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ARAŞTIRMA / RESEARCH

Vitamin D receptor and collagen type1α1 gene polymorphisms in patients with postmenauposal osteoporosis

Postmenopozal osteoporoz hastalarında vitamin D reseptör ve kollajen tip1α1 gen polimorfizmleri

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Abstract

Purpose: The iam of this study was to investigate the genotype and allele frequencies of osteoporosis related candidate genes, VDR and COL1A1 and their relationship between biochemical and anatomic parameters in patients with postmenopausal osteoporosis and healthy women in the Cukurova region of Turkey.

Materials and Methods: Eighty-four postmenopausal osteoporotic patients and fourty-five controls were recruited after obtaining informed consent. Four polymorphic restriction sites (*ApaI, TaqI, BsmI and FokI*) for the VDR gene and two polymorphic restriction sites (*Sp1 and -1997G/T*) for the COL1A1 gene were examined. All subjects were genotyped employing polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

Results: The frequencies of VDR and COL1A1 polymorphism genotypes, alleles and haplotypes were not statistically significant between patients and controls. The VDR gene BsmI, ApaI and TaqI polymorphisms, and the COL1A1 -1997G/T and Sp1 polymorphisms had a strong linkage disequilibrium. The most common haplotypes were "BAt" for patients and "baT" for controls in the VDR gene, the "TG" haplotype (also "TS") was the most common in two groups for COL1A1 gene.

Conclusion: The VDR and COL1A1 gene polymorphisms play key roles in osteoporosis susceptibility and BMD in postmenopausal women. Our findings may imply that polymorphisms of the VDR and COL1A1 genes were not associated with low BMD or fractures in postmenopausal Turkish women.

Keywords: Postmenopause, osteoporosis, VDR gene, COL1A1 gene, polymorphism

Öz

Amaç: Çalışmamızda, Çukurova bölgesinde, postmenopozal osteoporozlu hastalarda ve sağlıklı kadınlarda osteoporoz ile ilişkili aday genlerden VDR ve COL1A1 gen polimorfizmlerinin genotip ve allel sıklıkları ile biyokimyasal ve anatomik değişkenler arasındaki ilişkinin araştırılması amaçlanmıştır.

Yöntem: Seksen dört postmenopozal osteoporotik hasta ve kırk beş kontrol bilgilendirilmiş onam alındıktan sonra çalışıldı. VDR geni için dört polimorfik restriksiyon alanı (*ApaI, TaqI, BsmI ve FokI*) ve COL1A1 geni için iki polimorfik restriksiyon alanı (*Sp1 ve -1997 G / T*) incelendi. Tüm çalışma grubu polimeraz zincir reaksiyonu-restriksiyon fragman uzunluğu polimorfizmi (PCR-RFLP) kullanılarak genotiplendirildi.

Bulgular: Hastalar ve kontroller arasında VDR ve COL1A1 polimorfizm genotipleri, allel ve haplotip sıklıkları istatistiksel olarak anlamlı değildi. VDR geni için BsmI, ApaI ve TaqI polimorfizmleri ve COL1A1 geni için -1997G/T ve Sp1 polimorfizmleri arasında güçlü bir bağlantı vardır. VDR geninde en yaygın haplotipler, hastalar için "BAt" ve kontroller için "baT", COL1A1 geni için "TG" haplotipi (aynı zamanda "TS") her iki grupta da görülmüştür.

Sonuç: VDR ve COL1A1 gen polimorfizmleri postmenopozal kadınlarda osteoporoza yatkınlık ve BMD'de anahtar rol oynar. Bulgularımız, VDR ve COL1A1 genlerinin polimorfizmlerinin postmenopozal Türk kadınlarında düşük BMD veya kırıklarla ilişkili olmadığını düşündürmektedir.

Anahtar kelimeler: Postmenopoz, osteoporoz, VDR geni, COL1A1 geni, polimorfizim

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INTRODUCTION

Osteoporosis is a systemic multifactorial disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue leading to increased risk of fracture[1,2,3,4]. Most multifactorial diseases have a clear genetic component, including osteoporosis, are often called "polygenic" diseases, to emphasize their determination by multiple genetic factors[5,6]. The genetic factors play an important role in the pathogenesis of osteoporosis and about 50–85% of heritability for BMD is determined genetically, as suggested by twin and family studies. Most genetic determinants of osteoporosis are established with association studies, which are mostly based on polymorphisms[7,8,9].

