N-cadherin promoter polymorphisms and risk of osteoarthritis

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ABSTRACT **Osteoarthritis (OA) is the most common form of arthritis. It is characterized by cartilage destruction and bone remodeling, mediated in part by synovial fibroblasts (SFs). Given the functional significance of cadherins in these cells, we aimed at determining the role of genetic variants of N-cadherin (*CDH2*) in OA of the knee and hip. Six single-nucleotide polymorphisms in the genomic region of the *CDH2* gene were genotyped in 312 patients with OA and 259 healthy control subjects. Gene expression of *CDH2* was analyzed by qRT-PCR. Liquid chromatography-mass spectrometry was used to identify a transcription factor isolated by DNA pulldown. Its potential for binding to gene variants was examined by electrophoretic mobility shift assay, enzyme-linked immunosorbent assay, and chromatin immunoprecipitation. Genetic analysis identified a polymorphism located in the *CDH2* promoter region to be associated with risk of OA. The minor allele of rs11564299 had a protective effect against OA. Compared to carriers of the major allele, carriers of the minor allele of rs11564299 displayed increased N-cadherin levels in SFs. Based on *in silico* analysis, the minor allele was predicted to generate a novel transcription factor binding site, Direct-binding assays and mass spectrometric analysis identified hnRNP K as binding selectively to the minor allele. In summary, a *CDH2* promoter polymorphism influences the risk of OA, and hnRNP K was found to be involved in the regulation of elevated N-cadherin expression in patients with OA carrying the minor allele of rs11564299.—Ruedel, A., Stark, K., Kaufmann, S., Bauer, R., Reinders, J., Rovensky, J., Blažičková, S., Oefner, P. J., Bosserhoff, A. K. N-cadherin promoter polymorphisms and risk of osteoarthritis. *FASEB J.* 28, 683–691 (2014). www.fasebj.org**

Key Words: genetic association study · CDH2 · hnRNP K · rs11564299

Osteoarthritis (OA) is the most common form of arthritis. Cartilage destruction and bone remodeling characterize this painful and disabling disease. OA is a complex disease, and both environmental and genetic factors play a role in disease susceptibility. Several genetic loci with genome-wide significance have been identified for hip and knee OA (1–3). Three important points came up in these genetic studies: first, the effects of the common variants are low to moderate; second, differences between European and Asian study samples have been reported; and third, joint-specific genetic effects are involved (4, 5). Despite the replication of genetic risk variants, the molecular pathogenesis is still poorly understood. However, there is growing evidence that fibroblasts of the synovial membrane from OA patients [OA synovial fibroblasts (OASFs)] play an important role. It has been shown that activated OASFs are capable of migrating into and destroying the cartilage (6), thereby contributing to disease progression and severity.

Cadherins are a family of structurally related, calcium-dependent cell–cell adhesion proteins that play important roles in cell adhesion and migration in processes ranging from embryogenesis to cancer metastasis (7). Human *in vitro* and murine *in vivo* studies have established a critical role of cadherin 11 in the development and architecture of the synovium and in modulating the capacity of synovial fibroblasts (SFs) to produce cytokines, chemokines, and other inflammatory factors (8, 9). Less is known about the role of N-cadherin, which is encoded by the *CDH2* gene (10), has been found to be coexpressed with cadherin 11 on murine

Abbreviations: ChIP, chromatin immunoprecipitation; ds-oligonucleotides, double-stranded oligonucleotides; ELISA, enzyme-linked immunoabsorbent assay; EMSA, electrophoretic mobility shift assay; hnRNP K, heterogeneous nuclear ribonucleoprotein K; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; OA, osteoarthritis; OASF, osteoarthritis synovial fibroblast; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SF, synovial fibroblast; SNP, single-nucleotide polymorphism

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SFs (11), and has been reported to suppress or promote invasion in different kinds of cancer. For example, the loss of N-cadherin has been reported to lead to reduced cell–cell adhesion in osteosarcoma. Therefore, the absence of N-cadherin promotes metastasis in these cells (12). In contrast, it has been shown that N-cadherin expression in, for example, melanoma cells or pancreatic cancer cells leads to increased metastasis; therefore, overexpression promotes migration (13–15). Here, we describe changes of N-cadherin expression at the mRNA level in OASFs in association with a single-nucleotide polymorphism (SNP) in the N-cadherin promoter. Bringing together both genetic association studies and functional data could lead to the identification of novel pathways of OA onset and progression.

MATERIALS AND METHODS

Study population

A total of 571 Slovak individuals (127 males, 444 females) were included in the study: 312 patients with OA (60 males, 252 females) and 259 healthy control subjects (67 males, 192 females). OA disease status of the knee, hand, and hip were clinically and radiographically ascertained. The control subjects were selected by clinical exclusion of arthritic or degenerative symptoms. Further phenotype details are shown in **Table 1**. Our study case patients and control subjects did not differ in gender, but the latter were significantly younger ($P<0.001$).

