Association analysis of the COL1A1 polymorphism with bone mineral density and prevalent fractures in Polish postmenopausal women with osteoporosis

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Abstract

Introduction: Polymorphism in the promoter region of collagen type 1α (*COL1A1*) +1245G/T (Sp1, rs1800012) was in some studies shown to be relevant for bone mineral density (BMD) and low-energy fracture prediction. The aim of the study was to confirm this finding in a group of postmenopausal women diagnosed with osteoporosis.
Material and methods: We investigated 311 Caucasian women (mean age: 65.2 ±9.39 years) either after low-energy fractures (regardless of the location) or meeting World Health Organization (WHO) criteria for osteoporosis. All patients underwent clinical examination in order to exclude secondary osteoporosis; hip and lumbar spine DEXA was performed (Lunar). The three genotypes of Sp1 polymorphism were determined by RFLP (restriction fragment length polymorphism).
Results: Distribution of *COL1A1* genotypes (*SS/Ss/ss*) agreed with Hardy-Weinberg equilibrium. No relation between COL1A1 genotypes and hip/L1-L4 BMD was found. Fractures were reported in 26.3% of women. Prevalence of low-energy fractures, regardless of the type, was 50.0% in *ss* genotype carriers, 26.4% in *SS* homozygotes and 23.7% in *Ss* heterozygotes. There was no statistically significant recessive or dominant effect of any Sp1 genotype on fracture prevalence ($p = 0.613$).
Conclusions: We failed to observe that *COL1A1* Sp 1 genotypes contribute to BMD determination or are associated with prevalent low-energy fractures in a Polish cohort of postmenopausal osteoporotic women.

Key words: postmenopausal osteoporosis, bone mineral density, fractures, COL1A1 polymorphism.

Introduction

Osteoporosis is one of the most prevalent chronic conditions in the elderly population. Its progress is silent and lasts for years, with fractures as the most common first manifestation of the disease. In 2000, over

9 million low-energy fractures were reported worldwide (1.7 million Colles' fractures, 1.6 million hip fractures, and 1.4 million clinically overt vertebral fractures) [1]. Clinical consequences of such fractures, especially the hip, are severe: 50% of the affected patients lose the ability of unassisted motion; every fifth woman and every third man dies in the first year after the fracture [2].

Given the statistical data and the unquestionable growth in life expectancy, there is a clear need to find reliable markers of poor bone quality. Osteoporosis is a disease of multifactorial etiology, with a strong genetic background. Variance in peak bone mineral density (BMD) has been proven to be genetically dependent in 70% of cases [3]. Genetic and molecular tests are likely to identify subjects liable to low-energy fractures. Contemporary approaches used to find genes responsible for disease development include candidate gene studies. Numerous genes are being investigated with regard to bone metabolism: genes that encode growth factors and cytokines related to bone turnover (e.g. osteocalcin, transforming growth factor β1 – TGF-β1), encoding components of bone matrix (bone morphogenetic protein 2 – BMP-2, LDL receptor-related protein 5 – LRP5, osteoprotegerin), and genes related to receptor proteins of calciotropic hormones (vitamin D receptor – VDR, parathormone receptor – PTHR) [4–7]. The list also includes the gene that encodes the major bone protein type 1 collagen.

Collagen synthesis is complex and therefore prone to mutations. The protein is encoded by two genes – on chromosome 17 and 7 for chains α1(I) (*COL1A1*) and α2(I), respectively. Mutations in both of these genes have been demonstrated to be responsible for the autosomal dominant form of *osteogenesis imperfecta*, with severe osteoporosis [8]. The initial finding related to low bone mass in postmenopausal osteoporosis was reported in 1996 by Grant *et al.* [9], who described the Sp1 polymorphism in the *COL1A1* gene (+1245G/T, rs1800012). The change of a single nucleotide (G→T) was shown to result in polymorphism in intron 1, specifically in the binding site for transcription factor Sp1 – a site involved in the regulation of collagen transcription. The presence of the "s" allele is associated with increased mRNA transcription and a relative rise in the amount of α1(I) chain when compared to α2(I) chain. The normal ratio of α1 to α2 is 2 : 1. For heterozygotes of Sp1 polymorphism this proportion is approximately 2.3 : 1. An increased amount of transcript for α1 chain has been suggested to result in formation of collagen homotrimers, which are represented beside the normal protein [10]. Their presence is the reason for deteriorated bone microarchitecture.

