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Original Research Article

Investigation of the possible association of LRP5 gene polymorphisms with osteoporosis in an Indian subpopulation of Malda, West Bengal: a case-control study

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ABSTRACT

Background: The involvement of low-density lipoprotein receptor-related protein 5 (LRP5) in bone-related diseases with low bone mineral density like osteoporosis is scientifically well established. This study aims to explore the relationship between two LRP5 gene polymorphisms viz. rs3736228 (A1330V) and rs41494349 (Q89R) and the risk of osteoporosis in an Indian subpopulation.
Methods: This case-control study included 61 patients with osteoporosis, and 30 healthy controls from Malda Medical College and Hospital. The SNP analysis was performed by PCR-RFLP method with DraIII and AvaII enzymes for rs3736228 (A1330V) and rs41494349 (Q89R) respectively. The data is validated with DNA sequencing. The results are statistically evaluated.
Results: The distribution of the A1330V and Q89R genotypes in this population was as follows: AA 81.97%, AV 18.03%, and VV 0.00%; QQ 100%, QR 0.00%, and RR 0.0 0%. No homozygous mutant for A1330V and heterozygous or homozygous mutant for Q89Rare detected in this population. Both the polymorphisms in this population are in Hardy-Weinberg equilibrium. The genotype distributions of rs3736228 showed difference between the osteoporotic patients and control groups [odds ratio (OR):1.98, 95% confidence interval (CI): 0.51 to 7.71, p=0.374]. DNA sequencing of exon 18 not only confirms the presence of A1330V in Indian population but also identifies a novel mutation.
Conclusions: The odds ratio (OR) suggests a positive trend toward an association between the A1330V variant and osteoporosis risk. Exon 18 of LRP5 demands special scientific attention. No variation is detected for rs41494349 in the study population.

Keywords: Osteoporosis, LRP5, rs3736228, rs41494349, Indian population

INTRODUCTION

Osteoporosis, a multifaceted skeletal disorder is characterized by diminished bone mineral density (BMD) and degraded bone microarchitecture which considerably increases probability of low-trauma fractures commonly occurring at the hip, spine, or wrist.[1] It is generally asymptomatic until a fracture event marks its silent progression.[2]The pathophysiology of the disease is complex, involving environmental, hormonal, immunological and genetic factors. Despite being considered as largely hormonal, the genetic factors influencing osteoporosis encompass heritable BMD trait, single nucleotide polymorphisms (SNPs) of particular

genes, polygenic and multiple gene variants and many others.[3]

One of the genetic factors is low-density lipoprotein receptor-related protein 5 (LRP5). Multiple studies have demonstrated the involvement of low-density lipoprotein receptor-related protein 5 (LRP5) in bone-related diseases with low bone mineral density (BMD) such as osteoporosis.[4] The LRP5 gene encodes a co-receptor crucial for the Wnt/β-catenin signaling pathway essential for bone formation and maintenance.[5]Dysfunctional mutations in LRP5 result in extreme bone phenotypes like loss-of-function mutations cause osteoporosis–pseudoglioma syndrome (very low bone mass), while gain-of-function mutations lead to high-bone-mass conditions.[6] A very recent case study reports a frameshift pathogenic variant of LRP5 causing severe osteoporosis in a female patient with no family history of the disease before. Absence of optimal treatment for such patients has also been reported.[7] Identification of specific genetic variants of LRP5associated with osteoporosis not only will enhance the understanding of pathophysiology but also will contribute to risk prediction and development of targeted therapies for the disease.

