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Enhanced expression of the human chitinase 3-like 2 gene (YKL-39) but not chitinase 3-like 1 gene (YKL-40) in osteoarthritic cartilage

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Abstract

The knowledge of molecular alterations in osteoarthritic cartilage is important to identify novel therapeutic targets or to develop new diagnostic tools. We aimed to characterize the molecular response to cartilage degeneration by identification of differentially expressed genes in human osteoarthritic versus normal cartilage. Gene fragments selectively amplified in osteoarthritic cartilage by cDNA representational difference analysis included YKL-39 and the oesophageal-cancer-related-gene-4 (ECRG4). YKL-39 expression was significantly upregulated in cartilage from patients with osteoarthritis ($n = 14$) versus normal subjects ($n = 8$) according to real-time PCR (19-fold, $p = 0.009$) and cDNA array analysis (mean 15-fold, $p < 0.001$) and correlated with collagen 2 up-regulation. In contrast, the homologous cousin molecule YKL-40 (chitinase 3-like 1), which is elevated in serum and synovial fluid of patients with arthritis, showed no significant regulation in OA cartilage. Enhanced levels of YKL-40 may, therefore, be derived from synovial cells while modulation of YKL-39 and collagen 2 expression reflected the cartilage metabolism in response to degradation.

Keywords: Gene expression; RDA; Real-time PCR; cDNA-array; Chitinase 3-like gene family

Degeneration or the loss of normal structure and function of articular cartilage is an integral part of osteoarthritis (OA) and develops most commonly in the absence of a known cause. Many of the mechanisms responsible for the progressive loss of cartilage remain unknown and successive stages, like disruption of the extracellular matrix, the chondrocytic response to tissue damage, and the progressive loss of tissue, overlap [1]. Quantification of gene expression on the RNA level will allow to focus on the actual chondrocytic response to tissue changes in OA cartilage. Several studies addressed the expression pattern of known single genes, mainly collagens and proteoclycans, by RT-PCR [2,3] or in situ hybridization [4–7] but other methods like cDNA library construction and sequencing of expressed fragments [8] and cDNA array analysis have also been applied [9,10]. Due to the limitations in quantification and sensitivity of distinct techniques and the inherent heterogeneity of OA cartilage tissue, discordant results were obtained in previous studies for molecules like collagen 2 [2,6,10], collagen 1 [5,7], and MMP-1 and -3 [4,10]. Thus, although significant effort has been made in characterization of the molecular response to cartilage damage, clearly validated markers are still missing.

The aim of this study was to identify differences in gene expression between normal and OA cartilage tissue by a combination of several powerful techniques. cDNA representational difference analysis (RDA) [11], a PCR based subtractive hybridization method, was applied to allow for a highly sensitive detection of known and unknown genes that are differentially expressed between OA and normal cartilage. cDNA-RDA eliminates fragments present in both cell populations and leaves only the differences. It was combined with real-time PCR allowing exact quantification of expression levels of the identified genes and with cDNA array analysis which put expression of these genes into the context of other common molecules expressed in cartilage. The

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knowledge of molecular alterations in degenerated cartilage is important since it may allow to identify novel therapeutic targets in OA cartilage and aid development of robust diagnostic or prognostic tools.

Materials and methods

Human samples

Osteoarthritic cartilage tissue was obtained after written consent from patients undergoing total knee or hip arthroplasty. Cartilage tissue with macroscopic evidence of degeneration was collected from the tibial plateau and classified as OA cartilage. Normal cartilage was obtained after written consent of the relatives or patients from two crime victims and patients undergoing amputation for tumor resection. The study was approved by the Local Ethics Committee.

RNA extraction

Cartilage poly(A)$^+$-mRNA was directly isolated from the shock-frozen tissue using oligo(dT) coupled to magnetic beads (Dynabeads, Dynal, Oslo, Norway) according to the manufacturer's instructions.

