Human Molecular Genetics, 2005, Vol. 14, No. 8 ***1009–1017***
doi:10.1093/hmg/ddi093
Advance Access published on March 3, 2005

A functional single nucleotide polymorphism in the core promoter region of *CALM1* is associated with hip osteoarthritis in Japanese

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Received December 27, 2004; Revised February 14, 2005; Accepted February 22, 2005

Osteoarthritis (OA), a common skeletal disease, is a leading cause of disability among the elderly populations. OA is characterized by gradual loss of articular cartilage, but the etiology and pathogenesis of OA are largely unknown. Epidemiological and genetic studies have demonstrated that genetic factors play an important role in OA. To identify susceptibility genes for OA, we performed a large-scale, case–control association study using gene-based single nucleotide polymorphisms (SNPs). In two independent case–control populations, we found significant association ($P = 9.8 \times 10^{-7}$) between hip OA and a SNP (IVS3 − 293C > T) located in intron 3 of the calmodulin (CaM) 1 gene (*CALM1*). *CALM1* was expressed in cultured chondrocytes and articular cartilage, and its expression was increased in OA. Subsequent linkage-disequilibrium mapping identified five SNPs showing significant association equivalent to IVS3 − 293C > T. One of these (−16C > T) is located in the core promoter region of *CALM1.* Functional analyses indicate that the susceptibility −16T allele decreases *CALM1* transcription *in vitro* and *in vivo.* Inhibition of CaM in chondrogenic cells reduced the expression of the major cartilage matrix genes *Col2a1* and *Agc1.* These results suggest that the transcriptional level of *CALM1* is associated with susceptibility for hip OA through modulation of chondrogenic activity. Our findings reveal the *CALM1*-mediated signaling pathway in chondrocytes as a novel potential target for treatment of OA.

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INTRODUCTION

Osteoarthritis (OA; MIM165720) is a common disease and a leading cause of disability among the elderly populations. OA causes pain, deformity and loss of joint function, leading to reduced activity in daily living and decreased quality of life. OA affects various joints, most commonly spine, hand, knee and hip, with clinical features that are unique to each joint. About 20 million patients suffer from OA in the United States (1), and in Japan, the prevalence of hip OA has been reported as 3.5% (women) and 1.4% (men) (2). The cost of OA to society is estimated to exceed $15 billion in the United States, an amount roughly three times that of rheumatoid arthritis (3). Thus, OA is a serious medical and economical issue, particularly in the aging populations of developed countries.

OA is a degenerative disease characterized by gradual loss of articular cartilage. During the course of life, articular cartilage is lost gradually because of excessive mechanical stress and/or injuries to the joint. Decompensation of the capacity for cartilage repair by chondrocytes has been suggested as potential pathogenic factor in OA (4). In addition, aberration of cartilage matrix metabolism ultimately results in failure of its ability to respond to and withstand mechanical stress on the joint and hence contributing to OA.

The etiology and pathogenesis of OA remain largely undetermined. Epidemiological studies have demonstrated that OA is a genetic disease, with its major component transmitted as a complex, multifactorial trait (5). Many genetic studies have sought to identify susceptibility genes for OA, including candidate gene association studies and genome-wide linkage studies of hand, knee and hip OA (6–8). These efforts have resulted in the identification of several susceptibility loci for each type of OA, confirming that OA has a genetic component. However, the genetic components of OA remain largely uncharacterized because of limitations in current genetic approaches. Candidate gene association studies are severely limited by the requirement for *a priori* knowledge of 'candidacy'. The utility of linkage studies is limited by the fact that defined genomic regions, which contain hundreds of genes, are very broad to pinpoint single disease genes. Alternative methods for identifying susceptibility genes in common disease include the genome-wide association study, followed by linkage-disequilibrium (LD) mapping using single nucleotide polymorphisms (SNPs). This approach has been used successfully to identify susceptibility genes for myocardial infarction (9).

Through a large-scale association study followed by LD mapping, we show that the calmodulin (CaM) 1 gene (*CALM1*) is associated with hip OA in the Japanese population. We identified a functional SNP in the core promoter region of *CALM1*, which affects transcription of the gene through varying affinity for nuclear protein(s). Inhibition of CaM by specific antagonists decreases expression of the major cartilage matrix genes in chondroprogenitor cells. Our findings indicate that *CALM1* plays a crucial role in chondrocyte differentiation and that decreased *CALM1* expression might contribute to OA.

