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Comparative analysis of gene expression profiles between the normal human cartilage and the one with endemic osteoarthritis

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Summary

Objective: To investigate the differences in gene expression profiles of adult articular cartilage with endemic osteoarthritis (OA), Kashin–Beck disease (KBD), and the same regions in the normal joint.

Methods: The messenger RNA expression profiles of articular cartilage with KBD diagnosed according to "Diagnosing Criteria of Kashin–Beck Disease in China" were compared with the normal cartilage. Total RNA isolated separately from four pairs of the KBD and normal cartilage samples were evaluated by oligonucleotide microarray analysis. The microarray data were confirmed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) amplification and were compared with previously published experiments.

Results: About 4100 transcripts, which corresponded to 35% of the expressed transcripts, showed $\geq$twofold differences in expression between the cartilage tissues in pairs. Approximately 2% of the expressed genes (79, 55 genes expressed in KBD $>$ normal; 24 genes expressed in KBD $<$ normal) were commonly expressed in the four pairs of samples. The expression of some genes related to the metabolism, apoptosis, cell proliferation and matrix degradation activity was significantly different in KBD cartilage than in the normal, similar to the findings for genes that inhibit matrix degradation. Comparisons of qRT-PCR data and the previously reported data with the result of gene chips support the validity of our microarray data.

Conclusion: Differences between KBD cartilage and the normal exhibited a similar pattern among the four pairs examined, indicating the presence of common mechanisms mainly including chondrocyte metabolism and apoptosis that contribute to cartilage destruction in KBD.

Key words: Kashin–Beck disease, Cartilage, Gene expression, Microarray.

Introduction

Kashin–Beck disease (KBD) is an endemic osteochondropathy in China and other parts of Asia, affecting over 0.74 million patients with 10.342 million people at risk[1,2]. To date no consensus has been reached on the etiology, although three major environmental hypotheses have been proposed in the past 50 years: endemic selenium deficiency, serious cereal contamination by mycotoxin-producing fungi, and high humic acid levels in drinking water[3–7].

Previously, the histological and morphological changes, differing from those observed in osteoarthritic cartilage, have been seen in the articular cartilage of KBD, including significant alterations in chondrocyte phenotype, chondrocyte necrosis and apoptosis, and abnormal terminal chondrocyte differentiation[8–10]. KBD is considered to be a heterogeneous group of disorders with a variety of pathogenic factors, all of which result in similar patterns of cartilage degeneration and articular deformation[11]. The pathological changes in KBD articular cartilage include surface fibrillation, chondrocyte clustering, collagen II degradation and loss of proteoglycans from the matrix following a focal chondrocyte necrosis in deep zone. A correlation to chondronecrosis cartilage samples from children and adults with KBD has been reported[8,12]. It is an urgent need to better understand its pathogenesis to enable the development of new management and treatment strategies.

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Articular cartilage, which is an avascular deformable tissue including chondrocytes and the extracellular matrix (ECM) produced by them, plays a critical role in degeneration and deform of joints in KBD. To unravel the pathogenic mechanisms of KBD, the analysis of mRNA expression relevant to KBD can provide valuable insight into the physiopathological events or intracellular pathways involved with the disease.

Comprehensive mRNAs' expression profiles of human cartilage in KBD and the normal have never been compared, thus, the following questions remain unresolved. What percentage of the expressed transcripts shows obvious differences in expression levels in cartilage between KBD and the normal? Which transcripts show differential expression pattern in articular cartilage with KBD? What are the molecular functions involved in such groups of genes?

To provide answers to these questions, Agilent Human 1A 22 k high-density oligonucleotide array analysis was used to compare the gene expression profile of chondrocytes in KBD articular cartilage with the one in the normal cells. The validity of oligonucleotide array data was evaluated on the basis of the parallel analyses of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) amplification.

Materials and methods

CARTILAGE SAMPLES

Specimens of human articular cartilage were collected from a total of 15 KBD patients and nine normal. In order to match the age and sex, selected nine samples of the 15 patients' samples were used for microarray analysis and qRT-PCR analysis after histologic assessment (Table I). Patients were undergoing total knee replacement surgery at the hospital (three females/six males aged from 42 to 69 years old). KBD adults selected were diagnosed as the second and third degree, based on the Diagnosing Criteria of Kashin–Beck Disease in China[13] by radiography of the right hand, knee and hip joints and cartilage sections after hematoxylin and eosin (H&E) staining. The normal control group, which consisted of nine human articular cartilage samples (three females/six males aged from 34 to 60 years old), was collected from the fresh cadaver knees within 8 h of death, donated with a fairly large amount of cartilage from the same anatomical site as in KBD group. The cadaver donors, who died in traffic accidents, were from non-KBD areas and excluded KBD, and status of control cartilage samples was diagnosed by histological examination with H&E staining, and excluded the genetic bone and cartilage diseases, OA, and rheumatoid arthritis. Cartilage tissue on condyles of femur was dissected and rapidly frozen in liquid nitrogen and stored at −80 °C until RNA isolation was performed. This investigation was approved by the Human Ethics Committee, Xi'an Jiaotong University. All patients or relatives of donors provided an informed consent.

