

# CpG Methylation of the *GPX3* Promoter in Patients with Kashin-Beck Disease Potentially Promotes Chondrocyte Apoptosis

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## Research article

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# Abstract

**Objective:** To determine the methylation levels of CpGs in the *GPX3* promoter region and explore their potential effects on the apoptosis of chondrocytes.

**Methods:** Blood specimens were collected from 32 participants, including 16 KBD patients and 16 healthy subjects. Twenty-five CpGs in the promoter region of *GPX3* were identified and detected by MALDI-TOF-MS. Methylation levels of CpGs were compared between KBD patients and healthy subjects as well as among the KBD patients with different degrees. C28/I2 human chondrocytes were treated with tBHP and  $\text{Na}_2\text{SeO}_3$ . Apoptosis in chondrocytes was examined under a fluorescence microscope.

**Results:** The methylation levels of *GPX3*-8\_CpG\_11, *GPX3*-8\_CpG\_16, *GPX3*-8\_CpG\_20 in KBD patients were significantly higher than those of healthy subjects ( $P < 0.05$ ). The methylation levels of the other CpGs were not significantly different between the two groups ( $P > 0.05$ ). The methylation level of *GPX3*-8\_CpG\_24 in KBD patients with degree III was significantly higher than those in KBD patients with degree I/II ( $P < 0.05$ ). MSP-PCR analysis indicated that the methylation rate of KBD group (9.41%) was significantly higher than that of healthy subjects (1.18%), and that *GPX3* DNA methylation increased the risk of acquiring KBD 8 fold (OR = 8.000, 95% CI : 1.023-62.580); The mRNA expression of *GPX3* in whole blood of KBD patients was lower than that of healthy subjects ( $P < 0.05$ ); Compared with the control group, *GPX3*, *GPX1* and *GPX4* mRNA level of the tertbutyl hydroperoxide injury group decreased significantly ( $P < 0.05$ ), after supplementation with  $\text{Na}_2\text{SeO}_3$ . The logarithmic increase in mRNA levels of *GPX3*, *GPX1* and *GPX4* decreased the rate of apoptosis in chondrocytes.

**Conclusion:** The methylation patterns of CpGs in *GPX3* varied in KBD patients. The experiments indicated that the increased methylation of CpGs within the *GPX3* promoter may down-regulate the expression of *GPX3*, thereby reducing the antioxidant function of GPX3 and promoting chondrocyte apoptosis, both of which accelerates the development of KBD.

## Background

Kashin-Beck disease (KBD) is a common endemic, chronic and degenerative bone disease with the highest prevalence in China. KBD mainly affects the joints and cartilages of individuals[1, 2]. Geographically, KBD is mainly distributed from the northeast to the southwest of China in a belt shaped manner, as well as parts of Eastern Siberia in Russia, and North Korea[3, 4]. The recent epidemiological survey indicated that 0.54 million individuals were suffering from KBD by the close of 2017. Currently, it is estimated that 37.2 million individuals are at high risk of developing KBD in China[5]. KBD gravely threatens the health of the population; however, its pathogenesis remains elusive.

Selenium deficiency has been proposed for decades as the archetypal etiological theory of KBD[6-8]. However, the roles of selenium in the occurrence and development of KBD has not been ascertained. Selenium usually performs its biological roles in the human body as selenoprotein, of which its physiological function still needs to be systematically studied. The antioxidant effect of selenoprotein is

mainly attributed to the oxidation state of the selenium atom. Such antioxidant enzymes prevents DNA damage caused by oxidative stress, which is related to upregulated apoptosis and necrosis of KBD articular chondrocytes[9-11]. The above evidence indicates that the antioxidant properties of selenoproteins can protect chondrocytes from apoptosis and necrosis.

In recent years, studies have confirmed that, despite low environmental selenium concentrations, the individual's genetic characteristics also play an important role in the occurrence and development of KBD[12]. Therefore, the genetic factors contributing to KBD have received increasing attention. Consequently, a number of KBD susceptible genes were identified from some studies through modern molecular biology research technologies[13-15]. However, studies on the genetic factors contributing to KBD can only explain its pathogenesis partly. Researchers are rapidly discovering that epigenetic elements may also affect gene function by regulating gene expression levels[16], hence exhibiting a series of effects on human physiology.

Glutathione peroxidase family are key members of selenoproteins that catalyze the reduction of organic hydroperoxides and hydrogen peroxide ( $H_2O_2$ ) by glutathione, and thereby inhibiting oxidative cell damage[17, 18]. Experiments have showed that downregulation of *GPX3* expression by hypermethylation of its promoter, has been observed in a wide spectrum of human malignancies such as Osteoarthritis (OA) and KBD[16]. Therefore, genetic and epigenetic interactions of *GPX3* may affect human health. However, methylation levels of the *GPX3* promoter has not been quantified in KBD.

In our present study, CpGs of *GPX3* in the promoter region were identified and determined. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was employed to quantitate the methylation of CpGs explored their potential effect on the apoptosis of chondrocytes. Through these evaluations, the current study aimed to identify the divergent methylation of CpGs in *GPX3* promoter regions in KBD patients, and to further analyze the effect of *GPX3* promoter methylation in KBD.

## Methods And Materials

### 1.1 Study population

The study population was selected from KBD endemic areas of Shaanxi province, China. A biological specimen library was created from blood samples drawn from 226 KBD patients and 248 healthy subjects (Table 1). All the study participants received prudent radiographic examination. The national diagnosis criteria of KBD (WS/T207-2010) was used to select patients with KBD. Individuals with genetic bone and cartilage diseases, arthritis related disease and other skeletal disorders were excluded from this study. Individuals who met the selection criteria were recruited to participate in the study after submitting their signed informed consent. The proposed study design met the approval of the Human Ethics Committee of Xi'an Jiaotong University, People's Republic of China.

