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RESEARCH ARTICLE

Whole-transcriptome sequencing and bioinformatics analysis of calcified outer-layer tissue from cystic echinococcosis

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ABSTRACT

Echinococcosis is a common zoonotic disease in livestock; the type with the highest incidence is cystic echinococcosis (CE). In clinical management, patients with CE of the liver in which the cyst wall is calcified have been found to have better prognoses than those without calcification. In this study, we collected calcified and uncalcified cyst wall tissue from patients with hepatic CE and observed significant changes in the expression of 2336 messenger ribonucleic acids (mRNAs), 178 long noncoding RNAs (lncRNAs), 210 microRNAs (miRNAs), and 33 circular RNAs (circRNAs) using high-throughput sequencing (HTS). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of differentially expressed RNAs (DERNAs: DEmRNAs, DElncRNAs, DEmiRNAs, and DEcircRNAs) were performed to explore these RNAs' potential biological functions and signaling pathways. Ultimately, the results of hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining confirmed the correlation between calcification and apoptosis of the cyst wall. In summary, this study was an initial exploration of the molecular-biological mechanism underlying spontaneous calcification of the hydatid cyst wall, and it provides a theoretical basis for exploring new targets for drug treatment in CE.

Keywords: *Echinococcus granulosus*; calcification; cyst wall; RNA; apoptosis.

INTRODUCTION

Subtypes of the parasitic disease echinococcosis include cystic echinococcosis (CE), caused by *Echinococcus granulosus* (*E. granulosus*), and alveolar echinococcosis (AE), caused by *Echinococcus multilocularis* (*E. multilocularis*) (Ito & Budke, 2017). Echinococcosis is prevalent worldwide, especially in pastoral areas such as Xinjiang, Qinghai, Inner Mongolia, and Tibet in China, causing a huge economic burden and a serious health threat (Wen *et al*., 2019). The liver and lungs are the main organs parasitized by E. granulosus (McManus *et al*., 2012). Surgical resection is currently the clinical treatment of choice for CE. However, the operation is difficult and risky, and CE often recurs afterward (Ritler *et al*., 2019). For patients who cannot undergo surgery, albendazole (ABZ) and mebendazole (MBZ) are currently the most commonly used therapeutic agents. However, the poor solubility of these drugs in water and the barrier of the cyst wall block the absorption of the drug, which can cause side effects such as abnormal liver function, leukopenia, and hair loss after treatment (Wang *et al*., 2021a). Therefore, it is urgent to develop new drugs for CE.

It has been found that as the hydatid cyst swells in the liver, compression by the surrounding hepatic tissue causes the cyst wall to continuously produce fibers, which form a fibrous layer; this is the outer cyst wall (Czermak *et al*., 2008). Clinical examination can reveal the spontaneous calcification of this wall, often accompanied by degradation of cystic contents and a reduction in cyst size. During follow-up, patients with spontaneous calcification of the external cyst wall were found to have better prognoses than those without calcification. Such progressive calcification has been shown to significantly inactivate intracapsular protoscoleces (PSCs) because the calcified outer wall inhibits the hydatid's intake of nutrients from surrounding human tissues (Conchedda *et al*., 2018). Our preliminary *in vitro* studies revealed that certain factors can effectively cause spontaneous calcification of the outer cyst wall in CE and inhibit the growth of parasites by binding to bone morphogenetic protein receptor type II (BMPRII), insulin-like growth factor 1 receptor (IGF1R), and estrogen receptor alpha (Erα) (Zhang *et al*., 2016). Therefore, regulating the calcification of the outer cyst wall via molecular biology could become a new avenue of CE treatment, but because few studies have addressed the development of this pathological process, its underlying mechanism is still unclear.

New evidence suggests that miRNAs, lncRNAs, and circRNAs play key roles in various biological processes in CE (Santosh *et al*., 2015). Studies show that *miR-277*, *lethal-7 (let-7)*, *miR-71*, *miR-10*, *miR-2*, and *miR-9* are specifically expressed in the *E. granulosus* cyst wall, with *let-7*, *miR-71*, and *miR-2* helping regulate PSC development in the germinal layer (Cucher *et al*., 2011; Bai *et al*., 2014; Gao *et al*., 2022). LncRNAs and their targeted mRNA molecules

might play roles in the regulation of multiple signaling pathways. For example, lncRNAs are involved in biological processes such as apoptosis, terpene skeleton biosynthesis, carbon metabolism, and dorsoventral-axis formation in hydatids (Si *et al*., 2022; Xiao *et al*., 2022). Meanwhile, lncRNAs are important immune stress–related regulators that play a key role in CE (Yu *et al*., 2018); e.g., lncRNA028466 mediates host immune response by regulating the expression of T-helper 1 and 2 (Th1, Th2) cytokines (Wang *et al*., 2021b). Regulatory relationships between multiple lncRNAs and mRNAs constitute a vast regulatory network that is critical to various growth and development processes of *Echinococcus* (Yu *et al*., 2021; Gao *et al*., 2022).

