SCIENTIFIC ARTICLE

# Identification of Long Noncoding RNA Associated with Osteoarthritis in Humans

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**Objective:** Long noncoding RNAs (lncRNAs) are an important class of genes involved in various biological functions; however, knowledge about lncRNAs in osteoarthritis (OA) is limited. Therefore, the present study aimed to identify which lncRNAs are expressed in OA versus normal cartilage.

**Methods:** To identify lncRNAs specifically expressed in OA cartilage, expression of lncRNAs in OA cartilage was compared with that in normal cartilage using microarray analysis. The identified differences in expression of lncRNAs were validated by real time polymerase chain reaction (RT-PCR). Furthermore, expression of several key mRNAs associated with OA, including those for matrix metalloproteinase (MMP)-9, MMP-13, bone morphogenetic protein (BMP)-2, COL2A1 and ADAMTS5, was investigated by RT-PCR in OA and normal cartilage.

**Results:** Microarray analysis identified 121 lncRNAs that were up- or down-regulated in OA compared with normal tissue, 73 being upregulated and 48 downregulated compared with normal cartilage. Twenty-one of the above differently expressed lncRNAs were up-regulated twofold. Expression of six lncRNAs, including *HOTAIR*, *GAS5*, *PMS2L2*, *RP11-445H22.4*, *H19* and *CTD-2574D22.4*, was up-regulated in OA compared with normal tissue as validated by RT-PCR after microarray analysis. Expression of mRNA for MMP-9, MMP-13, BMP-2, and ADAMTS5 in OA was significantly greater than in normal cartilage. However, expression of mRNA for COL2A1 was lower in OA than in normal cartilage.

**Conclusion:** The differently expressed lncRNAs may be associated with the pathogenesis of OA. Further functional studies are critical to confirming the function of lncRNAs in OA and to exploring new potential targets for therapy.

**Key words:** Gene; Long noncoding RNAs; Microarray analysis; Osteoarthritis

## Introduction

Half of the world's population aged 65 years or older has osteoarthritis (OA), which is the most prevalent disease of articulating joints in humans[1]. OA is characterized by degradation of articular cartilage, thickening of subchondral bone and formation of osteophytes[2]. Clinically, OA results in severe physical disability with pain, stiffness and loss of mobility[3]. Pathologically, OA is characterized by local inflammation, synovitis and proteolytic degradation of cartilage, which is associated with alterations in the degree of expression by chondrocyte of genes involved in maintaining the integrity and function of cartilage[4,5]. However, the mechanism responsible for OA has not been fully clarified. Recent studies have shed light on the connection between genes, especially noncoding RNA genes, and the propensity to develop OA.

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Orthopaedic Surgery

| TABLE 1 Primers for targeted coding genes | | |
|---|---|---|
| Gene | Forward | Reverse |
| MMP-9 | 5'-GTGCTGGGCTGCTGCTTTGCTG-3' | 5'-GTCGCCCTCAAAGGTTTGGAAT-3' |
| MMP-13 | 5'-TGAGAGTCATGCCAACAAATTC-3' | 5'-CAGCCACGCATAGTCATGTAGA-3' |
| BMP-2 | 5'-TCATAAAACCTGCAACAGCCAACTCG-3' | 5'-GCTGTACTAGCGACACCCAC-3' |
| COL2A1 | 5'-CGAGGCAGACAGTACCTTGA-3' | 5'-TGCTCTCGATCTGGTTGTTC-3' |
| ADAMTS5 | 5'-GAACATCGACCAACTCTACTCCG-3' | 5'-CAATGCCCACCGAACCATCT-3' |
| GAPDH | 5'-GATCATCAGCAATGCCTCCT-3' | 5'-TGTGGTCATGAGTCCTTCCA-3' |

Noncoding RNAs include microRNAs and long noncoding RNAs (lncRNAs). It has become increasingly clear that lncRNAs, which are non-protein-coding transcripts longer than 200 nucleotides[6], may be involved in the regulation of various molecular and cellular functions. LncRNAs are widely distributed in the genome. A current study has shown that more than 90% of the human genome is composed of non-coding RNAs[7]. Many studies have indicated that changes in amounts of lncRNAs can result in aberrant expression of genes that contribute to a variety of disease states and biologic functions[8–15]. Thus far, 5446 lncRNA genes have been identified in the human genome; however, the functions of the vast majority of them remain unclear.

