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ORIGINAL ARTICLE

Analysis of methylation datasets identified significantly changed genes and functional pathways in osteoarthritis

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Abstract

Background Researches indicate that epigenetics was involved in osteoarthritis (OA). The purpose of this study was to describe the alterations of DNA methylation in hip and knee OA by comparing DNA methylome of OA cartilage and non-OA samples and to identify novel genes and pathways associated with OA.

Methods We gained two expression profiling datasets (GSE73626 and GSE63695) from the GEO dataset. The RnBeads in R package was used to identify differentially methylated CpG sites. Genes that showed significant differences in DNA methylation between OA and normal control groups underwent functional annotation analysis using the online tool of GeneCodis. Furthermore, we used the Sequenom MassARRAY platform (CapitalBio, Beijing, China) to perform the quantitative methylation analysis.

Results A total of 249 hypermethylated sites and 96 hypomethylated sites were obtained from OA samples compared with normal control samples. Functional analysis of differentially methylated genes obtained that embryonic skeletal system morphogenesis, cartilage development, and skeletal system development may be involved in the pathogenesis of OA. Eight genes including HOXB3, HOXB4, HOXB6, HOXC4, HOXC10, HOXD3, TBX3, and TBX5 were identified as potential novel biomarkers for OA.

Conclusion Taken together, our study found different molecular characteristics between OA patients and normal controls. This may provide new clues to elucidate the pathogenesis of OA.

Key Points
- *Embryonic skeletal system morphogenesis, cartilage development, skeletal system development may be involved in the pathogenesis of OA.*
- *Eight genes are identified as potential novel markers for OA.*
- *Our future in vivo molecular intervention experiments will extend our current findings.*

Keywords Biomarkers · DNA methylation · GEO dataset · Osteoarthritis

Introduction

Osteoarthritis (OA) is a chronic arthropathy that loads a great burden on the economy of health and reduces the quality of life [1, 2]. OA is now considered as a whole joint disease that involves degradation of the articular cartilage, thickening of the subchondral bone, osteophyte formation, variable degrees of synovial inflammation, degeneration of ligaments, and in the knee, the menisci, and hypertrophy of the joint capsule [3, 4]. Besides, there can also be changes in periarticular muscles, nerves, bursa, and local fat pads that may contribute to OA or the symptoms of OA [4]. Chondrocyte hypertrophy is an integrant developmental stage in endochondral ossification during normal bone formation and growth [5]. In OA, articular chondrocytes respond to the accumulation of injurious biochemical and biomechanical insults by shifting toward a degradative and hypertrophy-like state [3].

In recent years, epigenetics has become an important mechanism for gene regulation, control of gene expression, expression position, and expression time [6]. It is defined as a heritable

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change in genomic function without altering the DNA sequence. DNA methylation is a key process in epigenetics and plays an important role in the differentiation of multiple cell types in embryonic and adult tissues [7, 8]. Up to now, epigenetics playing a key role in the pathogenesis of cancer and leukemia has been researched widely, but few studies on OA have been reported. Studies have reported changes in the methylation status of genes that play a key role in OA pathogenesis, such as MMP3, MMP9, MMP13, ADAMTS4, IL1B, NOS2, GDF5, SOD2, and SOX9 [9–15]. It has been suggested that these methylation changes contribute to the differential gene expression in the OA cartilage. In short, those studies indicated that osteoarthritic chondrocytes have a regulated phenotype based on those up- or downregulated genes in osteoarthritic chondrocytes. But the regulatory regime responsible for these changes has not been illustrated. Because epigenetic changes are reversible, the possibility of reversing epigenetic markers may provide new molecular targets for emerging therapeutic interventions, compared with the genetic code [9, 10].

Taking all these into account, we integrated two GEO datasets of genome-wide DNA methylation profile of human articular chondrocytes from patients with OA to discover the relationships between DNA methylation and gene expression in osteoarthritic cartilage and to identify significantly changed genes and functional pathways in patients with OA.

Methods

Data collection

Datasets that meet the following criteria will be included in our study: (1) The selected dataset must be genome-wide methylation data. (2) These data come from articular cartilage tissue samples. (3) These datasets must be the original dataset. Following these three criteria, human DNA methylation microarray data of OA samples and normal samples were obtained from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) with an accession ID of GSE73626 and GSE63695. The data were produced by GPL13534 Illumina HumanMethylation450 BeadChip (HumanMethylation450_15017482). Consequently, 89 OA samples and 26 normal samples were included in the present study.

