Letter to the Editor

Polymorphism in rs2229783 of the Alpha 1(XI) Collagen Gene Is Associated with Susceptibility to but not Severity of Kashin-Beck Disease in a Northwest Chinese Han Population*

SHI Xiao Wei[1], ZHANG Feng[2], LI Zhi Yan[3], LYU Ai Li[2], and GUO Xiong[2,#]

The purpose of this study was to explore whether genomic polymorphisms in the alpha 1(XI) collagen gene (*COL*11A1) were associated with the risk and severity of Kashin-Beck disease (KBD). Twenty-two single nucleotide polymorphisms (SNPs) in *COL*11A1 were genotyped in 274 KBD cases and 249 healthy controls using the Sequenom MassARRAY system. The expression of type XI collagen (COL11A) in the knee articular cartilage of 22 KBD patients and 21 controls was analyzed by immunohistochemistry. Our results showed that the frequency distribution of genotypes of the rs2229783 polymorphism in *COL*11A1 was significantly different between the KBD and control groups (P = 0.0003). Moreover, the expression level of COL11A in cartilage was significantly lower in the KBD group than in the controls (t = 2.637, P = 0.02). However, no association was found between the rs2229783 and the severity of KBD, suggesting a role of *COL*11A1 in the susceptibility to but not the severity of KBD.

Kashin-Beck disease (KBD) is an endemic and chronic osteochondropathy mainly distributed over a limited area from southeastern Siberia extending to northeast and southwest China. A key pathological feature of KBD is chondrocyte necrosis in the deep zone of the growth plate of cartilage and articular cartilage[1]. The etiology of KBD remains unclear; recent epidemiological and genetic study results suggest that the interaction between environmental factors and susceptibility genes might play a role in the disease[2]. Certain susceptibility genes may affect susceptibility to environmental factors, such as selenium deficiency or other biologic factors[3].

Type XI collagen (*COL*11A), a cartilage-specific extracellular matrix (ECM) protein, is important for cartilage-collagen fibril formation and for ECM organization. It participates in fibril formation with other cartilage-specific collagens (type II and IX collagens) and regulates the diameter of cartilage collagen fibrils. Polymorphisms in the *COL*11A1 gene have been shown to associate with lumbar disc herniation (LDH)[4]. Mutations in *COL*11A1 have also been shown to result in relatively mild chondrodysplasias associated with osteoarthritis (OA). Given that KBD has been found to have overlapping phenotypes and pathologic changes similar to those of OA and rheumatoid arthritis (RA)[5], we hypothesized that, as in OA and RA, genetic variants, in addition to environmental factors, may play an important role in the etiology and pathogenesis of KBD, and phenotypic expression of types I, II, III, and X collagen in chondrocytes cultured *in vitro* were significantly different between KBD and control cultures[6]. However, the role of *COL*11A in the pathology of KBD remains unknown. In this study, for the first time, we evaluate the impact of genomic polymorphisms in *COL*11A1 on the risk and progression of KBD.

KBD was diagnosed according to the national diagnostic criteria of China (WS/T 207-2010). Patients with clinical symptoms, radiographic changes, or other osteochondropathies were excluded. A healthy control was defined as no KBD and no primary or secondary OA. A total of 274 KBD patients and 249 age- (53.37 ± 10.79 years *vs.* 51.81 ± 17.85 years, t = 1.27, P > 0.05) and sex-matched (male/female, 125/149 *vs.* 124/125, χ^2 = 0.91, P > 0.05) controls were collected from KBD-endemic

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1. Department of Pediatrics, The First Affiliated Hospital of Medical Collage of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, China; 2. School of Public Health, Health Science Center, Xi'an Jiaotong University, Key Laboratory of Environment and Gene Related Diseases of Ministry of Education, Key Laboratory of Trace Elements and Endemic Diseases of Ministry of Health, Xi'an 710061, Shaanxi, China; 3. Department of Emergency, The First Affiliated Hospital of Medical Collage of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, China

areas of the Linyou and Yongshou Counties of Shaanxi Province. In addition, the 274 KBD patients were classified as clinical stages I, II, and III, which comprised 57.66% (158/274), 31.02% (85/274), and 11.32% (31/274) of the total, respectively. Fresh blood (5 mL) was collected from each subject.

