Association study between polymorphisms in selenoprotein genes and susceptibility to Kashin-Beck disease

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Summary
Objectives: Kashin-Beck disease (KBD) is a disabling osteoarthropathy involving growth cartilage endemic to selenium (Se)-deficient regions in China. Associations between genetic variation in selenoprotein genes and susceptibility to many diseases have recently been investigated but few studies have been performed on KBD. We found four genetic polymorphisms in selenoprotein genes and assessed their association with increased susceptibility to KBD.

Methods: Four polymorphisms including GPX1 (rs1050450), TrxR2 (rs5748469), SEPP1 (rs7579) and DIO2 (rs225014) were analyzed for 161 KBD patients and 312 controls using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or tetra-primer amplification refractory mutation system PCR (Tetra-primer ARMS PCR). Glutathione peroxidase (GPX) activity in whole blood was measured using a GPX assay kit. The mRNA expression of GPX1, nuclear factor-κB (NF-κB) p65 and p53 in both whole blood and articular cartilage tissue were detected using Real-Time PCR.

Results: The genotypic and allelic frequency of GPX1 Pro198Leu was significantly different between KBD patients and controls (P = 0.013, 0.037). A significant increased KBD risk was observed in individuals with Pro/Leu or Leu/Leu (odds ratio = 1.781; 95% confidence interval: 1.127–2.814) compared with Pro/Pro. No association was observed between the other three single nucleotide polymorphisms (SNPs) and KBD risk. In addition, GPX enzyme activity in whole blood was lower in the KBD group (P < 0.01), and the GPX activity in whole blood decreased significantly in a subgroup of individuals representing Pro/Leu and Leu/Leu compared to Pro/Pro (P < 0.01). In whole blood and articular cartilage tissue samples of KBD patients, GPX1 and NF-κB p65 mRNA levels were lower (P < 0.01) while p53 levels were higher (P < 0.001).

Conclusion: GPX1 Pro198Leu is a potential genetic risk factor in the development of KBD and the GPX1 Leu allele is significantly associated with higher KBD risk among the Chinese Han population and with lower GPX enzyme activity. The expression of apoptosis related molecules in KBD patients significantly differs from controls.

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Introduction
Kashin-Beck disease (KBD) is a disabling osteoarthropathy involving growth cartilage that is endemic to certain areas of China, south-east Siberia and North Korea. The disease mainly affects children or teenagers, causing symmetrical thickening and deformity of joints of limbs which finally results in disturbed skeletal development. One of the most typical pathological characteristics of KBD is chondrocyte apoptosis and necrosis. Although its etiopathogenesis is still obscure, epidemiological studies demonstrate KBD is mainly common in low selenium (Se) areas where patients are in a Se-deficient condition. Furthermore, Se supplementation was effective in preventing a worsening of metaphysis change and in promoting repair. Therefore, Se
deficiency has been suggested to be an environmental factor causing KBD. However, KBD is focally distributed and shows a clustering of disease in families, and there is no KBD in some low Se areas such as Yunnan province in China and in Finland. Furthermore, Se supplementation could not entirely prevent KBD. These suggest that Se is not the only causative agent of KBD and that there must be some additional mechanism playing a role in KBD development.

Some susceptibility genes might lead to changes in susceptibility to environmental factors such as Se deficiency or other biological factors. It is likely that many of the effects of Se are mediated through its role as a constituent of Se-containing proteins. Selenium proteins contain the unusual selenocysteine amino acid residue incorporated by a specific tRNA. Ablation of this tRNA in mice results in chondronecrosis and abnormal skeletal development reminiscent of KBD. Also, Ebert et al. have found selenoproteins were important in bone metabolism since Se deficiency was associated with osteoarthropathy in humans. In the present study, we selected the four selenoproteins [e.g., glutathione peroxidases (GPXs), thioredoxin reductases (TrxRs) and selenoprotein P (SEPP)] responsible for antioxidative defense and maintaining a circulating level of thyroid hormone and regulation of bone metabolism. In addition to its antioxidative and bone metabolism regulating effects, these selenoproteins may be involved in cell signaling and reducing inflammatory processes. It has been reported that the cartilage degeneration and apoptosis induction pathways might be more important in KBD chondronecrosis. Some studies have demonstrated that p53 plays an important role in initiating apoptosis and that p53 gene expression is modulated by nuclear factor-xB (NF-xB) p65.