Analysis of differential methylation sites

The RnBeads in R package 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 previously described [16–18].

Functional annotation analysis of differentially methylated genes

Genes that showed significant differences in DNA methylation between the case and normal groups underwent functional annotation analysis using the online tool of GeneCodis (http://genecodis.cnb.csic.es/analysis) [19]. Gene ontology (GO) enrichment analysis was performed to classify the differentially methylated genes into the categories of cellular component, biological process, and molecular function [20]. In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed to detect the potential pathway of the differentially methylated genes [21].

Analysis of differential methylation regions at CpG island, promoter, and gene body levels

CpG island has an important role in recruiting transcription factors, and its hypermethylation state affects the recruitment of transcription factors, thereby reducing the transcriptional activity of downstream genes, that is, affecting gene expression. DNA methylation in the promoter region of the gene is one of the most common epigenetic phenomena that inhibit gene expression. The exact role of DNA methylation in the body region of the gene is currently unclear. However, studies have shown that the gene body region is closely related to gene transcription. Differential methylation region analysis of CpG island, promoter, and gene body levels was performed using R package RnBeads, the screening criteria were $|\Delta\beta| > 0.2$ and FDR < 0.05.

Methylation validation

We used the Sequenom MassARRAY platform (CapitalBio, Beijing, China) to perform the quantitative methylation analysis of blood samples (9 OA patients versus 8 normal control). The clinical information of these samples was shown in Supplementary Table 5. This system uses matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE) [22]. Primer designs for nine sites of 1–13 CpGs and 1–26 CpG (cg16559598, cg16458436, cg10281002, cg26196480, cg17645823, cg23827572, cg03877376), and 2–7 CpG (cg03255182 and cg07687119) were performed using Agena's EpiDesigner software. A 10mer tag was added to the 5′ end of the forward primer to balance the PCR reaction conditions. T7-Promoter sequence was added to the 5′ end of the reverse primer for subsequent transcription in vitro. After amplification, transcription, and RNase cleavage, all samples were spotted onto a 384-pad Spectro-CHIP (Sequenom) using a MassARRAYnanodispenser (Sequenom). Spectra peaks were

obtained with a MassARRAY Compact MALDI-TOF mass spectrometer (Sequenom). EpiTyper software (Sequenom) was used to analyze the resulting methylation calls.

In silico validation of the DNA expression in GSE117999

The GEO database (GSE117999) was used to validate the expression of selected genes. Gene transcripts differentially expressed in cartilage tissues were obtained from 12 patients with OA and from 12 patients without OA (arthroscopic partial meniscectomy). The expression of eight genes HOXB4, HOXB6, HOXC4, HOXC10, HOXD3, TBX3, PITX1, and SH3PXD2B was detected, with the difference of expression levels presented as box-plots.

Results

Identification of DMSs between case samples and control samples

Following the selection criterion of datasets, GSE73626 (including 5 hip osteoarthritic, 6 knee osteoarthritic, and 7 hip healthy cartilage samples) and GSE63695 (including 16 OA hip, 62 OA knee, and 19 healthy hip controls) were recruited in our study. Comparisons were performed between differential methylation sites (DMSs) in hip/knee OA samples and healthy samples. As shown in Fig. 1a, the average β values of all CpG sites in the microarray in OA hip and OA knee compared with healthy knee tissue were obtained. A Manhattan plot was produced to display *p* values that were generated by the −log10(FDR) function for each CpG site (Fig. 1b). As presented in the Manhattan plot, the DMSs were mostly distributed on chromosomes 2, 4, 12, and 17, while there were few DMSs on chromosomes 11, 13, 18, and 22.

A total of 249 hypermethylated sites and 96 hypomethylated sites were obtained from case samples compared with control samples. A heat map of the clustering analysis directly showed the all DMSs between the two kinds of samples, which was presented as Fig. 2. To investigate the details of the DMSs, we further listed the top 20 DMSs in Table 1.

Genomic features of DMSs between the case and normal groups

The CpG island and gene context of DMSs were analyzed in relationship with genomic locations. Significant methylated site differences were observed between case and normal groups according to the functional genomic distribution as well as the CpG content and neighborhood context (Fig. 3). In the gene context analysis, 3% were located in 3′UTR region, 23% were located in the gene body, 2% were located in 5′UTR, 3% were located in TSS1500, 2% were located in TSS200, and 67% were located in the intergenic region (Fig. 3a). In the CpG island analysis, 29% belonged to CpG island, 43% belonged to shore area, 9% belonged to shelf area, and the remaining 20% belonged to open sea area (Fig. 3b).

