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Therapeutic effects of acupuncture in rheumatoid arthritis are associated with centromere protein F expression

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Abstract
Rheumatoid arthritis is a globally common autoimmune inflammatory disease found especially in China. Acupuncture (AP), a traditional Chinese medicine (TCM) treatment method, is commonly used for treating rheumatoid arthritis. Many studies have demonstrated that acupuncture alone or in combination with other treatments is beneficial to treat clinical situation of rheumatoid arthritis, thus improving function and quality of life. In this study, we found that *centromere protein F* (*CENPF*) is a key gene in rheumatoid arthritis with acupuncture treatment by using differentially expressed genes (DEGs) and random forest model analysis of GSE57983 and GSE77298. Acupuncture helps to up-regulate the expression of CENPF in tissues in rheumatoid arthritis. Functionally, overexpression of CENPF inhibits monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, and Interleukin (IL)-6 expressions whereas deficiency of CENPF facilitates MCP-1, TNF-α, and IL-6 expressions in a collagen-induced arthritis (CIA) rat model. Furthermore, knocked down CENPF with acupuncture treatment antagonizes the inhibition of MCP-1, TNF-α, and IL-6 expressions in a CIA rat model. CENPF could be a crucial biomarker in regulating function of acupuncture in treating rheumatoid arthritis.
Objective: The objective of this study is to study the critical role of CENPF in regulation of rheumatoid arthritis with acupuncture treatment.
Methods: PCA was used to analyze the different expression genes between AP treatment group and control group. Volcano plot and random forest model were used to analyze the decreased and increased expression genes. RT-qPCR and IF were used to measure the expression of CENPF in CIA model rat with or without AP treatment. The expression of MCP-1, TNF-α

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and IL-6 was measured by western blotting. The pathology character and arthritis index were used to analyze the severity of joint injury.
Results: PCA data showed that the expression of genes was different between AP treatment group and control group from GEO datasets. Volcano plot and random forest model analysis indicated that CENPF is the most significantly increased expression gene after AP treatment. RT-qPCR and IF assay showed that CENPF is reduced expression in CIA model rat, while CENPF is upregulated expression in CIA model rat with AP treatment. Furthermore, overexpression of CENPF reduced the increasing of MCP-1, TNF-α and IL-6 in CIA model rat. On the contrary, CENPF deficiency induced the expression of MCP-1, TNF-α and IL-6 in CIA model rat. Additionally, the expression of MCP-1, TNF-α and IL-6 in CIA model rat was suppressed, whereas knockdown of CENPF antagonized the decrease of MCP-1, TNF-α and IL-6 in CIA model rat with AP treatment.
Conclusions: CENPF may be a key gene in regulation of the therapeutic effects of acupuncture in rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic, autoimmune inflammatory and debilitating disease that mainly affects the joints, but it should also be regarded as a type that includes extra-articular manifestations (such as rheumatoid nodules, lung involvement, or vascular inflammation) and systemic comorbid syndrome.[1] The long-term prognosis of rheumatoid arthritis is associated with significant morbidity, decreased quality of life, shortened life expectancy, and increased mortality.[2] The current rheumatic treatments include traditional anti-rheumatic drugs (such as leflunomide, methotrexate, etc.), biological agents, small-molecule drugs (such as tofacitinib), and glucocorticoids. These therapies mainly focus on suppressing immunity and inflammation, and most of them have limited efficacy with many adverse reactions.[3] Therefore, studying the pathogenesis and exploring more reasonable treatment strategies are of great significance for the diagnosis and treatment of rheumatoid arthritis.

Acupuncture (AP) and moxibustion in TCM with a wide range of theoretical and clinical presence have a history of more than 2,500 years.[4] In modern clinical applications, acupuncture also represents a potentially valuable strategic adjuvant therapy to relieve pain.[5] Chronic pain being the most common reason for seeking acupuncture treatment, more than 3 million American adults use this therapy.[6] Acupuncture is effective in inflammation caused by tissue injuries, and more and more evidence supports that it can be used to treat various inflammation types. Acupuncture is also effective for systemic inflammation, and inhibiting sepsis by activating the vagus nerve, leading to the production of dopamine in the adrenal medulla.[7] Acupuncture has advantages of treating rheumatoid arthritis by not only significantly reducing joint pain but also improving the patient's overall score and long-term prognosis.[8] A number of studies have confirmed that the therapeutic effect of acupuncture on rheumatoid arthritis is to reduce pro-inflammatory cytokines, such as Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) in serum and joints, and increase anti-inflammatory cytokines, including IL-4 and IL-10.[9] However, the specific mechanism of acupuncture to reduce inflammation in rheumatoid arthritis is not fully understood.

