Original Paper

Osteopontin, Bone Morphogenetic Protein-4, and Vitamin D Receptor Gene Polymorphisms in the Susceptibility and Clinical Severity of Spinal Tuberculosis

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Key Words

Spinal tuberculosis • Bone morphogenetic protein-4 • Osteopontin • Vitamin D receptor • Polymorphism • Expression

Abstract

Background/Aims: Spinal tuberculosis (TB) is a common and dangerous form of extrapulmonary TB with unclear mechanisms in its occurrence and progression. This study investigated the clinical significances of bone morphogenetic protein-4 (*BMP-4*), osteopontin (*OPN*), and vitamin D receptor (*VDR*) gene polymorphism, mRNA and protein expression in spinal TB patients. **Methods:** *BMP-4* and *OPN* gene polymorphisms were detected by direct DNA sequencing, while *VDR-FokI* polymorphisms were analyzed using PCR-RFLP. mRNA and protein expression was measured using real-time PCR and Western blot, respectively. **Results:** A significant lower frequency of TT genotype and T allele at 6007C>T polymorphism in *BMP-4* gene; higher frequency of GG genotype and G allele at -66T>G polymorphism in *OPN* gene, and higher frequency of the ff genotype and f allele at the *VDR-FokI* polymorphism were observed in patients with spinal TB compared to controls. TT genotype of 6007C>T polymorphism correlated with a lower *BMP-4* mRNA and protein expression, -66GG genotype correlated with a high *OPN* mRNA and protein expression, and ff genotype correlated with the lower *VDR* mRNA and protein levels in the intervertebral disc tissues. The TT genotype and low *BMP-4* gene expression; the -66GG genotype and high *OPN* gene expression; and the ff genotype and low *VDR* gene expression significantly correlated with the clinical severity of spinal TB. **Conclusion:** The 6007C>T polymorphism of *BMP-4*, -66T>G polymorphism of *OPN*, and *VDR-FokI* polymorphism are the susceptible factors of spinal TB and indicators of the clinical severity. These three genes may collaborate in the development of spinal TB.

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Introduction

The WHO's 2015 Global Tuberculosis Report demonstrated that tuberculosis (TB) killed 1.5 million people in 2014, including 0.4 million people with HIV-positive TB. An estimated 9.6 million people had fallen sick with TB in 2014 with 37% of new cases being undiagnosed or not reported [1]. Therefore, TB is still a leading cause of death worldwide. Although spinal TB occurs in less than 1% of TB patients, it is a common and dangerous extrapulmonary TB accounting for 50% of all patients with bone or joint TB [2]. Unfortunately, the susceptible population of spinal TB has not been identified. A previous study suggested that the interaction of host genes with environmental factors may determine the response of an individual to *M. tuberculosis* infection [3]. However, the molecular mechanisms driving the pathological response have not been evaluated.

The bone morphogenetic proteins (BMPs) are a family of multifunctional growth factors involved in the induction of bone formation. BMP-4 has been revealed to promote bone formation, cartilage formation, and chondrocyte maturation, while also playing potential roles in bone repair, fracture healing, and cartilage repair [4]. Wang et al. study revealed that the lower prevalence of TT genotype of 6007C > T polymorphism in the BMP-4 gene was significantly associated with a high risk and poor outcome of cervical spondylotic myelopathy [5]. The T allele in the 6007C > T polymorphism of *BMP-4* gene has been demonstrated to be a risk factor of ossification of the posterior longitudinal ligament (OPLL) in the cervical spine [6], while the TGGGCTT haplotype of *BMP4* gene, has been associated with OPLL-associated risk alleles and can increase the severity of OPLL [7]. However, the association of *BMP-4* SNPs with spinal TB has not been reported.

Osteopontin (OPN) is a noncollagenous matrix protein involved in bone formation and desorption [8]. OPN is also involved in many pathologic conditions, including cancer [9] and inflammatory response [10]. For example, *OPN* -443C/T polymorphism was found to be significantly associated with the susceptibility to develop gliomas and their prognosis [11] and the susceptibility and response to Ara-C based chemotherapy in AML patients [12]. The natural roles of OPN in bone have been widely studied. *OPN* -443C/T single nucleotide polymorphism is associated with the development of arterial calcification and bone density [13]. In addition, the *OPN* -66T>G genetic polymorphism contributes to patients' susceptibility to cervical spondylotic myelopathy and is an indicator of the outcome of anterior cervical corpectomy and fusion surgery [14]. Several studies reported the roles of OPN in tuberculosis infection. For example, OPN was found to be a marker for the acute infection of mycobacterium tuberculosis and for monitoring the course of the disease [15]. However, no report addressed the role of OPN expression or genetic polymorphism in spinal TB.