The majority of polymorphisms are due to the substitution of a single nucleotide, called single-nucleotide polymorphisms or SNPs. The SNPs are sites in the genome, where two or more alternative choices of a nucleotide are common in the population. A single SNP may cause a Mendelian disease, though for complex diseases, SNPs do not usually function individually, rather, they work in coordination with other SNPs to manifest a disease condition as has been seen in osteoporosis[10,11].

Several genes have been reported to be associated with bone mineral homeostasis, bone remodeling and bone matrix composition, e.g. vitamin D receptor (VDR), estrogen receptor alpha (ESR1), collagen type1 α 1 (COL1A1), transforming growth factor beta 1 (TGFB1), interleukin-6 (IL6), aromatase (CYP19), and LDL-receptor-related protein 5 through polymorphism based association studies[12,13,14].

Of these osteoporosis associated genes, VDR gene was the first candidate gene to be studied, and was chosen on the basis that it acts as an important regulator of calcium metabolism and bone cell function. Initial studies focused on polymorphisms in the 3' region of the VDR gene between exons 8 and 9, which are certainly non-functional[14,15]. A significant association was reported between a BsmI polymorphism (rs1544410) in intron 8 of VDR and BMD in a twin study and a population based study, but this association was later found to be much weaker than originally reported due to genotyping errors[16,17,18].

Likewise, type I collagen, which is the major protein of bone, is a heterotrimer consisting of α1(2) and α2(1) protein chains, is encoded by the COL1A1 and COL1A2 genes, respectively[17,19,20]. Polymorphisms have also been described in the promoter region of the COL1A1 gene which are in linkage disequilibrium with the Sp1 polymorphism (rs1800012), including an insertion/deletion polymorphism in a poly-T tract at position -1663 [-1663IndelT (rs2412298)] and a G/T polymorphism at position -1997 [-1997G/T (rs1107946)][17].

The VDR and COL1A1 gene polymorphisms have been reported to be associated with low BMD and an increased risk of osteoporotic fracture in several studies. However, some studies and meta-analyses have shown no association between these polymorphisms and BMD or osteoporotic fractures[14,17,21]. Due to this discrepancy in the literature, we need some additional information in order to elucidate the role of VDR and COL1A1 genes in osteoporosis pathogenesis[22].

In our study, our aim was to investigate the genotype and allele frequencies of osteoporosis related candidate genes, VDR gene TaqI, ApaI, BsmI, FokI polymorphisms and COL1A1 gene Sp1 and -1997G/T polymorphisms and their relationship between biochemical and anatomic parameters in patients with postmenopausal osteoporosis (n=84) and healthy women (n=45) in the southern part of Turkey, Cukurova region.

MATERIALS AND METHODS

Eighty-four postmenopausal osteoporotic women (patients) aged 44 to 84 years (mean 64.48±9.61 years), and fourty-five healthy postmenopausal women (controls) aged 47 to 90 years (mean 58.31±8.61 years) were recruited after giving informed consent. The study protocol was approved by the local Research Ethics Committee of Cukurova University School of Medicine.

Each individual had a history taken, physical examination, BMD of spine and hip, and biochemical tests that were used for diagnosis of the postmenopausal osteoporosis women at Osteoporosis Screening Unit of Cukurova Univesity Hospital. T-score levels of BMD were ≤-2.5 SD for the patients and were +1 SD (normal) or ±1 SD (osteopenic) for controls. The patients and controls were excluded from the study groups on the basis of the following criteria considered to cause secondary osteoporosis: Disorders of calcium and phosphorus

metabolism, endocrine, renal, liver and other chronic and malignancy illnesses, receiving any medicine known to influence bone mineralization, HRT (hormone replacement therapy), TRT (thyroid replacement therapy) and ER (estrogen receptor) modulation medicines. Surgical menopause was also excluded.

All studied groups were given questionnaire for age, height, weight, age at menarche and menopause, years since menopause, family history of osteoporosis, parity (live births), history of fractures, smoking habits, degree of physical activity, insufficient sun exposure, dairy products, caffeine and alcohol consumption.

Bone mineral density (in grams per square centimeter) was determined by dual-energy X-ray absorptiometry (DEXA, Hologic/Lunar) at the femoral neck and lumbar spine (vertebrae L1-L4). Serum calcium, osteocalcin, β-Crosslaps (Beta-CTX), $25(OH)D_3$, ALP (alkaline phosphatase), PTH (parathormone), InP (inorganic phosphate) like biochemical parameters were scored. Body mass index was calculated as an estimate of obesity [weight (kg)/height (m2)].