RESULTS

Large-scale association study

To identify susceptibility loci for hip OA, we performed a large-scale, case–control association study using 94 individuals with hip OA and 633 control subjects (first screen). We were able to genotype 75 253 of 81 398 gene-based SNPs selected from the JSNP database. Among these, we tested the association of 71 880 SNPs that were in Hardy–Weinberg equilibrium. In each of four models (allelic and genotypic frequencies in both recessive and dominant models), 2219 SNPs had *P*-values less than 0.01. The results were similar to those of a previous study that identified a susceptibility gene for myocardial infarction using the same approach (9). We then genotyped the qualifying SNPs from the first screen in a replication panel consisting of 334 individuals with hip OA and 375 control subjects and tested again for association. These independent tests identified several SNPs highly associated with hip OA (Mabuchi *et al.*, manuscript in preparation). Among these was a SNP (IVS3 − 293C > T) in intron 3 of *CALM1* on chromosome 14q24–q31. Association of the SNP was most significant in the recessive model ('TT' versus other genotypes) (Table 1). The association was significant in both male and female sub-populations (Supplementary Material, Table S1). When association was tested by stratifying the cases into sub-populations with/without acetabular dysplasia, each sub-population showed significant association. The significance was higher in the cases without acetabular dysplasia ($P = 0.000033$). The frequency of 'TT' genotype differed between the populations, but the difference was not statistically significant ($P = 0.19$). The association was not found in knee OA (data not shown).

To localize the susceptibility gene present in this region, we evaluated the LD extension with IVS3 − 293C>T. We genotyped 334 individuals with hip OA for SNPs in the 400 kb region around IVS3 − 293C>T, then examined the LD index (D') between IVS3 − 293C>T and SNPs having allele frequencies >10%. All SNPs showing D' scores greater than 0.99 were localized within *CALM1*, indicating that the LD extension with IVS3 − 293C>T was limited to this gene (Fig. 1A).

Expression analyses of *CALM1*

CALM1 encodes CaM, a ubiquitous eukaryotic calcium-binding protein, which is a principal mediator of the calcium signal (10). CaM participates in cartilage metabolism in response to mechanical stimulation (11). We found that *CALM1* was expressed in human articular chondrocytes in alginate bead culture and in human articular cartilage from OA patients (Supplementary Material, Fig. S1A). Microarray analysis showed higher levels of *CALM1* expression in hip and knee OA cartilage than in normal cartilage (Supplementary Material, Fig. S1B and C), further implicating *CALM1* in OA pathogenesis.

Search for susceptibility SNP

To identify a susceptibility SNP in the genomic region of *CALM1*, we examined SNPs for all exons of *CALM1*, their

Table 1. Association of the SNP in intron 3 of *CALM1* between cases and controls

Panel	Genotype										Odds ratio[a] (95% CI)	*P*-value[a]
	Case					Control						
	TT	CT	CC	Sum	Frequency[b]	TT	CT	CC	Sum	Frequency[b]		
First screen	18	35	40	93	0.19	55	278	298	631	0.09	2.51 (1.40–4.50)	0.0015
Replication	47	128	158	333	0.14	24	157	194	375	0.06	2.40 (1.43–4.02)	0.00065

CI, confidence interval
[a]Odds ratio and *P*-value were calculated on recessive model (TT versus CT + CC).
[b]Frequency of TT genotype.