It is important to study genetic variations of the selenoproteins that result in enhanced susceptibility to oxidative stress. Compelling evidence suggests there is an association between variants of the selenoprotein genes and an increased risk of cancer and other diseases, but few studies have been performed on the relationship between genetic polymorphisms in selenoproteins and susceptibility to KBD. We conducted a case-control study to explore the associations between KBD risk and four selenoprotein genetic polymorphisms [GPX1 (rs1050450), TrxR2 (rs5748469), SEPP1 (rs7579) and DIO2 (rs225014)] in a Chinese Han population which had not been previously studied. Furthermore, we also investigated GPX activity and mRNA expressions of GPX1, NF-xB p65 and p53. It is hoped that this study would help us screen for KBD susceptible genes and understand the molecular mechanism of chondrocyte apoptosis and necrosis in KBD.

Material and methods

Study population

According to the national diagnostic criteria of KBD of China (GB 16003-1995), 161 patients were enrolled in the study based on radiography examination (X-ray of the right hand) and clinical diagnosis (degree I-III) from six counties in KBD endemic areas (Xunyi, Linyou, Yongshou, Qianyang, Long and Changwu counties). A total of 312 healthy subjects with no signs or symptoms of arthritis or joint disease from Xi’an city were randomized to serve as a control group and were frequency matched by age and sex. All subjects in this study were Han Chinese and resided in Shaanxi Province, P.R. China. The study was performed in accordance with the Declaration of Helsinki and approved by the Human Ethics Committee of Xi’an Jiaotong University, P.R. China. Informed written consent was obtained from each subject.

Sample collection

Fresh blood (5 mL) was collected from the antecubital vein of all subjects in the fasting state. The blood samples were used for measurement of GPX activity in whole blood and for isolation of DNA and RNA. Articular cartilage tissue was obtained from knee joints from a total of nine KBD patients in excision of corpus librum and three control subjects during joint replacement surgery and immediately snap-frozen in liquid nitrogen and stored at −70°C until analyzed.

Genotyping analysis

DNA was isolated from peripheral blood lymphocytes using classical phenol/chloroform extraction. Four polymorphic variants of GPX1 (rs1050450), TrxR2 (rs5748469), SEPP1 (rs7579) and DIO2 (rs225014) were identified using PCR-restriction fragment length polymorphism (PCR-RFLP) and tetra-primer amplification refractory mutation system PCR (Tetra-primer ARMS PCR). Primers, annealing temperature and restriction endonucleases used in the study are listed in Table I. The reaction details are as follows:

The GPX1 (rs1050450) polymorphism was determined by PCR-RFLP using an Eppendorf gradient type mastercycler (Eppendorf, Germany) with a total volume of 12.5 μL containing 6.25 μL 2× Taq PCR MasterMix, 0.5 μL each primer (10 μM), 1.5 μL genomic DNA and 3.75 μL H2O. The reaction conditions were: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, and a final elongation step at 72°C for 5 min. The amplification products were digested with Apa I restriction endonuclease (Fermentas, MBI, Bioanalytical System, USA) and immediately snap-frozen in liquid nitrogen and stored at −70°C until analyzed.

