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Moxibustion attenuates inflammation and alleviates axial spondyloarthritis in mice: Possible role of APOE in the inhibition of the Wnt pathway

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ABSTRACT

Background and aim: Moxibustion is widely used in China and other East Asian countries to manage the symptom of ankylosing spondylitis (AS). This study investigated the effects of moxibustion intervention on protein expression through proteomics analysis in AS mice.
Experimental procedure: Proteoglycan-induced spondylitis (PGISp) was established in Balb/c mice. PGISp mice were intervened with daily moxibustion at ST36, BL23, and DU4 for four weeks. Various biochemical (including pro-inflammatory cytokines and bone metabolism indexes) and histopathological parameters were determined. The effects of *moxibustion* on protein changes in AS mice were analyzed using data-independent acquisition-mass spectrometry (DIA-MS). The target proteins were then confirmed by Western blot analysis.
Results: Moxibustion significantly decreased pro-inflammatory cytokine expression including IL-1β, TNF-α, IL-17, and IL-6, reduced the mRNA expression of RANKL, RANK, ALP, and OCN, and improved the histopathological examination in AS mice. DIA-MS proteomic technique has identified 25 candidate proteins involved in the mechanisms of moxibustion for AS mice, most of which are mainly associated with the regulation of Wnt/β-catenin. Integrated pathway analysis revealed that glycine, serine and threonine metabolism together with lipid metabolism were the most important canonical pathways involved in the anti-AS effect of moxibustion. In line with the multi-omic data, the levels of BPGM, $APOC_2$, APOE, and GPD1 modified in the AS mice, intervened with *moxibustion* as confirmed by Western blot. In particular, APOE may play a key role in linking the lipid metabolism and the Wnt/β-catenin pathway of new bone formation.
Conclusion: In conclusion, moxibustion may reduce pro-inflammatory cytokines and improve bone erosion for AS mice. The regulation of APOE by moxibustion may have a potential inhibitory effect on the Wnt/β-catenin pathway in AS mice. However, due to the lack of silencing or overexpression of key molecules of the signal pathway, whether the beneficial and positive effect of moxibustion involved in the regulation of Wnt/β-catenin signaling pathway by APOE or other aspects, needed to be explored in further study.

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List of abbreviations

AS	ankylosing spondylitis
ALP	Alkaline phosphatase
CAM	complementary and alternative medicine
CPG	clinical practice guideline
CACM	China Association of Chinese Medicine
DIA	data-independent acquisition
DEPs	different expressed proteins
FDR	false discovery rate
GO	gene ontology
HC	health control
ICAM-1	intercellular adhesion molecule-1
NSAIDs	nonsteroidal anti-inflammatory drugs
OCN	osteocalcin
PGISp:	proteoglycan-induced spondylitis
QC	quality control
RT	retention time
RT-PCR	reverse transcription-polymerase chain reaction
TNF-α:	tumor necrosis factor alpha

1. Introduction

Ankylosing spondylitis (AS), also known as axial spondyloarthritis (axSpA), is a chronic, systematic, and progressive autoimmune disease of unknown causes that predominantly influences patients' sacroiliac, axial joints, and even extra-articular structures (gut, eyes, skin, kidney, heart, etc.). It is typically characterized by sacroiliitis, enthesitis, axial inflammation, and the new bone formation.[1] The prevalence rate of ankylosing spondylitis in the United States of America is estimated as 0.55%, with an average age of onset less than 40 years, and in mainland China is likely to be approximately 0.2%–0.3%.[2,3]

Mounting evidence from the ASAS/EULAR international guidelines recommended that pharmacotherapies, including nonsteroidal anti-inflammatory drugs (NSAIDs) and FDA-approved biological agents (Tumor Necrosis Factor inhibitors and Interleukin-17A antagonist), are conventional and mainstream treatments for AS. The available evidence shows that NSAIDs are presently first-line medicine options for alleviating inflammatory back pain and joint swelling effectively for AS patients; however, existing research also suggests an association between NSAIDs use and increased risks of gastrointestinal hemorrhage and renal injury.[4] Tumor Necrosis Factor inhibitors and Interleukin-17A antagonists have revealed a remarkable and beneficial effect on reducing the disease activity and inflammatory markers. Nevertheless, the high cost of continuous biological agents' therapies brings a heavy burden on patients' families in remote districts.[5] Therefore, Chinese AS patients have turned their attention to traditional Chinese medicine (TCM).[6] Moxibustion, a main form of TCM, is widely used in China to manage the symptom of AS patients.[7,8]

The previous systematic review revealed that moxibustion could relieve the pain, improve physical function and reduce the disease activity for AS patients.[9] As moxibustion may have a beneficial effect on AS, possible mechanisms of action are of interest. Previous clinical research had proved that moxibustion at the governor vessel could decrease the serum levels of tumor necrosis factor-alpha (TNF-α) and intercellular adhesion molecule-1 (ICAM-1).[10] Moreover, Li et al. had reported that moxibustion could modulate the immune function of AS via balancing Th17/Treg.[11] Furthermore, our previous pre-clinical study revealed that moxibustion could exert a positive effect in AS model mice by regulating different metabolite expressions and regulating a wide variety of signaling pathways, including energy metabolism, lipid metabolism and intestinal flora metabolism.[12] These findings might provide a partial understanding of the effects of moxibustion on AS.

Great efforts have been made previously to explore the possible mechanism of moxibustion intervention for AS from the perspective of a handful of mRNA and proteins. However, moxibustion can exert multi-target effects and have an impact on various potential downstream targets.[13] Proteomic strategies fully comply with the holistic characteristics of moxibustion therapy, which have been used to discover the possible molecular mechanism of moxibustion for collagen-induced arthritis mice[14] and postherpetic neuralgia patients with herpes zoster.[15] There is still a lack of studies to explore the proteomic mechanisms of moxibustion for AS. Moreover, previous studies[14,15] all applied 2 DE-based proteomic strategies to characterize moxibustion-target holistic metabolic profiling. Notably, growing evidence has revealed that compared with the 2 DE approach, DIA-MS has become the dominant analytical instrument for proteomic research due to its high resolution, sensitivity, generality, and versatility features.[16] Thus, the aim of this research was to conduct a tissue-based DIA-MS proteomic analysis to identify novel moxibustion-related proteins for AS.

