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The association between knee synovial methylation and knee osteoarthritis in different ethnic populations from different altitude

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Title: The association between knee synovial methylation and knee osteoarthritis in different ethnic populations from different altitude.

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Abstract

Objective: To determine genome-wide DNA methylation profiles of knee cartilage from patients with osteoarthritis (OA) at different altitudes.

***Method*:** Twenty-four synovial tissue samples were selected. OA synovial tissue samples were obtained from 10 Tibetan OA patients at high altitude and 8 Han OA patients at low altitude. Six normal synovial tissue samples were used as the control group. Illumina Methylation Analyzer package was employed for identifying differentially methylated CpG sites.GO, Pathway and disease annotation enrichment analysis were performed for differential genes corresponding to differential methylation sites.

Results: In high-altitude osteoarthritis patients and normal controls, 413 methylation differential sites were associated with osteoarthritis, including 92 hypermethylated sites and 321 hypomethylated sites. In low-altitude osteoarthritis patients and normal controls, 723 methylation differential loci were associated with osteoarthritis, including 436 hypermethylated loci and 297 hypomethylated loci. There were 60 different loci in patients with osteoarthritis at different altitudes.
Conclusions: This multistage study identified an interferon-inducible gene interaction network associated with OA and highlighted the importance of gene in OA pathogenesis. The results enhanced our understanding of the important role of DNA methylation in pathology of OA.

Introduction

Osteoarthritis (OA) is one of the most common chronic joint diseases, which is characterized by the loss, degeneration and calcification of articular cartilage[1]. It often occurs in middle-aged and elderly people, and the incidence of women is significantly higher than that of men[2]. Studies have shown that in recent years, with the acceleration of the pace of life, the number of young people with this disease has begun to increase, eventually leading to a continuous growth of social burden[3].

Despite the unremitting efforts of many scholars, the pathogenesis of OA remains unclear. The present study shows that OA is a common degenerative disease caused by a combination and interaction of many factors such as natural and environmental

factors[4]. Increasing age, tissue and cell damage, obesity, overuse of joints, and genetic susceptibility are known as major risk factors for OA[5].

Osteoarthritis mainly affects the cartilage of weight-bearing joints, followed by the synovium. At present, it is generally believed that the central part of the OA mechanism is the degeneration of articular cartilage, which results from the imbalance between anabolism and catabolism in the extracellular matrix of cartilage caused by mechanical and biological factors[6]. Since these changes were reported to be caused by genetic changes in chondrocytes associated with OA epigenetic mechanisms, it was hypothesized that epigenetic changes in chondrocytes could be key drivers of OA[7].

Studies on the epigenetics of OA suggest that these mechanisms are important during the onset and progression of the disease[8]. Despite the presence of many epigenetic mechanisms such as DNA methylation, miRNA, and histone modifications[9], the involvement of DNA methylation in OA pathophysiology is the most studied topic[10-12]. DNA methylation occurs mainly at CpG dinucleotides, which are selectively added to cytosines to form 5-methylcytosine under the catalysis of DNA methyltransferase (DNMT). DNA methylation is involved in transcriptional repression by preventing protein binding to gene promoters and altering chromatin structure[13]. Changes in methylation status can accelerate the development of OA[14]. Therefore, further investigation of the mechanism of DNA methylation is another way to develop new OA therapeutic strategies. There have been several studies on DNA methylation of specific genes in OA cartilage. For example, expression of matrix metalloproteinase genes has been reported to be upregulated in OA chondrocytes, leading to extracellular matrix degradation[15]. In addition, genes associated with OA chondrocytes such as GDF-5, SOX-9, DIO 2, and ADAMTS-4 were also shown to be differentially expressed between OA cartilage and controls[16]. It has also been reported that the IL 1B promoter is demethylated in articular chondrocytes as a response to inflammatory cytokine signaling[17].

OA occurs mainly in the knee and hip joints. However, knee OA is more common than hip OA. Although articular cartilage of the hip and knee joints has essentially similar characteristics and function, disease progression and subsequent treatment may differ between the two joints[18]. Transcriptomic studies have shown that dysregulated genes in hip and knee OA are highly divergent[19], as are methylation patterns in hip and knee OA samples[20]. These findings highlight the importance of separating OA studies from skeletal sites and help us understand cartilage homeostasis. The treatment of osteoarthritis is mainly to relieve pain, improve the function of joints, and try to avoid the side effects of treatment. However, due to the slow progression of OA and the lack of sensitive detection methods to identify early OA changes, it is currently difficult to find disease-modulating drugs. These epigenetic markers can detect the phenotypes of various chondrocytes, including articular cartilage homeostasis, chondrogenic differentiation and OA occurrence, and provide new targets and strategies for the drug treatment of OA.

In this study, the synovial tissue samples of 10 high-altitude Tibetan osteoarthritis

patients, 8 low-altitude Han osteoarthritis patients and 6 Tu normal controls were collected. The illumina 850K methylation chip was used to screen and analyze the differential methylation sites to screen out the special genes that may be significantly related to the pathogenesis of osteoarthritis in plateau area. These genes may be used to identify novel DNA methylation sites important for RA, to fully understand the underlying pathological mechanisms, and to find osteoarthrotis-specific molecular markers of methylation

Materials and Methods

Cartilage specimen collection

Twenty-four synovial tissue samples were selected. OA synovial tissue samples were obtained from 10 Tibetan OA patients at high altitude and 8 Han OA patients at low altitude. Six normal synovial tissue samples were used as the control group.All subjects were age - and sex-matched. A questionnaire completed by nurses was used to obtain the clinical data of the subjects, including self-reported ethnicity, lifestyle characteristics, health status, family and medical history. Each subject underwent careful clinical and imaging examinations. Primary knee OA was diagnosed according to the Western Ontario 109 and McMaster Universities OA Index of pain, stiffness, and function, clinical 110 evaluation, and radiologic imaging. Hereditary bone and cartilage diseases, rheumatoid arthritis and other bone disorders were excluded. Synovial tissue samples were obtained from the same anatomical area of the femoral condyle of the knee joint. Synovial tissue samples from 10 high altitude Tibetan osteoarthritis patients, 8 low altitude Han osteoarthritis patients and 6 Tu normal controls were dissected and frozen in liquid nitrogen for genome-wide DNA methylation analysis. This study was approved by the Human Ethics Committee. All participants provided written informed consent.Genome-wide DNA methylation profiling。