Table I

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Primers*</th>
<th>Ta (°C)</th>
<th>REs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX1 (rs1050450)</td>
<td>Fw: 5'-TCCAGACCATGATGACTGAG-3'</td>
<td>58</td>
<td>Apa I</td>
</tr>
<tr>
<td></td>
<td>Rv: 5'-ACTGGCATAAACCAGACAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TrxR2 (rs5748469)</td>
<td>Fw: 5'-GGTGGAGCAGCTTGGTGCCT-3'</td>
<td>65</td>
<td>BsuR I</td>
</tr>
<tr>
<td></td>
<td>Rv: 5'-CCACACTCTTCTGGACCTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEPP1 (rs7579)</td>
<td>Fw(inner): 5'-TGACCCTCAAATACATAATACATCGG-3'</td>
<td>58</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Rv(inner): 5'-TGCTCTACATAATACATCGGAGGATTTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fw(outter): 5'-GACAGACATACAGTACCTGTCAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv(outter): 5'-CTTCACTATACAAAATATGTTGAGCAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIO2 (rs225014)</td>
<td>Fw: 5'-GATAGAAAGAATTAAACACCGTTCGCT-3'</td>
<td>58</td>
<td>Afl I</td>
</tr>
<tr>
<td></td>
<td>Rv: 5'-CAGCTATCTCTCTCGGATACCA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ta, annealing temperature; RE, restriction enzymes.
*All Primers used in this study were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China).
Lithuania) according to the manufacturer’s instructions with slight modification.

The TrxR2 (rs5748469) polymorphism was genotyped by PCR-RFLP with a total volume of 30 μL containing 0.12 μL Taq polymerase (5 U/μL), 3.0 μL 10 x PCR Buffer, 1.8 μL MgSO4 (25 mmol/L), 0.6 μL dNTP (10 mmol/L each), 0.18 μL each primer (100 μmol/L), 3 μL genomic DNA and 21.12 μL H2O. The reaction conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 30 s, and with a final elongation step at 72°C for 10 min. The amplification products were digested with BsuRI restriction endonuclease (New England Biolabs, China).

The genotyping of SEPP1 (rs7579) was performed using Tetra-primer ARMS PCR assay and both pairs of primers were used in the same PCR reaction. A 338 bp product was used for genotyping with the inner primers to selectively generate the corresponding polymorphism product. PCR was carried out with a total volume of 12.5 μL containing 6.25 μL 2 x Taq PCR MasterMix, 0.5 μL each primer (10 μM), 1.5 μL genomic DNA and 2.75 μL H2O. Thermocycling were the same as for GPX1 (rs1050450).

The DIO2 (rs225014) polymorphism was determined by PCR-RFLP. The PCR reaction was the same as for GPX1 (Pro198Leu). The amplification products were digested with Afa I restriction endonuclease (Takara, Japan).

DNA fragments were separated by 2% agarose electrophoresis stained with 0.5 μg/mL ethidium bromide and visualized with a Light Transilluminator (UVP, USA). In addition, about 20% of the DNA samples were randomly selected for re-genotyping.

GPX activity assays

The total GPX activity in whole blood was measured using the GPX assay kit (Nanjing Jiancheng Bio-Tek Co., Nanjing, China) according to manufacturer’s instructions. OD values were recorded at 412 nm on a model 752 ultraviolet and visible spectrophotometer (Beijing, China). One activity unit was defined as the concentration which consumed at 1 μmol/L of glutathione (GSH) in the reaction system per 4 μL whole blood reacted at 37°C for 5 min after subtracting non-enzymatic reaction.

Quantitative Real-Time PCR

A total of 24 blood samples and 12 articular cartilage samples were collected for mRNA quantification of GPX1, NF-κB p65 and p53 respectively. Total RNA was isolated from 3 mL whole blood or 100 mg articular cartilage tissues respectively using TRizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. Total RNAs were reverse transcribed using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, MBI, Lithuania) according to manufacturer’s instructions in the thermocycler to obtain the first strand cDNA.

The mRNA quantification of GPX1, NF-κB p65 and p53 was performed on iQ™SYBR Green 1 Real-Time PCR Kit (Bioer, Hangzhou, China) according to manufacturer’s instructions. Amplification primers are shown in Table II. Reactions were performed in a 25 μL mixture containing 2 μL cDNA, 0.5 μL each primer (10 μM), 12.5 μL 2 x SYBR Mix (with 4.0 mM MgSO4), 0.15 μL Taq DNA Polymerase and 9.35 μL ddH2O under the conditions of initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 61°C for 15 s and extension at 72°C for 30 s. All reactions were performed in duplicate. Results were normalized with total levels of β-actin expression and analyzed by iQ™SY software (version 2.0, Bio-Rad, USA) and SPSS 13.0.