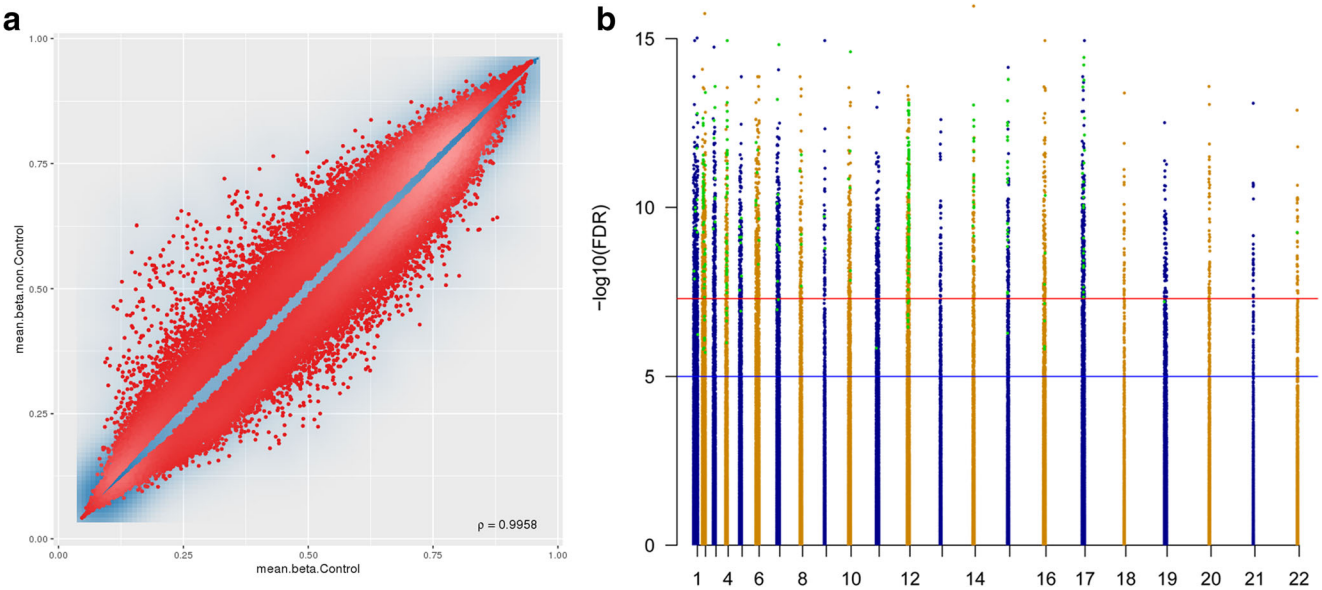

Fig. 1 The scatter plot and Manhattan plot of differentially methylated sites. **a** Scatter plot. Red indicates the site that conforms to FDR < 0.05. **b** Manhattan plot. Each point represents the observed −log10 (FDR) value at a differentially methylated site

