The Effect of Polymorphisms in SPP1 on Risk of Fracture: A Case-Control Study

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Background: The purpose of the study was to investigate the correlation between rs4754 and rs6840362 polymorphisms of secreted phosphoprotein 1 (SPP1) gene and fracture risk.

Material/Methods: rs4754 and rs6840362 were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 130 patients with fracture and 107 healthy controls matched with the former by age and sex. Hardy-Weinberg equilibrium (HWE) was assessed in the control group based on the genotype distributions of SSP1 polymorphisms. The differences in genotype, allele, and haplotype frequencies between cases and controls were detected by the chi-square test, and the relative risk of fracture is expressed by odds ratio (OR) and 95% confidence interval (CI). The linkage disequilibrium (LD) and haplotype analyses were conducted with HaploView software.

Results: The TT genotype in rs4754 had significant difference in patients with fracture and controls (10.77% and 4.59%, \(P=0.04\)) and the results showed that people carrying TT genotype of rs4754 were more susceptible to fractures than CC genotype carriers (OR=3.00, 95%CI=1.02–8.89). The T allele also had 1.54 times higher risk of fractures (OR=1.54, 95%CI=1.04–2.30), but this was not true for the rs6840362 polymorphism. LD between the 2 polymorphisms and haplotype C-T (rs6840362-rs4754) increased the susceptibility to fracture (OR=2.01, 95%CI=1.23–3.28).

Conclusions: SPP1 rs4754 polymorphism may be related to risk of fracture, but not rs6840362.

MeSH Keywords: Fractures, Bone • Haplotypes • Osteopontin • Polymorphism, Genetic

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Background

Non-traumatic fracture, spontaneous or occurring at low energy, is defined as a lesion caused by falling on a flat surface or due to an unbalanced center of gravity. It is usually seen in the elderly and the high rate of repeated fractures imposes a heavy burden on patients, their families, and society [1,2]. However, the pathology and etiology of non-traumatic fracture remain mysterious; like others disease, genetic, biological, and environmental factors are suspected and studied widely. Thiazolidinedione drugs, type 2 diabetes mellitus, chronic inflammation, and osteoporosis have been proved to be related to fracture [3–5]. Fragility fracture is the most serious outcome of osteoporosis, and over-expression of osteopontin (OPN) is considered as a risk factor for osteoporosis [6].

In 1979, Senger et al. discovered secreted phosphoprotein 1 (SPP1) [7], also called osteopontin (OPN), which is a kind of secreted protein in malignant epithelial cells. It is encoded by SPP1 gene located in chromosome 4. Animal osteoclasts, osteogenesis, vascular smooth muscle cell, and endothelial cell all can identify SPP1 expression [8,9]. SPP1, a non-collagenous protein of bone, widely exists in extracellular and intercellular sites in inflamed areas and bone tissue and can utilize polyaspartic acid salt and different receptor connection sequences to adsorb bone mineral as the cell adhesion protein [10]. Many studies showed that SPP1 is the modulator of crystal growth and nucleogenesis, and plays an important role in promoting the function exertion of osteoclasts [11–13]. In addition, when cell damage occurs and macrophages and T lymphocytes gather in the area of inflammation or infection, the expression quantity of SPP1 increases [14]. The above-mentioned evidence suggests that SPP1 is important for the development and function of bone.

Although fractures are a common orthopedic problem, there is little research about the correlation between SPP1 polymorphism and fractures. Research shows that extracellular SPP1 benefits the movement, fusion, and reabsorption of osteoclasts to compensate for the loss of sectional endogenous SPP1 [15]. Therefore, this study analyzed the correlation between single-nucleotide polymorphisms (SNP) in SPP1 rs4754 and rs6840362 polymorphisms and fracture to provide some theoretical foundations for the mechanism of fracture.

