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Original Article

Association of Receptor Activator of Nuclear Factor kappa B Ligand (RANKL) (rs9533155, rs9533156) Gene Polymorphism with its Circulatory Level and Bone Mineral Density in Postmenopausal Women with and without Osteoporosis

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Abstract

Introduction: The gene that codes for the receptor activator of nuclear factor kappa B ligand (RANKL) has been identified as a key regulator of osteoclastogenesis. It plays a key role in the remodeling of bones by affecting bone resorption. **Objectives:** The current study aims to evaluate the association pattern of single nucleotide polymorphism (SNP) of RANKL gene polymorphisms (rs9533155 (693C>G), rs9533156 (643T>C)) with its circulatory level and Bone Mineral Density (BMD) in North Indian postmenopausal women. **Methods:** In this study, 165 postmenopausal osteoporotic women were enrolled as patients (age 54.44 ± 6.00 years) and 165 postmenopausal non-osteoporotic women were enrolled as controls (age 54.47 ± 6.46 years). The BMD of all recruited subjects was determined, followed by genetic analysis by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) method. RANKL levels were also measured using Enzyme-Linked Immunosorbent Assay. **Result:** The current study demonstrated that the subjects with the GG genotype of rs9533155 have significantly decreased average BMD at the lumbar spine and forearm and increased RANKL serum levels as compared to homozygous wild-type CC and heterozygous CG genotypes. Furthermore, subjects with the homozygous wild TT genotype of rs9533156 showed significantly lower BMD at the femoral neck and higher RANKL serum level as compared to homozygous mutant CC and heterozygous TC genotypes. No significant difference was found in the frequency distribution of genotypes and alleles of rs9533155 and rs9533156 among osteoporotic patients and controls. **Conclusion:** Our results suggest that RANKL polymorphism may be linked to BMD variation and osteoporosis development in north Indian postmenopausal women.

Keywords: *BMD, Osteoporosis, Polymorphism, Postmenopausal Indian Women, Receptor Activator of Nuclear Factor Kappa-B Ligand*

Introduction

Osteoporosis, which is represented by low BMD, is a chronic skeletal disease, attributed to elevated osteoclastic activity (bone resorption) as compared to osteoblastic activity (bone growth). Increased bone turnover leads to microarchitecture worsening of the bone tissue and increased fracture risk at spine, forearm, hip, and other skeletal sites (Cummings & Melton, 2002). Osteoporosis is a widespread condition that impacts individuals of all genders and ethnic backgrounds, with elderly women being particularly susceptible (Kanis *et al.,* 1994). Global estimates indicate that around 200 million women are affected by osteoporosis, with prevalence rates of one-tenth among those over 60, one-fifth among those over 70, two-fifths among those over 80, and two-thirds among those over 90

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(Kanis *et al.,* 2012). In India, as of 2015, it was reported that 20% of the 230 million women aged over 50 are afflicted by this disease (Malhotra & Mithal, 2008; Khadilkar & Mandlik, 2015). Bone Mineral Density is a key contributing factor for osteoporosis. Research involving twins and families suggests that heritability accounts for up to 80% of BMD, with genetic influences responsible for approximately 60% to 80% of the observed variations in BMD (Ferrari, 2008; Seeman *et al.,* 2006; Pocock *et al.,* 1987; Nguyen, Blangero & Eisman, 2000)

