

CLINICAL SCIENCE

HIP FRACTURE RISK AND DIFFERENT GENE POLYMORPHISMS IN THE TURKISH POPULATION

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BACKGROUND: We aimed to discuss the risk assessments for both patients with hip fractures due to fall-related, low energy traumas and non-fractured control patients by examining bone mineral density and genetic data, two features associated with femoral strength and hip fracture risk.

METHODS: Twenty-one osteoporotic patients with proximal femur fractures and non-fractured, osteoporotic, age- and gender-matched controls were included in the study. Bone mineral density measurements were performed with a Lunar DXA. The COL1A1, ESR, VDR, IL-6, and OPG genes were amplified, and labeling of specific gene sequences was performed in a multiplex polymerase chain reaction using the osteo/check PCR kit from the whole blood of all subjects.

RESULTS: The bone mineral density (trochanteric and total bone mineral density values) of the fracture group was significantly decreased relative to the control group. We were not able to conduct statistical tests for the polymorphisms of the COL1A1, ESR, and VDR genes because our results were expressed in terms of frequency. Although they were not significant, we did examine differences in the IL-6 and OPG genes polymorphisms between the two groups. We concluded that increasing the number of cases will allow us to evaluate racial differences in femoral hip fracture risk by genotypes.

KEYWORDS: Hip fractures risk; Osteoporosis; Gene polymorphism.

INTRODUCTION

Proximal femoral fractures occur in both genders of the aging population as a result of osteoporosis as well as decreasing bone strength. The vast majority of hip fractures result from fall-related minor trauma to the proximal femur. Today, the most important approach to preventing this widespread, clinical problem involves the evaluation of bone strength and determination of fracture risks.¹⁻⁴ The pathogenesis of osteoporosis is complex, and is determined by the interaction of genetic, metabolic, and multiple

environmental factors.

All risk factors can be evaluated alone or together with bone mineral density (BMD) values; moreover, they can be combined for fracture risk assessment. Age and gender are two factors that affect the fracture risk independently of BMD values.^{2,5}

Genes have an important functional effect on bone metabolism and growth. Moreover, osteoporosis and hip fractures also appear to be under genetic control.⁶ Osteoporosis is polygenic in nature: multiple, common, polymorphic alleles interact both with one another and with environmental factors to determine bone mass.⁷ Rapid progress has been made in identifying the genes and alleles that may determine bone density in recent years, but little progress was achieved in identifying the genetic determinants of hip fracture risk.⁸ Although different studies have reported a relationship between several candidate polymorphic genes and BMD, some were inconclusive. The results of such studies among different populations have been mostly inconsistent, suggesting genetic heterogeneity of osteoporosis. It is likely that the cohort of

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genes indicating predisposition to the risk of osteoporosis may differ among populations with different ethnic backgrounds.⁷ These different results could reflect ethnic or racial differences in genetic makeup.

The genes under evaluation are those that code for major components of the bone matrix. Osteoporosis is strongly associated with Sp1 binding site polymorphisms in the first intron of the COL1A1 gene. This gene encodes collagen type I, an important component in the bone matrix.^{9,10}

The G to T polymorphism in COL1A1's first intron Sp1 site causes increased binding affinity for Sp1 and increased levels of COL1A1 transcript.¹¹ Alleles with a G-base at the Sp1 binding are defined as 'S', whereas alleles with a T-base are defined as 's'. Clinical studies in several populations have shown that patients with the 's' allele are likely to have reduced BMD and osteoporotic fractures.^{12,13}

Estrogens also play an important role in regulating bone homeostasis, bone turnover, and the maintenance of bone mass. The skeletal effects of estrogen are mediated by its binding to two different receptors that are members of the nuclear receptor superfamily of ligand-activated transcription factors. The individual contribution of these polymorphisms for osteoporosis remains to be universally confirmed. The human estrogen receptors (ESR1, chromosome 6q25) and (ESR2, chromosome 14q23-24) are likely candidate genes for osteoporosis. Both isoforms are expressed in osteoblasts, osteoclasts, and bone marrow stromal cells.¹⁴

The vitamin D receptor (VDR) is a major regulator of calcium and bone metabolism, and was the first candidate gene to be studied in relation to osteoporosis. A substitution in the 3'-region of the vitamin D receptor gene has been described, and the b [A] and B [G] alleles differentiated via BsmI RFLP analysis. The BB genotype is associated with decreased VDR function and increased osteoporosis risk.¹⁵

Osteoprotegerin (OPG), which is required for the maintenance of bone density, negatively regulates osteoclast differentiation and blocks pathophysiological induction of bone resorption by interacting with the receptor-activator of the nuclear factor kappa B ligand. The promoter region of the human OPG gene contains various binding sites that may mediate the stimulation of OPG gene expression.^{16,17} Polymorphisms in this and other regions of the OPG gene may contribute to genetic control of bone mass.¹⁸⁻²⁰ OPG and interleukin-6 (IL-6) are putative target genes for estrogen signaling and have been implicated both in cardiovascular diseases and osteoporosis.

