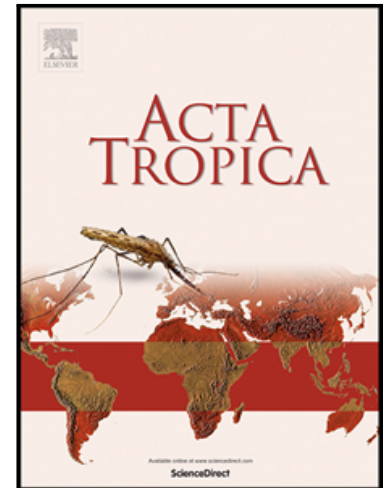


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Construction of ceRNA regulatory network in mice with *Echinococcus*-induced allergic reactions

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Highlights

1. The ceRNA network associated with *Cystic echinococcus* sensitization in mice was constructed;
2. The biological functions were specifically interacted with the immune inflammatory response in *Cystic echinococcus* sensitization;
3. CCL28 and IL-13 may be key factors regulated by let-7a-5p in the process of *Cystic echinococcus* sensitization.

Abstract

Background: Cystic echinococcosis (CE) is a common chronic zoonotic parasitic disease infected with the *Echinococcus granulosus*. This study aimed to construct the competitive endogenous RNA (ceRNA) network and investigate the mechanism of mice in echinococcosis sensitization.

Methods: The animal model of echinococcosis was established in mice, and the RNA sequencing was then performed using cysts. The differentially expressed RNAs (DERNAs: DEmRNAs, DE miRNAs, and DE lncRNAs) were screened between anaphylactic mice or non-anaphylactic mice and controls, respectively. The interactions of two DERNAs groups were identified and the ceRNA network was constructed. Moreover, the potential biological functions and pathways of the DEmRNAs were explored by enrichment analyses. Finally, the qRT-PCR and the western blot were performed to validate the expression levels of key RNAs and proteins.

Results: A total of 285 common DEmRNAs, 157 common DE lncRNAs and 4

common DEmiRNAs were observed. CeRNA network contained 3 DElncRNAs, 4 DEmiRNAs, and 27 DEMRNAs corporately. Enrichment results revealed that the functions of DEMRNAs focus on biological functions and pathways that specifically interact with the immune inflammatory response. In addition, the expression of 1700099I09Rik, let-7a-5p, Ccl28 and IL-13 was validated by RT-qPCR and western blot.

Conclusion: In this study, ceRNA network associated with CE sensitization in mice was constructed. The DEMRNAs in this network may be key clues for the immune mechanism of CE sensitization.

Keywords

Echinococcosis, sensitization, competitive endogenous RNA network, long non-coding RNA, Ccl28, IL-13

Introduction

As a neglected zoonotic disease, cystic echinococcosis (CE) caused by *Echinococcus granulosus* (*E. granulosus*) is an economic and public health problem in many developing countries (Soba *et al.*, 2020). China is recognized as a major endemic area for CE (Vuitton *et al.*, 2020). The entire life cycle of *E. granulosus* includes intermediate and definitive hosts, humans are accidental hosts, and occur mostly in the liver and lungs (M'Rad *et al.*, 2020).

E. granulosus host infection is a complex dynamic process that involves the recognition and interaction of multiple biomolecules including genes (Li *et al.*, 2021). *E. granulosus* in humans, triggers various allergic reactions, even anaphylactic shock (Ortona *et al.*, 2002). It is reported that in surgical treatment, rupture of the hepatic hydatid cyst causes an anaphylactic shock reaction (Davarci *et al.*, 2014). Anaphylactic shock is a serious complication of CE, with up to 4.6% of patients dying as a result of rigor and mishandling (Ye, Zhang, Ma, *et al.*, 2016). Since the occurrence of anaphylactic shock due to *E. granulosus* cyst ruptures during surgery is unpredictable, there are few strategies for its prevention and treatment (Wen *et al.*, 2010, Giorgio *et al.*, 2012). Immune responses have been shown to play an important role in *E. granulosus* induced anaphylactic shock (Ye, Zhang, Xuan, *et al.*, 2016). The immune mechanisms underlying CE sensitization may in-depth exploration to support the development of effective diagnostics and therapies.

In the past few years, competitive endogenous RNAs (ceRNAs) has become an important class of posttranscriptional regulatory factors, which can alter gene expression through miRNA mediated mechanism (Cesana *et al.*, 2011). The ceRNA network generally included microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and mRNAs (Liu *et al.*, 2021). At present, this indirect ceRNAs regulatory network has been discovered in many immunoregulation mechanisms (Chen *et al.*, 2018). Up to now, a series of lncRNAs and miRNAs, such as mmu-miR-378a-3p, mmu-miR-101b-3p, and mmu-miR-192-5p, have been identified

or speculated to be expressed in both parasites and hosts of echinococcosis, through which an unclear function and mechanism may perform large-scale biological functions (He *et al.*, 2020, Jin *et al.*, 2017).

Previously, we developed a suitable sensitized mouse experimental model by intraperitoneally injecting a cyst fluid suspension (Zhang, Ye & Zheng, 2016). The aim of this study was to obtain a deeper understanding of the molecular regulatory and reaction mechanism as well as the pathogenesis of echinococcosis sensitization in mice. We then screened the differentially expressed RNAs in sensitized mice models. Of note, there may be ceRNAs regulatory network in *E. granulosus*-infected mice related to an immune reaction. Ultimately, the qRT-PCR and Western blot validated the results, and this may provide robust and reliable theoretical support for clinical treatment.