Representational difference analysis

Preparation of representations. In order to identify differences independent of donor variability the mRNA from cartilage of two normal donors and from four OA donors was pooled. cDNA was synthesized by using reverse transcriptase (Superscript II, Invitrogen, Karlsruhe, Germany) and oligo(dT) primers. Two hundred microgram of the double-stranded cDNA was digested with *Dpn*II, phenol extracted and ethanol precipitated followed by a ligation with an adapter molecule (R-Bgl-12/24). The DNAs were diluted and PCR was amplified by using R-Bam-24 oligonucleotides as primers (20 cycles of 1 min at 95 °C and 3 min at 72 °C, each). To obtain enough starting material for the RDA these PCR products were reamplified in an additional PCR step with 5 cycles. The final PCR products were phenol extracted, ethanol precipitated and resuspended at 0.5 μg/μl to form the representations. Both tester and driver representations were digested with *Dpn*II (New England Biolabs, Frankfurt, Germany) to remove the R adapters and then phenol extracted and ethanol precipitated. The tester representations were gel purified and ligated to the J-Bgl-12/24 adapter.

Selective amplification. To isolate genes upregulated in OA, cDNA representations derived from osteoarthritic cartilage were used as tester and representations derived from normal cartilage were used as driver. Subtractive hybridization was performed according to Hubank and Schatz [11]. Briefly, for the generation of the first difference product (DP1) driver and tester were mixed at a 100:1 ratio, hybridized for 24 h at 67 °C, amplified by PCR with J-Bgl- 24 as primer, and treated with Mung Bean Nuclease (New England Biolabs) to digest ssDNA. A final PCR amplification step produced DP1. To generate the second difference products (DP2) the N-*Bgl* adapters were substituted against the J-*Bgl* adapters and the driver to tester ratio was 800:1.

Oligonucleotides

The primer and adapter oligonucleotides for RDA were R-*Bgl*-12: 5′-GATCTGCGGTGA-3′, R-*Bgl*-24: 5′-AGCACTCTCCAGCCTCTCACCGCA-3′, J-*Bgl*-12: 5′-GATCTGTTCATG-3′, J-*Bgl*-24: 5′-ACCGACGTCGACTATCCATGAAC-3′, N-*Bgl*-12: 5′-GATCTTCCCTCG-3′, N-*Bgl*-24: 5′-AGGCAACTGTGCTATCCGAGGGAA-3′. Oligonucleotides used for light cycler PCR were GAPDH forward: 5′-CCACCCATGGCAAATTCCATGGCA-3′, GAPDH reverse: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′, YKL-39 forward: 5′-GGCAGGTGTAGTGGTCTTG-3′, YKL-39 reverse: 5′-GCATATTCCACATTGTAGTAGG-3′, YKL-40 forward: 5′-CCAATATAAGCAACGATCACAT-3′, YKL-40 reverse: 5′-TCCAACACCAGTCTCAGAAG-3′, ECRG4 forward: 5′-ACGAGAAGCACCTGTTCCA-3′, ECRG4 reverse: 5′-TGGTTAGTAGTCATCGTAGTT-3′. All oligonucleotides were obtained from Interactiva (Ulm, Germany).

Sequence analysis

DP2 fragments were excised from a 3% agarose gel, reamplified by PCR, purified, and cloned into the pBluescript KS vector (Stratagene, Amsterdam, Netherlands) before transformation into *Escherichia coli* strain SR101. The plasmid DNA was prepared by using the Qiagen plasmid miniprep kit (Qiagen, Hilden, Germany) and given for insert sequencing to MWG-Biotech (Ebersberg, Germany). BLAST algorithms were used to screen for DNA and protein sequence similarities in public databases at the National Center for Biotechnology Information (NCBI).

Northern blot analysis

A human multiple tissue northern was obtained from Clontech (Heidelberg, Germany). Gene specific PCR fragments were labeled by random octamer priming (MBI Fermentas, St. Leon Roth, Germany) using [α-^{32}P]dCTP as a radioactive nucleotide. Hybridization was done in 0.5 M sodium phosphate buffer, 7% SDS, 1 mM EDTA at 68 °C overnight. Filters were washed in 40 mM sodium phosphate buffer, 1% SDS at 68 °C for 30 min two times.

