Int J Clin Exp Pathol 2015;8(11):15270-15274
www.ijcep.com /ISSN:1936-2625/IJCEP0015401

Original Article

Promoter polymorphism T-786C, 894G→T at exon 7 of endothelial nitric oxide synthase gene are associated with risk of osteoporosis in Sichuan region male residents

Zuchao Gu[1*], Yu Zhang[1,2*], Guixing Qiu[2]

[1]Department of Orthopedics, The First People's Hospital of Chengdu, Sichuan Province, P. R. China; [2]Department of Orthopedics, Peking Union Medical College Hospital, Beijing, 10005, P. R. China. [*]Equal contributors.

Received August 31, 2015; Accepted October 19, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Objective: To investigate the association between genetic polymorphism of T-786C in promoter region, 894G→T at exon 7 of endothelial nitric oxide synthase (eNOS) gene and osteoporosis (OP) disease. Method: The genotypes of 350 patients with osteoporosis and 350 healthy controls were detected by polymerase chain reaction (PCR) and DNA sequencing. The allele ratios and genotype distributions in the patients and controls were assessed using the Pearson χ^2-test. Odds ratios (OR) with two tailed *P*-values and 95% confidence intervals (CI) were calculated as a measure of the association of the eNOS genotypes with OP. Result: the C allele distribution frequency of T-786C eNOS gene in OP group (8.5%) was significantly higher than that in control group (3.9%), relative risk (OR) of OP associated with the CC genotype was 2.68 (95% CI, 0.92 to 1.37). The T allele frequency of 894G→T at exon 7 in eNOS gene in OP group (11.5%) was also significantly higher than that in control group (5.2%), OR of OP associated with the TT genotype was 2.60 (all $P<0.05$). Conclusion: The analysis results indicated that both T-786C in promoter region and 894G→T at exon 7 of eNOS gene might be genetic predisposal factors of OP, these polymorphisms may be independently or synergic with other loci to have an impact on the incidence of OP.

Keywords: T-786C, 894G→T, endothelial nitric oxide synthase gene, polymorphism, osteoporosis

Introduction

In recent years, considerable concern has been expressed about the osteoporosis [1]. Osteoporosis is the disease which characterized by the loss of bone mass and strength that induced the increasing of bone fragility and a series clinical problems [2, 3].

Nitric oxide (NO) is a pleiotropic signaling molecule with diverse effects on numerous physiologic and pathophysiologic processes including neurotransmission, vasodilatation, immune responses and bone cell function [4-6]. In bone cells, several factors including mechanical stress, estrogen have been found to regulate NO production by stimulating different isoforms of nitric oxide synthase (NOS). While mechanical stress and estrogen increase NO production by activation of endothelial NOS (eNOS) [7-9].

Materials and methods

Study subjects

A total of 700 subjects were studied. These consisted of 350 male OP patients (mean age 62.5 years, range 47-80 years) and 350 healthy male for control group. All patient samples were collected from Chengdu first people's hospital, were unrelated long-term middle-aged residents in Sichuan region of China. Excluded patients with various diseases which will affecting bone metabolism and eliminated the patients who took the drugs can affected bone metabolism in nearly 3 months. The inclusion criteria is following: Normal subject refers to bone mineral density (BMD) or bone mineral content (BMC) above 1 standard deviation (SD) than average of young adults; -2.5SD to -1SD for osteopenia; lower than -2.5SD is considered as osteoporo-

Table 1. The characteristics between patients with IVDD and healthy controls

Characteristics	Patients with LDD	Healthy controls	*P* value
Age (years)	55.12 ± 6.65	54.84 ± 6.35	0.504
BMI (means ± SD)	23.1 ± 2.5	23.3 ± 2.4	0.612

sis; and accompanied by one or more fracture sites for severe osteoporosis. All patients underwent ultrasound bone density measurement instrument to diagnose. Patients with osteopenia were not included in the present study, only lower than -2.5SD was considered as OP patients.

