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Identification of sequence polymorphisms in two sulfation-related genes, *PAPSS2* and *SLC26A2*, and an association analysis with knee osteoarthritis

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Abstract Osteoarthritis (OA) is one of the most common musculoskeletal disorders and is characterized by degeneration of articular cartilage. Sulfation of extracellular matrix proteins in articular cartilage is an important step in maintaining normal cartilage metabolism. Two sulfation-related genes have been reported as the causal genes of severe chondrodysplasias: mutations in *PAPSS2* (3'-phosphoadenosine 5'-phosphosulfate synthase 2) cause spondylo-epimetaphyseal dysplasia (SEMD), and mutations in *SLC26A2* (solute carrier family 26, member 2) cause diastrophic dysplasia. Given their critical roles in cartilage metabolism and the severe phenotypes that result from mutations in these genes, we examined *PAPSS2* and *SLC26A2* as candidate susceptibility loci for OA. We identified sequence polymorphisms in the coding and core promoter regions of these genes and analyzed their potential association with knee OA within the Japanese population. Ten sequence polymorphisms were detected in *PAPSS2* and five in *SLC26A2*. An association analysis showed suggestive association of one minor polymorphism in the promoter region of *SLC26A2*. This 4-bp adenine deletion allele, del4A, was over-represented in knee OA ($P = 0.043$, odds ratio = 3.43) and is thought to confer a minor susceptibility to knee OA within the Japanese population. Haplotype analysis showed no evidence of association with the

two genes, however, excluding them as major susceptibility loci for knee OA.

Key words Association analysis · Osteoarthritis · Polymorphisms · Sulfation · Haplotype analysis · *PAPSS2* (3'-phosphoadenosine 5'-phosphosulfate synthase 2) · *SLC26A2* (solute carrier family 26, member 2)

Introduction

Osteoarthritis (OA) is one of the most common musculoskeletal disorders and is characterized by degeneration of articular cartilage. The most common type of OA, idiopathic OA, usually occurs in the fifth or sixth decades of life, accompanied by pain and functional impairment. Idiopathic OA is a common, polygenic disease caused by the interaction of environmental and genetic factors. Although epidemiological studies have shown that OA is not an inevitable event of aging but has a strong genetic component (Stecher 1941; Kellgren et al. 1963; Spector et al. 1996; Hirsh et al. 1998; Felson et al. 1998), its nature is poorly understood. A number of genes can be considered as susceptibility loci for OA, given their postulated roles in established cartilage metabolism pathways; only a few of these have been previously examined, however (Hull and Pope 1989; Meulenbelt et al. 1997, 1998; Horton et al. 1998; Ushiyama et al. 1998).

Sulfation of extracellular matrix proteins in articular cartilage is an important step in maintaining normal cartilage metabolism. Two sulfation-related genes have been reported as the causal genes of severe chondrodysplasias in human and mouse. Mutations in *PAPSS2* (3'-phosphoadenosine 5'-phosphosulfate synthase 2) cause spondylo-epimetaphyseal dysplasia (SEMD) (ul Haque et al. 1998). Mutations in *SLC26A2* (solute carrier family 26, member 2) are responsible for diastrophic dysplasia (DTD) (Hastbacka et al. 1994), and an *Slc26a2* mutation has been found in the brachymorphic mouse (Sugahara and Schwartz 1979). These chondrodysplasias usually show early degen-

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erative changes in articular cartilage in multiple joints and thus can be considered monogenic models of idiopathic OA. PAPSS2 protein possesses both ATP sulfurylase and adenosine 5'-phosphosulfate (APS) kinase activities and generates 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the universal sulfate donor used in all known posttranslational sulfation reactions (Geller et al. 1987; Xu et al. 2000). SLC26A2 is a transmembrane glycoprotein with sulfate transporter activity (Hastbacka et al. 1994). In articular cartilage, proteoglycans such as aggrecan are main targets for sulfation. Aggrecan is the most abundant non-collagenous component of articular cartilage. Due to the high concentration of negative charge resulting from sulfation of its side chains, aggrecan provides articular cartilage with compressive properties and resiliency. Several studies have revealed changes in the sulfation status of proteoglycans in joints affected by conditions such as OA (Plaas et al. 1998; Bayliss et al. 2000).

Together, these findings implicate *PAPSS2* and *SLC26A2* as candidate susceptibility loci for OA. To examine this possibility, we identified sequence variations in these two genes and examined the potential association of these genes with knee OA in the Japanese population.

