Comparative analysis of signaling pathways in peripheral blood from patients with Kashin-Beck disease and osteoarthritis

YUJIE NING*, XI WANG*, SEN WANG and XIONG GUO

Key Laboratory of Trace Elements and Endemic Diseases, National Health and Family Planning Commission, School of Public Health, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China

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Abstract. The aim of the present study was to investigate the early diagnostic biomarkers of Kashin-Beck disease (KBD), and to compare the common signaling pathways of peripheral mononuclear cells between patients with KBD and those with osteoarthritis (OA). A total of 20 and 12 peripheral blood samples were separately collected from KBD patients and normal control subjects, respectively, in an endemic area according to the diagnosis criteria. Total RNAs were extracted and gene expression levels were determined using an Agilent whole genome expression microarrays. The gene expression data of OA were obtained from GEO published database. Significant different pathways between KBD and OA were analyzed using Ingenuity Pathway Analysis software. A total of 82 differentially expressed genes, 51 significant different signaling pathways and five significant biological functions were identified in KBD patient samples, while 89, 50 and five significantly different genes, pathways and functions were identified in OA. Nine common significant pathways and five common differentially expressed genes were identified between the KBD and OA. Nine common significant pathways and five common differentially expressed genes were found between the two diseases. The present results suggest that there are similarities in vascular microcirculation, immunoreactions and cell apoptosis between KBD and OA, which may contribute to the early diagnosis and pathogenetic study of KBD.

Correspondence to: Professor Xiong Guo, Key Laboratory of Trace Elements and Endemic Diseases, National Health and Family Planning Commission, School of Public Health, Health Science Center, Xi'an Jiaotong University, 76 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: guox@mail.xjtu.edu.cn

*Contributed equally

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Introduction

Kashin-Beck disease (KBD) is an endemic osteoarthropathy, which is similar with osteoarthritis (OA) in clinical manifestation, with pathological features including cartilage degeneration, cartilage extracellular matrix degradation, chondrocyte necrosis and apoptosis (1,2). In contrast with OA, instead of the middle aged and elderly people, KBD predominantly presents in 3-12 year old patients. KBD is characterized by swelling of digits, deformed limb joints and limited movement or potentially dwarfism (3), the majority of adult patients partly or completely lose work and self-care ability. KBD primarily occurs in the northeast and southwest regions of China where selenium deficiency has been associated with the disease pathogenesis (4). To date, there are more than 0.66 million KBD patients in China, while less than 30 million residents are at risk in endemic areas (4,5).

At present, diagnosis of KBD remains to be based on clinic manifestations and X-ray changes of metaphyseal (5). KBD may be classed as early, stage I, II and III, according to the severity (6). The clinic changes generally appear after cartilage degeneration with a slow process, thereby the method is not sensitive enough to diagnose and treat the early case which inevitably leads to irreversible aggravation, such as deformed limbs. Hence, effective early diagnosis is crucial for the therapy and prognosis of KBD.

Peripheral blood collection is rapid, with small trauma, which is why it is the ideal biological sample for the early diagnosis of various diseases (7). A number of studies have analyzed the differentially expressed genes of peripheral blood to identify marker genes for early diagnosis of diseases including cancer, coronary disease and OA (8-12). With regard to KBD, in prior studies differentially expressed genes in cartilage and peripheral blood have been screened using microarrays (1,7,13). Although single gene expression analyses are important for authenticating the related target genes of KBD and OA, KBD is a gene-environment interactional disease; therefore, gene network and pathway analyses may prove more comprehensive and effective for the early diagnosis and therapy of KBD and OA, as compared with single gene analyses.

In the present study, an Agilent 44k human microarray was applied to determine the gene expression profile of peripheral blood mononuclear cells (PBMCs) and to identify the different expressed genes between the KBD and normal control

Table I. Characteristics of the patients with KBD and control subjects used for microarray and RT-qPCR analysis.

