Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Relationships between endothelial nitric oxide synthase gene polymorphisms and osteoporosis in postmenopausal women*

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Received May 9, 2009; Revision accepted June 29, 2009; Crosschecked July 9, 2009

Abstract: Objective: To investigate the relationships between endothelial nitric oxide synthases (eNOS) G894T and 27 bp-variable number tandem repeat (VNTR) gene polymorphisms and osteoporosis in the postmenopausal women of Chinese Han nationality. Methods: In the present study, 281 postmenopausal women from Xi'an urban area in West China were recruited, and divided into osteoporosis, osteopenia, and normal groups according to the diagnostic criteria of osteoporosis proposed by World Health Organization (WHO). The bone mineral density (BMD) values of lumbar vertebrae and left hips were determined by QDR-2000 dual energy X-ray absorptiometry. Blood samples were tested for plasma biochemical indicators including testosterone, estradiol, calcitonin, osteocalcin, and procollagen type I amino-terminal propeptide by enzyme-linked immunosorbent assay (ELISA), tartrate-resistant acid phosphatase by spectrophotometric method, and the content of nitric oxide by Griess method. Genome DNA was extracted from whole blood, and G894T polymorphism of eNOS gene was analyzed by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and 27 bp-VNTR polymorphism of eNOS gene was genotyped by PCR method. Then the relationships between genotypes and biochemical indicators, genotypes and osteoporosis, and haplotypes and osteoporosis were analyzed. Results: The average BMD values of the femoral neck, ward's triangle and lumbar vertebrae 1~4 (L1~L4) in the subjects with T/T genotype in eNOS G894T locus were significantly higher than those in the subjects with G/T and G/G genotypes (P<0.05). The average BMD of the femoral neck in the subjects with a/a genotype of eNOS 27 bp-VNTR locus was evidently higher than that in the subjects with b/b genotype (P<0.05). The plasma testosterone and osteocalcin concentrations in the subjects of eNOS G894T G/T genotype were evidently higher than those in the subjects of other genotypes (P<0.05); the plasma estradiol concentration in the subjects of eNOS 27 bp-VNTR a/a genotype was obviously higher than that in the subjects of b/b genotype (P<0.01). eNOS G/G homozygous frequencies in osteoporosis women, osteopenia women, and normal women were 85.37%, 76.38%, and 83.87%, respectively (P>0.05). 0% osteoporosis woman, 0.79% osteopenia women, and 3.23% normal women were eNOS a/a homozygous (P<0.05). The frequencies of eNOS 27 bp-VNTR a allele were 5.33% in the osteoporosis group, 10.24% in the osteopenia group, and 16.13% in the normal group (P<0.05, odds ratio (OR)=0.29, 95% confidence interval (CI)=0.11~0.77), suggesting that a/a genotype and a allele might have protective effects on osteoporosis. The haplotype analysis showed that G-b was 87.7% (214/244) in the osteoporosis group (P<0.05, OR=2.48, 95% CI=1.18~5.18). G-a was 5.3% (13/244) in the osteoporosis group (P<0.05, OR=0.29, 95% CI=0.11~0.77). G-b was a risk factor for osteoporosis, and G-a a protective factor. Conclusion: eNOS G894T G/T genotype influenced the plasma testosterone and osteocalcin concentrations, and T/T genotype influenced BMD. eNOS 27 bp-VNTR a/a genotype increased plasma estradiol concentration to have a protective effect on osteoporosis.

Key words: Postmenopausal women, Osteoporosis, Endothelial nitric oxide synthase, Gene polymorphisms, Bone mineral density
doi:10.1631/jzus.B0920137 **Document code:** A **CLC number:** R68

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* Project supported by the National Natural Science Foundation of China (Nos. 30630058 and 30571725), the Xi'an Municipal Science and Technology Research Project Fund (No. GG06152), and the Shanxi Provincial Science and Technology Research and Development Project Fund (No. 2007K14-01), China

