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Original Article

Relationship between osteoporosis and osteoarthritis based on DNA methylation

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Abstract: The aim of this study was to investigate the relationship between osteoporosis and osteoarthritis by analyzing the DNA methylation in osteoporosis and osteoarthritis. The cancellous bone specimens were collected from a total of 12 hospitalized patients and divided into the osteoporosis group (OA), the osteoarthritis group (OP), the osteoporosis combined with osteoarthritis group (OA & OP), and the normal control group (N). The cancellous bone specimens of each group were detected and the differences in gene expression profiles by the MeDIP-chip technique were compared. Compared with Group OA & OP, the methylation levels in Group OA and Group OP were statistically higher, $P < 0.05$. In the microarray analysis, a total of 1,222 sites occurred hypermethylation. The analysis targeting the differentially expressed genes between Group OA & OP and Group N revealed that group OA and group OP had 4 common genes: PPIL3, NIF3L1, SMTN, and CALHM2. The level of genomic methylation is lower in the patients with osteoporosis and/or osteoarthritis. The common difference between osteoarthritis and osteoporosis is reflected in some specific promoters, which may participate in the processes of diseases through different pathways.

Keywords: Osteoporosis, osteoarthritis, mitochondria, DNA methylation

Introduction

Osteoarthritis (OA) and Osteoporosis (OP) are both degenerative diseases. There is controversy about whether these two diseases are unrelated, positively related, or negatively related [1-3]. To find prevalence of osteoporosis (OP) in postmenopausal females with primary knee osteoarthritis (OA) in India, where there is widespread Vitamin D deficiency (VDD), Dhaon et al. [4] considers that prevalence of osteoporosis in PMW with primary knee OA is similar to that in the general population. Yoshimura et al. [5] once reported, after 10 years of follow-up study, that osteoporosis (OP) at the lumbar spine might reduce the risk of subsequent OA in women while not in men. Roux et al. [6] has mentioned that hypertrophic osteophytes in lumbar osteoarthritis, to some extent, can reduce the incidence of lumbar fractures. However, the study did not explicitly state whether OA was achieved by directly affecting the progress of OP. In clinical practice, there are many elderly patients with OP and OA simultaneously. Geusens et al. [7] has mentioned that the common insights about convergent and divergent risk factors between OA and OP have resulted in new conclusions on the roles of BMD, BMI, falls, genetics, and epigenetics regarding the pathophysiology of diseases and increased fracture risks in OP and OA. Abundant research has proved the involvement of epigenetic mechanisms (post-translational modifications of histone tails, DNA methylation, and non-coding RNAs) in the differentiation of bone cells and mechanotransduction. Del Real et al. [8] points out that various epigenetic abnormalities have been elucidated in patients with OP, OA, and/or skeletal cancers, but the actual related pathogenetic roles are still not clear.

As a new research direction, the epigenetics provides new ideas for exploring the relationship of OA with OP. The epigenetics includes the DNA methylation, chromatin remodeling, histone modifications, or microRNA (miRNA). Its regulatory effects include the genomic imprinting, maternal effects, gene silencing, or nucleolar dominance [9]. Current research has shown that [10, 11] the occurrence and development

of osteoporosis and osteoarthritis both involve a variety of epigenetic mechanisms. This study analyzed the genomic differences in the mitochondrial characteristics, aiming to further investigate the relationship between OP and OA.

Materials and methods

Diagnostic criteria

The diagnosis of OP referred to the diagnostic criteria issued by WHO: Bone Mineral Density (BMD) of L2-4 measured by the dual-energy X-ray absorptiometry: T-value > -1.0 SD was defined as normal, -1.0 SD > T value > -2.5 SD was defined as bone loss; -2.5 SD > T value was defined as OP; -2.5 SD > T value, combined with osteoporosis fractures, was defined as severe OP.

The diagnosis of OA referred to the diagnosis and treatment guidelines of Osteoarthritis developed by the Chinese Society of Orthopedics in 2007: 1. Repeated knee pain within the past 1 month; 2. X-ray images (standing or weight-bearing position) showed the narrowing of joint space, subchondral bone sclerosis and (or) cystic degeneration, and callus formation at the edge of joints; 3. The joint fluid (at least 2 times) was clear and viscous, WBC < 2000/ml; 4. Middle-aged and elderly patients (≥ 40 years old); 5. With morning stiffness ≤ 3 minutes; 6. With the sound (feelings) of bone friction during activities.