Furthermore, LRP5 is located at chromosome 11q12-13 identified by Koller et al in 1998 as a quantitative trait locus (QTL) regulating BMD and playing important role in osteopororsis.[8] Till then many scientific groups around the world identified several SNPs of LRP5 associated with bone related diseases.[9] Researchers have identified seven new polymorphisms in the LRP5 gene, important for bone development. Out of these, two SNPs are predicted to be missense SNPs. They are rs41494349 (c.314A>G: Q89R) and rs3736228 (c.4037C>T: A1330V) located on Exon 2 and Exon 18 of LRP5 respectively.[10] In case of rs41494349, the wildtype glutamine (Q) at residue 89 resides within the first β-propeller domain gets replaced by an arginine (R).Q89R, though less thoroughly studied than A1330V, the replacement of glutamine with arginine is supposed to influence protein folding or ligand-receptor interactions, potentially modulating bone formation. On the other hand, in case of rs3736228, an alanine (A) residue at the position of 1330 in the LDL-receptor-like domain of LRP5is changed into valine (V) causing impairment in Wnt signaling in vitro by affecting LRP5 activity.[11]

The LRP5 gene polymorphisms rs41494349 (Q89R) and rs3736228 (A1330V) have been studied for their association with bone mineral density (BMD) and osteoporosis risk in different populations worldwide. The association studies of rs41494349 (Q89R) with osteoporosis generated diversified results among populations. A recent case-control study reported that rs41494349, particularly in combination with rs2306862 is significantly associated with abnormal bone mass in a Chinese population of postmenopausal women.[12]Conversely, a study in Thai menopausal women found no significant differences in BMD measurements between wild-type and risk alleles at rs41494349, suggesting population-specific variability in its association with osteoporosis risk.[11] On the other hand, CT/TT genotypes at rs3736228 were associated significantly with lower femoral neck BMD compared to CC homozygotes in Chinese postmenopausal population suggesting this variant's contribution to osteoporosis risk alongside age, BMI, and triglycerides as independent predictors.[13] Furthermore, in a large cohort of young Saudi females, the TT genotype conferred a 3.06-fold increased risk of osteopenia, supporting its role as a marker of early-onset of bone loss in this demographic.[14] In a cohort of postmenopausal Romanian women, a borderline association was observed between rs3736228 and lower BMD at the femoral neck and total hip, suggesting increased susceptibility to osteoporosis.[15] Functional genomics analyses further underscore the importance of rs3736228, revealing its cis-regulatory effects on genes differentially expressed between high and low BMD groups, thus suggesting a molecular mechanism underlying its association with osteoporosis.[16] Importantly, a meta-analysis of 19 studies encompassing over 25,000 subjects provided broader evidence for modest but consistent associations of both the polymorphisms with BMD. The rs3736228 AA genotype was linked to higher lumbar spine and femoral neck BMD compared to AV/VV genotypes, while the rs41494349 QQ genotype was associated with higher femoral neck BMD relative to QR/RR genotypes, suggesting their relevance for osteoporosis risk assessment across populations.[17] Despite of being an osteoporotic hub, the Indian population is rarely studied for these two polymorphisms.[18] Our study investigates the association of rs41494349 and rs3736228 with osteoporosis in an Indian subpopulation of Malda district, never been studied for this topic before.

METHODS

Study population and blood sample collection

This original research article comprised of 61 unrelated, osteoporotic patients and 30 healthy controls living in Malda district and surrounding regions in India. The mean age of the osteoporotic patients was 52.9±12.4 years, and for controls 49.966±12.620 years. All participants were of the Indian ethnic group. 1 ml of blood samples were collected in EDTA coated vials at room temperature from both the patients and control subjects with proper informed consent from the Department of Orthopaedics, Malda Medical College and Hospital, Malda, West Bengal, India. Some clinical information had also been collected using questionnaire about medical history, ongoing medication, and survey of the incidence of disease. Osteoporosis in patients is identified by X-ray reports or bone mineral density (BMD) from clinical reports or fracture history (hip, wrist and spine fracture with mild stress). The subjects undergoing a fitness checkup and having no reports of low BMD or fractures, other bone related complications were included as control subjects. No

participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as hyperthyroidism, diabetes mellitus, primary hyperparathyroidism, renal failure, pituitary and adrenal disease, or rheumatic disease. Postmenopausal women who had experienced early menopause (before 40 years of age) and those who had undergone ovariectomy or who were receiving estrogen replacement therapy were excluded from this study. The clinical information of the participants is presented in Table 1. The study was approved by the Institutional Ethics Committee, University of Gour Banga, Malda, West Bengal, India (Ref. No. UGB/ IEC (Human)/0005-21, dt.25/11/2021) and Institutional Ethics Committee of Malda Medical College and Hospital, Malda (No. P/MLD-MC/ IEC22/57). Study period is from November 2021 to August 2025.

