Involvement of MicroRNA-27a-3p in the Licorice-Induced Alteration of Cd28 Expression in Mice

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Abstract: Licorice has previously been shown to affect gene expression in cells; however, the underlying mechanisms remain to be clarified. We analyzed the microRNA expression profile of serum from mice treated by gavage with licorice decoction, and obtained 11 differentially expressed microRNAs (DEmiRNAs). We also screened differentially expressed genes (DEgenes) based on RNA-Seq data, and 271 common genes were identified by intersection analysis of the predicted target genes of 11 DEmiRNAs and the DEgenes. The miRNA–gene network showed that most of the hub genes were immune-related. KEGG enrichment analysis of the 271 genes identified three significant pathways, and the 21 genes involved in these three pathways, and the 11 DEmiRNAs, were constructed into a miRNA pathway–target gene network, in which mmu-miR-27a-3p stood out. Compared to ImmPort, there were 13 immune genes within the above group of 21 genes, and three intersected with the mmu-miR-27a-3p predicted target genes, Cd28, Grap2, and Cxcl12, of which the expression of Cd28 changed most significantly. We confirmed the regulation of Cd28 by mmu-miR-27a-3p using a dual-luciferase assay, and further confirmed that overexpression of mmu-miR-27a-3p could significantly downregulate the expression of Cd28 in lymphocytes. These results indicate that mmu-miR-27a-3p could be involved in the licorice-mediated regulation of the expression of Cd28 in mice.

Keywords: licorice; gene expression; regulation; miRNA; Cd28; mmu-miR-27a-3p

1. Introduction

Licorice (Glycyrrhiza uralensis Fisch) is one of the oldest and most frequently used medicinal plants in both the East and the West [1]. Classified as a form of tonic Chinese herbal medicine, licorice is used to treat splenic dysfunction, stomach weakness, fatigue, lack of strength, palpitation, dyspnea, cough, profuse sputum, acute pain in the abdominal cavity, limb contracture, carbuncles, and sores, as well as to alleviate drug toxicity [2–6]. Studies have shown that licorice has many pharmacological effects, and can exert anti-inflammatory, antiviral, and antitumor activities, as well as function in immune regulation [7–14].

It was reported that licorice can be used to mediate apoptosis and oxidative stress by regulating the p-STAT3 signaling pathway in the kidney, thereby alleviating the nephrotoxicity caused by strychnine [15]. Licorice can also promote the apoptosis of some cancer cells, inhibit their growth and reproduction, and reverse the drug resistance of ABCG2-mediated drug-resistant cancer cells in a concentration-dependent manner [16]. Furthermore, it was
shown that licorice can induce apoptosis and improve cell metabolism by regulating the expression of miRNA in cancer cells, which indicates the potential for its use in the prevention and treatment of cancer [17]. In addition, liquiritigenin, an active compound found in licorice roots, can inhibit IL-1β-induced inflammation and cartilage matrix degradation in rat chondrocytes [18]. Glycyrrhetinic acid induces Sirtuin 6 (Sirt6) overexpression by inhibiting the translocation of HMGB1 protein from the nucleus to the cytoplasm in human nasal epithelial cells, and reduces its extracellular accumulation. Thus, Sirt6 plays an important role in maintaining nasal mucosal homeostasis, suggesting that this compound may be useful for the treatment of chronic rhinosinusitis associated with nasal polyps [19]. Together, these studies indicate that licorice may significantly affect gene expression in the body.

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules composed of 21–24 nucleotides that regulate mRNA expression by recognizing homologous sequences and interfering with the transcription, translation, and epigenetic processes of protein-coding genes [20–22]. As one of the common regulators of gene expression, miRNAs participate in many biological processes, such as cell proliferation, differentiation, apoptosis, growth, and development [23,24]. At present, increasing evidence shows active components of traditional herbal medicine can regulate gene expression through miRNAs, underscoring the need to study these molecules for their potential mechanism of action in herbal medicines [25,26].

Here we report the effect of licorice decoction on the miRNA expression profile in mice, and further confirm the effect of licorice on the immune status of mice by exploring the regulatory relationship between mmu-miRNA-27a-3p and Cd28.

