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Use of integrative epigenetic and mRNA expression analyses to identify significantly changed genes and functional pathways in osteoarthritic cartilage

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Aim

Osteoarthritis (OA) is caused by complex interactions between genetic and environmental factors. Epigenetic mechanisms control the expression of genes and are likely to regulate the OA transcriptome. We performed integrative genomic analyses to define methylation-gene expression relationships in osteoarthritic cartilage.

Patients and Methods

Genome-wide DNA methylation profiling of articular cartilage from five patients with OA of the knee and five healthy controls was conducted using the Illumina Infinium Human-Methylation450 BeadChip (Illumina, San Diego, California). Other independent genome-wide mRNA expression profiles of articular cartilage from three patients with OA and three healthy controls were obtained from the Gene Expression Omnibus (GEO) database. Integrative pathway enrichment analysis of DNA methylation and mRNA expression profiles was performed using integrated analysis of cross-platform microarray and pathway software. Gene ontology (GO) analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Results

We identified 1265 differentially methylated genes, of which 145 are associated with significant changes in gene expression, such as *DLX5*, *NCOR2* and *AXIN2* (all p-values of both DNA methylation and mRNA expression < 0.05). Pathway enrichment analysis identified 26 OA-associated pathways, such as mitogen-activated protein kinase (MAPK) signalling pathway ($p = 6.25 \times 10^{-4}$), phosphatidylinositol (PI) signalling system ($p = 4.38 \times 10^{-3}$), hypoxia-inducible factor 1 (HIF-1) signalling pathway ($p = 8.63 \times 10^{-3}$), pantothenate and coenzyme A (CoA) biosynthesis ($p = 0.017$), ErbB signalling pathway ($p = 0.024$), inositol phosphate (IP) metabolism ($p = 0.025$), and calcium signalling pathway ($p = 0.032$).

Conclusion

We identified a group of genes and biological pathways which were significantly different in both DNA methylation and mRNA expression profiles between patients with OA and controls. These results may provide new clues for clarifying the mechanisms involved in the development of OA.

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Keywords: Osteoarthritic cartilage, DNA methylation profiles, mRNA expression profiles, Integrative analysis

Article focus

■ We explored the relationship between DNA methylation and mRNA expression profiles of osteoarthritic cartilage from

the entire genome perspective. To what extent are aberrant genes of DNA methylation consistent with those of mRNA expression profiles?

- Which biological functions do identified significant common genes in our study involve?

Key messages

- A total of 145 common aberrant genes for DNA methylation and mRNA expression profiles, and 26 pathways were detected for osteoarthritis. It has been suggested that some of the identified genes and pathways, such as DLX5 and mitogen-activated protein kinase signalling pathway, are associated with the development of osteoarthritis.
- These results yielded new insights into the pathogenesis of osteoarthritis.

Strengths and limitations

- This study was unique in that it combined genome-wide DNA methylation and gene expression profiles of osteoarthritic cartilage to identify significantly changed genes and functional pathways.
- Considering the sample size, further biological studies are required to validate these findings.

The pathogenesis of osteoarthritis (OA) remains unclear. Many factors have been implicated in its development, including age, gender, obesity, physical activity and genetic factors.¹⁻⁴ The prevalence of OA remains high due to the ageing population and rising levels of obesity,³ and its prevention and treatment involves a huge burden on public health resources.⁵⁻⁷ High-throughput analysis, such as genomics and transcriptomics, has been used in pathogenetic studies of OA.⁸⁻¹¹ For example, a gene expression study observed specific profiles in damaged and undamaged cartilage and determined the relevant genes and pathways in the progression of OA.¹⁰ A genome-wide DNA methylation study of osteoarthritic cartilage identified epigenetic dysregulations of a host of genes and pathways associated with OA.⁹ However, the molecular mechanisms of OA are very complicated, involving abnormal alterations of multilevel biological macromolecules. Therefore, single-omics studies cannot reveal the complicated pathogenesis of OA. In order to address this issue, multi-omics integrative analysis is currently receiving much attention¹²⁻¹⁴ and has been used in pathogenetic studies of many conditions.¹⁵⁻¹⁷