Table I
Normal/KBD sample pairs used for microarray and qRT-PCR analysis

	Normal	KBD
Set 1	54 y (M)	52 y (M)
	55 y (M)	55 y (M)
	48 y (F)	49 y (F)
	37 y (M)	42 y (M)
Set 2	60 y (M)	69 y (M)
	58 y (M)	51 y (M)
	56 y (M)	50 y (M)
	58 y (F)	57 y (F)
	34 y (F)	44 y (F)

Set 1: microarray analysis and set 2: real-time quantitative PCR analysis. KBD – Kashin–Beck disease, F – female, and M – male.

RNA EXTRACTION, NORMALIZATION OF THE AMOUNT OF RNA AND MICROARRAY ANALYSIS

Samples from the KBD or the control group were divided into four pairs and underwent microarray analysis (Table I). In brief, frozen cartilage tissue of each sample was rapidly ground in liquid nitrogen with freezer mill (SPEX CertiPrep, Metuchen, NJ, USA)[14]. Total RNA was isolated separately using the Agilent total RNA isolation minikit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The Human-Actin Competitive PCR Set (Takara Bio, Kyoto, Japan) was used according to the manufacturer's instructions to normalize the RNA amount that would be available for microarray analysis. To confirm the integrity of the total RNA, 60 ng (normalized value) of denatured total RNA was loaded in each lane of a 1% non-denaturing (formaldehyde-free) agarose gel, subjected to electrophoresis, and stained with ethidium bromide. Smaller amounts of total RNA can be detected in non-denaturing agarose gels than in formaldehyde-denaturing agarose gels. It was further transcribed *in vitro* into cRNA and labeled with CyDye using Amino Allyl MessageAmp aRNA Kit (Ambion) according to the manufacturer's protocol. Then, 0.5 μg of each labeled cRNA of reference and experimental sample were purified, combined, and mixed with hybridization buffer before being applied on the microarray. The hybridization solution was prepared using *in situ* hybridization kit plus (Agilent Technologies). The fluorescent labeled cRNA was used for oligo microarray hybridization. Hybridizations of each pair were carried out using Agilent Human 1A 22 k oligo microarray (Agilent Technologies, G4110B) containing 22,575 oligonucleotides that represent more than 20,173 human genes. Hybridization was performed in the hybridization chamber (GeneMachines, San Carlos, CA, USA). Conditions of hybridization and washing were according to the protocol of Agilent Oligonucleotide Microarray Hybridization (Agilent Technologies).

ANALYSIS OF THE DATA

The quality of the fluorescence spots of the microarray data was evaluated as "present" or "absent" and selected through automatic recognition. The data were recorded and transferred into text files using Feature Extraction 9.3 Software (Agilent Technologies) and Spotfire 8.0 (Spotfire Inc., Cambridge, MA, USA). The spots that failed to pass quality control procedures of these softwares were flagged and excluded from further analysis. A possible dye bias was eliminated using an algorithm for the same extraction software (Extraction 9.3 Software, Agilent) that applies normalization factors (global normalization, location normalization). Generated files were imported into spreadsheets (Excel, Microsoft Corp., Redmond, WA, USA) for downstream data analysis and statistical evaluation. The more detailed process can be accessed from the Agilent Company. Then, individual genes were classified as "up-regulation" or "down-regulation" when the fold change of spot labeling intensity was more than twofold or less than 0.5-fold in pairs, respectively. The "fold change" value represents the ratio of the individual signal intensity in KBD to the individual signal intensity in the normal of all four pair samples.

To identify the genes that showed the same expression pattern in different groups, the selection criteria were defined so that selected transcripts had to show a more than twofold difference in the level of expression among all four pair samples (Fig. 1). The standard error of the mean (S.E.M.) was calculated from the standard deviation (SD) of the fold change value.

qRT-PCR

Total RNA was prepared in the same way as for the oligonucleotide array analysis. These RNA samples were converted into cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primer. qRT-PCRs were performed using the ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primer and probe sets were purchased as TaqMan Gene Expression Assays for the set of genes to be analyzed: NM_001236 (*CBR3*), NM_006475 (*POSTN*), NM_001003940 (*BMF*), NM_006283 (*TACC1*), NM_003376 (*VEGF*), NM_021992 (*TMSL8*), NM_012115 (*CASP8AP2*), NM_001015880 (*PAPSS2*). Results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold change was calculated as described above.