It is widely known that vitamin D plays a crucial role in bone metabolism. The active form of 1,25-(OH)₂D₃ exerts its role through the vitamin D receptor (VDR). The *VDR* gene contains four polymorphic regions and three of them are located at the 3'-end of the *VDR* gene and can be detected by the restriction enzymes *BsmI*, *ApaI*, and *TaqI* [16]. Another polymorphic region is located at the start codon and can be detected by *FokI*. The *FokI* polymorphism is the only one that results in changes in VDR protein structure, thereby causing a 1.7-fold decrease in transcriptional efficiency [17]. Few studies have reported the frequency of *VDR* gene polymorphisms in spinal TB patients with controversial findings. For example, Selvaraj et al. study on *BsmI*, *ApaI*, *TaqI*, and *FokI* polymorphisms of *VDR* gene in 64 spinal TB patients and 103 controls showed that the frequencies of Bb genotype of *BsmI* polymorphic variants and FF genotype of *FokI* polymorphism were significantly higher in spinal TB patients than in healthy controls [18]. Zhang et al. study in 110 spinal TB patients and 102 controls showed that the frequency of the ff genotype of *VDR-FokI* polymorphism was significant higher than that in the healthy controls [19].

Osteopontin (*OPN*), bone morphogenetic protein-4 (*BMP-4*), and vitamin D receptor (*VDR*) gene are three crucial genes playing important roles in bone formation and metabolism. The roles of these three gene polymorphisms in the susceptibility and clinical severity of

spinal TB have not been well addressed. In this study, we investigated the genotype and allele frequencies of *BMP-4*, *OPN*, and *VDR-FokI* polymorphism and their protein levels in intervertebral disc tissues in 150 patients with spinal TB and 149 healthy controls. We further analyzed the associations of genotypes and protein expression of these genes with the clinical severity of TB.

Material and Methods

Subjects

150 patients diagnosed with spinal TB were enrolled in this study from January 2013 to December 2015 at Hunan Provincial People's Hospital and Xiangya Hospital. Spinal TB was diagnosed according to findings from a medical history of tuberculosis, laboratory tests, such as a positive skin test and elevated erythrocyte sedimentation rate (ESR), histological analysis of pathogen, computed tomography (CT), and magnetic resonance imaging (MRI). The spinal TB patients with comorbid disorders or other complications, such as rheumatoid arthritis, congenital cervical anomalies, trauma, prior spinal cervical surgery, HIV-positive, and ankylosing spondylitis were excluded from this study. 149 age- and sex-matched healthy subjects were enrolled as controls. The clinical characteristics including sex, age, body mass index (BMI), smoking and alcohol consumption, family history of TB, daily desk work time, residence, and GATA (Gulhane Askeri Tip Akademisi) classification were collected. The GATA classification was made by two well-trained clinicians (YZ, XS) who did not involve in the laboratory study by following previously published criteria [20, 21] and this information was blind to the investigators who performed the experiments. Briefly, spinal TB is classified into three main types: I, II, III, and the Type I is subdivided into Type IA and Type IB. Type I only includes "one-level disc involvement and soft tissue infiltration without abscess, collapse and neurologic deficit" with Type IA that "lesions only limited to vertebra", and Type IB that "abscess formation exceeds the vertebra". Type II included the involvement of "one- or two-level disc degeneration, abscess formation and mild kyphosis correctable" with possible neurological deficit. Type III included the involvement of "one- or two-level disc degeneration, abscess formation, instability and deformity" that can only be corrected with instrumentation [21]. 96 patients accepted surgical therapy, and the intervertebral disc tissues were collected. The study protocol was approved by the Ethics Committees at Hunan Provincial People's Hospital and Xiangya Hospital. Signed informed consent was obtained from all patients and this study was carried out in compliance with the Helsinki Declaration.