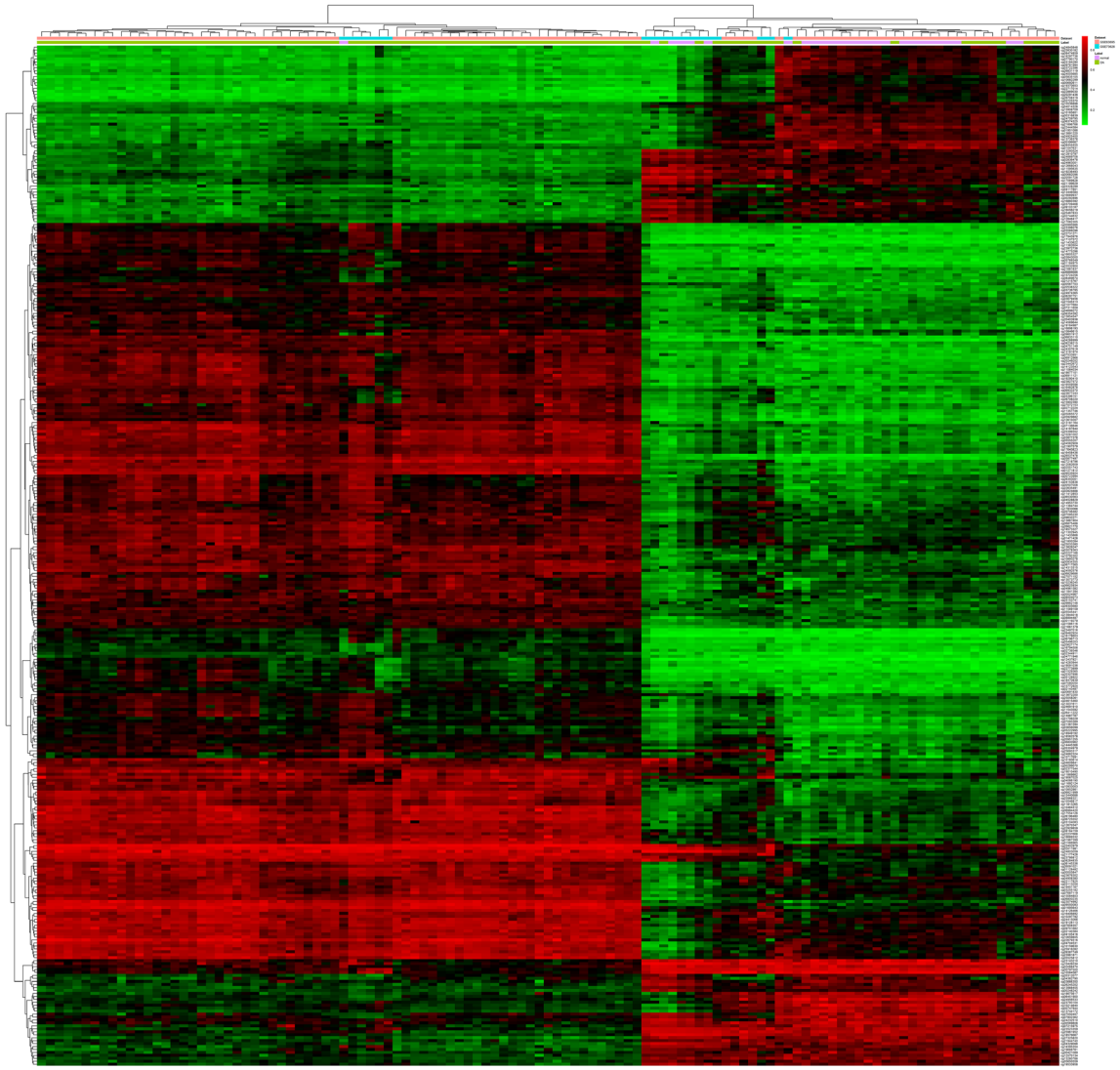

Fig. 2 Unsupervised hierarchical clustering heatmap of all differentially methylated sites between OA patients and normal controls. Each row represents a differentially methylated site and each column, a sample. Differentially methylated sites clustering tree is shown on the right. The color scale illustrates the relative level of differentially methylated sites expression

Functional annotation analysis of differentially methylated genes in the case and normal groups

A total of 72 differentially methylated genes (DMGs) that correspond to the DMSs was obtained, which include 42 hypermethylated genes and 31 hypomethylated genes. Among them, there are two methylated sites (hypomethylated cg18395354 and hypermethylated cg04609841) on the gene of SDK1. The above DMGs were functionally enriched using GeneCodis online software. In Table 2, the results showed that the DMGs were significantly enriched in embryonic skeletal system morphogenesis (HOXB3, HOXB4, HOXB6, GSC, and HOXD3), cartilage development (PITX1, HOXB3, HOXC4, and HOXD3), and skeletal system development (PITX1, TBX3, SH3PXD2B, HOXC10, and EN1).

Identification of DMSs at CpG island, promoter, and gene body levels

Differential methylation region analysis of CpG islands, promoter, and gene body levels was performed using R package RnBeads. As a result, 16 differentially methylated CpG islands were obtained, including 12 hypermethylated CpG islands and 4 hypomethylated CpG islands at CpG island level (Supplementary Table 1). The scatter plot of CpG islands was shown in Fig. 4a. A total of 102 DMGs were obtained, including 41 hypermethylated genes and 61 hypomethylated genes at the promoter level (Supplementary Table 2). The scatter plot of DMGs is shown in Fig. 4b. At the gene body level, 84 DMGs were obtained, including 41 gene-level hypermethylated genes and 43 gene-level hypomethylated genes (Supplementary Table 3). The scatter plot of DMGs is shown in Fig. 4c.

