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Large-scale gene expression profiles, differentially represented in osteoarthritic synovium of the knee joint using cDNA microarray technology

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Abstract

Osteoarthritis (OA) is one of the most common age-related chronic disorders of articular cartilage, joints and bone tissue. Diagnosis of OA commonly depends on clinical and radiographic findings. However, changes in cartilage associated with the early stage of OA cannot be detected using radiographs, because significant cartilage degeneration must occur before radiographic findings show alterations of the appearance of cartilage. To identify new biomarkers of OA, we analysed gene expression profiles of synovium from 43 patients with OA, ten patients with rheumatoid arthritis (RA), and eight non-OA/non-RA patients using a novel cDNA microarray chip. We identified 21 genes with simultaneous significant differences in expression between OA and non-OA/non-RA groups and between OA and RA groups. Linear discriminant analysis showed that the three groups could be well separated using those 21 genes. Statistical analysis also revealed that several of the 21 genes were associated with disease progression and clinical presentation. The graphical modelling method indicated that some of the 21 genes are significantly associated with a particular clinical presentation, suggesting biological relationships among those genes. This is the first report of the use of cDNA microarray technology to create large-scale gene expression profiles differentially expressed *in situ* in OA synovium of the knee joint.

Keywords: *Biomarker, osteoarthritis, knee joint, gene, synovium, microarray*

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Introduction

Osteoarthritis (OA) is a progressive, debilitating disease of the joints characterised by degradation of articular cartilage with associated changes in ligaments, synovium and bone. In a previous epidemiological study of residents of the mountain village

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Miyagawa, in Mie prefecture, Japan, we found that the prevalence of OA increases with age, and that about 30% of the village residents older than 65 years have OA of the knee joint (unpublished communication). Because OA of the knee joint sometimes leads to substantial disability, it can have severe socioeconomic effects.

Diagnosis of OA commonly depends on clinical and radiographic findings. However, changes in cartilage associated with the early stage of OA cannot be detected using radiographs, because significant cartilage degeneration must have already occurred before radiographic findings show alterations of the appearance of cartilage. Therefore, a diagnostic molecule that can be used to non-invasively and quantitatively assess patient status, particularly at the early stages of OA, would greatly contribute to developing an effective therapeutic strategy.

Because OA is mainly considered a degenerative disease of cartilage, much attention has been focused on developing assays for cartilage-derived molecules that are released into the circulation from the joint and reflect disturbances of cartilage turnover (Vilim et al. 2001, Reijman et al. 2004, Sharif et al. 2004, Garnero et al. 2003). However, such markers have not previously been reliably identified. Recent findings suggest that several interconnected events that occur in the OA synovium play important roles in initiation and progression of degeneration of the articular cartilage (Hogan et al. 1994, Smith et al. 1997, Parsonage et al. 2003, Scharstuhl et al. 2003, Blom et al. 2004, Kriegsmann et al. 2004, Scaife et al. 2004, van Lent et al. 2004). The exact mechanisms of synovial interconnection in OA pathogenesis are unclear. However, evidence suggests that synovial cells can be activated (e.g. via Toll-like receptors) by matrix debris that leaks from the cartilage due to unsupportable loading on the joint or enhanced enzyme activity (Huhtala et al. 1995, Amin & Abramson 1998). In addition, this debris can induce expression of matrix-degrading enzymes such as matrix metalloproteinase (MMP) by various cell populations (Dang et al. 2003). Despite this evidence that molecules in joint fluid, mainly secreted from synovium, contribute to progression of OA, there has been little effort to identify biomarkers secreted into peripheral circulation from synovium (Saxne & Heinegard 1992, Lohmander et al. 1993, Johansen et al. 1996, Laurent et al. 1996, Naito et al. 1999, Chevalier et al. 2001, Pavelka et al. 2004, Elliott et al. 2005). In the present study, we conducted a series of experiments using OA synovium to identify biomarkers useful for diagnosis and monitoring of disease progression of OA.

Recent advances in molecular biology and biotechnology have led to the development of nucleic acid-based medicines that directly target arthritis-related genetic alterations or molecular mechanisms. Several methods for examining coordinated gene expression have been developed, including differential subtraction method (Seki et al. 1998, Justen et al. 2000), ribonuclease protection assay (RPA) (Ruger et al. 2004), differential display and sequencing of cDNA libraries (Scaife et al. 2004), and expressed sequence tags (ESTs) (Kumar et al. 2001). Microarray technology was developed to analyse the expression of thousands of genes in a very short time (Aigner et al. 2003, Tardif et al. 2004), and it has become a powerful tool for analysing genetic expression on a large scale, to obtain novel information about etiology (Aigner et al. 2001, Neaumann et al. 2002, Thornton et al. 2002, Zhang et al. 2002, Aigner et al. 2003, Ochi et al. 2003, van der Pouw Kraan et al. 2003, Rihl et al. 2004, Tardif et al. 2004).

The present report is unique in that it presents the results of the first systematic large-scale gene expression profile of *in situ* OA synovium, which were created in order to find candidate biomarkers differentially expressed in OA synovium. In addition, molecules selectively expressed in OA synovium may become novel molecular targets for control of the progression of OA. The goal of this study was to identify genes that are differentially expressed in OA synovium *in situ*, and to analyse gene expression patterns of synovium in patients at various stages and clinical presentation, in order to identify molecules that can serve as targets for diagnostic and therapeutic purposes.

