

Association of *Paraoxonase 1 (PON1)* polymorphisms with osteoporotic fracture risk in postmenopausal Korean women

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Abbreviations: BMD, bone mineral density; ht, haplotype; LD, linkage disequilibrium; MAF, minor allele frequency; OF, osteoporotic fracture; PON1, paraoxonase 1; YSM, years since menopause

Abstract

There is increasing evidence of a biochemical link between lipid oxidation and bone metabolism. Paraoxonase 1 (PON1) prevents the oxidation of low-density lipoprotein (LDL) and metabolizes biologically active phospholipids in oxidized LDLs. Here, we performed association analyses of genetic variation in

PON1 to ascertain its contribution to osteoporotic fractures (OFs) and bone mineral density (BMD). We directly sequenced the *PON1* gene in 24 Korean individuals and identified 26 sequence variants. A large population of Korean postmenopausal women ($n = 1,329$) was then genotyped for eight selected *PON1* polymorphisms. BMD at the lumbar spine and femoral neck was measured using dual-energy X-ray absorptiometry. Lateral thoracolumbar (T4-L4) radiographs were obtained for vertebral fracture assessment, and the occurrence of non-vertebral fractures (i.e., wrist, hip, forearm, humerus, rib, and pelvis) was examined using self-reported data. Multivariate analyses showed that none of the polymorphisms was associated with BMD at either site. However, $+5989A > G$ and $+26080T > C$ polymorphisms were significantly associated with non-vertebral and vertebral fractures, respectively, after adjustment for covariates. Specifically, the minor allele of $+5989A > G$ exerted a highly protective effect against non-vertebral fractures (OR = 0.59, $P = 0.036$), whereas the minor allele of $+26080T > C$ was associated with increased susceptibility to vertebral fractures (OR = 1.73, $P = 0.020$). When the risk for any OFs (i.e., vertebral or non-vertebral) was considered, the statistical significance of both polymorphisms persisted ($P = 0.002-0.010$). These results suggest that *PON1* polymorphisms could be one of useful genetic markers for OF risk in postmenopausal women.

Keywords: arylalkylphosphatase; bone density; fractures, bone; polymorphism, single nucleotide; postmenopause

Introduction

Osteoporotic fractures (OFs) among elderly people are effectively a worldwide epidemic, and the predicted aging of populations will further increase the burden of these minimal trauma fractures on healthcare systems (Melton, 1993). In addition to high costs, OFs are associated with high morbidity and disability, high risk for long term institutionalization, and increased risk of death (Cooper *et al.*, 1993; Melton, 1993). One central objective of bone biology research is to identify all important factors that underlie OFs, with the ultimate goal of in-

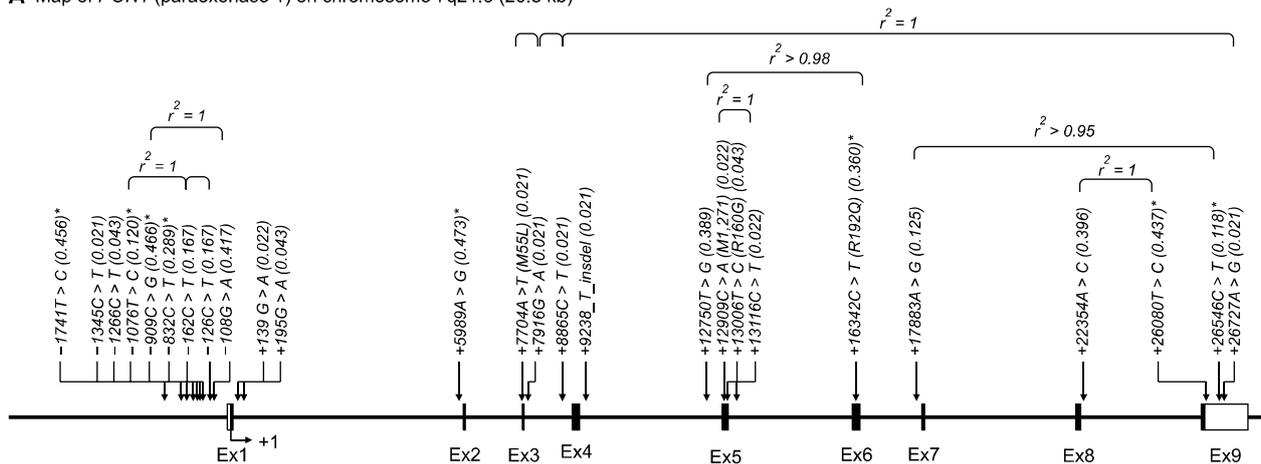
tervening effectively and reducing the risk and incidence of OFs. Most previous studies have concentrated on extrinsic and non-genetic environmental factors, and studies on the genetic determinants of OFs are relatively rare, although recent study has reported that the age-adjusted heritability of OFs is about 27% (Michaelsson *et al.*, 2005). Furthermore, genetic studies of bone have largely been confined to a consideration of bone mineral density (BMD) because this parameter is relatively easy to measure (Kanis, 1997). However, these results may not accurately reflect the heritability of OFs, because BMD is not the only important risk factor for OF; many other identified and/or unidentified intrinsic factors are also important (Kleerekoper *et al.*, 1985; Faulkner *et al.*, 1993; Harris *et al.*, 1998). Therefore, studies of the genetic influence on OFs per se are

essential.

