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The study on polymorphisms of Sep15 and TrxR2 and the expression of AP-1 signaling pathway in Kashin-Beck disease

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ABSTRACT

The aim of the study was to investigate the association between rs5859 in Sep15, rs1139793 in TrxR2 polymorphisms with the risks of KBD and to detect the expression of AP-1 pathway in KBD subjects and in vitro. 208 KBD and 206 control subjects were included. PCR-Restriction Fragment Length Polymorphism (RFLP), Amplification Refractory Mutation Specific-PCR (ARMS-PCR) and Western Blotting were conducted. The results showed the minor A-allele frequency of rs5859 in KBD was statistically significantly higher than that in the control group ($P < 0.05$). The cases carrying A-allele had a 2-fold (95%CI: 1.064–3.956) increased risk of developing KBD compared with the G-allele carriers. There was no significant difference in genotype and allele distribution of rs1139793 between KBD patients and controls ($P > 0.05$). The frequency of the minor A allele of rs5859 was significantly different in Chinese healthy population compared with European, African and American. The frequency of the minor A allele of rs1139793 showed significant difference when compared with African and American. The levels of JunB, JunD, P65 proteins in KBD group were higher than those in control group ($P < 0.0001$). The expression of JunB, JunD, P65 proteins all increased in tBHP-induced C28/I2 oxidative damage model compared with control group ($P < 0.05$) and decreased after Se supplementation. Our finding indicated Sep15 is a possible candidate susceptibility gene for KBD. Combined with the in vitro study, our studies reveal novel insights into the mechanism of Se supplementation as an antioxidant via inhibiting the AP-1 signaling pathway in patients with KBD.

Kashin-Beck Disease (KBD) is known as an endemic, chronic and disabling osteoarthropathy, which was named after the two Russian Cossack doctors Nikolai Kashin and Evgeny Beck who first described it between 1859 and 1906 [1,2]. KBD occurs predominately in regions from Sichuan-Tibet Plateau to the northeast China, Eastern Siberia of Russia and North Korea [3–5]. One of the primary features of KBD is apoptosis and necrosis of chondrocytes resulting from excessive oxidative stress. Although the etiopathogenesis of KBD is still unknown, three major environmental etiologies of KBD have been suggested: Selenium (Se) deficiency, contamination of mycotoxins such as Trichothecene mycotoxin (T-2) and high humic acids in drinking water [6–8]. Epidemiological investigation of environmental risks has shown that selenium deficiency may contribute to the etiopathogenesis of KBD [9]. Se supplementation could significantly decrease the incidence of KBD [10]. Thus, it is considered that Se deficiency is a main environmental factor of KBD. However, the selenium supplementation cannot fully prevent the incidence of KBD [10]. Furthermore, an obvious familial aggregation of the distribution of KBD was found and the genetic factors account for > 25% of the risks of KBD [1,11]. These findings suggest that environment-gene interaction may play an important role in the development of KBD.

As a unique antioxidant, selenium exerts its biological activities as a constituent of selenium-containing proteins [12]. Currently, a total of 25 selenoproteins have been described in human, most of which are involved redox reactions protecting against the oxidative stress. Among them, the 15-kDa selenoprotein (Sep15) and Thioredoxin reductase 2

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(TrxR2) are similar with each other structurally and both of them exert the effect on cellular redox regulation and apoptosis. Our previous study found that Pro198Leu of Gpx1 might be a genetic risk factor in the development of KBD [5]. In recent years, associations between the SNPs of rs5859 (1125G/A) in Sep15 gene and rs1139793 in TrxR2 gene with risks of cancer, subcutaneous fibrosis and other diseases have been studied [13–18]. It indicated that single nucleotide polymorphism (SNP) in the genes encoding the selenoproteins may influence the susceptibility to KBD.

The previous studies have been reported that apoptosis of articular chondrocytes is one of the most common mechanisms resulting in cartilage destruction in KBD [17]. Additionally, SNP variants may affect the binding and expression of AP-1 signaling pathways [18]. AP-1 is comprised of members of the JUN and FOS proto-oncogene family and activated by the activation of proteins, involving the major pathways of ERKs, JNKs, and p38K. JNKs (Jun B, Jun D) plays a pivotal role in activation of a number of transcription factors interacting with the promoter regions of regulated genes which are integral to apoptotic pathway activation in chondrocytes. Taken together, in this study, we hypothesized that SNPs (rs5859 in Sep15 gene and rs1139793 in TrxR2 gene) and oxidative stress related signaling pathway AP-1(JUN B, JUN D and P65) may affect the articular chondrocytes in KBD.