Material and Methods

The case and the control groups

This case-control study investigated the correlation between SPP1 polymorphisms and fracture. The research subjects were all unrelated Han Chinese. Both groups were informed about the study and provided signed informed consent. Afterwards, the orthopedic investigators, who had been trained professionally, recorded the relevant information of all subjects and collected blood samples. This research was reviewed and authorized by the Ethics Committee of the Chinese PLA General Hospital. The process of sample collection was conducted according to the national ethics criteria for human genome research.

The 130 patients in the case group were non-traumatic fracture patients in the Orthopedics Department of the Chinese PLA General Hospital during October 2012 to January 2014, including 82 postmenopausal women and 48 men. Their age range was 52–78 years, with average age of 63.58±10.29 years. The cases were checked by X-ray and read and diagnosed by an orthopedist or trained radiologist. We excluded patients who had calcium or phosphorus metabolism diseases, pathologi cal fractures caused by traumatic fracture, or cancer and the other bone diseases. Patient clinical data were also recorded, including sex, age, symptoms, part affected, and severity of fracture, and then entered the data into an Excel form.

The healthy control group included healthy people who had a medical examination and were also in the Chinese PLA General Hospital during the same period. There were a total of 107 people, including 73 postmenopausal women and 34 men, ages 50–73 years. The inclusion criteria for the healthy control group were healthy examinees with normal physical examination results. They were matched with the case group for age and sex without medical history. The patients with tumor, diabetes, hepatic disease, or severe osteoporosis (past or present) were excluded.

Methods

DNA extraction

We collected 3 ml of venous blood from every subject into an anticoagulative tube with ethylene diamine tetraacetic acid (EDTA) after obtaining informed consent. According to the manufacturer’s instructions, peripheral blood leucocyte genome DNA of all samples was extracted using Beijing TIANGEN biochemical blood genome DNA extraction kit, and then stored in −20°C a refrigerator for later use.

SPP1 genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was applied for the genotyping of SPP1 rs4754, rs6840362 polymorphisms. For primer design, we searched GeneBank database on the NCBI website to refer to the published SPP1 gene sequence in human chromosome 4. Complying with general primer design principles, we designed the PCR primers using Primer Premier 5.0 software. The detailed sequences are listed in Table 1. A total of 25 µl solution
in PCR system included 1.0 µl DNA template, each 1.0 µl of forward and reverse primers, 2.5 µl 10× buffer, 1.5 µl MgCl₂, 1.0 µl dNTPs, 0.5 µl Taq enzyme, and 16.5 µl ddH₂O. The amplification conditions of PCR were 95°C pre-denaturation for 5 min; followed by 38 cycles of 94°C degeneration for 30 s, 60°C annealing for 60 s, 72°C extension for 60 s, and finally 72°C extension for 10 min. The PCR products were checked in 1% agarose gel electrophoresis (AGE). Enzyme reaction system was a volume of 20 µl, including 4 µl restriction enzyme (BpiI for rs4754 and AluI for rs6840362), 8 µl PCR products, 2 µl 10× buffer solution, and 6 µl double-distilled water. Then the mixture was digested in a 37°C water bath overnight. The enzyme-digested products were separated by 3% AGE and we observed the final outcome in the imaging system. 

**Statistical analysis**

PASW Statistics 18.0 software was used to analyze the data and P<0.05 was considered as the statistical significance. Measurement data are expressed by x±s and%. The genotype frequencies in the control group were tested by χ² to confirm if their distribution matched Hardy-Weinberg equilibrium (HWE). The comparison of genotypes, alleles and haplotypes between two groups were tested by chi-square test. Linkage disequilibrium (LD) and its correlation coefficient (D' value) were calculated with HaploView. Haplotypes were analyzed between rs4754, rs6840362 if LD existed. The effect of SPP1 polymorphisms on fracture was evaluated with odd ratio (OR) and 95% confidence interval (CI).