The Illumina Infinium MethylationEPIC BeadChip (850K chip) was used to detect methylation in the synovial tissue samples of 10 high-altitude Tibetan OA patients, 8 low-altitude Han OA patients and 6 Tu normal controls. More than 850,000 methylation sites were included. The 500ng DNA samples were treated with bisulfite using the EZ DNA Methylation kit (Zymo Research, USA). The bisulfite converted DNA samples were then amplified, hybridized to the Methylation850 chip, stained and washed according to standard procedures. Raw image intensity was scanned using an iScan SQ scanner (iScan System, Illumina,USA). Raw image intensity data obtained were processed using GenomeStudio software (Illumina, USA). The average percentage of methylated cytosines at a specific CpG site is expressed as a β value, which ranges from 0(completely unmethylated) to 1(fully methylated).Differential methylation analysis

The empirical Bayes moderated t-test in limma[21] was called by Illumina Methylation Analyzer package[22] for identifying differentially methylated CpG sites.Benjamini-Hochberg method was used to calculate a false discovery rate (FDR)

adjusted P value for each CpG site. Significant CpG sites were defined as: (1) FDR adjusted P value (P_{adj}) ≤ 0.05; (2) |β-value difference (Δ_β)| ≥ 0.2. For quality control, the CpG sites with missing values, or detection P values > 0.05 in more than 90% of cartilage specimens were eliminated.

Functional annotation and enrichment analysis

In the first step, the input gene sets are mapped to the database genes, and then the pathways, diseases, and functions in which these gene sets are involved are annotated.

For the mapping of gene ids, the input ids were directly mapped to GENES through cross-linked links in the KEGG GENES database. Alternatively, the ID can be mapped onto the KO ontology if desired. For sequence similarity mapping, BLAST sequence similarity alignment was performed between the input sequences and the sequences of KEGG GENES.

In the second step, the results obtained in the previous step are compared with the background (usually the genes in the whole genome, or all the probes on the chip) to uncover statistically significantly enriched pathways, diseases, or functions. Binomial test, chi-square test, Fisher exact test or hypergeometric distribution test were used for statistical analysis. Then, FDR correction was performed using QVALUE, Benjamini-Hochberg, or Benjamini-Yekutieli methods. The purpose of performing FDR correction is to reduce the probability of type I error events. When a gene is enriched to a large number of pathways, diseases or functional nodes, even a relatively tightly controlled p-value will lead to a high proportion of type I error events. Therefore, multiple hypothesis testing methods are needed to correct the p-value.

Result

Differentially methylated positions between OA and Normal controls

We measured DNA methylation levels at 742 543 methylation sites in knee cartilage sample from 10 high altitude group OA patients and 6 healthy controls using Illumina 850K methylated chip(Fig.1and2)Clustering heatmap of differential loci. The ordinate is -log10(p-values), and the abscissa is DeltaBeta. Among them, the hypermethylated sites are marked in red, the hypomethylated sites are marked in blue, and the sites with no significant difference are marked in gray. The volcano plot can visually reflect the number, significance and reliability of the difference sites. After quality controland screening procedure In high-altitude osteoarthritis patients and normal controls, 413 methylation differential sites(FDR adjusted P value (P_{adj}) ≤ 0.05; |β-value difference (Δ_β)| ≥ 0.2.) were associated with osteoarthritis, including 92 hypermethylated sites and 321 hypomethylated sites(Fig.3).

GO enrichment analysis of DMG identified 30 significant GO terms for biological process(Fig.4) such as single-organism developmental process (Corrected P-Value= 6.261439E-41), anatomical structure development (Corrected P-Value= 1.868629E-39) and multicellular organismal development (Corrected P-Value= 4.795432E-37). For cellular component we identified 30 significant GO terms such as cell periphery(Corrected P-Value= 2.52904E-22), plasma membrane(Corrected P-Value=

1.19169E-21) and cell projection(Corrected P-Value= 1.03925E-19).For molecular function, we identified 30 significant GO such as ion binding(Corrected P-Value= 1.32513E-12), anion binding(Corrected P-Value= 8.10204E-11) and transmembrane receptor protein kinase activity(Corrected P-Value= 2.19994E-10) IPA network analysis observed rich functional connections among identified DMG.The top 30 significantly enriched GO nodes of the three GO levels and all parent nodes of these nodes were selected to draw the GO functional hierarchical network diagram. The points in the graph represent GO nodes, and the edges represent the relationships between nodes. The first row in each node describes the GO node name, the second row describes the node number in GO, and the third row describes the corrected p-value. The node color indicates the significance of the corrected p-value, with darker colors indicating more significant corrected p-values(Fig.5).The differentially expressed genes were enriched in tyrosine kinase receptor signal transduction pathway, Wnt signaling pathway, extracellular matrix organization pathway, etc(Fig6)