Genotyping and SNPs

Five ml of peripheral blood was collected into EDTA tubes. Genomic DNA was isolated from white blood cells using Qiagen Genomic DNA Isolation Kit (Qiagen, Valencia, CA, USA). The *BMP-4* gene was amplified by polymerase chain reaction (PCR) using forward primer: 5'-TGAAGGCAAGATGTCTGACACA-3' and reverse primer: 5'-CCTTCCTGCATTTCTATCCTA-3' for -5826G>A (rs1957860) polymorphism, and forward primer: 5'-ATTGCCCCAACCTGAGCTATC-3' and reverse primer: 5'-TGGGGGCTTCATAACCTC-3' for 6007C>T (rs17563) polymorphism. The promoter region of *OPN* gene including -66T>G (rs28357094), -156G>/GG (rs17524488), and -443C>T (rs11730582) polymorphisms was amplified using forward primer: 5'-ACCTGTATGACACTGTGTAGGC-3' and reverse primer: 5'-CACTGCAATTCTCATGGTAGTGAG-3'. The DNA polymorphisms in *BMP-4* and *OPN* gene were detected by direct DNA sequencing for all samples using an ABI Prism® 7500 Sequence Detection System (Applied Biosystems).

The *VDR-FokI* polymorphisms were analyzed using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. Briefly, the *VDR* gene containing the C/T polymorphism at the *FokI* recognition site (GGATG) was amplified using forward primer: 5'-AGCTGGCCCTGGCACTGACTCTGCTC-3' and reverse primer: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3' and produced a 265-bp DNA fragment. PCR products were purified and digested with *FokI* restriction enzyme for 2 h at 37°C. The digestion products were separated on 7% SDS-PAGE gel and stained with silver. At the C/T polymorphism site, the presence of T residue can allow the 265-bp DNA fragment to be digested by *FokI* and yielded a 69-bp fragment and a 196-bp fragment, representing the "f" genotype. In contrast, the presence of C residue does not allow the digestion of *FokI*, representing the "F" genotype. Thus, there were three genotypes: FF (1 band at 265 bp); ff (2 bands at 196 and 69 bp); and Ff (3 bands at 265, 196, and 69 bp).

Real-time PCR

Total RNA was extracted from intervertebral disc tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) by following the user manual. Reverse transcription was performed using SuperScript™ IV First-Strand Synthesis System (Invitrogen). The human *BMP4* gene was amplified using forward primer: 5'-GCCAAGTCCTGCTAGGAGGC-3' and reverse primer: 5'-TCAGGGCCCTGGTCCACCTGC-3". The *OPN* gene was amplified using forward primer: 5'-CATCTCAGAAGCAGAATCTC-3' and reverse primer: 5'-CGTCTGTAGCATCAGGGTACTG-3'. the *VDR* gene was amplified using forward primer: 5'-CATCACCAAGGACAACCGACG-3" and reverse primer: 5'-GAAGCTGGGAGTGTGTCTGGAG-3'. Human GAPDH was amplified using forward primer: 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse primer: 5'-CTGGGTGGCAGTGATGGCATGG-3' as internal control. mRNA expression was measured by SYBR green qPCR assay (Takara, Dalian, China) and quantitatively analyzed using the comparative CT method ($\Delta\Delta CT$).

Western blot

The intervertebral disc tissues were collected from spinal TB patients during surgery. Tissues were homogenized as previously described [22]. 20- μ g of total protein was separated on SDS-polyacrylamide gels and transferred to PVDF membranes (BioRad). After blocking with 1 $\times$ PBST butter (PBS+0.01% Tween-20) containing 5% non-fat milk for 45 min, the membranes were incubated with primary rabbit anti-human BMP-4 (ab155033), OPN (ab181440, detecting the cleaved OPN), VDR (ab109234), and β -actin (ab3280) antibody (Abcam, USA) for overnight at 4°C. After washing with 1 $\times$ PBST for 3 times, the membranes were then incubated with HRP-conjugated goat-anti-rabbit secondary antibody (Abcam, USA) for 2 hrs at room temperature. The densitometry of the specific bands was analyzed using adobe photoshop. The BMP-4, OPN, and VDR protein levels were normalized to β -actin.

