

Selenoprotein P gene r25191g/a polymorphism and quantification of selenoprotein P mRNA level in patients with Kashin-Beck disease

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Kashin-Beck disease (KBD) is an endemic and deformable osteoarthritis. Epidemiological study has revealed that lower Se level is the principal environmental factor in the pathogenesis of KBD. Selenoprotein P (SEPP1) is a special selenoprotein, which is the primary form of Se *in vivo*. Our aim was to investigate the putative association of SEPP1 r25191g/a single nucleotide polymorphism (SNP) with KBD risk and the SEPP1 transcriptional levels in whole blood and articular cartilage tissue of KBD cases and controls, respectively. One hundred and sixty-seven cases with KBD and 166 control subjects from Shaanxi province of China were included in the present study. The detection of SNP r25191g/a in the 3' untranslated region was performed using an efficient technique, tetra-primer amplification refractory mutation system PCR. A quantitative analysis of SEPP1 mRNA in KBD and control groups by real-time PCR was also performed. The present results show no significant difference in genotype and allele distribution of SNP r25191g/a between individuals with KBD and controls ($P=0.279$ and 0.428 , respectively). There was also no association between SNP r25191g/a and risk of KBD (OR 1.153; 95% CI 0.533, 2.496). However, the frequency of the rare genotype AG of SNP r25191g/a was significantly lower in Chinese population than in the Caucasians. It was shown that the SEPP1 mRNA expression in whole blood was lower in KBD patients than in the control group (0.149-fold, $P<0.001$), but that it was much higher in articular cartilage tissue (4.53-fold, $P=0.012$). Our aim was to lay a foundation for us to further study the association between the pathogenesis of KBD and SEPP1.

Kashin-Beck disease: Selenoprotein P: Polymorphisms: mRNA

Kashin-Beck disease (KBD) is a chronic endemic osteoarthropathy with articular cartilage degeneration and necrosis. It occurs predominately in the northern part of China, Russia and a few northern areas of North Korea⁽¹⁾. Presently, the aetiology is not clear. Epidemiological study has revealed that low Se may play a crucial role in the pathogenesis of KBD^(2–4). Essential trace element Se exerts its biological functions *in vivo* mainly through selenoproteins. Selenoprotein P (SEPP1) is one of the rather special selenoproteins. In recent years, the study of SEPP1 is receiving increasing attention, especially the possible association between genetic polymorphisms of SEPP1 and disease susceptibility.

Recently, two relatively common single nucleotide polymorphisms (SNP) of SEPP1 have been reported. It was shown that one SNP, which is located in the 3' untranslated region (position 25 191) may predict the behaviour of biomarkers of Se status and response to supplementation and thus susceptibility to the disease⁽⁵⁾. The results obtained from the studies done on trypanosomiasis indicate that SEPP1 plays a role in protection against infectious illnesses⁽⁶⁾. Three variants of SEPP1 gene (rs3797310,

rs2972994, –4166) were significantly associated with advanced adenoma risk⁽⁷⁾.

Selenocysteine is the active form of Se that is responsible for various important biological functions of SEPP1. Furthermore, the SEPP1 mRNA contains two functional selenocysteine elements in its 3' untranslated region⁽⁸⁾. Normally, the SEPP1 is down-regulated in a subset of human prostate tumours, mouse tumours and prostate carcinoma cell lines⁽⁹⁾. There is a significant reduction or loss of SEPP1 mRNA expression in colon cancers⁽¹⁰⁾. Although all these genetic studies suggest a possible association of some SEPP1 SNP in individuals with some disease, the association between SEPP1 gene polymorphism and susceptibility to KBD and the SEPP1 mRNA expression level in different tissues have not been studied.

Therefore, the present study was designed to investigate the role of polymorphism in the 3' untranslated region of the SEPP1 gene, which is at position 25 191 and described as r25191g/a, in KBD patients in endemic areas of Shaanxi province of China, and to detect the SEPP1 mRNA level in whole blood and articular cartilage tissue of the KBD patients and healthy subjects, respectively.

Abbreviations: ARMS PCR, amplification refractory mutation system PCR; KBD, Kashin-Beck disease; SEPP1, selenoprotein P; SNP, single nucleotide polymorphism.

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Materials and methods

Study population

The study group consisted of 167 KBD patients (eighty-nine males and seventy-eight females; mean age 52.1 (SD 5.4) years) and 166 healthy controls (eighty-one males and eighty-five females; mean age 52.2 (SD 4.6) years), who were all Han Chinese and were from the same geographical area (Shannxi, China). KBD was diagnosed on the basis of clinical and radiological findings according to the national diagnostic criteria of KBD. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Human Ethics Committee of Xi'an Jiaotong University, People's Republic of China. Written informed consent was obtained from all the subjects.