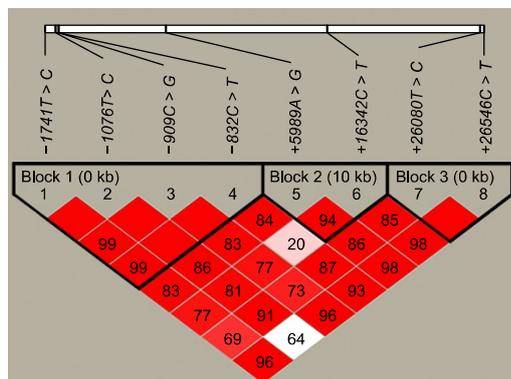
There is increasing evidence of a biochemical link between lipid oxidation and bone biology. Increased lipid oxidation causes oxidative stress and reduces Wnt signaling, thereby decreasing the differentiation and survival of osteoblasts (Almeida *et al.*, 2009). In addition, oxidized lipids have direct adverse effects on cellular components by increasing adipogenesis of marrow stromal cells at the expense of their osteogenic differentiation (Parhami *et al.*, 1999) and inducing osteoclastic differentiation *via* a cAMP-mediated pathway (Tintut *et al.*, 2002). Therefore, genes related to lipid oxidation could be good candidates for genetic studies of bone health.

Paraoxonase 1 (PON1), a product of the *PON1* gene which is clustered in tandem on the long arms of human chromosome 7 (q21.22), is a

A Map of *PON1* (paraoxonase 1) on chromosome 7q21.3 (26.8 kb)



B LDs among *PON1* polymorphisms



C Haplotypes in *PON1*

	Block1					Block2			Block3				
	-1741T > C	-1076T > C	-909C > G	-832C > T	Freq.	Hap.	+5989A > G	+16342C > T	Freq.	Hap.	+26080T > C	+26546C > T	Freq.
ht1	C	T	G	C	0.454	ht1	A	C	0.517	ht1	T	C	0.445
ht2	T	T	C	C	0.245	ht2	G	T	0.350	ht2	C	C	0.437
ht3	T	T	C	T	0.168	ht3	G	C	0.123	ht3	T	T	0.118
ht4	T	C	C	T	0.120	ht4	A	T	0.010				
ht5	T	T	G	C	0.012								
ht6	C	T	C	C	0.0008								
ht7	C	T	C	T	0.0008								

Figure 1. Gene maps, haplotypes, and linkage disequilibrium (LD) coefficients of *PON1*. (A) Polymorphisms identified in *PON1* on chromosome 7q21.3. Coding exons are marked with black rectangles, and 5'- and 3'-UTRs with white rectangles. The first base of the translation start site is denoted nucleotide '+1'. Asterisks indicate SNPs that were genotyped in a larger population ($n = 1,329$). The frequencies of SNPs that were not subjected to larger-scale genotyping are based on sequence data ($n = 24$). (B) LD blocks and correlation coefficients among *PON1* polymorphisms. Red squares indicate statistically significant allelic association between the pair of SNPs, as measured by the D' statistic; darker shades of red indicate higher values of D' , up to a maximum of 1. (C) Haplotype frequencies of *PON1* polymorphisms.

Table 1. Frequencies of *PON1* polymorphisms in 1,329 postmenopausal Korean women

Loci	Position	Amino acid change	rs#	Genotype			MAF	HWE*
-1741T > C	Promoter		rs757158	TT	CT	CC	0.456	0.696
				388	670	271		
-1076T > C	Promoter		rs854573	TT	CT	CC	0.120	0.732
				1027	284	17		
-909C > G	Promoter		rs854572	CC	CG	GG	0.466	0.647
				375	669	285		
-832C > T	Promoter		rs854571	CC	CT	TT	0.289	0.997
				674	543	112		
+5989A > G	Intron1		rs2074351	AA	AG	GG	0.473	0.297
				362	677	290		
+16342C > T	Exon6	R192Q	rs662	CC	CT	TT	0.360	0.194
				532	636	160		
+26080T > C	Exon9 (3'UTR)		rs3917577	TT	CT	CC	0.437	0.852
				422	652	255		
+26546C > T	Exon9 (3'UTR)		rs3735590	CC	CT	TT	0.118	0.721
				1037	270	22		

*P values of deviation from Hardy-Weinberg Equilibrium (HWE) among subjects without osteoporotic fractures ($n = 1,165$). MAF, minor allele frequency.

calcium-dependent glycoprotein that is closely associated with high-density lipoprotein (HDL) in serum (Costa *et al.*, 2005). PON1 preserves HDL functions and contributes to the antioxidant effect of HDL by hydrolyzing lipid peroxidase and thereby preventing the oxidation of low-density lipoprotein (LDL) (Mackness *et al.*, 1991). It also metabolizes biologically active phospholipids in oxidized LDLs (Costa *et al.*, 2003). These actions suggest a critical role for PON1 in bone metabolism in addition to its known effects on cardiovascular diseases (CVDs) and atherosclerosis. However, the possible association of *PON1* polymorphisms with OFs or BMD has received little research attention. Therefore, in the present study, we performed extensive screening of the *PON1* gene by direct sequencing to detect polymorphisms, and investigated their influence on OF risk and BMD in postmenopausal Korean women.

Results

In this study, we investigated a population of Korean postmenopausal women for *PON1* gene polymorphisms by directly sequencing all exons and exon-intron boundaries in the *PON1* gene, including -2,000 bp of the 5' flanking region. This analysis identified 26 SNPs: nine in the promoter region, four in the coding regions of exons, three in the 3' untranslated regions (UTRs), and ten in the introns. The frequencies of these polymorphisms are shown in Figure 1A. Of the 26 polymorphisms

identified, five (+139G > A, +195G > A, +9238T *insdel*, +12909C > A, and +13116C > T) had not been previously reported. We selected eight polymorphisms (1741T > C, 1076T > C, 909C > G, -832C > T, +5989A > G, +16342C > T, +26080T > C, and +26546C > T) for larger-scale genotyping ($n = 1,329$) based on minor allele frequency (MAF > 0.1), LDs, and haplotype-tagging status. All genotype distributions were in Hardy-Weinberg equilibrium ($P > 0.05$; Table 1). After calculating $|D'|$ and r^2 among polymorphisms, *PON1* could be parsed into three haplotype blocks, with each block having strong LD spine (Figure 1B). There were four, three, and three common haplotypes (frequency > 0.1) in block1 (BL1), block2 (BL2) and block3 (BL3), respectively (Figure 1C). Among the common haplotypes in three blocks, BL1-ht1, BL1-ht4, BL2-ht1, BL2-ht2, BL3-ht2 and BL3-ht3 were not further analyzed because they were almost equivalent to -1741T > C, -1076T > C, +5989A > G, +16342C > T, +26080T > C and +26546C > T, respectively.