DNA analysis

DNA was extracted from peripheral blood lymphocytes using conventional salting out extraction method modified from Miller's method[23].All subjects were genotyped employing polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

Genotyping

Four polymorphic restriction sites (*ApaI, TaqI, BsmI and FokI*) for VDR gene and two polymorphic restriction sites (*Sp1* and *-1997G/T*) for the COL1A1 gene were examined. The allele designation was made according to the presence or absence of the restriction enzyme site where the presence was indicated by a lowercase letter while the absence by an uppercase letter (for ApaI, BsmI, FokI and Sp1 polymorphisms) but if both alleles had restriction sites i.e., TaqI the allele having common restriction sites designated by uppercase letter. For the -1997G/T polymorphism nucleotide symbols were directly used to indicate the alleles.

VDR genotyping

A 740 bp fragment was amplified by polymerase chain reaction (PCR) with forward (INT8 F:5'-CAGAGCATGGACAGGGAGCAAG-3') and reverse (EX9 R:5'-GCAACTCCTCATGGCTGAGGTCTCA-3') primers to detect *ApaI* and *TaqI* restriction sites[24,25]. PCR was carried out in a 25 µL reaction mixture containing 100-200 ng genomic DNA, 1X PCR buffer, 1.5 mM magnesium chloride, 250 µM dNTPs, 10 pmol/µl of each primer and 0.5 U Taq DNA polymerase. After an initial denaturation period of 5 min at 94°C PCR was performed as follows: 10 cycles of amplification for 20 s 94°C, annealing at 40 s 65°C, and extension of 1 min at 72°C, 25 cycles of amplification for denaturation at 20 s 94°C, annealing at 40 s 63°C, and extention of 1 min at 72°C, followed by a final extension step of 6 min at 72°C[26].

After amplification, the PCR products were digested with restriction endonucleases *ApaI* and *TaqI* (Roche Diagnostics) respectively for 3 hours at 37°C and 65°C, electrophoresed on 3% agarose gel (Sigma Aldrich), stained with ethidium bromide, and visualized under ultraviolet light.

Digestion of the 740 bp PCR product with *ApaI* yield fragments of 211 and 529 bp for the presence of the restriction site (thus designated the allele as "a"), if the PCR product undigested (designated the allele as "A"). The presence of TaqI restriction site produced three bands of 291 bp, 247 bp and 202 bp length fragments thus designating the allele as "t", whereas the absence of TaqI restriction site produced two fragments of 493 bp and 247 bp length respectively designating the allele as "T".

For the BsmI polymorphic site, the forward primer sequence 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and the reverse primer sequence was 5'-AACCAGCGGAAGAGGTCAAGGG-3' were used as modified from Morrison et al[27]. PCR procedure was the same as with ApaI and TaqI restriction polymorphic sites. The PCR cycling protocol was as follows: initial denaturation period of 5 min at 94°C, 32 cycles of amplification for 45 s at 94°C, annealing at 40 s 64°C, and 1 min at 72°C, followed by a final extension step of 7 min at 72°C. Subsequently, the 825 bp PCR products were digested with restriction enzyme *BsmI* (Roche Diagnostics) for 3 hours at 37°C. The digestion products were underwent electrophoresis on a 3% agarose gel, stained by ethidium bromide, and visualized under ultraviolet light. The seperation patterns were 650 bp and 175 bp for "b" alleles and 825 bp for "B" allele (no restriction site).

FokI polymorphic site was amplified with following primer sequences:

VDR2a (Forward):5'-AGCTGGCCCTGGCACTGACTCTG**G***CTCT-3'

VDR2b (Reverse):5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'.

These primers were modified from Lucotte et al[28]. The PCR cycling protocol was as follows: initial denaturation period of 5 min at 94°C, 30 cycles of amplification for 30 s at 94°C, annealing at 1 min 60°C, and 30 s at 72°C, followed by a final extension step of 7 min at 72°C. The 265 bp PCR products were digested with *FokI* (Roche Diagnostics), restriction enzyme at 37°C for 3 hours and then electrophoresed through a 3% agarose gel, stained by ethidium bromide, and visualized under ultraviolet light. The digestion products were 196 bp and 69 bp for "f" alleles and 265 bp for "F" alleles (FokI restriction site).

