Detection of Unsaturated Disaccharides, Pyridinoline, and Hydroxyproline in Urine of Patients with Kashin-Beck Disease: Comparison with Controls in an Endemic Area

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ABSTRACT. ***Objective.*** To investigate the pathologic status of adult patients with Kashin-Beck disease (KBD) in an endemic area of China through detection of 5 biochemical markers in their urine, and to study the correlations between these markers and KBD.

Methods. A total of 55 patients with KBD over age 40 years were recruited and divided into groups, Grade 1 and Grade 2, according to clinical diagnosis criteria for KBD and our inclusion criteria; 25 healthy persons were enrolled into a control group. The first-time urine of the 80 participants was collected in the morning. Three unsaturated disaccharides, pyridinoline (PYD), and hydroxyproline (HYP) were detected in urine samples with high performance liquid chromatography, ELISA, and a chemical kit. Mean levels of these markers were compared in the 3 groups.

Results. The mean concentrations of 3 unsaturated disaccharides and PYD in the Grade 2 group were significantly higher than levels in the Grade 1 group and controls ($p < 0.05$). There was no significant difference between findings in the Grade 1 group and controls. Levels of 3 unsaturated disaccharides correlated with each other ($p < 0.01$). The correlation coefficient between PYD and HYP was 0.470 ($p < 0.01$). Except for HYP, the other markers all correlated with grade of KBD, rather than age or sex of subjects.

Conclusion. The cartilage degradation of patients with Grade 2 KBD was more severe than that of Grade 1 patients and controls. The pathologic condition of Grade 1 patients was mild. Except for HYP, the markers we investigated specifically reflected the pathologic bone metabolism of adult patients with KBD. Trial registration number ChiCTR-TRC-00000140. (First Release March 15 2009; J Rheumatol 2009;36:816–21; doi:10.3899/jrheum.080642)

Key Indexing Terms:
KASHIN-BECK DISEASE DISACCHARIDES PYRIDINOLINE
HYDROXYPROLINE URINE

Kashin-Beck disease (KBD) is a degenerative disease of the peripheral joints and spine in childhood that is believed to be caused by ingestion of cereal grains infected with the fungus *Fusarium sporotrichiella*. The disposition of KBD endemic areas in China were from northeast to Sichuan province and the Tibet plateau. This region also extended to a few areas of Russia and North Korea. KBD outbreaks prevailed in China several times in the past. Pandemic situations at the end of the 1950s and 1970s were severe and extensive; 14 provinces, cities, and autonomous regions were involved. The population in the endemic area was over 30 million[1]. By the end of 2003, there were about 810,000 patients all over the country[2]. It is necessary to study the current status of patients with KBD and therapeutic remedies.

KBD is a specific kind of osteoarthritis (OA) and its pathologic changes are identical with those of OA in principle[3]. Some OA markers reflecting the matrix and collagen degradation of cartilage may also be used in evaluation of patients with KBD. The markers we selected included unsulfated chondroitin sulfate disaccharide (ΔDi-0S), chondroitin-4 sulfate disaccharide (ΔDi-4S), chondroitin-6 sulfate disaccharide (ΔDi-6S), pyridinoline (PYD), and hydroxyproline (HYP). There are no reports on these biochemical markers for adult KBD patients to date.

ΔDi-0S, ΔDi-4S, and ΔDi-6S are products of chondroitin sulfate (CS). In articular cartilage, CS and keratan sulfate chains are the main kinds of glycosaminoglycans (GAG). GAG chains are covalently attached to the protein cores of

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individual proteoglycan molecules, and proteoglycans are the major components of the extracellular matrix of articular cartilage[4,5]. In the process of articular cartilage breakdown, there is increased damage to resident matrix molecules, with upregulation of synthesis of collagen and proteoglycan. These new molecules are also subject to degradation. Degradation products are released to body fluids, where they can be detected[6].

The hydroxypyridinium crosslinks of collagen, PYD and deoxypyridinoline (DPD), are formed during the extracellular maturation of fibrillar collagens and are released upon the degradation of mature collagens. The 2 components show a high specificity for skeletal tissues. While PYD is found in cartilage, bone, ligaments, and blood vessels, DPD is found in bone and dentin only[7]. Once bone matrix degradation occurs, PYD and DPD as the breakdown products of collagen are excreted in urine. The 2 markers change prior to bone density and can be monitored successively for observing the longterm changes of bone metabolism.

