Cis- and *Trans*-Acting Gene Regulation Is Associated with Osteoarthritis

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Osteoarthritis (OA) is a complex disease of the skeleton and is associated with aging. Both environmental and genetic factors contribute to its pathogenesis. We set out to identify novel genes associated with OA, concentrating on regulatory polymorphisms allowing for differential expression. Our strategy to identify differentially expressed genes included an initial transcriptome analysis of the peripheral blood mononuclear cells of six patients with OA and six age-matched healthy controls. These were screened for allelic expression imbalances and potentially regulatory single-nucleotide polymorphisms (SNPs) in the 5′ regions of the genes. To establish disease association, disparate promoter SNP distributions correlating with the differential expression were tested on larger cohorts. Our approach yielded 26 candidate genes differentially expressed between patients and controls. Whereas *BLP2* and *CIAS1* seem to be *trans*-regulated, as the absence of allelic expression imbalances suggests, the presence of allelic imbalances confirms *cis*-regulatory mechanisms for *RHOB* and *TXNDC3*. Interestingly, on/off–switching suggests additional *trans*-regulation for *TXNDC3*. Moreover, we demonstrate for *RHOB* and *TXNDC3* statistically significant associations between 5′ SNPs and the disease that hint at regulatory functions. Investigating the respective genes functionally will not only shed light on the disease association but will also add to the understanding of the pathogenic processes involved in OA and may point out novel therapeutic approaches.

Osteoarthritis (OA [MIM 165720]) is the most common musculoskeletal disease associated with aging.[1] Clinical problems include pain and joint stiffness, which often lead to significant disability and joint replacement. Although OA has previously been considered a mere consequence of age-related cartilage degeneration, it is now accepted that OA is the result of active disease processes. Imbalances between the synthesis and degradation of the extracellular matrix and between the proliferation and apoptosis of the articular chondrocytes are the most widely discussed disease processes.[2–5]

OA is a complex disease with multiple environmental and genetic factors contributing to its pathogenesis.[6–9] To elucidate the genetics, whole-genome linkage analyses and candidate-gene approaches have been performed.[9] Whereas the former may provide large QTLs without revealing the disease-associated genes themselves, the latter can yield only small aspects of the genetics.[10–15]

The description of differential gene expression as a driving force in evolution led to an increased appreciation of regulatory gene polymorphisms.[16] Although the differential expression of genes allows for a flexible response to a changing environment and, thus, may be beneficial for the population as a whole, adverse effects for the individual may occur. Indeed, regulatory gene polymorphisms are likely candidates for an association with complex diseases: moderate alterations in gene expression can implement subtle differences over a long period of time and may render the organism susceptible to cumulative pathogenic impacts.[17] In OA, regulatory gene polymorphisms within the *IL1* locus have been described elsewhere to be associated with the disease.[18,19]

We set out to identify novel regulatory gene polymorphisms associated with OA. To that end, we performed transcriptome analyses of peripheral blood mononuclear cells (PBMC) from OA patients and controls, and we identified genes that were differentially expressed. We based our study on the assumption that regulatory SNPs leading to differential expression in PBMC may do so in articular chondrocytes as well. Since we were primarily interested in regulatory polymorphisms acting in *cis,* we screened our candidate genes for allelic expression imbalances and promoter SNPs associated with the disease. Indeed, we found two genes exerting allelic expression imbalances, which confirmed the presence of *cis*-regulatory polymorphisms. In addition, we found, in the same two genes, 5′ SNPs that show a statistically significant association with OA. We, therefore, consider our approach suitable to identify disease-

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Table 1

Primers for Amplification and Sequencing of the 5′ UTRs

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

associated differential gene expression resulting from regulatory gene polymorphisms.

Subjects and Methods

Study Population

For microarray analyses, six patients with knee OA awaiting joint replacement surgery and six age-matched healthy controls were analyzed. Whereas the controls were without any previously recorded joint complaints, the patients' diagnosis of OA was based on clinical evaluations, excluding metabolic causes. All six patients with OA were recruited from the Charité University Hospital in Berlin and were selected for comparable and low synovitis scores of the joints replaced. Determining the synovitis score included screening the tissues for hyperplasia/enlargement of the synovial lining cell layer, activation of resident cells/synovial stroma, and inflammatory infiltrations.[20]

For the OA association study, SNP analysis was performed on 171 patients undergoing joint replacement surgery and 182 healthy controls. Patients with OA as well as healthy controls were of European white ethnicity. All patients fulfilled the criteria of the American College of Rheumatology regarding OA and were recruited from different hospitals within the Berlin area. The mean age of the patients was 69.4 ± 10.9 years, with a male-to-female ratio of 1:2.9 and a hip-to-knee ratio of 1:2.1. The mean age of the controls was 39.6 ± 9.8 years, with a male-to-female ratio of 1:1.9. The study was approved by the local ethics committee, and informed consent was obtained from all participants.

Microarray Analyses

Fifty milliliters of peripheral blood was obtained, and erythrocytes were lysed using ammoniumchloride/Tris at 37°C. Leukocytes were then stained on ice with monoclonal antibodies and Microbeads (Miltenyi Biotec). The $CD15^+$ cells were depleted using automated separation with autoMACS (Miltenyi Biotec). From the $CD15^-$ population, $CD14^+$ monocytes, $CD19^+$ B cells, $CD4^+$ T helper cells, and $CD8^+$ cytotoxic T cells were positive selected by fluorescence-activated cell sorting, with use of the DiVantage (BD Biosciences) or the MoFlo (DakoCytomation). The purities of the isolated cell fractions were >98%. All samples were treated equally. Cells were lysed directly after sorting, with the use of RLT lysis buffer (Qiagen) containing β-mercaptoethanol. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and was treated with DNAse (RNase free DNase Set [Qiagen]), to remove genomic DNA. Double-stranded cDNA was synthesized using SuperScript II RNase H^- Reverse Transcriptase and the SuperScript II Kit (Invitrogen) and was converted to Biotin-labeled cRNA via in vitro transcription, with use of the BioArray HighYield Transcription Labeling Kit (Enzo). The labeled cRNA was then fragmented and hybridized to Affymetrix HG-U133A chips. All preparation procedures and quality controls were performed in accordance with the Affymetrix standard protocol.

