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Comparing gene expression profiles of Kashin-Beck and Keshan diseases occurring within the same endemic areas of China

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In this study, differentially expressed genes in peripheral blood from patients with Kashin-Beck disease and Keshan disease were compared to further investigate the etiology and pathogenesis of both diseases, which occur in a common endemic area of China. Twenty Kashin-Beck disease patients and 12 healthy controls, and 16 Keshan disease patients and 16 healthy controls, were grouped into four pairs. Patients and controls were selected from common endemic areas for the two diseases. Total RNA was isolated from peripheral blood mononuclear cells from all patients and controls, and gene expression profiles analyzed by oligonucleotide microarrays. Sixteen genes differentially expressed in both Kashin-Beck disease and Keshan disease (versus controls) were identified, and comprised nine genes showing synchronous and seven asynchronous expression. The Comparative Toxicogenomics Database shows that expression and biological function of these genes can be affected by multiple environmental factors, including mycotoxin and selenium content, potential environmental risk factors for the two diseases. Thus, these shared differentially expressed genes may contribute to the distinct organ lesions, caused by common environmental risk factors of Kashin-Beck disease and Keshan disease.

Kashin-Beck disease, Keshan disease, microarray, peripheral blood mononuclear cells, common environmental factors, differential gene expression

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Kashin-Beck disease (KBD) and Keshan disease (KD) are major endemic diseases in China. The two diseases are distributed in a diagonal belt ranging from the northeast to the southwest of China, a region coincident with low environmental selenium content. In China, there are >0.66 million KBD, 0.04 million KD patients, and with approximately 30 million residents at risk [1,2]. KBD is an endemic osteoarthropathy, characterized by significant alterations in chondrocyte phenotype, necrosis and apoptosis, and abnormal terminal chondrocyte differentiation [3]. KD is an endemic myocardosis that mostly occurs in women and preschoolers, and is characterized by multifocal myocardial necrosis and fibrosis that can result in cardiogenic shock and congestive heart failure [4]. Because of articular cartilage and myocardium deformities, the majority of KBD and KD patients partially or completely lose their working capacity, and even self-care abilities, which seriously impacts on their quality of life, and also increases the medical burden to society.

The etiology and pathogenesis of KBD and KD are still

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unclear. However, as both diseases typically occur in the same regions of China, and therefore share similar environmental conditions, a common environmental etiological hypothesis has been proposed, with selenium deficiency and cereal contamination by mycotoxins likely factors [5–8]. Investigations have shown the selenium content is low in the internal and external environments of the endemic KBD and KD areas, resulting in residents with lower selenium content in blood, urine and hair than residents from non-endemic areas [9,10]. Mycotoxicosis has also been proposed as an etiological factor in both diseases, although the pathogenic fungus contributing to the two diseases is different; namely the T-2 toxin for KBD [11] and citreoviridin for KD [8]. In addition to a shared endemic area, the living conditions of those affected by KBD and KD are similar, for example, patients tend to live either in remote rural areas or areas of backward transportation, have a low income and monotonous diet.

Over the past decades, KBD and KD research has predominantly focused on pathogenic environmental factors, but recently gene expression microarray analyses have identified a set of abnormally expressed genes in KBD [12,13], but to date none has been identified for KD. Consequently, the interaction between genes and common environmental factors in KBD and KD development is now under debate. Comparing gene expression patterns should provide mechanistic evidence for the differing organ lesions of KBD and KD that are caused by common environmental risk factors. To our knowledge, no research has yet been conducted comparing KBD and KD gene expression profiles.

In this study, we used the Agilent Human 1A Oligo microarray to compare gene expression profiles of peripheral blood mononuclear cells (PBMCs) between KBD or KD patients and healthy controls, and identified the common genes differentially expressed in both diseases groups. Our aim was to investigate the underlying molecular mechanisms and determine how common environmental factors affect KBD and KD pathology. Validity of identified oligonucleotide array data was performed by parallel analyses of selected transcripts using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR).