Moreover, as one of the major epigenetic modifications, the methylation of DNA plays an important role in the regulation of gene expression. Several authors have investigated the relationship between levels of gene expression and DNA methylation status in osteoarthritic chondrocytes.¹⁸⁻²⁰ For example, downregulation of collagen type IX alpha 1 chain (COL9A1) expression was associated with hypermethylation in osteoarthritic cartilage.¹⁹ The loss of methylation in cytosine-phosphate-guanine (CpG) sites in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) enhancer elements of inducible nitric oxide synthase

Table I. Basic characteristics of the patients for genome-wide DNA methylation profiling

Osteoarthritis		Normal	
Gender	Age (yrs)	Gender	Age (yrs)
Female	58	Female	47
Female	62	Female	62
Male	72	Male	55
Male	66	Male	46
Male	65	Male	45

(NOS) is responsible for gene induction in human articular chondrocytes.¹⁸ Nevertheless, previous studies only focused on specific genes, rather than the whole genome. Integrating genome-wide DNA methylation and mRNA expression profiles has the potential to detect more comprehensive aberrant changes related to OA.

In this study, we aimed to explore the relationships between DNA methylation and gene expression in osteoarthritic cartilage, and to identify significantly changed genes and functional pathways in patients with OA, hoping that the findings would provide new clues about the pathogenesis of OA.

Methods and Materials

Ten specimens of knee cartilage were used, five specimens from patients with primary OA who had undergone total knee arthroplasty (TKA) and five normal specimens from healthy patients who had undergone amputation following a road traffic accident. All patients were Han Chinese and were collected at the Xi'an Red Cross Hospital. They came from the Xi'an City of Shaanxi Province and were genetically unrelated. The basic characteristics of the patients for genome-wide DNA methylation profiling are shown in Table I. Nurse-administered questionnaires were used to obtain general clinical data, including ethnicity, lifestyle characteristics, health status and family and medical histories. Each patient underwent clinical and radiological examination. Primary OA of the knee was diagnosed according to the Western Ontario and McMaster Universities OA Index (WOMAC) for pain, stiffness, function, and clinical and radiological assessment.²¹ We excluded those who were obese or who had bone or cartilage disease, rheumatoid arthritis, or any other skeletal disorder.

The specimens of articular cartilage were harvested from the same anatomical areas of the femoral condyles. For genome-wide DNA methylation profiling, the specimens from the ten patients were dissected and rapidly frozen in liquid nitrogen. The DNA was then extracted using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) following the standard protocol. Agarose gel electrophoresis was used to evaluate the quality of extracted DNA.

Genome-wide DNA methylation profiling. Genome-wide DNA methylation profiling of the articular cartilage from