The patients with the item 1+2 or 1+3+5+6 or 1+4+5+6 can be diagnosed as knee osteoarthritis.

Inclusion criteria

1. Menopausal female patients, with menopause for more than 1 year, aged 55-75 years old; 2. Patients meeting the above diagnostic criteria of western medicine and traditional Chinese medicine syndrome and diagnosed as knee osteoarthritis; 3. Volunteers with complete relevant information, can sign the informed consent, and agree to retain bone specimens.

Exclusion criteria

1. with severe heart, liver, kidney, or other system diseases, mental illness, or cancer; 2. with secondary osteoporosis, history of acute trauma, knee meniscus injury, lateral collateral ligament injury, cruciate ligament injury, traumatic synovitis, or hematoma of knee(s); 3. with heart, liver, kidney, or other systemic diseases, mental illness, or cancer during treatment; 4. combined with rheumatic bronchitis, rheumatoid arthritis, gout, or other painful joint diseases; 5. with bone marrow/hematopoietic diseases.

Case data

A total of 12 female patients from March 2015 to December 2015 were selected, aging 60-70 years and meeting the above standards. These 12 patients were divided into Group OA (n = 3), Group OP (n = 3), Group OA & OP (n = 3), and Group N (n = 3). The bone mineral density of each patient was measured using a bone densitometer (GE LUNAR Prodigy, USA, cv = 1.0%). Before the study, all the patients signed the informed consent. The age and weight ratio of each group was strictly controlled to make it statistically insignificant.

Specimen collection and preservation

1 cm^3 of lumbar spinal cancellous bone was sampled during lumbar surgery, placed in a freeze-dried tube, and preserved at -80°C.

Analysis of methylation chips

The Arraystar Human RefSeq Promoter Microarray was selected and Shanghai Kangcheng Co. Ltd. was commissioned for the gene chip hybridization. It is a chip that can detect apparent methylation and the binding sites of transcription factors within the promoter region of the RefSeq gene. The chip uses approximately 180,000 probes to cover approximately 23,148 gene promoter regions with 210 bp as the gap (the designed region is approximately -1300 bp to +500 bp of TSS). The specific method was as follows:

Extraction and Fragmentation of genomic DNA: The genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue Kit (69506, QIAGEN, Fremont, CA) and the purified DNA was then quantified and evaluated using the NanoDrop ND-1000 (Thermo Scientific, Massachusetts, USA), with 10 cycles of Bioruptor sonicator (diagenode, Liege, Belgium) in the “low” mode

(30 seconds "ON" and 30 seconds "OFF") to interrupt the genomic DNA to approximately 200-1000 bp. Agarose gel (EEO015, Gene Company LTD, Hong Kong, China) electrophoresis was then performed to detect gDNA and cut DNA.

Immunoprecipitation: (1) 1 μg of ultrasound-cleaved genomic DNA was immunoprecipitated using a 5-methylcytosine mouse monoclonal antibody (MAB-31HMC-100, diagenode, Liege, Belgium). (2) The DNA was denatured at 94°C for 10 min and then rapidly placed onto ice for overnight culture with 1 μL of primary antibody and 400 μL of immunoprecipitation buffer (0.5% BSA in PBS) at 4°C. (3) The antibody-bound DNA fragments were recovered and then mixed with 200 μL of mouse anti-IgG magnetic beads (11201D, Invitrogen, California, USA) for 2 h at 4°C. (4) After the hybridization with antibodies, the mixture was washed 5 times at 4°C. (5) After washing, the beads were resuspended in a TE buffer containing 0.25% SDS and 0.25 mg/mL proteinase K (127149304, QIAGEN, Fremont, CA) at 65°C for 2 hr and then cooled to room temperature. The MeDIP DNA was recovered using the Qiagen MinElute columns (QIAGEN, Fremont, CA).

