

SMAD3 Is Upregulated in Human Osteoarthritic Cartilage Independent of the Promoter DNA Methylation

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ABSTRACT. Objective. To compare *SMAD3* gene expression between human osteoarthritic and healthy cartilage and to examine whether expression is regulated by the promoter DNA methylation of the gene.

Methods. Human cartilage samples were collected from patients undergoing total hip/knee joint replacement surgery due to primary osteoarthritis (OA), and from patients with hip fractures as controls. DNA/RNA was extracted from the cartilage tissues. Real-time quantitative PCR was performed to measure gene expression, and Sequenom EpiTyper was used to assay DNA methylation. Mann-Whitney test was used to compare the methylation and expression levels between OA cases and controls. Spearman rank correlation coefficient was calculated to examine the association between the methylation and gene expression.

Results. A total of 58 patients with OA (36 women, 22 men; mean age 64 ± 9 yrs) and 55 controls (43 women, 12 men; mean age 79 ± 10 yrs) were studied. *SMAD3* expression was on average 83% higher in OA cartilage than in controls ($p = 0.0005$). No difference was observed for DNA methylation levels in the *SMAD3* promoter region between OA cases and controls. No correlation was found between *SMAD3* expression and promoter DNA methylation.

Conclusion. Our study demonstrates that *SMAD3* is significantly overexpressed in OA. This overexpression cannot be explained by DNA methylation in the promoter region. The results suggest that the transforming growth factor- β /SMAD3 pathway may be overactivated in OA cartilage and has potential in developing targeted therapies for OA. (First Release December 15 2015; J Rheumatol 2016;43:388–94; doi:10.3899/jrheum.150609)

Key Indexing Terms:

OSTEOARTHRITIS CARTILAGE *SMAD3* GENE EXPRESSION DNA METHYLATION

Osteoarthritis (OA), affecting 250 million people worldwide, is the most common form of arthritis¹. It presents with joint pain, stiffness, joint deformity, and disability², and imposes

a high socioeconomic burden³. Despite the high prevalence and socioeconomic burden, the pathogenesis of OA remains elusive^{4,5}. Evidence suggests that the *SMAD3* gene (Similar to Mothers Against Decapentaplegic type 3) plays a role in the development of OA⁶.

SMAD3 is one of the important intracellular signal transducers of the transforming growth factor- β (TGF- β) signaling pathway, which is known to play a critical role in the development, homeostasis, and repair of cartilage⁷. A lack of TGF- β /SMAD3 signaling activity is suggested to contribute to development of OA. *SMAD3*-deficient chondrocytes exhibit decreased TGF- β activity and an enhanced inappropriate terminal maturation⁸. Mice overexpressing SMURF-2, an E3 ubiquitin ligase known to inhibit TGF- β signaling, spontaneously develop an OA-like phenotype and have decreased levels of SMAD3 phosphorylation⁹. Consistent with these findings is that *SMAD3* knockout mice develop degenerative joint disease similar to human OA¹⁰. These mice are severely deficient in both type II collagen and aggrecan as a result of increased proteolytic activity of matrix metalloproteinase 13¹¹. Related to this, a patient with knee OA was found to have a missense mutation in the linker region of SMAD3 protein and an elevated serum level of matrix metalloproteinase (MMP-2 and MMP-9)¹².

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Eight missense point mutations in *SMAD3*^{13,14} have been reported to be the cause of the aneurysm-osteoarthritis syndrome, a rare syndromic disease characterized by early-onset polyarticular OA, aneurysms of the main arteries, and several connective tissue disorders. A single-nucleotide polymorphism (SNP) mapping to the first intron of *SMAD3* was reported to be involved in the risk of both hip and knee OA in European populations¹⁵, and in a population from northeast China¹⁶. Recently, we found that a SNP located in the last intron of *SMAD3* was significantly associated with the total burden of radiographic OA¹⁷. Given the location of this SNP in the gene, it is more likely that the effect of the *SMAD3* on later-onset OA is quantitative, rather than qualitative. We therefore investigated whether gene expression of *SMAD3* is different between OA-affected and healthy cartilage and whether the different expression is due to changes in promoter DNA methylation.