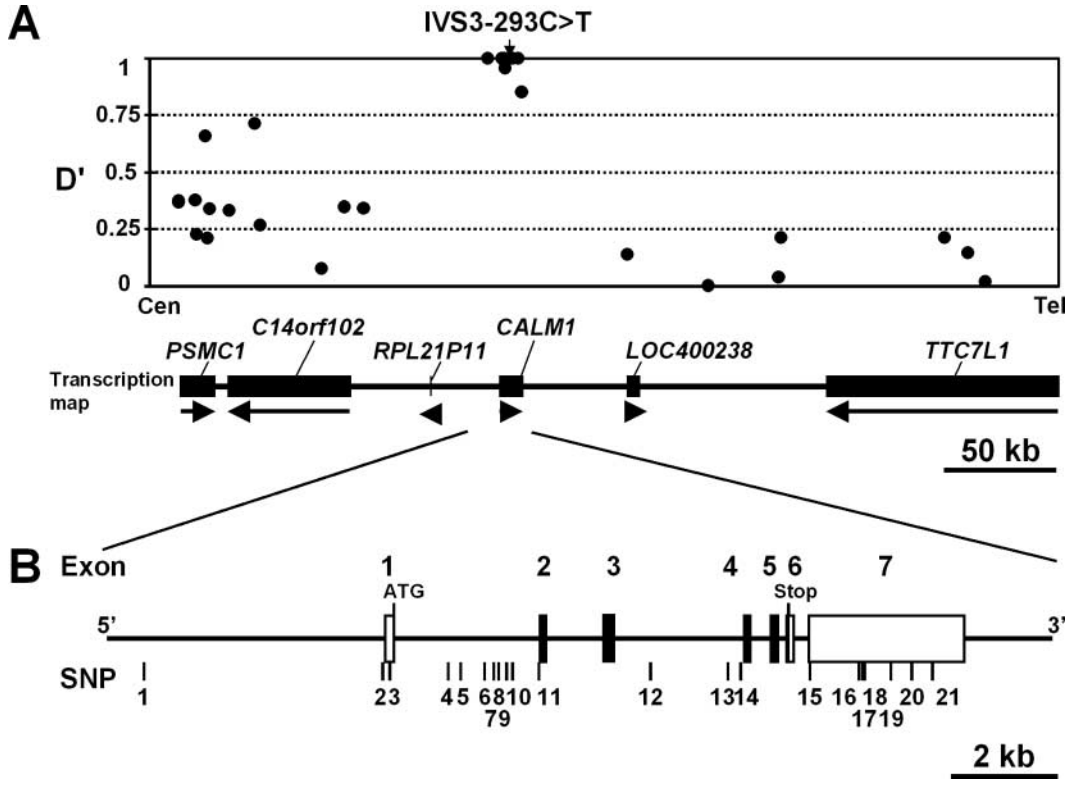

Figure 1. *D′* block and genomic structure of the *CALM1* gene. (**A**) Linkage disequilibrium around *CALM1*. *D′* scores between the SNP in the *CALM1* locus (IVS3 − 293C>T) and other SNPs in the region are shown with a transcriptional map. Horizontal arrows indicate the orientation of each gene (5′ → 3′). (**B**) Exon–intron structure and SNPs of the *CALM1*. Boxes indicate exons. White fields indicate the untranslated region, and black fields indicate the coding regions. SNPs in this region are shown as axial bars with ID number.

5′ and 3′ flanking sequences and a 1.4 kb promoter region using DNA from 16 hip OA patients. Within the *CALM1* region, we confirmed a total of 21 SNPs (Fig. 1B). We genotyped and divided the SNPs that were in absolute LD relationship with each other (pairwise LD index, $\Delta > 0.97$) into eight groups (Supplementary Material, Table S2). For each SNP group with minor allele frequencies >10%, we genotyped representative SNPs in 334 case and 375 control individuals and analyzed the haplotype structure of the region. This analysis identified four common haplotypes, which covered >90% of the population haplotypes in both groups (Table 2). For each haplotype, we examined the association with hip OA and found a significant association with haplotype II (Table 2). This haplotype contained only group D SNPs, represented by the original marker SNP, IVS3 − 293C>T (SNP 13 in Fig. 1B). No other haplotype had a *P*-value lower than haplotype II. Moreover, three SNPs not included in the haplotype analyses (SNPs 14, 16 and 17 in Fig. 1B) showed no significant association in the same case–control population (data not shown). Therefore, we concluded that the association with susceptibility to hip OA originated from the group D SNPs. We confirmed that all five SNPs in group D had significant association equivalent to IVS3 − 293C>T (Table 3), indicating that these SNPs are all candidate susceptibility

Table 2. Haplotype association analysis using representative SNPs

Haplotype	Group (representative SNPs)						Haplotype frequency		*P*-value
	A (6)	B (8)	C (11)	D (13)	E (18)	F (19)	Case	Control	
I	T	C	T	C	C	T	0.451	0.460	0.72
II	T	G	T	T	C	T	0.331	0.268	0.01
III	C	G	C	C	G	T	0.137	0.161	0.20
IV	T	C	T	C	C	G	0.035	0.032	0.81

Table 3. Association of the SNPs of *CALM1* in group D

SNP	Location	Sequence	dbSNP reference[b]	Allele		Genotype								Odds ratio[a] (95% CI)	*P*-value[a]
						Case				Control					
				1	2	11	12	22	Frequency[c]	11	12	22	Frequency[c]		
2	Promoter	−16[d]	rs12885713	T	C	46	128	160	0.14	22	154	199	0.06	2.56 (1.50–4.36)	0.00036
5	Intron	IVS1 + 1271	rs2300496	C	A	46	129	159	0.14	23	155	197	0.06	2.44 (1.44–4.12)	0.00061
9	Intron	IVS1 − 692	rs2300500	G	C	47	128	159	0.14	23	156	196	0.06	2.51 (1.49–4.23)	0.00040
13[e]	Intron	IVS3 − 293	rs3213718	T	C	47	128	158	0.14	24	157	194	0.06	2.40 (1.43–4.02)	0.00065
20	3′-UTR	Exon 7 + 1996	rs3179089	G	C	45	131	158	0.13	20	160	195	0.05	2.76 (1.59–4.78)	0.00018

CI, confidence interval.
[a]Odds ratio and *P*-value were calculated on recessive model (11 versus 12 + 22).
[b]www.ncbi.nlm.nih.gov/projects/SNP.
[c]Frequency of '11' genotype.
[d]The first nucleotide of the exon 1 start site is designated as position 1, on the basis of the reference sequence GenBank accession no. NM_006888.2.
[e]Original marker for which association was detected in the first screen.

