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Original Article

Polymorphism of LRP4 Gene (rs9667108) among Post Menopause Women with Osteoporosis

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Abstract

Background: Many studies have been done to identify the factors that influence the development and progression of osteoporosis. One genetic factor is polymorphisms of *LRP4* gene. Regarding the lack of comprehensive study on polymorphisms of *LRP4* gene in the north of Iran, mainly Mazandaran Province, we decided to investigate the polymorphism of this gene in postmenopausal women with osteoporosis.

Methods: This case-control study has been conducted at GhaemShahr Valiasr Hospital on 100 female patients with osteoporosis (average age of 58.1) and 90 healthy females without osteoporosis (average age of 55.2). After sampling and extraction of genomic DNA via of the salt deposition method, the genotype and SNP (rs9667108) polymorphism of *LRP4* gene were evaluated with the PCR-RFLP method. Restriction enzymes cut the PCR products. In order to identify patients, their bone mineral density was tested by the DEXA method .The results of digestion (digestion enzyme) were analyzed by MedCalc, SPSS software, Hardy-Weinberg equilibrium, and Chi2.

Results: The statistical analysis has shown the significant relationship between SNP (rs9667108) polymorphism and the risk of osteoporosis disease in patients and control groups ($P<0.05$). In SNP (rs9667108), the GC genotype, compared to GG, increased the risk of disease significantly (1.556 time). Similarly, CC genotype, compared to GG genotype, increased the risk of this disease by 2.091 time.

Conclusion: The existence of mutation in the *LRP4* gene could increase susceptibility to osteoporosis disease. Moreover, determining this patient's genotype in SNP (rs9667108) can be used to identify individuals who are in endanger osteoporosis.

Keywords: Osteoporosis; PCR; Polymorphism genetics; Single nucleotide polymorphisms (SNPs)

Introduction

Osteoporosis increases the risk of bone fracture in people with reduced bone mass (1). The prevalence of osteoporosis in the United States will increase by 50% in 2025 (2). About 40% of women and 10% of men are at risk for osteoporosis during their lifetime (3). One of the significant concerns about osteoporosis is its asymptomatic occurrence in most cases (4). Early diagnosis and Prevention are essential for this disease (1-4).

In general, bone density index and bone quality play an essential role in bone strength. Bone quality index refers to the structure, reconstruction, architecture, and geometry of bone. There is currently no international unit of measurement for bone strength. Bone minerals (BMD), which provide 70% of bone strength, are often used as a good indicator of bone strength (5).

Follow-up of risk factors can lead to early detection of osteoporosis, but the only assuring way to diagnose the process is to test bone mineral density (6). It helps to improve the quality of life and treatment to reduce risk factors and delay fracture (7). The maximum bone mass is detected between the ages of 20 and 30, which is determined by statistical calculations from among the same-sex population (6).

The most important thing about osteoporosis is its prevention (6). Prevention of osteoporosis is divided into primary and secondary categories. Primary prevention is considered as modifiable factors in life (7). These factors include adequate mobility, proper daily exercise, non-smoking, proper intake of calcium and vitamin D in the diet (8).

Preventing the early onset of adverse health outcomes in people with osteoporosis is identified as secondary prevention. In other words, you have the disease, and you want to prevent something wrong from happening (11) in the case of osteoporosis, the "bad" or negative health result is the bone fracture. Bone mineral density testing is the best way to prevent secondary osteoporosis (9).

Various studies have been performed to identify genes in charge of osteoporosis that show that at least 30 genes are involved in the disease. Variables in 56 regions of the genome were affected by bone mineral density, while 14 of these variables increased the risk of fractures (10-12).

One of the most critical gene families involved in osteoporosis is the *LRP4* gene. A study has focused on finding effective therapeutic compounds in the WNT/ B catenin signaling pathway, one of the basic foundations in ontogenesis. The external building blocks (extra-cytoplasmic component) of *LRP4* are very similar to *LRP5 / 6,* two members of the *LRP* receptor subgroup, and serve as auxiliary receptors in the WNT-signaling stream (13).

The proposed role of *LRP4* as a canonical WNT signaling antagonist is probably through the translocation of *LRP5/6* hemodarg proteins into a joint complex formed by the FZ protein with *LRP5 / 6* which is the process for binding of canonical proteins to the translocation of canonical proteins (14). Bone is an essential method in the assessment of osteoporotic fractures. In a meta-analytic partnership, at least 20 genes were identified about pelvic and lumbar bone with BMD, such as *LRP5, LRP4*, and osteoprotegerin (OPG), sclerostin (sost), and these genes were found to play a role in genetic structure and BMD (15). Accordingly, important molecules with new biological internal pathways could express the effects of BMD differences in individuals, especially WNT signaling pathways (14).