Genotyping

From each blood sample, a leukocyte cell pellet was obtained by centrifugation of 1 ml of whole blood and then used for genomic DNA isolation according to the previously described [10]. The extracted DNA was stored at -20°C until analysis.

The PCR was performed in a final reaction volume of 25 μl containing 100 ng of genomic DNA, 10 pmol of each primer, 5 U Taq Polymerase, 1.5 mmol/L $MgCl_2$, and 2.5 μl of 10x PCR buffer. After initial denaturation at 94°C for 4 minutes, the PCR products underwent 35 cycles at 94°C for 30 sec for denaturation, 65°C for 30 sec for annealing, and 72°C for 1 min for extension. There action was completed by a final extension of 5 min at 72°C. After affinity membrane purification using the QIAquick Gel Extraction kit (Qiagen, Carlsbad, CA, USA), the PCR products were subjected to cycle sequencing with the respective forward and reverse primer using an automated ABI 3100 DNA sequencer by GeneCore Bio Technologies (Shanghai China). A 15% blind, random sample of study subjects was genotyped twice by different persons and the reproducibility was 100%.

Statistical analysis

All the data are expressed as mean ± standard deviation (SD). The clinical and demographic characteristics among all groups were compared by the Student's unpaired *t*-test. Differences in genotype prevalences from that expected for Hardy-Weinberg equilibrium were checked using the χ^2-test. The allele ratios and genotype distributions in the patients and controls were assessed using the Pearson χ^2-test. Odds ratios (OR) with two tailed *P*-values and 95% confidence intervals (CI) were calculated as a measure of the association of the eNOS genotypes with OP.

All statistical analyses were carried-out using SPSS software package (SPSS Inc., Chicago, IL), version 19.0 for Windows and P<0.05 was considered statistically significant.

Results

Comparison between baseline parameters

No significant differences in age and BMI were found between OP patients and normal controls. The characteristics of the two groups are presented in **Table 1**.

The association between T-786C, 894G→T at exon 7 polymorphisms of the eNOS gene and the risk of OP

The polymorphism distribution ratios of T-786C and 894G→T gene were demonstrated in **Table 2**. The genotype distribution of the two polymorphisms in controls was in with the Hardy-Weinberg equilibrium (*P*=0.056 for T-786C, and *P*=0.062 for 894G→T). The frequency distributions of eNOS T-786C genotypes were 79.7% for TT, 18.6 for TC and 1.7% for CC in OP group while the control group genotypes were 92% for TT, 8% for TC and 0 for CC. The frequency of C heterozygous and homozygous for T was significantly higher in OP group when compared to controls (10.4% vs. 4.0%; *P*<0.05). CC and CT genotype had significantly higher relative risk compared to TT type, and C allele had significantly higher risk than T allele (**Table 2**).

894G→T polymorphism The frequency distributions of the eNOS G894T genotypes in OP group were 2.57% for TT, 19.1% for GT, and 78.3% for GG, while the control group genotypes were 0.3% for TT, 9.1% for GT, and 90.6% for GG. The frequency of the 894T allele increased in the OP patient group compared to the control group (12.1% vs. 4.9%; *P*<0.001). T allele at 894G→T site significantly increased the relative risk of OP (**Table 2**, *P*<0.001). All samples' genotype are comply with Hardy-Weinberg equilibrium which indicated that the genic polymorphism of T-786C in promoter region, 894G→T at exon 7 of endothelial nitricoxide synthase (eNOS) gene distributed in the survey population genetic equilibrium has been reached.