Table 1 The details of top 20 differential methylation sites

cgid	Chromosome	Start	Strand	Δβ	FDR	Hyper/ hypomethylation
cg07311659	chr4	174442784	–	− 0.242737873	1.14773E−15	Hypermethylation
cg20951255	chr7	131291256	–	− 0.201924262	1.51466E−15	Hypermethylation
cg14953730	chr10	134498830	–	− 0.235705988	2.47544E−15	Hypermethylation
cg07093389	chr17	70112097	–	− 0.237326238	3.64317E−15	Hypermethylation
cg03656099	chr17	59481954	+	− 0.238897852	6.10109E−15	Hypermethylation
cg03328299	chr15	74281983	+	0.217288223	1.60381E−14	Hypomethylation
cg14445366	chr17	70112050	–	− 0.250105645	1.68476E−14	Hypermethylation
cg03679456	chr3	169377946	+	− 0.232320399	2.58978E−14	Hypermethylation
cg17833066	chr17	29836341	–	− 0.227237853	2.68933E−14	Hypermethylation
cg24232510	chr2	240582629	–	0.202769844	3.89094E−14	Hypomethylation
cg11384744	chr7	16794623	+	− 0.202042382	6.28958E−14	Hypermethylation
cg06933370	chr15	37387523	–	− 0.270049453	6.875E−14	Hypermethylation
cg04699272	chr4	174447055	–	− 0.200844465	7.75034E−14	Hypermethylation
cg21800284	chr12	115107215	–	− 0.211970092	7.95167E−14	Hypermethylation
cg16406892	chr12	115118777	–	− 0.230581737	9.18245E−14	Hypermethylation
cg16056219	chr14	75043777	+	0.260774927	9.18245E−14	Hypomethylation
cg06800962	chr3	149096029	–	− 0.210631562	1.09272E−13	Hypermethylation
cg00567703	chr12	54413101	+	− 0.274153318	1.36203E−13	Hypermethylation
cg21381594	chr12	114773533	–	− 0.224677116	1.36203E−13	Hypermethylation
cg05545441	chr4	174442812	–	− 0.20641687	1.41695E−13	Hypermethylation

Functional annotation analysis of DMGs at the promoter and gene body levels

Functional enrichment analysis of DMGs at the promoter and gene body levels was performed using GeneCodis online software. At the promoter level, the DMGs were significantly enriched in embryonic skeletal system morphogenesis (FDR = 6.59E−04), bone marrow development (FDR = 2.12E−02) and other biological processes, vitamin D binding (FDR = 1.81E−02), MHC protein binding (FDR = 1.70E−02), and other molecular function (Supplementary Table 4). At a gene body level, the DMGs were significantly enriched in biological processes such as embryonic skeletal system morphogenesis (FDR = 1.21E−04).

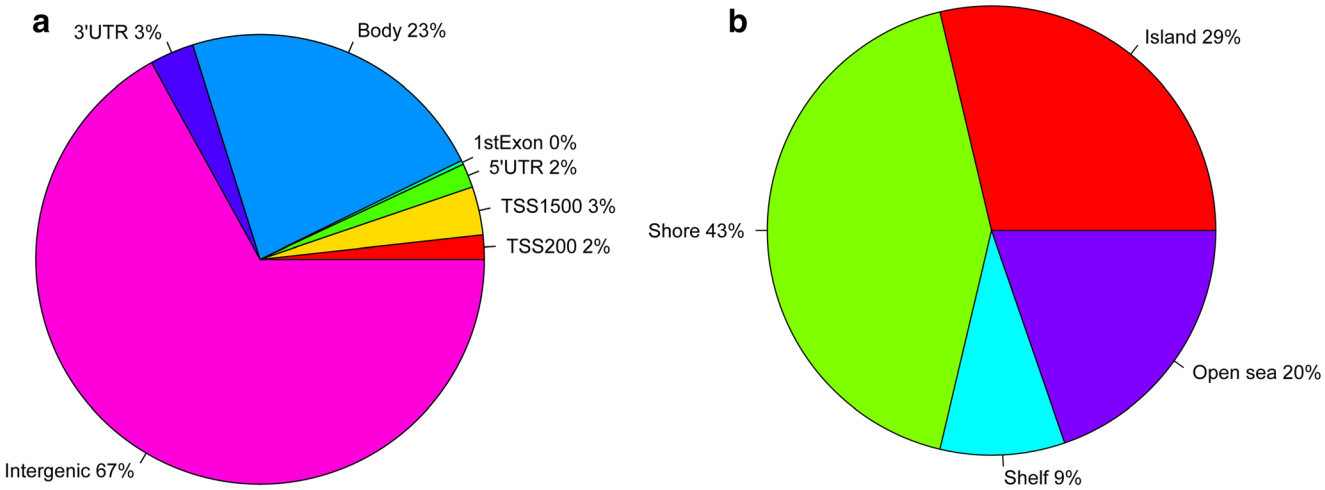

Fig. 3 Genomic features of differentially methylated sites between OA patients and normal controls. **a** Graph showing percentages of differentially methylated sites according to their functional genomic distribution. **b** Graph showing percentages of differentially methylated sites according to their CpG content/neighborhood context

