LRP5rs121908669, Genetic regulation of bone mass and susceptibility to osteoporosis

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Abstract

Background and aims: Genetic epidemiological studies are necessary to develop diagnostic and treatment strategies for various diseases, including osteoporosis. The LRP5G171R single nucleotide polymorphism SNP is one of the pathologically proven SNPs related to bones , and there is no genotyping study for it in all over the world. Methods: LRP5rs121908669 was detected using PCR-RFLP and DNA sequencing in EDTA blood samples. The distribution of genotypes was studied in all bone states(normal, abnormal) i.e. abnormal= osteopenia/ osteoporosis Results: The frequency of genotypes were 32.65% vs 67.34% for GG, 65.62% vs 34.37% for GC and 75% vs 25% for CC in normal and abnormal groups, respectively. Conclusions: There was an association between LRP5G171R normal and low bone mineral density BMD.

Keywords: Genetic epidemiological studies, LRP5rs121908669, LRP5G171R.

INTRODUCTION

Osteoporosis is a complex disease, and allelic variation in many other candidategenes including those that encode growth factors, cytokines, calciotropic hormones, and bone matrix proteins are likely to also play a role and warrant systematic investigation. Bmd1/ Genetic factors play an important role in the pathogenesis of osteoporosis. Twin- and family-based studies have indicated that 60–85% of the variance in bone mineral density (BMD) is genetically determined[1,2]. Most family and association studies to date have focused on the genetic contributions to bone density, a major determinant of bone strength and fracture risk. Bone density is not the only determinant of skeletal fragility, however, and genetic influences on fracture risk are independent of bone density[3]. The microarchitectural properties and overall size and geometry of bone also influence skeletal strength and the genetic influences on these phenotypes should be investigated more rigorously. Even fewer studies have assessed the association between candidate-gene variation and the risk of fracture, the most important clinical outcome of osteoporosis. The genes and allelic variants conferring osteoporotic risk are largely undefined, but the number of candidates has increased steadily in recent years[4].

Osteoporosis is a complex, multifactorial disease, and most candidate-gene association studies have had limited statistical power to assess gene-gene and gene-environment interaction. Although gender plays an important role in the development of osteoporosis, genetic studies have almost exclusively focused on women, and have not tested whether gender modifies the association between genetic variation and osteoporotic risk. Therefore, future genetic studies will need to recruit larger samples of individuals including men. Rapid additional progress in our understanding of the molecular basis of osteoporosis can be expected in the near future as ongoing genome-wide linkage.Large-scale molecular epidemiologic studies will be increasingly necessary in the future to quantify the relative, absolute and attributable risks of fracture associated with specific genetic variants[5].

The LRP5 gene turned out to be an important regulator of peak bone mass in vertebrates [6]. Single nucleotide polymorphisms SNPs in the LRP5 gene may cause high or low bone mass[7,8]. Both may lead to osteoporosis and fractures[7].

LRP5rs121908669 (G171R) that causes the high bonemass phenotype is located in the aminoterminal part of the gene. Some

genetic information about this SNP is explained in the table below table.1[9]

Table.1: Some genetic information about LRP5 G171R(LRP5rs121908669) from NCBI

Name	**Gene ID: 4041 /LRP5**
Description	LDL receptor-related protein 5
Location	11:68312591-68449275
Cytogenetic region	11q13.2
SNV	G>C 511
EXON	3/23
RefSeqGene	143636 bp
Protein change	G171R
OMIM:	603506
Condition	ADO1

A genetic epidemiological study of this mutation may provide an added value to genetics studies in osteoporosis that serve to develop new diagnostic and therapeutic strategies[10].

MATERIALS AND METHODS:

The study included 150 participants who visited rheumatology clinic at Tishreen University Hospital, Lattakia, Syria, throughout the period between March 2019 and September 2021,which was interspersed with interruptions due to the Corona pandemic. The work was approved by the Ethics Committee in Syrian Ministry of High Education, and prior written consents were obtained from all the participants.