	KBD				Normal			
Sample pair	*n*	Age, years (range)	Male	Female	*n*	Age, years (range)	Male	Female
Microarray								
1	5	47.20 (41-55)	2	3	3	45.00 (35-63)	1	2
2	5	50.40 (43-58)	2	3	3	48.67 (39-68)	1	2
3	5	50.20 (40-67)	2	3	3	44.67 (37-59)	1	2
4	5	46.60 (38-58)	3	2	3	51.00 (27-54)	0	3
RT-qPCR								
1	5	59.60 (52-69)	3	2	5	56.00 (48-65)	3	2

KBD, Kashin-Beck disease; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

subjects. The data of different expressed genes in peripheral blood of OA was obtained from GEO database (http://www.ncbi.nlm.nih.gov/geo/). Ingenuity Pathway Analysis (IPA) was used for network and pathway analyses. The comparison of commonly expressed genes and signaling pathways between KBD and OA patients may help to identify the key genes and pathways associated with KBD, for improved early diagnosis and elucidation of its pathogenesis.

Materials and methods

Ethical approval. This study was approved by the human ethics committee of Xi'an Jiaotong University (Xi'an, China) and all subjects provided informed consent.

Disease diagnosis and grouping. *S*ubjects were selected randomly from the Center for Disease Control, Yongshou County (Xianyang, China), an endemic area with a KBD prevalence of 17.4% (14). Patients were diagnosed according to WS/T 207-2010 criteria (6), while those with any history of other osteoarticular diseases were excluded. Finally, 20 KBD patients and 12 normal controls were selected (age and gender matched) and divided into four groups (KBD, n=5 subjects/pair; control, n=3 subjects/pair) (Table I). In additional, 5 KBD patients and 5 normal controls were selected to validate the results of the microarray (Table I).

Peripheral blood sample collection. A total of 3 ml peripheral blood was collected from each subject into heparinized vacutainer tubes (BD Biosciences, San Jose, CA, USA) for gene expression analysis. The number of leukocyte cells was determined by using a Hemovet 950 (Drew Scientific, Inc., Oxford, CT, USA). PBMCs were separated from the plasma by centrifuging blood at 1,500 x g for 20 min. The cell pellet was resuspended in Hank's balanced salt solution (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell suspension was layered over 5 ml Lympholyte-H (Cedarlane Laboratories Ltd., Hornby, ON, Canada) in a 15-ml Falcon tube and centrifuged for 40 min at 1,500 x g. After rinsing twice with cold Hank's balanced salt solution, they were stored in RNAlater (Ambion, Inc., Austin, TX, USA) until RNA isolation.

RNA preparation. The total RNA was isolated from PBMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) following the manufacturer's recommended protocol. The quality and integrity of extracted total RNA were evaluated using a high resolution electrophoresis system (Agilent 2100 bioanalyzer; Agilent Technologies, Palo Alto, CA, USA). To avoid individual differences among subjects, 30 μl total RNA was extracted from each individual subject and mixed to form four pairs of microarrays, according to the four groups in Table I.

Microarray hybridization. Isolated total RNA was mixed with RNase-free DNase I (Thermo Fisher Scientific, Inc.) and incubated at 37˚C for 30 min, followed by mixing with EDTA at 65˚C for 10 min, to remove residual genomic DNA. The isolated total RNA of KBD and control in each pair was first transcribed into cDNA, which were labeled with CyDye, using an Amino Allyl Message Ampa RNA Kit (Ambion, Inc.) according to the manufacturer's protocol. For each pair, 0.5 μg labeled cDNA was purified separately and mixed with hybridization buffer (LifeGen Technologies, LLC, Madison, WI, USA) prior to application to the microarrays. The Agilent 44K human whole-genome oligonucleotide microarray (Agilent Technologies) consisting of 21,073 (60-mer) oligonucleotide probes, which span conserved exons across the transcripts of the targeted full-length genes, was then applied for microarray hybridization following the Agilent recommended protocol. The microarray slides were scanned using Gene-Pix 4000B (Axon Instruments, Inc., Foster City, CA, USA). GenePixPro 3.0 software package (Axon Instruments, Inc.) was used to analyze the 16-bit TIFF images produced by the Axon scanner. The ratio image of all spots was defined by accessing the gene list file which described the location of each gene on the microarray. The gene expression data were imported into Excel spreadsheets (Microsoft Corporation, Redmond, WA, USA) using Unigene (http://www.ncbi.nlm.nih.gov/unigene) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) descriptors after the average signal intensity was subtracted from the median background intensity. Global normalization was conducted to calculate the relative expression level between two samples using all detected genes.