The relationship between *COL1A1* gene polymorphism and BMD was previously investigated, but various studies generated discrepant results. Moreover, data on the Polish population are scarce.

The aim of the study was to assess the association of Sp1 polymorphism of the *COL1A1* gene with BMD and fracture prevalence in women with postmenopausal osteoporosis.

Material and methods

The study group comprised 311 postmenopausal, non-institutionalized Caucasian women from the Wielkopolska region (Poland), patients of the Endocrinology Outpatient Clinic, University Hospital No. 2. The diagnosis of osteoporosis was based on previous low-energy fracture or the WHO criteria (DEXA hip or lumbar *T*-score < –2.5 SD). Low-energy fracture was classified as such in cases when the following occurred:

1. Minor trauma or fall from standing height or less, preceding the fracture.
2. Fracture at the age of ≥ 50.

A subgroup of patients with a history of fracture was subject to further, separate analysis.

In the majority of patients prevalent low-energy fractures were self-reported, and in some cases hospital discharge charts or radiographs were available.

Validity of self-report has been reported to be high in the assessment of hip fracture [11] but lower for vertebral fractures. The latter is believed to result from underestimation of vertebral fractures, which often are either unreported by patients or referred to as non-specific back pain.

All study participants underwent a physical examination, including anthropometric measurements. Medical history was taken and women with suspicion of secondary osteoporosis (e.g. with hyperthyroidism, rheumatoid arthritis, chronic kidney diseases, pituitary gland diseases, taking systemic steroids) were excluded from the study. The studied individuals did not receive any pharmacotherapy for osteoporosis or other drugs influencing bone metabolism. The clinical data are presented in Table I.

Table I. General characteristics of study group (n = 311)

Parameter	Result
Age [years]	65.2 ±9.39
Body weight [kg]	62.4 ±11.0
Height [m]	1.589 ±0.059
BMI [kg/m^2]	24.7 ±4.32
BMD FN [g/cm^2]	0.700 ±0.088
BMD LS [g/cm^2]	0.799 ±0.126

Data are presented as mean ± SD unless otherwise indicated. BMI – body mass index, BMD – bone mineral density, FN – femoral neck, LS – lumbar spine.

Bone mineral density of the lumbar spine (L1–L4) and the femoral neck (FN) was measured by dual energy X-ray absorptiometry (DEXA) (Lunar, Lunar Inc., Madison, WI, USA). The apparatus was calibrated daily. Measurements were performed using standard procedures.

Genotyping

DNA was isolated from peripheral blood leukocytes by the guanidinium isothiocyanate method. The PCR reaction was carried out in 20 µl with 500 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 7.5 pmol of each starter and 0.5 U of Taq polymerase (Sigma).

Conditions for the PCR reaction of the fragment containing intron 1 of collagen 1α gene were as follows: initial denaturation at 94°C for 4 min, 40 cycles of amplification (denaturation at 94°C for 40 s, starters attachment at 67°C for 40 s, starters elongation at 72°C for 60 s). The final stage was elongation at 72°C for 5 min. For Sp1 polymorphism a 260 bp fragment was amplified using the primers F 5'-TAACTTCTGGACTATTTGCGGACTTTTTGG-3' and R 5'-GTCCAGCCCTCATCCTGGCC-3' [9].