2. Methods and materials

2.1. Animals

All experimental procedures were approved by the Animal Welfare Committee of ZCMU and also in accordance with NIH laboratory animals' guidelines. A total of 32 retired female Balb/c mice supplied by ZCMU were housed in the Animal Research Centre with the following environment conditions: 12-h/12-h light and dark cycle and controlled humidity (57 ± 2)% and temperature (25 ± 1)°C. Before the animal experiments, all mice were housed in the Animal Research Centre for one-week of adaptive feeding (providing adequate food and water). Eight mice were randomly assigned to the health control (HC) group, and the remaining twenty-four mice were used for AS mice modeling construction.

2.2. AS model construction

The AS model construction was adopted from our previous publishing study.[12] Briefly, the mice were i. p. injected with an emulsion of 100 μg of cartilage proteoglycans (Sigma-Aldrich, St. Louis, MO, USA) and 2 mg of dimethyldioctadecylammonium (Sigma-Aldrich, St. Louis, MO, USA) three times at 21-day intervals. The AS mice model was considered successful if axial skeleton ankyloses were detected via digital radiography fourteen weeks after the last i. p. injection.[12,17] The successful AS modeling mice were further randomly assigned to the AS modeling mice group (AS group), acupuncture points' moxibustion group (MA group), and non-acupuncture points' moxibustion group (MNA group), each of which consisted of eight mice.

2.3. Moxibustion intervention and biological sample collections

The protocol of moxibustion intervention was performed according to our previously published article.[12] Briefly, in the MA group, moxa sticks (diameter 7 mm, length 120 mm, Han Medicine, Moxa Co Ltd) were lit and burned for 5 s. The distance between the moxibustion and the skin was about 2 cm. The acupuncture points were selected as follows: ST36 (Zusanli), BL23 (Shenshu), and DU4 (Mingmen). Two pieces to ST36 (Zusanli) form one pair each, and two pieces to BL23 (Shenshu) also form one pair each. The location

of mentioned acupuncture points was referred to the 'Veterinary Acupuncture & Moxibustion Atlas',[18] and moxibustion at ST36, BL23, and DU4 lasted approximately 35 min. Each mouse received moxibustion therapy daily by an experienced moxibustion therapist in our research team for a total of four weeks. In the MNA group, the non-acupuncture points were located on the hypochondrium, 3 mm and 5 mm above the iliac crest.[19] Besides acupuncture point selection, moxibustion intervention was carried out following the same protocol in the MA group. After a 4-week moxibustion intervention, mice in each group were sacrificed, and tissue ligaments from the spine were collected and immediately transferred to a −80 °C refrigerator for further analysis.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissue ligaments by applying a tissue RNA purification kit plus (ES Science, RN002plus, China). The concentration of isolated total RNA was quantified by Nanodrop one (Thermo, IL, USA). Subsequently, total RNA was reverse transcribed to cDNA using Thermo Scientific Revert Aid First Strand cDNA synthesis kit (Thermo, K1622, IL, USA) according to the manufacturer's protocol. The resulting cDNA was then amplified by PowerUp™ SYBR™ Green kit (Thermo, IL, USA), and the specific primer pairs are listed in Supplementary Table 1. The PCR conditions in LightCycle 96 SW 1.1 instrument (Roche AG, Switzerland).

2.5. Histopathology

The dissected spines in each group were fixed in formalin for 24 h and decalcified for 30 days. After that, specimens were stained with hematoxylin and eosin (H&E) to visualize cartilage enlargement, inflammatory cell infiltration, intervertebral disc destruction and syndesmophyte formation.

2.6. DIA-MS based proteomics analysis

Briefly, cell lysis buffer (PMSF 1:100) for Western and IP (Beyotime, China) was added to tissue ligaments and then homogenized by the homogenizer. The supernatant total protein was quantified with a BCA assay (Bio-Rad, Hercules, CA) according to the manufacturer's procedure. The sample was then stored at −80 °C. An equal aliquot from each sample in this experiment was pooled into one sample for DDA library generation and quality control (QC). Protein digestion was further carried out with a FASP method.[20] Next, digested pool peptides were fractionated using a commercial peptide fractionation kit (Thermo, IL, USA). Afterward, all resulting peptides were desalted on C18 Cartridges (Empore™ SPE Cartridges C18, Sigma) following the manufacturer's protocol. Finally, the iRT-Kits were applied to correct relative retention time (RT).

Subsequently, the resulting peptides were separated with an EASY-SprayTM C18 Trap column (Thermo, IL, USA) using a linear gradient of buffer at an appropriate flow rate. MS detection method was selected as a positive ion model, and the detailed process program of MS1 scan and MS2 scan were based on the manufacturer's instruction. Finally, all collective fractions and peptides were generated for the AS ligament tissue-based DDA library. The parameters of MS1 Scan and MS2 Scan of DIA were also set following the manufacturer's instruction. In order to monitor the stability of MS performance, QC samples were injected at every 5 detected samples. The DDA data were searched with MaxQuant Version 1.5.2.8 analysis software. Then, DIA data were analyzed with Spectronaut software (Biognosys AG, Switzerland), searching the previously constructed DDA library.

2.7. Protein identification, quantification and the integrated multi-omic analysis

The differentially expressed proteins (DEPs) were selected according to: (1) for multiple testing corrections, False Discovery Rate (FDR) <0.05 in one-way ANOVA testing. (2) Post hoc comparisons were performed with Tukey's HSD comparison for four possible two-group comparisons (HC vs. AS, AS vs. MA, MA vs. MNA); (3) fold change (FC) >1.33 or <0.77.

Some novel bioinformatic analysis methods were adopted to explore the functional annotation of DEPs altered by moxibustion in AS mice. (1) Gene Ontology (GO) enrichment analysis adopted by DAVID software and Cytoscape visualization platform software; (2) Canonical pathway enrichment analysis was performed using KEGG pathway databases (https://www.kegg.jp/); (3) The Protein-Protein interaction (PPI) network was constructed by the STRING online software (https://www.string-db.org/). FDR<0.05 was used to filter the possible statistically significant enriched GO terms and candidate KEGG pathways.

Previously, we obtained tissue-based metabolic profiling of moxibustion for AS. Next, we performed the protein–metabolite interaction network via OmicBean software and the integrated canonical pathway through MetaboAnalyst 5.0. FDR<0.05 was used to filter the integrated network and integrated signaling pathway.