The characteristics of the study subjects are shown in Table 2. OFs of any kind (i.e., vertebral and non-vertebral fractures combined) were noted in 164 of 1,329 postmenopausal women. Of the 164 subjects with OFs, 99 exhibited vertebral fractures and 73 exhibited non-vertebral fractures. Women with OFs were significantly older (62.1 ± 6.4 yr vs. 58.7 ± 7.4 yr) and their YSM interval was longer (12.8 ± 7.3 yr vs. 9.3 ± 7.8 yr) than those without OFs. Lumbar spine and femoral neck BMD were marginally lower in women with OFs

Table 2. Clinical characteristics of subjects by osteoporotic fracture (OF) status

Variables	Subjects with any OFs* (n = 164)	Subjects without any OFs (n = 1,165)	P value [†]
Age (yr)	62.1 ± 6.4	58.7 ± 7.4	< 0.001
Weight (kg)	56.2 ± 7.5	56.2 ± 7.0	0.974
Height (cm)	154.7 ± 4.6	155.0 ± 5.4	0.497
Years since menopause (yr)	12.8 ± 7.3	9.3 ± 7.8	< 0.001
Lumbar spine bone mineral density (g/cm ²)	0.862 ± 0.151	0.883 ± 0.160	0.096
Femoral neck bone mineral density (g/cm ²)	0.715 ± 0.110	0.731 ± 0.122	0.083
History of cardiovascular diseases (%)	7 (4.3%)	61 (5.2%)	0.708

Values are mean ± SD unless otherwise specified.

*Any OFs include either vertebral or non-vertebral (wrist, hip, forearm, humerus, rib, and pelvis) fractures.

[†]By Student *t* test for continuous variables and Fisher's exact test for categorical variables.

(0.862 ± 0.151 g/cm² and 0.715 ± 0.111 g/cm², respectively) than in those without (0.883 ± 0.160 g/cm² and 0.731 ± 0.122 g/cm², respectively). There were no significant differences in weight, height, or history of CVDs between the two groups. An examination of the independent effect of each variable on OF status by multiple logistic regression analysis revealed that only age was significantly associated with increased risk of OFs of any kind ($P = 0.040$, OR = 1.05, 95% CI = 1.01-1.11).

Independent associations of lumbar spine and femoral neck BMD with age, weight, height, and YSM are shown in Table 3. As expected, age and YSM were inversely associated with BMD at both the lumbar spine and femoral neck regions,

whereas weight and height were positively associated with BMD at both sites. A Pearson's correlation coefficient was also calculated to examine the correlation between the BMDs at the two bone loci. The BMDs of the lumbar spine and femoral neck correlated strongly and positively with each other (correlation coefficient = 0.632, $P < 0.001$).

The association of *PON1* polymorphisms with BMD at the lumbar spine and femoral neck was analyzed using multiple regression analysis, controlling for age, YSM, weight, and height. However, none of the polymorphisms or haplotypes showed a significant association with BMD at either site (Table 4).

The genetic effects of *PON1* polymorphisms on the risk of vertebral and non-vertebral fractures were assessed by multiple logistic regression analysis after controlling for age, YSM, weight, and height (Table 5). The minor allele of +26080T > C was more frequent in subjects with vertebral fracture (frequency = 0.490) than in those without fracture (frequency = 0.433), and was significantly associated with a higher probability for the occurrence of vertebral fracture in the recessive model ($P = 0.020$, OR = 1.73, 95% CI = 1.09-2.76). An extension of this analysis to a consideration of any OFs (i.e., vertebral or non-vertebral) showed that the association between the minor allele of +26080T > C and increased susceptibility to OFs persisted (co-dominant model: $P = 0.010$, OR = 1.36, 95% CI = 1.08-1.72; recessive model: $P = 0.002$, OR = 1.80, 95% CI = 1.23-2.63). On the other hand, the minor allele of +5989A > G was less frequent in subjects with non-vertebral fracture (frequency = 0.432) than in those without fracture (frequency = 0.475), and showed an association with decreased risk of non-vertebral fracture in the dominant model ($P = 0.036$, OR = 0.59, 95% CI = 0.36-0.97). The minor allele of +5989A > G also showed a protective effect against the occurrence of any OFs (dominant model: $P = 0.005$, OR = 0.60, 95% CI = 0.43-0.86), as was observed for

Table 3. Multiple regression analyses on bone mineral density (BMD) (g/cm²) in 1,329 postmenopausal Korean women

Variables	Lumbar spine BMD (g/cm ²)			Femoral neck BMD (g/cm ²)		
	β	SE	P value	β	SE	P value
Age (yr)	-0.005	0.001	< 0.001	-0.005	0.001	< 0.001
Weight (kg)	0.005	0.001	< 0.001	0.003	0.000	< 0.001
Height (cm)	0.002	0.001	0.003	0.001	0.001	0.100
YSM (yr)	-0.003	0.001	0.004	-0.003	0.001	< 0.001
	Adjusted R ² = 0.246			Adjusted R ² = 0.277		

Values are adjusted for all other variables in the Table.