Results

FINDINGS OF THE GENOME-WIDE SCREENING

The Agilent Human 1A high-density oligonucleotide array system was used to compare gene expression profiles of chondrocytes in KBD joint cartilage with the profile of chondrocytes in normal joint cartilage. There are 22,575 probe

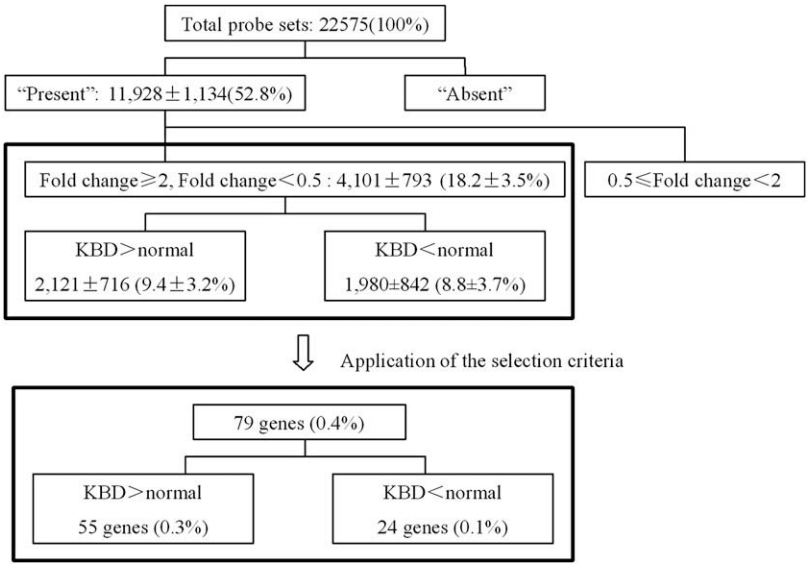

Fig. 1. Flow chart showing the procedure for identifying genes with different expression levels in human cartilage of KBD and the normal. The terms "present," and "absent" represent expression levels of the transcripts described in Materials and methods. Values are the mean ± SD number and percentage of transcripts. See Materials and methods for details of the selection criteria.

sets on the Agilent Human 1A chip including 918 positive probes, 162 negative control probes and 422 ignore probes, covering more than 18,000 transcripts variants that represent 21,073 human genes. The expression analysis detected ≈55% (mean ± SD 11,928 ± 1134) of all probe sets corresponding to the transcripts in each cartilage sample (Fig. 1). However, little difference was noted between the percentage of transcripts expressed in KBD and the normal tissues: KBD 53.9 ± 4.6% (12,158 ± 1038) vs normal 51.8 ± 8.1% (11,697 ± 1830). More than twofold difference in expression per sample was noticed in 18.2 ± 3.5% (4101 ± 793) of all probe sets: expression was higher in KBD samples in 9.4 ± 3.2% (2121 ± 716) of the transcripts, and lower in 8.8 ± 3.7% (1980 ± 842) of the transcripts. This corresponds to ≈35% of the expressed transcripts (4101/11,928 = 0.344). Furthermore, ≈2% of differentially expressed genes were commonly detected in all four pairs (79/4101 = 0.019).

Thus, we identified 79 genes, which consisted of 55 genes expressed at higher and 24 genes expressed at lower level in KBD than in normal cartilage. These genes were then subdivided into functional categories: the genes with higher expression in KBD group were subdivided into 12 categories, including metabolism, ECM, secreted inhibitors/antagonists, transcription regulator/factors, G protein correlated, acceptor, DNA modification, cytokines/growth factors, proteases, oncogene correlated, signal transduction and miscellaneous, and the genes with higher expression in normal group into 14 categories, including DNA modification, signal transduction, transcription correlated, oncogene correlated, cytokines/growth factors, ECM, development correlated, apoptosis, cell cycle, membrane proteins/receptor, cytoskeleton and cell movement, metabolism and miscellaneous. Data for all of the selected genes are shown in Tables II and III.

RESULTS OF REAL-TIME QUANTITATIVE PCR OF SELECTED EIGHT GENES

To validate the oligonucleotide array data, qRT-PCR was performed on eight selected genes using additional five pairs of cartilage samples. For these eight genes, the groups of high expression as determined by qRT-PCR were consistent with the groups of high expression as determined by oligonucleotide array analysis. We found that the levels of mRNA expression for *POSTN*, *CBR3*, *BMF*, *TACC1*, *VEGF*, *TMSL8*, *CASP8AP2* and *PAPSS2* genes in KBD and the normal were significantly different as determined by qRT-PCR methods (Fig. 2), and more importantly, the changes were rather consistent with the array data.

EXPRESSION PATTERNS OF GENES REPORTED TO BE DIFFERENTIALLY EXPRESSED IN KBD, AND IN OA VS THE NORMAL CARTILAGE

The expression level of some of the genes that, on the basis of the present microarray data or shown in the previous studies, were differentially expressed in KBD cartilage vs the normal were further assessed. We confirmed elevated gene expression of Bcl-2, Bax, vascular endothelial growth factor (VEGF), Col5 and Col1 in KBD articular cartilage. Our list of differentially expressed genes was compared with a previously published list of differences between intact-looking vs damaged OA cartilage[15]. We could identify 11 up-regulated and three down-regulated genes behaving similarly both in KBD and OA. The names and symbols of these genes are shown in Table IV. Despite the presence of similarly behaving genes, there were still a number of differentially expressed genes that were specific for KBD.

