SCIENTIFIC ARTICLE

Identification of Long Noncoding RNA Associated with Osteoarthritis in Humans

Dan Xing, MD[1]*, Jin-qian Liang, MD[2]*, Yan Li, MD[3], Jun Lu, MD[3], Hao-bo Jia, MD[1], Li-yan Xu, MD[1], Xin-long Ma, MD[1]

[1]Department of Orthopaedics, Tianjin Hospital, [3]Department of Orthopaedics, Tianjin Gongan Hospital, Tianjin and [2]Department of Orthopaedic Surgery, Peking Union Medical College Hospital, Beijing, China

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.

Address for correspondence Xin-long Ma, MD, Department of Orthopaedics Institute, Tianjin Hospital, Jiefangnan Road, Tianjin, China 300211
Tel: 0086-22-60362062; Fax: 0086-22-60362062; Email: tjmuld@gmail.com

Disclosure: Each author certifies that he or she has no commercial associations that might pose a conflict of interest related to the submitted article. This study was supported by grants from the National Natural Science Foundation of China (No. 81301596, 81373839), the Scientific and Technological Project of Tianjin Public Security Bureau (No. 2013KYSGAY033, 2013KYSGAY032) and the Key Technologies & Program of Tianjin (No. 13JCZDJC31800).

*These two authors contributed equally to this work and are first co-authors.

Received 16 October 2014; accepted 20 October 2014

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

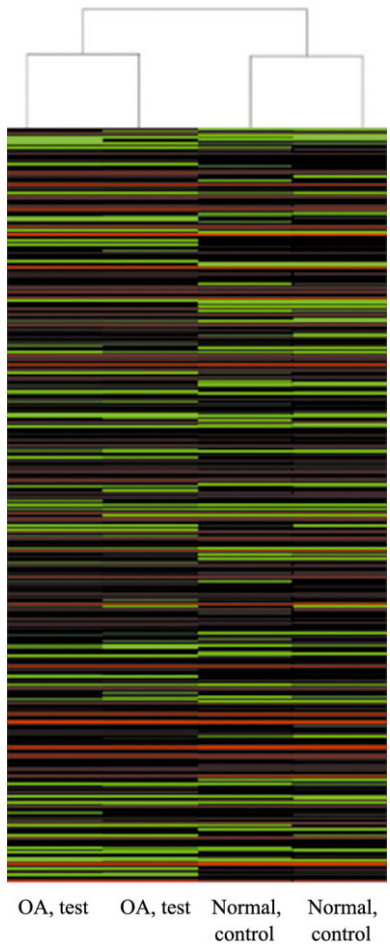

Fig. 1 Differences in expression of lncRNAs. Heat map presentation of the expression profile of lncRNAs. Each column represents a sample and each row a gene. High relative expression is indicated by red and low relative expression by green.