Analyses of Allelic Imbalances

Genomic DNA was extracted from peripheral blood with use of the Invisorb Blood Giga Kit (Invitek). Identification of a SNP to be used as allelic marker within the transcribed region was performed via PCR amplification and sequencing of the UTRs. Information about 5′ and 3′ UTRs were derived from public databases. UTRs were sequenced for their full lengths. Primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research). The resulting 18–24mer primers were suitable for amplifying and sequencing genomic DNA fragments of ~700 bp (for detailed primer sequence information, see tables 1 and 2). In cases where 3′ UTRs were longer than 700 bp (*PADI2* and *VNN1* [MIM 603570]), multiple primer pairs were used. The PCR was performed using the High Fidelity PCR Master (Roche), and the conditions were 30 cycles of 94°C for 1 min, 55°C–68°C for 1 min, and 72°C for 1 min. Before sequencing, the PCR products were purified using the Invisorb PCRapid Kit (Invitek), to remove primers and PCR reagents.

Pyrosequencing technology (Biotage AB) was used to confirm differential gene expression at the allelic level. The cDNA was synthesized using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and a mixture of random hexamere and anchored oligo-dT primers. The RNA templates used were the same as those used for microarray analysis. cDNA fragments between 165 and 253 bp in length were PCR amplified using the primers *RHOB* (MIM 165370) 5′ Bio-TGTGTGTCTGTTCGACTCCC-3′ (forward), *RHOB* 5′-GCAGTACCCGGGGTCTATGT-3′ (reverse), *BLP2* 5′ Bio-CAGGCCCACAGTATTTCTTA-3′ (forward), *BLP2* 5′-GTGGGAGTGTTTCTGTTGTT-3′ (reverse), *CIAS1* (MIM 606416) 5′ Bio-ACAGCTCTGTGATCCTTCCG-3′ (forward), *CIAS1* 5′-TACATGAGGTCACCAAGAGG-3′ (reverse), *TXNDC3* (MIM 607421) 5′ Bio-ATGGAACTTCGCCGAGGTCA-3′ (forward), and *TXNDC3* 5′-CGGTGTAGAACAGGGGTCAG-3′ (reverse), and the PCR conditions were 45 cycles of 95°C for 15 s, 50°C–60°C for 30 s, and 72°C for 15 s. For each gene and each individual, four PCR samples were run in parallel. The PCR products were then purified via the Invisorb PCRapid Kit (Invitek). The sequencing primers—*RHOB* 5′-CCGCTGGTGAAGG-3′, *BLP2* 5′-AGTTTTGCAGTGTAATACAT-3′, *CIAS1* 5′-TGAGGCGCTGTGAT-3′, and *TXNDC3* 5′-CCTCTAAGCTAGACTG-3′—were defined using the SNP Primer Design software version 1.0.1 (Biotage AB). Before pyrosequencing analysis, the biotinylated DNA strand was prepared using

Table 2

Primers for Amplification and Sequencing of the 3′ UTRs

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 3

Primers for Amplification and Sequencing of the Promoter Regions

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

streptavidin-coated paramagnetic beads (Dynal), in accordance with the manufacturer's instructions. Measurements were performed with the PSQ96 system (Biotage AB) and, in the case of the *CIAS1* gene, with the PSQ96HS system as well. Each sample was run in quadruplicates (see above).

Genotyping

SNPs in the promoter regions and in the 3′ UTRs of the genes were identified via PCR amplification and sequencing of genomic DNA fragments. Promoter fragments were located 5′ of the transcription start site and were at least 500 bp long. Information on the 3′ UTR was derived from public databases, and 3′ UTRs were sequenced in full length. For detailed primer information, see tables 2 and 3. To establish SNP associations, 171 patients and 182 controls were analyzed for the presence of the promoter SNPs, excepting *VNN1,* via an allele-specific PCR assay. Two different forward primers were designed with the most 3′ base representing either the SNP or the wild-type allele. Each forward primer was combined with a different reverse primer, so that the PCR products differed in size. For detailed primer information, see table 4. PCR was performed using the PCR Master Mix (Promega), with 45 cycles of 94°C for 1 min, 62°C–65°C for 1 min, and 72°C for 1 min. The PCR products were analyzed on 2% agarose gels (GTQ, Roth).

Because the presence of the SNP at position −137 of the *VNN1* gene generates a recognition sequence for the endonuclease *Mnl*I, this SNP was genotyped via PCR and restriction analysis. First, a 301-bp fragment was amplified using the primers 5′-GCAGCCCTAGCGAACTAATG-3′ and 5′-CAGCTGCAGTGAAAGTGTCC-3′. PCR was performed using the PCR Master Mix (Promega), with 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCR products were then purified using the Invisorb PCRapid Kit (Invitek), before 4 μl of the purified PCR product was digested using 10 units of *Mnl*I (MBI Fermentas) at 37°C. After that, the DNA fragments were analyzed on 2.5% agarose gels (GTQ, Roth).