Materials and methods

Patient characteristics

The present study was conducted after obtaining approval of the Ethics Committee of both Mie University and Shionogi & Co., Ltd, and all patients gave informed consent preoperatively. Synovial tissue of the knee was collected from the following patients: 43 patients with OA (aged 28–85 years, mean 69.6 years); ten patients with rheumatoid arthritis (RA) (aged 54–75 years, mean 66.0 years); and eight patients without OA or RA (non-OA/non-RA) (aged 19–35 years, mean 25.9 years). A medical history and physical examination was performed on all patients by one author (HK). Knee OA was diagnosed based on clinical symptoms and radiological examination according to the American College of Rheumatology criteria for knee OA (Altman et al. 1986). RA was diagnosed according to revised criteria of the American College of Rheumatology (Arnett et al. 1988). Anterior–posterior radiographs of the knee joint were obtained at a supine position under standardised conditions. Severity of knee OA was categorised according to the Kellgren–Lawrence radiographic rating system (Menkes 1991). A summary of the clinical characteristics of the patients, including osteophytes, synovitis, hydrarthrosis and crystal deposition, are listed in Table I. The presence of osteophyte and crystal deposition was evaluated by radiographs. Synovitis and hydrarthrosis were assessed by intraoperative findings. The eight non-OA/non-RA patients comprised six patients with meniscal injury, one with an anterior cruciate ligament (ACL) injury and one with a femoral medial chondyle fracture. At the time of surgery, no patients were on the medications acetaminophen, non-steroidal anti-inflamatory drugs, or selective cyclooxygenase 2 inhibitors. None had received intraarticular steroid injections within 3 months prior to surgery.

Preparation of synovium

Synovial tissue was obtained during arthroscopy (six meniscal injury, one ACL injury and early and moderate OA), knee replacement surgery (severe OA and RA) and open reduction and internal fixation (one femoral medial chondyle fracture). The time span between knee injury and the collection of the tissue sample was 2–5 days (mean 3.3 days) in the patients with meniscal injury, 7 days in the patient with ACL injury, and 1 day in the patient with the femoral fracture. Synovial tissues were separated from associated connective tissue or fat. Only tissue samples with a synovial lining layer were used in this study. The synovial samples were frozen in liquid nitrogen immediately after removal and were stored at -80°C. The frozen tissues were crushed with a Cryo-Press CP-100W (Microtec Nition,

Table I. Summary of demographic data.

Characteristics	OA	RA	Non-OA/non-RA
Total number	43	10	8
Gender			
Male	3	1	5
Female	40	9	3
Age (years) (mean)	28–85 (69.6)	54–75 (66.0)	19–35 (25.9)
Grade			
I	5		
II	4		
III	7		
IV	27		
Clinical record			
Osteophyte			
Yes	36	4	0
No	7	6	8
Synovitis			
Yes	14	8	1
No	29	2	7
Hydrarthrosis			
Yes	18	4	1
No	25	6	7
Crystal deposit			
Yes	5	0	0
No	38	10	8

OA, osteoarthritis; RA, rheumatoid arthritis.

Chiba, Japan) and suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with the homogeniser Polytron2100 (Kinematica, Bethlehem, PA, USA), followed by preparation of total RNA as described in the manufacturer's protocol.

Generation of cDNA microarray

Messenger RNA was prepared from human mesenchymal stem cells using a QuickPrep Micro mRNA Purification Kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's instructions. cDNA was synthesised from the mRNA template using SuperScript Choice System for cDNA Synthesis (Invitrogen) according to the manufacturer's protocol, and was cloned into plasmid pMX (donated by Dr T. Kitamura). Plasmid DNA was prepared from each clone, and the cDNA inserts were sequenced using a MegaBACE 1000 DNA sequencer (Amersham Biosciences). We selected 3265 cDNA clones whose sequences appeared to be independent from each other, and amplified them by polymerase chain reaction. The Lucidea Universal ScoreCard (GE Healthcare Bio-Sciences) was used as control DNA for normalisation of both intra- and inter-microarray data. The DNA fragments were spotted onto Type 7-star glass slides (GE Healthcare Bio-Sciences) using a Microarray System Generation III Spotter (GE Healthcare Bio-Sciences), such that each cDNA fragment was spotted at the same position on both the right and left sides of each glass slide, in order to perform duplicate experiments.

Microarray hybridization

We amplified 2.5 μg of total RNA from each specimen using a MessageAmp aRNA Kit (Ambion, Austin, Texas, USA) according to manufacturer's instruction. The amplified RNA was mixed with Lucidea Microarray ScoreCard control reagent (Amersham Biosciences) and labelled with Cy5. Hybridisation to the microarray was performed in an Automated Slide Processor (Amersham Biosciences) with Hybridization Buffer Ver.2 (Amersham Biosciences) at 48°C for 12 h according to the protocol for Type7-star slides. The arrays were washed with 1 × SSC (75 mM NaCl, 7.5 mM sodium citrate, pH 7.0) containing 0.2% SDS at 45°C for 10 min, 0.1 × SSC containing 0.2% SDS at room temperature for 10 min, and 0.1 × SSC at room temperature for 4 min. The washed arrays were scanned using a Microarray System Generation III scanner (Molecular Dynamics), and the signal intensity of each spot was recorded.

Normalisation of the level of gene expression

We transformed the level of gene expression using base 2 logarithmic transformation; hereafter, the transformed level of gene expression is referred to as the gene expression level. We used a dynamic range control gene (3DR) as the reference control gene. Variations in the expression of the reference control gene, allocated to each of the pen groups in a slide, were compensated systematically. In order to reduce systematic variation, we first transformed the expression of the reference control gene in each pen group with an appropriate transformation, so that the expression of the reference control gene was equal in all the pen groups in a slide. Second, we transformed the expression of other genes using the same transformation that we used for the reference control gene. We used the ratio of the expression of each gene to that of the control gene in the subsequent analyses. The ratio corresponds to the difference in expression between respective genes and the reference control gene on the original scale.

