

Original Article

Anti-TNF Therapy Regulates Phagosome Pathway by Inhibiting NCF4 Expression to Treat Ankylosing Spondylitis

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Abstract

Objectives: Ankylosing spondylitis (AS) is challenging to diagnose in its early stages, and treatment options are limited. **Methods:** GEO2R analysis and weighted gene co-expression network analysis (WGCNA) were used to identify DEGs and key modules. Kyoto Encyclopedia of Genes and Genomes analysis and Protein-protein interactions were used to identify core genes. Receiver operating characteristic curve, chi-square and t-test were used to analyze the correlation between gene expression and clinicopathological characteristics. Gene expression was detected using Real-time polymerase chain reaction and western blotting. **Results:** GEO2R analysis and WGCNA identified 1100 DEGs and brown module. The KEGG analysis revealed that 444 core genes were closely associated with specific pathways. PPIs demonstrated that a key module, consisting of 6 genes, was linked to the phagosome pathway. NCF4, identified as an effective biomarker, was selected for diagnosing AS. Bioinformatics analyses indicated that NCF4 could be associated with important clinical markers. RT-PCR and western blotting showed increased expression of NCF4 in AS, which decreased after anti-TNF therapy. **Conclusions:** Anti-TNF therapy may exert its therapeutic function by inhibiting NCF4 expression, hence controlling the phagosome pathway. NCF4 has the potential to function as a diagnostic and prognostic biomarker for AS.

Keywords: Ankylosing Spondylitis, Anti-TNF Therapy, Biomarker, Phagosome

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory condition primarily affecting the axial skeleton and entheses. The typical clinical manifestations of AS include inflammatory back pain and stiffness, with the most common features being syndesmophytes and spinal ankylosis¹. Prolonged inflammation leads to progressive ossification of the vertebral column, resulting in irreversible loss of spinal mobility and decreased quality of life². Due to nonspecific symptoms, lack of objective clinical findings, and the slow progression of the disease, early diagnosis, assessment of disease activity, and

monitoring radiographic progression in AS pose significant challenges. The average delay in diagnosing AS ranges from 8 to 11 years³. Since delayed diagnosis hinders early treatment, identifying new biomarkers for early diagnosis becomes crucial, potentially improving disease activity, prognosis, and treatment response in AS⁴.

Currently, the treatment goal for AS is to alleviate symptoms, maintain function, and slow disease progression. The primary treatment approach involves a combination of exercise and nonsteroidal anti-inflammatory drugs (NSAIDs)⁵. Over the past decade, tumor necrosis factor (TNF) inhibitors have emerged as the cornerstone of treatment for patients with limited benefit from NSAIDs. These inhibitors aid in achieving better functional outcomes and reducing disease activity⁶. However, anti-TNF therapy has limitations, and there are still many unanswered questions regarding TNF's role as a mediator in AS. Thus, further research is needed to comprehend TNF's function in AS pathogenesis, optimize anti-TNF therapy, and develop safer and more effective treatment strategies⁷.

DNA microarray technology has evolved as a versatile biomedical tool with various diagnostic applications⁸. In this

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study, we utilized gene expression profiles from AS patients to identify genes and pathways associated with TNF inhibitor treatment, aiming to identify potential biomarkers and provide new insights and methods for the clinical treatment of ankylosing spondylitis.

Materials and methods

Clinical Specimens

A total of 10 patients from The First Hospital of Qiqihar were included in this study and divided into two groups: the AS group (n=5) and the Control group (n=5). The exclusion criteria were as follows: (1) patients who did not meet the diagnostic criteria for AS (Diagnosis of AS followed the 1984 modified New York criteria⁹); (2) pregnant women; (3) patients with acute and critical illnesses; (4) patients with other autoimmune diseases. The investigation protocol was approved by the Ethics Committee of Qiqihar First Hospital. All assays were performed in accordance with the applicable regulations, and patients provided written informed consent.

Data collection

The Gene Expression Omnibus (GEO) primarily comprises gene expression data obtained through DNA microarrays. For this study, we selected the datasets GSE25101, GSE73754, and GSE141646 from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) and obtained both the platform files and the original files (.CEL files) for further analysis.

Analysis of differentially expressed genes (DEGs)

The GEO2R web application, accessible at <http://www.ncbi.nlm.nih.gov/geo/geo2r/>, represents a significant upgrade introduced by GEO. This user-friendly interface allows users to conduct complex R-based analysis on GEO data, aiding in the identification and display of differentially expressed genes (DEGs)¹⁰. GEO2R analysis was performed on the GSE25101 and GSE73754 datasets, as they included both normal and case sequenced specimens. DEGs between the AS and control groups were identified for each dataset. Finally, genes with a P-value <0.05 were selected for further investigation.

Construction of the weighted gene co-expression network analysis (WGCNA)

Gene co-expression networks were constructed using the Weighted Gene Co-expression Network Analysis (WGCNA), a systems biology approach that transforms co-expression measures into connection weights or topology overlap measures, enabling the investigation of gene interactions¹¹. In this study, the WGCNA was applied to the GSE141646 dataset, which included detailed clinical characteristics of AS patients. Firstly, samples were clustered to identify any potential outliers. Secondly, the co-expression network was constructed using the automatic network construction function. The R function 'pickSoftThreshold' was employed to

determine the soft thresholding power (β), which is multiplied by the co-expression similarity to establish adjacency. Thirdly, modules were identified using hierarchical clustering and the dynamic tree cut function. Fourthly, to associate modules with clinical traits, gene significance (GS) and module membership (MM) calculations were performed. Relevant information regarding the genes within each module was extracted for further investigation. Lastly, we visualized the eigengene network¹².