Measurement of urinary HYP excretion is used to estimate collagen catabolism. Degradation of bone collagen releases free 4-HYP and peptides containing 4-HYP into the plasma. Most of the free HYP is reabsorbed by the tubules, while essentially all the filterable HYP-containing peptides pass through the kidneys. Urinary HYP measurement is particularly useful in monitoring treatment and progression in metabolic bone diseases[8].

Our aim was to evaluate the present pathologic conditions of patients with KBD and collect baseline data for a future prevention study. We also studied correlations between the markers and the disease, and discuss the prospects for application of those indexes.

MATERIALS AND METHODS

Diagnosis and grading criteria for KBD (GB 16003-1995)[9]. The premise for adult KBD was that patients had ever lived in an endemic area and were sick during childhood.

Grade 1 disease. On the basis of the above premise, patients were classified as having Grade 1 disease if they showed multiple symmetrical joint thickening of fingers or other limbs, had difficulty in bending and stretching activities, and had slight muscular atrophy and pain. Physical signs were characterized by interphalangeal joint thickening.

Grade 2. Patients were classified as having Grade 2 disease if, on the basis of grade 1 disease, symptoms and physical signs were aggravated. Patients flex their fingers with difficultly and are unable to touch the palm with fingertips when they clench a fist. The joint movements of wrist, elbow, knee, and ankle are plainly difficult, and muscle atrophy of all 4 limbs is severe. The physical signs are significant thickening of interphalangeal joints, brachydactyly, and phocomelic limbs.

Examples of hands of patients with Grade 1 and Grade 2 KBD are shown in Figure 1.

Selection of patients. We recruited patients with KBD from Guanghui village, Yabuli town, Heilongjiang province, China, one of the areas endemic for KBD. A total of 1047 adult residents lived in the village (538 men, 509 women) and 282 were aged over 40 years. Of these 282 residents, 80 were diagnosed as having KBD through clinical examination. Finally, 55 patients were selected, 33 classified for the Grade 1 group and 22 for the Grade 2 group. The criteria for inclusion were age over 40 years, and grading according to the clinical diagnosis criteria for KBD[9].

The exclusion criteria were history of or active presence of other bone or cartilage disease or other illness known to affect bone metabolism (i.e., Paget's disease, osteoporosis, osteomalacia, cancer, or alcoholism); severe articular inflammation confirmed by examination; traumatic knee lesions; substantial abnormalities in hepatic, renal, or metabolic functions; overweight, defined as a body mass index > 30; and use of intraarticular or systemic corticosteroids in the 3 months preceding enrolment. The exclusion methods included examination of medical records, physical examination, and routine urine test.

Participants for the control group were 25 healthy local residents over 40 years old, with no evidence of KBD, and following the exclusion criteria for patients. Further, controls were not taking hormone treatments or any other drugs known to affect bone metabolism.

The total number of participants was 80 persons.

The study was approved by the ethics committee of Harbin Medical University Endemic Control Center and the local village committee, according to the Helsinki Declaration. All subjects provided oral informed consent to participate. The Chinese clinical trial registration number for the study is ChiCTR-TRC-00000140 (http://www.chictr.org/).

Sample collection. The first-time urine of participants was collected in the morning; 2% chlorhexidine acetate was added to urine samples as antiseptic, and samples were stored at –80°C until measurement.

Purification of unsaturated disaccharides from urine. Purification was performed as described with minor modifications[10]. A 200-µl quantity of 15 mM acetic acid containing 10% NaCl was added to 100 µl of sample. The mixture was heated in a boiling water bath for 5 min and cooled in an ice bath, and the solution was centrifuged at 4000 rpm for 20 min. A 260-µl portion of the supernatant was transferred to a centrifugal filter containing 20 µl of 0.10 M NaOH on the filter. After centrifugation at 4000 rpm for 40 min, the retained sample was washed twice with 0.20 M Tris-HCl buffer (pH 8.0) and then dissolved in the buffer. The purified sample was digested with 5 µl CS ABC lyses (0.1 U) for 3 h at 37°C. After digestion was completed, the samples were again centrifuged at 4000 rpm for 30 min, and the unsaturated disaccharides passing through the filter were recovered and analyzed by postcolumn derivatization high performance liquid chromatography (HPLC).

