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Sp1 binding site polymorphism of a collagen gene (rs 1800012) in women aged 45 and over and its association with bone density

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Background/aim: Sp1 polymorphism of type I collagen genes is accompanied with bone collagen disorders and severe clinical phenotypes such as osteogenesis imperfecta. The aim of this study was to study the association between COLIA1 Sp1 polymorphism and bone density rate.

Materials and methods: In this descriptive, analytical study conducted in 2013 in southwestern Iran, 200 blood samples, per the Cochran sample size formula, were taken from women aged 45 and older. DNA was extracted from the samples using the phenol–chloroform method and the genomic fragments in question were proliferated using the polymerase chain reaction (PCR) method.

Results: The genotype distribution of Sp1 polymorphism for the SS, Ss, and ss genotypes was 57.1%, 31.4%, and 11.4%, respectively, in the control group and 9.2%, 75.4%, and 15.4%, respectively, in the patients. Statistically, Sp1 polymorphism in patients had a significant deviation ($P = 0.001$, $\chi^2 = 34.25$) and there was no Hardy–Weinberg equilibrium. In the control group, there was no significant deviation for Sp1 polymorphism ($P = 0.226$, $\chi^2 = 2.97$). Sp1 polymorphism was significantly associated with bone density. Women with the SS genotype had the highest bone density.

Conclusion: Sp1 gene polymorphism is associated with bone density rate in women aged 45 and over, and is more commonly observed in homozygosity. Determining this genotype's polymorphism is valuable to identify the women at risk of developing osteoporosis.

Key words: Bone mineral density, collagen type 1α1 Sp1 polymorphism, osteoporosis, polymerase chain reaction

1. Introduction

Osteoporosis is a common skeletal disorder that is accompanied by a decrease in bone mass, deterioration of bone microstructures, and an increase in susceptibility to fracture (1). Osteoporosis has a complex etiology and is considered to be a multifactorial polygenic disease with genetic factors alongside hormonal, environmental, and nutritional ones contributing to its incidence (2).

Fracture leads to increases in morbidity and mortality and imposes high costs on individuals and society. On average, one of every three women and one of every 12 men develop fractures during their lifetime (3,4).

One of the conditions causing osteoporosis is a postmenopausal decrease in estrogen. After 35 years, the amount of bone density gradually declines in both men and women, but since estrogen decreases in postmenopausal women, their bone mass declines more rapidly compared to men, so that 5–10 years after menopause one third of the bone density is lost. In ages over 65 years, the amount of bone loss equalizes in women and men and bone mass continues to decline until death (1,5). Genetic differences are likely to have a role in bone mineral density (BMD) diversity in different ethnicities (6).

An extracellular matrix is the main component of bone and develops a scaffolding and three-dimensional network from collagen type I, on which minerals precipitate (7). Collagen is a fibrous protein that comprises about 30% of body weight and is frequently seen in connective tissues like bones, tendons, and skin. This protein has an important contribution to wound healing, cell proliferation, cell differentiation, and cell migration. Proteins of the collagen family are the most frequent proteins of the extracellular matrix (8,9).

Collagen type I is the main collagen and the highest protein in the bone matrix, comprising approximately 80% of the total bone tissue's proteins. Variations in collagen characteristics and the relative amount in the bone matrix could influence the mechanical properties of the bone

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tissue and cause fragility and increased susceptibility to fracture. Collagen consists of two chains, alpha 1 and alpha 2 (at a 2:1 ratio in the collagen fibers), which are encoded by two different genes, collagen type 1α1 (COLIA1) and collagen type 1α2 (COLIA2) (10–12). The gene COLIA1 is on the long arm of chromosome 17 ($17q^{21-22}$) and has 51 exons; the gene COLIA2 is on chromosome 7 and has 52 exons (13). An imbalanced ratio of collagen chains could be a potential risk factor for bone fragility and a predisposing factor for osteoporosis (12).

Different polymorphisms have been described in several genes (14). Although many studies have been conducted to date, the unique contributions of each one of these polymorphisms to osteoporosis pathogenesis have still remained unconfirmed worldwide (15).