Gene Expression Analyses in Articular Chondrocytes

Total RNA was isolated from the articular cartilage of patients with OA, via the guanidinium-isothiocyanate method. Directly after joint replacement surgery, cartilage pieces were obtained from the joint surfaces and were frozen in liquid nitrogen. Then, the tissue was minced in liquid nitrogen using a mortar and pestle, was resuspended in guanidinium-isothiocyanate solution, and was homogenized using a syringe and needle. After centrifugation, the supernatant was added to a cesium chloride solution and was ultracentrifugated for 15–20 h at 20,000 rpm. The RNA pellet was then resuspended in diethyl pyrocarbonate–H_2O, followed by the removal of remaining proteins via a phenol/chloroform extraction. Finally, the RNA was precipitated by the addition of yeast tRNA, sodium acetate, and ethanol. cDNA synthesis was performed as described above. Cartilage-specific gene expression was analyzed via PCR amplification of cDNA, with use of the exon-overlapping primers *CIRBP* (MIM 602649) 5′-CCGCTTGCGTCAGGGACCTG-3′ (forward), *CIRBP* 5′-CAGCCATTGGAAGGACGATCT-3′ (reverse), *BLK* (MIM 191305) 5′-GAAGTTGCTCGTTGTCGCT-3′ (forward), *BLK* 5′-TCATCCAGGTGTTCATC-3′ (reverse), *DKFZP434O1427* 5′-CGAGGCCAGAGAGAAAAGAC-3′ (forward), *DKFZP434O1427* 5′-CCAGCTACTCCCTTCTTTGG-3′ (reverse), *FIZZ3/RETN* (MIM 605565) 5′-TGCAGGATGAAAGCTCTCTG-3′ (forward), *FIZZ3/RETN* 5′-TCTCCAGGTTTATTTCCAGCTC-3′ (reverse), *VNN1* 5′-ACGTGGCAATTTTGCTTTTC-3′ (forward), *VNN1* 5′-TCACCAAGGTAACAGCAGGA-3′ (reverse), *TXNDC3* (forward) 5′-GATGAGGCAGCTGAAGAACA-3′, and *TXNDC3* (reverse) 5′-CACTAAAGAGAAAAACAGGTTCACA-3′. The PCR conditions were 45 cycles of 94°C for 1 min, 50°C–55°C for 1 min, and 72°C for 1 min. PCR amplification of β-actin–specific cDNA was used as a positive control, with the primers 5′-CGGGAAATCGTGCGTGACAT-3′ (forward) and 5′-GAACTTTGGGGGATGCTCGC-3′ (reverse) and 20 cycles of 95°C for 1 min, 65°C for 2

Table 4

Primers for Analyzing SNP Associations via Allele-Specific PCR

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 5

Summary of Microarrays Probed

Array	Probe
1	Pool of $CD4^+$ cells from controls 1–6
2	$CD4^+$ cells from control 1
3	$CD4^+$ cells from control 2
4	$CD4^+$ cells from patient 1
5	$CD4^+$ cells from patient 2
6	$CD4^+$ cells from patient 3
7	$CD4^+$ cells from patient 4
8	$CD4^+$ cells from patient 5
9	$CD4^+$ cells from patient 6
10	Pool of $CD8^+$ cells from controls 1–5
11	$CD8^+$ cells from control 1
12	$CD8^+$ cells from control 2
13	Pool of $CD8^+$ cells from patients 1–3, 6
14	$CD8^+$ cells from patient 4
15	$CD8^+$ cells from patient 5
16	Pool of $CD14^+$ cells from controls 1–4, 6
17	$CD14^+$ cells from control 1
18	$CD14^+$ cells from control 2
19	$CD14^+$ cells from patient 1
20	$CD14^+$ cells from patient 4
21	$CD14^+$ cells from patient 5
22	$CD14^+$ cells from patient 6
23	Pool of $CD19^+$ cells from control 1–4
24	$CD19^+$ cells from control 2
25	Pool of $CD19^+$ cells from patients 1, 2, 4–6
26	$CD19^+$ cells from patient 3

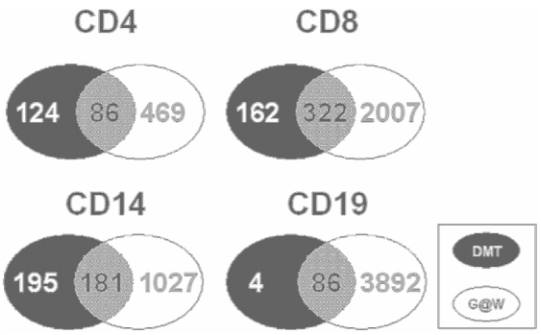

Figure 1 Combination of two software tools, which reduces the number of differentially expressed candidate genes and lends more weight to genes identified via two different methods. For each cell type, lists of differentially expressed genes were compiled using DMT and Genes@work (G@W), before intersections were formed. Black numbers on the gray intersection indicate the results.

min, and 72°C for 1.5 min. PCR products were visualized via agarose gel electrophoresis.

Bioinformatic and Statistical Analyses

Differences in mRNA expression levels between patients with OA and healthy controls were assessed via Affymetrix technology. For bioinformatics analyses, we combined two different software tools, the Affymetrix Data Mining Tool (DMT) (v. 3.01) and Genes@work (v. October 2002). With use of the DMT, comparisons were made between the signal log ratios of individual chips, followed by two-sided Mann-Whitney tests, to identify P values <.05. The only genes considered for further analysis were those that showed a statistically significant differential expression for 50% of the comparisons made for $CD4^+$, $CD8^+$, and $CD14^+$ cells, and for 100% of the comparisons made for $CD19^+$ B cells. For the Genes@work analysis, we preselected "present" genes, performed pattern analyses based on the signal values, and chose the pattern containing the highest number of genes.

We assessed SNP associations by performing the χ^2 test, including Yate's continuity correction, based on the number of alleles. P values were subsequently corrected in accordance with Bonferroni. Testing for Hardy-Weinberg equilibrium (HWE) was performed using the software Arlequin v2.0.