1 Materials and methods

All studies were approved by the Human Ethics Committee of Xi'an Jiaotong University. All patients and controls provided informed consent.

1.1 Disease diagnosis and group categories

All subjects were randomly chosen from Yongshou and Xunyi counties, both within Shaanxi Province, and therefore the same endemic area for both diseases. The prevalence rate of KBD and KD in the two counties above are 17.4% and 13.9%. KBD and KD patients were diagnosed by using the National Diagnostic Criteria of Kashin-Beck Disease (WS/T 207-2010) or Keshan Disease (WS/T 210-2011) in China, respectively. Controls and KBD patients with a history of other bone and joint diseases were excluded from the study. Similarity controls and KD patients with a history of any other heart diseases were excluded from the study. Following study enrolment and exclusion criteria, 20 KBD patients and 12 normal subjects and 16 KD patients and 16 normal subjects, chosen from endemic areas, were identified. The participants were divided into four pairs of KBD and control (KBD, *n*=5/pair; control, *n*=3/pair; Table 1), or KD and control (KD, *n*=4/pair; control, *n*=4/pair; Table 2). All subjects were of Chinese Han lineage, and were matched for age, gender and area.

Table 1 KBD-control pairs used for microarray analysis (set 1) and quantitative RT-PCR (set 2) analyses[a)]

Sample pair	KBD				Normal			
	n	Age (range) (years)	Male	Female	*n*	Age (range) (years)	Male	Female
Set 1								
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
Total	20	48.60 (38–67)	9	11	12	44.84 (27–68)	3	9
Set 2								
1	3	57.67 (55–60)	1	2	3	49.67 (47–53)	1	2
2	3	56.67 (50–58)	1	2	3	50.00 (45–55)	1	2
Total	6	57.17 (50–60)	2	4	6	49.83 (45–55)	2	4

a) Characteristics of the patients with KBD and control according to sets of sample pairs used for microarray and quantitative RT-PCR.

Table 2 KD-control pairs used for microarray analysis (set 1) and quantitative RT-PCR (set 2) analyses[a)]

Sample pair	KD				Normal			
	n	Age (range) (years)	Male	Female	*n*	Age (range) (years)	Male	Female
Set 1								
1	4	44.75 (33–50)	4	0	4	52.00 (42–56)	4	0
2	4	41.25 (30–46)	0	4	4	39.75 (36–45)	0	4
3	4	49.75 (47–51)	0	4	4	48.25 (47–49)	0	4
4	4	57.12 (55–62)	0	4	4	58.75 (52–64)	0	4
Total	12	48.60 (38–67)	4	12	16	44.84 (36–64)	4	12
Set 2								
1	3	47.00 (42–50)	3	0	3	44.00 (40–52)	3	0
2	3	47.33 (42–52)	0	3	3	42.33 (27–54)	0	3
Total	6	47.16 (42–52)	3	3	6	43.16 (27–54)	3	3

a) Characteristics of the patients with KD and control according to sets of sample pairs used for microarray and quantitative RT-PCR.

1.2 Blood collection and PBMC isolation

Peripheral blood (3 mL) from each subject was collected into heparinized vacutainer tubes (Becton Dickenson, San Jose, CA, USA) for gene expression analysis. Leukocyte cell numbers were determined using a Hemovet 950 (Drew Scientific, Oxford, CT, USA). PBMCs were separated from plasma by centrifuging blood at 1500×*g* for 20 min. Cell pellets were resuspended in Hanks' balanced salt solution (Gibco BRL/Invitrogen, Carlsbad, CA, USA). Cell suspensions were layered over 5 mL of Lympholyte-H (Cedarlane Labs, Hornby, Canada), in a 15 mL Falcon tube, and centrifuged for 40 min at 1500×*g*. After rinsing twice with cold Hanks' balanced salt solution, cells were stored in RNA later (Ambion Inc., Austin, TX, USA) until RNA isolation.