### 1.2 Quantifying the mRNA expression in KBD patients and chondrocytes

Total RNA from KBD patients and controls (n=8 in each group), as well as chondrocytes (n=3 in each group), was extracted using the Trizol KIT (Life Technologies, Carlsbad, CA). RNA extracts were reverse-transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (MBI, Fermentas, Vilnius, Lithuania) following the manufacturer's instructions. Relative quantification of *GPX3*, *GPX1* and *GPX4* mRNA was performed by iQe5 quantitative real-time PCR Detection Systems (qRT-PCR) (Bio-Rad, Philadelphia, PA) with *β-actin* as a reference. The sequences of primers are listed in Table 2. qRT-PCR was performed in a 20 μL reaction mixture containing cDNA (1.6 μL), each primer (0.8 μL), 2×SYBR Premix Ex Taq (10 μL) (Takara, Mountain View, CA), and ddH<sub>2</sub>O (6.8 μL) using the TaqMan method (94°C for 2 min, and 40 cycles of 94°C for 10 s and 72°C for 30 s). All reactions were performed in duplicate. Relative expression levels of *GPX3*, *GPX1* and *GPX4* mRNAs were normalized to *β-actin* and analyzed by iQe5 software (version 2.0, Bio-Rad, Philadelphia, PA).

### 1.3 Quantitative methylation analysis

#### 1.3.1 The design and synthesis of primers

Primers covering CpGs for quantitative methylation analysis were designed by Agena's software (<http://www.epidesigner.com/index.html>) and synthesized by Liuhe Huada Gene Technology Co., Ltd (Beijing, China). The primer sequences are as follows: the forward primer, Fw: GGAATAAGAAATGTTTTTTAGAATGGA, and the reverse primer, Rv: ACCAAAAACAAAAAAACAAACAAA, which are also illustrated in Figure 1A. The target fragment of *GPX3* contained 25 CpGs, which is showed in Figure 1B.

#### 1.3.2 Quantitative methylation by MALDI-TOF MS

EZ-96 DNA methylation kit (Zymo Research) was used to treat the genomic DNA (200 ng) of each participant with bisulfite according to the manufacturer's instructions. The Sequenom Mass ARRAY platform (CapitalBio, Beijing, China) containing a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer and RNA base-specific cleavage (Mass CLEAVE), were both employed to quantify the methylation of *GPX3* CpGs. Quantitative methylation data was obtained via a Spectro CHIP (SEQUENOM) and a Mass ARRAY Compact System (SEQUENOM). The EpiTYPER software version 1.0 (SEQUENOM) was used to analyze and display the results.

### 1.4 Qualitative methylation analysis

Genomic DNA of the study population was extracted from whole blood samples (n=80 in each group) using TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). The genomic DNA was treated with bisulfite using EZ-96 DNA methylation kit (ZYMO Research, Irvine, CA) according to the manufacturer's instructions and amplified by methylation-specific polymerase chain reaction (MSP). The sequences of primer are as follows: methylated primers (F: 5'-TATGTTATTGTCGTTTCGGGAC-3'; R: 5'-GTCCGTCTAAAATATCCGACG-3', products size: 177bp) and unmethylated primers (F: 5'-TTTATGTTATTGTTGTTTTGGGATG-3'; R: 5'-ATCCATCTAAAATATCCAACACTCC-3', products size: 186bp).

Next, a 50 $\mu$ L PCR mixture containing 5 $\mu$ L 10 $\times$ PCR buffer, 4 $\mu$ L dNTP mixture, 1 $\mu$ L of each primer, 3 $\mu$ L bisulfite-modified DNA, 35.75 $\mu$ L ddH<sub>2</sub>O, and 0.25 $\mu$ L hot-start Taq-polymerase (Takara, Mountain View, CA) for each blood sample was prepared. PCR conditions were set as: 94 °C for 10 min (initial denaturation), followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 10 min. Finally, PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and visualized in ultraviolet light (GBox F3, Syngene, UK).

### 1.5 C28/I2 human chondrocytes injured by tBHP

Human chondrocyte cell line named C28/I2, was provided by Professor Mary B Goldring (Hospital for Special Surgery, Weill Cornell Medical College, New York, USA). C28/I2 was cultured in DMEM/F-12 (Hyclone, Logan, UT) supplemented with 12% FBS (SiJiQing, Zhejiang, China) and 1% penicillin/streptomycin solution in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Upon reaching 90% confluence, chondrocytes were seeded on 96-well culture plates. Our experiment contained 6 groups: control group (C), purely Selenium (Se) group (S2, 0.10  $\mu$ g/mL Na<sub>2</sub>SeO<sub>3</sub>), tertbutyl hydroperoxide (tBHP) injury group (O, 150  $\mu$ mol/L tBHP), low Se group (OS1, 0.05  $\mu$ g/mL Na<sub>2</sub>SeO<sub>3</sub> + 150  $\mu$ mol/L tBHP), medium Se group (OS2, 0.10  $\mu$ g/mL Na<sub>2</sub>SeO<sub>3</sub> + 150  $\mu$ mol/L tBHP) and high Se group (OS3, 0.15  $\mu$ g/mL Na<sub>2</sub>SeO<sub>3</sub> + 150  $\mu$ mol/L tBHP). OS1, OS2 and OS3 were treated with increasing concentrations of Na<sub>2</sub>SeO<sub>3</sub> (0.05, 0.10 and 0.15  $\mu$ g/mL) for 24 h as pre-protection, and then treated with 150  $\mu$ mol/L tBHP for 24 h. Chondrocytes were stained with 2% Hoechst 33342 in DMEM/F-12 containing 12% FBS and incubated at 37°C for 30 min. Apoptosis in chondrocytes was monitored under a fluorescence microscope. Five high-power fields (100 $\times$ ) were selected randomly to count apoptotic cells and calculate the apoptotic rate in each group (n=3).

### 1.6 Statistical analysis

Quantitative data was presented as mean  $\pm$  standard deviation (SD). Groups were compared using the student's *t*-test. Curve-fitting method was used for obtaining the correlation between *GPX1*, *GPX3* and *GPX4* mRNA expressions and apoptosis rate of chondrocytes. All statistical analyses were performed using SPSS 23.0, and the significance level  $\alpha=0.05$  were considered to have statistical significance (SPSS Inc., Chicago, IL, USA).

## Results

### 2.1 Baseline characteristics of study population

226 KBD patients and 248 healthy controls with matched frequency of age and gender were recruited in this study. No statistically significant differences were seen between the cases and controls in age ( $t=1.305$ ,  $P=0.193$ ) and sex ( $\chi^2=1.914$ ,  $P=0.167$ ). The demography of the two groups are shown in Table 1.