The PCR product was then subjected to restriction fragment length polymorphism (RFLP) analysis using MlsI restrictive enzyme (Fermentas). Owing to the fact that the effectiveness of PCR reaction in that particular case was very low, the division of digestion products was carried out with ALFexpress (Amersham Pharmacia Biotech). Fragments obtained after enzymatic digestion differed only in 18 bp. Eight microliters of digestion product was mixed with 4 µl of ALFexpress aggravating buffer with internal markers of 113 bp and 525 bp. Next, thermal denaturation was performed at 94°C for 5 min, and after that the product was immediately chilled on ice. Every 12 µl of mixture was put on gel. Disjunction took place in 6.15% denaturizing polyacrylamide gel, in which the relation of acrylamide to methylenebisacrylamide was 20 : 1. Division conditions were as follows: temperature 55°C, power 25 W, 0.6 × concentrated buffer TBE. Detection on the ALFexpress set was performed using inducement of fluorescent marker Cy5.

Table II. Analysis of conformity of genotype distribution of examined polymorphism with Hardy-Weinberg equilibrium

Variable		Numbers observed		Numbers expected	
		N	%	*N*	%
COL1A1 Sp1					
Genotype	SS	204	65.5	205.0	65.9
	Ss	97	31.1	94.99	30.5
	ss	10	3.2	11.00	3.5
				p = 0.709	

Statistical analysis

All data are expressed as mean ± SD, unless otherwise stated. The analyzed data came from the interval and nominal scale. Student's *t*-test was used to compare the two groups, and the Mann-Whitney test was applied in the absence of compliance with the required assumptions (normality and homogeneity of variance). Nominal data were analyzed with the χ^2 test. When more than two groups were compared simultaneously, univariate analysis of variance with the Tukey post-hoc test was performed. In case of non-compliance with normal distribution or lack of homogeneity of variance, the Kruskal-Wallis test with Dunn's post-hoc test was performed. All tests were analyzed at the significance level of $\alpha = 0.05$. Statistical analysis was performed using Statistica 8.0 software (Stat Soft Inc, Tulsa, USA).

The analyzed genotypes were tested for Hardy-Weinberg equilibrium (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl and Statistica 8.0).

The association analysis was performed for three possible effects of a given polymorphism: the allele, the recessive and the dominant effect.

In the analysis of associations (*case-control* type) with the occurrence of fractures, three possible effects of Sp1 polymorphism were also analyzed: the effect of allele dose (χ^2 Armitage test for a trend), the effect of recessive/dominant action (Pearson's χ^2) and the odds ratio (OR) were calculated. Additionally, frequencies of alleles were also compared between the groups with and without fractures. In all cases, Pearson's χ^2 and the odds ratio for the risk alleles of Sp1 polymorphism were determined. The number of single alleles was calculated as the sum of a double number of alleles in homozygotes (dominant or recessive) and a single number of alleles in heterozygotes.

Results

Mean age in the study group was 65.2 ±9.3 years. Out of all studied women, 82 (26.3%) were reported to have a fracture. Clinical data are presented in Table I. The *COL1A1* Sp1 genotypes were in Hardy-Weinberg equilibrium (p = 0.709). The frequency of genotypes of *COL1A1* in the study group is shown in Table II.

There was no statistically significant difference in BMD between carriers of different genotypes of *COL1A1* polymorphism (Table III). No evidence for an allele dose effect was found, either for FN or L1–L4 BMD.

Similarly, no proof of a recessive or dominant effect of any allele was found – the presence of "s" or "S" did not influence BMD in any of the skeletal sites (Table III). The relation between fracture risk and *COL1A1* polymorphism is shown in Table IV.

Prevalence of low-energy fractures, regardless of type, was 50.0% in *ss* genotype carriers, 26.4% in *SS* homozygotes, and 23.7% in *Ss* heterozygotes. There was no significant recessive or dominant effect of any Sp1 genotype on fracture prevalence ($p = 0.613$).