2.8. Western blot analysis

Four cytokines (IL-1β, TNF-α, IL-17 and IL-6), four DEPs (BPGM, GPD1, APOE and $APOC_2$) were identified by the integrated Omic analysis, and five key proteins (LRP5, DVL1, β-catenin, Cacybp, Skp1) involved in the Wnt/β-catenin signaling pathway were further validated by Western blot analysis. Briefly, total proteins were extracted using Cell lysis buffer (PMSF 1:100) for Western and IP (Beyotime, China). The total protein concentrations were quantified with a BCA assay (Bio-Rad, Hercules, CA). Next, the target proteins were separated by 8–20% Precast-Glgel Tris-Glycine PAGE (Sangon, Biotech), transferred to PVDF membrane. Then the PVDF membrane was blocked for 1 h. Next, the PVDF membrane was incubated with β-actin, IL-1β, TNF-α, IL-17, IL-6, BPGM, GPD1, APOE, APOC, LRP5, DVL1, β-catenin, GSK-3β, Cacybp and Skp1 overnight at 4 °C. Subsequently, the PVDF membrane was washed three times in TBST and incubated with either secondary antibody goat anti-mouse IgG-HRP (diluted 1: 2000, absin, abs20001, China) or goat anti-rabbit IgG-HRP (diluted 1: 2000, absin, abs20002, China) for 1 h. Protein bands were detected with the help of a FluorChem Q scanner (Proteinsimple, CA, USA).

2.9. Statistical analysis

SPSS statistical software version 22.0® (SPSS Inc, CA, USA) was used to analyze the proteomic data and other data, including mRNA expression levels and Western blot validation. All data were calculated by one-way analysis of variance (ANOVA) followed by Tukey's HSD comparison. A value of P less than 0.05 was considered statistically significant.

3. Results

3.1. Analysis of pro-inflammatory cytokines and bone metabolism indexes

AS is characterized by the marked expression of pro-inflammatory cytokines.[21] Inhibitory effect of moxibustion on the tissue level of pro-inflammatory cytokines (IL-1β, TNF-a, IL-17, and IL-6) were measured in proteoglycan-induced spondylitis (PGISp)

mice model by RT-PCR and Western blot. As shown in Fig. 1 and Fig. S1, when compared with the HC group, the tissue mRNA and protein expressions of IL-β, TNF-α, IL-17, and IL-6 were significantly up-regulated in the AS group ($p < 0.01$). Acupuncture points' moxibustion can significantly down-regulate the mRNA and protein expression of these pro-inflammatory cytokines ($p < 0.05$). However, non-acupuncture points' moxibustion can only exert favorable but not significant effects on down-regulating the mRNA and protein expression of these pro-inflammatory cytokines ($p > 0.05$). RANKL, RANK, ALP, and OCN could be used as typical markers to characterize the bone resorption and bone formation of AS mice. In order to evaluate the bone metabolism regulatory effects of moxibustion on AS mice, RANKL, RANK, ALP, and OCN were determined by RT-PCR. As shown in Fig. S2, when compared with the HC group, mRNA expression of RANKL, RANK, ALP, and OCN are significantly increased in the AS group ($P < 0.05$). After moxibustion intervention, the mRNA expressions of RANKL, RANK, ALP, and OCN were significantly down-regulated ($P < 0.05$). The above results suggested that moxibustion could inhibit pro-inflammatory cytokines and regulate bone metabolism for AS mice.

3.2. Histological examinations

The severity of disease and effects of moxibustion on spine histology was assessed in photomicrographs of sections stained with H&E. As shown in Fig. 2, the normal intervertebral joint showed no signs of inflammation, intervertebral disc (IVD) destruction or excessive tissue production and syndesmophyte formation (Fig. 2 A). The histopathological results of the lumbar vertebra samples from AS model group (Fig. 2 B) showed that the IVD, including annulus fibrosus and nucleus pulposus, had been completely destroyed with cartilaginous matrix deposition with chondrocyte expansion evident, which further resulted in the joint space narrowing and syndesmophyte formation. Moreover, mice in the MNA group revealed that the discs were significantly destroyed, and excess tissue production and extensive osteoproliferation were observed, particularly at the vertebral corners (Fig. 2 D). However, moxibustion-intervened mice exhibited mild inflammatory cell infiltration at the periphery of the lumbar vertebra and incomplete IVD destruction. Moreover, moxibustion significantly reverted the spondylitis-induced reduced intervertebral space and chondrocyte expansion (Fig. 2 C). Taken together, these findings indicate that acupuncture points' moxibustion inhibits the development and severity of spondylitis in mice.

3.3. DIA-MS based proteomics results

To explore the mechanism of action of moxibustion on AS, a global analysis of proteomics was used to discover the proteins whose expression was affected by moxibustion intervention. In summary, moxibustion-target protein profiling was measured by a DIA-MS based proteomic platform. According to the pre-defined criteria, DEPs that changed >1.33 or <0.77 and FDR <0.05 found from the HC group and AS group were forty-two. Twenty-five proteins of these forty-two DEPs were significantly altered after moxibustion at specific acupuncture points, which included 3 overexpressed proteins and 22 down-regulated proteins. However, only six of these twenty-five candidate proteins (Kng1, Krt19, Psmc6, Crip1, Uggt1, Erp44) were also significantly altered in moxibustion at non-acupuncture points. Quantitative comparison of these twenty-five DEPs is listed in Supplementary Table 2 and Fig. S3.

Next, to understand the biological mechanisms associated with the key proteins in important modules, gene-ontology (GO) analysis of 25 candidate moxibustion targeted DEPs were performed using Cytoscape software (Fig. 3). The top 10 most enriched biological functions were related to cellular homeostasis, macromolecular complex remodeling, lipoprotein and phospholipid metabolic process. The top 10 molecular function terms were functionally associated with the antioxidant reaction and glycolysis metabolism (UPD-glucose and BPGM activity). Additionally, the cellular component analysis revealed that 25 candidate DEPs were mainly linked with the lipoprotein particle.

After that, we employed bioinformatic analysis to elucidate the key biological signaling pathways that involved in the mechanism of moxibustion for AS. KEGG enrichment analysis showed that ten