RANKL, which is alternatively named TRANCE (TNF-Related Activation-induced Cytokine), belongs to the tumor necrosis factor superfamily and is specifically categorized as TNFSF11 (Manolagas, 2000; Boyle, Simonet & Lacey, 2003; Jones, Kong & Penninge, 2002; Teitelbaum, 2004). The RANKL gene is on chromosome 13 (13q14.11), and it is expressed on the surface of osteoblasts. The RANKL gene encodes a 317-amino acid glycoprotein and is a leading member in osteoclastogenesis of bone remodeling, which attaches to its receptor RANK (Receptor Activator of Nuclear Factor-kB) resulting in increased bone loss and resorption (Nakashima, Hayashi & Takayanagi, 2012; Okamoto *et al.,* 2017; Gravallese *et al.,* 2000; Teitelbaum *et al.,* 2003; Saidenberg-Kermanac'h *et al.,* 2004; Lacey *et al.,* 1998; Kong *et al.,* 1999). Mencej *et al.* (2008) demonstrated a positive relation of these SNPs with imbalanced bone metabolism, low BMD, & osteoporosis onset. It is considered a probable gene that controls osteoporosis susceptibility (Ralston & de Crombrugghe, 2006). Genome-wide association studies (GWAS) suggested the RANKL gene has a susceptible locus that has been implicated to have a pioneer role in the regulation of bone mass (Styrkarsdottir *et al.,* 2008). The RANKL gene's promoter and untranslated region have also been demonstrated to contain single nucleotide polymorphisms that are linked to trauma fractures and bone mineral density (Hofbauer, 1999; Hsu *et al.,* 2006; Mencej *et al.,* 2008; Hofbauer *et al.,* 2000). Hofbauer *et al.* (2000) study reveals that the RANKL gene is a major candidate gene for the predisposition of postmenopausal osteoporosis. According to Wang *et al.* (2016), race and ethnicity are attributes of osteoporosis gene polymorphisms. The association between RANKL gene polymorphisms and bone mineral density has been reported globally, but the results vary and are controversial due to race and ethnic variations (Hsu *et al.,* 2006; Haryono *et al.,* 2019). The TNFSF11 gene polymorphisms & their correlation with bone mineral density at different bone sites in the north Indian population have not been investigated. From this perspective, new research on polymorphisms in different ethnic populations is required to substantiate the role of susceptibility loci of the RANKL gene. In order to determine whether there may be a link between the RANKL gene (rs9533155, rs9533156) polymorphisms & the incidence of postmenopausal osteoporosis in north Indian women, the current case-control study was designed.

Material & Methods

A total of 165 postmenopausal osteoporotic women (patients) (age: 54.44± 6.00) and 165 healthy postmenopausal women (controls) (age: 54.47± 6.46) who visited the orthopedics department of King George's Medical University, Lucknow, from December 2018 to April 2021 were recruited. Informed written consent was obtained from all enrolled subjects. We recorded each enrolled subject's medical history, demographic profile, and physical parameters on a predetermined study questionnaire. Subjects with osteoporosis as per WHO criteria and with age ≥ 45 years and who had not experienced menstruation for the last 1 year were enrolled as patients. Controls included age-matched (≥ 45 years) postmenopausal women without osteoporosis (normal BMD). Women on steroid therapy or hormone replacement therapy, women with diabetes, cancer, or any other degenerative disease that needs long-term treatment, and women with gynecological diseases that affect the release of female sex hormones were not included in the study. Ethical clearance was obtained from the ethical committee of King George's Medical University, Lucknow (KGMU- 97thECM II A/P3).

BMD Measurements

BMD (g/cm2) was measured by Dual energy X-ray absorptiometry (DXA) (Lunar Prodigy, Madison, WI, USA) in all subjects at the left skeletal sites of the femoral neck, hip, forearm, and lumbar spine L1-L4. As per the WHO (World Health Organization) criteria, bone mass is represented as BMD measurement; if the T score (peak bone density) is at or above -1 SD (standard deviation), then

young adult women are considered healthy, and women are considered osteoporotic if the T score is below -2.5 SD.

RANKL gene:

DNA isolation and RANKL genotyping

For genotyping of the RANKL gene, 5 ml of peripheral venous blood was collected from each enrolled subject; 2 ml of blood was withdrawn into EDTA vials (Ethylene Diamine Tetra Acetic Acid vials) which were used to isolate DNA by the salting-out method for genetic polymorphism study. Centrifugation at 3000 rpm for 5 minutes separated the serum from 3 ml of blood collected in a plain vial. The separated serum was stored at −20ºC for ELISA. Single Nucleotide Polymorphisms of RANKL gene (rs9533155 and rs9533156) were determined using PCR-RFLP method. To confirm the results, samples were reanalyzed.