Table 2. The genotype and allele distributions of polymorphisms in the OP and control groups

		OP group		Control group				
Location	Genotype	n	%	n	%	P^*	OR (95% CI)	$P^\#$
T-786C	TT	279	79.7	322	92.0	<0.001	1	
	CT	65	18.6	28	8.0		2.679 (1.673-4.292)	0.001
	CC	6	1.7	0	0		2.154 (1.977-2.347)	0.01
	T	623	89.6	672	96.0	<0.001	1	
	C	77	10.4	28	4.0		2.970 (1.902-4.630)	<0.001
894G→T	GG	274	78.3	317	90.6	0.001	1	
	GT	67	19.1	32	9.1		2.422 (1.543-3.803)	0.001
	TT	9	2.57	1	0.3		10.412 (1.311-82.706)	0.006
	G	615	87.9	666	95.1	0.001	1	
	T	85	12.1	34	4.9		2.780 (1.843-4.195)	0.001

Note: $^*P$ value was calculated by Chi test among all the different genotypes; $^\#P$ value was calculated by unconditional logistic regression.

Discussions

Osteoporosis is an important cause of morbidity and mortality among men [11]. Approximately 40% of all osteoporotic fractures worldwide occur in men older than 50 years [12]. The bone metabolism abnormality is the pathological basis of OP occurrence. The abnormal of bone metabolism is occurred by the imbalance between osteoclast and osteoblast activities, the bone mass loss during the bone remodeling process and the bone structural damage resulted in OP [13]. The occurrence of OP associated with the environment, genetic and other factors [14]. Currently, the molecular basis for genetic susceptibility is still unclear, NO as a regulatory factor in the bone formation process which may have an effect on the prevention and treatment of OP has attracted widespread attention [15]. According to Pennisi and Simoncini' study [16, 17], OP were significantly alleviated in menopausal women who taking the cardiovascular drugs that can release eNOS, compared to the patients without taking such medicine. However, the association between eNOS gene polymorphism with OP is still unclear.

Miyamoto has reported that a mutation (-786-T→C) in the promoter region of the eNOS gene reduced transcription of gene [18]. The molecular mechanism for the reduced eNOS gene transcription, the key factor is replication protein A1 (RPA1), known as a single-stranded DNA binding protein essential for DNA repair, replication and recombination. RPA1 can specifically bind to the mutant allele. The functional importance of the diminished eNOS expression was revealed by the finding that serum nitrite/nitrate levels among individuals carrying the -786T→C mutation were significantly lower than among those without the mutation. RPA1 thus apparently functions as a repressor protein in the -786T→C mutation-related reduction of eNOS gene transcription associated with the development of OP.

Asakimori et al [19] reported for the -786T→C, genotype CC, TC and TT among Japanese population are respectively 0%, 8.6% and 91.4%, while in Americans the ratio is 2.0%, 21.7% and 76.3% respectively. In this study, among 350 cases of healthy people, we found that T allele frequency was 96%, C allele frequency was 4.0%. The genotype of CC, TC and TT frequencies were 0%, 8.0% and 92% respectively. T allele was similar with the Japanese but significantly lower than the Americans.

We analyzed the distribution of genotype of -786T→C among 350 OP patients, compared to healthy controls, the results showed that the C allele frequency of OP group was 10.4% compared with 4.0% in the control group, the difference was significant (P<0.05). The risk of OP occurrence in C allele homozygous carriers is 2.970 times higher than T allele homozygous carriers (P<0.001). The data indicated that the upstream promoter region T-786C polymorphism may be a genetic susceptibility gene of OP.

As for the 894G→T exon 7 polymorphism, Timothy investigate the its distribution among healthy and young Americans [20]. Among Healthy white women 120 cases, GG, GT and TT genotypes frequencies were 47%, 42% and