DNA Labeling and Chip Hybridization: (1) The purified DNA was quantified using NanoDrop ND-1000. (2) After labeling the DNA using the Roche Dual-Color DNA Labeling Kit (the experimental procedure referred to the standard Roche MeDIP-chip regulations), 1 μg of DNA and 10 D of Cy5-9mer primer (IP sample) or the Cy3-9mer primer (Input sample) were incubated at 98°C for 10 min, after that, 100 pmol of dNTPs and 100 U of Klenow fragment were added and mixed at 37°C for 2 h (PCR:MJ Mini, BIO-Rad, California, USA). (3) 0.1-time volume of 0.5M EDTA (E7889-100ml, Sigma, Missouri, USA) was added to terminate the reaction, and the labeled DNA was recovered with isopropanol or ethanol. (4) The labeled DNA and the chips were hybridized at 42°C for 16 to 20 h in a hybridization cassette using the Roche hybridization kit (Hybridization System-Nimblegen Systems, Inc., Madison, WI, USA). (5) After the hybridization was completed, the chip was washed using the Roche washing buffer pack (Nimblegen Systems, Inc., Madison, WI, USA).

Analysis of chip data

Methylation Enrichment and Binding Site Determination: Starting from the standardized log 2-ratio data, a sliding window (1500 bp) provided by NimbleScan v2.5 (Roche-NimbleGen) was used to determine the binding sites for analyzing the MeDIP-chip data. A one-sided Kolmogorov-Smirnov (KS) test was used to determine whether these probes had a significant normal distribution to the ratio of the logarithm of the probes on the other arrays. Each probe can obtain a *P*-value of log 10 from the window KS test surrounding a particular probe. If the *P* values of several neighboring probes were significantly above a set threshold, this region can then be considered to be a peak. The peak data file was thus obtained from the *P* value data file. NimbleScan was used to detect the peak by searching the minimum *P* value which had at least 2 probes above the threshold, and the peaks within the 500 bp in distance were merged.

Analysis of differentially enriched peaks using the median method: When comparing the differentially enriched areas between two groups of samples, the ratio of average logarithm, 2 of each probe in each group, was calculated first (for example, the experimental group and the control group) to calculate the M' value (the calculation method referred to the formula). NimbleScan Sliding Window was then re-run to determine the binding site algorithm to find the differentially enriched peaks.

M' = Average (log2 MeDIPE/Input E) - Average (log2 MeDIPC/Input C)

The criteria of NimbleScan algorithm for identifying the differential enhanced peaks (DEPs) were as follows: at least one of the two samples had a median ≥ 0.3, (M') > 0; at least half the probes of one peak-corresponded two samples had a coefficient of variation ≤ 0.8.

Statistical analysis

SPSS22.0 was used for the statistical analysis. The data obtained from the experiments were first tested for the normal distribution and homogeneity of variance. The data with normal distribution and homogeneity of variance were expressed as the mean ± standard deviation. The comparison of mean values among groups

Table 1. Comparison of lumbar bone mineral density among different groups (g/cm^2, $\bar{x} \pm s$)

Group	n	Lumbar BMD (g/cm^2)
OA	3	1.22±0.09
OP	3	0.59±0.14$^{\Delta}$
OA & OP	3	0.62±0.19$^{\Delta}$
N	3	1.12±0.11

Note: The data followed the normal distribution with a uniform variance. LSD test, $^{\Delta}$indicates $P < 0.05$ between group OA and group N; when compared with group OP. $\alpha = 0.05$.

was performed using one-way analysis of variance (one-way ANOVA). The pairwise comparison of mean values among groups used the LSD test. The data with non-normal distribution and heterogeneity of variance were expressed as the median (P25-P75). Multiple comparisons among groups used the Kruskal-Wallis test, with $P < 0.05$ (bilateral) considered as statistical significance.

Results

Analysis of general information

The one-way analysis of variance for the bone mineral density among groups was $F = 16.53$, $P = 0.001 < 0.05$, indicating statistical significance in the bone mineral density among the groups while comparable. The LSD examination targeting the lumbar spine BMD revealed significance, the lumbar spine BMDs in Group OP and group OA & OP were significantly lower than Group OA and Group N (N) ($P < 0.05$). There was comparability among the groups. No statistical significance was observed in the bone mineral density between group OA and group N, OP, or OA & OP, $P > 0.05$, and the baseline characteristic was consistent (**Table 1**).

Analysis of specimen quality

Analysis of Sample-related Coefficient Matrix: The matrix analysis of each specimen in all the groups showed that the relationship among the specimens of different groups was relatively consistent, among which the relationship coefficients of the specimens in Group OA, Group OP, and Group N were all above 90% (**Figure 1**).