The blood samples were collected from the KBD and control groups. Articular cartilage tissue was also obtained from knee joints from a total of nine KBD patients (during excision of corpus liberum) and three control subjects (during joint replacement surgery).

Genotype analysis

Genomic DNA was isolated from whole-blood samples from 167 KBD patients and 166 unrelated volunteers, and was extracted by the traditional phenol/chloroform extraction method. Optimisation of tetra-primer amplification refractory mutation system PCR (ARMS PCR) for the detection of SNP r25191g/a was done empirically using the primers designed by the original software available on the website: http://cedar.genetics.soton.ac.uk/public_html/primer.html.

The PCR primer sequences were as follows: forward inner primer (G allele), 5'-TGACCTTCAAATAAATATTTAAA-ATCGG-3', and reverse inner primer (A allele), 5'-TGTGTC-TAGACTAAATTGGGGAGTATTTT-3'; forward outer primer (5'-3'), GAGGAGAACATAACTGAATCTTGT-CAGT-3', and reverse outer primer (5'-3'), 5'-CTCCATCAT-AAAAAATATGGTTTGGAGTC-3'. The G and A alleles at SNP r25191g/a in the 3' untranslated region of the SEPP1 gene were identified using the tetra-primer ARMS PCR methodology. Two primers recognised 'G' and 'A' alleles, and the other two primers were used for inner control. PCR was carried out using an Eppendorf gradient type mastercycler (Eppendorf, Germany) with a total volume of 12.5 µl, containing 6.25 µl of 2 × Master Mix, 0.5 µl of each primer (10 µM), 1.5 µl of genomic DNA and 2.75 µl of H₂O. PCR were performed under the following conditions: 95°C for 3 min, followed by thirty-five cycles at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s, and final elongation step at 72°C for 5 min.

The amplified ARMS PCR products (PCR product size: GG = 213 bp and AA = 183 bp, and PCR control fragment size: 338 bp) were identified by gel electrophoresis, which was carried out on 2% agarose gels stained with ethidium bromide (0.5 µg/ml).

Quantitative real-time PCR

Real-time PCR was used to assess SEPP1 mRNA expression from a total of twenty whole-blood samples randomly

(KBD: 10, and control: 10) and twelve articular cartilage tissue samples (KBD: 9, and control: 3).

Total RNA was isolated from 3 ml of whole blood and 100 mg of articular cartilage tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA were reverse transcribed using the RevertAid™ First-Strand cDNA Synthesis Kit (MBI, Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions to obtain first-strand complementary DNA.

The mRNA quantification of SEPP1 in whole blood and articular cartilage tissue was performed on iQ™5 Real-Time PCR Detection Systems (Bio-Rad, Philadelphia, PA, USA) using BioEasy SYBR Green I Real-Time PCR Kit (Bioer, Hangzhou, People's Republic of China) according to the manufacturer's instructions. Amplification primers that were used are as follows: SEPP1 (forward: 5'-GATGGAGCAACT-GAAAGGTG-3', and reverse: 5'-CCCCTAGGTCATAGTT-TACG-3')⁽⁹⁾ and β-actin, internal standard (forward: 5'-GAA-CGGTGAAGGTGACAGCAG-3', and reverse: 5'-GTGGAC-TTGGGAGAGG ACTGG-3'). Reactions were performed in a 20 µl mixture containing 2 µl of complementary DNA, 0.5 µl of each primer (10 µM), 12.5 µl of 2 × SYBR Mix (with 4.0 mM Mg²⁺), 0.15 µl of Taq DNA Polymerase and 9.35 µl of double distilled H₂O under the conditions of initial denaturation at 94°C for 2 min, followed by forty cycles of denaturation at 94°C for 10 s, annealing at 58°C for 15 s and extension at 72°C for 30 s. All reactions were performed in duplicate. Results were normalised with total levels of β-actin expression, and analysed using iQ™5 software (version 2.0; Bio-Rad) and SPSS 13.0 (SPSS, Chicago, IL, USA).

Statistical analysis

All data were analysed using SPSS software version 13.0. Deviation from the Hardy–Weinberg equilibrium was analysed by χ^2 goodness-of-fit test. The distribution normality was analysed using the Kolmogorov–Smirnov test. Differences without skewness between the KBD and control groups were assessed by two-tailed Student's *t* test. Categorical variables were presented using frequency counts and compared by χ^2 test. All *P*-values were of two ways at 0.05% level of significance. The risk of developing the disease was expressed as OR and 95% CI.