Subjects' written consent was obtained according to the current Declaration of Helsinki. The study was approved by the ethics committees of the Slovakia National Institute of Rheumatic Diseases (Piestany, Slovakia) and the University of Regensburg (Regensburg, Germany).

Marker selection and genotyping

SNPs in the *CDH2* genomic region (NM_001792.3) on chromosome 18 were selected to cover the gene without redundancy with respect to linkage disequilibrium (LD) structure: 1 SNP at the distal promoter (rs11083271), 1 SNP at the proximal promoter (rs11564299), 1 SNP in intron 2 (rs11564392), and 1 SNP at the 3′ end of the gene (rs2871593). In addition, rs4131805, located 302 kb upstream of the *CDH2* 5′ end, was included because of a reported association with bone density and geometry (16). Therefore, 542 kb of the genomic region was analyzed with ≥1 SNP in each distinct LD block (**Fig. 1*A***). After a first round of association testing, SNP rs11083255 was analyzed as an additional marker in the *CDH2* promoter region. For information on all SNP markers analyzed, see **Table 2**.

Genomic DNA was isolated from whole-blood specimens with the PureGene DNA Blood Kit (Qiagen, Hilden, Germany). DNA samples were genotyped with 5′ exonuclease TaqMan technology (Life Technologies; Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. In brief, for each genotyping experiment, 10 ng DNA was used in a total volume of 5 μl containing 1× TaqMan Genotyping Master Mix. Polymerase chain reaction (PCR) and post-PCR end point plate reading was performed on an Applied Biosystems 7900HT Real-Time PCR System. Sequence Detection System 2.3 software (Life Technologies) was used to assign genotypes applying the allelic discrimination test. Case and control DNA were genotyped together on the same plates with duplicates of samples (10%) to assess intraplate and interplate genotype quality. No genotyping discrepancies were detected. Assignment of genotypes was performed by a person without knowledge of the proband's affection status.

Cell culture, RNA extraction, and real-time quantitative reverse transcription PCR (qRT-PCR) analysis

Synovial tissue specimens were obtained from patients with OA immediately after the knee joint capsule was opened (17). Synovial cells from patients with OA were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Munich, Germany), 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany), and penicillin and streptomycin (P/S) (PAA, Piscataway, NJ, USA). The same medium was used to grow the cell lines HSE (immortalized rheumatoid SFs) and K4Im (immortalized normal SFs), which were kindly provided by Prof. W. K. Aicher (University of Tübingen, Tübingen, Germany). Total cellular RNA was isolated from cultured cells with the RNeasy kit (Qiagen), and cDNAs were generated by reverse transcription. In parallel, genomic DNA from cultured cells for genotyping was isolated with the QIAamp DNA Mini Kit (Qiagen). qRT-PCR was performed with SYBR Green master mix (Qiagen), with the primer pair 5′-TGGATGAAGATGGCATGG-3′ and 5′-AGGTGGCCACTGTGCTTAC-3′, targeting exon A to exon B of *CDH2*. Relative gene expression was normalized to β-actin mRNA levels by using

TABLE 1. *Characteristics of study population for association analyses*

Variable	OA case patients	OA-free control subjects
n	312	259
Gender, [% female (*n*)]	80.8 (252)	74.1 (192)
Age at inclusion [yr (range)]	57.9 ± 13.5 (21–83)	39.3 ± 15.1 (18–78)***
Age of onset [yr (range)]	50.8 ± 12.8 (14–82)	NA
Isolated knee OA (*n*)	68	–
Isolated hip OA (*n*)	74	–
Isolated hand OA (*n*)	19	–
Combined knee and hip OA (*n*)	79	–
Combined knee and hand OA (n)	7	–
Combined hip and hand OA (*n*)	5	–
Combined knee, hip and hand OA (*n*)	5	–
Not classified (*n*)	55	–

Values are means ± SD. NA, not applicable. ***$P < 0.001$.