Statistical analysis

The candidate genes, which would be regarded as having significant association with the prevalence of OA, were selected as follows: (1) for each gene, we compared the mean of the normalised expression between OA and non-OA/non-RA groups and between OA and RA groups by Dunnett's multiple comparison at 0.05 significance level, on the right and on the left of a slide, respectively; (2) considering the possibility of heterogeneous variation among groups, we also similarly compared mean absolute deviation from the median of the non-OA/non-RA group; and (3) We selected the gene where statistical significances were detected in both comparisons with non-OA/non-RA group at least on one side of a slide in (1) or (2). We only adjusted the multiplicity derived from three groups.

To examine the performance of the selected genes to classify into OA, RA or non-OA/non-RA, we conducted linear discriminant analysis. In order to evaluate the relationship between the severity of OA and the level of gene expression, we subjected the expression data of the genes to one-way ANOVA with five levels (four grades of OA (Kellgren–Lawrence radiographic rating system: I, II, III and IV) and control) at 0.05 significance level. To examine relationships between the expression of the

selected genes and the clinical characteristics, we compared the expression of the selected genes between 'none' and 'present' groups on the right and left side of a slide, respectively, using Welch's t-test at the 0.05 significance level. To evaluate the inter-relationships between the selected genes, we employed graphical modelling and drew a straight line to connect each pair of genes for which the partial correlation coefficient was statistically significant ($p < 0.05$).

Results

Identification of genes differentially expressed in OA synovium

To identify genes differentially expressed in synovium from OA, RA and non-OA/non-RA patients, we used a cDNA microarray. After intra-slide and inter-slide normalisation (see Materials and methods), we compared the means of the normalised expression levels of each gene among the OA, RA and non-OA/non-RA groups. Because of the possibility of differences in degree of variation among the three groups, we also compared mean absolute deviation from the median of the non-OA/non-RA group. These comparisons were performed between the OA group and the non-OA/non-RA group and between the OA and RA groups, using Dunnett's multiple comparison. Each subject was tested two to six times. The intra-class correlation coefficient (ICC) was estimated for each gene to assess intra-subject and inter-subject reliability in our system. The median ICC was 0.7968 and ICC above 0.7 accounted for 83.2% of the genes (data not shown). This result indicated that the reliability of this system was high.

Out of the 3265 genes that were analysed, 85 genes (2.6%) were found to have differences in expression levels between OA synovium and non-OA/non-RA synovium (data not shown). For 64 of those 85 genes, differences in expression between OA synovium and RA synovium were not statistically significant. Thus, we identified 21 genes with simultaneous significant differences between OA and non-OA/non-RA groups and between OA and RA groups, by comparing the mean and/or mean absolute deviation on the right or left side of each slide (Table II).

The 21 genes were grouped into the following seven categories according to previous findings (Kumar et al. 2001). Eight of the genes were enzyme/enzyme enhancer/inhibitors: *PPP2R5C* (protein phosphatase 2, regulatory subunit B, gamma isoform), *PGK1* (phosphoglycerate kinase), *SORD* (sorbitol dehydrogenase), *EXT1* (exostoses 1), *HS6ST* (heparan sulfate 6-sulfotransferase), *HARSL* (histidyl-tRNA synthetase-like), *LIMK2* (LIM domain kinase 2) and *GSTT1* (glutathione *S*-transferase). Four genes were extracellular matrix protein: *FN1* (fibronectin), *FBLN3* (fibulin-3), *P4HA2* (procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), α polypeptide II) and *COL3A1* (collagen type III, α1). Three genes were receptor/signalling proteins: *IL1R1* (interleukin-1 receptor), *TBCC* (tubulin-folding cofactor C) and *CXCL2* (chemokine, CXC motif, ligand 2). Three genes were, respectively, a growth suppressor/tumour suppressor, ribosomal protein and transcription factor: *BCSC-1* (breast cancer suppressor candidate 1), *RPL15* (60S ribosomal protein L15) and *TCF17* (transcription factor 17). Three genes were of unknown function: *APEX* (APEX nuclease), *PCBP1* (poly(rC) binding protein 1) and *EVER1* (Lak-4p).

Table II. The genes selected as the candidate biomarker of osteoarthritis (OA). (A) Genes selected by comparing the means of the normalised gene expression. (B) Genes selected by comparing the mean absolute deviation with the median.

A

Gene name	Accession number	Location[a]	Functional classification[b]	Mean[c]	
				OA	RA
FBLN3	U03877	sp	ECM	1.7641	0.9475
TCF17	AF116030	np	TF	1.8975	1.139
PGK1	AB062432	sp	E/EE/I	2.3093	9.8283
SORD	BC021085	cp	E/EE/I	1.5498	2.6248
HS6ST	AB006179	sp	E/EE/I	2.3865	1.1678
HARSL	U18937	cp	E/EE/I	1.8238	1.986
IL1R1	M27492	mp	R/SP	2.1446	1.0097
LIMK2	D45906	cp	E/EE/I	1.2985	0.9389
P4HA2	U90441	sp	ECM	2.1745	1.1317
COL3A1	X14420	sp	ECM	2.2221	1.1209
BCSC-1	AF002672	Unknown	G/TS	1.8756	3.0458
EXT1	BC001174	sp	E/EE/I	2.1138	3.4442

B

Gene name	Accession number	Location[a]	Functional classification[b]	Mean absolute deviation[c]	
				OA	RA
RPL15	AF283772	cp	RP	1.4017	1.028
APEX	BC004979	np	Unknown	1.4541	1.0276
CXCL2	M36820	sp	R/SP	1.5602	1.1776
FN1	BX640802	sp	ECM	1.4215	1.0381
PCBP1	X78137	sp	Unknown	1.3928	0.9278
GSTT1	X79389	sp	E/EE/I	1.3148	1.0745
PPP2R5C	U37352	np	E/EE/I	1.4543	1.0526
TBCC	U61234	cp	R/SP	1.7065	1.0962
EVER1	BC023597	cp	Unknown	1.5955	1.047

[a]Location: sp, secretory protein; np, nuclear protein; cp, cytoplasmic protein; mp, membranous protein.
[b]Functional classification: ECM, extracellular matrix protein; TF, transcription factor; E/EE/I, enzymes/enzyme enhancer/inhibitor; R/SP, receptor/signalling protein; G/TS, growth/tumour suppressors; RP, ribosomal protein.
[c]Relative gene expression level compared to that of non-OA/non-RA group.
RA, rheumatoid arthritis.

