Research Article

Identification of Differentially Expressed circRNAs, miRNAs, and Genes in Patients Associated with Cartilaginous Endplate Degeneration

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Background. Intervertebral disc degeneration (IDD) disease is a global challenge because of its predominant pathogenic factor in triggering low back pain, whereas cartilaginous endplate degeneration (CEPD) is the main cause of IDD. Accumulating evidence have indicated that the differentially expressed microRNAs (DEMs) and differentially expressed genes (DEGs) have been determined to be involved in multiple biological processes to mediate CEPD progression. However, the differentially expressed circular RNAs (DECs) and their potential biofunctions in CEPD have not been identified.

Methods. GSE153761 dataset was analyzed using R software to predict DECs, DEMs, and DEGs. Pathway enrichment analysis of DEGs and host genes of DECs and protein-protein interaction network of DEGs were conducted to explore their potential biofunctions. Furthermore, we explore the potential relationship between DEGs and DECs.

Results. There were 74 DECs, 17 DEMs, and 68 DEGs upregulated whereas 50 DECs, 16 DEMs, and 67 DEGs downregulated in CEPD group. Pathway analysis unveiled that these RNAs might regulate CEPD via mediating inflammatory response, ECM metabolism, chondrocytes apoptosis, and chondrocytes growth. A total of 17 overlapping genes were predicted between the host genes of DEGs and DECs, such as SDC1 and MAOA. Moreover, 6 upregulated DECs, of which hsa_circ_0052830 was the most upregulated circRNA in CEPD, were derived from the host genes SDC1, whereas 8 downregulated DECs were derived from the host genes MAOA.

Conclusion. This will provide novel clues for future experimental studies to elucidate the pathomechanism of CEPD and therapeutic targets for CEPD-related diseases.

1. Introduction

Intervertebral disc degeneration (IDD) diseases are a global challenge that contributed to dyskinesia in patients and the increased of socioeconomic burden [1–3]. The intervertebral disc (IVD) is divided into 4 regions: the annulus fibrosus, the transitional zone, the central nucleus pulposus (NP), and up-down cartilaginous endplates (CEP) [4, 5]. IVD is an avascular structure on account of the blood vessels derived from the lumbar artery terminate at the CEP [5]. The CEP acts as an exchange channel, which is responsible for the metabolic waste transportation and nutrient intake of IVD [5–7]. During IDD, the water in NP tissue continually decreases, causing the pyknotic substances deposited in the CEP and gradually calcifies, then the calcified CEP leads to the decrease of CEP permeability [8]. CEP degeneration (CEPD) caused by endplate chondrocyte death and calcification can block this exchange channel, which further aggravates IDD and the failure of endogenous repair, and ultimately forms a vicious cycle [9, 10]. Additionally, the abnormal increase of proinflammatory cytokine secretion and extracellular matrix (ECM) degradation is another characteristic of CEPD [9, 11]. Thus, it is very necessary to find a novel treatment method to mitigate CEPD.

In recent years, noncoding RNAs (ncRNAs) have drawn growing attention because of the role which they play in a spectrum of chronic degenerative diseases of the spine and joints, encompassing IDD [12–16], ankylosing spondylitis...
microRNAs (DEMs) were selected based on the criterion of log_{2} value > 2 and p value < 0.05. The box plot is performed using the R software package ggplot2; the heatmap is performed using the R software package pheatmap. Volcano plot, bubble diagram, and chord plot were performed using the R software package limma [23]. The differentially expressed circRNAs (DECs) and differentially expressed genes (DEGs) were identified using the criterion of |log_{2}(fold – change)| > 2 and p value < 0.05, while the differentially expressed microRNAs (DEM) were selected based on the criterion of |log_{2}(fold – change)| > 1 and p value < 0.05. The box plot is performed using the R software package ggplot2; the heatmap is performed using the R software package heatmap.2. Venn Analysis. The DEGs and the host genes of DECs were intersected to select overlapping genes using Venn plot analysis by Sangerbox tools.