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

Understanding the biological function of core modules is crucial. Enrichment analysis was performed to evaluate the significance of the key module genes identified through WGCNA analysis. Subsequently, KEGG pathway enrichment analyses were conducted using the WEB-based GENE Set Analysis Toolkit (<http://www.webgestalt.org/option.php>) to identify the most relevant module genes. A false discovery rate (FDR) ≤ 0.1 was considered statistically significant.

Protein-protein interactions (PPIs) network construction

Information on PPIs can be obtained from the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://string-db.org/>)¹³. In order to gain a better understanding of the cellular functions and biological behaviors of the selected genes, a PPI network was constructed using STRING, with a significant cutoff threshold set at a confidence score ≥ 0.7 . Finally, the Cytoscape program was utilized to visualize the PPI networks.

Western blot

Protein lysis was performed using RIPA buffer (Thermo Fisher Scientific, USA). Protein concentrations were measured using the Pierce Protein Assay Kit. After running the proteins through SDS-PAGE, they were transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Primary antibodies were applied to identify the proteins of interest. Protein concentrations were determined using chemiluminescence (Bio-Rad, USA) following the manufacturer's protocols. The primary antibodies used in this study included MMP2 (Abcam, UK, ab37150), MMP9 (Abcam, ab73734), PLEKHG5 (Sigma-Aldrich), N-cadherin (Abcam, ab76011), Vimentin (Abcam, ab8069), mDia (Santa Cruz, USA, sc-373895), and GAPDH (Cell Signaling Technology, USA, 5174). Protein quantification was performed using chemiluminescence (Bio-Rad, USA) according to the manufacturer's instructions.

RT-PCR (Real-time polymerase chain reaction)

Total RNAs were extracted using TRIzol (Invitrogen, USA) following the manufacturer's instructions. For reverse transcription, 1 μ g of extracted RNA was converted to cDNA using the Moloney Leukemia Virus Reverse Transcriptase