YSM, years since menopause; β, regression coefficient; SE, standard error.

Table 4. Regression analysis of bone mineral density (BMD) (g/cm^2) at lumbar spine and femoral neck with PON1 polymorphisms in postmenopausal Korean women

Skeletal sites	Loci	C/C*	C/R	R/R	P_a^\dagger	P_b	P_c	
Lumbar spine	-1741T > C	388 (0.884 \pm 0.149)	670 (0.880 \pm 0.161)	271 (0.878 \pm 0.169)	0.777	0.614	0.938	
	-1076T > C	1027 (0.879 \pm 0.162)	284 (0.889 \pm 0.152)	17 (0.858 \pm 0.144)	0.898	0.950	0.776	
	-909C > G	375 (0.881 \pm 0.149)	669 (0.882 \pm 0.161)	285 (0.878 \pm 0.170)	0.994	0.830	0.824	
	-832C > T	674 (0.877 \pm 0.168)	543 (0.888 \pm 0.152)	112 (0.870 \pm 0.147)	0.942	0.611	0.278	
	BL1_ht2	755 (0.881 \pm 0.158)	498 (0.883 \pm 0.164)	76 (0.869 \pm 0.146)	0.945	0.848	0.819	
	BL1_ht3	920 (0.880 \pm 0.164)	371 (0.884 \pm 0.150)	38 (0.864 \pm 0.148)	0.972	0.751	0.444	
	+5989A > G	362 (0.880 \pm 0.166)	677 (0.883 \pm 0.159)	290 (0.876 \pm 0.151)	0.867	0.725	0.923	
	+16342C > T	532 (0.884 \pm 0.162)	636 (0.876 \pm 0.157)	160 (0.889 \pm 0.163)	0.572	0.183	0.396	
	BL2_ht3	1028 (0.882 \pm 0.163)	275 (0.878 \pm 0.151)	26 (0.866 \pm 0.118)	0.824	0.778	0.929	
	+26080T > C	422 (0.869 \pm 0.155)	652 (0.888 \pm 0.158)	255 (0.883 \pm 0.171)	0.221	0.137	0.669	
	+26546C > T	1037 (0.883 \pm 0.162)	270 (0.873 \pm 0.152)	22 (0.867 \pm 0.122)	0.599	0.624	0.754	
	BL3_ht1	411 (0.884 \pm 0.162)	654 (0.881 \pm 0.158)	264 (0.874 \pm 0.159)	0.381	0.444	0.509	
	Femoral neck	-1741T > C	388 (0.730 \pm 0.117)	670 (0.727 \pm 0.124)	271 (0.733 \pm 0.120)	0.642	0.757	0.248
		-1076T > C	1027 (0.727 \pm 0.122)	284 (0.740 \pm 0.119)	17 (0.721 \pm 0.138)	0.496	0.459	0.993
-909C > G		375 (0.730 \pm 0.115)	669 (0.728 \pm 0.125)	285 (0.733 \pm 0.119)	0.638	0.719	0.230	
-832C > T		674 (0.729 \pm 0.121)	543 (0.728 \pm 0.121)	112 (0.741 \pm 0.121)	0.891	0.742	0.363	
BL1_ht2		755 (0.732 \pm 0.121)	498 (0.729 \pm 0.124)	76 (0.715 \pm 0.100)	0.458	0.572	0.469	
BL1_ht3		920 (0.730 \pm 0.120)	371 (0.730 \pm 0.125)	38 (0.716 \pm 0.106)	0.701	0.898	0.386	
+5989A > G		362 (0.733 \pm 0.121)	677 (0.729 \pm 0.122)	290 (0.726 \pm 0.119)	0.605	0.282	0.777	
+16342C > T		532 (0.729 \pm 0.122)	636 (0.730 \pm 0.121)	160 (0.729 \pm 0.121)	0.756	0.601	0.880	
BL2_ht3		1028 (0.732 \pm 0.121)	275 (0.723 \pm 0.123)	26 (0.712 \pm 0.105)	0.591	0.637	0.681	
+26080T > C		422 (0.726 \pm 0.118)	652 (0.731 \pm 0.121)	255 (0.732 \pm 0.127)	0.651	0.930	0.482	
+26546C > T		1037 (0.731 \pm 0.121)	270 (0.724 \pm 0.124)	22 (0.722 \pm 0.106)	0.674	0.689	0.823	
BL3_ht1		411 (0.728 \pm 0.126)	654 (0.732 \pm 0.120)	264 (0.725 \pm 0.118)	0.861	0.879	0.627	

The number of subjects and means \pm standard deviation of the BMDs are shown.

*C/C, C/R, and R/R represent homozygotes for the common allele, heterozygotes, and homozygotes for the rare allele, respectively.

$^\dagger P_a$, P_b , and P_c represent the P values of co-dominant, dominant, and recessive models for multiple regression analysis, respectively, after controlling for age, years since menopause, weight, and height.

non-vertebral fractures. Importantly, the associations of +26080T > C and +5989A > G with OFs of any kind were still significant after strictly adopting a Bonferroni correction ($P = 0.016$ in the recessive model and $P = 0.038$ in the dominant model, respectively).

Because there was no association between BMD at the lumbar spine and femoral neck and PON1 polymorphisms, we supposed that the association of the genotypes with fracture might be independent of variations in BMD. To confirm this hypothesis, we further analyzed the association of +26080T > C and +5989A > G with OFs after additional adjustment for BMD values at the lumbar spine and/or femoral neck as covariates. As shown in Table 6, the associations of +26080T > C with vertebral fracture, +5989A > G with non-vertebral fracture, and both polymorphisms with any OFs were persistently significant even after additional adjustments for BMD values ($P = 0.003$ - 0.032 in co-dominant, dominant, and recessive models).