MATERIALS AND METHODS

Subjects. The study was part of the ongoing Newfoundland Osteoarthritis Study (NFOAS) initiated in 2011, aiming at identifying novel genetic, epigenetic, and biochemical markers for OA^{18,19}. Patients with OA were recruited from those who underwent total knee or hip joint replacement due to severe primary OA between November 2011 and December 2013 in St. Clare's Mercy Hospital and Health Science Centre General Hospital in St. John's, Newfoundland. Healthy controls were recruited in the same hospitals from those who underwent hemiarthroplasty of the hip due to hip fracture with no evidence of OA. OA diagnosis was based on American College of Rheumatology criteria²⁰ and the judgment of the attending orthopedic surgeons. The pathology report on the removed cartilage for all subjects was reviewed to ensure accuracy of diagnosis and the status of any cartilage degeneration in the controls. The consent rate of the study was 90%. The study protocol was approved by the Health Research Ethics Authority of Newfoundland and Labrador and written consent was obtained from all participants.

Demographic and anthropometric data. Demographic information was obtained by a self-administered questionnaire with the help of research staff if necessary. Anthropometric data including height and weight were retrieved from hospital admission and medical records and body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters. Age was calculated at the time of the surgery.

DNA/RNA isolation. Four pieces (~200 mg each) of cartilage tissues were retained from either tibial plateaus or femoral heads during the surgery. Samples were then flash-frozen and stored in liquid nitrogen until the experiment. DNA and RNA were extracted from the same piece of the cartilage tissue to avoid sampling bias. Up to 200 mg frozen cartilage tissue was transferred to the homogenizing cylinder together with 1 ml TRIzol lysis reagent and 200 µl guanidine thiocyanate and homogenized using a cryogenic mill (Spex Freezer Mill, model 6770, Metuchen, NJ, USA) with the following procedure: 2 cycles of 2 min grinding at maximum frequency with 10 min for cooling between grinding cycles. The homogenate was then transferred to a new 2 ml RNase-free tube and incubated 5 min at room temperature. Then 200 µl chloroform was added and the mix was vortexed vigorously, before being incubated 2-3 min, followed by centrifugation at 12,000 g at -4°C for 15 min. After centrifugation, the sample separated into 3 phases: the aqueous phase containing RNA, the interphase, and the organic phase containing DNA. The RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, Netherlands) was then used for extracting total RNA from the aqueous phase according to the manufacturer's protocol. The DNA was extracted using the phenol-chloroform method from the interphase and organic phase.

***SMAD3* gene expression measurement.** Complementary DNA (cDNA) synthesis from the extracted RNA was done using the Maxima H Minus First Strand cDNA Synthesis Kit (K1682; Thermo Scientific, Vilnius, Lithuania). One hundred nanograms of RNA from each sample primed with 0.5 µl of random primer was denatured at 65°C for 5 min and chilled on ice before addition of a reverse-transcription solution containing 2 µl of 5× buffer, 0.5 µl Ribolock, 1 µl of 10 mM dNTPs mix (Invitrogen, Carlsbad, CA, USA), and 0.5 µl Maxima polymerase in a final volume of 20 µl. The cDNA synthesis reaction was performed for 60 min at 42°C and then 5 min at 70°C. One microliter of the converted cDNA was subject to quality control by PCR amplification of the *SMAD3* and *GAPDH* genes followed by agarose gel electrophoresis.