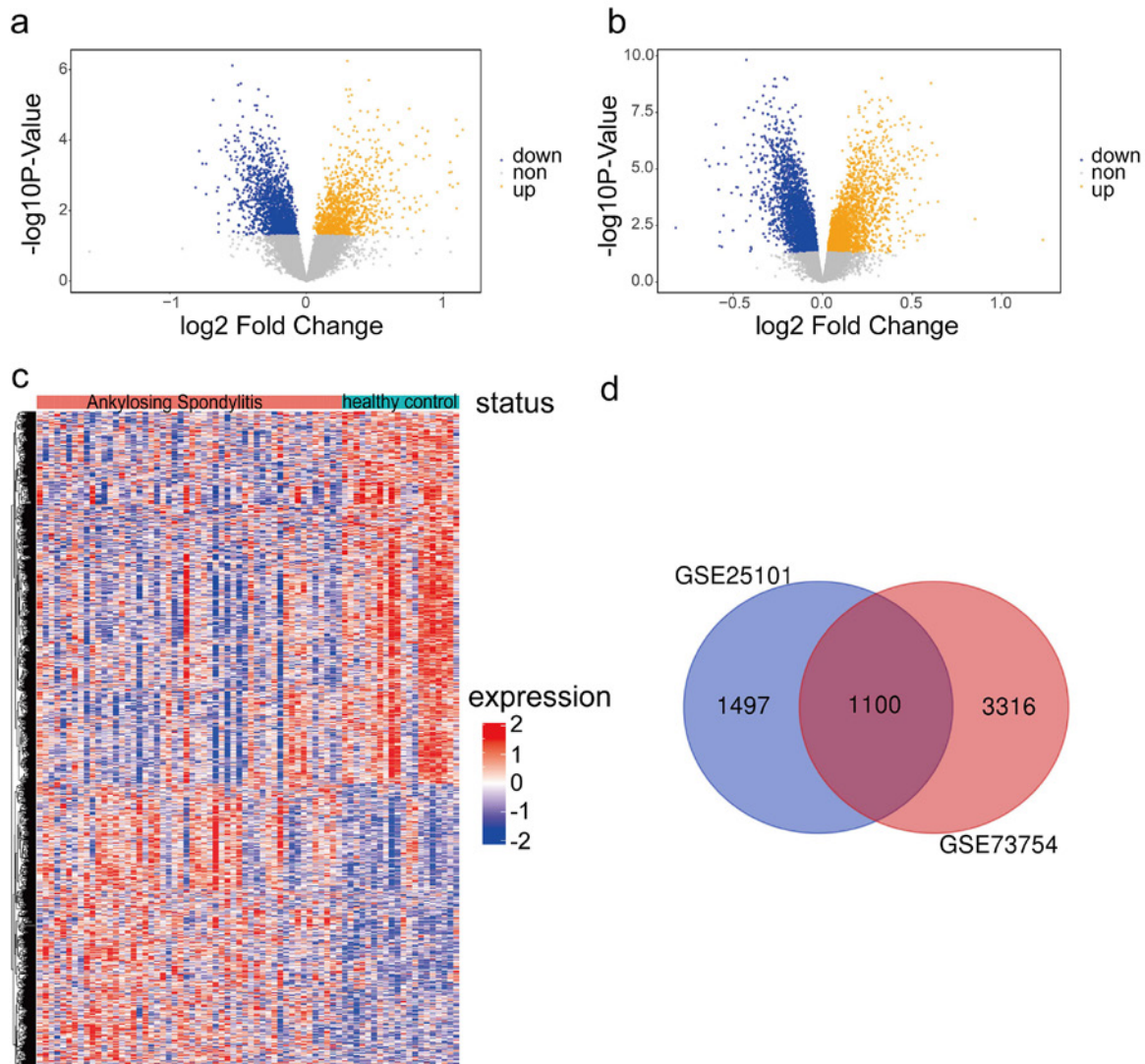


Figure 1. Identification of differentially expressed genes. a-b) Volcano plots displaying the gene expression profiles in GSE25101 and GSE73754 datasets. The red/blue symbols classify the upregulated/downregulated genes based on the criteria: P-value <0.05. c) Common DEGs identified in both GSE25101 and GSE73754 datasets. d) Expression matrix of 1100 common DEGs in 72 pairs of AS and adjacent normal tissues, represented by unsupervised hierarchical clustering in GSE72754. Blue, red, and white colors indicate lower expression, higher expression, and no expression difference among the genes, respectively.

Kit (Madison, USA). Subsequently, RT-PCR was performed to amplify the samples and analyze mRNA expression levels using the Green Mix SYBR (Promega) kit. Each experiment was repeated three times to determine the fold change (FC) in relative expression levels, and the data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The accuracy of AS prediction for the target gene was evaluated using receiver operating characteristic (ROC) analysis. The ROC curve was generated using the 'pROC' package in R software. The two-tailed t-test was used to

analyze the correlation between gene expression erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and Anti-TNF. Statistical visualization was performed using R software, and a significance level of $p < 0.05$ was used to determine statistical significance in our analysis.

Results

Identification of DEGs and related pathways in AS after data integration

Gene expression profiles from GSE25101 revealed that 1140 genes were upregulated and 1457 genes were

