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Correlating HRMAS NMR Spectroscopy and Gene Analysis in OA Cartilage

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Abstract

Osteoarthritis (OA) is a common multifactorial and heterogeneous degenerative joint disease, and biochemical changes in cartilage matrix occur during the early stages of OA before morphologic changes occur. Thus, it is desired to measure regional biochemical changes in the joint. High-resolution magic angle spinning (HRMAS) NMR spectroscopy is a powerful method of observing cartilaginous biochemical changes ex vivo, including the concentrations of Alanine and N-acetyl, which are markers of collagen and total proteoglycan content, respectively. Previous studies have observed significant changes in chondrocyte metabolism of OA cartilage via the altered gene expression profiles of ACAN, COL2A1, and MMP13, which encode aggrecan, type II collagen, and matrix metalloproteinase 13 (a protein crucial in degrading type II collagen), respectively. Employing HRMAS, this study aimed to elucidate potential relationships between N-acetyl and/or Alanine and ACAN, COL2A1, and/or MMP13 expression profiles in OA cartilage. Thirty samples from the condyles of five subjects undergoing total knee arthroplasty to treat OA were collected. HRMAS spectra were obtained at 11.7 T for each sample. RNA was subsequently extracted to determine gene expression profiles. A significant negative correlation between N-acetyl metabolite and ACAN gene expression levels was observed; this provides further evidence of N-acetyl as a biomarker of cartilage degeneration. The Alanine doublet was distinguished in the spectra of 15 of the 30 specimens of this study. Alanine can only be detected with HRMAS NMR spectroscopy when the collagen framework has been degraded such that Alanine is sufficiently mobile to form a distinguished peak in the spectrum. Thus, HRMAS NMR spectroscopy may provide unique localized measurements of collagenous degeneration in OA cartilage. Identifying imaging markers that could provide a link between OA pathology and chondrocyte metabolism will facilitate the development of more sensitive diagnostic techniques and will improve methods of monitoring treatment for patients suffering from OA.

Graphical abstract

The significant negative trend observed between the HRMAS NMR metabolite N-acetyl and the ACAN gene expression profile provides further evidence of N-acetyl as a biomarker of OA progression. This could lead to the subsequent identification of non-invasive markers that would

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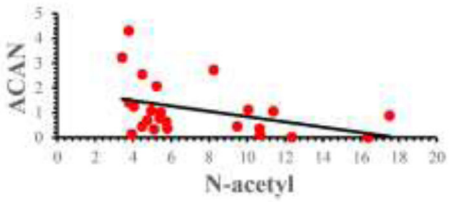
allow for earlier diagnoses and more clinically accessible methods of tracking disease development. Evidence was also found suggesting the unique potential of HRMAS NMR spectroscopy to analyze regional biochemical changes in cartilage collagen during OA.



Keywords

Osteoarthritis; Cartilage; HRMAS NMR Spectroscopy; Gene expression; N-acetyl; Alanine; Proteoglycan; Collagen

Introduction

Osteoarthritis (OA) is a prevalent, debilitating, and degenerative disease of the joint that results in changes in cartilage composition (1-7). Although its pathology has not yet been fully elucidated, it is widely accepted that multiple underlying physical and biochemical mechanisms affect the integrity of the cartilage matrix via an overall imbalance between the joint's catabolic and anabolic processes (7-9).

Because of the heterogeneous nature of OA progression (10), it is of great importance to develop methods to measure localized compositional changes of the knee joint noninvasively with MR imaging. To achieve this goal, potential imaging biomarkers of OA pathology must first be identified. High-resolution magic angle spinning (HRMAS) NMR spectroscopy is a powerful and sensitive tool for detecting and quantifying metabolites in articular cartilage ex vivo (11-15). Specifically, HRMAS NMR spectroscopy has previously been used to detect decreased levels of N-acetyl (a marker of total proteoglycan content) and Alanine (a potential marker of collagen concentration) in human OA cartilage compared to control (11). However, it is unknown how the levels of these HRMAS-detectable metabolites relate to changes in chondrocyte gene expression in that tissue.