Materials and Methods

Ethics approval

The animal experiments strictly abided by the welfare rules and regulations of the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University (No. K202104-11).

Animals and treatments

Nine specific pathogen free female BALB/c mice (4-8weeks, 20±2g) were purchased from the Animal Laboratory Center of the First Affiliated Hospital of Xinjiang Medical University. They were randomly divided into three groups: (1) healthy control (Ctrl; n=3); (2) *E. granulosus* infection induced anaphylactic mice (ANPC; n=3); and (3) *E. granulosus* infection non-anaphylactic mice (CE; n=3). The larval stage (cyst) fluid and protoscoleces were extracted aseptically from sheep livers infected with *E. granulosus* as previously described (Baz *et al.*, 1995). Phosphate buffered saline (PBS) was used to rinse the protoscoleces three times, and the collected protoscoleces were inoculated in the plate by density of 2000/mL at 37°C and 5% CO₂ for 40 days until the diameter of small cysts measured 200-300 µm. The small cysts, which were rinsed three times with PBS, were intraperitoneally inoculated in BALB/c mice (anaphylactic group and non-allergic group) by 50 *E. granulosus* PSC/mouse, while the control group was inoculated with NaCl solution. After six months, the anaphylactic group were intraperitoneally inoculated with the cyst fluid extracted from sheep of a 0.1-mL/10g body weight to induce sensitization, while the control group and non-allergic mice were inoculated with NaCl solution. Mice were humanely slaughtered by cervical dislocation after 1h of anaphylactic response. The spleen was extracted after the peritoneal cavity opened and then stored at -80 °C.

RNA extraction

The total RNA of spleen samples in three groups were obtained using Trizol based on the manufacturer's instructions with modifications. Briefly, aqueous supernatants of samples were centrifuged after samples were mixed with chloroform and completely homogenized, were kept overnight at -20°C . After a repeated centrifugation, the pellets were air-dried and suspended in nuclease-free water. The quality and concentration of RNA were determined using Nanodrop 2000 and formaldehyde-denaturing electrophoresis.

RNA sequencing

For small RNA sequencing, the libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2500/2000 platform and 50bp single-end reads were generated. Raw data (raw reads) of fastq format were processed through custom perl and python scripts and estimated by TPM (transcript per million).

For RNAs sequencing, quantification was performed using Qubit 2.0 after library construction, followed by Illumina PE150 sequencing. Raw data (raw reads) of fastq format were estimated by fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM).

Screening of differentially expressed RNAs

In this study, the differentially expressed analysis was conducted by employing edgeR package with installation of R (version 3.6.1, www.r-project.org). The results were quantified by RSEM and the cutoff criterion of $|\log_2\text{FC}| > 2$ (FC, fold change) and $p < 0.05$ [31]. Specifically, we explored the differentially expressed lncRNAs (DElncRNAs), mRNAs (DEmRNAs), and miRNAs (DEmiRNAs) between CE vs Ctrl, as well as ANPC vs Ctrl.

Enrichment analysis

The functional roles of DEmRNAs were detected by Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The Database for Annotation Visualization and Integrated Discovery (DAVID) online tool (<https://david.ncifcrf.gov/>) was employed for potential biological functions and pathways in the common DEmRNAs that overlapped in three groups, and a threshold of a p value < 0.05 was set for the significant functional categories [32]. To clarify the functions concretely, GOBP, GOCC, and GOMF were involved in the GO analysis.

Prediction of mRNA and lncRNA targeted by miRNA

The target mRNAs and lncRNAs of differentially expressed miRNA were screened by database prediction. To clarify the interactions within miRNA-mRNA, RAID (version 2.0) was used to predict all target mRNAs for DE miRNAs. The mRNAs targeted by miRNA were utilized to build miRNA-mRNA regulation network by intersecting with the common DEMRNAs.

The lncRNA-mediated binding as well as sequestration of targeted lncRNAs of DE miRNAs were detected by the database of DIANA. Moreover, the DELncRNAs were also cooperatively intersected with the potential target lncRNAs.

Construction of the ceRNA network

The lncRNA-miRNA-mRNA network were constructed and visualized by Cytoscape (version 3.5.1, www.cytoscape.org) based on RNAs interactions network, including DELncRNAs, DEMRNAs, and DE miRNAs.

qRT-PCR and Western blot validation

The relative expression of key mRNAs, miRNAs, and lncRNAs were detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Specifically, the threshold cycle (Ct) values were normalized based on GAPDH as an internal control to quantify the expression levels of genes. We took each group had three replicates to ensure quantitative accuracy into consideration and the relative gene expression were calculated with $2^{-\Delta\Delta C_t}$ method. For the Western blot analysis, the immunoblot expression of interleukin-13 and chemokine (C-C motif) ligand 28 (Ccl28) was determined. Protein extracted from spleen samples. Subsequently, 25 μ g protein in each sample was loaded with a 12% SDS-PAGE separation gel and then transferred to PVDF membranes. The blots were detected with the Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher), and specific protein bands were visualized by the LAS-3000 Imaging-System (Fujifilm). Quantitative analyses of each protein band were performed employing Image J software.