All of the participants were interviewed using a structured questionnaire. The questionnaire included socio-demographic characteristics, work habits, physical activity, medication history, age, age of beginning and end of menstrual, pregnancy and number of children, history of family orthopedic complaint, clinical history of bone pain , measurements of height and weight, body mass index BMI (kg/m2), data of fractures, lumbar and femur Z-score, lumbar and femur T-score. All participants were women with pre-menopause or post-menopause. They were from different families. Blood phosphorous and calcium values were collected from patients' files. The controls had high or normal T-score for both femur and lumbar T-score. All patients with hypertension, diabetes, osteomalacia, surgical menopause and cancer were excluded.

Bone densitometry:

The bone mineral density (BMD; g/cm2) of the lumbar spine (L1-L4) and left femur as measured by dual energy X-ray absorptiometry (DXA) (Medix DR, France). All DXA scans were conducted by a specially trained specialist. BMD Results were converted to age- and gender-specific Z-score that matched normal Caucasians. The samples were classified into 3 groups (normal, osteopenia, and osteoporosis) according to the World Health Organization classification of T-score values.

Insilico Study:

An Insilico study on NCBI was done in 2021.It was found that there were thousands of mutations for 569 genes associated with osteoporosis. There were two proven pathogenic SNPs for osteoporosis only without any other diseases with a predictive effect on protein of 87% according to the bioinformatics application of SNP PREDICT[11,12]. Currently, there are greater numbers of genes related to osteoporosis, numbering 855 genes, and dozens of SNPs that are pathogenic proven for osteoporosis without other diseases as shown in table.2[11]

Table.2: results of an Insilico study(2021) to determine the pathogenetic SNPs for osteoporosis

Number of genes	**Number of SNPs**	**Clinical significance**	**Names of genes**
569	Thousands	Coding/noncoding protein	-
483	Thousands	Coding protein	-
7	Dozens	Pathogenic for osteoporosis and other diseases	BMND7,BMND8,BMND4, CALCR,COL1A1,COL1A2,LRP5
2	3	Pathogenic only for osteoporosis	COL1A2,LRP5
The chosen SNP is LRP5rs121908669			

DNA Extraction:

Blood samples were collected using EDTA anticoagulant container tubes (2.5 ml blood from each participant) in Tishreen University Hospital, Lattakia, Syria. The samples were kept at -20 c . Work had been completed in the biotechnology laboratories of the Atomic Energy Authority, Damascus, Syria, where DNA was isolated from samples using the (QIAamp DNA Blood Mini kit , Qiagen, Germany) according to the manufacturer's procedures and was stored at -20°C. The total DNA of each sample was measured by using a spectrophotometer followed by a of quantity Ultraviolet light.

LRP5rs121908669 SNP analysis:

The studied SNP was selected using the software https://loschmidt.chemi.muni.cz/predictsnp/. The Prediction ratio for its effect on the protein was 87%. In 2021, an Insilico study was conducted on NCBI concerning the genes of osteoporosis. It was found that there are only two genes with 3 SNPs proven pathogenic for osteoporosis without other diseases. One of them is ofLRP5rs121908669.

LRP5rs121908669 polymorphism of exon 3 was amplified using a specific forward primer: (5'-TCTGTGTTAGCTGCTTCTCTT-3') and Reverse primer 5′- CCAGGACTGCGTGGGTA -3′

Primers were designed using https://www.ncbi.nlm.nih.gov/tools/primer-blast,and https://bioinfo.ut.ee/primer3-0.4.0/. The primers were manufactured using (a polygon primer designer device, in Germany). The stock concentration was 52.51 n.mol/ml for reverse primer and 63.60 n.mol/ml for forward primer. Both were diluted with dual distillation water(ddw) (10X).