2.1. Analysis of GSE153761 Microarray Dataset. Opening the accession number GSE153761 in GEO database, then these raw data were normalized and log2-transformed using R software package limma [23]. The differentially expressed circRNAs (DECs) and differentially expressed genes (DEGs) were identified using the criterion of |log_{2}(fold – change)| > 2 and p value < 0.05, while the differentially expressed microRNAs (DEM) were selected based on the criterion of |log_{2}(fold – change)| > 1 and p value < 0.05. The box plot is performed using the R software package ggplot2; the heatmap is performed using the R software package heatmap.2. Volcano plot, bubble diagram, and chord plot were performed using the Sangerbox tools, a free online platform for data analysis. All the above analysis methods and R package were performed by R foundation for statistical computing.

2.2. Venn Analysis. The DEGs and the host genes of DECs were intersected to select overlapping genes using Venn plot analysis by Sangerbox tools.

2.3. Pathways Enrichment Analysis. GO, KEGG, and Reactome pathway enrichment analysis were conducted by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) online tools (https://david-d.ncifcrf.gov/) [24]. The analytical results were visualized by bubble and chord plot using Sangerbox tool. A value of p < 0.05 was set as a statistically significant difference.

2.4. PPI Network Construction. The Search Tool for the Retrieval of Interacting Genes (STRING) database was utilized to seek the interactions between multiple proteins [25]. The PPI network was constructed by STRING to predict the potential relationship among those DEGs. Furthermore, those proteins that interact with each other were extracted from the PPI network and visualized by Cytoscape software [26]. The CytoHubba plugin in Cytoscape software was utilized to find the hub genes [27].

2.5. Statistical Analysis. Statistical analysis was conducted by R (version 3.6.2) software. For all data analyses, a value of p < 0.05 was set as a statistically significant difference.

3. Results

3.1. Identification of the DECs, DEMs, and DEGs from GEO Database. GSE153761 microarray dataset was downloaded from GEO database, which included circRNA, miRNA, and gene expression data. The fundamental information of GSE153761 dataset was shown in Table 1. As shown in Figure 1(a), the box plot revealed the distribution trends of the 6 different samples were the same, that is, they are all on the same line, suggesting the data have already been standardized. Then, the R software limma package was utilized to identify DECs, DEMs, and DEGs. The results of analysis unveiled that a total of 88731 circRNAs, 1309 miRNAs, and 18655 genes were identified (Supplementary Table 1). Compared with the expression of circRNAs, miRNAs, and genes in the normal CEP group, a volcano plot reflected that there were 74 DECs, 17 DEMs, and 68 DEGs upregulated whereas 50 DECs, 16 DEMs, and 67 DEGs downregulated in CEPD group with the screening criteria of |log_{2}(fold – change)| > 2 and p value < 0.05 (Figures 1(b)–1(d), Supplementary Table 2). Furthermore, we displayed the top 25 DECs and DEGs as well as the 33 DEMs through heatmap (Figures 1(e)–1(g)), from which we found that the 3 CEPD samples can be remarkably separated from the 3 control samples, suggesting that the results of analysis were trustable.

3.2. Functional Enrichment Analysis of the DEGs. To find the potential etiological factors and key genes that correlated with the pathomechanism of CEPD, the analysis of GO,
Group