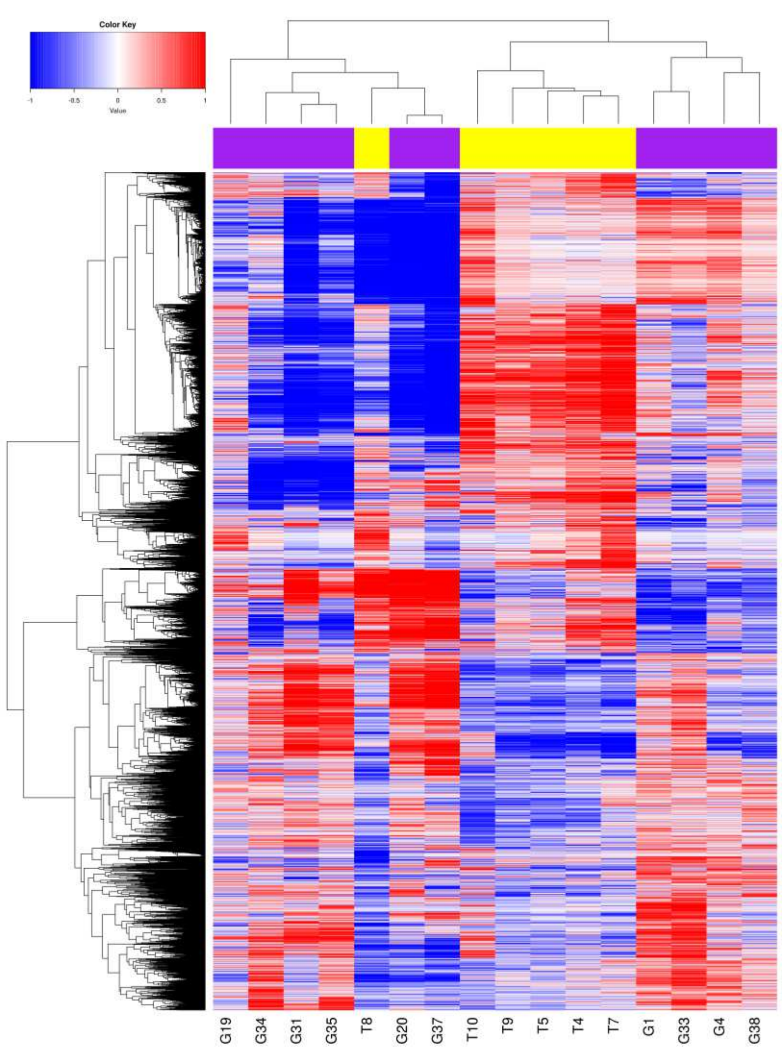


Figure1: Clustering heatmap of differential loci

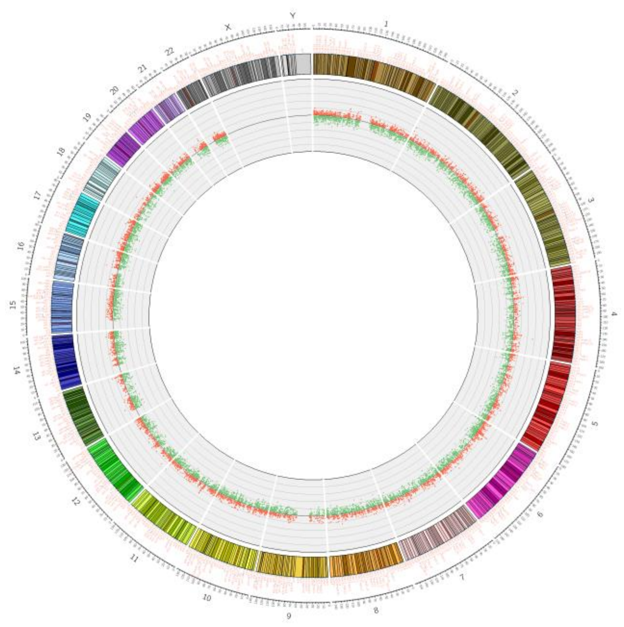


Figure2:circos plot of differential CpG distributionThe colored bands in the outer circles indicate different chromosomes, and the inner circles indicate the degree of difference in the differential CPGS at that position by chromosomal position. Hypermethylated sites are shown in red, and hypomethylated sites are shown in green. The distance of the points in the figure from the dividing line indicates the difference in methylation levels of the sites, with greater distance indicating greater degree of
difference.

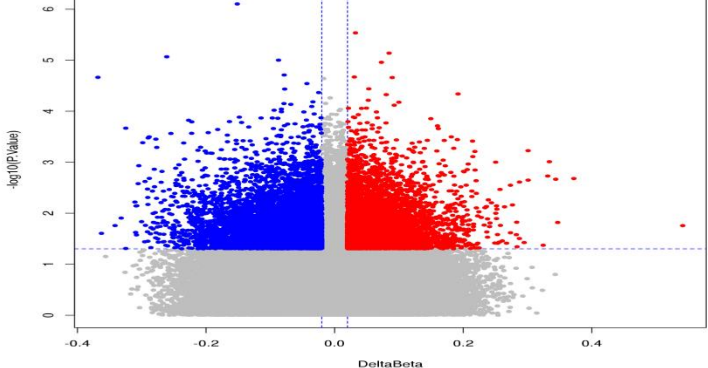


Figure3: Significant CpG sites were defined as: (1) FDR adjusted P value (P_{adj}) ≤ 0.05; (2) |β-value difference (Δ_β)| ≥ 0.2.