Detection of RANKL Gene rs9533155 Polymorphism

To identify rs9533155 polymorphism of the RANKL gene, PCR was performed using primer F-5´ TTGTTTTCTCACTAAGAGCCACA 3´ and R-5´ TCCCAAATCCCTATTTCTGC 3'. 25 µl PCR reaction mixture was prepared containing 12.5 µl PCR master mix (GeneDireX), 2 µl genomic DNA, 1.5 µl of each primer (Forward & Reverse) (Eurofins), and 7.5 µl HPLC water. PCR was performed for 5 min at 94ºC followed by 30 cycles of 94ºC for 30 sec, 45ºC for 30 sec, extension at 72ºC for 1 min & final extension for 7 minutes at 72ºC. All the reactions were performed in an automated thermal cycler (BIO-RAD T100). C stands for the wild allele in the rs9533155 polymorphism, and G for the mutant allele. The BsaJI restriction enzyme digested the PCR product (299 bp) at 60°C and analyzed it using 3% agarose gel electrophoresis. 2 bands of 159 bp and 140 bp represent the wild-type genotype (CC); 3 bands of 299 bp, 159 159bp, 140 bp represent the heterozygous genotype (CG); and only one band of 299 bp represents the mutant genotype (GG).

Detection of RANKL Gene rs9533156 Polymorphism

RANKL gene (rs9533156) polymorphic site was determined by Polymerase chain reaction amplification with Forward Primer F=5´TTGTTTTCTCACTAAGAGCCACA3´ and Reverse Primer R=5´TCCCAAATCCCTATTTCTGC3´. 25 µl PCR reaction mixture was prepared containing 12.5 µl PCR master mix (GeneDireX), 2 µl genomic DNA, 1.5 µl of each primer (Forward & Reverse) (Eurofins), and 7.5 µl HPLC water. PCR was performed for 5 min at 94ºC followed by 30 cycles of 94ºC for 30 sec, 45ºC for 30 sec, extension at 72ºC for 1 min & final extension for 7 minutes at 72ºC. All the reaction were performed in an automated thermal cycler (BIO-RAD T100). After RANKL gene (rs9533156) amplification, the final PCR product (299bp) was digested with TspRI restriction enzyme at 65°C and analysed by 3% agarose gel electrophoresis. T represents wild allele and C represents mutant allele. 2 bands of 193 bp and 106 bp represents wild genotype (TT), whereas only 1 band of 299bp represent mutant genotype (CC). 3 bands of 299bp, 193bp and 106bp represents heterozygous genotype (CT).

Estimation of circulatory RANKL level

RANKL serum level was estimated using ELISA Kit obtained from Wuhan Fine Biotech Co., Ltd, Wuhan, China. The tests were done in duplicate and as per the instructions mentioned in manufacturer protocol. The detection limit of the assay was 78.125-5000 pg/ml. Intra-assay variation was CV<8% and variation of inter assay was CV< 10%. The assay sensitivity was 46.875pg/ml.

Statistical Analysis

We performed statistical analysis using INSTAT version 3.05 (Graph Pad Software, CA, USA). We represented numerical variables as means and SD. All categorical data were presented as percentages. BMD results as per the T-score were represented in g/cm². The power of the study was 90%. Genotype distributions were in Hardy Weinberg equilibrium. A Chi-square test was applied to compare the difference in frequency distribution of alleles & genotypes among patients and controls.

An independent sample t-test was used to analyse the difference in meaning between patients & controls. Moreover, to analyse the difference in means between two or more groups, one way ANOVA was performed. A 95% confidence interval (CI) and adjusted odds ratio (OR) were employed to analyse the association of genetic, anthropometric, and demographic factors with osteoporosis. To calculate 95% CI & OR of genotypes, we used logistic regression, Year Since Menopause (YSM), Age, and Body Mass Index (BMI) & were adjusted by odd ratio. P-values ≤ 0.05 were expressed as statistically significant.

Results

Table 1 depicts the baseline parameters of all subjects enrolled in our study. It was observed that the bone mineral density (BMD) across different skeletal sites were found to be significantly lower in the patients when compared with controls. Moreover, both body mass index and serum levels of RANKL were higher in patients when compared with controls ($p = 0.046$; $p < 0.0001$). We did not find any significant differences in age, weight & years since menopause.

