Analysis of circular RNA expression profile of pathological bone formation in ankylosing spondylitis

Abstract

Objective: To screen and analyze the function of specific CircRNAs involved in pathological bone formation in patients with ankylosing spondylitis (AS).

Methods: From September 2019 to October 2020, hip capsule tissues obtained from 3 patients with AS developed hip joint fusion and 3 patients with femoral neck fracture (FNF) were obtained. The circular RNA expressions of hip capsule were analyzed by Arraystar CircRNA chip. qRT-PCR analysis was performed to identify the expression patterns of differentially expression CircRNAs.

Results: Our findings showed that there were 25 up-regulated and 39 down-regulated differential CircRNAs. Among these CircRNAs, we screened 10 highest up-regulated CircRNAs and 13 lowest down-regulated CircRNAs (Fold Change≥2, P<0.05). In further verification analysis, hsa_circ_0067103, hsa_circ_0004496, and hsa_circ_0002649, ACTG1 were significantly upregulated, while hsa_circ_0020273, hsa_circ_0005699, and hsa_circ_0048764 were markedly downregulated in AS tissues than FNF controls.

Conclusion: The expression of CircRNAs involved of pathological bone formation in AS were significantly different from those of control group. These differentially expressed Circular RNAs may be closely related to the occurrence and development of pathological bone formation in AS.

Ankylosing spondylitis (AS) is an immune-mediated chronic inflammatory disease. AS may develop ankylosis and fibrosis of spinal deformities in the late stage, and even cause serious functional damage. To seek out potential factors that cause abnormal new bone formation in AS, it would help to facilitate early diagnosis, treatment and favorable prognosis of AS. Circular RNA (circular RNA) is a single-stranded or double-stranded non-coding RNA with closed ring structure and it is structurally stable and not easy to degrade. So far, it has been demonstrated that the functions of circRNA include regulating transcription, binding protein, being a micro RNA (miRNA) sponge, protein complex and protein induction and stabilization, which are closely related to the occurrence and development of tumor, cardiovascular, diabetes, rheumatoid arthritis and other diseases. To the best of our knowledge, the expressions and functions of circRNAs involved in pathological bone formation in AS have been investigated in the spinal ligaments but not hip joint capsules. Therefore, this study aims to figure out the circRNA expression profile in AS pathological bone formation.

The hip joint capsules were obtained from 3 male patients with AS who developed hip joint ankylosis and fusion and received total hip arthroplasty. Meanwhile, the hip joint capsules were also obtained from 3 male patients with femoral neck fracture (FNF) were enrolled in the control group (excluding diffuse idiopathic skeletal hyperostosis, osteoarthritis, rheumatoid arthritis, other spondyloarthritis and potential immune system diseases). Five milligram hip joint capsules separated from each specimen in both AS and FNF groups were obtained and stored in liquid nitrogen, and then total RNA was extracted using TRIzol (Life Technologies). Then the RNA quantification and quality was examined by using a Nanodrop ND-1000 spectrophotometer. Total RNAs of the 2 groups were digested with Rnase R (Epicenter, Inc.) to remove linear RNAs and enrich circRNAs, respectively. Then the enriched circRNAs were amplified and transcribed into fluorescent complementary RNA (cRNA) utilizing a random priming method (Arraystar). The labeled cRNAs were hybridized onto the Arraystar Human CircRNA Array (Arraystar). Finally, after washing the slides, the arrays were scanned by Agilent Scanner G2505C. Data are presented as mean± standard deviation. SPSS 20.0 was used for all statistical analyses. CircRNA expression profiles in hip joint capsule tissues between the AS and control groups were analyzed using paired t test. P<0.05 was considered statistically significant.

The aberrantly expressed circRNAs were analyzed by hierarchical cluster analysis, and the related scatter diagram was drawn. Red represents relatively highly expressed circRNAs, and green indicates low-expressed circRNA. There were 25 up-regulated and 39 down-regulated differential circRNAs. Among these circRNAs, 10...
up-regulated circRNAs and 13 down-regulated circRNAs with fold change ≥2 were screened (Figure 1).

The quantitative real-time polymerase chain reaction in 10 pairs of AS and FNF hip joint samples to identify the expression of potential circRNAs was next performed. Our findings demonstrated that several indicated circRNAs including hsa_circ_0067103, hsa_circ_0004496, and hsa_circ_0002649 were significantly up-regulated ($P<0.05$; Figure 2A-C), while hsa_circ_0020273, hsa_circ_0005699, and hsa_circ_0048764 were markedly down-regulated ($P<0.05$; Figure 2D,E). The level of the distinguished circRNAs showed similar tendencies as indicated in previous screening. However, no significant changes of hsa_circ_007874 and hsa_circ_0089153 were observed between the AS and FNF hip capsule joint samples (Figure 2G,H).