2. Materials and Methods
2.1. Preparation of Licorice Decoction
Considering that water decoction is the traditional processing method of Chinese herbal medicine, in order to better simulate the therapeutic effect of licorice in practice, decoction was prepared to treat mice. Radix Glycyrrhizae Preparata (25 g) was incubated in 100 mL pure water and soaked at room temperature for 20 min, then boiled and kept at 60 °C for 2 h after boiling. Supernatant was then collected and the sediment was resuspended in 100 mL pure water, the above decocting steps repeated, and supernatant collected again. The supernatants were combined and concentrated to 25 mL with a rotary evaporator (90 °C, 0.08 MPa, 14 r/min) to prepare the licorice decoction at a concentration of 1 g/mL.

2.2. Animal Experiments
All experiments using mice were performed using protocols approved by the Guangdong Pharmaceutical University Institutional Animal Care and Use Committee. Specific pathogen-free (SPF) BALB/c male mice (Animal Experiment Center, Guangzhou University of Traditional Chinese Medicine, Guangzhou, China), 7–8 weeks old (about 18–22 g), were maintained in an SPF environment with a 12 h light/dark cycle, and provided with autoclaved mouse chow and water ad libitum for the duration of the experiment. After 3 days of adaptive feeding, mice in the experimental group were given 0.2 mL/d licorice decoction by gavage for 7 days, and the mice in the control group were given normal saline under the same conditions. There were no less than three mice in each group. At 24 h after the last gavage, mice were sacrificed and the tissue samples were collected.

2.3. MiRNA Expression Profile
The total RNA of mouse serum was extracted using TRIzol and subjected to small RNA sequencing (sRNA-seq) analysis (Guangzhou Ribo, Guangzhou, China). TMM (trimmed mean of M-values) in the BioConductor Edger software package (Edger installation package for R language) was used to normalize the miRNA expression and perform statistical analysis to identify differentially expressed miRNAs (DEmiRNAs). The screening crite-
ria were $|\log(\text{fold change})| \geq 1.5$, $p$-value $\leq 0.01$, and “ggplot2” was used to perform data visualization.

2.4. Transcriptome Analysis

Total RNA was extracted from mouse small intestine using TRIzol and subjected to RNA-seq analysis (Novogene, Beijing, China). TMM (trimmed mean of M-values) in the BioConductor Edger software package was used to normalize gene expression and perform statistical analysis to identify the differentially expressed genes (DEgenes). The screening criteria were $|\log(\text{fold change})| \geq 1.5$, $p$-value $\leq 0.01$. The STRING 11.5 online tool was used to analyze the interaction network of DEgenes with a confidence level of 0.95, and the network was imported into Cytoscape3.9.0 software to further identify the core genes using the Cytohubba plugin. The DEgenes were subjected to gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis using the R packages “clusterProfiler” and “pathview”.

2.5. MiRNA Target Gene Prediction

The multiMiR R package containing eight major databases (DIANA-microT, EIMMo, MicroCosm, miRanda, miRDB, PicTar, PITA, TargetScan) was used to predict the miRNA target genes. Meanwhile, miRWalk3.0 (http://mirwalk.umm.uni-heidelberg.de/, accessed on 22 March 2021) was used for online prediction of miRNA target genes, and the results were compared with the R package to screen for common target genes.

2.6. Identification of Key miRNAs

The predicted target genes were intersected with the DEgenes, and the common genes were subjected to GO and KEGG enrichment analysis as above. Genes from significantly enriched KEGG pathways and DEmiRNAs were used to construct the miRNA–gene network with Cytoscape software (v3.9.0). Genes with expression levels negatively correlated with DEmiRNA were selected and counted, and the DEmiRNAs exhibiting regulatory relationships with several of these genes were selected as candidate target miRNAs. In addition, the screening criteria of DEmiRNAs were further improved to reduce the number of DEmiRNAs, which were then compared with the above candidate target miRNAs.

2.7. Identification of mmu-miR-27a-3p Target Genes

The genes significantly enriched in KEGG pathways were compared with the immune-related genes from ImmPort (https://www.immport.org/home, accessed on 9 April 2021), and the common genes were then compared with the predicted target genes of mmu-miR-27a-3p. The miRNA binding sites on target genes were predicted using starBase 3.0 (http://starbase.sysu.edu.cn/index.php, accessed on 9 April 2021).

2.8. Construction of Dual-Luciferase Reporter Plasmid

The miRNA binding site within the Cd28 3′UTR was predicted using starBase 3.0 (http://starbase.sysu.edu.cn/index.php, accessed on 9 April 2021), and primers were designed accordingly (Supplementary Table S1). As a control, a mutant Cd28 3′UTR was prepared by replacing the sequence of the miRNA binding site by overlap PCR with mutation primers (Supplementary Table S1). After PCR amplification and sequencing verification, either wild-type or mutant Cd28 3′UTR were inserted between the Xho I and Not I sites of psiCHECK-2.