GSE153761

Figure 1: Continued.
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KEGG, and reactome enrichment pathways was conducted for DEGs. GO function annotations included terms in the biological process (BP), molecular function (MF), and cellular component (CC) categories. As shown in Figure 2(a), the 19 BP terms were enriched, such as positive regulation of apoptotic cell clearance, inflammatory response, neutrophil chemotaxis, endochondral ossification, angiogenesis, chemokine production, regulation of endothelial cell proliferation, and collagen catabolic process. Extracellular space, extracellular exosome, proteinaceous extracellular matrix, blood microparticle secretory granule, and cell junction were the enriched important CC terms (Figure 2(b)). The MF were predicted to be mainly enriched in the following terms: receptor for advanced glycosylation end products (RAGE) receptor binding, serine-type endopeptidase activity, arachidonic acid binding, endopeptidase inhibitor activity, growth factor activity, and Toll-like receptor 4 binding (Figure 2(c)). KEGG analysis revealed those DEGs were predominantly enriched in the salivary secretion, complement and coagulation cascades, systemic lupus erythematosus, amoebiasis, and staphylococcus aureus infection (Figure 2(d)). Moreover, the reactome pathways in which these genes were enriched included regulation of complement cascade and activation of C3 and C5, alpha-defensins, aefensins, and erythrocytes take up oxygen and release carbon dioxide (Figure 2(e)). It is not hard to see that those DEGs were predicted to participate in inflammatory response, ossification, angiogenesis, ECM catabolic process, cell proliferation, and apoptosis, while these pathological processes were the crucial pathogenesis of CEPD.

3.3. Construction of PPI Network. PPI is an invaluable tool for studying the potential interrelationship among multiple proteins, so we mapped the 135 DEGs into STRING website to analyze the interaction among them. The PPI network stats consisted of 134 nodes and 207 edges, of which 85 nodes were found to be involved in the PPI pairs. Subsequently, these genes with interaction were ranked using betweenness method and visualized via cytoHubba plug-in of Cytoscape software (Figure 3(a)). Among them, haptoglobin (HP), scleraxis (SCX), lactoferrin (LTF), TNFα-stimulated gene-6
Cell adhesion
Extracellular matrix organization
Collagen fibril organization
Collagen catabolic process
Aptotic process
Neutrophil aggregation
Negative regulation of endodermal cell differentiation
Skin development
Blood vessel development
Chemokine production
Cell migration
Protein autophosphorylation
Regulation of cell growth
Leukocyte migration involved in inflammatory response
Activation of cysteine-type endopeptidase activity involved in apoptotic process

Gene count
2
4
6
8
10
12

Gene Ratio
0.00 0.01 0.02 0.03 0.04

(a)
(b)

Figure 2: Continued.
Figure 2: Functional enrichment analysis of the DEGs. (a–c) Most enriched Gene Ontology (GO) terms in biological process (BP), cellular component (CC), and molecular function (MF). The x-axis represents the p value, the left y-axis represents the GO terms, and the right y-axis represents the gene ratio (up) and gene count (down). (d) Bubble diagram exhibited the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis results of the DEGs. (e) The results of reactome pathway analysis of the DEG was shown by bubble diagram.
Figure 3: Continued.
(TNFAIP6), syndecan 1 (SDC1), leptin (LEP), interleukin 18 receptor accessory protein (IL18RAP), cathelicidin antimicrobial peptide (CAMP), apolipoprotein D (APOD), C4b-binding protein α-chain (C4BPA), S100 calcium-binding protein A8 (S100A8), S100A12, S100A9, complement C8 beta chain (C8B), phospholipase A2 group IIA (PLA2G2A), serum amyloid A1 (SAA1), complement C8 alpha chain (C4A), and C4B were associated with inflammatory response, dentin matrix protein 1 (DMP1), asporin (ASPN), growth and differentiation factor-5 (GDF5), metalloproteinase-8 (MMP8), LEP, IL18RAP, SCX, and LTF were associated with ossification, as well as GDF5, MMP8, arginase-1 (ARG1), and A disintegrin and metalloproteinase with thrombospondin motif 14 (ADAMTS14) were associated with ECM metabolism. As shown in Figure 3(b), the top 5 key hub genes were HP, LTF, GDF5, S100A12, and TNFAIP6. Furthermore, the important GO functional analysis results of all DEGs which contained genes not appearing in PPI pairs that associated with cartilage metabolism were displayed through chord plot (Figure 3(c)). The results indicated that collagen type III α 1 chain (COL3A1) is involved in “endochondral ossification,” “collagen catabolic process,” and “extracellular region,”...
as well as fibroblast growth factor 18 (FGF18) is involved in “endochondral ossification,” “angiogenesis,” “regulation of endothelial cell proliferation,” “extracellular region,” and “growth factor activity.”