COL1A1 genotyping

The intronic polymorphism (Sp1) of the COL1A1 gene was detected by PCR with a mismatched primer that introduced a diallelic restriction site, as previously described by Grant et al[19]. The forward and reverse primer sequences were 5'-TAA CTT CTG GAC TAT TTG CGG ACT TTT TGG-3' and 5'-GTC CAG CCC TCA TCC TGG CC-3' respectively. The test discriminates two alleles, "S" and "s", which correspond to the presence of guanine and thymidine , respectively, as the first bases in the Sp1 binding site in the first intron of the gene for COL1A1[29]. The PCR amplification was performed in 25 µL of reaction mixture containing 200 ng genomic DNA, 2.5 µl of 10X PCR buffer, 2mM $MgCl_2$, 5 pmol of each primers, 200 µM dNTP, 1 unit of Hot Start Taq DNA polymerase (Sigma Aldrich). A 265 bp fragment was obtained in 35 cycles as follows: initial denaturation at 95°C for 10 min afterwars denaturation at 94°C for 1 min, annealing at 60°C for 1 min and at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The PCR products were digested with *MscI* (NEB) restriction enzyme overnight at 37°C. These fragments were underwent electrophoresis on 8% polyacrylamide gel (Sigma Aldrich). Then, digestion products were stained by ethidium bromide, and visualized under ultraviolet light. The digestion products were designated as "s" (for 247 bp and 18 bp fragments) and "S" (for the absence of restriction fragments). **(**"S" allele can indicate as G", "s" allele as "T"[30].

The -1997G/T polymorphism is in the upstream regulatory region of COL1A1 gene. This polymorphism was amplified using the following primers 5'-CAC CCT GCC CTA GAC CAC -3'(forward) and 5'-GAA AAT ATA GAG TTT CCA GAG-3' (reverse) as previously reported by Garcia Giralt et al[20]. The PCR conditions were as follows in a 25 µL of reaction mixture containing 200 ng genomic DNA, 2.5 µL of 10X PCR buffer with $MgCl_2$, 5 pmol of each primers, 250 µM dNTP, 1 unit of Taq DNA polymerase (Sigma Aldrich).

The PCR cycle were consisted of 35 cycles each with following procedure: 3 min initial denaturation at 94°C, 40 s denaturation at 94°C, 45 s annealing at 57°C, 30 s extension at 72°C and final extension 7 min 72°C. After amplification, the PCR product was digested with restriction endonuclease *Eco31I* (Fermentas) for overnight at 37°C, electrophoresed in 3% agarose BasicaLE (Promega) gel, stained by ethidium bromide, and visualized under ultraviolet light. A 293 bp PCR product was obtained and if digestion sites present the PCR product was splitted into 212 bp and 81 bp fragments thus designated as "T" allele, while "G" allele for the absence of restriction site.

Statistical analysis

SPSS 18.0 software was used for statistical analysis of the data. Categorical measurements were summarized as number and percentage, numerical measurements were summarized as mean and standard deviation together with median and minimum-maximum. Chi square and Fisher Exact test statistic was employed for comparison of genotype groups. Mann Whitney U test was used to compare the numerical measurements between two groups without normal distribution. Kruskal Wallis test was used for general comparison between two groups without normal distribution. Statistical significance level was p=0.05 in all tests.

RESULTS

Basic clinical and biochemical characteristics of patients and controls are shown in Table 1. Body mass index (BMI), age at menopause (years), lumbar spine BMD (g/cm^2), lumbar spine T score, femoral neck T score were lower in patients as compared with controls. Ca (mg/dL), Vit D3, PTH (pg/mL), lumbar spine and femoral neck BMD, T score, Z score clinical and biochemical characteristics were significantly different among groups.

Table 1. Clinical and biochemical parameters of patients and controls.