Results

Identification of differentially expressed lncRNAs, mRNAs, and miRNAs

By investigating aberrant expression of RNAs in CE as well as ANPC, we identified differential expression of RNAs in CE vs Ctrl, and ANPC vs Ctrl. Compared to Ctrl, A total of 743 DEMRNAs, 422 DELncRNAs, and 39 DE miRNAs were identified to have an aberrant expression in ANPC. Which included 568 upregulated and 175 downregulated DEMRNAs (Figure 1A), 233 upregulated and

189 downregulated DElncRNAs (Figure 1B), and 24 upregulated and 15 downregulated DEMiRNAs (Figure 1C). Meanwhile, a total of 555 DEMRNAs, 389 DElncRNAs, and 30 DEMiRNAs were detected in CE, compared with Ctrl. Among them, 393 upregulated and 162 downregulated DEMRNAs were found (Figure 1D), and there were 189 upregulated and 200 downregulated DElncRNAs (Figure 1E) and 8 upregulated and 22 downregulated DEMiRNAs (Figure 1F).

RNAs related to allergic reaction

To identify the most important RNAs associated with *E. granulosus* sensitization, we filtered the DEMRNAs, DElncRNAs, and DEMiRNAs to determine which represented in the ANPC by obtaining the common DERNAs. The results suggested that 285 DEMRNAs, 157 DElncRNAs, and 4 DEMiRNAs were identified as common RNAs (Figure 2A-C). The heatmap was used to display the expression of common DEMRNAs and DElncRNAs in ANPC, CE and Ctrl group (Figure 2D-E). In the common DEMiRNAs, mmu-let-7a-5p, mmu-miR-147-5p, mmu-miR-190b-5p, and mmu-miR-26b-3p were included (Figure 2F). Therefore, common RNAs should be explored for a better understanding of the biological function and regulatory networks.

Functional enrichment analysis of DEMRNAs

Then the results of biological functions for common DEMRNAs were obtained. The top 20 most enriched biological processes (BP) (Figure 3A), cellular components (CC) (Figure 3B), and molecular functions (MF) (Figure 3C) in GO terms were selected. The results indicated that the BP terms of common DEMRNAs included neuropeptide signaling pathway, hemostasis, and cellular response to interleukin-1, which were connected with immune and inflammatory responses. The results of CC terms were related to proteinaceous extracellular matrix, extracellular space, and cell periphery. Moreover, MF terms which significantly enriched by DEMRNAs were type 1 fibroblast growth factor receptor binding, calcium ion binding, and serine-type endopeptidase activity.

In addition, eighteen KEGG pathways of common DEMRNAs were significantly enriched according to enrichment analysis. The three most enriched pathways were complement and coagulation cascade, PI3K-Akt signaling pathway, and IL-17 signaling pathway (Figure 4).

Prediction of ceRNA networks for miRNAs

To establish miRNA-mRNA networks, we predicted the target mRNAs for 4 DEMiRNAs by RAID databases. The target mRNAs were compared with DEMRNAs and eventually networks were created, as shown in Figure 5A. In total, four miRNAs and 27 mRNAs were included in the miRNA-mRNA networks. In addition, the

DIANA database was adopted to explore the target lncRNAs of DE miRNAs. By compared with DE lncRNAs, we screened 3 target DE lncRNAs (1700099I09Rik, A730082K24Rik, and Gm16499). Cytoscape software was employed to construct and visualize the lncRNA-associated ceRNA network (Figure 5B).

qRT-PCR and Western blot detection

In mice after sensitization, we identified a large number of inflammatory biological functions and signaling pathways. Therefore, we validated the expression of inflammatory markers in target mRNAs. The expression of Ccl28 and IL-13 were significantly upregulated in the anaphylactic group, compared with that in controls (Figure 6A-B). The expression of 1700099I09Rik was also evaluated in the CE and anaphylactic groups (Figure 6C), while the expression of let-7a-5p was significantly downregulated, compared with control group (Figure 6D). The Western blot results showed that the protein levels of Ccl28 and IL-13 were both significantly increased in the anaphylactic group compared with the control group (Figure 6E-G).

Discussion

Anaphylactic shock caused by echinococcosis is a special type of allergic reaction caused by the hydatid cyst fluid, and it has become the main cause of death in patients infected with *E. granulosus*. Currently, the roles of ncRNAs have been elucidated by many researchers, they are regarded as a paramount part of the ceRNA regulatory network by acting as miRNAs sponges, as shown in an increasing number of studies (Bossi & Figueroa-Bossi, 2016, Bosia *et al.*, 2017). However, the ceRNA regulatory network related to immune mechanisms underlying CE sensitization still remain largely unclear and poorly explored, though there are some studies based on lncRNA and miRNA associated with the immune response of CE.

Previous research has illustrated that Th1 and Th2 responses are associated with chronic CE infection and sensitization (Rigano *et al.*, 2004, Vuitton, 2004). IL-17 with secretion is restricted to specific subsets of T cells and plays an important role in host immunology, but the receptor for IL-17 is widely expressed throughout the whole body (Li *et al.*, 2019). In recent years, it has been found that Th17 and Treg cells and the related IL-17 are involved in *E. granulosus* infections (Ma *et al.*, 2014). It was found that CE cyst fluid may attenuate Th2 allergic inflammatory responses via Treg cells (Kim *et al.*, 2019, Jeong *et al.*, 2021). Study has shown that changes in Treg cells and IL-17 are associated with CE induced anaphylactic shock (Zhang, Ye, Ma, *et al.*, 2016). After *E. granulosus* infections, the PI3K/Akt pathway was activated and promotes the alternative activation of macrophages, this is considered as one of the host immune regulation mechanisms (Wang *et al.*, 2019). If immune cells encounter parasitic antigens after the second exposure, it is likely that a strong immune response will be generated, which is one of the reasons for the higher incidence of anaphylaxis.

in CE patients (Pan *et al.*, 2013).