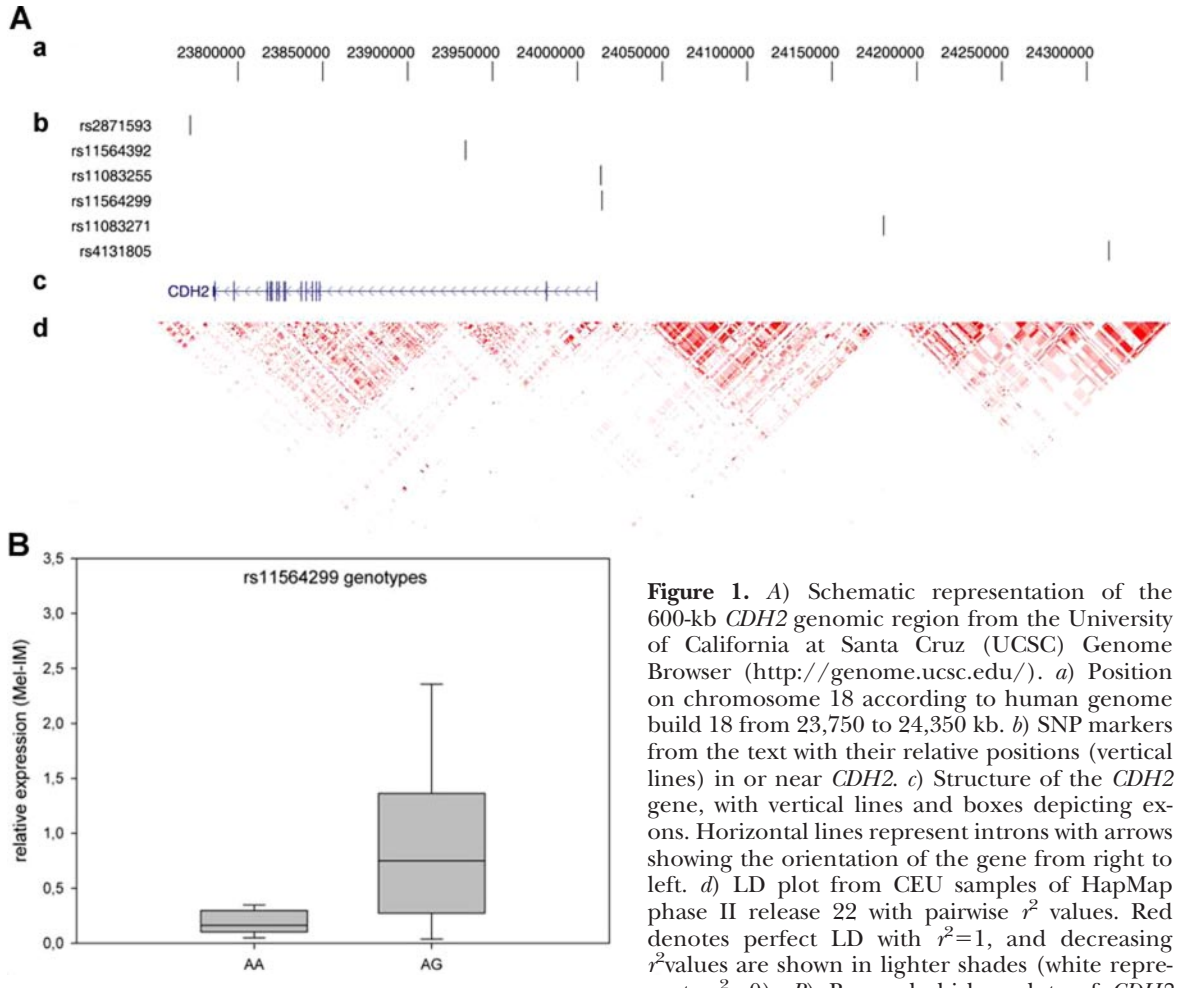


Figure 1. *A*) Schematic representation of the 600-kb *CDH2* genomic region from the University of California at Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/). *a*) Position on chromosome 18 according to human genome build 18 from 23,750 to 24,350 kb. *b*) SNP markers from the text with their relative positions (vertical lines) in or near *CDH2*. *c*) Structure of the *CDH2* gene, with vertical lines and boxes depicting exons. Horizontal lines represent introns with arrows showing the orientation of the gene from right to left. *d*) LD plot from CEU samples of HapMap phase II release 22 with pairwise r^2 values. Red denotes perfect LD with $r^2=1$, and decreasing r^2values are shown in lighter shades (white represents $r^2=0$). *B*) Box-and-whisker plots of *CDH2* mRNA expression for rs11564299 genotypes. Boxes represent the 25th to 75th percentiles; horizontal lines within boxes represent median values. Whiskers show the 5th and 95th percentiles.

the comparative cycle threshold (C_t) method. The Mel-Im cell line was used as a calibrator sample.

Preparation of nuclear extracts

Nuclear extracts from OASFs were prepared by using the method of Dignam *et al.* (18).

Enzyme-linked immunoabsorbent assay (ELISA)

For quantitative measurement of the binding capacity of transcription factors, a sandwich ELISA was established based on a previously published protocol (19). 5′-Biotinylated (Btn) forward oligonucleotides that contained either variant A [5′-(Btn)-TATTCCTTCTTGTAA-3′] or variant G of rs11564299 [5′-(Btn)TATTCCTCCTTGTAA-3′] were annealed overnight to reverse complementary, nonbiotinylated oligonucleotides (5′-TTACAAGAAGGAATA-3′ and 5′-TTACAAGGAGGAATA-3′, respectively) and then were bound onto streptavidin-coated plates. After 3 washes of the plates with PBS, nonspecific binding was blocked with incubation buffer (MIA ELISA; Roche Applied Sciences, Mannheim, Germany) for 1 h. Nuclear proteins from HSE cells, 6 μg in 150 μl of incubation buffer, were allowed to bind to the oligonucleotides for 1 h.