Knee articular cartilage was collected from 22 KBD patients and 21 healthy controls. The KBD patients, consisting of 10 males and 12 females with an average age of 51.00 ± 8.30 (32-66) years, underwent knee debridement or arthroplasty at a hospital. The healthy control subjects, consisting of 11 males and 10 females with an average age of 48.23 ± 7.65 (33-61) years, had no history of osteochondropathy, but they had undergone amputation because of traffic accidents. No significant differences were observed between KBD and control group in age (t = 1.13, P > 0.05) and sex (χ^2 = 0.21, P > 0.05). None of the KBD patients or controls were diagnosed with bone or cartilage genetic diseases or RA.

The study was performed in accordance with the Declaration of Helsinki and approved by the Human Ethics Committee of Xi'an Jiaotong University, China. Written informed consent was also obtained from the subjects or their relatives.

Based on the available information on SNPs in *COL*11A1 that are considered predisposing factors in osteochondropathy[7-8], 22 SNPs were selected from the NCBI SNP and HapMap database and evaluated in this study. The selected SNPs were required to have a minor allele frequency (MAF) ≥ 5%. Genomic DNA was extracted from the peripheral blood of the 274 KBD patients and 249 healthy controls using a blood DNA extraction kit (TIANGEN, Beijing, China). Genotyping was performed using the Sequenom MassARRAY system. Primers were designed using Sequenom SNP Assay Design software version 3.0 for iPLEX reactions. The protocol and reaction conditions were as outlined by the manufacturer. Data management and analysis were conducted by Sequenom Typer 4.0 Software (http://bioinfo.iconcologia.net/SNPstats_web).

Following collection, the cartilage tissues were immediately fixed in 4% (w/v) paraformaldehyde, washed in phosphate-buffered saline (PBS), decalcified, embedded in paraffin, and cut into 5-8-μm-thick slices for immunohistochemistry and hematoxylin and eosin (HE) staining. Immunochemical identification was performed using the streptavidin-peroxidase (SP) method. Briefly, after deparaffinization, endogenous peroxidase was blocked with 3% H_2O_2 for 15 min, after which the slides were washed with PBS. The slides were then predigested using a digestive complex followed by rinsing with PBS. After blocking using 10% normal goat serum, the sections were incubated with a primary antibody recognizing COL11A at a 1:100 dilution (polyclonal rabbit anti-COL11A, Bioss Co, Beijing, China) or with PBS as a negative control, at 4 °C overnight. Next, the sections were incubated with 1:200 biotinylated goat anti-rabbit IgG (ZSGB-Bio Co, Beijing, China) at 37 °C for 20 min, followed by incubation with horseradish peroxidase-labeled streptavidin solution at 37 °C for 15 min. Color development was continued for 5 min at room temperature using diaminobenzidine followed by rinsing with distilled water. Counterstaining was performed with hematoxylin. The positive cells were counted at 40× magnification in six randomly selected fields; an average of six fields was observed for each tissue. A comparison of the positive rate of COL11A-stained cells between the two groups showed that the percentage of stained cells in the KBD group was 18.53% ± 8.41%, significantly lower than that of 26.58% ± 11.47% in the controls (t = 2.637, P < 0.05).

The Hardy-Weinberg equilibrium (HWE) of each SNP was tested by the goodness-of-fit χ^2 test to compare the expected and observed genotype frequencies in the control group; SNPs with P > 0.05 were considered to be in HWE. An unconditional logistic regression analysis model was used to evaluate the relationships between different genotypes and disease risk [odds ratios (*OR*), 95% confidence intervals (95% *CI*)] adjusted by age and gender[9]. To account for multiple testing, a Bonferroni correction was applied, and a significant association was defined at P < 0.002 (0.05/22)[9].

A case-control comparison of both genotype and allele frequencies for the 22 SNPs is presented in Table 1. All tested SNPs were in HWE (P = 0.16-1.00). When the allele frequencies were compared between the KBD patients and controls, a significant χ^2 value was detected for rs2229783 (P = 0.006). The distribution frequencies of rs2229783 genotypes was significantly different between the two groups (P = 0.0003). However, the significant association of allele frequency of rs2229783 did not persist following Bonferroni correction (P > 0.002).