Statistical Analysis

Quantitative data were presented as means $\pm$ SD (standard deviations) and analyzed using the Statistical Package for the Social Sciences, version 16.0 (SPSS 16.0). The frequency of various genotypes in controls and patients was subjected to Hardy-Weinberg equilibrium analysis. The qualitative data were expressed as absolute numbers and/or percent values. Differences in demographic characteristics between patients and controls were compared using Student's t test or the χ^2 test. Genotypes and allele frequencies were compared using χ^2 analysis or Fisher's exact test. Protein and mRNA expression in different genotypes were analyzed using one way ANOVA. Correlations between BMP-4, OPN, and VDR protein expression levels with clinical severities were analyzed using linear regression (GraphPad Prism). Multivariate logistic regression analysis was used to determine the influence of gene polymorphisms on spinal TB and adjusting for age, sex, BMI, smoking status, family history status, daily desk work time, and residence. The adjusted odds ratio (OR), 95% confidence interval (CI), and adjusted p value were calculated. The results were considered statistically significant at $P < 0.05$.

Results

General clinical characteristics

There were no significant differences in age, sex, body mass index (BMI), smoking, daily desk working time, and residence between patients with spinal TB and healthy control subjects (Table 1). Significantly more subjects had heavy alcohol consumption ($P < 0.05$) and had a history of TB ($P < 0.001$) in patients than in controls. Among the 150 patients, 48 patients were Type IA of GATA classification, 47 were Type IB, 34 were Type II, and 21 were Type III (Table 1). Intervertebral disc samples were collected from 96 spinal TB patients, including all type III, Type II and 41 type IB patients.

Genotype distributions and allele frequencies

Hardy-Weinberg equilibrium analysis showed no significant differences in the genotype frequencies for all polymorphisms in *BMP-4*, *OPN*, and *VDR* gene ($P > 0.05$). In *BMP-4* gene, rs-5826G>A and 6007C>T polymorphism were measured. Spinal TB patients showed no significant differences in genotype and allele frequencies in rs-5826G > A polymorphism

Table 1. Demographic characteristics of subjects

	Case n=150	Control n=149	P
Age (mean±SD, year)	46.3±3.9	45.8±4.1	0.112
Male (n, %)	88(58.7%)	89(59.7%)	0.842
BMI (mean±SD, Kg/m ²)	23.6±2.4	23.5±2.1	0.434
Smoking, n, (%)	54(35.5%)	51(34.2%)	0.752
Heavy Alcohol user, n, (%)	17(11.3%)	6(4.0%)	0.018
Family history, n, (%)	31(20.7%)	9(6.0%)	<0.001
Desk working time (day/hour)	4.5±0.6	4.4±0.7	0.078
Residence			
Countryside, n, (%)	79(52.7%)	78(52.3)	1
Town, n, (%)	49(32.7%)	52(34.9%)	0.680
City, n, (%)	22(14.7%)	19(12.8%)	0.631
GATA classification			
Type IA	48(32.0%)		
Type IB	47(31.3%)		
Type II	34(22.7%)		
Type III	21(14.0%)		

Table 2. The genotypic and allelic frequencies of BMP-4, OPN, and VDR-FokI gene polymorphisms between spinal tuberculosis and controls

		Control (n=149) N(%)	Case (n=150) N(%)	Adjusted OR	95%CI	Adjusted P
BMP-4						
-5826G>A	GG	33(22.1%)	36(24.0%)	1		
	GA	70(47.0%)	74(49.3%)	0.91	0.698-1.730	0.68
	AA	46(30.9%)	40(26.7%)	0.72	0.493-1.345	0.42
	G	136(45.6%)	146(48.7%)	1		
6007C>T	A	162(54.4%)	154(51.3%)	0.71	0.608-1.157	0.29
	CC	29(19.5%)	44(29.3%)	1		
	CT	74(49.6%)	73(48.7%)	0.75	0.594-1.472	0.07
	TT	46(30.9%)	33(22.0%)	0.52	0.383-1.082	0.02*
	C	133(44.6%)	161(53.7%)	1		
	T	165(53.4%)	139(46.3%)	0.54	0.518-0.986	0.04*
OPN						
-66T>G	TT	46(30.9%)	30(20.0%)	1		
	TG	79(53.0%)	79(52.7%)	1.07	0.626-1.553	0.95
	GG	24(16.1%)	41(27.3%)	1.96	1.113-3.448	0.02*
	T	171(57.4%)	139(46.3%)	1		
-156G/GG	G	127(42.6%)	161(53.7%)	2.58	1.160-2.214	<0.001**
	GG	58(38.9%)	56(37.3%)	1		
	GGG	64(43.0%)	68(45.3%)	1.18	0.706-1.761	0.64
	GGGG	27(18.1%)	26(17.3%)	1.01	0.527-1.730	0.88
	G	180(60.4%)	180(60.0%)	1		
	GG	118(39.6%)	120(40.0%)	1.23	0.797-1.536	0.55
-443C/T	CC	38(25.5%)	45(30.0%)	1		
	CT	83(55.7%)	75(50.0%)	0.59	0.464-1.164	0.19
	TT	28(18.8%)	30(20.0%)	0.86	0.609-1.917	0.79
	C	159(53.4%)	165(55.0%)	1		
	T	139(46.6%)	135(45.0%)	0.88	0.678-1.291	0.69
VDR-FokI						
	FF	39(26.2%)	22(14.7%)	1		
	Ff	68(45.6%)	53(35.3%)	0.65	0.409-1.036	0.07
	ff	42(28.2%)	75(50.0%)	2.55	1.577-4.115	<0.001**
	F	146(49.0%)	97(32.3%)	1		
	f	152(51.0%)	203(67.7%)	2.79	1.462-3.679	<0.001**