Results

Transcriptome Analysis of PBMC Reveals 26 Genes Differentially Expressed in Patients with OA

To identify genes differentially expressed in patients with OA and in healthy controls, we performed transcriptome analyses on PBMC from six patients and six age-matched controls. In detail, we isolated the $CD4^+$ and the $CD8^+$ T cells, the $CD19^+$ B cells, and the $CD14^+$ monocytes, to >98% purity each. From these 48 cell populations, first-strand cRNA was synthesized. For the controls, two individual cRNAs of each cell type and cell type–specific pools were hybridized to HG-U133A gene chips. For the patients, our goal was to hybridize as many individual cRNAs as possible. However, since some individual yields of mRNA were <5 μg, samples had to be pooled. In total, 26 HG-U133A gene chips were probed, as outlined in table 5.

For bioinformatic analyses, we combined two different software programs, to generate lists of genes either up- or down-regulated in patients with OA. We used the DMT to calculate statistically significant expression differences between patients and controls. The second software program, Genes@work, was used to search for similarities in gene expression among patients or controls and to produce lists of genes for which the expression is similar among the patients but different from the controls. Since both methods yield long lists of differentially expressed genes, we formed the intersections to lend more weight to genes identified via two different methods. Figure 1 illustrates our results. In total, the DMT revealed 210 differentially expressed genes for the CD4, 484 for the CD8, 376 for the CD14, and 90 for the $CD19^+$ cells. In contrast, larger lists resulted from the Genes@work analysis, yielding 555 differentially expressed genes for the CD4, 2,329 for the CD8, 2,208 for the CD14, and 3,978 for the $CD19^+$ cells. The intersections contain 86, 322, 181, and 86 genes, respectively.

It was our goal to identify among these differentially expressed genes those candidates that carry *cis*-regulatory gene polymorphisms. Assuming that *cis*-regulation will be manifested in any cell type expressing the gene of interest, we disregarded all the genes that, even though present in all four cell types (see table 6), did not show differential expression in all cell types. However, we did allow for cell type–specific differential gene expression, provided that these genes were expressed exclusively in that particular cell type. To avoid mere inflammation markers, we excluded those genes that also showed a differential expression in PBMC from patients suffering from chronic inflammatory joint diseases (A. Gruetzkau, personal communication). We thus reduced the list of candidate genes to the 26 listed in table 7. The signal values for these 26 genes obtained from all gene chips are listed in table 6. The mean fold changes were 1.37 for the $CD19^+$ B cells, 1.48 for the $CD4^+$ T helper cells, 1.53 for the $CD8^+$ cytotoxic T cells, and 1.72 for the $CD14^+$ monocytes. The corresponding SDs were 0.73, 0.76, 1.06, and 0.57, respectively.

Table 6

Signal Values and Detection Calls Obtained from All Gene Chips

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 7

Differentially Expressed Genes in OA

Gene	Protein
Expressed in $CD4^+$ cells only:	
FBXL8	F-box and leucine-rich repeat protein 8
Expressed in $CD8^+$ cells only:	
S100B	S100 calcium-binding protein beta (neural)
Expressed in $CD14^+$ cells only:	
ASGR2	Asialoglycoprotein receptor 2
CIAS1	Cold autoinflammatory syndrome 1
DYSF	*DYSF*erlin, limb girdle muscular dystrophy 2B
FIZZ3/RETN	Found in inflammatory zone 3
HM74	Putative chemokine receptor, GTP-binding protein
ICAM4	Intercellular adhesion molecule 4
IRAK3/IRAK-M	Interleukin-1 receptor-associated kinase 3
KIAA0994/PADI2	Peptidyl arginine deiminase, type II
MGAM	Maltase-glucoamylase (alpha-glucosidase)
TXNDC3	Thioredoxin domain containing 3
VNN1	Vanin 1, vascular non-inflammatory molecule
Expressed in $CD19^+$ cells only:	
BLK	B lymphoid tyrosine kinase
GH1	Growth hormone 1
Expressed in all four cell types:	
RHOB	*RHOB,* GTP-binding protein
BLP2	BBP-like protein 2
CIRBP	Cold inducible RNA binding protein
DKFZP434O1427	Dactylidin
DUSP1	Dual specificity phosphatase 1
EIF4A1	Eukaryotic translation initiation factor 4A1
GPRK6	G-protein-coupled receptor kinase 6
H2AFO	H2A histone family, member O
H3F3B	H3 histone, family 3B (H3.3B)
SUI1	Putative translation initiation factor
TRC8	Patched-related protein translocated in renal cancer

Figure 2 outlines the experimental workflow pursued to identify *cis*-regulation and disease association among the 26 candidate genes. First, the genomic DNA of the six patients and six controls were screened for allelic differences in the 5′ and 3′ UTRs of all candidate genes. Any SNP detected was considered for pyrosequencing, which would reveal allelic imbalances and proof of *cis*-regulation. Moreover, SNPs in the 5′ regions were directly analyzed for disease association, provided that they showed a disparate distribution among the six patients and six controls that correlated with the differential expression seen on the gene chips. Disease association then prompted the analysis of gene expression in articular chondrocytes, to strengthen a functional relevance for the pathogenesis of OA. Genes carrying a disease-associated SNP and expressed in chondrocytes will be considered for further functional analysis. This analysis could be overexpression or knockdown in chondrocytic cells, for the investigation of the genes' impact on apoptosis and cytokine response. Genes showing neither disparate promoter SNP distribution nor disease association underwent no further analysis.