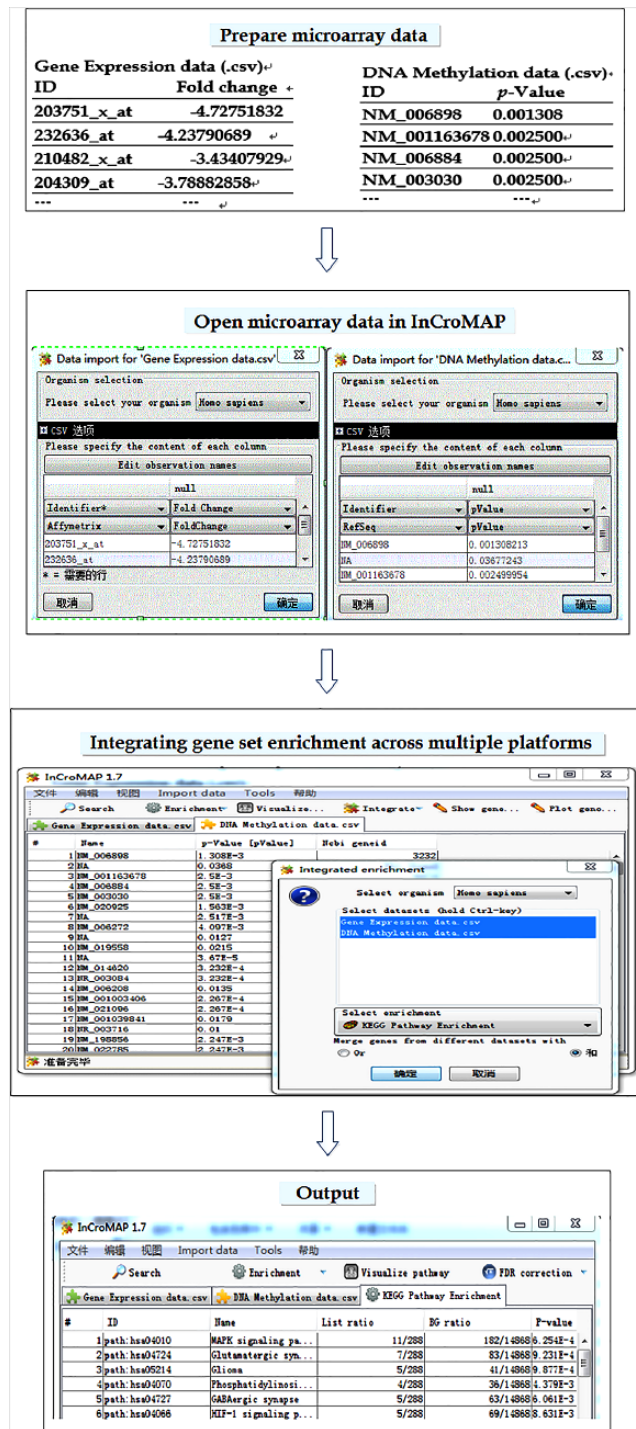


Fig. 1

Integrative pathway enrichment analysis of DNA methylation and mRNA expression profiles using integrated analysis of cross-platform microarray and pathway (InCroMap).

the specimens was performed using the Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, California), covering more than 485 000 sites of methylation across the entire genome. Bisulfite treatment of 500 ng DNA specimens was performed using the EZ DNA

Methylation Kit (Zymo Research Corp., Irvine, California). The bisulfite-converted DNA specimens were then amplified, hybridized to HumanMethylation450 array, stained and washed following a standard protocol. The raw image intensities were scanned using the iScan SQ Scanner (iScan System, Illumina) and the data were processed by GenomeStudio Software (Illumina). The mean percentage of methylated cytosine at a given CpG site was expressed as a β value, varying from 0 (completely unmethylated) to 1 (completely methylated).

Differential methylation analysis. The empirical Bayes moderated *t*-test of limma package²² in Bioconductor was used to identify differentially-methylated CpG sites. The Benjamini-Hochberg method was used to calculate a false discovery rate (FDR) adjusted p-value for each CpG site. Significant sites were defined as: (1) FDR adjusted p-value (Padj) ≤ 0.05 ; (2) β -value difference ($|\Delta\beta|$) ≥ 0.2 . The sites with missing values, or detection p-values > 0.05 in more than 90% of cartilage specimens were eliminated. These parameters are widely used quality control parameters for DNA methylation analysis, and have been used in previous methylation profile studies.²³⁻²⁵ As a result, 618 of the CpG probe sites were eliminated, which did not result in significant information missing.

mRNA expression profiles. Microarray data used here have been deposited in the National Centre for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE16464. Chondrocytes cultured in monolayer (passage 2) were subjected to gene expression profiling using genome-wide oligonucleotide microarrays HGU133plus2.0 (Affymetrix, Santa Clara, California) according to the manufacturer's recommendations. Detailed information about the patients, cell culture, and microarray analysis have been previously reported.²⁶

Statistical analysis. Significantly common altered genes were screened by comparing genes with p-value < 0.05 identified by analysis of genome-wide DNA methylation profiling and mRNA expression profiles. The results of single gene analysis were further subjected to gene ontology (GO) enrichment analysis to identify OA-associated GO terms by means of the Database for Annotation, Visualization and Integrated Discovery 6.7. Integrative pathway enrichment analysis of DNA methylation and mRNA expression profiles was conducted by analysis of cross-platform microarray and pathway (InCroMAP) (Fig. 1). InCroMAP can perform pathway enrichment analysis using heterogeneous, cross-platform data sets. We used Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Following the parameters recommended by the developers of InCroMAP, we used a threshold for each data set, that is, "p-value < 0.05 " for DNA methylation profiles and "fold-change > 1.5 " for mRNA expression profiles. A p-value was calculated by InCroMAP for each analyzed pathway.