LRP4 is a vital regulator in the kidneys (15). It is also involved in tooth development (16) and is an essential coreceptor for arginine in the formation of neuromuscular junction (17). The proposed role of *LRP4* as a canonical wnt signaling antagonist was probably through the translocation of *LRP5/6* hemodialysis proteins in a joint complex formed by the FZ protein *with LRP5/6*. This process is required for the binding of wnt proteins to transmit the wnt signal to the descending elements of the canonical cascade (18).

Our previous study examined polymorphisms related to *LRP4* gene in the database (NCBI) among the existing polymorphisms according to criteria such as validation, the percentage of mutant allele frequency in the population, and its place in the population to study *rs 4752947* gene (19) and recent study has assayed *rs9667108* gene polymorphism of *LRP4* in patients with osteoporosis. We aimed to investigate the relationship between *LRP4* gene (*rs9667108*) polymorphism and osteoporosis in postmenopausal women in northern Iran.

Materials and Methods

This study was a case-control study. The statistical population included 190 people in the age range of 45-60 years among postmenopausal women referred to Vali-e-Asr Hospital in Ghaemshahr from at GhaemShahr Valiasr Hospital, Mazandaran province, Iran. Overall, 100 patients with osteoporosis were diagnosed by osteoporosis radiographic tests these people and 90 patients in the control group (without osteoporosis) with the help of medical records, interviews, and according to ethical principles with the written consent by questionnaires were reviewed.

Inclusion criteria includeed age, sex, clinical and pathological factors such as body mass index (BMI), and output criteria include alcohol and tobacco consumption and stomatal diseases such as rheumatoid arthritis and bone cancer.

The study was conducted by obtaining license number 94413 in the Research Council of the Islamic Azad University, Tonekabon Branch, and the consent of the participants.

DNA extraction

After collecting whole blood samples, genomic DNA was extracted from peripheral blood leukocytes by a standard salt deposition method. After extracting DNA from the studied samples, to evaluate the quantity and quality of DNA and to know its concentration and purity, the absorption of the DNA optical sample was examined by spectrophotometry at 260 nm and 280 nm with 1% agarose gel.

Primer design and synthesis

The *LRP 4* gene polymorphism was investigated by a replication-based method. The region from one microliter of genomic DNA was amplified. The desired two mmol MgCl 2, 1.5 µmol dNTPs, one picomole of each specific primer (designed by Allele ID 6 software), two units of DNA polymerase tag enzyme in the final volume of 25 microliters were made using a German-made Indorf thermocycler (Table 1).

The general PCR program includes initial spinning at 94 °C for 3 minutes, cyclic spinning at 94 °C for 30 seconds, binding of primers at 61 °C for 1 minute, and elongation at 72 °C. C in one minute at 35 cycles and finally the final elongation of 5 minutes at 72 °C. To verify the accuracy of the obtained product, PCR was performed on 2% agarose gel and electrophoresis with ethidium bromide staining next to the marker. For enzymatic digestion, seven µl of PCR product with one µl of ApoI enzyme (Thermo Scientific) and two µl of required enzyme buffer in the final volume of 20 µl was mixed at 37 °C for 16 hours according to the instructions of the mixed enzyme. Genotypic analysis: The product was placed on 2% agarose gel, and electrophoresis was performed along with marker and ethidium bromide staining. Finally, to confirm the results of enzymatic digestion by PCR - RELP method on ten% of the samples. Sequencing was performed.

Enzymatic digestion

For enzymatic digestion, seven µl of PCR product was mixed with one µl of ApoI (Thermo Scientific) restriction enzyme and two µl of required enzyme buffer in a final volume of 20 µl according to the enzyme instructions and placed at 37 °C for 16 h. The enzyme cuts the mutant genotype (TT) to produce a 175 bp product. For genotypic analysis, the product was placed on 2% agarose gel, and electrophoresis was performed along with marker and ethidium bromide staining. Also, the results of enzymatic digestion were confirmed by PCR-RFLP sequencing on ten percent of the samples.

Table 1: Specifications of enzymes and primers used

	Primer sequence
Rs:9667108	Forward: *ACTTTGAGGTGGAGGAACTAGG* Reverse: *CTCATGTCGCCTTAGTTCTCTTG*

Statistical analysis

The statistical software used was SPSS version 22 (IBM Corp., Armonk, NY, USA) and MedCalc. A two-way analysis of variance was used to investigate the effect of polymorphism on BMD. A parametric *t*-test was used to compare the means of quantitative variables in the two groups. Quantitative variables were expressed as mean ± standard deviation, and qualitative variables were expressed as percentages (%). The disease has been used. A significant *P*-value of 0.05 was considered.