Characteristics	Patients (n=84) (Mean ±SD)	Controls (n=45) (Mean ±SD)	p
Age (Year) ***	64.48±9.61	58.31 ±8.61	< 0.001
Height (cm) ***	156.49 ±5.79	160.07±6.02	< 0.001
Weight (kg) ***	63.96 ±11.82	78.20±12.90	< 0.001
BMI (kg/m^2) ***	26.13±4.89	30.56±4.99	< 0.001
Age at menarche (years)****	13.52 ± 1.3	13.07±0.91	0.037
Age at menopause (years)**	45.87±5.103	48.60±3.54	0.002
Years since menopause (years) ***	18.63±10.66	9.71±8.32	< 0.001
Lumbar spine BMD (g/cm^2) ***	0.73±0.11	0.92±0.08	< 0.001
Lumbar spine T score***	-2.90±1.05	-1.09±0.76	< 0.001
Lumbar spine Z score***	-1.23±1.25	0.11±0.86	< 0.001
Femoral Neck BMD (g/cm^2) ***	0.67 ± 0.09	0.86±0.09	< 0.001
Femoral Neck T score***	-2.0 ±0.84	-0.63±0.81	< 0.001
Femoral neck Z score***	-0.58±1.05	0.16±0.83	< 0.001
Ca (mg/dL) ***	9.74 ±0.39	9.43 ±0.36	< 0.001
InP (mg/dL) *	3.57 ±0.53	3.77±0.55	0.049
ALP (U/L) ****	178.23 ±66.45	193.13±39.27	0.170 NS
Osteocalcin (ng/mL) ****	20.69±15.10	20.54 ±6.75	0.950 NS
β-Crosslaps (ng/mL) ****	0.29 ±0.20	0.26±0.13	0.371 NS
Vit D3***	34.84±18.77	17.97±10.27	< 0.001
E2 (pg/mL) ****	18.98±7.95	15.34±19.67	0.139 NS
PTH (pg/mL) ***	61.57±23.25	47.43±15.51	< 0.001

BMI= Body mass index, BMD = Bone mineral density, ALP = Alkaline phosphatase; Ca= Calcium, InP= Inorganic phosphorus E2= Estradiol PTH = Parathyroid hormone.; Values are means ± SD. * P ≤ 0.05. ** p≤ 0.01 *** p≤ 0.001 **** Not significant (NS)

The frequencies of VDR and COL1A1 genotypes and alleles are summarized in Table 2. The genotype frequencies were 49.4% for "Aa", 39.2% for "AA", 11.4% for "aa" in the patients, and 62.2% for "Aa", 28.9% for "AA", 8.9% for "aa" in the controls respectively. In controls, "Aa" genotype frequency was higher (62.2%) than patients (49.4%) but "AA" genotype frequency was higher in patients (39.2%) than controls (28.9%). This genotype frequency differences, however, were not statistically significant (p=0.38). The "A" allele was higher in patients (63.9%) than controls (60%) whereas "a" allele in controls was lower (40%) than patients (36.1%). As a result "A" allele showed an increase in patients, which was statistically significant (p=0.05).

In FokI polymorphism, FF genotype distribution was 51.2% for patients and 61.4% for controls. When the patients and controls compared for "FF" genotype, controls had higher "FF" genotype. However, genotype and allele frequency were not statistically significant. Sp1 polymorphism is in the promoter region of the COL1A1 gene. This polymorphism genotype frequencies were 34.1% for "Ss/GT", 59.8% for "SS/GG", 6.1% for "ss/TT" in the patients and 26.8% for "Ss/GT", 65.9% for "SS/GG" and 7.3% for "ss/TT" in the controls. The genotype and allele frequency p values were 0.82 and 0.73, respectively and no significant differences was found among patients and controls. ***** ("S" allele can indicate as G", "s" allele as "T" [30]). The -1997G/T polymorphism of COL1A1 gene genotype frequencies were 60.8% for "GT", 26.6% for "TT", 12.6 for "GG" in the patients and 44.2% for "GT", 39.5% for "TT", 16.3 for "GG" in the controls. The patients had a higher percentage for "GT" genotype (60.8%) than controls (44.2%). But, we did not observe differences in terms of genotype and allele frequencies (p=0.20 and 0.73 respectively).

