Identification of HIF-1α/VEGFA signaling pathway and transcription factors in Kashin-Beck disease by integrated bioinformatics analysis

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Abstract. Kashin-Beck disease (KBD) is a chronic and endemic osteoarthropathy. The pathogenesis of KBD has yet to be fully elucidated, although previous studies have shown that its etiology may be associated with low selenium abundance and high exposure to mycotoxins, such as T-2 toxin. In the present study, the Comparative Toxicogenomics Database was used to identify key genes associated with KBD, T-2 toxin and selenium. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to identify the biological processes and pathways that key genes may be associated with. By searching the Search Tool for the Retrieval of Interacting Genes database and the Molecular Complex Detection plug-in with Cytoscape, it was possible to construct a KBD-associated protein-protein interaction (PPI) network, and screen the core modules and genes. Western blot analysis was subsequently used to verify the expression levels of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor A (VEGFA), two components that are associated with the HIF-1 signaling pathway in KBD disease. Via this approach, a total of 301 key genes were identified that were associated with KBD, T-2 toxin and selenium. The results of the GO and KEGG enrichment analyses demonstrated that these key genes were mainly involved in the process of apoptosis. Previous studies have demonstrated that excessive apoptosis of chondrocytes plays a crucial role in the pathophysiology of KBD, and that HIF-1α has an important role in chondrocyte apoptosis; therefore, the present study was focused on the expression level of HIF-1α in KBD. By analyzing the PPI network constructed from the key genes, a total of 10 core genes were obtained that may be associated with KBD. The results of western blotting experiments revealed that, after treating chondrocytes with different concentrations of T-2 toxin, the expression levels of HIF-1α and VEGFA were markedly downregulated. The iRegulon plug-in for Cytoscape was used to predict the transcription factors that may regulate HIF-1α and VEGFA in the HIF-1 signaling pathway. Using this approach, 10 core genes and 15 transcription factors were obtained. These results may help to clarify the pathogenesis of KBD, thereby providing further avenues for the therapeutic treatment of KBD.

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Introduction

Kashin-Beck disease (KBD) is a chronic and endemic osteoarthropathy characterized by chondrocyte necrosis in growth plates and articular cartilage (1). The geographical distribution of KBD includes southeastern Siberia in Russia, the northern region of North Korea and a long narrow zone from northeast to southwest China (2). Patients with KBD often exhibit clinical features such as arthralgia, restricted mobility and an enlarged metaphysis (1). In severe cases, short stature and dwarfism may occur (3). The pathogenesis of KBD has yet to be fully elucidated, although it is generally considered that its cause is multifactorial, including such contributory factors as selenium deficiency, iodine deficiency, food contaminated with mycotoxins and drinking water contaminated with humic acid (4).

Mycotoxins are a group of toxic secondary metabolites produced by fungal species. According to present statistics, ~25% of the world's food crops are contaminated with mycotoxins (5). T-2 toxin is a mycotoxin widely found in grains in the geographical regions where KBD is prevalent, and has been shown to induce apoptosis of human chondrocytes, oxidative stress and mitochondrial damage (6). When compared with normal subjects, the expression levels of apoptosis-associated molecules, including Bcl-2, Bax, Fas and inducible nitric oxide synthase, in the articular cartilage of patients with KBD have been shown to be elevated (7). In addition, T-2 toxin contamination and selenium deficiency have been shown to be widespread in drinking water and cereals in KBD-endemic areas (8). Selenium is an essential biological trace element that has previously been used in the treatment of KBD (9).

The 2019 Nobel Prize in Physiology or Medicine was awarded to scientists for their discovery of how cells sense and adapt to oxygen supply, and also for their contribution towards understanding of the molecular machinery that regulates the activity of genes in response to varying levels of oxygen (10). Previous studies have demonstrated that hypoxia and hypoxia-associated signaling pathways exert an important role in the progression of KBD disease (11,12). Under low-O_2 (hypoxic) conditions, cells activate various adaptive responses to match the oxygen demands of metabolic, bioenergetic and redox processes (13). Hypoxia-inducible factor (HIF) is considered to be a key regulator of the transcriptional response to hypoxic stress. HIF is a heterodimeric transcription factor that consists of either HIF-1α or HIF-2α and HIF-1β/ARNT subunits (14). It is a key transcription factor that is activated in a hypoxic environment, subsequently regulating the expression of a series of genes that are responsible for cell metabolism, migration, proliferation, angiogenesis and inflammation (15). Previous studies have shown that T-2 toxin is able to induce the production of reactive oxygen species in chondrocytes, and activate the expression of both NF-κB and HIF-2α (16-18).