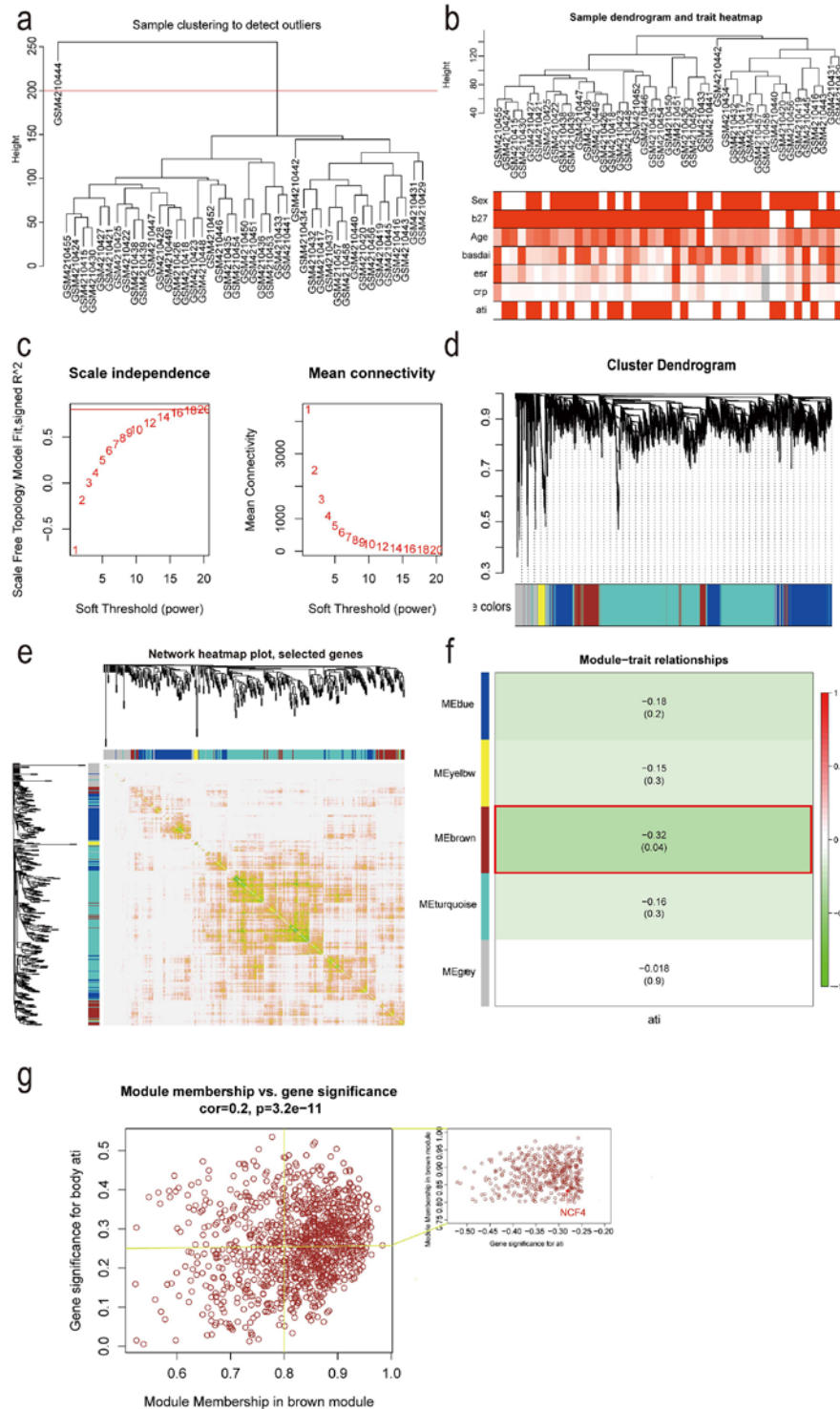


Figure 2. Weighted co-expression network construction and identification of the key module. a) Hierarchical clustering dendrogram of samples from GSE141646 dataset. b) Hierarchical clustering dendrogram of samples displaying clinical traits at the bottom. c) Analysis of scale-free fit index and mean connectivity for different soft-thresholding powers. Testing the scale-free topology with $\beta=18$. d) Hierarchical clustering dendrogram of genes based on topological overlap. Modules are represented by branches in the clustering tree. e) Heatmap illustrating the topological overlap matrix (TOM) among 400 selected genes in WGCNA. Darker colors indicate higher overlap, while lighter colors indicate lower overlap. The gene dendrogram and module assignment are shown on the left side and top, respectively. f) Correlation between module eigengenes and clinical traits. Each row corresponds to a module eigengene, and columns represent clinical traits. Each cell contains the correlation and P-value. The brown module was selected. g) Scatter plot of genes in the brown module. The vertical line represents the cutoff for module membership (0.8), and the horizontal line represents the cutoff for gene significance in anti-TNF therapy (0.25). Genes located in the upper right quadrant include NCF4.