Genomic DNA isolation

1 ml of whole blood obtained from each participant by intravenous injection was stored in EDTA vacutainer tubes at 4°C. 50-70 µl sample was pipetted in a sterilized microcentrifuge tube containing 500 µl TES buffer (TES, pH 8,1M Tris-HCl, 0.5M EDTA and 5M NaCl). To the above sample 50 µl of 10% SDS and 10 µl of proteinase K (100 mg/ul) had been added and were mixed thoroughly for few seconds and it was then incubated at 56°C for 30 minutes. After incubation, 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed thoroughly. Centrifugation was carried out at 12000 rpm for 10 minutes. The supernatant was transferred to 1.5 ml microcentrifuge tube. 500 µl of chloroform-isoamyl alcohol (24:1) was added and mixed thoroughly. Centrifugation was carried out at 12000 rpm for 10 minutes. The supernatant was collected and absolute alcohol with double the amount of it was added and kept at -20°C overnight for precipitation. Centrifugation was further carried out at 12000 rpm for 10 minutes. Ethanol was discarded and pellet was retained. The pellet was resuspended in 70% ethanol and centrifuged at 7000 rpm for 10 minutes to remove excess salt. After discarding the ethanol, the pellet was dried and dissolved in 30 µl of nuclease free water and incubated at 56°C for 15 minutes. Thus, the genomic DNA was obtained and ready to be quantified. DNA quality was checked by running aliquots on 0.8% agarose gel and quantified by reading absorbance at 260 nm with a spectrophotometer. DNA was visualized on agarose gels and photographed using Gel-DOC system (BioVision, India). The DNA samples were stored at -20°C for further use.

PCR-RFLP

Genomic DNA, extracted by the above-mentioned method was used for genotyping of the proposed SNPs, Q89R (rs41494349) located in exon 2, and A1330V (rs3736228) located in exon 18 of LRP5 with polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method as described by Okubo et al. (2002).[10] The exon specific primers, restriction enzymes and fragment lengths for each SNP are provided in Table 2. Each PCR reaction was carried out in a total volume of 20 µl containing 10 µl 2X GoTaq® Green Master Mix (Promega), 1 µl each of forward and reverse primers (20 pmol/µl), 3 µl of genomic DNA (50-100 ng) and nuclease-free water to make up the final volume. The PCR conditions for both the primer sets are optimized through temperature gradients. The thermal cycling profile for the amplification of exon 2 of the LRP5 gene containing the rs41494349 (Q89R) included an initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 45 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 10 minutes, and the reactions were held at 4°C. On the other hand, the PCR thermal profile for the amplification of exon 18 of the LRP5 gene containing the rs3736228 (A1330V) involved annealing at 56°C, while the rest of the steps remained the same.

Genotyping of the PCR-amplified products was performed using restriction fragment length polymorphism (RFLP) analysis. The PCR products were digested with the appropriate restriction enzymes for 8 hours at 37°C in a water bath. For rs41494349, AvaII (Eco47I) enzyme (Thermo Scientific, India) was used, with an inactivation step at 65°C for 20 minutes. For rs3736228, DRAIII (AdeI) enzyme (Thermo Scientific, India) was used without a heat inactivation step. A 100 bp DNA ladder was run alongside the samples to verify fragment sizes. Digested fragments were resolved by electrophoresis on a 3% agarose gel at 100 V in 1X TAE buffer for 1–1.5 hours. Controls without enzymes were included to confirm complete digestion.

Sequencing of the purified PCR amplified DNA

Purification of the PCR products from agarose gels was done by Wizard® SV Gel and PCR Clean-Up System (Promega, India) following manufacturer's protocol. Purified samples were dissolved in 10 µl of 50% Hi-Di formamide and analysed in SeqStudio Genetic Analyzer (Applied Biosystems).