To better understand the regulatory role of relevant RNAs on spontaneous calcification in CE, in this study we analyzed their functions by comparing DERNAs in calcified and uncalcified cystic walls in CE patients. We also constructed lncRNA–mRNA and circRNA–miRNA–mRNA co-expression networks using GO and the KEGG to investigate the regulatory roles of these RNAs in the biological process of wall calcification. Finally, histological differences between calcified and non-calcified external capsule walls were observed via H&E staining, and the level of apoptosis in calcified tissues was detected via TUNEL assay, to verify that spontaneous calcification was an important endpoint of tissue inactivation and apoptosis in external cyst walls. Through these experiments, we were able to identify calcification-related RNAs and signaling pathways, providing a potential molecular means of actively inducing cyst wall calcification to treat CE.

MATERIALS AND METHODS

Ethical approval

This study strictly complied with the relevant rules and regulations of the Medical Ethics Committee of the First Affiliated Hospital of Shihezi University of Medicine (Shihezi, China; No. KJX2022-108-02).

Cyst wall tissue source

From the pathology laboratory of the First Affiliated Hospital of Shihezi University of Medicine, we collected tissue samples of cyst walls from CE patients and identified whether they were uncalcified or calcified. The clinical samples were analyzed to determine patients' mRNA, miRNA, lncRNA, and circRNA profiles. None of the patients in this study were found to have any disease other than hepatic CE.

RNA extraction and sequencing

Per the manufacturer's protocol, we extracted total RNA from cyst walls using the RNA Isolation Kit (Ambion; Thermo Fisher Scientific, Waltham, MA, USA). The suspension was stored at –80°C until use. RNA quality and concentration were determined by NanoDrop 2000 (Thermo Fisher). After purification, liganding, and amplification, we sequenced the RNAs on an Illumina HiSeq 4000 sequencing platform (Illumina, Inc., San Diego, CA, USA).

Screening of differentially expressed RNAs

We used the edgeR software (v4.1.3; www.r-project.org) to analyze DERNAs. Results were quantified by the cutoff criterion of |log2 fold change (FC)|>1, and significance was set at $P < 0.05$. Specifically, we compared differentially expressed mRNAs, lncRNAs, miRNAs, and circRNAs (respectively, DEmRNAs, DElncRNAs, DEmiRNAs, and DEcircRNAs) between uncalcified and calcified cyst walls.

Enrichment analysis

We used GO and KEGG pathway enrichment analyses to detect the functional roles of DERNAs. Biological functions and pathways of DERNAs between the two groups were analyzed using the Database for Annotated Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) with a threshold *P*-value of < 0.05 for significant functional categories. To clarify the functions concretely, we employed the categories of Gene Ontology Biological Process (GOBP), Gene Ontology Cellular Component (GOCC), and Gene Ontology Molecular Function (GOMF) in our GO analysis.

RNA co-expression network construction

We constructed and visualized lncRNA-mRNA and circRNA-miRNA-mRNA networks using Cytoscape version 3.5.1 (www.cytoscape.org) based on the RNA interaction network, including DElncRNAs, DEmRNAs, DEmiRNAs, and DEcircRNAs.

Protein-protein interaction network construction and analysis

STRING (Search Tool for the Retrieval of Interacting Genes) is an interactive gene database search tool that can obtain protein-protein interaction (PPI) information. We imported the filtered differentially expressed genes (DEGs) into STRING (version 11.0; https://string-db.org/), setting the confidence score at ≥0.4. The corresponding data were obtained and imported into Cytoscape for Molecular Complex Detection (MCODE; version 1.4.2;) analysis. The screening parameters of MCODE were degree cutoff = 2, *k*-score = 2, node score cutoff = 0.2, and max depth = 100, which filtered out the most tightly connected clusters from the PPI. We then set the score to >3 to obtain the 14 most tightly connected clusters. The results were subjected to GO/KEGG functional enrichment analysis using Metascape genes.

H&E staining

We fixed calcified and uncalcified cyst wall tissue with formaldehyde fixative for 24 h. Paraffin-embedded tissue and 4-μm-thick paraffin sections were prepared, dewaxed, and subjected to H&E staining per standard protocols, and liver histopathological changes were observed under a microscope (E600, Eclipse, Nikon, Japan).