Results

Genotype analysis

To evaluate the accuracy of the assigned SEPP1 genotypes, a total of fifty samples were randomly chosen, and the results were confirmed by repeating the ARMS PCR. Notably, no discrepant results were detected when the ARMS PCR assay was repeated using the same samples. The genotype distribution of the SNP r25191g/a in patients with KBD and the control group was in the Hardy–Weinberg equilibrium ($P > 0.05$).

In the present study, we analysed SNP r25191g/a in 333 individuals. The genotype and allele frequencies of individuals for SNP r25191g/a were determined. As shown in Table 1, no significant difference in the distribution of the genotype of SNP r25191g/a was found between the individuals with KBD and controls (50.9% G/G, 40.1% G/A and 9.0%

Table 1. Single nucleotide polymorphism (SNP) r25191g/a genotype and allele frequencies in patients, controls and other populations and risk of developing Kashin-Beck disease (KBD)

(No. of subjects and percentages; odds ratios and 95 % confidence intervals)

SNP r25191g/a	Controls* (n 166)		Caucasian† (n 46)		South Asian† (n 47)		KBD (n 167)		P	OR	95 % CI
	n	%	n	%	n	%	n	%			
Genotype											
GG	98	59.0	22	47.8	29	61.7	85	50.9	0.279	1.000	
GA	53	32.0	21	45.6	16	34	67	40.1		1.457	0.917, 2.315
AA	15	9.0	3	6.5	2	4.3	15	9.0		1.153	0.533, 2.496
Allele											
G-allele	249	75.0	65	70.7	74	78.7	237	71.0	0.428	1.000	
A-allele	83	25.0	27	29.3	20	21.3	97	29.0		1.153	0.533, 2.496

* Control groups in the present study.

† Data reported by Calvo Alfonso *et al.*⁽⁹⁾.

A/A v. 59.0, 32.0 and 9.0 %, respectively, $P=0.29$), but the genotype frequency of GA heterozygote tended to be higher in patients with KBD than in the controls. The distribution of the A allele frequencies was also not different between cases and controls (29.0 v. 25.0 %, respectively, $P=0.428$). The OR value showed no association between the SNP r25191g/a and risk of KBD (OR 1.153; 95 % CI 0.533, 2.496). In addition, when the data were adjusted for potential confounding factors (adjusted for age and sex) also, no significant association was found (data not shown).

Furthermore, the distributions of the SEPP1 genotype and allele in the control groups were compared with that in other population groups (Table 1). Interestingly, the genotype distribution for the homozygous GG and AA genotypes for the Chinese population were similar to those reported for the Caucasian and South Asian populations⁽¹⁰⁾. In all ethnic groups, the frequency of the AA genotype was rare compared with the GG homozygote or GA heterozygote. However, the distribution of the heterozygous GA genotype was notably different in the Caucasian group than in the Chinese control group (46 v. 32 %). In addition, a decreased frequency of the SEPP1 gene A allele and an increased frequency of G allele were observed in the Chinese population group than in the Caucasian group.

Quantitative real-time PCR

The SEPP1 mRNA expression in the whole blood was lower in the KBD group than in the control group (0.149-fold), but it was much higher in articular cartilage tissue in the KBD group than in that in the control group (4.525-fold). The results indicate that differences were highly significant ($P<0.001$ and $P=0.012$, respectively) in both whole blood and articular cartilage tissue between the two groups (Fig. 1).

Discussion

Genotype analysis

SEPP1, found predominately in the plasma, is also expressed in other tissues and is presumably secreted by them⁽¹¹⁾. Some authors propose that it might also act as an antioxidant and heavy-metal chelator in the extracellular matrix⁽¹²⁾. With the identification of a complex repeat structure within the SEPP1 promoter and analysis of this regulatory DNA sequence,

a complex repeat structure within the SEPP1 promoter may be of functional relevance to SEPP1 gene expression⁽¹³⁾.

Epidemiological studies have shown that the distribution of KBD was identical to that found in Se-deficient regions in China, and that children in KBD-endemic areas who are at the risk of developing the disease come under the nutritional status of Se deficiency with lower Se in blood, urine and hair compared with children free from the disease in endemic areas⁽¹⁴⁾. Therefore, we aimed to clarify whether polymorphisms of SEPP1, a special selenoprotein, were correlated with KBD risk. According to the present results, we were unable to confirm this possibility and found no correlation between SNP r25191g/a in SEPP1 gene and the risk of developing KBD among the Chinese population. As this report is the first to investigate the association of SNP r25191g/a in SEPP1 gene with KBD and the number of subjects in the present is limited, further study is necessary to draw a conclusion.