In the present study, a classic case control study was designed and conducted to explore the relationship between polymorphisms of rs5859 in Sep15 gene and rs1139793 in TrxR2 gene and KBD in order to understand the pathogenesis of the disease. In addition, the role of AP-1 signaling pathway and P65 in tert-Butyl hydroperoxide (tBHP) induced chondrocytes oxidative damage and the protective effect of Na_2SeO_3 were studied.

1. Materials and methods

1.1. Study population

The study group included 208 KBD patients and 206 healthy controls, who were all Han Chinese and were from Shaanxi province of China. According to the national diagnosis of KBD (WS/T207-2010, Ministry of Health, people's Republic of China), KBD patients were recruited based on radiographic examination (X-ray of the right hand) and clinical diagnosis (degree I-III) from KBD areas (Linyou, Qianyang, Xunyi, Changwu, Yongshou and Long counties). The healthy controls with no signs or symptoms of arthritis or joint disease from Shaanxi Xi'an were randomly selected and frequency matched with KBD group by age and sex. The present study was approved by the Institutional Review Board of Xi'an Jiaotong University. Subjects were given a written informed consent before commencing the study.

1.2. Selection of SNPs

It has been reported that rs5859 of Sep15 could reduce the efficiency of selenium incorporation into Sep15 and the minor allele frequency of rs5859 is 4.9% (http://phase3browser.1000genomes.org/Homo_sapiens/Variation/Population?db=core; r = 1:87328025-87329025; v = rs5859; vdb = variation; vf = 5592). Therefore, rs5859 was included into our study.

The NCBI SNP database (national center of biotechnology information, NCBI)was referenced to obtain all the cSNPs information of TrxR. The bioinformatics databases and three bioinformatics programmers, namely, SIFT (http://sift.jcvi.org/), PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SNP3d (http://www.snps3d.org/) were employed to analyze and predict the impact of SNP changes on the structure and function of related proteins. SIFT score for TrxR2 rs1139793 was 0, predicting that the amino acid substitution is harmful to the protein. After running the PolyPhen, this variant was predicted to be benign. In addition, SNPs3d prediction scores(svm frofile score = −1.11 < 0 and svm structure score = −0.47 < 0)suggested a loss of hydrogen and the change of protein function. Therefore, Sep15 rs5859 and TrxR2 rs1139793 was selected as the candidate SNPs in this study.

Table 1
Primers of two SNPs for PCR-RFLP and ARMS-PCR.

SNPs	Primers (5′-3′)*
rs5859	Fw: CAGACTTGCGGTTAATTATG
	Rw: GCCAAGTATGTATCTGATCC
rs1139793	Fw(inner): GCCTCCCGGCCATGATCGTTA
	Rv(inner): GCGGCCTGAGCTGACACCAAC
	Fw(outer): GGACCAGATGCGCCCAGAGC
	Rv(outer): GCTCGGTGCCAGTGTTGGGG

* All the primers used in this study were synthesized by Beijing Liuhe Huada gene technology company (Beijing, China).

1.3. Genotyping analysis

Genomic DNA was isolated from whole-blood samples from 208 KBD patients and 206 controls using proteinase K method. Rs5859 in the Sep15 was detected by PCR-Restriction Fragment Length Polymorphism and rs1139793 in TrxR2 by Amplification Refractory Mutation Specific-PCR (ARMS-PCR). All the primers used in this study were synthesized by Beijing Liuhe Huada gene technology company (Beijing, China). Primers were seen in Table 1.

For rs1139793, PCR reaction was optimized to amplify the desired region at the following conditions: 94 °C for 3 min, following by 35 cycles at 94 °C for 30s, annealing 15 cycles of 94 °C for 30s, 67.5 °C(minus 0.4 °C each cycle) for 30s, 72 °C for 30s, 20 cycles at 94 °C for 30s, 61.5°Cfor 30s, 72 °C for 30s. About 1.5 μl DNA (0.30 μg/μl) was used as template in 12.5 μl reaction mixture containing 6.25 premix Taq, 0.2 μl of each outer primer, 1.2 μl of each inner primers DNA and 1.95 μl H_2O. Rs5859 polymorphism was determined in the Eppendorf gradient-type mastercycler (Eppendorf, Hamburg, Germany) with a total volume of 12.5 μl, containing 6.25 μl 2 × Taq PCR Master Mix, 0.5 μl each primer, 1.5 μl genomic DNA (0.30 μg/μl) and 3.75 μl double distilled water (ddH2O). PCR conditions were as follows: 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 51 °C for 30 s and extension at 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The amplification products from rs5859 were digested at 37 °C for 3 h with a *Bfa* I restriction endonuclease (Takara, Beijing, China).