Discussion

Despite considerable limitations such as low sensitivity and insensitivity to alternative splicing, posttranscriptional regulation, and posttranslational modification, cDNA array technology provides a powerful tool, which we can exploit to obtain an overview of gene expression patterns, hardly achievable with other techniques. This has been shown to be true for the analysis of known genes as well as the discovery of new genes

Table II
List of genes with lower expression in KBD than normal cartilage in microarray data

Category	Gene name	Public ID	Gene symbol	Fold change	S.E.M.
Metabolism	Cathepsin K	NM_000396	*CTSK*	0.47	0.12
	Carbonyl reductase 3	NM_001236	*CBR3*	0.36	0.09
	Homeodomain interacting protein kinase 2	NM_022740	*HIPK2*	0.32	0.08
	Fatty acid binding protein 4, adipocyte	W60781	*FABP4*	0.38	0.09
	HtrA serine peptidase 3	NM_053044	*HTRA3*	0.38	0.08
ECM	Fibulin-1	NM_006486	*FBLN1*	0.14	0.06
	Tenascin XB	NM_019105	*TNXB*	0.36	0.13
Secreted inhibitors/antagonists	BMP-binding endothelial regulator precursor protein	NM_133468	*BMPER*	0.33	0.14
Transcription regulator/factors	Transforming, acidic coiled-coil containing protein 1	NM_006283	*TACC1*	0.43	0.03
	Homeo box C10	NM_017409	*HOXC10*	0.42	0.04
	Homo sapiens checkpoint suppressor 1	NM_005197	*CHES1*	0.28	0.10
G protein correlated	Regulator of G protein signaling 10	NM_001005339	*RGS10*	0.43	0.05
	Frizzled homolog 8 (Drosophila)	NM_031866	*FZD8*	0.41	0.10
Acceptor	Retinal outer segment membrane protein 1	NM_000327	*ROM1*	0.34	0.10
DNA modification	Zinc finger with KRAB and SCAN domains 1	AY260738	*ZKSCAN1*	0.40	0.08
Cytokines/growth factors	Periostin, osteoblast specific factor	NM_006475	*POSTN*	0.27	0.12
	Synaptopodin 2-like	NM_024875	*SYNPO2L*	0.32	0.11
	Cathepsin C	NM_001814	*CTSC*	0.29	0.12
Development correlated	Aortic preferentially expressed gene 1	NM_005876	*APEG1*	0.40	0.08
Oncogene correlated	Bcl2 modifying factor	NM_001003940	*BMF*	0.37	0.09
	RAS, dexamethasone-induced 1	NM_016084	*RASD1*	0.41	0.03
Miscellaneous	Regulator of G protein signaling 5	NM_003617	*RGS5*	0.27	0.14
	Chromosome 11 open reading frame 8	NM_001584	*C11orf8*	0.39	0.07
	Unknown	A_23_P142113	*A_23_P142113*	0.39	0.04

See Materials and methods for the definition of the selection criteria and for the method of calculating the "fold change" and the S.E.M. Public ID = accession number in public databases (RefSeq or GenBank).

of interest. It is increasingly being used to investigate the basis of disease, leading to the generation of gene profiles for disease screening and gene targets for drug therapy[16,17].

In this study, we compared the gene expression profiles in KBD cartilage vs the normal one using oligonucleotide microarray. Transcripts with a twofold difference in mRNA expression between these two samples accounted for an average of 34% of all expressed transcripts per pairs of cartilage tissue sample, ≈2% of which were commonly detected in the four sample pairs. The former observations have indicated that some gene expressions of chondrocytes in KBD cartilage were quite different from those of chondrocytes in the normal tissue, even though both paired sample groups were harvested from the same anatomic regions and the patients at similar ages. This finding suggests that the gene expression profiles of chondrocytes in KBD cartilage changes in a specific manner. Thus, there may be a common molecular mechanism of KBD development, assuming that the changes in gene expression patterns of chondrocytes lead to cartilage deformity. However, based on gene array data alone it is difficult to evaluate, which of changes observed derive from primary processes of KBD and which result from secondary responses due to cartilage degeneration.

Using our selection criteria, we identified 79 genes that were differentially expressed in KBD vs the normal cartilage. The validity of our data was confirmed using qRT-PCR and by comparing our present findings with those of the previous studies. The possible roles of these genes with significantly altered mRNA expression are discussed here in order to reveal possible clues of the underlying mechanisms that may participate in the pathogenesis or progression of KBD, such as chondrocyte metabolism, ECM, proliferation and apoptosis activity of chondrocytes.