from the control subjects. In contrast, the univariate and multivariate logistic regression analysis showed that the frequency of TT genotype ($P<0.05$) of 6007C>T polymorphism was significantly lower in spinal TB patients than that in controls after adjusting for age, sex, BMI, smoking status, family history status, daily desk work time, and residence ($p<0.05$). With the

Fig. 1. Association analysis of gene polymorphisms and clinical severity. The Type IA, IB, II, and III was scored for 1, 2, 3, and 4, respectively and the mean score was calculated. A) The associations between BMP-4 genotypes and GATA classification scores. ** $P < 0.001$ vs. CC and TT genotype of 6007C>T polymorphism. B) The associations between OPN genotypes and GATA classification scores. ** $P < 0.001$ vs. TT and TG genotype of -66T>G polymorphism. C) The associations between VDR-FokI genotypes and GATA classification scores. ** $P < 0.001$ vs. FF and Ff genotypes.

Fig. 2. Real-time PCR of *BMP-4*, *OPN*, and *VDR* mRNA expression. A) Relative mRNA levels of *BMP-4* gene in spinal TB with *BMP-4* CC, CT, and TT genotypes. ** $P < 0.001$ vs. CC and CT genotypes. B) Relative mRNA levels of *OPN* gene in spinal TB with *OPN* -66TT, -66GT, and -66GG genotypes. ** $P < 0.001$ vs. -66TT and -66GT genotypes. C) Relative mRNA levels of *VDR* gene in spinal TB with *VDR* FF, Ff, and ff genotypes. * $P < 0.01$, ** $P < 0.001$ vs. FF genotypes.

CC genotype as reference, TT genotype was a low risk factor for spinal TB (adjusted OR=0.52, 95% CI: 0.383-1.082, adjusted $P < 0.05$). The CT genotype also increased the risk of spinal TB, ($P = 0.07$). With C allele as reference, T allele was a lower risk for spinal TB (adjusted OR=0.54, adjusted $P < 0.05$) (Table 2). In *OPN* gene, no significant differences in the frequencies of genotypes and alleles for -156G/GG and -443C/T polymorphisms were observed (Table 2). The adjusted univariate and multivariate logistic regression analysis showed that spinal TB patients had a higher GG genotype frequency of the -66T/G polymorphism than the controls (adjusted OR=1.96, 95%CI:1.113-3.448, adjusted $P < 0.05$) compared with the TT genotype. With T allele as reference, G allele had a higher risk for spinal TB (adjusted OR=2.58, adjusted $P < 0.001$) (Table 2).

In spinal TB patients, significantly low frequency of FF and high frequency of ff genotype were observed. The adjusted univariate and multivariate logistic regression analysis showed that spinal TB patients had a higher ff genotype frequency than the controls (adjusted OR=2.55, 95% CI:1.577-4.115, adjusted $P < 0.001$) compared with the FF genotype. With F genotype as reference, f genotype was a higher risk for spinal TB (adjusted OR=2.79, adjusted $P < 0.001$) (Table 2).