All the PCR products and enzyme-digested products were loaded on 1.5% agarose gel and stained with ethidium bromide (0.5 mg/ml). The gel was visualized by a Light Transilluminator (UVP, Upland, CA, USA). Repeat genotyping of 20% of the DNA samples was performed randomly.

1.4. Western blotting

Total protein from whole blood and cartilage cells was extracted using Trizol method, quantified with BCA assay (Bi Yun Tian Company, China) and separated by SDS-PAGE (10% gels). After the separated proteins were transferred onto NC membranes (Millipore Ireland BV, Cork, Ireland), they were pre-incubated in blocking buffer (5% non-fat dry milk with Tween 20) for 60 min at room temperature, rinsed three times with TBST for 5 min followed by incubation with primary antibodies against JunB, JunD, p65 (1:1000, Sigma Company, USA) overnight at 4 °C. After washing, the membrane was incubated with secondary antibody (1:10 000, Pierce Company, USA) for 30 min. The blots were visualized by enhanced chemiluminescence and exposure to X-ray film. The signals were quantified by gel imaging system (GDS-8000). Each independent experiment repeated four times.

1.5. Oxidative damage of C28/I2 human chondrocytes induced by tBHP

C28/I2 chondrocytes (human articular chondrocytes cell line), provided by Dr. Mary B. Goldring from the Cornell University USA, were cultured in DMEM Ham's F-12 with a ratio of 1:1 (v/v) (DMEM/F 12; Hyclone, Logan, UT, USA) containing 12% fetal calf serum (SiJiQing Bio-Technique Co. Ltd., ZheJiang, China) and Penicillin-Streptomycin Solution (100×) in a humidified atmosphere with 5% CO_2 at 37 °C. The cell experiments included tBHP injury group (O, tBHP 300 μmol/l), low Se pre-protection group (OS1, 0.05 μg/ml Na_2SeO_3 + 300 μmol/l tBHP), high Se pre-protection group (OS2, 0.10 μg/ml Na_2SeO_3 + 300 μmol/l tBHP) and control group (C). These doses used here were based on the experiments published on our previous study [19]. Group OS1, OS2 were firstly pre-protected with different concentrations of Na_2SeO_3 for 24 h and then treated with 300 mmol/L tBHP for 24 h. Cell apoptosis was analyzed with Hoechst 33342 staining. Chondrocytes were stained with 2% Hoechst 33,342 in DMEM/F-12 contain 12% fetal bovine serum, followed by incubation at 37 °C for 30 min. Apoptotic chondrocyte with clear condensation and small bright nucleus was evaluated using a fluorescence microscope.

1.6. Statistical analysis

The values are presented as the mean ± SD. Distribution normality of quantitative data was analyzed using the Kolmogorov-Smirnov test. Differences of quantitative data without skewness between the KBD and control groups were measured using an independent sample Student's *t*-test.A one-way ANOVA test followed by the least significant difference test (LSD) for post hoc comparisons was performed for the apoptosis rates. Hardy-Weinberg equilibrium was assessed using Pearson's goodness-of-fit test. The case-control association studies were analyzed using x^2 tests on 2 × 2 and 2 × 3 contingency tables for genotype and allele frequencies, respectively. x^2 was carried out to calculate odds ratios, 95% confidence intervals (CI) and *P* values. Odds ratio (OR) and 95% confidence interval (CI) were calculated as an estimate of risk. All statistical analyses(Kolmogorov-Smirnov test, Student's *t*-test, Pearson's goodness-of-fit test, x^2 test, ANOVA) were performed using SPSS 20.0 statistical software; P values < 0.05 were considered statistically significant. All *P* values were two-sided.

2. Results

2.1. Demographic characteristics of the study groups

A total of 208 patients with KBD and 206 healthy controls matched frequently with age and gender were included in this study. The 208 KBD patients were further divided into I, II and III degrees based on clinical diagnosis. The baseline characteristics were shown in Table 2. There were no statistically significant differences observed between KBD and control group in age (47.9 ± 4.4 vs. 48.4 ± 6.7, $P = 0.664$) and gender (male/female, 114/94 vs. 135/61, $P = 0.245$).

Table 2
Characteristics of the study populations.

Characteristics	Cases ($n = 208$)	Control ($n = 206$)	Values	*P*
Sex, male/female	114/94	135/61	1.353	0.245
Age, mean ± SD	47.9 ± 4.4	48.4 ± 6.7	0.437	0.664
Degree				
I	37	–		–
II	55	–		–
III	116	–		–

No statistically significant differences were observed between KBD and control group in age and gender (all $P > 0.05$).