TABLE 2. *SNP markers*

SNP	TaqMan assay	Position on chromosome 18[a]	Localization	Alleles		Call rate[b]
				Major	Minor	
rs2871593	C_15841206_20	23,771,322	3′ Intergenic	A	G	0.95
rs11564392	C_30810483_10	23,933,730	Intron 2	T	C	0.95
rs11083255	C_27837839_10	24,013,162	Promoter	T	C	0.97
rs11564299	C_30810437_20	24,014,026	Promoter	A	G	0.95
rs11083271	C_417876_10	24,180,057	5′ Intergenic	C	T	0.95
rs4131805	C_26772835_20	24,313,110	5′ Intergenic	T	C	0.95

[a]National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) build 36.3 (human genome build 18).[b]Based on n = 571 samples.

For displacement analysis, double-stranded oligonucleotides (ds-oligonucleotides) without the biotin label were incubated with the nuclear extracts for 10 min before incubation on the plate. The plates were then washed 3 times with incubation buffer, followed by incubation with a specific antibody against ETS-1 (1:500; Novocastra; Leica Biosystems, Wetzlar, Germany) or heterogeneous nuclear ribonucleoprotein K (hnRNP K; 1:500; Acris Antibodies, San Diego, CA, USA) for 1 h. After 3 washes with incubation buffer, a secondary antibody, anti-mouse or anti-rabbit (1:1000; Cell Signaling Technology, Danvers, MA, USA) conjugated with horseradish peroxidase, was added for 30 min. The binding was measured by using ABTS solution (Roche Applied Sciences).

Pulldown assay

5′-Biotinylated forward oligonucleotides containing either variant A or G of rs11564299 were annealed overnight to their respective complementary, nonbiotinylated strands [5′-(Btn)TATTCCTTCTTGTAA-3′ and 5′-TTACAAGAAGGAATA-3′; and 5′-(Btn)TATTCCTCCTTGTAA-3′ and 5′-TTACAAGGAGGAATA-3′]. Streptavidin beads (GE Healthcare Europe GmbH, Freiburg, Germany) were washed with 1 ml of ice-cold PBS. Then, the beads were incubated with the annealed 5′-biotinylated oligonucleotides overnight. Nuclear extracts from the HSE cells were precleared by incubation with streptavidin beads overnight. On the next day, the precleared lysate was incubated with the oligonucleotides bound to streptavidin beads for 4 h. The samples were analyzed by SDS-PAGE, followed by silver staining. The bands selectively occurring in the samples of the minor variant of rs11564299 were cut out and analyzed by mass spectrometry (MS).

MS

Respective bands were excised and washed according to Shevchenko *et al.* (20) with slight modifications. Briefly, gel pieces were washed 3 times alternately with 50 μl of 50 and 25 mM NH_4HCO_3 in 50% acetonitrile. Subsequently, the gel slices were dried in a vacuum centrifuge. Trypsin solution (5 μl; 12.5 ng/μl in 50 mM NH_4HCO_3) was added to each gel piece and incubated at 37°C overnight for in-gel digestion. The obtained peptides were eluted with 20 μl of 5% formic acid and subjected to nano-LC-MS/MS-analysis on an Ultimate 3000 nano-HPLC system (Dionex GmbH, Idstein, Germany). The samples were preconcentrated on a 300 μm i.d., 5 mm C18-PepMap precolumn (5 μm, 300 Å; Thermo Fisher Scientific, Idstein, Germany), with 0.1% formic acid and a flow rate of 40 μl/min. The peptides were then separated on a 75 μm i.d., 15 cm, C18-PepMap-column (flow rate 300 nl/min; Thermo Fisher Scientific), with a 1 h binary gradient from 4 to 40% of solvent B (solvent A: 0.1% formic acid; solvent B: 0.1% formic acid in acetonitrile). The nano-HPLC was directly coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (QStar XL; AB Sciex, Darmstadt, Germany) acquiring 1 full MS and 2 MS/MS spectra of the most intense ions in the respective full-MS scan, repeatedly. The MS/MS spectra were searched against the Uniprot database with the Mascot Daemon and the Mascot algorithm (version 2.2; Matrix Science Ltd., London, UK), with trypsin used as the protease with a maximum of 1 missed cleavage site, oxidation of methionine as a variable modification, and 0.2 Da tolerance for MS and MS/MS signals. Only proteins with ≥2 significantly scored peptide spectra that passed manual inspection were considered to be positive identifications.