Quantification of *SMAD3* was performed using the ABI-7900HT Fast Real-Time PCR system on a 96-well plate. *GAPDH* was used as an internal reference gene to normalize the relative quantification of the targeted gene *SMAD3*. *GAPDH* and *SMAD3* amplification primers were designed using the NCBI primer-blast tool for the shortest isoforms of the genes, and the sequences were blasted in the NCBI Blast tool to ensure 100% coverage of all the isoforms as well as minor similarities to other genomic sequences. Primers were validated using a 4-point dilution series of 2 random cDNA samples. The primer efficiencies were found to be within the acceptable range, i.e., 106% and 110% for *GAPDH* and *SMAD3*, respectively. Table 1 presents the primer sequences used and the size of PCR products. qPCR was then performed in triplicate using 5 µl of cDNA, 10 µl SYBR Green (Power SYBR Green PCR Master Mix, Applied Biosystems, Waltham, MA, USA), and 0.4 µl of forward and reverse primers in a final volume of 20 µl. Cycling conditions were as follows: 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, repeated in 45 cycles, followed by melt-curve analysis. One of the control samples was selected as calibrator and the relative quantification (RQ) of *SMAD3* expression in each sample was calculated as fold-changes in relation to the calibrator using the Livak method²¹.

***SMAD3* promoter methylation assay.** Bisulfate conversion of DNA was conducted using the EpiTect Bisulfite Kit (Qiagen, Venlo, Netherlands). Briefly, 50 ng genomic DNA in 2 µl water was mixed with 38 µl RNase-free water, 85 µl bisulfate mix, and 15 µl DNA protect water in a final volume of 140 µl. The conversion reaction and DNA cleanup were conducted according to the manufacturer's instructions.

SMAD3 promoter DNA methylation was quantified using the Sequenom EpiTyper platform²². Primers for the target region were designed using Sequenom EpiDesigner, and the PCR target sequence was tested with the RSeqMeth program implemented in R programming language. Table 1 gives the primer details. The converted DNA was subject to PCR amplification, shrimp alkaline phosphatase treatment, *in vitro* RNA transcription, base-specific cleavage, and mass spectrometry analysis. The mass signals generated were translated into quantitative DNA methylation levels (beta value range 0-1) by MassArray EpiTyper Analyzer software. On every bisulfite plate, standard DNA samples with 0%, 50%, and 100% methylations were included as controls for the technical steps of the experiment.

Statistical analysis. Descriptive statistics were summarized using either mean or percentage, and comparisons between OA cases and controls were performed using Student T-test or chi-squared test where appropriate. Nonparametric Mann-Whitney test was used to compare gene expression and methylation levels between OA cases and controls, and nonparametric regression model was used to adjust for potential confounders. Spearman rank correlation coefficient (*rho*) was calculated to examine the relationship between the promoter DNA methylation and gene expression. Analysis was conducted using STATA/SE 11.2 (Stata Corp., College Station, TX, USA). Significance was defined as α level of 0.05.

RESULTS

A total of 113 participants were included in the study, 58 (47 hip OA, 11 knee OA) categorized as OA cases and 55 as healthy controls (hip fracture patients). Overall, 69% of

Table 1. Primers used in quantitative PCR (qPCR) and EpiTyper experiments.

	Primer Sequence (5' > 3')	Product Size, bp
<i>SMAD3</i> reverse primer, qPCR	GGC TCG CAG TAG GTA ACT GG	91
<i>SMAD3</i> forward primer, qPCR	GCA TGG ACG CAG GTT CTC C	
<i>GAPDH</i> reverse primer, qPCR	TCG CCC CAC TTG ATT TTG G	106
<i>GAPDH</i> forward primer, qPCR	GCA AAT TCC ATG GCA CCG T	
<i>SMAD3</i> reverse primer, EpiTyper	CAG TAA TAC GAC TCA CTA TAG GGA GAA GGC TTC CAA CCA TTA AAA AAT AAC CAA AA	208
<i>SMAD3</i> forward primer, EpiTyper	AGG AAG AGA GAA AGG ATT TGA ATT ATA GGA GGA TAG	

participants were female and 31% were male. Controls were on average 15 years older than OA cases ($p < 0.0001$) and had a lower BMI than OA cases ($p < 0.0001$). Table 2 presents the characteristics of the study population. Pathological examination of the joint cartilage confirmed all the OA cases. It also confirmed that 21 controls had healthy cartilage, but the other 34 controls had age-related minor degenerative changes. The gene expression experiment was performed on 38 patients with OA (32 hips, 6 knees) and 28 controls, and the methylation analysis was conducted for 49 patients with OA (38 hips, 11 knees) and 51 controls. Since 52 subjects had data on both methylation and expression, they were included in the methylation expression correlation analysis.