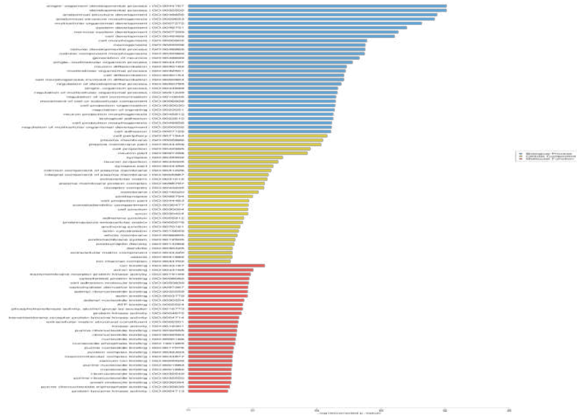
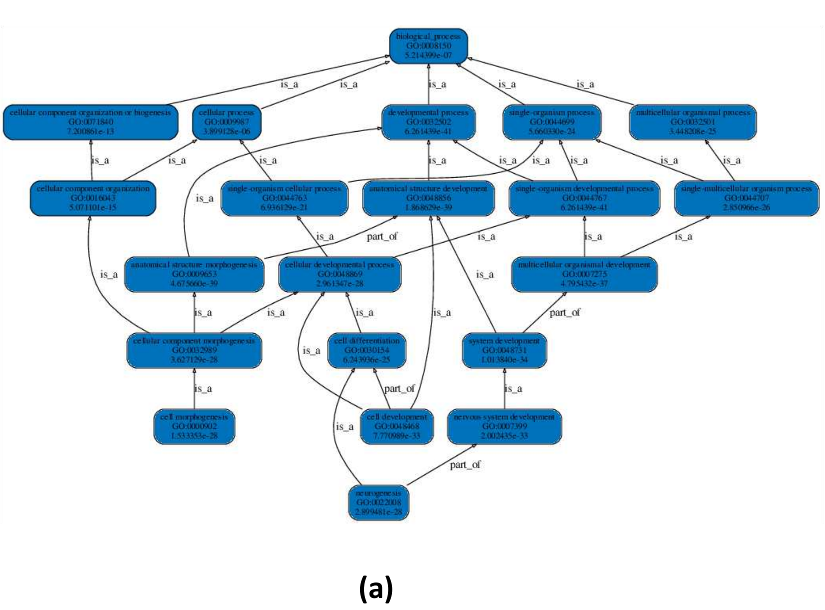
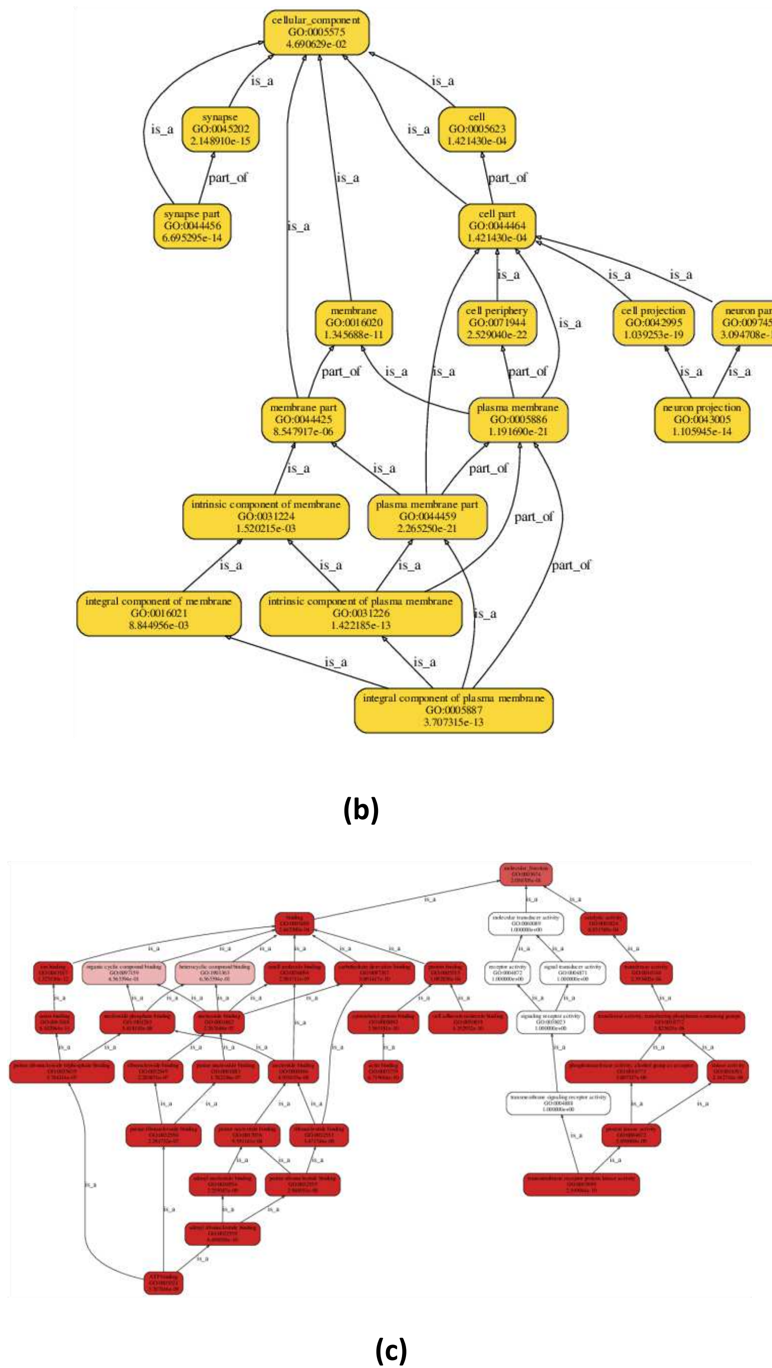


Figure4:GO enrichment analysis of DMG identified 30 significant GO terms for biological process cellular component and molecular function.



(a)



(b)

(c)

Fig5: The top 30 significantly enriched GO nodes of the three GO levels and all the parents of these nodes are plotted as a network of GO functional levels. The points in the graph represent GO nodes, and the edges represent the relationships between nodes. The first row in each node describes the GO node name, the second row describes the node number in GO, and the third row describes the corrected p-value. The node color indicates the significance of the corrected p-value, with darker colors indicating more significant corrected p-values.**a** Biological processes.**b** Cell components.**c** Molecular function.