To understand chondrocyte function, it is important to first appreciate the complexity of gene expression in knee cartilage. Three genes in particular have been studied extensively – ACAN (encodes aggrecan), COL2A1 (encodes type II collagen), and MMP13 (encodes matrix metalloproteinase 13, which degrades type II collagen, among other proteins (16-18)). The results of previous research on these three genes have elucidated that their regulation is dynamic, multifactorial, and dependent on the progression of OA. In early OA, several studies have observed ACAN to be up-regulated (19-22), down-regulated (23), or unchanged (24-26). COL2A1 mRNA expression similarly has been noted to be significantly induced (21-22, 26) or to have remained stable (23, 25). MMP13 expression has largely been reported to be markedly enhanced in early OA (23, 25-26), which corresponds to its significant catabolic role during the early stages of cartilage degeneration (10). During late

OA, Cs-Szabo et al (27) observed an up-regulation in ACAN expression while Yagi et al (28) and Brew et al (29) noted a down-regulation of ACAN mRNA levels and Aigner et al (24) saw no change. COL2A1 has been documented to be up-regulated (24, 30-31). And, as with early OA, several studies have reported MMP13 expression to be induced in late OA (18, 24, 29).

Finding imaging markers that could provide a link between OA pathology and chondrocyte metabolism is quintessential to developing more sensitive diagnostic methods and improving the treatment of patients suffering from OA. Therefore, the goal of this study was to elucidate potential relationships between the metabolites N-acetyl and Alanine detectable by HRMAS NMR spectroscopy and the gene expression profiles of ACAN, COL2A1, and MMP13.

Methods

Subjects

Femoral condyles were collected from five patients with primary OA (four female, one male; ages 51-71 years with average of 63±8 years) undergoing total knee arthroplasty at the University of California, San Francisco Moffitt Hospital. Patients were excluded from the study if they screened positive for standard bloodborne pathogens. This study was approved by the Committee for Human Research at the University of California, San Francisco. Informed consent was obtained from all of the subjects.

Cartilage Collection

As shown in Figure 1, six samples were collected from each subject, with each sample consisting of a pair of adjacent 3 mm cartilage biopsy punches to ensure that an adequate amount of tissue was acquired for analysis. The adjacent punches were taken within close proximity to one another to maximize the homogeneity of the samples. Two samples were collected from each the lateral and medial inferior femoral condyles (LIFC and MIFC, respectively). One sample was collected from each the lateral and medial posterior femoral condyles (LPFC and MPFC, respectively) due to the smaller size of the LPFC and MPFC. The punches were immediately flash frozen and stored in liquid nitrogen until the time of HRMAS NMR analysis and RNA extraction.

HRMAS NMR Spectroscopy Analysis

For each sample (or pair of cartilage punches) collected, HRMAS NMR spectra were obtained with a 11.7 T (500 MHz for ^{1}H) Varian INOVA spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 4 mm gHX nanoprobe at 1° C. This study followed the protocols established in previous studies that focused on optimizing the quality of HRMAS NMR spectra and maximizing tissue integrity (11, 32). In brief, water pre-saturated 1-D spectra were acquired with 40,000 complex points over a 90° pulse, 20,000 Hz spectral width, a TE of 144 ms, and a TR of 4.2 s. In total, sample preparation, tuning, shimming, pulse width calibration, and spectral acquisition required approximately 1.5 hours per sample. Metabolite signals were quantified using the Electronic Reference to access In vivo

Concentrations (ERETIC) method as established by Swanson, et al (33). The resulting spectra were referenced to 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP) at 0 ppm.

For this study, the N-acetyl peak (2.04 ppm) and, when present, the Alanine peak (1.47 ppm) were quantified, as shown in Figure 2. Previous work identified N-acetyl and Alanine as markers for total proteoglycan and collagen content in cartilage, respectively (11).

RNA Extraction

Each sample of two 3 mm frozen biopsy punches were ground into a fine powder with a liquid nitrogen-cooled mortar and pestle and added to TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The RNA was further purified using the Invitrogen PureLink RNA Mini Kit (Carlsbad, USA) with on-column DNase, also per manufacturer's instructions. RNA concentration was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was determined with a bioanalyzer for quality control (Agilent 2100, Agilent Technologies, Santa Clara, CA, USA).