Discrimination between OA, RA and non-OA/non-RA groups using the 21 selected genes

Next, we performed linear discriminant analysis for discrimination among OA, RA and non-OA/non-RA groups, and used the leave-one-out cross-validation to evaluate the performance of the discriminator based on the 21 selected genes. For genes that exhibited significant differences only in comparison of absolute deviations, we used the absolute deviation to generate a discriminator. Discriminant scores of each subject for OA vs. RA and OA vs. non-OA/non-RA were plotted in the two-dimensional space as shown in Figure 1. The linear discriminant analysis indicated that the OA, RA and non-OA/non-RA groups were obviously distinguished by the 21 identified genes. The discriminant analysis gave a misclassification rate of 5.66% (Table III). The adequacy

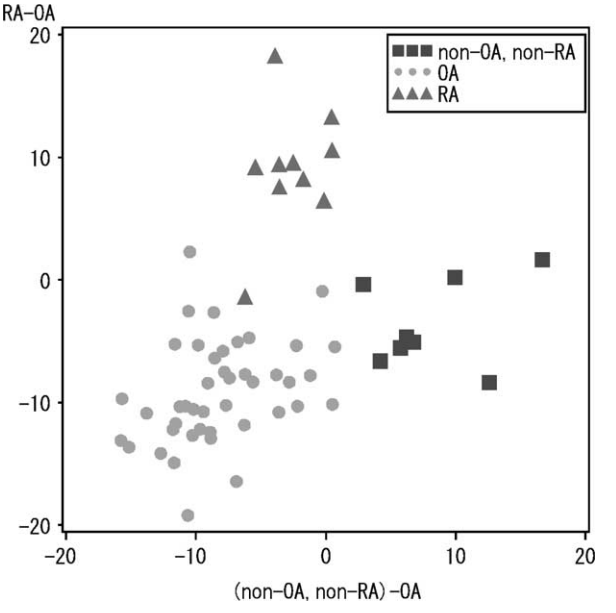

Figure 1. Discrimination between osteoarthritis (OA), rheumatoid arthritis (RA) and non-OA/non-RA groups using the 21 selected genes. Linear discriminant analysis shows that the OA, RA and non-OA/non-RA groups are well separated using the 21 genes identified by the univariate analysis.

of using their genes was evaluated using a leave-one-out cross-validation. The misclassification rate obtained using the cross-validation was 26.09% (Table III).

Relationships between disease severity and the expression of selected genes

In order to evaluate the relationship between the radiographic severity of OA and the level of gene expression, we performed one-way ANOVA to compare severity among five levels (four grades of OA (I, II, III and IV) and a control) for each of the selected gene' expressions. Consequently, the variations between levels were significant for four of the 21 genes. We then divided the variations into linear trend and other components with orthogonal contrasts. Table IV shows that the linear trend of the level of gene expression over severity was significant ($p < 0.05$) for each of the four genes: *PPP2R5C*, *EXT1*, *HS6ST and GSTT1*. For three of the four genes (*PPP2R5C, EXT1* and *HS6ST*), the level of gene expression was clearly increased as severity progressed, whereas that of *GSTT1* decreased (Figure 2).

Relationships between clinical characteristics and the expression of selected genes

We examined relationships between expression of the 21 selected genes and four clinical characteristics: osteophyte formation, hydrarthrosis, crystal deposits and synovitis. All clinical characteristics were given binary variables: 'none' and 'present'. We compared means of expression of selected genes between 'none' and 'present' groups, using Welch's *t*-test. Seven genes were associated with osteophyte formation: *HARSL*, *P4HA2*, *PGK1*, *SORD*, *EXT1*, *HS6ST* and *LIMK2*. Two genes were associated with the presence of synovitis: *PCBP1* and *GSTT1*. Seven genes were closely associated with hydrarthrosis: *BCSC-1*, *PPP2R5C*, *IL1R1*, *TBCC*, *EVER1*,

Table III. Classification by the linear discriminant analysis or the leave-one-out cross-validation.

	Predicted						
	Linear discriminant analysis			Cross-validation			
Original	Non-OA/ non-RA	OA	RA	Non-OA/ non-RA	OA	RA	Total
Non-OA/non-RA	8	0	0	6	1	1	8
	100.00%	0.00%	0.00%	75.00%	12.50%	12.50%	100.00%
OA	2	40	1	9	33	1	43
	4.65%	93.02%	2.33%	20.93%	76.74%	2.33%	100.00%
RA	0	1	9	1	2	7	10
	0.00%	10.00%	90.00%	10.00%	20.00%	70.00%	100.00%

OA, osteoarthritis; RA, rheumatoid arthritis.

CXCL2 and *HARSL* (Table V). None of the genes was associated with crystal deposits.

Graphical modelling

The graphical modelling connects each pair of genes for which partial correlation was significant ($p < 0.05$). Figure 3 also shows the relationships between the levels of gene expression and the three specified clinical characteristics: hydrarthrosis, osteophyte formation and synovitis. Some genes that were significantly associated with particular clinical characteristics correlated closely with each other, suggesting biological relationships among those genes.