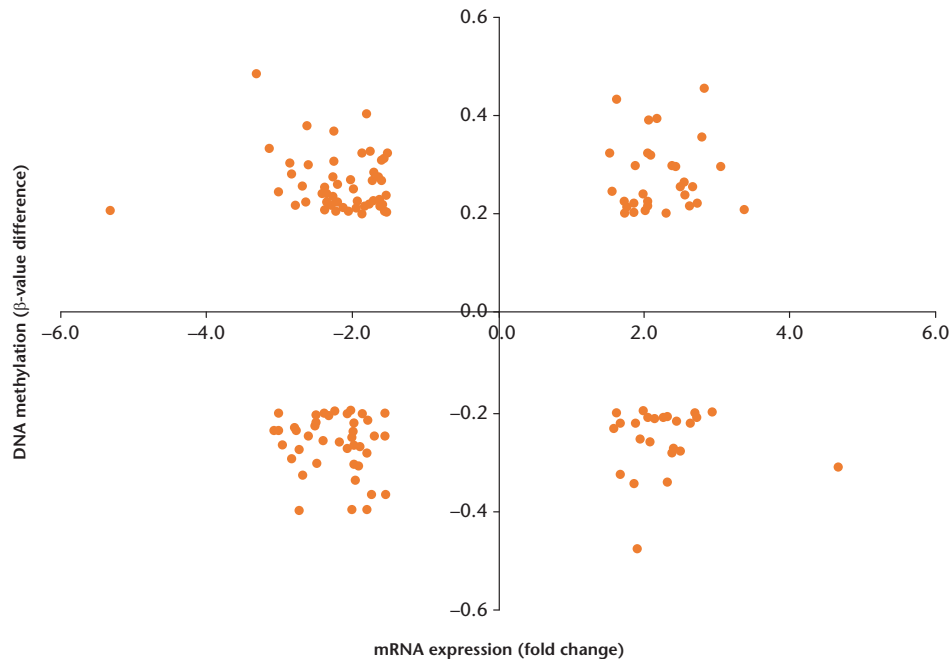


Fig. 2

A scatter plot of the 145 common aberrant genes of DNA methylation and mRNA expression profiles.

Results

Single gene analysis. We identified 1265 differentially methylated genes, including 678 methylated and 587 demethylated genes. Integrating the DNA methylation of mRNA expression profiles detected 145 genes significantly altered in both (Supplementary Table i and Fig. 2). Among these, 48 were significantly hypermethylated and downregulated genes from the methylated group, and 24 were hypomethylated and upregulated genes from the demethylated group.

GO enrichment analysis. Further enrichment analysis of the 145 genes identified 127 significant GO terms for biological process, 11 significant GO terms for cell component, and 50 significant GO terms for molecular function, such as cytoskeleton organization (p -value = 1.47×10^{-11} , false discovery rate (FDR) = 2.47×10^{-8}), F-actin capping protein complex (p -value = 9.20×10^{-10} , FDR = 1.20×10^{-6}) and skeletal system development (p -value = 9.88×10^{-10} , FDR = 1.72×10^{-5}). More detailed results of GO enrichment analysis with FDR < 0.01 are shown in Supplementary Table ii.

Pathway enrichment analysis. KEGG pathway enrichment analysis identified 26 significant biological pathways (Supplementary Table iii), such as the mitogen-activated protein kinase (MAPK) signalling pathway (p = 6.25×10^{-4} , Fig. 3), phosphatidylinositol (PI) signalling system (p = 4.38×10^{-3}), hypoxia-inducible factor 1 (HIF-1) signalling pathway (p = 8.63×10^{-3}), pantothenate and coenzyme A (CoA) biosynthesis (p = 0.017), ErbB signalling pathway (p = 0.024), inositol phosphate (IP) metabolism (p = 0.025), and calcium signalling pathway (p = 0.032).