### 2.2 The CpGs methylation of *GPX3* of KBD

16 KBD patients and 16 healthy subjects with no significant differences in age and gender, were selected randomly from the study population to detect CpG methylation detection (Table 3). Figure 2 illustrates the methylation profiles of 14 CpGs (or CpG Units) in the promoter region of *GPX3*. The methylation levels of *GPX3-8\_CpG\_11*, *GPX3-8\_CpG\_16*, *GPX3-8\_CpG\_20* in KBD patients were significantly higher than those in the healthy subjects ( $P < 0.05$ ). The methylation levels of the other CpGs were not significantly different between the two groups ( $P > 0.05$ ) (Figure 3).

### 2.3 The CpGs methylation of *GPX3* in KBD patients with different degrees

The methylation levels of 14 CpGs (or CpG Units) in the promoter region of *GPX3* between KBD patients with degree I/II and III are shown in Figure 4. The methylation level of *GPX3-8\_CpG\_24* in KBD patients with degree III was significantly higher than those in KBD patients with degree I/II ( $P < 0.05$ ).

### 2.4 Validation by MSP-PCR

MSP-PCR was used to detect the methylation status of *GPX3* promoter region in KBD patients and healthy subjects (n=85/group) quantitatively (Table 4). The amplicons were resolved by 2% agarose gel electrophoresis with band patterns showing that 8 (9.41%) patients with KBD and 1 (1.18%) healthy controls developed methylation in *GPX3*. After  $\chi^2$  test, the difference of methylation rates of *GPX3* between the two groups was significant ( $\chi^2 = 4.396$ ,  $P = 0.036$ ), indicating that the methylation rate of KBD group was significantly higher than that of control group. Also, *GPX3* DNA methylation increased the risk of KBD 8-fold (OR = 8.000, 95% CI: 1.023-62.580) (Table 5). The results of MSP-PCR and MALDI-TOF-MS were harmonious.

### 2.5 The mRNA transcription level of *GPX3* in KBD patients

*GPX3* mRNA level in whole blood was detected by qRT-PCR with  $\beta$ -actin as a quantitative control. Results showed the mRNA expression of *GPX3* in whole blood of KBD patients was lower than that of healthy subjects ( $P < 0.05$ ) (Figure 5).

### 2.6 The effect of Oxidative damage and Selenium on chondrocytes apoptosis

The results showed that the apoptosis rate in group C, O, S2, OS1, OS2, OS3 was  $(1.30 \pm 0.32)\%$ ,  $(65.96 \pm 3.98)\%$ ,  $(2.03 \pm 0.36)\%$ ,  $(14.54 \pm 2.02)\%$ ,  $(4.15 \pm 0.19)\%$ , and  $(14.74 \pm 2.56)\%$ , respectively. The apoptosis rates of the six groups was significantly different from each other ( $F = 595.157$ ,  $P < 0.001$ ). Pairwise comparison using LSD analysis suggested that the apoptosis rate in group O was significantly higher than that in group C ( $P < 0.001$ ) while apoptosis rates of group OS1, OS2, OS3 were significantly lower than that of group O ( $P < 0.001$ ). These results indicate that tBHP-induced oxidative damage can increase apoptosis of chondrocytes, while in contrast, supplementation with  $\text{Na}_2\text{SeO}_3$  can decrease apoptosis of chondrocytes.

### 2.7 The mRNA transcription levels of *GPX3* in chondrocytes

The changes of *GPX3* mRNA levels in chondrocytes treated with tBHP and Na<sub>2</sub>SeO<sub>3</sub> was further examined. The results showed that *GPX3* mRNA level of group O decreased significantly compared with group C ( $P < 0.05$ ). However, *GPX3* mRNA levels were elevated upon supplementation with Na<sub>2</sub>SeO<sub>3</sub> (Figure 6A).

## 2.8 The mRNA transcription levels of *GPX1* and *GPX4* in chondrocytes

The changes in mRNA levels of *GPX1* and *GPX4*, the two main members of GPXs family, in chondrocytes treated with tBHP and Na<sub>2</sub>SeO<sub>3</sub> was also examined to observe whether they exhibited similar trends in mRNA transcription. The results showed that *GPX1* and *GPX4* mRNA levels both decreased significantly in group O in comparison with group C ( $P < 0.05$ ). However, *GPX1* and *GPX4* mRNA levels were increased after supplementation with Na<sub>2</sub>SeO<sub>3</sub> (Figure 6B-C).

## 2.9 The effect of *GPX1*, *GPX3* and *GPX4* mRNA transcription levels on chondrocytes apoptosis

In order to understand the effect of *GPX1*, *GPX3* and *GPX4* mRNA levels on chondrocytes apoptosis, curve fitting was used to correlate *GPX1*, *GPX3*, *GPX4* mRNA levels with apoptosis rate of chondrocytes. The results showed that the rate of apoptosis was decreased with the elevation of *GPX1*, *GPX3*, *GPX4* mRNA levels. In addition, the increasing trends of *GPX1*, *GPX3*, *GPX4* mRNA levels exhibited similar "L" curves (Figure 7).

# Discussion

We aimed to evaluate the CpG methylation level of *GPX3* of KBD quantitatively. Primers targeting the 25 CpGs were therefore designed to test the CpG methylation levels by MALDI-TOF-MS. Significant variation in methylation of *GPX3* CpGs in KBD blood were identified. To substantiate the pathogenetic implication for KBD, C28/I2 human chondrocytes treated with tBHP were cultured to explore the potential effect of methylation patterns in the CpGs of *GPX3* on KBD development. The results showed that the mRNA transcription levels of *GPX3* in KBD patients and chondrocytes treated with tBHP were all decreased significantly, similarly, the apoptosis rates of chondrocytes were decreased with logarithmic elevation of *GPX3* mRNA levels. The results indicated that increased levels of methylation in *GPX3* CpGs may down-regulate the *GPX3* mRNA transcription levels, thereby reducing its antioxidant function, promoting apoptosis of chondrocytes and consequently accelerating the development of KBD.

Classic chondrocytic necrosis in articular cartilage, excessive apoptosis, dedifferentiation of chondrocytes, and chronic inflammation are the main pathological features of KBD, which are widely accepted. Findings about the beneficial effects of various antioxidants on KBD are gradually increasing. Selenium, assimilated in humans as selenocysteine (Sec) in selenoproteins such as glutathione peroxidases (GPXs), participates in antioxidant defense in human bodies [19]. More than 50 selenoproteins have currently been identified and 25 of them can be found in humans [19, 20]. Nevertheless, many of their structure and specific biological functions remains an enigma. Glutathione

peroxidases (GPXs) are a big family of antioxidant enzymes which can actively reduce H<sub>2</sub>O<sub>2</sub> and organic hydroperoxides to inhibit the ROS-induced damage to cell membranes and proteins[21]. Possibly, GPXs protect cells from oxidative damage. In mammals, eight glutathione peroxidases (GPX1-GPX8) have been identified so far, of which GPX1-GPX4 and GPX6 are selenium-containing GPXs[22].