In this study, we constructed the model of sensitization using cyst fluid of *E. granulosus* and screened differentially expressed mRNAs, lncRNAs, and miRNAs through a microarray analysis. We obtained large numbers of DEmRNAs, DElncRNAs, and DEMiRNAs among three experimental groups. Importantly, DEmRNAs we identified were associated with inflammatory immune responses. This suggests that the process of sensitization may be associated with inflammation and immunity.

Interestingly, among the differentially expressed RNAs, ceRNA networks were established, including lncRNA-miRNA interactions and miRNA-mRNA interactions in CE sensitization, through biological prediction. This further confirmed the existence of ceRNAs regulation during sensitization. Furthermore, we selected key mRNA, miRNA, and lncRNA associated with immune mechanisms in the anaphylaxis after analyzing KEGG pathway and ceRNA networks. They were verified through qRT-PCR and the Western blot experiments.

Among them, Ccl28 and IL-13 are helper T cell immune response cytokines, and up-regulated in sheep infection with *E. granulosus* (Hui *et al.*, 2015). Ccl28 has recently been recognized as a potent factor promoting cellular immune responses in the systemic circulation and mucus (Yan *et al.*, 2021). Up regulation of Ccl28 has been reported to be associated with multiple allergic responses, including allergic dermatitis (Maeda *et al.*, 2008), allergic rhinitis (Nagakubo *et al.*, 2016, English *et al.*, 2006), allergic asthma. Immunological tolerance was induced by Th2 cells after a cyst fluid sensitization infection with *E. granulosus* (Fallon & Mangan, 2007). Interleukin-13 (IL-13) is a marker cytokine produced in the process of Th2 immune response. It is essential to protect the body from the infection of extracellular parasites and is associated with allergic reactions (Zhu, 2015). Moreover, the Western blot analysis showed that Ccl28 and IL-13 were both significantly upregulated in the anaphylactic group, which is similar to previous studies. Targeted regulation of Ccl28 and IL-13 during re-exposure to the cyst fluid of *E. granulosus* may be an effective agonist for the prevention and treatment of CE sensitization.

It is found that miRNAs may involve in the pathogenesis of CE (Cucher *et al.*, 2015, Jin *et al.*, 2017). It is possible that many host proteins closely related to anti-infection processes may be interfered with by parasites by disturbing miRNA biogenesis pathways (Zheng, 2013). Parasite secreted extracellular vesicles containing miRNAs involved in sensitization in a mouse model of experimental allergic asthma, which suppressed allergen induced type 2 innate immune responses (Buck *et al.*, 2015). Among these miRNAs, let-7a regulates the anti-inflammatory process of sepsis by inhibiting specific genes that target downstream signaling pathways (Chen *et al.*, 2007). let-7a level was decreased in sepsis-induced liver injury patients (Zhang & Niu, 2019). The downregulated let-7a contributes to the development of allergic diseases by regulating the expression of target genes (Yu *et al.*, 2017, Specjalski & Jassem, 2019). The decreased expression of miR-147 has also been observed in chronic myelocytic leukemia (CML) patients who have a bone marrow clone malignancy, and different studies have shown that miR-147 could be either an oncogene or a tumor

suppressive gene in different types of human cancers (Li *et al.*, 2018). Moreover, Ying *et al.*, revealed that mmu-miR-26b inhibited liver fibrosis and angiogenesis in mice and enhanced T cell responses in the activation of the immune system (Shangguan *et al.*, 2020). Decreased miR-26b regulates the expression of target genes and thereby promotes allergic inflammatory responses (Kwon *et al.*, 2015).

To our knowledge, this is the first study on the sequencing analysis of differentially expressed lncRNA-mediated ceRNA regulatory network for sensitization infected with *E. granulosus*. These key RNAs may play significant roles in echinococcosis sensitization. However, our study also has limitations. first, the specific regulatory mechanism of these ceRNAs response to an *E. granulosus* sensitization infection also need to be experimentally established in the future. Then, the study of anaphylactic regulatory response in the host following initial exposure to the antigen of cyst fluid was lack. Moreover, whether the experiments have clinical translation value deserves further exploration.

Conclusions

In summary, we constructed ceRNA networks of *E. granulosus* sensitization in mice. In the network, 1700099I09Rik, Ccl28, IL-13, and let-7a-5p were identified and validated to be associated with *E. granulosus* sensitization. Inflammatory immune response may be the potential molecular deregulation mechanism of sensitization. The ceRNAs-based study may provide a novel biomarker discovery approach for *E. granulosus* sensitization, and contribute to the prevention and treatment in the future.

Author statement

All authors agree to submit.

Declarations of Competing Interest

All authors declare no competing interests.

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Ethical Approval

The study was approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University (K202104-11).