INTRODUCTION

Osteoporosis is a systemic metabolic bone disease characterized by decrease of bone mass and degeneration of bone microstructure resulting in increasing bone fragility prone to fracture (Lewiecki, 2008). Osteoporosis occurs to people with low peak-value of bone mass during their body development or to aged people with abnormal maintenance of bone mass (Walker, 2008). Many factors are involved in the etiology of primary osteoporosis, and it has been widely believed that environmental factors, such as age, nutrition, exercises, and living habits, play important roles in development of osteoporosis (Ilich and Kerstetter, 2000; Prentice, 2004; Manios *et al.*, 2007; Haas and Moore, 2007). Smith *et al*.(1973) first proposed that genetic factors participate in gain of bone mass. Afterward, numerous twin and pedigree studies reported that genetic factors are involved in osteoporosis and the secondary fracture of osteoporosis, and that 80% of bone mass variance in human populations is determined by genetic factors (Ongphiphadhanakul, 2007; Williams and Spector, 2006; Pocock *et al*., 1987). Osteoporosis has been believed as a multigenetic disorder in which many genes influence bone mass gain and regulation of bone turnover. The candidate genes for osteoporosis include genes for vitamin D receptor, estrogen receptor, androgen receptor, calcitonin receptor, type I collagen, and transforming growth factor (TGF)-β1 (Williams and Spector, 2007; Ralston, 2007). Mutations or polymorphisms of these genes may increase susceptibility to osteoporosis. It is still uncertain that which specific gene plays a key role in the development of osteoporosis; therefore, more work needs to be done.

Nitric oxide (NO), a signaling molecule synthesized from L-arginine by nitric oxide synthases (NOS), is an important factor in regulating bone metabolism (van't Hof and Ralston, 2001). The impact of NO on bone metabolism is two-way directional, affecting not only the function of osteoclasts, but also the differentiation and proliferation of osteoblasts (Brennan *et al*., 2003; van't Hof *et al*., 2004). It has been found that NO is related with osteoporosis development in postmenopausal women (Ozgocmen *et al*., 2007; Rosselli *et al*., 1995). During menstrual cycle, blood NO metabolite concentrations correlate with estrogen levels, being higher in the follicular phase than in the secretory phase (Cicinelli *et al*., 1996). NO metabolites decrease in postmenopausal women and are increased by estrogen replacement (Rosselli *et al*., 1995; Wimalawansa, 2008). NO may modulate anabolic effects of estrogen on bone homeostasis by restraining osteoclast-mediated bone resorption and stimulating osteoblast activity. Accordingly, NO donated by organic nitrates, including nitroglycerin, is thought to protect from bone loss associated with estrogen deficiency (Wimalawansa, 2008).

At the present, three NOS isozymes, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), have been found in mammalians (Ricciardolo *et al*., 2006; Sun *et al*., 2005). Studies on NOS gene-deficient mice provided insight on the functional significance and the relative contributions that the different NOS isoforms and NO may exert on skeletal remodeling (Kozak and Kozak, 2003). eNOS is the predominant constitutive isoform of NOS within bone. Histomorphometric and bone mineral density (BMD) analyses of young adult eNOS gene-deficient mice revealed marked abnormalities in bone volume and formation rate and the reduced BMD that are mainly related to dysfunctional osteoblasts (Aguirre *et al*., 2001). Ovariectomized eNOS gene-deficient mice have the bone lose comparable to that observed in wild-type controls but show a blunted anabolic response to high dose of estrogen (Armour *et al*., 2001).

Human eNOS gene located at 7q35-36 includes 26 exons and 25 introns with a total length of 21 kb. There is a 27 bp-variable number tandem repeat (VNTR) polymorphism in the 4th intron of eNOS gene, and based on the number of repeats, 2 kinds of alleles are obtained. The allele with 4 repeats is a allele and the allele with 5 repeats is b allele. It is reported in recent years that 27 bp-VNTR polymorphism in eNOS gene locus is related with many kinds of clinic diseases, such as primary hypertension, type 2 diabetes mellitus, cerebral infarction, coronary heart disease, asthma, and other ischemic cardiovascular and cerebrovascular diseases (Benjafield and Morris, 2000; Hoffmann *et al*., 2005; Matyar *et al*., 2005; Mearin *et al*., 2006; Uthra *et al*., 2007). G894T polymorphism in exon 7 of eNOS gene as a missense mutation, affects eNOS protein and is related with the spasm of coronary arteries, myocardial infarction,

primary hypertension, left ventricular hypertrophy, artherosclerosis, erectile dysfunction, and cerebral infarction (Li *et al*., 2005; Tang *et al*., 2008; Lee *et al*., 2007; Antoniades *et al*., 2007; Reali *et al*., 2008). Recently, an eNOS polymorphism, Glu298Asp, is implicated in osteoporosis. However, it is rarely reported whether the polymorphisms of eNOS 27 bp-VNTR and G894T gene loci are related with bone metabolism and osteoporosis, so it remains very unclear.