Currently and collectively, the biological functions of GPX1, GPX3, and GPX4 are widely and mostly studied. GPX1 is activated during cartilage formation and is responsible for eliminating reactive oxygen species (ROS), which are essential for chondrogenic induction[23]. Results reported here showed that GPX1 knockdown can impair gene expression of Col II and aggrecan and also suppress the proliferation of chondrocytes. Therefore, GPX1 is necessary for chondrogenic induction in ATDC5 cells[24]. Another study showed that *GPX3* was significantly increased in bone samples from hip fracture patients, which suggested increased antioxidative activity in the samples[25]. The latest evidence showed that the methylation and expression of *GPX3* vary in KBD blood samples[16], which may have an important role in this bone disease occurrence and development. Furthermore, the mRNA level of *GPX4* was strikingly decreased in the blood of KBD patients and the polymorphisms of two SNPs (rs713041, rs4807542) of *GPX4* may be related to the development of KBD. On that account, *GPX4* can serve as a potential susceptibility biomarker for KBD[26]. The above evidence strongly suggests that these selenium-containing GPXs may play essential roles in the occurrence of KBD, which is worth exploring further.

In addition to the genetic factors of GPXs, their epigenetic studies are also an important hotspot for study. However, quantitative research on the methylation levels of GPXs in KBD have not been reported. In our present study, we paid special attention to the quantitative assessment of *GPX3* CpG methylation levels and their potential effect on apoptosis of chondrocytes. The results indicated that increased levels of *GPX3* CpGs methylation may down-regulate *GPX3* mRNA transcription and induce chondrocyte apoptosis. Our study provided valuable clues for exploring the potential interaction between the CpG methylation (epigenetics) and mRNA expression (genetics) of *GPX3* in KBD. Integrated analysis of the genetic and epigenetics information of genes will unravel the complexity of chronic bone diseases, such as KBD[27, 28].

It has been recognized that epigenetic deregulation through genetic and environmental elements could result in delayed or blocked cancer development[29]. The genetic profile of an individual could provide the underlying susceptibility factors and triggers for the evolution of a complex disease (cancer, bone disease, etc.). Epigenetic regulation also has very important implications for development of such diseases[30]. Increasing evidence showed that unraveling the interplay between genetic and epigenetic parameters becomes increasingly essential for interpreting the etiology of complex diseases[27, 28, 30]. The findings of our present study provide preliminary clues and new evidence that could illustrate the interplay between genetic and epigenetic parameters of *GPX3* in the onset of KBD.

## Conclusions



The methylation profiles of CpGs in the promoter region of *GPX3* in the whole blood of KBD patients were detected and their potential effect on the apoptosis of chondrocytes was explored. It was initially suggested that there may be an interplay between the CpGs methylation in the promoter region of *GPX3* and *GPX3* expression in KBD patients, which stimulated chondrocyte apoptosis. Our present study provides a new perspective and new clue for mastering the pathogenic process of KBD.

## Declarations

### Ethics approval and consent to participate

Individuals were recruited to participate in the study after submitting their signed informed consent. The proposed study design met the approval of the Human Ethics Committee of Xi'an Jiaotong University, People's Republic of China.

### Consent for publication

Not applicable.

### Availability of data and material

All data generated or analyzed during this study are included in this published article.

### Authors' contributions

Study design and conception: Yongmin Xiong, Rongqiang Zhang

Acquisition of the data: Rongqiang Zhang, Di Zhang, Xiaoli Yang, Dandan Zhang, Qiang Li, Chen Wang, Xuena Yang

Analysis and interpretation of the data: Rongqiang Zhang, Yongmin Xiong

All authors were involved in reading the draft and revising it carefully, and all authors approved the final version to be published.

### Competing interests

The authors declared no conflict of interest.

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## Tables

Table 1  
Baseline characteristics of study population

Characteristics	KBD (n = 226)	Controls (n = 248)	t/ $\chi^2$	P
Age, $\bar{x} \pm SD$	54.20 $\pm$ 5.40	53.50 $\pm$ 6.20	1.305	0.193
Gender, F/M	116/110	143/105	1.914	0.167

Table 2  
The information of primers for mRNA expression detection

Genes	Primer Sequence (5'-3')	Length(bp)
GPX1	Fw: CCTTCCACTTCATCTGTCCTG	149
	Rv: GATTCTTAGCCTCATCCGCC	
GPX3	Fw: TTCACGACATCCGCTGGAA	101
	Rv: CATCTTGACGTTGCTGACCGT	
GPX4	Fw: CTCTATGCAGGTTCCACTGTC	120
	Rv: TTCCCCAAACTTCTCCACAG	
$\beta$ -actin	Fw: GAACGGTGAAGGTGACAGCAG	200
	Rv: GTGGACTTGGGAGAGGACTGG	

Table 3

Baseline characteristics of study population for CpG methylation

Characteristics	KBD (n = 16)	Controls (n = 16)	t/ $\chi^2$	P
Age, $\bar{x} \pm SD$	54.26 $\pm$ 5.61	52.35 $\pm$ 8.79	0.733	0.469
Gender, F/M	6/10	8/8	0.508	0.476
Degree, n(%)				
I	1(6.25)	-		
II	7(43.75)	-		
III	8(50.00)	-		