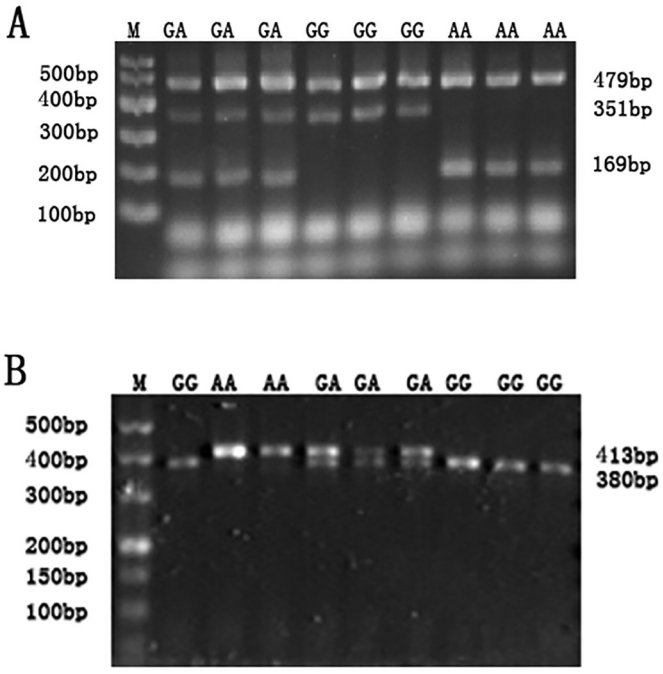

Fig. 1. Agarose gel electrophoretograms for polymorphism analysis. (A) The PCR product of TrxR is 479 bp in length. Two fragments of 479 and 351 bp for GG genotype, three fragments of 479, 351 and 169 bp for GA heterozygote, while 479 and 169 bp for AA genotype. (B) PCR fragment of Sep15 was digested with Bfa I (CTAG). The 413 bp PCR product was cleaved into two fragments of 380 and 33 bp in the presence of GG homozygous, three fragments of 413, 380 and 33 bp for GA heterozygote (the 33 bp fragment is typically not visible on the gels), while the AA homozygous remained uncleaved showing only the 413 bp PCR product.

2.2. Distribution of Sep15 rs5859 and TrxR2 rs1139793 genotype in KBD patients

In the present study, Sep15 rs5859 and TrxR2 rs1139793 were genotyped by PCR-RFLP and ARMS-PCR. Agarose gel electrophoretograms of the two SNPs for polymorphism analysis were shown in Fig. 1. The frequency of genotypes of rs5859 and rs1139793 in the controls did not deviate significantly from those expected under the Hardy-Weinberg equilibrium, suggesting that the samples we selected can be representative of population (Table 3).

The distribution of Sep15 rs5859 genotypes in two groups was shown in Table 4. The frequency of the GG, GA and AA genotypes for rs5859 was 88.9%, 8.7% and 2.5% in the patients with KBD, and 93.2%, 6.8% and 0.0% in the controls, respectively. The frequency of the G-allele and minor A-allele was 93.3% and 6.7% in patients with KBD, correspondingly, and 96.6% and 3.4% in the control group respectively. The x^2-test results showed that the frequency of GG, GA and

Table 3
Results of Hardy-Weinberg equilibrium.

SNPs	Oa			Eb			χ2	P^c
	A/G	G/G	A/A	A/G	G/G	A/A		
Rs1139793								
Control	76	71	10	76.1	67.0	13.9	3.090	0.079
KBD	50	40	13	49.9	44.0	9.1	0.186	0.666
Rs5859								
Control	14	192	0	13.5	192.2	0.2	0.255	0.614
KBD	18	185	5	26.1	180.9	0.9	20.086	< 0.0001

a: Observed frequency; b: Expected frequency; c: $P > 0.05$ means statistically compliance with H-W equilibrium.

Table 4
Comparison of genotype and allele frequencies for rs5859 and rs1139793 between KBD patients and controls.