In the present study, we examined the polymorphisms of eNOS 27 bp-VNTR and G894T gene loci, the blood contents of sex hormones, and the related biochemical indicators of postmenopausal women from urban areas of Xi'an, China, and analyzed the relationships between the two kinds of polymorphisms of eNOS genes and BMD and biochemical parameters in order to provide help for early diagnosis of the susceptible population and prevention of osteoporosis.

MATERIALS AND METHODS

Subjects

Included in the study were 281 postmenopausal women aged 45 to 65 years, who were Chinese Han nationality. They had lived in Xi'an urban areas more than 10 years, and had been in natural menopause for more than 6 months, with no diseases that might influence bone metabolism, severe chronic diseases that needed long-term therapies, or gynecological diseases that could influence the secretion of female sex hormones, and no hormone drugs intake or osteoporosis treatment 6 months before the investigation. According to a pilot study, the estimated population morbidity rate of osteoporosis was 40% and the relative allowable error was 20% (α=0.05). Therefore, using Epi-Info software, the sample size was 145. But considering the cluster sampling and possible visiting failures, the sample size was amplified to 290 with multistage cluster sampling method (Liu *et al*., 2008). Informed consent was obtained from all the subjects and the investigation was conducted in accordance with humane and ethical research principles from our university.

BMD determination

BMD values in lumbar vertebrae and left hips of all subjects were determined by using QDR-2000 dual energy X-ray absorptiometry (DEXA) (Hologic Company, USA), which was controlled by computers with auto-position fixing, auto-detecting, and auto-data manipulating (Liu *et al*., 2002; Mutlu *et al*., 2007). The relative error of repeated detection was 0.5%. In practical operation, the subject lay down in the middle of the detecting bed, with the distance of 3 cm between her head and the bed top, her both hands on the sides of the body, and her two legs straightening and separating gently. Through the fan-shaped scanning, the BMD values of 8 special parts, such as lumber vertebrae (L1~L4), femoral neck, ward's triangle, greater trochanter and intertrochanter of femur, were calculated.

The diagnostic criteria of osteoporosis proposed by World Health Organization (WHO) in 1994 were used (Imashuku *et al*., 2007; Miller, 2006), in which a loss of bone mass$\leq$1*SD* was considered as normal, 1*SD*<loss of bone mass$\leq$2.5*SD* as osteopenia, and loss of bone mass>2.5*SD* as osteoporosis. Among the determined 8 parts of a subject, if T-score of any part <–2.5*SD*, she was diagnosed as osteoporosis, and for all parts, if T-score$\geq$–1.0*SD*, she was normal, and the rest should be diagnosed as osteopenia.

Biochemical indicators detection

10 ml superficial vein blood was collected from the elbow of the fasting women, added with heparin anticoagulant, and centrifuged at 3000 r/min for 10 min, and then the plasma was isolated and stored at –70 °C for use. Seven biochemical indicators in the plasma samples were tested for testosterone (T), estradiol (E_2), calcitonin (CT), osteocalcin (OC), and procollagen type I amino-terminal propeptide (PINP) by enzyme-linked immunosorbent assay (ELISA), tartrate-resistant acid phosphatase (TRAP) by spectrophotometric method, and the content of NO by Griess method (Liu *et al*., 2009). The ELISA kits of testosterone, estradiol, and osteocalcin were purchased from Cayman Chemical Company (USA), and the kits of calcitonin and PINP from Shanghai Xitang Bio-Tech Company (China). The TRAP assay kit was purchased from Nanjing Jiancheng Bio-Tech Company (China).

Genomic DNA extraction

0.2 ml anticoagulant blood was used to extract genomic DNA following the instruction in the TIANamp Blood genomic DNA extraction kit (Qiagen Company, USA). 50~200 ng/μl DNA (optical density ratio of 1.6~1.9 at 260/280 nm) was obtained.