2.9. Cell Culture and Transfection

HEK-293T cells (purchased from ATCC) were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37 °C under 5% CO2. Freshly isolated spleens from C57 mice were subjected to grinding, filtering, and centrifuging. Lymphocytes were then isolated from cell pellets using mouse spleen lymphocyte isolation solution (Mouse Organ Tissue Lymphocyte Separation Liquid KIT, Tianjin, China) according to the manufacturer’s protocol. After
transfection, the splenic lymphocytes were cultured in 1640 medium containing 10% mouse autologous serum at 37 °C under 5% CO₂.

1 × 10⁶ HEK-293T cells (100 µL) were transfected with 3 µg of the psiCHECK2 construct and 100 pmol mimics (Guangzhou Ribo, Guangzhou, China), using the NEPA21 Super Electroporator (NEPA GENE, Ichikawa, Japan) instrument. In addition, 1 × 10⁶ splenic lymphocytes (100 µL) were transfected with mimics (Guangzhou Ribo, Guangzhou, China) using NEPA21 Super Electroporator.

2.10. Luciferase Reporter Assay

Cells were collected 24 h after transfection, and Renilla and Firefly luciferase activity was assessed using the TransDetect® Double-Luciferase Reporter Assay Kit (TransGen Biotech, Beijing, China) according to manufacturer’s protocol. Each sample was repeated in triplicate. RL/FL ratio = Renilla luciferase reading/Firefly luciferase reading; normalized ratio = RL/FL ratio of mimic group divided by the RL/FL ratio of control group.

2.11. Flow Cytometry

Splenic lymphocytes were collected 29 h after transfection and stained with anti-Cd28 (BioLegend, San Diego, CA, USA). Each sample was repeated in triplicate. The expression of Cd28 was detected by flow cytometry (Beckman, Brea, CA, USA).

2.12. Quantitative Real-Time PCR (qRT-PCR)

The Cd28 expression level was determined by qRT-PCR using specific primers (Supplementary Table S2). The qPCR reaction was performed using Hieff® qPCR SYBR Green Master Mix (Yeasen Bio, Shanghai, China). Each sample was repeated in triplicate. The relative expression level was quantified using the 2⁻ΔΔCt method, and the GAPDH gene was used as the control.

2.13. Statistical Analysis

SPSS 26.0 statistical software was used to analyze the data. The graphs were made using R package ggplot2, Cytoscape 3.9.0, or GraphPad Prism 8. The values were compared by one-way analysis of variance with 0.05 as the minimum level of significance.

3. Results

3.1. Screening of Differentially Expressed microRNAs and Prediction of Their Target Genes

According to certain screening criteria, and the relatively large expression baseline (including at least one group of expression baseline ≥ 1000), 11 DEmiRNAs were identified, including five upregulated miRNAs and six downregulated miRNAs (Figure 1A,B). Two methods, multiMiR R package and miRWalk3.0 online tools, were used to predict the target genes of the 11 DEmiRNAs, resulting in the identification of 10,224 and 9501 candidate target genes, respectively, of which 5712 genes were shared (Figure 1C).

The shared candidate target genes were subjected to GO and KEGG enrichment analysis. The GO enrichment results show that, with respect to molecular functions (MF), target genes were mainly enriched in DNA-binding transcription activator activity, RNA polymerase II-specificity, and nucleoside-triphosphatase regulatory activity. For cellular components (CC), target genes were mainly enriched in the neuron-to-neuron synapse, postsynaptic density, or the synaptic membrane. For biological processes (BP), target genes were mainly enriched in synapse organization, dendrite development, regulation of cell growth, and axonogenesis (Figure 1D). In addition, KEGG enrichment results showed that these target genes were mainly related to the MAPK signaling pathway, axon guidance, and the Ras signaling pathway (Figure 1E).
showed that these target genes were mainly related to the MAPK signaling pathway, axon guidance, and the Ras signaling pathway (Figure 1E).