Quantitative Reverse Transcription PCR

RNA was reverse transcribed into cDNA (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA) for quantitative reverse transcription polymerase chain reaction (qRTPCR; CFX96 Real-Time Systems, Bio-Rad, Hercules, CA, USA). The expression levels of four genes were evaluated in this study – beta-2-microglobulin (B2M), ACAN, COL2A1, and MMP13. TaqMan primer set (Life Technologies) assay identification numbers are shown in Table 1. Following the precedent established by two previous studies (34-35), B2M was used as the endogenous gene to which the levels of expression of ACAN, COL2A1, and MMP13 were normalized. ACAN, COL2A1, and MMP13 were specifically chosen for this study because of the extensive past research performed on these three genes (16-31). Two technical replicates were run for each sample in adjacent plate wells for reproducibility.

The contents of the PCR plate were first held at 50° C for two minutes for Uracil-N Glycosylase (UNG) incubation to optimize UNG activity. They then were held at 95° C for 10 minutes for AmpliTaq Fast DNA Polymerase activation. The contents lastly were amplified with 40 PCR cycles (consisting first of a denaturation step at 95° C for 15 seconds and then an annealing/extending step at 60° C for one minute).

Analysis of Gene Expression

The data were analyzed with the delta Ct method. Replicate ACAN, COL2A1, and MMP13 Ct values were first averaged and, following the precedent established by two previous studies (34-35) normalized to corresponding endogenous B2M expression levels, yielding delta Ct values. Normalization to an endogenous gene allows for the consideration of global changes in chondrocyte metabolism when determining the changes in expression levels of individual genes. Delta Ct values were then normalized to the corresponding ACAN, COL2A1, and MMP13 data from a reference cartilage specimen to yield delta delta Ct values. The reference, a calibration standard used in the lab for multiple experiments, is a cartilage plug derived from intact cartilage physically distant from an osteonecrotic lesion

on a 49-year-old woman undergoing total hip arthroplasty, also at the University of California, San Francisco Moffitt Hospital. The fold inductions for each sample were then calculated as 2^(-delta delta Ct) to describe changes in gene expression levels in OA cartilage compared to the reference.

Statistical Analysis

Spearman's rank correlation tests were performed using the R statistical computing software between gene expression and HRMAS NMR spectral data, yielding p and rho values. Correlation tests were run between (i) the expression levels of each gene and (ii) the N-acetyl and Alanine HRMAS NMR values and the expression levels of each gene.

Results

Due to the variability in tissue degeneration and in assay sensitivity, some gene or HRMAS measurements were not available for every sample. Table 2 shows the number of samples with HRMAS NMR spectroscopy and gene data available for analysis.

ACAN, COL2A1, and MMP13 Expression Levels

To quantify the extent of OA-dependent changes in chondrocyte gene expression, the mRNA levels of ACAN, COL2A1, and MMP13 were evaluated. The fold changes (mean ±standard deviation) of ACAN, COL2A1, and MMP13 in OA cartilage compared to the reference cartilage are shown in Table 3.

Furthermore, to elucidate any potential interrelationships among the expression levels of these three genes of interest, we also performed analyses to correlate the fold-changes in ACAN, COL2A1, and MMP13 expression (Table 4a). The data for ACAN and COL2A1 showed a non-significant trend towards a positive relationship ($p = 0.065$, rho = 0.36). Also observed were a non-significant positive trend between COL2A1 and MMP13 ($p = 0.27$, rho = 0.35) and a non-significant negative trend between ACAN and MMP13 ($p = 0.61$, rho = −0.19). Table 4b highlights the results of another study (29) in which the same analysis produced similar results.

Gene Expression Levels vs. N-acetyl and Alanine Values

The mean normalized concentrations of N-acetyl and Alanine were 7.20 and 0.94, respectively.

To identify a potential link between imaging markers and gene expression levels, analyses were performed to uncover correlations between N-acetyl and Alanine metabolite values and the fold-changes in ACAN, COL2A1, and MMP13 mRNA levels. As shown in Table 5, a significant negative correlation between N-acetyl and ACAN ($p = 0.016$, rho = −0.49), whose scatter plot is shown in Figure 3, and a non-significant positive correlation between Alanine and COL2A1 ($p = 0.19$, rho = 0.36) were obtained. Other results showed nonsignificant negative relationships between N-acetyl and COL2A1 ($p = 0.31$, rho = −0.21), N-acetyl and MMP13 ($p = 0.30$, rho = −0.35), and Alanine and MMP13 ($p = 0.54$,

rho = −0.26). We found no observable trend between Alanine and ACAN (p = 0.91, rho = −0.038).