Fig. 3. BMP-4, OPN, and VDR protein expression. BMP-4, OPN, and VDR protein in the intervertebral disc tissues of 96 spinal TB patients was analyzed by Western blot. A) Representative Western blot of BMP-4 protein. B) Semi-quantitative analysis of BMP-4 protein expression in patients. ** $P < 0.001$ vs. CC and TT genotype of 6007C>T polymorphism. C) Representative Western blot of OPN protein. D) Semi-quantitative analysis of OPN protein levels in patients. ** $P < 0.001$ vs. TT and TG genotype of -66T>G polymorphism. E) Representative Western blot of VDR protein. F) Semi-quantitative analysis of VDR protein expression in patients. * $P < 0.05$ vs. FF and ff genotype, ** $P < 0.001$ vs. FF genotype.

Association of gene polymorphisms with clinical severity

The association of *BMP-4*, *OPN*, and *VDR* gene polymorphisms with clinical severity at diagnosis was evaluated. The clinical severity was evaluated with GATA classification. The Type IA, IB, II, and III were scored as 1, 2, 3, and 4 and the average score was compared between different genotypes for each polymorphism. Higher scores represented more severe clinical symptoms. The CT genotype of the 6007C>T polymorphism in *BMP-4* gene, the GG genotype of the -66T>G polymorphism in *OPN* gene, and the ff genotype of *FokI* polymorphism in *VDR* gene were significantly associated with the clinical severity compared to other two genotypes of the same polymorphism ($p < 0.001$). No significant association between genotypes and clinical severity was observed at other polymorphisms in *BMP-4* and *OPN* genes (Fig. 1).

BMP-4, *OPN*, and *VDR* mRNA and protein level in intervertebral disc and association with clinical severity

The intervertebral disc tissues were collected in 96 patients with Type IB, II, and III spinal TB. The mRNA expression of *BMP-4*, *OPN*, and *VDR* gene was measured by real-time PCR (Fig. 2) and their protein expression was measured by Western blot (Fig. 3). The

Fig. 4. Correlations of BMP-4, OPN, and VDR protein expression with clinical severity. The Type IA, IB, II, and III was scored for 1, 2, 3, and 4, respectively. A) Correlation of BMP-4 protein levels with GATA classification scores. B) Correlation of OPN protein levels with GATA classification scores. C) Correlation of VDR protein levels with GATA classification scores.

genotype distributions of 6007C>T polymorphism in *BMP-4* gene, -66T>G polymorphism in *OPN* gene, and *FokI* polymorphism in *VDR* gene were analyzed because significant differences in genotype frequencies were observed in these polymorphisms. The other polymorphisms without significant difference in genotype frequencies were not compared. For 6007C>T polymorphism, *BMP-4* mRNA (Fig. 2A) and protein expression (Fig. 3A, 3B) were significantly lower in CT genotype than that in CC and TT genotypes ($P<0.001$). For -66T>G polymorphism, *OPN* mRNA (Fig. 2B) and protein expression (Fig. 3C, 3D) were significantly higher in GG genotype than that in TG and TT genotypes ($P<0.001$). For *FokI* polymorphism, *VDR* mRNA (Fig. 2C) and protein expression (Fig. 3E, 3F) was significantly lower in Ff and ff genotype than that in FF genotypes ($P<0.05$, $P<0.001$).

The association between *BMP-4*, *OPN*, and *VDR* mRNA and protein expression with clinical severity was analyzed. In 96 patients with collected intervertebral disc tissues, significant association between *BMP-4* mRNA and protein expression, *OPN* mRNA and protein expression (cleaved OPN), and *VDR* mRNA and protein expression were observed ($p<0.001$) (data not shown). Low *BMP-4* mRNA (Fig. 4A) and protein (Fig. 4B), high *OPN* mRNA (Fig. 4C) and cleaved protein (Fig. 4D), and low *VDR* mRNA (Fig. 4E) and protein expression (Fig. 4F) significantly correlated with more severe clinical symptoms ($p<0.001$).

Associations between BMP-4, OPN, and VDR protein level

The correlations between BMP-4, cleaved OPN, and VDR protein level were analyzed in 96 patients with spinal TB (Fig. 5). In intervertebral disc tissues, BMP-4 protein levels negatively correlated with cleaved OPN protein levels (Fig. 5A, $P<0.001$), cleaved OPN protein

levels negatively correlated with VDR protein levels (Fig. 5B, $P < 0.001$), and BMP-4 protein levels positively correlated with VDR protein levels (Fig. 5C, $P < 0.001$).