Statistical analysis

Quantitative data was expressed as mean ± standard deviation (SD). The distribution normality was analyzed using the Kolmogorov–Smirnov test. Differences without skewness between the KBD and control groups were assessed by Student’s t-test (two tailed). Differences of measurement data departing from normal distribution between two groups were analyzed with the Mann–Whitney U-test. Categorical variables were presented using frequency counts and compared by a χ2-test. Deviation from Hardy–Weinberg equilibrium was analyzed by a χ2 goodness-of-fit test. Odds ratio (OR) and 95% confidence interval (CI) were calculated as an estimate of risk. P < 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 13.0 software.

Results

Baseline characteristics

A total of 161 KBD patients and 312 frequency age and sex matched controls were included in this study. No significant differences were observed between KBD and control group in age (52.2 ± 6.2 vs 52.0 ± 6.1, P = 0.717) and sex (male/female, 86/75 vs 157/155, P = 0.523). In addition, the clinical stages of the 161 KBD patients were divided into I, II and III. The baseline characteristics are shown in Table III.

Genotype analysis

In this study, four single nucleotide polymorphisms (SNPs) were genotyped by PCR-RFLP and ARMS-PCR. Agarose gel electrophoretograms for polymorphism analysis are shown in Fig. 1. The electrophoresis bands were clear and the brightness was uniform in Fig. 1 by which different genotypes could be discerned. All of the SNP genotype frequencies in the study were in Hardy–Weinberg equilibrium (P > 0.05). The genotypic and allelic distribution of GPX1 (rs1050450, Pro198Leu) and TrxR2 (rs5748469) were presented in Table IV. The results show that Pro198Leu of GPX1 displayed significant differences in genotypic and allelic frequency between the KBD and control groups. The other three SNPs detected were not significantly different from control. The frequency of variant genotypes, the 198Pro/Leu heterozygote and the 198Leu/Leu mutant homozygote, in KBD cases (27%) was significantly higher

Table II: List of sequences of primers used in Real-Time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX1</td>
<td>5'-CGCTTCCAGACCCATTGACATC-3'</td>
<td>5'-CGAGGTTGATTTCTCTGAAGATCA-3'</td>
</tr>
<tr>
<td>NF-κB p65</td>
<td>5'-CGCATCCAGACCCATTTGACATC-3'</td>
<td>5'-AGAGGCCTCAGACCATTCAGG-3'</td>
</tr>
<tr>
<td>p53</td>
<td>5'-TCCGGTGGGATTTTGATGAC-3'</td>
<td>5'-TCAGTGATCATGTCGAGTCCAGG-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GACGCTGGAAGCTGACCCAG-3'</td>
<td>5'-CTGAGCCTTGGGAGGACTAG-3'</td>
</tr>
</tbody>
</table>
The codon 198 of GPX1 among KBD cases was significantly higher than that in control group (17%, $P = 0.013$). The Leu allele frequency at codon 198 of GPX1 among KBD cases was significantly higher than that in control group (13% and 9%, respectively, $P = 0.037$). The distributions of genotype and allele of GPX1 198Pro/Leu in different subgroups of age, sex and clinical stages were further analyzed in KBD cases. Results showed that there were no significant differences of genotype distribution in different subgroups ($P = 0.437$, 0.468 and 0.952 for age, sex and clinical stages, respectively) and no significant differences were observed for allele frequency ($P = 0.506$, 0.505 and 0.993, respectively). It is suggested that the genotypic and allelic frequency of KBD group were not influenced by age, sex or clinical stage (data not shown).

However, no significant differences were found in genotypic and allelic frequency of the SNPs of TrxR2 (rs5748469, AC and CC) ascribed to a single group respectively during $\chi^2$-test.