Discussion

There has been much research aimed at identifying biochemical markers for use in diagnosis and monitoring of the progression of OA. The following biomarkers for OA have been identified in the serum or urine of OA patients, in comparisons with healthy control subjects: proteoglycan monomer fragments (Lohmander et al. 1993), hyaluronic acid (Laurent et al. 1996, Pavelka et al. 2004, Elliott et al. 2005), pentosidine (Pavelka et al. 2004), collagen type II C-telopeptide (Reijman et al. 2004, Garnero et al. 2003), CRP (Spector et al. 1997), cartilage oligometric matrics protein (COMP) (Clark et al. 1999, Sharif et al. 2004), MMP (Naito et al. 1999), tissue inhibitors of metalloproteinases (TIMP) (Naito et al. 1999, Chevalier et al. 2001) and YKL-40 (Johansen et al. 1996). However, these markers are clearly not

Table IV. The genes in which linear trend of severity of osteoarthritis and gene expression was significant.

	p Value		
Genes	ANOVA	Linear trend	Residual
PPP2R5C	0.0192	0.0108	0.1254
EXT-1	0.0206	0.0034	0.3482
HS6ST	0.0118	0.0012	0.4677
GSTT1	0.0187	0.0081	0.1542

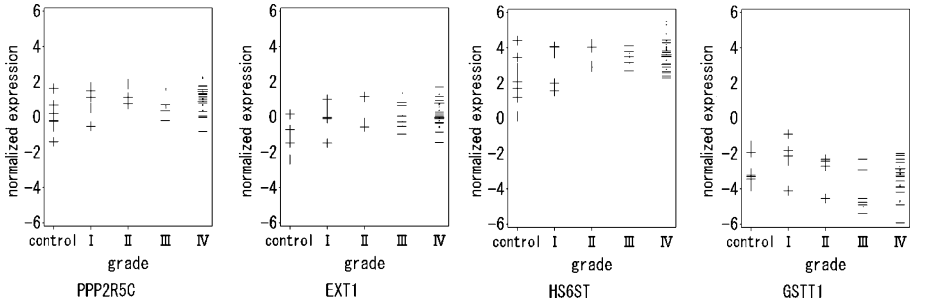

Figure 2. Relationships between osteoarthritis (OA) grade and gene expression. For three of the four genes (*PPP2R5C*, *EXT1* and *HS6ST*), the level of gene expression clearly increased as severity progressed, whereas that of *GSTT1* decreased. Severity of OA with four levels was categorised according to the Kellgren–Lawrence radiographic rating system.

specific for OA (Vignon et al. 2001). Therefore, more OA-specific biomarkers are urgently needed.

Global scale molecular profiling of osteoarthritic tissues is an important first step in identifying candidate target molecules that are involved in pathogenesis of a disease. Although research using cDNA microarray chips has been conducted in various fields of study (Eisen et al. 1998), there have been few cDNA microarray studies of gene expression profiles of *in situ* synovium of patients with OA of the knee joints (Rihl et al. 2004). The present study is the first in which gene expression profiles of *in situ* OA synovium were obtained using large-scale cDNA microarray technology to identify OA-specific biomarkers.

Table V. Relationships between clinical characteristics and the expression of selected genes. The values in bold highlight the genes that are statistically associated with the clinical characteristics.

	Clinical characteristics					
	Osteophyte		Synovitis		Hydrarthrosis	
Gene	Left[a]	Right[b]	Left[a]	Right[b]	Left[a]	Right[b]
BCSC-1	0.089	0.139	0.404	0.655	0.045	0.002
PPP2R5C	0.115	0.060	0.431	0.632	0.035	0.068
IL1R1	0.092	0.080	0.555	0.328	0.019	0.025
TBCC	0.344	0.378	0.103	0.205	0.002	0.001
EVER1	0.786	0.949	0.079	0.054	0.025	0.032
CXCL2	0.148	0.132	0.090	0.103	0.044	0.055
HARSL	0.020	0.046	0.535	0.934	0.015	0.027
P4HA2	0.008	0.015	0.963	0.932	0.591	0.518
PGK1	0.000	0.001	0.710	0.736	0.554	0.615
SORD	0.063	0.035	0.589	0.694	0.288	0.254
EXT1	0.006	0.022	0.363	0.330	0.142	0.052
HS6ST	0.007	0.007	0.855	0.851	0.960	0.773
LIMK2	0.031	0.030	0.409	0.507	0.878	0.886
PCBP1	0.811	0.816	0.021	0.038	0.713	0.605
GSTT1	0.177	0.068	0.022	0.053	0.514	0.459

[a,b]The same cDNAs were spotted at the same positions on the right and left sides of each slide glass, in order to perform duplicate experiments.
Numeric characters are the p values of Welch's t-test.