Gene expression analysis. Significance analysis of microarrays (SAM) (15) is a statistical technique used to identify differentially expressed genes. The cutoff for significance is determined by a tuning parameter, delta, selected by the user based on the false-positive rate (<0.05). The SAM algorithm performs unsupervised calculations based on Student's *t*-test to identify significant genes in a set of microarray data. SAM computes a statistic 'di' for each gene 'i', measuring the strength of the association between gene expression and the response variable. It uses repeated permutations of the data to determine if the expression levels of selected genes are significantly associated with the response.

Microarray data of PBMCs in OA. The PBMCs of OA microarray data GSE48556, published by Ramos *et al* (12), was downloaded from GEO database in NCBI and performed using IPA. The data GSE48556 included 106 patients with OA and 33 normal controls that were age and sex-matched. In total, 1 ml peripheral blood was collected from each sample for the microarray analysis, conducted using an Illumina HumanHT-12 v3 BeadChip platform (Illumina, San Diego, CA, USA).

IPA. IPA uses Fisher's exact test to determine which biofunctions and canonical pathways are significantly associated with the genes of interest compared with the whole Ingenuity knowledge base (http://www.ingenuity.com/science/knowledge-base). In the present study, IPA was used to identify the signaling pathways and biofunctions of KBD and OA in PBMCs. Gene symbols of PBMCs of KBD and OA patients were imported into IPA, version 8.5 (Ingenuity Systems; Qiagen, Hilden, Germany) web-based software, respectively. IPA is a repository of biological interactions and functional annotations including proteins, genes, complexes, cells, tissues, metabolites, drugs and diseases. EntrezGene (http://jura.wi.mit.edu/entrez_gene/), Ref-Seq (http://www.ncbi.nlm.nih.gov/refseq/), OMIM (http://www.ncbi.nlm.nih.gov/omim), TargetScan (www.targetscan.org), GWAS (http://www.gwascentral.org/) and KEGG (http://www.genome.jp/kegg/) were selected by IPA. The trial version of IPA software was used. Genes from the dataset that met the log ratio cutoff of 2.0 were considered for analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation. To validate the microarray results from different experimental groups, four significant differentially expressed genes in PBMCs were selected as target genes to undergo RT-qPCR. Total RNA was isolated from PBMCs (Table I), and prepared in the same method as for oligonucleotide microarray analysis. mRNA was converted into cDNA using superscript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and random primers. qPCR was performed using 2.0 μl cDNA and the SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc., Otsu, Japan) on the ABI7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The PCR cycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primer sequences were as follows: CCL3 forward, 5'-TTCCGTCACCTGCTCAGAAT-3' and reverse, 5'-TGGCTGCTCGTCTCAAAGTA-3'; B2M forward, 5'-TGGGTTTCATCCATCCGACA-3' and reverse, 5'-ATGCGGCATCTTCAAACCTC-3'; HBA2 forward, 5'-CAGCTCTTGCTGCTGCTGTG-3' and reverse, 5'-AAGGATGATCTTGCAGGCAGAA-3'; BIRC3 forward, 5'-GACTCAGGTGTTGGGAATCTGGA-3' and reverse, 5'-TGAGGGTAACTGGCTTGAACTTGAC-3'; and β-actin forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. Relative fold-change for each individual gene was calculated using the comparative threshold cycle equation $2^{-\Delta\Delta Cq}$. Cq values of target genes were normalized against the Cq values of β-actin. The PCR results were analyzed using the Bio-Rad iQ5 software, version 2.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mann-Whitney Wilcoxon Test was performed to determine significance levels of expression differences for the selected genes between KBD and healthy controls.

Results

Different expressed genes between KBD and OA. Using the SAM 4.01 analysis, 82 differentially expressed genes were identified from microarrays of KBD compared to the control. Among these genes, 78 were upregulated and four downregulated. These genes are primarily involved in eight functions, including transport, transmembrane receptor, cytokine, ion channel, transcription regulator, enzymes and growth factors. Compared with the 89 differentially expressed genes of OA identified by Ramos *et al* (12), BIRC3, IL-1β, NFKBIA, EGR1 and CXCL8 are identified as common differentially expressed genes (Table II).