The Polymerase chain reaction (PCR) was performed in a total volume of 25 µl containing 5 µl of genomic DNA, 5 µl PCR buffer, 1 µl dNTPs, 2 µl of each primer, and 1 µl of Taq DNA polymerase. PCR program included initial denaturation at 95 °C for three minutes followed by 40 cycles of 95 °C for 45 seconds, 52°C for 45 seconds, and 72 °C for 60 seconds with a final extension at 72 °C for 7 minutes. PCR reaction was conducted in a PCR T100 thermocycler (Mastercycler, Eppendorf, Germany). The amplification PCR products were run on 2% agarose gel stained with DNA Safe Stain Dye and visualized under UV light. The positive result produced bands 259 base pair (bp) (= 259 bp) which indicates the presence of the fragment which was chosen to detect this SNP.

Restriction enzymes for RFLP were chosen from https://nc2.neb.com/NEBcutter2/. The Restriction Fragment Length Polymorphisms (RFLP) of the LRP5 gene was carried out by PCR product gestion for 16h at 37 °C with 0.8 µl Bfi1 (MBI Fermentas, Vilnius, Lithuania). Then, 15 µl of the digested PCR products were added to 3 (6X) loading dye and loaded on 3.5% agarose gel, and run at 80V for 60 minutes. PCR products for L 5rs121908669 were then visualized using the gel documentation system BIO-RAD (Gel-DocSy1-L8-M5). The lengths of the digested product were 192pb*67bp; 259pb; 259bp*192*67bp for the normal genotype GG, Hhomozygous genotype CC, and heterozygous genotype GC, respectively. The ladder is 20pb. There was no positive or negative control sample.

All were confirmed by direct sequencing using SeqStudio Genetic Analyzer (Applied Biosystems, USA). The cycle-sequencing reaction was performed in a 10 µl volume containing 1 µl of the ready reaction of the terminator, 5 p.mol of either the forward or reverse primer, and 10 ng of purified PCR product (ExoSAP-IT kit; Amersham BioSciences, Piscataway, NJ, USA). The thermal cycle protocol was 95˚C for 4 minutes followed by 30 cycles at 96˚C for 10 seconds, 50˚C for 5 seconds, and 60˚C for 4 minutes (ABI GeneAmp PCR System 9700, Applied Biosystems). Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) were used for the effective and reliable removal of excess dye terminators (DyeEx 2.0, Qiagen, Germany) from completed DNA sequencing reactions. Data were compared and aligned with different sequences using the NCBI BLAST Assembled Genomes tool(http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS:

All data about age, age of beginning and end of menstrual, pregnancy and number of children, history of family orthopedic complaint, bone complaint, measurements of height and weight, body mass index BMI (kg/m2), data of fractures, classification of cases according to WHO*, are contained in the table below table.3. All participants had normal blood concentrations of calcium and phosphorous.

Table.3: Clinical, laboratory, demographic and radiological information for participants

Variable	**Case**
Total number	150
Age	60(40, 80)
Age of beginning of menstrual	14(11, 17)
Age of end of menstrual	50.5(46, 55)
Weight	69.5(40,99)
Height	165(150,180)
BMI	29.69(17.99, 41.4)
Data on fractures(YES/NO)	85/65
History of family orthopedic complaint(YES/NO)	56/94
Clinical history of bone complaint(YES/NO)	139/11
L2-L4(lumbar) Z-score	(-4.1, 3.1)
L2-L4 (lumbar)T-score	(-5.6, 1.2)
Femur Z-score	(-1.9, 1.1)
Femur T-score	(-2.2, 1.1)

Normal(T-score ≥1) *	74
Osteopenia (-2.5) < T-score <(-1)	48
Osteoporosis T-score ≤ (-2.5)	28
Total	150

* World Health Organization Definition of Osteoporosis by T-score values

LRP5rs121908669 detection and genotyping were determined using PCR-RFLP figure.1 and DNA sequencing figure.2. It was found that there were 97(64.66%) GG genotype, 20 (13.3%) homozygous genotype CC, 33 (22%) heterozygous genotype GC.