Table 2 Functional annotation analysis of DMGs in the case and normal groups

Items	Items_Details	Support	FDR
Biological process			
GO:0007275	Multicellular organismal development	24	2.70E−18
GO:0009952	Anterior/posterior pattern specification	9	4.36E−11
GO:0048704	Embryonic skeletal system morphogenesis	5	1.86E−06
GO:0060021	Palate development	5	1.01E−05
GO:0035115	Embryonic forelimb morphogenesis	4	1.16E−05
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	9	1.19E−05
GO:0008595	Anterior/posterior axis specification, embryo	3	3.62E−05
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	9	9.21E−05
GO:0030900	Forebrain development	4	1.37E−04
GO:0061325	Cell proliferation involved in outflow tract morphogenesis	2	1.48E−04
GO:0003253	Cardiac neural crest cell migration involved in outflow tract morphogenesis	2	1.48E−04
GO:0051216	Cartilage development	4	1.58E−04
GO:0045893	Positive regulation of transcription, DNA-dependent	8	1.64E−04
GO:0001501	Skeletal system development	5	2.00E−04
GO:0006355	Regulation of transcription, DNA-dependent	13	3.09E−04
Cellular component			
GO:0005634	Nucleus	34	1.49E−09
GO:0005667	Transcription factor complex	7	7.58E−06
GO:0005730	Nucleolus	9	4.26E−02
Molecular function			
GO:0003700	Sequence-specific DNA-binding transcription factor activity	28	5.16E−25
GO:0043565	Sequence-specific DNA binding	24	6.57E−25
GO:0042803	Protein homodimerization activity	8	2.37E−04
GO:0000976	Transcription regulatory region sequence-specific DNA binding	3	2.16E−03
GO:0003677	DNA binding	12	3.03E−03
GO:0008188	Neuropeptide receptor activity	2	3.59E−03
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	2	3.69E−03
GO:0001078	RNA polymerase II core promoter proximal region sequence-specific DNA-binding transcription factor activity involved in negative regulation of transcription	2	1.33E−02
GO:0005115	Receptor tyrosine kinase-like orphan receptor binding	1	1.89E−02
GO:0043924	Suramin binding	1	1.89E−02
GO:0008134	Transcription factor binding	4	2.09E−02
GO:0005516	Calmodulin binding	3	2.47E−02
GO:0001077	RNA polymerase II core promoter proximal region sequence-specific DNA-binding transcription factor activity involved in positive regulation of transcription	2	2.58E−02
GO:0044212	Transcription regulatory region DNA binding	3	2.81E−02
GO:0042610	CD8 receptor binding	1	2.97E−02

Validation of the DNA methylation using MassARRAY

To validate the observations in the DNA methylation array, we detected DNA methylation change of CpGs in the three methylation regions (1–13 CpGs, 1–26 CpG, and 2–7 CpG) using MassARRAY. As shown in Supplementary fig. S1, in total, 18, 10, and 29 detectable CpG sites were examined in the methylation regions of 1–13 CpGs, 1–26 CpG, and 2–7 CpG, respectively. The percentages of methylation at four different loci were noted in OA ($n = 3$) and normal control ($n = 3$). Clustering analysis of these three methylation regions is shown in Supplementary fig. S2.