In this study, we proposed to explore the critical role of CENPF in regulating rheumatoid arthritis with acupuncture treatment. By analyzing Gene Expression Omnibus (GEO) series number (GSE)57983 and GSE77298 chips in the GEO database, we found that the level of some transcriptomes in the synovium and muscles of rheumatoid arthritis rats after acupuncture stimulation was related to disease risk, especially CENPF. The expression of CENPF was significantly increased after acupuncture treatment. It has been reported that CENPF is related to the pathogenesis of rheumatoid arthritis,[10] and the level of CENPF is also increased in rheumatoid arthritis if treated with TNF inhibitors.[11] Therefore, we investigated the effects of CENPF on rheumatoid arthritis with acupuncture treatment.

Materials and methods

Principal component analysis, volcano plot, and random forest model analysis

Principal component analysis (PCA) was used as described by Pennathur et al.[12] GEO dataset GSE57983 and GSE77298 was analyzed by R package (Limma analysis) after batching correction process. Data between acupuncture treatment group and control group of rheumatoid arthritis were demonstrated with PCA. For volcano plot (1), the vertical lines correspond to 2 times change of DEGs. The horizontal line indicated P = 0.05. Red dots indicated significantly increased group, and blue dots indicated significantly decreased group. According to a Venn diagram, 14 differentially up-regulated genes and 16 down-regulated genes were obtained by intersection of differentially regulated genes. Random forest model was constructed to analyze the genes associated with clinical manifestations (2).

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol reagent (Ambion, CA, USA). A total of 1-µg RNA was reverse-transcribed into complementary DNA (cDNA) using the ImProm-IITM Reverse Transcription System (Promega, WI, USA). Real-time

RT-qPCR was performed using SYBR GREEN qPCR Super Mix (Invitrogen, CA, USA) and processed by QuantStudio 3. Primers were listed as follows:

CENPF forward: 5′-CTCTCCCGTCAACAGCGTTC-3′;

CENPF reverse: 5′-GTTGTGCATATTCTTGGCTTGC-5′;

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5′-AGACAGCCGCATCTTCTTGT-5′;

GAPDH reverse: 5′-CTTGCCGTGGGTAGAGTCAT-5′.

Immunofluorescence assay

The immunofluorescence assay was performed as described by Dong et al..[13] Cells were washed, fixed, permeabilized, blocked, and incubated with anti-CENPF antibody and Alexa Fluor goat anti-mouse IgG(H+L) antibody, and images were visualized under confocal microscope. Staining results were measured by fluorescent microscope.

Western blotting assay

Western blotting was performed as described by Yu et al.[14] Cells were washed with phosphate buffer solution (PBS) and proteins were isolated with radioimmunoprecipitation assay (RIPA) buffer and phenylmethylsulfonyl fluoride (PMSF). Total proteins were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred with polyvinylidene fluoride (PVDF), and incubated with the corresponding primary antibodies: anti-rabbit CENPF (1:1,000, ab5; abcam, England), anti-mouse MCP-1 (1:1,000, sc-32771; Santa, USA), anti-rabbit TNF-α (1:1,000, ab183218; abcam, USA), anti-mouse IL-6 (1:1,000, sc-130326; Santa), and anti-mouse GAPDH (1:1,000, sc-47724; Santa). The PVDF membranes were washed, and secondary antibodies were applied in ratio of 1:5,000 for 1 h at room temperature. Immunoreactions were visualized with enhanced chemiluminescent (ECL) reagent.

Collagen-induced arthritis (CIA) models

The CIA rats model was constructed with 4-mg/mL bovine type II collagen. On day 0, 0.1 mL of emulsion was injected subcutaneously in mouse's back and base of the tail. On 21st day, a booster injection of bovine type II collagen and incomplete Freund's adjuvant (IFA) emulsion was injected into the tail. On every 5th day, CIA was evaluated by visually scoring the paws, including adjacent areas. In addition, digital calipers were used to measure ankle swelling. The score for each paw ranges from 0 to 4, as follows: 0 = normal; 1 = erythema, mild swelling; 2 = erythema and swelling, extending to the ankle joint, and first and second toes; 3 = erythema and swelling extending to the metatarsal joint and beyond two toes; and 4 = ankylosing deformity with joint swelling. The scores of each paw were added to get a cumulative score between 0 and 16. Ethical approval was obtained from the Ethics Committee of Shaanxi University of Traditional Chinese Medicine.