Known HIF-1 signaling pathways include the PI3K-Akt/HIF-1α, MAPK/HIF-1α and HIF-1α/vascular endothelial growth factor A (VEGFA) signaling pathways (19-22). VEGFA is a member of the VEGF platelet-derived growth factor family of structurally related mitogens (23). Previous studies have shown that VEGF in the articular cartilage of patients with KBD may be abnormally expressed (24,25). Moreover, HIF has been shown to regulate the expression levels of several hundred genes, and VEGF is one of the primary target genes (26). In the past decade, extensive research has clarified the key role of HIF and VEGF in controlling the survival of hypoxic cartilage [for a review on this topic, see (27)]. It was therefore possible to hypothesize that the HIF-1α/VEGFA signaling pathway may be associated with the pathogenesis and progression of KBD.

In the present study, the Comparative Toxicogenomics Database (CTD) was used to identify genes associated with KBD, T-2 toxin and selenium. After identifying which of the genes were intersecting, those genes were further selected for subsequent enrichment analysis and protein-protein interaction (PPI) network construction, and western blotting was then performed to verify the expression levels of HIF-1α and VEGFA in chondrocytes treated with T-2 toxin.

Materials and methods

Target identification using the CTD. The CTD (http://ctdbase.org) is a useful public resource featuring extensive information regarding exposure to numerous types of chemicals and human health (28). The database includes in excess of 38 million toxicogenomic relationships that may be explored further in terms of analytical investigations and the development of scientific hypotheses. In the present study, all key genes associated with KBD, T-2 toxin and selenium were predicted using CTD database that was updated to June 2020 (inference score, >3.07).

Gene Ontology (GO) and pathway enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov) is an online bioinformatics tool designed to identify the functions of a large number of genes or proteins (11). In the present study, KBD-associated key genes were uploaded, and GO enrichment results were collected (http://geneontology.org/), including biological processes (BP), cellular component (CC), molecular function (MF) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis (https://www.kegg.jp/). $P<0.05$ was considered to indicate a statistically significant result in this analysis.

PPI network analysis. Information regarding PPIs may be evaluated using an online tool, the Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org) (29). To estimate the interactions of KBD-associated key genes, these genes were first analyzed by STRING, and subsequently Cytoscape software (version 3.6.1; https://cytoscape.org) was used to construct a PPI network. The Molecular Complex Detection (MCODE; http://apps.cytoscape.org/apps/mcode) plug-in for Cytoscape was used to investigate modules of the PPI network (degree cutoff=2; maximum depth=100; k-core=2; and node score cutoff=0.2). Similarly, the STRING database and Cytoscape software were used to construct a PPI network of genes associated with the HIF-1 signaling pathway.

Chondrocyte culture and experimental protocol. Human C28/I2 normal chondrocytes were purchased from the BeNa Culture Collection and cultured in DMEM Nutrient Mixture F-12 (DMEM/F12; 1:1) (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS (Hyclone; Cytiva) in a humidified incubator containing 5% CO_2 at 37˚C. All cells were used for subsequent experiments between the fifth and tenth passages. Cells were cultured in 6-well plates and used for protein extraction once the cell density had reached $6x10^4$ cells/well. The medium was replaced every other day. T-2 toxin was provided by MedChemExpress and dissolved in DMSO to make up a working solution with a concentration of 100 μg/ml T-2 toxin. The cells were plated and incubated for 24 h to allow them to adhere prior to treatment with T-2 toxin. Subsequently, cells were exposed to fresh medium containing various doses of T-2 toxin (0, 0.001, 0.005, 0.01, 0.02 and 0.05 μg/ml). Proteins were then extracted by ultrasonic disruption after the C28/I2 chondrocytes had been incubated at 37˚C with T-2 toxin for 3 days, as detailed in previous studies (30,31). Incubation with each concentration of T-2 toxin was repeated five times, and DMSO-treated cells were used as a control.