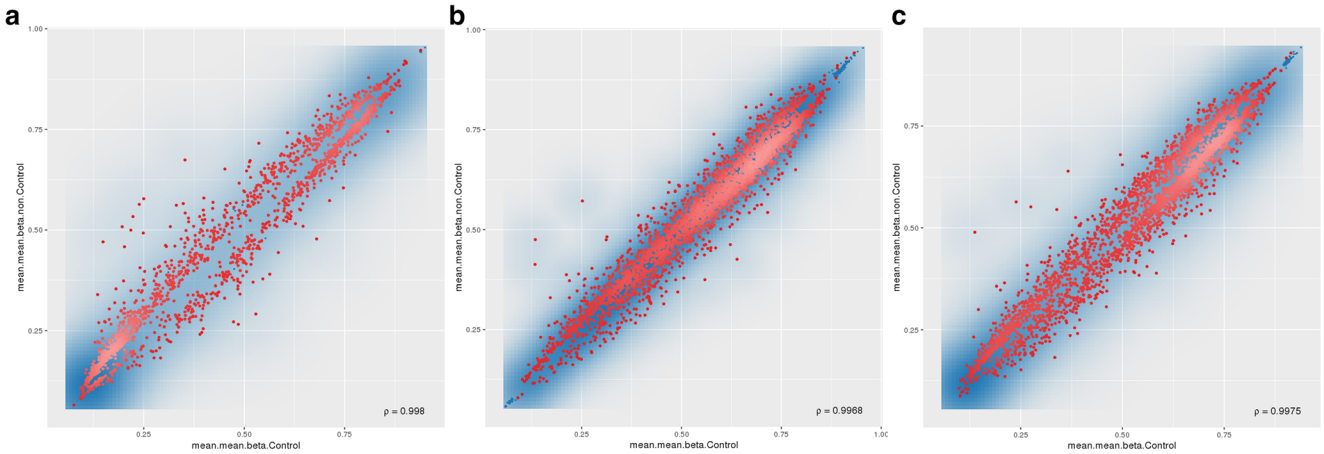

Fig. 4 The scatter plot of differentially methylated sites at **a** CpG island, **b** promoter, and **c** gene body levels

Validation of genes in GEO GSE117999 dataset

To define the key genes that play crucial roles in OA compared with normal control, GEO GSE117999 repository was searched for high-throughput gene expression data and hybridization arrays, chips, and microarrays. As shown in Supplementary fig. S3, the expression levels of HOXB4, HOXB6, and HOXD3 were upregulated, and the expression levels of HOXC4, HOXC10, TBX3, PITX1, and SH3PXD2B were downregulated. In our integrated analysis, the genes of HOXB4, HOXB6, and PITX1 were hypomethylated, and the other genes of HOXC4, HOXC10, HOXD3, TBX3, and SH3PXD2B were hypermethylated. These results of HOXB4, HOXB6, HOXC4, HOXC10, TBX3, and SH3PXD2B were consistent with the analysis in the GSE73626 and GSE63695 datasets.

Discussion

OA is one of the most general chronic osteoarthropathy and degenerative joint disease [23]. In recent years, epigenetic regulation of gene expression dynamics has become increasingly important in OA [24, 25]. The development and progression of OA have been reported to be related to many factors, including age, joint damage, obesity, and chronic inflammation, as well as genetic factors, including epigenetic modifications and changes in gene or miRNA expression [26]. In-depth understanding of gene regulation in OA may help to diagnose and treat OA. Based on this, DNA methylation analysis of hip and knee cartilage of OA patients was performed, and potential targets for OA were identified and verified in the present study. A total of 249 hypermethylated sites and 96 hypomethylated sites were obtained from case samples compared with control samples. We also identified the pathways enriched in the sites and attempted to classify hip and knee OA and OA-free cartilage according to their genome-wide DNA methylation profiling.

GO enrichment analysis revealed that the DMGs are mainly enriched in embryonic skeletal system morphogenesis, cartilage development, and skeletal system development. It is reasonable and consistent with the results reported in the literature. Changes in DNA methylation are also related to gene activation during chondrogenesis [27, 28]. Recent studies focused on articular chondrocytes have uncovered a number of epigenetic alterations in OA disease, involving SIRT1 [29], DOT1L [30], and other histone modifiers [31]. In our study, the genes of HOXB3 (hypomethylation), HOXC4 (hypermethylation), and HOXD3 (hypermethylation) were enriched in the pathway of cartilage development. Thus, we indicated that the epigenetic alterations of HOXB3, HOXC4, and HOXD3 may play a crucial role in the pathogenesis of OA. As previously described, genes involved in skeletal developmental pathways and embryonic organ morphogenesis may be a potential area for further OA studies [32]. The genes of HOXB3, HOXB4, HOXB6, and HOXD3 were enriched in the pathway of embryonic skeletal system morphogenesis. HOXC10 was enriched in the skeletal system development pathway. The main functions of the HOX family genes can regulate the rate and timing of chondrocyte proliferation and differentiation and the proliferation of undifferentiated mesenchyme, participating in the primordium of mesenchymal protoplasts and the reconstruction of chondrocytes. Previous literature reported that HOXA10 mutations can cause femoral deformation and knee degeneration. Nguyen NC et al. indicated that the HOXD9 gene is exclusively expressed in rheumatoid arthritis (RA) synovium but also suggested that the HOXD9 gene contributes to the pathology of RA through the fibroblast-like synoviocytes (FLS). Our study found that the promoter region of HOXB1 gene contains 12 methylation sites, and the promoter region of HOXB4 gene contains four methylation sites, which is hypomethylated in cartilage tissue of patients with OA. The promoter region of HOXD11 gene contains 7