Discussion

This study endeavored to elucidate potential links between imaging markers and levels of gene expression by quantifying the expression levels of ACAN, COL2A1, and MMP13 by qRT-PCR and the concentrations of N-acetyl and Alanine by HRMAS NMR spectroscopy in OA cartilage. We detected mean normalized concentrations of N-acetyl and Alanine in our OA cartilage samples to be 7.20 and 0.94, respectively, which were lower than the control cartilage data reported by Shet et al (8.5 for N-acetyl and 1.5 for Alanine; (11)).

The main finding of our study is the observed significant negative correlation between the HRMAS NMR spectroscopy metabolite N-acetyl (an established marker of total proteoglycan content (11)) and the ACAN gene expression profile (p = 0.016, rho = −0.49). It is well known that proteoglycan loss occurs during cartilage degeneration in OA, and it has been previously reported that N-acetyl levels as measured by HRMAS NMR decrease in OA cartilage (11). In contrast, the regulation of ACAN gene expression is considerably more multifactorial (19-29). As noted by Brew et al (29), while ACAN has been noted to be up-regulated in early OA (19), they reported a down-regulation of ACAN expression in late OA. As such, the correlation between N-acetyl and ACAN may depend on OA severity. Considering Brew et al's claims, we hypothesize that a negative correlation may be present between N-acetyl and ACAN in early OA while a positive trend may be observed in late OA.

Given this information, one may find our observation of a significant negative correlation between N-acetyl and ACAN surprising since most individuals undergoing total knee arthroscopy as a treatment for OA typically present with late-stage OA. However, the spectrum of disease severity that is present even within one osteoarthritic knee (10) may affect the observed interrelationship between N-acetyl and ACAN. Namely, the regulation of gene expression in areas of visibly less degraded cartilage, which was where we harvested our cartilage plugs in order to ensure that sufficient cartilage was collected for analysis, may more closely resemble those in cartilage during the relatively early stages of OA. Lorenz et al in fact has proposed that the degenerative changes occurring in cartilage neighboring OA lesions appear to mirror those in early OA (10). This line of reasoning not only explains the negative trend between N-acetyl and ACAN that we observed, but also suggests the unique power of HRMAS NMR spectroscopy as a tool to track and analyze regional biochemical changes in OA cartilage.

At this time it is too preliminary to hypothesize about the relationships among Alanine and ACAN, COL2A1, and MMP13 because of the lack of significant correlations among these variables. However, it is worth discussing the fact that we were able to detect Alanine on the HRMAS NMR spectra of only 15 of the 30 collected and processed samples. In cartilage with intact collagen, the Alanine peak is too broad to be differentiated in HRMAS NMR spectra due to the very short T_2 relaxation time associated with macromolecules. Alanine can only be detected when it is mobile enough to have its own distinguished peak, which is a

result of the degradation of the collagen framework. This suggests the potential of a second unique ability of HRMAS NMR spectroscopy to delineate localized collagen degeneration in relation to the stage and extent of OA progression.

While not the main goal of this study due to our small sample size, we also endeavored to contribute to the existing literature regarding gene expression levels in OA cartilage, which requires large-scale studies due to the established dynamic nature of gene expression in OA. Assuming our cartilage specimens do in fact resemble early OA cartilage due to reasons elucidated above, like several previous studies, we observed no consistent OA-dependent changes in ACAN (24-26) or COL2A1 (23, 25) expression levels and a considerable induction in MMP13 expression (23, 25-26). We also ran correlations among the levels of ACAN, COL2A1, and MMP13 mRNA and found results similar to those of a prior study (29), which are shown in Table 4. Specifically, we found positive trends between both ACAN and COL2A1 and COL2A1 and MMP13, although the correlations were not significant, and a non-significant negative trend between ACAN and MMP13.

Despite the fact that many groups have investigated the changes in chondrocyte gene expression profiles with large subject cohorts, there remains a lack of consensus about the change in gene expression in OA, especially regarding ACAN and COL2A1. As Lorenz and Richter have comprehensively summarized in (10), one major reason for this is the fact that there is no single common methodology employed to conduct this analysis. Different labs favor using human samples or different animal models to study OA at different stages of disease progression (10). Additionally, they prefer to normalize their target gene expression data to different standards – including DNA or total RNA levels, or the expression levels of various housekeeping genes (10).