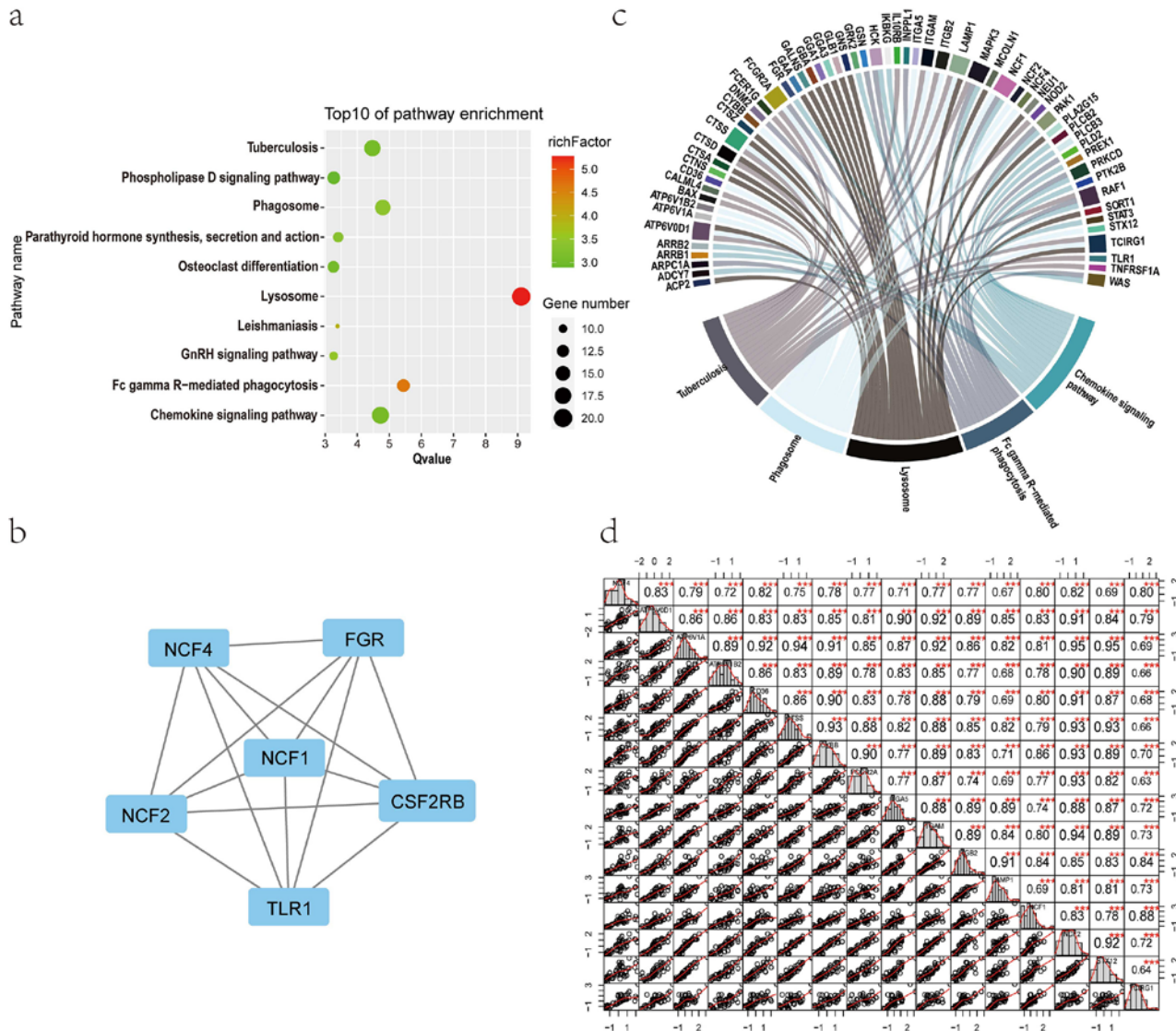


Figure 3. KEGG analysis and PPI network of brown module genes. a) KEGG analysis of 444 core genes from the brown module, showing the top 10 pathways with the most significant enrichment (based on P-value). b) Relationships between the five most significant pathways and their corresponding genes. c) Key module selected from the protein-protein interaction (PPI) network, consisting of NCF1, NCF2, NCF4, FGR, CSF2RB, and TLR1. d) Correlation among genes involved in the phagosome pathway.

downregulated ($p < 0.05$) in peripheral blood samples of individuals with AS compared to those in normal controls, as depicted in the volcano plots (Figure 1a). In the GSE73754 dataset, we identified 4416 differentially expressed genes (DEGs) ($p < 0.05$), with 2024 genes upregulated and 2392 genes downregulated in AS (Figure 1b). Based on the p-value threshold, we selected DEGs for integrated analysis and found 1100 overlapping DEGs in the two GSE datasets (Figure 1c). Using the GSE73754 dataset, we further examined the expression of the 1100 identified DEGs in peripheral blood samples of individuals with AS and normal controls (Figure 1d).

Construction of WGCNA and identification of key modules, especially the brown module

Co-expression analysis is commonly employed to investigate the association between gene expression levels. Genes involved in the same pathway or functional network may exhibit similar expression patterns¹⁴. After identifying sample GSM4210444 as an outlier and excluding it from further analysis in the GSE141646 dataset, we included the remaining 43 samples with clinical data in the Weighted Gene Co-expression Network Analysis (WGCNA) (Figure 2a-b). To construct a scale-free network, we selected a soft-thresholding power of $\beta = 18$ (scale-free $R^2 = 0.80$) (Figure