In this study, we aimed to investigate the risk assessments of both patients with hip fractures and non-fractured, control patients by examining BMD values and possible allelic influences of the COL1A1, ESR, VDR, IL-6, and OPG genes.

MATERIAL AND METHODS

Twenty-one osteoporotic patients with proximal femur fracture due to simple low-energy trauma falls (8 male, 13 female, mean age 74.47 ± 8.91 years) were included in the study along with 21 osteoporotic volunteers with no history of fracture at any anatomic localization serving as age- and gender-matched controls. All subjects were of Turkish origin from different regions of Turkey. The study was approved by the Hospital Ethics Committee. No patients had neoplastic bone pathology, long-term corticosteroid usage, bone metabolism disease, or arthritis. Control subjects did not have any history of disease related to bone metabolism or structural integrity. In addition, we ensured that no fixed deformity or restricted joint movement was found in the fracture-free and radiologically-measured joints.

Proximal femur bone mineral density measurements

BMD measurements were performed with a Lunar DXA for both fracture and control group patients. The measurements were taken from the intact side of the hip in the fracture group and from the right side of the hip in the control group. BMD measurements were obtained as femoral neck, Ward's triangle, trochanteric, and total BMD values.

Genotyping

Genomic DNA was extracted from whole blood samples using an Invisorb® Spin Blood Mini Kit according to the manufacturer's instructions. The COL1A1, ESR, VDR, IL-6, and OPG genes were amplified, and labeling of specific gene sequences was performed using biotinylated primers in a multiplex polymerase chain reaction (PCR) using the osteo/check PCR kit (The Ogham Diagnostic System). The PCR reaction included an initial activation step (15 min., 95°C), 3-step-cycling of 35 cycles (denaturing 1 min. at 94°C, annealing 2 min. 56°C and elongation 1 min. 68°C), and terminal elongation (5 min. at 68°C and cooling to 4°C). Detection of amplified DNA sequences by means of allele-specific hybridization was done in the Array Tube®. Denatured PCR products were added onto the chip of the Array Tube®, and hybridization was performed with sequence-specific probes to distinguish between wild-type and mutated DNA sequences. After washing to remove the non-bound PCR products, free binding sites were blocked on the chip. Bound biotin-labeled PCR products were coupled with streptavidin-conjugate. After washing to remove non-bound streptavidin, staining and densitometric detection were conducted in a Solas 1 reader. Finally, an automatic calculation of patient genotypes was performed.

Data Analysis:

In both groups, mean ± standard deviation (SD) values were calculated for anthropometric data such as weight, height, BMI, and BMD. In order to establish a difference in these parameters between the two groups, an independent sampling test (t test) was then used. Parameters with values of p<0.05 were considered statistically significant. Results for the polymorphisms of the COL1A1, ESR, and VDR genes are given as frequencies, and Fisher’s Exact Test was used for tests of IL-6 and OPG gene polymorphisms. All statistical analysis was performed with the SPSS (Statistical Package for Social Sciences) for Windows v. 10 (SPSS Inc, Chicago, IL) program.

RESULTS

The means and standard deviations for the anthropometric and BMD values for the fracture and control groups are listed in Table 1. There were no significant differences between the fracture and control groups with regard to age, weight, length, or BMI. BMI showed a slight but non-significant difference between the control and fracture groups.

The average BMD values of the control group were higher than those of the fracture group in all anatomical regions. However, the difference was statistically significant only for the trochanteric and total BMD values (p=0.039 and p=0.012 respectively). The differences for the neck and Ward’s BMD values were not significantly different between the two groups.

The distributions of the COL1A1 gene Sp1 genotypes as well as the ESR1 gene PvuII and XbaI genotypes among

Table 1 - Means and Standard Deviations of Anthropometric and Femoral Bone Mineral Density (BMD) Parameters in the Fractured and Control Groups

	Fractured n=21 Mean ± SD	Control n=21 Mean ± SD	p
Anthropometric parameters			
Age (years)	74.47 ± 8.91	75.47 ± 7.44	0.695
Height (cm)	164.71 ± 8.28	160.66 ± 9.91	
Weight (kg)	68.80 ± 12.45	70.95 ± 13.95	
BMI	25.46 ± 4.82	27.36 ± 4.14	0.179
BMD parameters			
Neck BMD (gr/cm ²)	0.67 ± 0.09	0.67 ± 0.17	0.657
Wards BMD (gr/cm ²)	0.51 ± 0.11	0.52 ± 0.19	0.450
Trochanter BMD (gr/cm ²)	0.55 ± 0.07	0.64 ± 0.18 *	0.039
Total BMD (gr/cm ²)	0.72 ± 0.09	0.84 ± 0.21 *	0.012