Electrophoretic mobility shift assay (EMSA)

Two double-stranded oligomeric binding sites with either variant A (5′-TACAAGAAGGAA-3′) or variant G (5′- TACAAGGAGGAA-3′) of rs11564299 were generated. The ds-oligonucleotides were end labeled with T4 polynucleotide kinase (Roche Applied Sciences) and [^{32}P]ATP[γP] (Amersham-GE Healthcare, Munich, Germany). Band shifts were performed by incubating 5 μg recombinant *hnRNP K* (Cusabio Biotech, Wuhan, China) in 5× mobility shift buffer [1 μg of poly(dI-dC)(dI-dC), 40% glycerol, 25 mM $MgCl_2$, 1 mM EDTA, 25 mM dithiothreitol, 250 mM KCl, and 25 mM HEPES/KOH, pH 7.9) with the DNA probe for 10 min before separation on a 6% nondenaturing polyacrylamide gel. DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel at 250 V, 50 mA, and 100 W for 1.5 h (21).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described by the manufacturer (ChIP-IT Enzymatic; Active Motif, La Hulpe, Belgium). Specific antibodies against hnRNP K (Sigma-Aldrich), ETS-1 (Novocastra), polymerase II (Active Motif), and IgG (Active Motif) were used. For qRT-PCR, primers spanning the region around rs11564299 were designed (hCDH2 rs11564299 forward, 5′-CTTCTTGTAATCAGAGGCC-3′, and hCDH2 rs11564299 reverse, 5′- ATTGTTTTAGCATCTTGCC-3′). For quantification of the positive control, commercially available GAPDH primers (Active Motif) were used.

Statistical analyses and bioinformatics tools

Differences between dichotomous traits were calculated by χ^2 test. Differences in continuous variables between groups were calculated with a 2-tailed *t* test for normally distributed values or with the nonparametric Wilcoxon rank sum test for variables that failed normal distribution, as determined by the Shapiro-Wilk test. Expression data between the 3 genotype groups were compared by Kruskal-Wallis test. Association analysis was carried out in a logistic regression model with gender as a covariate. When age of onset was included as a covariate, for the controls age at inclusion was used. Prevalence ORs with their 95% CIs were reported. Values of $P < 0.05$ were considered significant. To determine whether the genotypes of case patients and control subjects of all SNPs deviated from Hardy-Weinberg equilibrium (HWE), actual and predicted genotype counts of both groups were compared by an exact test (22). Association analyses were performed with PLINK 1.07 (23), and the statistical software package JMP 7.0.2 (SAS Institute, Inc., Cary, NC, USA) was used for other analyses. For LD testing, HaploView 4.2 was used (24) with HapMap phase II data from releases 22, 24, and 27 (25). MatInspector 8.04, and SNPInspector 2.2 (Genomatix Software GmbH, Munich, Germany) were used to analyze the potential functional effects of SNPs on transcription factor binding sites.

RESULTS

Testing for genetic association

All genotyped markers fulfilled our criteria of a ≥95% call rate and showed no deviation from HWE (**Table 3**).

TABLE 3. *Results of an SNP association analysis in an OA case–control sample*

Association	SNP					
	rs2871593	rs11564392	rs11083255	rs11564299	rs11083271	rs4131805
OA case patient genotypes						
11	275	290	286	200	145	189
12	35	22	22	99	134	104
22	2	0	0	12	31	18
MAF	0.0625	0.0353	0.0357	0.1977	0.3161	0.2251
P, HWE	0.337	1	1	1	1	0.515
OA control subject genotypes						
11	225	246	209	140	140	146
12	31	13	36	92	98	102
22	3	0	3	27	20	11
MAF	0.0714	0.0251	0.0847	0.2819	0.2674	0.2394
P, HWE	0.127	1	0.396	0.064	0.634	0.233
Results						
P^a	0.5024	0.3597	0.0012	0.0015	0.0644	0.5843
P^b	0.8894	0.7640	0.0107	0.0050	0.0221	0.9739
OR for minor allele [OR (95% CI)]	1.04 (0.61–1.76)	1.14 (0.49–2.62)	0.48 (0.23–0.83)	0.63 (0.46–0.87)	1.44 (1.05–1.96)	0.99 (0.71–1.39)

Numbers of the genotypes (*i.e.*, 11, 12, 22) are according to the alleles in Table 1. MAF, minor allele frequency. [a]Logistic regression adjusted for sex.[b]Logistic regression adjusted for age at inclusion and sex.

The 5 SNPs genotyped initially were not in tight LD (maximum r^2=0.03 in the entire study sample of 571 persons). There was a significant association (P=1.5×10^{-3}) between rs11564299 and risk of OA in logistic regression analysis after adjustment for gender. Significance decreased to $P = 5.0 \times 10^{-3}$ after additional adjustment for age of recruitment (Table 3). In the fully adjusted model, SNP rs11083271 was nominally associated with OA risk (P=0.02; Table 3). Because rs11564299 is localized in the promoter region 2583 bp upstream of the *CDH2* 5′ end, we searched the HapMap data for polymorphisms closer to the *CDH2* transcription start site. The SNP marker rs11083255 is localized 1719 bp upstream of *CDH2* and was included in a second round of genotyping. Both rs11564299 and rs11083255 were in weak LD in our study sample (r^2=0.215). A significant association between rs11083255 and OA risk was detected (P=1.2×10^{-3} for logistic regression adjusted for gender; P=1.1×10^{-2} for logistic regression adjusted for gender and age at recruitment; Table 3). The effect direction for both SNPs was the same, with a protective effect of the minor allele (Table 3). Taking multiple testing with a total of 6 markers into account, only rs11564299 showed significant association with OA risk in our study (P=5.0×10^{-3}×6 tests=0.03).