Table 1: Biochemical and anthropometric profile of patients & controls

Sl. No.	Variables	Postmenopausal women with osteoporosis (*N* = 165)	Postmenopausal women without osteoporosis (*N* = 165)	*p* value
1.	Age	54.44± 6.00	54.47±6.46	0.959
2.	Weight	57.67± 10.06	56.78±7.03	0.355
3.	Height	152.07± 6.48	153.64±6.98	0.035*
4	BMI	24.86± 3.98	24.07±3.02	0.046*
5.	Year since Menopause (YSM)	9.33± 5.92	9.53±6.50	0.770
6.	BMD L1-L4 Lumbar spine (g/cm^2)	0.69± 0.12	0.89±0.12	<0.0001*
7.	BMD Hip (g/cm^2)	0.54± 0.13	0.84±0.11	<0.0001*
8.	BMD Forearm (g/cm^2)	0.58±0.13	0.81±0.11	<0.0001*
9.	BMD Femoral Neck (g/cm^2)	0.66±0.11	0.78±0.09	<0.0001*
10.	RANKL (pg/ml)	212.20±39.70	114.50±43.09	<0.0001*

The data are expressed as the average ± standard deviation (SD). $p< 0.05$ is considered statistically significant, Body mass index (BMI), Year since menopause (YSM), Bone mineral density (BMD), Receptor activator of nuclear factor kappa B ligand (RANKL), Kilogram (kg), Centimetre (cm), Kilogram per square metre (kg/m^2), Gram per square centimetre (g/cm^2), Picograms Per millilitre (pg/ml)

Figure 1 depicts the average BMD at various skeletal sites, and RANKL levels among patients according to different genotypes (rs9533155 and rs9533156) of RANKL gene. Our study showed subjects possessing homozygous mutant GG genotype at RANKL rs9533155 had considerably lower average BMD at lumber spine, forearm as compared to CC (homozygous wild) and CG (heterozygous) genotypes. The BMD was also low at hip and femoral neck in patients with GG genotype but result was not statistically significant. Additionally homozygous wild TT genotype at RANKL rs9533156 gene polymorphism showed statistically significant low BMD at femoral neck in comparison to CC (homozygous mutant) and TC (heterozygous) genotypes. BMD was low at the lumber spine, hip, forearm with TT genotype but was not statistically significant. Higher levels of RANKL were found in the GG genotype of rs9533155 compared to CC and CG and in the TT genotype of rs9533156 compared to CC and TC.