IPA analyses of KBD and OA. Based on 82 different expressed genes in KBD, 51 significant pathways and five most common molecular and cellular biofunctions, including cell death and survival (29 genes were involved), lipid metabolism, molecular transport, micro-molecule biochemistry and cell movement (Table III) were identified by IPA. Regarding OA, based on 89 different expressed genes, 50 significant pathways and five most common molecular and cellular biofunctions were identified by IPA. The five most common molecular and cellular biofunctions were primarily associated with cell growth and proliferation (42 genes were involved), intercellular signaling and interaction, cell migration, gene expression and cell movement (Table III). There are nine common significant pathways between KBD and OA including NF-κB, IL-6, IL-10, apoptosis, death receptor, TWEAK, p38 MAPK, TNFR1 and TNFR2 signaling pathways (Table IV). In additional, five most common networks of KBD and five of OA were identified using IPA, including cell-to-cell signaling and interaction, cellular movement, immune cell trafficking; cellular development, cellular growth and proliferation, organ morphology; lipid metabolism, molecular transport, small molecule biochemistry; hematological disease, hereditary disorder, organism injury and abnormalities; cancer, reproductive system disease and cell morphology (Table V).

RT-qPCR verification. To validate the microarray data, four differentially expressed genes were selected for RT-qPCR analysis using PBMC samples from an additional 10 KBD

Table II. List of five common expression genes in PBMCs of KBD and OA patients.

Gene	Symbol	NCBI ID	Fold change	
			KBD	OA
Baculoviral IAP repeat containing 3	BIRC3	NM_001165	2.23	2.01
Interleukin 1, beta	IL-1β	NM_000576	2.03	1.76
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	NM_020529	2.01	1.75
Early growth response 1	EGR1	NM_001964	2.13	5.35
Chemokine (C-X-C motif) ligand 8	CXCL8	NM_000584	2.11	3.07

KBD, Kashin-Beck disease; OA, osteoarthritis.

patients. The expression levels of BIRC3, CCL3, B2M and HBA2, in the PBMCs of KBD patients were shown to be significantly different from the normal controls (Fig. 1). Notably, the changes were consistent with those indicated by the microarray data.

Discussion

In the present study, five differentially expressed genes and nine significant pathways in common between KBD and OA were identified, which may prove useful for investigating the early diagnosis and pathogenesis of each condition. Compared with cartilage, peripheral blood is easier to collect and results in reduced wounding. In addition, peripheral blood may respond to rapidly to changes in disease status (8). Therefore, differentially expressed genes in the peripheral blood may be useful results for improving the early diagnosis of KBD.

The present study identified five genes that were recurrently differentially expressed in the peripheral blood of patients with KBD and OA. BIRC3 is a member of BIRC family, apoptosis regulation is among the most important functions of their encoded proteins (16,17). Furthermore, BIRC2, BIRC3 and TRAF2 have been identified as critical genes for the activation of NF-κB signaling in TNF pathway (18). In the present study, BIRC3 was significantly upregulated in KBD and OA compared with control, suggesting that BIRC3 may be a critical gene involved in NF-κB signaling.

The protein encoded by EGR1 is a type of C_2H_2 zinc finger of the EGR family, primary functions of which involve transcriptional regulation (19). EGR1 is able to activate the expression of TGF-β, which has been associated with the proliferation and differentiation of chondrocyte (19). It has been observed that EGR1 is upregulated in the synovial cells of patients with rheumatoid arthritis (RA) (20), but downregulated in chondrocytes of OA patients (21). However, in the present study, EGR1 was upregulated in the peripheral blood of KBD and OA patients. Therefore, this gene may play a role in the etiology of KBD; however, further studies involving chondrocytes may be required to assess whether EGR1 could be a biomarker of KBD.

Inflammation as manifested in OA and RA has not been observed in KBD to date; however, associated genes such as CXCL8 and IL-1β were identified as being differential expressed commonly in OA and KBD in the present study. Chemokine CXCL8 is involved in decomposition pathway of cartilage extracellular matrix, and IL-1β may cause cartilage damage by inducing the decomposition of extracellular matrix (22-25). These genes showed dysregulated expression in the serum and synovial fluid of patients with OA and rheumatic arthritis (26). According to the IPA analyses, inflammation associated pathways such as IL-6 and IL-10 were identified in the peripheral blood of KBD and OA patients, indicating that inflammatory change occurs in the peripheral blood of KBD in degree I or advanced patients. These findings require further study to verify whether there is similar inflammatory change in the peripheral blood and cartilage of KBD in the early stage.