Determination of unsaturated disaccharides by HPLC. Unsaturated disaccharides were detected by HPLC with fluorometric postcolumn derivatization using 2-cyanoacetamide as a fluorogenic reagent as described[10]. The postcolumn HPLC system was constructed with 3 LC-6A independent pumps (Shimadzu Corp., Kyoto, Japan), a sample injector with a 20 µl loop (model 7725i; Reodyne, Cocati, CA, USA), a fluorescence spectro-photometer (RF-530; Shimadzu Corp.), a column thermocontroller (CTO-6A; Shimadzu Corp.), an N2000 chromatographic workstation (ZheJiang University, China), and a constant-temperature desktop dry box (202-AO; Zhejiang Ketong Instrument, China). A DOCOSIL column (150 mm × 4.60 mm id; 60°C; S.A.S. Corp., Tokyo, Japan) was used at a flow rate of 0.90 ml/min. Unsaturated disaccharides were eluted by the use of 0.80 mM tetrabutylammonium hydrogen sulfate and 2 mM NaCl in 8-acetonitrile as the eluting agent. Aqueous 1% 2-cyanoacetamide solution and 1 M NaOH at the same flow rate of 0.15 ml/min were added to the effluent by 2 independent pumps. The mixture was passed through a thermostable stainless steel coil (11 m × 0.50 mm id) set in a constant-temperature desktop dry box thermostated at 120°C and then through a cooling coil (3 m × 0.25 mm id). The effluent was monitored fluorometrically (ex. 346 nm, em. 410 nm). A 20-µl portion of sample solution was loaded onto the HPLC.

Human PYD ELISA. A human PYD ELISA kit (USCNlife, Missouri City, TX, USA) was employed using the quantitative sandwich enzyme immunoassay technique. The assay was performed as recommended by the manufacturer.

HYP chemical detection. The chemical principle of the HYP determination kit (Jiancheng Bioengineering Institute, Nanjing, China) is that HYP-oxi-