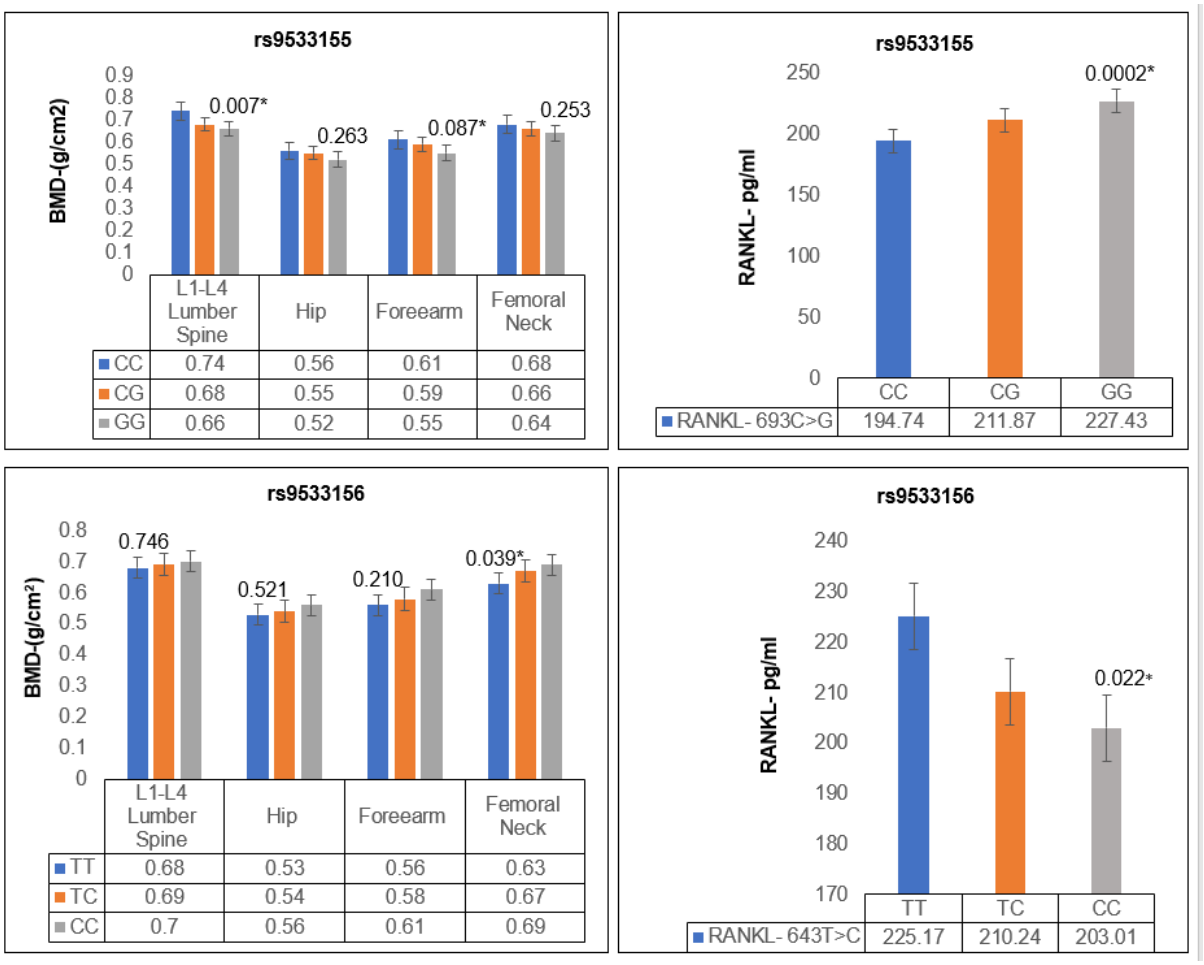


Figure 1: The comparison of BMD at different skeletal sites and RANKL levels among patients according to different genotype of rs9533156 and rs9533156

Figure 2 shows the average BMD at various skeletal sites and RANKL levels among controls according to different genotypes (rs9533155 and rs9533156) of the RANKL gene. In people with the GG genotype, low BMD was found in the lumbar spine, hip, forearm, and femoral neck. However, the results were only statistically significant in the lumbar spine. Moreover, statistically significant increased RANKL levels were also found in controls with the GG genotype. Additionally, BMD was low at all different skeletal sites in controls with the TT genotype of rs9533156 compared to CC and TC, but the result was not statistically significant; moreover, higher levels of RANKL were found in the TT genotype compared to CC and TC.