***SMAD3* expression in cartilage.** We first examined *SMAD3* expression between the controls with intact healthy cartilage and those with minor age-related degeneration, and found no difference among the 2 groups. The average RQ values were 1.25 ± 1.05 and 1.35 ± 0.69 for these 2 groups ($p = 0.37$), respectively. We therefore combined these 2 groups and treated them as controls in the subsequent analyses.

The mean RQ values of the *SMAD3* were 2.37 ± 1.30 in OA cartilage and 1.30 ± 0.89 for controls. This represents 83% increased expression of *SMAD3* in OA cartilage compared to controls ($p = 0.0005$) (Figure 1). Similar results were observed when analyses were done separately for knee and hip OA (72% increase for knee OA, $p = 0.01$; 84% increase for hip OA, $p = 0.001$), but no difference was found between knee OA and hip OA ($p = 1.00$) (Figure 1).

We found that *SMAD3* expression was not associated with age and BMI in either OA or healthy cartilage (all $p > 0.07$). However, we found that women tended to have lower expression than men, but this was significant only in OA

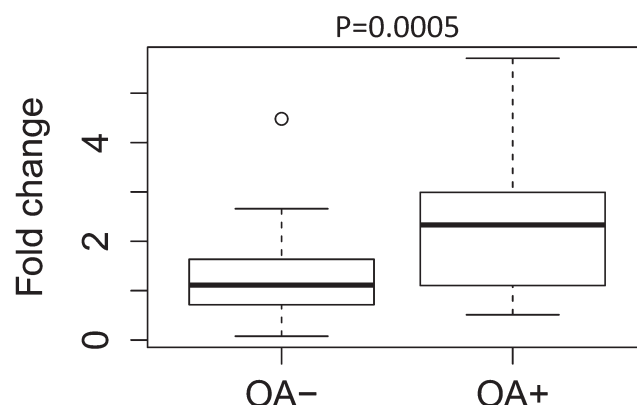


Figure 1. Relative quantification of *SMAD3* expression in human cartilage from osteoarthritis (OA) cases and controls (Mann-Whitney test for comparisons).

cartilage ($p = 0.05$; see Supplementary Figure 1-3, available online at jrheum.org). The significant difference in the *SMAD3* expression between OA cases and controls remained after adjustment for sex using nonparametric regression ($p = 0.001$).

DNA methylation in the SMAD3 promoter region. The upstream 600 bp sequence of the first exon of the longest isoform of *SMAD3* (Figure 2) was retrieved from the Ensemble genome browser (ENST00000327367) and copied into the EpiDesigner²², from which a 208 bp region was identified as optimal for the experiment design, containing the largest number of detectable CpG sites. The identified sequence was subsequently blasted in UCSC Blast and confirmed to be part of the active promoter. It contained 5 CpG sites, 4 of which were successfully assayed by the EpiTyper²². These 4 sites are located 413 bp, 442 bp, 455 bp, and 475 bp upstream of the first exon of *SMAD3* (Figure 2). The methylation levels at these 4 CpG sites were similar between OA cases and controls (Figure 3) (all $p > 0.05$). The same results were observed when the analyses were done for knee OA and hip OA ($p > 0.05$). We also calculated Spearman rank correlation coefficients between each CpG site and the *SMAD3* expression, and found no correlations (all $p > 0.05$) (Table 3; and Supplementary Figure 4, available online at jrheum.org).

Table 2. Characteristics of the study population. Values are mean \pm SD, unless indicated otherwise.