With regard to chondrocyte metabolism, *ERH*, *PAPSS2*, *GSTT2*, *FABP4*, *CTSC*, *HIPK2*, *HTRA3*, *PDE4B*, *PDK3*, *IDH2*, *PDE8B*, *etc.*, genes were found to be differentially expressed between KBD and the normal human cartilage. Sulfation of proteoglycans is a very important posttranslational modification in chondrocyte growth and development. The enzyme 3′-phosphoadenosine 5′-phosphosulfate synthase (PAPSS) catalyzes the biosynthesis of PAPS (3′-phosphoadenosine 5′-phosphosulfate), which serves as the universal sulfate donor compound for all sulfotransferase reactions[18]. Mutations of *PAPSS2* gene in the murine and human are responsible for diseases affecting the skeletal system and play an important role of the gene product for cartilage growth and development in mouse embryo[19]. Overexpression of PAPSS could lead to higher activity of PAPS, which might cause higher sulfation of cartilage proteoglycans. However, it is difficult to evaluate its role in KBD, since it is not clear whether the sulfation degree of KBD cartilage differs from normal.

Glutathione *S*-transferase theta 2 (GSTT2) belongs to a family of detoxification enzymes, and its overexpression is thought to provide the cells with protection against oxidative stress and various drugs[20]. The fact that serious cereal contamination by T-2 toxin, moniliformin and butenolide (BUT) and high humic acid levels in drinking water have been found in KBD areas[5,12,21] suggests that the pathogenetic mechanisms in chondrocytes of KBD might be related to poison. It has also been reported that low selenium level may cause oxidative DNA base damage in KBD[3,22–24]. Previous data have suggested that enhancer of rudimentary homolog (ERH) might be an important transcription regulator that also functions in the control of cell-cycle progression[25]. Fatty acid binding proteins (FABPs) are proteins that reversibly bind fatty acids and other lipids. So far, nine tissue-specific cytoplasmic FABPs have been identified. Adipose tissue FABP (FABP4) has been suggested as a bridge between inflammation and other pathways related to the metabolic syndrome[26]. Adipocyte fatty acid binding protein, aP2 (FABP4) is expressed in adipocytes and macrophages, and integrates inflammatory and metabolic

Table III
List of genes with higher expression in KBD than normal cartilage in microarray data