Ad-CENPF and Ad-shCENPF treatment assay

Ad-CENPF (adenovirus) and Ad-shCENPF were purchased from Sigma (Bochum, Germany). Mice were injected subcutaneously in joints with indicated Ads. Mice were randomly assigned to the following seven groups: Control, CIA model, Ad-CENPF treatment group, CIA+Ad-CENPF overexpression treatment group, CIA+Ad-shCENPF treatment group, CIA+acupuncture treatment group, and CIA+combined acupuncture and AAV-shCENPF treatment group.

Hematoxylin and eosin (H&E) staining assay

H&E staining assay was performed as described by Yu et al.[15] The CIA rat model was fed for 8 weeks, and then sacrificed. Their arthritis tissues were collected, fixed, and sectioned. The sections were stained with H&E, and examined for histological changes under light microscope.

Statistical analysis

Statistical analyses were performed using One-way ANOVA and Student's *t*-test. Data were presented as mean values ± standard error of mean (SEM) of at least three independent experiments. $P \leq 0.05$ were considered as significant.

Results

Analysis of differential expression genes (DEGs) in rheumatoid arthritis model with acupuncture treatment based on GEO database

In order to identify crucial molecules in regulation of rheumatoid arthritis, we first performed de-batch correction and normalization of GEO data to make it easy for comparison. Then PCA was used to compare differences between acupuncture treatment group and control group. As shown in Figure 1A, there was remarkable difference between acupuncture treatment group and control group by PCA of GSE57983. Similarly, we also found differences between RA group and healthy control group by PCA of GSE77298 (Figure 1B). Next, we analyzed DEGs in GSE57983 and GSE77298 by Volcano plot. As shown in Figure 2A, 14 differentially up-regulated genes (red) and 16 down-regulated genes (blue) were screened in these two GEO datasets. We used random forest model to analyze these DEGs, and *CENPF* gene (NM_001100827) was found to play the most significant role in clinical features (Figures 2B and C).

Collectively, the data suggested that CENPF plays a critical role in acupuncture for treating rheumatoid arthritis.

Acupuncture treatment increases CENPF expression in rheumatoid arthritis model

Based on the result of bioinformatic analysis, we discovered that CENPF plays an important role in acupuncture treatment of rheumatoid arthritis. Therefore, we first analyzed the expression of CENPF in both acupuncture treatment group and untreated group of rheumatoid arthritis. As shown in Figure 3A, the expression of CENPF was significantly up-regulated in acupuncture treatment group compared with acupuncture untreated group of rheumatoid arthritis in GSE57983, while there was no difference between rheumatoid arthritis group and HC (health control)