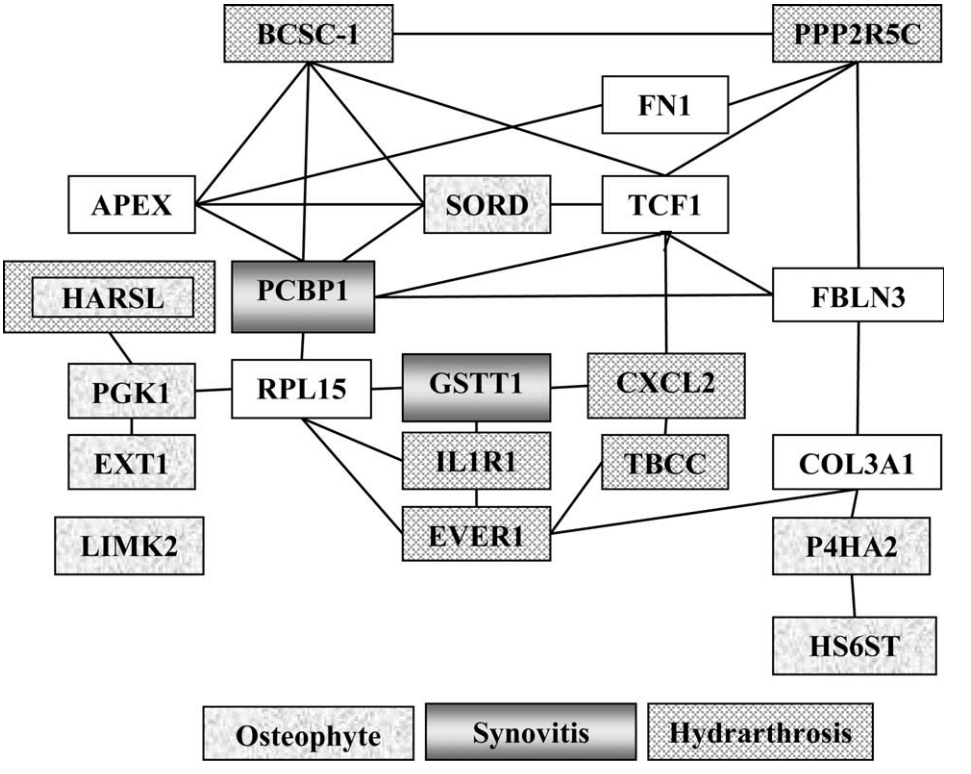

Figure 3. Relationships between the levels of gene expression and the three specified clinical characteristics. The graphical modelling connects each pair of genes for which partial correlation was significant ($p < 0.05$). Some genes that were significantly associated with particular clinical characteristics correlated closely with each other, suggesting biological relationships among those genes.

Two selection criteria were set for finding two types of characteristics of OA-specific genes. One type is that the averages of gene expression are different among the OA, RA and non-OA/non-RA groups. On the other hand, it will be not the case that all genes which characterise OA group always have this characteristic. Some OA-specific genes may have another type of characteristic. The average gene expression may not differ among OA, RA, and non-OA/non-RA groups. However, the variability in expression may be become greater in the OA group than in the non-OA/non-RA group, owing to heterogeneity in the OA population. The gene with the greater variability cannot be detected by comparing the averages of gene expression. It is necessary to compare the size of the dispersion of the distribution of gene expression. Therefore, the absolute deviation from the median of the non-OA/non-RA group was defined as a measure of the dispersion. The genes in which the size of the dispersion was different from the non-OA/non-RA group were found by comparing the averages of this absolute deviation. Consequently, 12 genes were chosen from the viewpoint that the average of the gene expression was different between OA and non-OA/non-RA groups, and nine genes were chosen from the viewpoint that the size of the dispersion of the gene expression was different between the OA group and non-OA/non-RA groups. The set of the genes chosen based on each criterion has a mutually

different feature, and there is no overlap between the two sets of selected genes. Therefore, we think that the method used to select the genes was appropriate.

Among the 21 genes differentially expressed in OA synovium, seven genes (*FBLN3*, *IL1R1*, *LIMK2, COL3A1*, *CXCL2*, *FN1* and *GSTT1*) have previously been implicated in the pathogenesis of arthritis (Haskill et al. 1990, Hogan et al. 1994, Justen et al. 2000, Mattey et al. 2000, Aigner et al. 2001, Kumar et al. 2001, Thornton et al. 2002, Zhang et al. 2002, Ochi et al. 2003, van der Pouw Kraan et al. 2003, Onodera et al. 2004, Scaife et al. 2004). For example, recent findings suggest that *FN1* is an important regulator of matrix turnover activity of chondrocytes during disease development (Aigner et al. 2001, Peters et al. 2002), and that different molecular forms of *FN1* can induce variable responses in chondrocytes according to the respective components at the integrin recognition site (Peters et al. 2002). Evidence of interaction between genotypes of *GSTT1* and manganese superoxide dismutase (MnSOD) has also been described previously, with the MnSOD VV/GSTT1-null combination being associated with more severe radiological outcome in RA (Mattey et al. 2000). The fact that seven out of the 21 genes are already known to be related to arthritis suggests the validity of our cDNA microarray approach to the study of synovial gene expression. More importantly, we identified 14 genes that have not previously been reported to be expressed in OA synovium: *TCF17*, *PGK1*, *SORD*, *HS6ST*, *HARSL*, *P4HA2*, *BCSC-1*, *EXT1*, *RPL15*, *APEX*, *PCBP1, PPP2R5C*, *TBCC* and *EVER1*. Further investigations are needed to find out the mechanisms of OA progression concerning these 14 genes.

Out of the 21 genes differentially expressed in OA synovium, the following ten genes encode secretory proteins that can be expected to be released into the extracellular space from certain cells located in synovium: *PGK1*, *FN1*, *COL3A1*, *CXCL2*, *GSTT1*, *EXT1*, *HS6ST*, *FBLN3*, *PCBP1* and *P4HA2*. Accordingly, we believe that proteins encoded by these genes could be possible candidate biomarkers for OA; this can be verified by determining whether the over-expression of these genes is specific to OA synovium and if the gene products are secreted into the peripheral circulation from synovium.

We performed linear discriminant analysis to determine whether a subset of the 21 genes we identified is useful in classifying arthritis. We found that the OA, RA and non-OA/non-RA groups were well separated by performing a linear discriminant analysis using the 21 genes we identified. These results support the validity of the strategy we formulated to identify biomarkers exclusively expressed in OA synovium.