3.2. Screening of DEgenes Based on RNA-Seq

According to certain screening criteria, 1398 DEgenes were identified, including 1033 upregulated genes and 365 downregulated genes (Figure 2A,B). In order to explore the interaction between the DEgenes, a protein–protein interaction (PPI) network was generated using the STRING online tool. Then, 212 core genes were identified using Cytoscape 3.9.0 software with the Cytohubba plugin. A majority of the top 20 genes were immune-related genes (Figure 2C). The GO analysis showed that the DEgenes were mainly enriched in antigen binding and immunoglobulin receptor binding, in terms of MF; immunoglobulin complex and circulating, in terms of CC; and B cell activation and immunoglobulin production, in terms of BP (Figure 2D). KEGG analysis showed that the DEgenes were mainly involved in immune-related pathways, such as cytokine–cytokine receptor interactions and cell adhesion (Figure 2E). These results show that the DEgenes identified are closely related to immune function. Therefore, the immune status of the body could be affected by licorice treatment.
3.3. Comprehensive Analysis of DEgenes and Predicted Target Genes of DEmiRNAs

Figure 2. Screening of DEgenes based on RNA-Seq. (A) Volcano plot of differential miRNAs with $|\log (\text{fold change})| \geq 1.5, p \leq 0.01$ as screening criteria. (B) Distribution of upregulated and downregulated DEgenes. (C) The interaction network between DEgenes generated using the STRING online tool and visualized with Cytoscape 3.9.0, in which the core genes were identified using Cytohubba plugin. (D) GO enrichment of DEgenes. (E) KEGG enrichment of DEgenes.
3.3. Comprehensive Analysis of DEgenes and Predicted Target Genes of DEmiRNAs

We compared the predicted target genes of DEmiRNAs with the DEgenes based on transcriptomes, and found 271 common genes (Figure 3A). Correlation analysis was carried out between these 271 genes and the 11 DEmiRNAs, resulting in the identification of 243 target genes with negative correlation. The miRNA–mRNA relationship was further visualized using Cytoscape 3.9.0, and scored using the Cytohubba plugin (Supplementary Table S3). Figure 3B shows the top 20 hub target genes. In order to explore the biological functions of the 271 potential target genes, GO and KEGG enrichment analyses were performed. GO enrichment analysis showed that DEgenes were mainly enriched in growth factor binding, sulfur compound binding, sialyltransferase activity, in terms of MF; intrinsic components of synaptic membranes, and intrinsic components of postsynaptic membranes, in terms of CC; and T cell activation, regulation of leukocyte cell–cell adhesion, and other processes closely related to immunity, in terms of BP. On the whole, the enriched genes are mainly involved in immune-related processes, of which the activation of T cell is the most significant (Figure 3C). In the KEGG enrichment analysis, there were only three significant enrichment pathways, cell adhesion molecules, viral protein interaction with cytokines and cytokine receptors, and T cell receptor signaling (Figure 3D). These results further indicate that licorice may exert effects through affecting immune activity.

Figure 3. Comparative analysis of DEgenes and the predicted target genes of 11 DEmiRNAs. (A) The intersection between DEgenes and the predicted target genes of 11 DEmiRNAs. (B) Correlation analysis between 271 common genes and 11 DEmiRNAs identified 243 negatively correlated genes. The miRNA–mRNA relationship was visualized with Cytoscape 3.9.0, in which the core genes were identified using the Cytohubba plugin. (C) GO enrichment of 271 common genes. (D) KEGG enrichment of 271 common genes.

3.4. Identification of mmu-miR-27a-3p as a Key miRNA and Prediction of Its Target Genes

The 21 DEgenes involved in the above three KEGG pathways were collected (Supplementary Table S4), and Cytoscape 3.9.0 was used to identify the miRNA–gene network between them and the 11 DEmiRNAs (Figure 4A). Of these, five DEmiRNAs known to exert negative regulatory effects on a number of genes were selected (Supplementary Table S5,
In addition, the screening criteria of DEmiRNAs were further restricted to $|\log(\text{fold difference})| \geq 2$, $p \leq 0.01$, and another five DEmiRNAs were identified (Supplementary Table S6, Figure 4B). Comparison of the two groups of DEmiRNAs resulted in the identification of one miRNA, mmu-miR-27a-3p (Figure 4B).

Considering the prominent enrichment of genes with immune functions, the 21 DEgenes involved in the above three KEGG pathways were compared with 1541 immune-related genes derived from the ImmPort database, resulting in the identification of 13 common genes (Figure 4C, upper). These genes were compared with the 67 predicted target genes of mmu-miRNA-27a-3p found among the 271 common genes mentioned above, this resulted in the identification of three target genes (Figure 4C, middle). Changes in expression of the three common genes are shown.