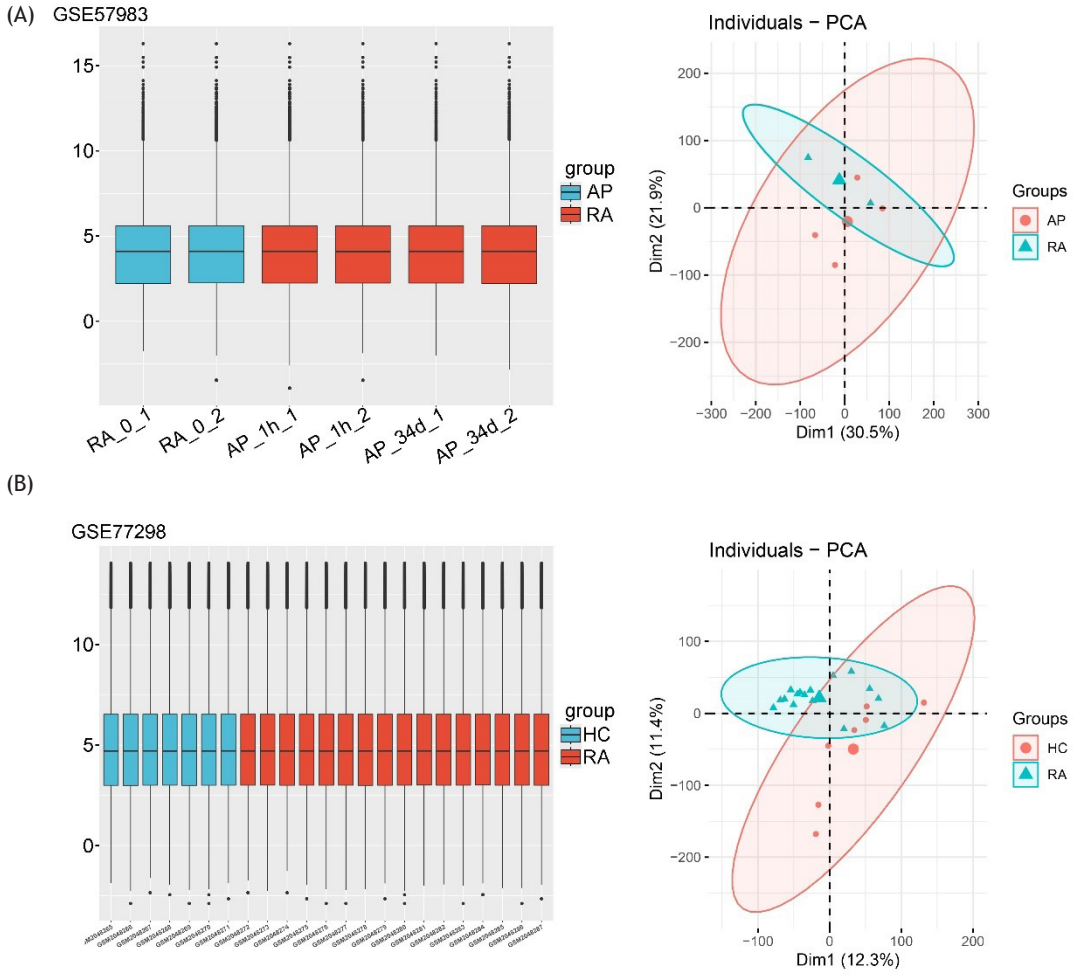

Figure 1 GEO chip data normalization correction process. (A) Principal component analysis (PCA) of GSE57983 was performed to analyze differences between acupuncture treatment group and control group. (B) PCA of GSE77298 was performed to analyze differences between rheumatoid arthritis treatment group and control group.

group in GSE77298. In addition to GEO dataset analysis of CENPF expression, we further analyzed the messenger RNA (mRNA) expression of CENPF in CIA model. As shown in Figure 3B, CENPF decreased in CIA rat model tissues compared with normal rat tissues, while CIA rat model with acupuncture treatment abolished the inhibition of CENPF compared with untreated CIA rat model, which was confirmed by IF analysis (Figure 3C). Taken together, our data suggested that acupuncture treatment positively regulated CENPF expression.

Up-regulating CENPF to alleviate joint damage in rheumatoid arthritis rat model

In order to clarify the function of CENPF in joint injury, we constructed a CIA rat model and injected it with Ad-CENPF. Generally, pro-inflammatory cytokines, including MCP-1, TNF-α, and IL-6, were up-regulated in CIA rat model, and the expressions of MCP-1, TNF-α, and IL-6 were increased but the expression of CENPF was reduced in this model tissues. However, there was no difference in the expressions of MCP-1, TNF-α, and IL-6 in Ad-CENPF treatment group and Ad-NC (negative control) group. Moreover, the increased expressions of MCP-1, TNF-α, and IL-6 were inhibited in the tissues of CIA rat model with Ad-CENPF treatment (Figure 4A).

Next, we used arthritis index to measure the severity of arthritis. As shown in Figure 4B, there were no macroscopic signs of arthritis in control group with Ad-NC or Ad-CENPF treatment. The arthritis index was 12 in CIA rat model with AD-NC treatment whereas after treated with Ad-CENPF in CIA model, the arthritis index was 7, and CENPF overexpression reduced the injury of arthritis. In addition, we used H&E staining to analyze the pathological condition of knee joint injury. As shown in Figure 4C, the joint tissue of control group was normal with about one to three layers of neatly arranged synovial cells. In CIA group, the synovial cells of the joints proliferated excessively, accompanied by high levels of lymphocyte infiltration, abundant pannus formation, and obvious cartilage erosion. After Ad-CENPF treatment, the pathological performance of CIA group improved significantly, with decreased pathological

