Investigation of the common paraoxonase 1 variants with paraoxonase activity on bone fragility in Turkish patients

Bahar Toptaş · Özlem Kurt · Hülya Yılmaz Aydoğan ·
Ilhan Yaylim · Ümit Zeybek · Ayse Can · Bedia Agachan ·
Mehmet Uyar · M. Kerem Özyavuz · Turgay İsbir

Abstract There is increasing evidence of a biochemical link between oxidative stress and bone metabolism. Oxidative stress has been shown to be involved in bone resorption as it causes loss of bone mineral density (BMD). Paraoxonase 1 (PON1), can prevent these effects of the oxidative stress on bone formation. It has been suggested that the PON1 gene as possibly implicated in reduced BMD in bone fragility cases. It has been hypothesized that PON1 gene polymorphisms may influence both the risk of osteoporosis and osteopenia occurrence and prognosis. The aim of our study is to evaluate the relationship between PON1 polymorphisms and bone fragility development. Seventy-four osteoporotic, 121 osteopenic and 79 nonosteoporotic postmenopausal women were recruited. For detection of the polymorphisms, polymerase chain reaction-restriction fragment length polymorphism techniques have been used. BMD was measured at the lumbar spine and hip by dual-energy X-ray absorptiometry. Distributions of PON1 (PON 192 and PON 55) polymorphisms in study groups were not significantly different. But, there was medium strength connection between in the osteopenic with control groups regarding *PON1* 55–*PON1* 192 haplotypes and we found a power strength connection between in the osteoporosis with control groups regarding *PON1* 55–*PON1* 192 haplotypes. Furthermore, subjects with PON1 192RR and PON1 55LL genotypes had lower PON activity values of osteoporotic subject compared to healthy control and this difference was statistically significant ($p < 0.05$). This result suggest that PON1 genotypes could be higher risk for osteoporosis, as determined by reduced BMD.

Introduction

Osteoporosis is a systemic skeletal disorder defined as a diminished in bone mineral density (BMD) and a deterioration in the micro-architecture of bone, with a consequent susceptibility to fracture and enhance in bone fragility [1–3]. It has been reported that osteoporosis was related with a nearly four times higher fracture rate, while, osteopenia was associated with a 1.8-fold higher rate compared to that of normal BMD in women [4, 5]. It is widely accepted that risk factors for development of osteoporosis and osteopenia have been recognized such as diet, physical activity, increased oxidative stress and diminished antioxidant capacity [2]. Although the underlying causes of some changes in bone fragility in osteoporosis are still unclear, several possible mechanisms are known in bone growth modelling and remodelling [6–8]. Process of bone remodelling related with bone renewed to maintain bone strength

B. Toptaş · H. Y. Aydoğan · I. Yaylim (✉) · Ü. Zeybek ·
B. Agachan
Department of Molecular Medicine, The Institute of
Experimental Medicine (DETAE), İstanbul University, Capa,
34390 Istanbul, Turkey
e-mail: ilhanyaylim@gmail.com

Ö. Kurt · A. Can
Department of Biochemistry, Faculty of Pharmacy, Istanbul
University, Istanbul, Turkey

M. Uyar · M. K. Özyavuz
Physical Therapy and Rehabilitation Clinic, Üsküdar State
Hospital, Istanbul, Turkey

T. İsbir
Department of Medical Biology, Faculty of Medicine, Yeditepe
University, Istanbul, Turkey

and mineral homeostasis. BMD and other determinants of osteoporotic fracture are managed by strong genetic control and this relationship is well established [1, 6, 9, 10]. The oxidative stress is associated with osteoporosis or osteopenia, as it causes loss of BMD. Lipid peroxidation products, which cause oxidative stress, is one of the main factor.

Lipid peroxidation products, can inhibit differentiation of osteoblasts by changing mineral content, decreasing formation, and inhibiting mineralization and therefore this situation may cause osteoporosis [11–14]. Various antioxidant molecules such as PON1, can prevent these effects of oxidative stress on bone formation. PON1, confers antioxidant properties high density lipoprotein (HDL), with which it is associated by reducing the accumulation of lipid peroxidation products. PON1 activity has been suggested to be inversely related to oxidative stress in plasma. Serum PON1 activity is influenced by nutrition, several environmental factors, and PON1 polymorphisms [14, 17].