Light cycler analysis

Reverse transcription was performed with 10 μl poly(A)$^+$-mRNA prepared from cartilage from six normal and six OA donors by using reverse transcriptase Superscript II (Invitrogen) and oligo(dT) primers. The first strand cDNA was subjected to light cycler PCR (Roche Diagnostics, Mannheim, Germany) using gene specific upstream and downstream primers. To quantify the mRNA levels with the LightCycler (Roche Diagnostics) aliquots of first-stranded cDNAs were amplified and real-time fluorimetric intensity of CYBR green I was monitored. The cycling parameters were optimized according to the LightCycler protocol from Roche (LightCycler Operator's Manual, Version 3.5, October 2000). The concentration of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control reference gene since it is considered to be of stable expression in different tissues. It was detected at the same level in normal and OA cartilage. The GAPDH concentration was determined once for each cDNA sample and used to normalize all other genes (YKL-39, YKL-40, and ECRG4) tested for the same cDNA sample. Melting curves of the PCR products were used as quality control. One normal and one OA sample were excluded from YKL-39 quantification due to invalid melting curves.

cDNA array analysis

An in-house made cDNA array consisting of 24 genes including 19 cartilage related and 2 housekeeping genes as well as 3 Arabidopsis specific negative controls [12] was used for hybridization. ^{32}P-labeled cDNA probes were prepared from isolated mRNA from cartilage samples of 8 normal and 14 OA subjects according to manufacturer's protocol (SuperScript II, Invitrogen). The labeled cDNAs were denatured and hybridized to cDNA arrays in 0.5 M phosphate buffer, pH 7.2/7% SDS overnight at 68 °C. Arrays were washed 3 × 30 min in 0.04 M phosphate buffer, pH 7.2/1% SDS at 68 °C. Arrays were exposed to a phosphor imaging plate for up to 72 h. Images were captured on a Bio-Imaging Analyser BAS-1800 II using BAS Reader 2.26 beta software (Fuji/Raytest, Straubenhardt, Germany) and analyzed using the AIDA software (Fuji/Raytest). The relative expression level for a gene of interest was calculated as a mean value of the signal

intensities of this gene (spotted in duplicates) and normalized to the intensity of the housekeeping genes GAPDH and β-actin.

Statistical analyses

Mean and standard deviations (SD) were calculated for all values. Due to the size and distribution of the samples in this expression study, the Mann–Whitney *U* test was chosen to evaluate significant differences in expression levels. This non-parametric two-tailed test is not based on assumptions about the distribution of expression values (e.g., normal distribution) or the equality of variance. The Mann–Whitney *U* test uses the ranks of the data to calculate the statistic. For all tests a p value $\leqslant 0.05$ was considered significant. Data analysis was performed with SPSS for Windows 10.0 (SPSS, Chicago, IL, USA).

Results

ECRG4 and YKL-39 gene fragments selectively amplified in OA cartilage

OA-specific gene expression was analyzed by RDA with osteoarthritic cartilage serving as tester and normal cartilage as driver. The reverse experiment (tester: normal, driver: OA) was designed as internal control. While a smear of cDNA products was evident in the starting material of both cartilage samples, a distinct band pattern of the final difference products appeared for the OA- and the normal-specific amplification after two cycles of RDA with different tester:driver ratios (Fig. 1). Sequence analysis of fragments selectively amplified in OA cartilage revealed that one fragment was derived from the human oesophageal cancer related protein 4 gene (ECRG4), a 148 amino-acid protein of unknown function. ECRG4 was first described after mRNA differential display analysis of normal oesophageal epithelia and primary squamous cell carcinoma [13]. We evaluated tissue specific expression of ECRG4 by a human multiple tissue Northern with heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. All tissues revealed a ECRG4 transcript of approximately 1 kb with the strongest expression detected in heart and kidney (Fig. 2).

Two of the three identified fragments belonged to the gene for human chitinase 3-like 2 (CHI3L2, YKL-39) [14]. This molecule is one of the two known chondrocyte