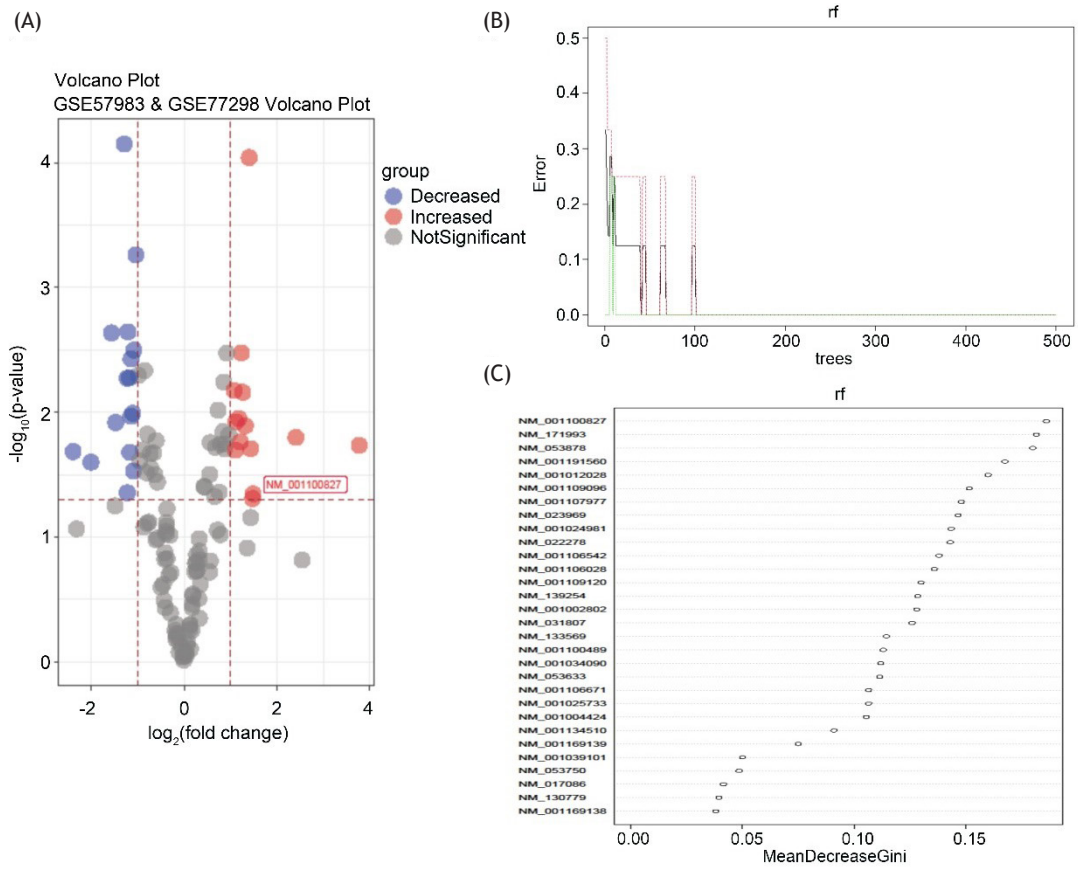

Figure 2 Analysis of differential genes (DEGs) in rheumatoid arthritis model under acupuncture treatment based on GEO database. (A) Volcano plot was used to measure DEGs between acupuncture treatment group and control group in GSE57983 and GSE77298. Blue: decreased expression genes; red: increased expression genes; gray: no significant change expression genes. (B) and (C) Random forest analysis of decreased and increased DEGs in GSE57983 and GSE77298.