In the present study, we compared the distribution of our control study group with that of other populations to identify whether the distribution of the SNP r25191g/a in

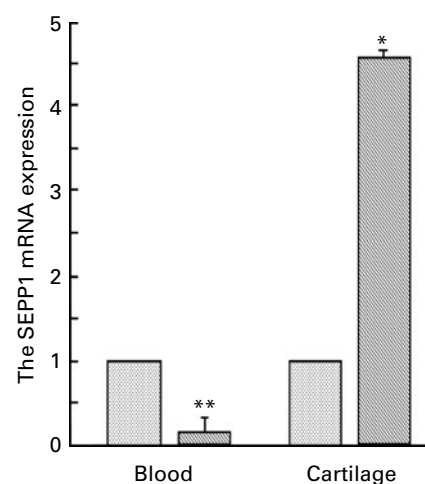


Fig. 1. The selenoprotein P (SEPP1) mRNA expression in whole blood and articular cartilage tissue. The SEPP1 mRNA expression in whole blood was lower in the Kashin-Beck disease (KBD) group (□) than in the control group (■) (0.149-fold), but it was much higher in articular cartilage tissue of the KBD group than in that of the control group (4.525-fold). Mean value was significantly different from that of the control group: * $P=0.012$, ** $P<0.001$.

SEPP1 genes is different in other healthy population groups (Table 1). The distribution of the GA and AA genotypes at position SNP r25191g/a in SEPP1 genes in the present control study group was different from that found in a Caucasian group, and was similar to that observed in a South Asian population. Although this result should be considered with caution as the sample size of the Caucasian group was very small, the data highlight the possible variability of protein gene frequencies in different population groups and hence influence the disease outcome. Further study is necessary as the distribution of the AG of SNP r25191g/a was notably different between the control groups of Chinese and some other populations. At the same time, some genetic polymorphisms associated with an increased risk of multi-factorial diseases occur only among particular ethnic populations, and other co-factors such as environmental factors, viral load, lifestyle factors and other transmitted diseases have also been established as risk co-factors for KBD. It is recommended that the same study be carried out in other ethnic populations with KBD.

Selenoprotein P mRNA expression

The results of real-time PCR study revealed a lower expression of SEPP1 mRNA in whole blood in KBD patients than in that in the healthy controls, with a higher expression in articular cartilage tissue. SEPP1 is a Se-rich plasma protein that supplies Se to the brain, testis, liver and other tissues. On account that SEPP1 is the major plasma selenoprotein, the synthesis of SEPP1 is a priority compared with that of glutathione peroxidase in supplementary Se; SEPP1 has ten selenocysteine, which may be more important in storage, transport and maintenance of Se homeostasis, so the concentration of plasma SEPP1 can better reflect the status of human Se⁽¹⁵⁾. In the present study, the expression of mRNA in whole blood decreased in the KBD group than in that in the control group (0.149-fold, $P < 0.001$) (Fig. 1), indicating that the KBD patients were in a condition of Se deficiency. Under the condition of Se deficiency, GSH metabolism is affected and glutathione peroxidase activity decreases, which will increase the oxidation to damage bone cells and articular cartilage tissue⁽¹⁶⁾.

The mRNA expression of lesioned cartilage tissue increased in the KBD group (4.525-fold, $P = 0.012$) (Fig. 1), and this result makes sense. SEPP1 binds to ~60% of the Se in the plasma⁽¹⁷⁾. SEPP1 is thought to mediate two important functions: (a) the protection of tissues against oxidative stress and (b) the transport of Se in serum and possible intracellular binding of Se⁽¹⁸⁾. Therefore, the SEPP1 in the whole blood could be transported to the impaired cartilage tissue in order to play the role of an antioxidant. As described previously, KBD is a chronic osteoarthropathy with articular cartilage degeneration and necrosis. Thus, the significant increase in SEPP1 mRNA levels in impaired articular tissues of KBD patients may be a reflection of cartilage cell damage repair. In this process, some specific indistinct cytokines and receptors may play a crucial role. Several studies have indicated that SEPP1 mRNA and protein expression can be influenced by different cytokines such as IL-1b, TNF- α , transforming growth factor- β 1 and interferon- γ ^(19,20). So, additional multi-centre follow-up studies such as SEPP1 expression detection are necessary to confirm the difference in SEPP1 mRNA level in different tissues between KBD patients and controls.

The present study found no correlation between SNP r25191g/a in SEPP1 gene and the risk of developing KBD among the Han Chinese population. However, the frequency of the rare high-producing allele AG of SNP r25191g/a was significantly lower in the Chinese population than in the Caucasian group. SEPP1 mRNA in the KBD group was down-regulated in blood and up-regulated in articular cartilage tissue (0.149-fold and 4.525-fold, respectively), and the difference was statistically significant ($P < 0.001$ and $P = 0.012$, respectively). Considering the interaction of SEPP1 mRNA expression with some specific indistinct cytokines and receptors, a clear understanding of the underlying association between the pathogenesis of KBD and SEPP1 is required.

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