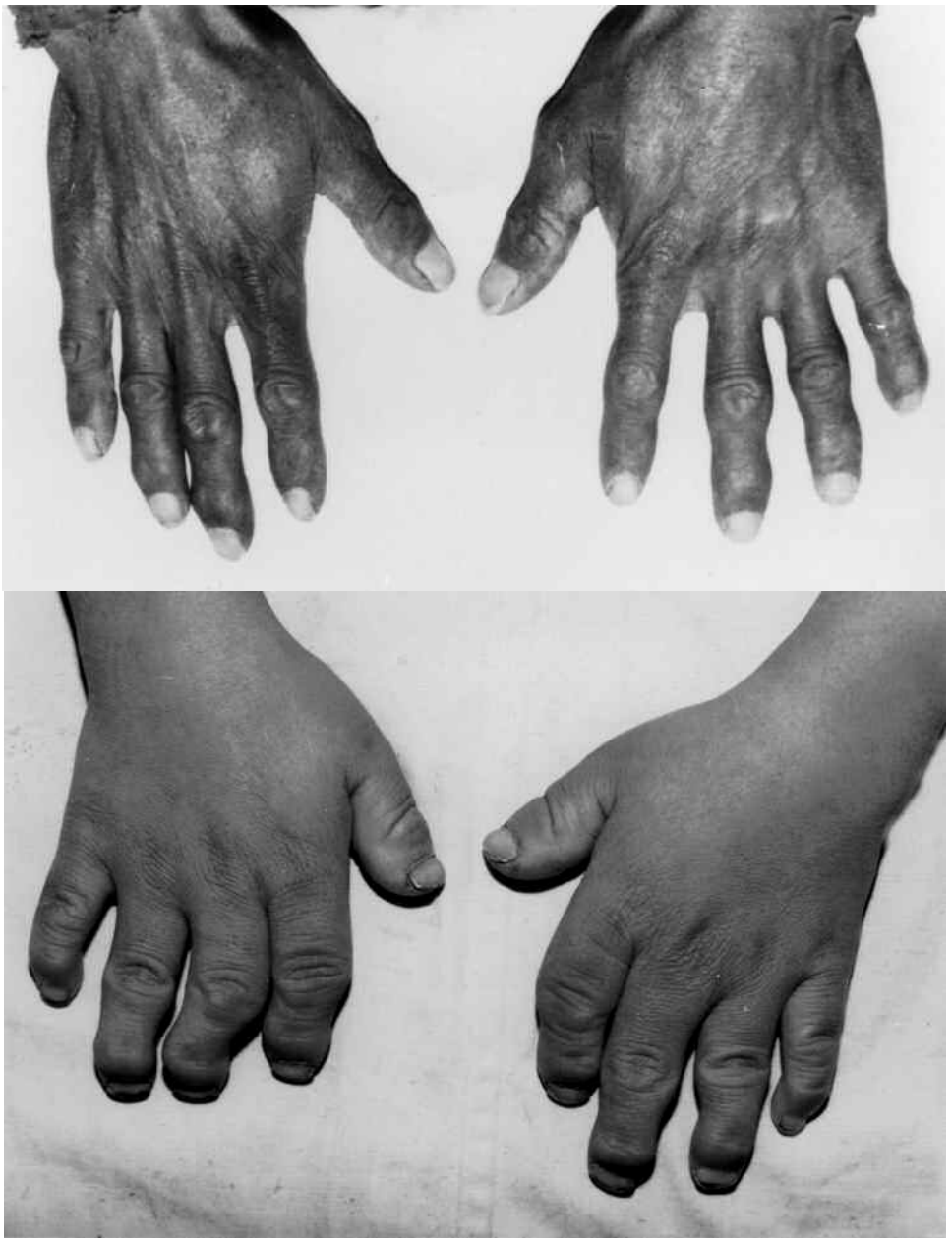

Figure 1. A. An adult patient with Grade 1 KBD; the knuckles are plainly thickened. B. Brachydactylia abnormality of a patient with Grade 2 KBD.

dized products act with dimethylaminobenzaldehyde and show amaranth. The assay was performed as recommended by the manufacturer.

Statistical analyses. We used variance analysis to compare the means of the groups and correlation analysis to determine the relations of the markers. All statistical analysis was performed with SPSS (version 10.0). A 2-sided p value < 0.05 was considered statistically significant.

RESULTS

The age and sex characteristics of Grade 1 and Grade 2 patients and controls are described in Table 1.

Variance analysis of disaccharides, PYD, and HYP. A typical chromatogram of 3 disaccharides is shown in Figure 2.

Concentrations of 3 disaccharides showed progressive tendencies in controls and the Grade 1 and Grade 2 groups. The mean concentrations of patients in Grade 2 group were significantly higher than the levels of the other 2 groups. The F values for ΔDi-0S, ΔDi-4S, and ΔDi-6S variance analysis were 85.700, 19.609, and 13.855, respectively ($p < 0.01$). However, there was no significant difference between Grade 1 group and controls.

PYD and HYP levels were revised by urinary creatinine (CR). There was a significant difference of PYD/CR in the 3 groups ($p < 0.05$). The mean PYD/CR of the Grade 2

Table 1. Age and sex composition of the participants.

Group	N	Age Interval	Mean Age, yrs	Men N	Men Age, yrs	Men Mean	Women N	Women Age, yrs	Women Mean
Control	25	42–69	53.20	14	44–69	54.97	11	42–62	51.18
Grade 1	33	41–75	50.97	16	42–75	53.56	17	41–56	48.53
Grade 2	22	40–74	52.82	17	40–74	52.41	5	43–74	54.20
Total	80	40–75	52.18	47	40–75	53.51	33	41–74	50.27

The age and sex matches of the subgroups are close, except there are fewer female patients of Grade 2 group.