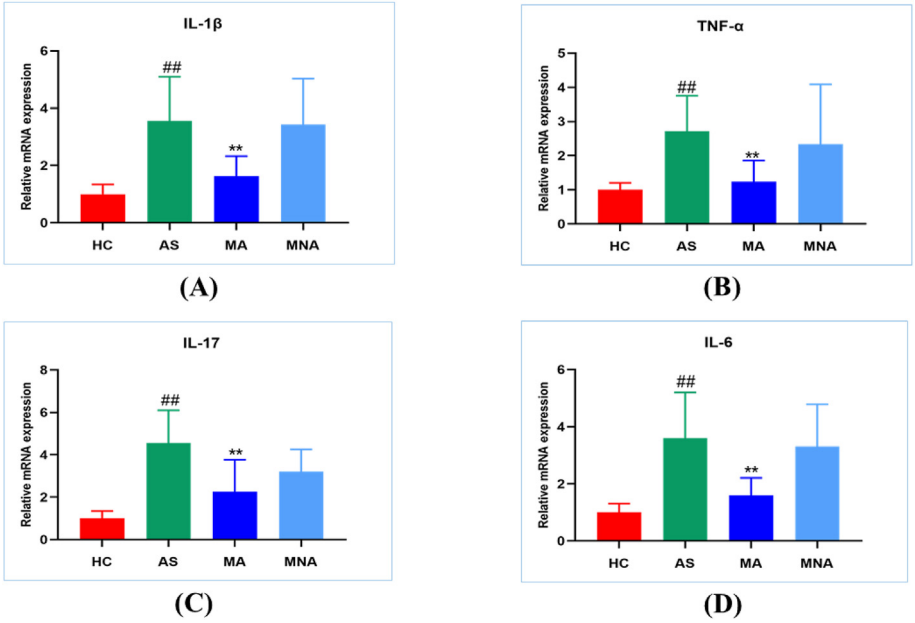

Fig. 1. IL-1β, TNF-α, IL-17 and IL-6 gene mRNA expression levels in ligament tissue of mice, as assessed by reverse transcription-polymerase chain reaction (RT-PCR) (HC: n = 8; AS: n = 8; MA: n = 8; MNA: n = 8). Data are presented as fold change compared to that of the HC group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: HC vs. AS, $^{*}p < 0.05$, $^{**}p < 0.01$: AS vs. MA.

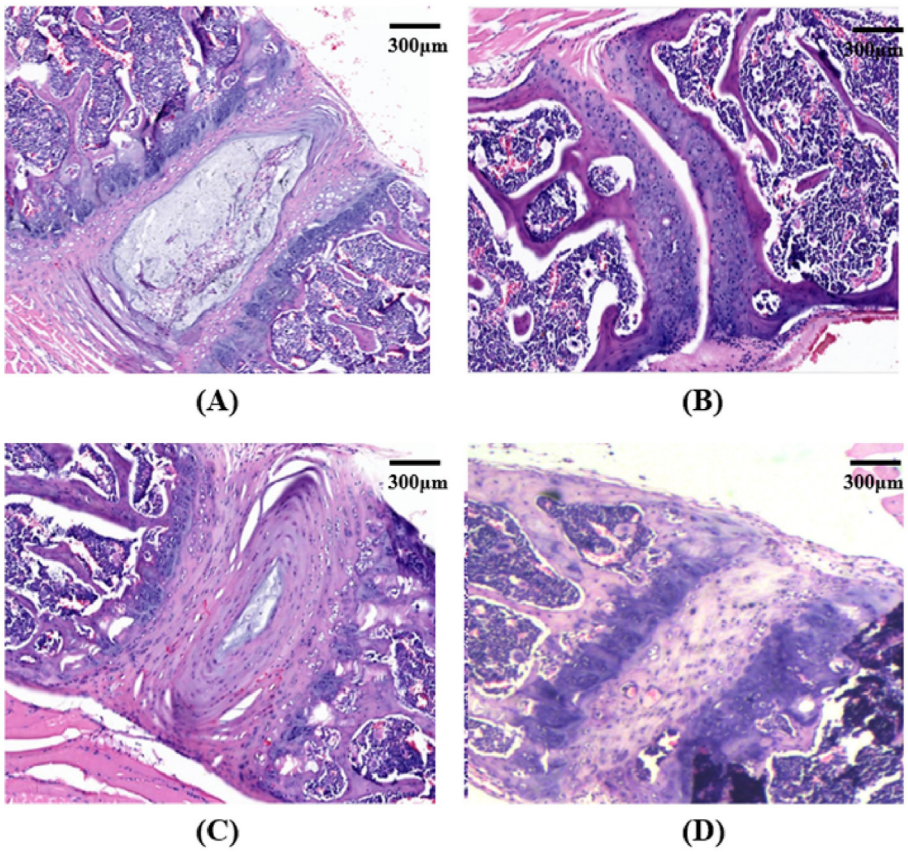

Fig. 2. Histopathological features of the lumbar vertebra at four different groups with hematoxylin and eosin staining (H&E) (Scale bars represent 300 μm in each group). A: HC group, B: AS group, C: MA group, D: MNA group.

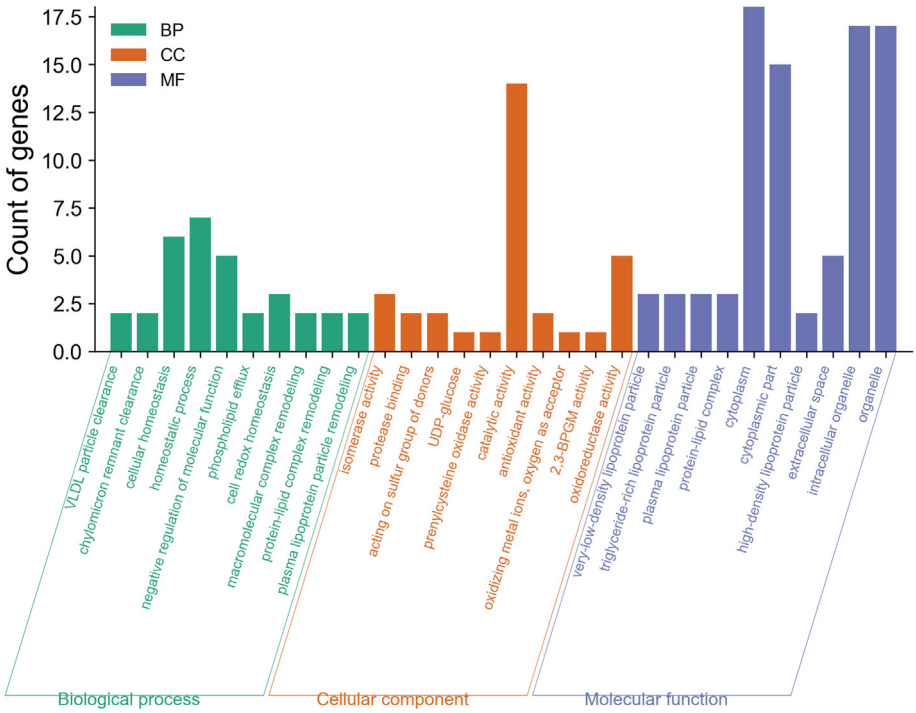

Fig. 3. Gene ontology (GO) annotation of identified tissue ligament proteins according to biological function category, molecular function category, and subcellular localization category. The bars show the count of genes in each GO terms, and the top 10 GO terms were shown.

canonical pathways including Wnt signaling pathway, Terpenoid backbone biosynthesis, processing in the endoplasmic reticulum, proteasome, mineral absorption, glycine, serine and threonine metabolism, glycerophospholipid metabolism, complement and coagulation cascades, cholesterol metabolism, and arachidonic acid metabolism were significantly altered by moxibustion therapy (Fig. 4).