3.4. Finding the Potential Relationship between DEGs and DECs. Growing evidence has unveiled that circRNAs not only can regulate the expression levels of their host genes in a miRNA-dependent manner [28, 29] but also can mediate host gene alternative splicing or transcription in a miRNA-independent manner [30, 31]. For instance, circ-F-box and WD repeat domain-containing 7 (FBXW7) regulates the mRNA and protein levels of FBXW7 via sponging miR-197-3p [28]. Bai et al. [29] reported that circ-filamin-binding LIM protein 1 (FBLIM1) acts as a ceRNA to positively modulate its host gene Fblim1 expression by repressing miR-346. A circRNA derived from Sepallata3 gene was validated to mediate its alternative splicing through R-loop formation. Li et al. [31] demonstrated that circ-EIF3J and circ-PAIP2 can interact with U1 small nuclear ribonucleoprotein and RNA polymerase II to promote their host gene transcription. To explore whether there was a relationship between DEGs and DECs, Venn analysis was conducted to predict that there were 17 overlapping genes between the host genes of DEGs and DECs, such as SDC1 and monoamine oxidase A (MAOA) (Figure 4(a)). Intriguingly, the DECs, including hsa_circ_0052828, hsa_circ_0052829, hsa_circ_0052830, hsa_circ_0052832, hsa_circ_0052833, and hsa_circ_0052834, were all predicted upregulated and derived from the host genes SDC1, of which hsa_circ_0052830 was predicted to be the most upregulated circRNA in CEPD (Figure 5(b) and Supplementary Table 3). Additionally, the DECs predicted to
Figure 5: Continued.
Figure 5: Continued.
be downregulated in CEPD, such as hsa_circ_0090314, hsa_circ_0090315, hsa_circ_0090316, hsa_circ_0090317, hsa_circ_0090318, hsa_circ_0090319, hsa_circ_0090320, and hsa_circ_0090321, were all derived from the host genes MAOA (Figure 4(b) and Supplementary Table 3). Moreover, 5 upregulated DECs, hsa_circ_0081056, hsa_circ_0081065, hsa_circ_0081091, hsa_circ_0081167, and hsa_circ_0081108, were all derived from COL1A2, a key component of ECM (Figure 4(b) and Supplementary Table 3). Figure 4(c) shown the expression of key DEGs in CEPD group compared with the normal group. For example, GDF5, fibroblast growth factor (FGF18), COL13A1, and SDC1 were upregulated, whereas S100A8, S100A9, MMP8, and MAOA were downregulated in CEPD group.

3.5. Functional Enrichment Analysis of the Host Genes of DECs. circRNAs are derived from precursor (pre)-mRNAs and formed by back-splicing, namely, a downstream 5′ splice site is covalently binded with an upstream 3′ splice site [32]. Moreover, the abundance of circRNAs are negatively correlated with their linear host gene mRNAs because there is a competition between circRNA biogenesis and pre-mRNA splicing [33]. Thus, whether the host genes of DECs are involved in the regulation of IDD is a meaningful issue worth studying. First, the host genes of DECs were displayed in Supplementary Table 3. Subsequently, GO and KEGG pathway enrichment analyses were conducted to predict the potential biological functions of their host genes. GO function annotations included terms in the biological process (BP), molecular function (MF), and cellular component (CC) categories. The bubble diagram shown a total of 15, 12, and 11 important GO terms were significantly enriched in BP, CC, and MF, respectively (Figures 5(a)–5(c)). The BP aspect encompassed cell adhesion, extracellular matrix organization, collagen catabolic process, apoptotic process, neutrophil aggregation, chemokine production, regulation of cell growth, and leukocyte migration involved in inflammatory response (Figure 5(a)). The CC were found to be mostly enriched in the following terms: extracellular matrix, extracellular matrix, proteinaceous extracellular matrix, extracellular exosome, extracellular region, and collagen trimer (Figure 5(b)), whereas the most enriched in MF were extracellular matrix structural constituent, RAGE receptor binding, Toll-like receptor 4 binding, collagen binding, insulin-like growth factor binding, and metalloendoproteinase activity (Figure 5(c)). The results of KEGG pathway analysis uncovered the host genes of DECs were predominantly associated with 5 different signaling pathways, such as ECM-receptor interaction, focal adhesion, PI3K-Akt signaling pathway, protein digestion and absorption, amoebiasis, and platelet activation (Figure 5(d)).