The possible associations between polymorphisms in selenoprotein genes and KBD risk were estimated by calculating OR and 95%CI (Table IV). The results showed there was a 1.781-fold (95%CI: 1.127–2.814) increased risk of KBD for individuals with at least one variant allele (Pro/Leu or Leu/Leu genotype) compared with homozygous wild-type individuals (Pro/Pro). In addition, individuals with the variant T-allele (Leu) for this SNP had a 1.563-fold (95%CI: 1.127–1.855) increased risk of KBD. These data suggested that Pro198Leu of GPX1 was significantly associated with an increased risk of KBD. However, ORs and 95%CIs data indicated there were no significant associations of TrxR2 (rs5748469), SEPP1 (rs7579) and DIO2 (rs225014) between KBD and control group (Table IV).

The GPX activity of whole blood are shown in Fig. 2. The GPX activities were 176.36 ± 12.45 U and 199.23 ± 11.49 U for KBD and control group respectively. The difference was significantly different ($P < 0.01$) between the two groups. Furthermore, when subdivided by GPX1 Pro198Leu genotype, the GPX activity in subgroups of age, sex and clinical stages was significantly higher than that in control group (17%, $P = 0.013$). The Leu allele frequency at codon 198 of GPX1 among KBD cases was significantly higher than that in control group (13% and 9%, respectively, $P = 0.037$). The distributions of genotype and allele of GPX1 198Pro/Leu in different subgroups of age, sex and clinical stages were further analyzed in KBD cases. Results showed that there were no significant differences of genotype distribution in different subgroups ($P = 0.437$, 0.468 and 0.952 for age, sex and clinical stages, respectively) and no significant differences were observed for allele frequency ($P = 0.506$, 0.505 and 0.993, respectively). It is suggested that the genotypic and allelic frequency of KBD group were not influenced by age, sex or clinical stage (data not shown).

However, no significant differences were found in genotypic and allelic frequency of the SNPs of TrxR2 (rs5748469), SEPP1 (rs7579) and DIO2 (rs225014) between KBD and control group (Table IV).

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Fig. 1. Agarose gel electrophoreograms for polymorphism analysis. (A) PCR fragment of GPX1 was digested with ApaI (GGGCC). The 222 bp PCR product was cleaved into two fragments of 170 and 52 bp in the presence of CC homozygous, three fragments of 222, 170 and 52 bp for CT heterozygote (the 52 bp fragment is typically not visible on the gels), while the TT homozygous remained uncleaved showing only the 222 bp PCR product. (B) The PCR-amplified fragment of TrxR2 was subjected to digestion with BsuRI (GGCC). The 254 bp PCR product was cleaved into two fragments of 209 and 45 bp for AA genotype, four fragments of 209, 122, 87 and 45 bp for AC genotype, while 122, 87 and 45 bp for CC genotype (the 45 bp fragment is typically not visible on the gels). (C) The PCR product of SEPP1 is 338 bp in length. Two fragments of 338 and 213 bp for GC genotype, three fragments of 338, 213 and 183 bp for GA heterozygote, while 338 and 183 bp for AA genotype. (D) PCR fragment of DIO2 was subjected to digestion with AfaI (GTAC). The 412 bp PCR product was cleaved into 363 and 49 bp fragments for TT, three fragments of 412, 363 and 49 bp for CT heterozygote (the 49 bp fragment is typically not visible on the gels), whereas the CC genotype remained uncleavable. M, molecular size marker; P, PCR product. PCR fragments were separated on 2% agarose gels.

Table III

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (n = 161)</th>
<th>Controls (n = 312)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD</td>
<td>52.2 ± 6.2</td>
<td>52.0 ± 6.1</td>
<td>0.717</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>86/75</td>
<td>157/155</td>
<td>0.523</td>
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<tr>
<td>Degree</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>93</td>
<td></td>
<td></td>
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</tbody>
</table>

* The comparison between two groups was performed using t-test and no significance.