Characteristic	Cases, n = 58	Controls, n = 55	p
Age, yrs	64.2 \pm 10.2	79.3 \pm 9.4	< 0.0001
Body mass index, kg/m ²	31.7 \pm 0.9	23.5 \pm 0.8	< 0.0001
Female, %	62	78	0.06

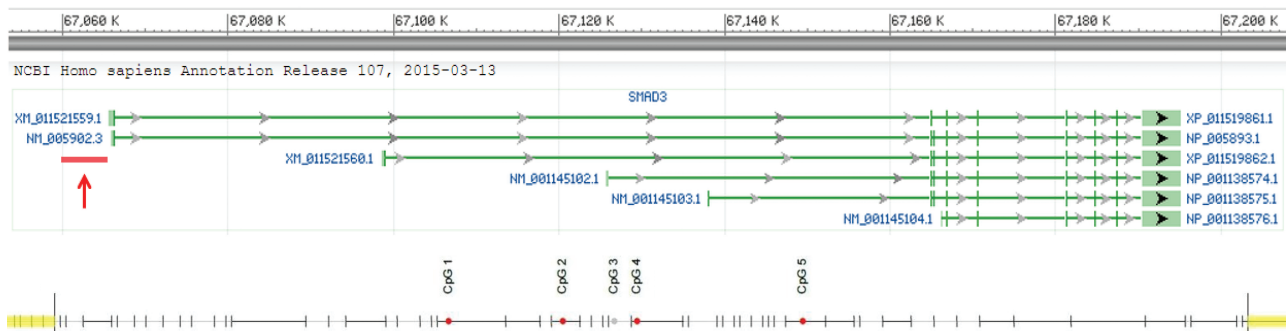


Figure 2. Location of 4 CpG sites in the promoter region of SMAD3. DNA segment within the 600 bp region (red arrow) upstream of the first exon of SMAD3 was the targeted region for the methylation assay using EpiTyper. A 208 bp segment within the region (lower horizontal line) containing 5 CpG sites was amplified, of which 4 CpG sites were successfully assayed by EpiTyper (red dots) and one was not analyzed (gray dot). The breaks represent fragmentation in MassCleave reaction sites. Locations of the CpG sites on Ensembl-Havana Gencode gene set (release 22) as follows: CpG1: chr15:67,062,688; CpG2: chr15:67,062,708; CpG4: chr15:67,062,721; CpG5: chr15:67,062,750.