Table 2. Polymorphisms of VDR and COL1A1 genes, genotype and allele frequencies

Polymorphisms of VDR Gene		**Patients**		**Controls**				**Patients**		**Controls**	
		n	**%**	**n**	**%**			**n**	**%**	**n**	**%**
Bsm I	Bb	35	42.7	22	48.9	TaqI	Tt	39	48.2	22	48.9
Genotype	BB	20	24.4	9	20	Genotype	TT	30	37	19	42.2
Frequency	bb	27	32.9	14	31.1	Frequency	tt	12	14.8	4	8.9
Bsm I	B	75	45.7	40	44.4	TaqI	T	99	61.1	60	66.7
Allele	b	89	54.3	50	55.6	Allele	t	63	38.9	30	33.3
Frequency						Frequency					
Apa I	Aa	39	49.4	28	62.2	FokI	Ff	32	38.1	14	31.8
GenotypeFr	AA	31	39.2	13	28.9	Genotype	FF	43	51.2	27	61.4
equency	aa	9	11.4	4	8.9	Frequency	ff	9	10.7	3	6.8
Apa I	A	101	63.9	54	60	FokI	F	118	70.2	68	77.3
Allele	a	57	36.1	36	40	Allele	f	50	29.8	20	22.7
Frequency						Frequency					
Polymorphisms of COL1A1 Gene		Patients		Controls				Patients		Controls	
		n	%	n	%			n	%	n	%
Sp1	Ss/GT	28	34.1	11	26.8	-1997G/T	GT	48	60.8	19	44.2
Genotype	SS/GG	49	59.8	27	65.9	Genotype	TT	21	26.6	17	39.5
Frequency	ss/TT	5	6.1	3	7.3	Frequency	GG	10	12.6	7	16.3
Sp1	S/G*	126	76.8	65	79.3	-1997G/T	G	68	43	33	38.4
Allele		38	23.2	17	20.7	Allele	T	90	57	53	61.6
Frequency	s/T					Frequency					

* ("S" allele can indicate as G", "s" allele as "T".)

Table 3. Haplotypes of BsmI, ApaI and TaqI polymorphisms genotype combinations at VDR gene and -1997G/T and Sp1 polymorphisms genotype combinations at COL1A1 gene

	Haplotypes	**Patients**		**Controls**	
		n	%	n	%
BsmI, ApaI and TaqI polymorphisms genotype combinations at VDR gene *	BAT	12	12.7	6	13.1
	BAt	29	**30.8**	12	26.1
	bAT	23	24.5	11	23.9
	baT	28	29.8	17	**36.9**
	bAt	1	1.1	0	0
	bat	1	1.1	0	0
	BaT	0	0	0	0
	bAt	0	0	0	0
-1997G/T and Sp1 polymorphisms genotype combinations at COL1A1 gene **	GG	40	34.5	21	31.8
	TG	58	**50**	35	**53**
	GT	6	5.2	3	4.6
	TT	12	10.3	7	10.6

P values for * 0.89 and **0.95.

Among BsmI, ApaI and TaqI polymorphisms, there was a strong linkage disequilibrium. In the VDR gene, the genotype combinations of BsmI, ApaI and TaqI polymorphisms were BbAaTt, BBAATT, BbAATt, bbAaTT, BBAAtt, bbaaTT, BbAATT, BBAATt, bbAATT, BbAaTT, bbaaTt, BBAaTt. The BbAaTt (30.7 % in patients, 35.5 % in controls), bbAaTT (15.4% in patients, 20% in controls), BBAAtt (14.1% in patients, 8.9% in controls) genotype combinations were most frequently observed. In patients and controls genotype combinations were not statically significant (p=0.77). Genotypes, which have multiple heterozygous alleles in genotype combinations, were not included in the calculations since some haplotypes cannot be predicted.

The COL1A1 gene, genotype combinations of -1997G/T and Sp1 polymorphisms were TTGG, GTGG, GGGG, TTGT, GTGT, GGGT, TTTT, GTTT, and GGTT. The GTGG (35.61% in patients, 30.7% in controls), GTGT (23.3% in patients, 15.4% in controls) TTGG (16.4% in patients, 25.6% in controls) genotype combinations were the most frequently observed. In patients and controls genotype combinations were not statically significant (p=0.84).

As for VDR gene, all homozygote genotype

combinations (BBAATT, BBAAtt, bbaaTT, bbAATT) and heterozygote allele genotype combinations (bbAaTT, BbAATT, BBAATt, bbaaTt) estimating haplotype distributions were shown in Table 3. In patients and controls haplotype frequencies were not statically significant (p=0.89). The most common haplotypes were **"BAt"** for patients and **"baT"** for controls.

The COL1A1 gene, -1997G/T and Sp1 polymorphisms genotype combination haplotype distributions were shown in Table 3. The "GTGT" genotype had two heterozygote allele so it was not suitable for haplotype analyses. There was no significant difference for patient and control groups (p=0.95). The **"TG"** haplotype (also **"TS"**) was the most detected haplotype in both groups.