Figure 5: Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of host genes of the DECs. (a–c) Most enriched GO terms in biological process (BP), cellular component (CC), and molecular function (MF). The x-axis represents the p value, the left y-axis represents the GO terms, and the right y-axis represents the gene ratio (up) and gene count (down). (d) Bubble diagram exhibited the KEGG enrichment analysis results of the host genes.
together, these results suggested that the host genes of DECs possibly regulate ECM metabolism, chondrocytes apoptosis, and chondrocytes growth.

4. Discussion

The pathomechanism of CEPD is the results of multifactorial dysregulation, of which chondrocytes apoptosis, CEP calcification, and inflammatory response are the main factors [8, 9, 11]. Increasing evidence indicated that CEPD is the predominant cause of triggering IDD [34–37]. Recently, experimental research displayed that CEPD significantly aggravated IDD progression by promoting inflammatory cytokine expression or apoptosis in NP cells [34, 36]. Luo et al. [36] also observed that the recurrence rate of patients is remarkably higher with IDD with CEP inflammation after surgery. Liu et al. [37] validated that CEP matrix stiffness can promote CEP calcification which had positively correlated with the degree of IDD. Berg-Johansen et al. [35] measured CEP thickness through ultrashort echo-time MRI and reported that CEP thickness variation is associated with IDD. In this study, we displayed the landscape of RNAs in human cervical CEPD and performed an analysis of circRNA, miRNA, and gene expression profiles to identify new RNAs that may exert crucial role in modulating CEPD.

The dysregulation of RNA expression levels is a hallmark pathological characteristic of CEPD. Abundant evidence has uncovered the DEMs exert protective or destructive role in the occurrence and progression of CEPD. As previously verified by Chen and colleagues [38], miR-34a enhanced CEP chondrocytes apoptosis by directly repressing Bcl-2. miR-20a overexpression was demonstrated to enhance CEP calcification by targeting ankylosis progressive homolog [35]. These data suggest that miR-34a and miR-20a positively regulate CEPD. Among the 33 DEMs, several of them had been demonstrated to play a pivotal role in IDD. MiR-675, predicted to be the most upregulated miRNA in our study, was demonstrated to inhibit human chondrocytes apoptosis and ECM degradation [39], implying that its might protect against CEPD. Niu et al. [40] found hyperbaric oxygen treatment can elevate miR-107 to inhibit MMP3/9/13 expression in degenerated NP cells. Yu et al. [41] verified miR-10b promote NP cell proliferation by targeting the HOXD10-RhoC-Akt signaling pathway, and its levels in IDD were correlated with IDD grade. As reported by Wang et al. [42] miR-223 overexpression could repress NF-kB signaling and inflammation response in NP cells by repressing IRAK1. Additionally, miR-431, a direct downstream target of circSEMA4B, could regulate NP cells proliferation, senescence, and ECM degradation via mediating Wnt signaling pathway [43]. Given that the pathogenesis of CEPD is similar to IDD, we guess that these DEMs or other miRNAs whose functions have not been identified might exert important role in mediating CEPD.