Production of pre-inflammatory cytokines and other inflammatory mediators in synovial fluid of OA joints is well documented (Smith et al. 1997, Dang et al. 2003, Parsonage et al. 2003, Scaife et al. 2004, van Lent et al. 2004). However, there have been few studies of relationships between expression of inflammatory parameters in OA knee joints and radiographic severity of OA knee joints (Saxne & Heinegard 1992, Brenner et al. 2004, Reijman et al. 2004, Elliott et al. 2005). In order to evaluate the relationship between the levels of gene expression and severity of OA, we applied the linear trend test based on one-way layout ANOVA to the expression data of each of the 21 selected genes, with five levels (four grades of OA (I, II, III, and IV) and control). The results of the test showed an increasing trend between levels of gene expression and the severity of OA to be significant in each of three genes (*PPP2R5C* (0.0108), *EXT1* (0.0034), *HS6ST* (0.0012)) in the synovium while a decreasing trend

was significant in *GSTT1* (0.0081). The figures in parentheses are the *p* values for linear trend shown in Table IV. However, the gene expressions for each of *EXT-1* and *HS6ST* were significantly different between the OA group and the RA group with regard to the mean, while those for each of *PPP2R5C* and *GSTT1* were significantly different with regard to the mean absolute deviation. Therefore the level of gene expression for each of *EXT-1* and *HS6ST* increased with the severity of OA. As for the trend in *PPP2R5C* and *GSTT1*, the degree of variability in the gene expressions affected the severity of OA. The four genes are associated with the radiographic progression of OA knee joints. It is unclear why they are associated with radiographic disease progression, but these results indicate that they are promising candidate biomarkers for monitoring of OA progression.

The major clinical characteristics of OA are degeneration of the articular cartilage and osteophyte formation, and the minor characteristics are joint effusion, crystal deposition and synovial membrane proliferation. Accordingly, we examined relationships between expression of the 21 selected genes and four clinical characteristics: osteophyte formation, hydrarthrosis, crystal deposition and synovitis. Osteophytes are thought to form via a process of enchondral ossification. The cells responsible are most likely periosteal cells that have the potential to undergo chondrogenesis (Scharstuhl et al. 2003, Blom et al. 2004, van Lent et al. 2004). In a recent study, synovial macrophages are considered to mediate osteophyte formation during experimental OA and other OA-related pathology (Blom et al. 2004). It was demonstrated that multiple intra-articular injections of transforming growth factor-α (TGF-α or bone morphogenetic protein-2 (BMP-2) induced considerable osteophyte formation and synovial thickening in murine knee joints (van Beuningen et al. 2000), and that over-expression of TGF-α/BMP inhibitors led to a significant reduction in osteophyte formation and synovial thickening (Scharstuhl et al. 2003). The synovial lining macrophages are considered to play a crucial role in the promotion of TGF-β-mediated osteophyte formation (van Lent et al. 2004). In the present study, expression of TGF-β in OA synovium was significantly up-regulated, compared with the non-OA/non-RA group (data not shown). However, TGF-β was not chosen as one of the molecules closely associated with OA, because it was also up-regulated in RA synovium. Seven genes (*HARSL*, *P4HA2*, *PGK1*, *SORD*, *EXT1*, *HS6ST* and *LIMK2*) were associated with osteophyte formation. Interestingly, heparan sulfate, which is produced by the heparin sulfate biosynthesis enzymes EXT1 and HS6ST, mediates TGF-β/BMP activity via intermolecular association (Nishiwaki et al. 2004). This suggests that both *EXT1* and *HS6ST* may play important roles in osteophyte formation. Although our result showed that expression of EXT1 is important for osteophyte formation, it was higher in the synovium from the RA patients, where osteophyte formation is not usually observed. We thought that there was no inconsistency. Since the degradation of bone and cartilage is ordinarily coupled to an attempt at repair in RA, anabolic molecule for bone and cartilage is sometimes up-regulated. For example, bone sialoprotein (BSP) is a molecule produced by osteoblasts, and positively regulates the mineralisation process mainly in the cartilage–bone interfaces in OA/RA. In the synovial fluid of RA patients, the BSP level increased and was correlated with the degree of knee damage (Saxne et al. 1995). We assumed that, the higher expression of EXT1 protein in RA patients compared with that in OA patients may be the result of the high bone turnover in RA as well as BSP expression. Because no specific biomarker of osteophyte formation has previously

been reported, some of the genes identified in the present study may turn out to be the first confirmed biomarkers of osteophyte formation. Further study is needed.

The genes *PCBP1* and *GSTT1* were associated with the presence of OA synovitis. There have been few previous reports of gene products associated with OA synovitis. Serum cartilage oligometric protein (COMP) is one of the molecules that is associated with clinically diagnosed synovitis in patients with OA of the knee (Vilim et al. 2001). COMP is produced not only by cartilage but also by synovium, and is released into the circulation (Di Cesare et al 1999). However, the *COMP* gene was not represented in the cDNA chip used in the present study. Thus, further study is needed to determine the molecular mechanisms including COMP, PCBP1 and GSTT1 in the generation of synovitis.

Seven of our 21 selected genes (*BCSC-1*, *PPP2R5C*, *IL1R1*, *TBCC*, *EVER1*, *CXCL2* and *HARSL*) are closely associated with hydrarthrosis. IL1R1 is the cell surface receptor for the two forms of interleukin-1 (IL1-α and IL1-β), which are mediators of inflammation. In culture, IL-1 induces cleavage and loss of proteoglycan aggrecan and denaturation of type II collagen in porcine, bovine and human cartilage (Billinghurst et al. 2000). A recent report indicated that autocrine/paracrine activities of IL1 in articular cartilage play important roles in cartilage matrix degradation not only in RA, but also in OA (Smith et al. 1997, Amin & Abramson 1998). IL-1 activity requires signalling via the cytoplasmic domain of IL1R1 (IL-1 receptor type 1). In OA, IL1R1 is up-regulated in synovial fibroblasts possibly conferring on these cells a greater sensitivity to IL1-β-induced stimulation (Sadouk et al. 1995). In spite of this evidence showing the significance of IL-1 signalling in cartilage degradation both in OA and RA, there are no reports that have compared the amount of IL1R1 gene expression between OA and RA synovium. Our data are the first reported that indicate that IL1R1 gene expression in OA synovium is higher than in both RA and non-OA/non-RA. Why the *IL1R1* gene is more expressed in OA than in RA remains unresolved. Further *in vitro* study is needed. CXCL2 is a member of the chemokines, which mediate recruitment of leucocytes and are induced by inflammatory cytokines, growth factors and pathogenic stimuli. Some reports indicate that expression of *CXCL2* is markedly up-regulated in synovial fibroblasts derived from tissue involved in murine passive collagen-induced arthritis (Onodera et al. 2004) or OA (Hogan et al. 1994). During the progression of OA, synovial cells and articular chondrocytes may communicate by releasing molecules such as IL-1 and CXCL2 into the synovial fluid and the resulting inflammatory responses may cause the progression of hydrarthrosis.