Conditional logistic regression analysis with each nominally associated SNP showed that rs11083255 and rs11564299 were not fully independently associated with OA risk (P=0.040, OR=0.69 for rs11564299, after adjustment for rs11083255; P=0.166, OR=0.61 for rs11083255, after adjustment for rs11564299)—that is, loss of significance of ≥1 order of magnitude was observed. As assumed by LD, rs11083271 did not fully explain the association results for rs11564299 (P=0.018, OR=0.67) and rs11083255 (P=0.020, OR=0.46).

Because our study sample included different phenotypes of OA (Table 3), we performed separate analyses on samples of knee OA and hip OA. A comparison of 68 patients with isolated knee OA with 259 control subjects showed that 3 markers from the combined analysis (rs11083255, rs11564299, and rs11083271) remained significantly associated (logistic regression using gender and age at inclusion as covariates; P=0.016, P=0.036, and P=0.042, respectively). For hip OA (n=74 cases), no association was detected (P=0.639, P=0.071, and P=0.887, respectively).

Expression analysis

A total of 29 synovial cell specimens were available from patients with knee OA. Comparison of *CDH2* mRNA expression levels between heterozygotes (n=3) and homozygotes (n=26) for the major allele of rs11083255 did not reach the significance level (P=0.43). The 3 genotypes of rs11564299 were strongly associated with *CDH2* mRNA expression (P=0.0086). Comparison between rs11564299 heterozygotes (n=9) and homozygotes (n=19) for the major allele yielded $P = 0.0073$ (Fig. 1*B*). For both SNPs, the mean expression of *CDH2* was higher in carriers of the minor alleles (*e.g.*, rs11564299: heterozygotes, mean=1.04±0.94; homozygotes, mean=0.26±0.33).

In silico data

That the G allele of rs11564299 was associated with elevated *CDH2* mRNA levels in OASF led to the hypothesis that this allele may generate a novel transcription factor binding site. *In silico* analysis with Genomatix (Genomatix Software GmbH) predicted the loss of a TEF (26) binding site and a gain in sites for ETS-like transcription factors (27) and ZNF35 (28).

Analysis of a potential transcription factor binding site

A quantitative ELISA with ds-oligonucleotides containing either variant A or G of rs11564299 as bait was established to verify the predicted ETS-like binding site. After incubation with nuclear proteins and detection with a specific ETS-1 antibody and peroxidase-labeled secondary antibodies, no differences in binding efficiency to one of the variants were observed (**Fig. 2*A***). Therefore, ETS-1 was excluded as a transcription factor binding selectively to the minor allele of rs11564299. Subsequently, a pulldown assay was performed for identification of the transcription factor responsible for the higher *CDH2* mRNA levels observed with the minor rs11564299 allele. The pulldown was performed with nuclear extracts of the cell line HSE, which had tested heterozygous for rs11564299, by using the specific oligonucleotides for alleles A and G of rs11564299. Pulled down proteins were separated on an acrylamide gel and stained silver, followed by excision, tryptic digestion, and MS analysis of those protein bands that had been pulled down selectively with ds-oligonucleotides containing variant G (Fig. 2*B*). This method revealed hnRNP K as a potential binding protein. Expression of hnRNP K in early (P3) and late (P8) passages of OASFs was determined by qRT-PCR, and no significant differences were observed between the different groups (Fig. 2*C*). For analysis of differential binding of hnRNP K to the minor allele (G) compared to the major allele (A) of rs11564299, a quantitative ELISA with a specific hnRNP K antibody was established. The ELISA showed that hnRNP K bound only to ds-oligonucleotides containing variant G, but not to those containing variant A (**Fig. 3*A***). The binding was inhibited by preincubation with ds-oligonucleotides containing variant G but not variant A (Fig. 3*A*). As shown before, performing the ELISA with an ETS-1 antibody revealed no differential binding of ETS-1 to ds-oligonucleotides containing either variant of the SNP rs11564299 (Fig. 2*A*). As a negative control, a setting with the forward biotinylated ssDNA oligonucleotides was performed, but did not show any binding of hnRNP K to the ssDNA (data not shown). EMSAs of similar oligonucleotides yielded the same results. A shift was observed only for probes carrying variant G after incubation with recombinant hnRNP K (Fig. 3*B*). This result showed that hnRNP K bound exclusively to the minor allele of rs11564299, indicating that hnRNP K may be responsible for elevated *CDH2* mRNA expression. To further elucidate the binding of hnRNP K to the minor variant of rs11564299, a ChIP assay was performed. Antibodies against ETS-1 (positive control 1), pol II (positive control 2), and IgG (negative control) were used in addition to the anti-hnRNP K