Discussion

In an effort to identify and characterize polymorphisms associated with bone metabolism in postmenopausal women, we focused on the role of PON1 polymorphisms in the determinations of OFs and bone mass. Although we could not establish that the genetic polymorphisms influenced BMD at the lumbar spine and femoral neck, direct sequencing and association analyses revealed that PON1 +5989A > G and +26080T > C polymorphisms were significantly associated with non-vertebral fractures and vertebral fractures, respectively. Specifically, the minor allele (G) of +5989A > G had a highly protective effect against non-vertebral fractures, whereas the minor allele (C) of +26080T > C increased susceptibility to vertebral fractures. To our knowledge, this is the first clinical report demonstrating a relationship between PON1 polymorphisms and OFs, and this relationship suggests that PON1 polymorphisms could be one of genetic markers with predictive

Table 5. Association of *PON1* polymorphisms with the risk of osteoporotic fractures (OFs) in postmenopausal Korean women

		Vertebral fractures							
Loci	Position	Case	Control	Co-dominant		Dominant		Recessive	
				OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>
-1741T > C	Promoter	0.480	0.454	1.12 (0.83-1.50)	0.463	1.02 (0.65-1.61)	0.924	1.33 (0.82-2.15)	0.244
-1076T > C	Promoter	0.152	0.117	1.42 (0.94-2.14)	0.099	1.40 (0.88-2.24)	0.160	2.53 (0.70-9.13)	0.158
-909C > G	Promoter	0.490	0.464	1.12 (0.83-1.49)	0.461	0.97 (0.62-1.53)	0.905	1.39 (0.87-2.22)	0.165
-832C > T	Promoter	0.313	0.287	1.16 (0.84-1.58)	0.371	1.12 (0.74-1.69)	0.589	1.45 (0.75-2.83)	0.272
<i>BL1_h12</i>		0.197	0.248	0.72 (0.50-1.04)	0.075	0.75 (0.49-1.14)	0.177	0.30 (0.07-1.26)	0.099
<i>BL1_h13</i>		0.162	0.169	0.94 (0.63-1.40)	0.756	0.86 (0.55-1.36)	0.525	1.54 (0.53-4.50)	0.432
+5989A > G	Intron1	0.434	0.476	0.82 (0.61-1.11)	0.198	0.69 (0.44-1.06)	0.093	0.91 (0.55-1.51)	0.711
+16342C > T	Exon6 (R192Q)	0.342	0.361	0.92 (0.67-1.26)	0.607	0.90 (0.59-1.38)	0.636	0.89 (0.46-1.72)	0.732
<i>BL2_h13</i>		0.101	0.125	0.75 (0.46-1.20)	0.225	0.69 (0.40-1.18)	0.171	1.00 (0.23-4.36)	0.996
+26080T > C	Exon9 (3'UTR)	0.490	0.433	1.28 (0.96-1.71)	0.098	1.13 (0.72-1.77)	0.594	1.73 (1.09-2.76)	0.020
+26546C > T	Exon9 (3'UTR)	0.091	0.120	0.70 (0.42-1.16)	0.164	0.68 (0.40-1.17)	0.165	0.59 (0.08-4.48)	0.606
<i>BL3_h11</i>		0.419	0.447	0.90 (0.67-1.20)	0.459	0.82 (0.53-1.26)	0.367	0.93 (0.55-1.57)	0.791
		Non-vertebral fractures							
Loci	Position	Case	Control	Co-dominant		Dominant		Recessive	
				OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>
-1741T > C	Promoter	0.445	0.457	0.96 (0.69-1.35)	0.830	0.99 (0.59-1.66)	0.963	0.91 (0.50-1.66)	0.747
-1076T > C	Promoter	0.137	0.119	1.22 (0.74-2.00)	0.438	1.26 (0.73-2.17)	0.412	1.10 (0.14-8.59)	0.930
-909C > G	Promoter	0.486	0.465	1.10 (0.79-1.54)	0.581	1.08 (0.63-1.84)	0.780	1.20 (0.69-2.08)	0.522
-832C > T	Promoter	0.253	0.291	0.82 (0.56-1.21)	0.325	0.83 (0.52-1.35)	0.457	0.61 (0.22-1.72)	0.352
<i>BL1_h12</i>		0.260	0.244	1.09 (0.74-1.60)	0.680	1.01 (0.63-1.64)	0.959	1.54 (0.64-3.71)	0.337
<i>BL1_h13</i>		0.116	0.171	0.62 (0.36-1.04)	0.068	0.59 (0.33-1.04)	0.070	0.47 (0.06-3.53)	0.464
+5989A > G	Intron1	0.432	0.475	0.80 (0.57-1.13)	0.204	0.59 (0.36-0.97)	0.036	1.03 (0.58-1.81)	0.921
+16342C > T	Exon6 (R192Q)	0.322	0.362	0.83 (0.58-1.19)	0.308	0.68 (0.42-1.09)	0.107	1.13 (0.56-2.26)	0.737
<i>BL2_h13</i>		0.116	0.123	0.87 (0.52-1.47)	0.604	0.87 (0.49-1.56)	0.646	0.68 (0.09-5.13)	0.705
+26080T > C	Exon9 (3'UTR)	0.507	0.433	1.36 (0.97-1.90)	0.071	1.50 (0.87-2.60)	0.147	1.52 (0.88-2.63)	0.132
+26546C > T	Exon9 (3'UTR)	0.110	0.119	0.88 (0.51-1.50)	0.639	0.87 (0.48-1.56)	0.631	0.87 (0.11-6.69)	0.894
<i>BL3_h11</i>		0.384	0.448	0.77 (0.55-1.09)	0.135	0.72 (0.44-1.18)	0.191	0.69 (0.35-1.33)	0.262
		Any OFs*							
Loci	Position	Case	Control	Co-dominant		Dominant		Recessive	
				OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>
-1741T > C	Promoter	0.470	0.454	1.07 (0.85-1.36)	0.554	1.02 (0.71-1.47)	0.897	1.19 (0.81-1.77)	0.378
-1076T > C	Promoter	0.143	0.116	1.33 (0.94-1.87)	0.105	1.31 (0.90-1.92)	0.163	2.23 (0.70-7.11)	0.174
-909C > G	Promoter	0.494	0.462	1.15 (0.91-1.45)	0.250	1.04 (0.72-1.50)	0.840	1.40 (0.96-2.04)	0.081
-832C > T	Promoter	0.284	0.289	0.98 (0.76-1.27)	0.887	0.96 (0.69-1.33)	0.794	1.04 (0.58-1.89)	0.887
<i>BL1_h12</i>		0.223	0.248	0.85 (0.64-1.12)	0.243	0.82 (0.59-1.15)	0.251	0.80 (0.37-1.71)	0.558
<i>BL1_h13</i>		0.140	0.172	0.77 (0.55-1.08)	0.127	0.70 (0.48-1.03)	0.067	1.13 (0.43-2.98)	0.804
+5989A > G	Intron1	0.430	0.479	0.79 (0.62-1.00)	0.054	0.60 (0.43-0.86)	0.005	0.96 (0.64-1.43)	0.824
+16342C > T	Exon6 (R192Q)	0.331	0.364	0.86 (0.67-1.11)	0.236	0.76 (0.55-1.07)	0.114	0.99 (0.59-1.64)	0.958
<i>BL2_h13</i>		0.107	0.125	0.79 (0.54-1.14)	0.209	0.75 (0.49-1.13)	0.170	0.92 (0.27-3.14)	0.890
+26080T > C	Exon9 (3'UTR)	0.500	0.428	1.36 (1.08-1.72)	0.010	1.27 (0.88-1.84)	0.196	1.80 (1.23-2.63)	0.002
+26546C > T	Exon9 (3'UTR)	0.098	0.121	0.76 (0.51-1.11)	0.156	0.73 (0.48-1.12)	0.147	0.73 (0.17-3.23)	0.683
<i>BL3_h11</i>		0.402	0.451	0.82 (0.65-1.04)	0.101	0.73 (0.52-1.03)	0.076	0.83 (0.54-1.28)	0.396