TUNEL assay for apoptosis

Cystic-tissue samples were fixed with 4% paraformaldehyde (PFA) for 48 h, sequentially dehydrated, paraffin-embedded, and sectioned (4 μm). We then added 100 μl (concentration, 20 μg/mL) of working solution and incubated the mixture for 10 min at room temperature. After staining and observation under a fluorescence microscope (E600, Eclipse, Nikon, Japan), cells were counted using ImageProPlus version 6.0 (Media Cybernetics, Silver Spring, MD, USA), and we took the average value to calculate the apoptosis index (AI):

$$AI = \text{number of positive cells/total number of cells} \times 100\%$$

Statistical analysis

We used SPSS version 26.0 (IBM Corp., Armonk, NY, USA) for all statistical analyses. All data were expressed as means ± standard deviations (SD). We measured differences between the two groups using a *t* test, and between-group comparisons were evaluated via one-way analysis of variance (ANOVA). $P < 0.05$ was considered to suggest a statistically significant difference.

RESULTS

Identification of differentially expressed RNAs

We first identified differential expression of RNAs in uncalcified (S1) and calcified (S2) outer cyst wall tissue. Expression of 2336 mRNAs, 178 lncRNAs, 210 miRNAs, and 33 circRNAs in total was significantly altered in calcified versus uncalcified cyst wall tissue. These included 442 upregulated and 1894 downregulated mRNAs (Figure 1A), 91 upregulated and 87 downregulated LncRNAs (Figure 1B), 117 upregulated and 93 downregulated miRNAs (Figure 1C), and 12 upregulated and 19 downregulated circRNAs (Figure 1D). We also analyzed the expression of mRNAs, lncRNAs, miRNAs, and circRNAs in uncalcified (S1) and calcified (S2) outer cyst wall tissue using heat maps (Supplementary Figure 1A-D). In the differential