Analyses of Large Cohorts Reveal Disease Associations for 5′ SNPs in the RHOB *and* TXNDC3 *Genes*

To identify new polymorphisms or to confirm previously published ones in the 5′ regions of our candidate genes, we sequenced the genomic DNA from the six patients and the six controls. In detail, we sequenced up to 700 bp upstream of the respective transcriptional start sites, and we also sequenced the 5′ UTRs if polymorphisms had been indicated. For seven genes, no polymorphisms were found. All the other 5′ SNPs were tested, patient by patient, for a correlation with the respective expression signals (see table 6). Figure 3 summarizes the most promising results. Whereas, for *RHOB, BLK, CIRBP,* and *VNN1,* an increased expression in the patients correlates with an increased frequency of the minor promoter allele, the situation is reversed for *DKFZP434O1427*. Here, the decreased expression in the six patients is paralleled by a decreased frequency of the minor promoter allele. For *FIZZ3/RETN,* an increased expression in the patients correlates with a decreased frequency of the minor allele. *TXNDC3* represents yet another situation, since the

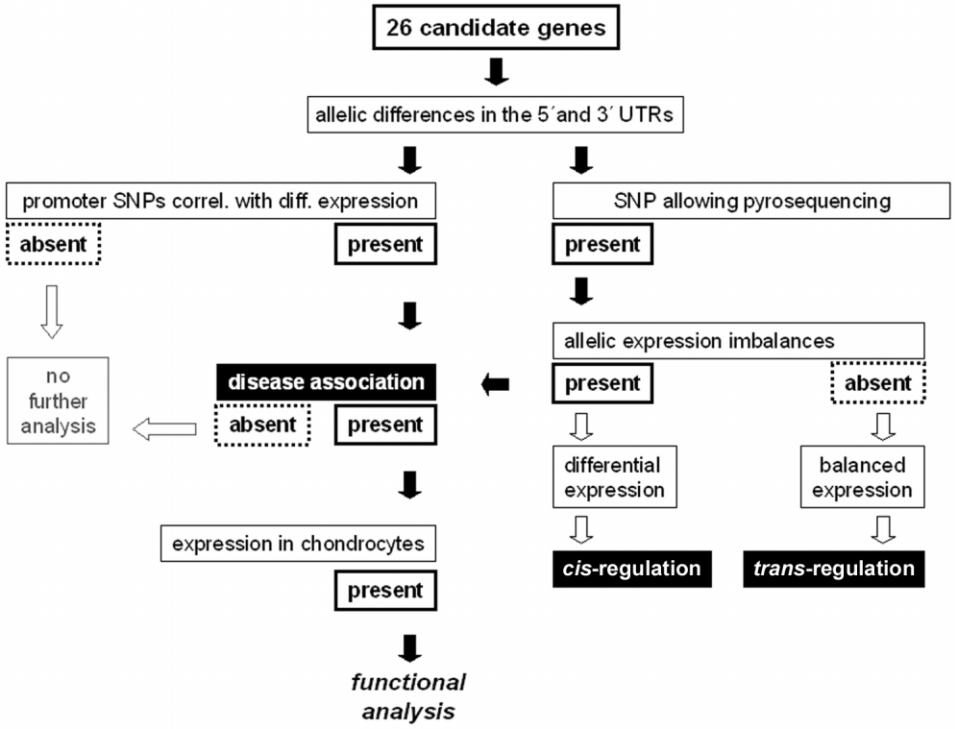

Figure 2 Workflow for the identification of *cis*-regulated and disease-associated genes among the 26 candidates. Our screening focuses on allelic expression imbalances, disease-associated promoter polymorphisms, and expression in articular chondrocytes.

gene is switched off in the controls, as shown by mean signal values of 79.6 in monocytes. However, in the patients, elevated signal values directly correlate with the presence of the T allele at position 428. Signal values are 96.5 and 99 for the CC, 178.6 for the CT, and 208.5 for the TT genotype (see table 6).

To screen a large cohort of patients with OA for an association with any of these 5′ SNPs, we established high-throughput PCR assays for the A/G SNP at position −165 of *RHOB,* for the C/T SNP at position −1218 of *BLK,* for the G/A SNP at position −342 of *CIRBP,* for the G/T SNP at position −81 of *DKFZP434O1427,* for the C/G SNP at position −180 of *FIZZ3/RETN,* and for the C/T SNP at position 428 of *TXNDC3*. For the A/C SNP at position −137 of *VNN1,* a combined PCR/restriction assay was established. A total of 171 patients and 182 controls were analyzed, and table 8 summarizes the results. Allele distributions for all controls, except for *DKFZP434O1427,* and for all patients, except for *RHOB* and *VNN1,* conform to HWE. Whereas no disease association was found for *BLK, CIRBP, DKFZP434O1427, FIZZ3/RETN,* and *VNN1,* statistically significant associations with OA resulted for the −165 promoter SNP of *RHOB* and for the 428 SNP of *TXNDC3,* with corrected *P* values of .0007 each.

Gene Expression in Chondrocytes Supports a Role of Candidate Genes in the Pathogenesis of OA

Because our initial transcriptome analysis was performed on PBMC, formal proof of gene expression in chondrocytes would lend weight to the role of the candidate genes in the pathogenesis of OA. To that extent, we extracted mRNA from the cartilage of patients with OA undergoing joint replacement surgery, transcribed it into cDNA, and assayed for the presence of *BLK*-, *CIRBP*-, *DKFZP434O1427*-, *FIZZ3/RETN*-, *VNN1*-, and *TXNDC3*-specific transcripts. *RHOB* has been shown elsewhere to be expressed in articular chondrocytes.[21] Figure 4 summarizes the data. Whereas *CIRBP, DKFZP434O1427,* and *TXNDC3* are expressed abundantly, *BLK, FIZZ3/RETN,* and *VNN1* are expressed at lower levels, and sequence-specific PCR products could be visualized only in samples with high concentrations of cDNA. We thus conclude that our differentially expressed candidate genes *RHOB, BLK, CIRBP, DKFZP434O1427, FIZZ3/RETN, VNN1,* and *TXNDC3* are expressed in articular chondrocytes.