Subjects and methods

Sample collection

One hundred and sixty-five knee OA patients and 127 controls were selected from the population participating in the Genetic Study Program of Bone and Joint Disease at Tokyo University Hospital and related institutions. The control population consisted of volunteers who had undergone treatment for injuries or other orthopedic diseases. To minimize population admixture, all individuals included in this study were Japanese, living in or around Tokyo. The study protocol was approved by the ethical committees of the participating institutions, and written informed consent was obtained from each participant. Blood samples were obtained from all participants, and genomic DNAs were prepared from peripheral leukocytes according to standard protocols.

Assessment of OA

Knee OA was assessed by physical examination and confirmed by radiographs. For each patient, standard three-direction knee radiographs (anteroposterior, lateral, and skyline views) were taken and assessed by a single expert observer (AF). To assess OA, we used a new radiographic scale (Table 1), which is a modification of the Kellgren scale (Kellgren and Lawrence 1957). The Kellgren scale has been widely used in previous genetic studies; however, it is not suitable for the genetic association study. Originally invented for population-based epidemiological studies, the Kellgren scale estimates osteophytes and joint-space narrowing compositely and puts emphasis on the detection of early changes in OA. The true pathogenesis of OA resides in degeneration and subsequent loss of articular cartilage, however; osteophytes only reflect the reactive change. The dominance of osteophytes in diagnosing radiographic OA using the Kellgren scale has been pointed out (Hart and Spector 1995; Uitterlinden et al. 1997). In contrast, our new three-part scale emphasizes joint-space narrowing and estimates the severity of osteophytes independently. The primary scale is based on estimations of the severity of joint-space narrowing, and osteophyte severity and the involvement of the patellofemoral joint are estimated independently to use as secondary scales. This simple, clear-cut system allows us to evaluate the pathologic status of the OA joint more directly, and is useful for classifying the more severe cases. A population rich in severe phenotypes might raise the statistical power of a case-control association study, because the population could have stronger genetic backgrounds (Lander and Schork 1994). Therefore, we believe that our new radiographic scale is more useful for case-control association studies.

A diagnosis of OA was confirmed by detection of joint-space narrowing of grade 3 or more by this scale. Therefore, only symptomatic and definite radiographic OA was included in the study. We excluded rheumatoid arthritis and other polyarthritis caused by autoimmune diseases, as well as post-traumatic OA and infection-induced OA. We also excluded chondrodysplasias on the basis of the presence of short stature, positive family history, and involvement of multiple joints.

Table 1. A new radiographic scale for the evaluation of osteoarthritis

Main scale		Additional scales			
Grade	Joint-space narrowing	Grade	Osteophytes	Grade	Patello-femoral joint
1	Minimal	1	None	1	No evidence of OA
2	Definite, but more than half of the normal joint remains	2	Minimal	2	Osteophytes or joint-space narrowing
3	Less than half of the normal joint remains	3	Definite but small		
4	Diminished	4	Massive		
5	Loss of bone stock around the joint				

Identification of polymorphisms in *PAPSS2* and *SLC26A2*

A total of 16 knee OA patients were initially screened for nucleotide sequence variations in *PAPSS2* and *SLC26A2*. The exon–intron junction and the transcription start site of *PAPSS2* had been previously determined (Xu et al. 2000), and the reference sequence was obtained from the GenBank database (AF160505-9). All 12 exons and their flanking regions, as well as a 1-kb sequence upstream of the transcription start site, were analyzed by direct sequencing of polymerase chain reaction (PCR) products from genomic DNA. Because the precise genomic structure of *SLC26A2* had not been previously determined, exon–intron junctions were predicted by comparing the mRNA sequence (U14528) and the genomic sequence (AC008427) in the public database. We analyzed the two predicted exons of *SLC26A2* and their flanking regions, as well as a 1-kb sequence upstream of the transcription start site, by direct sequencing. The primer sets used for PCR (Table 2) were designed using the Primer3 program ([http://www-genome.wi.mit.edu/genome_software/other/primer3.html](http://www.genome.wi.mit.edu/genome_software/other/primer3.html)). Amplification was carried out as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Direct sequencing on both strands was performed using a BigDye Terminator cycle sequencing kit (ABI, Foster City, CA, USA) and analyzed on an ABI Prism 3700 DNA analyzer.

Genotyping

Single-nucleotide polymorphisms (SNPs) were genotyped using the TaqMan assay (ABI) in 96-well plates after optimization for each primer set (Afonina et al. 1997; Livak 1999). The reaction was run on an ABI GeneAmp PCR system 9700 (50°C for 2 min, 95°C for 10 min, and 95°C for 15 s; and 62°C for 1 min, for 40 cycles) and read on an ABI

Prism 7700 sequence detector. Complex insertion–deletion polymorphisms that could not be genotyped by the TaqMan assay were genotyped by direct sequencing.