Detection of gene polymorphisms

The eNOS gene is composed of 26 exons on chromosome 7q35-36 (Via *et al*., 2003), and there is a 27 bp-VNTR polymorphism in the 4th intron (Smith, *et al*., 2006). Polymerase chain reaction (PCR) method was used to detect eNOS 27 bp-VNTR gene polymorphism. The forward primer was 5′-AGGCCCTATGGTAGTGCCTTT-3′ and the reverse primer 5′-TCTCTTAGTGCTGTGGTCAC-3′ (Beijing Aoke Company, China). A 25-μl reaction contained 50~200 ng of genomic DNA, 10 μl of 2.5× Taq buffer, 0.4 μmol/L of each primer, 0.1 U of Taq DNA polymerase (Qagen Company, USA), and 100 μmol/L of each dNTP. PCR mixture was incubated at 95 °C for 5 min, and then run at 95 °C for 1 min, 63 °C for 30 s, and 72 °C for 30 s for 31 cycles, and a final extension of 72 °C for 10 min. The extended products were electrophoresed on 3% (w/v) agarose gels and visualized after ethidium bromide staining under ultraviolet transillumination.

Because of the G on the 894th position of the 7th exon of the eNOS gene mutated to T (G894T), the Asp replaces Glu on the 298th position of the protein. The genomic DNA was amplified using PCR and digested by restriction enzyme. Then PCR-restriction fragment length polymorphism (RFLP) method was used to detect eNOS G894T gene polymorphism. This region was amplified by PCR using the forward primer 5′-AAGGCACAGGAGACAGTGGATGGA-3′ and the reverse primer 5′-CCCAGTCAATCCC TTTGGTGCT-3′ (Beijing Aoke Company, China). A 25-μl PCR mixture contained 50~200 ng of genomic DNA, 10 μl of 2.5× Taq buffer, 100 μmol/L of each dNTP, 0.4 μmol/L of each primer, and 0.1 U of Taq DNA polymerase. After the first 95 °C for 5 min, the PCR reaction was run at 95 °C for 1 min, 59 °C for 30 s, and 72 °C for 40 s for 30 cycles, and a final extension of 72 °C for 10 min. 12 μl of PCR product was mixed with 5 U of restriction enzyme *Ban*II, 2 μl of 10× restriction enzyme digestion buffer, and 5μl of deionized water, and incubated at 37 °C for 3 h. The digested PCR products were electrophoresed on 2.5% (w/v) agarose gels and visualized after ethidium bromide staining under ultraviolet transillumination.

Finally, we randomly selected 10% of the samples to repeat the detection of eNOS G894T and eNOS 27 bp-VNTR gene polymorphisms, and the results were fully confirmed.

Statistical analysis

For the case-control analysis, chi-squared (χ^2) tests were performed to compare genotypes and allele frequencies using the 2002 version of Epi_Info (http://www.cdc.gov/epiinfo/). Values for D', the normalised linkage disequilibrium statistic, were calculated by 2LD software. Haplotype frequencies were estimated using the program PHASE version 2.2, a software that implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (Ma *et al*., 2006). The distribution of global haplotype frequencies in cases and controls was compared by the Epi_Info program.

RESULTS

Basic information of subjects

Complete BMD values were obtained from 281 subjects, among whom 280 had gene polymorphism results and 244 had plasma biochemical indicator results. According to the diagnostic criteria of osteoporosis proposed by WHO in 1994 (Imashuku *et al*., 2007; Miller, 2006), the 281 subjects were divided into normal group, osteopenia group, and osteoporosis group. The incidence of osteoporosis was found to be 43.77% (123/281), the osteopenia rate 45.20% (127/281), and the normal rate 11.03% (31/281). The BMD values in different body positions are listed in Table 1.

Gene polymorphism detections

The PCR products of eNOS intron 4 27 bp-VNTR polymorphism included a/a genotype with a 393-bp band, b/b genotype with a 420-bp band, and a/b heterozygous genotype with 393-bp and 420-bp bands (Fig.1a).

The PCR product of G894T polymorphism detection was 248 bp long. As *Ban*II restriction enzyme recognized G, but not T, the digested product of T/T

Table 1 BMD in different positions of subjects (g/cm^2)

Group	*n*	NBMD	TBMD	WBMD	LBMD
Normal	31	0.882±0.072	0.746±0.072	0.906±0.205	1.126±0.074
Osteopenia	127	0.756±0.068	0.629±0.062	0.705±0.134	0.951±0.095
Osteoporosis	123	0.633±0.073*	0.530±0.061*	0.548±0.094*	0.794±0.105*

NBMD: BMD of femur neck; TBMD: BMD of greater trochanter of femur; WBMD: BMD of ward's triangle of femur; LBMD: BMD of lumber vertebrae (L1~L4). * P<0.05, significantly different compared with each two groups