To investigate possible protein-protein interactions among selected DEPs, we performed a proteomic analysis with STRING online software. Finally, one predicted proteomic network of 25

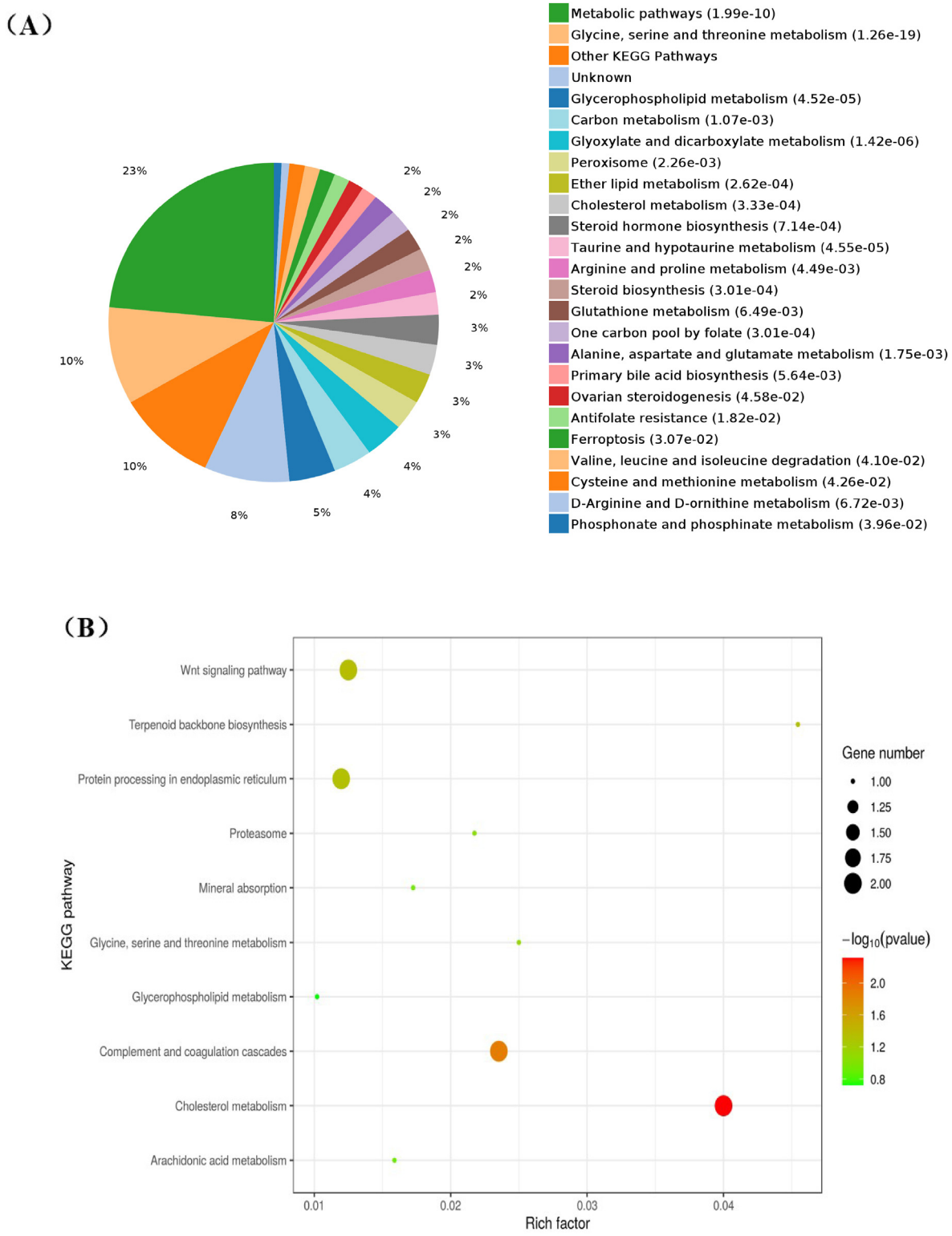

Fig. 4. KEGG classification of the identified proteins in ligament tissue samples using the Omicbean classification system. (B) KEGG enrichment scatter plot of differential expressed proteins in the ligament tissue. The x-axis indicates the rich factor, and the y-axis indicates the name of the pathway. The dot size means the gene number, and the dot color represents the *P* value. The top 10 KEGG pathways were shown.

candidate DEPs was established in Fig. S4. Interestingly, most of these KEGG pathways were associated with metabolic pathways (23%) (Fig. 4A), and the Wnt signaling pathway was the top pathway involving in the effect of moxibustion for AS mice (Fig. 4B).

3.4. Multi-omic analysis and Western blot and RT-PCR validation

Our preliminary tissue-based metabolomics research had found some important small-molecule metabolites and metabolic pathways that are involved in the mechanism of moxibustion for AS mice.[12] To provide further insights into the regulatory effects of moxibustion intervention in AS mice, integrated pathway analysis and multi-omic interaction network analysis were employed based on significantly differentially expressed proteins and metabolites via OmicBean software and MetaboAnalyst 5.0. Here, the protein-metabolomic integrated KEGG pathway analysis showed that glycine, serine, and threonine metabolism and two lipid metabolism pathways, including glycerophospholipid metabolism and cholesterol metabolism, were the most key canonical pathways involved in the anti-AS effect of moxibustion (Fig. 5A). The detailed moxibustion targeted protein-metabolite network diagraph was depicted in Fig. 5B.

Notably, BPGM, $APOC_2$, APOE, and GPD1 played a vital role in the moxibustion targeted multi-omic network picture, and were chosen for further Western blot and RT-PCR validation. The results showed that BPGM, $APOC_2$, and GPD1 protein and mRNA expression were significantly increased, and APOE significantly decreased, in the AS mice group ($P < 0.05$). However, the levels of the above four proteins were significantly reversed by moxibustion at specific acupuncture points, consistent with the results of the quantitative DIA-MS screening (Fig. 6 and Fig. S5).