Discussion

Spinal tuberculosis is the most common and dangerous skeletal tuberculosis, which results from a hematogenous spread of *M. tuberculosis* into the dense vasculature of cancellous bone. The progression of spinal TB can result in vertebral collapse, spinal cord compression, sinus formation, and paraplegia [23]. The genetic susceptibility to tuberculosis has been widely studied and many genetic factors have been identified [24]. However, only a few studies have recently demonstrated the genetic susceptibility of spinal tuberculosis, including two studies on vitamin-D receptor gene polymorphism in spinal tuberculosis with controversial findings [18, 19], and three studies on the polymorphism of monocyte chemoattractant protein-1 in spinal tuberculosis [25-27]. In this study, we confirmed the contribution of *FokI* polymorphism in the vitamin-D receptor gene in the susceptibility to spinal tuberculosis, and explored *VDR* gene expression in intervertebral disc and its association with clinical severity. Importantly, we first identified the roles of *BMP-4* and *OPN* gene polymorphisms in the susceptibility to spinal tuberculosis and indication of clinical severity.

The structure and transcriptional regulation of *BMP-4* gene have been well documented [28]. The single nucleotide polymorphisms at -5826 G>A have been reported to influence *BMP-4* gene transcription, while 6007C>T polymorphism codes for an amino acid change from valine (with the T allele) to alanine (with the C allele) [29]. The critical roles of *BMP-4* in bone osteoblast differentiation, bone formation, and fracture healing have been well addressed [5, 6]. The CC genotype of *BMP-4* 6007C>T polymorphism was reported to be associated with lower total and intertrochanteric hip bone mineral density in postmenopausal women [30]. The lower frequencies of TT genotype of 6007C>T polymorphism have been associated with a high risk and poor outcome of cervical spondylotic myelopathy [5, 6]. In this study, rs-5826G > A and rs6007C>T polymorphisms of *BMP-4* gene were selected to evaluate their associations with the susceptibility and the clinical severity of spinal TB. Results showed significantly lower frequencies of TT genotype and T allele in the 6007C>T polymorphism in patients with spinal TB than that in healthy controls. Logistic regression analysis suggested that T allele carriage exhibits a protective effect against the occurrence of spinal TB. In addition, the TT genotype correlated with a low level of *BMP-4* mRNA and protein expression

Fig. 5. Correlations between BMP-4, OPN, and VDR protein level. The correlations between BMP-4, OPN, and VDR protein level in intervertebral disc tissues were analyzed in 96 spinal TB patients. A) BMP-4 protein levels negatively correlated with OPN protein levels. B) OPN protein levels negatively correlated with VDR protein levels. C) BMP-4 protein level positively correlated with VDR protein levels.

in the intervertebral disc, suggesting that 6007C>T polymorphism significantly affected the *BMP-4* gene expression. Moreover, low frequency of TT genotype significantly correlated with severe clinical symptoms (high GATA classification grade). The *BMP-4* mRNA and protein expressions in intervertebral disc tissues were significantly lower in the TT genotype of *BMP-4* 6007C>T carriers compared to CC and CT carriers and negatively associated with the clinical severity of spinal TB. Our findings suggest that the *BMP-4* genetic polymorphisms could be used as a biomarker for the susceptibility to spinal TB and a predictor of clinical severity of spinal TB.

