Original Article

Molecular genetic variations in vitamin D receptor gene with risk of osteoporosis in postmenopausal women

Sumbal Zahid, Mumayyiza Tahir, Sameen Ahmed

Emergency Department, Sir Ganga Ram Hospital, Lahore and Sheikh Zayed Hospital, Lahore University Institute of Physical Therapy, The University of Lahore, Institute of Biochemistry & Biotechnology, University of the Punjab, Lahore and Centre for Applied Molecular Biology (CAMB), University of the Punjab, Lahore, Pakistan

Objective: To investigate the association of Vitamin D receptor (VDR) gene polymorphisms and development of osteoporosis.

Methodology: This case-control study was conducted at Sir Ganga Hospital and Sheikh Zayed Hospital, Lahore, Pakistan from January 2015 to August 2015. A total of 136 postmenopausal women between 46-75 years of age were included in the study while women with serious internal disease and premature (surgical) menopause before the age of 45 years were excluded. Genotyping of VDR *Apa*I, *Taq*I and *Bsm*I loci was done using polymerase chain reaction-restriction fragment length polymorphism. Levels of ionized calcium, C-reactive protein, alkaline phosphatase were measured and body mass index was calculated. Statistical analysis was done by using SPSS version 16.0.

Results: Percentage of AA genotype was higher (28%) as compared to controls (16.6%). The postmenopausal cases showed 54% TT, 42%Tt and 4% tt genotype. The Bb genotype (42.6 %) was most frequent in both cases and controls. Postmenopausal cases and controls showed non-significant difference in alkaline phosphatase, C-reactive protein and ionized calcium levels.

Conclusions: Findings explained the earlier inconsistent association results and no particular genetic variation in Vitamin D receptor gene had pronounced effect in predisposition to osteoporosis. (Rawal Med J 201;42:286-290)

Keywords: Osteoporosis, postmenopausal women, vitamin D receptor gene polymorphisms.

INTRODUCTION

Osteoporosis is a bone disease that is characterized by decreased bone density.[1] According to the World Health Organization, 200 million men and women have T score less than -2.5 SD in the hip or lumbar spine and suffer from osteoporosis.[2,3] Changes in the production of estrogen, due to menopause, play role in regulation of the bone forming and bone dissolving cells.[4] Estrogen has an impact on density of bone by slowing down bone resorption. Deficiency of estrogen leads to decreased activity of enzyme 25(OH) Vitamin D α hydroxylase, converting 25-OH Vitamin D to 1, 25, $(OH)_2$ Vitamin D (potent Vitamin D). This vitamin D is a major regulator of bone and calcium homeostasis and maintains bone mineral density.[5,6]

See also pages 279, 281, 425

Bone mineral density (BMD) is a genetically linked trait and more than 100 candidate genes are found to be associated with it.[7,8] Among these candidate genes, the first extensively investigated one is vitamin D receptor (VDR).[2] The polymorphisms in the VDR gene were suggested to calculate the spinal and femoral BMD in Caucasian women.[9] VDR belongs to the nuclear receptor super-family of ligand-inducible transcription factors.[10] VDR is approximately 75 kb in length and is present on the long arm of 12 chromosome (3q11 locus). It consists of 11 exons, 29 of which are actively transcribed.[2] The three adjacent RFLPs for *Bsm*I, *Apa*I and *Taq*I, respectively, in intron 8/exon 9 at the 3' end of the VDR, have been most frequently studied so far.[11] The goal of present research was to study polymorphic sites in one of the osteoporosis predisposition genes i.e. *Apa*I, *Taq*I and *Bsm*I of the VDR and investigate the possibility of an association of these polymorphisms with menopausal osteoporosis.

METHODOLOGY

This case-control study was conducted at Emergency Department, Sir Ganga Ram Hospital,

Lahore and Outpatient Department, Sheikh Zayed Hospital, Lahore from January 2014 to August 2014. A total of 136 postmenopausal women between 46-75 years of age were included in the study through non-probability convenience sampling with their informed consent. Women having serious internal disease and premature (surgical) menopause before the age of 45 years were excluded. Information regarding physical investigation, physiological and demographic data of each subject was recorded.

Out of 136 postmenopausal women, 100 were cases and 36 were controls. Total subjects were divided into three age groups. i.e. 46-55 years, 56-65 years and 66-75 years. Out of 36 PM controls, 20 subjects were of 46-55 years, 14 were of 56-65 and 2 were of 66-75 years. The frequency of PM cases of age group 46-55 and 56-65 years was 50 and 34 respectively. Age group of 66-75 years had 16 cases.