Statistical analysis

For each identified SNP, a χ -squared test was performed between knee OA and the control groups for both genotypic and allelic frequencies. Statistical significance was inferred as $P < 0.05$ without Bonferonni's correction for multiple testing. The correction was not applied because when the SNP loci are in strong linkage disequilibrium with one another, the correction tends to be too conservative, yielding false negative results. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated with respect to the minor versus major allele. Maximum-likelihood haplotype frequencies for each gene were computed using an expectation-maximization (EM) algorithm (Excoffier and Slatkin 1995) on both the knee OA and control groups, and the population difference was calculated using a χ -squared test. Computations were performed using Arlequin software (<http://lgb.unige.ch/arlequin/>).

Results

Characteristics of sample populations

The sample population for this study is described in Table 3. Although the mean age of the control group was approximately four years younger than that of the knee OA group, the age of onset of the knee OA group was more than six years earlier than the mean age of the control group. Therefore, this group is considered appropriate as a non-OA control. Forty-two percent of OA patients were associated with Heberden's nodes and 46% had at least one first-

Table 2. Method for polymerase chain reaction amplification of *PAPSS2* and *SLC26A2*

	Primer (5'→3')		Product size (bp)	Annealing temperature (°C)
Target	Sense	Antisense		
<i>PAPSS2</i>				
Promoter and exon 1	CACCATGTGTGATGATGCTG	CACAGAGCCTCTTTCTCTTGC	988	55
Exon 2	TGGAAGCAAAACTGCTTATGAA	ACATCTCCCAGCCTCCTTCT	536	60
Exon 3	TATGAAGCCTGCTTGCTTTG	CTTCCCCTAGCAGTTGGAGA	544	55
Exon 4	TTTCTGGACTTTCTCTCAAGCA	GAACGTTATTGCATATGGCTGA	509	55
Exons 5–6	CAAGGATGGCTGTTTGACCT	ATTGATCAGCTGCCTTGTGG	566	55
Exon 7	AGCATAACAGGTGGGGACAC	TGGAGCACACTGTAAATGATCC	396	55
Exon 8	TGGATTTGGGTCTTAATGCTTC	AACATCTCCTGAGGCAAAGC	490	60
Exons 9–10	CCCGAGATTGGTCTAAAAGG	GGAAACAGAAAGGATCCCAGA	2500	50
Exon 11	CATGAAGAGTGCACGGAGTC	ACTCAGTCCACTTGGGAGCA	513	60
Exon 12	TGGGTCTCAAAAACCATAACCT	ACATCGCCACATTGTTTGAC	1100	55
<i>SLC26A2</i>				
Promoter	TGGCTATTTTTACCTCCTTTGTTT	AGTTCCAGATGGATCCCAGA	1085	55
Exon 1	TCCAAAACCTGAATTCCTTTAACTCT	TTGAATTTTGCCTCTACCCTTC	982	60
Exon 2	AGCTCTGACATTCTGTGATGCT	GACTCTTCCACCAAGCCAAG	1101	55
Exon 2	GCCTACTTGTGGGGTTTGT	TGCCTTAAGGACAGGAATGG	1205	55

PAPSS2, 3'-phosphoadenosine 5'-phosphosulfate synthase 2; *SLC26A2*, solute carrier family 26, member 2

Table 3. Characteristics of the study population

Population (Number of individuals)	Sex		Age of onset	Family history		Heberden's nodes		Radiographic grade	
	Male/Female	Age		+/-	+/-	+/-	+/-	JSN	Osteophytes
Knee OA (165)	20/145	72.1 ± 7.0	62.0 ± 10.1	76/89	69/96	3.5 ± 0.6	3.2 ± 0.7	—	—
Control (127)	33/94	68.4 ± 8.0	—	—	—	—	—	—	—

Age and age of onset are shown by mean ± SD (years)