1.3 RNA preparation

Total RNA was isolated from PBMCs using Trizol reagent (Life Technologies Inc., Carlsbad, CA, USA) following the manufacturer's recommended protocol. The quality and integrity of extracted total RNA were determined using a high resolution electrophoresis system (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). To avoid individual differences among subjects, 30 μL of total RNA was extracted from each individual subject and then mixed to form eight pairs of microarrays (Tables 1 and 2).

1.4 Microarray hybridization

Isolated total RNA from each KBD/control or KD/control pair was transcribed into complementary DNA (cDNA), and then reverse transcribed into cRNA and labeled with CyDye using Amino Allyl MessageAmp aRNA Kit (Ambion) according to the manufacturer's instructions. Before reverse transcription, RNase-free DNase I was used to remove residual genomic DNA from total RNA. For each pair, 0.5 μg of labeled cRNA was purified separately and then mixed together with hybridization buffer before applying to microarrays. The Agilent Human 1A Oligo microarray, consisting of 21073 (60-mer) oligonucleotide probes spanning conserved exons across transcripts of targeted full-length genes, was used for microarray hybridization following the recommended protocol. Microarray slides were scanned using Gene-Pix 4000B (Axon Instruments Inc., Foster City, CA, USA), and GenePixPro 3.0 software (Axon Instruments Inc.) used to analyze the 16-bit TIFF images produced by the scanner. Ratio images for all spots were defined by accessing the gene list file describing the microarray location of each gene. After the average signal intensity was subtracted from the median back intensity, gene expression data were imported into Excel spreadsheets (Microsoft Corp., Redmond, WA, USA) using Unigene and GenBank descriptors. Global normalization was conducted to calculate relative expression levels between two samples using all detected genes. A detailed description of this normalization process can be obtained at www.aglient.com.

1.5 Gene expression analysis

To identify differentially expressed genes, expression ratios were calculated for each gene, with ⩽0.5 or ⩾2.0 regarded as statistically significant. *P*-values were calculated using the Agilent *P*-value log ratio algorithm:

$$P = 1 - Erf\left(\frac{|xdev|}{\sqrt{2}}\right) = Erfc\left(\frac{|xdev|}{\sqrt{2}}\right),$$

with *Erf(x)* calculated as

$$Erf(x) = \frac{2}{\sqrt{Pi}}\int_0^x e^{-t^2}\,dt.$$

Erf(x) represents twice the integral of the Gaussian distribution, with a mean value of 0, variance of 0.5, and *xdev* the deviation of the log ratio from 0. This calculation provides the statistical significance of the log ratio for each feature (i.e., transcript level) between red and green channels. Only

P-values ⩽0.05 were regarded as significant.

1.6 Quantitative real-time PCR validation

For quantitative real-time PCR of our microarray analysis, seven genes with expression ratio ⩽0.5 or ⩾2.0 were selected. Total RNA was isolated from an additional 24 subjects, and prepared as for the oligonucleotide microarray analysis. RNA was then converted into complementary DNA (cDNA) using superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) and random primers. Quantitative RT-PCR was performed using the ABI7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All primer and probe sets (HBA1, NM_000558; PF4, NM_002619; SAPS1, NM_014931; B2M, NM_004048; HBB, NM_000518; APOA1, NM_000039; HBA2, NM_00517) were supplied by TaqMan Gene Expression Assays (Applied Biosystems). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was simultaneously assayed as an endogenous invariant control for data normalization. Paired *t* tests were performed to determine significance levels of expression differences for seven genes, between KBD or KD patients and normal controls.

2 Results

2.1 Comparative analysis of differentially expressed genes between KBD and KD

We used the Agilent Human 1A Oligo microarray system to compare gene expression profiles in PBMCs from KBD or KD patients versus controls. We identified 136 differentially expressed genes (53 up-regulated and 83 down-regulated), between KBD and normal controls, with an average ratio in 20381 genes. Moreover, comparing KD and normal controls, we identified 3310 differentially expressed genes (3154 up-regulated and 156 down-regulated), with an average ratio in 43376 genes. Comparing all identified differentially expressed genes, we found 16 genes showing differential expression in both diseases, including nine with synchronous and seven with asynchronous expression (Tables 3 and 4). These 16 genes were subdivided into 11 categories, namely metabolism, cytochrome enzymes, transcription related, G-protein related, receptor, cytokine factor, ion channel transport protein, signal transduction, hematopoietic related, interleukin and immune related.