Western blot analysis. The protein concentration was determined by using the BCA method (Biyuntian Biotechnology). SDS-PAGE loading buffer was added and the protein sample boiled in 100˚C water. Protein samples of chondrocytes treated with different concentrations of T-2 toxin were separated using SDS-PAGE (10% gels) and transferred to PVDF membranes (EMD Millipore). After blocking with 5% skimmed milk diluted in TBS containing 0.1% Tween-20 (TBST) overnight at room temperature, the membranes were incubated with primary antibodies, as detailed below, and then incubated with the secondary antibodies conjugated with horseradish peroxidase. Blots were visualized by using a hypersensitivity ECL chemiluminescence detection kit (Biyuntian

Biotechnology). The anti-HIF-1α (1:1,000; cat. no. ab82832) and anti-VEGFA (1:1,000; cat. no. ab46154) antibodies were purchased from Abcam, whereas the rabbit anti-β-actin (1:3,000; cat. no. bs-0061R) and goat anti-rabbit IgG (1:3,000; cat. no. bs-0295G-HRP) antibodies were purchased from BIOSS. The primary antibodies were incubated for 30 min at 37˚C and then overnight at 4˚C. The membrane was subsequently incubated with a secondary antibody for 1 h at room temperature after washing three times in TBST. The relative intensities of the blots featuring the target proteins of interest were calculated by normalizing against β-actin. Densitometric analysis of western blots was performed using ImageJ software (v1.52; National Institutes of Health).

Transcription factor prediction. IRegulon (http://apps.cytoscape.org/apps/iregulon) was developed using a genome-wide ranking-and-recovery approach as a Cytoscape plug-in for the purpose of detecting enriched transcription factor motifs and their optimal sets of direct targets (32). This technology was used to perform the enrichment of transcription factor motifs in target sequences with a position matrix method to identify transcription factors that were associated with HIF-1α, VEGFA and the HIF-1 signaling pathway. A minimum identity between orthologous genes was defined as 0.05, and the maximum false discovery rate (FDR) value of motif similarity was set to 0.001. Associations with the normalized enrichment score (NES) were used for further analysis.

Statistical analysis. The results are expressed as the mean ± SD for experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA and the means were compared by Dunnett's post hoc test using GraphPad Prism statistical software (version 8.01; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of genes associated with KBD, T-2 toxin and selenium. Using the CTD database, 3,676 genes were identified to be associated with KBD, 2,299 to be associated with T-2 toxin and 1,669 to be associated with selenium. In order to obtain the KBD-associated key genes, the above genes were selected for intersection analysis. A set of 301 key genes were revealed to be held in common among the genes of the KBD, T-2 toxin and selenium groups, as shown by the Venn diagram in Fig. 1.

Functional enrichment analysis of KBD-associated key genes. The results revealed that the majority of key genes included in the GO enrichment analysis were associated with the BP component term 'negative regulation of apoptotic process' (GO: 0043066; Fig. 2A). The BP terms 'response to hypoxia' and 'response to reactive oxygen species' were also in the top 10 results of the GO enrichment analysis. The majority of key genes were associated with the CC parameter term 'cytosol' (GO: 0005829) and MF parameter term 'protein binding' (GO: 0005515). Furthermore, KEGG enrichment analysis of the key genes found the term 'pathways in cancer' (hsa05200) to be most significantly enriched (Fig. 2B). In addition,

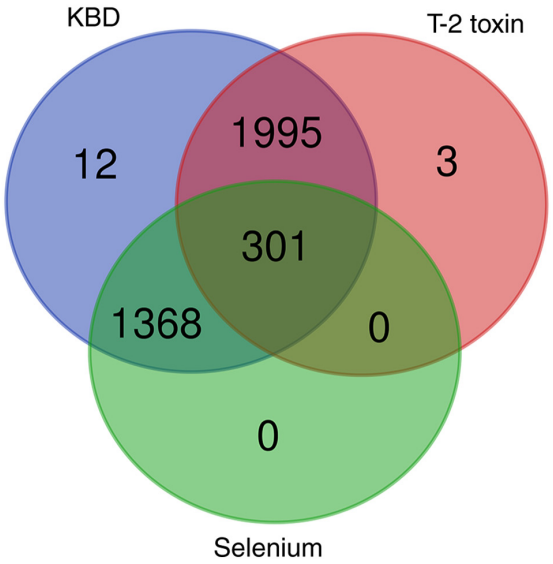

Figure 1. Intersection analysis of genes associated with KBD, T-2 toxin and selenium. A Venn diagram is presented showing the similarities among KBD, T-2 toxin and selenium-associated genes, and only 301 genes were held in common. KBD, Kashin-Beck disease.