Genotyping and statistical analysis

Allele and genotype frequencies for both SNPs were determined by direct gene counting. These genotype categorizations were further analysed under dominant, recessive, and additive genetic models to investigate potential associations with osteoporosis susceptibility.

For rs41494349, genotypes were classified based on nucleotide substitution as GG (wild-type, Gly/Gly), GA (heterozygous, Gly/Ala), and AA (homozygous variant, Ala/Ala).

For rs3736228 (A1330V), genotypes were categorized as wild-type CC (corresponding to AA at the protein level,

Ala/Ala), CT (heterozygous, Ala/Val), and TT (homozygous variant, Val/Val).

The Hardy-Weinberg equilibrium (HWE) was tested using the Chi-square test to verify population-level consistency in genotype distributions. To determine the significance of the observed genotype frequency differences between the case and control groups, Fisher's exact test was applied due to small sample sizes in some genotype categories.

Additionally, odds ratios (ORs) were calculated with 95% confidence intervals (CIs) to measure the strength of association between the presence of the variant genotype (AV + VV) and osteoporosis risk. A two-proportion Z-test was also conducted to compare the prevalence of the AV genotype between cases and controls, testing for statistical significance at the 0.05 level.

All statistical analyses were carried out using freely available tools such as: MedCalc Online: https://www.medcalc.org/calc/odds_ratio.php. Social Science Statistics (Z-Test Calculator): https://www.socscistatistics.com/tests/ztest/default2.aspx. Graphpad (Fisher Exact Test): https://www.graphpad.com/quickcalcs/contingency2/, p-value calculator: https://calculator-online.net/p-value-calculator/.

To evaluate the association between LRP5 gene variants and osteoporosis risk, genotype data for two single nucleotide polymorphisms (SNPs) rs3736228 (A1330V) and rs41494349 were analyzed under genetic inheritance models. For rs3736228 (A1330V), the dominant model was employed, wherein the heterozygous (AV or CT) and homozygous mutant (VV or TT) genotypes were grouped together and compared against the wild-type (AA or CC). This model was selected because no homozygous mutant (VV/TT) individuals were observed in the study population, making the dominant approach more suitable for detecting potential associations.

In contrast, genotyping of the rs41494349 SNP revealed no heterozygous or homozygous variant genotypes within the study population, with all subjects exhibiting the wild-type (GG) genotype. As a result, statistical analysis under dominant or recessive models was not applicable for rs41494349 due to the absence of genotype variation. Nonetheless, its inclusion in the study allowed for comprehensive screening of potentially relevant LRP5 polymorphisms associated with osteoporosis susceptibility.

RESULTS

Demographic characteristics and anthropometric comparison of the participants

The participants were not categorized on the basis of gender or menopausal status due to small sample size. The demographic and anthropometric characteristics of the study participants were analysed between osteoporotic cases (n=61) and non-osteoporotic controls (n=30). The mean age of cases was 52.90±12.41 years, slightly higher than that of controls (49.97±12.62 years), but not statistically significant ($p>0.05$). Similarly, mean height was comparable between groups (155.44±8.77 cm in cases vs. 154.69±3.67 cm in controls; $p>0.05$). Notably, the case group exhibited a lower mean body weight (56.05±6.85 kg) compared to controls (58.37±7.29 kg), although this difference also did not reach statistical significance. Despite the lack of significance, the trend toward lower weight in osteoporotic individuals suggests a possible association between reduced body mass and increased osteoporosis risk. These observations highlight the importance of including body composition as a potential risk factor in osteoporosis studies and support further research on weight-related bone health parameters. The body mass index (BMI), an important anthropometric parameter linked to bone health, was calculated for both osteoporotic cases and control participants. The mean BMI in the case group was 23.27±3.36 kg/m², while in the control group, it was slightly higher at 24.38±2.99 kg/m². An independent samples t-test was conducted to determine whether this difference was statistically significant. The result yielded a t-statistic of -1.60with 54 degrees of freedom, and a corresponding P value of approximately 0.115, indicating no statistically significant difference between the groups ($p>0.05$).