Significant results between two groups are noted with bold type and asterisks. (**p<0.05)

Table 2 - Distribution of COL1A1 gene Sp1 as well as ESR gene PvuII and XbaI polymorphisms in the two groups

	Patient		Control	
	n=19	%	n=20	%
COL1A1 Sp1				
SS	5	26.3	9	45.0
Ss	11	57.9	9	45.0
ss	3	15.8	2	10.0
ESR 1 PvuII				
PP	5	26.3	8	40.0
Pp	9	47.4	8	40.0
pp	5	26.3	4	20.0
ESR 1 XbaI				
XX	4	25.0	9	47.4
Xx	8	50.0	7	36.8
xx	4	25.0	3	15.8

The restriction fragment length polymorphisms (RFLPs) were coded as Ss (Sp1), Pp (PvuII), and Xx (XbaI). The uppercase letter signifies absence of the site, and the lowercase letter signifies presence of the site.

patients and controls are shown in Table 2. The frequency of the COL1A1 gene SS genotype was higher than that of the ss genotype in both the fracture and control groups, whereas the frequency of the Ss heterozygote genotype in each group was similar or higher than that of either the SS or ss genotypes (57.9 vs. 45.0%, respectively). The frequencies of ESR1 PvuII (PP/pp) and XbaI (XX/xx) genotypes were similar in the fracture group (26.3 vs. 25.0%, respectively), but the frequencies of PP and XX were increased (40.0 vs. 47.4% respectively) and pp and xx were decreased (20.0 vs. 15.8% respectively) in the control group relative to fracture group. The Pp heterozygote frequencies accounted for 47.4% of the fracture group and 40% of the control group. Xx heterozygote frequencies accounted for 50% of the fracture group and 36.8% of the control group.

The distributions of the VDR gene BsmI genotypes among patients and controls are shown in Table 3. The frequency of the BB genotype was somewhat lower among

Table 3 - Distribution of VDR gene BsmI polymorphism in patients and controls

	Patient		Control	
	n=19	%	n=21	%
VDR BsmI				
BB	2	1.5	0	0.0
Bb	11	57.9	14	66.7
bb	6	31.6	7	33.3

The restriction fragment length polymorphisms (RFLPs) were coded as Bb (Bsm I), where the uppercase letter signifies presence of the site and the lowercase letter signifies absence of the site.

Table 4 - Distribution of OPG genes G209A and T245G as well as IL-6 gene G-174C polymorphisms in the groups

	Patient		Control		p value
	n=19	%	n=21	%	
OPG G209A					
GG					
GA	3	15.8	1	4.8	>0.05
AA	16	84.2	20	95.2	>0.05
polymorphism were termed GG, GA, and AA; G indicates the presence of the restriction site at -209					
OPG T245G	n=20	%	n=20	%	p value
TT					
TG	3	15.0	0	0	>0.05
GG	17	85.0	20	100	>0.05
polymorphism were termed TT, TG, and GG; T indicates the presence of the restriction site at -245					
IL-6 G-174C	n=20	%	n=17	%	p value
GG					
GC	10	50.0	7	41.2	>0.05
CC	10	50.0	10	58.8	>0.05
polymorphism were termed GG, GC, and CC; G indicates the presence of the restriction site at -174					

fracture patients, whereas that of the Bb genotype was higher (BB: 1.5 vs. 0%; Bb: 57.9). The frequency of bb homozygotes was similar between fractured patients and controls (31.6 vs. 33.3%, respectively).

The distributions of the OPG gene G209A and T245G as well as IL-6 gene G-174C polymorphisms among patients and controls are shown in Table 4. For OPG, the GG and TT genotypes were not observed in either group. The frequencies of the GA and TG genotypes were lower than those of the AA and GG genotypes, which showed the highest frequencies. No statistically significant differences were observed among these genotype between the fracture and control groups. For IL-6, the GC and CC genotypes had approximately equal frequencies within the groups. No statistically significant differences between groups were noted.

DISCUSSION

Bone constantly undergoes remodeling to repair and replace existing bone tissue. In aging, the amount of bone tissue gradually declines as structural elements are lost. Osteoporotic proximal femur fractures are typically characterized by a decrease in bone strength below a threshold level. However, studies show that the risk of fracture formation in the trochanteric region increases markedly as the trochanteric BMD value decreases. This finding provoked us to hypothesize that the amount of bone mass loss, which is accompanied by a decrease in bone strength, is far more important than the increase in resistance resulting from cortical thickening.