SNPs for hip OA. The *P*-value of the haplotype association is less than those of respective SNPs. Therefore, the possibility that the haplotype II itself is implicated in OA susceptibility is unlikely.

In vitro functional analyses of the susceptibility SNP located on the promoter region

One of the five candidate susceptibility SNPs, −16C>T (SNP 2 in Fig. 1B), was localized within a core promoter region of *CALM1*, which includes the TATA element. To investigate possible allelic differences in promoter activity generated by this SNP, we generated fusion constructs which express the luciferase gene under the control of a 1.4 kb segment of *CALM1* (nucleotides −1231 to +202, shown in the left panel of Fig. 2B) that has previously been reported to have promoter activity (12). Reporter constructs containing the −16C or −16T allele were transfected in parallel into the OUMS-27 cells or Huh-7 cells. OUMS-27 cells are derived from a human chondrosarcoma and retain differentiated chondrocytic phenotypes (13); Huh-7 cells are derived from a hepatocellular carcinoma (14). In OUMS-27 cells, the construct containing the −16C allele showed >2-fold luciferase activity than that containing the −16T allele (Fig. 2A). Similar results were observed in Huh-7 cells (Fig. 2B, top).

To investigate the critical region responsible for differences in transcriptional activity between the −16C and −16T alleles, we generated three deletion mutants of the *CALM1* promoter construct. Approximately 2-fold differences in luciferase activity between the two alleles were observed with each construct, including the shortest 61 bp construct (nucleotides −53 to +8), in Huh-7 cells (Fig. 2B). These results indicate that the difference in transcriptional activity results from the sequence immediately surrounding the SNP.

Using a gel-shift assay, we examined whether a nuclear extract from Huh-7 cells could bind to the sequence surrounding −16C>T (Fig. 2C). The band corresponding to the −16T allele was more intense than that corresponding to the −16C allele, indicating that one or more nuclear factors bound to the −16T allele more tightly than to the −16C allele. Taken together with the reduced transcriptional activity of the −16T allele relative to the −16C allele, this result suggests the presence of suppressor(s) of *CALM1* transcription, which bind more tightly to the −16T allele.

Allelic differences in the expression of *CALM1 in vivo*

To investigate the effects of −16C>T on *CALM1* transcription *in vivo*, we measured the amount of *CALM1* transcripts under the control of each allele using RNA difference plot analysis (15). Using fluorescence-labeled primers, we amplified cDNA and genomic DNA from the cells of an individual who was heterozygous with respect to −16C>T and evaluated the allelic difference of the linked marker in the transcribed region (+114G>A in the 5′ untranslated region, SNP 3 in Fig. 1B). Molecular haplotyping confirmed the haplotype structures of −16 to +114 as C–G and T–A.