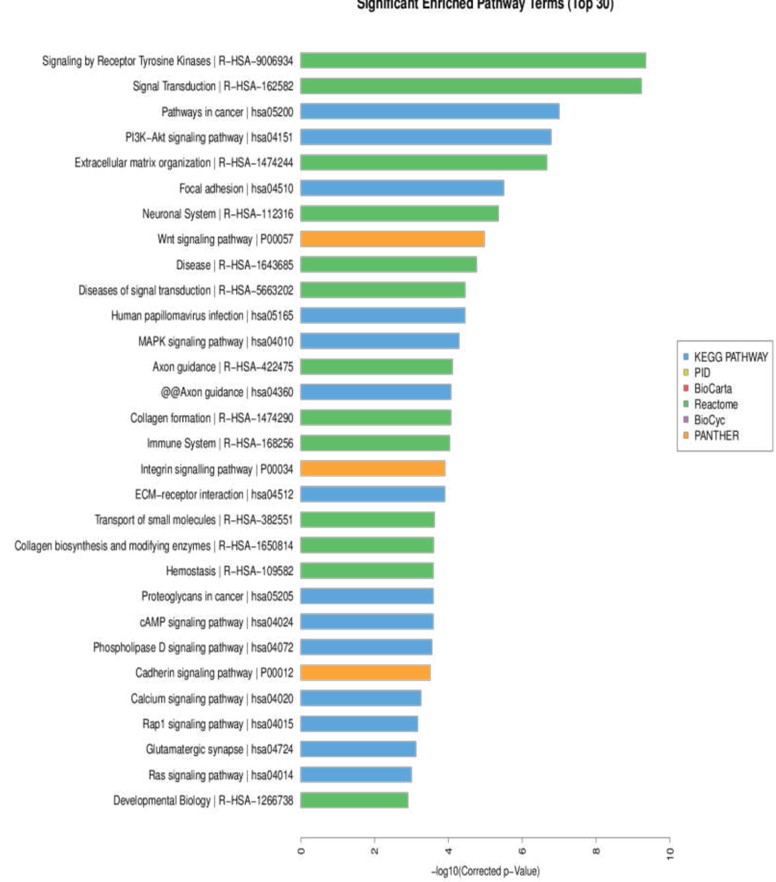


Figure6: Significantly enriched KEGG pathways

Comparative analysis of DNA methylation profiles of Osteoarthritis patients at different altitudes

Significant differences in 413 methylation sites, including 92 hypermethylated sites and 321 hypomethylated sites, were associated with osteoarthritis in both high-altitude osteoarthritis patients and healthy controls. Significant differences in 723 methylation sites, including 436 hypermethylation sites and 297 hypomyelinization sites, were associated with osteoarthritis between patients with low-latitude osteoarthritis and normal controls. There were 5,200 sites of methylation differences among osteoarthritis patients at different altitudes, of which 60 sites were significantly different(Fig7and8). GO enrichment analysis of 60 differential methylation positions identified 30 significant GO terms for biological process, 30 for molecular function and 30 for cellular component(Fig9).The top 30 significantly enriched GO nodes of the three GO levels and all parent nodes of these nodes were selected to draw the GO functional hierarchical network

diagram(Fig10).At the same time, the differential genes were input into the database genes, and the pathways, diseases and functions involved in these gene sets were annotated(Fig11).