Table 3 List of nine synchronous expression genes in KBD and KD patients[a)]

Gene name	Symbol	Public ID	Fold change	
			KBD	KD
Carbonic anhydrase I	CA1	NM_001128829	2.95±0.88	3.76±1.70
Cytochrome c oxidase subunit Vla polypeptide 2	COX6A2	NM_005205	2.10±3.70	2.10±1.10
CREB regulated transcription coactivator 1	CRTC1	NM_015321	2.93±2.80	2.62±1.83
Ladybird homeobox 1	LBX1	NM_006562	2.42±2.07	2.05±1.06
G protein-coupled receptor 153	GPR153	NM_207370	2.29±2.12	2.23±1.33
Hemoglobin, delta	HBD	NM_000519	2.41±0.98	2.04±0.83
Synuclein, alpha	SNCA	NM_000345	2.95±1.52	2.37±0.22
Pleiotrophin	PTN	NM_002825	2.11±1.10	2.75±0.48
Solute carrier family 9, subfamily A	SLC9A9	NM_173653	0.37±0.06	0.49±0.29

a) Common differential expression genes between the KBD vs. controls and the KD vs. controls were assessed using the selection criteria described in Materials and methods. According to the fold change value, only those genes showing significant differences (*P*-value<0.05) in expression and screening the common differential expression genes in KBD and KD are listed. Fold change, the mean and standard error of the mean (SEM) of the fold change in expression of each gene.

Table 4 List of seven asynchronous expression genes in KBD and KD patients[a)]

Gene name	Symbol	Public ID	Fold change	
			KBD	KD
ATP/GTP binding protein-like 4	AGBL4	NM_032785	0.46±0.005	2.22±1.72
Ankyrin repeat domain 20 family, member A2	ANKRD20A2	NM_001012421	0.43±0.07	2.28±2.55
Calcium channel, voltage-dependent, gamma subunit 6	CACNG6	NM_145814	2.47±2.06	0.45±0.21
Hemoglobin, alpha2	HBA2	NM_000517	4.68±2.76	0.28±0.17
Hemoglobin, alpha1	HBA1	NM_000558	4.32±2.65	0.31±0.21
Interleukin 1 family, member 10	IL1F10	NM_032556	0.48±0.11	2.68±1.88
Leukocyte-associated immunoglobulin-like receptor2	LAIR2	NM_002288	0.34±0.20	2.17±1.25

a) Common differential expression genes between the KBD vs. controls and the KD vs. controls were assessed using the selection criteria described in Materials and methods. According to the fold change value, only those genes showing significant differences (*P*-value<0.05) in expression and screening the common differential expression genes in KBD and KD are listed. Fold change, the mean and standard error of the mean (SEM) of the fold-change in expression of each gene.

2.2 Quantitative RT-PCR analysis

To validate the microarray data, seven differentially expressed genes were selected for quantitative RT-PCR analysis using PBMC samples from an additional 12 KBD and 12 KD subjects. Expression levels of HBA1, PF4, SAPS1, B2M, HBB, APOA1 and HBA2 mRNA in KBD and KD PBMCs, were significantly different from normal control PBMCs (Figure 1). Thus, our quantitative RT-PCR analysis confirms the oligonucleotide array results.

3 Discussion

KBD and KD are distributed within the same geographical area, although the clinical manifestation and target pathological organs are not identical (articular cartilage and epiphyseal plates mainly injured in KBD, and myocardial damage present in KD). In this study, we used oligonucleotide microarrays to compare PBMC gene expression profiles between KBD or KD patients and normal controls. We identified differentially expressed genes in both KBD and KD (versus healthy controls), which may help identify the underlying mechanisms of the distinct organ lesions, caused by common environmental risk factors of KBD and KD.