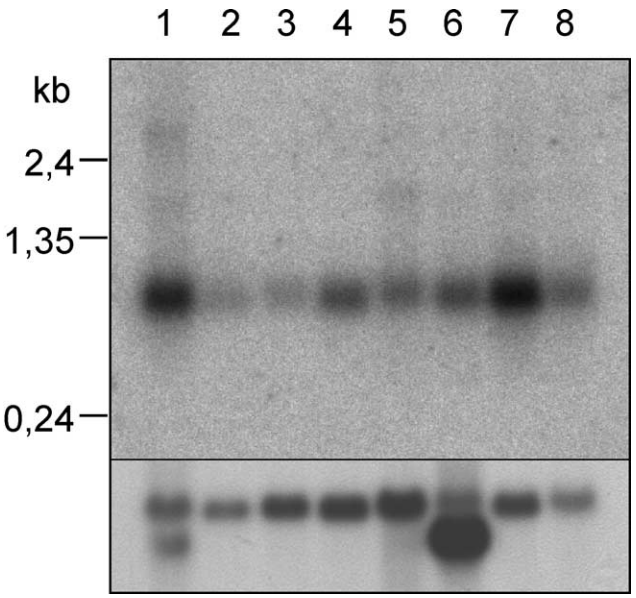


Fig. 2. Transcript length and tissue specific expression of ECRG4. Northern blot analysis was performed on multiple human tissues (Clontech) with an ECRG4 specific probe. An approximately 1 kb transcript was present in all tested tissues with the strongest expression observed for heart and kidney. Hybridization with β-actin is shown in the lower panel indicating equal amounts of mRNA in each lane. 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas (kb, kilobases).

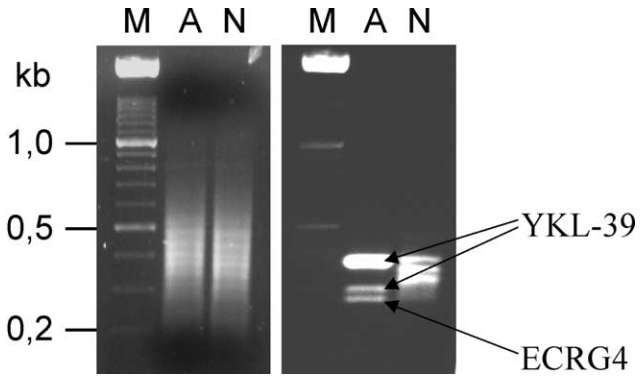


Fig. 1. Representational difference products obtained from osteoarthritic and normal human cartilage. Agarose gel electrophoresis of the starting material for cDNA-representational difference analysis (RDA) (left) and the final difference products after the second RDA-cycle (right). The different band pattern obtained with osteoarthritic (A) and normal (N) cartilage as tester demonstrates the high specificity during the subtractive hybridization process (M, 100 bp ladder; kb, kilobases).

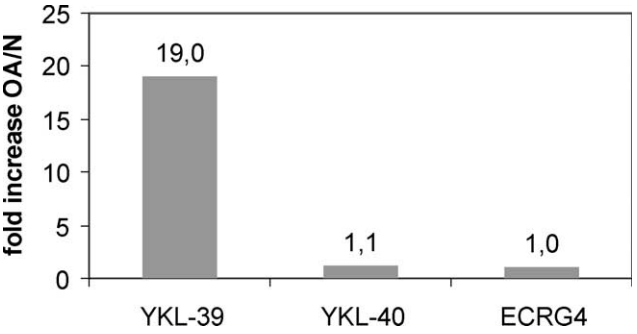
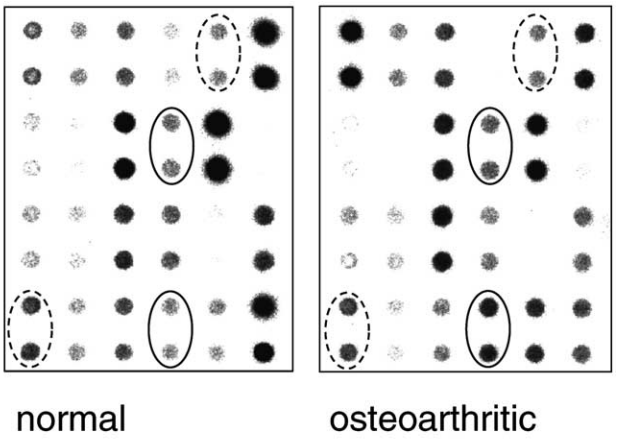


Fig. 3. Quantification of YKL-39, YKL-40, and ECRG4 gene expression by real-time PCR. RNA was derived from 6 independent cartilage samples from each group (OA and normal). The gene expression was quantified in each sample by real-time RT-PCR with GAPDH as the reference housekeeping gene. The mean change of YKL-39, YKL-40, and ECRG4 expression in OA versus normal cartilage is shown. A significant upregulation was evident for YKL-39 ($p = 0.009$) confirming the RDA result in a larger cohort.

expressed members of the chitinase 3-like gene family. The other is CHI3L1, also known as YKL-40 or human cartilage glycoprotein HC gp-39 [15]. Enhanced expression of YKL-39 and ECRG4 was confirmed in the OA versus the normal starting material.

