Calcif Tissue Int (2000) 66:409–413

Calcified
Tissue
International

Lack of Influence of Collagen Type Iα1 Sp1 Binding Site Polymorphism on the Rate of Bone Loss in a Cohort of Postmenopausal Danish Women Followed for 18 Years

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Received: 25 January 1999 / Accepted: 12 January 2000

Abstract. A polymorphism in an Sp1 site in the collagen Iα1 (COLIA1) gene has recently been identified and the Ss and ss genotypes were shown to be potentially important determinants of low bone mass in postmenopausal women. Additionally, in a Dutch population, the association of the COLIA1 polymorphism with low bone mineral density (BMD) was more pronounced with increasing age, suggesting a genotype effect on the rate of bone loss. We have investigated the relationship between the COLIA1 Sp1 polymorphism and the rate of bone loss in a longitudinal study with a total of 133 postmenopausal women followed for 18 years. The frequencies of the genotypes were SS 70.7%, Ss 27.8%, ss 1.5% and were in Hardy-Weinberg equilibrium. No association of the COLIA1 genotype with rate of bone loss was detected and there was no difference between the genotype groups with respect to BMD at the femoral neck or lumbar spine. Women with the Ss or ss genotypes, who have been postulated to have low BMD, had even higher BMD at the lower forearm than women with the SS genotype. The levels of serum osteocalcin and urinary collagen type I degradation products were not found to be associated with the COLIA1 Sp1 polymorphism. In conclusion, this study does not support the hypothesis that the Ss COLIA1 genotype predisposes women to increased rate of bone loss or low BMD. However, because of a low absolute number of the ss genotype, it was not possible to reach a conclusion on this particular genotype with regard to an association with low BMD or rate of bone loss.

Key words: COLIA1 — Collagen — Sp1 — Polymorphism — Bone loss.

Osteoporosis is a debilitating bone disease that is expressed clinically by fragility fractures of predominantly the distal radius, the proximal femur, or the spine [1]. A major risk factor is low bone mineral density (BMD) and studies of the contribution of heredity to bone mass have shown a strong genetic component [2, 3]. Osteoporosis can, in most if not all cases, be prevented by hormone replacement therapy [4], however, the available methods for assessing the future risk of the disease are lacking in accuracy [5]. The availability of a genetic marker would therefore be a valuable supplement to the existing methods. Recently the relationship between bone density and polymorphisms within several possible osteoporosis candidate genes such as the vitamin D receptor gene and the estrogen receptor gene has been investigated but with conflicting results [6–9]. There are many explanations for this including the fact that BMD is affected by a multitude of factors and that there might be differences in the nature and frequency of a genetic polymorphism among the different populations [10]. This emphasizes the importance of investigating the supposed effect of a genetic polymorphism on BMD in many different populations.

Another likely candidate for a gene where a polymorphism might influence bone mass is type I collagen. Mutations in the pro-α_1 and pro-α_2 chains of this gene give rise to the disease osteogenesis imperfecta [11, 12] which is characterized by osteopenia associated with recurrent fracture and skeletal deformity. The severity of the clinical expression of the disease is extremely variable and ranges from stillbirth to no symptoms [13]. More subtle mutations in the type I collagen gene might therefore be involved in the etiology of osteoporosis. Recently, a G → T functional polymorphism in a binding site for the transcription factor Sp1 in the collagen type Iα1 (COLIA1) gene was identified [14]. The T allele could be detected by analysis of the DNA by the polymerase chain reaction with a mismatched primer thereby generating a site for the restriction endonuclease Mscl. The G/T (Ss) and T/T (ss) genotypes [but not the G/G homozygotes (SS)] were shown to be associated with low bone mass and were more prevalent in patients with osteoporotic fractures in a Scottish and an English population. The association with an increased risk of fractures has since been confirmed in several other populations [15, 16]. An overrepresentation of the Ss and ss genotypes or the ss genotype among postmenopausal osteoporotic patients were described in a French and Danish study. Additionally, a Dutch [17] cross-sectional study showed that women with the Ss and ss genotypes had lower BMD and that the risk of vertebral fractures was slightly increased in women with the Ss genotype. In the latter study, the association of the s allele with low BMD was stronger with increasing age, suggesting that the polymorphism at the Sp1 site in the COLIA1 gene might be a marker for accelerated bone loss in older women. In this study, we have therefore

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investigated the relationship between the COLIA1 Sp1 polymorphism and rate of bone loss in a longitudinal study with a follow-up period of 18 years.