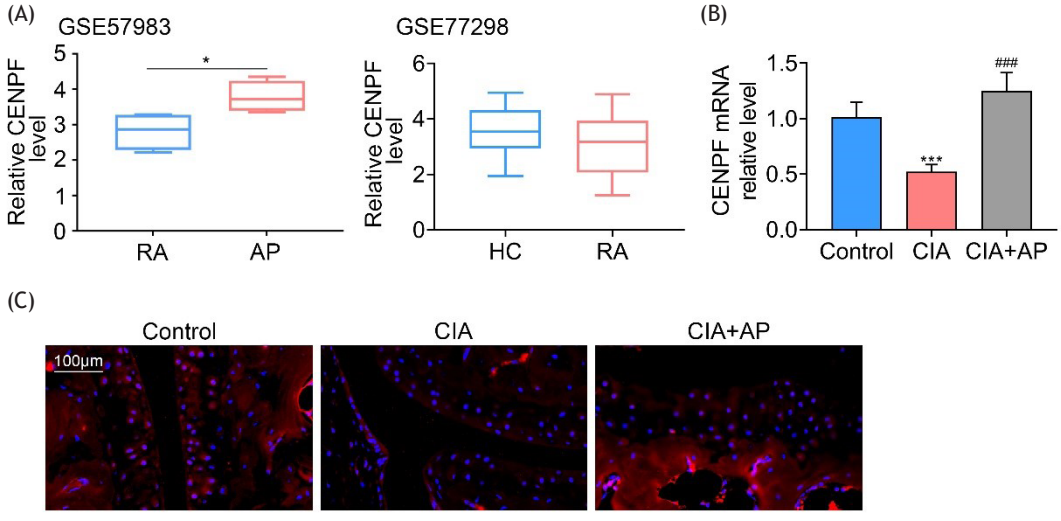

Figure 3 Acupuncture treatment increases CENPF expression in rheumatoid arthritis model. (A) GEO dataset analysis of the expression of CENPF in rheumatoid arthritis model with acupuncture treatment. (B) Expression of CENPF mRNA in CIA model with electroacupuncture stimulation was analyzed by RT-qPCR. (C) Immunofluorescence assay was used to measure the expression of CENPF in the indicated treatment. *P < 0.05, ***P < 0.001. ###P < 0.001.*Compared with control group; #compared with CIA group.

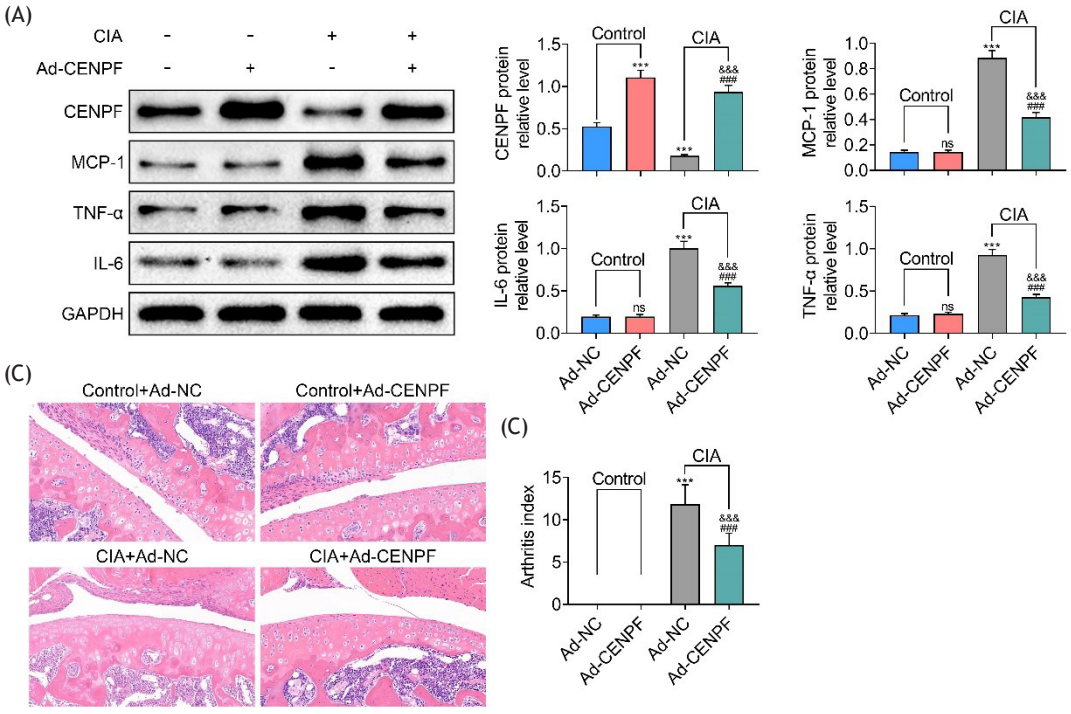

Figure 4 Up-regulating CENPF to alleviate joint damage in rheumatoid arthritis rat model. (A) Levels of CENPF, MCP-1, TNF-α, and IL-6 in arthrosis tissues with the indicated treatment were detected by Western blotting assay. (B) The severity of rheumatoid arthritis was measured by arthritis index. (C) H&E staining was used to analyze the pathology of arthrosis tissues with the indicated treatment. ***P < 0.001, &&&P < 0.001, ###P < 0.001. *Compared with control Ad-NC group; #compared with control Ad-CENPF group; &compared with CIA Ad-NC group.

score. However, Ad-CENPF had no obvious effect on control group.