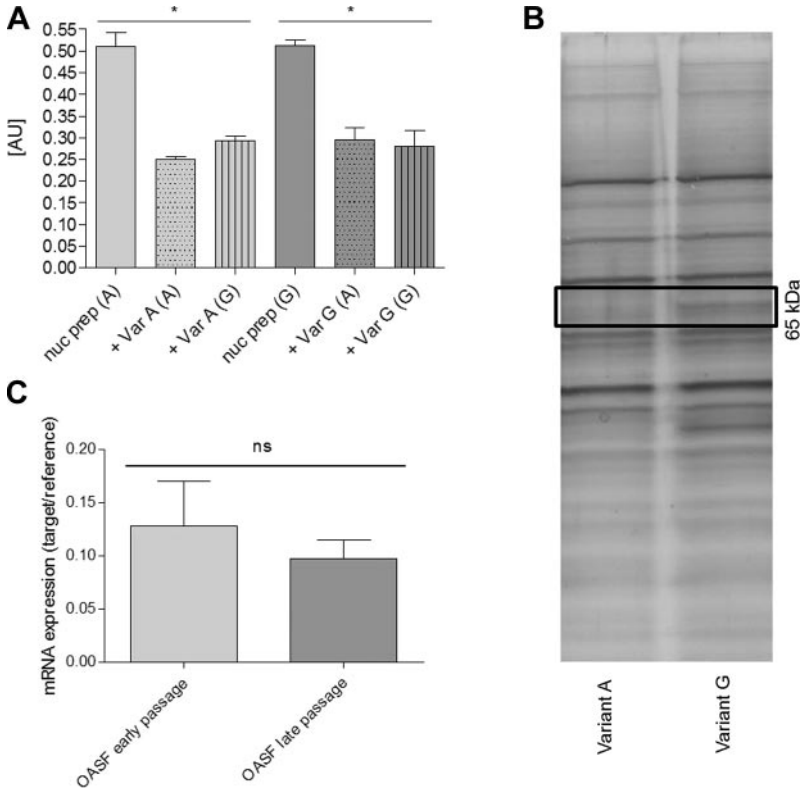
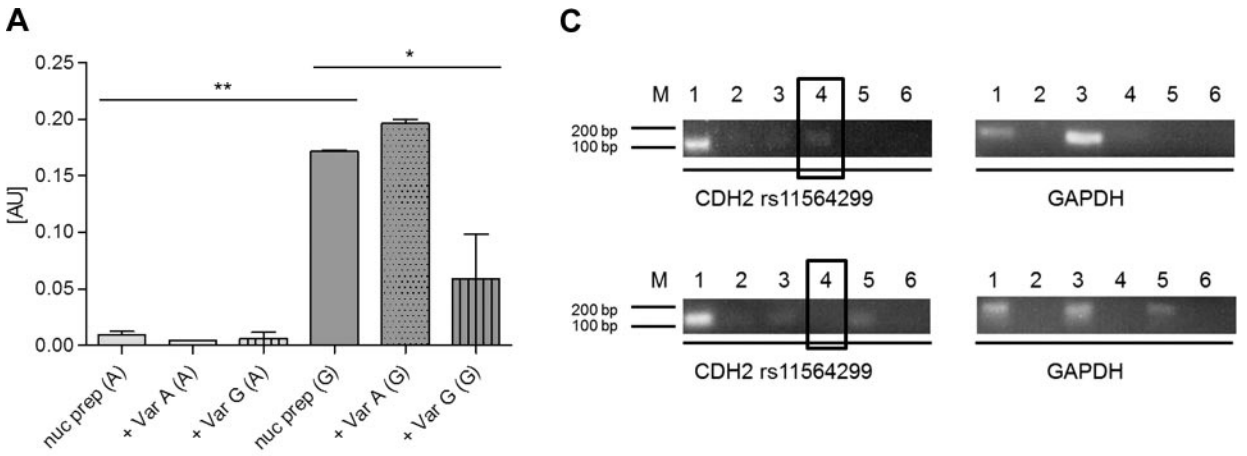


Figure 2. *A*) ELISA, performed with an ETS-1 antibody, did not show preferential binding to one of the 2 alleles of rs11564299. Here, competition was possible with variants A and G. *B*) Silver-stained acrylamide gel of a pulldown assay using oligonucleotides containing either allele A or G of rs11564299. A band, showing only the pulldown with variant G, was analyzed by MS and revealed hnRNP K as a potential transcription factor binding to the *CDH2* promoter with the minor allele G. *C*) mRNA expression of hnRNP K was not altered during cell culture cultivation of OASFs.