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

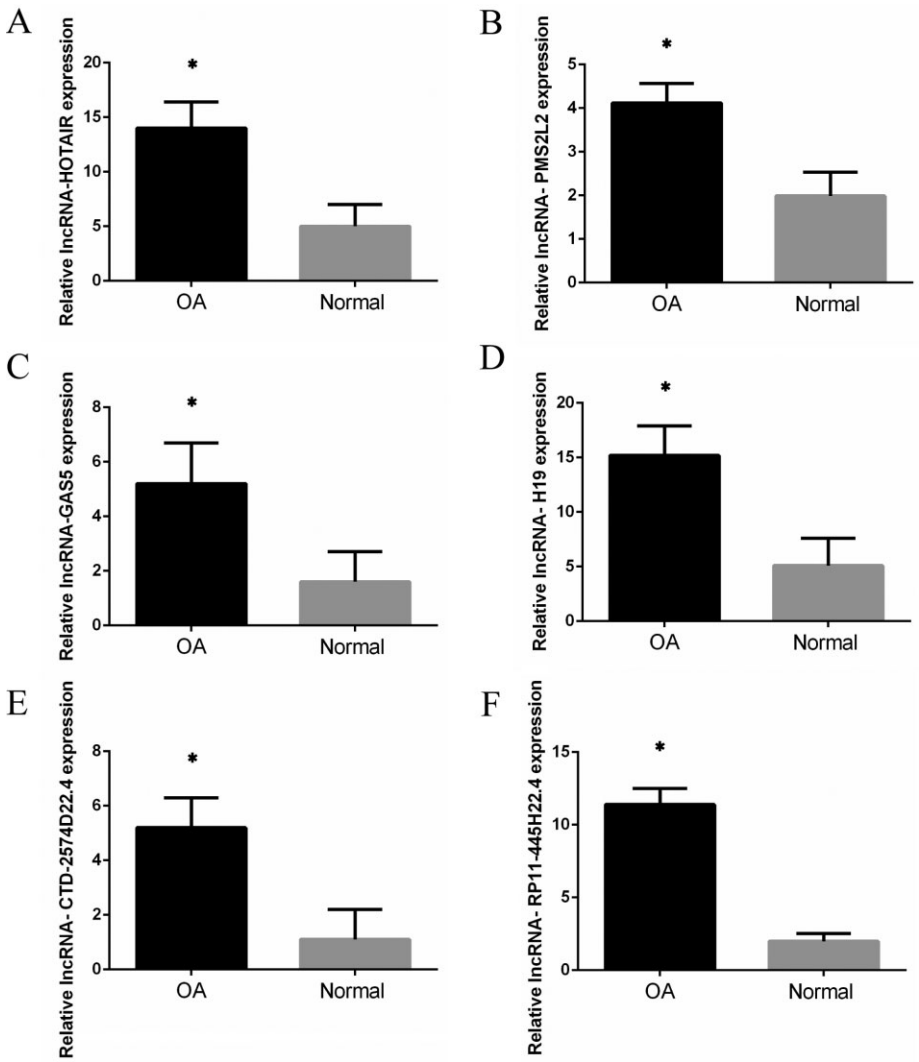

Fig. 2 Validation of differences in expression of six lncRNAs. The expression in samples from OA and normal tissues of (A) *HOTAIR*, (B) *PMS2L2*, (C) *GAS5*, (D) *H19*, (E) *RP11-445H22.4* and (F) *CTD-2574D22.4* was analyzed by RT-PCR. Gene expression was determined by the ΔCt method; results were 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

