International Journal of Biomedical Research
ISSN: 0976-9633 (Online); 2455-0566 (Print)
Journal DOI: https://dx.doi.org/10.7439/ijbr
CODEN: IJBRFA **Original Research Article**

Vitamin D Receptor Polymorphisms of ApaI (rs7975232) and TaqI (rs731236) in South Indian Postmenopausal Osteoporotic and Healthy Women

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***Article History:**
Received: 16/01/2017
Revised: 01/02/2017
Accepted: 01/02/2017
DOI: https://dx.doi.org/10.7439/ijbr.v8i1.3859

Abstract

Two adjacent single nucleotide polymorphisms of the vitamin D receptor gene (VDR) ApaI (rs7975232) and TaqI (rs731236) are commonly studied in several pathologies. We aimed to evaluate the distribution of VDR ApaI, and TaqI allele and genotype frequencies in 50 cases of postmenopausal osteoporotic (cases) and 50 postmenopausal healthy women (controls). The polymorphisms were detected using PCR – RFLP. ApaI (rs7975232) polymorphism genotype frequencies were AA 68.08%, Aa 25.53% and aa 6.38% in postmenopausal osteoporotic women and AA 38.00%, Aa 46.00% and aa 16.00% in postmenopausal healthy controls respectively ($\chi 2 = 8.96$, $p = 0.011$). Taq l (rs731236) polymorphism genotype frequencies were TT 40.00%, Tt 56.00% and tt 4.00% postmenopausal osteoporotic women and TT 4.00%, Tt 64.00% and tt 32.00% in postmenopausal healthy women ($\chi 2 = 25.88$, $p = 0.000002$). The Odds ratio for ApaI (rs7975232) polymorphism was 2.8148 with 95 % CI: 1.1207 to 7.0696 and a significance level $p = 0.0276$ and for TaqI (rs731236) polymorphism was 3.7778 with 95 % CI: 1.6496 to 8.6514 and a significance level $p = 0.0017$. Our study confirmed that the odds of getting osteoporosis were 2.8 fold greater when the 'A' allele is inherited for ApaI (rs7975232) polymorphism and 3.7 fold greater when the 'T' allele is inherited for TaqI (rs731236). In conclusion our study has proved to a certain extent that the VDR gene polymorphisms ApaI (rs7975232) and TaqI (rs731236) might be important genetic markers in determining postmenopausal risk of osteoporosis in a population of south Indian women.

Keywords: Osteoporosis, Polymorphisms, VDR, ApaI, TaqI, PCR-RFLP.

1. Introduction

Osteoporosis is a disorder in the skeletal system caused by a decrease in bone mass and micro-architectural deterioration of the bone tissue, which results in bone fragility and increases the risk of fracture. The main problem with osteoporosis is that it is a very common disorder, progresses silently and an often remains asymptomatic and undetected until fracture occurs to indicate a possibility of the disease. The amount of bone tissue in the skeleton, known as bone mass, drops shortly after puberty, at about 18 years, and then increases gradually until age 30. This is known as peak bone mass for at that point, bones have reached their maximum strength and density. Gender is an important factor affecting bone mass. Peak bone mass tends to be higher in men than in women. While before puberty, both boys and girls acquire bone mass at similar rates, after puberty, however, men tend to acquire greater bone mass than women [1]. Moreover, women tend to experience minimal change in total bone mass between age 30 and menopause. But in the first few years after menopause, most women go through rapid bone

loss which then slows but continues throughout the postmenopausal years. This loss of bone mass can lead to osteoporosis. Osteoporosis leads to bones with less tensile strength and significantly more susceptibility to fracture with less force. At some point, the amount of bone available for mechanical support falls below ‘‘fracture threshold’’ and the patient may sustain a fracture [2].