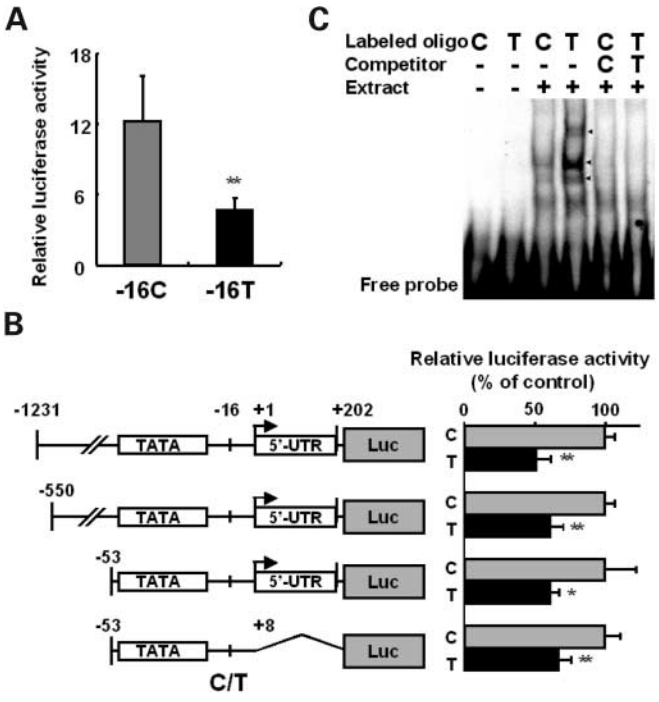

Figure 2. Transcriptional regulatory activity affected by the −16C>T SNP. (**A**) Luciferase assay in OUMS-27. DNA fragments of the 1.4 kb promoter region (−1231 to +202) containing the −16C or −16T allele were ligated into the promoterless/enhancerless firefly luciferase expression vector pGL3-basic. The OA-susceptible −16 allele shows reduced activity. (**B**) Identification of the critical region for the difference in transcriptional activity. The 1.4 kb reporter constructs containing the −16C or −16T allele, and their deletion mutants are shown in the left panel. These construct were co-transfected with pRL-TK into Huh-7 cells. Data are mean ± SEM of values from six transfections. Single asterisk and double asterisks indicate $P < 0.05$ and 0.01 by Student's *t*-test, respectively. (**C**) Gel-shift assay. Nuclear extract from Huh-7 cells was incubated with double-stranded, DIG-labeled oligonucleotides (C or T). For competition studies, the nuclear extract was pre-incubated with unlabeled oligonucleotides (125-fold excess) before adding labeled oligonucleotide. Arrows indicate the bands that show tighter binding to the T allele. The experiments were repeated three times with similar results.

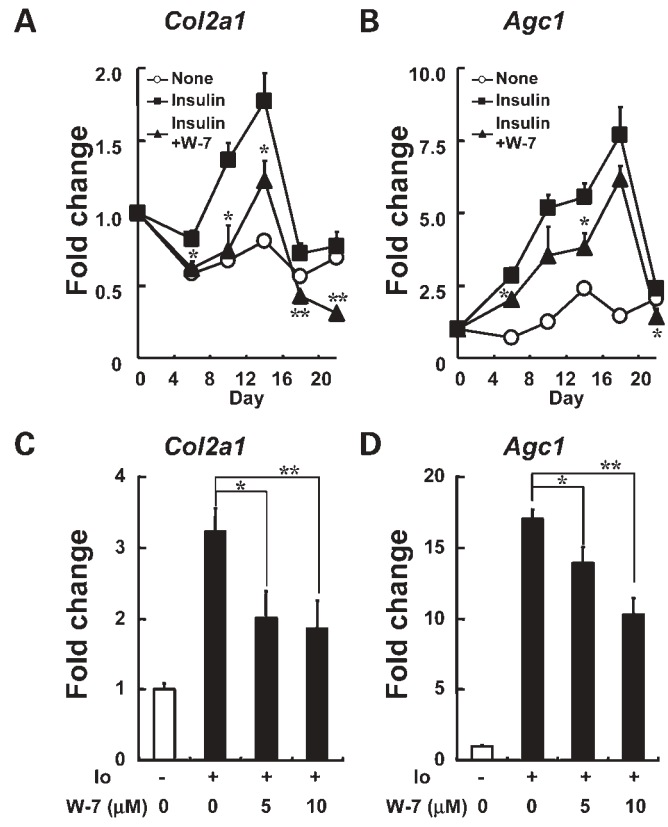

Figure 3. Effect of the CaM antagonist W-7 on chondrogenic activity. (**A** and **B**) Effect of W-7 on ATDC5 differentiation. ATDC5 cells were untreated (open circles) and exposed to 10 μg/ml insulin alone (closed squares) or to 10 μg/ml insulin plus 20 μM W-7 (closed triangles) for 6, 10, 14, 18 and 22 days. At each time point, cells were harvested and mRNA levels of *Col2a1* (A) and *Agc1* (B) were quantified. (**C** and **D**) Effect of W-7 on transcription of cartilage matrix genes stimulated by ionomycin in RCJ3.1C5.18 cells. Cells were untreated (white columns) or exposed to 2 μM ionomycin (Io) (black columns) with various concentration of W-7. After 24 h, cells were harvested and levels of *Col2a1* (C) and *Agc1* (D) mRNA were quantified. Copy numbers of these genes were measured by quantitative real-time PCR analysis and normalized relative to the amount of total RNA. Expression levels are shown as fold change when compared with levels at day 0 (A and B) or with non-ionomycin stimulated conditions (C and D). Values are average ± SEM ($n = 3$). Statistical tests were performed between insulin alone and insulin plus W-7 (A and B). Single asterisk and double asterisks indicate $P < 0.05$ and 0.01 by Student's *t*-test, respectively. Similar results were obtained in three independent studies.

Amplified products were separated by electrophoresis specialized for polymerase chain reaction (PCR)- single-strand conformation polymorphism analysis, and the signal intensity of each allele was measured. The expression ratio of the +114G to +114A allele corresponding to that of −16C and −16T, respectively, was 1.09 (99% CI = 1.04–1.15). This result was replicated through a similar experiment using another marker (exon 7 + 1996 G > C, SNP 20 in Fig. 1B; the expression ratio: 1.12; 95% CI = 1.05–1.20). Thus, the susceptible −16T allele showed decreased *in vivo* expression levels relative to the non-susceptible −16C allele.