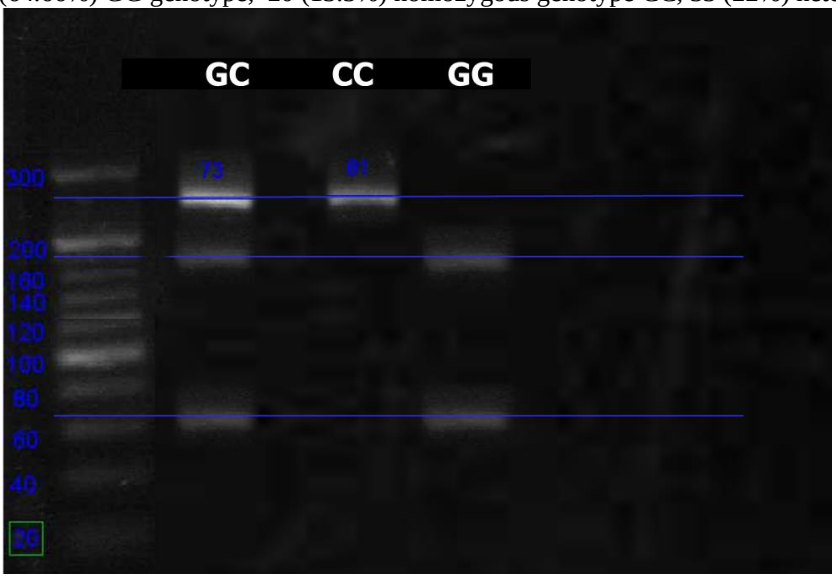

Figure.1: Agarose gel electrophoresis of PCR products for the Bfi1 polymorphism: the far left lane, 20bp DNA ladder, The rest of lanes,GG genotye, CC genotype, GC genotype.

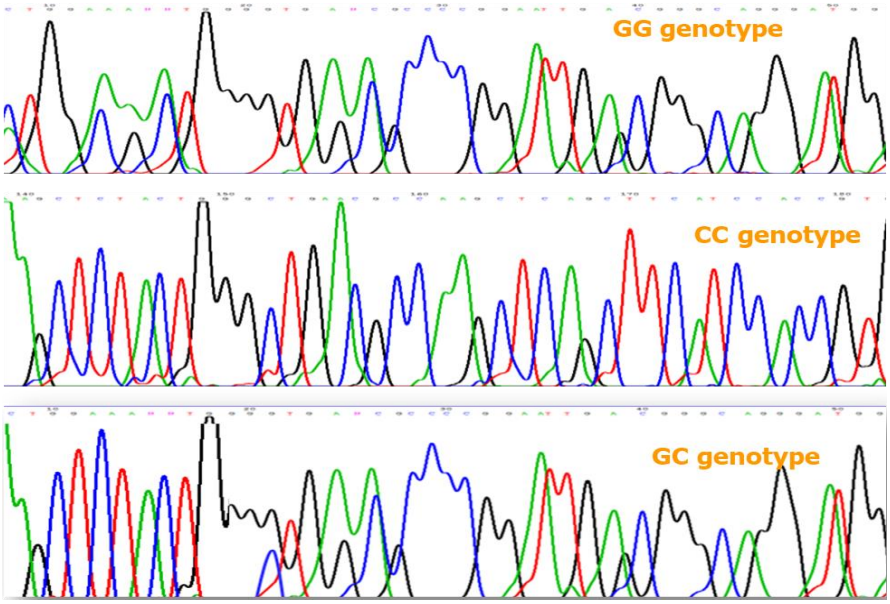

Figure.2: GG(GGGGT),CC(GGGCT),GC(GGG(G/C)T) genotypes of LRP5G171R by DNA sequencing analysis

This study is the first of its kind in Syria related to LRP5. It is the first of its kind worldwide related to LRP5rs121908669 genotyping.