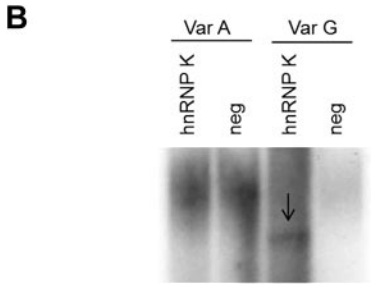


Figure 3. *A*) For quantitative measurement of the binding capacity of hnRNP K, an ELISA was established with specific oligonucleotides containing variant A or G. A clear binding to variant G was observed, whereas no binding K to variant A was measured. Binding was repressed only in competition experiments with variant G oligonucleotides, not with variant A. *B*) EMSAs with the variant A or G probes showed binding only to variant G. *C*) Specific binding of hnRNP K to variant G was confirmed by ChIP. In heterozygous HSE cells, binding was observed, whereas in K4Im cells, which are homozygous for variant A, only binding to ETS-1 was detected. Top panel: HSE. Bottom panel: K4Im. Lane 1, input; lane 2, IgG antibody; lane 3, Pol II antibody; lane 4, hnRNP K antibody; lane 5, ETS-1 antibody; lane 6, H_2O control. (Differential binding of ETS-1 in the control setting is due to base pair changes in the *GAPDH* promoter region of K4Im, which resulted in an ETS-1 binding site; data not shown.)

antibody. The ChIP assay was performed with HSE cells, which are heterozygous for rs11564299, and K4Im, a cell line of immortalized SFs that are homozygous for the major allele of rs11564299. hnRNP K bound only to chromatin in HSE cells, whereas no binding was observed when the homozygous wild-type K4Im cells were tested (Fig. 3*C*). These findings confirmed that hnRNP K bound exclusively to the N-cadherin promoter in the presence of the minor allele of rs11564299, therefore making hnRNP K a candidate for inducing the elevated levels of N-cadherin.

DISCUSSION

Genetic association studies have shown that SNPs contribute significantly to the susceptibility and severity of many diseases (29). Also, associations of genetic variants with OA onset and progression have been published (2, 30). As shown before, SNPs in the promoter region of a gene can influence disease susceptibility (31, 32). Our results indicate that *CDH2* SNP rs11564299 influences susceptibility to OA prevalence, as the minor G allele is more common among the control population than the OA population. We found that the allelic state of rs11564299, which is located 2583 bp upstream of the *CDH2* transcription start site, significantly affected mRNA expression levels of *CDH2* in SFs, with the AG heterozygotes (HSE cells) expressing significantly more *CDH2* than the AA homozygotes (K4Im cells). This result indicates stronger activity of the *CDH2* promoter in patients carrying the minor allele, probably caused by an allele-specific transcription factor binding site. However, none of the transcription factors predicted *in silico* to bind to the variant promoter sequence showed differential binding to the minor allele of rs11564299. Rather, MS analysis identified hnRNP K as a factor that bound exclusively to the G allele of rs11564299 and not to the A allele. hnRNP K is one of the major pre-mRNA binding proteins. It has also been described to bind dsDNA and to act as a transcription factor (33). Binding of hnRNP K was confirmed by a specific ELISA and ChIP. Thus, hnRNP K appears to contribute to the increased N-cadherin expression levels in SFs carrying the G allele of rs11564299. As N-cadherin is a core component of adherens junctions, the increased levels of it may affect cell–cell contacts, tissue architecture, and cell motility (34, 35). It has been described before that N-cadherin levels are critical for cell invasion and that N-cadherin re-expression in glioma cells and in human arterial smooth muscle cells leads to less migration (36, 37). Therefore, higher N-cadherin levels in SFs may lead to a decreased migratory potential of the cells. As shown by Schubert *et al.,* (6) migration of SF into cartilage leads to the degradation of the extracellular matrix and cartilage loss. Thus, inhibition of migration by enhanced cell–cell contact may prevent or reduce cartilage destruction. All evidence combined, our study implicates for the first time *CDH2* encoding N-cadherin as a susceptibility gene for OA, especially of the knee. Some limitations of our study should be discussed. First, we had no replication study for the genetic association data, and therefore our finding of rs11564299 as a risk

factor for OA susceptibility could be a false positive. However, the apparent link between rs11564299 allelic state and *CDH2* mRNA expression lends functional support to this hypothesis. Second, the effect size of the rs11564299 minor allele on OA risk is small (OR=0.63, *i.e.*, ~1.6 times increased risk in carriers of the major allele). However, it has become obvious that common variants are often associated with OR < 1.5 (38). Third, because the control subjects were selected by clinical exclusion of arthritic or degenerative diseases, apparent OA was excluded. However, the preclinical state of joint disorders within the controls could have affected the association results. Fourth, because of our marker selection, we did not fully cover the whole *CDH2* gene when tagging SNPs. This deficiency is one that could be solved by screening existing genome-wide association data on OA (2). Since SFs are only 1 candidate cell type in the joint that is potentially responsible for OA onset and progression, future studies should expand N-cadherin analysis to other cells and their interaction within the joint.

The exact role of N-cadherin for OA susceptibility has yet to be defined. Further analyses should be aimed at the interaction of genetic variants in *CDH2* with known risk factors for OA, such as age, increased body-mass index, mechanical stress, and microinjuries of the joint. FJ

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