Materials and Methods

Subjects

In 1977, 315 healthy, early postmenopausal, Danish women aged 45–54 were recruited through questionnaires for a 2-year follow-up study on the prevention of postmenopausal bone loss [18]. The women had no diseases known to affect bone metabolism and had not, at any time after the menopause, received drugs known to affect bone metabolism, including hormone replacement therapy (HRT). Twelve years later the women were invited to a follow-up examination. Two hundred and sixty-five turned up and a group of 182 women were found eligible for repeat measurements. The exclusions were mainly due to HRT use [19]. In 1995, 18 years after the first visit, the 260 women from the original population that were still alive and living in the area were contacted with an invitation to participate in a study on the influence of the vitamin-D-receptor polymorphism on the rate of bone loss [7]. Of these, 149 women turned up for examination and 13 were excluded because of diseases with a known influence on calcium metabolism. This left a group of 136 women for repeat bone mass measurements, spinal radiography, and blood and urine sample collection. Of these, 133 were available for the present study. These women did not differ significantly from the 179 women who were not included in terms of weight, height, age, and years since menopause at baseline.

The rate of bone loss was initially studied for a 2-year period using nine repeated measurements of bone mineral content (BMC) at the lower forearm. Twelve and 18 years after inclusion, BMD was measured at the spine, the hip, and the lower forearm. From all these measurements, the total bone loss in percent was calculated for three periods: (1) early postmenopausal bone loss at the distal forearm over 2 years using simple linear regression on the 9 BMC measurements; (2) late postmenopausal bone loss at the distal forearm, at the proximal femur, and at the lumbar spine over 6 years; (3) long-term postmenopausal bone loss over 18 years at the lower forearm. Participants receiving HRT in period 1 and period 2 were excluded from the calculation of the rate of bone loss. In period 3 those participants having received HRT totaling more than 3 years were excluded. This left a total of 107 subjects in the three time periods.

DNA Analysis

DNA was extracted from peripheral blood leukocytes using standard procedures, and 200 ng was amplified by the polymerase chain reaction (PCR) in a 100 μl reaction volume containing 0.2 mM of each of the dNTPs, 1 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS-HCl, 0.1% Triton X-100, 0.5 μM of each of the two collagen type Iα1 primers (sequences as previously described [14]), and 2.5 U Taq DNA polymerase. The reactions were performed on a GeneAmp® PCR system 9600 instrument with a cycling protocol of one initial cycle at 94°C for 4 minutes, 60°C for 10 seconds, and 72°C with ramp for 1.4 minutes and hold for 15 seconds. This was followed by 38 cycles of 94°C for 50 seconds, 60°C for 10 seconds, and 72°C with ramp for 1.4 minutes and hold for 15 seconds. Finally, 1 cycle of 94°C for 50 seconds, 60°C for 10 seconds, and 72°C for 5 minutes. Ten μl of the PCR reaction was digested with the restriction endonuclease MscI at 25°C and resolved by electrophoresis on a 3% agarose gel. To confirm the accuracy of the genotyping, a subset of samples was reanalyzed by another laboratory. No discrepancies were found.

Bone Mass Measurements

Bone mass measurements were performed on commercially available scanners as previously described [7]. In 1977–89, BMC and BMD of the forearm were measured by single photon absorptiometry using iodine-125 (Bone Mineral Analyzer 1100; Mølsgaard Medical A/S, Hørsholm, Denmark) and in 1995 by single energy X-ray absorptiometry (DTX100; Osteometer A/S, Denmark). The correlation between the two techniques in measuring BMC at the distal forearm is good (R = 0.99), with the measurement points lying along the line of equality. Furthermore, the same external standard and calibration system has been used by the center since 1977, thus making the results obtained at the different time points comparable. The BMDs of the lumbar spine and proximal femur were measured by dual-energy X-ray absorptiometry (QDR 1000, Hologic Inc., Waltham, Massachusetts, USA).

Table 1. Characteristics of subjects at time of inclusion in relation to COLIA1 genotype (mean values and SDs)

Vital statistics	SS n = 94	Ss, ss n = 39	*P*-value
Age (yr)	50.7 ± 2.5	51.3 ± 2.2	0.18
YSM	1.7 ± 0.9	1.7 ± 0.8	0.97
Height (cm)	162.6 ± 5.7	162.1 ± 6.0	0.63
Weight (kg)	63.2 ± 11.1	65.7 ± 12.9	0.29
Age at menarche (yr)	14.1 ± 1.6	13.7 ± 1.5	0.13

YSM = years since menopause

Radiography of Spine and Fracture Diagnostics

Lateral radiography of the thoracic and lumbar spine was performed standing, using standard X-ray equipment. Vertebral deformities (from T4 to L4) were assessed by an expert radiologist and graded by quantitative morphometry where the ratio of the anterior and posterior heights of each vertebral body was determined. A more than 25% reduction of vertebral height was graded as a fracture.