Sizes of DNA fragments: After the genomic DNA was fragmented, the fragmented DNA of each sample was quantified using NanoDrop ND-1000, and then we examined the size agarose gel electrophoresis. The results showed that the sizes of the DNA fragments ranged from 200 to 1000 bp and met the requirements of follow-up experiment (**Figure 2**).

Analysis of differences in DNA methylation levels

The one-way analysis of variance (ANOVA) was performed to compare the average DNA methylation level among the groups. $F = 22.95$, $P = 0.000 < 0.05$. There were differences in the DNA methylation level among groups and there was comparability among groups. The LSD test showed that Group N had more of a statistical significance in the DNA methylation level than Group OA, Group OP, and group OA & OP ($P < 0.05$). The DNA methylation level in Group N was significantly higher than the other groups. Compared with group OA & OP, the methylation levels in group OA and group OP were higher than group OA & OP ($P < 0.05$) and the differences were statistically significant. The comparison of the mean DNA methylation level between Group OA and Group OP showed no statistical significance, $P > 0.05$, indicating that the mean DNA methylation level between Group OA and Group OP was comparable (**Table 2**).

Analysis of methylation chip results

Many studies have confirmed that transcriptional inhibition of promoter downstream genes is related to the methylation of promoters and it is known that mammalian promoters with different GC content have different methylation profiles. Based on the CpG ratio, the GC content, and the length of the CpG enrichment region, the promoters can be divided into the following three categories: high CpG-density promoter (HCP), low CpG-density promoter (LCP), and intermediate CpG-density promoter (ICP). Most ICP are methylated either in the active state or in the inactive state, which implies that the low density of methylated cytosine does not hinder the gene activity, so it is considered that only the methylation of HCP and ICP is considered to be meaningful. Compared with Group OP and group OA, the CpG island of 1222 gene promoters occurred hypermethylation ($P < 0.05$), among which 501 were on LCP, 191 were on ICP, and 530 were on HCP (**Figure 3**); most of the differential genes