Genotype distributions and *P* values for logistic analyses of three alternative models (co-dominant, dominant, and recessive) after controlling for age, weight, height, and years since menopause as covariates are shown.

*Any OFs include either vertebral or non-vertebral (wrist, hip, forearm, humerus, rib, and pelvis) fractures.

Table 6. Association of *PON1* polymorphisms with the risk of osteoporotic fractures (OFs) after additional adjustment for bone mineral density (BMD) (g/cm²) in postmenopausal Korean women

Vertebral fractures*									
Loci	Position	Case	Control	Co-dominant		Dominant		Recessive	
				OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
+26080T > C	Exon9 (3'UTR)	0.490	0.433	1.27 (0.95-1.70)	0.107	1.12 (0.71-1.75)	0.631	1.73 (1.08-2.75)	0.022
Non-vertebral fractures [†]									
Loci	Position	Case	Control	Co-dominant		Dominant		Recessive	
				OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
+5989A > G	Intron1	0.432	0.475	0.80 (0.56-1.12)	0.193	0.58 (0.35-0.95)	0.032	1.03 (0.58-1.81)	0.922
Any OFs ^{‡, #}									
Loci	Position	Case	Control	Co-dominant		Dominant		Recessive	
				OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
+5989A > G	Intron1	0.430	0.479	0.79 (0.62-1.00)	0.055	0.61 (0.43-0.86)	0.006	0.96 (0.64-1.43)	0.825
+26080T > C	Exon9 (3'UTR)	0.500	0.428	1.36 (1.07-1.72)	0.011	1.28 (0.88-1.85)	0.195	1.79 (1.23-2.62)	0.003

P values for logistic analyses of three alternative models (co-dominant, dominant, and recessive) after adjustment for BMD in addition to age, weight, height, and years since menopause as covariates, are shown.

*After additional adjustment for lumbar spine BMD.

[†] After additional adjustment for femoral neck BMD.

[‡] After additional adjustment for lumbar spine and femoral neck BMD.

[#] Any OFs include either vertebral or non-vertebral (wrist, hip, forearm, humerus, rib, and pelvis) fractures.

value for the risk of OFs in postmenopausal women.

Recent study by a large consortium (Richards *et al.*, 2009) showed that some of investigated genes may have effects on fracture risk that are not mediated through BMD alone, and Andrew *et al.* (Andrew *et al.*, 2005) reported a small genetic correlation (~10%) between OF and BMD. These observations raise two related points. First, although there is evidence that genetic risk factors are important in the etiology of both OF (Michaelsson *et al.*, 2005) and BMD (Arden and Spector, 1997; Eisman, 1999), such genetic factors are likely to be specifically linked to each phenotype. Of course, a low genetic correlation between OF and BMD does not exclude the possibility that these two traits might share candidate genes in common. However, more effort is required to determine the direct relationships between gene polymorphisms and OF risk in addition to their potential pleiotropic effects, and genes found to be associated with BMD need to be validated for their relationship to fractures. Second, genetic influences are known to contribute to fall-related factors, such as postural balance (Pajala *et al.*, 2004), muscle function (Tiainen *et al.*, 2004, 2005), cognitive abilities (Wright *et al.*, 2001), and physical activity (Frederiksen and Christensen, 2003); thus, genetics may also contribute indirectly to OFs through increased fall risk. Accordingly, knowledge of the role of genetics in

fall susceptibility in older people is useful in designing effective fall-prevention programs.