Category	Gene name	Public ID	Gene symbol	Fold change	S.E.M.
DNA modification	Heme binding protein 2	NM_014320	*HEBP2*	2.67	0.49
	ADP-ribosylation factor-like 7	NM_005737	*ARL4C*	5.07	3.61
Signal transduction	ABI gene family, member 3 (NESH) binding protein	NM_015429	*ABI3BP*	2.83	0.66
Transcription correlated	LIM and cysteine-rich domains 1	NM_014583	*LMCD1*	4.27	1.66
	Goosecoid	NM_173849	*GSC*	3.33	0.92
	Homeo box B2	NM_002145	*HOXB2*	5.08	2.28
Oncogene correlated	B-cell translocation gene 1, anti-proliferative	NM_001731	*BTG1*	8.48	6.20
Cytokines/growth factor	Insulin-like growth factor binding protein 4	NM_001552	*IGFBP4*	4.13	1.53
	IGF-II mRNA-binding protein 2	NM_000597	*IGFBP2*	9.70	3.73
	Transforming growth factor, beta-induced, 68 kDa	NM_000358	*TGFBI*	7.86	3.44
	LIM domain only 4	NM_006769	*LMO4*	2.69	0.35
	Scrapie responsive protein 1	NM_007281	*SCRG1*	2.55	0.32
	Chordin-like 2	NM_015424	*CHRDL2*	3.60	0.58
	Vascular endothelial growth factor	NM_003376	*VEGF*	2.99	0.52
ECM	Collagen, type V, alpha 2	NM_000393	*COL5A2*	8.85	6.83
	Collagen, type I, alpha 1	NM_000088	*COL1A1*	4.92	1.80
	Thrombospondin 1	NM_003246	*THBS1*	2.95	0.76
	Hyaluronan and proteoglycan link protein 1	NM_001884	*HAPLN1*	7.50	4.70
	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III	NM_182904	*P4HA3*	3.42	1.40
Development correlated	Brain expressed, X-linked 1	NM_018476	*BEX1*	4.99	1.19
	Likely ortholog of mouse limb-bud and heart gene	NM_030915	*LBH*	3.31	1.39
	Eyes absent homolog 2 (Drosophila)	NM_172113	*EYA2*	2.87	0.28
Apoptosis	Growth-arrest-specific 2-like 1	NM_152236	*GAS2L1*	7.63	3.76
	Tumor necrosis factor, alpha-induced protein 6	NM_007115	*TNFAIP6*	5.94	1.52
	Death-associated protein kinase 1	NM_004938	*DAPK1*	3.09	0.98
	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	NM_002546	*TNFRSF11B*	8.53	8.01
	BCL2-antagonist/killer 1	NM_001188	*BAK1*	5.97	1.57
	B-cell CLL/lymphoma	NM_004324.2	*BCL2*	3.23	0.97
	PERP, p53 apoptosis effector	NM_022121	*PERP*	5.59	2.38
	CASP8 associated protein 2	NM_012115	*CASP8AP2*	5.43	3.64
	Apoptotic protease activating factor	NM_181861	*APAF1*	5.70	3.92
	Homo sapiens caspase 6, apoptosis-related cysteine peptidase, transcript variant alpha, mRNA	NM_001226	*CASP6*	8.33	5.50
	BCL2-associated X protein	NM_004324.2	*BAX*	3.79	1.53
	Tumor necrosis factor (ligand) superfamily member 14	NM_003807	*TNFSF14*	2.72	0.58
Cell cycle	CDC37 cell division cycle 37 homolog	NM_007065	*CDC37*	2.67	0.37
Membrane proteins/ receptor	Frizzled homolog 1 (Drosophila)	NM_003505	*FZD1*	3.67	1.25
	Frizzled homolog 10 (Drosophila)	NM_007197	*FZD10*	2.49	0.18
Cystoskeleton and cell movement	Thymosin-like 8	NM_007059	*TMSL8*	9.65	8.50
	Tubulin, beta 2	NM_001069	*TUBB2A*	3.27	0.70
	Stathmin-like 2	NM_007029	*STMN2*	11.17	3.84
Metabolism	PAPSS2	NM_001015880	*PAPSS2*	3.79	1.21
	Phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	NM_001037341	*PDE4B*	3.38	1.27
	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	NM_002168	*IDH2*	4.31	0.71
	Phosphodiesterase 8B	NM_003719	*PDE8B*	8.47	4.08
	Pyruvate dehydrogenase kinase, isoenzyme 3	NM_005391	*PDK3*	3.00	0.67
	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 1 (GalNAc-T1)	XM_031104	*GALNTL1*	8.16	2.62
	Glutathione *S*-transferase theta 2	NM_000854	*GSTT2*	4.01	1.51
	Enhancer of rudimentary homolog (Drosophila)	NM_004450	*ERH*	7.50	2.12
Miscellaneous	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	A_23_P123234	*A_23_P123234*	5.70	2.27
	FAT tumor suppressor homolog 4 (Drosophila)	AK026709	*FAT4*	3.53	0.25
	Suppressor of cytokine signaling 3	NM_003955	*SOCS3*	3.01	0.71
	Gremlin 1 homolog, cysteine knot superfamily *Xenopus laevis*)	NM_013372	*GREM1*	6.79	4.81
	Fer-1-like 3, myoferlin	NM_013451	*FER1L3*	2.82	0.61
	Nipsnap homolog 3A	NM_015469	*NIPSNAP3A*	2.97	0.61
	Vestigial-like 3	NM_016206	*VGLL3*	4.10	1.83

See Materials and methods for the definition of the selection criteria and for the method of calculating the "fold change" and the S.E.M. Public ID = accession number in public databases (RefSeq or GenBank).

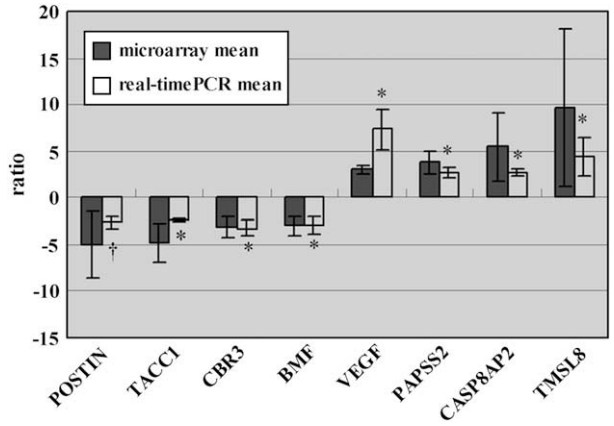

Fig. 2. Histogram showing levels of expression of eight selected genes, as measured by oligonucleotide array (solid bars: $n = 4$) and real-time quantitative polymerase chain reaction (open bars: $n = 5$).Values are the mean ± SD (see Materials and methods for further details).*$P < 0.05$, †$P > 0.01$ for KBD vs the normal, by paired *t*-test.

responses. Targeting aP2 with small-molecule inhibitors is possible, and may lead to a new class of powerful therapeutic agents to prevent and treat metabolic diseases[27].