LRP5rs121908669 detection and genotyping were determined using PCR-RFLP and DNA sequencing. It was found that there were 52 (34.66%) mutant cases (CC, GC), 98(65.33%) normal cases GG. The mutant cases were 20 (13.3%) homozygous genotype CC, 32 (21.33%) heterozygous genotype GC. The mutant cases (CC,GC) were 16 (19.51%) vs 36 (52.94%) in (osteoporosis and osteopenia) patients, and controls, respectively.

The homozygous genotype CC was 20 (13.3%). They distributed within 5(25%) vs 15 (75%) in (osteoporosis and osteopenia) patients, and controls, respectively.

The heterozygous genotypes GC were 32 (21.33%). They distributed among 11(34.37%) vs 21 (65.62%) in (osteoporosis and osteopenia) patients, and controls, respectively.

The normal genotype cases GG were 98 (65.33%). They distributed among 66(67.34%) vs 32 (32.65%) in osteoporosis and osteopenia patients, and controls, respectively.

DISCUSSION:

CC, GC genotypes are associated with normal BMD values in a higher percentage than the decreased BMD values. The idea of an association between mutant cases with high BMD regardless of genotype consists with Liesbeth Van Wesenbeeck et al. study,2002 [12]. But, It differs from studies that report ethnic differences in the expression of mutations [13].

The CC, GC genotype carriers with decreased BMD values cannot be neglected. It conflicts with Liesbeth Van Wesenbeeck et al. study,2002 [12]. It can be attributed to the presence of protective factors for expression (genetic or environmental factors), or presence of anti-genetic factors, or the presence of interaction between genes. Thus, the different genotypes of mutation may lead to a difference in its effect on bone mineral density [14].

The CC,GC genotypes carriers with low BMD don't all show clinical symptoms with ADO1, although it is a proven pathogenic mutation of ADO1. This conflicts with Liesbeth Van Wesenbeeck et al.2001 study [12]. They report that LRP5rs121908669 is associated with ADO1, which is often asymptomatic and sometimes associated with pain and hearing loss, but sure, it is not associated with fractures[7,12,15, 16]. That can be explained to differences in gene expression related to race.

The clinical features for LRP5G171R are likely related to HBM more than ADO1, and more clinical genetic studies are needed to resolve the issue. High bone mass HBM and ADOI are diseases from the osteopetrose group. The radiological features of ADOI and HBM are strikingly similar but clinically HBM patients do not have any complaints and are fully asymptomatic[17,18] whereas at least some ADOI patients suffer from severe pain[17,19].ADOI is the only type of osteopetrosis that is not associated with an increased fracture rate but HBM is associated with an increased fracture rate [6,7,17].

The prevalence of mutant cases is 52 (34.66%). It is a significant percentage. It conflicts with studies that report that it is a rare mutation with recessive transmitted [15].

CONCLUSION AND RECOMMENDATIONS:

LRP5rs121908669 may be with high or low BMD in variable clinical features, not only with ADO1. So, it is important to change its clinical significance on the NCBI platform from proven for ADO1 to conflicting information. The clinical features of the mutation are closer to HBM than ADO1, but this needs more studies to be approved.

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Declaration: I confirm that this work is a part of an approved PhD thesis which was approved by university board's decision No.1698 of 05/02/2019, and this work is an original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Ethical approval statement: The work was approved by the Ethics Committee in Syrian Ministry of Higher Education and written informed consent was obtained from all the participants according to the Declaration of Helsinki.

Informed Consent Statement:"Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author (Eiman M. Shahrour), upon reasonable request. All relevant material is included in this publication

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Conflict of Interests

Eiman shahrour , Bassel AL-Halabi , Amir N Dabboul, Walid Al-achkar, Abd Alrazak Hassan, Atieh Khamis, and Haissam Yazigi declare that they have no conflict of interest.