Although *PON1* can affect bone metabolism through its involvement in lipid oxidation, the exact mechanism by which it influences OF risk independently of BMD in postmenopausal women is not entirely clear at present. However, bone fragility depends on factors besides BMD, including the morphology, architecture, remodeling and quality of bone (Garnero *et al.*, 2005). Therefore, *PON1* may be involved in bone metabolic pathways other than those reflected in BMD. Further studies are needed to clarify the associations between these BMD-independent OF risk factors and *PON1* polymorphisms.

Despite the presumptive influence of *PON1* on bone metabolism, we have been able to identify only one study that focused on the relationship between *PON1* polymorphisms and osteoporosis-related phenotypes. In 2003, Yamada *et al.* (2003) reported that two polymorphisms in the coding region-L55M and Q192R-were associated with BMD at the lumbar spine and/or femoral neck after controlling for age in postmenopausal Japanese women. In our study, the L55M polymorphism was not prevalent (MAF = 0.021), mainly due to ethnic differences, and the Q192R polymorphism had no association with BMD at any sites in postmenopausal Korean populations. However, the Japanese study did not adjust for weight, which is

an important determinant of both BMD (Kanis, 1990) and PON1 activity (Bajnok *et al.*, 2008). Moreover, this study did not consider other identified polymorphisms besides L55M and Q192R and did not address the influence of L55M or Q192R on OF; thus, it could not completely evaluate the associations of genetic variations in *PON1* with bone phenotypes.

The *PON1* +5989A>G and +26080T>C polymorphisms are located in the intron and exon 9/3'UTR, respectively, and do not cause amino acid changes, suggesting that these polymorphisms may be markers that lie close to and/or in linkage disequilibrium with other functional genes. However, it has been shown previously that 3'UTRs regulate mRNA stability and thereby influence gene expression (Frittitta *et al.*, 2001; Di Paola *et al.*, 2002). Therefore, the +26080T>C polymorphism, which is located in the 3'UTR of the *PON1* gene, may have a genetic role by regulating *PON1* mRNA stability.

OFs and CVDs are known to be related in postmenopausal women (Sennerby *et al.*, 2007). Because *PON1* can affect lipid metabolism, an important risk factor for CVDs, we additionally adjusted for history of CVDs to exclude the effects of this possible confounding factor. The significance after adjustment persisted in both +26080T>C ($P=0.024$ in the recessive model) and +5989A>G ($P=0.035$ in the dominant model) polymorphisms, suggesting that *PON1* polymorphisms and OF risk are related independently of CVDs.

In almost all of studied related to OFs, vertebral and non-vertebral fractures were analyzed separately, and there might be several reasons for that. Firstly, spine is mainly composed of trabecular architecture, whereas non-spinal skeletal part, such as femur neck, is usually composed of cortical architecture. Secondly, while at least 90% of non-vertebral fractures results from a fall, falls precede only ~25% of vertebral fractures, with many resulting from apparently insignificant everyday activities (Cooper *et al.*, 1992; Youm *et al.*, 1999). Thirdly, subjects with non-vertebral fractures promptly go to hospital due to the pain and disability, when the event occurs. However, those with vertebral fractures sometimes do not know the fact for a long time until undergoing radiographs for any reasons, such as routine health examination. Because of these reasons, our group also analyzed vertebral and non-vertebral fractures separately and together.

There are several potential limitations to this study. Most importantly, the power to draw any definite conclusions was limited by the small

sample size due to difficulty in gathering information and relatively low incidence of OFs. Secondly, although we attempted to consider as many confounding factors as possible, there might be a possibility that the observed association could be attributable to uncontrolled factors. Thirdly, the study population, comprised of women who had visited a university hospital, may not be representative of the general community, thus possibly resulting in selection bias. Fourthly, because non-vertebral fractures were obtained by self-report and not validated by radiographs, the results could be affected by recall bias. However, self-report has previously been demonstrated to be accurate. Specificity is above 80% for self-report of fracture (Nevitt *et al.*, 1992; Ivers *et al.*, 2002; Hundrup *et al.*, 2004), with underreporting rare (Ivers *et al.*, 2002). Fifthly, because we did not measure *PON1* activity, we do not know whether *PON1* activity varied according to the absence or presence of polymorphisms and haplotypes. Therefore, we cannot assert that the genotypes are functionally relevant. Lastly, the genetic component of fracture may ultimately be a combination of polygenic effects and gene-gene and genetic-environmental interactions. Additional multiple analyses with other possible candidate genes would be needed to resolve these more complex relationships.

In summary, we found that *PON1* +5989A>G and +26080T>C polymorphisms were significantly associated with non-vertebral fractures and vertebral fractures, respectively, without influencing BMD in postmenopausal Korean women. Our observations suggest that *PON1* polymorphisms could be a genetic marker with predictive value for OF risk in postmenopausal women.

Methods

Subjects

The study population comprised apparently postmenopausal Korean women ($n=1,329$) who visited the Asan Medical Center (AMC) in Seoul. Menopause was defined as the absence of menstruation for at least one yr, and was confirmed by measurement of serum follicle-stimulating hormone levels. Women displaying premature menopause (< 40 yr of age), and those taking drugs that might affect bone metabolism for more than 6 months or within the previous 12 months, such as glucocorticoid, sex hormone, bisphosphonate or other treatments for osteoporosis, were excluded. Additionally, subjects were excluded if they had suffered from any disease that might affect bone metabolism, such as diabetes, cancer, hyperparathyroidism, or rheumatoid arthritis. Women were also excluded if they had osteophyte formation above the fourth grade of the Nathan classification (Nathan, 1962) and/or severe facet

joint osteoarthritis in the lumbar spine as determined by conventional spine radiographs. The study was approved by the AMC ethics review committee, and written informed consent was obtained from all subjects.