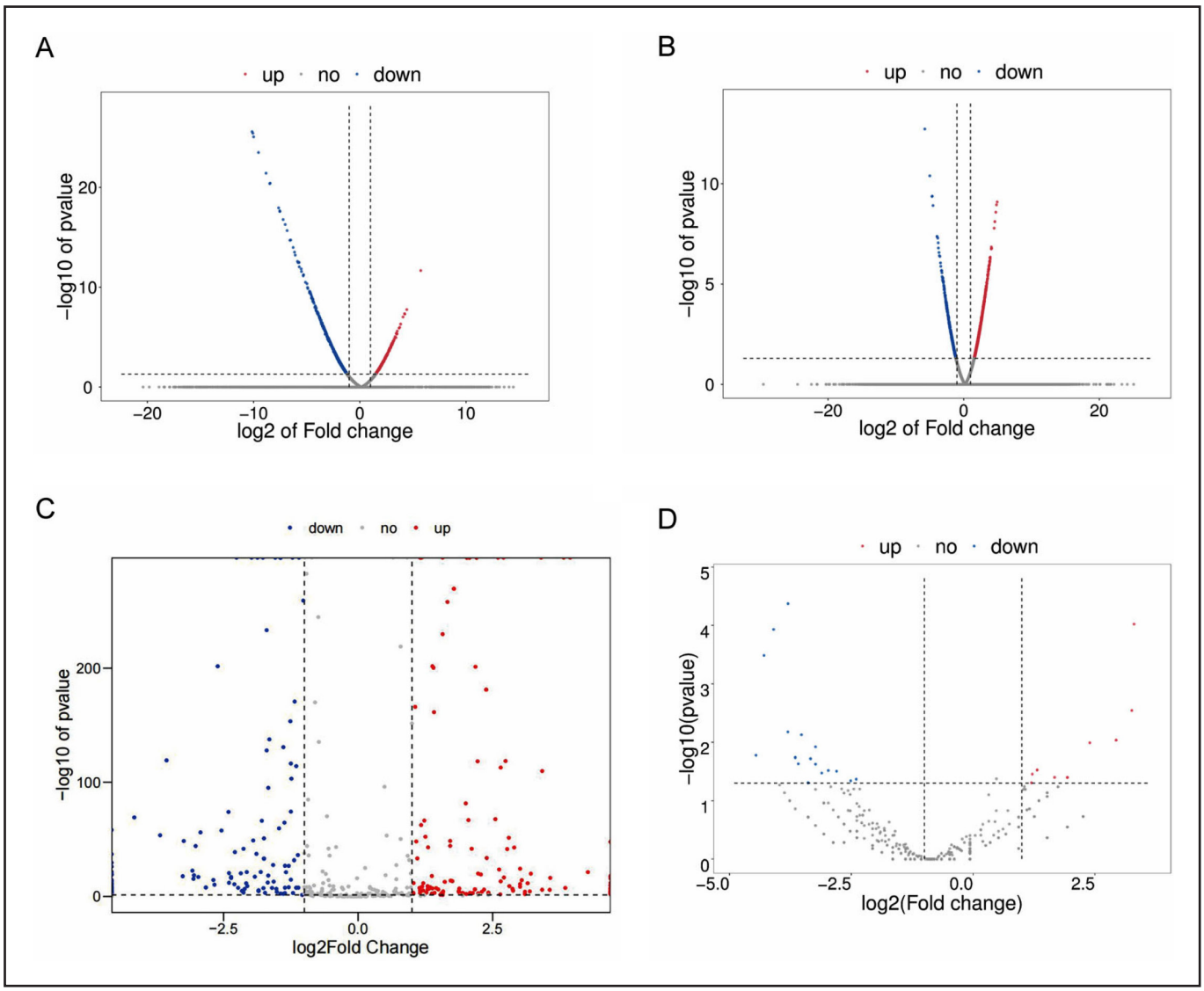

Figure 1. Differentially expressed RNAs. Volcano plots of differentially expressed mRNA (A), lncRNA (B), miRNA (C), and circRNA (D) between uncalcified and calcified cyst wall tissue.Red dots represent upregulation and blue dots represent downregulation.

Table 1. Differentially expressed mRNAs associated with calcification

Gene name	Description	Log2(fc)	Regulation
ASAH1	N-acylsphingosine amidohydrolase 1	-1.74	down
CP	ceruloplasmin	-7.54	down

expression of mRNA *N-acylsphingosineamidohydrolase1* (*ASAH1*) and *ceruloplasmin* (*CP*) caught our attention (Table 1). Initially, we validated RNAs that were significantly differentially expressed between uncalcified and calcified cyst wall tissue.

GO and KEGG pathway analyses

We performed GO and KEGG pathway analyses of significantly differentially expressed RNAs. GO annotations corresponding to these genes were classified into three categories: biological process (BP), molecular function (MF), and cellular component (CC). Selected TOP20 GO analysis showed (Figure 2A-D) that DEmRNAs were enriched in protein binding, cytosol, extracellular exosome, oxidoreductase activity, oxidation-reduction process, collagen-containing extracellular matrix, and peroxisome. DElncRNAs are mostly enriched in CD95 death-inducing signaling complex, methylation, ripoptosome, and regulation of the necroptotic process. DEmiRNAs are mostly enriched in the CD95 protein binding process. DEcircRNAs are enriched in NAADP-sensitive calcium-release channel activity. KEGG enrichment analysis shows that DEmRNAs are enriched in Complement and coagulation cascades, PPAR signaling pathway, and Ferroptosis. We found that DEmiRNAs were significantly enriched in the PI3K-AKT signaling pathway, Calcium signaling pathway, and Jak-STAT signaling pathway (Figure 3A-D).

Co-expression network of differentially expressed genes

We constructed a lncRNA-mRNA co-expression network of differentially expressed genes (DEGs) to reflect the interrelationship between lncRNAs and mRNAs (Figure 4A). The circRNA-miRNA-mRNA co-expression network also revealed interactions between these RNAs (Figure 4B). *Circ_0001346* completed regulation of the mRNA ring finger protein 13 (*RNF13*) by mediating *miRNA-16-5p*, *miRNA-17-5p*, and *miRNA-93-5p*. The mRNA, integrin subunit alpha 3 (*ITGA3*), was affected by four circRNA-miRNA pathways.