Genomic DNA was isolated from leucocytes by phenol chloroform extraction method.[12] The Ap and Bs fragment were amplified by polymerase chain reaction. A single amplification was performed with primers spanning *Apa*I (intron 8) and *Taq*I (exon 9) polymorphisms PCR mixture was prepared by using 2.5µl of 10X PCR buffer, 2.5µl of 25mM $MgCl_2$, 1µl of 10mM dNTPs, 1.25µl of 20 pmol forward and reverse primer, 13µl of PCR water, 0.5µl of 5 U/µl *Taq* DNA polymerase (Thermo Scientific) and 3µl of template DNA. PCR products were analyzed on 2% agarose gel.

After amplification, PCR products were digested with ApaI and Mva1269I at 37 ?C and TaqI at 65?C (Fast digest, Thermo Scientific). The digested products were analyzed for the presence or absence of recognition sites by ethidium bromide staining of fragments separated through 8 % native polyacrylamide gel.

BMI was calculated and 18.524.9 was considered as normal. Ionized calcium was determined using ion-sensitive electrode (EDT Direct Ion Ltd, Part # 3041). Alkaline phosphatase (Randox, Catalogue # AP 542) and C-reactive protein (immunoassay test kit by BioCheck, Inc. Catalogue # BC-1119) were estimated from serum of 8 to 12 hours fasting postmenopausal women.

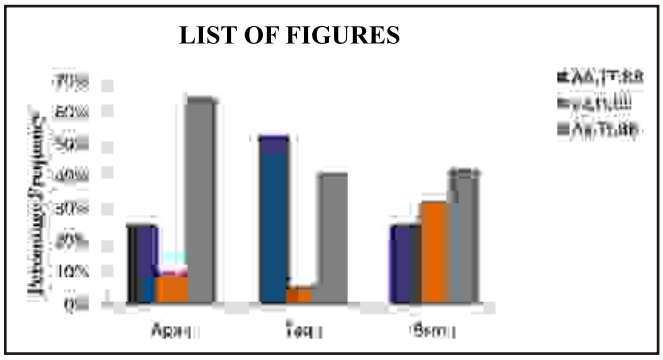
Statistical analysis was done by using SPSS 16.0. P<0.001 equalled highly significant difference and Pearson's correlation coefficient (r) was used to measure the strength of relationship between two variables.

RESULTS

A total of 136 postmenopausal subjects (controls n=36 and cases n=100) were examined. The study subjects were divided into three age groups; 46-55 years, 56-65 years and 66-75 years.

In total study population, *Apa*I and *Bsm*I homozygotes (AA and BB) were less (25%) in contrast to *Taq*I homozygotes (TT=52.9%). The percentage frequency of aa, tt and BB genotypes were found to be least (Fig.)

Fig 1: Distribution of *Apa*I, *Taa*I and *Bsm*I genotypes in PM subjects.



Genotypic distribution of Ap fragment of VDR gene showed that in all age groups, the most frequent genotype was Aa, 65.7% in age group of 46-55 years, 62.5% in age group of 56-65 years and 66.6% in 66-75 years of age group.

Table 1. Genotypic distribution of *Apa*I, *TaqI and BsmI* between postmenopausal cases and controls of three age groups.

	Genotypes	Age groups					
		46-55 (n=70)		56-65 (n=48)		66-75 (n=18)	
		Cases (n=50)	Controls (n=20)	Cases (n=34)	Controls (n=14)	Cases (n=16)	Controls (n=2)
ApaI	AA	16	2	10	2	2	2
	Aa	32	14	18	12	12	-
	aa	2	4	6	-	2	-
TaqI	TT	36	10	16	8	2	-
	Tt	14	10	16	2	12	2
	tt	-	-	2	4	2	-
BsmI	BB	16	2	8	-	8	-
	Bb	18	12	16	8	4	-
	bb	16	6	10	6	4	2

Table 2. Physiological and biochemical markers of bone turnover in relation to different age groups in the PM cases.

Variables	PM Cases (n=100)		
	46-55 years	56-65 years	66-75 years
Menopausal Age **	5.6 ± 5.0	12.1 ± 4.1	23.8 ± 7.5
BMI	25.42 ± 4.35	25.19 ± 2.59	27.93 ± 4.32
Alkaline Phosphatase (U/l)	671.3 ± 643	764 ± 618	557.4 ± 601.9
C-Reactive Protein (mg/l)	1.1 ± 0.43	1.02 ± 0.38	1.75 ± 2.7
Ionized Calcium (ppm)	24.2 ± 8.53	24.95 ± 8.18	23.3 ± 14.5

**p < 0.001=Highly significant difference

In three different groups of postmenopausal subjects based on age, TT genotype was high in age groups of 46-55 years (65.7%) and 56-65 years (50%). 66-75 years of age group had the most heterozygotes (Tt=77.7%). No tt was observed in 46-55 years of age group. Distribution of genotypes of *Bsm*I between postmenopausal cases and controls showed that % age frequency of BB genotype was least (25%) while Bb genotype was the major one found in both cases and controls. Frequency of Bb genotype was greater in all age groups except in age group of 66-75 years (Table 1).