Table 4

Baseline characteristics of study population for MSP-PCR

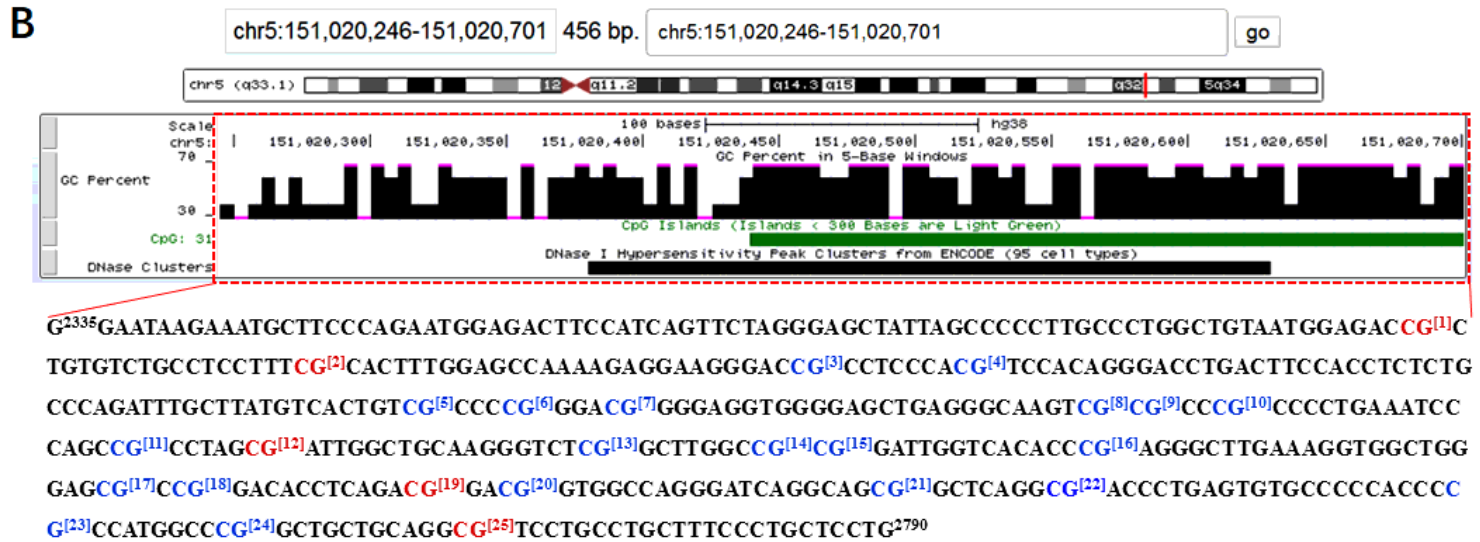
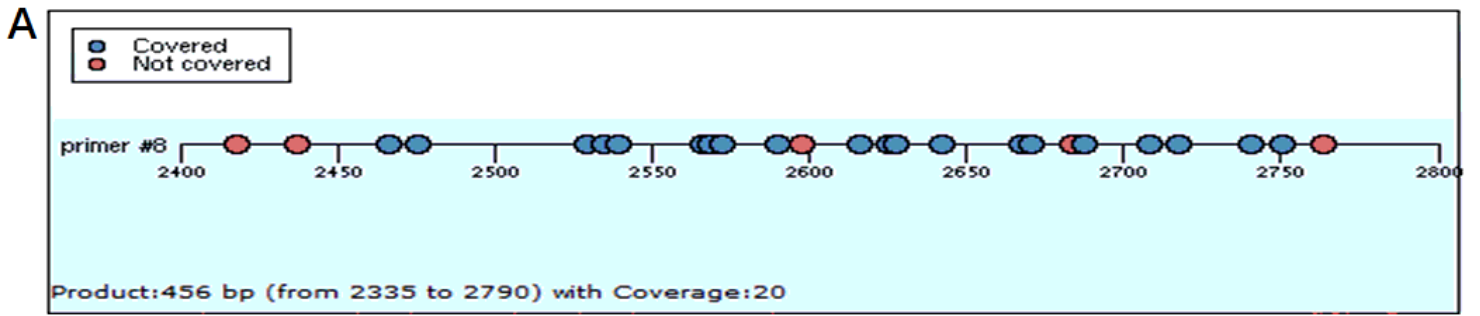
Characteristics	KBD (n = 85)	Controls (n = 85)	t/ $\chi^2$	P
Age, $\bar{x} \pm SD$	55.28 $\pm$ 6.34	54.32 $\pm$ 8.74	0.820	0.414
Gender, F/M	25/60	30/55	0.672	0.412

Table 5

Methylation rate of GPX3 between KBD patients and controls, n (%)

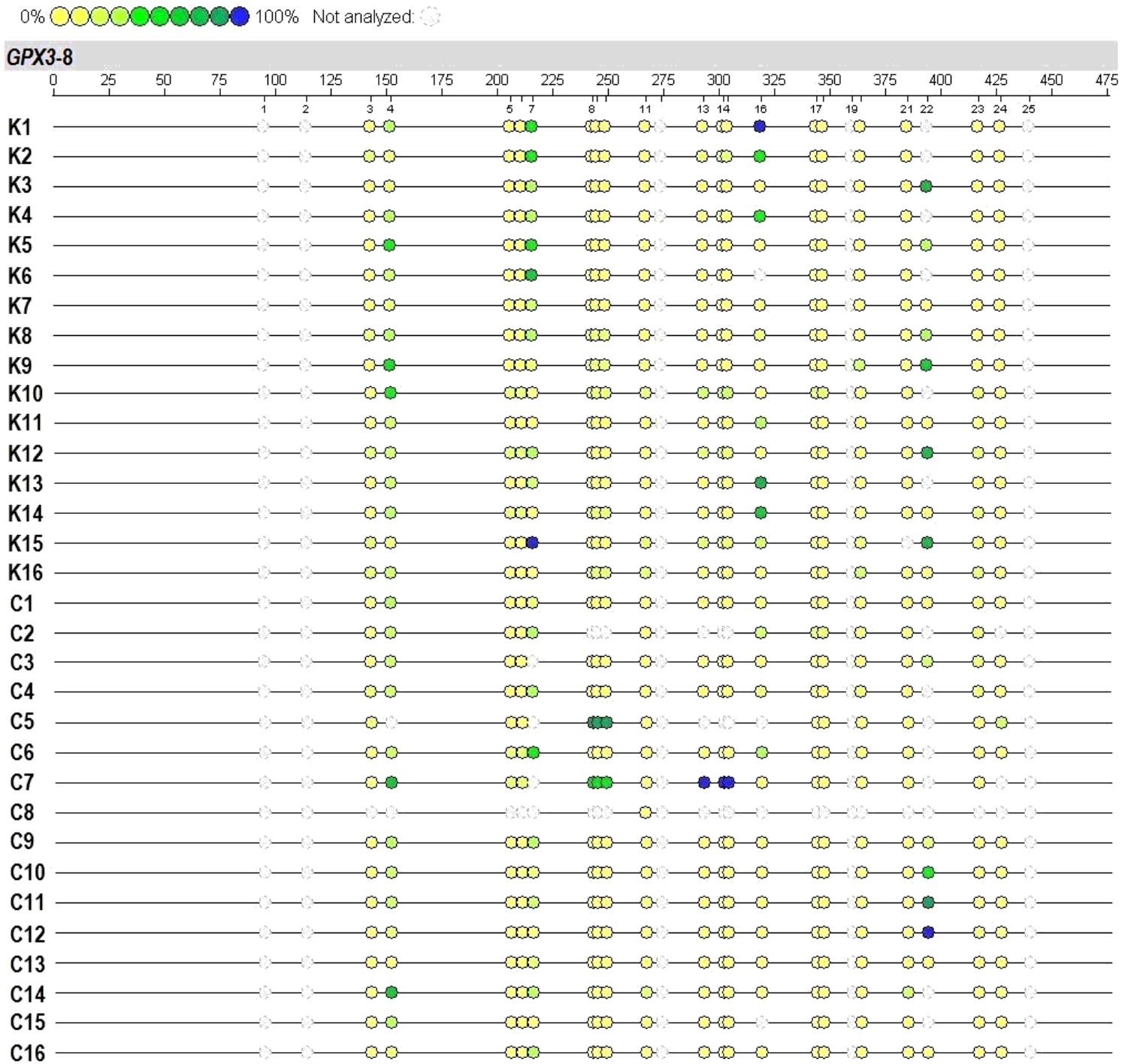
Group	Methylation	Unmethylation	$\chi^2$	P	OR (95%CI)
KBD	8(9.41)	77(90.59)	4.396	0.036	8.000(1.023, 62.580)
Control	1(1.18)	84(98.82)			