SNPs	Control	KBD	P	OR[a]	OR. 95% C.I.[b]
rs5859 genotype(n)	206	208			
GG	192(93.2)	185(88.9)		1	–
GA	14(6.8)	18(8.7)	0.436	1.334	0.645, 2.761
AA	0(0.0)	5(2.5)	–	–	–
Allele					
G	398(96.6)	388(93.3)	0.029	1	–
A	14(3.4)	28(6.7)		2.052	1.064, 3.956
rs1139793 genotype (n)	157	103			
GG	71(45.2)	40(38.8)		1	–
GA	76(48.4)	50(48.5)	0.564	0.856	0.506, 1.450
AA	10(6.37)	13(12.6)	0.067	0.433	0.174, 1.078
Allele					
G	218(69.4)	130(63.1)	0.134	1	–
A	96(30.6)	76(36.9)		1.328	0.916, 1.924

[a] *OR*: Odds ratio; [b]: 95% C.I.: 95% confidence interval; The frequency of GG, GA and AA genotypes for rs5859 between the KBD and control had no significant difference ($P > 0.05$), the minor A-allele frequency in KBD was significantly higher than that in the control group ($P < 0.05$), the variant A-allele was associated with an increased risk for KBD (OR: 2.052, 95% CI: 1.064–3.956). No statistical differences were found in the frequency of genotypes and minor A-allele for TrxR2 rs1139793 between KBD patients and controls (all $P > 0.05$).

AA genotypes for rs5859 between the KBD and control had no significant difference ($P > 0.05$), however, the minor A-allele frequency in KBD was significantly higher than that in the control group (6.7% vs. 3.4%, $P < 0.05$). Compared with the G-allele, the variant A-allele was associated with an increased risk for KBD (OR: 2.052, 95% CI: 1.064–3.956).

The frequency of TrxR2 rs1139793 genotypes in KBD and control group was also examined (Table 4). There was no statistical differences, however, in individuals with KBD and controls (38.8% GG, 48.5% GA and 12.6% AA vs. 45.2%, 48.4% and 6.37%, respectively, $P = 0.188$). Although the frequency of minor A-allele was higher in KBD than that in control group, no significant difference was found (30.6%, 36.9%, respectively, $P = 0.134$).

2.3. Polymorphism of rs5859 and rs1139793 in different population

To examine if the polymorphism of rs5859 and rs1139793 are associated with specific population, the genotype and allele frequencies of rs5859 and rs1139793 in the control group were analyzed in Han Chinese in Beijing, Japanese in Tokyo, European, African and American published from the 1000 genomes (http://www.1000genomes.org/). As was shown in Table 5, the genotype and allele frequencies of rs5859 in the control group in the present study were similar to the results from Han Chinese in Beijing, Japanese in Tokyo($P > 0.05$), indicating that the control samples in the present study were representative. The differences of the genotype and allele frequencies, however, between the control group and the other people such as European, African and American were statistically significant ($P < 0.05$). Compared with African and American, the genotype and allele frequencies of rs1139793 from the control group were statistically significant ($P < 0.05$) and similar to the results from Han Chinese in Beijing, Japanese in Tokyo, and European. Risk allele frequencies of rs5859 and rs1139793 were shown in Fig. 2.

2.4. Association of KBD with AP-1 signaling pathway

The protein expression levels of AP-1 signaling pathway in whole blood from 20 KBD patients and 20 healthy controls were detected by Western blotting. Results showed the protein expression levels of JunB, JunD, P65 in KBD group were higher than those in control group ($P < 0.0001$), suggesting that AP-1 signaling pathway was up-regulated in KBD patients (Fig. 3).

2.5. Effects of selenium on expression of AP-1 and P65 in chondrocytes

We further conducted a tBHP-induced chondrocytes oxidative damage model to examine chondrocyte apoptosis and the role of AP-1 signaling pathway. The results showed that the rate of chondrocyte apoptosis (Table 6) and the protein expressions of JunB, JunD, P65 all increased in tBHP-induced C28/I2 oxidative damage chondrocytes compared with control group (all $P < 0.05$), while these were decreased after either low and high dose of Se treatment, indicating that the Se supplementation exerts beneficial antioxidant function on chondrocytes oxidative damage via AP-1 signaling (Fig. 4).

3. Discussion

In present study, Sep15, located primarily in endoplasmic reticulum, exhibits redox activity and is involved in protein folding mechanisms [20,21]. The SNP rs5859(1125 G/A) in 3′-UTR region of Sep15 was found to decrease the efficiency of the selenocysteine insertion sequence element and may influence expression levels of the selenoprotein gene product in a selenium-dependent manner [22]. Data from genetic association studies connecting variants in the Sep15 gene with the risk of diseases have shown that the investigated SNP may

Table 5
The genotype and allele frequencies for rs5859 and rs1139793 in different ethnic groups.