Another major reason for the inconsistent findings in the literature is the fact that gene expression is multifactorial and dynamic, and the development of OA is not uniform throughout the joint cartilage. As such, gene expression levels also vary throughout the joint even for a single individual (10, 26). Fully elucidating the pathology of OA will require both a local and global understanding of the disease. At the present time, HRMAS NMR Spectroscopy requires explanted specimens for analysis. Thus, future technological development is required if one desires to directly use in vivo MR spectroscopy techniques to quantify cartilage matrix biochemical profiles for non-invasive study. Regardless, our observation of a significant negative correlation between N-acetyl and ACAN is suggestive of HRMAS NMR Spectroscopy's power to help identify potential biomarkers that are relevant to OA pathology, which can subsequently be used to study the relationship between imaging biomarkers and regional biochemical changes at the RNA and protein level in vivo using MR's clinical and translational applications.

The limitations of this study include a small subject sample size: a total of 30 cartilage samples were collected from a cohort of five subjects. Also, we did not perform corrections for T1 and T2 because we did not measure those values; however, we selected TR and TE values previously used in the literature (11) to minimize T1 weight and the baseline distortion caused by macromolecules with short T2 values, respectively. In addition, a nontrivial amount of RNA degradation inevitably occurs during the HRMAS NMR

procedure despite specimens being scanned at 1° C to minimize degradation; however, our tests for quality control suggested that the concentration and integrity of our RNA yield were sufficient for robust data analysis. Another limitation was that we did not request the operating surgeon to grade the severity of the OA subjects. Also, an ideal reference cartilage would have been a cartilage sample from an individual who had to undergo an above-knee amputation for reasons not involving knee health. Therefore, it would be interesting to repeat the analysis described above with a larger cohort of subjects whose stages of OA progression have been clinically determined.

Conclusion

To our knowledge, for the first time, cartilage matrix metabolism at the level of both gene expression and imaging biomarkers was studied simultaneously. We found evidence suggesting an intrinsic link between the HRMAS NMR spectroscopy metabolite N-acetyl and ACAN gene expression. This shows promise of N-acetyl as a possible marker of disease progression that may lead to the subsequent identification of novel non-invasive imaging markers that would allow for earlier diagnoses and easier clinical monitoring of treatment with MR imaging. Lastly, evidence was found suggesting the potential of HRMAS NMR spectroscopy as a powerful tool to analyze localized biochemical changes in OA cartilage.

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Abbreviations used

OA	osteoarthritis
HRMAS	high-resolution magic angle spinning
ACAN	gene that encodes aggrecan
COL2A1	gene that encodes type 2α1 collagen
MMP13	gene that encodes matrix metalloproteinase 13
B2M	gene that encodes beta-2-microglobulin
LIFC, MIFC	lateral and medial inferior femoral condyles, respectively
LPFC, MPFC	lateral and medial posterior femoral condyles, respectively
ERETIC	Electronic Reference to access In vivo Concentrations
TSP	3-(trimethylsilyl)propionic-2,2,3,3-d4 acid
qRT-PCR	quantitative reverse transcription PCR
UNG	Uracil-N Glycosylase

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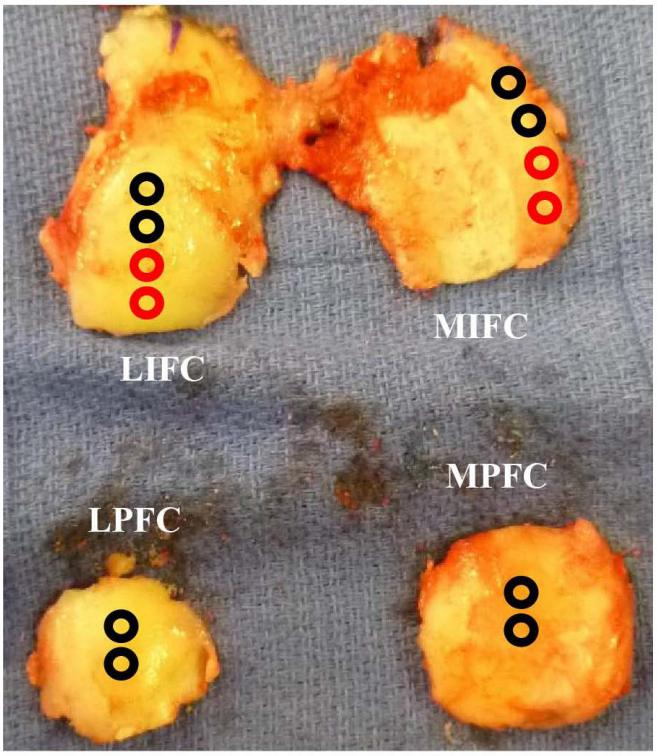

Figure 1.
Diagram depicting how and where cartilage punches were collected for each subject. Two samples (with each sample representing a pair of adjacent punches and visually depicted in this figure by different colored circles) were collected from each the LIFC and MIFC, and one sample was collected from each the LPFC and MPFC.