COLIA1 and COLIA2 mutations are accompanied by bone collagen disorders and severe clinical phenotypes including osteogenesis imperfecta. A substitution mutation of G/T in intron 1 of gene COLIA1 (rs 1800012, G>T substitution) probably influences gene transcription, resulting in a variation in the alpha 1 sequence/alpha 2 sequence ratio and collagen adult protein; this is called COLIA1 Sp1 polymorphism (16,17).

The genotypes of COLIA1 Sp1 consist of SS, Ss, and ss. Allele s inheritance is recessive. The presence of allele s increases the tendency toward Sp1 transcription factor. The ratio of collagen α1(I) to α2(I) was 2.3 to 1 in Ss heterozygotes compared with the expected value of 2 to 1 in SS homozygotes (18). (For osteoblasts of homozygous SS, the alpha 1/alpha 2 ratio is similar to the rate in question, i.e. 2 to 1). The variation in the alpha sequence ratios is due to the increase in mRNA COLIA1 compared to mRNA COLIA2. As a result, the strength of a heterozygous Ss individual's bone declines in comparison to one with homozygous SS. Homozygous ss has the least bone density with a higher risk of fracture. Of course, this polymorphism is more associated with vertebral and wrist fractures. In gene COLIA1, there are other polymorphisms that are associated with bone density, including PCOL2, which causes G/T substitution in the promoter region (12,17,19).

Recent advances in detecting the genetic basis of diseases have opened new horizons, and disease diagnosis through determining genotype is gradually increasing. The pharmacologic and interventional methods for preventing osteoporosis in the individuals at risk add to the significance of the issue. Different studies have demonstrated the association of COLIA1 Sp1 polymorphism with bone density rate (20), but no such association has been specified in Iranians. Since no study, to the best of our knowledge, has yet been done on the association of osteoporosis having a molecular basis with this polymorphism, this study aimed to investigate the association between Sp1 polymorphism and bone density rate in women aged 45 and over in an accessible Iranian subpopulation.

2. Materials and methods

In this descriptive, analytical study conducted between December 2012 and September 2013, blood samples were taken for bone density measurement from women aged 45 and over in referring centers in southwestern Iran, based on convenience sampling.

In this study, the age of 45 was regarded as the age of onset of menopausal changes (21).

In this study, 200 samples were investigated. Sample size was calculated using the Cochran equation. Exclusion criteria included corticosteroid consumption history, ovariectomization or premature ovarian failure, thyroid disease, calcium intake disorders, and gastrointestinal and renal diseases.

After consent was obtained from the participants and questionnaires related to clinical data were filled out, a 5 mL sample of peripheral blood was taken from each patient and put in tubes containing EDTA (0.5 M). The blood samples were sent to the Cellular and Molecular Research Center of Shahrekord University of Medical Sciences for molecular analysis. For this, DNA was extracted using the phenol–chloroform method (22). The quality, quantity, concentration, and purity of the extracted DNA were examined by spectrophotometry.

The chain relevant to the collagen 1 gene was extracted from the NCBI database (23). The primers for Sp1 polymorphism were designed using Primer3, which examines the primers in terms of primer–dimer formation or their self-supplementation and provides the operator with the best chain. The primers used are shown in Table 1.

Polymorphism proliferation was done by polymerase chain reaction (PCR). In the present study, for proliferation

Table 1. The primer chains used in this study.

Tm	Primer type	5′–3′ primer sequences
70	F	CTGGACTATTTGCGGACTTTTTGG
60	R1	GGCGAGGGAGGAGAGAAG
68	R2	GTCCAGCCCTCATCCTGGCC

of the regions in question and PCR conduction, firstly the main reaction solution was prepared in a microtube (vial). At this step, the DNA sample was not added to the solution. After distributing the solution among the special PCR vials, we added the necessary amount of DNA sample to them. In each PCR analysis, a vial was regarded as a negative control that indicated lack of material contamination with the DNA sample. The final volume of reaction solution was considered to be 25 µL in each microtube. A thermocycler (PC818, ASTEC, Japan) was used.