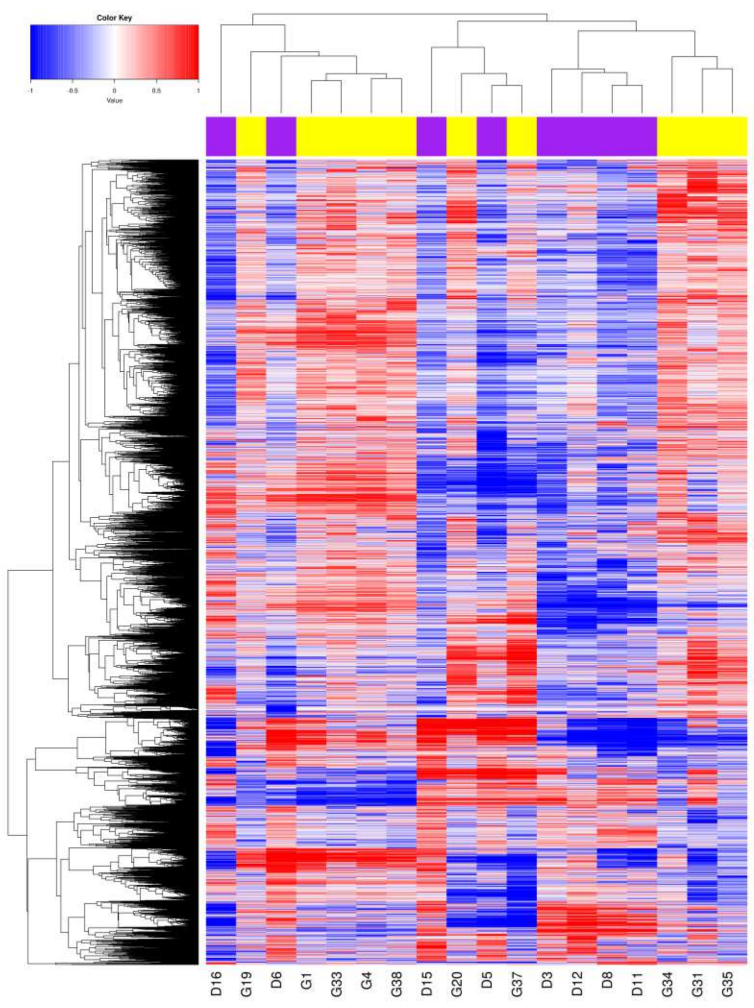


Figure7: Clustering heatmap of differential loci

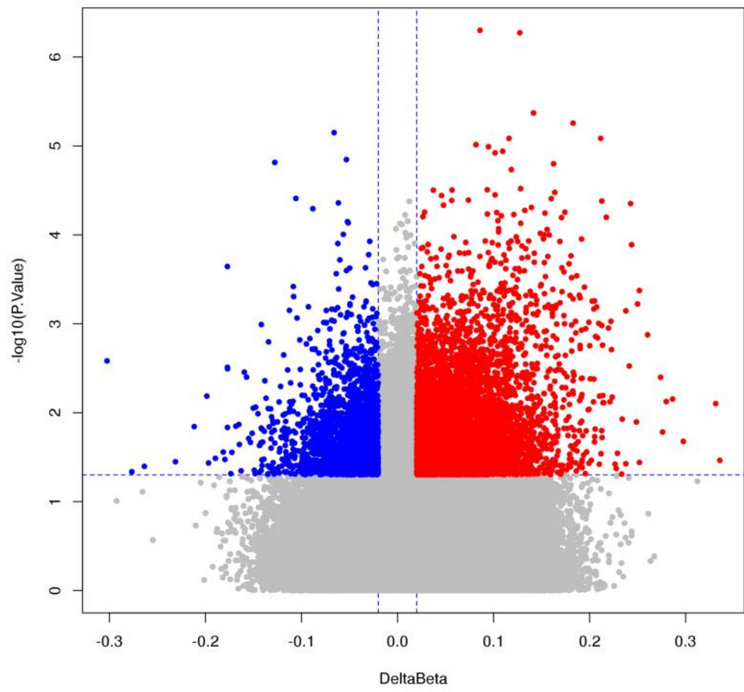


Figure8: Significant CpG sites were defined as: (1) FDR adjusted P value (P_{adj}) ≤ 0.05; (2) |β-value difference (Δ_β)| ≥ 0.2.

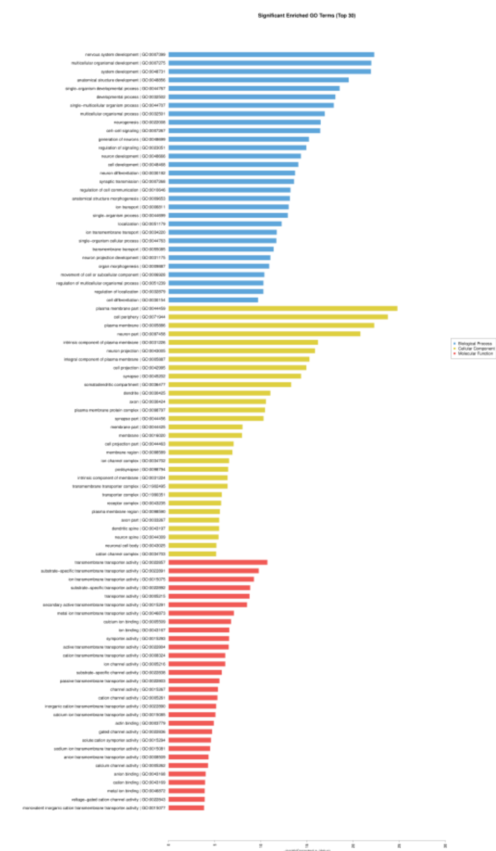
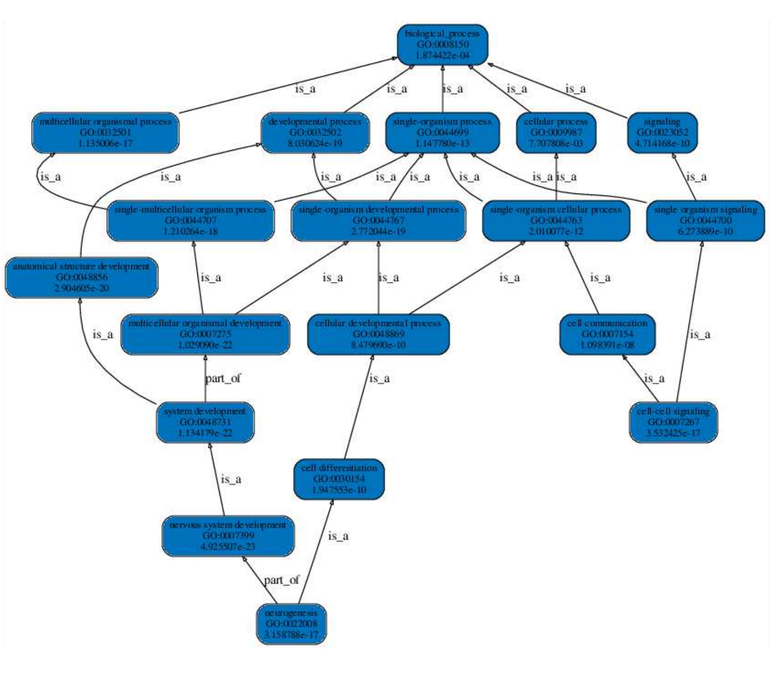
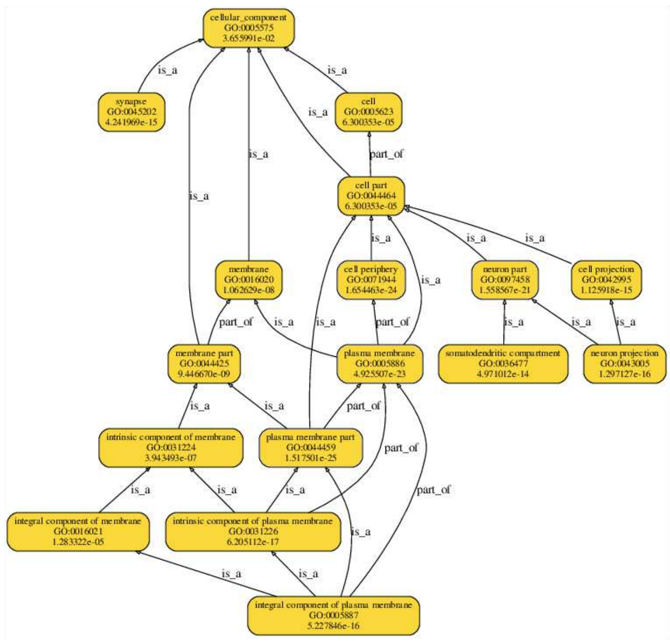