There were 82 differentially expressed genes and 51 significant pathways identified in KBD, while 89 differentially expressed genes and 50 significant pathways were identified

Table III. Biological functions of genes identified in PBMCs of KBD and OA patients compared with healthy controls[a].

Function	Molecules	P-value range
KBD		
Cell death and survival	29	$2.67x10^{-7}$-$1.43x10^{-2}$
Lipid metabolism	12	$6.55x10^{-7}$-$1.43x10^{-2}$
Molecular transport	20	$6.55x10^{-7}$-$1.43x10^{-2}$
Small molecule biochemistry	15	$6.55x10^{-7}$-$1.43x10^{-2}$
Cellular movement	15	$1.06x10^{-6}$-$1.43x10^{-2}$
OA		
Cellular growth and proliferation	42	$5.35x10^{-7}$-$4.24x10^{-3}$
Cell-to-cell signaling and interaction	20	$5.91x10^{-7}$-$4.24x10^{-3}$
Cellular movement	23	$5.91x10^{-7}$-$4.24x10^{-3}$
Gene expression	26	$1.24x10^{-6}$-$4.24x10^{-3}$
Cellular development	38	$1.75x10^{-6}$-$4.24x10^{-3}$

[a]Molecules and P-values were determined using Ingenuity Pathway Analysis. PBMC, peripheral blood mononuclear cell; KDB, Kashin-Beck disease; OA, osteoarthritis.

Table IV. Nine common significantly canonical pathways in PBMCs of KBD and OA patients[a].

Signaling pathway	Genes in pathway (n)	Genes regulated (n)		P-value	
		KBD	OA	KBD	OA
NF-κB	164	5	3	$1.58x10^{-3}$	$3.25x10^{-2}$
IL-6	116	5	3	$3.02x10^{-2}$	$1.32x10^{-2}$
IL-10	68	4	2	$1.05x10^{-3}$	$3.37x10^{-2}$
Apoptosis	87	3	3	$2.38x10^{-3}$	$6.00x10^{-3}$
Death receptor	91	2	3	$1.04x10^{-2}$	$6.80x10^{-3}$
TWEAK	33	3	3	$5.00x10^{-3}$	$3.64x10^{-4}$
P38 MAPK	115	3	4	$1.24x10^{-3}$	$1.44x10^{-3}$
TNFR1	47	2	3	$4.18x10^{-4}$	$1.04x10^{-3}$
TNFR2	28	3	2	$2.10x10^{-2}$	$6.24x10^{-3}$

[a]Number of genes regulated and P-value were calculated by IPA. PBMC, peripheral blood mononuclear cell; KBD, Kashin-Beck disease; OA, osteoarthritis; NF-kB, nuclear factor-kappa B; IL, interleukin; TWEAK, TNF-related weak inducer of apoptosis; MAPK, mitogen-activated protein kinase; TNFR, tumor necrosis factor receptor.

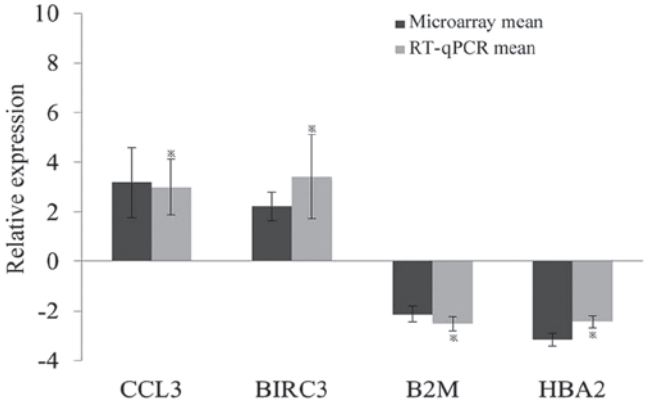

Figure 1. Histogram showing expression ratios of four selected genes, as determined using microarray and reverse transcription-quantitative polymerase chain reaction analyses. Bars show the mean ± standard deviation. *P<0.05, KBD vs. healthy controls, calculated by Mann-Whitney Wilcoxon test. RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