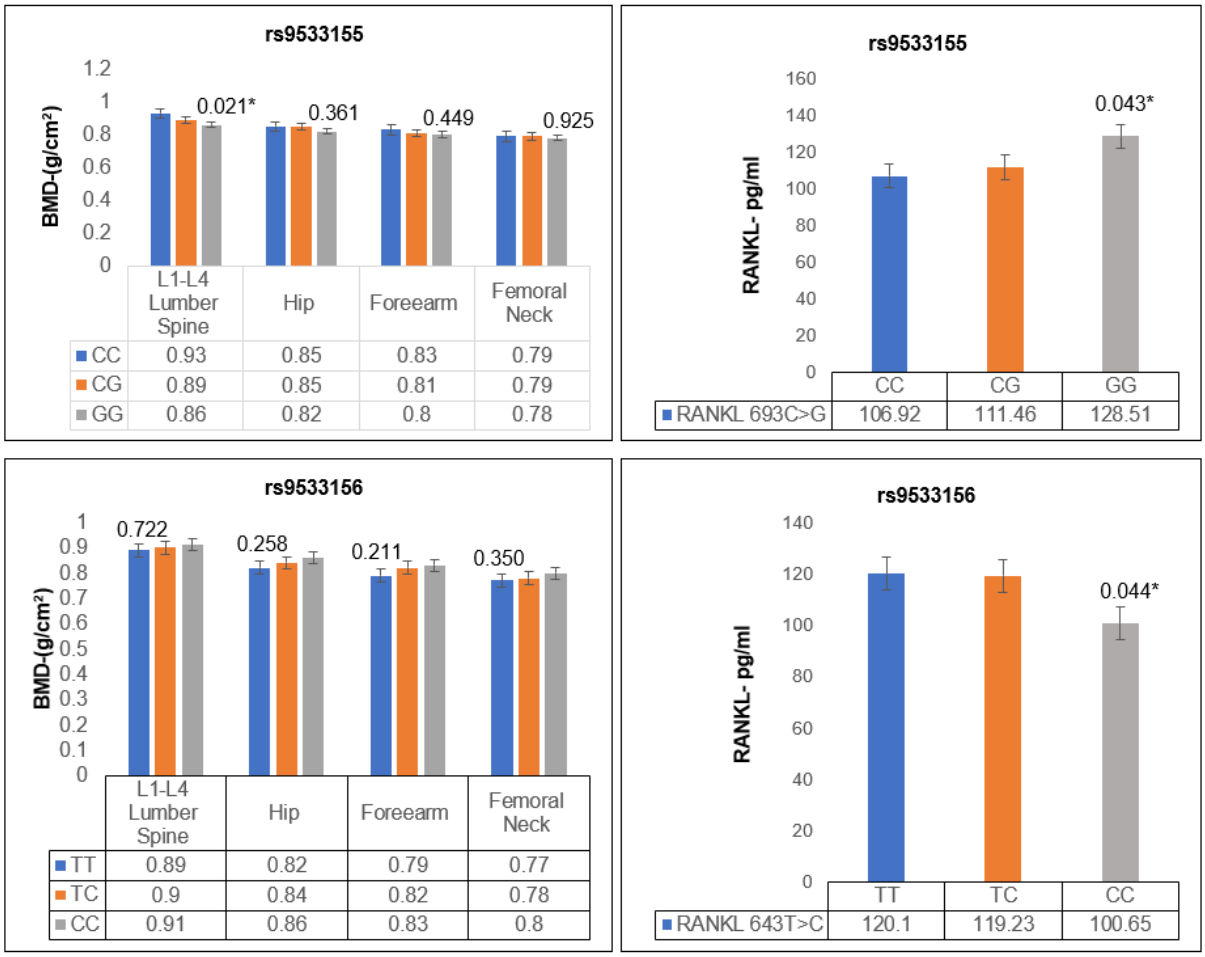


Figure 2: The comparison of BMD at different skeletal sites and RANKL levels among controls according to different genotype of rs9533155 and rs9533156.

Table 2: Frequency distribution profile of alleles and genotypes of rs9533155 & rs9533156 in patient and control group using univariate analysis

	rs9533155		
Genotypes	**Patients (165)**	**Controls (165)**	*p* value
CC	44(26.67%)	47(28.48%)	
CG	69(41.82%)	76(46.10%)	0.472
GG	52(31.52%)	42(25.45%)	
Alleles			
C	157(47.58%)	170(51.52%)	
G	173(52.42%)	160(48.48%)	0.350
rs9533156			
Genotypes	Patients (165)	Controls (165)	*p* value
TT	45(27.27%)	46(27.88%)	
TC	72(43.64%)	75(45.45%)	
CC	48(29.09%)	44(26.67%)	0.884
Alleles			
T	162(49.09%)	167(50.61%)	
C	168(50.91%)	163(49.39%)	0.756

p< 0.05 is considered statistically significant
Data is presented in number and percentage

Table 2 depicts the frequency distribution of RANKL (rs9533155 and rs9533156) genotypes and alleles in patient and control groups through univariate analysis. We did not find any significant difference in frequency distribution of different genotypes of RANKL rs9533155 (p= 0.472) and

rs9533156 (p= 0.884) gene polymorphism among patients and controls. At RANKL rs9533155 the genotype frequencies of CC, CG, GG in patients & controls were 26.67%, 41.82% and 31.52% & 28.48%, 46.10% and 25.45% respectively. In RANKL rs9533156 the genotype frequencies of TT, TC, CC in patients & controls were 27.27%, 43.64%, 29.09% & 27.88%, 45.45%, 26.67% respectively. We did not find any significant difference in the frequency of allelic distribution at RANKL rs9533155 & rs9533156 polymorphism in patients and controls (*p*= 0.350; 0.756).