Adaptive changes in proximal femoral anatomy are related to old age and decreased BMD. Other adaptive changes have been thought to arise from genetic properties regarding bone morphology and mineral distribution. These different adaptations result in different bone strengths and degrees of fracture formation risk. In order to establish a relationship between proximal femoral geometric parameters and fracture risk, more studies should be carried out on a broader and more ethnically diverse population.

Although our study was limited in its statistical power to detecting differences in polymorphisms, we discussed the frequencies of polymorphisms and compared our results with others from the Turkish population which are limited to only a few studies.

The COL1A1 Sp1 binding site polymorphism appears to be an important susceptibility allele for osteoporotic fractures. Results from studies in several populations and meta-analyses show that carriers of the s allele have an increased risk of fracture.^{21,22} This allele can predispose one to osteoporotic fractures by affecting bone strength through mechanisms that are partly independent of differences in BMD. A frequency distribution study on 111 healthy, postmenopausal Turkish women showed that seventy-nine (71.2%) were homozygous for SS, 30 (27.0%) were heterozygous carrying Ss, and two (1.8%) were homozygous for ss. In our study, the ss and Ss frequencies were higher in the fracture group than the control group; accordingly, the SS frequency in the control group was higher than that in the fracture group (Table 2). Another study performed by Simsek et al. showed no significant differences between the COL1A1

genotype groups in terms of age, months since menopause, weight, height, baseline BMD at the femoral neck, or lumbar spine. All patients in that study were osteopenic, with a T score of <2.5 SD.²³

According to the meta-analysis of the estrogen receptor gene from different study groups, XX homozygotes for XbaI might have a higher BMD as well as a decreased risk of fractures in comparison to carriers of the x allele. In contrast, PvuII polymorphisms were not associated with either BMD or fracture risk.²⁴ In our study, the XX frequency in the control group was higher than that in the fracture group (Table 2); however, we were not able to perform statistical tests. As we observed from another study of the Turkish population conducted by Yilmaz et al. with regard to coronary artery disease (CAD), a statistically significant relationship between the ESR1 c.454-397T>C (PvuII) polymorphism and CAD was found to be independent of known CAD risk factors.²⁵

VDR gene polymorphisms associated with osteoporosis, osteomalacia, breast cancer, hypocalcemia, and psoriasis have been reported in the Turkish population.²⁶⁻³⁰ Gunes et al. found no statistically significant differences in the allelic and genotypic frequencies of BsmI between patient (n=110) and control (n=150) groups. Similar to our findings, these authors also observed that the heterozygote genotype was the most frequent among both patients and controls (Table 3).³¹ Our data regarding allelic frequencies were also similar to those found in other countries, including Denmark,³² Slovenia,³³ Brazil,³⁴ and Italy.³⁵ Those results may indicate a lack of correlation between BMD or fracture risk and VDR gene polymorphisms.

IL-6 is also a candidate cytokine that has been implicated in enhanced osteoclastogenesis, bone resorption, and accelerated bone loss. In our study, we did not observe any GG genotypes in either group. The GC and CC frequencies were equal, and statistically non-significant differences were reported between the groups. Budak et al. reported frequencies that differed from those in our study. In their control group of 50 healthy subjects, they observed 55.1% GG, 26.5% GC, and 18.4% CC polymorphisms.³⁶ Moffett

et al. analyzed the relationship between the IL-6 G-174C polymorphism and osteoporosis phenotypes in 3376 older women. Women with the CC genotype had a significantly slower rate of decline in hip BMD and a 33% lower risk of wrist fracture than women with the GG genotype. Variation at the IL-6 locus may contribute to genetic susceptibility to bone fragility.³⁷ Additionally, Feng et al. suggested a negative relationship between femoral neck BMD and IL-6 levels in the CC genotype.³⁸

Osteoprotegerin plays an important role as an inhibitor of osteoclast differentiation, and polymorphisms in the gene coding for OPG might influence the bone remodeling process. OPG could thus be a candidate gene for identifying individuals at risk for developing low bone mass or osteoporosis. The G to A substitution at position 209 and the T to G substitution at position 245 in the promoter region of the OPG gene have been well described. As with the IL-6 gene, we did not observe any statistically significant differences in the OPG gene between the fracture and control groups. Carriers of the G (for G209A) and T alleles (for T245G) have been found to be at risk for osteoporosis, but our study did not include any subjects homozygous for GG or TT. Subjects heterozygous for GA or TG were also rare (Table 4). Ohmori et al. found no evidence of a significant linkage between OPG and osteoporosis, but they did note the possible association of osteoporosis and a single-nucleotide polymorphism located in the promoter region of the gene.³⁹

Due to multifactorial inheritance and influential environmental factors, the molecular-genetic basis of osteoporosis is currently not clearly understood. We hope that the differences in individual responsiveness to treatment, when clarified at the genomic level, will enable more effective, individualized guidance in clinical practice.

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