Changes in the expression levels of these three genes are shown in the heat map (Figure 4C, lower). Of these, due to the significant expression changes, Cd28 was selected for further investigation because of its regulatory relationship with mmu-miRNA-27a-3p.
The qRT-PCR detection showed that after treatment with licorice decoction, the expression in healthy mice showed significant upregulation (Supplementary Figure S1).

3.5. Regulation of Cd28 Gene Expression by mmu-miRNA-27a-3p

The binding site for miRNA-27a-3p in the Cd28 3′UTR was predicted using starBase 3.0, and a mutant binding site was designed as a control (Figure 5A). The sequences of both the Cd28 3′UTR and the mutant Cd28 3′UTR were amplified by overlap PCR (Figure 5B). The wild-type and mutant Cd28 3′UTR sequences were inserted into the 3′ end of Renilla Luciferase (RLuc) in the psiCHECK-2 vector, and the recombinant vectors were confirmed by sequencing (Supplementary Figure S2).

Figure 5. Regulation of Cd28 gene expression by mmu-miRNA-27a-3p. (A) A miRNA-27a-3p binding site with the Cd28 3′UTR was predicted using starBase 3.0 and a mutant binding site was designed as...
a control. (B) Amplification of the Cd28 3′UTR and the preparation of mutant Cd28 3′UTR by overlap PCR. (C) The RL/FL ratio of each group. (D) The normalized ratio of each group. (E) Flow cytometric analysis of the expression of Cd28 on splenic lymphocytes from C57 mice transfected with mmu-miR-27a-3p mimics. The ratio and mean fluorescence intensity (MFI) were analyzed. (F) qRT-PCR detection of Cd28 in C57 spleen lymphocytes. **, \( p < 0.01; \) ***, \( p < 0.001; \) ****, \( p < 0.0001 \).

HEK-293 T cells were co-transfected with reporter plasmids and the mmu-miRNA-27a-3p mimic or a control mimic. The luciferase assay showed a significant reduction in the RL/FL ratio in the Cd28 3′UTR + miR-27a-3p mimic transfected group (\( p < 0.01 \)) (Figure 5C). The normalized ratio of the Cd28 3′UTR + miR-27a-3p mimics group was also significantly lower than that of mutant Cd28 3′UTR + miR-27a-3p mimics group (Figure 5D). There was no significant difference between the psiCHECKTM-2 vector control and mutant Cd28 3′UTR control (\( p > 0.05 \)). These results indicate that mmu-miR-27a-3p can directly regulate the expression of Cd28.

To confirm these findings, the mmu-miR-27a-3p mimic was transfected into splenic lymphocytes from C57 mice. Flow cytometry results showed that overexpression of mmu-miR-27a-3p reduced the expression level of Cd28 (Figure 5E). Furthermore, qRT-PCR results also showed that Cd28 was significantly downregulated by overexpression of mmu-miR-27a-3p (Figure 5F). These results verify that mmu-miR-27a-3p can regulate the expression of Cd28.

4. Discussion

Although the medicinal value of licorice has been suggested for the treatment of inflammation [27], for liver protection [28], as an antiviral [29], and as an anticancer therapeutic [30], the underlying mechanisms of these processes have not been clarified. In recent years, many studies have shown that the active components of traditional herbal medicine regulate gene expression by way of miRNAs, which may also be the mechanism through which licorice exerts its effects [31–34].

In this study, we found significant changes in the miRNA expression profile of mouse serum after treatment with licorice, including 11 miRNAs with significant differences (Figure 1B). The predicted target genes of these 11 differential miRNAs were subjected to GO enrichment analysis, and the results show the enrichment of genes with DNA-binding transcription activator activity, RNA polymerase II-specificity, involvement in neuron-to-neuron synapses, and synapse organization (Figure 1D). Thus, this implies that licorice potentially exerts comprehensive effects on the gene expression profile of mice.