A bone mineral density (BMD) test is used to identify osteoporosis and determine risk for fractures. The most widely recognized BMD test is called a central dual-energy x-ray absorptiometry, or central DEXA test. A BMD test measures bone mineral density and compares it to that of a standard to give a score. Although no bone density test is 100-percent accurate, the BMD test is an important predictor of whether a person is susceptible to having a fracture in the future. BMD test results are compared to the peak bone mineral density of a postmenopausal healthy 30-year-old adult, and give a T-score. A score of 0 means BMD is equal to the norm for a postmenopausal healthy young adult. Differences between a BMD and that of the postmenopausal healthy young adult norm are measured in units called standard deviations (SDs). The more standard deviations below 0, indicated as negative numbers, the lower BMD and the higher the risk of fracture. The World Health Organization (WHO) defines the categories for diagnosis as

- Normal (T-score -1.0 and above)
- Low bone mass, referred to as osteopenia (T-score between -1.0 and -2.5)
- Osteoporosis (T-score -2.5 and below)
- Severe osteoporosis (T-score -2.5 and below with history of a fracture) [3]

Osteoporosis is a serious disease all over the world and it affects more than 75 million people in Europe, Japan and the USA [4]. Osteoporosis causes more than 8.9 million fractures annually worldwide, of which more than 4.5 million occur in the Americas and Europe. In India the number of people affected with osteoporotic fracture above the age of 50 is approximately 163 million. This number is expected to increase to 230 million in 2015 and such more in the years to come [5]. Given the knowledge that high peak bone density reduces osteoporosis risk later in life, it makes sense to pay more attention to those factors that affect peak bone mass.

Risk factors for osteoporosis include unchangeable risks like sex, age, race, family history; hormonal factors including sex hormones, thyroid, parathyroid and adrenal glands; dietary factors like low calcium intake, long term medications like steroid; life style choices like sedentary life style, excessive consumption of alcohol and tobacco etc. Of these genetic factors are considered to be major contributors to the pathogenesis of postmenopausal osteoporosis. Various candidate genes have been reported to be associated with osteoporosis including genes involved in matrix protein molecules, matrix associated enzymes, Calciotropic (Steroid) Hormone/Receptors/Enzymes, Growth Factors/Cytokines/Receptors, Wnt-signalling pathway and Homocystein pathway [6].

1.2 Vitamin D (1,25- Dihydroxyvitamin D3) Receptor (VDR) Gene

Bone cells are regulated for growth and differentiation by 1,25-Dihydroxyvitamin D3 (1,25(OH) 2D3, calcitriol), the biologically most active naturally occurring metabolite of vitamin D. Vitamin D deficiency is associated with several common diseases, including bone related diseases, diabetes, cardiovascular diseases, autoimmune diseases, tuberculosis and cancer as indicated by epidemiological and laboratory investigations [7].

The vitamin D (1, 25- dihydroxyvitamin D3) receptor (VDR) gene is a prominent candidate gene for the regulation calcitriol entry into the cell and hence of bone mass remodelling particularly in postmenopausal women. Polymorphism of the vitamin D receptor (VDR) gene was considered as a risk factor for osteoporosis [8]. The association of VDR genotype and BMD may be different in various ethnic and geographical groups. The action of vitamin D depends on the functional status of the VDR, and its polymorphic variants. Studies have shown a close relationship between VDR gene polymorphism and the immunological action. The active hormonal form of 1,25-Dihydroxyvitamin D3 (1,25(OH) 2D3, calcitriol) plays an important role in activating monocytes, stimulating cell-mediated immunity, and suppressing lymphocyte proliferation [8]. Studies have proved that polymorphic variations at the VDR locus accounted for up to 75% of the genetic contribution to loss of bone mass but subsequent studies though suggesting a strong association between vitamin-D receptor (VDR) genotype and bone mineral density (BMD) in osteoporosis, conflict on the possible association between postmenopausal bone loss and the VDR genotype [9].

1.3 VDR Polymorphism

Subtle DNA sequence variations called mutations are abundant in the human genome and can have modest but real biological effects. A mutation becomes a polymorphism when the allele frequency reaches 1% in a mendelian population. The high frequencies of these polymorphisms are often studied extensively to explain the link between these variations in the risk for common disease. The humans genome carries a large number of polymorphisms in the form of single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs) and copy number variation (CNV) which may lead to different cellular effects due to various mechanisms altering gene expression by enhanced/reduced transcription involving promoter/

enhancer/ silencer, altered posttranscriptional or posttranslational activity affecting mRNA stability or changes in the tertiary structure of the gene product influencing the functional protein [10].