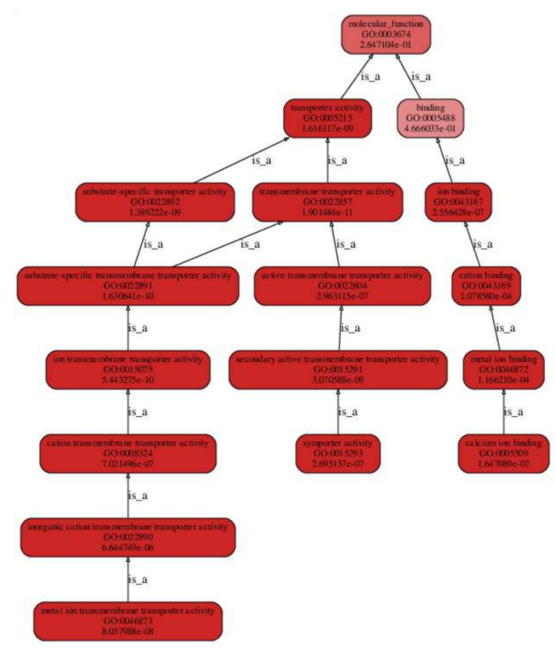
Figure9:GO enrichment analysis of DMG identified 30 significant GO terms for biological process cellular component and molecular function.



(a)



(b)



(c)

Fig10: The top 30 significantly enriched GO nodes of the three GO levels and all the parents of these nodes are plotted as a network of GO functional levels

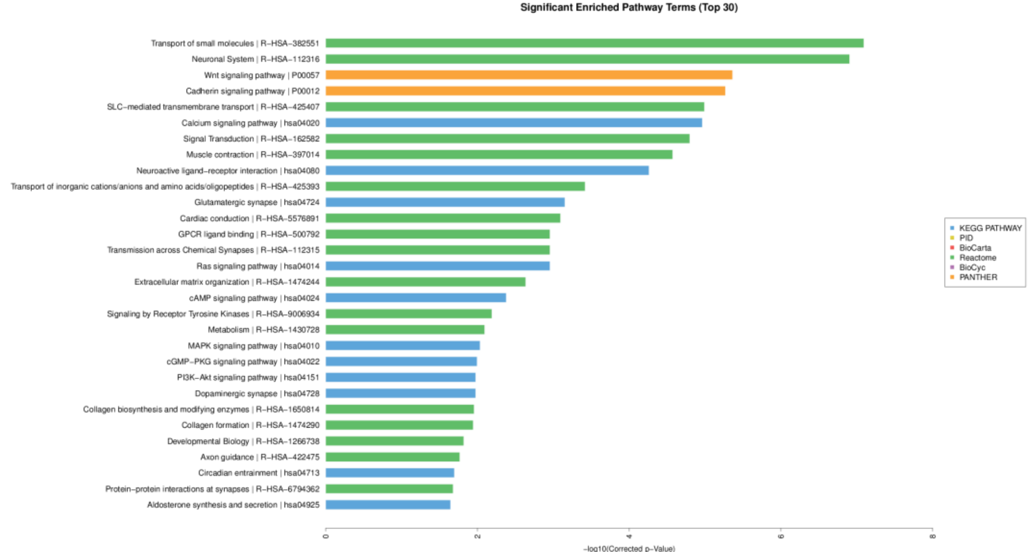


Figure11: Significantly enriched KEGG pathways

Key genes in the pathway were selected for further detection

Among the 30 significant enrichment pathway items, Signaling by Receptor Tyrosine Kinases|R-HAS-9006934, PI3K-Akt signaling pathway|hsa04151, Wnt signaling pathway|P0057. 3 significant enrichment items related to osteoarthritis were selected. Among them, the WNT pathway has attracted our attention. Some studies have confirmed that WNT10A could specifically clear these senescent OA-SMSCs in vitroexperiments and naturally occurring OA models via proapoptotic effects. Mechanistically, WNT10A activatednoncanonical Wnt/calcium signaling in senescent OA-SMSCs, which in turn induced histone deacetylase 5(HDAC5) phosphorylation and nuclear export via its downstream Ca 2+ /calmodulin-dependent protein kinase II(CaMKII) to regulate cell fate. The regulation of this pathway significantly improved the regenerative micro-environment of OA, exhibiting its potential as a novel clinical disease-modifying OA drugs (DMOADs) target[34]. Five genes, CTNNA1, FGF1, GAB1, GSK3B and MTOR with high frequency and high association with osteoarthritis, were selected for further verification(Fig12).