SNPs	Control	CHB	JPT	EUR	AFR	AMR
rs5859 genotype(n)	206	103	104	503	661	347
GG	192(93.2)	93(90.3)	96(92.3)	309(61.4)*	71(10.7)*	236(68.0)*
GA	14(6.8)	10(9.7)	8(7.7)	177(35.2)*	311(47.0)*	97(28.0)*
AA	0(0.0)	0(0.0)	0(0.0)	17(3.4)*	279(42.2)*	14(4.0)*
Allele						
G	398(96.6)	196(95.1)	200(96.2)	795(79.0)*	453(34.3)*	569(82.0)*
A	14(3.4)	10(4.9)	8(3.8)	211(21.0)*	869(65.7)*	125(18.0)*
rs1139793 genotype(n)	157	103	104	503	661	347
GG	71(45.2)	46(44.7)	47(45.2)	262(52.1)	601(90.9)*	133(38.3)*
GA	76(48.4)	42(40.8)	47(45.2)	202(40.2)	56(8.5)*	167(48.1)*
AA	10(6.37)	15(14.6)	10(9.6)	39(7.8)	4(0.6)*	47(13.5)*
Allele						
G	218(69.4)	134(65.0)	141(67.8)	726(72.2)	1258(95.2)*	433(62.4)*
A	96(30.5)	72(35.0)	67(32.2)	280(27.8)	64(4.8)*	261(37.6)*
				280(27.8)		

Control: Han Chinese in the present study from Shaanxi; CHB: Han Chinese in Beijing, China; JPT: Japanese in Tokyo, Japan; EUR: European; AFR: African; AMR: American; These data were from http://www.1000genomes.org/ except for controls. *: vs control: $P < 0.05$.

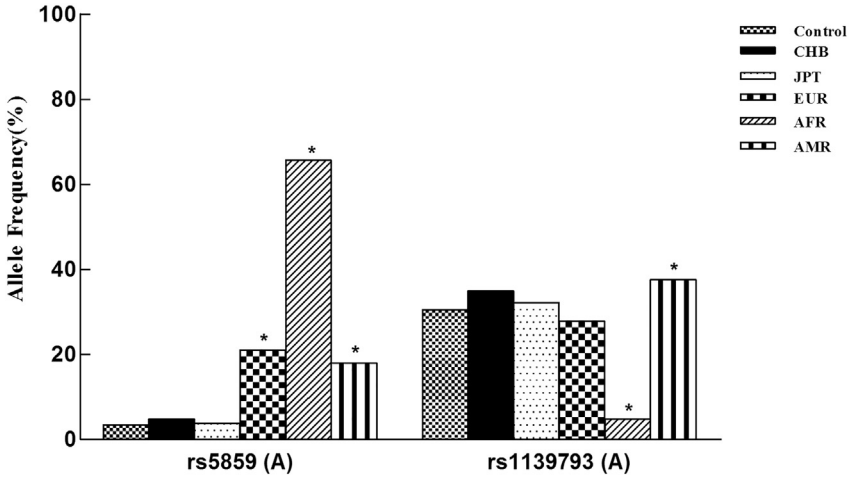

Fig. 2. The allele frequencies for rs5859 and rs1139793 in different ethnic groups. CHB: Han Chinese in Beijing, China; JPT: Japanese in Tokyo, Japan; EUR: European; AFR: African; AMR: American. There were statistically significant between the control group and populations such as European, African and American ($P < 0.05$). *compared with C group $P < 0.05$.

