying mechanism by which these genetic differences influence BMD remains to be clearly determined (Thakkinstian et al. 2004b). How the receptor functions are influenced by an intronic polymorphism of the ER-α gene is not well defined, although there is a probability that its position in an intron near the gene promoter could play a key role in either transcriptional regulation of mRNA processing or the transcript's stability.

Polymorphisms in the PTH Gene and BMD

The regulating effect of PTH on bone metabolism and its role as an anabolic agent was considered as the basis to analyse the hypothesis that variations in the *BstB*1 polymorphism of the PTH gene are associated with low BMD in postmenopausal women. Association analysis of polymorphisms of candidate genes can be a useful tool to identify the genetic risk of osteoporosis (2006). The present study the osteoporotic women with *BstB*I-AA recessive genotype has a lowered BMD at the calcaneus than the osteoporotic subjects with the GG and GA genotype. A similar finding was reported amongst Asian Indians by Vupputuri et al. (2006) but differed from those reported in the Chinese and Japanese populations (Hosoi et al. 1999; Li et al. 2003). However the study made by Wynne et al. (2003) on Irish population reported a linkage contribution of the PTHR1 locus to low BMD. Evaluating SNPs in the PTHR1 gene, Giroux et al. (2010) did not find any association with either lumbar spine or femoral neck BMD. A similar study conducted by Zhang et al. (2006) on SNPs for linkage association of the PTHRI gene BMD with 1873 subjects from 405 Caucasian nuclear families showed no significant results for individual

SNP. We made a study of 300 unrelated postmenopausal women with limited range of life style variations in one of the prefectures (Madurai district) in south India to examine the *BstB*I RFLP in the PTH gene. Our findings revealed that the effects of environmental factors, e.g. food intake and other life style variation did have a considerable impact on BMD and bone metabolism which cannot be ignored. The *BstBI* polymorphism used in this study could be a genetic marker for low BMD and susceptibility to osteoporosis. To identify the genetic risk factors for osteoporosis on a molecular basis, an association study with polymorphisms of candidate genes could be of value. However, the limitations observed in this approach ought to be considered because of the polygenic characteristics of BMD. Hence, a more in depth study of the *BstB*I PTH gene polymorphism will be ideal.

Polymorphisms in the COLIA1 Gene and BMD

The study in Caucasian populations reported the Sp1 binding site polymorphism of the collagen type Iα1 gene to be associated with reduced BMD and increased susceptibility to bone fractures. The study conducted by Grant et al. (1996) on two female British populations reported that a lower BMD at the lumbar spine in the G/T heterozygotes subjects than G/G homozygotes, whilst a still lower BMD was found in the T/T homozygotes and the presence of the T allele was a higher risk factor for osteoporotic fractures. A comparative study of BMD levels at the lumbar spine and femoral neck region was made by Uitterlinden et al. (2001) on a larger population of postmenopausal women with various genotypes. The subjects with GT genotypes comprising 44% of the population had only 2% lower BMD at the lumbar spine; TT subjects had 6% lower BMD at the lumbar spine and 4% lower BMD at the femoral neck when compared to their GG genotypes counterparts. However, the study made by Liden et al. (1998) on a sample population of healthy and osteoporotic Swedish women reported no such association of the GT and TT genotypes with BMD or the risk of osteoporotic fractures. A similar study evaluating the differences in the BMD between various genotypes in a healthy female postmenopausal French population were small and became negligible in the subsequent multivariate analysis (Garnero et al. 1998). In our study the GT genotype was of very low frequency and there was no TT genotype reported in any of the subjects. However, there was no significant association with BMD. The results of studies amongst the black subjects of south Africa (Ojwang et al. 2001) and those of the Sikkimese men and women in north-east India (Soibam et al. 2019) and our study show relative similarities indicating no significant association with low BMD. This study is not in tune with the results of Singh et al. (2013) amongst the women of North West India, which reports a significant association between this polymorphism and low bone density. A similar association was also reported in postmenopausal Korean women (Han et al. 1999b) and a study group of healthy north-eastern Chinese women (Beavan et al. 1998). A favourable genetic background is a major contributing factor. Genetic differences could probably play a role in ethnic diversity as observed amongst Caucasian population amongst whom the major part of the studies were performed. With regard to bone metabolism, the Asian population differs from the Caucasians in many aspects.

Reduced fracture risk as observed amongst the Asians may be due to the influence of factors such as increased fractional intestinal calcium absorption (Kung et al. 1998), shorter hip axis length and differences in lifestyle (Lau and Cooper 1996). Of the 300 samples analysed concerning the sp1 polymorphism of the collagen type I α 1 gene, none of the samples in our study indicated the presence of the TT genotype. These findings lead to the assumption that, differences observed in the genotypic background concerning the sp1 polymorphism could be a governing factor for the lower fracture risk in Asians where the T allele is found to be very rare as against the western population where there is a high frequency of the T allele.

Gene-to-gene Interactions by Multiple Linear Regression Analysis

The establishment of gene interaction model amongst the VDR, ER α1, PTH and COLIA1 genes for BMD susceptibility by multiple linear regression analysis was an added breakthrough in our study. The best gene–gene interaction model identified amongst the seven SNPs of the four candidate genes was a two-way interaction model which included the SNPS as variables. The combination of *Apa*I × *Bsm*I and *Xba*I × *BstB*I was seen to be associated with low BMD in this model. The interaction of *Apa*I × *Bsm*I was also found to be significant in a study of north Indian women reported by Mitra et al. (2006a). A synergistic interaction amongst these SNPs further indicates an influencing role of epistasis on BMD.

Conclusion

This study supports the significant role of rs1544410 and rs6254 polymorphisms and its significant allelic association with BMD either individually or in different combinations pertaining to osteoporosis susceptibility amongst post-menopausal women from the south Indian population of Tamil Nadu. Reviews of data from previous meta-analysis on this topic of study lacks relevant data from the Indian subcontinent. Thus, this study with the objective to determine the influencing effect of these polymorphisms individually and cumulatively on BMD could be considered as the first of its kind reported from south Indian population from Tamil Nadu. However, it is mandatory that further replication studies are undertaken to establish and validate newer susceptibility loci obtained from large scale GWAS in independent samples from other ethnic groups.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Not applicable.

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