3.1 Functions of genes with synchronous expression in KBD and KD

The nine genes showing synchronous expression in KBD and KD include CA1, COX6A2, HBD, LBX1 and PTN. These genes belong to various functional categories, including metabolism, transcription related, ion channel transport protein, hematopoietic related and immune related.

Carbonic anhydrases (CA1) belong to a large family of metalloenzymes that catalyze reversible hydration of carbon dioxide (CO_2). They participate in a variety of biological processes, including respiration calcification, acid-base balance, and bone resorption. Overexpressed CA1 in synovial tissue of ankylosing spondylitis contributes to abnormal bone calcification and bone resorption [14]. Myocardium respiratory disturbances, such as anoxia, cell ischemia and abnormal lipid-conformation of the erythrocyte membrane, are pathological of KD [15]. The main physiological function of CA is transportation of CO_2 from tissues to plasma [16]; therefore CA1 overexpression in KD patients may be requirement for increased CO_2 transportation via carbonic anhydrase, because of increased CO_2 concentration as a result of anoxia and cardiomyocyte ischemia. Thus, CA1 may play an important role in progress of both KBD and KD, but there is little research on the relationship between CA1 and environment interactions.

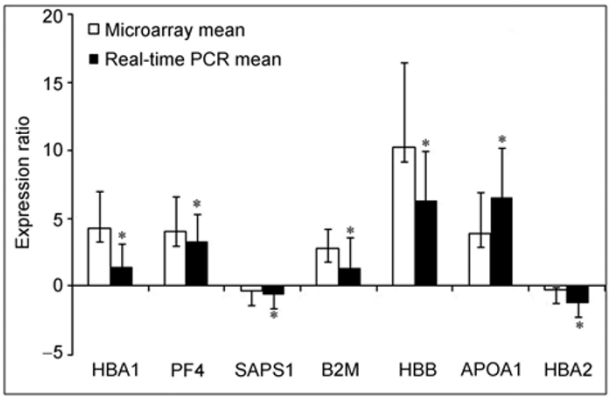

Figure 1 The expression ratio comparison of the selected gene between the microarray and quantitative RT-PCR. Histogram shows the expression ratio of the seven selected genes, as measured by microarray and quantitative RT-PCR. * indicates $P<0.05$, calculated by Student's paired *t*-test.

COX6A2 encodes cytochrome c oxidase, the terminal enzyme in the mitochondrial respiratory chain which catalyzes electron transfer from reduced cytochrome c to oxygen, and reduction of oxygen to water molecules to maintain reactive oxygen within mitochondria at a normal physiological level [17]. In KBD patients, reactive oxygen content is increased in articular chondrocytes, and cytochrome c is released from mitochondria to cytoplasm which stimulates apoptosis [18]. Moreover, in the cardiomyocyte of KD patients, cytochrome c oxidase activity is reduced [15], and animal experiments have shown cytochrome c oxidase activity through increased citreoviridin dosage [19]. Furthermore, low selenium has an inhibitory action on cytochrome c oxidase activity in mouse cardiomyocytes [20]. Therefore, COX6A2 overexpression, interacting with the same environment factors, may play a key role in pathogenesis of the two diseases.

Pleiotrophin (PTN) is a cell growth factor that promotes angiogenesis, cell proliferation and differentiation. PTN contributes to chondrogenesis and prevents cartilage degeneration [21,22]. PTN expression is elevated during the early stages of osteoarthritis in patients [23], and PTN may be involved in synovial angiogenesis, as it is the angiogenic factor generated in the inflammatory reaction of rheumatoid arthritis [24]. Moreover, PTN overexpression occurs in dilated cardiomyopathy, supporting neovascularization due to heart damage, and can also reduce the cardiomyocyte reaction to hypoxia and promote neonatal cardiomyocytes apoptosis by suppressing activity of AKT/PKB [25]. Therefore, PTN may play an important role in KBD and KD development.