BMD measurement and osteoporotic fracture assessment

Areal BMD (g/cm^2) at the lumbar spine (L2-L4) and femoral neck were measured by dual-energy X-ray absorptiometry in 834 women using Lunar equipment (Lunar, Expert XL, Madison, WI). In the remaining 495 women, BMD was estimated using Hologic equipment (Hologic, QDR 4500-A, Waltham, MA). The precision levels of the Lunar and Hologic equipment, presented as coefficients of variation, were 0.82% and 0.85% for the lumbar spine, and 1.12% and 1.20% for the femoral neck, respectively. These values were obtained by scanning 17 volunteers who were not part of the study. Each volunteer underwent five scans on the same day, getting on and off the table between examinations. Cross-calibration equations between the two systems were derived by measuring BMD values in 109 healthy Korean women (mean age, 55 ± 11 yr, range 31-75 yr) using the two machines, and were calculated as follows (Jo *et al.*, 1999):

L2-L4 BMD (g/cm^2): Lunar = $1.1287 \times$ Hologic - 0.0027

Femoral neck BMD (g/cm^2): Lunar = $1.1556 \times$ Hologic - 0.0182

We examined prevalent morphological vertebral fractures in all study subjects by means of obtaining lateral thoracolumbar (T4-L4) radiographs. The assessment of vertebral fractures was made in accordance with the recommendations of the Working Group on Vertebral Fractures (Kiel, 1995). Radiographs were assessed at AMC by expert radiologists blinded to this study. A vertebral fracture was defined quantitatively as more than a 20% reduction in any measured vertebral height (i.e., anterior, middle, or posterior) (Genant *et al.*, 1993). Non-vertebral fractures (i.e., wrist, hip, forearm, humerus, rib, and pelvis) were assessed using self-reported data. Fractures clearly caused by major trauma, such as motor vehicle accidents or a fall from more than a standing height, were excluded.

Sequencing analysis of the *PON1* gene

We sequenced all exons, including exon-intron boundaries, and the promoter region (~ 1.5 Kb) to detect single nucleotide polymorphisms (SNPs) in twenty-four Korean DNA samples using the ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Twenty-four DNA samples from Korean subjects for initial sequencing were randomly selected from unrelated local residents with no history of familial disease (13 females and 11 males; mean ages, 55.0 ± 8.3 yr; range 41-69 yr). Fifteen primer sets were designed for amplification and sequencing analyses based on GenBank sequences (reference sequence of contig: NT_007933.15). Sequence variants were verified with automated sequencing chromatograms. SNPs were detected by multiple sequence alignment using the Phred/

Phrap/Consed package and Polyphred (Ewing *et al.*, 1998; Gordon *et al.*, 1998).

Genotyping with fluorescence polarization detection

For genotyping of polymorphic sites, amplifying primers and probes were designed for TaqMan (Livak, 1999). Primer Express (Applied Biosystems) was used to design both the polymerase chain reaction (PCR) primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. PCRs were run in the TaqMan Universal Master mix without UNG (Applied Biosystems), with PCR primer concentrations of 900 nM and TaqMan MGB-probe concentrations of 200 nM. Reactions were performed in a 384-well format in a total reaction volume of 5 μl using 20 ng of genomic DNA. The plates then were placed in a thermal cycler (PE 9700, Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The TaqMan assay plates were transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity in each well of the plate was read. Fluorescence data files from each plate were analyzed using automated software (SDS 2.1, Applied Biosystems).

Statistics

The χ^2 test was used to determine whether individual variants were in Hardy-Weinberg equilibrium at each locus in the subjects without OF (Chae *et al.*, 2009). We examined Lewontin's D' ($|D'|$) and the linkage disequilibrium (LD) coefficient r^2 between all pairs of biallelic loci (Hedrick, 1987). Haploview version 3.2 (Whitehead Institute for Biomedical Research, Cambridge, MA) was used to examine the structure of the LD block (Barrett *et al.*, 2005). This program uses two-marker expectation maximization to estimate the maximum-likelihood values of the four gamete frequencies from which the D' and log of odds (LOD) values are derived. Haplotypes (ht) of each individual were inferred using the algorithm developed by Stephens *et al.* (PHASE), which uses a Bayesian approach incorporating a priori expectations of haplotypic structure based on population genetics and coalescent theory (Stephens *et al.*, 2001). Phase probabilities of all polymorphic sites for hts were also calculated for each individual using this software. Individuals with phase probabilities of less than 97% were excluded from the analysis. The genetic effects of inferred hts were analyzed using the same method described for the analysis of polymorphisms. Associations between OF status and each clinical variable were determined using Student t test for continuous variables with a normal distribution and Fisher's exact test for categorical variables. Multiple regression analyses of BMD at the lumbar spine and femoral neck with *PON1* polymorphisms were performed using age, years since menopause (YSM), weight, and height as covariates. The genotype and ht distributions between participants with and without OFs were analyzed with a logistic regression model controlling for age, YSM, weight, and height. History of CVD was additionally adjusted in this model because of the possible effect as a confounding

factor. Genotypes of major homozygotes, heterozygotes, and minor homozygotes were given codes of 0, 1, and 2; 0, 1, and 1; and 0, 0, and 1 in the co-dominant, dominant, and recessive models, respectively. All statistical analyses were conducted using SAS (SAS Institute, Cary, NC).

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