To the best of our knowledge, the potential role and mechanism of circRNAs in CEPD have not been identified; our current research was the first to identify the circRNA expression profile in CEPD. Zheng et al. [44] found Hipk3 gene could produce multiple circRNA isoforms, whereas cir-

chIPK3 is the predominant circRNA isoform and could regulate cell growth. In this study, we found Sdc1, Maoa, and Col2a1 gene could produce 6, 8, and 5 DECs, respectively. There was an important feature that Sdc1 and Col2a1 gene-produced DECs are all upregulated, while the DECs derived from Maoa are all downregulated in CEPD. Furthermore, hsa_circ_0052830 derived from Sdc1 is predicted to be the most upregulated DEC in CEPD. Regarding the multiple DECs derived from the same host gene, it is interesting to know which DEC is the most important isoform, or what is the difference between the functions of these DECs, and what role each DEC plays, which need future experiments to elucidate.

This study also identified a series of DEGs that exert crucial roles in regulating CEPD and IDD. LTF was observed to suppress IL-1β-induced apoptosis by activating cAMP responsive element-binding protein 1 in human chondrocytes [45]. FGF18 acts as a chondrocyte protective factor and can stimulate chondrogenesis and cartilage repair, which is regulated by circPDE4D-miR-103a-3p pathway [46, 47]. IL18RAP encodes a proinflammatory cytokine interleukin-18 (IL18) protein; IL18 can activate macrophages to secrete TNF-α and IL-1β, which further increased ECM degradation via upregulating MMPs during IDD [48]. Dolor et al. [49] validated that MMP8 treatment can enhance the transport properties of the human CEP to improve intervertebral disc nutrition. Zhu et al. [50] demonstrated that GDF5 can attenuate IDD in a rat model via promoting ECM synthesis.

Interestingly, SDC1 and MAOA were not only DEGs but also host genes of DECs. Literature reported SDC1 can repress inflammatory cytokine expression in epithelial [51] and epithelial apoptosis [52]. MAOA was also reported to promote tumor cell proliferation [53]. Furthermore, functional enrichment analysis of DEGs and host genes of DECs unveiled there are overlapping terms, such as inflammatory response, regulation of cell proliferation, collagen catabolic process, extracellular matrix, extracellular region, Toll-like receptor 4 binding, and RAGE receptor binding. The above phenomenon suggested that certain DECs might regulate their host gene expression, splicing, or transcription to mediate CEP chondrocytes apoptosis and proliferation as well as ECM metabolism and inflammatory response. Additionally, there is a DECs-DEMs-DEGs ceRNA regulatory network in CEPD, which needs further investigation.

However, several limitations existed in this study. Firstly, we did not conduct RNA sequencing. Secondly, we did not collect normal and degenerated NP tissues for qRT-PCR verification. At last, more molecular experiments, including qRT-PCR, Western blot, luciferase reporter assays, and RNA immunoprecipitation, as well as animal experiments, such as in situ tissue immunohistochemical tests, will be conducted to elucidate the role of key CEPD-related DECs, DEMs, and DEGs in the future.

5. Conclusion

Collectively, we successfully identified CEPD-related DECs, DEMs, and DEGs as well as predicted their potential biofunctions by bioinformatics analysis of the RNA expression
profile. These results provide evidence that DECs, DEMs, and DEGs might have an important role in regulating the pathological process of CEPD. In fact, this is the first paper studying the DECs in CEPD, which contributes to us have a new understanding of the pathogenesis of CEPD. In the future, these information might be useful for guiding further experimental studies and provide novel therapeutic targets for CEPD-related diseases.

Data Availability

The datasets supporting the conclusions of this article are included within the article.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

HX, YL, and JL conceived and designed the research; ZH and GL analyzed the data; LD and LT searched the reference literatures; HX drafted the manuscript; BX reviewed and revised the manuscript. All the authors read and approved the final manuscript. Haiwei Xu, Yongjin Li, Jianhua Li, Zhenxin Huo, and Guowang Li contributed equally to this work.

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Supplementary Materials

Supplementary 1. Supplementary Table 1 Total circRNAs, miRNAs, and genes were identified in GSE153761 dataset.

Supplementary 2. Supplementary Table 2 The DECs, DEMs, and DEGs were identified in GSE153761 dataset.

Supplementary 3. Supplementary Table 3 The host genes of DECs.

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