For separation, identification, and purification of the DNA fragments, an electrophoresis technique on 8% polyacrylamide gel was used with TBE and loading X6 (Fermentas) buffers (24). The fragments were dissolved using restriction enzymes (Fermentas). The detection of DNA size was done with a ready-to-use GeneRuler™ DNA ladder. Silver nitrate staining was used to observe the obtained bands. The data were analyzed using SPSS with the chi-square and analysis of variance (ANOVA) tests.

3. Results

Considering that all women over 45 years old have some proportion of bone density decline, we assigned the individuals with normal bone density and/or mild osteopenia as the control group (comprising 70 individuals) and those with severe osteopenia and osteoporosis as the patient group (consisting of 130 individuals), based on radiograph t-scores (Table 2). In this study, a t-score > −1 was determined as normal, a t-score ≤ −1 to ≥ −2 as mild osteopenia, a t-score < −2 to ≥ −2.5 as severe osteopenia, and a t-score < −2.5 as osteoporosis (25).

To investigate the quality and quantity of the extracted DNA, NanoDrop was used. In most cases, the quality of the extracted DNA samples was desirable and had an A260/A280 value between 1.75 and 2.

For Sp1 polymorphism, the PCR reaction is a seminested type; that is, through the first step of the PCR, the fragment containing Sp1 polymorphism is proliferated. The size of the fragments yielded by PCR of the Sp1 polymorphism gene COLIA1 in the first step was 287 bp; the second PCR was conducted on the 287 bp product obtained from the first PCR and the cutting site was developed using the second PCR. The size of the fragments obtained from the second PCR was 254 bp. From enzymatic dissolution of Sp1 polymorphism of the collagen gene by enzyme restrictor MscI, two fragments of 18 and 236 bp were developed. The 18 bp fragment had a high speed because of its small size and it is not observed in Figure 1.

Genotype and allele frequency were calculated for the polymorphism in question in the two groups. To examine the difference in genotypic frequency between the patients and the control group, chi-square and ANOVA tests were used. The confidence interval (CI) was set to 95% in all experiments and $P < 0.05$ was considered significant (Tables 3 and 4).

The chi-square test was independently calculated for the patients and control group with a degree of freedom of 2 for the polymorphism in question. The P value for Sp1 polymorphism in the patients was calculated as 0; that is, statistically there was a significant deviation for the Sp1 polymorphism and no Hardy–Weinberg equilibrium was noted ($P = 0.001$, $\chi^2 = 34.25$). In the control group, the P value obtained was 0.266; that is, in the control group there was no significant deviation for Sp1 polymorphism and a Hardy–Weinberg equilibrium was seen ($P = 0.226$, $\chi^2 = 2.97$).

The ANOVA test indicated a significant difference in the Sp1 polymorphism between the control group and the patients.

Table 2. Characteristics of the control and patient groups in this study.

Variable	Mean ± SD (patients)	Mean ± SD (control group)	P value
Number	130	70	
Lumbar vertebrae t-score	2.8- ± 0.67	0.9- ± 0.8	< 0.0001
Femoral neck t-score	1.3- ± 0.78	0.34- ± 0.5	< 0.0001
Age (years)	59.9 ± 8.4	51.3 ± 5.7	< 0.0001
Height (cm)	154.8 ± 3.6	158.5 ± 4.9	< 0.0001
Weight (kg)	66.3 ± 8.09	71.7 ± 9.1	< 0.0001
Lumbar vertebrae BMD* (g/cm^2)	0.73 ± 0.1	0.92 ± 0.15	< 0.0001
Femoral neck BMD (g/cm^2)	0.76 ± 0.1	0.88 ± 0.11	< 0.0001

* = Bone mineral density.

