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

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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 χ²-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 osteopo-
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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, 1.5 mmol/L MgCl₂, 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 χ²-test. The allele ratios and genotype distributions in the patients and controls were assessed using the Pearson χ²-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 nitric oxide synthase (eNOS) gene distributed in the survey population genetic equilibrium has been reached.

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with IVDD</th>
<th>Healthy controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.12 ± 6.65</td>
<td>54.84 ± 6.35</td>
<td>0.504</td>
</tr>
<tr>
<td>BMI (means ± SD)</td>
<td>23.1 ± 2.5</td>
<td>23.3 ± 2.4</td>
<td>0.612</td>
</tr>
</tbody>
</table>

### Table 2. The association between T-786C, 894G→T at exon 7 polymorphisms of the eNOS gene and the risk of OP

<table>
<thead>
<tr>
<th>Genotype</th>
<th>OP Group</th>
<th>Control Group</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>79.7%</td>
<td>92%</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>18.6%</td>
<td>8%</td>
<td>1.6 (1.2-2.3)</td>
<td>0.007</td>
</tr>
<tr>
<td>CC</td>
<td>1.7%</td>
<td>0%</td>
<td>9.3 (5.9-14.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>OP Group</th>
<th>Control Group</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>78.3%</td>
<td>90.6%</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>19.1%</td>
<td>9.1%</td>
<td>1.8 (1.4-2.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TT</td>
<td>2.57%</td>
<td>0.3%</td>
<td>7.9 (4.4-14.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Polymorphism of eNOS with risk of osteoporosis

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].

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

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

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

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References


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