The human PON1 gene located on the long arm of chromosome 7 (q21.22) [13, 17]. PON1 coding region contains two functional polymorphic variants, which are characterized by the location of glutamine (Q genotype) and arginine (R genotype) at position 192, and leucine (L genotype) and methionine (M genotype) at position 55. Previous studies show modular activity of PON1, hence it has different kinetics and distinct activities in different physiological conditions. Furthermore, polymorphic variation may lead to change the enzyme activity. There are more than 160 defined polymorphisms, both in introns, exons and regulatory regions of the gene. Some studies reveal that some polymorphisms may cause high, intermediate or low paraoxonase activity [14–20]. Paraoxone hydrolysis activity varies among individuals due to the these polymorphisms of the PON1 gene. A major risk factor for osteoporotic or osteopenic fractures is low BMD, which is under strong genetic control. Therefore, this polymorphisms may be a crucial role for PON1 in bone metabolism [13]. The purpose of this study was to determine any relationship between serum PON1 activity and 584A>G(Gln192Arg) and 172T>A(Leu55Met) polymorphisms of the PON1 gene in osteoporosis.

Materials and methods

Patients selection and clinical investigation

PON1 activity, PONI 55/192 gene polymorphism has been studied among 74 osteoporotic, 121 osteopenic and 79 nonosteoporotic women. Patients were selected from Üsküdar State Hospital, Physical Therapy and Rehabilitation Clinic, İstanbul.

Quantification of paraoxonase activity

Paraoxonase activities was measured according to Furlong et al. [20]. The assay buffer contains 0.132 M Tris HCl (pH 8.5), 1.32 mM $CaCl_2$ and 2.63 M NaCl. Addition of 200 μl of 6 mM freshly prepared paraoxon (0,0-diethyl-0-p-nitrophenylphosphate; Sigma, Poole, UK) and 40 μl of serum initiated the assay. The rate of generation of p-nitrophenol was determined at 37 °C, with the use of a continuously recording spectrophotometer at 405 nm. A molar extinction coefficient of 18.05×10^3 was used for calculation using paraoxon as substrate. Pararaoxonase activity is expressed as units/liter (unit: μmol paraoxon hydrolyzed/min).

Isolation of DNA

Blood samples from all subjects were collected into tubes containing EDTA. DNA was prepared from leukocyte pellet by SDS lysis ammonium acetate extraction and ethanol precipitation [21].

Genotyping methods of the paraoxonase 55/192 polymorphism

For the 192 polymorphism, sense primer 5′ TAT TGT TGC TGT GGG ACC TGA G 3′ and antisense primer 5′ CAC GCT AAA CCC AAA TAC ATC TC 3′ which encompass the 192 polymorphic region of the human PON1 gene, were used. For the 55 polymorphism, sense primer 5′ GAA GAG TGA TGT ATA GCC CCA G 3′ and antisense primer 5′ TTT AAT CCA GAG CTA ATG AAA GCC 3′ were used. The PCR reaction mixture contained 100 ng DNA template, 0.5 M of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 1 U *Taq* DNA polymerase (MBI Fermentas). After denaturing the DNA for 5 min at 94 °C, the reaction mixture was subject to 35 cycles of denaturation for 1 min at 95 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C for the 192 genotype. The 99 bp PCR product was digested with 8 U Bspl restriction endonuclease (MBI Fermentas, Lithuania) overnight at 55 °C and the digested products separated by electrophoresis on a 4 % metaphore agarose gel and visualized using ethidium bromide. The R-genotype (arginine) contains a unique Bspl restriction site which results in 66- and 33-bp products and the Q-genotype (glutamine) can not be cut, allowing the 192 genotype to be determined. For the PON1 55 polymorphism, the PCR reaction and the cycling conditions were the same as above. The PCR product (170 bp) was digested with Hsp19211 (Promega, USA) in the presence of BSA (0.1 μg/μl final concentration; 37 °C, overnight) and the digested products were separated and identified as above. Allele L (leucine) did not contain the

Hsp19211 site whereas M (methionine) contained the Hsp19211 site giving rise to 126- and 44-bp products [18, 22, 23].

Statistical analyses

Statistical analyses were performed using the SPSS software package, revision 10.0. Clinical laboratory data are expressed as mean ± SD. Mean values were compared between patients and control subjects by the unpaired Student's t test. Differences in the distribution of PON genotypes or alleles between cases and controls were tested using the Chi square statistic, respectively. PON allele frequencies were estimated by gene counting methods. $p < 0.05$ were considered statistically significant.

Results

Characteristics of patients with osteoporotic and osteopenic cases and healthy controls are shown in Table 1. The patient and control groups had similar distributions for age. The *PON* 192 and *PON* 55 genotypes and allele frequencies for osteoporotic and osteopenic patients and control subjects are shown in Table 2. Frequencies of *PON* 192 QQ, RR and QR genotypes among the patients with osteoporosis were 43.2, 9.5 and 47.3 %, respectively; among the osteopenia, they were 53.7, 10.7 and 35.5 % respectively; among the control, they were 43.0, 13.9 and 43.0 % respectively. The gene frequency for the *PON1* 192 and *PON1* 55 polymorphisms in study groups were not significantly different. The frequencies of *PON* 55LL, MM, and LM genotypes among the patients with osteoporosis were 40.5, 6.8 and 52.7 % respectively; among the

osteopenic subjects, they were 52.9, 5.0 and 42.1 %, respectively: among the control, they were 50.6, 8.9 and 40.5 % respectively. However, we observed a medium strength connection between in the osteopenic with control groups regarding *PON1* 55–*PON1* 192 haplotypes and we found a power strength connection between in the osteoporosis with control groups regarding *PON1* 55–*PON1* 192 haplotypes.