DISCUSSION

Osteoporosis, a common condition characterized by low bone mass and defects in the microarchitecture of bone tissue, is a significant public health burden. Many factors influence the risk of osteoporosis, including diet, physical activity, medication, and coexisting diseases, but one of the most important clinical risk factor is the positive family history, emphasizing the importance of genetics in the pathogenesis of the disease[17].

In this study, it was found that patients had higher familial osteoporosis story (p=0); interestingly controls had a better educational background level (p=0.001) and their daily caffeine consumption (p=0.001) was also higher. There was no significant difference between the groups in terms of daily consumption of dairy products, smoking and sun exposure. Eventhough the patients were older and years since menopause was longer than controls, this issue does not interfere with genotype or allele frequencies.

In this study, no statistically significant difference was found between the genotype distributions of VDR gene TaqI, ApaI, BsmI and FokI polymorphisms or COL1A1 gene Sp1 and -1997G/T polymorphisms, and lumbar spine and femur neck BMD, T score and Z score variables (p>0.05).

The active metabolite of vitamin D, calcitriol, plays an important role in regulating the function of bone cells and the maintenance of serum calcium homeostasis. Calcitriol binds to VDR and regulates gene expression of the genes involved in osteoporosis[6,16,31]. A significant number of RFLP polymorphisms have been identified in the human VDR gene as well.

The BsmI polymorphism is in strong linkage disequilibrium with 2 other polymorphisms, detected as ApaI and TaqI restriction fragment length polymorphisms[21]. Some of these alleles, alone or in combination (haplotypes), could modify the stability and expression level of its mRNA[13]. Another common polymorphism has been described in exon 2 of the VDR gene, which is a T→C transition recognized by the *FokI* restriction enzyme[17]. This introduces an alternative translational start codon that results in a shorter isoform of the VDR gene[17,32].

In our study, the differences between genotype and allele frequencies of VDR gene polymorphisms (BsmI, ApaI, TaqI, FokI) in patients and controls were not statistically significant. However, although the distribution of ApaI genotype frequency in patient and control populations was not statistically significant, there was an increasing genetic shift in the "A" allele frequency in patients (p=0.05). This result can be interpreted as an increase in the "A" allele in ApaI polymorphism in patients with osteoporosis.

There are two main phenotypes known as "low BMD" and "fracture" in osteoporosis. One of the extensive studies that examined the linkage of VDR polymorphisms and osteoporosis-related phenotypes, performed by Fang et al.[29] in Rotterdam in 2005, with 6148 female and male participants. As a result of this study it was found that haplotypes in the VDR gene promoter (Cdx) and 3 'UTR region (BsmI, ApaI, and TaqI) were associated with increased risk of bone breakage. The risk of fracture in the group of individuals bearing risk alleles (polymorphisms) in both regions increased by 15-48% compared to controls. To our surprise, in this study the risk alleles for fracture were not associated with BMD[29].

Several association studies subsequently performed for the association between VDR alleles, BMD and fractures, which have resulted with conflicting results[17]. Uitterlinden et al.[21] have concluded that there was no relationship between VDR gene FokI, BsmI, ApaI and TaqI polymorphisms and BMD or fracture risk in an extensive study involving 9 European countries named GENOMOS (Genetic Markers for Osteoporosis) Consortium including 26242 samples, another VDR polymorphism, however, Cdx2 has been shown to have a low association with spine fractures[21].

Many researchers have made functional analyzes of VDR polymorphisms and haplotypes. Reporter gene constructs prepared from the 3 'region of the VDR genome showed haplotype specific differences in gene transcription in different individuals. This finding increases the possibility that polymorphisms in this region are involved in the regulation of RNA stability[27]. Allele-specific transcription differences in the VDR gene were observed in cell lines that were heterozygous for TaqI polymorphism. In this study, "t" alleles were 30% more common than "T"s[33]. These results were the opposite of Morrison's results. In another study (MrOs') it was observed that 3'VDR haplotypes in male bones were associated with allele-specific transcription[34]. In particular, the presence of haplotype 1 (baT) is associated with the accumulation of VDR gene mRNA, and this haplotype has been reported to be associated with increased risk of fracture in men.