Results

A total of 190 postmenopausal women in the age range of 45-60 years participated in this study, of which 100 patients with osteoporosis (mean age 58.17 ± 0.44) and 90 healthy controls (mean age 55.29 ± 0.54) were enrolled. According to the chi-square test shown in Table 2, the mean age difference between the patient and control groups was statistically significant (P <0.001). Age distribution in control and patient groups had a normal distribution (P <0.001).

The bone mineral density in the lumbar vertebrae and hip bone in patients was significantly different from the healthy group (P <0.001) (Table 2). All extracted DNAs were of good quality, and the amount of light absorption at 260 to 280 nm was more than 1.8. Fig. 1 shows the electrophoresis results of PCR products before and after enzymatic cleavage, respectively. Fig.1 A shows the efficiency of chain reaction performance in the studied samples. In Fig.1 B, the amplification product was digested enzymatically, and the following banding pattern was obtained.

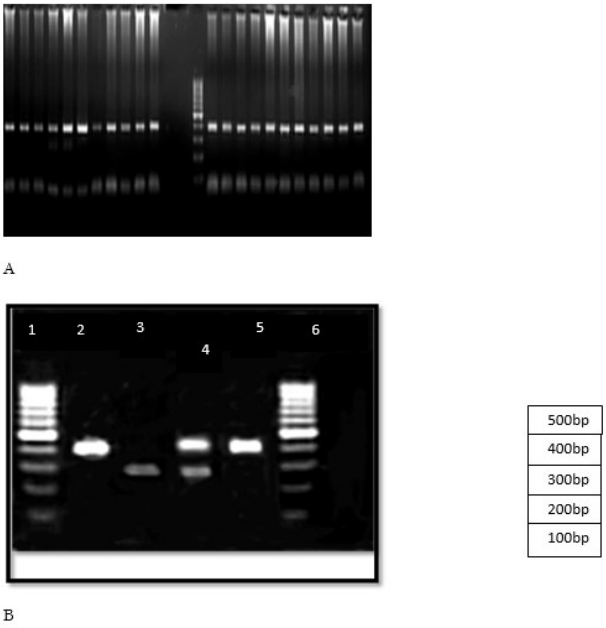

Fig. 1: Electrophoresis of PCR product before (A) and after enzymatic digestion (B) on 2% gel. In order from left to right: 1-Ladder 500bp - 2-Undigested product - 3-Wild homozygous genotype GG (+ / +) - 4- Heterozygous genotype GC (+/-) – 5-Homozygous mutant CC (- / -) - 6 -Ladder500bp

The distribution of genotypic and allelic frequencies in the two groups of women with osteoporosis and the healthy group is summarized in Table 3. GC genotype compared to GG genotype increased the chance of disease by 1.556 times, and CC genotype increased by 3.5 times compared to GG genotype. Also, the C allele increases the chance of disease by 2.091 times compared to the G allele.

Table 2: Statistical analysis of age and BMD results in patient and healthy groups (P<0.001)

Variable	*Patient group*	*Control group*
Age (yr)	58.17±0.44	55.29±0.54
Lumbar vertebral bone density (g / cm2)	0.804±0.01	1.23±0.01
Lumbar T-score	3.057±0.06	0.386±0.09
Lumbar Z-score	2.019±0.07	0.858±0.1
T-score right hip bone	1.521±0.11	0.78±0.08
T-score left hip bone	1.411±0.08	0.79±0.08
Z-score right hip bone	0.612±0.08	1.229±0.08
Z-score left hip bone	0.609±0.08	1.238±0.08

Table 3: Distribution of genotypic and allelic frequencies studied in two groups of patients and controls

Polymorphism	*Genotype*	*Number of patients (percentage)*	*Number of controls (percentage)*	*chi square*	P *value*	*OR*	*CI 95%*
RS 9667108	GG	79(79)	79(87.8)	0.106		1	
	GC	14(14)	9(10)	0.399	0.33	1.556	(802.3 – 637.0)
	CC	7(7)	2(2.2)	0.175	0.13	3.5	(373.17 – 705.1)
Allele	G	172(86)	167(92.8)				
	C	28(14)	13(7.2)	0.033	0.04	2.091	(175.4 – 047.1)

Discussion

The aim of this study was to investigate the association between rs 9667108 polymorphism and *LRP4* gene with osteoporosis in postmenopausal women.