signaling pathways known to be associated with KBD were identified among the top 15 enrichment terms, including 'apoptosis' (hsa04210), and the 'TNF signaling pathway' (hsa04668), 'PI3K-AKT signaling pathway' (hsa04151) and 'HIF-1 signaling pathway' (hsa04066).

Construction of the PPI network for KBD-associated key genes. A PPI network of key genes was constructed in the online database STRING, and contained 299 nodes and 2,830 edges. Subsequently, the interaction pairs were entered into Cytoscape software to construct multiple PPI networks. The core network module was then selected using the Cytoscape MCODE plug-in. The first-ranked module was extracted under the default parameters and contained 42 nodes and 716 edges (Fig. 3). The top 10 genes with MCODE scores in this module were revealed to be *BCL2L1*, *MMP9*, *CASP8*, *HSP90AA1*, *IL10*, *HSPA4*, *CXCL8*, *CASP9*, *MTOR* and *TNF*.

Downregulation of HIF-1 α and VEGFA in T-2 toxin-treated chondrocytes. In order to study the expression of HIF-1α and VEGFA in KBD, different concentrations of T-2 toxin were used to treat chondrocytes for 3 days. Western blotting results revealed that the expression levels of HIF-1α and VEGFA in the T-2 toxin-treated group were significantly reduced in comparison with the control group. As the concentration of T-2 toxin was increased, the expression levels of HIF-1α and VEGFA were gradually reduced. However, when the concentration of the T-2 toxin added was 0.01 and 0.02 μg/ml, the expression level of HIF-1α showed a tendency to increase (Fig. 4).

Construction of the PPI network associated with the HIF-1 signaling pathway and prediction of transcription factors. Including HIF-1α and VEGFA, all molecules of the HIF-1 signaling pathway identified by KEGG enrichment analysis were used to construct a PPI network (Fig. 5). Subsequently,