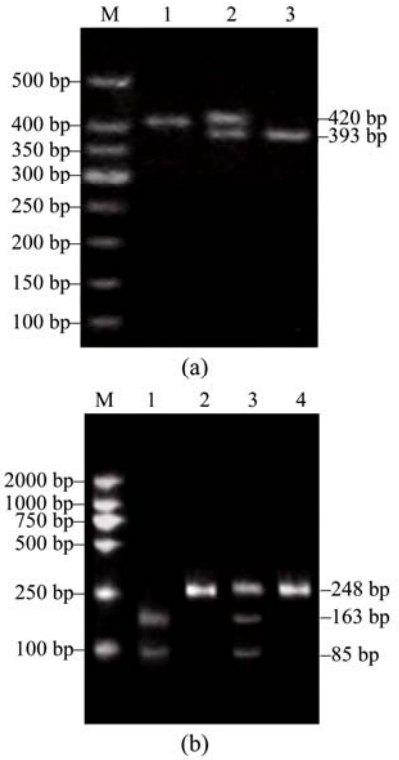

Fig.1 Results of (a) PCR amplified fragments of eNOS 27 bp-VNTR polymorphism and (b) enzyme digested products of eNOS G894T polymorphism

(a) PCR results of the eNOS intron 4 27 bp-VNTR polymorphism. Lane M: 50 bp DNA Ladder Marker; Lane 1: b/b homozygous genotype; Lane 2: a/b heterozygous genotype; Lane 3: a/a homozygous genotype; (b) Enzyme digested results of the eNOS G894T polymorphism. Lane M: D2000 DNA Ladder Marker; Lane 1: G/G homozygous genotype; Lanes 2 and 4: T/T homozygous genotype; Lane 3: G/T heterozygous genotype

homozygous genotype was a 248-bp band. The G/G homozygous genotype had two bands, 163 bp and 85 bp, and the G/T heterozygous genotype had three bands, 248, 163, and 85 bp (Fig.1b).

Relationship between gene polymorphism and BMD

The average BMD of the femur neck in T/T genotype women was (0.817±0.143) g/cm^2, significantly higher than that in G/G ((0.714±0.109) g/cm^2) genotype women and that in G/T ((0.717±0.099) g/cm^2) genotype women (P<0.05). The BMD of ward's triangle in T/T genotype women was (0.789±0.200) g/cm^2, significantly higher than that in G/G ((0.648±0.155) g/cm^2) genotype women (P<0.05). The average BMD of L1~L4 in T/T genotype women was (1.067±0.183) g/cm^2, significantly higher than that in G/G ((0.899±0.145) g/cm^2) genotype women and that in G/T ((0.904±0.136) g/cm^2) genotype women (P<0.05). The average BMD of the femur neck in a/a genotype was (0.867±0.087) g/cm^2, significantly higher than that in b/b ((0.714±0.109) g/cm^2) genotype women (P<0.05). The BMD of some parts in T/T genotype women and a/a genotype women increased (Table 2).

Relationship between gene polymorphisms and biochemical indicators

The plasma testosterone concentration of G/T genotype was (38.2±4.4) ng/dl, significantly higher than that of G/G genotype ((36.1±5.8) ng/dl) (P<0.05). The plasma osteocalcin of G/T genotype is (6.2±2.0) ng/ml, significantly higher than that of G/G genotype ((5.1±1.9) ng/ml) and that of TT genotype ((4.2±1.9) ng/ml) (P<0.05). The plasma estradiol concentration of a/a genotype was (101.0±18.4) pg/ml, significantly higher than that of b/b genotype ((70.7±18.6) pg/ml) (P<0.01). Therefore, the plasma testosterone and osteocalcin concentrations of G/T genotype significantly increased; the plasma estradiol concentrations of a/a genotype significantly increased (Table 3).

Relationship between genotypes and allelic frequencies and osteoporosis

The results showed that eNOS G/G homozygotes accounted for 86.06% (105/122) in osteoporosis patients, 76.38% (97/127) in osteopenia women, and 83.87% (26/31) in normal group (P>0.05). The eNOS a/a homozygous was zero in the osteoporosis group,

0.79% (1/127) in the osteopenia group, and 3.23% (1/31) in normal group (P<0.05). In the osteoporosis group a allele accounted for 5.33% (13/244), in the osteopenia group 10.24% (26/254), and in the normal group 16.13% (10/62) (P<0.05, odds ratio (OR)= 0.29, 95% confidence interval (CI)=0.11~0.77). The a/a genotype and the a allele had a protective effect on osteoporosis (Tables 4 and 5).