3.2 Function of genes with asynchronous expression in KBD and KD

The seven genes showing asynchronous expression in KBD and KD include HBA1, HBA2, IL1F10 and CACNG6. These genes belong to various functional categories, including metabolism, signal transduction, ion channel transport protein, hematopoietic related, interleukin and immune related.

Hemoglobin alpha chains 1 and 2 (HBA1 and HBA2) are up-regulated in KBD, and down-regulated in KD patients. The HbA molecule comprises two alpha and two beta chains, which during normal adult life comprises approximately 97% of total hemoglobin. Abnormal erythrocyte membrane structures is present in both KBD and KD [26]. Hemoglobin form can affect erythrocyte membrane structure [27], and low selenium levels can disorder the ultrastructure and function of erythrocyte membrane [28]. It has been well reported that hemoglobin content in patients with heart failure, as found in KD, is significantly lower than normal controls [29–31]. In addition, the selenium content of erythrocyte and erythrocyte membranes in KBD patients is lower than normal controls [32]. Therefore, it is feasible that co-influences from environmental factors (low selenium and mycotoxins) and different alterations in gene expression (HBA1 and HBA2) may contribute to abnormal erythrocyte membrane structures in KBD and KD patients. The reason for differential expression of HBA1 and HBA2 (up-regulated in KBD, and down-regulated in KD) may involve the differing disease pathogenesis and target organs.

Interleukin-1 (IL-1) is a pleiotropic cytokine and determining factor in the mechanism of inflammatory pathogenesis. In osteoarthritis patients, IL1 is involved in cartilage degradation and the inflammatory reaction between synovium and cartilage [33]. IL-1 content in the synovium and serum of KBD and osteoarthritis patients is significantly elevated [34,35], and KBD is associated with suppressed immunity in PBMCs [36]. An IL-1 polymorphism has been found associated with KBD and rheumatoid arthritis [37,38]. IL1F10 expression is found in heart, placenta and spleen [39], and increased IL-1 expression occurs in coronary heart disease, myocardial infarction and cardiac failure patients [40], consistent with IL1F10 overexpression in KD patients. IL1 is also an important moderator of hypertrophy, fibrosis and myocardium insufficiency. However, the relationship between IL1F10 down-regulation and increasing IL1 is unclear, but our microarray results suggest IL1 cytokines, encoded by IL1F10, play an important role in KBD and KD pathogenesis.

CACNG6 encodes a gamma subunit of the calcium channel, a channel specifically for calcium ion transport across cell membranes. Calcium channels are crucial for osteoblast differentiation to osteocytes and osteoclasts, and both calcium ions and calcium channels play critical roles in apoptosis [41]. A number of hormones and cytokines that act directly on osteoblasts are regulated by calcium ions (via calcium channels) [42]. It has been reported that decreased calcium channel density is present in myocardial hypertrophy and cardiac failure [43]. We detected up-regulated and down-regulated CACNG6 expression in KBD and KD patients, respectively, suggesting that CACNG6 is involved in pathogenesis of both endemic diseases.

Using the Comparative Toxicogenomics Database, we found that among the 16 genes differentially expressed in KBD and KD, expression and function of some genes are affected by selenium and mycotoxins, the environmental risk factors for KBD and KD. For example, HBA2 and CRTC1 expression is altered by selenium content *in vivo*, and mycotoxins can affect SLC9A9, COX6A2, GPR153 and PTN gene expression. A detailed description of the genes listed can be obtained (www.ctdbase.org). Based on our results, and those of previous studies, we suggest that exposure to common environmental risk factors (mycotoxins and low selenium) leads to dysfunction of key genes, and contributes to specific organ lesions in KBD and KD.

In summary, we compared PBMC gene expression profiles between KBD or KD patients and normal controls to investigate the same hypothesis and epidemiological characteristics of both two diseases at the molecular level. In KBD and KD, identical environmental susceptibility genes varying in expression levels may combine on a common environmental background to play an important role in disease pathogenesis through an environment-gene related interaction.

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