Performing Logistic regression analysis after adjusting for age, BMI, and years since menopause for rs9533155 and rs9533156, we found no significant difference in patients and controls in the frequency distribution of genotypes. (Table 3 and Table 4).

Table 3 : Comparison of frequency distribution of rs9533155 genotypes among patients and controls after adjusting for Age, Year since menopause (YSM) & Body mass index (BMI)

rs9533155					
Genotype	**Patients (*N*=165)**	**Controls (*N*=165)**	***p***	**OR**	**95 %CI**
Codominant Model					
CC	44(26.67%)	47(28.48%)	Ref.	Ref.	Ref.
CG	69(41.82%)	76(46.10%)	1.091	0.970	(0.574-1.639)
GG	52(31.52%)	42(25.45%)	0.343	1.323	(0.742-2.358)
Dominant Model					
CC	44(26.67%)	47(28.48%)	Ref.	Ref.	Ref.
CG+GG	121(73.33%)	118(71.52%)	0.712	1.095	(0.676-1.776)
Recessive Model					
CC+CG	113(68.48%)	123(74.55%)	Ref.	Ref.	Ref.
GG	52(31.52%)	42(25.45%)	0.223	1.348	(0.834-2.178)

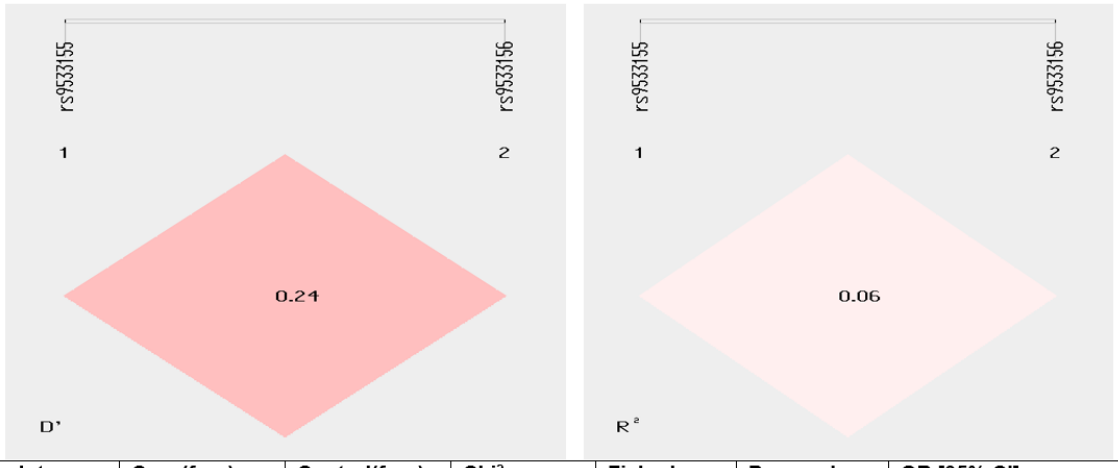
p< 0.05 is considered statistically significant
Data is expressed in number and percentage

Table 4: Comparison of frequency distribution of rs9533156 genotypes among patients and controls after Adjusting for Age, Year since menopause (YSM) & Body mass index (BMI)

rs9533156					
Genotype	**Patients (*N*=165)**	**Controls (*N*=165)**	***p***	**OR**	**95 %CI**
Codominant Model					
TT	45(27.27%)	46(27.88%)	Ref.	Ref.	Ref.
TC	72(43.64%)	75(45.45%)	1.056	0.981	(0.582-1.655)
CC	48(29.09%)	44(26.67%)	0.713	1.115	(0.624-1.991)
Dominant Model					
TT	45(27.27%)	46(27.88%)	Ref.	Ref.	Ref.
TC+CC	120(72.73%)	119(72.12%)	0.902	1.031	(0.636-1.671)
Recessive Model					
TT+TC	117(70.91%)	121(73.33%)	Ref.	Ref.	Ref.
CC	48(29.09%)	44(26.67%)	0.623	1.128	(0.697-1.826)

p< 0.05 is considered statistically significant
Data is expressed in number and percentage

The linkage disequilibrium analysis revealed relatively moderate LD degree between two polymorphism (Figure 3) (D:0.24, R^2:0.06). The haplotype frequency of GC was 1.048-fold higher in patients when compared with controls but not to a statistically significant level (*p* value = 0.777) moreover, the haplotype CT frequency distribution was greater in control group when compared with patient group (*p* value = 0.137) but did not reach to a statistically significant level.