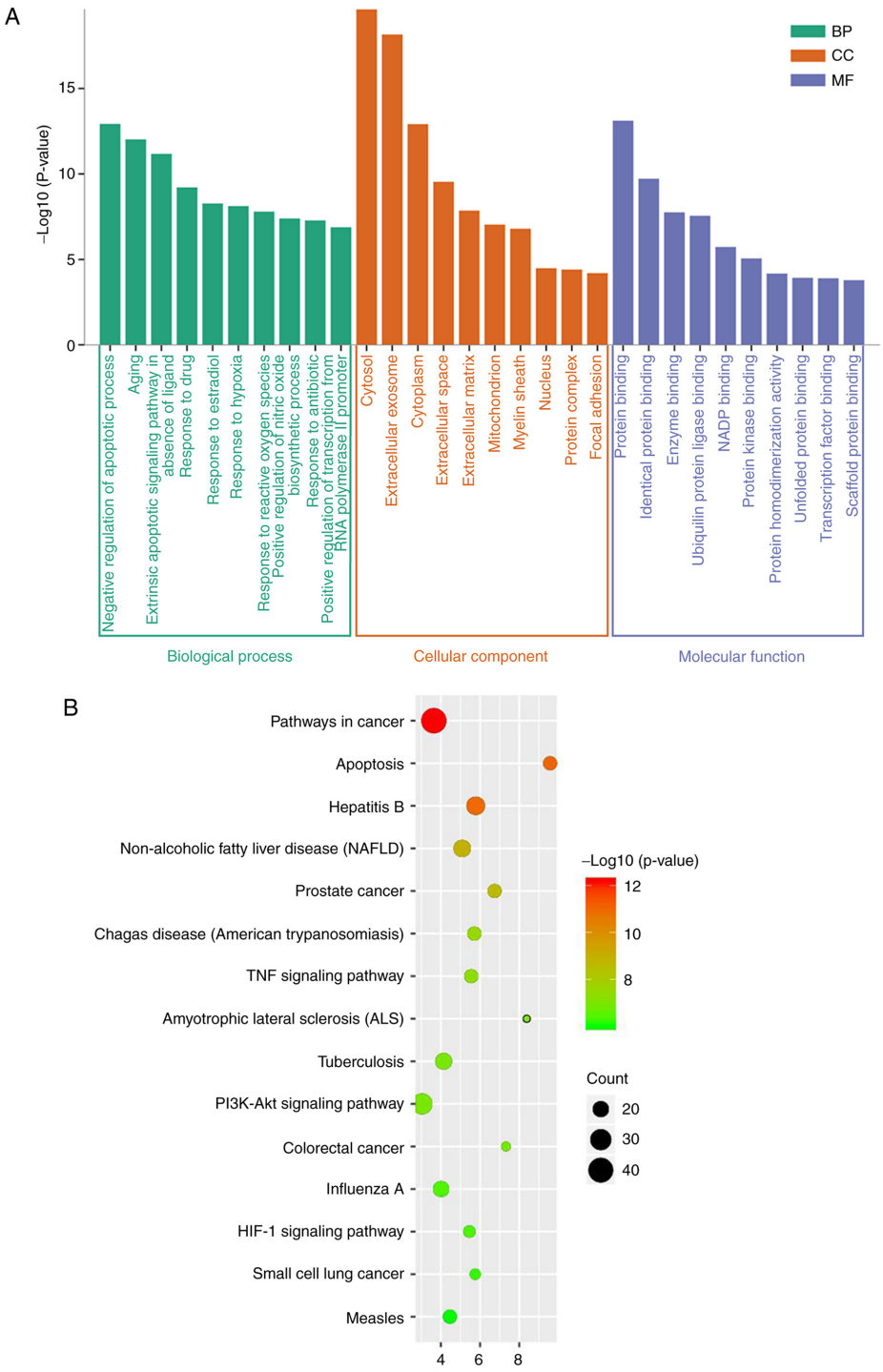

Figure 2. Enrichment analysis of key genes. (A) Gene Ontology enrichment analysis. Green, orange and blue represent the BP, CC and MF parameters, respectively. The x-axis shows the terms of biological function, whereas the y-axis shows the P-value after log transformation. (B) Kyoto Encyclopedia of Genes and Genomes enrichment analysis. The size of the circles represents the number of genes enriched in the various pathways. BP, biological processes; CC, cellular component; MF, molecular function.

the constructed network was imported into Cytoscape, and the iRegulon plug-in was used to predict the transcription factors that may regulate these target genes (NES=7.152). The results obtained showed that 7 targets (*NOS3*, *VEGFA*, *IL6*, *RELA*, *TIMP1*, *GAPDH* and *HMOX1*) in the PPI network composed of 17 genes were regulated by the predicted 15 transcription factors (*MAFK*, *MAFG*, *NFE2*, *NFE2L2*, *MAFF*, *BACH1*, *MAFA*, *JUNB*, *FOS*, *JUND*, *FOSL1*, *JUN*, *FOSB*, *MAF* and *DBP*).

Discussion

KBD is a chronic and severe progressive bone and joint degenerative disease of unknown etiology. An elevated prevalence of KBD has been shown in populations living in geographic areas with low selenium abundance and high exposure to mycotoxins (8). An accumulating body of evidence, together with recent scientific discoveries, have indicated that mycotoxins, including T-2 toxin, have the potential to trigger cell hypoxia (9,10).