Since the production and resorption of bone mass tends to increase bone resorption by osteoclast cells with aging, it is predictable that with increasing age in postmenopausal women, we will see a decrease in bone mass density and thus an increase in the incidence of osteoporosis. This prediction was also seen in the results of our study (19).

The effects of *LRP* family members on bone diseases have been discussed in several studies. In addition to *LRP5*, other members of this family also play an important role in bone homeostasis. In the meantime, *LRP6* has a similar role to *LRP5*, but other members of this family, such as *LRP4* and *LRP8*, have less roles. These genes modulate the WNT signaling pathway, so mutations in these genes cause interference in the pathway. As a result, it disrupts bone cells (20). The immunohistochemical assay indicates that the *LRP4* gene impress in the sclerostin protein of osteoblast and osteocyte target cells (13). Sclerostin protein regulates osteoblast and osteocyte target cells. It actually slows down or stops the production of new bones (21). The association between sclerostin and *LRP4* is essential for the inhibitory function of sclerostin in bone formation, it shows that *LRP4* plays a crucial role in bone homeostasis (22). Dysfunction of the *LRP4* gene increases the protein sclerostin and thus decreases bone mass (23-26). LRP4 directly associate with sclerostin in the bone and therethrough helps ability of sclerosis to antagonize the identification of WNT ligands through *LRP5/6* in the osteoblast and prevent bone formation (27).

A meta-analysis of genomic studies performed on 19,195 Nordic individuals showed an association between *LRP4* and lumbar spine and femur BMD variability (28). They reported a connection between mutations in the *LRP4* gene and osteoporosis. The two polymorphisms rs2306029 and

rs6485702 decrease BMD in all parts except the lumbar vertebrae. The results of this study were similar to our study except for the lumbar vertebrae. Our study showed that rs9667108 polymorphism can also reduce BMD in the lumbar vertebrae (29). In this study, by analyzing the *LRP4* gene, we showed that there is a significant relationship between rs9667108 polymorphism with BMD of the lumbar spine and hip bone, which were similar to previous studies.

There was a significant relationship between rs381661 and rs6485702 polymorphisms in the *LRP4* gene with BMD after controlling for confounders and the Wnt signaling pathway (30). Examination of 113 polymorphisms in 16 osteoporosis-related genes showed that rs898604 and rs17790156 polymorphisms in the *LRP4* gene were associated with bone fractures in osteoporotic individuals (31).

Analysis of 150 candidate genes and evaluation of 36016 SNPs revealed that SNPs from 9 gene loci (*ESR1, LRP4, ITGA1, LRP5, SOST, SPP1, TNFRSF11A, TNFRSF11B,* and *TNFSF11*) were associated with BMD in the femoral neck and lumbar spine. SNPs from *LRP5, SOST, SPP1*, and *TNFRSF11A* loci were significantly associated with fracture risk, and other loci were not statistically significant (32, 33). One study examined the effect of *SOST*(rs851056) and *DKK1*(rs1569198) polymorphisms on osteoporosis in Mexican postmenopausal women. The results show that these polymorphisms, unlike rs9667108, have no effect on osteoporosis in postmenopausal women (34). Another studies in postmenopausal women noted *TNFRSF11B, SPTBN1, ESR1*and *LRP4* as a sensitive sites for osteoporotic fractures. The protein encoded by *TNFRSF11B* is osteoprotegerin, which plays as a pretence receptor for RANKL and inhibits bone resorption. Evidence of invivo shows that *LRP4* in osteoblasts can repress bone formation and increase osteoclastogenesis by acting as a sclerostin receptor. These studies, similar to our study, shows the effect of *LRP4* gene on osteoporosis (31, 35).

According to this study, by examining the genotypic and allelic distribution among patient and healthy groups, there was a significant relationship between polymorphism SNP (rs9667108) and the chance of osteoporosis ($P<0.001$). Our study found that mutations in the C allele increase the chance of developing the disease, and the CC phenotype is susceptible to osteoporosis. In our study, the association between the *LRP4* gene and osteoporosis was similar to previous studies. However, more research is needed to link it to fracture risk and suggested that this study be performed with more samples and more *LRP4* gene subtypes.

Conclusion

In our study, the association between the *LRP4* gene and osteoporosis has been shown. There was a significant relationship between polymorphism SNP (rs9667108) and the chance of osteoporosis. Mutations in the C allele have increased the chance of developing the disease and the CC phenotype is susceptible to osteoporosis. More research is needed to link it to the risk of fracture. It is suggested that this study be performed with more samples and more *LRP4* gene subtypes.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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