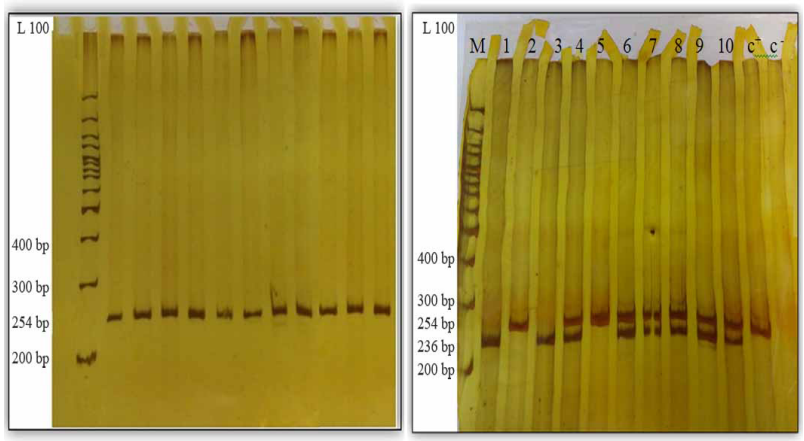

Figure 1. A: PCR product of Sp1 polymorphism in the second step (the size of the proliferated section was 254 bp). B: The results relevant to enzymatic dissolution of Sp1 polymorphism. M = DNA size marker, C+ = positive control, and C– = negative control. The samples numbered 1 and 3 have homozygous genotype ss, the samples numbered 2 and 5 have homozygous genotype SS, and the samples numbered 4, 6, 7, 8, 9, and 10 have heterozygous genotype Ss.

Table 3. Sp1 genotype and allele frequency in the two groups.

Sp1 polymorphism of the collagen gene		Genotypic frequency		OR (95% CI)	P value
		Control group	Patients		
Genotypes	SS	40 (57.1%)	12 (9.2%)	2.69 (1.47–4.93)	<0.0001
	Ss	22 (31.4%)	98 (75.4%)	0.64 (0.32–1.29)	0.17
	ss	8 (11.4%)	20 (15.4%)	1.00	
	Total	70 (100)	130 (100%)		
Alleles		Allelic frequency			
	S	0.73 (73%)	0.47 (47%)	1.80 (1.28–2.53)	<0.0001
	s	0.27 (27%)	0.53 (53%)	1.00	
	Total	1 (100%)	1 (100%)		

Table 4. The number of expected and observed individuals for Sp1 polymorphism in the two groups.

Sp1 polymorphism of the collagen gene		Patients		OR (95% CI)	P value	Control group		OR (95% CI)	P value
		observed	expected			observed	expected		
Genotypes	SS	12	28.7	0.82 (0.45–1.48)	0.52	40	37.3	0.84 (0.52–1.36)	0.54
	Ss	98	64.8	1.68 (1.16–2.44)	0.003	22	27.6	0.71 (0.42–1.22)	0.41
	ss	20	36.5	1.00		8	5.1	1.00	
	Total	130	130			70	70		

The mean t-score for the lumbar vertebrae was –2.425 in 12 patients with genotype SS, –2.761 in 98 with genotype Ss, and –3.395 in 20 patients with genotype ss. The df for both lumbar vertebrae and femoral neck was 2 while the P value for lumbar vertebrae was 0.00 and that of femoral neck 0.007, indicating that the Sp1 polymorphism was significantly associated with t-score, i.e. bone density. The individuals with genotype SS had the highest density, followed by the individuals with genotypes ss and Ss (Table 5).

4. Discussion

Polymorphism or multiformity is a change in nucleotides in a gene sequence that leads to no particular disease and has a frequency of higher than 1% in the communities. In fact, this polymorphism can develop or destroy an enzyme's identifying site and develop various genotypes (26). The results of the present study highlight that Sp1 polymorphism is an important index for low bone mass and vertebrae fracture and determining the genotypes of this polymorphism is likely to be valuable in identifying the women at risk of osteoporosis (27).

Different studies on the association of polymorphism of several genes with osteoporosis have been conducted, including the vitamin D receptor gene, collagen type 1, calcitonin receptor, insulin-like growth factor, and interleukin 6 (28).

In the present study, the association of Sp1 polymorphism of the collagen gene with bone density was investigated in women aged 45 and older. In view of the obtained results, homozygous ss and heterozygous Ss individuals had the lowest t-scores, lower in the homozygous ss individuals compared to the heterozygous Ss. There is a significant association between bone density and Sp1 polymorphism of the collagen gene and a statistically considerable difference in genotype and allelic frequency was observed between the patients and the control group ($P < 0.05$). In the patients with severe osteoporosis and vertebral fracture, genotypes ss and Ss are prevalent (approximately 54%) (29,30).