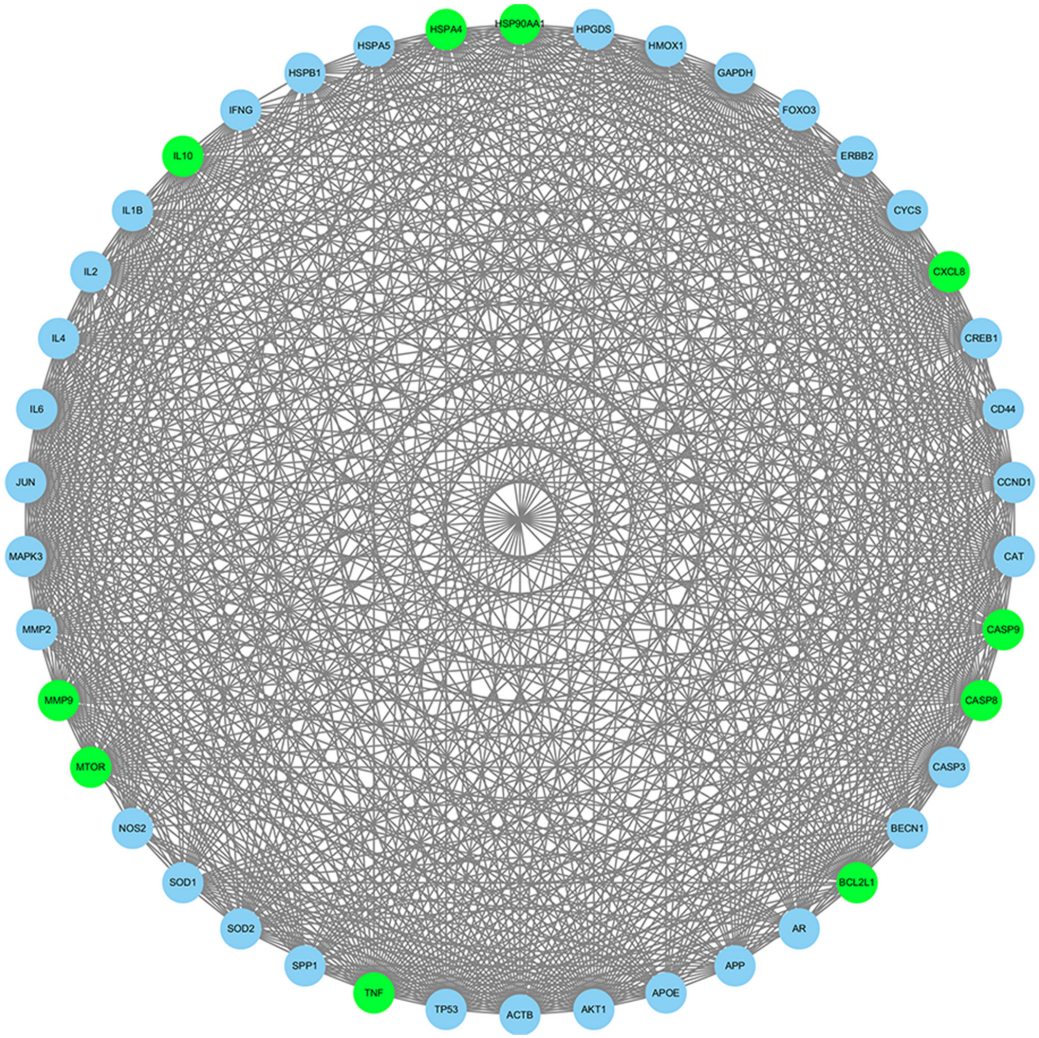

Figure 3. PPI network construction and module analysis. The densest connected regions (42 nodes and 716 edges) in the PPI network were identified with the MCODE plug-in Cytoscape. The green nodes represent the top 10 genes based on MCODE score. MCODE, Molecular Complex Detection; PPI, protein-protein interaction.

In the present study, the target genes associated with KBD, T-2 toxin and selenium were first obtained from the CTD database. These targets were associated with the GO BP terms of 'apoptosis' and 'hypoxia', as well as the 'TNF signaling pathway', 'PI3K-AKT signaling pathway' and 'HIF-1 signaling pathway'. These results suggested that TNF-α was associated with inflammation and apoptosis in KBD; this is in agreement with previous studies that indicated that the levels of TNF-α in the serum and cartilage of patients with KBD were markedly higher compared with those of healthy controls (33,34). PI3K-AKT is the main signaling pathway for chondrocyte survival and apoptosis, and the core hub for transmitting external signals (35). A previous study indicated that oxidative stress-induced chondrocyte apoptosis may be mediated via upregulation of the PI3K-AKT signaling pathway (36). An additional study indicated that the regulation of HIF-1α by components of the PI3K-AKT signaling pathway may directly regulate the stability of HIF-1α protein via its downstream effects (37).