in OA. Among these pathways, nine significant pathways were found to be common between KBD and OA, including NF-κB, IL-6, IL-10, apoptosis, death receptor etc., as described in the results. The NF-κB signaling pathway consists of numerous types of transcription factors which specifically bind to the enhancer κB on κ light chain of immunoglobulin (27). NF-κB transcription factor has an important role in the cell response to damage, stress and inflammation (27). In addition, it has been associated with tissue damage, stress, cell differentiation and apoptosis (27-29). The pathological activation of NF-κB signaling pathway is involved in various inflammatory and rheumatic diseases, including OA and RA (30). NF-κB1 and RelA proteins were observed to be significantly increased in the cells of OA synovium (31). A previous animal study indicated that NF-κB and ERK1/2 of MAPK participate in reducing the expression of collagen type II while promoting the expression of TNF-α protein gene in prechondroblasts and articular chondrocytes (32). Furthermore, during OA pathogenesis, NF-κB signaling pathway was shown to be significant in maintaining the normal physiological conditions of chondrocytes, mRNA and protein expression patterns, and the metabolic balance of cartilage matrix (31). Consequently, it may be considered to be a critical pathway for the etiological study and targeted therapeutic investigation of KBD and OA.

Apoptosis is programmed death under physiological or pathological conditions in which a series of mechanisms have been enacted by multiple signal transmissions (33). In recent years, chondrocyte apoptosis has been demonstrated to be crucial in cartilage matrix degradation and in the inhibition of matrix synthesis during the articular cartilage degradation process of OA (34). Chondrocyte apoptosis has been detected in the middle layer of articular cartilage of KBD and significantly decreased number of chondrocyte in infant patients, and notably decreased synthesis of collagenase type II, which may induce excessive apoptosis of chondrocytes (34-36). In the present study, the apoptosis signaling pathway was the common different signaling pathway in PBMCs from patients with KBD and OA, and the apoptosis signaling pathway was identified in the differentially expressed genes in PBMCs of infant KBD patients using performed IPA analysis (unpublished). Therefore, the cell apoptosis was identified in the cartilage, the target organ of KBD, in addition to the PBMCs.

In conclusion, nine signaling pathways and five differentially expressed genes were screened using IPA analysis, which were common between peripheral blood of KBD and OA patients. NF-κB and apoptosis may be critical signaling pathways of KBD in etiology investigation. Furthermore, changes in the IL-6 and IL-10 pathways suggest that there may be inflammatory reaction in the peripheral blood of patients KBD, which may indicate a diagnosis of KBD in the early stages of the disease.

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Table V. Ingenuity pathway analysis of the most common networks in KBD and OA.