Plasma PON1 activity of osteoporotic and osteopenic patients were significantly lower than in control subjects (32.31 ± 11.78 U/ml, 88.95 ± 44.24 U/ml and 243.30 ± 43.86 U/ml, $p = 0.004$). The association between respectively serum total PON concentration and PON 55/192 polymorphisms are shown in Table 3. In the osteoporotic women with PON1 192RR genotype in serum PON activity

Table 2 PON1 192 and PON1 55 Genotype and allele frequencies

Genotypes and alleles	Osteoporotic subjects	Osteopenic subjects	Control
PON 192 Genotypes			
QQ	43.2 % ($n = 32$)	53.7 % ($n = 65$)	43.0 % ($n = 34$)
RR	9.5 % ($n = 7$)	10.7 % ($n = 13$)	13.9 % ($n = 11$)
QR	47.3 % ($n = 35$)	35.5 % ($n = 43$)	43.0 % ($n = 34$)
PON 192 Alleles			
Q	66.9 ($n = 99$)	71.5 ($n = 173$)	97.2 ($n = 102$)
R	33.1 ($n = 49$)	28.5 ($n = 69$)	50.8 ($n = 56$)
PON 55 Genotypes			
LL	40.5 % ($n = 30$)	52.9 % ($n = 64$)	50.6 % ($n = 40$)
MM	6.8 % ($n = 5$)	5.0 % ($n = 6$)	8.9 % ($n = 7$)
LM	52.7 % ($n = 39$)	42.1 % ($n = 51$)	40.5 % ($n = 32$)
PON 55 Alleles			
M	66.9 % ($n = 99$)	74 % ($n = 179$)	70.9 % ($n = 112$)
L	33.1 % ($n = 49$)	26.03 % ($n = 63$)	29.1 % ($n = 46$)

Table 1 General demographic informations and parameters of patient and control groups (values as average ± standard deviation)

Parameters	Osteoporotic $n = 74$ (mean ± SD)	Osteopenia $n = 121$ (mean ± SD)	Control $n = 79$ (mean ± SD)	p
Age (years)	61.23 ± 7.44	58.41 ± 7.64	58.88 ± 7.20	$p \geq 0.05$
BMI (kg/m²)	27.93 ± 4.18	30.41 ± 4.71	30.49 ± 5.89	$p \geq 0.05$
PON1 act. (U/l)	32.31 ± 11.78	88.95 ± 44.24	243.30 ± 43.86	$p < 0.05$
L2-4 BMD (g/cm²)	0.83 ± 0.10	1.20 ± 0.93	1.20 ± 0.11	$p < 0.05$
Neck BMD (g/cm²)	0.77 ± 0.09	0.84 ± 0.08	0.95 ± 0.09	$p < 0.05$
Wards BMD (g/cm²)	0.58 ± 0.10	0.67 ± 0.09	0.80 ± 0.10	$p < 0.05$
Trochanter BMD (g/cm²)	0.66 ± 0.08	0.84 ± 0.10	0.84 ± 0.10	$p < 0.05$
Total BMD (g/cm²)	0.81 ± 0.09	1.04 ± 0.09	1.04 ± 0.01	$p < 0.05$
Total Z score of BMD (g/cm²)	−0.75 ± 0.72	−0.86 ± 0.75	0.86 ± 0.75	$p < 0.05$
Age of menopause (years)	46.04 ± 5.70	46.46 ± 5.23	46.46 ± 5.23	$p \geq 0.05$
Weight (kg)	65.28 ± 10.45	73.42 ± 12.03	81.33 ± 13.48	$p \geq 0.05$
Height (cm)	1520.05	155.0 ± 0.04	156 ± 0.06	$p \geq 0.05$

Table 3 Changes in PON1 activities in control and patient groups due to genotypes (values as average ± standard deviation)

	PON1 activity (U/ml)			Statistical significance
	Control group	Osteoporotic subjects	Osteopenic subjects	
PON1 192 QQ	242.32 ± 50.47	37.79 ± 16.76	170.12 ± 89.96	$p < 0.05$**
PON1 192 RR	342.09 ± 112.83	7.2 ± 2.2	13.4 ± 3.6	$p < 0.05$**
PON1 192 QR	202.09 ± 85.34	33.5 ± 21.1	13.36 ± 2.61	$p \geq 0.05$
PON1 55 LL	286.67 ± 71.50	11.71 ± 4.01	93.80 ± 61.04	$p < 0.05$
PON1 55 MM	399.08 ± 214.72	6.23*	6.74 ± 3.44	$p \geq 0.05$
PON1 55 LM	164.89 ± 42.00	53.22 ± 22.44	93.36 ± 73.5	$p < 0.05$**