### Results

**General conditions of research objects**

This study collected a total of 239 subjects that met inclusion requirements, with complete data in case and control groups. In the case group, women accounted for 63.08%, and the sex ratio was 1.71:1 (women: men); the group of 109 healthy people included 75 women and 34 men. There was no significant different between the 2 groups by sex. The controls (mean age 62.13±9.24 years) were similar to the cases (63.58±10.29) in terms of age (P>0.05). Furthermore, the genotypes distribution

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**Table 1.** Primer sequences of SPP1 gene in rs4754, rs6840362.

| SNP      | Primer sequence          |
|----------|--------------------------|
| rs4754   | For. 5’TCTCCGCCATCTTTTTCA-3’ |
|          | Rev. 5’AAAATCGTGTGTGGCAGG-3’ |
| rs6840362| For. 5’CCGTTGGAGGACAGTATG-3’ |
|          | Rev. 5’TTTAGATGACCTGAGAATG-3’ |

**Table 2.** Frequency comparisons of genotypes and alleles in SPP1 gene polymorphisms.

| Genotype/allele | Case, n=130 (%) | Control, n=109 (%) | χ² | P     | OR (95% CI) |
|-----------------|-----------------|--------------------|----|-------|-------------|
| rs4754 CC       | 55 (42.31)      | 59 (54.13)         |    |       | 1.00        |
| TC              | 61 (46.92)      | 45 (41.28)         | 1.91| 0.17  | 1.45 (0.85–2.48) |
| TT              | 14 (10.77)      | 5 (4.59)           | 4.22| 0.04  | 3.00 (1.02–8.89) |
| C               | 171 (65.77)     | 163 (74.77)        |    |       | 1.00        |
| T               | 89 (34.23)      | 55 (25.23)         | 4.56| 0.03  | 1.54 (1.04–2.30) |
| rs6840362 CC    | 107 (82.30)     | 86 (78.90)         |    |       | 1.00        |
| CT              | 18 (13.85)      | 20 (18.35)         | 0.83| 0.36  | 0.72 (0.36–1.45) |
| TT              | 5 (3.85)        | 3 (2.75)           | 0.16| 0.69  | 1.34 (0.31–5.76) |
| C               | 232 (89.23)     | 192 (88.07)        |    |       | 1.00        |
| T               | 28 (10.77)      | 26 (11.93)         | 0.16| 0.69  | 0.89 (0.51–1.57) |
of SPP1 polymorphisms both conformed to HWE, suggesting that our population similar in genetic background to a Mendelian population.

The distribution of SPP1 gene SNP in case and control groups

From Table 2, we could see clearly that the TT, TC, and CC genotype frequencies in rs4754 were 42.31%, 46.92%, and 10.77% in the case group and 54.13%, 41.28%, and 4.59%, respectively, in the control group. The C and T allele frequencies were 65.77% and 34.23% in the case group, and 74.77% and 25.23%, respectively, in the control group. The TT genotype and T allele distributions had statistically significant differences in the 2 groups (P=0.04, 0.03) and their carriers were easily subject to fractures compared with people with CC genotype and C allele (TT vs. CC: OR=3.00, 95%CI=1.02–8.89; T vs. C: OR=1.54, 95%CI=1.04–2.30). The genotypes frequencies of rs6840362 were 82.30%, 13.85%, and 3.85% in cases and 78.90%, 18.35%, and 2.75% in controls, respectively. There were no significant differences between the 2 groups based on genotypes (P=0.36, 0.69) or was allele (P=0.69).

Haplotype analysis of SPP1 rs4754, rs6840362 polymorphisms

LD of SPP1 rs4754 and rs6840362 was analyzed by HaploView software. Strong LD was discovered between them (D'=1.0, r²=0.227) and 3 haplotypes were identified in our population, namely C-C, C-T, and T-T (rs6840362-rs4754) (Table 3). The frequencies of C-C, C-T, and T-T haplotype were 65.77%, 23.46%, and 10.77% in case group, and 74.77%, 13.30%, and 11.93%, respectively, in the control group. The distributions of C-T haplotype had obvious differences between the 2 groups (P=0.01), which indicated that it could increase the risk of fractures (OR=2.01, 95%CI=1.23–3.28).