Transcriptome analysis of the mouse intestine identified 1398 DEgenes following treatment with licorice, with a majority of these top 20 hub genes being immune-related (Figure 2C). GO and KEGG analysis showed that the enriched genes are known for biological functions and their involvement in pathways mainly related to the immune system (Figure 2D,E). In order to investigate the association between DEMiRNAs and DEgenes, we compared the predicted target genes of DEMiRNAs with DEgenes, and found 271 common genes. KEGG analysis of these genes identified three significantly enriched pathways (Figure 3D). The miRNA pathway–gene interaction network comparison of 21 genes involved in the three pathways, and the 11 DEMiRNAs identified five DEMiRNAs with known negative regulatory relationships with a number of genes. Furthermore, five DEMiRNAs using higher screening criteria were identified based on sRNA-seq data. As the only common miRNA between the two groups of DEMiRNAs, mmu-miR-27a-3p was particularly noteworthy (Figure 4B), as it reportedly regulates immune cell function [35]. Moreover, overexpression of miR-27a-3p can inhibit the inflammatory response during spinal cord injury, by reducing TLR4 and inhibiting the expression of TNF-α and IL-6 [36]. In breast cancer, exosomal miR-27a-3p was used as an intervention target to inhibit PD-L1 expression in macrophages, and to block the activation of the PTEN-AKT/PI3K pathway to inhibit immune escape in breast cancer cells [37]. In addition, it was reported that adipocyte-derived exo-miR-27a-3p can inhibit ICOS+ T cell proliferation and IFN-gamma secretion [38]. There-
fore, it is reasonable to speculate that mmu-miR-27a-3p could be involved in the regulation of gene expression, and especially of immune-related genes, by licorice.

To identify mmu-miR-27a-3p target genes, we compared the 21 genes involved in the above three pathways with 1541 immune genes derived from the ImmPort database, and found 13 common genes. These 13 genes were then compared with predicted target genes of mmu-miRNA-27a-3p, and three common genes, Cd28, Grap2, and Cxc112, were identified (Figure 4C). Combined with the expression changes, Cd28 was selected for further exploration of its regulation by mmu-miR-27a-3p.

A luciferase assay showed that mmu-miR-27a-3p could affect the expression of a reporter gene downstream of the Cd28 3′UTR (Figure 5C,D). Furthermore, both flow cytometry and qRT-PCR showed that overexpression of mmu-miR-27a-3p in the splenic lymphocytes of C57 mice could significantly downregulate the expression of Cd28 (Figure 5E,F). These results confirm that Cd28 is a target gene of mmu-miR-27a-3p.

It is well known that Cd28 plays a pivotal role in T cell activation. In addition, Cd28 is necessary for humoral responses to viral infection, vaccination, and in allergy models [39–41]. It was reported that Cd28 signaling in plasma cells (PC) is essential for promoting plasma cell survival and sustaining antibody titers [42,43]. Cd28 also promotes the functional maturation of NKT cells and the development of innate-like CD8+ T cells [44]. Boesteanu et al. showed that both CD4+ and CD8+ memory T cells require Cd28 co-stimulation for maximal expansion and pathogen clearance [45]. However, it was reported that the B7/Cd28/CTLA4 pathway has the ability to both positively and negatively regulate immune responses [46]. Moreover, Cd28 plays a crucial role in the maintenance of regulatory T cell (Treg) pool size through promoting the development and proliferation of these cells [47]. B7-Cd28 co-stimulation modulates central tolerance via thymic clonal deletion and Treg generation [48]. Interestingly, it was reported that glycyrrhizin can regulate T and B cell proliferation, and improve cognition in aging mice [49]. Furthermore, it was reported that glycyrrhizin may reverse the increased susceptibility of thermally injured mice to HSV infection through the induction of CD4+ CD28+ contrasuppressor T cells [50], and that glycyrrhizic acid can upregulate Foxp3 and enhance the activity of Treg cells [51,52]. Therefore, further study of the role of mmu-miR-27a-3p and Cd28 in the regulation of immune responses following treatment with licorice is warranted.

5. Conclusions

In conclusion, our findings reveal that treatment with licorice can significantly alter the gene expression profile of mice and affect the expression of both miRNAs and genes. Moreover, we identify a regulatory relationship between mmu-miR-27a-3p and Cd28, which lays the foundation for further elucidating the mechanism of action of licorice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13071143/s1. Figure S1. qRT-PCR detection of Cd28 in mice treated with licorice decoction; Figure S2. Sequencing results of the recombinant dual-luciferase reporter vectors; Table S1. Primers used for vector construction; Table S2. Primers used for qRT-PCR; Table S3. The hub genes involved in the miRNA–mRNA network; Table S4. The DEgenes involved in the three enriched KEGG pathways; Table S5. The DEmiRNAs involved in regulating the 21 DEgenes; Table S6. The DEmiRNAs with screening criteria |log(fold difference)| ≥ 2, p ≤ 0.01; Table S7. The target genes of mmu-miRNA-27a-3p among 271 common genes.

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