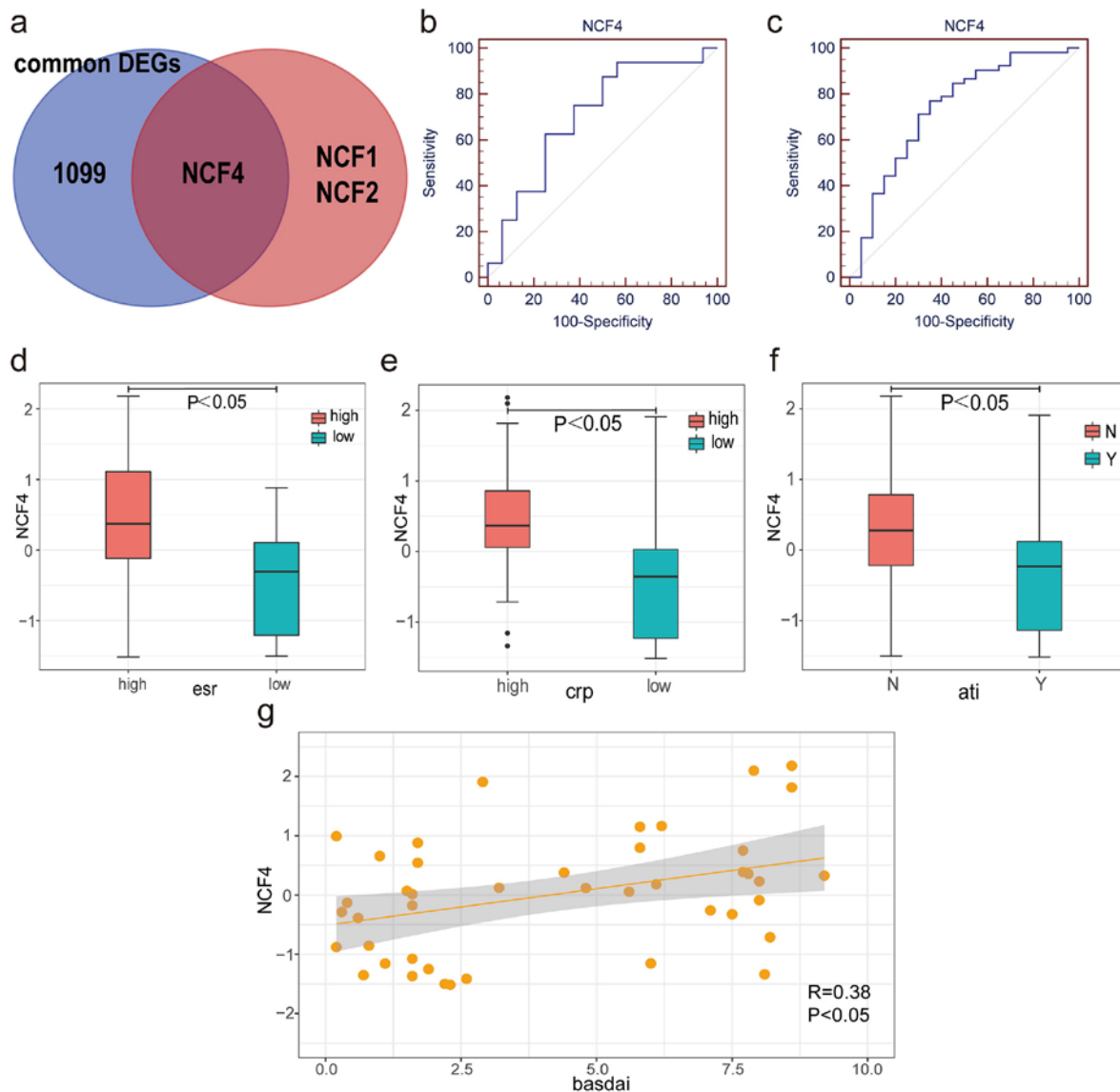


Figure 4. Identification of NCF4 and its relationship to clinical characteristics. a) NCF4 was identified by integrating genes related to the phagosome pathway and 1100 common DEGs. b-c) ROC curve analysis of NCF4 in GSE25101 and GSE73754 datasets. d) Quantification of NCF4 mRNA levels in patients with high erythrocyte sedimentation rate (ESR) and low ESR. e) Quantification of NCF4 mRNA levels in patients with high C-reactive protein (CRP) and low CRP. f) Quantification of NCF4 mRNA levels in patients receiving Anti-TNF therapy and those who did not. g) Positive correlation between NCF4 mRNA expression level and the bath ankylosing spondylitis disease activity index (BASDAI).

2c). Using the merged dynamic tree cut, we identified four gene co-expression modules after excluding the grey module (Figure 2d). A heat map of the Topological Overlap Matrix (TOM) was generated for 400 analyzable genes, revealing that each module served as an independent validation for one another (Figure 2e). Among these modules, the brown module showed the highest correlation with AS Anti-TNF therapy ($R^2 = -0.32$, $P = 0.04$, Figure 2f). We further examined the brown module by plotting gene significance (GS) against module membership (MM) using the AS patient data on Anti-

TNF therapy, revealing a correlation of 0.2 ($P = 3.2 \times 10^{-11}$). This analysis identified 444 genes, including NCF4, that met the criteria of $MM > 0.80$ and $GS < 0.25$, designating them as hub genes within the brown module (Figure 2g, Supplemental Table S1).

The KEGG pathway enrichment analyses and PPI network of brown module genes

To further investigate the gene-related pathways involved in Anti-TNF therapy in AS patients, we analyzed the KEGG