## Figures



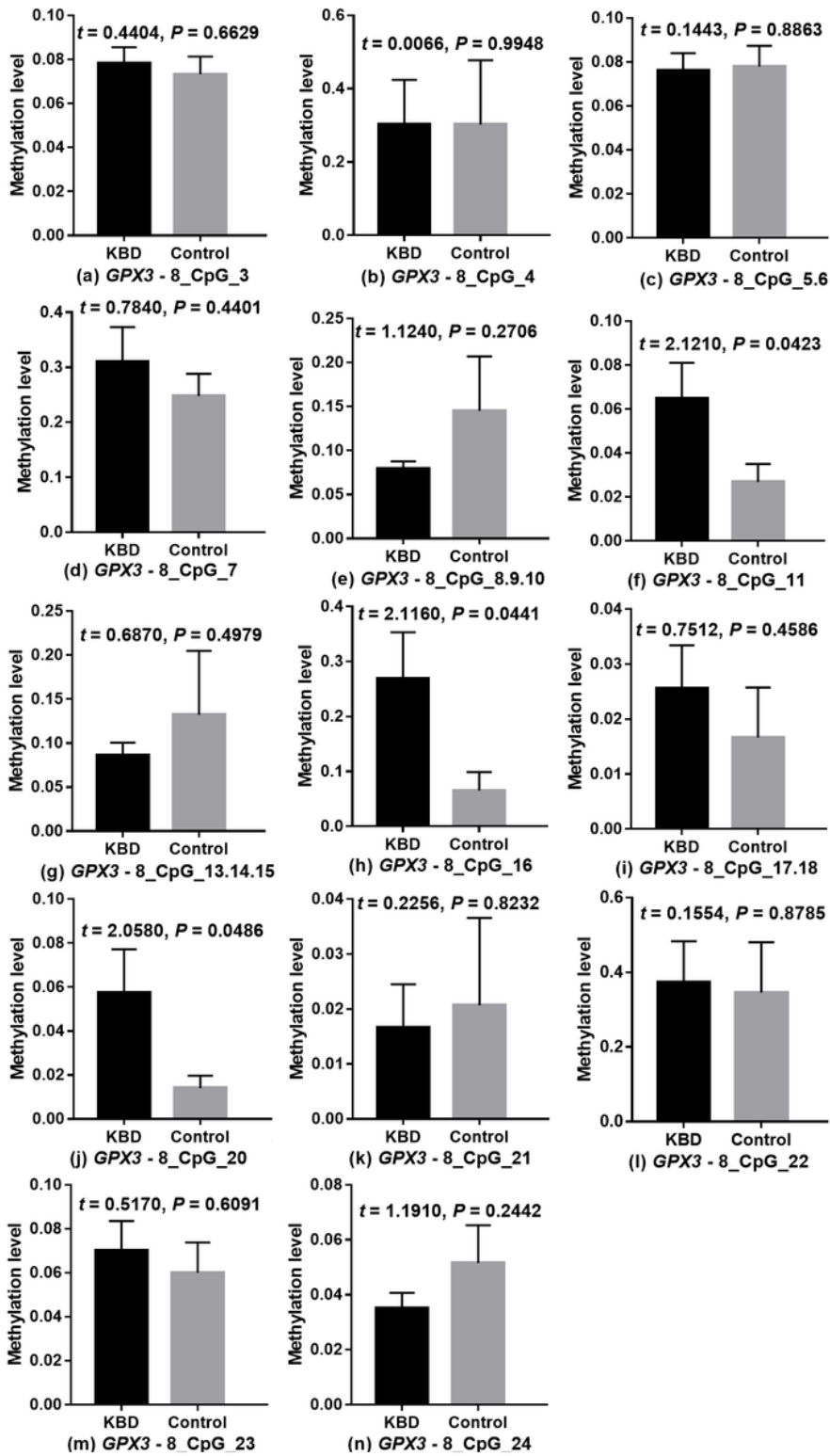
**Figure 1**

The information of CpG methylation sites in primer GPX3 #8 and the sequence of target fragment in the promoter region of GPX3 (A: The dots represent the CpG sites to be tested in the target fragment; B: The blue bases represent CpG sites to be tested).