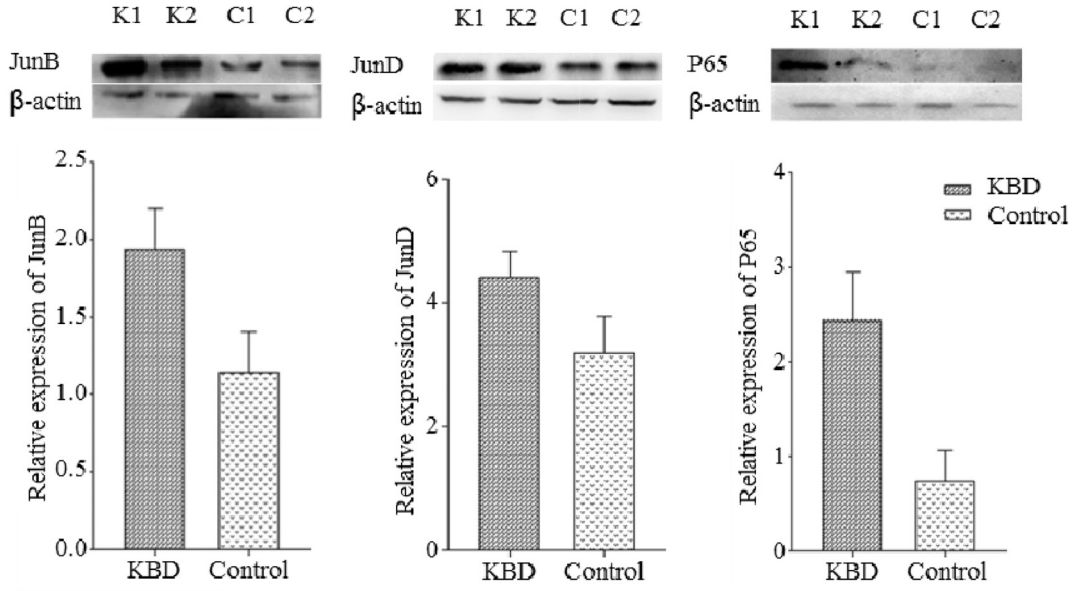

Fig. 3. The expression of AP-1 signaling pathway in KBD patients. The protein expression levels of JunB, JunD, P65 in KBD group were higher than those in control group ($P < 0.0001$), suggesting that AP-1 signaling pathway was up-regulated in KBD patients. *compared with C group $P < 0.05$.

Table 6
The apoptosis rates in each group.

Groups	Apoptosis rate (%)	*F*	*P*
C	0.78 ± 0.06	304.400	< 0.0001
O	92.27 ± 3.44*		
OS1	71.38 ± 5.22#		
OS2	44.31 ± 9.16#		

C: control group; O: tBHP injury group (tBHP 300 μmol/L); OS1: low Se pre-protection group (0.05 μg/mL Na_2SeO_3 + 300 μmol/L tBHP); OS2: high Se pre-protection group (0.10 μg/mL Na_2SeO_3 + 300 μmol/L tBHP); *Compared with C, $P < 0.05$; #Compared with O, $P < 0.05$. The rate of chondrocyte apoptosis was increased in O group compared with C group, while decreased in OS1 and OS2 groups.

increase the risk of developing multiple diseases. Association between selenium status and lung cancer risk in smokers in a case-control study was modified by the polymorphism of rs5859 [17]. The study about Korean population showed that the minor allele A for rs5859 was associated with increased rectal cancer risk in men [15]. It was reported that the cultured cells containing A instead of G allele is responsible for a lower responsiveness to Se [19,20]. Se deficiency has been recognized as a main environmental factor of KBD. In addition, TrxR2 deficiencies enhanced chondrogenic differentiation and apoptosis of ATDC5 cells, which could increase cell sensitivity to exogenous oxidative stress [23]. However, it remains unclear if the TrxR2 SNPs activates the apoptosis of articular chondrocytes and affects the occurrence of KBD. Therefore, we investigated the association between KBD risk and the two SNPs: rs5859 in Sep15 gene and rs1139793 in TrxR2 gene. The results showed that for the SNP rs5859 in the Sep15 gene, the minor A-allele frequency in KBD was statistically significant higher than that in control group. The cases carrying A-allele had a 2-fold increased risk of developing KBD compared to the G-allele carrier. Our results raised concern about the possible association between rs5859 of Sep15 with KBD and that minor A-allele could be a risk factor in patients with KBD. The frequency of TrxR2 rs1139793 genotypes also showed that the frequency of minor A-allele was higher in KBD than that in control group, suggesting that minor A-allele of rs1139793 in TrxR2 gene may increase the risk of KBD occurrence.

The results suggested that rs5859 was associated with the occurrence of KBD, but whether this was through an improper protein folding function of Sep15 caused by the variant allele and then lead to additional damage within the chondrocyte remains unknown. In addition, malignant mesothelioma cell lines with the A allele of rs5859 were less