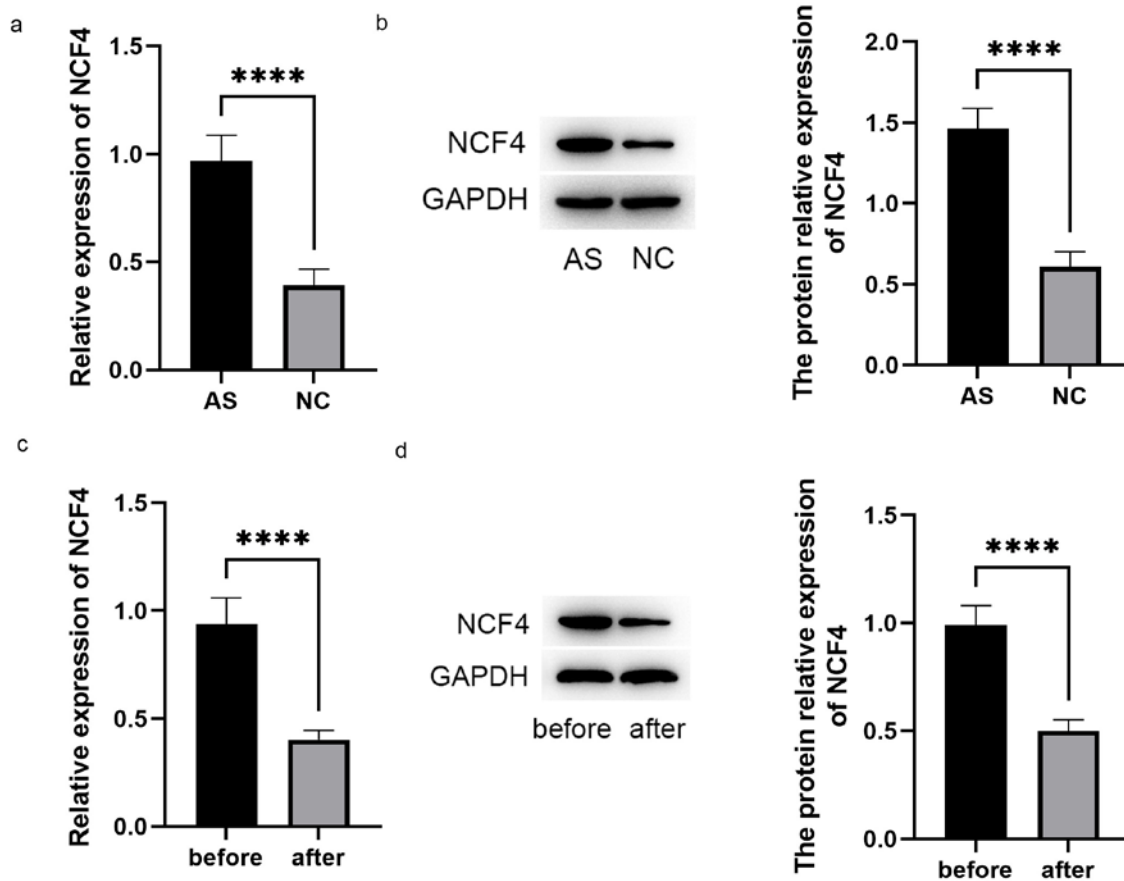


Figure 5. Clinical validation of NCF4 as the target gene in AS. (a) QT-PCR showed that the mRNA expression of NCF4 was significantly higher in the AS group compared to the normal control group (NC group). (b) Western blot analysis confirmed higher protein levels of NCF4 in the AS group compared to the NC group. (c) QT-PCR demonstrated that the mRNA expression of NCF4 was significantly higher in the pre-therapy group (Pre group) compared to the post-therapy group (Post group). (d) Western blot analysis confirmed higher protein levels of NCF4 in the Pre group compared to the Post group (n=5).

pathways of the 444 hub genes obtained from the brown module and selected the top 10 most significant pathways for visualization (Figure 3a). The analysis revealed a close association between genes connected to Anti-TNF therapy and the phagosome and lysosomal pathways. Based on this finding, we hypothesized that Anti-TNF treatment may exert its efficacy by regulating phagocytosis.

Furthermore, we explored the gene-pathway correspondence and identified key genes, such as NCF4 and FCGR2A, that play essential roles in the most significant pathways for Anti-TNF therapy (Figure 3b). Subsequently, we performed protein-protein interaction (PPI) analysis on the 444 genes from the Brown module. Using the STRING database, the PPI network was constructed, consisting of 441 nodes and 1553 edges, with a confidence score above 0.7. The PPI network was visualized using Cytoscape software, and the MCODE algorithm was employed to identify the most important gene modules for clustering. Finally, we identified a

core module composed of NCF1, NCF2, NCF4, FGR, CSF2RB, and TLR1 (Figure 3c). Notably, the genes NCF1, NCF2, and NCF4 within the core module showed a close association with the phagosome pathway, indicating their involvement in this pathway (Figure 3d).

Identification of the hub gene and its relationship to clinical characteristics

The phagosome is a vesicle formed around a phagocyte during endocytosis. It is a common cellular structure involved in the immune process. Phagocytes play a crucial role in killing and digesting pathogenic microorganisms that invade the body. Through integrating phagocytic pathway-related genes in KEGG pathway analysis with differentially expressed genes (DEGs), NCF4 was identified as a biomarker gene (Figure 4a).

To assess the sensitivity and specificity of NCF4 for AS diagnosis, we performed a ROC curve analysis and compared

the AUC values. The ROC curves of NCF4 in the GSE25101 and GSE73754 datasets were displayed in Figure 4b-c, demonstrating adequate sensitivity and specificity with average AUC values of 0.707 and 0.736, respectively. It should be noted that the expression and function of NCF4 in AS are still largely unknown. Therefore, we propose NCF4 as a target gene and aim to investigate its role in AS, providing a potential new therapeutic option.

Furthermore, we conducted further investigations into the relationship between the hub genes and clinical features. The results revealed that NCF4 expression was closely associated with the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels (Figure 4d-e). Moreover, NCF4 expression significantly differed between patients before and after Anti-TNF medication, showing a considerable decrease after treatment (Figure 4f). In terms of the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), NCF4 expression showed a positive linear correlation with the scores (Figure 4g).

NCF4 has been clinically validated as a target gene in AS

To confirm the accuracy of NCF4 diagnosis in clinical samples (n=5) and normal controls (n=5), we performed qRT-PCR and Western blot analysis. The qRT-PCR results showed that the mRNA expression of NCF4 in the AS clinical samples group (AS group) was significantly higher than that in the normal control group (NC group). Similarly, the Western blot analysis revealed higher protein levels of NCF4 in the AS group compared to the NC group (Figure 5a-b). These findings indicate that NCF4 is a target gene for AS.

Furthermore, we investigated whether anti-TNF therapy regulates the phagosome pathway through NCF4 expression for the treatment of AS. qRT-PCR and Western blot were performed on peripheral blood samples collected before anti-TNF therapy (before group) and after anti-TNF therapy (after group). The qRT-PCR results showed that the mRNA expression of NCF4 in the before group was significantly higher than that in the after group. Similarly, the Western blot analysis showed higher protein levels of NCF4 in the before group compared to the after group (Figure 5c-d). These results suggest that anti-TNF therapy regulates the phagosome pathway for the treatment of AS by inhibiting NCF4 expression.