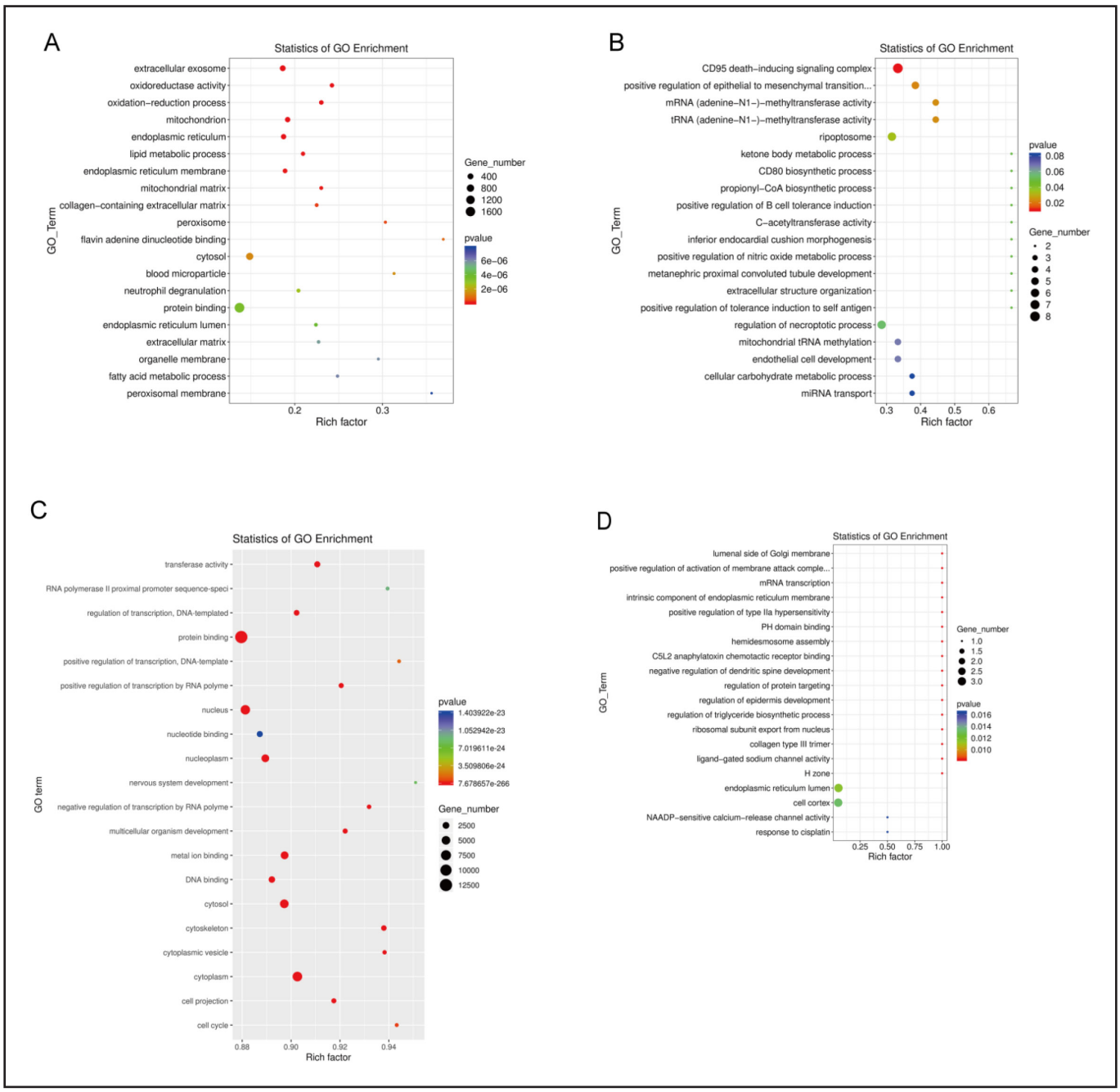

Figure 2. GO enrichment analysis. Results of functional enrichment analysis of the top 20 enriched words for differentially expressed mRNA (A), lncRNA (B), miRNA (C), and circRNA (D).

Protein-protein interaction network analysis of differentially expressed genes

We used STRING and Metascape to construct a PPI network, which included 142 upregulated DEGs and 406 downregulated DEGs (|logFC|>2.0, $P < 0.05$; Figure 5A-B). We selected the three best-scoring terms by *P*-value and retained them as functional descriptions of the corresponding components (Table 2).

Table 2. GO enrichment analysis in PPI network and significant clusters TOP3

GO	Description	Log10 (P)
hsa04610	Complement and coagulation cascades	-20.1
GO:0032787	Monocarboxylic acid metabolic process	-18.0
GO:0006954	inflammatory response	-17.5

Histopathological changes

We observed morphological differences between calcified and uncalcified cyst wall tissue via H&E staining. Clear foci of calcification were seen in calcified versus uncalcified tissue. We observed fibrous-tissue hyperplasia in the walls of non-calcified capsules. Some fibrous connective tissue had undergone glassy degeneration, with a small amount of lymphocyte-dominated inflammatory-cell infiltration visible in the interstitial space (Figure 6A). Foci of necrosis were seen within the wall of the calcified capsule, with calcium salt deposits within these foci. A small amount of cellular debris and inflammatory-cell infiltration appeared around the necrotic foci, surrounded by a large amount of fibrous connective-tissue hyperplasia with lymphocytic infiltration (Figure 6B).