Pairs	Molecules in network	Score[a]	Molecules	Most common functions
KBD				
1	Ap1, BCR, ↑†BIRC3, ↑CAMP, caspase, ↑CCL3, ↑CCL4, CD3, ↑CD69, Cg, ↑CXCL1, ↑CXCL8, ↑DEFA1, Fcer1, Focal adhesion kinase, ↑GOS2, ↑GZMB, Hsp27, IgG, ILL, ↑IL7, IL12, ↑IL1B, ↑IL1R2, Immunoglobulin, ↑IVL, Jnk, Mapk, NFκB, ↑NFKBIA, Nr1h, PI3K, Pkc(s), ↑RUNX1T1, TCR	28	16	Cell-to-cell signaling and interaction, cellular movement, immune cell trafficking;
2	↑ALK, ↑BDP1, ↓Cebp, ↓‡CELF1, ↓DYRK1A, ↑GMNM, ↓HBA1/HBA2, ↓HBB, ↑HBG1, ↓HGF, IL2, ↑IL10, ↑IRX5, ↑KLRC1, ↑KLRC2, ↑KRR1, ↑MAPK1, ↓MAPK8, ↑MCM6, ↑MTA2, ↑NR3C1, ↓PGK1, ↑PLK2, ↑PMS1, ↓PPP2R1A, ↑RBBP6, ↑RFC1, ↑S100P, ↑SMARCA4, ↑TNFAIP2, ↑TNFSF9, ↓TP53, ↓TPT1, ↓UBE2D1, ↑ZNF678	23	14	Cellular development, cellular growth and proliferation, organ morphology
3	26s Proteasome, ↑ADRBK2, Akt, ↓B2M, ↑CCL21, ↑CD1D, Creb, ↑EGR1, ERK, ERK1/2, estrogen receptor, ↑FGF19, ↑FGF21, ↑GBA, ↑GRP, ↓HBB, Histone h3, Histone h4, HNF4A, ↓HPSE, ↑KLB, ↓NPC1L1, P38 MAPK, PDGF BB, PI3K(family), PTGFR, ↑PTPRC, RNA polymerase II, SHP, ↑SMG1, ↓SNCA, ↑TGFA, ↑TTAL1, Vegf, ↓ZFP36	11	8	Lipid metabolism, molecular transport, small molecule biochemistry
4	miR-146a-5p, ↑IL1F10	2	1	Hematological disease, hereditary disorder, organismal injury and abnormalities
5	NUPR1, ↑TCEANC2	2	1	Cancer, reproductive system disease, cell morphology
OA				
1	↑AP3M1, ↑AKAP17A, ↑BCDIN3D, CXXC4, C19orf70, CCDC71, EVX1, EZH2, ↑FRYL, ↑GPR18, ↑HIST2H2BE, ↑HCP5, ↑HIST1H3H, HLX, ↑H3F3A/H3F3B, ↑LCMT2, MAD2L2, MT1A, ↑PTPN4, PARP10, ↑PSMC6, ↑PLEKHF1, P2RY6, ↑PELO, PRG2, ↑RF2BP2, ↑RAB22A, RB1, SATB1, ↑SIAH1, TSPY1, UBC, VRK1, ↑ZNF217, ↑ZNF564	41	19	Cellular assembly and organization, cellular function and maintenance, cardiovascular system development and function
2	26s Proteasome, ↑ABCA7, ↑BIRC3, ↑caspase, Cytokine, cytochrome C, ↑CASP3, ↑CDKN2D, Calcineurin protein, ↑Hsp70, Hsp90, HDL, Hdac, ↑HSPA1A/HSPA1B, Interferon alpha, Ifn gamma, ↑Ikb, IKK, Jnk, ↑NFE2L2, NfkB-RelA, NFκB, ↑NFKBIA, ↑Proinflammatory Sod, PRKAA, ↑PPM1B, RAB4, ↑RABEP1, ↑TINF2, ↑TGFBR3, TCR, Tlr, U biqutin, ↑UBE2L3	23	16	Cell death and survival, renal necrosis/cell death, post-translational modification
3	Adaptor protein 2, ↑Atf, ↑ATHL1, ↑CSRNP1, CHRM4, Ck2, ↑CX3CR1, CCKAR, CARTPT, ↑DUB, GCHFR, H35, hydroquinone, hemoglobin, Ifi47, ↑IL1B, IL26, IL37, Icam, KLK1, mi-R-3065-3p, mir-375, ↑MAX, ↑NBPF10, ↑OSM, Proinsulin, RPAD, RNA polymerase II, ↑TM2D3, ↑USP24, ↑USP36, UCN3, USP33, USP50	18	12	Endocrine system development and function, molecular transport, small molecule biochemistry

Table V. Continued.

Pairs	Molecules in network	Score[a]	Molecules	Most common functions
4	Ap1, Alp, Akt, ↑ATF4, ↑ADRB, Alpha catenin, ADCY, Cg, CaMKII, ↑CSE1L, Collagen(s), Collagen type IV, Collagen type I,Cbp/p300, ↑Creb, ↑EGR1, Fibrinogen, Growth hormone, Hsp27, ↑IL1, Importin alpha, Lamnin, Lh, ↑LIMS1, Mek, ↑MPL, Mapk, Notch, PP2A, ↑PPP1R15A, PDGFBB, Pdgf, ↑SRSF5, ↑ RNMT, ↑VCL	16	12	Cell morphology, tissue morphology, hematological system development and function
5	ADRA1D, ADAM21, ADRA1A, ADRA2C, ADCY2, AIFM3, ARF4, ARRDC3, ARF4, CACNA1H, CNNM3, CHRM2, CRYGS, DCY3, ELAVL1, ↑FAM117B, ↑FAM43A, GPRC5B, ↑KCNJ2, ↑LFNG, ↑LEPROT, mi-149-3p, miR-96-5p, miR-92-3p, miR-296-5p, NUP93, ↑P2RY13, ↑PVRIG, ↑PLAGL2, PRDX5, RAB8A, ↑RSBN1, RGS17, SLC12A5, STARD3, TMX1	16	9	Respiratory disease, inflammatory disease, cardiac arrhythmia

[a]Scores were based on the number of focus genes and network size. †, ‡:↑ and ↓ indicate significantly upregulated and downregulated genes in the networks, respectively. KBD, Kashin-Beck diease; OA, osteoarthritis.

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