Allelic Expression Imbalances Confirm Cis*-Regulation for* RHOB *and* TXNDC3

To provide direct proof for *cis*-regulation, we screened for expression imbalances. We used the pyrosequencing

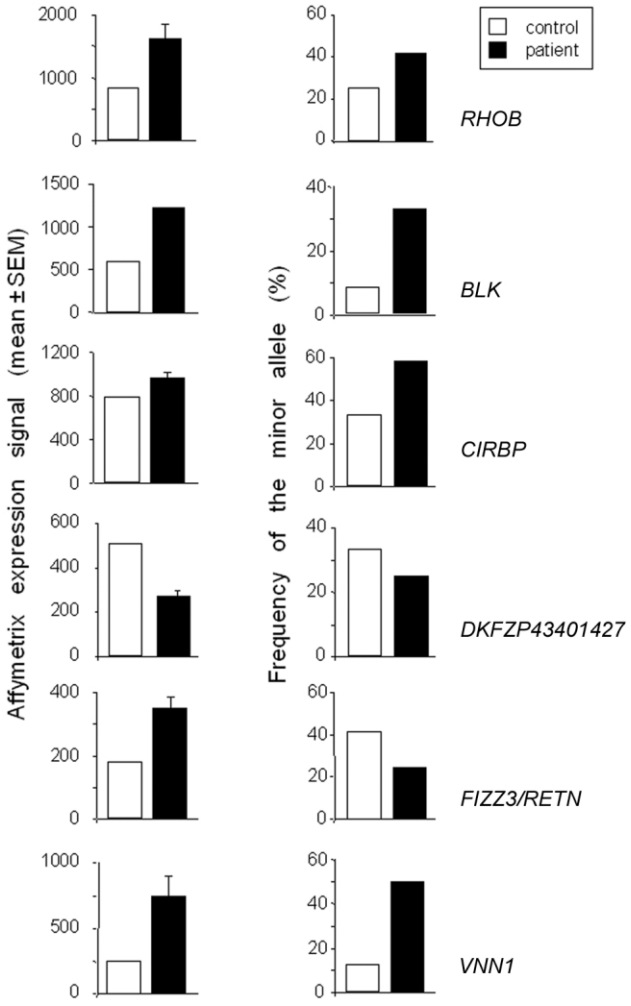

Figure 3 Correlation between the presence of promoter SNPs and expression signals, which hints at disease-associated regulatory polymorphisms. Expression signals from $CD14^+$ monocytes (*RHOB, CIRBP, DKFZP434O1427, FIZZ3/RETN,* and *VNN1*) and $CD19^+$ B cells (*BLK*) were averaged for the patients and were compared to the pooled data from the controls in the left panels, detailing the differential expression. The right panels compare the frequencies of the minor alleles of promoter SNPs unevenly distributed between patients and controls. Whereas *BLK* is expressed exclusively in B cells and *FIZZ3/RETN* and *VNN1* in monocytes, *RHOB, CIRBP,* and *DKFZP434O1427* are expressed in all four cell types. The *P* values of .012, .009, and .002 summarize their differential expression and result from a Mann-Whitney test performed on the transcriptional values from all cell types.

method, to quantify the amount of transcript arising from each allele. The requirements for this method—a suitable SNP allowing for the discrimination of both alleles on the cDNA level and individuals heterozygous for this particular SNP—were met by 16 of our candidate genes. However, because of homopolymeric stretches close to the SNP, sequences leading to template loops during the assay, absence of sufficient amounts of mRNA, and very low frequency of that particular SNP allowed for the successful analysis of *RHOB, BLP2, CIAS1,* and *TXNDC3* only. Table 9 lists the dbSNP accession numbers of the SNPs used, their positions with respect to the transcriptional start site, the heterozygous donors, and the relative expressions arising from the sets of two alleles. Whereas no allelic imbalances could be observed for *BLP2* and *CIAS1,* differential expression on the allele level and, thus, *cis*-regulation is confirmed for both *RHOB* and *TXNDC3*.

Absence of Allelic Imbalances and On/Off–Switching of Genes Suggests Trans*-Regulation*

The absence of allelic imbalances for *BLP2* and *CIAS1* suggest *trans*-regulatory elements controlling the gene expression (table 4). Alternative evidence for *trans*-regulation is provided for *FBXL8* (MIM 609077), *MGAM* (MIM 154360), and *TXNDC3*. These genes are switched off in the healthy donors, as indicated by "absent" and "marginal" expression signals on the gene chips. At the same time, they are switched on in the patients (see table 6). Interestingly, *TXNDC3* gene regulation occurs both in *cis* and in *trans*.

Discussion

Ever since differential gene expression has been implicated in driving evolutionary processes,[16] regulatory gene polymorphisms have gained increasing attention. Although the differential expression of genes allows for a flexible response to a changing environment and may thus be beneficial for the population as a whole, adverse effects for the individual may occur.[22] Indeed, regulatory gene polymorphisms within the *IL1* locus have been described elsewhere to be associated with OA.[18,19]

Our approach to identify novel OA-associated regulatory gene polymorphisms is based on an initial transcriptome analysis used to define genes that are differentially expressed between patients and controls. We chose PBMC for our transcriptome analysis for two reasons: (1) Patient and control material is easily accessible and can be obtained under identical conditions, and (2) even though gene expression may be strictly cell type–specific, numerous genes are expressed in a spatially less-exquisite fashion. Indeed, data from our own lab and the Affymetrix technical notes confirm that 40%–60% of all genes represented on the HG-U133A gene are transcribed in any tissue or cell type tested.[23] The overlap of genes simultaneously expressed in various cell types is all the larger the closer the cells are related. Indeed, both immune cells and chondrocytes are of mesodermal origin, the development of the articular cartilage/bone system and of the adaptive immune system is phylogenetically tightly linked, and both systems use the same