The *s* allele was chosen as the risk allele because its frequency was higher in the fracture group (6% vs. 4.3%), although without statistical significance ($p > 0.05$). Odds ratio for having a fracture in *s* allele carriers was 1.12 (95% CI:

Table III. Association between Sp1 polymorphism COL1A1 and hip/L1–L4 BMD

***COL1A1* Sp1 FN**		**BMD corrected for age, height, body mass [g/cm²]**		
		Percentage	**BMD**	**SD**
Allele dose	SS	65.4	0.700	0.007
	Ss	30.8	0.698	0.010
	ss	3.8	0.716	0.028
Significance			$p = 0.862$	
Recessiveness and dominance effect:				
Genotype	SS + Ss	96.2	0.700	0.006
	ss	3.8	0.716	0.028
Significance			$p = 0.570$	
Genotype	SS	65.4	0.700	0.007
	Ss + ss	34.6	0.700	0.009
Significance			$p = 0.979$	
***COL1A1* Sp1 LS**				
Allele dose effect:				
Allele dose	SS	66.3	0.799	0.012
	Ss	28.9	0.797	0.017
	ss	4.8	0.820	0.043
			$p = 0.813$	
Recessiveness and dominance effect:				
Genotype	SS + Ss	95.2	0.798	0.010
	ss	4.8	0.820	0.043
Significance			$p = 0.629$	
Genotype	SS	66.3	0.799	0.011
	Ss + ss	33.7	0.800	0.016
Significance			$p = 0.944$	

Table IV. Association analysis of COL1A1 Sp1 polymorphism with fracture prevalence

Parameter		**Patients without fractures**	**Patients with fractures**		**Recessiveness and dominance effect**		**Patients without fractures**		**Patients with fractures**	
		n	***n***	**%**			***n***	**%**	***n***	**%**
Genotype	SS	*n* = 204	150	54	26.4	SS vs. Ss + ss	$\chi^2 = 0.003$ $p = 0.954$	Alleles S	374	81.7	131	79.9
	Ss	*n* = 97	74	23	23.7	ss vs. Ss + SS	$\chi^2 = 2.972$ $p = 0.085$	s	84	18.3	33	20.1
	ss	*n* = 10	5	5	50.0	ss vs. Ss + SS		Pearson's χ^2	$\chi^2 = 0.251$	$df = 1$	$p = 0.616$	
Pearson's χ^2		$\chi^2 = 3.230$	$df = 2$	$p = 0.199$					OR = 1.12, 95% CI: 0.72–1.76			
Armitage's χ^2 for trend		$\chi^2 = 0.256$	$df = 1$	$p = 0.613$					Risk allele s			

0.72–1.76) when compared to the *S* allele, but it was not statistically significant.

Discussion

Different contemporary approaches used in the search for the loci/locus responsible for osteoporosis provide only a partial answer to all the questions posed and confirm the complexity of the genetic trait of osteoporosis. The disease is known to be of polygenic nature, and a single gene has potentially little effect on the phenotype. The phenotype is further modified by environmental factors, which partly explains the lack of reproducibility of molecular analyses in osteoporosis. However, the value of genetic assessment is undoubtedly high-molecular markers do not depend on the time of diagnosis and remain unchanged during an individual's lifetime. They may allow one to identify people with high or even the highest risk for fractures and perhaps help to prevent them. Prevention forms the core component of osteoporosis management.

The hypothesis that the polymorphism within a gene encoding a vital bone protein might serve as a reliable marker of bone quality is very promising. Unlike the VDR gene, the *COL1A1* gene affects the final protein product. However, in a homogeneous group of postmenopausal women we did not observe an association between *COL1A1* alleles and osteoporotic phenotype measured by BMD, both at the hip and the lumbar spine. BMD used in the analyses was age, height and BMI corrected, which highlights the statistical power of this association.

Our results are consistent with several published reports [12–15], although not all of them [16–18], including the GENOMOS study [19], which was a very large, multicenter study involving over 20 000 individuals from different European countries. The authors found that *ss* homozygotes of *COL1A1* had significantly lower L1–L4 BMD than other genotypes.