methylation sites and exhibits a hypermethylation state in cartilage tissue of patients with OA. We also performed functional annotation analysis of DMGs at the promoter levels.

TBX5 belongs to the T-box transcription factor family, and its sequences have a T-box DNA-binding domain. It plays an important role in tissue development and cancer [33]. Interestingly, in colon cancer cell lines, inactivation of the TBX5 gene occurs through DNA hypermethylation. In contrast, TBX5 is active and hypomethylated in RA patients. Hypomethylation of gene promoters is often crucial in autoimmune diseases [34, 35]. The previous study reported that hypomethylation is responsible for the activated phenotype of rheumatoid arthritis synovial fibroblasts (RASF) [36]. Karouzakis E et al. [33] compared the synovial tissue and synovial fibroblasts in OA and RA based on epigenomics; the degree of methylation of TBX5 was higher and the expression level of TBX5 was lower in OA patients than that in RA patients. The current study showed that TBX5 was hypermethylated in OA patients compared with normal control.

The T-box gene TBX3 is closely related to TBX5, and the homology between mouse and human is 98% and 96%, respectively [37]. TBX5 functions as a transcription activator [38], whereas TBX3 acts as a transcriptional repressor. However, TBX3 contains both an activation domain and a suppression domain, which may be regulated in different cellular environments [39, 40]. Among other functions, both proteins are involved in bone development [41] and remodeling [42]. Mutations in the human TBX3 and TBX5 genes cause ulnar-mammary syndrome (UMS, OMIM 181450) and Holt-Oram syndrome (HOS, OMIM 142900), respectively. Both syndromes cause defects in limb development [38, 43], which asserts their link to bone pathways recently recognized to interact with immune pathways [44]. However, the role of the TBX3 and TBX5 proteins in the immune system, their link to RA, and their biomarker potential remain unexplored. Our GO enrichment analysis showed that TBX3 was enriched in the skeletal system development pathway. Previous studies reported similar results [32, 45–47]. Embryonic organ and skeletal system morphogenesis have been reported to be major pathways involved in the development of OA. A. HE et al. identified a group of OA-related GO terms, functionally involved in apoptosis, the development of the skeletal system and ossification [48].

Our results of GO enrichment analysis revealed that PITX1 was enriched in the "cartilage development" and "skeletal system development" pathways. The PITX1 gene provides instructions for the production of a protein that plays a key role in the development of lower limbs. The PITX1 protein is mainly found in the developing legs and feet [49]. As previously reported, PITX1 was upregulated in OA articular chondrocytes, which was modulated by E2F1 and TFDP1 [50]. In our results, PITX1 was hypomethylated and the expression level of PITX1 was increased in OA patients compared with normal control. EN1 was enriched in the "skeletal system development" pathway and was hypermethylated in OA compared with normal control. EN1 is a homeobox gene that contributes to the development in the dorsal midbrain and anterior hindbrain of human beings [51]. Haohuan Li showed that EN1 rs4144782 was significantly associated with an increased risk of OA [52].In conclusion, we identified that embryonic skeletal system morphogenesis (HOXB3, HOXB4, HOXB6, GSC, and HOXD3), cartilage development (PITX1, HOXB3, HOXC4, and HOXD3), and skeletal system development (PITX1, TBX3, SH3PXD2B, HOXC10, and EN1) may be involved in the pathogenesis of OA. In addition, eight genes including HOXB3, HOXB4, HOXB6, HOXC4, HOXC10, HOXD3, TBX3, and TBX5 are identified as potential novel markers for OA. This may potentially act as methylation-based biomarkers for detection, prognosis, monitoring, and predicting therapeutic responses in OA. In the present study, we are currently screening the potential candidate genes and pathways of OA. Our future in vivo molecular intervention experiments will extend our current findings.

Compliance with ethical standards

Disclosures None.

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