Table 8

5′ SNPs in the *RHOB* and *TXNDC3* Genes Show Association with OA

Gene	Position	dbSNP Accession Number	SNP	Allele Distribution (Controls)	HWE *P* Value (Controls)	Allele Distribution (Patients)	HWE *P* Value (Patients)	SNP Association *P* Value
BLK	−1218	*rs27366344*	C/T	84.2/11.1	.491	85.6/14.4	.294	.243
CIRBP	−342	*rs12980707*	G/A	85.4/14.4	.124	80.3/19.7	.572	.1058
DKFZP434O1427	−81	*rs877661*	G/T	77.7/22.3	.025	74.2/25.8	.46	.312
VNN1	−137	*rs4897612*	A/C	70.5/29.5	.863	69.2/30.8	.011	.7882
FIZZ3/RETN	−180	*rs1862513*	C/G	68.9/31.1	.699	75.9/24.1	.786	.3353[a]
RHOB	−165	*rs49846015*	A/G	76.1/23.9	.569	60.8/39.2	.005	.0007[a]
TXNDC3	428	*rs4720262*	C/T	86.6/13.4	.251	74.1/25.9	.149	.0007[a]

[a] *P* values were corrected according to Bonferroni; *P* values <.05 show deviation from HWE.

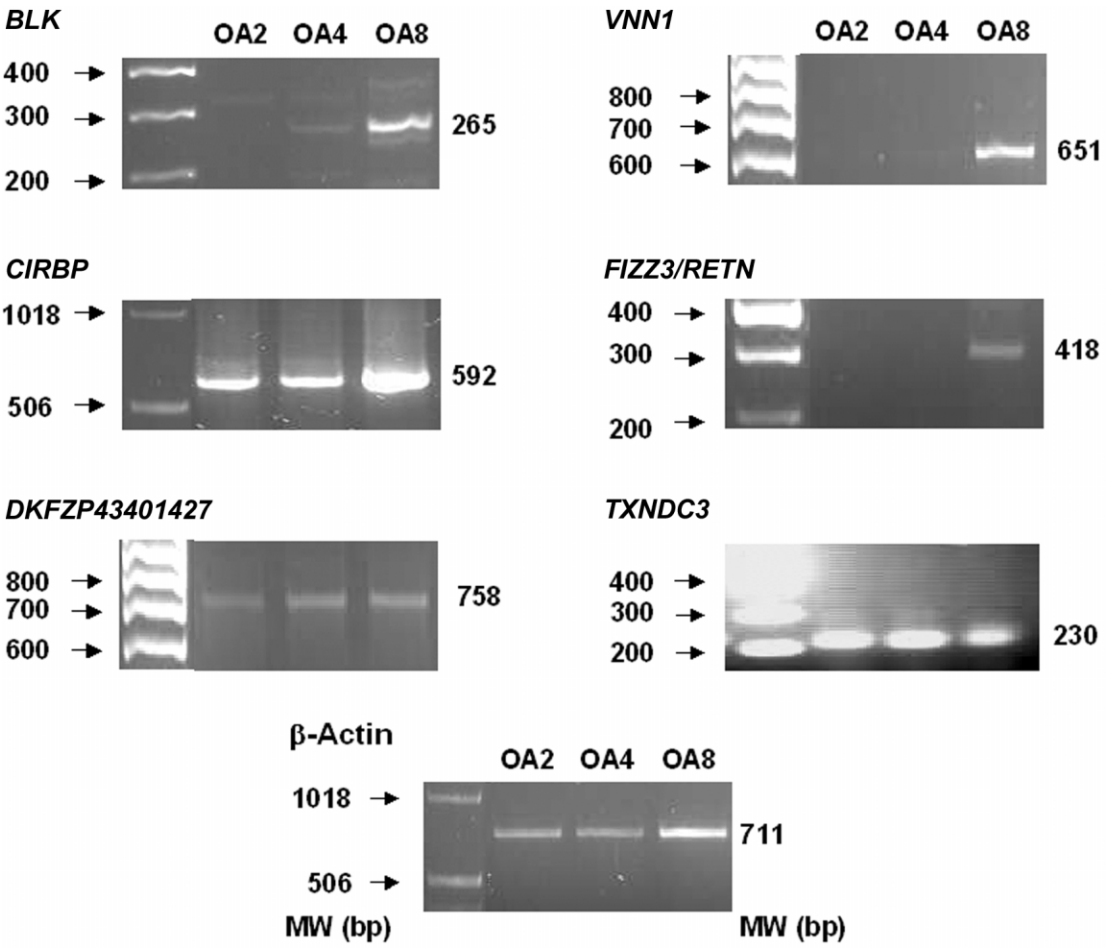

Figure 4 Gene expression in chondrocytes supporting a role for candidate genes in the pathogenesis of OA. RNA isolated from the cartilage of patients with OA undergoing joint replacement surgery was transcribed into cDNA and was assayed for the presence of specific transcripts of *BLK*, *CIRBP*, *DKFZP434O1427*, *FIZZ3/RETN*, *VNN1*, and *TXNDC3*. The comparison to β-actin indicates the use of unequal amounts of template cDNA for the three patients. Size markers are indicated on the left of each panel, and the sizes of the specific PCR products are given on the right. MW = molecular weight.

Table 9

***RHOB* and *TXNDC3* Show Allelic Imbalances**

Gene and Donor	Allelic Expression[a]	dbSNP Accession Number	Position
RHOB:		*rs1062292*	+2749
Control 1	G 46.6/53.4 T		
Control 4	G 35.8/64.2 T		
Patient 3	G 27.4/72.6 T		
Patient 6	G 49.7/50.3 T		
BLP2:		*rs708394*	+10401
Control 3	T 47.0/53.0 C		
Control 4	T 49.8/50.2 C		
Patient 4	T 48.4/51.7 C		
CIAS1:		*rs10754558*	+30685
Control 1	C 50.3/49.7 G		
Control 2	C 50.1/49.9 G		
Control 3	C 52.6/47.4 G		
Patient 1	C 46.5/53.5 G		
TXNDC3:		*rs4720262*	+428
Control 1	G 48.1/51.9 A		
Patient 6	G 33.3/66.7 A		

[a] Allele 1 percentage/allele 2 percentage.

cytokines and growth factors as messenger molecules.[2] Our approach is, therefore, likely to identify genes associated with the systemic character of OA but is bound to miss those expressed in a cartilage-specific manner. Indeed, IL-1β was upregulated in some patients' monocytes; however, the difference between patients and controls was not significant enough to pass our stringent bioinformatics analyses.