Most studies of lncRNA genes have been in the field of cancer[8–15]. However, a few studies of OA have been published[16–18]. New epigenetic studies in OA are likely to reveal novel aspects of chondrocyte and cartilage biology and potentially help to sub-characterize OA phenotypes[19–21]. Therefore, the purpose of the present study was to identify candidate lncRNAs that are up-regulated or down-regulated in OA compared with normal cartilage.

## Materials and Methods

### Articular Cartilage Tissue Donors
Osteoarthritis cartilage was isolated from the knee joints of eight patients (two men, six women; aged 54–86 years) undergoing total knee arthroplasty for primary OA. Normal articular cartilage was isolated from the knees of eight patients (4 men, 4 women; aged 24–51 years) after trauma or death. The normal donors were significantly younger than those with OA because of the difficulties in obtaining intact articular cartilage with no degenerative changes from elderly subjects. All 16 tissue donors included in this study provided written informed consent. The study was approved by the ethics committees of Tianjin Hospital and Peking Union Medical College Hospital.

### RNA Extraction
Joint tissue was isolated with a surgical scalpel from condyles and tibia plateaus within 24 h after the joints had been removed from the donor. The material was immediately shock-frozen in liquid nitrogen and the samples stored at −80 °C until RNA isolation. The samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA isolated according to the manufacturer's instructions. RNA quality and quantity were measured with a NanoDrop
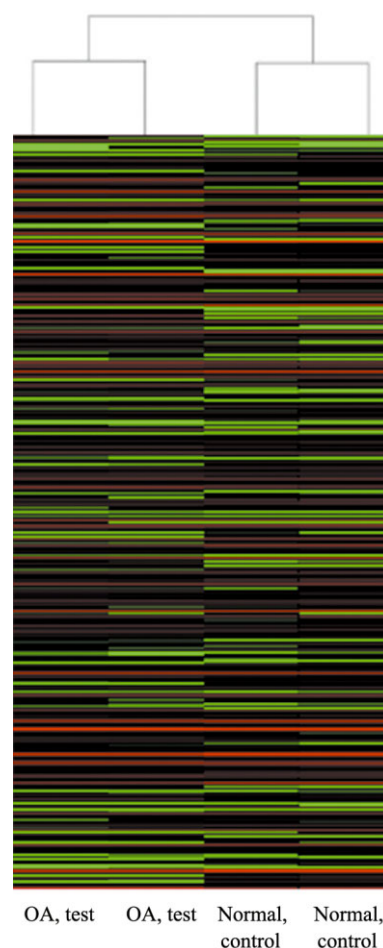


**Fig. 1** Differences in expression of lncRNAs. Heat map presentation of the expression profile of lncRNAs. Each column represents a sample and each row a gene. High relative expression is indicated by red and low relative expression by green.

| TABLE 2 Twenty-one candidate lncRNAs expressed > twofold differently in OA than in normal tissues | | | |
|---|---|---|---|
| HGNC symbol | Ensemble gene ID | Relationship | Fold change |
| HOTAIR | ENST00000422207 | Intergenic | 21.22342 |
| GAS5 | ENST00000442067 | Intergenic | 17.00201 |
| TNFSF10 | NR_033994 | Exon sense-overlapping | 12.03719 |
| PMS2L2 | uc011kep.2 | Intergenic | 10.56100 |
| ARFRP1 | NR_051956 | Natural antisense | 8.10922 |
| RP11-445H22.4 | ENST00000427303 | Intronic antisense | 7.42091 |
| RP11-611D20.2 | ENST00000429224 | Bidirectional | 5.56102 |
| H19 | NR_002196 | Intergenic | 4.57201 |
| RP11-69E11.4 | ENST00000440190 | Natural antisense | 3.97400 |
| HOTTIP | ENST00000243766 | Bidirectional | 3.10582 |
| MEG3 | ENST 00000214548 | Intergenic | 3.45011 |
| PVT1 | ENST00000520913 | Intergenic | 2.94523 |
| CTD-2574D22.4 | ENST00000567795 | Intron sense-overlapping | 2.70800 |
| AC019117.2 | ENST00000454003 | Intergenic | 2.70023 |
| HMlincRNA858 | HMlincRNA858 | Intergenic | 2.55209 |
| AC002465.2 | ENST00000436097 | Intronic antisense | 2.45535 |
| AX747599 | uc021upk.1 | Exon sense-overlapping | 2.29832 |
| WNT2 | NR_024047 | Exon sense-overlapping | 2.25401 |
| PRR24 | NR_037675 | Exon sense-overlapping | 2.10008 |
| LA16c-361A3.3 | ENST00000569670 | Intronic antisense | 2.09202 |
| FAS-AS1 | NR_028371 | Intronic antisense | 2.02001 |
| HGNC, HUGO Gene Nomenclature Committee. | | | |

spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity determined by gel electrophoresis.