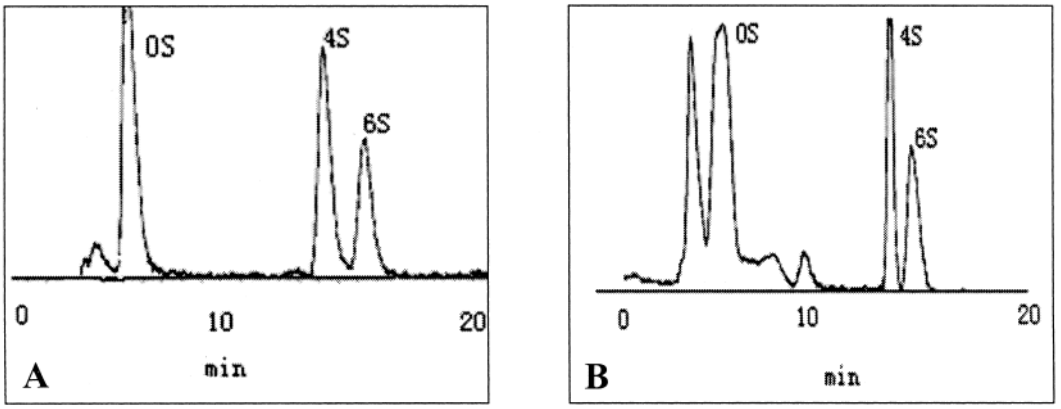

Figure 2. Chromatograms of unsaturated disaccharides on HPLC. A. Standard unsaturated disaccharides. B. Unsaturated disaccharides purified from human urine. Horizontal axis represents retention time (min), each peak is integrated by peak area. Retention times of ΔDi-0S, ΔDi-4S, and ΔDi-6S are 6.740 min, 16.757 min, and 18.690 min, respectively.

group was significantly higher than the levels of Grade 1 group and the control group. However, there was no statistical difference between Grade 1 and the control group. There was no difference for the means of HYP/CR in the 3 groups.

Mean values for ΔDi-0S, ΔDi-4S, ΔDi-6S, PYD/CR, and HYP/CR of the 3 groups are shown in Table 2.

Correlation analysis. There were highly significant correlations among the 3 disaccharides ($p < 0.01$). The correlation coefficients between ΔDi-0S and other 2 disaccharides were 0.572 and 0.576, respectively. Between PYD and HYP, the correlation coefficient was 0.470 ($p < 0.01$), whereas, unsaturated disaccharides did not correlate with PYD or HYP. Correlation coefficients are shown in Table 3.

Except for HYP, other markers all correlated with KBD grading and were independent of subjects' age or sex. These correlation coefficients are shown in Table 4.

Table 2. The means and F values of 3 unsaturated disaccharides, PYD/CR, and HYP/CR in the 3 study groups.

Marker	Group	N	Mean (SD)	F
ΔDi-0S†, μg/ml	Control	22	1.123 (0.204)	85.700**
	Grade 1	29	1.177 (0.286)	
	Grade 2	21	2.045 (0.285)	
ΔDi-4S†, μg/ml	Control	22	0.838 (0.206)	19.609**
	Grade 1	29	0.847 (0.331)	
	Grade 2	21	1.320 (0.316)	
ΔDi-6S†, μg/ml	Control	22	0.930 (0.102)	13.855**
	Grade 1	29	1.014 (0.347)	
	Grade 2	21	1.381 (0.366)	
PYD/CR††	Control	24	0.117 (0.092)	3.431*
	Grade 1	33	0.100 (0.106)	
	Grade 2	20	0.310 (0.566)	
HYP/CR	Control	25	0.010 (0.006)	0.072
	Grade 1	33	0.009 (0.011)	
	Grade 2	22	0.009 (0.008)	

† In disaccharide detection, we excluded 8 samples that might be unqualified. †† In PYD ELISA determination, 3 samples were excluded because they were out of the kit detection range. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

Based on our results, we conclude that the pathologic status of articular cartilage of patients with Grade 2 KBD was more severe than those of the Grade 1 group or controls. However, Grade 1 patients' conditions were mild and they exhibited no significant differences from the controls. Levels of unsaturated disaccharides and PYD in the urine could definitely reflect the severe collagen and matrix metabolic changes of Grade 2 KBD. Except for HYP, the markers correlated with KBD grading specificially, and could be used as indications for evaluation of therapy.

The integrity of articular cartilage is impaired in a num-

Table 3. Correlation coefficients of the markers.

	PYD/CR	HYP/CR	ΔDi-0S	ΔDi-4S	ΔDi-6S
PYD/CR	1.000	0.470**	0.113	0.122	0.069
HYP/CR	0.470**	1.000	–0.061	0.004	–0.024
ΔDi-0S	0.113	–0.061	1.000	0.572**	0.576**
ΔDi-4S	0.122	0.004	0.572**	1.000	0.341**
ΔDi-6S	0.069	–0.024	0.576**	0.341**	1.000

* Significant at the 0.01 level (2-tailed).

Table 4. Correlation coefficients among markers and KBD grading, age, and sex.