PM cases (n=100) of all three age groups had highly significant difference in years since menopause. All women were overweight with a slight difference of BMI. The age group of 56-65 years had highest Alkaline Phosphatase (764 ± 618) and ionized calcium level (24.95 ± 8.18) while CRP levels (1.75 ± 2.7) and BMI (27.93 ± 4.32) were reported highest in 66-75 years of age group. Ionized calcium level (23.3 ± 14.5) was least in age range of 66-75 years as compared to other two groups (Table 2).

DISCUSSION

Osteoporosis is a major health problem in women after menopause generally over the age of 50.[13] Allelic variants of gene encoding VDR have been studied for genetic regulation of postmenopausal bone mass but the results are contradictory. The genotypic distribution of *Apa*I showed that the most prevalent genotype was Aa. These findings are contradictory to those reported by Xu et al., who observed aa as the major genotype in postmenopausal women.[14] In case of *Taq*I genotypes, the postmenopausal cases showed 54% TT, 42%Tt and 4% tt. These results were in accordance to Rizzoli et al.[15] Distribution of genotypes of *Bsm*I between postmenopausal cases and controls showed that percentage frequency of BB genotype was least (25%).The present study is in harmony with earlier findings of Zhang et al.[16]

Bone metabolic profile analysis showed that C-reactive protein (1.29±1.17) level and Alkaline phosphatase (664.2±620.9) level was higher in cases as compared to controls (0.96±0.65 and 515.9±337, respectively whereas ionized calcium level (24.15±10.4) was lower in cases than controls (25.86±7.06). CRP and ALP can be helpful as an early predictor of osteoporosis.[17] Higher levels of C-reactive protein (CRP) are associated with low bone mineral density in elderly females while Alkaline phosphatase higher levels are also indicative of menopausal alteration of bone turn over.[18,19]

In postmenopausal subjects (age 46-55 years), cases were overweight having BMI (25.42±4.35) comparative to controls (24.62±4.34). A highly significant difference ($p<0.001$) was present in menopausal age of this group. Early menopause of the cases can be the reason for susceptibility to joint pain. Results showed that 92% of the postmenopausal cases in this age group had positive record of joint pain. In this age group, Ionized calcium and BMI were found to be positively correlated (r= 0.1173). These findings are in contrast with earlier ones.[20] The ratio of sedentary to active cases was high in this age group leading to the possible explanation for positive correlation of ionized calcium and BMI.

In age group of 56-65 years, highest ALP levels (764±618) and ionized calcium levels (24.95±8.18) as compared to other age groups was observed. This discrepancy here enforces us to consider that these high ALP levels do not contribute to increased turnover of bone associated with menopause. The present study proposed that the reason for highest ALP in PM cases was due to other disorders as 8.2% cases of 56-65 years of age had renal problems while 6.3% had GIT infections.[1] In this age group, ionized calcium and ALP, CRP and BMI were negatively correlated.

The PM cases of the age group 66-75 years had

highest BMI (27.93±4.32). These subjects had least ionized calcium level (23.3±14.5). Reduced calcium levels lead to predisposition to bone related disorders including osteoporosis. Many factors, including pro-inflammatory cytokines, have been implicated in the pathogenesis of osteoporosis. Therefore, inflammatory marker like CRP increases in response to tissue damage, inflammation and infection.[19] Highest CRP levels (1.75 ± 2.7) in this age group were indicator of age related joint inflammation as compared to other groups.

CONCLUSION

No particular genotype of *VDR* had found to produce a relationship with osteoporosis. The inconsistencies found in the literature and the results obtained in the present work suggest us that other genetic and non-genetic factors are involved in the occurrence of osteoporosis, confounding the results of the possible association of osteoporosis and *VDR* polymorphisms.