REFERENCES

1. Cummings SR, Nevitt MC, Browner WS, Stone K, Fox KM, et al. 1995. Risk factors for hip fracture in white women. Study of Osteoporotic Fractures Research Group. N Engl J Med 332:767–773
2. Krall EA, Parry P, Lichter JB & Dawson-Hughes B (1995) Vitamin D receptor alleles and rates of bone loss: influence of years since menopause and calcium intake. Journal of Bone and Mineral Research 10, 978–984.
3. Gueguen R, Jouanny P, Guillemin F, Kuntz C, Pourel J & Siest G (1995) Segregation analysis and variance components analysis of bone mineral density in healthy families. Journal of Bone and Mineral Research 12, 2017–2022.
4. Bouxsein ML, Myers ER, Hayes WC. 1996. Biomechanics of age-related fractures. In: Marcus R,Feldman D, Kelsey J, editors. Osteoporosis. San Diego: Academic Press, p 373–393
5. Stuart H. Ralston1,3 and Benoit de Crombrugghe2 Genetic regulation of bone mass and susceptibility to osteoporosis 2015 GENES & DEVELOPMENTdoi:10.1101/gad.1449506
6. Van Wesenbeeck E, Cleiren E, Gram J, Beals R, Benichou O, Scopelliti D, Key L, Renton T, Bartles C, Gong Y, Warman M, Vernejoul M, Bollerslev J, Van Hul W (2003) Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with and increased bone density. Am J Hum Genet 72:763–771.
7. Mark J., Kimberley H., Roel N. and Wim HU.(2004) LRP5 and Wnt Signaling: A Union Made for Bone J Bone Miner Res. Vol.19 DOI: 10.1359/JBMR.040816.
8. Whyte MP (1999) Sclerosing bone disorders. In: Favus M-J (ed) Primer on the metabolic bone diseases and disorders of mineral metabolism, 4th ed. Lippincott Williams and Wilkins, Philadelphia, pp 367–383
9. https://www.ncbi.nlm.nih.gov/
10. Joseph M. Zmuda, et al.(1999) Recent Progress in Understanding the Genetic Susceptibility to Osteoporosis. Genetic Epidemiology 16:356–367
11. Mark J., Kimberley H., Roel N. and Wim HU.(2004) LRP5 and Wnt Signaling: A Union Made for Bone J Bone Miner Res. Vol.19 doi: 10.1359/JBMR.040816
12. Van Wesenbeeck E, Cleiren E, Gram J, Beals R, Benichou O, Scopelliti D, Key L, Renton T, Bartles C, Gong Y, Warman M, Vernejoul M, Bollerslev J, Van Hul W (2003) Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with and increased bone density. Am J Hum Genet 72:763–771.
13. Lei, S. F. ; et,al.(2006) Ethnic difference in osteoporosis-related phenotypes and its potential underlying genetic determination; J. Musculoskelet Neuronal Interact. 6; 36-46
14. Xiaowei Zhu., Weiyang Bai., and Houfeng Z(2021)Twelve years of GWAS discoveries for osteoporosis and related traits: advances, challenges, and applications Bone Research 9:23; https://doi.org/10.1038/s41413-021-00143-3
15. Little RD, Carulli JP, Del Mastro RG, et al.(2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant highbone-mass trait. Am J Hum Genet 70:11-9.
16. Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K, Lifton RP (2002) High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med 346: 1513–1521.
17. Van Hul E, Gram J, Bollerslev J, VanWesenbeeck L, Mathysen D, Andersen PE, Vanhoenacker F, Van Hul W (2002) Localization of the gene causing autosomal dominant osteopetrosis type I to chromosome 11q12–13. J Bone Miner Res 17:1111–1117
18. Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB, Recker RB (1997) Linkage of a gene causing high bone mass to human chromosome 11q12–13. Am J Hum Genet 60:1326–1332.
19. Bollerslev J (1989) Autosomal dominant osteopetrosis: bone metabolism and epidemiological, clinical, and hormonal aspects. Endocr Rev 101:45–67.