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Figure 1. Identification of differentially expressed RNAs. Volcano plot of differentially expressed mRNA (A), lncRNA (B), and miRNA (C) between the anaphylactic group (ANPC) vs the control group (Ctrl). Volcano plot of differentially expressed mRNA (D), lncRNA (E), and miRNA (F) between CE group vs the control group (Ctrl). The red dots represent upregulation, and blue dots represent downregulation, while the green dots mean no diversification.

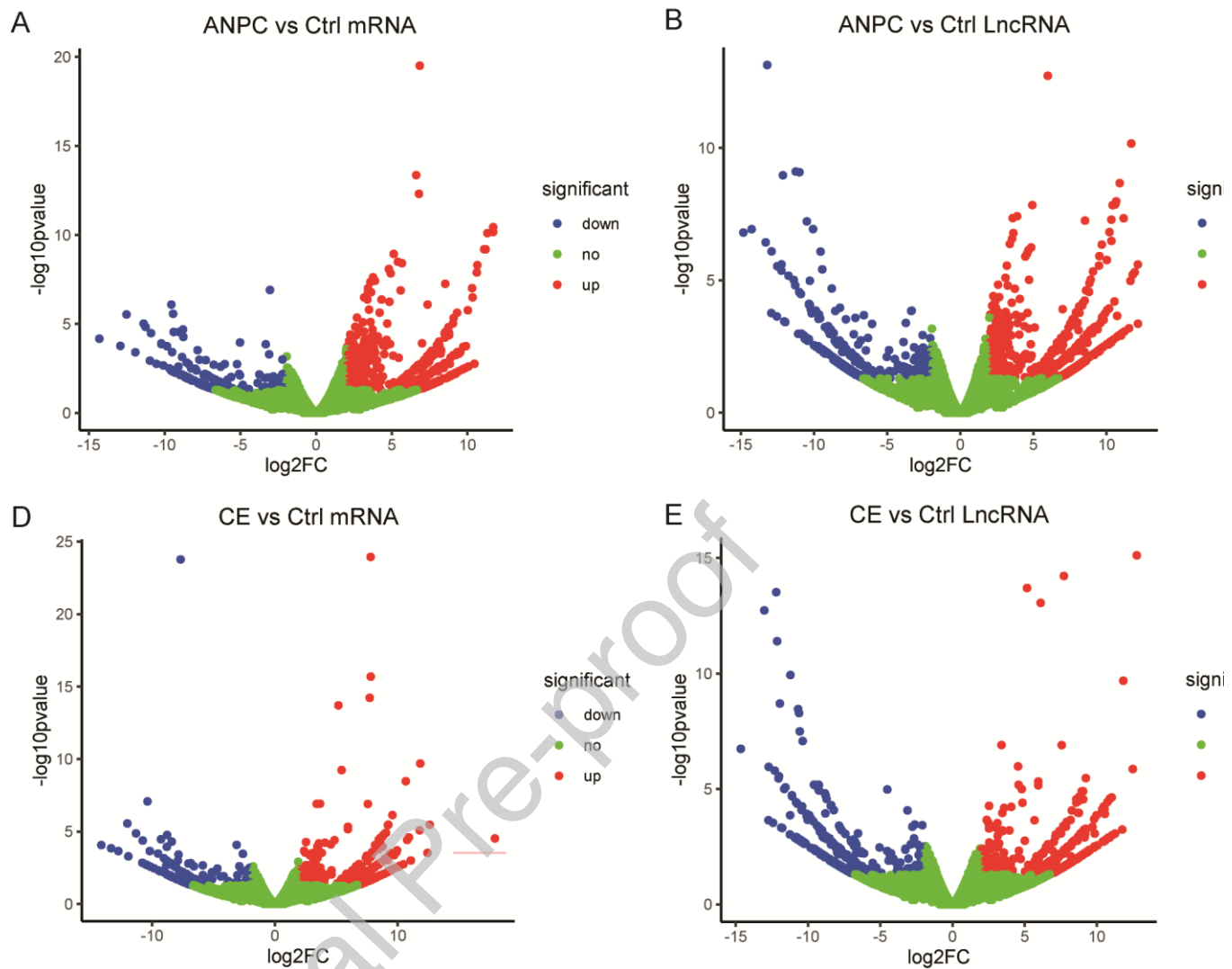


Figure 2. The Venn plot analysis of differentially expressed RNAs. A. Common DEMRNAs of the two DEMRNAs groups were identified using Venn diagrams. B. Common DEMiRNAs of the two DEMiRNAs groups. C. Common DELncRNAs of the two DELncRNAs. Heatmap of common differentially expressed mRNA (D) and lncRNA (E) in the ANPC, CE, and Ctrl groups, respectively. Up- and downregulated RNAs are colored in red and blue. F. The common differentially expressed miRNAs in ANPC, CE, and Ctrl groups, respectively.