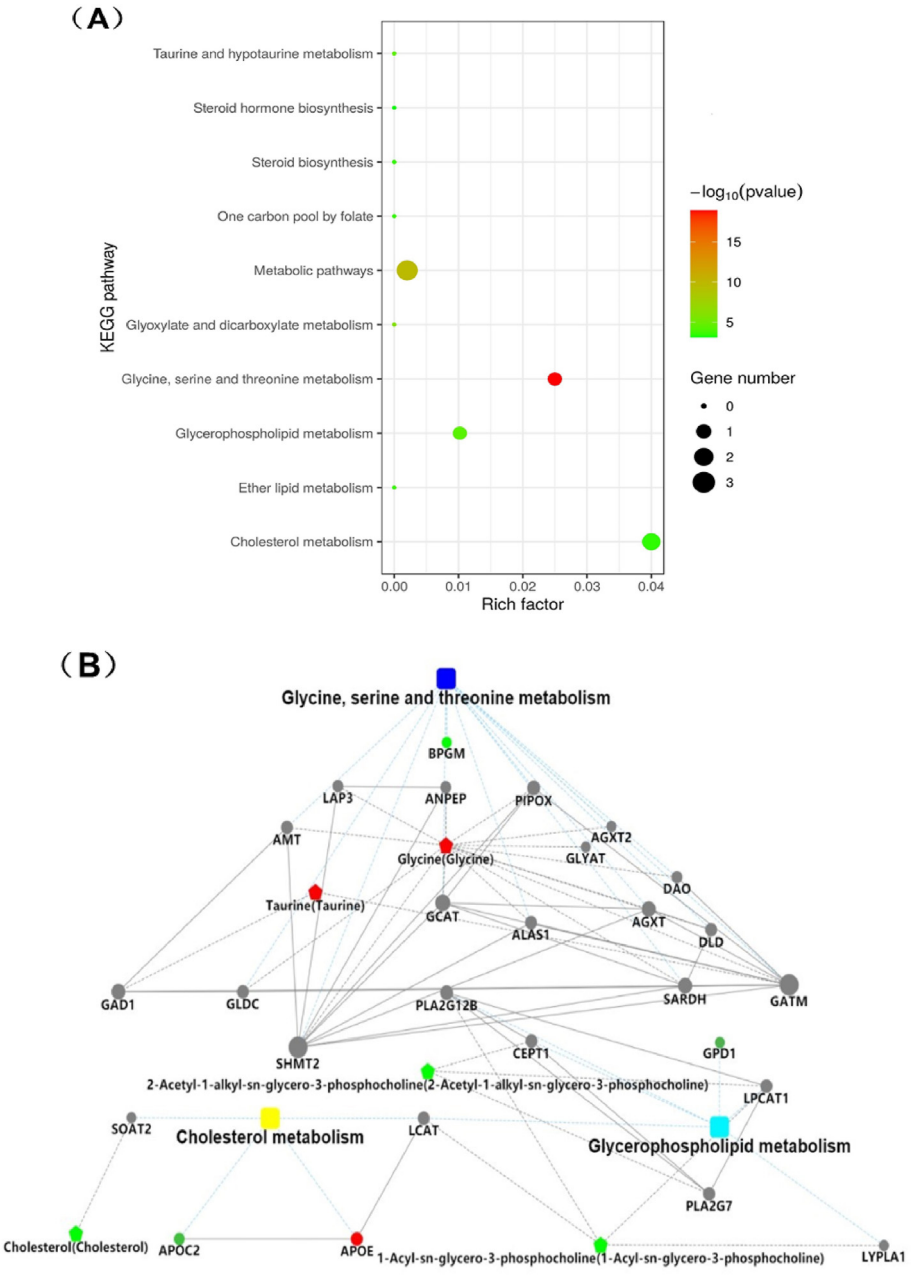

Fig. 5. (A) Four canonical metabolomic pathways categories of the differential metabolites and proteins affected. (B) Protein-metabolite interaction network.

3.5. Moxibustion may attenuate AS mice via inhibition of canonical Wnt/β-catenin signaling pathway by APOE

Among four validated proteins, APOE was an important protein involved in cholesterol metabolism, which might bridge the lipid metabolism and bone metabolism via binding lipid protein LRP5. Interestingly, LRP5 was also a Wnt coreceptor, which mainly modulated the osteoclastogenesis and bone formation of AS. Thus, moxibustion may attenuate AS mice via prohibiting the Wnt signaling pathway by APOE. DIA-MS proteomic analysis had demonstrated that Skp1 and CacyBP were involved in ubiquitination degradation of the Wnt/β-catenin pathway (Supplementary Table 2). Thus, as shown in Fig. 7, the Western blotting was further used to detect the inhibitory effect of moxibustion on the Wnt/β-cateninsignaling pathway. The results showed that compared to the HC group, the expression of upstream proteins of the Wnt/β-catenin pathway, including LRP5, DVL1, and core protein β-catenin in AS group, were significantly up-regulated ($P < 0.05$); the expression of GSK-3β, CacyBP, and Skp1, involved in the ubiquitination degradation of the Wnt/β-catenin pathway, was significantly down-regulated ($P < 0.05$). However, the levels of the above six proteins were significantly reduced by moxibustion at specific acupuncture points ($P < 0.05$) (Fig. 7).

4. Discussion

In general, the results of this study clearly showed that moxibustion might significantly reduce the levels of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-17. Moreover, moxibustion may also alleviate bone erosion and prohibit the new bone formation by decreasing the mRNA expression of RANKL, RANK, ALP, and OCN. DIA-MS based proteomic study showed that a total of 25 DEPs seemed as the moxibustion-target proteins for AS mice, and multi-omics analysis further proved that glycine, serine, and threonine metabolism and lipid metabolism might be the key pathways of moxibustion treatment of AS mice.