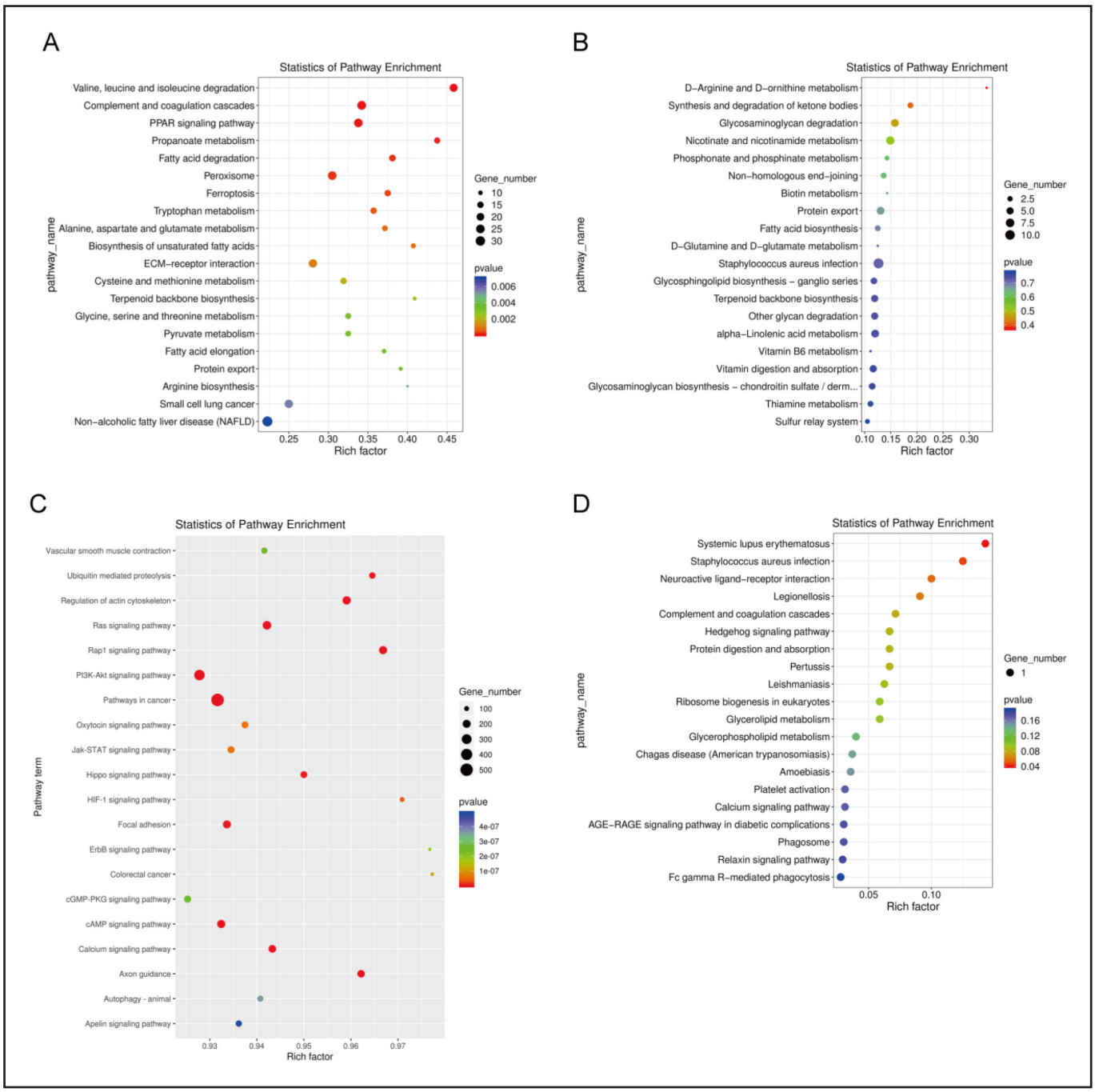

Figure 3. KEGG pathway analysis of DERNAs. Commonly differentially expressed mRNA (A), lncRNA (B), miRNA (C), and circRNA (D). Each line indicates a gene, and the number of lines shows the genes enriched.

Apoptosis among groups

To clarify the relationship between the development of cyst wall calcification and apoptosis, we performed TUNEL staining on different tissue (Figure 7). The results showed that the green fluorescence intensity and apoptotic index of calcified cyst wall tissue were significantly stronger than those of non-calcified cyst wall tissue. This suggested the involvement of apoptosis in outer cyst wall calcification in CE, which might also be a causal factor both in the inactivation of cystic contents and in hydatid shrinkage.

DISCUSSION

CE is a zoonotic parasitic disease that is severely neglected worldwide, posing a health risk to the public and affecting economic development. Although surgical techniques to remove hydatid capsules have advanced in recent years, they do not effectively prevent postoperative complications, and the postsurgical recurrence rate is high. Therefore, non-invasive targeted molecular therapy is of great importance in treating this disease (Craig & Larrieu, 2006; Budke *et al.*, 2013). CE is clinically classified into five types based on imaging features. In types I and II, cysts are mostly solitary, with clear borders and high viability. Type III is characterized by significant collapse of the internal cyst, type IV is marked by gradual fibrotic changes in the wall of the cyst, and type V features calcification of the cyst wall after necrosis (Cong, 2012). Spontaneous calcification of the external wall results from the combined actions of the host organism and the parasite. This suggests that during host immune resistance, the parasite's microenvironment changes in a way that is not conducive to its survival. Pathological calcification is classified as either dystrophic or metastatic; these categories