** The comparison between two groups was performed using $\chi^2$-test and no significance.

Table IV

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Controls, n (%)</th>
<th>KBD, n (%)</th>
<th>P</th>
<th>OR (95%CI)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX1 (rs1050450)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>259 (83)</td>
<td>118 (73)</td>
<td>1.000</td>
<td>(–)</td>
</tr>
<tr>
<td>CT</td>
<td>50 (16)</td>
<td>43 (27)</td>
<td>0.013*</td>
<td>1.781 (1.127–2.814)</td>
</tr>
<tr>
<td>TT</td>
<td>3 (1)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-allele</td>
<td>568 (91)</td>
<td>279 (87)</td>
<td>0.037*</td>
<td>1.000 (–)</td>
</tr>
<tr>
<td>T-allele</td>
<td>56 (9)</td>
<td>43 (13)</td>
<td>1.563 (1.025–2.385)</td>
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<tr>
<td>TrxR2 (rs5748469)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>AA</td>
<td>81 (74)</td>
<td>70 (83)</td>
<td>1.000</td>
<td>(–)</td>
</tr>
<tr>
<td>AC</td>
<td>28 (26)</td>
<td>13 (16)</td>
<td>0.132</td>
<td>0.579</td>
</tr>
<tr>
<td>CC</td>
<td>0</td>
<td>1</td>
<td>(0.283–1.185)</td>
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</tr>
<tr>
<td>A-allele</td>
<td>190 (87.2)</td>
<td>153 (91.1)</td>
<td>0.225</td>
<td>1.000 (–)</td>
</tr>
<tr>
<td>C-allele</td>
<td>28 (12.8)</td>
<td>15 (8.9)</td>
<td>0.665 (0.343–1.290)</td>
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<tr>
<td>SEPP1 (rs7579)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>98 (59)</td>
<td>82 (50.9)</td>
<td>1.000</td>
<td>(–)</td>
</tr>
<tr>
<td>GA</td>
<td>53 (31.9)</td>
<td>65 (40.4)</td>
<td>0.272</td>
<td>1.466 (0.919–2.337)</td>
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<tr>
<td>AA</td>
<td>15 (9.1)</td>
<td>14 (8.7)</td>
<td>1.115 (0.509–2.446)</td>
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<tr>
<td>G-allele</td>
<td>249 (75)</td>
<td>229 (71.1)</td>
<td>0.263</td>
<td>1.000 (–)</td>
</tr>
<tr>
<td>C-allele</td>
<td>83 (25)</td>
<td>93 (28.9)</td>
<td>1.218 (0.862–1.722)</td>
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<tr>
<td>DIO2 (rs225014)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>64 (30.6)</td>
<td>48 (29.8)</td>
<td>1.000</td>
<td>(–)</td>
</tr>
<tr>
<td>TC</td>
<td>106 (50.7)</td>
<td>78 (48.5)</td>
<td>0.761</td>
<td>0.981 (0.610–1.578)</td>
</tr>
<tr>
<td>CC</td>
<td>39 (18.7)</td>
<td>35 (21.7)</td>
<td>1.197 (0.663–2.159)</td>
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</tr>
<tr>
<td>T-allele</td>
<td>234 (56)</td>
<td>174 (54)</td>
<td>0.598</td>
<td>1.000 (–)</td>
</tr>
<tr>
<td>C-allele</td>
<td>184 (44)</td>
<td>148 (46)</td>
<td>1.082 (0.808–1.449)</td>
<td></td>
</tr>
</tbody>
</table>

* represents significance at $P < 0.05$.

** The comparison of genotype and allele frequency between two groups was performed using $\chi^2$-test.

† The variant genotypes of GPX1 (rs1050450, CT and TT) and TrxR2 (rs5748469, AC and CC) were ascribed to a single group respectively during $\chi^2$-test.

‡ 95%CI, 95% confidence interval.
whole blood decreased significantly in the subgroup of individuals representing Pro/Leu and Leu/Leu compared to Pro/Pro ($P < 0.01$).