**Figure 2**

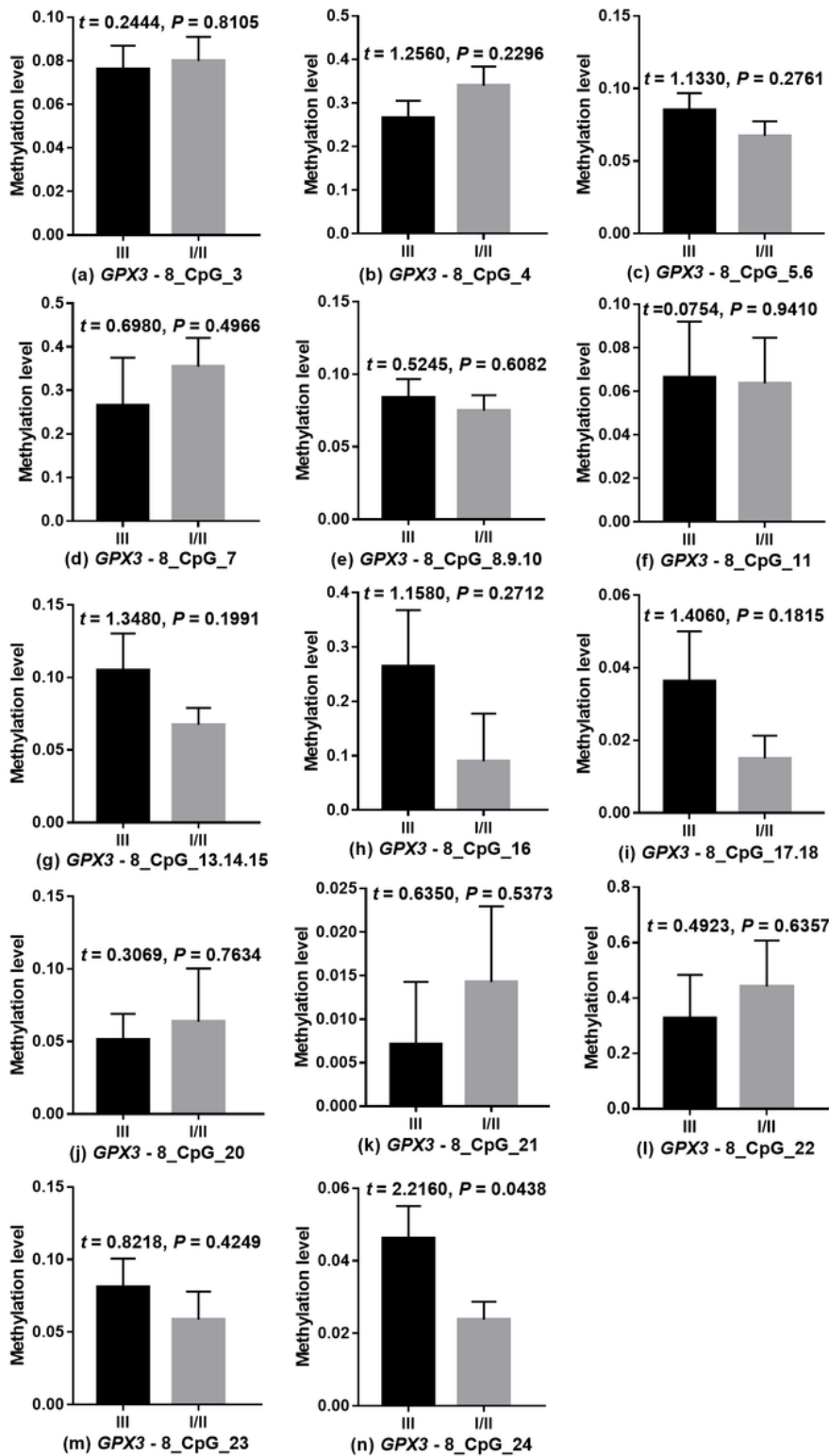
Methylation levels of CpGs in the promoter region of GPX3 in KBD and Controls (The color of the dots indicates the methylation level. The darker the color, the higher the methylation level; otherwise, the lower the methylation level. The numbers 1-25 in the figure represent CpGs GPX3-8\_CpG\_1-25; KBD: KBD patients, C: healthy control group).



**Figure 3**

Comparison of methylation levels of four CpGs in the promoter region of GPX3 between KBD and Controls.





**Figure 4**

Comparison of methylation levels of CpGs in the promoter region of GPX3 between KBD patients with different degree.

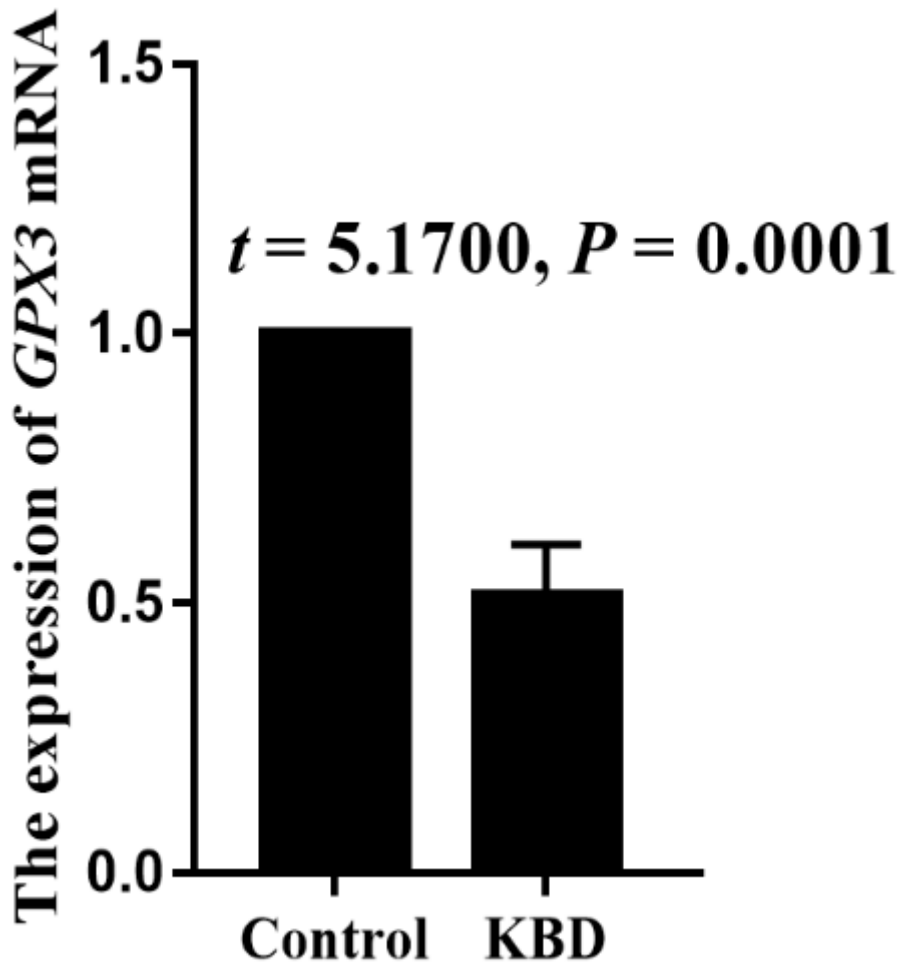


Figure 5

GPX3 mRNA levels in peripheral blood of KBD patients.