Haplotype	Case(freq)	Control(freq)	Chi²	Fisher's p	Pearson's p	OR [95% CI]
CC	64(0.193)	60(0.182)	0.13	0.765	0.717	1.074 [0.726~1.588]
CT	93(0.281)	110(0.335)	2.21	0.151	0.137	0.777 [0.558~1.083]
GT	67(0.203)	56(0.17)	1.129	0.317	0.287	1.237 [0.835~1.833]
GC	106(0.321)	102(0.31)	0.079	0.801	0.777	1.048 [0.754~1.456]

Figure 3: Linkage Disequilibrium pattern & Haplotype analysis among two SNVs rs9533155 and rs9533156 for association with postmenopausal osteoporosis patients and controls.

Discussion

Present The present study demonstrated that promoter gene polymorphism (rs9533156 & rs9533155) in RANKL is linked to lower BMD at the forearm, lumbar spine, hip, and femoral neck and a higher serum RANKL level, which is in concordance with the results of studies conducted by Sassi et al. (2017), Mencej *et al.* (2006), and Shang, Lin, & Cui (2013). Takács *et al.* (2010) also said that in the additive model, the TT genotype of rs9533156 led to a drop in BMD on the hip. Wawrzynaik et al. (2020) showed that the rs9533156 TT genotype lowers BMD in Polish women who have gone through menopause, which supported our study. González-Mercado *et al.* (2019) demonstrated that postmenopausal osteoporosis women with 643TT (rs9533156) genotype had low BMD at the femoral neck. Wolski et al. (2016) conducted a study that linked the 643C>T (rs9533156) RANKL gene to osteoporosis risk. There was a 693 G>C (rs9533155) case where the GG genotype had lower BMD at all skeletal sites. This matches what we found (Mencej *et al.,* 2008). In contrast, Shang, Lin & Cui (2013) reported that the CC genotype of rs9533155 is linked to lower BMD. Haryono *et al.* (2019) did a study that showed RANKL gene polymorphisms did not change BMD in Indonesian women who were going through menopause.

Study by Stern *et al.* (2007) demonstrated that high serum RANKL level was linked to low BMD. Abdi *et al.* (2021) he demonstrated that osteoporotic Arabian women with TT genotype of rs9533156 showed increased RANKL level as compared to TC and CC genotypes. Yunaini *et al.* (2018) in his study on Indonesian population demonstrated that subjects with TT genotype had increased RANKL levels while CC genotype had decreased levels of RANKL. An analysis by Tu *et al.* (2015) reported significant associations between RANKL and BMD. Conversely studies by Styrkarsdottir *et al.* (2009), Liu *et al.* (2010), Oelzner *et al.* (2007), Xu *et al.* (2012), Nabipour *et al.* (2009) and Chiba *et al.* (2009) demonstrated there exists no link between RANKL rs9533156 (C>T) and BMD.

Conclusion

We can conclude that individuals with the homozygous mutant GG genotype of RANKL (rs9533155) and the homozygous wild genotype TT (rs9533156) have lower BMD at the forearm, lumbar spine,

hip, and femoral neck and higher serum RANKL levels. RANKL is also one of the main things that makes postmenopausal north Indian women more likely to get osteoporosis. Possibly this is the first study of this kind of north Indian population with some limitations that subjects were enrolled from one medical centre within the same area and showed homogenous ethnicity, which may not accurately describe the risk of osteoporosis in the Indian population. Therefore, more studies need to be conducted with a larger sample size and include other ethnic groups within the Indian population.

Conflict of Interest

The authors declare that they have no competing interests.

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