### Microarray Analysis
Four samples were amplified and transcribed into fluorescent complementary RNA (cRNA) along the entire length of the transcripts, without 3′ bias, by a random-priming method. The labeled cRNAs were hybridized onto a 4 × 180K SBC human lncRNA Microarray V5.0 Array that contained about 63,431 lncRNAs and 39,887 protein-coding transcripts. After the slides had been washed, the arrays were scanned using an Agilent G2505C Scanner (Santa Clara, CA, USA). Raw data were extracted using Agilent Feature Extraction Software.

### Quantitative Real-time Polymerase Chain Reaction (RT-PCR) Analysis
An RT_PCR kit (Promega, Madison, WI, USA) was used to reverse transcribe 500 ng total RNA to complementary DNA. Amplification of matrix metalloproteinases (MMP)-9, MMP-13, bone morphogenetic protein (BMP)-2, COL2A1 and ADAMTS5 was performed using a kit (Promega). The specific primer sequences are shown in Table 1 and were designed by Sangon Biotech (Sangon Biotech, Shanghai, China). Sequence specificity was verified using the BLAST algorithm available online at the National Center for Biotechnology Information. All experiments were performed according to the

manufacturer's protocol in triplicate using three independent samples. Quantitative real-time PCR data were calculated by the $2^{(-\Delta\Delta CT)}$ methods[22], the reference gene being glyceraldehyde phosphate dehydrogenase (GAPDH).

### Statistical Analysis
Differences in expression were defined by fold changes in up- or down-regulation of expression. Data are presented as mean ± standard deviation (SD) and were analyzed by the two-tailed Student $t$-test. $P < 0.05$ was considered statistically significant.

## Results

### Differences between Normal and OA Cartilage in lncRNA Expression
One hundred and twenty-one lncRNAs were identified as expressed differently in OA than in normal cartilage. Seventy-three of these lncRNAs were up-regulated and 48 down-regulated in OA tissue (Fig. 1). Of the lncRNA with different expression, there was a greater than twofold change in expression of twenty-one candidate lncRNA in OA tissue compared with normal tissue (Table 2).

### Validation of Microarray Data by RT-PCR
To validate the microarray assay findings, a random selection of six lncRNAs were examined. Consistent with the microarray
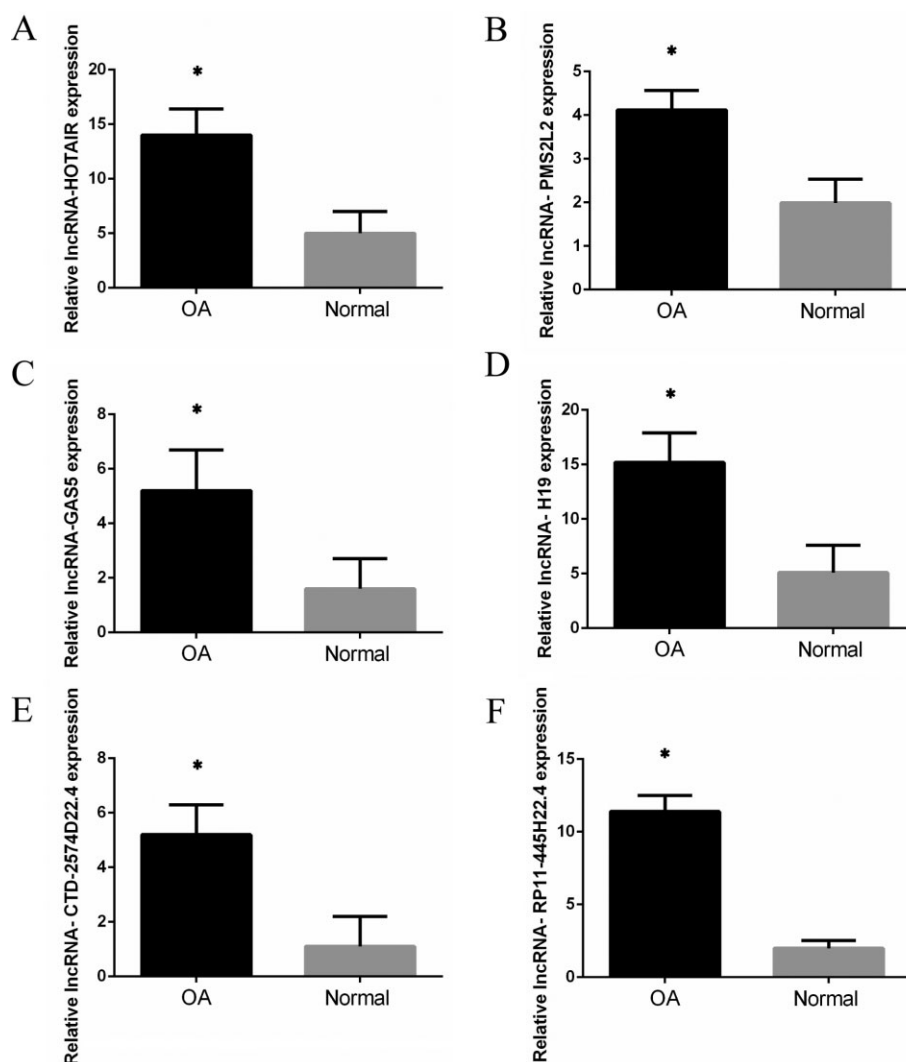
**Fig. 2** Validation of differences in expression of six lncRNAs. The expression in samples from OA and normal tissues of (A) *HOTAIR*, (B) *PMS2L2*, (C) *GAS5*, (D) *H19*, (E) *RP11-445H22.4* and (F) *CTD-2574D22.4* was analyzed by RT-PCR. Gene expression was determined by the ΔCt method; results were normalized to GAPDH expression. *$P < 0.05$ versus normal.