In addition to the PI3K-AKT/HIF-1α pathway, HIF-1 signaling also includes the MAPK/HIF-1α signaling pathway and the HIF-1α/VEGFA signaling pathway (38). A previous study revealed that excessive apoptosis of chondrocytes and oxidative stress served a crucial role in the pathophysiology of KBD (39). Earlier research also indicated that under hypoxic or normoxic conditions, the level of apoptosis of HIF-1α-deficient chondrocytes was significantly increased in osteoarthritis (40,41). Therefore, it was hypothesized that T-2 toxin-induced chondrocyte apoptosis was associated with HIF-1α, and experiments were devised to assess the expression of HIF-1α in KBD. The results indicated that HIF-1α expression was reduced in chondrocytes treated with different concentrations of T-2 toxin in a dose-dependent manner. By contrast, VEGF-A expression was shown to be reduced in a dose-dependent manner following treatment with different concentrations of T-2 toxin. However, the precise mechanism via which HIF-1α regulates VEGFA in KBD requires further exploration.

Through the PPI network constructed by the key genes associated with KBD and the MCODE plug-in of Cytoscape, 10 molecules were screened out that may be associated with KBD. The genes *CASP8* and *MTOR* have been previously associated with KBD (42-44). Using Cytoscape software and its plugin to analyze the HIF-1 signaling pathway led to the prediction of 15 transcription factors and 7 target genes. Of these, the genes *IL6* (45), *RELA* (46), *TIMP1* (47) and *HMOX1* (48), as well as

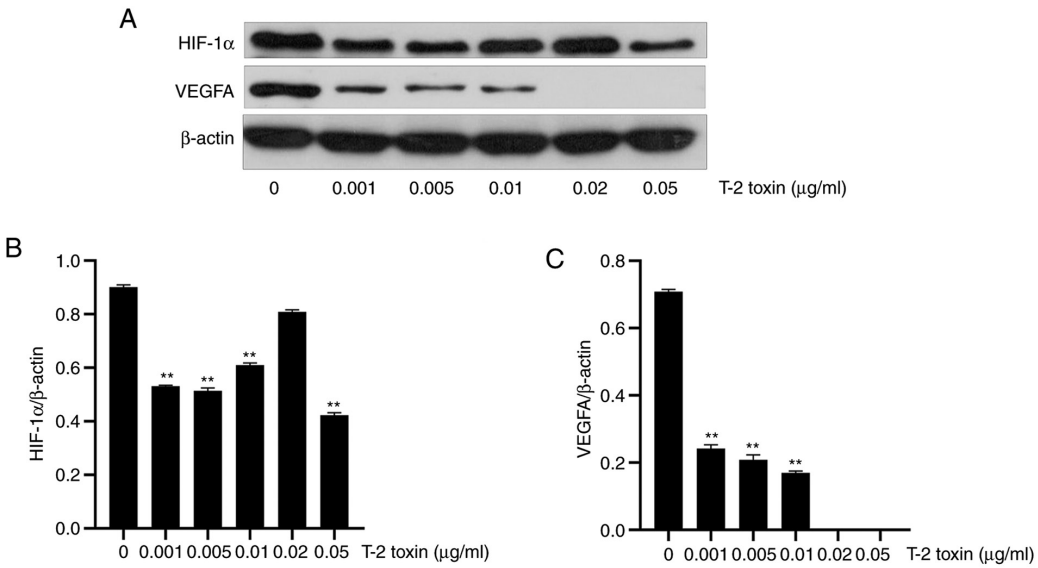

Figure 4. Effects of T-2 toxin on the expression levels of proteins associated with the HIF-1α/VEGFA signaling pathway. (A) C28/I2 chondrocytes were pretreated with the indicated concentrations (0.001, 0.005, 0.01, 0.02 and 0.05 μg/ml) of T-2 toxin for 3 days, followed by western blotting analysis to examine the protein expression levels of HIF-1α and VEGF-A. β-actin was used as the loading control. Ratios of (B) HIF-1α and (C) VEGFA protein expression values normalized to β-actin expression. Data are shown as the mean ± SD from three independent experiments. **P<0.01 vs. control group (0 μg/ml T-2 toxin). HIF-1α, hypoxia-inducible factor/1α; VEGFA, vascular endothelial growth factor A.