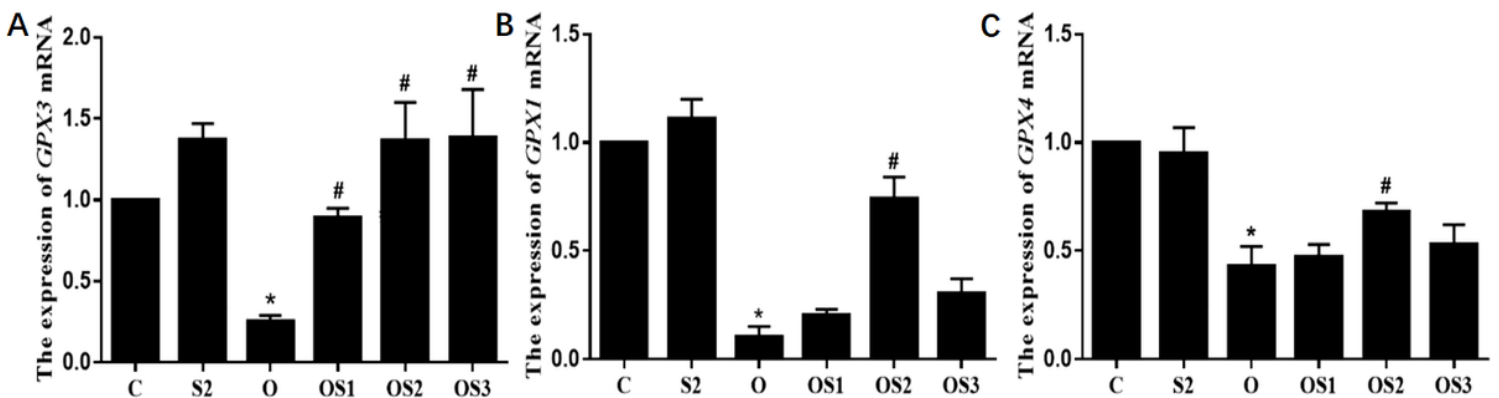
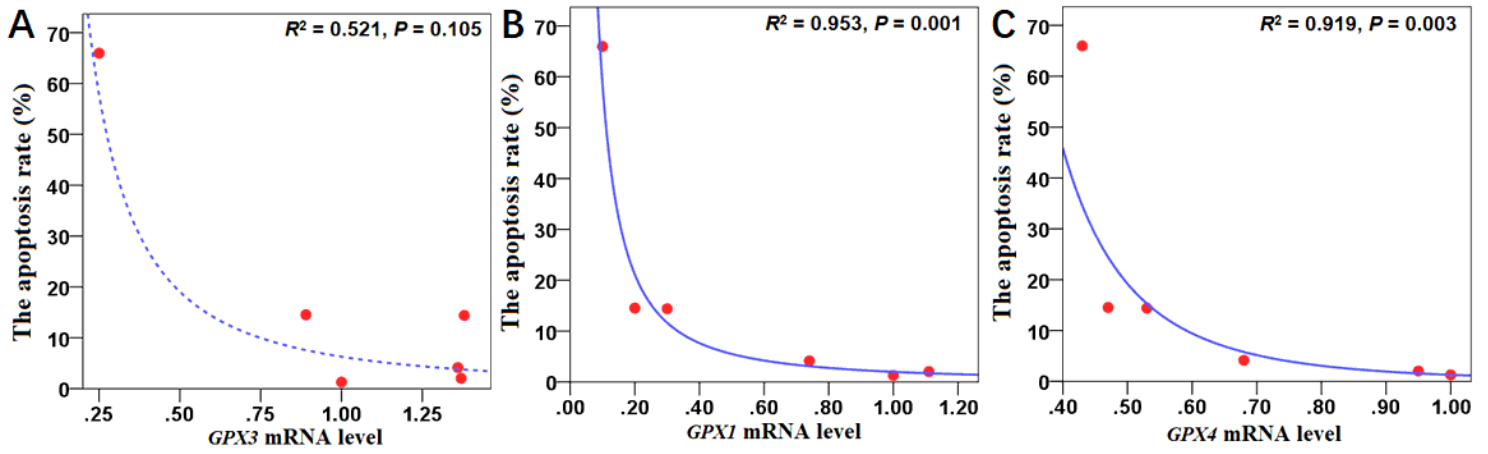


Figure 6

GPX1, GPX3, GPX4 mRNA levels in chondrocytes treated by tBHP and Na<sub>2</sub>SeO<sub>3</sub> (C: control group; S2: treated by 0.10 μg/mL Na<sub>2</sub>SeO<sub>3</sub>; O: treated by 150 μmol/L tBHP; OS1: treated by 0.05 μg/mL Na<sub>2</sub>SeO<sub>3</sub>+150 μmol/L tBHP; OS2: treated by 0.10 μg/mL Na<sub>2</sub>SeO<sub>3</sub>+150 μmol/L tBHP; OS3: treated by 0.15 μg/mL Na<sub>2</sub>SeO<sub>3</sub>+150 μmol/L tBHP).



**Figure 7**

The correlation between GPX1, GPX3, GPX4 mRNA expressions and apoptosis rate of chondrocytes (C: control group; S2: treated by 0.10  $\mu\text{g/mL}$   $\text{Na}_2\text{SeO}_3$ ; O: treated by 150  $\mu\text{mol/L}$  tBHP; OS1: treated by 0.05  $\mu\text{g/mL}$   $\text{Na}_2\text{SeO}_3$ +150  $\mu\text{mol/L}$  tBHP; OS2: treated by 0.10  $\mu\text{g/mL}$   $\text{Na}_2\text{SeO}_3$ +150  $\mu\text{mol/L}$  tBHP; OS3: treated by 0.15  $\mu\text{g/mL}$   $\text{Na}_2\text{SeO}_3$ +150  $\mu\text{mol/L}$  tBHP).