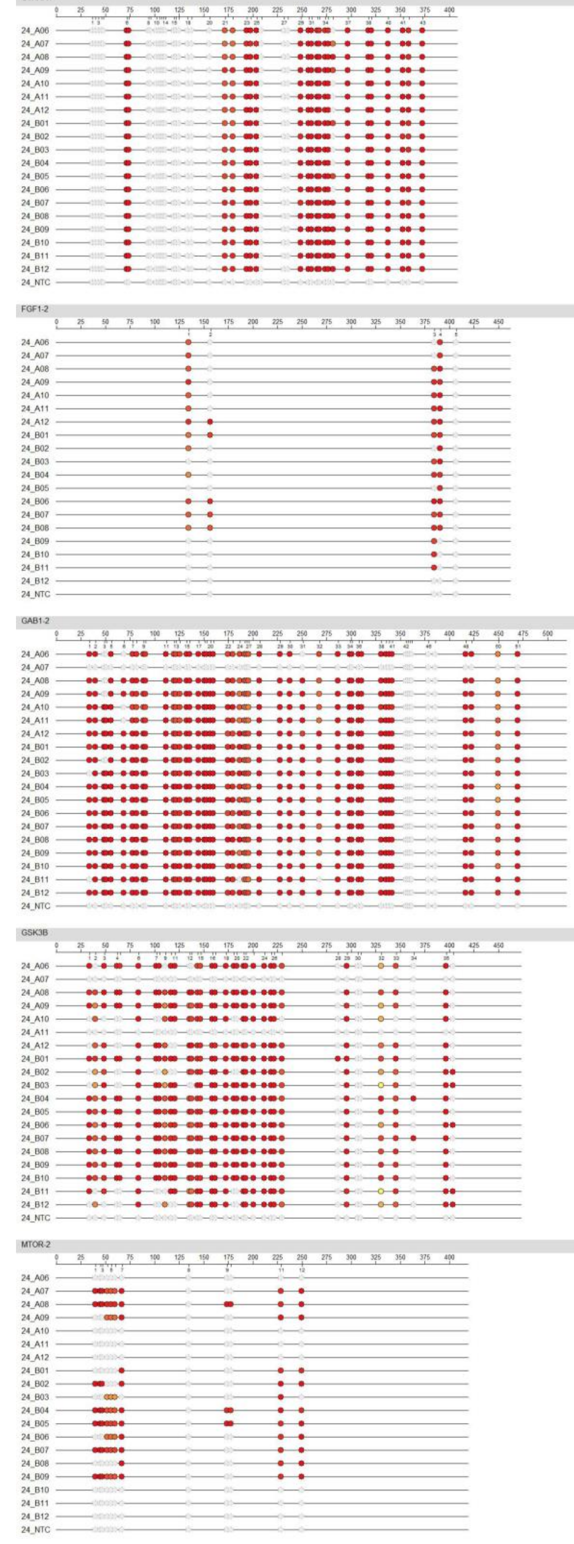


Figure12： DNA fragments were methylated per sample, per CpG Unit.A01-A10

represents high-altitude OA samples, A11-A12 and B01-B06 represent low-altitude OA samples, B07-B12 represents normal control samples, and NTC represents negative control samples.

Discussion

A variety of non-genetic factors have been demonstrated as key contributors to the development of OA[23]. Among many cellular mechanisms responsible for the physiological integration of these environmental signals, epigenetics has emerged as one of the most significant, and has been linked to both development and progression of OA[24]. The canonical epigenetic control mechanisms include cytosine genomic DNA methylation, noncoding RNA, and histone post-translational modifications[25]. DNA methylation can orthotopically prevent the binding of some transcription factors (TFs) to DNA motifs[26] or it can recruit proteins with methyl-binding domains that recognize methylated CpG dinucleotides and regulate transcription initiation[27].
Because osteoarthritis has its own specific variety of highly methylated genes, that is, methylation spectrum and transcription spectrum. At present, some progress has been made in the early diagnosis of osteoarthritis by using DNA methylation analysis and transcriptional expression profile, but there are few clinical markers available. Besides, few data exist regarding the prevalence of OA in plateau in China. Z. Zhang et.al investigated the prevalence of osteoarthritis (OA) in Jiuhe village, which is located about 3.5 km high of Qonggyai County of Tibet and the associated factors and draw the conclusion: The prevalence of osteoarthritis in Jiuhe village, plateau of Tibet was significantly high, which provides relevant basis for our study[28]. This study intends to collect synovial tissue samples from patients with osteoarthritis and normal controls. Illumina 850K methylation chip was used to screen and analyze the differential methylation sites, and the selection of five special genes may be significantly related to the incidence of osteoarthritis in plateau areas. These genes may be used to identify new DNA methylation sites that are important for rheumatoid arthritis, to fully understand the underlying pathologic mechanisms, and to find methylation molecular markers specific for osteoarthritis, and to guide the early diagnosis, risk assessment and better treatment of osteoarthritis.
To determine genome-wide DNA methylation profiles of knee cartilage from patients with osteoarthritis (OA), We measured DNA methylation levels in knee cartilage sample from 10 high altitude group OA patients and 6 healthy controls.
In high-altitude and low-latitude osteoarthritis patients compared with normal controls,

there were 5,200 sites of methylation differences among osteoarthritis patients at different altitudes, of which 60 sites were significantly different. GO enrichment analysis of 60 differential methylation positions identified 30 significant GO terms for biological process, 30 for molecular function and 30 for cellular component, which has not been published before.

In the biological process, genes enriched in Nervous system development are the most, and genes enriched in plasma membrane part in cellular component are the most. The genes enriched in transmembrane transporter activity are the most in molecular function. In the research of Courties A et.al, they suggest that the ANS is involved in joint homeostasis and OA pathogenesis[29]. In the transmembrane transporter, Glutamate may be one of the endogenous autocrine/paracrine factors that play a role in intercellular communications within the bone-related cells[30].

Five genes, CTNNA1, FGF1, GAB1, GSK3B and MTOR with high frequency and high association with osteoarthritis, were selected for further verification.

Manipulation of individual FGF signaling molecule causes or ameliorate articular cartilage degeneration in mouse osteoarthritis models created by Chen L et.al, indicating its critical role in cartilage maintenance, and FGF signaling is a therapeutic target for osteoarthritis[31]. Many studies showed that growth factor receptor binding 2-associated binding protein 1 (GAB1), a member of associated binder family, acted as a key regulator in signal transduction through interacting with cytokine and growth factor receptors[32]. The PI3K/AKT/mTOR signaling pathway plays an essential role in maintaining cell homeostasis[33], and inflammatory factors can activate this pathway, which inhibits autophagy and exacerbates the inflammatory response[34].

Our study explored the correlation between different genetic information and the incidence of osteoarthritis by discovering the expression of different ethnospecific methylation sites, aiming to provide a theoretical basis for the diagnosis and treatment of cartilage degenerative diseases in plateau areas in the future.

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Contributions

L.Z.w. and X.J. was in charge of the design of the work and Z.D. wrote the main manuscript text and R.R. have drafted the work and L.Z.l. and L.G. and G.T. and L.J were responsible for the collection and interpretation of data.

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Ethics declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Qinghai University, and the study protocol number was 2019-ZJ-876. This study was conducted in accordance with the Declaration of Helsinki

Consent to participate

Informed consent was obtained from all the participants prior to the study following presentation of the nature of the procedures.

Competing interests

The authors declare no competing interests.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, X.J., upon reasonable request.

Consent for publication

Not applicable for this study

Reference:

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