RANKL and its cell surface binding receptor RANK, are mainly expressed on OC precursor cells, which play an essential role in osteoclastogenesis. The previous systematic review, including 20 original studies, had revealed that the expression of RANKL/RANK in AS patients was significantly higher than that in healthy volunteers, which may be mainly attributed to the inflammation cytokines-mediated bone erosion.[22] To further explore this issue, on one hand, some pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, may directly enhance osteoclast differentiation and lead to structural damage of AS through binding with cytokines-receptor.[23] On the other hand, under systematic inflammation condition of AS,

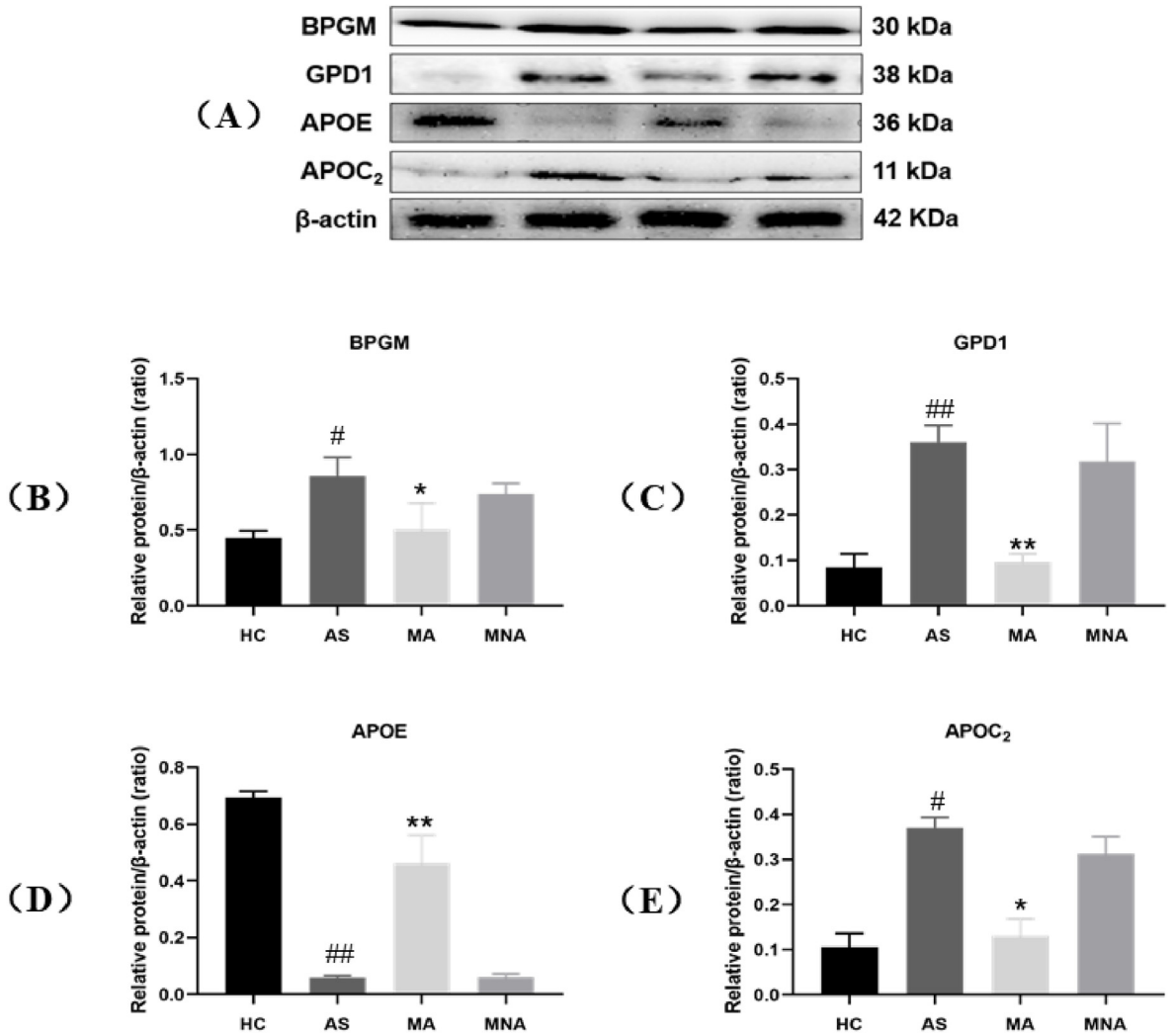

Fig. 6. Western blot study of BPGM, GPD1, APOE and $APOC_2$ of ligament tissue under moxibustion interventions: (A) Representative Western blot of each target protein expression & (B–E) Graph representing semi-quantitative analysis of target proteins in each group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: HC vs. AS, $^{*}p < 0.05$, $^{**}p < 0.01$: AS vs. MA.

Th17 cells can secrete IL-17, which further induce local inflammation releasing on the tissue ligament of AS and result in exaggerating RANKL/RANK expression on fibroblasts of tissue ligament and bone erosion.[23] The previous clinical review had demonstrated that moxibustion might improve the physical function and decrease the disease activity of AS patients via decreasing the expression of RANKL and RANK in serum.[24] In line with the previous clinical study, moxibustion may alleviate bone erosion and protect the spine structure of AS mice via downregulating the inflammatory cytokines and further inhibiting the RANKL/RANK signaling pathway.

BPGM is a vital energy metabolic enzyme that is primarily involved in energy metabolism. It has been well-reported that energy proteins are significantly elevated under the systematic inflammation conditions in AS patients.[25] Previous *in vivo* studies had demonstrated that moxibustion could increase the serum levels of energy proteins so as to modulate the energy metabolism in CIA rats.[14] In line with the previous *in vivo* studies, moxibustion can also down-regulate the tissue levels of BPGM in AS mice, which show a critical role in correcting the impairment of energy metabolism.

According to recent studies, the deterioration of lipid profile is very prevalent in AS patients, and abnormal lipid metabolism, especially the dysregulation of cholesterol metabolic pathway, is closely related to the cartilage dysfunction and new bone formation of AS patients.[26] In this research, three key lipid proteins, involved in the cholesterol metabolism pathway of AS mice, were screened by multi-omics analysis (Fig. 5). Notably, Schilling et al. had found out that APOE is expressed by late differentiated osteoblasts isolated from the calvaria of 3-day-old mice through microarray differential expression analysis and RT-PCR verifying analysis.[27] Moreover, Schilling et al.[27] pointed out that APOE, rather than other apolipoproteins, plays a unique physiological role in regulating bone formation. Previous clinical study[28] showed that the expression level of AOPE in serum of AS patients was significantly lower than that of healthy people. Consistent with the previous clinical study,[28] the multi-omic analysis and subsequent Western blot validation analysis also showed that the expression level of APOE protein in ligament tissue of AS group was significantly lower than that of the HC group (Figs. 5 and 7).