Collectively, our data suggested that CENPF overexpression released injury of the joint.

Knockdown of CENPF weakens the therapeutic effect of acupuncture on rheumatoid arthritis

Additionally, we constructed a CIA rat model treated with acupuncture or/and injected with Ad-shCENPF. As shown in Figure 5A, Ad-shCENPF treatment further increased expressions of MCP-1, TNF-α, and IL-6 in CIA rat model. Besides, CENPF was up-regulated whereas MCP-1, TNF-α, and IL-6 were lowly expressed in acupuncture treatment CIA model compared with control CIA model. However, inhibited expressions of MCP-1, TNF-α, and IL-6 were alleviated in acupuncture treatment CIA model with Ad-shCENPF injection.

Next, we also used arthritis index to measure the severity of arthritis. As shown in Figure 5B, compared with control group, the arthritis index was significant in all four indicated treatment groups. Among these, deficiency of CENPF increased arthritis index in CIA model, but acupuncture treatment decreased arthritis index in CIA model, and arthritis index of acupuncture and Ad-shCENPF treatment CIA group was between arthritis indices of two single treatments. Pathologically, deficiency of CENPF induced viability of the synovial cells of joints in acupuncture treatment CIA group compared with Ad-shNC group.

Taken together, our data indicated that deficiency of CENPF attenuated the therapeutic effect of acupuncture on rheumatoid arthritis.

Discussion

In Chinese medicine, rheumatoid arthritis is named as the category of “arthralgia” that has the characters of meridian deficiency and stagnation, accompanied with diverse pathological changes, including phlegm, dampness, and stasis blocking the meridians. With prolongation of rheumatoid arthritis, the disability rate of patients increases, which seriously affects their quality of life. Over the past two decades, Chinese medicine, as a traditional method of therapy, is commonly used for treating this disease, especially the use of acupuncture.[16] In the present study, we screened 30 differentially expressed genes of acupuncture treatment patients and control group by bioinformatics analysis. We concluded that CENPF was the most significantly increased gene with acupuncture treatment. We also established that expression of CENPF decreased in CIA rat model whereas it was up-regulated in CIA rat model with acupuncture treatment.

Currently, acupuncture has become a complementary and alternative therapy for rheumatoid arthritis. Previous study conducted in Taiwan by Wu et al. reported that acupuncture reduced the risk of coronary heart disease in patients with rheumatoid arthritis.[17] Acupuncture inhibits its expression of nuclear factor kappa B (NF-κB) signaling