Table 1: Patients characteristics.

Group	Age (years) (mean ± SD)	Height (cm) (mean ± SD)	Weight (kg) (mean ± SD)	BMI (kg/m²) (mean ± SD)
Case	52.90±12.41	155.44±8.77	56.05±6.85	23.27±3.36
Control	49.97±12.62	154.69±3.67	58.37±7.29	24.38±2.99

The characteristic features of the participants can be observed at a glance in Table 1.

DNA quality and quantity

Genomic DNA was isolated from peripheral blood samples and assessed for quality using both spectrophotometric and fluorometric approaches. DNA concentration was measured with the Qubit 4 fluorometer using 2 µl of DNA mixed with 198 µl of working solution (total 200 µl). The average DNA concentration recorded was 10 ng/µl. Further assessment of DNA integrity was done by running samples on a 0.8% agarose gel (Figure

1A), and absorbance at 260 nm was recorded using a spectrophotometer to ensure purity.

PCR-RFLP

The rs41494349 polymorphism in Exon 2 of the LRP5 gene was analyzed using PCR-RFLP. The expected fragment sizes following AvaII digestion were 436 bp for the wild-type genotype (Q/Q) and 274 bp+162 bp for the homozygous variant (R/R). Heterozygous individuals (Q/R) would exhibit all three fragments (436 bp, 274 bp, and 162 bp). However, in the present study, all samples displayed a single undigested band at 436 bp, corresponding exclusively to the wild-type genotype. No heterozygous (Q/R) or homozygous variant (R/R) genotypes were detected in either the case or control groups, indicating the absence or extremely low frequency of the variant allele in this population (Figure1B).

Table 2: Particulars of PCR-RFLP applied in this study.

Location	Sequence alteration	Amino acid change	PCR primer (5'3') for RFLP detection	Restriction enzyme	Fragment size /base pairs (bp)
Exon 2	c.314 A>G	Q89R	Forward: 5'TCTGGGCATAGTGCTCCATC3' Reverse: 5'TTCCGGGATGTGCCATTGAG3'	*Ava II*	Wild (Q): 436 Variant (R): 274+162
Exon 18	c.4037 C>T	A1330V	Forward: 5'GACTGTCAGGACCGCTCACACG3' Reverse: 5'AAGGTTTTCAGAGCCCCTAC3'	*Dra III*	Wild (A): 143 Variant (V): 119 +24

Table 3: genotypic distribution of rs3736228.

Group	CC (Wild-type)	CT (Heterozygous)	TT (Mutant)	Total	Variant (CT+TT) %
Cases	50	11	0	61	18.03
Controls	27	3	0	30	10

Table 4: Summary of key statistical values.

Statistic	Value
Odds ratio (OR)	1.98
95% confidence interval	0.51 to 7.71
Fisher's exact P value	0.374
Chi-square test P value	0.4906
Hardy-Weinberg χ^2	0.599 (non-significant)

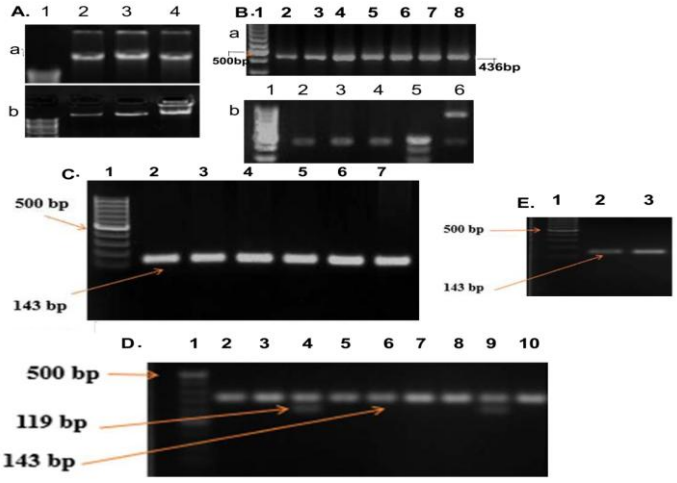

Figure 1: Agarose gel electrophoresis. A. DNA from cases (a), controls (b). B. amplification of exon 2 in cases, lane 1, 100bp marker (a), RFLP of exon 2, lane 1, 100bp marker, lane 5, 20 bp marker, lane 6 positive control for AvaII enzyme (b). C. amplification of exon 18 in blood samples, lane 1, 500bp marker. D. RFLP of exon18 in cases, lane 1, 100bp marker. E. PCR clean-up products of exon 18 from case samples.