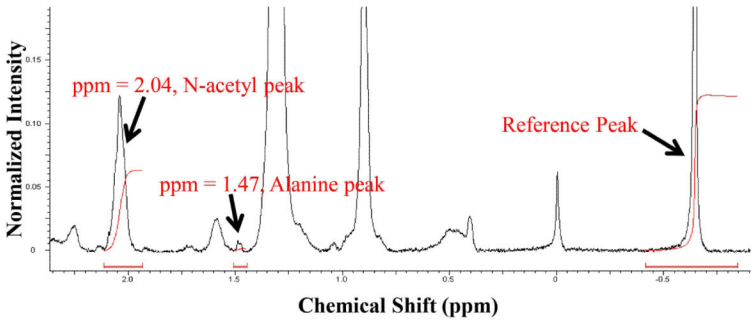

Figure 2.

Example of HRMAS NMR spectrum acquired from a pair of OA cartilage plugs. Spectral data was referenced to TSP at 0 ppm. Peaks quantified include the N-acetyl singlet at ppm = 2.04 and, when present, the Alanine doublet at ppm = 1.47. The red curves under each quantified peak were generated by the program to depict their integration visually.

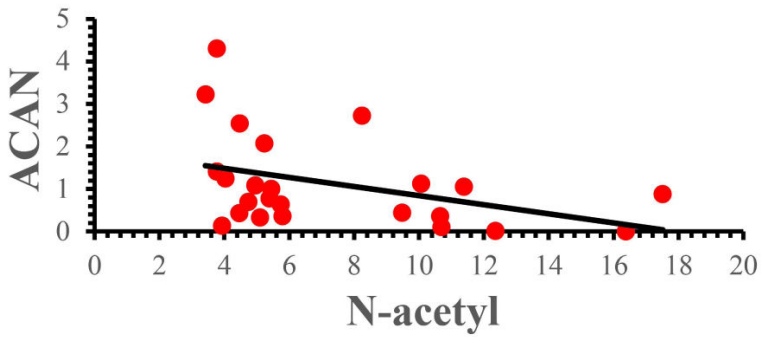

Figure 3.

Scatter plot of the significant negative correlation between N-acetyl metabolite measured by HRMAS and ACAN gene expression levels ($p = 0.016$, $rho = -0.49$).

Table 1

TaqMan primer set assay identification numbers for the genes studied in this project.

Gene	Assay Identification Number
B2M	Hs99999907_m1
ACAN	Hs00153936_m1
COL2A1	Hs00156568_m1
MMP13	Hs00233992_m1

Table 2

Number of samples whose data was used for analysis for each variable considered in this study.

ACAN	COL2A1	MMP13	N-acetyl	Alanine
27	29	12	26	15

Table 3

Expression levels of ACAN, COL2A1, and MMP13 in OA compared to the reference cartilage plug, expressed in terms of fold induction (mean ± standard deviation).

Gene	Fold Induction
ACAN	1.06±1.06
COL2A1	1.01±1.16
MMP13	75.74±96.71

Table 4

Correlations observed between ACAN, COL2A1, and MMP13 gene expression profiles in **a.** this study and **b.** a previous study (29).

Correlation	a. p	rho	b. p	r
ACAN vs. COL2A1	0.065	0.36	<0.01	0.314
ACAN vs. MMP13	0.61	−0.19	NS	−0.233
COL2A1 vs. MMP13	0.27	0.35	<0.001	0.46

Table 5

The p and rho values of the correlations between N-acetyl and Alanine values and ACAN, COL2A1, and MMP13 gene expression levels.

	N-acetyl		Alanine	
	p	**rho**	**p**	**rho**
ACAN	* **0.016**	**− 0.49**	0.91	−0.038
COL2A1	0.31	−0.21	0.19	0.36
MMP13	0.30	−0.35	0.54	−0.26

* $p < 0.05$