Marker	KBD Grading	Age	Sex
ΔDi-0S	0.735**	0.012	0.144
ΔDi-4S	0.513**	–0.024	0.063
ΔDi-6S	0.497**	–0.074	0.140
PYD/CR	0.227*	–0.035	0.051
HYP/CR	–0.041	–0.173	–0.245*

* Significant at the 0.05 level (2-tailed). ** Significant at the 0.01 level (2-tailed).

ber of rheumatic diseases, leading to the release of cartilage matrix synthetic and degradation products into the synovial fluid and subsequently into the serum and urine[11]. The coinstantaneous increase of 3 unsaturated disaccharides demonstrated that adult KBD patients' articuli were processing breakdown and repair procedures simultaneously. Increased detection of disaccharides in Grade 2 patients' urine revealed the degradation and synthesis of their cartilage matrix was more active than in patients with Grade 1 disease and controls. In other words, the pathologic change of patients with Grade 2 disease was more severe.

It is commonly accepted that occurrence of the collagen hydroxypyridinium crosslinks PYD and DPD in urine is an indication of the breakdown of mature collagen[12]. They have been shown to be useful measures of bone resorption in conditions such as osteoporosis, Paget's disease, and hyperparathyroidism[13,14], and have also been demonstrated to be elevated in various rheumatic diseases including rheumatoid arthritis and OA[15,16]. The discovery that PYD is excreted in urine[12,17,18] raised the possibility of using this compound as an index of collagen degradation. The fact that PYD is present only in mature collagen provides major advantages in specificity over other markers of collagen breakdown. In our studies, PYD levels of the Grade 2 group were higher than values of the Grade 1 group and controls, illustrating that the collagen degradation of Grade 2 patients was significantly more severe than the cartilage damage of Grade 1 patients and controls.

For the 3 disaccharides and PYD, there was no significant difference between patients of the Grade 1 group and controls. The major reason for this finding might be that impairment of Grade 1 patients' matrix and collagen was not severe and the pathologic changes were mild. Nonetheless, we also noted that numeric values of the levels of the 3 disaccharides of Grade 1 patients were higher than the means of the control group, but were without statistical significance. In addition, there was another possible reason. We assumed that our controls were recruited in the endemic area and that they had had the chance to be affected by the mycotoxin; these adult controls had no symptoms or signs of KBD. But their urinary bone markers might not be in the normal range because of the toxin infection. The biomarkers we studied properly reflected the minor bone metabolic abnormalities of the controls. Thus, we observed similar levels of the markers in the 2 groups and no differences between them. However, we are not able to exclude this kind of control subject because currently we do not have laboratory techniques and criteria for detection of asymptomatic individuals. This problem should be resolved in a future large-scale study with the goal of comparing biochemical markers between control subjects from endemic and non-endemic areas.

HYP is found mainly in collagens, making up about 13% of the amino acid content of these proteins[19-21] About 90% of the HYP is released by the breakdown of collagen in tissues[22,23]. Since half of human collagen resides in bone, excretion of HYP in urine is regarded as a marker of bone resorption[21,24,25]. However, the urinary total HYP represents only a small fraction of total collagen catabolism. There are a number of issues that can lead to a lack of tissue specificity[19-21,24,26]. HYP is also liberated by the breakdown of complement[27]. Inflammatory conditions can also cause dramatic increases in urinary excretion of HYP[19,20]. HYP levels are also influenced by dietary intake of food products containing gelatin[19,28,29]. In our studies, there was no significant difference of HYP levels in the 3 groups. This might be because HYP is not a specific marker for collagen degradation and tends to be influenced by various factors, as described. Indeed, we obtained significant results from a more specific marker of bone resorption, PYD.

The markers varied with subjects' KBD grading and correlated with each other. The correlations of the 3 disaccharides were significant, as was the correlation between PYD and HYP. Moreover, matrix metabolic disaccharides were independent of the collagen-degradation product PYD. Except for HYP, the markers correlated uniquely with KBD gradings and were not influenced by subjects' age or sex. Thus we might conclude that these markers could represent the pathologic changes of KBD in some degree, and might be used as indicators for assessment of therapies for KBD.

Our study evaluated the current pathologic status of adult patients with KBD through detection of 5 urinary biochemical markers. The proteoglycan and collagen degradation of patients with Grade 2 KBD was more severe than that in Grade 1 KBD patients and controls. The pathologic condi-

tion of patients with Grade 1 disease was mild. Except for HYP, the markers correlated specifically with KBD grading, and might be utilized to evaluate the curative effects of some drugs. At best this was a preliminary study. The way forward may be to take the most specific markers and study a disease population with more attention to clinical detail. The prospective value of these markers also remains to be proved by a further prevention study.

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