Odds ratios and 95% *CI*s for KBD were calculated from an unconditional logistic regression model to evaluate relative risk (Table 2). Weakly increased KBD risks were observed among individuals with the

homozygote mutant genotype AA at rs1676486 (*OR* = 1.96, *P* = 0.03) and CC at rs12035138 (*OR* = 1.84, *P* = 0.04), compared with the homozygous wild type GG (rs1676486) and TT (rs12035138). The two SNPs also showed an elevated KBD risk in the recessive model (*OR* = 2.13, 95% *CI* = 1.18-3.84 and *OR* = 1.81, 95% *CI* = 1.04-3.12), but the significance did not remain after Bonferroni correction. However, rs2229783 was associated with a decreased risk of KBD in the dominant model (*OR* = 0.49, 95% *CI* = 0.34-0.71, *P* = 0.0001), which remained significant after Bonferroni correction for multiple testing. These results suggest that polymorphism in *COL*11A1 is associated with susceptibility to KBD: carriers of the mutant allele 'A' in rs2229783 have a decreased risk for KBD relative to those homozygous for the wild type allele 'G'.

To explore whether rs2229783 contributes to the severity of KBD, the effect of rs2229783 on the clinical stage of KBD was further observed in the KBD group. When the allele and genotype frequencies were compared according to clinical stage of KBD, no significant association was detected. Dominant and recessive models were also applied to rectify this, and the results showed a lack of association between polymorphisms of rs2229783 and the severity of KBD (Table 3).

KBD is an endemic OA with a specific geographical distribution. In the present study, we first investigated the associations between 22 SNPs in the *COL*11A1 gene and the corresponding risk of KBD in a Han Chinese population. A coding region single nucleotide polymorphism (cSNPs) rs2229783 (Ile1602Ile) showed a significant association with the risk of KBD. The results suggested that polymorphisms in the *COL*11A1 gene play an important role in the risk of KBD in the Han Chinese population. Subjects who carry allele 'A' in rs2229783 have a lower risk of KBD than those who do not. The polymorphic locus rs2229783, which is located in exon 62 of *COL*11A1, has been reported to be a susceptibility locus for LDH[4]. However, there are limited studies on the function of rs2229783 in osteoarthropathy. The effects of rs2229783 on clinical characteristics of KBD (clinical stages of KBD) were further observed in the KBD group in our study;

Table 1. Comparison of Genotype and Allele Frequencies between Cases and Controls

SNPs	Genotype (%)*			Minor Allele Frequency (%)		
	KBD (*N* = 274)	Controls (*N* = 248)	*P* Values	KBD	Controls	*P* Values
rs2615977	85.0/14.6/0.4	82.3/16.9/0.8	0.61	8.0	9.0	0.36
rs1415359	86.5/13.2/0.4	83.5/15.7/0.8	0.58	7.0	8.6	0.31
rs4908273	66.8/30.3/2.9	70.2/26.2/3.6	0.58	18.1	16.7	0.57
rs3753844	61.3/35.0/3.6	67.5/28.1/4.4	0.23	21.2	18.5	0.28
rs1676486	47.8/37.9/14.3	47.4/45.3/7.3	0.02	33.2	29.8	0.24
rs3753841	52.0/42.1/5.9	53.2/37.4/9.3	0.24	26.9	27.9	0.71
rs1463048	60.9/35.8/3.3	68.4/28.7/2.9	0.20	21.2	17.1	0.10
rs2229783	70.4/27.7/1.8	53.9/41.3/4.9	0.0003	16.3	26.1	0.006
rs1337185	83.9/15.7/0.4	81.8/17.4/0.8	0.63	8.2	9.5	0.47
rs3767275	44.5/45.6/9.9	45.3/46.4/8.1	0.79	31.1	33.1	0.57
rs1012282	84.6/15.1/0.4	81.4/17.8/0.8	0.56	7.9	9.6	0.31
rs11164634	61.2/35.2/3.7	67.1/28.1/4.8	0.20	21.2	18.8	0.33
rs1903787	83.8/15.8/0.4	81.4/17.8/0.8	0.67	8.3	9.7	0.43
rs2126642	74.5/23.4/2.2	69.2/29.6/1.2	0.22	13.8	15.9	0.35
rs945748	84.6/15.1/0.4	82.1/17.1/0.8	0.66	7.9	9.3	0.42
rs9659030	61.2/35.2/3.7	67.3/27.8/4.8	0.18	21.2	18.7	0.29
rs1676498	82.8/16.4/0.7	83.9/16.1/0.0	0.29	8.9	8.5	0.78
rs3767274	88.0/11.7/0.4	86.7/13.3/0.0	0.47	6.2	6.9	0.68
rs1463035	88.0/11.7/0.4	83.8/16.2/0.0	0.17	6.2	8.0	0.24
rs1463034	87.2/12.4/0.4	83.8/16.2/0.0	0.25	6.6	8.1	0.35
rs12035138	47.8/37.1/15.1	48.6/42.5/9.0	0.08	33.7	30.0	0.21
rs6691654	83.9/15.7/0.4	81.8/17.4/0.8	0.63	8.2	9.5	0.47