**mRNA expression of GPX1, NF-κB p65 and p53**

GPX1 mRNA level was determined by Real-Time PCR. β-actin cDNA was the reference in all experiments and was used to normalize GPX1 mRNA expression. mRNA levels of GPX1 in both whole blood and articular cartilage tissue in KBD group were lower than that in control group (0.715- and 0.980-fold, respectively). Results showed a significant difference ($P = 0.026$) in whole blood and no significant difference in articular cartilage tissue ($P = 0.780$) between the two groups (Fig. 3). GPX1 mRNA level showed no significant difference between subgroups of Pro/Leu, Leu/Leu and Pro/Pro but it tended to decrease in individuals with Pro/Leu and Leu/Leu ($P > 0.05$).

The mRNA expression of NF-κB p65 in whole blood and articular cartilage decreased in the KBD group compared to controls (0.403- and 0.122-fold, respectively). Statistical analysis showed that both differences are significant [$P < 0.001$, (Fig. 4A)].

The mRNA level of p53 in whole blood and articular cartilage tissue increased significantly in the KBD group (3.922- and 7.357-fold, respectively) compared to controls and the differences were statistically significant [$P = 0.010$ and 0.022, respectively, (Fig. 4B)].

**Discussion**

Epidemiological studies have shown that the distribution of KBD overlaps the Se deficiency regions in China and KBD children in endemic areas had lower Se in blood, urine and hair than that of children free from this disease in endemic areas.$^{20-22}$ It is believed that genetic factors as well as environmental Se deficiency underlie the pathogenesis of KBD. GPX, TrxR, SEPP and DIO are important antioxidative selenoproteins responsible for cell growth, immune function and metabolism of bone. Furthermore, one of the primary features of KBD is apoptosis and necrosis of chondrocytes resulting from excessive oxidative stress. The genetic coding for these selenoproteins are getting more attention from researchers. In recent years, possible associations between genetic variations in these selenoprotein genes and risk of cancer, cardiovascular disorder and other diseases have been studied.$^{16-19,23}$ The findings suggested some functional SNPs of selenoprotein genes were related with susceptibility to diseases, but it is not known whether these genetic variations play a role in the development of KBD.

To clarify whether polymorphisms in selenoproteins were correlated with KBD risk we conducted a case–control study to investigate four genetic polymorphisms across selenoprotein genes. These potential SNPs of the genes were GPX1 (rs1050450), TrxR2 (rs5748469), SEPP1 (rs7579) and DIO2 (rs225014)$^{24-26}$ which are all tag SNPs in coding regions. Genotype analysis was performed using PCR-RFLP and ARMS-PCR. The two methods for SNP typing are simple, low cost, label-free and reproducible. Results showed that the genotypic and allelic frequency of GPX1 198Pro/Leu was statistically different between the KBD and control groups ($P = 0.016$, 0.043, respectively), whereas no significant differences were found in the other three SNPs (Table IV). In the four polymorphisms, the Pro198Leu variation of GPX1 was significantly associated with an increased risk of KBD.

In mammalian cells, GSH and GPX constitute the principal antioxidant defense system.$^{27}$ There are at least six different GPX isoenzymes in GPX family of which the most abundant is GPX1, an intracellular Se-dependent antioxidative selenoenzyme within most cells. The human GPX1 gene possesses several genetic polymorphisms, one of which, GPX1 Pro198Leu, is supposed to be functional$^{16,18,28}$ as the amino acid change from proline to leucine at codon 198 may cause some important conformational changes of GPX1.$^{29}$ Interestingly, some epidemiologic studies indicated that frequency of Leu allele of GPX1 was strongly associated with an increased risk of various cancers.$^{30-33}$ Though these correlations have not been consistently observed in all populations.$^{34}$ The current study showed that an increased risk of KBD was observed in carriers of Pro/Leu and Leu/Leu compared with homozygous wild-type (Pro/Pro) individuals (OR = 1.781; 95%CI: 1.127–2.814), and that GPX1 198Pro/Leu was associated with increased KBD risk in a Chinese Han population. Correspondingly, the Leu allele of the GPX1 polymorphism was correlated with increased KBD risk (OR = 1.563; 95%CI: 1.025–2.385). It is
plausible that GPX1 Pro198Leu might be a genetic risk factor for the development of KBD. In this study, no associations were found between KBD risk and three other gene polymorphisms of TrxR2, SEPP1 and DIO2. We also investigated the GPX enzyme activity and mRNA expression of GPX1. Results showed that GPX enzyme activity and mRNA level in whole blood were lower in the KBD group compared with controls (both \( P < 0.01 \)). No significant difference was found in the GPX1 mRNA expression in articular cartilage tissue between KBD and control group which is in accordance with the results of Wang et al.\(^\text{15}\). It is possible that functional polymorphisms in selenoprotein genes influenced selenoproteins expression, stability or activity\(^\text{25}\). Ravn-Haren et al. reported that GPX1 Pro198Leu genotype and GPX activity were strongly correlated and GPX activity was lower in individuals with the variant T-allele\(^\text{29,32}\). In our study, when subdivided by GPX1 Pro198Leu genotype, the GPX activity in whole blood decreased significantly in the subgroup of individuals with Pro/Leu and Leu/Leu compared to Pro/Pro (\( P < 0.01 \)). This was in accordance with Ravn-Haren's results. GPX1 mRNA level was not significantly different between subgroups of Pro/Leu, Leu/Leu and Pro/Pro but tended to decrease in individuals with Pro/Leu and Leu/Leu (data not shown).