The vitamin D (1, 25-dihydroxyvitamin D3) receptor (VDR) is a member of the steroid hormone receptor superfamily of ligand-activated transcription factors. The VDR locus has been mapped to chromosome 12q13.1 and its genomic organisation shows that it is about 100 kb with an extensive promoter region capable of generating tissue specific transcripts. The gene is comprised of 11 exons, the noncoding 5'-end of the gene includes exons 1A, 1B, and 1C and eight additional exons (exons 2-9) that encode the structural portion of the VDR gene product. To date more than hundred polymorphisms have been reported within its 67076-bp sequence including the promoter region, in and around its eight exons 2-9, six alternatively spliced regions (1a–1f) and in the 3'UTR region. Only few polymorphisms of VDR gene have been studied through restriction fragment length polymorphisms (RFLP) and it has been indicated that these RFLPs may be linked to truly functional polymorphisms in the VDR gene (or in a nearby gene), which explains some of the associations observed for the VDR gene and various diseases [7]. VDR gene variants that were detected using the conventional restriction enzyme approach are the CDX-2, FokI, BsmI, Tru9I, ApaI, and TaqI discovered in the 3' end of the VDR gene (Figure 1). CDX-2 is located in the promoter region and can affect gene regulation. The start codon polymorphism of the FokI (rs2228570) site in exon 2 alters the start codon leading to a 424 amino acid protein instead of 427 amino acids. The VDR gene TaqI polymorphism (rs731236) is an RFLP at codon 352 in exon 9 of the VDR gene and is a synonymous mutation. The ApaI (rs 7975232) and the BsmI (rs 1544410) polymorphisms are RFLPs in intron 8 at the 3' end of the VDR gene [10].

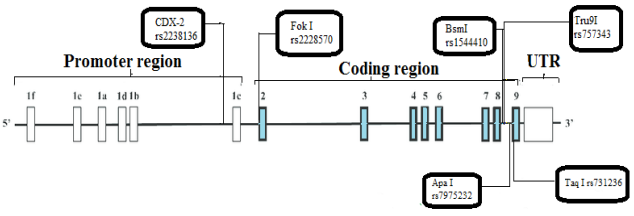

Figure 1: Structure of the genomic region of the VDR and location of the ApaI and TaqI polymorphisms.

Conflicting results were obtained from studies performed in different populations for association of VDR gene polymorphisms to osteoporosis. This lack of concordance can be attributed to a lack of statistical sample power, heterogeneity between different populations, population stratification, effects of other confounding factors and technical problems, such as lack of harmonisation of methodologies used in different studies. The present study has been taken up with an aim to determine the role of VDR ApaI (rs7975232) and TaqI (rs731236) polymorphisms in the development of osteoporosis in postmenopausal south Indian women from Tamil Nadu. Our preliminary study on BMD values showed that South Indian women were affected by osteoporosis in large numbers, especially in Hills of Kodaikanal because calcium level is lesser than the recommended level for potable water (Unpublished data). This case-control "association study" has been attempted to evaluate the contribution of VDR ApaI (rs7975232) and TaqI (rs731236) polymorphisms to the phenotype of interest at the population level in south Indian postmenopausal osteoporotic women (cases) and postmenopausal healthy women (controls).

2. Materials and Methods

2.1 Subjects

A case-control study was performed with 50 postmenopausal osteoporotic women (cases) and 50 postmenopausal healthy women (controls) with and age range of 50 – 70 years from the southern regions of Tamil Nadu. The investigation was done in accordance with the ethical principles outlined by the Indian Council of Medical Research (ICMR) guidelines for medical research involving human subjects. Ethical clearance was obtained from the Institutional Ethics Committee of Mother Teresa Women's University, Kodaikanal. All subjects were informed about the contents and aims of the study and gave their written consent. A detailed questionnaire was used when collecting samples to record their family history of fractures, osteoporosis symptoms such as join pains, back pain, arthritis etc. All volunteers were above the age of 18 years. The postmenopausal osteoporotic women (cases) were selected for the study from the health camps conducted by local hospitals using the BMD measurements and with a family history of fracture. The BMD values were obtained by Sunlight MiniOmni bone sonometer. Patients with the T score of 2.5 SD i.e <-2.5 below the young adult mean and postmenopausal were included in the study as post menopausal osteoporotic women patients and those with 1 SD (+1 or −1) of the young adult mean were used as postmenopausal healthy controls. Women with the conditions known to affect bone metabolism, i.e., diseases such as Paget's disease, osteogenesis imperfecta, rheumatoid arthritis, etc., or those using medications (glucocorticosteroids) were excluded from the study. The corresponding study controls were postmenopausal healthy women and did not have the following symptoms. Of these samples 47 postmenopausal osteoporotic women (cases) and 50 postmenopausal healthy women (controls) were used