Relationship between haplotype and osteoporosis

The haplotype analysis showed that G-b accounted for 87.7% (214/244) in the osteoporosis group (P<0.05, OR=2.48, 95% CI=1.18~5.18). G-a accounted for 5.3% (13/244) in the osteoporosis group (P<0.05, OR=0.29, 95% CI=0.11~0.77). The results show that G-b was an osteoporosis risk factor, and G-a a protective factor for osteoporosis (Table 6).

Table 2 Relationship between eNOS G894T and 27 bp-VNTR gene polymorphisms and BMD (g/cm^2)

Genotype	NBMD		TBMD		WBMD		LBMD	
	n	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*
G894T								
G/G	228	0.714±0.109	228	0.596±0.093	228	0.648±0.155	226	0.899±0.145
G/T	46	0.717±0.099	46	0.603±0.096	46	0.693±0.230	45	0.904±0.136
T/T	6	0.817±0.143*	6	0.671±0.110	6	0.789±0.200**	5	1.067±0.183*
27 bp-VNTR								
a/a	2	0.867±0.087***	2	0.722±0.118	2	0.764±0.064	2	1.050±0.168
a/b	45	0.724±0.105	45	0.606±0.098	45	0.666±0.135	45	0.910±0.132
b/b	233	0.714±0.109	233	0.597±0.093	233	0.656±0.179	229	0.900±0.148
Total	280	0.716±0.109	280	0.599±0.094	280	0.659±0.172	276	0.903±0.146

n: number of samples. NBMD: BMD of femur neck; TBMD: BMD of greater trochanter of femur; WBMD: BMD of ward's triangle of femur; LBMD: BMD of lumber vertebrae (L1~L4). $^{*}P$<0.05, significantly different compared with G/G, G/T; $^{**}P$<0.05, significantly different compared with G/G; $^{***}P$<0.05, significantly different compared with b/b

Table 3 Relationship between eNOS G894T and 27 bp-VNTR gene polymorphisms and biochemical indicators

	T (ng/dl)		OC (ng/ml)		TRAP (U/L)		E_2 (pg/ml)		PINP (ng/ml)		CT (pg/ml)		NO (nmol/L)	
	n	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*	*n*	Mean±*SD*
G894T														
G/G	195	36.1±5.8	195	5.1±1.9	191	5.95±2.89	182	71.6±18.2	188	23.9±19.1	186	37.6±26.3	190	107.3±52.6
G/T	44	38.2±4.4*	44	6.2±2.0**	44	6.39±2.73	40	71.4±21.0	41	20.2±16.5	44	56.8±37.0	42	117.3±52.3
T/T	5	36.8±8.5	5	4.2±1.9	5	7.15±4.03	5	70.0±16.3	5	22.2±13.6	5	21.7±14.9	5	117.6±60.1
27 bp-VNTR														
a/a	2	36.7±1.29	2	3.3±0.7	2	4.84±1.44	2	101.0±18.4***	2	–	2	–	2	52.5±14.8
a/b	40	36.0±6.2	40	4.9±1.7	39	5.48±3.18	37	74.7±17.5	39	30.4±31.3	39	36.1±26.4	38	108.5±51.0
b/b	202	36.6±5.6	202	5.3±2.0	199	6.18±2.83	188	70.7±18.6	193	22.0±19.0	194	42.3±36.4	197	110.0±53.0

n: number of samples; $^{*}P$<0.05, significantly different compared with G/G; $^{**}P$<0.05, significantly different compared with G/G, T/T; $^{***}P$<0.05, significantly different compared with b/b

Table 4 Relationship between genotypes of eNOS G894T and osteoporosis

Group	*n*	Genotype[a]			χ^2 (P value)[b]	Allele[a]		χ^2 (P value)[b]	OR (95% *CI*)
		G/G	G/T	T/T		G	T		
Osteoporosis	122	105 (86.06%)	17 (13.82%)	0 (0%)	3.97 (0.138)	227 (93.03%)	17 (6.97%)	0.52 (0.470)	1.43 (0.48~4.09)
Osteopenia	127	97 (76.38%)	25 (19.69%)	5 (3.94%)	0.84 (0.658)	219 (86.22%)	35 (13.78%)	0.74 (0.389)	0.67 (0.24~1.77)
Normal	31	26 (83.87%)	4 (12.9%)	1 (3.23%)		56 (90.32%)	6 (9.68%)		

n: number of samples. [a]Percentage for each single nucleotide polymorphism (SNP) is given in parentheses; [b]Compared with the normal group. D'=1.0