We identified 26 genes that are differentially expressed between patients with OA and controls. Whereas *RHOB* and *S100B* (MIM 176990) have been directly linked to OA before,[21,24] *GH1* (MIM 139250) has been shown to be involved in chondrocyte differentiation in the growth plate and to be associated with short stature (SS [MIM 604271]).[25] Other genes—among them *RHOB, BLP2, CIAS1, DUSP1* (MIM 600714), *DYSF* (MIM 603009), and *S100B*—have been implicated in apoptosis and regulation of cell cycle and repair.[21,26–30] A link to the OA characteristic of imbalance of cartilage degeneration and regeneration is, therefore, plausible. Other feasible candidates are transcription and translation factors like *DKFZP434O1427, FBXL8, EIF4A1* (MIM 602641), and *SUI1,*[31–34] even though their specific targets need to be identified before the link to OA can directly be explained. Another candidate, *VNN1,* is a glycosyl-phosphatidylinositol–anchored cell surface molecule, which has, so far, been implicated in the homing of T-cell progenitors to the thymus and in sexual development in the mouse.[35,36] Interestingly, in mouse testis, *VNN1* is regulated by Sox-9, a transcription factor involved in chondrocytic differentiation during development and still expressed in articular chondrocytes.[37,38] An additional interesting candidate is *TXNDC3,* which shows *cis*-regulation and carries a 5′ SNP with a statistically significant association with OA. Likewise, the thioredoxin protein NM23-H8 encoded by *TXNDC3* has been described elsewhere to be expressed in testis.[39,40] Here, we add the expression in monocytes and chondrocytes; however, the exact role of *TXNDC3* for the pathogenesis of OA needs to be further investigated.

To identify regulatory gene polymorphisms responsible for the differential expression, we pursued two different routes. The first one focused directly on polymorphisms in the 5′ regions of the genes, which were screened (1) for a disparate distribution among patients and controls and (2) for a correlation between the SNP haplotype and the signal value. Even though the analysis of six patients and six controls uses too few subjects for any firm conclusions to be drawn, we demanded an obvious correlation between the presence of a regulatory gene polymorphism and the differential expression documented in signal values. This correlation is graphically documented for six genes and their SNPs in figure 3. These six SNPs and the 5′ SNP of *TXNDC3* were subsequently analyzed for disease associations, with the use of larger cohorts. Interestingly, except for the *DKFZP434O1427* gene, the tendencies for the rare alleles found in the six patients and six controls initially analyzed were again reflected in the larger cohorts. The 5′ SNP in the *TXNDC3* gene is located in the second exon, which is noncoding, and the direct correlation of the T allele with an elevated expression hints either at a tight linkage disequilibrium with a regulatory SNP or at some regulatory function. The latter is supported by the observation that *TXNDC3* transcripts seem to be alternatively spliced in monocytes and chondrocytes, with the chondrocyte transcripts lacking exon 2 (data not shown). In total, we identified two OA-associated polymorphisms in the genes encoding *RHOB* and *TXNDC3*. And, whereas no direct statistical significance could be observed for the other five genes tested, the genotype distributions for *VNN1* and *DKFZP434O1427* and the resulting differences in the Hardy-Weinberg disequilibria between patients and controls suggest that the analysis of larger cohorts may yield more disease associations. The second route pursued the identification of OA-associated regulatory gene polymorphisms and focused on differential expression detectable on the allele level. To that end, we performed pyrosequencing analyses and used SNPs in the transcribed regions of the genes, to differentiate both alleles. We did not, however, assume that a SNP in the 3′ UTR might be the regulatory polymorphism itself, nor do we know about any linkage disequilibrium with a 5′ regulatory SNP. Any negative result could consequently be a false negative one. Even though pyrosequencing is a very powerful method, there are numerous technical restrictions, and, among our 26 candidates, pyrosequencing analysis could be performed

for 4 genes only. Two of them, *RHOB* and *TXNDC3,* showed allelic imbalances and, thus, direct proof of a *cis*-regulated gene expression. In contrast, *BLP2* and *CIAS1* showed a balanced expression hinting at *trans*-regulation. It should be mentioned, though, that balanced expression per se does not exclude the possibility of *cis*-regulation and differences in allelic expression in individuals not tested here. Supporting *trans*-regulation for *BLP2* and *CIAS1* are the lack of suggestive 5′ polymorphisms in both genes, yet it was beyond the scope of this article to scan more distantly for SNPs. Also beyond scope was the follow up on *trans*-regulated gene expression.

In summary, we identified 26 genes that are differentially expressed in OA patients and that may contribute to the pathogenesis of the disease. We confirmed allelic imbalances and, hence, a *cis*-regulated gene expression for *TXNDC3* and *RHOB*. For both, 5′ SNPs that show a statistically significant association with OA have been identified. Whether these SNPs are true regulatory gene polymorphisms needs to be confirmed with molecular methods. The elucidation of the specific function of the various genes in the context of OA will help the understanding of the pathogenesis of the disease and will hint at novel therapeutical approaches.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Affymetrix DMT, http://www.Affymetrix.com/support/technical/technotesmain.affx

dbSNP, http://www.ncbi.nlm.nih.gov/SNP/

Genes@work, http://www.research.ibm.com/FunGen/FGDownloads.htm#GAW

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for OA, *VNN1, RHOB, CIAS1, TXNDC3, CIRBP, BLK, FIZZ3/RETN, FBLX8, MGAM, S100B, GH1,* SS, *DUSP1, DYSF,* and *EIF4A1*)

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