Other in vitro studies[35,36,37] did not show any association between BsmI polymorphism and allele-specific transcription, mRNA stability, or ligand binding[6]. Due to their close location in the 3'UTR region of VDR gene, there is a strong Linkage Disequilibrium (LD) between BsmI, ApaI and TaqI polymorphisms. In our study, it was found that, for these polymorphisms, "BbAaTT" was the most common combination between patients and controls and followed by "BbAATT", "BBAAtt". In Asian populations bbAATT" and "bbaATT" combinations are the most common while in Caucasian populations "BbAaTt" and "BBAAtt" combinations were reported[38]. In light of this information, we can comment that our data is consistent with the Caucasian population.

In our study, there was no significant difference in allelic frequency distributions of haplotypes between patients and controls (p=0.89). According to this although the difference in the incidence between patients and controls is not significant, our findings may imply that "BAt" and "baT" haplotypes are the most common in patients and controls, respectively.

Type 1 collagen is the main structural protein of the bone, and the genes (COL1A1 and COL1A2) encoding this protein are important candidate genes for osteoporosis pathogenesis. Genetic studies have shown that mutations in the coding regions of the type I collagen genes (COL1A1, COL1A2) leads osteogenesis imperfecta (OI)[6,20,39,40]. In studies with probands with this disease, 200 mutations were defined in the coding regions of COL1A1 and COL1A2 genes[41]. Polymorphisms in the coding sequences in type 1 collagen genes are very rare and are not associated with osteoporosis. However, polymorphisms in the COL1A1 regulator region have been shown to be associated with bone mass and possible osteoporotic fractures[6].

Many studies have focused on the molecular mechanism of the association between Sp1 polymorphism and osteoporosis susceptibility. In both in vitro and ex vivo studies, Sp1 polymorphism, "T/s" allele, has been shown to promote COL1A1 transcription which leads to increased production of the collagen α1 protein. The increase in collagen alpha1 protein leads to an abnormal α1 and α2 chain ratio, which leads to defects in bone mineralization, decreased bone strength and increased risk fracture[6,17].

In the COL1A1 promoter region, Garcia-Giralt et al.[20] define 2 polymorphisms (-1663IndelT and -1997G/T) in 2002 and it was showed that these polymorphisms had strong linkage disequilibrium (LD) with each other and with Sp1[20]. In this study, it was determined that postmenopausal Spanish women had a significant relationship between -1997G/T polymorphism and lumbar vertebra BMD. This relationship was later shown in British women as well[44].

In summary, the data so far indicate that the common allelic variants at the 5 'end of the COL1A1 gene are associated with bone mass, osteoporotic fractures, and predisposition to spinal fractures. Three polymorphisms affecting the Sp1 transcription binding site has been described in the intron at position 1245 and in the promoter -1997G/T and -1663IndelT polymorphisms. At least two of these polymorphisms have a functional effect on DNA binding and COL1A1 transcription. This affects the production of collagen protein and bone strength[6].

Previous studies have indicated that there is a strong Linkage Disequilibrium (LD) between the COL1A1 promoter and intron 1 polymorphisms[20,42,43]. In our study, among

-1997G/T and Sp1 polymorphisms, "GTGG (or GTSS)" genotype combination was the most common in patients and controls. Subsequently, combinations of genotypes "GTGT" and "TTGG" were also observed. There was no statistically significant difference between the patients and controls regarding the distribution of genotype combinations (p=0.84). In patients and controls, the

most common haplotypes were "TG" and followed by "GG" haplotype and there was no significant difference between the groups (p=0.95). Our findings are inconsistent with other studies.

Although numerous association studies relating polymorphisms in these genes to BMD have been published, results are conflicting possibly because of variations in study design, small sample sizes, and heterogeneous populations. Until now, population-specific genetic differences and environmental factors have been shown as the main cause of the differences between the positive and negative findings obtained in studies on the relationship of VDR and COL1A1 gene polymorphisms to osteoporosis in various populations and this relationship is yet to be fully explained.A major limitation so far of association studies using VDR polymorphisms in relation to complex-disease end points has been the small number of analyzed polymorphisms and, thus, the lack of knowledge about the influence of and relation between other polymorphisms in the gene[29].

In conclusion, VDR gene polymorphisms play key roles in osteoporosis susceptibility and BMD in postmenopausal women, although different VDR gene polymorphisms might have significantly different influences on the risk of osteoporosis and BMD in postmenopausal osteoporotic women with different ethnicities[44].

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