Enhanced expression of YKL-39 in OA cartilage

To quantify the differential expression of ECRG4 and YKL-39 in relation to a housekeeping gene (GAPDH), cartilage samples from 12 additional donors (6 OA and 6 normal) were analyzed independently by quantitative RT-PCR with gene specific primers (Fig. 3). The analysis of YKL-40 was included for comparison since increased levels of YKL-40 protein were reported in serum and synovial fluid of patients with rheumatoid arthritis (RA) and OA [16–18]. A strongly enhanced expression of YKL-39 was evident in OA compared to normal cartilage (mean 19-fold, $p = 0.009$) (Fig. 3) while expression levels of YKL-40 and ECRG4 were similar in both groups. Discordant results for ECRG4 in the initial sample pool compared to a larger independent collective underline the necessity for analysis of gene expression in many donors. Remarkably, the ECRG4 signal was 5- to 37-fold (mean 16-fold) higher than GAPDH in all cartilage samples and, in spite of this strong expression, it has so far not been detected in cartilage.



COL2A1	CRTL1	AGC1	Arab.16	ACTB	DCN
COL2A1	CRTL1	AGC1	Arab.16	ACTB	DCN
COL9A1	Arab.5	FMOD	CHI3L1	COMP	CSPG2
COL9A1	Arab.5	FMOD	CHI3L1	COMP	CSPG2
COL11A1	CHM1	BGN	CRTAC1	Arab.21	LUM
COL11A1	CHM1	BGN	CRTAC1	Arab.21	LUM
GAPDH	GDF5	MIA	CHI3L2	CILP	PRELP
GAPDH	GDF5	MIA	CHI3L2	CILP	PRELP

Fig. 4. cDNA array analysis of normal versus OA cartilage. A representational result of the gene expression profile of normal and OA cartilage is shown. The grid on the bottom summarizes the genes spotted at each position (standard abbreviations according to the OMIM database). Arab. 5, 16, and 21 indicate negative controls from plant specific *Arabidopsis* genes. () highlights the positions for YKL-39 (CHI3L2) and YKL-40 (CHI3L1) and the dotted lines indicate the positions of the housekeeping genes GAPDH and β-actin (ACTB). The remaining abbreviations represent the following genes: collagen type II α-1 (COL2A1), collagen type IX α-1 (COL9A1), collagen type XI α-1 (COL11A1), cartilage link protein (CRTL1), chondromodulin 1 (CHM1), growth/differentiation factor 5 (GDF5), aggrecan 1 (AGC1), fibromodulin (FMOD), biglycan (BGN), melanoma inhibitory activity (MIA), cartilage acidic protein 1 (CRTAC1), cartilage oligomeric matrix protein (COMP), cartilage intermediate layer protein (CILP), decorin (DCN), chondroitin sulfate proteoglycan 2 (CSPG2), lumican (LUM), and proline arginine-rich end leucine-rich repeat protein (PRELP).

Expression of YKL-39 and YKL-40 in relation to common cartilage molecules

A self-designed cartilage cDNA array composed of 24 genes was used to compare gene expression levels of YKL-39 and YKL-40 with those of common cartilage molecules including collagen type 2, aggrecan, biglycan, and the cartilage oligomeric protein (COMP). Fig. 4 shows one representative cDNA array hybridization result for normal ($n = 8$) and OA ($n = 14$) cartilage which included the 12 samples of the light cycler analysis. While most cartilage relevant genes including YKL-40 did not show a significant alteration between OA and normal cartilage specimen, YKL-39 was significantly elevated (mean 15-fold, $p < 0.001$, Mann–Whitney U test) (Fig. 5). Upregulation of collagen type 2 occurred in parallel (normal $n = 5$, OA $n = 13$, mean 8.8-fold, $p = 0.003$). This indicates an independent regulation of the chitinase 3-like molecules YKL-39 and YKL-40 in response to cartilage degradation and a correlation of YKL-39 and collagen type 2 expression in damaged tissue (correlation coefficient: 0.61).