12%, respectively; healthy black women 78 cases, GG, GT and TT genotypes were 68%, 32% and 0%, respectively. One of Chinese population study showed the 894G→T allele frequency of 8.9%, GG, GT, TT genotype frequency was 84%, 14% and 2%, respectively, G allele frequency of 91%, T allele frequency of 9% [21]. We analyzed 350 cases of healthy people and found that T allele frequency was 95.1%, T allele frequency was 4.9%. The genotype of GG, GT and TT frequencies were 90.6%, 9.1% and 0.3%. T allele was similar with the domestic research and significantly lower than the Americans. We compared 350 cases of OP patients with healthy controls, the results showed that the T allele frequency of OP group was 12.1% compared with 4.9% in the control group, the difference was significant (P<0.05). The risk of OP occurrence in T allele homozygous carriers is 2.78 times higher than the G allele, OR is 2.78, 95%; CI=1.843-4.195. The data indicated that the 894G→T exon 7 of eNOS region polymorphism may be a genetic susceptibility gene of OP.

Based on the above, our research shows that the T-786C in promoter region and 894G→T at exon 7 of eNOS gene associated with the onset of OP, since there is no linkage disequilibrium between these two loci which indicated that the T-786C and 894G→T may be independently or with other sites synergy effect on the incidence of OP and act as the genetic risk factor of OP, at least in the Sichuan region of China population. But the occurrence of OP affected by the environmental and genetic factors. So the upstream promoter region eNOS T-786C and 894G→T at exon 7 remains to be further expanded the objects of research. There also need to establish more stringent screening criteria to avoid the influence caused of grouping and stratification, and strengthen the research of multi-chain-related genes.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zuchao Gu, Department of Orthopedics, The First People's Hospital of Chengdu, Sichuan Province, P. R. China. Tel: 86-13520040187; E-mail: zuchao66gu@163.com

References

[1] Hodgson SF, Watts NB, Bilezikian JP, Clarke BL, Gray TK, Harris DW, Johnston CC Jr, Kleereko-per M, Lindsay R, Luckey MM, McClung MR, Nankin HR, Petak SM, Recker RR; AACE Osteoporosis Task Force. American Association of Clinical Endocrinologists medical guidelines for clinical practice for the prevention and treatment of postmenopausal osteoporosis: 2001 edition, with selected updates for 2003. Endocr Pract 2003; 9: 544-64.

[2] Kanis JA, Black D, Cooper C, Dargent P, Dawson-Hughes B, De Laet C, Delmas P, Eisman J, Johnell O, Jonsson B, Melton L, Oden A, Papapoulos S, Pols H, Rizzoli R, Silman A, Tenenhouse A; International Osteoporosis Foundation; National Osteoporosis Foundation. A new approach to the development of assessment guidelines for osteoporosis. Osteoporos Int 2002; 13: 527-536.

[3] Kanis JA, Melton LJ 3rd, Christiansen C, Johnston CC and Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res 1994; 9: 1137-1141.

[4] Garthwaite J and Boulton CL. Nitric oxide signaling in the central nervous system. Annu Rev Physiol 1995; 57: 683-706.

[5] Garcia-Cardena G, Oh P, Liu J, Schnitzer JE and Sessa WC. Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. Proc Natl Acad Sci U S A 1996; 93: 6448-6453.

[6] van't Hof RJ and Ralston SH. Nitric oxide and bone. Immunology 2001; 103: 255-261.

[7] Renella R, Schlehe JS, Selkoe DJ, Williams DA and LaVoie MJ. Genetic deletion of the GATA1-regulated protein alpha-synuclein reduces oxidative stress and nitric oxide synthase levels in mature erythrocytes. Am J Hematol 2014; 89: 974-7.

[8] Klein-Nulend J, van Oers RF, Bakker AD and Bacabac RG. Nitric oxide signaling in mechanical adaptation of bone. Osteoporos Int 2014; 25: 1427-1437.

[9] Charytan DM, Padera R, Helfand AM, Zeisberg M, Xu X, Liu X, Himmelfarb J, Cinelli A, Kalluri R and Zeisberg EM. Increased concentration of circulating angiogenesis and nitric oxide inhibitors induces endothelial to mesenchymal transition and myocardial fibrosis in patients with chronic kidney disease. Int J Cardiol 2014; 176: 99-109.