Discussion

mRNA expression profiling is often used to identify disease-related genes and gene sets. DNA methylation can regulate gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA. Through integrative analysis of genome-wide DNA methylation profiles and mRNA expression profiles of osteoarthritic cartilage, we can learn more about the significant changes that occur between them, and how they play a role in the development of OA. In this study, we first identified 145 common aberrant genes in both DNA methylation and mRNA expression levels, and 26 associated biological pathways in osteoarthritic cartilage.

Some of the 145 common aberrant genes are thought to be associated with OA. For example, distal-less homeobox 5 (DLX5) is a transcription factor, which, it has been suggested, not only controls skeletal development, but also participates in ectopic chondrocyte hypertrophy during the development of OA.²⁷ Furthermore, overexpression of DLX5 in chicken or mouse cartilage promoted chondrocyte hypertrophy, while DLX5 deficiency resulted in delayed hypertrophy.²⁸⁻³⁰ It was also found that DLX5 could induce the expression of runt related transcription factor 2 (RUNX-2) in animal models by means of specifically trans-activating the RUNX-2 P1 promoter,³¹ and thus influence matrix metalloproteinase 13 (MMP-13) expression in osteoarthritic chondrocytes,³² which is associated with abnormal gene expression and cartilage degeneration processes connected with OA. Nuclear receptor corepressor 2 (NCOR2), a transcription factor, was significantly associated with three OA-related traits