There might be several reasons for the discordance. Uitterlinden *et al.* concluded that the effect of polymorphism 1245 G/T on BMD is most marked in the oldest age group (> 80) [17]. Women included in our group did not fit into that category (65.2 ±9.3 years). Secondly, two additional polymorphisms within the collagen gene promoter have been identified in recent years: –1997G/T (PCOL2, rs107946) and –1663indelT (PCOL1, rs2412298). They both were proven to be associated with BMD, lumbar as well as hip [20, 21]. Also, because they are in linkage disequilibrium with the analyzed Sp1 polymorphism, haplotype analysis may provide new information. Gonzalez-Bofill *et al.* found that haplotype –1997T-1663ins+1245G had a negative impact on BMD, which persisted for the next 10 years, regardless of hormonal replacement therapy [22]. These authors failed to demonstrate increased risk of fracture related to this haplotype. The test group, however, consisted of healthy perimenopausal women. In contrast, Jin *et al.*, in their meta-analysis, demonstrated an association of low bone mass and high fracture risk with different haplotype (–1997G-1663del + 1245T) [23]. In the view of these conflicting reports, it seems likely that regulation of bone biomechanics involves an interaction with other, hitherto unknown, genes and their polymorphic variants.

The frequency of COL1A1 Sp1 genotypes in our group of Caucasian origin was similar to that described by other authors. Langdahl *et al.* identified 5.3% *ss* homozygotes in a group of 372 Danish men and women [18], Uitterlinden *et al.* reported a rate of 3.3% in over 1 770 women in the Netherlands [17], and Ismail *et al.* reported 3.5% among participants of EPOS [24]. Distribution of other Sp1 genotypes was also close to other investigated European populations, as shown by the GENOMOS consortium [19]. Therefore, our group seems representative. Notably, race was shown to impact the distribution of allelic variants. In a group of Korean women with osteoporosis the presence of *ss* homozygotes was not demonstrated at all [25], similarly to Japanese individuals [26].

Our study indicates that in our selected cohort of Polish women there is no trend toward higher frequency of low-energy fractures (regardless of location) among any genotypes of Sp1 polymorphism. Presumably, the lack of statistical significance was the consequence of a very small number of individuals with *ss* genotype. However, our results are consistent with the findings of Ralston *et al.*, who evaluated the largest number of osteoporotic individuals of both sexes so far and demonstrated no association between fracture and any Sp1 allele, including *s*. A significant association was found only for incident vertebral fractures in the female subgroup ($p = 0.05$, OR = 1.33 (CI: 1.0–1.77)) [19]. Despite such convincing data, some authors have reported an association between Sp1 polymorphism and fractures. We were able to show such a trend for prevalent fractures when we included 41 men in our cohort ($p = 0.058$, OR = 2.92 (95% CI: 0.92–9.29); data not shown). Bernad *et al.* reported higher fracture (Colles' and vertebral) prevalence in a cohort of Spanish osteoporotic women with *ss* genotype [27]. Tran *et al.* reached the same conclusion with regard to hip and vertebral fractures (Australian cohort) [28], and Weichetová *et al.* did so for Colles' fracture [29]. Also, a 2001 meta-analysis [30], covering 16 Sp1 polymorphism studies, showed that the *s* allele was strongly associated with susceptibility to fracture (OR = 1.52, 95% CI: 1.27–1.81).

In 2009, Tran *et al.* suggested including individual information on carrying the Sp1 allelic variant in the nomogram for osteoporotic fracture risk assessment based on the Dubbo study [28]. Their goal was to assess whether this information could lead to more "efficient" identification of patients at risk of fracture. It turned out that taking the fact of being homozygous for *ss* into account resulted in a significant (approximately 5%) change in the threshold of both vertebral and hip fracture risk. Calculated in this way, the risk has changed the indications for antiresorptive therapy.

In the light of our findings, we need to conclude that it is not only BMD that has an absolute effect on fracture risk, but potentially other elements as well. Firstly, there are other factors regulating bone strength that are genetically determined, for example body weight, age at menopause, bone geometry or bone turnover rate. Secondly, most low-energy fractures have been known to occur in women with osteopenia, who do not meet the WHO criteria for osteoporosis. Thirdly, the lack of statistical significance of our calculations might result from insufficient sensitivity of the method. Moreover, our study was not population-based, which might have been a limitation. Still, there is a consensus that the susceptibility to fracture is determined by complex interaction of many genes. Further studies are needed to determine the gene contribution to low-energy fracture risk.

Conflict of interest

The authors declare no conflict of interest.

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