Discussion

SPP1 was first found as an acidic protein of converting specificity, and later was found to be expressed in bone. It serves as a potential bridge connecting osteocytes and hydroxyapatite, which is why human SPP1 (hSPP1) is also called OPN [16]. This protein is rich in arginine, glutamic acid, and serine, and includes 1 N-glycosylation, 5-6 O-glycosylation side chains, several serine phosphorylation sites, and threonine phosphorylation sites [16,17]. Studies show that SPP1 inhibits the formation of calcium oxalate crystals in urine production, which effectively protects kidney from calcium deposits and urinary calculus [18]. Hasegawa et al. has suggested that OPN is highly expressed in osteoarthritis (OA) and synovial fluid [19]. Sanchez et al. demonstrated that the expression of OPN in osteosclerosis areas of subchondral bone in OA patients is clearly higher than in the normal group [20]. Sakata et al. verified that serum osteopontin cultivated by stem cells is an autoantigen of OA and rheumatoid arthritis (RA) [21]. SPP1 is a low phosphorylated protein with 5500 molecular weight in preosteoblasts and it may be related to the formation of bone matrix [22]. Osteoblasts can synthesize the high phosphorylated proteins and take part in regulating the growth of light apatite crystal, so that OPN is regarded as a sign of osteoblast maturation and differentiation [23]. In addition, the amino terminal of SPP1 is related to exocriinosity, and carboxy terminal participates in regulating the adhesion function [24].

OPN is a kind of matrix protein found in multiple tissues composed of collagen and non-collagen, and SPP1 is an important component of the latter. SPP1, which is an important extracellular matrix protein, is also an important cytokine. It plays a major role in the process of diffusion and movement between cells, adherence of crossing cytoplasm, mineralization and reestablishment of bone tissue, immunoregulation, and signal transduction. Some foreign scholars have found that SPP1 polymorphisms also influence urinary calculus. Gao et al. tested SPP1 polymorphism by TaqMan probe technique and analyzed the relationship between SPP1 polymorphism and urinary calculus; the results showed that SPP1 A9402G polymorphism might be a genetic marker and risk factor for forming calculus [25]. Later, Gao et al. also discovered an obvious correlation between 2 new SNPs in the SPP1 promoter region and the risk of kidney stones [26]. Liu et al., in a study of the correlation between 3 SNPs of the SPP1 gene promoter region and the risk of calcium kidney stones in Taiwan, proved that SPP1 gene -156delG/G polymorphism can be used as a
genetic marker to check for risk of calcium kidney stones [27]. Miyazaki et al. showed that SPP1 polymorphism is involved in the pathogenetic process of lupus nephritis [28]. These study results are not completely consistent, and the correlation between SPP1 gene polymorphism and several diseases has ethnic heterogeneity.

In this study, genotypes and allele frequencies of rs6840362 polymorphism had no statistically significant difference between the group of patients with fractures and the healthy control group. However, this study only aimed at the Han population in northern China. Therefore, we need to get further verification with larger sample size and more ethnic groups to determine whether rs6840362 polymorphism is related to fracture in other regions. In contrast, we found that the genotype and allele distributions of rs4754 polymorphism had statistical difference between the 2 groups in this study. Moreover, the result was verified by study of the correlation between haplotype of SPP1 rs6840362, rs4754 polymorphisms, and fracture, in which C
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 haplotype was discovered to obviously increase the risk fracture in older adults. Results of this article prove that SPP1 gene polymorphisms are associated with the occurrence of fractures.

Conclusions

The results of the present study support the correlation between SPP1 polymorphisms and fracture in the Han population of north China. Nevertheless, research with larger sample scale and different populations is required to enrich the study results. It will be important to check all joints and bones of the body by zeugmatography to achieve the aims of precaution, early diagnosis, and timely treatment.

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