for ApaI (rs7975232) polymorphism genotyping and 50 postmenopausal osteoporotic women (cases) and 50 postmenopausal healthy women (controls) were used for TaqI (rs731236) polymorphism genotyping.

2.2 DNA analysis

DNA was extracted from the mouth wash collected using a modified protocol of Ausubel *et al*. Genotyping TaqI (rs731236) and ApaI (rs7975232) polymorphisms (2000bp restriction site polymorphism) was done using PCR-RFLP with specific primers to amplify the fragment from the isolated DNA as per Flugge *et al*[11]. ApaI / TaqI primers F 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′ R 5′-CAC TTC GAG CAC AAG GGG CGT TAG C-3′ were used. Initial denaturation was at 95^{0}C for 2 minutes followed by 30 cycles of denaturation at 96^{0}C for 30 seconds, primer annealing at 56^{0}C for 60 seconds and extension at 72^{0}C for 135 seconds. A final extension of 72^{0}C for 5 minutes was given to complete the reaction in a Biorad T 100 ^{TM} Thermal Cycler. For restriction digestion of the PCR products the reaction mix was prepared as follows: 10X buffer 2X, restriction enzymes ApaI (rs7975232) and TaqI (rs731236) polymorphism 1U for a total volume of 20µl of the PCR product respectively in separate reaction tubes and incubated at 25°C for 1 hour and the product were analyzed by electrophoresis on 1.5% of agarose gel.

The genotypes for ApaI (rs7975232) and TaqI (rs731236) polymorphism were defined using the restriction endonucleases and were denoted A, and T indicating the absence of the restriction site or as a, and t indicating the presence of the restriction site respectively. The aa homozygous individual with the presence of the ApaI (rs7975232) polymorphism restriction site generates two fragments 1700 and 300 bp long, while the tt homozygous individual with the presence of the TaqI restriction site generates two fragments of 1800 and 200 bp lengths (Figure 2a and 2b). The absence of ApaI and TaqI restriction sites (AA and TT, respectively) leaves a 2000bp undigested fragment. A heterozygous individual exhibits all three bands, for the restriction sites Aa (2000, 1700, 300 bp) and Tt (2000, 1800, 200 bp) [12].

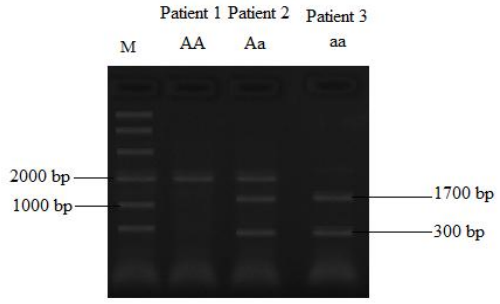

Figure 2a: Representative Gel of the Genotypes observed in ApaI (rs7975232) polymorphism.

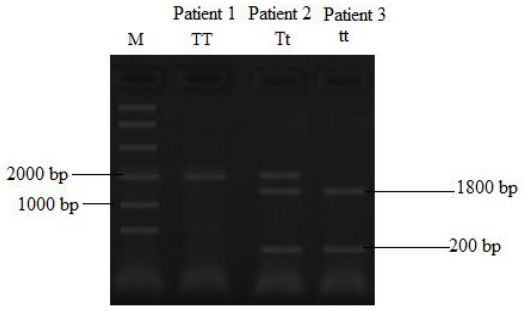

Figure 2b: Representative Gel of the Genotypes observed in TaqI (rs731236) polymorphism.