Discussion

Ankylosing Spondylitis (AS) is a prevalent type of immune-mediated inflammatory arthritis that significantly impacts a person's quality of life and has a notable familial predisposition¹⁵. Moreover, the chronic functional impairment caused by post-inflammatory new bone formation, particularly in the spine, contributes to the long-term burden of the disease¹⁶. As TNF is a key cytokine involved in the pathophysiology of AS, TNF inhibitors have been introduced in clinical practice. Adalimumab, golimumab, infliximab, certolizumab, and etanercept are currently approved TNF

inhibitors used in AS clinical trials². TNF inhibitors have demonstrated efficacy in alleviating clinical symptoms¹⁷ and even preventing structural damage progression with long-term use in AS patients¹⁸. However, there are limitations to Anti-TNF therapy in AS, and several unresolved questions remain regarding TNF's role as a mediator in AS⁷.

The discovery of biomarkers has gained considerable attention in recent years as a potential approach to enhance early diagnosis of AS and aid in patient selection for treatment. Accurately assessing disease activity, predicting therapy response, and predicting radiographic progression in AS could all benefit from the utilization of biomarkers. Currently, apart from established biomarkers such as C-reactive protein (CRP) and human leukocyte antigen (HLA)-B27 subtype, most biomarkers for AS are still in the research phase and are not yet routinely employed in clinical practice¹⁹. In our study, we identified a total of 1100 common Differentially Expressed Genes (DEGs) in AS samples from two Gene Expression Omnibus (GEO) datasets. Through Weighted Gene Co-expression Network Analysis (WGCNA), we screened and analyzed 444 core genes related to Anti-TNF therapy, and examined their association with the KEGG pathway. Protein-Protein Interactions (PPIs) analysis revealed a key module consisting of six genes that were connected with the phagosome pathway, which was also identified in the KEGG pathway analysis. The genes involved in the phagosome pathway overlapped with the common DEGs, leading to the identification of a potential biomarker, NCF4. NCF4, an upregulated gene in AS patients, showed a significant reduction in expression in patients undergoing Anti-TNF therapy. Additionally, NCF4 demonstrated a close relationship with clinical characteristics such as erythrocyte sedimentation rate (ESR) and CRP.

The role of NCF4 in the phagocytic pathway has been confirmed. The gene product functions as a cytosolic regulator of phagocyte NADPH-oxidase, which is an essential enzyme system for host defense. Chronic granulomatous disease (CGD) is a primary phagocytic immunodeficiency caused by a deficiency of NADPH oxidase (PHOX), resulting from genetic variations in NCF4 and the subsequent deficiency of Nox2 oxidase in phagocytic cells^{20,21}. Phagocytosis is the process by which particles are ingested and enclosed within a phagosome, which initially resembles a miniature plasma membrane. However, phagosomes undergo rapid remodeling during maturation by fusing with endosomes and lysosomes, eventually forming phagolysosomes. In the acidic and degradative environment of phagolysosomes, particles are broken down²². The process of effective phagocytosis relies on the interaction between phagosomes and lysosomes, which aligns with the findings of our KEGG pathway analysis.

A healthy equilibrium between bone formation and bone resorption is crucial for maintaining the homeostasis of hard tissues. However, in pathological conditions, this balance is disrupted, resulting in either excessive production or resorption of bone matrix. Localized excessive bone resorption is observed in autoimmune arthritis and giant cell bone tumors (GCTB)²³. Experimental findings indicate a

significant increase in osteoclast formation in patients with AS, particularly in those with ankylosis of the sacroiliac joint. Additionally, studies have reported a decrease in bone density among AS patients, with increased osteoclasts and enhanced phagocytosis potentially contributing to the osteopenia observed in AS patients²⁴.

Based on our work, we propose the potential biological role and clinical significance of NCF4 in AS through WGCNA and KEGG pathway analyses. Anti-TNF therapy may regulate the process of phagocytosis by inhibiting the expression of NCF4, thereby alleviating the condition of AS patients.

Our study has limitations. We did not perform a clinical trial or cytological experiment to further verify the role and function of NCF4. Additionally, we did not conduct in-depth analysis at the animal level, which limits the available evidence for clinical translation.

Conclusion

The mechanism of action for Anti-TNF therapy in AS may involve the inhibition of NCF4 expression and regulation of the phagocytosis process. Therefore, NCF4 has the potential to serve as a biomarker for both the diagnosis and prognosis of AS.

Authors' contributions

Sha Liu designed the study and drafted the manuscript. Hui Zhu was responsible for the collection and analysis of the experimental data. Both authors revised the manuscript critically for important intellectual content. Both authors have read and approved the final manuscript.

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Supplemental Table S1. Information of datasets from GEO.

Dataset	Samples	Case/Control	Study	Country	Platforms	Gene #	Gene chip
GSE25101	32	16/16	Pimentel-Santos FM et al. (2011) ²⁵	Australia	GPL6947	15117	Illumina HumanHT-12 V3.0 expression beadchip
GSE73754	72	52/20	Gracey E et al. (2016) ²⁶	Canada	GPL10558	31280	Illumina HumanHT-12 V4.0 expression beadchip
GSE141646	44	44/0	Wang XB et al. (2017) ²⁷	Australia	GPL20301	60669	Illumina HiSeq 4000 (Homo sapiens)