Author Contributions:
Conception and design: Sumbal Zahid
Collection and assembly of data: Sumbal Zahid, Mumayyiza Tahir
Analysis and interpretation of the data: Mumayyiza Tahir, Sameen Ahmed
Drafting of the article: Sumbal Zahid, Mumayyiza Tahir
Critical revision of the article for important intellectual content: Sumbal Zahid, Sameen Ahmed
Statistical expertise: Sameen Ahmed
Final approval and guarantor of the article: Sumbal Zahid
Corresponding author email: Sumbal Zahid (sumbal_zahid3@hotmail.com
Conflict of Interest: None declared
Rec. Date: Feb 16, 2017 Revision Rec. Date: May 1, 2017 Accept Date: May 13, 2017

REFERENCES

1. Garnero P, Munoz F, Borel O, Sornay-Rendu E, Delmas PD. Vitamin D Receptor Gene Polymorphisms Are Associated with the Risk of Fractures in Postmenopausal Women, Independently of Bone Mineral Density. J Clinical Endocrinol Metabolism 2005;90:482935.
2. Horst-Sikorska W, Dytfeld J, Wawrzyniak A, Marcinkowska M, Michalak M, Franek E, et al. Vitamin D receptor gene polymorphisms, bone mineral density and fractures in postmenopausal women with osteoporosis. Mol Biol Rep 2013;40:38390.
3. Uzzan B, Cohen R, Nicolas P, Cucherat M, Perret GY. Effects of statins on bone mineral density: a meta-analysis of clinical studies. Bone 2007;40:1581-7.
4. Kosa JP, Balla B, Speer G, Kiss J, Borsy A, Podani J, et al. Effect of menopause on gene expression pattern in bone tissue of nonosteoporotic women. Menopause 2009;16:367-77.
5. Mitra S, Desai M, Khatkhatay MI. Vitamin D receptor gene polymorphisms and bone mineral density in postmenopausal Indian women. Maturitas 2006;55:2735.
6. Bolland MJ, Grey A, Avenell A, Gamble GD, Reid IR. Calcium supplements with or without vitamin D and risk of cardiovascular events: reanalysis of the Women's Health Initiative limited access dataset and meta-analysis. BMJ 2011;342:d2040.
7. Marozik P, Mosse I, Alekna V, Rudenk E, Tamulaitiene M, Ramanau H, et al. Association between Polymorphisms of VDR, COL1A1, and LCT Genes and Bone Mineral Density in Belarusian Women with Severe Postmenopausal Osteoporosis. Medicina (Kaunas) 2013;49:177-84.
8. Uysal AR, Sahin M, Gursoy A, Sevim GS. Vitamin D Receptor Gene Polymorphism and Osteoporosis in the Turkish Population. Genetic testing 2008;12:591-4.
9. Moran JM, Rodriguez-Velasco FJ, Roncero-Martin R, Rey-Sanchez P, Martinez M, Pedrera-Zamorano JD. The Relationship between Polymorphisms in the Vitamin D Receptor Gene and Bone Mineral Density in Postmenopausal Women. ISRN Genetics 2014. doi:10.1155/2014/549457
10. El-Badrawy ES, Ibrahim ZS, Aziz AA, Kamel MM, Shehab GM, Kamal A. Association of Vitamin D Receptors Genes Polymorphism (Apa I, and Taq I) with type 1 diabetes in Saudi Arabia (KSA). Life Sci J 2013;10:1555-62.
11. Massart F, Reginster JY, Brandi ML. Genetics of menopause associated diseases. Maturitas 2001;40:103116.
12. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucl Acid Res 1988;16:1215.
13. Ginecol M. Osteoporosis management in post-menopausal women. Minerva Ginecologica 2012;64:181-94.
14. Xu H, Xiong D, Xu F, Zhang Y, Lei S, Deng H. Association between VDR ApaI Polymorphism and Hip Bone Mineral Density Can Be Modified by Body Mass Index: A Study on Postmenopausal Chinese Women. Acta Biochimica Et Biophysica Sinica 2005;37:61-7.
15. Rizzoli R, Bonjour JP, Ferrari SL. Osteoporosis, genetics and hormones. J Molecular Endocrinol 2001;26:79-94.
16. Zhang H, Tao G, Wu Q, Liu J, Gao Y, Chen R, et al. Studies on the frequencies of vitamin D receptor gene polymorphism in postmenopausal women of Han and Kazak nationality in China. J Bone Miner Metab 2001;19:3213.

17. Jenkins DK. Bone alkaline phosphatase: a serum bone turnover assay: usefulness in managing postmenopausal woman receiving to prevent and treat osteoporosis. Quidal corporation 2001;1-8.
18. Cumming SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. N Engl J Med 2009;361:756-65.
19. Ganesan K, Teklehaimanot S, Tran TH, Asuncion M, Norris K. Relationship of C-Reactive Protein and Bone Mineral Density in Community-Dwelling Elderly Females. J National Med Assoc 2005;97:329-33.
20. Hooshmand S, Metti DM, Garcia SF, Vereda YS, Chai SC, Saadat RL, et al. Negative association between habitual dietary calcium intake, BMI, and body fat mass in postmenopausal women. The FASEB Journal 2012;26:lb401.