Role of CaM in chondrogenesis

To investigate the role of CaM in chondrogenesis, we used an *in vitro* chondrogenesis model, the ATDC5 cell line, which mimics chondrocyte differentiation in the presence of insulin (16). During differentiation, the expression of *Col2a1* and *Agc1*, which encode major cartilage extracellular matrix proteins, increased in a time-dependent manner (Fig. 3A and B). Addition of the W-7, a naphthalene sulfonamide derivative which directly binds to and inhibits CaM, suppressed the expression of these genes by half (Fig. 3A and B). This observation indicates that CaM transduces one or more signals that induce expression of cartilage matrix genes during chondrocyte differentiation. To further investigate the role of CaM in chondrogenesis, we examined the effect of W-7 on Ca^{2+}-regulated cartilage matrix gene expression in the rat chondrogenic cell line RCJ3.1C5.18. In these cells, expression of *Col2a1* and *Agc1* increased in the presence of ionomycin, a Ca^{2+} ionophore that increases the intracellular concentration of Ca^{2+} (Fig. 3C and D). These increases were suppressed by W-7 in a dose-dependent manner (Fig. 3C and D). This observation indicated that the Ca^{2+} signal induces cartilage

matrix gene expression and is regulated by CaM in chondroprogenitor cells.

Analyses of combinatorial effect of *CALM1* and other genes

To examine the relation of *CALM1* to other genes in the etiology of hip OA, we estimated the combinatorial effect of *CALM1* and other genes by combining genotype data on pair of loci in 323 cases and in 374 controls. We found significant combinatorial association between the *CALM1* SNP and the asparatic acid repeat polymorphism (D14 allele) in the asporin gene (*ASPN*) (17), with regard to hip OA susceptibility. The two loci are not linked physically. The genotype that was homozygous with respect to the *CALM1* allele and homozygous or heterozygous with respect to D14 allele in *ASPN* showed highest odds ratio (Table 4).

Table 4. Combinatorial effect between *CALM1* and *ASPN*

		ASPN[a]				Odds ratio (95% CI)	
		Case[b]		Control[b]			
		D14[c]	Others	D14	Others	D14	Others
CALM1[d]	TT	10	37	1	23	13.16 (1.66–104.06)	2.12 (1.20–3.73)
	CT	23	99	15	142	2.02 (1.01–4.02)	0.92 (0.65–1.29)
	CC	21	133	18	175	1.54 (0.79–3.00)	1

CI, confidence interval.
[a]Genotype of D-repeat.
[b]Number of cases and controls are 323 and 374, respectively.
[c]Number of individuals who are heterozygous or homozygous with respect to the allele encoding the D14 variant.
[d]Genotype of IVS3 − 293C>T.

DISCUSSION

Through a large-scale association study using SNPs and subsequent LD mapping, we have identified *CALM1* as a susceptibility gene for hip OA. Multiple testing that might produce false-positive results is a considerable problem in large-scale association study because it uses a huge number of markers as in this study. The final *P*-value for the association of the SNP was calculated as 9.8×10^{-7} by multiplying the *P*-values of the two independent tests. When Bonferroni's correction was applied to this result, we obtained only marginally significant association (the corrected $P = 0.070$). Therefore, the possibility remains that the association of *CALM1* might be false positive. However, it is widely recognized that Bonferroni's correction is overly conservative. If we exclude the makers that are in almost complete LD ($\Delta > 0.97$) with others ($n = 19\,794$), the corrected *P*-value becomes 0.051. In addition to the association data, we have shown that *CALM1* is expressed in normal articular cartilage, with increased expression in OA cartilage. *CALM1* encodes CaM, which plays a crucial role in facilitating chondrocyte differentiation and maintaining the cartilage matrix. We have also shown that a SNP in the core promoter region of *CALM1* exerts an allele-specific effect on gene expression *in vitro* and *in vivo*. These functional significances in cartilage and OA support the candidacy of *CALM1* as a susceptibility gene for OA.

CaM binds to intracellular Ca^{2+} and mediates various signals involved in cellular function including proliferation, motility, cell-cycle progression and transcription (10). In chondroprogenitor cells, the Ca^{2+} signal regulates chondrogenic differentiation. The elevation of intracellular concentration of Ca^{2+} by ionomycin induces expression of *Agc1* in the rat chondrogenic cell line RCJ3.1C5.18 (18). Ionomycin also induces chondrogenesis in the limb bud mesenchyme of chicken (18). Our experiments using W-7, specific antagonist of CaM, indicate that this signal is mediated by CaM. The crucial role of CaM in chondrogenic differentiation was also confirmed in ATDC5. Chondroprogenitor cells are present in human articular cartilage (19) and might be involved in cartilage repair. Decompensation of the cartilage repair capacity in chondrocyte function has been suggested as potential pathogenic factor for OA (4). Therefore, aberration of the Ca^{2+}–CaM signal regulating chondrogenesis over time is likely to promote the onset and progression of OA.