Somewhat at potential variance with our results are the results with the GPX1 knockout mice. These mice have no apparent osteoarthritis, as do KBD patients, but rather have an increased susceptibility to paraquat and other oxidizing chemicals\(^\text{36}\). More likely though, the GPX1 polymorphism observed here to be correlated with KBD is coincident with some other defect, perhaps by genetic linkage to a nearby gene or causes secondary effects which are more directly related to pathogenesis. Reactive oxygen species also serve as signaling molecules in cells and can affect other cell signaling pathways.

Decreased GPX enzymic activity would unbalance the redox system. Excessive oxidative stress could result in apoptosis and necrosis of chondrocytes which could then play a crucial role in the pathogenesis of KBD\(^\text{37,38}\). It is known that NF-\(\kappa\)B p65 is an important subunit of NF-\(\kappa\)B which is a well-known transcription factor family involved in the progress of inflammation and anti-apoptosis and its inhibition would increase apoptosis\(^\text{39}\). p53 was reported to play an important role in starting apoptosis and controlling the cellular response to DNA-damaging agents and mutation\(^\text{40}\). Furthermore, p53 can be regulated by the NF-\(\kappa\)B p65\(^\text{41}\). Excessive peroxides might lead to chondrocyte apoptosis and necrosis which could be reflected by NF-\(\kappa\)B p65 and p53 expression. To understand the relevant molecular mechanism in development of KBD we investigated mRNA expression of NF-\(\kappa\)B p65 and p53 in both whole blood and articular cartilage tissue from KBD and control subjects. The mRNA level of NF-\(\kappa\)B p65 was lower in whole blood and articular cartilage tissue in KBD patients (\( P < 0.001 \)), whereas the mRNA level of p53 increased (\( P < 0.05 \)). This suggests that apoptosis signaling pathways were activated in KBD patients. These results could be a further clue in elucidating the molecular mechanism of KBD.

This was the first study on the association between these four SNPs and KBD risk and further research is needed to confirm and expand the present results. Our sample size is relatively small and a larger sample study is needed to verify and refine our conclusions. In addition, studies on other genetic variations, SNP–SNP interactions and gene function of selenoprotein would also be beneficial to determine susceptibility genes of KBD.

In summary, results of our study demonstrated that Pro198Leu of GPX1 might be a genetic risk factor in the development of KBD. It is indicated that GPX1 Leu allele might be associated with higher KBD risk among Chinese Han population with lower GPX enzyme activity. In addition, alteration of NF-\(\kappa\)B p65 and p53 mRNA expression suggests that apoptosis signaling might be activated in KBD patients. This study is essential for elucidating the etiology and pathogenesis of KBD and searching for new treatment targets for KBD.

Conflict of interest
The authors have no conflict of interest and certify this to be a true and original work.

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