In the present study, older patients were shorter and thinner and the BMD of these individuals was lower compared to the control group. Some other researchers obtained similar results. For example, in a study on British women, this polymorphism was associated with BMD. As such, BMD in the homozygous genotype SS was higher than in the heterozygous genotype Ss and the homozygous genotype ss (29).

Langdahl et al. investigated the association of Sp1 polymorphism of the collagen gene osteoporosis-associated fractures in Danish men and women and found that Sp1 polymorphism of COLIA1 was notably associated with lumbar vertebral fractures in both men and women (31).

Inconsistent findings have been obtained on the association between Sp1 polymorphism of the collagen gene and osteoporosis-related fractures in Caucasian postmenopausal women (32–35). The results of four meta-analyses in this field indicated that Sp1 polymorphism of COLIA1 gene is associated with osteoporosis-related fractures and BMD in Caucasian postmenopausal women (18,27,36,37). This polymorphism contributes importantly to osteoporosis pathogenesis, which is mediated by affecting BMD and other predictors of osteoporosis-associated risk of fracture such as the ultrasound characteristics of bone and skeletal geometry (38).

One of the above meta-analyses is indicative of an association between the presence of the s allele of Sp1 polymorphism and a decline in BMD of the lumbar vertebrae and femoral neck, and an increase in vertebral fracture (36). Ralston et al. argued that fracture risk in individuals with the homozygous ss genotype was about 40% higher than that in those with the genotype SS (39). Ji et al. conducted a study on menopausal Caucasian women and concluded that Sp1 polymorphism was likely to be associated with osteoporotic fracture (40). A study in Turkey indicated that bone density in the individuals with genotype Ss was lower compared to those with genotype SS (41), consistent with the present study. Some

Table 5. The relationship between Sp1 polymorphism and t-score.

	Genotypes	Number	T-score (Mean ± SD)	Standard error	P value
Lumbar vertebrae	SS	12	–2.425 ± 0.48	0.13	0.001
	Ss	98	–2.761 ± 0.64	0.06	
	ss	20	–3.395 ± 0.62	0.13	
Femoral neck	SS	12	–1.250 ± 0.55	0.16	0.007
	Ss	98	–1.300 ± 0.81	0.08	
	ss	20	–1.850 ± 0.55	0.12	

researchers have obtained opposite results. For example, in a study of postmenopausal Swedish women, Liden et al. argued that COLIA1 Sp1 polymorphism was not significantly associated with femoral neck BMD, but closely associated with vertebral fracture (42). In an investigation by McClean et al. in Northern Ireland, osteoporosis-associated fractures and Sp1 were not associated (43). Bandres et al. investigated the association between BMD and COLIA1 in Spanish postmenopausal women and stated that although no association was found between COLIA1 and BMD, this gene was significantly associated with the prevalence of osteoporotic fractures. This should be examined in a larger sample size (44). The association between osteoporosis and Sp1 polymorphisms (COLIA1) in postmenopausal women in Turkey was investigated and no statistically considerable difference in genotype and allelic frequency was observed between the patients and the control group (45). As stated by Bandres et al., in studies in which opposite results were obtained, a small sample size was offered as the reason. The other reason for this inconsistency could be the study of different races. In view of the numerous potentially identifiable genetic markers and clinical outcomes to which these may be related, testing and validation of statistical hypotheses in genetic epidemiology is an unprecedented task (27).

Since collagen I has numerous polymorphisms, it is recommended that the association of other collagen I polymorphisms with bone density be examined to achieve a complete haplotype. The association of bone density with other polymorphisms involved in osteogenesis regulation should be studied as well.

Sp1 gene polymorphism is associated with bone density rate in women aged 45 and over, more commonly observed in homozygosity. Determining this genotype's polymorphism is valuable to identify the women at risk of developing osteoporosis.

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