JSN, joint-space narrowing

Table 4. Sequence polymorphisms in *PAPSS2* and *SLC26A2*

Location	GenBank accession no.	Position in genomic sequence	Nucleotide change	Amino acid change	Frequencies in screening
<i>PAPSS2</i>					
Promoter	AF160503	98	-656A > C	—	11/32
Promoter	AF160503	233	-521delG	—	1/32
Intron 4	AF160505	1494	IVS4 + 133C > T	—	14/32
Intron 7	AF160505	3039	IVS7 + 17G > A	—	4/32
Intron 7	AF160505	3063	IVS7 + 41ins A	—	4/32
Intron 9	AF160507	608	IVS9 + 179C > T	—	14/32
Intron 9	AF160507	760	IVS9 + 331A > G	—	2/32
Exon 12	AF160509	1491-1499	3'UTR + 139-148 del or ins A or T	—	20/32
Exon 12	AF160509	1790	3'UTR + 438insCA	—	4/32
Exon 12	AF160509	1939	3'UTR + 587C > T	—	12/32
<i>SLC26A2</i>					
Promoter	AC0088427	10282	-716 to -713del4A	—	1/32
Promoter	AC0088427	9795	-226G > A	—	2/32
Exon 2	AC0088427	4777	c.2046G > A	L682L	1/32
Exon 2	AC0088427	4758	c.2065A > T	T688S	3/32
Exon 2	AC0088427	4433	+170C > T	—	1/32

Positions in promoter sequences are shown for transcriptional start site = 1

UTR, untranslated region

degree relative who also had knee OA. The mean radiographic grade estimated using joint-space narrowing was 3.5, indicating that the group had relatively severe OA.

Sequence polymorphisms in *PAPSS2* and *SLC26A2*

A total of 15 polymorphisms were detected in *PAPSS2* and *SLC26A2* by sequencing analysis (Table 4). We identified 10 polymorphisms in *PAPSS2*: 7 SNPs, 2 simple insertions or deletions, and 1 complex insertion-deletion. Two of the polymorphisms resided in the promoter region, five were in introns, and three were found in the 3'-untranslated region (UTR). We identified no cSNPs (coding-region SNPs) in *PAPSS2*. In *SLC26A2*, we identified five polymorphisms: four SNPs and one 4-bp deletion. Two of the polymorphisms resided in the upstream region of the translation start site, two were in the coding region (cSNPs), and one was located in the 3'-UTR. One of the cSNPs identified was nonsynonymous (T689S). The mean interval between the neighboring SNPs was 9.7kb in *PAPSS2* and 1.5kb in *SLC26A2*.

Association analysis

Sequence polymorphisms used in the association analysis were selected by their genomic localization and allelic fre-

quencies determined by the screening of polymorphisms (Table 4). We genotyped five SNPs and a complex insertion-deletion polymorphism in the 3'-UTR of *PAPSS2*; for *SLC26A2*, we genotyped two promoter polymorphisms and a nonsynonymous cSNP (Table 5). A promoter polymorphism in *SLC26A2* showed significant association with OA. This 4-bp adenine deletion allele, del4A, was overrepresented in the OA group ($P = 0.043$, odds ratio = 3.43). Other biallelic polymorphisms showed no significant difference between the OA and control groups. We identified seven different alleles of the complex insertion-deletion polymorphism in the 3'-UTR of *PAPSS2* (delA, delT, insA and delT, delAT, insAT, and insATA), but we found no differences in this polymorphism between the knee OA and control groups. All polymorphisms were in Hardy-Weinberg equilibrium.

Haplotype analysis

Haplotype 211 in *SLC26A2*, representing the combination of the del4A allele and the major alleles of two other loci, was significantly overrepresented in the OA population (Table 6). Because 212 and 221 haplotypes were not observed, however, the 211 haplotype was thought to simply reflect the del4A allele itself. No *PAPSS2* haplotypes were associated with OA.

Table 5. Association analysis of *PAPSS2* and *SLC26A2* (bi-allelic polymorphisms)

Gene	Location	Nucleotide change	Genotype (11/12/22)		Allelic frequencies		χ -squared test	Odds ratio
			OA	Control	OA	Control	<i>P</i> value	(95% CI)
<i>PAPSS2</i>	Promoter	−656A > C	56/83/26	47/59/21	0.41	0.40	0.78	1.05 (0.75–1.46)
<i>PAPSS2</i>	Intron 4	IVS4 + 133C > T	61/83/21	47/57/23	0.38	0.41	0.51	0.89 (0.64–1.25)
<i>PAPSS2</i>	Intron 7	IVS7 + 17G > A	129/34/2	100/25/2	0.12	0.11	0.97	1.01 (0.60–1.25)
<i>PAPSS2</i>	Intron 9	IVS9 + 179C > T	41/82/42	42/60/25	0.50	0.43	0.093	1.33 (0.95–1.84)
<i>PAPSS2</i>	Exon 12	3′-UTR + 587C > T	68/79/17	46/64/17	0.34	0.39	0.28	0.83 (0.59–1.16)
<i>SLC26A2</i>	Promoter	−716 to −713 del 4A	152/13/0	124/3/0	0.039	0.012	0.043	3.43 (0.97–12.17)
<i>SLC26A2</i>	Promoter	−226G > A	142/22/1	107/20/0	0.073	0.079	0.78	0.92 (0.49–1.70)
<i>SLC26A2</i>	Exon 2	c.2065A > T	141/23/1	107/20/0	0.076	0.079	0.89	0.96 (0.52–1.77)