The Ca^{2+}–CaM signal also has a crucial function in maintaining the cartilage phenotype in response to mechanical stimuli in mature chondrocytes. Mechanical stimuli can change intracellular concentration of Ca^{2+} in chondrocytes in seconds to minutes (20,21). Cyclic or dynamic loading stimulates the synthesis of cartilage extracellular matrix components (22,23). CaM is required for the transient elevation of *Agc1* mRNA in bovine articular cartilage explants by low-level compressive loading (11). These findings suggest that daily mechanical stimuli might maintain healthy articular cartilage via the Ca^{2+}–CaM signaling mechanism. Therefore, aberration of this mechanism in articular chondrocytes would also eventually spur the onset and progression of OA.

The identity of downstream regulators of CaM in chondrocytes remains unclear. CaM might bind directly to SOX9, a key regulator of chondrogenesis, and facilitate its nuclear translocation (24). CaM also could facilitate chondrogenesis through activating calcineurin, a Ca^{2+}–CaM dependent phosphatase, which is involved in maintaining the chondrocyte phenotype (18). Calcineurin also plays a role in the pathway mediating the transient elevation of *Agc1* mRNA in response to low-level compressive loading in bovine articular cartilage explants (11). The contribution of CaM-dependent protein kinase II in this pathway has also been established (11). Further study is necessary to characterize the signaling pathway downstream of CaM. Clarification of CaM-mediated pathways would present a new paradigm for the etiology and pathogenesis of OA, as well as a new target for treatment of OA.

With regard to complex genetic traits, each related gene contributes disease susceptibility to some extent, but such genes may also interact with each other. Most recently, we have found *ASPN* as a susceptibility gene for both knee and hip OA (17). Asporin is an extracellular matrix protein, which inhibits chondrocyte differentiation by binding TGF-β1 and suppressing its signal. The combinatorial effect

between *CALM1* and *ASPN* found in this study suggests presence of the epistatic effect of the two loci. There may be a cross-talk between the TGF-β and CaM signals in chondrocyte. The relationship between CaM and asporin should be clarified in future work.

MATERIALS AND METHODS

Subjects

We recruited 428 individuals affected with hip OA (24 males and 404 females, mean age: 53.7 years) and 1008 controls (517 males and 491 females, mean age: 46.7 years) from several medical institutions in Japan. All subjects were Japanese. Most cases were female (94%). All individuals with hip OA were symptomatic and were treated in these medical institutions on a regular basis. Hip OA was diagnosed by clinical symptoms and radiological findings of narrowing of the joint space and/or osteophytes of the hip joint. Because acetabular dysplasia is the most common cause and predisposing factor of hip OA in Japan (25), 40% of the cases had acetabular dysplasia (center-edge angle $<25^\circ$ or acetabular angle $>25^\circ$ in the anteroposterior radiograph of the hip). We obtained informed consent from each subject as approved by the ethical committees of the participating medical institutions and the SNP Research Center of The Institute of Physical and Chemical Research (RIKEN). Genomic DNA was extracted from peripheral blood leukocytes of each subject, using a standard protocol.

Large-scale association study

For the association study, we used our own SNP database (JSNP database, http://snp.ims.u-tokyo.ac.jp/) and carried out screening of SNPs as described previously (9,26). First, we genotyped 81 398 SNPs in genomic DNA from 94 individuals with hip OA and 633 control individuals. For the successfully genotyped SNPs, we calculated *P*-values as previously described (the first screen) (9). SNPs that passed the first screen were further genotyped in a second replication panel consisting of individuals with hip OA (334) and control (375) subjects, as an independent test. For both screens, *P*-values less than 0.01 were considered significant.

SNP discovery and genotyping

For a fine-scale association study and LD mapping of the 400 kb region containing *CALM1*, we detected SNPs in the region by direct sequencing of genomic DNA from 16 affected individuals as described previously (9,26). We genotyped the SNPs using the Invader assay, TaqMan assay and direct sequencing of PCR products with capillary sequencers (ABI3700, Applied biosystems) and tested for the association.

Statistical analysis

We carried out statistical analysis for the association study, Hardy–Weinberg equilibrium, calculation of LD coefficient (D') and LD index (Δ) as described previously (27,28). We estimated haplotype frequencies using the expectation-maximization algorithm (29). We examined combinatorial effects of *CALM1* and other loci with regard to OA susceptibility as previously described (27). Odds ratio was defined against the genotype consisted of homozygotes of non-susceptibility alleles from both loci. Luciferase assay data were analyzed by Student's *t*-test using Excel software (Microsoft).