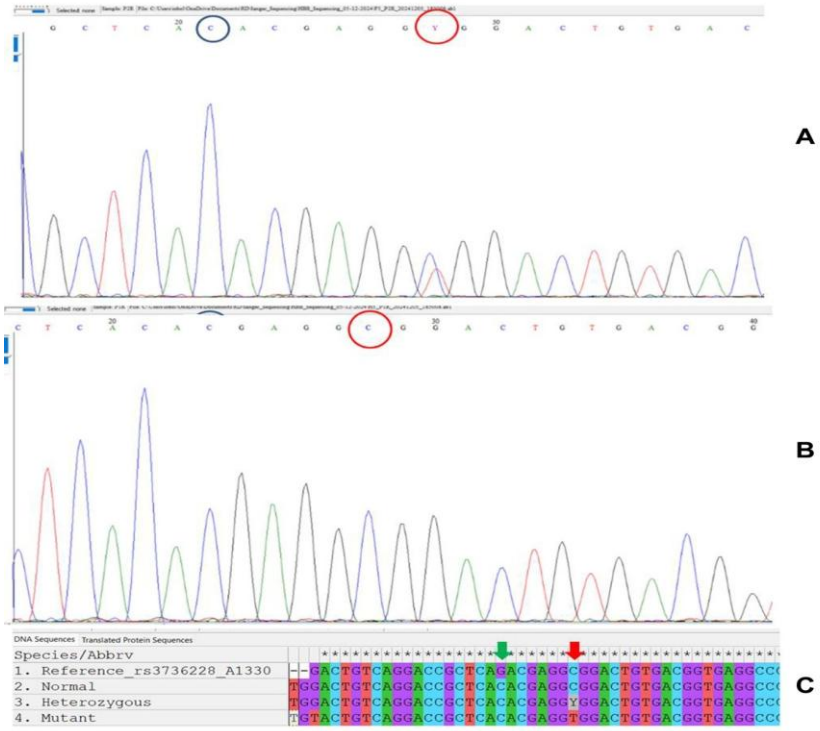

Figure 2: Electropherogram of exon 18 of LRP5. (A) Representative heterozygous Dra III status (encircled with red) and novel mutant (encircled with blue); (B) representative homozygous DraIII status. Multiple sequence alignment of query sequences with reference sequence (rs3736228from dbSNP) and (C) the marked region (Red down arrow) shows rs3736228of LRP5 gene and a novel mutant (Green down arrow).

PCR amplification targeting the region surrounding the rs3736228 (A1330V) SNP in Exon 18 of the LRP5 gene produced a produced a fragment of 143 bp. After restriction digestion, clear amplicon of 119 bp is produced in heterozygotes, as visualized on the gel (Figure 1, C, D). However, the expected 24 bp digested fragment, resulting from RFLP enzymatic cleavage, was not observable, likely due to its small size falling below the detection threshold of standard gel electrophoresis.

DNA sequencing

To overcome this limitation and validate the genotypes, Sanger sequencing for purified amplicons of Exon 18 (Figure 1, E) of representative samples was performed. The sequencing results were consistent with the expected A1330V variant alleles. Interestingly, an unreported (novel) mutation was detected in proximity to the rs3736228 site. This novel variant was not the focus of the current study and warrants further investigation (Figure 2).