Note. *Homozygote of the major allele/ heterozygote /homozygote of the minor allele.

dominant and recessive models were also applied to rectify it. However, our data showed a lack of association between *COL*11A1 gene polymorphism and severity of KBD in the northwest Han Chinese population. These results suggest a role of *COL*11A1 in the susceptibility to but not in the severity of KBD. Polymorphisms of the *COL*11A1 gene may play an important role in determining the expression of *COL*11A and should be correlated with cartilage destruction via changes in the expression of *COL*11A related to KBD. In the present study, the expression of *COL*11A in articular cartilage of the knee was further analyzed by immunohistochemistry. Our results showed that the percentages of cells that stained positive for *COL*11A was lower in the KBD group than in the controls. *COL*11A is a quantitatively

Table 2. Analysis of Association of the 22 SNPs Gene Polymorphism with the Risk of KBD

SNPs	Genotype Model*			Dominant Model	Recessive Model	Allele Model**
	AA	AB	BB			
	OR (95% *CI*)	*OR* (95% *CI*)	*OR*	*OR* (95% *CI*)	*OR* (95% *CI*)	*OR* (95% *CI*)
rs2615977	0.45 (0.04-5.07)	0.75 (0.45-1.22)	1.00	0.82 (0.51-1.31)	0.45 (0.04-5.01)	0.74 (0.47-1.16)
rs1415359	0.46 (0.04-5.20)	0.81 (0.49-1.35)	1.00	0.80 (0.49-1.29)	0.47 (0.04-5.20)	0.79 (0.49-1.26)
rs4908273	0.92 (0.33-2.55)	1.24 (0.83-1.86)	1.00	1.16 (0.80-1.68)	0.79 (0.30-2.09)	1.13 (0.81-1.58)
rs3753844	0.95 (0.38-2.37)	1.40 (0.95-2.08)	1.00	1.30 (0.90-1.86)	0.81 (0.34-1.94)	1.21 (0.88-1.67)
rs1676486	**1.96 (1.07-3.59)**[a]	0.76 (0.51-1.12)	1.00	0.98 (0.69-1.39)	**2.13 (1.18-3.84)**[a]	1.17 (0.89-1.53)
rs3753841	0.74 (0.36-1.51)	1.07 (0.74-1.56)	1.00	1.05 (0.74-1.48)	0.60 (0.37-1.17)	0.95 (0.72-1.27)
rs1463048	1.22 (0.44-3.40)	1.40 (0.95-2.07)	1.00	1.39 (0.97-2.00)	1.17 (0.43-3.19)	1.28 (0.93-1.76)
rs2229783	0.48 (0.16-1.47)	0.71 (0.47-1.05)	1.00	**0.49 (0.34-0.71)**[b]	0.35 (0.12-1.02)	0.73 (0.52-1.00)
rs1337185	0.44 (0.03-5.57)	0.88 (0.54-1.41)	1.00	0.86 (0.55-1.36)	0.45 (0.04-4.99)	0.85 (0.55-1.32)
rs3767275	1.04 (0.54-2.02)	0.99 (0.68-1.42)	1.00	1.03 (0.73-1.46)	1.24 (0.67-2.27)	1.00 (0.77-1.32)
rs1012282	0.45 (0.04-5.08)	0.74 (0.46-1.20)	1.00	0.80 (0.51-1.27)	0.47 (0.04/5.80)	0.74 (0.47-1.15)
rs11164634	0.93 (0.38-2.29)	1.41 (0.95-2.09)	1.00	1.29 (0.90-1.85)	0.74 (0.31-1.74)	1.21 (0.88-1.67)
rs1903787	0.45 (0.04-5.09)	0.77 (0.48-1.25)	1.00	0.85 (0.54-1.34)	0.47 (0.04-5.18)	0.76 (0.49-1.19)
rs2126642	1.47 (0.36-6.03)	0.69 (0.46-1.04)	1.00	0.78 (0.53-1.15)	1.85 (0.46-7.48)	0.79 (0.56-1.14)
rs945748	0.45 (0.04-5.11)	0.76 (0.47-1.25)	1.00	0.84 (0.53-1.34)	0.45 (0.04-5.01)	0.76 (0.48-1.19)
rs9659030	0.89 (0.36-2.17)	1.44 (0.97-2.13)	1.00	1.31 (0.91-1.87)	0.74 (0.31-1.74)	1.21 (0.88-1.67)
rs1676498	/	0.99 (0.61-1.63)	1.00	1.08 (0.68-1.72)	/	1.07 (0.68-1.69)
rs3767274	/	0.81 (0.47-1.38)	1.00	0.89 (0.53-1.49)	/	0.87 (0.52-1.45)
rs1463035	/	0.62 (0.37-1.05)	1.00	0.71 (0.43-1.17)	/	0.69 (0.42-1.12)
rs1463034	/	0.66 (0.39-1.10)	1.00	0.76 (0.47-1.25)	/	0.72 (0.44-1.17)
rs12035138	**1.84 (1.01-3.36)**[a]	0.79 (0.54-1.16)	1.00	1.03 (0.73-1.45)	**1.81 (1.04-3.12)**[a]	1.16 (0.88-1.52)
rs6691654	0.44 (0.03-5.57)	0.88 (0.54-1.410	1.00	0.85 (0.54-1.34)	0.41 (0.04-4.56)	0.85 (0.55-1.32)