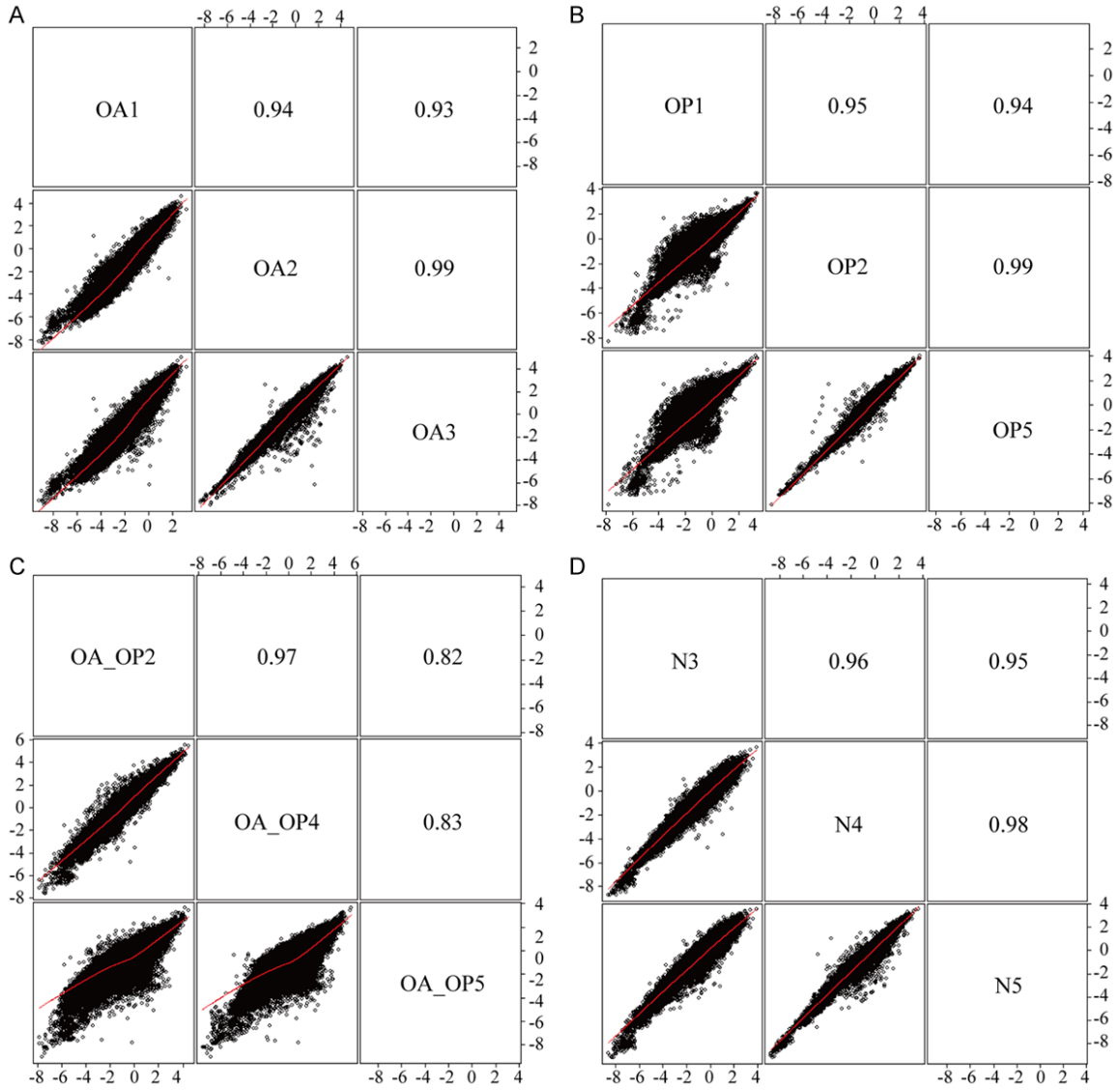

Figure 1. Analysis of relationship coefficient matrix of specimens among different groups.

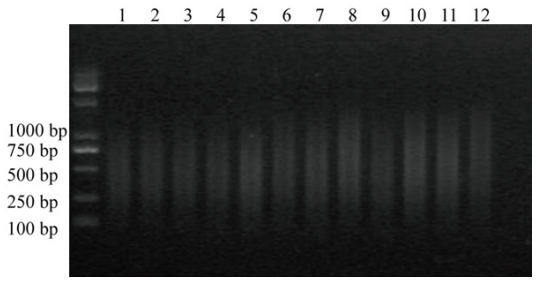

Figure 2. Agarose gel electrophoresis analysis of sizes of genomic DNA fragments of each specimen (BP).

were located on chr I, chr 3, chr I1, chr I6, chr I7, and chr I9 (**Figure 3**). The above genes were analyzed and classified by the GO typing for the enrichment test. It can be seen that the genes involved in methylation involve almost all major biological processes and common signal pathways.

Comparison of differential genes

The comparison of the methylated genes in HCP and ICP among groups revealed that there were 644 differential genes in Group OA, 669 differential genes in Group OP, and 492 differential genes in Group OA & OP than Group N. The comparative analysis of differentially expressed genes in Group OA & OP and Group N revealed 4 common differential genes in these

Table 2. DNA methylation levels in different groups (%, $\bar{x} \pm s$)

Group	n	Level of DNA methylation (%)
OA	3	$3.47\pm0.13^{\Delta,\blacktriangle,\blacklozenge}$
OP	3	$3.39\pm0.53^{\Delta,\blacktriangle}$
OA & OP	3	$2.02\pm0.29^{\Delta}$
N	3	5.04 ± 0.64

Note: The data followed the normal distribution with a uniform variance. The average DNA methylation level among groups was analyzed by one-way ANOVA: $F = 22.95$, $P < 0.05$. The pairwise comparison of DNA methylation level among groups was performed using the LSD test, $^{\Delta}$indicates $P < 0.05$ when compared with group N; $^{\blacktriangle}$indicates $P < 0.05$ when compared with group OA & OP; $^{\blacklozenge}$indicates $P > 0.05$ when compared with group OP. $\alpha = 0.05$.

two groups (NIF3L1 coexisted in HCP and ICP): PPIL3, NIF3L1, SMTN, and CALHM2, among which PPIL3, NIF3L1, and SMTN were all located in HCP. The specific information of differential genes is as follows (**Table 3**).