Discussion

By analysis of gene expression in OA versus normal cartilage we have identified a strong and significant upregulation of YKL-39 and collagen 2 in OA and describe abundant expression of an unknown gene ECRG4 in cartilage independent of an OA status. Expression of YKL-39 and YKL-40, two mammalian members of the chitinase 3-like gene family lacking chitinase activity, has, to our knowledge, never been compared in OA versus normal cartilage. In this study, three different methodological approaches detected significant differences between normal and OA cartilage only for YKL-39 but not for YKL-40 expression. Both mature human chitinase-like proteins, named according

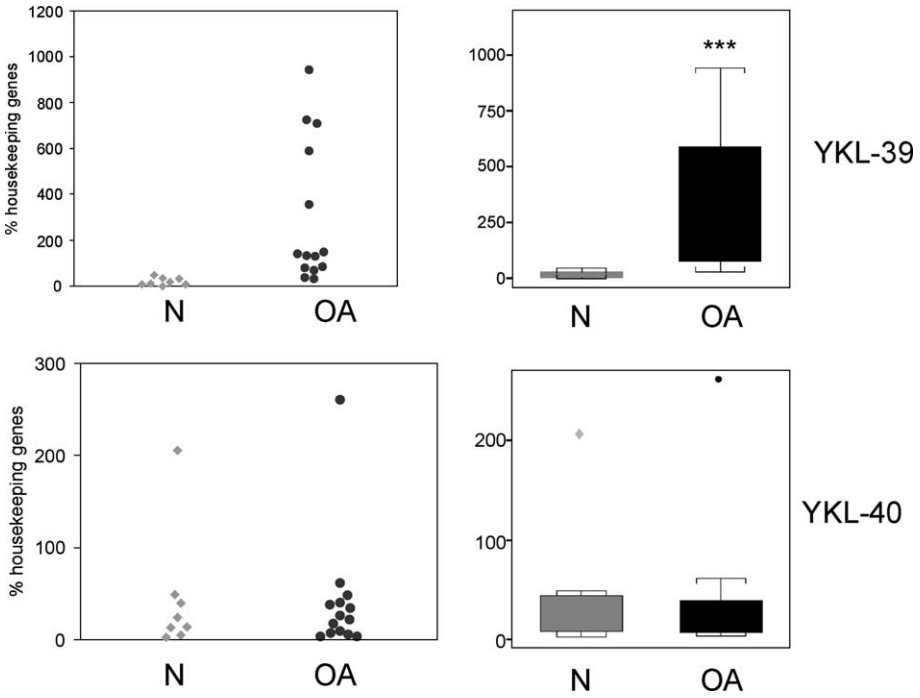


Fig. 5. Quantification of YKL-39 and YKL-40 gene expression by cDNA-array analysis. Gene expression of YKL-39 and YKL-40 was quantified in cartilage samples from 8 normal (N) subjects and 14 OA patients in relation to the housekeeping genes GAPDH and β-actin. YKL-39 but not YKL-40 expression was significantly elevated (15-fold, $p < 0.001$) in OA versus normal cartilage. On the right the lower boundary of the box is the 25th percentile and the upper boundary is the 75th percentile. The whiskers show the highest and lowest values.

to their N-terminal amino-acid sequence YKL, show a sequence homology of 51% on the amino-acid level and are abundant in media from cultured chondrocytes and synoviocytes [14,15,19].