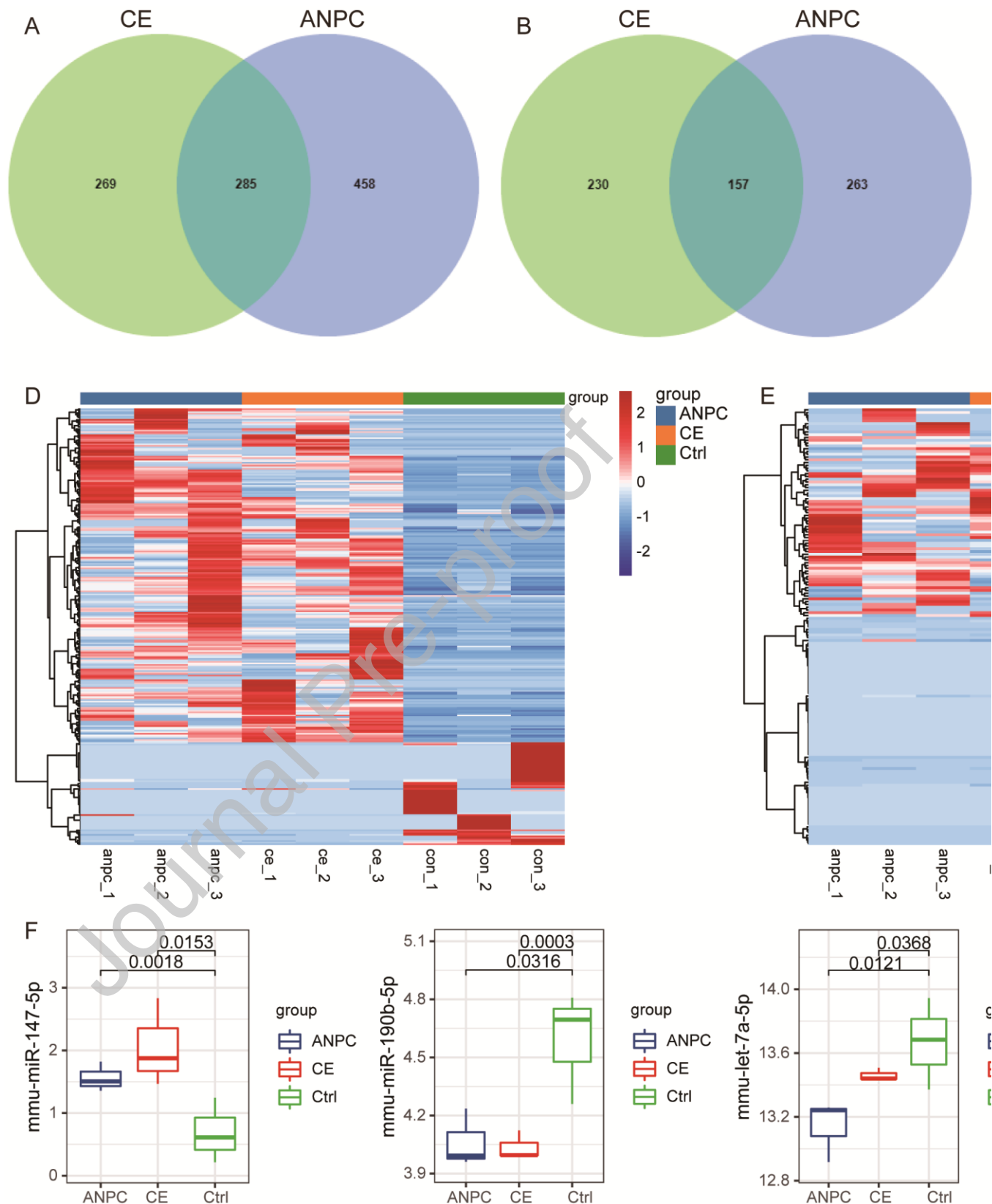


Figure 3. GO enrichment analysis results of common DEMRNAs. Functional enrichment analysis of GOBP (A), COCC (B), and GOMF (C). Enriched bubble diagrams of the top 20 enrichment GOBP, COCC and GOMF terms of DEMRNAs. The scale of balls represents gene counts. The color shows the P-value, and the

horizontal bar represents the corresponding p-values.