Discussion

The concept of epigenetics was first proposed by Waddington CH in 1942, who considered epigenetics as a discipline for studying the mechanisms of biological development. In the middle of 1970s, the concept proposed by R. Holliday [12] was that epigenetics studied the expression changes of heritable genes that were caused by non-DNA sequence changes. With the continuous progress of epigenetic studies, it has revealed that the occurrence and development of many systemic diseases, such as tumors, degenerative diseases, nervous system diseases, or cardiovascular diseases, have very close relationship with epigenetics. Related mechanisms cover such fields as post-translation histone modification, miRNA-mediated post-transcription regulation, and DNA methylation [13]. DNA methylation is mainly a process of adding the methyl group to DNA molecules. In mammalian cells, DNA methylation predominantly occurs on the cytosine (C) of dinucleic acid in cytosine and guanylate (CpG) [14, 15]. Under the action of catalytic enzymes, DNA methyltransferase can move the methyl group (S-adenosylmethionine) to the 5th carbon atom (cytosine in the DNA duplex) and form the 5-methyl Cytosine (5-m C), and this is the process of DNA methylation. The DNA methyltransferases (DNMTs) that can catalyze this reaction include: DNMT1, DNMT3A, DNMT3B, and DNMT3L, among which DNMT1, DNMT3A, and DNMT3B can directly catalyze the methylation reaction while DNMT3L mainly catalyzes other transferases to complete this activity. In eukaryotes, DNA methylation has three states, namely sustained hypomethylation, induced demethylation, and hypermethylation.

In the genome, CpG is the site that maintains methylation and both the C-terminus in 5'-CpG-3' and 3'-GpC-5' of the DNA duplex are methylated. The CpG islands is one CpG cluster being composed of a large number of unmethylated CpGs, most of which are present in the transcription start site and promoter of structural genes. The hypermethylation of DNA first causes the 3D changes of the major groove in the DNA duplex and then blocks the DNA binding activity of methylation-sensitive transcription factors (TFs, including E2F, CREB, AP2, cMyc/Myn, NF-kB, cMyb, or ETS), thus causing gene silencing.

Zhang et al. [16] studied the interaction between DNA methylation and osteogenic differentiation of MSCs. The osteogenesis model of MSCs was pretreated with 5-azacytidine 24 hours before the induction of osteogenic differentiation. The results revealed that as the DNA methylation modification of the whole genome was continuously reduced, the osteogenic differentiation efficiency was also significantly improved. The determination targeting the fluctuation of methylation modification level on the key regulatory osteogenic differentiation gene Dlx5 at the single gene level revealed that after the CpG island shore on the Dlx5 gene was pretreated, its methylation level was significantly lower than other sites. Del Real et al. [17] studied the hMSCs obtained from the femoral head of females undergoing hip replacement due to hip fractures and hip osteoarthritis and used the Infinium 450 K Bead Array to explore the DNA methylation. The transcriptome analysis was accomplished by RNA sequencing. The genomic analysis shows that most differentially-methylated loci are located in the genomic region with enhancer activities while far away from the genosomes and promoters. These regions are associated with the genes that are differentially expressed in the pathways and rich in the growth of hMSC and the differentiation of osteoblasts. The hMSCs obtained from

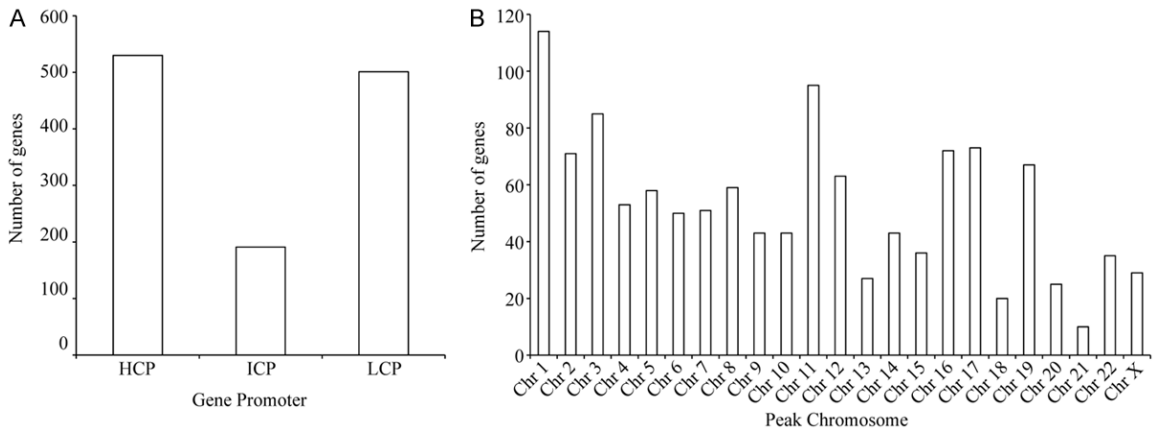

Figure 3. Type and chromosome distribution of 1222 hypermethylated promoters in group OA and OP. A: Distribution of promoter types; B: Chromosome distribution.