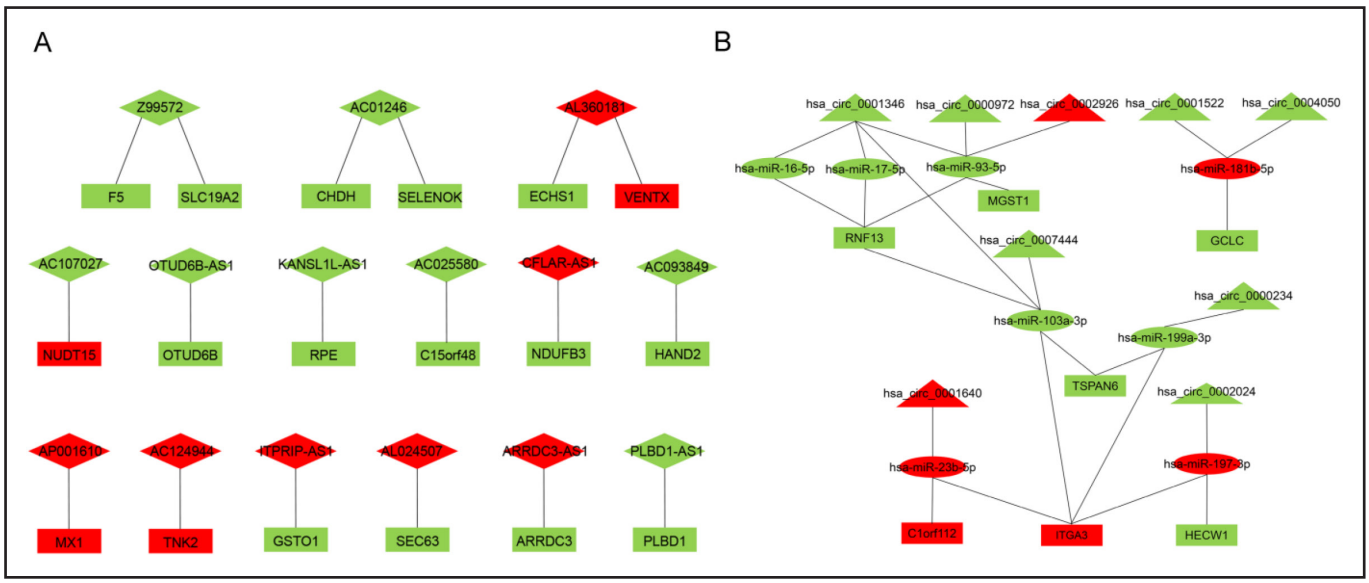

Figure 4. RNA co-expression network. LncRNA-mRNA co-expression network is shown in A, and circRNA-miRNA-mRNA co-expression network is shown in B. Triangular nodes represent circRNA, diamond nodes represent lncRNA, circular nodes represent miRNA, and square nodes represent lncRNA. The triangular nodes represent circRNA, the diamond nodes represent lncRNA, the circular nodes represent miRNA, and the square nodes represent lncRNA. red and green of the circular nodes represent up-regulation and down-regulation, respectively.

