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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 poylmorphisms. 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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2042 3 — 28

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Background

Non-traumatic fracture, spontaneous or occurring al 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 moderator 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, pathological 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 *SSP1* 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

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

SNP		Primer sequence
rs4754	For.	5'-TTCCCGGCCATCTTAATTTTCA-3'
	Rev.	5'-AAAACTTCGGTTGCTGGCAGGT-3'
rs6840362	For.	5'-CCGTGGGAAGGACAGTTATG-3'
	Rev.	5'-TTTAATTGACCTCAGAAGATG-3'

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

Genotype/allele	Case, n=130 (%)		Control, n=109 (%)		$\chi 2$	*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)

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 $MgC1_2$, 1.0 μl dNTPs, 0.5 μl Taq enzyme, and 16.5 μl ddH_2O. 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 (*Bpi*I for rs4754 and *Alu*I 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 $\bar{x}\pm s$ and%. The genotype frequencies in the control group were tested by χ^2 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 *SSP1* 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 3. Analyses of LD and haplotypes in *SPP1* rs4754, rs6840362 polymorphisms.

Haplotype SNP1-SNP2	Case group 2 n=260 (%)	Control group 2 n=218 (%)	$\chi 2$	*P*	OR (95% CI)
CC	171 (65.77)	163 (74.77)	–	–	1.00
CT	61 (23.46)	29 (13.30)	7.87	0.01	2.01 (1.23–3.28)
TT	28 (10.77)	26 (11.93)	0.01	0.93	1.03 (0.58–1.83)

SNP1 – rs6840362; SNP2 – rs4754.

of *SSP1* 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 difference 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, r^2=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 difference 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 expresses 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 exocrinosity, 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

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genetic marker to check for risk of calcium kidney stones [27]. Miyazak 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 $C_{rs6840362}$-T_{rs4754} 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.

References:

1. Cauley JA: Public health impact of osteoporosis. J Gerontol A Biol Sci Med Sci, 2013; 68: 1243–51
2. Banu J: Causes, consequences, and treatment of osteoporosis in men. Drug Des Devel Ther, 2013; 7: 849–60
3. Rathmann W, Kostev K: Fracture risk in patients with newly diagnosed type 2 diabetes: a retrospective database analysis in primary care. J Diabetes Complications, 2015; 29: 766–70
4. Schett G, Saag KG, Bijlsma JW: From bone biology to clinical outcome: state of the art and future perspectives. Ann Rheum Dis, 2010; 69: 1415–19
5. Bazelier MT, Vestergaard P, Gallagher AM et al: Risk of fracture with thiazolidinediones: disease or drugs? Calcif Tissue Int, 2012; 90: 450–57
6. Ermakov S, Leonov A, Trofimov S et al: Quantitative genetic study of the circulating osteopontin in community-selected families. Osteoporos Int, 2011; 22: 2261–71
7. Senger DR, Wirth DF, Hynes RO: Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell, 1979; 16: 885–93
8. Denhardt DT, Guo X: Osteopontin: a protein with diverse functions. FASEB J, 1993; 7: 1475–82
9. Weber GF: The metastasis gene osteopontin: a candidate target for cancer therapy. Biochim Biophys Acta, 2001; 1552: 61–85
10. McKee MD, Nanci A: Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. Microsc Res Tech, 1996; 33: 141–64
11. Boskey AL, Spevak L, Paschalis E et al: Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcif Tissue Int, 2002; 71: 145–54
12. Kavukcuoglu NB, Denhardt DT, Guzelsu N, Mann AB: Osteopontin deficiency and aging on nanomechanics of mouse bone. J Biomed Mater Res A, 2007; 83: 136–44
13. Ishijima M, Tsuji K, Rittling SR et al: Resistance to unloading-induced three-dimensional bone loss in osteopontin-deficient mice. J Bone Miner Res, 2002; 17: 661–67
14. Giachelli CM, Lombardi D, Johnson RJ et al: Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli *in vivo*. Am J Pathol, 1998; 152: 353–58
15. Suzuki K, Zhu B, Rittling SR et al: Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. J Bone Miner Res, 2002; 17: 1486–97
16. Sodek J, Ganss B, McKee MD: Osteopontin. Crit Rev Oral Biol Med, 2000; 11: 279–303
17. Johnson GA, Burghardt RC, Bazer FW: Osteopontin: a leading candidate adhesion molecule for implantation in pigs and sheep. J Anim Sci Biotechnol, 2014; 5: 56
18. Sorensen S, Justesen SJ, Johnsen AH: Identification of a macromolecular crystal growth inhibitor in human urine as osteopontin. Urol Res, 1995; 23: 327–34
19. Hasegawa M, Segawa T, Maeda M et al: Thrombin-cleaved osteopontin levels in synovial fluid correlate with disease severity of knee osteoarthritis. J Rheumatol, 2011; 38: 129–34
20. Sanchez C, Deberg MA, Bellahcene A et al: Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone. Arthritis Rheum, 2008; 58: 442–55
21. Sakata M, Tsuruha JI, Masuko-Hongo K et al: Autoantibodies to osteopontin in patients with osteoarthritis and rheumatoid arthritis. J Rheumatol, 2001; 28: 1492–95
22. Sodek J, Chen J, Nagata T et al: Regulation of osteopontin expression in osteoblasts. Ann NY Acad Sci, 1995; 760: 223–41
23. Zhou S, Zu Y, Sun Z et al: Effects of hypergravity on osteopontin expression in osteoblasts. PLoS One, 2015; 10: e0128846
24. Takahashi K, Takahashi F, Tanabe KK et al: The carboxyl-terminal fragment of osteopontin suppresses arginine-glycine-asparatic acid-dependent cell adhesion. Biochem Mol Biol Int, 1998; 46: 1081–92
25. Gao B, Yasui T, Okada A et al: A polymorphism of the osteopontin gene is related to urinary calcium stones. J Urol, 2005; 174: 1472–76
26. Gao B, Yasui T, Itoh Y et al: Association of osteopontin gene haplotypes with nephrolithiasis. Kidney Int, 2007; 72: 592–98
27. Liu CC, Huang SP, Tsai LY et al: The impact of osteopontin promoter polymorphisms on the risk of calcium urolithiasis. Clin Chim Acta, 2010; 411: 739–43
28. Miyazaki T, Ono M, Qu WM et al: Implication of allelic polymorphism of osteopontin in the development of lupus nephritis in MRL/lpr mice. Eur J Immunol, 2005; 35: 1510–20

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