** One of them patient group according to the control group for PON1 genotypes are significant
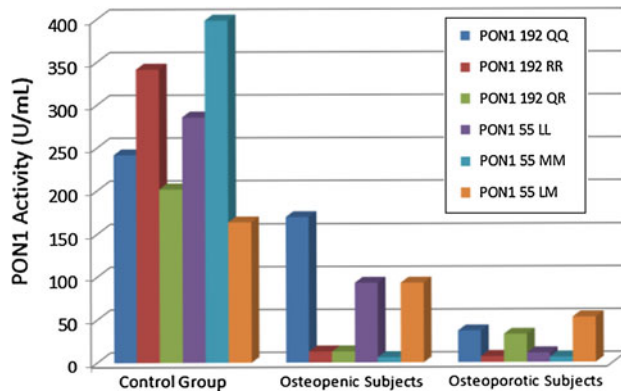

Fig. 1 The distribution of the activity of paraoxonase genotypes

levels were observed between 7.2 ± 2.2 U/ml, while these levels in healthy women with the same genotype were between 342.09 ± 112.83 U/ml and this result were statistically significant ($p < 0.05$). Furthermore, in the osteoporotic women with PON1 55LL genotype, in serum PON activity levels were observed 11.71 ± 4.01 U/ml, while these levels in healthy women with the same genotype were between 286.67 ± 71.50 U/ml and this difference were statistically significant ($p < 0.05$). Plasma PON activity according to genotypes are shown in Fig. 1.

Discussion

In this study, we demonstrated the distribution of *PON1* 192 and *PON1* 55 polymorphisms and paraoxonase activity in Turkish patients with osteoporotic and in healthy control individuals, which has not been studied before.

PON1 activity was measured by using paraoxone substrate [23]. Human PON1 protein is a 43 kDa molecular mass and containing 354 amino acids. PON1 gene is located on the long arm of chromosome 7 between q21.3 and q22 [23]. Previously it was shown that the *PON1* gene has important two single nucleotide polymorphisms (SNPs) which result in amino acid substitutions, that can change its activity. At position 55, leucine (Leu) is replaced by methionine and the other causing GlnArg at the 192nd position. The genotypes 55LL and 192RR demonstrate high PON1 activity, as determined by the ability to hydrolyze paraoxone [15, 16, 19, 20, 25–28]. It has been demonstrated that PON1 192Q genotype is six times more active than PON1 192R genotype and PON1 55 M genotype causes low serum activity and also decreases serum levels than PON1 55L genotype [15, 16, 19, 25, 27, 28]. PON1 55 genotypes are associated with high serum PON1 activity than PON1 192 genotypes [16]. In parallel with these studies, we found similar results.

PON1 activity changes in some other diseases such as oxidative stress, diabetes and cardiovascular diseases [29]. BMD is associated with the morphology, architecture, remodeling and quality of bone. The antioxidant capacity of PON1 may prevent these effects of oxidative stress on bone development. Therefore, PON1 may be relationship in bone metabolism and as a result of osteoporosis [30]. Yamada et al. [2] studied that two polymorphisms in the coding region-L55 M and Q192R-. They found that these polymorphisms were associated with BMD in Japanese elderly women [2]. For PON 55 genotypes, in study groups with the LL genotype had significantly lower BMD than carriers of the MM genotype. For PON 192, participants with the R allele carriers had significantly lower BMD than carriers of the Q allele carriers. As a result of these studies, they implicated that, PON 55 and 192 genotypes associated with lower BMD (55LL and 192RR) are the least effective in protecting LDL against oxidation [2, 31]. This observation suggests a potential mechanism by which PON1 affects risk of osteoporosis [2]. Kim et al. [30] reported that, the L55 M polymorphism was not prevalent, and the Q192R polymorphism had no association with BMD at in postmenopausal Korean populations. This result may be associated with genetic differences. But they suggest that other polymorphisms in the PON1 gene polymorphisms may be associated with osteoporosis in postmenopausal women [30]. However some previous studies have reported that there is no association between PON1 concentrations and osteoporosis [2, 32]. Yamada et al. found that those with the PON 55LL genotype were affected favorably by high serum PON activity in healthy women, with lower

bone resorption in osteoporosis patients. Our result was similar to the these studies [2]. Our findings suggest a role of PON1 genotypes which confer a higher risk for osteoporosis, as determined by reduced BMD. One of the potential limitations of this study is the relatively small sample size. A larger sample size would strengthen the present study findings and provide further verification.

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