1 | DISCUSSION

AS is a rheumatic and immune disease, and its pathogenesis is related to the interaction between genetic regulation, interaction of molecules and signal pathways. Any single gene protein or signal pathway is difficult to explain this complex syndrome in AS. The current diagnostic methods of AS pathological bone formation are not specific. Moreover, the effect of drug treatment remains debatable.
Hence, there is an urgent need for a sensitive and specific diagnostic index to deepen our understanding of the occurrence, development and pathological bone formation of AS, and it also can provide potential targets for the development of novel diagnostic and therapeutic strategies against AS.

The current study aimed to screen and analyze the function of specific circRNAs involved in pathological bone formation in patients with AS. It was shown that there were 25 up-regulated and 39 down-regulated differential circRNAs. Among these circRNAs, 10 highest up-regulated circRNAs and 13 lowest down-regulated circRNAs were screened.

So far, limited studies have been conducted on the interaction between circRNA and miRNA in AS. However, some studies have shown that circRNA is related to inflammation and the expression of osteoblasts and osteoclasts in rheumatic immune system diseases, such as osteoarthritis and osteoporosis.\(^7\) Has-circ-0005105 competitively binds with miR-26a in chondrocytes and up-regulates the target gene NMPT and enhances the expression of osteogenesis-related genes in bone marrow mesenchymal stem cells and enhance alkaline phosphatase (ALP) activity.\(^8\) CircRNA-0048211/miRNA-3-5p/BMP2 axis plays a role in the progression of postmenopausal osteoporosis. Its overexpression can significantly up-regulate osteogenesis-related genes in bone marrow mesenchymal stem cells and enhance alkaline phosphatase (ALP) activity and mineralization ability.\(^9\) CircRNA-33287 may increase the expression of Runx3, Runx2, osterix and ALP through competitive binding with MIR-214-3p.\(^10\) Therefore, it was speculated that the differences in circRNAs may be related to the

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**FIGURE 2** Circular RNAs (circRNAs) are differently expressed in 10 pairs of ankylosing spondylitis (AS) and femoral neck fracture (FNF) hip capsule joint samples. Bar chart shows the expression levels of specific circRNAs in necrotic bone of AS patients \((n = 10)\) compared with FNF control group patients \((n = 10)\). The expression of hsa_circ_0067103 (A), hsa_circ_0004496 (B), hsa_circ_0002649 (C) hsa_circ_0020273 (D), hsa_circ_0005699 (E), and hsa_circ_0048764 (F) were found to be significantly different \((^*P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001 \text{ for the comparison indicated by Mann–Whitney U test})\). No significant changes of hsa_circ_0007874 (G) and hsa_circ_0089153 (H) were observed between the AS and FNF groups \((^*P > 0.05)\). Potential gene ACTG1(I) modulated by specific circRNAs in hip joint capsule samples of AS patients. Scatter plots reveals gene expression levels in hip joint capsule tissue of AS patients \((n = 10)\) in comparison to the FNF control group \((n = 10)\). It is demonstrated that ACTG1 was up-regulated in AS compared with FNF \((^{***}P < 0.001 \text{ for the comparison indicated by Mann–Whitney U test})\).
initiation and progression of inflammation in AS and local bone destruction and fibrosis formation.

There are some limitations that should be taken into account. First, this is a preliminary study of circRNA expressions in hip joint capsule in AS patients. Since the pathogenesis of AS is attributed to the complex interaction of genetic, environmental and immune factors as mentioned above, hip capsule tissue selection alone was not able to fully explain the whole problem of AS pathological bone formation; further intensive investigations are needed with regard to the identification of whether screening circRNAs are deregulated in other tissues including sacroiliac joint or spinal facet joint. Second, only 3 samples in the AS and control groups were collected; a larger sample may reveal much more information in the future.

KEYWORDS
ankylosing spondylitis, circRNA, gene chip, pathological bone formation

AUTHOR CONTRIBUTIONS
ZYC, WJ and CZ: sample collection; experiment work; data analyze; and write the manuscript. WJ and LZR: experiment work and bioinformatics design. ZYC: research design, manuscript review and project administration. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT
The authors declare they have no competing interests.

ETHICS APPROVAL
The present study was approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University.

INFORMED CONSENT
Written informed consent was obtained.