Crystals are detected in the synovial fluid of 52% of OA patients with effusions (Nalbant et al. 2003). It has been suggested that crystals, whether primary or secondary to tissue degeneration, may accelerate progression of OA (Nalbant et al. 2003). However, in the present study, there was no correlation between crystal deposition and the genes expressed in synovial tissue. It may be partially because it is difficult to detect fine crystal deposition by radiography.

After basic processing of raw experimental data and initial identification of candidate relevant genes, several statistical methods are available for further analysis. We performed graphical modelling to analyse relationships among our 21 selected genes (Figure 3), and found relationships between levels of expression of the 21 genes and clinical characteristics of three specified symptoms: hydrarthrosis, osteophytes

and synovitis. These data indicate that some genes significantly associated with particular clinical presentations strongly correlated with each other, suggesting biological relationships among those genes. Genes that are differentially expressed in OA synovium are also promising subjects for study of post-translational activity, functional investigation using animal models (e.g. knock-out or transgenic strategies), and study of genetic polymorphisms.

Our study has several limitations. The first is the heterogeneity of cell populations present in synovial tissue. The synovium used in the present study contains many different cell populations, including synoviocytes, blood vessel cells, adipocyte and fibroblastic cells. In some experiment, microdissection methods were used in preparation of tissue samples in order to select cell types of interest (Firestein & Pisetsky 2002). On the other hand, the validity of cDNA-based microarrays due to amplification of RNA and heterogeneity of the synovial tissue was evaluated for examination of synovium from patients with spondyloarthropathy (Rihl et al. 2004). It was thus concluded that microarrays are useful for analysing synovial tissue biopsies, and that they provide gene expression profiles with high reproducibility and low variability (Rihl et al. 2004). The purpose of the present study was to identify target molecules for use in diagnosis of OA. Therefore, as an initial screening, profiling the comprehensive gene expression in whole synovial tissue should be more meaningful than selecting genes expressed in specific types of cells. The second limitation of our study is that transcript levels do not necessarily reflect the amount of protein that represents the actual functional entity of a gene. Discrepancies between transcript and the protein were considered as a result of translational control, post-translational processing and regulation of protein stability. Therefore, further investigations based on proteomics are needed to approach physiological function of the identified gene concerning pathogenesis of osteoarthritis. The third point is that there were some limitations in the present study regarding the differences in confounding factors between the OA, RA and non-OA/non-RA groups. One of the most important confounding factors is the patient age. Table I shows that the distributions of age were different among the three groups, i.e. OA, RA and non-OA/non-RA groups, and the possibility of a confounding effect between influence of group and that of age on the amount of gene expression was worrying. Therefore, we compared the amount of gene expression of the 21 genes between OA and non-OA/non-RA groups and between OA and RA groups, taking into account the influence of age. Namely, we compared the amount of gene expression using analysis of covariance (ANCOVA) with age as a covariate. Table VI shows the results (p values) obtained from the comparisons among the three groups with ANCOVA. The assumption that slopes of regression lines for the three groups are equal was not found to be wrong for any one of the 21 genes. Significant differences in the amount of gene expression were recognised between OA and non-OA/non-RA groups for *BCSC-1, EXT1, RPL15, APEX, CXCL2, FN1, GSTT1, PPP2R5C* and *TBCC* and between OA and RA groups for all the genes except for *HARSL*, *LIMK2* and *GSTT1*. For *BCSC-1, EXT1, RPL15, APEX, CXCL2, FN1, PPP2R5C* and *TBCC*, significant differences were recognised in both comparisons. Further study including consideration of confounding factors is needed to obtain reliable biomarkers of OA.

Table VI. p-Value in analysis of covariance for each of the 21 genes.

Gene name	Accession number	Parallel test	Comparison between groups	
			OA vs. non-OA/non-RA	OA vs. RA
Raw data				
FBLN3	U03877	0.6116	0.1551	0.0083
TCF17	AF116030	0.8388	0.0517	0.0068
PGK1	AB062432	0.9690	0.3048	0.0006
SORD	BC021085	0.9098	0.3833	0.0069
HS6ST	AB006179	0.1901	0.3180	0.0043
HARSL	U18937	0.6772	0.1388	0.7778
IL1R1	M27492	0.6966	0.0988	0.0053
LIMK2	D45906	0.2293	0.8576	0.2524
P4HA2	U90441	0.4358	0.3944	0.0113
COL3A1	X14420	0.8012	0.0775	0.0069
BCSC-1	AF002672	0.8871	0.0260	0.0399
EXT1	BC001174	0.5758	0.0077	0.0254
Absolute deviation				
RPL15	AF283772	0.4462	0.0314	0.0155
APEX	BC004979	0.9668	0.0164	0.0110
CXCL2	M36820	0.6879	0.0098	0.0392
FN1	BX640802	0.6008	0.0127	0.0224
PCBP1	X78137	0.8122	0.0850	0.0016
GSTT1	X79389	0.4928	0.0497	0.1203
PPP2R5C	U37352	0.1849	0.0122	0.0139
TBCC	U61234	0.9108	0.0224	0.0043
EVER1	BC023597	0.6762	0.1472	0.0126

OA, osteoarthritis; RA, rheumatoid arthritis.

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