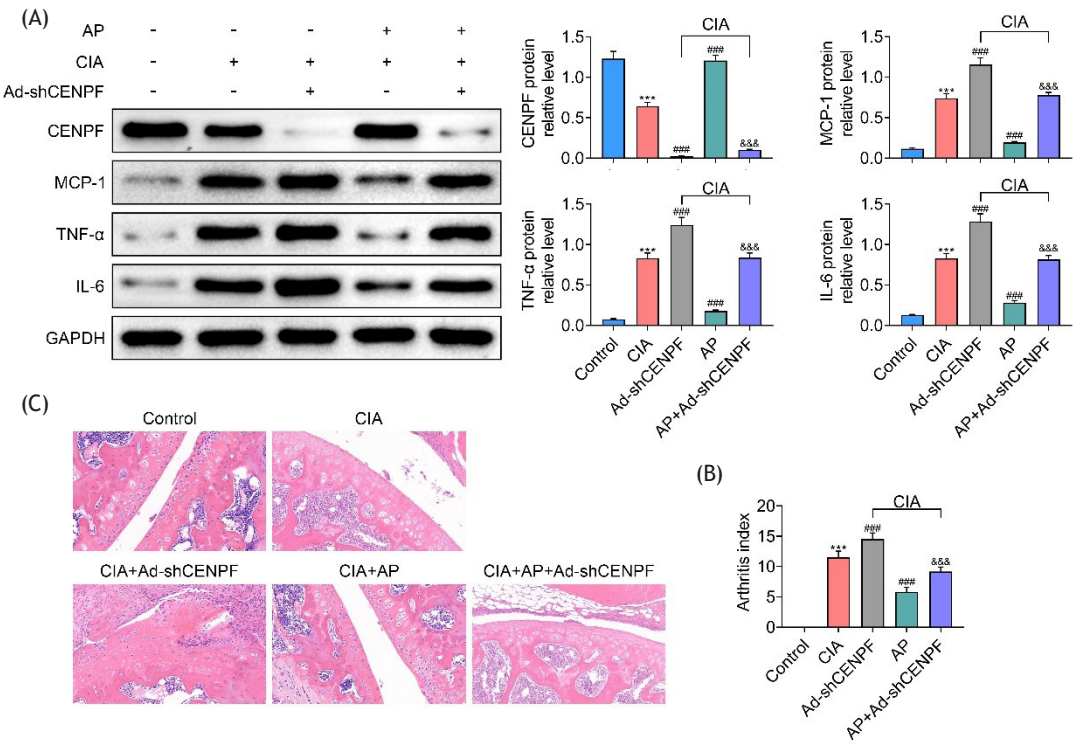

Figure 5 Knock down of CENPF weakens the therapeutic effect of acupuncture on rheumatoid arthritis. (A) Levels of CENPF, MCP-1, TNF-α, and IL-6 in CENPF-knockdown CIA model with acupuncture treatment were detected by Western blotting assay. (B) Severity of rheumatoid arthritis from the indicated group was measured by arthritis index. (C) H&E staining was used to analyze the pathology of arthrosis tissues from CENPF-knockdown CIA model with acupuncture treatment. ***$P < 0.001$, $^{\&\&\&}P < 0.001$, $^{\#\#\#}P < 0.001$. *Compared with control group; $^{\#}$compared with control group; $^{\&}$compared with acupuncture group.

pathway and mediates expressions of related inflammatory factors in rheumatoid arthritis patients, thereby alleviating the clinical manifestations of arthritis.[18] Thus, we concluded that acupuncture treatment in rheumatoid arthritis patients was associated with increased CENPF expression.

CENPF is an outer kinetochore component and a member of centromere-associated protein family. CENP family proteins from A to F are all known at present. Previous studies have demonstrated that CENPF is associated with cell cycle, cell death, embryogenesis, and tumorigenesis.[19-21] In rheumatoid arthritis, CENPE is up-regulated in rheumatoid synovial fibroblasts, which reflects a phenotype of RA fibroblast activation.[22] In the present study, expression of CENPF was down-regulated in CIA rat model; however, high expression of CENPF was confirmed in CIA model with acupuncture treatment.

Generally, rheumatoid arthritis is an illness associated with the joints, and is involved in systemic autoimmune inflammation with much extra-articular manifestation and systemic immune network imbalance.[4] Recent studies have reported that acupuncture has anti-inflammatory and analgesic effects on CFA rat model, a common animal model utilized in rheumatoid arthritis. Furthermore, the dynamic distribution of immune cytokines is consistent with the pathophysiology of rheumatoid arthritis, and acupuncture stimulates immune cytokines at an early stage. Acupuncture helps to reduce inflammation and pain of infected joints. Furthermore, acupuncture effectively induces innate and adaptive immune cytokines as a major part of rheumatoid arthritis response and damage repair.[23] IL-6, IL-7, IL-18, and TNF-α are involved in innate immunity. In terms of molecular regulation mechanism, we established that acupuncture treatment and overexpression of CENPF inhibited expressions of MCP-1, TNF-α, and IL-6 in a CIA rat model. However, deficiency of CENPF can inhibit expressions of MCP-1, TNF-α, and IL-6 in acupuncture-treated CIA rat model. Furthermore, knock down of CENPF attenuated the therapeutic effect of acupuncture on rheumatoid arthritis by H&E staining. In addition, it has been reported that acupuncture alleviates rheumatoid arthritis by modulating immune network.[24]

Conclusion

We are the first to report that CENPF is up-regulated in rheumatoid arthritis after acupuncture treatment. However, CENPF deficiency negatively regulated the effects of acupuncture in rheumatoid arthritis. Thus, *CENPF* gene has the potential to be explored as a marker in regulating rheumatoid arthritis with acupuncture treatment.

Availability of Data and Materials

All data generated and analyzed in this study are included in the published article.

Competing interests

The authors stated that there was no conflict of interest to disclose.

Contribution of authors

Weitao Dong and Bin Wang designed the study, and supervised data collection. Rongchao Zhang analyzed and interpreted the data. Junyi Cao and Rong Wu prepared and reviewed the manuscript for publication. All authors read and approved the final manuscript.

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