Biochemical Measurements

Urinary degradation products of the α_1-chain of the C-telopeptides of type I collagen were determined by ELISA (CrossLaps, Osteometer BioTech, Denmark) and normalized to urinary creatinine assessed by a standard colorimetric method. Serum osteocalcin was measured by ELISA (Osteocalcin NMID, Osteometer BioTech, Denmark), as previously described [20].

Statistical Analysis

Simple and multiple linear regression analyses were carried out to determine the relationship among BMD, age, weight, height, years since menopause, and COLIA1 genotype. Standard *t*-tests were used for comparison between the COLIA1 genotypes (SS versus Ss and ss combined), employing a significance level of 5%.

Results

The frequencies of the SS, Ss, and ss genotypes were 70.7%, 27.8% and 1.5%, respectively, and were in Hardy-Weinberg equilibrium ($\chi^2 = 0.593$). The Ss and ss groups were analyzed together because of the low absolute number of the ss genotype (n = 2). The clinical characteristics of the SS and Ss/ss groups at baseline are shown in Table 1. No significant differences were found between the two geno-

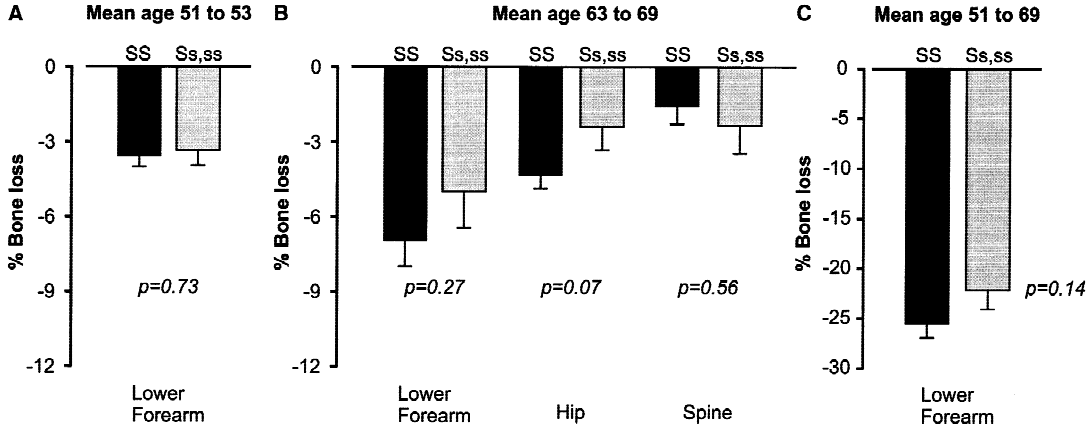

Fig. 1. Postmenopausal bone loss and COLIA1 Sp1 genotype. **(A)** Early postmenopausal bone loss for a 2-year period. **(B)** Late postmenopausal bone loss for a 6-year period. **(C)** Long-term postmenopausal bone loss over 18 years.

type groups with respect to age, years since menopause, height, weight, and age at menarche.

Bone loss was calculated for three different periods: early postmenopausal loss at the lower forearm (a 2-year follow-up); late postmenopausal loss at the lower forearm, lumbar spine, and femoral neck (a 6-year follow-up); and long-term postmenopausal loss at the lower forearm (an 18-year follow-up). No association of the COLIA1 genotypes with rate of bone loss was found for any of the three periods (Fig. 1). In addition, BMD at the lumbar spine or femoral neck at age 69 did not significantly differ between the two genotype groups (Table 2). However, at the lower forearm, women with the genotype SS had significantly lower BMC at baseline ($P = 0.01$) and significantly lower BMD after 18 years ($P = 0.01$) (Table 2). A multivariate analysis of variance showed that the rate of bone loss is significantly related to height, weight, and biochemical markers of bone turnover, but not to COLIA1 alleles. Adjustments for height, weight, and biochemical markers did not change the results and there was still no effect of the COLIA1 alleles on the rate of bone loss. For the BMD values from 1995 and the values for BMC for the lower forearm for 1977, in addition to the above-mentioned factors, age was also a contributory factor. However, adjustment for these factors did not in any way change our results (data not shown).

Spinal radiography was performed and markers of bone turnover were measured at the 18-year follow-up visit. Thirteen percent of the women had vertebral fractures. The frequencies of the COLIA1 genotypes were the same in the group of women with spinal fractures compared with the entire study population (Table 2). No differences were found for the levels of serum osteocalcin or urinary collagen type I degradation products among the COLIA1 genotypes (Table 2).