Table 5 Relationship between genotypes of eNOS 27 bp-VNTR and osteoporosis

Group	*n*	Genotype[a]			χ^2 (*P* value)[b]	Allele[a]		χ^2 (*P* value)[b]	*OR* (95% *CI*)
		a/a	a/b	b/b		a	b		
Osteoporosis	122	0 (0%)	13 (10.66%)	109 (89.34%)	9.04 (0.01)	13 (5.33%)	231 (94.67%)	8.30 (0.004)	0.29 (0.11~0.77)
Osteopenia	127	1 (0.79%)	24 (18.9%)	102 (80.31%)	2.04 (0.362)	26 (10.24%)	228 (89.76%)	1.71 (0.190)	0.59 (0.25~1.41)
Normal	31	1 (3.23%)	8 (25.81%)	22 (70.97%)		10 (16.13%)	52 (83.87%)		

n: number of samples. [a]Percentage for each single nucleotide polymorphism (SNP) is given in parentheses; [b]Compared with the normal group. *D'*=1.0

Table 6 Relationship between haplotypes of eNOS G894T and 27 bp-VNTR and osteoporosis

Haplotype	Osteoporosis[a]	Osteopenia[a]	Normal[a]	Osteoporosis-normal		Osteopenia-normal	
				χ^2 (*P* value)	*OR* (95% *CI*)	χ^2 (*P* value)	*OR* (95% *CI*)
G-b	214 (87.7%)	195 (76.8%)	46 (74.2%)	7.07 (0.008)	2.48 (1.18~5.18)	0.18 (0.749)	1.15 (0.58~2.27)
T-b	17 (7.0%)	33 (13.0%)	6 (9.7%)	0.52 (0.470)	0.70 (0.24~2.09)	0.51 (0.477)	1.39 (0.52~3.91)
G-a	13 (5.3%)	24 (9.4%)	10 (16.1%)	8.30 (0.004)	0.29 (0.11~0.77)	2.32 (0.128)	0.54 (0.23~1.30)
T-a	0 (0%)	2 (0.8%)	0 (0%)			0.49 (0.483)	Undefined
Globe				9.22 (0.027)		3.04 (0.385)	

[a] Percentage for each haplotype is given in parentheses

DISCUSSION

Human gene polymorphisms are derived from the different copying numbers of repeat sequences in genome, and also come from the variation of a single nucleotide. They are usually divided into three types: restriction fragment length polymorphism (RFLP), the polymorphisms of DNA repeat sequences, and single nucleotide polymorphism (SNP) (Zhang *et al*., 2005; Blair *et al*., 2003; Malone *et al*., 2008). In recent years, the research of the relationship between the gene polymorphisms and the susceptibility to diseases has drawn more and more attentions, providing meaningful references for clinical diagnosis, treatment, and prognosis (Shah, 2007; Yang *et al*., 2008). It has been reported that polymorphisms are associated with osteoporosis (Williams and Spector, 2006; 2007; Pocock *et al*., 1987; Ralston, 2007). Strong associations between the risk of osteoporosis and specific polymorphisms of the VDR, ESR1 and COL1A1 genes have been reported (Williams and Spector, 2006; 2007; Pocock *et al*., 1987; Ralston, 2007). However, the relationship between the polymorphism of eNOS gene and osteoporosis has just recently been studied, remaining unclear (Taylor *et al*., 2006; Cho *et al*., 2008; Firat *et al*., 2009).

NO is an important factor in regulating bone metabolism. A large number of studies have shown a close relation between the bone metabolism and the NO level (van't Hof and Ralston, 2001; Brennan *et al*., 2003; van't Hof *et al*., 2004). Low NO concentration is necessary for the maintenance of the normal function of osteoblast and osteoclast. A slightly elevated NO concentration often shows an inhibition for osteoclast, while for the osteoblast it promotes a formation. High NO concentrations inhibit the formation and differentiation of osteoblast and osteoclast (Brennan *et al*., 2003; van't Hof *et al*., 2004; Wimalawansa, 2008). The effect of NO-mediated estrogen on the bone tissue has been extensively studied and confirmed (Wimalawansa, 2008).