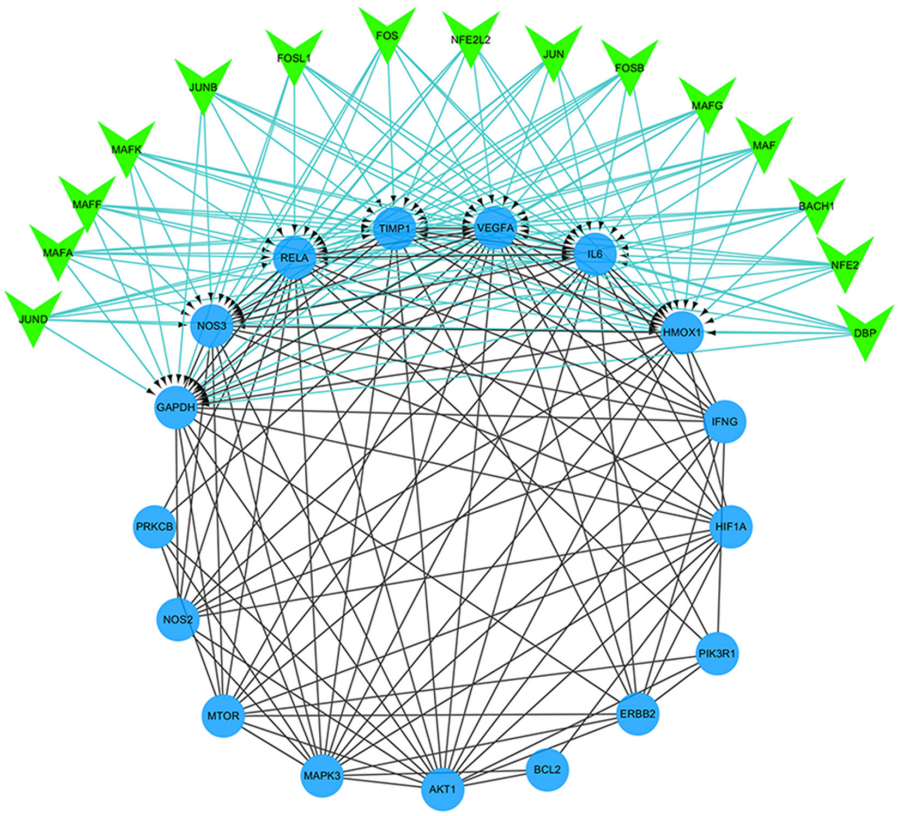

Figure 5. Regulatory networks of transcription factors and genes associated with HIF-1α, VEGFA and the HIF-1 signaling pathway. Circles represent genes or target genes, and green 'V' shapes represent transcription factors. HIF-1α, hypoxia-inducible factor/1α; VEGFA, vascular endothelial growth factor A.

the transcription factors *NFE2L2* (48), *JUNB* (49), *FOS* (50), *JUND* (49) and *JUN* (47), have been reported to be associated with KBD.

There are several limitations of the present study that should be acknowledged. Follow-up experiments on selenium deficiency were not performed. Whether selenium deficiency or selenium deficiency combined with T-2 toxin is able to affect the expression of HIF-1α and VEGFA following treatment of the chondrocytes requires further experimental verification. In addition, further experiments, such as inhibitor studies or

transfection experiments, are required to determine the association between HIF-1α and VEGFA.

In conclusion, a total of 10 core genes and 15 transcription factors associated with KBD were identified in the present study. The results also indicated that the expression levels of HIF-1α and VEGFA in T-2 toxin-treated chondrocytes were downregulated. Therefore, the results of the present study suggested that the HIF-1α/VEGFA signaling pathway is involved in KBD, and this knowledge may help to both further elucidate the pathogenesis of KBD and provide possible avenues for treatment of KBD in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL and GW conceived and designed the experiments, performed the experiments, analyzed the data, contributed materials and analytical tools, prepared the figures and authored and reviewed drafts of the paper. BX and HH analyzed the data and authored and reviewed drafts of the paper. All authors have read and approved the final manuscript. BX and WL confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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