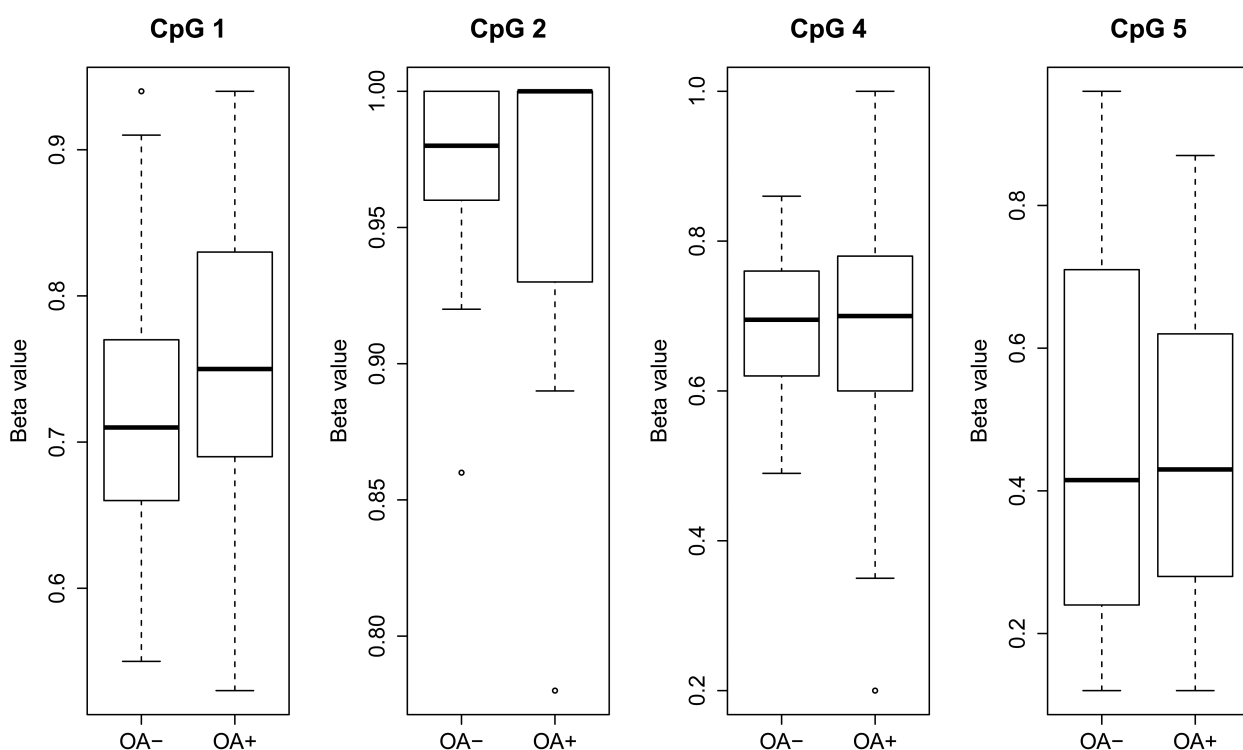


Figure 3. Methylation levels of 4 CpG sites in the SMAD3 promoter from osteoarthritis (OA) cases and healthy controls. None of the comparisons was significant (all $p > 0.05$) (Mann-Whitney test for comparisons).

DISCUSSION

To our knowledge, this is the first study investigating *SMAD3* gene expression and its promoter DNA methylation in human osteoarthritic and healthy cartilage tissues. We found that *SMAD3* was significantly overexpressed in the OA cartilage compared to healthy cartilage. The overexpression was independent of the DNA methylation in the *SMAD3* promoter region, and appeared not to be joint-specific.

SMAD3 is one of the intracellular mediators of the TGF- β

signaling pathway, which is involved in diverse cellular processes including proliferation, differentiation, migration, and apoptosis, as well as extracellular matrix (ECM) synthesis and degradation^{23,24}. Its activity is essential to the maintenance of cartilage²⁵. The signaling suppresses the catabolic effects of interleukin 1 and tumor necrosis factor- α on cartilage, and prevents degradation of ECM molecules by enhancing the production of protease inhibitors, such as tissue inhibitors of metalloproteinase²⁶. Decreased phospho-

Table 3. Spearman correlation coefficients between methylation levels and gene expression.

	CpG 1	CpG 2	CpG 4	CpG 5
Controls, n = 22	-0.14	-0.31	0.18	-0.06
OA, n = 30	0.04	-0.20	0.11	-0.01
Combined, n = 52	0.12	-0.02	0.16	0.01

p > 0.05 for all tests.

ylation of SMAD3 was previously observed during the progression of murine models of OA^{27,28}, and it was also shown that SMAD3 knockout mice developed OA-like features¹⁰. These observations indicate that a lack of TGF- β /SMAD2/3 signaling activity is involved in the development of OA, particularly early-onset OA¹⁰. Thus, it was expected that SMAD3 expression would be diminished in OA cartilage. However, we observed that SMAD3 was significantly overexpressed in OA cartilage. Potential confounders may bias our results, as a previous study reported that women had lower SMAD3 expression than men²⁹. Consistently, we also found that women had lower expression of SMAD3 in cartilage than men. However, the significant difference in SMAD3 expression between OA-affected cartilage and controls was not altered after adjustment for sex, indicating that the observed association cannot be explained by sex. We also examined the effects of age and BMI on SMAD3 expression, and found there was no significant association. However, there was a trend to a decrease in SMAD3 expression with increasing age in the OA cases only, and there was an increase of SMAD3 expression with increasing BMI in both OA cases and controls (Supplementary Figure 2, available online at jrheum.org). It is possible that this trend might become significant with a larger sample size. Further studies with a larger sample size are needed to rule out the confounding effects of age and BMI on the observed association.