While clinical correlates to YKL-39 expression are unknown, YKL-40 had been linked with tissue remodeling [20,21], joint injury [16], monocyte to macrophage maturation during inflammation [22–24] metastasis in breast cancer [25] and with fibrosis in liver cirrhosis [26]. It was detected in situ in inflammatory macrophages associated with rheumatoid synovium [27,28] and significantly elevated levels of YKL-40 protein occurred in serum and synovial fluid from patients with rheumatoid arthritis [29] and OA [29,30] compared to normal individuals [16,29]. Kawasaki et al. [28] demonstrated that YKL-40 levels were significantly higher in synovial fluid from patients with failed total hip arthroplasty compared to OA patients, although in all cases of failed total hip arthroplasty, the cartilage tissue in the joints had been completely removed during the previous surgery. Since, for this reason, the YKL-40 source in synovial fluid could not be articular cartilage, it was concluded, that YKL-40 in synovial fluid derives more abundantly from synovium than from cartilage. As YKL-40 levels correlated with severity of synovial inflammation [29] but not with OA grading [28] YKL-40 levels in synovial fluid and serum rather reflect the inflammatory state of the joint than cartilage degradation.

Quantitative data about YKL-39 and YKL-40 expression in cartilage are sparse. On the protein level, semiquantitative immunohistochemical studies revealed YKL-40 in chondrocytes of the superficial and intermediate zone of cartilage, but attempts to detect this secretory protein in the matrix failed. While 81% of OA cartilage sections with histological changes contained chondrocytes positive for YKL-40, such cells were seen in only 23% of normal cartilage sections [31]. No difference in YKL-40 staining was obvious between cartilage samples from OA and RA patients [17]. On the RNA level, in situ hybridization (4 cartilage samples) [32] and Northern blot analysis (4 samples) [15] detected no YKL-40 expression in normal cartilage specimens while advanced OA cartilage samples ($n = 4$ and $n = 2$) were positive. Due to the low sample size no statistical evaluation or account for tissue and donor heterogeneity was included in these studies. One comprehensive report on gene expression analysis of 1185 genes by cDNA array analysis in normal, early OA, and late OA cartilage [10], however, described significantly enhanced expression of RNA from a chitinase-like gene fragment called ''chitinase precursor'' in OA, which was interpreted as upregulation of YKL-40 by citing Hakala et al. [15] and Garnero et al. [33]. In fact, the gene fragment immobilized on the filter was derived from YKL-39 corroborating our results on upregulation of YKL-39 expression in OA cartilage samples.

To our knowledge, our study is the first comparing gene expression levels of YKL-39 and YKL-40 in normal and OA cartilage samples and we describe significant upregulation in OA cartilage only for YKL-39 but not for YKL-40 expression. Our detection of significant levels of YKL-40 in normal cartilage is in contrast to the above-mentioned Northern blot and in situ hybridization studies [15,32] which may have failed to detect YKL-40 RNA in normal cartilage due to the much lower sensitivity of these methods and the restricted number of donors included in the studies. Discrepancy of our data to immunohistochemical results for YKL-40 in OA versus normal cartilage may have several reasons. Since RNA has a very short half-life, RNA levels depict only the actual transcription of the YKL-40 gene, in our case, late in OA. Protein half-life in articular cartilage, in contrast, may be quite long as evident from collagen type 2 which was estimated to have a half-life in cartilage of 360 years [34]. Increased levels of YKL-40 protein in OA cartilage may, therefore, represent older molecules that may have been synthesized during earlier stages or inflammatory episodes of OA, which were not analyzed in this study. Alternatively, detection of YKL-40 protein in OA cartilage may be improved due to unmasking of YKL-40 epitopes in a cartilage tissue depleted of proteoglycans, a situation found especially in middle and superficial layers of OA cartilage and, thus, in the region where YKL-40 was described. Finally, YKL-40 expression may be differently regulated on the RNA and on the protein level. Longitudinal studies and antibodies specific for YKL-39 will be required to further elucidate such possible discrepancies between RNA and protein detection.

In sum, our results suggest that YKL-39 and collagen 2 but not YKL-40 are specifically up-regulated in response to cartilage degeneration in late OA. This suggests that elevated YKL-40 protein levels in synovial fluid and serum from patients with joint disease are derived from cells of the synovial membrane rather than cartilage and, thus, do not reflect the cartilage metabolism in late stages of the disease. The distinct regulation of YKL-39 and YKL-40 in normal versus OA cartilage promotes the view that both proteins may display different functions in cartilage. Further studies with combined analysis of YKL-39 and YKL-40 are required to elucidate whether the two chitinase-like molecules may complement each other in discrimination of cartilage degradation from joint inflammation in arthritis.

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