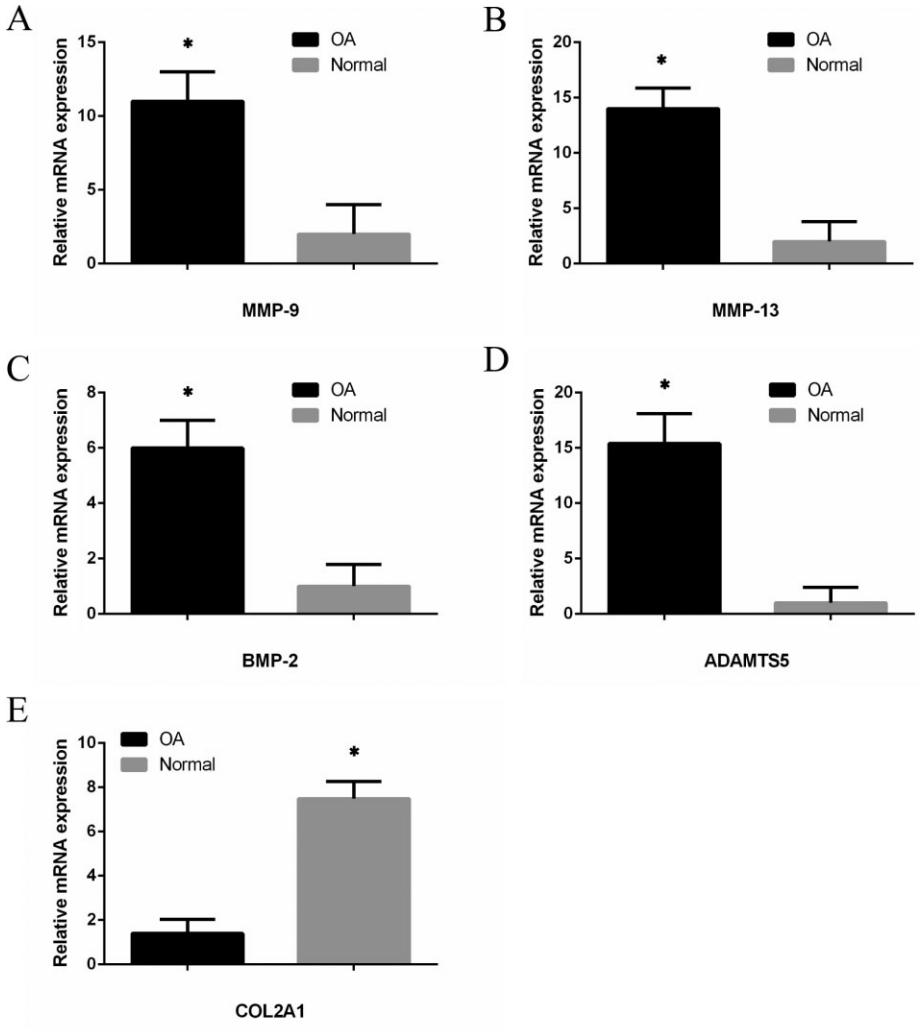

Fig. 3 Differences in mRNA expression between OA and normal tissue. The expression in OA and normal tissues of mRNA (A) *MMP-9*, (B) *MMP-13*, (C) *BMP-2* and (D) *ADAMTS5* (D) was analyzed by RT-PCR. Gene expression was determined by the ΔCt method; results were normalized to GAPDH expression. The expression 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* inthe 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.

Acknowledgement

The authors thank Professor Zhao Meng, Tianjin Medical University, Tianjin, China for his helpful advice regarding microarray analysis.

References

1. Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. Lancet, 2011, 377: 2115–2126.

2. Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, Duong le T. Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. Bone, 2006, 38: 234–243.

3. Findlay DM, Atkins GJ. Osteoblast-chondrocyte interactions in osteoarthritis. Curr Osteoporos Rep, 2014, 12: 127–134.

4. Scanzello CR, Umoh E, Pessler F, *et al*. Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease. Osteoarthritis Cartilage, 2009, 17: 1040–1048.

5. Brooks P. Inflammation as an important feature of osteoarthritis. Bull World Health Organ, 2003, 81: 689–690.

6. Perkel JM. Visiting "noncodarnia". Biotechniques, 2013, 54: 301, 303–304.

7. Birney E, Stamatoyannopoulos JA, Dutta A, *et al*. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature, 2007, 447: 799–816.

8. Eades G, Wolfson B, Zhang Y, Li Q, Yao Y, Zhou Q. lincRNA-RoR and miR-145 regulate invasion in triple-negative breast cancer via targeting ARF6. Mol Cancer Res, 2014, pii: molcanres.0251.2014. [Epub ahead of print]

9. Militti C, Maenner S, Becker PB, Gebauer F. UNR facilitates the interaction of MLE with the lncRNA roX2 during Drosophila dosage compensation. Nat Commun, 2014, 5: 4762. doi: 10.1038/ncomms5762

10. Conde L, Riby J, Zhang J, Bracci PM, Skibola CF. Copy number variation analysis on a non-hodgkin lymphoma case-control study identifies an 11q25 duplication associated with diffuse large B-cell lymphoma. Plos ONE, 2014, 9: e105382. DOI: 10.1371/journal.pone.0105382

11. Malouf GG, Zhang J, Yuan Y, *et al*. Characterization of long non-coding RNA transcriptome in clear-cell renal cell carcinoma by next-generation deep sequencing. Mol Oncol, 2014, pii: S1574-7891(14)00162-8. doi: 10.1016/j.molonc.2014.07.007. [Epub ahead of print]

12. Quenet D, Dalal Y. A long non-coding RNA is required for targeting centromeric protein A to the human centromere. Elife, 2014, 3: e3254. doi: 10.7554/eLife.03254.