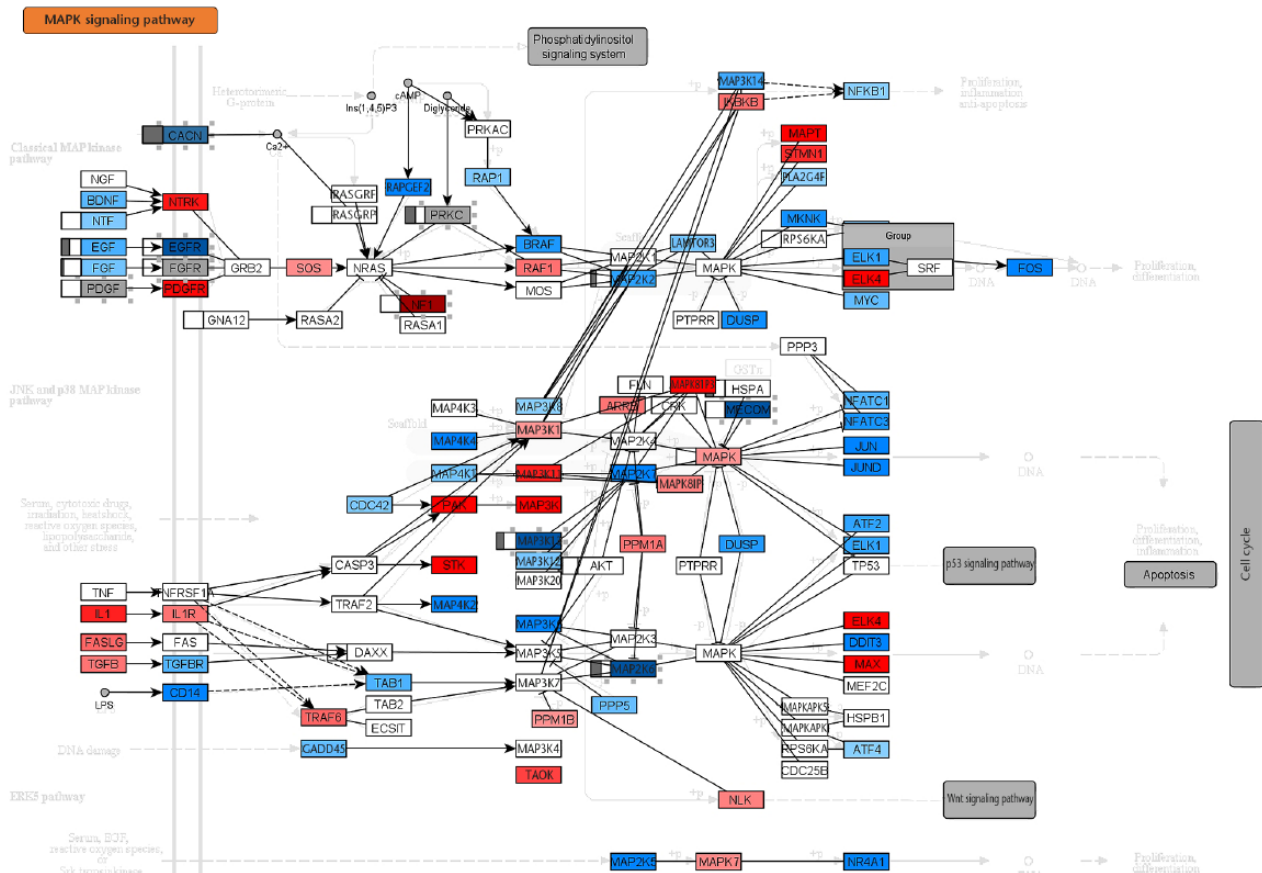


Fig. 3

Diagram of the mitogen-activated protein kinase signalling pathway. Rectangular nodes correspond to genes or gene families and their colour indicates mRNA expression (blue means downregulated, red means upregulated). The box to the left of those nodes indicates promoter DNA methylation. The largest peak is shown and an empty box indicates no differential methylation. The bar to the left indicates hypomethylation and the bar which goes from the middle of the box to the right indicates hypermethylation. Small rectangular boxes below the mRNA nodes indicate protein and protein modification expression. Each of those boxes is red, which indicates upregulation.

(presence/absence of joint space narrowing, presence/absence of osteophytes, and Kellgren-Lawrence score).³³ In this study, we observed that NCOR2 was significantly hypermethylated and downregulated in osteoarthritic cartilage, which was consistent with a previous study.³⁴ The mechanism of NCOR2 implicated in the pathogenesis of OA is largely unknown and needs to be studied in greater detail. Axis Inhibition Protein 2 (AXIN2) is another interesting gene identified in this study. Previous authors observed that disruption of AXIN2 expression resulted in the accelerated maturation of chondrocytes.³⁵ Furthermore, AXIN2 in the Wnt pathway was upregulated in osteoarthritic bone, and involved not only in the distortion of cartilage, but also in changes in the subchondral bone.³⁶

GO enrichment analysis of 145 common affected genes also identified a group of OA-related GO terms, functionally involved in apoptosis, the development of the skeletal system and ossification. Previous authors reported similar results.³⁷⁻⁴⁰ Embryonic organ and skeletal system morphogenesis have been reported to be major pathways involved in the development of OA.³⁷ Several biomolecules participated in the progress of

apoptosis and embryonic skeletal growth of osteoarthritic chondrocytes. For example, insulin-like growth factor 1 (IGF-1) may downregulate the expression of programmed cell death 5 (PDCD5) and thus inhibit apoptosis of osteoarthritic chondrocytes.⁴¹ casein kinase 2 (CK2) downregulation facilitates TNF- α -mediated chondrocyte death through apoptosis and autophagy.⁴² Hypoxia-inducible factor 2 alpha (HIF-2 α) was essential for the endochondral ossification of cultured chondrocytes and embryonic skeletal growth in mice.⁴³

Integrative pathway enrichment analysis identified several significant functional pathways for OA, of which the MAPK signalling pathway is the most significant. The MAPK cascade is a highly conserved module that is involved in various cellular functions, including proliferation, differentiation and migration. Specifically, inhibition of the p38-MAPK signalling pathway could suppress the apoptosis and expression of proinflammatory cytokines in human osteoarthritic chondrocytes.⁴⁴ This pathway is implicated in the production of matrix metalloproteinases and the regulation of biological responses of apoptosis, fatalization, calcification and proliferation of cartilage cells in OA.^{45,46} Matrix metalloproteinases