Cathepsin C (CTSC), member of the papain family of cysteine proteinases, is a lysosomal aminopeptidase. Cathepsins are synthesized in the endoplasmic reticulum as pre-proproteins consisting of a signal peptide, a propeptide and a catalytic region of the enzyme. In addition to the intracellular degradation of collagens, cathepsins can also degrade matrix proteins extracellularly[28]. Homeodomain interacting protein kinase 2 (HIPK2) is an evolutionary conserved serine/threonine kinase that regulates gene expression by phosphorylation of transcription factors and accessory components of the transcription machinery. HIPK2 is activated in response to DNA-damaging agents or morphogenic signals and accordingly HIPK2-guided gene expression programs trigger differentiation and development or alternatively apoptosis[29]. High temperature requirement A (HtrA) and its homologues constitute the HtrA family proteins, a group of heat shock-induced serine proteases. HtrA proteins perform crucial functions with regard to protein quality control in the periplasmic space, functioning as both molecular chaperones and proteases[30]. HtrA serine peptidase 3(HtrA3) bounds to various TGF-beta proteins and inhibits the signaling of BMP-4, -2 and TGF-beta 1. The inhibition occurs upstream of the cell surface receptor. HtrA3 also shows proteolytic activities toward beta-casein and some ECM proteoglycans. The protease activity is absolutely required for the TGF-beta signal inhibition activity. The data suggest that HtrA3 has the overlapping biological activities and the function in complementary fashion in certain types of tissues[31].

With regard to ECM, *COL5A2*, *COL1A1*, *THBS1*, *HAPLN1*, *P4HA3*, *FBLN1* and *TNXB* genes were found to be differentially expressed between KBD and the normal human cartilage. The developmental pattern of col5a2 expression closely resembles that of the type I collagen, thus further substantiating the notion that these macromolecules cooperate in the formation of fibrillar networks in abnormal-cartilaginous matrices in KBD[32]. These consisted with the result of our study on abnormal chondrocyte differentiation and abnormal expression of collagen types in articular cartilage from patients with KBD[10]. Fibulins, and particularly the splice variants of fibulin-1 (FBLN1), play a critical role in a number of developmental and pathological processes. As an example, FBLN1 has been implicated in limb malformations. It was reported that patients in a family affected with a complex type of synpolydactyly have been found to carry a balanced chromosomal translocation that involves an alternatively spliced exon of FBLN1 [33]. A human gene termed *XB(TNXB)* overlaps the *P450c21B* gene encoding steroid 21-hydroxylase and encodes a protein that closely resembles ECM proteins[34]. Hyaluronan and proteoglycan link protein 1 (HAPLN1), an ECM-linking protein act as a stabilizers of the interaction between versican and hyaluronan (HA) in various tissues[35]. Matrix synthesis by articular chondrocytes is a critical factor in

Table IV
List of genes behaving similarly both in the present KBD microarray data, and in data set obtained comparing intact vs damaged regions of OA cartilage[15]

Comparison, expression	Gene symbol	Gene name	Public ID	Fold change	S.E.M.
Intact < damaged	*ABI3BP*	ABI gene family, member 3 (NESH) binding protein	NM_015429	2.83	0.66
	COL1A1	Collagen, type I, alpha 1	NM_000088	4.92	1.80
	COL5A1	Collagen, type V, alpha 1	NM_000093	8.85	6.83
	FZD1	Frizzled homolog 1 (Drosophila)	NM_003505	3.67	1.25
	FZD10	Frizzled homolog 10 (Drosophila)	NM_007197	2.49	0.18
	GALNTL1	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-like 1	XM_031104	8.16	2.62
	TGFBI	Transforming growth factor, beta-induced, 68 kDa	NM_000358	7.86	3.44
	THBS1	Thrombospondin 1	NM_003246	2.95	0.76
	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	NM_007115	5.94	1.52
	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	NM_003701	8.53	8.01
	P4HA3	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III	NM_182904	3.42	1.40
Intact > damaged	*BMPER*	BMP-binding endothelial regulator precursor protein	NM_133468	0.33	0.14
	CTSC	Cathepsin C	NM_001814	0.29	0.12
	C11orf8	Chromosome 11 open reading frame 8	NM_001584	0.39	0.07

The fold change and S.E.M. listed are the result of our microarray data. See Materials and methods for the definition of the selection criteria and for the method of calculating the fold change and the S.E.M. Public ID = accession number in public databases (RefSeq or GenBank).

the maintenance of cartilage integrity in healthy joints. The relationship between these genes and the apparent joint deformity in KBD need to be investigated in future.

In previous studies, it was found that the levels of tumor necrosis factor-alpha (TNF-alpha), VEGF and interleukin-beta (IL-beta) in KBD group were higher than those in the normal group[36], and that chondrocyte apoptosis and different expression of Bcl-2, Bax, Fas, and iNOS existed in articular cartilage of KBD[9]. The contents of NO, iNOS and sFas/APO-1 in sera of the adults with KBD were significantly higher than in normal one, suggesting that both NO- and Fas-induced pathways may play a role in the pathogenesis in adult KBD[37]. With regard to chondrocyte proliferation and apoptosis, *TNFRSF11B*, *TNFSF11*, *TNFAIP6*, *GAS2L1*, *BAK1*, *BCL2*, *CASP6*, *CASP8AP*, *PERP* and *APAF1* genes were found differentially expressed in KBD in this experiment. Programmed cell death is essential for generation of the complex structural and functional organisation of the cell. Most cells that undergoing programmed cell death need genes that are essential for their death to be transcriptionally activated. The product of TNF-stimulated gene 6 (TSG-6), alternatively termed TNFAIP6 (for TNF-alpha-induced protein 6), which specifically binds to HA and to inter-alpha-inhibitor (I alpha I), shows potent anti-inflammatory activity in acute and chronic inflammation, notably in several models of autoimmune arthritis[38].