“11” and “22” under Genotype denote homozygotes of the major and minor alleles, respectively. “12” denotes heterozygotes
CI, confidence interval

Table 6. Haplotype analysis of *PAPSS2* and *SLC26A2*

Haplotype	Haplotype frequencies		χ -squared test <i>P</i> value	Odds ratio (95% CI)
	KOA <i>N</i> = 165	Control <i>N</i> = 127		
<i>PAPSS2</i>				
111121	0.040	0.049	0.64	0.83 (0.37–1.84)
111131	0.032	0.040	0.70	0.84 (0.35–2.01)
111221	0.117	0.099	0.45	1.23 (0.72–2.09)
111231	0.091	0.098	0.76	0.92 (0.52–1.60)
222231	0.048	0.040	0.60	1.24 (0.55–2.79)
121112	0.185	0.219	0.29	0.8 (0.53–1.20)
211221	0.110	0.080	0.22	1.43 (0.81–2.54)
211231	0.049	0.061	0.45	0.76 (0.37–1.55)
212231	0.040	0.027	0.44	1.45 (0.57–3.68)
221112	0.103	0.131	0.31	0.77 (0.46–1.28)
Others	0.183	0.155	—	—
<i>SLC26A2</i>				
111	0.88	0.91	0.34	0.77 (0.44–1.32)
122	0.073	0.079	0.78	0.92 (0.49–1.70)
211	0.039	0.012	0.043	3.43 (0.97–12.17)

All haplotypes that showed allelic frequencies greater than 3% in either population are shown

“1” denotes the major allele and “2” the minor allele

Each locus is shown from left to right, −656A > C, IVS4 + 133C > T, IVS7 + 17G > A, IVS9 + 179C > T, 3′UTR + 139–148 del or ins A or T, 3′UTR + 587C > T in *PAPSS2*, and −716 to −713del4A, −226G > A, T688S in *SLC26A2*

In the 5th locus of *PAPSS2*, 3′UTR del-ins A or T, “1” denotes the wild-type allele, “2” the delA allele, and “3” the delT allele

Discussion

In this study, we identified 15 DNA polymorphisms in two sulfation-related genes, *PAPSS2* and *SLC26A2*. Our study shows that a minor polymorphism in the promoter region of *SLC26A2*, del4A, was significantly overrepresented in patients with knee OA compared with the control. Because this polymorphism lies within the upstream region of *SLC26A2* and has considerable sequence changes, it seems likely that *SLC26A2* is expressed differently between the two alleles.

The del4A allele has only a minor contributing effect on knee OA, however. Its frequency is only 4%, even in the population with the disease, reducing the statistical power to detect the population difference at this locus. The *P* value

from a two-sided exact test showed only marginal significance, and the 95% CI was too wide to confirm the association. Therefore, it is more appropriate to state that this risk allele showed only suggestive association. In addition, we found no differences in clinical features between the knee OA patients with/without the del4A allele, including age of onset, family history, Heberden’s nodes, and radiographic grade. Further association analyses in larger populations or functional analyses will be needed to determine the true significance of the polymorphism.

We found no population difference between knee OA and the control group in our haplotype analysis, so we have excluded *PAPSS2* and *SLC26A2* as major susceptibility loci for knee OA in the Japanese population. Haplotype analysis is a powerful tool for detecting small genetic differences between two populations and predicting unknown sequence variants associated with common polygenic diseases (Jeunemaitre et al. 1997; Chakravarti 1999). Although we did not examine relatively rare polymorphisms, the negative results of our haplotype analysis justify this strategy.

Sulfate conjugation is an important pathway in the biotransformation of a number of exogenous and endogenous compounds. Many hormones, neurotransmitters, carbohydrates, and drugs are included in this pathway (Leyh 1993). *PAPSS2* and *SLC26A2* are expressed ubiquitously in systemic organs (Hastbacka et al. 1994; Xu et al. 2000). Polymorphisms detected in this study may affect other systemic functions such as drug sensitivity. As such, they will be useful markers in future association studies for other common diseases.

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