2.3 Statistical Analysis:

The Hardy–Weinberg equation was used for the assessment of the predicted genotype frequencies of the VDR gene polymorphism in postmenopausal osteoporotic women and postmenopausal healthy controls in south Indian population. Two-tailed Fisher's exact test was done to compare the allele and genotype frequencies using VassarStats. Odds ratio was calculated using Medcalc.

3. Results

Genotypic analysis in the VDR gene locus using the ApaI (rs7975232) and TaqI (rs731236) restriction endonuclease polymorphisms revealed six single genotypes. The distribution of the analyzed gene polymorphisms in the controls and in the patients with postmenopausal osteoporosis were in correspondence with the one expected from the Hardy-Weinberg equilibrium (P>0.05 in all cases), showing that the analyzed groups were selected correctly. The frequencies (%) of genotypes and alleles of the two loci in the groups studied are presented in Table 1 and 2.

Table 1: Genotype frequencies of VDR ApaI (rs 7975232) and Taq I (rs 731236) polymorphisms in the postmenopausal osteoporotic and postmenopausal healthy women

Subjects	ApaI (rs7975232) Genotype AA	Aa	aa	Total (N)	$\chi 2$	p Value
Postmenopausal Osteoporotic Women N (%)	32 (68.08)	12 (25.53)	3 (6.38)	47	8.96	0.011
Postmenopausal healthy Controls N (%)	19 (38.00)	23 (46.00)	8 (16.00)	50		
Subjects	TaqI (rs731236) Genotype TT	Tt	tt	Total (N)	$\chi 2$	p Value
Postmenopausal Osteoporotic Women N (%)	20 (40.00)	28 (56.00)	2 (4.00)	50	25.88	0.000002
Postmenopausal healthy Controls N (%)	2 (4.00)	32 (64.00)	16 (32.00)	50		

Out of the 94 subjects ApaI (rs7975232) polymorphism showed the following genotype frequencies: AA 68.08% (32/47), Aa 25.53% (12/47) and aa 6.38% (3/47) in postmenopausal osteoporotic women (cases) and AA 38.00 % (19/50), Aa 46.00% (23/50) and aa 16.00%

(8/50) in postmenopausal healthy women (controls) respectively. The observed ApaI (rs7975232) polymorphism genotype frequencies in cases and controls when analysed for chi square statistic using the Fisher's exact test with Yates correction showed $\chi 2 = 8.96$, $p = 0.011$ proving a statistically significant deviation in the two groups. Of the 100 subjects analyzed for TaqI (rs731236) polymorphism the genotype frequencies were as follows: TT 40.00% (20/50), Tt 56.00% (28/50) and tt 4.00% (2/50) for postmenopausal osteoporotic women (cases) and TT 4.00% (2/50), Tt 64.00% (32/50) and tt 32.00% (16/50) for postmenopausal healthy women (controls). The observed TaqI (rs731236) polymorphism genotype frequencies in cases and controls when analysed for chi square statistic using the Fisher's exact test with Yates correction showed $\chi 2 = 25.88$, $p = 0.000002$ proving a statistically significant deviation in the two groups.

The most frequently identified allele of ApaI (rs7975232) polymorphism was 'A' 80.00% (38/47) in postmenopausal osteoporotic women (cases) and 60.00% (30/50) in postmenopausal healthy women (controls). For TaqI (rs731236) polymorphism the most frequent allele was 'T' 68.00% (34/50) in postmenopausal osteoporotic women (cases) and t allele 64.00% (32/50) in postmenopausal healthy women (controls).

Table 2: Allele frequencies of VDR ApaI (rs 7975232) and Taq I (rs 731236) polymorphisms in the postmenopausal osteoporotic and postmenopausal healthy women

Subjects	ApaI (rs7975232) Allele A	a	Total (N)	$\chi 2$	p Value
Postmenopausal Osteoporotic Women N (%)	38 (80.00)	9 (19)	47	6.87	0.003
Postmenopausal healthy Controls N (%)	30 (60.00)	20 (40.00)	50		
Subjects	TaqI (rs731236) Allele T	t	Total (N)	$\chi 2$	p Value
Postmenopausal Osteoporotic Women N (%)	34 (68.00)	16 (32.00)	50	12.06	0.0005
Postmenopausal healthy Controls N (%)	18 (36.00)	32 (64.00)	50		

The Odds ratio for ApaI (rs7975232) polymorphism was 2.8148 with 95 % CI: 1.1207 to 7.0696 and a significance level p = 0.0276. The Odds ratio TaqI (rs731236) polymorphism allele was 3.7778 with 95 % CI: 1.6496 to 8.6514 and a significance level p = 0.0017. This proved that the odds of getting osteoporosis were greater with the 'A' allele for ApaI (rs7975232) polymorphism and 'T' allele for TaqI (rs731236) polymorphism (Table 3).