The eNOS isoforms seem to play a key role in regulating osteoblast activity and bone formation since eNOS knockout mice have osteoporosis due to defective bone formation (Aguirre *et al*., 2001; Armour *et al*., 2001). Studies have indicated that the NO derived from the eNOS pathway acts as a mediator of the oestrogen effects on bone (van't Hof and Ralston, 2001). eNOS also mediates the effects of mechanical loading on the skeleton where it acts along with prostaglandins to promote bone formation and suppress bone resorption (van't Hof and Ralston, 2001). The previous studies found that eNOS gene polymorphisms can change the activity of eNOS protein, thereby changing the NO concentration in tissues, and NO can impact the trabecular bone volume and structure by influencing the function of osteoclast and osteoblast (van't Hof and Ralston, 2001; Grassi *et al*., 2006). Few studies on the relationships between polymorphisms of eNOS gene and osteoporosis have been reported (Taylor *et al*., 2006; Cho *et al*., 2008; Firat *et al*., 2009). The study by Taylor *et al*.(2006)

failed to demonstrate an association between the eNOS polymorphism, Glu298Asp, and osteoporotic fracture and BMD, while they found a relatively weak association between the eNOS genotypes and hip fracture. Also, Cho *et al*.(2008) investigated 18 polymorphisms including Glu298Asp and found that the Glu298Asp polymorphism was not associated with BMD. Firat *et al*.(2009) suggested that eNOS gene polymorphisms, T-786C and Glu298Asp, were not major contributors to adult BMD in the postmenopausal Turkish women.

In the present study, through literature reviews we chose two polymorphism loci of the closest relationship between the eNOS and other diseases as the detection sites, which were located at the eNOS gene exon 7 G894T mutation and intron 4 27 bp-VNTR loci (Aguirre *et al*., 2001; Cho *et al*., 2008). We performed clinical testing to examine the associated biochemical markers. The results show that the two eNOS gene polymorphisms affected the BMD of various body parts, and that the average BMD of T/T genotype from the femur neck, ward's triangle and lumbar vertebrae increased significantly, and the average BMD of a/a genotype of the femoral neck was significantly higher than that of b/b genotype. But there was no correlation between the gene polymorphism and plasma NO. This suggests that the concentration of plasma NO did not fully reflect the local bone tissue. Activities of iNOS and cNOS also influence blood NO concentration (van't Hof and Ralston, 2001).

Our study indicates that the women with genotypes of eNOS G894T G/T have significantly higher plasma concentrations of testosterone and osteocalcin. It is generally believed that androgen may be related to the osteoblast differentiation (Alexandre, 2005). Testosterone is the most active androgen in women, and with the decrease of testosterone levels, the direct effect of androgen on maintaining bone quality will be lowered (Tok *et al*., 2004). Almost all circulating osteocalcin is produced by osteoblasts; therefore, the concentration of blood osteocalcin may specifically reflect the activity of osteoblasts (Lee *et al*., 2000). The increases of testosterone and osteocalcin should reflect the activation of osteoblasts, enhancement of bone turnover, and increase of BMD. However, we observed that the BMD in women with G/T genotype was significantly lower than that in those with T/T genotype. The relationship between G894T polymorphism and osteoporosis may be complicated and influenced by other uncertain factors.

Our results also show that the blood concentration of estradiol increased significantly in women with eNOS 27 bp-VNTR a/a genotype. The ovary function of postmenopausal women declines gradually with the decrease of estrogen level. However, the deficiency of estrogen, as a very important risk factor, leads to increasing bone absorption and affects the formation of extracellular matrix and the deposition of calcium salt (Gambacciani and Vacca, 2004; Riggs *et al*., 2003). The women with a/a genotype increased blood estradiol and had significant enhancement of local BMD. In addition, the frequency of a allele in normal women was significantly higher than that in osteopenia and osteoporosis women, suggesting that a/a genotype or a allele of eNOS 27 bp-VNTR locus may protect the women from osteoporosis. The protective effects may result from influencing estrogen level or interaction between estrogen and NO. The haplotype analysis in our study showed that G-b was an osteoporosis risk factor, and G-a a protective factor.

Taken together, the present study shows that eNOS G894T G/T genotype influences the plasma testosterone and osteocalcin concentrations, and T/T genotype influences BMD. The a/a genotype and a allele of eNOS 27 bp-VNTR may have protective effects on osteoporosis through influencing estrogen level and interaction of estrogen with NO. The findings provide a useful reference for early clinical intervention and prevention of osteoporosis.

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