To explore this issue, LRP5 has behaved as an APOE receptor among the LDLR family, which may bind APOE-containing lipoproteins both *in vivo* and *in vitro*, and have an impact on APOE levels.[29–31] Interestingly, APOE may also act as Wnt negative

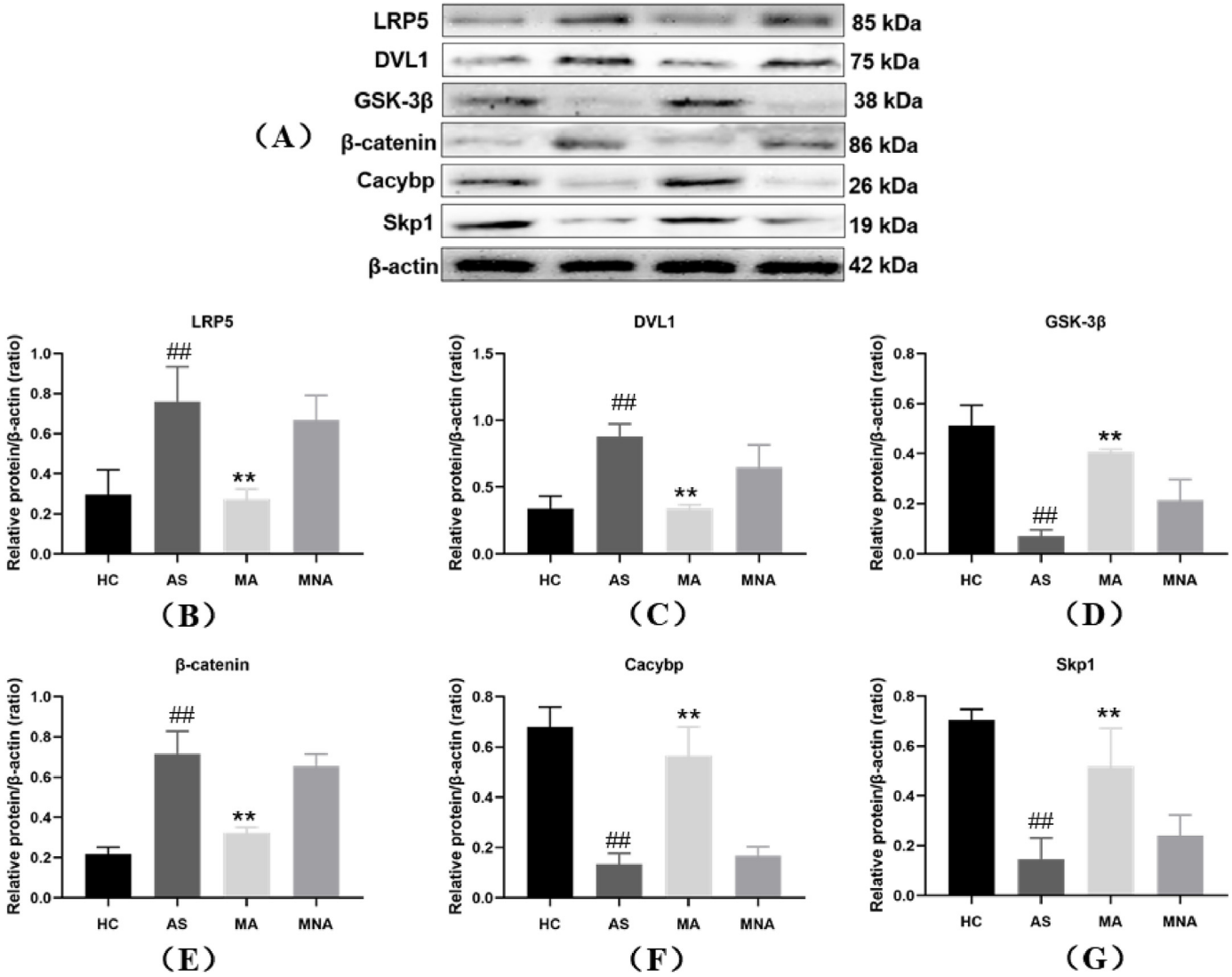

Fig. 7. Western blot study of LRP5, DVL1, GSK-3β, β-catenin, Cacybp and Skp1 of ligament tissue under moxibustion interventions: (A) Representative Western blot of each target protein expression & (B–G) Graph representing semi-quantitative analysis of target proteins in each group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: HC vs. AS, $^{*}p < 0.05$, $^{**}p < 0.01$: AS vs. MA.

regulator to be implicated in modulating the Wnt signaling pathway by interacting with LRP5 during the osteoblast differentiation development.[32] So far, several recent papers have demonstrated that the Wnt/β-catenin signaling pathway might play a critical role involving the osteoclastogenesis of AS.[33] Interestingly, enriched KEGG pathway analysis in this study also have demonstrated that the Wnt signaling pathway was the top pathway, involving in the pathogenesis of AS and some downstream proteins of the Wnt signaling pathway (Cacybp and Skp1), were significantly altered following AS mice modeling (Fig. 4 and Supplementary Table 2). In the physiological condition, β-catenin in the Wnt signaling pathway is degraded by a complex composed of glycogen synthase kinase-3β (GSK-3β). Moreover, CacyBP interacting with Skp1 is also involved in the ubiquitination and degradation of β-catenin.[34] However, when the Wnt/β-catenin signaling pathway was activated in bone, Wnts can bind to Frizzled-LRP5 receptors as the complex molecular at the cell surface, which may further lead to the inhibition of GSK3β and CacyBP/Skp1 complex mediated by disheveled (Dvl) protein. In this condition, the degradation capacity of β-catenin was decreased, and the β-catenin was accumulated in the cytoplasm and translocated into the nucleus, leading to up-regulating the levels of ALP and OCN.[35]

However, inhibition of the Wnt/β-catenin signaling pathway by down-regulating the levels of TNF-α and subsequently up-regulating the levels of APOE following moxibustion therapy might result in alleviating the pathological cascade leading to new bone formation in AS because the activation of Wnt/β-catenin signaling has been closely linked with osteoclastogenesis of AS. Similarity, in addition to the proteins in the Wnt/β-catenin signaling pathway (Fig. 7) altered following moxibustion intervention, the downstream specific osteoblast proteins, including ALP and OCN were also suppressed following moxibustion intervention (Supplementary Fig. S2).

5. Conclusion

In conclusion, moxibustion may reduce pro-inflammatory cytokines and improve bone erosion for AS mice. DIA-MS proteomic analysis identified 25 differentially expressed proteins, and pathway analysis revealed that the Wnt/β-catenin pathway played a key role in the regulation of AS by moxibustion. Moreover, combined analysis of differential proteins and metabolites suggested that lipid metabolism, especially APOE protein, might be involved in mediating the effects of moxibustion for AS mice. Western blot verifying analysis further showed that regulation of APOE by moxibustion might have a potential inhibitory effect on the Wnt/β-catenin pathway in AS mice. However, due to the lack of silencing or overexpression of key molecules of the signal pathway, the beneficial and positive effect of moxibustion involved in the regulation of the Wnt/β-catenin signaling pathway by APOE and other aspects are needed to be explored in further study.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2022.04.002.

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