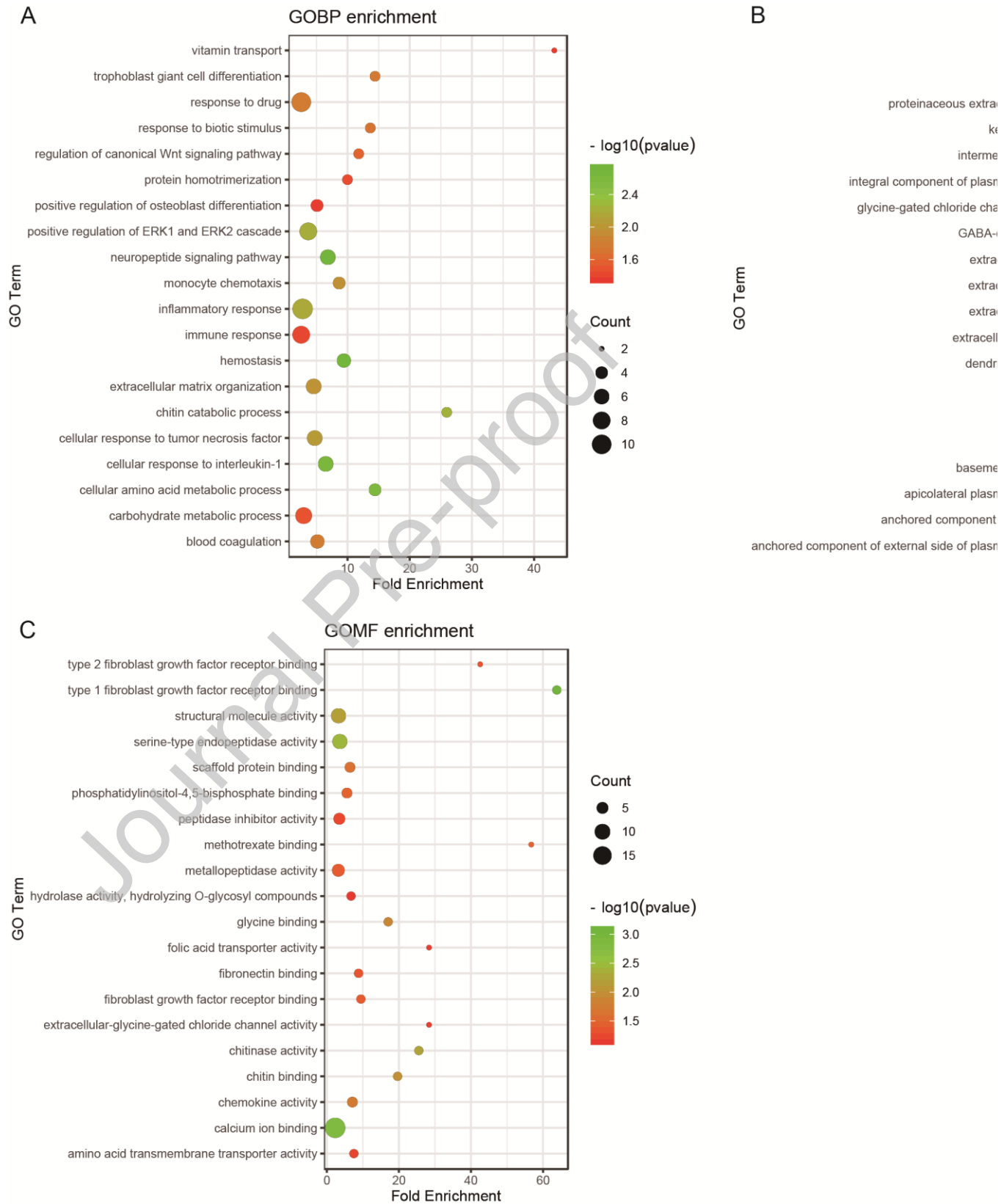


Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

of common DEmRNAs. Each line indicates a gene, and the number of lines shows the genes enriched. The KEGG analysis was conducted using the DAVID database.