data, RT-PCR showed that *HOTAIR*, *GAS5*, *PMS2L2*, *RP11-445H22.4*, *H19* and *CTD-2574D22.4* were up-regulated compared with normal tissue (Fig. 2A to F).

### Differences between Normal and OA Cartilage in mRNA Expression

The expression of mRNA for MMP-9, MMP-13, BMP-2 and ADAMTS5 was significantly greater in OA than in normal tissue. However, expression of mRNA for COL2A1 was lower in OA than in normal tissue (Fig. 3A to E).

### Discussion

Osteoarthritis is the most prevalent disease of articulating joints in humans[23]. Its causes(s) are as yet unclear[21,24,25]. Several studies have focused on the epigenetic regulation of its

pathogenesis and potential targets for therapy, specifically noncoding RNAs[26]. Some studies have demonstrated that lncRNAs may be involved in the regulation of various molecular and cellular functions. Most studies have focused on associations between the function of lncRNAs and various cancers. However, a few relevant studies have investigated the effects of lncRNAs in the pathogenesis of OA[16–18]: we designed the present study to identify lncRNAs that may be associated with pathogenesis of OA.

In the present study, we identified a number of lncRNAs that are expressed significantly differently in OA than in normal cartilage, including both up-regulation and down-regulation. Furthermore, we selected six of these lncRNAs for validation by RT-PCR. Although the changes in expression of lncRNA detected by RT-PCR and by microarray analysis
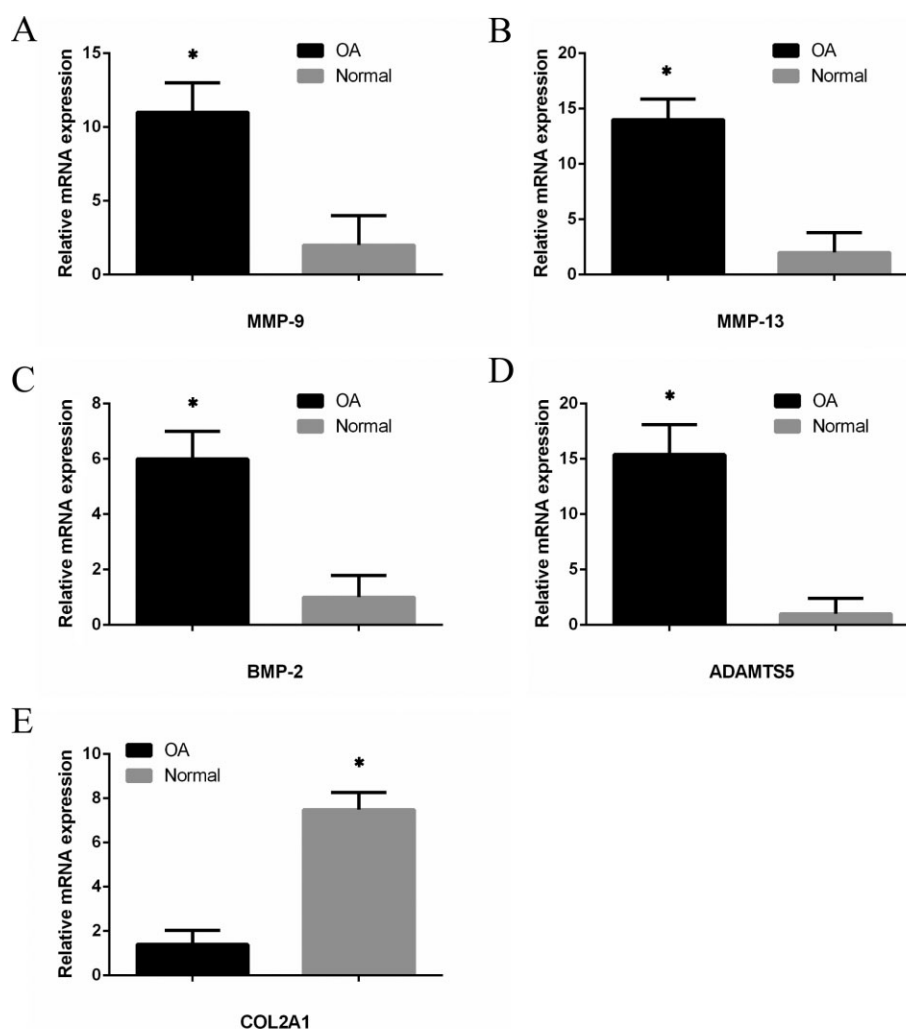
**Fig. 3** Differences in mRNA expression between OA and normal tissue. The expression in OA and normal tissues of mRNA (A) *MMP-9*, (B) *MMP-13*, (C) *BMP-2* and (D) *ADAMTS5* (D) was analyzed by RT-PCR. Gene expression was determined by the ΔCt method; results were normalized to GAPDH expression. The expression in samples from OA and normal tissues of mRNA for (E) *COL2A1* was analyzed by RT-PCR. Values in A, B C, D and E are expressed as mean ± SD. *, $P < 0.05$ versus normal.

showed a similar trend, the fold changes were not the same. This may be attributable to differences in sample sizes and the sensitivity of two methods. Currently, validation of microarray data by RT-PCR or other methods is the routine approach in the identification of RNA.