Discussion

The purpose of this study was to investigate whether postmenopausal women with the Ss or ss COLIA1 genotypes loose bone faster than women with the SS genotype. This was based on a recent paper which, in a cross-sectional study, showed that increasing postmenopausal age resulted in an increased difference in BMD among the COLIA1 genotypes. In that paper, Uitterlinden et al. [17] found no significant genotype-related difference in BMD for women 55–69 years of age, however, among older women, the BMDs in the Ss and ss groups were significantly lower than for the SS group both at the femoral neck and the lumbar spine indicating that the s allele might be a marker for accelerated bone loss. This hypothesis has been tested in the present study which is the first longitudinal study to investigate a possible association of the Sp1 COLIA1 polymorphism with postmenopausal bone loss. No association of the s allele with bone loss was found at either the lumbar spine, femoral neck, or lower forearm. If anything, a tendency towards slower long-term bone loss for the Ss, ss population was found.

The study had a power of 90% (at the 5% significance level) to detect a 0.5% per year difference in bone loss between the SS and Ss groups. However, if a smaller, but less clinically relevant effect of the Ss genotype on bone loss should be detected, a larger sample size would be required. A small effect of the s allele on rate of bone loss can therefore not be entirely excluded, but it seems unlikely since the results showed an opposite trend. A possible explanation for the lack of a relationship between bone loss and COLIA1 may have been the age of the patients. In the Dutch study, the increased divergence of BMD values in patients with different COLIA1 genotypes was most marked over the age of 70, whereas here, the average age of the patients with 18-year follow-up was 68. On the other hand, it is possible that the differences observed in the Dutch study were due to a cohort effect.

Low BMD at the lumbar spine has, in several studies [14, 16, 17, 21], been associated with the Ss and ss COLIA1 genotypes, whereas only one large study [17] has been able to detect a significant relation at the femoral neck, and a Swedish group found no correlation to BMD at either of these two sites [22]. In the current study we also did not find any association of the COLIA1 genotypes with BMD at either the lumbar spine or femoral neck. At the lower forearm, however, women with the Ss genotype had even higher bone mass than the women with the SS genotype. This was surprising and has, to our knowledge, not been described before.

Taken together, this indicates that there may be both site-dependent, age-dependent, and population-dependent [23] variations in the effect of the Sp1 COLIA1 genotype on

Table 2. Bone mass, biochemical markers of bone turnover, and frequencies of vertebral fractures in relation to COLIA1 genotype. Bone mineral density (BMD) measurements at mean age 69 at the lower forearm, hip and spine and BMC at the lower forearm at mean age 51. Mean values and the standard error of the mean are shown

	SS	Ss, ss	*P*-value
Number[a]	75/94	33/39	
Spine BMD (g/cm^2) Age 69	0.855 ± 0.017	0.871 ± 0.134	0.6
Hip BMD (g/cm^2) Age 69	0.753 ± 0.015	0.776 ± 0.020	0.4
Lower forearm BMD (g/cm^2) Age 69	0.339 ± 0.007	0.373 ± 0.009	0.01
Lower forearm BMC (g) Age 51	3.233 ± 0.056	3.477 ± 0.065	0.01
Collagen degradation products (μg/mmol Cr)	346.1 ± 14.1	337.9 ± 6.9	0.8
Osteocalcin (ng/ml)	28.7 ± 15.7	27.6 ± 3.2	0.5
Vertebral fractures	13% (12/94)	13% (5/39)	—

[a] Number of individuals that had determined BMD, biochemistry/BMC, fracture evaluation

BMD and that the most consistent evidence of an association of the s allele with bone disease is the relation to vertebral fractures. This has been reported for British, French, Danish, and Dutch populations [14–17] where, in all cases, an increased frequency of the s allele was found in patients with osteoporosis. In this present study we did not find any difference in genotype distribution in the group with vertebral fractures (Table 2), however, the number of women with moderate or severe vertebral fractures was too small ($n = 17$) to allow any conclusions to be drawn regarding this issue.

In conclusion, in a longitudinal study with a maximum of 18 years follow-up, we did not detect any association of the COLIA1 Sp1 genotype with rate of bone loss. This, together with the reports of only a weak correlation of the COLIA1 genotype with BMD, but a more consistent relation to vertebral fracture, support the hypothesis that the COLIA1 genotype might be more associated with bone quality than with bone mass. Despite the increased research efforts to discover genes involved in the pathology of osteoporosis, most have yet to be described and it will be interesting to see how the COLIA1 Sp1 polymorphism in combination with other genetic markers could contribute to the prediction of osteoporosis and fracture risk.

Acknowledgments. We thank Ms. Grace Taylor for excellent technical assistance.

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