[10] Fukuda S, Fukuda Y, Ishitsuka M, Kajita T, Kameda J, Kaneyuki K, Kobayashi K, Koshio Y, Miura M, Moriyama S, Nakahata M, Nakayama S, Obayashi Y, Okada A, Okumura K, Sakurai N, Shiozawa M, Suzuki Y, Takeuchi H, Takeuchi Y, Toshito T, Totsuka Y, Yamada S, Earl M, Habig A, Kearns E, Messier MD, Scholberg K, Stone JL, Sulak LR, Walter CW, Goldhaber M, Barszczak T, Casper D, Gajewski W, Kropp WR, Mine S, Price LR, Smy M, Sobel HW, Vagins MR, Ganezer KS, Keig WE, Ellsworth RW, Tasaka S,

Kibayashi A, Learned JG, Matsuno S and Takemori D. Tau neutrinos favored over sterile neutrinos in atmospheric muon neutrino oscillations. Phys Rev Lett 2000; 85: 3999-4003.

[11] Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA and Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. JAMA 2009; 301: 513-21.

[12] Center JR, Nguyen TV, Schneider D, Sambrook PN and Eisman JA. Mortality after all major types of osteoporotic fracture in men and women: an observational study. Lancet 1999; 353: 878-882.

[13] Robinson LJ, Mancarella S, Songsawad D, Tourkova IL, Barnett JB, Gill DL, Soboloff J and Blair HC. Gene disruption of the calcium channel Orai1 results in inhibition of osteoclast and osteoblast differentiation and impairs skeletal development. Lab Invest 2012; 92: 1071-1083.

[14] Larsen ER, Mosekilde L and Foldspang A. Vitamin D and calcium supplementation prevents osteoporotic fractures in elderly community dwelling residents: a pragmatic population-based 3-year intervention study. J Bone Miner Res 2004; 19: 370-378.

[15] Nichols SP, Storm WL, Koh A and Schoenfisch MH. Local delivery of nitric oxide: targeted delivery of therapeutics to bone and connective tissues. Adv Drug Deliv Rev 2012; 64: 1177-1188.

[16] Simoncini T and Genazzani AR. Non-genomic actions of sex steroid hormones. Eur J Endocrinol 2003; 148: 281-292.

[17] Pennisi P, D'Alcamo MA, Leonetti C, Clementi A, Cutuli VM, Riccobene S, Parisi N and Fiore CE. Supplementation of L-arginine prevents glucocorticoid-induced reduction of bone growth and bone turnover abnormalities in a growing rat model. J Bone Miner Metab 2005; 23: 134-139.

[18] Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, Harada M, Kajiyama N, Kishimoto I, Kuwahara K, Hino J, Ogawa E, Hamanaka I, Kamitani S, Takahashi N, Kawakami R, Kangawa K, Yasue H and Nakao K. Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T-->C mutation associated with coronary spastic angina. Hum Mol Genet 2000; 9: 2629-2637.

[19] Asakimori Y, Yorioka N, Taniguchi Y, Ito T, Ogata S, Kyuden Y and Kohno N. T(-786)-->C polymorphism of the endothelial nitric oxide synthase gene influences the progression of renal disease. Nephron 2002; 91: 747-751.

[20] Howard TD, Giles WH, Xu J, Wozniak MA, Malarcher AM, Lange LA, Macko RF, Basehore MJ, Meyers DA, Cole JW and Kittner SJ. Promoter polymorphisms in the nitric oxide synthase 3 gene are associated with ischemic stroke susceptibility in young black women. Stroke 2005; 36: 1848-1851.

[21] Li YY, Zhai ZG, Yang YH, Pang BS, Wang HY, Zhang W, Zhao L, Wang J and Wang C. Association of the 894G>T polymorphism in the endothelial nitric oxide synthase gene with risk of venous thromboembolism in Chinese population. Thromb Res 2011; 127: 324-327.