(MMPs), in particular, can accelerate the degradation of articular cartilage.⁴⁵

The PI signalling system belongs to a class of environmental information processing or signal transduction and includes various molecular and signalling pathways, some of which have been shown to be related to the development of OA. For example, it has been shown that the disruption of phosphoinositide-specific phospholipase γ 1 (PLC γ 1) contributes to the synthesis of extracellular matrix (ECM) in human osteoarthritic chondrocytes through regulating the expression of ECM-related signalling molecules, including matrix metalloproteinase 13 (MMP-13), type II collagen (Col II), tissue inhibitor of metalloproteinase 1 (TIMP1), sex determining region Y-box 9 (Sox-9), and a negatively charged proteoglycan (AGG). Furthermore, the PLC γ 1/IP3/Ca²⁺/CaMK II signalling axis regulated the ECM synthesis of human chondrocytes by triggering the mTOR/P70S6K/S6 pathway.⁴⁷ Another study found that through the phosphoinositide 3-kinase (PI3K)/AKT and extracellular signal-regulated kinase 1/2 pathway, osteopontin (OPN) could upregulate the expression of vascular endothelial growth factor (VEGF).⁴⁸ The expression of OPN and VEGF were associated with the severity of the destruction of osteoarthritic cartilage.⁴⁹⁻⁵¹ Based on previous results and our own, we suspect that the PI signalling system is involved in the development of OA. More functional studies are needed to confirm our findings.


Hypoxia-inducible factor 1 is a transcription factor that functions as a master regulator of oxygen homeostasis. Despite its name, HIF-1 is induced not only in response to the reduced availability of oxygen, but also by other stimulants, such as nitric oxide or various growth factors. It exhibits protective activity in chondrocytes during the development of OA.⁵² It has been shown that HIF-1 is important for the formation and maintenance of cartilage tissue in conditional knockout mice, in which deletion of the oxygen-sensitive subunit HIF-1 α severely interfered with skeletal development and led to massive cell death within the centre of the forming cartilaginous elements.⁵³ However, HIF-1 α and -2 α exert differing, even opposite, effects in chondrocytes and cartilage. For example, HIF-2 α acts as a catabolic factor in the destruction of cartilage by regulating the expression of target genes in chondrocytes.⁵⁴ Our results further confirm the importance of HIF-1 signalling in the development of OA.

We used five patients with OA and five healthy controls for genome-wide DNA methylation profiling. This sample size is relatively small. Articular cartilage specimens were collected from patients with advanced OA who had undergone TKA, and from healthy controls who had undergone amputation following a road traffic accident. Thus, it was difficult to use a large sample for DNA methylation profiling. However, the sample size is generally acceptable for microarray-based studies and has

been used in recent studies on OA.⁵⁵⁻⁵⁷ The DNA methylation and mRNA expression profiles were also derived from different cartilage specimens. This may affect the results, which should be interpreted with caution. Further studies are needed to confirm our findings, and reveal the potential roles of identified genes and pathways in the development of OA.

In summary, we conducted an integrative analysis of genome-wide DNA methylation and gene expression profiles of osteoarthritic cartilage. Compared with a single-omics study, an integration study can provide more detailed information to detect common defective functional genes or pathways related to OA, especially those in which genes are low-expressed because of underlying DNA methylation, or over-expressed due to DNA demethylation. In addition, not all of these DNA methylations are correlated with altered mRNA transcription.^{58,59} Further biological studies are required to validate these results.

Supplementary material

 Tables providing further information on 145 significantly common changed genes and their gene ontology enrichment analysis results, together with integrative pathway enrichment results of DNA methylation and mRNA expression profiles by integrated analysis of cross-platform microarray and pathway.

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- F. Zhang takes responsibility for the integrity of the work as a whole, from inception to the finished manuscript.

Author Contributions

- A. He, Collection and assembly of data, Analysis and interpretation of the data, Drafting and final approval of the manuscript.
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- Y. Wen, Collection and assembly of data.
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- K. Xu, Collection and assembly of data.
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- J. Han, Drafting and final approval of the manuscript.
- L. Liu, Collection and assembly of data.
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- X. Liang, Collection and assembly of data.

- P. Li, Analysis and interpretation of data.
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- X. Wang, Drafting and final approval of the manuscript.
- X. Guo, Conception and design of the study.
- T. Ma, Drafting and final approval of the manuscript.
- F. Zhang, Contributed to conception and design, drafting and final approval of the manuscript.

Conflict of Interest Statement

- None declared

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