The present study showed that several mRNA (MMP-9, MMP-13, BMP-2 and ADAMTS5) are up-regulated and one mRNA (COL2A1) down-regulated in OA compared with normal cartilage. Our findings concerning expression of mRNA are consistent with those of previous studies of OA[27–29]. Thus, the present RT-PCR results supported cartilage degeneration in the OA group. It also demonstrated that the different expression of the above mRNA may be associated with differences in expression of lncRNAs filtered from microarray analysis.

Steck *et al.* reported than lncRNA *H19* acts as a metabolic correlate in cartilage and cultured chondrocytes[18]. Liu *et al.* demonstrated that lncRNA *CIR* contributes to extracellular matrix degradation and plays a key role in the pathogenesis of OA[17]. Song *et al.* showed that *GAS5* contributes to the pathogenesis of OA by acting as a negative regulator of miR-21 and thereby regulating cell survival[16]. The above differences in expression of lncRNAs were confirmed in the present study. However, *HOTAIR*, a newly identified lncRNA found in the present study, was expressed 21-fold in OA tissue compared with normal tissue according to microarray analysis. lncRNA *HOTAIR* has been investigated in the area of cancer[30–32]. Thus far, no studies have assessed the role of lncRNA *HOTAIR* in the pathogenesis of OA. Therefore, further studies on the regulation of *HOTAIR* and its functions in OA occurrence are still required.

The limitations of the present study include the following: (i) the small sample size decreases the reliability of the

results, individual patient's characteristics may have influenced results of microarray analysis and RT-PCR; (ii) most of the lncRNAs, especially those down-regulated in OA tissue, were not validated by RT-PCR; (iii) although differences in expression of lncRNAs were reported in the present study, the mechanisms of these lncRNAs need to be confirmed by further specific studies; and (iv) GO analysis and pathway analysis are required to investigate the relationships between noncoding RNAs, coding RNAs and proteins.

In summary, the present study identified 121 lncRNAs that are up- or down-regulated in OA tissue compared with normal tissue according to microarray analysis. The lncRNA with the most strongly up-regulated expression was *HOTAIR*. These differences in expression of lncRNAs may be associated with the pathogenesis of OA. Further functional studies are critical to confirming the function of lncRNAs in OA and to exploring new potential targets for therapy.

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