13. Tseng YY, Moriarity BS, Gong W, *et al*. PVT1 dependence in cancer with MYC copy-number increase. Nature, 2014, 512: 82–86.

14. Reddy MA, Chen Z, Park JT, *et al*. Regulation of inflammatory phenotype in macrophages by a diabetes-induced long non-coding RNA. Diabetes, 2014, pii: DB_140298. [Epub ahead of print]

15. Li Z, Li X, Wu S, Xue M, Chen W. Long non-coding RNA UCA1 promotes glycolysis by upregulating hexokinase 2 through the mTOR-STAT3/microRNA143 pathway. Cancer Sci, 2014, 105: 951–955.

16. Song J, Ahn C, Chun CH, Jin EJ. A long non-coding RNA, GAS5, plays a critical role in the regulation of miR-21 during osteoarthritis. J Orthop Res, 2014, 32: 1628–1635.

17. Liu Q, Zhang X, Dai L, *et al*. Long noncoding RNA related to cartilage injury promotes chondrocyte extracellular matrix degradation in osteoarthritis. Arthritis Rheumatol, 2014, 66: 969–978.

18. Steck E, Boeuf S, Gabler J, *et al*. Regulation of H19 and its encoded microRNA-675 in osteoarthritis and under anabolic and catabolic *in vitro* conditions. J Mol Med (Berl), 2012, 90: 1185–1195.

19. Barter MJ, Young DA. Epigenetic mechanisms and non-coding RNAs in osteoarthritis. Curr Rheumatol Rep, 2013, 15: 353. doi: 10.1007/s11926-013-0353-z.

20. van der Kraan PM. Understanding developmental mechanisms in the context of osteoarthritis. Curr Rheumatol Rep, 2013, 15: 333. doi: 10.1007/s11926-013-0333-3.

21. Gonzalez A. Osteoarthritis year 2013 in review: genetics and genomics. Osteoarthritis Cartilage, 2013, 21: 1443–1451.

22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 2001, 25: 402–408.

23. Li Y, Wei X, Zhou J, Wei L. The age-related changes in cartilage and osteoarthritis. Biomed Res Int, 2013, 2013: 916530. doi: 10.1155/2013/916530. Epub 2013 Jul 22.

24. Seidel MF, Lane NE. Control of arthritis pain with anti-nerve-growth factor: risk and benefit. Curr Rheumatol Rep, 2012, 14: 583–588.

25. Hardingham T. Extracellular matrix and pathogenic mechanisms in osteoarthritis. Curr Rheumatol Rep, 2008, 10: 30–36.

26. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem, 2012, 81: 145–166.

27. Weng X, Lin P, Liu F, *et al*. *Achyranthes bidentata* polysaccharides activate the Wnt/beta-catenin signaling pathway to promote chondrocyte proliferation. Int J Mol Med, 2014, 34: 1045–1050.

28. Wu C, Zheng J, Yao X, *et al*. Defective autophagy in chondrocytes with Kashin-Beck disease but higher than osteoarthritis. Osteoarthritis Cartilage, 2014, Aug 27. [Epub ahead of print]

29. Weng T, Xie Y, Yi L, *et al*. Loss of Vhl in cartilage accelerated the progression of age-associated and surgically induced murine osteoarthritis. Osteoarthritis Cartilage, 2014, 22: 1197–1205.

30. Wu Y, Zhang L, Wang Y, *et al*. Long noncoding RNA HOTAIR involvement in cancer. Tumour Biol, 2014, pii: S1063-4584(14)01223-0. doi: 10.1016/j.joca.2014.08.010. [Epub ahead of print]

31. Wu Y, Liu J, Zheng Y, You L, Kuang D, Liu T. Suppressed expression of long non-coding RNA HOTAIR inhibits proliferation and tumourigenicity of renal carcinoma cells. Tumour Biol, 2014, [Epub ahead of print]

32. Zhang H, Cai K, Wang J, *et al*. MiR-7, inhibited indirectly by LincRNA HOTAIR, directly inhibits SETDB1 and reverses the EMT of breast cancer stem cells by downregulating the STAT3 pathway. Stem Cells, 2014, 32: 2858–2868.