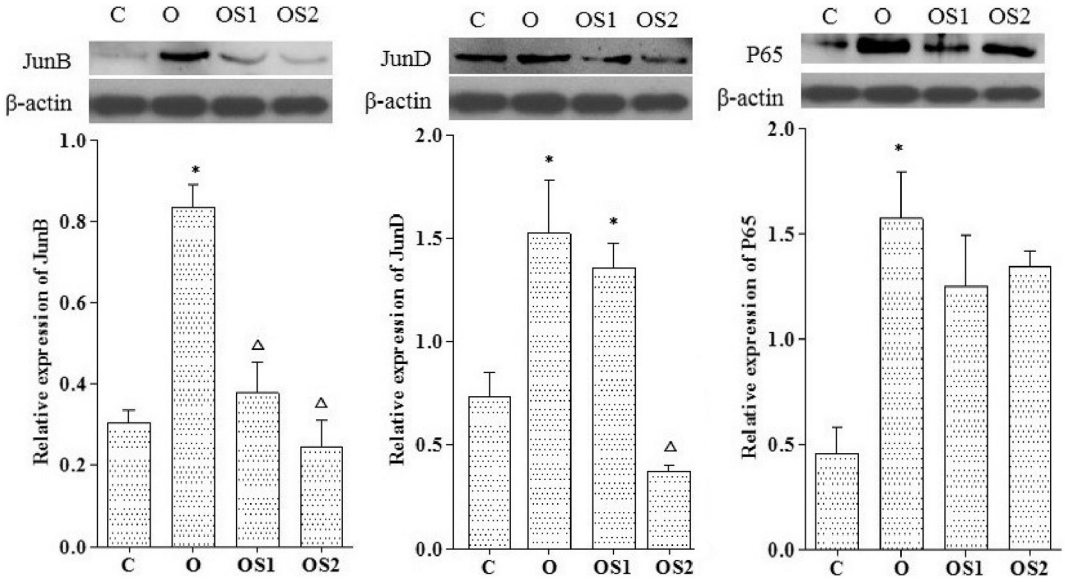

Fig. 4. The expression of AP-1 signaling pathway in chondrocytes. The protein expressions of JunB, JunD, P65 all increased in tBHP-induced C28/I2 oxidative damage chondrocytes compared with control group (all $P < 0.05$), while these were decreased after either low and high dose of Se treatment. C: control group. O: tBHP injury group (tBHP 300 μmol/L). OS1: low Se pre-protection group (0.05 μg/mL Na_2SeO_3 + 300 μmol/L tBHP). OS2: high Se pre-protection group (0.10 μg/mL Na_2SeO_3 + 300 μmol/L tBHP). *compared with C group $P < 0.05$; △compared with O group $P < 0.05$.

responsive to the growth-inhibiting and apoptotic effects of added selenium than those with the homozygous G genotype [19]. Therefore, we supposed that individuals carrying A allele could lead to abnormal expression of Sep15 protein and disorder of redox and apoptosis in KBD patients easily, but this hypothesis needs further investigation.

Comparative analyses were also conducted for frequency and allele distributions of the two SNPs with other populations from 1000 genomes (http://www.1000genomes.org). Ethnic difference was found in distribution of genotypes and alleles of SNP rs5859 in different healthy population groups. The minor A-allele frequency was similar with the two Asian populations (CHB and JPT), while showed significant difference when compared with other three populations (EUR, AFR and AMR). In the six populations listed, compared with the G, A was rare allele except the AFR population. As the first time proposing possible association between rs5859 of Sep15 and KBD, confirmation of this association is needed in other populations and in larger sample size. Moreover, further clinical and functional investigations are needed to confirm the present conclusion.

Researchers reported that SNP mutations may affect the binding and expression of AP-1 signaling pathways [18]. Cell experiment results suggested that oxidative damage could lead to up-regulation of AP-1 signaling pathway and p65, and the expression of AP-1 and p65 could be down-regulated after Na_2SeO_3 supplement. Previous studies have been reported that Na_2SeO_3 has cytoprotective effect due to its ability to resist oxidative damage [24]. The results showed that the expression of AP-1 was down-regulated by Na_2SeO_3, which might play important roles against apoptosis in chondrocytes.

In conclusion, the allele frequency of SNP rs5859 in the Sep15 gene differs statistically significant between KBD patients and healthy controls, and A allele is associated with increased KBD risk. There is no significant differences in either genotype or allele frequencies of rs1139793 in TrxR2 gene between healthy and KBD subjects. The minor A allele of rs5859 in Sep15 may be a possible candidate susceptibility gene for KBD, suggested that individuals carrying A allele could lead to abnormal expression of Sep15 protein and disorder of redox and apoptosis in KBD patients easily. Furthermore, Selenium might exert anti-apoptotic effects by mediating the expression of AP-1 signaling pathway.

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The authors' contributions are as follows: RP Wu, YY Li and RQ Zhang carried out the genotyping of the patients and the controls. RP Wu, YY Li, Wenyan Sun, and RQ Zhang participated in the statistical analysis and drafted the manuscript. XX Dai, YY Li, HM Wang, LX Han, JF Liu, XL Du, H Guo, XY Mo collaborated in collection of samples and acquisition of data. RP Wu and RQ Zhang completed the writing of the manuscript. YM Xiong conceived of the whole study and critically revised the manuscript. All authors read and approved the final manuscript.

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