Osteopontin is an abundant non-collagenous protein in bone matrix and participates in bone resorption and formation [31]. High OPN protein levels have been suggested as a risk factor for low bone mineral density in postmenopausal women [32]. *OPN* gene expression has been reported to be significantly influenced by the genetic polymorphisms at its promoter region [33]. Among the *OPN* gene polymorphisms, -156G>GG, -443C>T and -66T>G polymorphisms were mostly studied. The -443T>C is at the binding site of MYT1 zinc finger factor [34], while -156G>GG is at the binding site of RUNX2 [35]. Polymorphisms at these sites can affect the regulation of other proteins on *OPN* gene expression. The polymorphisms of the *OPN* -66T>G and -443C>T were suggested to be a risk factor of several inflammatory conditions, such as osteoarthritis [36]. In this study, the -66G>T, -156G>GG, and -443T>C polymorphisms were detected in patients with spinal TB. Our study indicated that the frequencies of -156G>GG and -443T>C polymorphisms in patients with spinal TB were not different from healthy controls. In contrast, higher frequency of the -66GG genotypic carriers of -66T>G polymorphisms was observed among the spinal TB patients. Logistic regression analysis suggested that G allele carriage is a risk factor for the occurrence of spinal TB. In addition, the GG genotype correlated with a high *BMP-4* mRNA and protein expression, whereas TT and TG correlated with a low *BMP-4* mRNA and protein expression in the intervertebral disc, suggesting that -66G>T polymorphism significantly affected the *OPN* gene expression. In addition, high frequency of GG genotype of -66G>T polymorphism significantly correlated with severe clinical symptoms. The *OPN* mRNA expression and cleaved protein level in the intervertebral disc tissues were significantly higher in GG genotype carriers compared to TG and TT carriers of -66G>T polymorphism. Both GG genotype, *OPN* mRNA and cleaved protein levels positively correlated with the clinical severity of spinal TB. Our findings suggest that the osteopontin genetic polymorphisms can be used as a biomarker for the susceptibility and a predictor of clinical severity of spinal TB. Besides the roles in bone metabolisms, osteopontin also functions as a pro-inflammatory cytokine [37]. Osteopontin is found to regulate inflammatory responses of T and B cells, macrophages, and neutrophils [38]. In the clinic, plasma OPN levels are found to correlate with chronic inflammatory diseases and autoimmune diseases [39]. However, a previous study in Japanese patients with pulmonary TB showed that the plasma OPN was significantly higher in TB patients than that in healthy controls, but the levels of both the full length OPN and cleaved OPN in TB patients were not significantly higher than that in the healthy controls [40]. In this study, OPN may also reflect the immune responses against the pathogens, but not pathogenicity. Thus, further study should verify its pathogenicity in animal models.

Vitamin D exerts various biological activities through its binding to intranuclear VDR. The *VDR* gene contains three polymorphic regions at the 3'-end and one polymorphic region at the start codon, which can be detected by *BsmI*, *ApaI*, *TaqI* and *FokI*, respectively [16, 17]. Many studies have reported the role of single nucleotide polymorphisms of the *VDR* gene as a risk factor of pulmonary TB. However, the association of *VDR* gene polymorphism with spinal TB was reported in only two studies with small sample sizes. One study showed that the frequencies of FF genotype of *FokI* polymorphism were significantly higher [18], but in another study, the frequency of ff genotype of *FokI* polymorphism was significant higher in patients with spinal TB compared to the healthy controls [19]. In this study, significantly lower FF and higher ff genotype were observed in spinal TB patients compared to the controls. Also, the f allele correlated with low *VDR* mRNA and protein expression in intervertebral disc tissues, and the *VDR* mRNA and protein expression in ff genotype carriers was significantly

lower than that in FF and Ff genotype carriers. The ff genotype, low *VDR* mRNA and protein expression in intervertebral disc tissues negatively correlated with the clinical severity. A previous study demonstrated that the plasma *VDR* mRNA expression were significantly decreased in Rheumatoid Arthritis and Systemic Lupus Erythematosus [41], which supports an association of low *VDR* gene expression level with pathogenesis.

As stated above, osteopontin, bone morphogenetic protein-4, and vitamin D receptor are three crucial molecules playing important roles in bone formation and metabolism. This study showed that in intervertebral disc tissues, BMP-4 protein levels negatively correlated with OPN, but positively correlated with *VDR* protein levels. Also, OPN protein levels negatively correlated with *VDR* protein levels. This finding suggested that expression of these three genes was closely associated with each other and collaboratively contributed to the pathogenesis and disease progression of spinal TB. This finding also suggests that multiple genetic factors are involved in the susceptibility and pathology of spinal TB.

In conclusion, the TT genotype of 6007C>T polymorphism in *BMP-4* gene and the FF genotype of *VDR-FokI* polymorphism are protectors from spinal TB, whereas the GG genotype of -66T>G polymorphism in *OPN* gene was a risk factor for the occurrence of spinal TB. These three genes are the susceptibility genes of spinal TB and they may collaborate in the development of spinal TB. The TT genotype of 6007C>T polymorphism, and low *BMP-4* mRNA and protein expression, the ff genotype of *VDR-FokI* polymorphism, and low *VDR* mRNA and protein expression, and the GG genotype of -66T>G polymorphism, and high *OPN* mRNA and protein expression are indicators of clinical severity in spinal TB patients.

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Disclosure Statement

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