Chondrocytes are a source of TSG-6 which may play a role in cartilage remodeling and joint inflammation[39]. That TSG-6 expression in KBD articular chondrocytes possibly were implications in joint inflammation and cartilage degradation consisted with primal research[40]. The *GAS2* (growth-arrest-specific 2) gene, localized on human chromosome 11p14.3−p15[41] is a multifunctional gene involved in the regulation of apoptosis and chondrogenesis[42]. Expression of BCL2-antagonist/killer 1 (BAK1) was higher in KBD samples[43]. BAK1 promotes cell death and counteracts the protection from apoptosis that is provided by B-cell CLL/lymphoma (BCL2)[44]. Caspases (caspase 6, apoptosis-related cysteine protease; CASP8AP, CASP8 associated protein 2) are a growing family of cysteine proteases involved with apoptosis. They are crucial for execution of apoptosis, since inhibition of caspases by zVAD-fmk, a broad spectrum caspase inhibitor, does not prevent death, but results in necrosis rather than apoptosis[45]. APAF1 (apoptosis signaling; death receptor signaling) activates procaspase-9 to caspase-9 initiating the apoptotic pathway involving the downstream activation of caspases 3, 6, and 7[46]. It has been reported that mitochondrial cytochrome *c* functions as a cofactor with APAF-1 to activate caspase-9[47]. In addition, APAF-1 has been suggested as an essential downstream effector of p53-mediated apoptotic pathway after the DNA damage induced by radiation or doxorubicin[47]. Besides the passive apoptosis pathway, caspase-8 may directly induce the Fas−FasL pathway or be activated in a caspase-9-dependent manner[48]. The *PERP* (p53 apoptosis effector) gene is a mediator of p53-dependent apoptosis and is highly expressed in cells undergoing p53-dependent apoptosis[49,50]. These findings suggest that apoptotic pathways and anti-apoptotic reactions that participate in both repair and apoptosis induction were present in chondrocytes necrosis with KBD.

The apparent joint impairment in adults will continue to deteriorate in older KBD patients due to the development of OA as a result of joint deformity. We subsequently compared our microarray result with the data of comparative analysis of gene expression profiles in intact and damaged regions of human osteoarthritic cartilage. Fourteen genes, including ABI gene family, member 3 (NESH) binding protein (ABI3BP), types I,V collagen, transforming growth factor, beta-induced (TGFBI), TNF, alpha-induced protein 6 (TNFAIP6), TNF (ligand) superfamily, member 11 (TNFSF11), and BMP-binding endothelial regulator precursor protein (BMPER) were found expressions in the same tendency. This suggests that during the transition from normal cartilage to KBD lesional cartilage, the gene expression profile changes before there is any apparent damage to the cartilage.

Because the expression levels of some genes probably change during the transitional period from normal to normal appearing cartilage, our study design might have allowed us to miss some important genes that show no differences in expression levels between normal and KBD cartilage. To overcome this problem, further studies comparing intact with damaged cartilage from same joints are needed.

Although we used rather high criteria to estimate a differential gene expression in KBD chondrocytes, and we could confirm many changes by parallel analyses, there are several limitations of this experiment. Cartilages from the same anatomical locations were collected from KBD patients and the normal control tissues, and were used to look at the difference on genetic expression. Thus, we cannot completely exclude that some genetic effects might be due to the different genetic background that were present in the KBD and the normal groups. We also cannot discriminate the difference between pathological tissue and unaffected parts in the same arthrodial cartilage. Since the microarray analysis is only a screening tool, and we have learned that no one statistical algorithm is sufficient to derive an accurate view of the genes significantly overexpressed or underexpressed in one population compared with another, further studies are necessary to identify these genes. Transgenic animal models can also be helpful to confirm the effect on these genetic changes.

In conclusion, a clear difference in the gene expression profile existed in KBD cartilage compared with normal one. A group of genes appear to behave similarly in KBD and OA cartilage, however, differential gene expression specific for KBD was also observed. Their expressions may imply a special mechanism responsible for the destruction of the articular cartilage in KBD. This also suggests that the cartilage degeneration, apoptosis induction pathways and matrix metabolism might be more important in KBD cartilage. Elucidation of this mechanism is essential for the development of effective treatments for KBD.

Conflicts of interest

None declared.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2008.05.008.

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