Luciferase assay

DNA fragments corresponding to the promoter and 5′-untranslated region of *CALM1* (left panel in Fig. 2B) were amplified by PCR using genomic DNA containing the −16T or −16C allele as template, then cloned into the *Nhe*I–*Xho*I sites of pGL3-basic (Promega) in the 5′–3′ orientation. The primer sequences used for PCR are listed in Supplementary Material, Table S3. We grew OUMS-27 and Huh-7 cells (JCBR cell bank, Osaka, Japan) in Dulbecco's Modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FBS containing antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin). We then transfected cells (5×10^4 cells/well) with 0.2 μg of the constructs and 4 ng of pRL-TK vector (Promega) as an internal control for transfection efficiency, using Fugene-6 (Roche). After 24 h, cells were solubilized and luciferase activity was measured using the Pikkagene dual luciferase assay system (Toyo Ink, Tokyo, Japan).

Gel-shift assay

Nuclear extract was prepared from Huh-7 cells as previously described (30). We incubated the nuclear extract with 34 bp double-strand oligonucleotide probes for the −16C and −16T alleles for 20 min at room temperature. Oligonucleotide sequences are listed in Supplementary Material, Table S4. Probes were labeled using the digoxigenin gel-shift kit (Roche). For competition studies, we pre-incubated the nuclear extract with unlabeled oligonucleotides (125-fold excess) before adding labeled oligonucleotide. Protein–DNA complexes were separated by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE buffer, followed by transfer to nitrocellulose membrane and detection using a chemiluminescent signal detection system (Roche) according to the manufacturer's instructions.

RNA difference plot analysis

RNA difference plot analysis was performed as previously described (15). The cDNA and genomic DNA from normal human articular chondrocytes (nHAC, Cambrex Corporate, East Rutherford, NJ, USA) were used as templates for PCR. Fragments containing +114G or +114A were amplified using specific primers listed in Supplementary Material, Table S5. The 5′ ends of the forward primers were labeled with Texas Red. PCR was carried out using AmpliTaq DNA polymerase (Applied biosystems) in 10 μl of reaction volume, followed by treatment with 0.5 U of Klenow fragment (Takara, Tokyo, Japan). For single-strand conformation polymorphism analysis, an aliquot was diluted with 10 volumes of a loading solution containing 90% deionized formamide and 0.01% fuchsin (Sigma), then denatured and applied to a 0.5× MDE gel solution (Cambrex bio science Rockland,

Inc., Rockland, ME, USA) containing 1 × TBE. Electrophoresis was carried out at 20°C and 30 W for 2 h using an SF5200 autosequencer (Hitachi Ltd, Tokyo, Japan). The signal intensity of the polymorphic fragments was analyzed using Allele Links (Hitachi Ltd). Experiments were repeated nine times independently. The expression ratio between two alleles was calculated by dividing the RNA ratio by the DNA ratio with confidence interval.

Chondrogenic differentiation of ATDC5 cells

A chondrogenic mouse embryonic carcinoma cell line, ATDC5 (RIKEN cell bank, Tsukuba, Japan), was maintained in a 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12, Invitrogen) supplemented with 5% FBS containing antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin). Cells were plated at 3×10^4 cells/well in 12-well plates. After cells reached confluence, the culture medium was replaced with differentiation medium (DMEM/F12 supplemented with 5% FBS, antibiotics, 10 μg/ml bovine insulin, 10 μg/ml human transferrin and 3×10^{-8} M sodium selenite) in the presence (20 μM) or absence of the CaM inhibitor W-7 (Wako, Osaka, Japan) and cultured for several time periods (0, 6, 10, 14, 18 and 22 days). The culture medium was replaced every other day.

Ionomycin stimulation of RCJ3.1C5.18 cells

RCJ3.1C5.18 cells were generously provided by Jane Aubin (University of Toronto) (31). Cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were plated at 1×10^5 cells/well in 12-well plates. After cells reached confluence, the culture medium was replaced with medium containing 2 μM ionomycin (Sigma) with or without various concentrations of W-7 and was cultured for an additional 24 h.

Quantitative real-time PCR analysis

We extracted total RNA from ATDC5 and RCJ3.1C5.18 cells and synthesized first-strand cDNA. We carried out real-time PCR on an ABI PRISM 7700 sequence detection system (Applied biosystems) using QuantiTect SYBR Green PCR (Qiagen) according to the manufacturer's instructions. First-strand cDNA was amplified using primers specific for the mouse and rat α1 chain of the type II collagen (*Col2a1*) gene, the aggrecan 1 (*Agc1*) gene and rodent *Gapdh* (Applied biosystems). Copy numbers of *Col2a1* and *Agc1* were calculated by referring standard curves and normalized by the total RNA using *Gapdh* as an internal control. Specific primer sequences are listed in Supplementary Material, Table S6.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank patients for participating the study. We also thank Jane Aubin, Hideki Kizawa, Ryo Yamada, Ikuyo Kou, Eiji Nakashima and Koji Yoshimura for help in performing the study and Tomoko Kusadokoro and Mizuho Kosuge for excellent technical assistance. This work was supported by Japanese Millennium Project.

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