Genotyping and statistical association analysis

Genotyping of the rs3736228 (A1330V) SNP in the LRP5 gene was performed on 61 osteoporotic cases and 30 control subjects using PCR-RFLP (Table 3). The observed genotypes were AA (n=50), AV (n=11), and VV (n=0) in cases, and AA (n=27), AV (n=3), and VV (n=0) in controls. In the dominant model (AV + VV vs. AA), the variant genotype frequency was 18.03% in cases and 10.00% in controls. The odds ratio (OR) was 1.98, indicating that individuals with the AV genotype had nearly twice the odds of osteoporosis compared to those with the AA genotype. However, Fisher's exact test yielded a p-value of 0.374, which is not statistically significant ($p>0.05$). A chi-square test also supported this, yielding $\chi^2=0.4752$ ($p=0.4906$). The key statistical values are displayed on Table 4. The observed genotype distribution for the LRP5 gene polymorphisms in this study was as follows: for A1330V (rs3736228), the frequencies were AA (wild-type): 81.97%, AV (heterozygous): 18.03%, and VV (homozygous variant): 0.00%. For Q89R (rs41494349), all individuals exhibited the QQ (wild-type) genotype, with no QR or RR variants detected (QR: 0.00%, RR: 0.00%), indicating complete conservation of the wild-type allele in the study population. Hardy-Weinberg equilibrium was tested in the case group, yielding allele frequencies of C=0.91 and T=0.09, and a non-significant deviation ($\chi^2=0.599$), indicating that genotype distributions were in equilibrium.

DISCUSSION

This study proves no statistical significance due to small sample size but indicates a number of important points regarding current osteoporosis status in Indian population.

India holding a varied ethnicity shows demographic variations. This study selects a population that was never been studied for osteoporosis. Despite the lack of significance, the trend toward lower weight in osteoporotic individuals suggests a possible association between reduced body mass and increased osteoporosis risk. These observations highlight the importance of including body composition as a potential risk factor in osteoporosis studies and support further research on weight-related bone health parameters. The lower average BMI observed in osteoporotic individuals may point to a possible biological association between reduced body mass and decreased bone mineral density. While this trend was not conclusive within the scope of the present sample size, it aligns with previous literatures suggesting that lower BMI may be a risk factor for osteoporosis due to reduced mechanical loading on bones and potential deficiencies in fat-related estrogen production.[19,20,21]These findings underscore the importance of including BMI in osteoporosis risk assessments and support further investigation with larger, more diverse cohorts. Regarding the association of osteopororsis with A1330V genotype, though statistical significance was not reached, this trend aligns with prior studies that reported an association between the variant and reduced bone mineral density (BMD).[17,22,23] This biological plausibility supports the need for further investigation in larger and more diverse cohorts. Importantly, no VV genotype was detected in either group, consistent with the rarity of this homozygous variant in Asian populations, including prior studies of South Asian genotypes from NCBI dbSNP. Despite of being statistically insignificant, the calculated odds ratio (OR = 1.98) indicates that individuals carrying the AV genotype of the LRP5 rs3736228 (A1330V) polymorphism had nearly twice the odds of developing osteoporosis compared to those with the AA genotype. The wide confidence interval (95% CI: 0.51 to 7.71) reflects the limited sample size, yet the direction of effect is consistent with prior studies linking the V allele to lower BMD and the upper bound (7.71) suggests a potentially strong effect that a larger sample might detect. In contrast, the absence of the Q89R mutation in our population may limit its utility as a biomarker for osteoporosis risk in this specific genetic background. Interestingly, besides validating the A1330V mutation in the population, a novel polymorphic site (not mentioned in the reference sequence) has been detected through DNA sequencing of exon 18 of LRP5 gene. This particular polymorphic site needs special attention of the researchers. The consequences of homozygous mutant for A1330Vare also a matter of curiocity.

Smaller sample size is one of the major limitations of this study. Lack of BMD scores of the patients prohibits the chance of investigation of genetic correlation with BMD and osteoporosis risk.

CONCLUSION

Overall, this study identified potential association of rs3736228 of LRP5 gene in the population of Malda for the first time. No variation has been found for rs41494349 in this population as all the individuals are found to carry wild type amino acid. The study reports a novel mutation in exon 18 of LRP5 for the first time. In conclusion, exon 18 of LRP5 may play an important role in its function as well as in osteoporosis pathophysiology.

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