Note. *AB stands for the minor/major alleles, BB as the reference genotype; **Major allele as the reference allele; [a]The significance did not remain after correction for multiple testing; [b]The significance remained after correction for multiple testing.

Table 3. Comparison of Genotype and Allele Frequencies of rs2229783 in *COL*11A1 among Subjects of Different Clinical Stages of KBD

KBD Stages	Alleles (%)		Genotypes (%)			Recessive Model (%)		Dominant Model (%)	
	A	G	AA	AG	GG	AA + AG	GG	AA	AG + GG
I (*n* = 158)	15.19	84.81	1.27	27.85	70.88	29.11	70.89	1.27	98.73
II (*n* = 85)	15.29	84.71	3.53	23.53	72.94	27.06	72.94	3.53	96.47
III (*n* = 31)	19.35	80.65	0.00	38.71	61.29	38.71	61.29	0.00	100.00
χ^2 values	0.70			4.53		1.52		2.23	
P values	0.71			0.34		0.47		0.33	

minor component of cartilage collagen fibrils, but it is essential for the interaction between proteoglycan (PG) aggregates and collagens, and the highly oriented network of the fibrillar collagens offers tensile strength[10]. However, the expression of *COL*11A was lower in knee articular cartilage of KBD, and the reduction in *COL*11A, the critical organizer of ECM, ultimately causes disintegration of the ECM and joint degeneration related to KBD. Therefore, we believe that the A allele of rs2229783 may induce a relative increase in *COL*11A expression compared with the G allele, and this increase may be associated with a reduced risk of KBD; further studies should be carried out to explore it.

In conclusion, the rs2229783 polymorphism of the *COL*11A1 gene is significantly associated with the risk of KBD but not the severity of KBD in the northwest Han Chinese population, and the expression level of COL11A in cartilage was significantly lower in the KBD group, the A allele of rs2229783 may induce a relative increase in *COL*11A expression and be implicated as a protective factor against KBD. Our results should lead to a better understanding of the pathogenic mechanism of KBD. In addition, due to the relatively small number of subjects, our findings are considered preliminary and need to be validated in further studies using larger sample sizes of these subpopulations.

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[#]Correspondence should be addressed to Prof GUO Xiong, Tel: 86-29-82655091, E-mail: guox@mail.xjtu.edu.cn

Biographical note of the first author: SHI Xiao Wei, female, born in 1971, PhD, majoring in molecular mechanisms of cartilage and bone disease.

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