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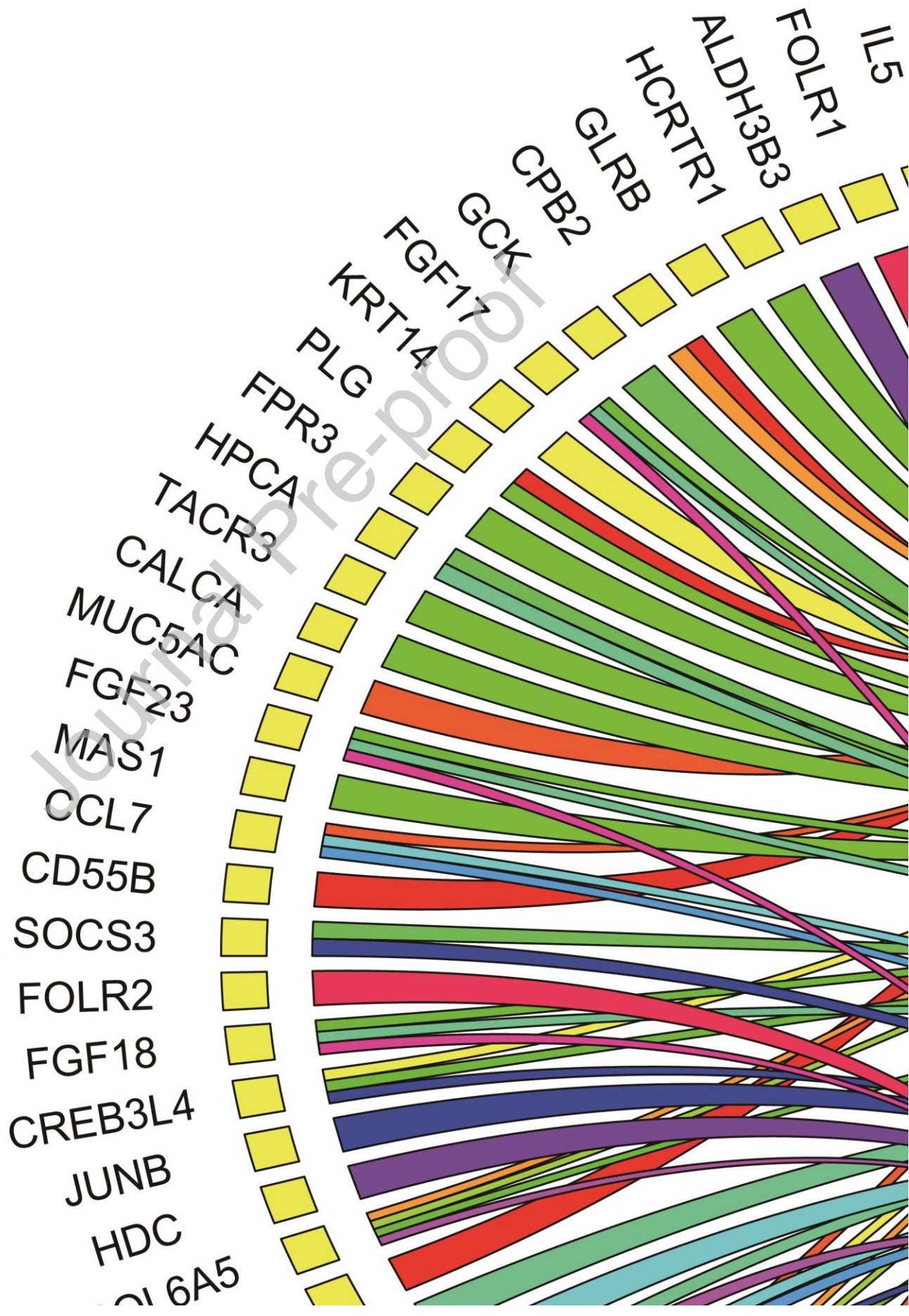
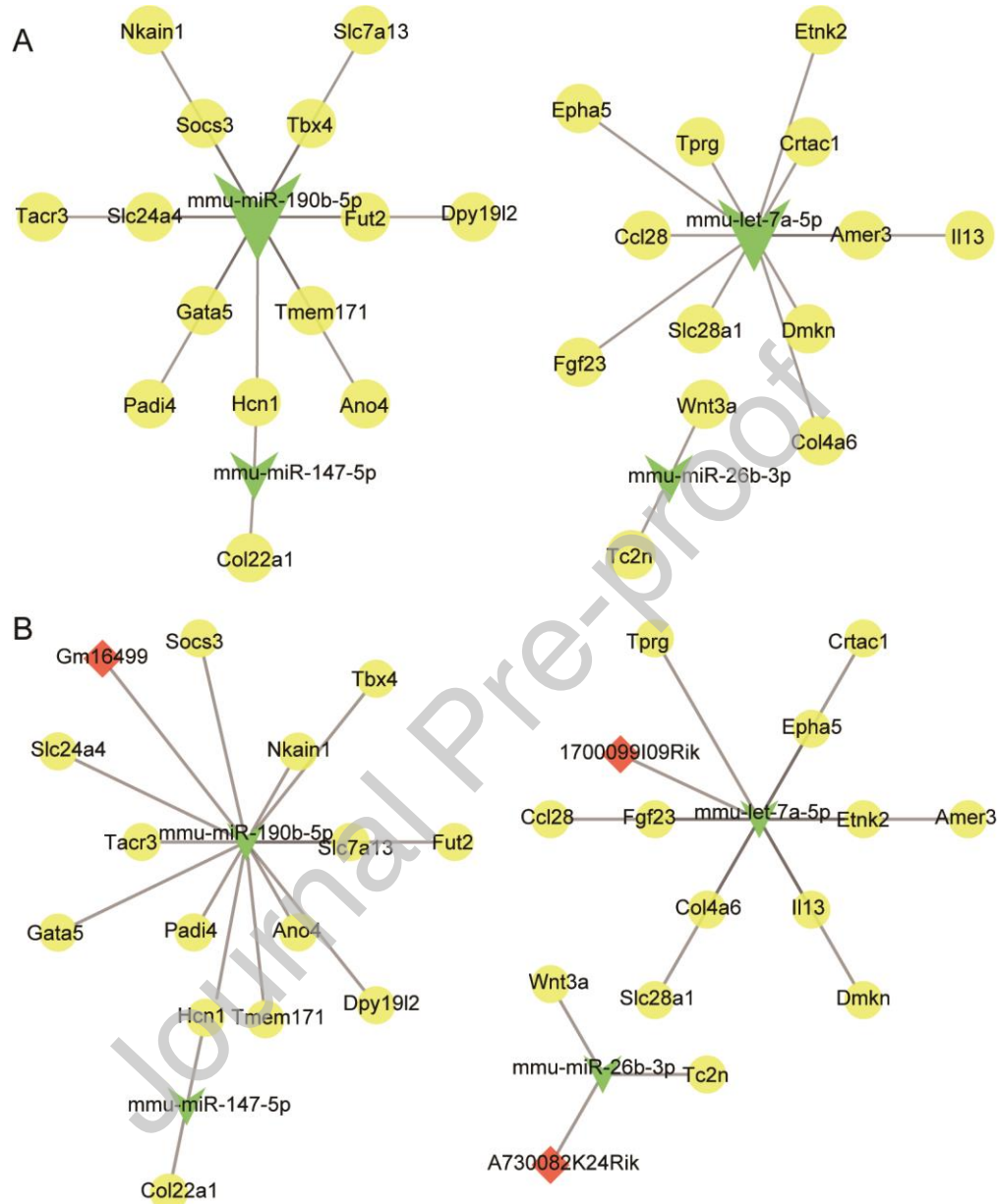


Figure 5. Construction of ceRNA network. A. The interaction of miRNA-mRNA. B. The network plot illustrating a regulatory landscape of ceRNAs network. The green arrows represent miRNAs, the yellow circles represent mRNAs, the red diamonds represent lncRNAs.



Figure

6. RT-qPCR and western blot validation. The relative expression of Ccl28 (A), IL-13 (B), 1700099I09Rik (C), and let-7a-5p (D) in the control, CE, and anaphylactic group samples were detected by qRT-PCR. The Western blot analysis of Ccl28 and IL-13 (E) and the statistics of the Western blot for Ccl28 (F) and IL-13 (G). * $p < 0.05$, ** $p < 0.01$.