The observed paradox can be interpreted in terms of 2 possible mechanisms during the development of OA. First, the overactivity of the TGF- β pathway could indicate an attempt in the cartilage to repair damage occurring during OA development. Initiation of OA is thought to be caused by an attempt to repair an initial cartilage injury. The response results in an overproduction and accumulation of collagen and proteoglycans, leading to cartilage swelling and breakage, subchondral bone cavity formation and osteophyte growth, and finally the involvement of the whole joint and OA presentation^{30,31}. The second possible explanation could be related to the hypothesis that only a narrow range of bioactive TGF- β levels can maintain cartilage health, and any concentrations below or above this range may lead to aberrant alterations in TGF- β pathways, resulting in abnormal cartilage function³². In accord with this hypothesis, multiple intraarticular injections of TGF- β in joints of mice resulted in changes in articular cartilage that strongly

resembled both experimental and spontaneous OA³³. Enhanced expression of TGF β 1 and TGF β 3 was detected in developing osteophytes and articular cartilage in murine experimental OA, and the inhibition of endogenous TGF- β prevented osteophyte formation²⁷. Increased activity of TGF- β was also observed in other joint tissues. High concentration of active TGF- β 1 in subchondral bone of mice was reported to initiate osteoarthritic changes in the bone and cartilage³⁴. Induced expression of TGF β 1 from the synovial lining layers resulted in OA-like changes in murine knee joints, including hyperplasia of synovium and chondro-osteophyte formation³⁵. Increased activity of TGF- β can also enhance the expression of cartilage degradative enzymes such as MMP13. A study showed that TGF- β can upregulate the levels of MMP13 in normal cartilage *in vitro* and mimic the *in situ* distribution of the increased MMP13 in both OA and rheumatoid arthritis-affected cartilage³⁶. This phenomenon has also been observed in other tissues. Activation of the TGF- β /SMAD3 pathway enhanced MMP13 expression in squamous carcinoma cells³⁷, breast cancer cells³⁸, human gingival fibroblasts³⁹, and osteoblastic cells⁴⁰. Our results favor this hypothesis, but further studies are required to investigate SMAD3 and MMP13 simultaneously in human joint tissues.

It is not clear yet what causes increased SMAD3 expression in OA cartilage. DNA methylation is thought to regulate gene expression. We examined the correlation between the promoter DNA methylation and SMAD3 expression, but found no correlation. A recent study by Raine, *et al*⁴¹ examined whether the expression of SMAD3 in OA cartilage was correlated with rs12901499, a SNP that was reported to be associated with OA¹⁵. They found no correlation between this SNP and SMAD3 expression in OA cartilage, but identified another SNP, rs8031440, located at 3'UTR that was associated with the expression of SMAD3. The SNP was weakly associated with OA. The study also found that SMAD3 expression in OA knee cartilage was different from that of OA hip cartilage, in contrast to what we found in our study. The reason for this discrepancy is not clear. The study by Raine, *et al*⁴¹ did not include healthy control cartilage, making interpretation of their results difficult.

The strength of our study is the use of human cartilage rather than animal models or cultured cells, thus having direct application to patients with OA. We extracted DNA and RNA from the same sample and minimized bias in examining the correlation between DNA methylation and gene expression due to differential sampling. However, we measured expression levels of mRNA only, which may not reflect the corresponding protein levels or their phosphorylated SMAD3 isoforms. This is particularly significant since the function of SMAD3 protein depends on not only its expression levels but also its phosphorylation status. Further, only 4 CpG sites in the SMAD3 promoter were investigated, and we cannot rule

out the association with DNA methylation at other CpG sites in the gene. We observed the promoter region of the longest isoform of the *SMAD3* gene, whereas there are several different isoforms of the gene and possibly multiple promoters, and we may have overlooked the isoform-specific CpG sites. Obtaining cartilage tissue samples from healthy individuals is ethically not acceptable, and we used cartilage samples from patients with hip fracture as controls, which may not necessarily represent true healthy cartilage. However, we performed pathological examinations and confirmed the healthy status of the control samples. Patients with OA were all at the end stage of the disease; thus the findings may not be related to OA initiation and progression. Finally, we studied only cartilage tissue, limiting the generalizability of the findings to other joint tissues.

We have demonstrated that *SMAD3* is overexpressed in osteoarthritic cartilage, independent of the promoter DNA methylation, suggesting that the TGF- β /SMAD3 pathway may be overactivated in OA cartilage; this may represent potential for developing targeted therapies for OA.

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ONLINE SUPPLEMENT

Supplementary data for this article are available online at jrheum.org

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