Table 3: Odds Ratio for ApaI (rs 7975232) and TaqI (rs 731236) polymorphism in postmenopausal osteoporotic and postmenopausal healthy women

	ApaI (rs7975232) allele	TaqI (rs731236) allele
Odds ratio	2.8148	3.7778
95 % CI:	1.1207 to 7.0696	1.6496 to 8.6514
z statistic	2.203	3.144
Significance level	P = 0.0276	P = 0.0017

4. Discussion

Osteoporosis is considered as a complex interaction between subtle genetic polymorphisms and environmental influences and has become an important issue in this century as a consequence of improved health care [13]. Vitamin D functions as a potent regulator of calcium homeostasis and plays a prominent role in immunomodulation, cellular differentiation and replication in different target tissues [14]. A trans-acting transcriptional activator the VDR, through its dihydroxylated metabolite (1, 25-dihydroxyvitamin D) regulates expression of target genes. Thus, VDR gene polymorphisms have been associated with multiple traits and disease phenotypes like osteoporosis, Type I diabetes mellitus, Grave's disease, and primary hyperparathyroidism [1,15-19].

However, contrasting results of association of VDR polymorphisms to osteoporosis have been reported in some populations. Zajockove *et al*., 2002, Duman *et al.*, 2004, Mercado *et al.*, 2013 and Martinez *et al*., 2015 on VDR ApaI (rs7975232) polymorphism have found no association of this polymorphism to osteoporosis risk. Marozik *et al*., 2013 and Mitra *et al*., 2006 reported an association of AA genotype with osteoporosis while, Douroudis *et al*., 2003 reported an association of aa genotype with osteoporosis. For the TaqI (rs731236) polymorphism Zajockove *et al*., 2002, Gursoy *et al.*, 2008, Marozik *et al*., 2013 and Mercado *et al*., 2013 showed no association to osteoporosis, while Douroudis *et al*., 2003 showed an association of the TT genotype with osteoporosis. Mitra *et al*., 2006 and Duman *et al*., 2004 showed the association of tt genotype with osteoporosis. It has been suggested some of these contrasting results may have been due to insufficient sample size or because of possible genetic effects were masked by different gene-gene and gene-environment interaction. The inconsistent findings between our study and the data reported in literature are likely related to both the ethnic differences among the study populations and to the different inclusion criteria, as well as to the lack of a standardized approach to define pathological phenotypes [28].

This was the first study to explore the association of VDR genotypes with osteoporosis in postmenopausal women from south Indian region that are not only ethnically different from other Asian or South East Asian populations but also different from North and East Indian populations. The result shows that there is a significant association between the presence of ApaI (rs7975232) polymorphism AA genotype (p = 0.011) and TaqI (rs731236) polymorphism TT genotype (p = 0.000002) variants and the risk for osteoporosis. Our study confirmed the odds of getting osteoporosis is 2.8 fold greater when the 'A' allele is inherited for ApaI (rs7975232) polymorphism and 3.7 fold greater when the 'T' allele is inherited for TaqI (rs731236).

In conclusion our study has proved to a certain extent that the VDR gene polymorphims ApaI (rs7975232) and TaqI (rs731236) polymorphisms might be important genetic markers in determining postmenopausal risk of osteoporosis in a population of south Indian women. Furthermore, the observation that the association outcomes between VDR ApaI and TaqI genotypes and the exact mechanism underlying the associations we describe here remains to be elucidated, as does the relative importance of other genetic and environmental variables, our observations here should be considered as preliminary.

Acknowledgements

This research was supported by the University Grants Commission - Major research project funding. F.No.43-92/2014 (SR) dated 6.10.2015.

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