Table 3. Basic information of differential genes

Peak ID	Peak Score	Peak DM Value	Gene Name	Promoter Classification	Chromosome
975	3.61	0.455965469	PPIL3	HCP	chr2
3494	2.69	0.239787114	NIF3L1	HCP	chr2
2071	2.52	0.212739393	SMTN	HCP	chr22
1773	2.59	0.170241887	CALHM2	ICP	chr10

fractured patients showed the proliferation and upregulation of enhanced osteoblast driver RUNX2/OSX. In addition, they also showed the signs of accelerated methylation aging.

According to certain studies regarding genomic expression profiling, the inactivation and mutation in the miRNA coding regions may be involved in the pathogenesis of OP. Regardless of the upregulation or inhibition of miRNAs, it is undeniable that significant changes happen in the expressions of multiple miRNAs in the OA and OP cells. Therefore, the profile of the expressions of specific miRNAs in such patients can be seen as new markers for the diagnosis of cartilage lesions.

According to Nicolas, inhibiting the endogenous miR-22 in the OA chondrocytes can inhibit the expressions of IL-1β and MMP-13 by increasing the expressions of PPARα and BMP-7, thereby increasing the content of proteoglycan that can protect the cartilages [18]. Certain *in vitro* studies by Mizoguchi have confirmed that the absence of specific Dicer enzymes will induce the inhibition of osteoclast-mediated bone resorption. The cause of this process reflects the role of the miR-22 osteoclasts, indicating that miRNAs not only play roles in osteoclastogenesis but also play positive regulatory roles in the bone resorption process [19]. Other studies have shown epigenetic dysregulation of many genes and pathways in OA, including some OA-susceptible genes. In addition, the CpG methylation in OA is related to the histological severity [20]. Alvarez et al. [21] demonstrated the role of hypoxia in regulating the expressions of anabolic and catabolic genes, as well as the effects on the changes of DNA methylation. These results further support the role of epigenetics in osteoarthritis and critically highlight the complex relationship between the physiological environment of chondrocytes and the process of osteoarthritis, as well as the effects on therapeutic intervention and the understanding of the pathophysiology of OA.

Although the reasons for inter-group differences and specific mechanisms have not been clarified yet, the expression significance of other related genes on OA or OP has been initially verified. Reppe et al. [22] considered that bones exposed to stress or not showed different gene expressions, reflecting the differences in bone turnover and remodeling; the comparative histological analysis among group N, group OP, and group OA revealed the transcriptomics, DNA methylation of epigenomics, proteomics, and metabonomics. These studies, along with the genome-wide association studies, in vitro observations, and transgenic ani-

mal models, have identified a number of genes and gene products that function through the Wnt and other signaling systems and are closely related to bone density and fractures. Delgado-Calle et al. [23] studied the genome-wide methylation profile of the bones in patients with hip OA and OP, and the final results suggested that the genome-wide methylation analysis of bone samples revealed the regions with differential methylation in OP and OA. These regions are rich in the genes associated with cell differentiation and skeletal embryogenesis, such as those in the homoeotic superfamily, suggesting the presence of developmental components predisposed to these diseases.

Conclusions

This study reveals that the patients with OP/OA have low levels of genomic methylation. The common difference between OA and OP is in certain specific promoters. These genes involve almost all *in vivo* biological processes and lay the theoretical foundation for further exploring the molecular regulation mechanisms. Four differential genes (PPIL3, NIF3L1, SMTN, and CALHM2) have been identified in OA/OP compared with Group N, which may be involved in the development of diseases through different pathways, and it also confirms that OA and OP have certain relationship at the level of gene methylation, which provides theoretical basis for the common pathogenesis of these two diseases. However, due to the small number of samples and the large number of genes in this experiment, there may be some bias in screening common differential genes. We believe that with the continuous progress of research, the relationship between OA and OP will be further revealed, and more problems will arouse everyone's attention and thinking. At the same time, it will have very important significance toward the diagnosis or related treatment research against diseases.

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Disclosure of conflict of interest

None.

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