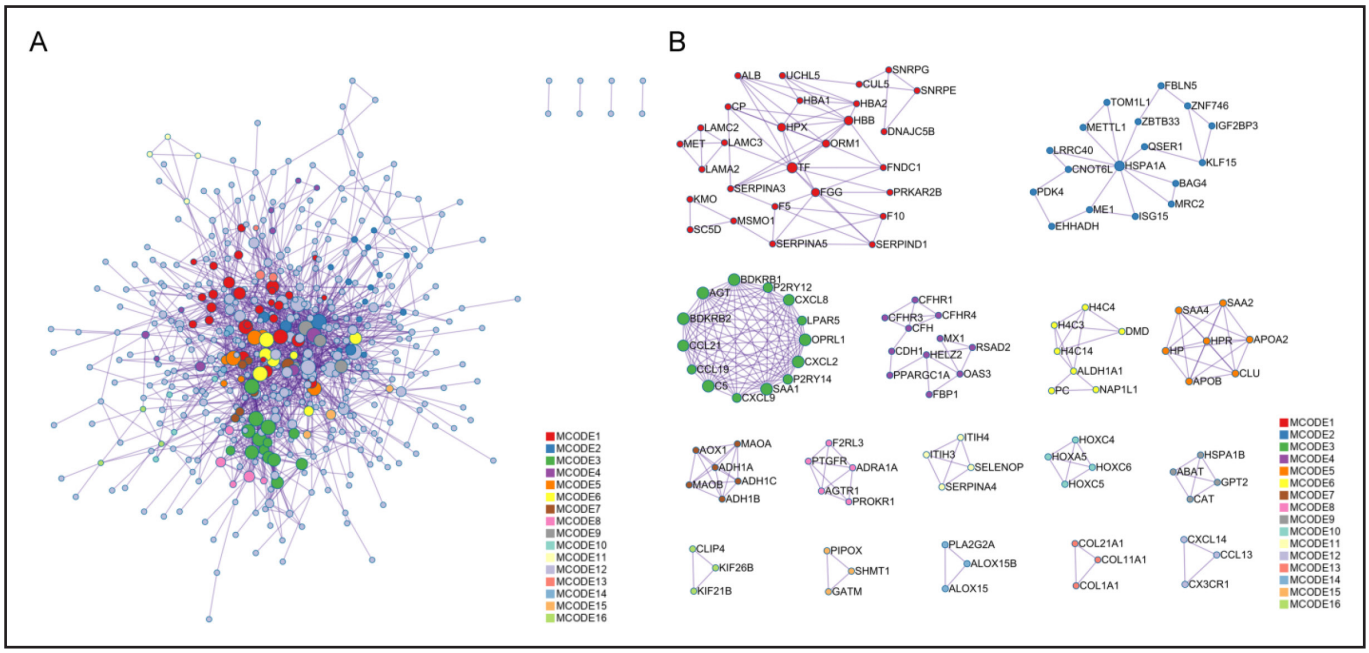

Figure 5. PPI network of differential genes. Protein-protein interaction network of differentially expressed genes constructed used STRING and Metascape (A). The top 16 gene hubs expressed in the PPI network (B).

are often used to describe terminal manifestations of local-tissue necrosis in the fields of cardiovascular disease and oncology (Zhao *et al*., 2016; Li *et al*., 2021).

In some patients with prolonged disease, calcified nodules of varying sizes are often seen in the outer cyst wall. This is dystrophic calcification caused by the immune response, indicating that in these cases the hydatid infection has tended to heal itself (Peng *et al*., 2005; Govindaraj *et al*., 2021). Calcification of the outer cyst wall can induce degeneration and death of PSCs. Complete calcification of the wall means that the CE has largely resolved on its own and no drug or surgical treatment is needed at this time, which avoids the trauma and side effects of treatment and therefore has obvious economic benefits. In addition, ABZ treatment has been shown to effectively accelerate the onset and progression of external–cyst wall calcification in CE, further suggesting the important role of such calcification in hydatid inactivation and necrosis (Sanei *et al*., 2021). Therefore, a comprehensive assessment of hydatid-tissue calcification on CE patients is important not only to the choice of treatment but also to the patient's prognosis.

ASAH1 has been shown to play a vital role in vascular calcification. Researchers found that arterial vascular-wall calcification was significantly increased in *ASAH1* knockout (KO) mice. *ASAH1* gene deletion significantly enhanced differentiation of mesenchymal stem cells (MSCs) to osteoblasts with high expression of osteogenic markers such as osteopontin (OPN) and runt-related transcription factor 2 (RUNX2) (Bhat *et al*., 2020,

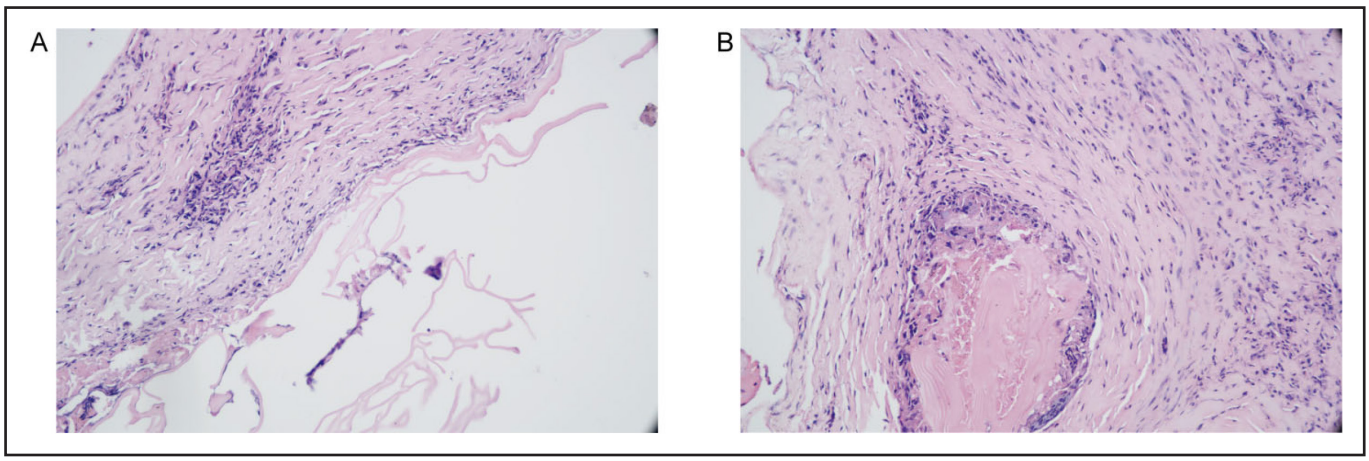

Figure 6. Hematoxylin and eosin staining of outer cyst wall tissue. Comparison of pathological changes between uncalcified (A) and calcified (B) outer cyst wall tissue (H&E, ×200).

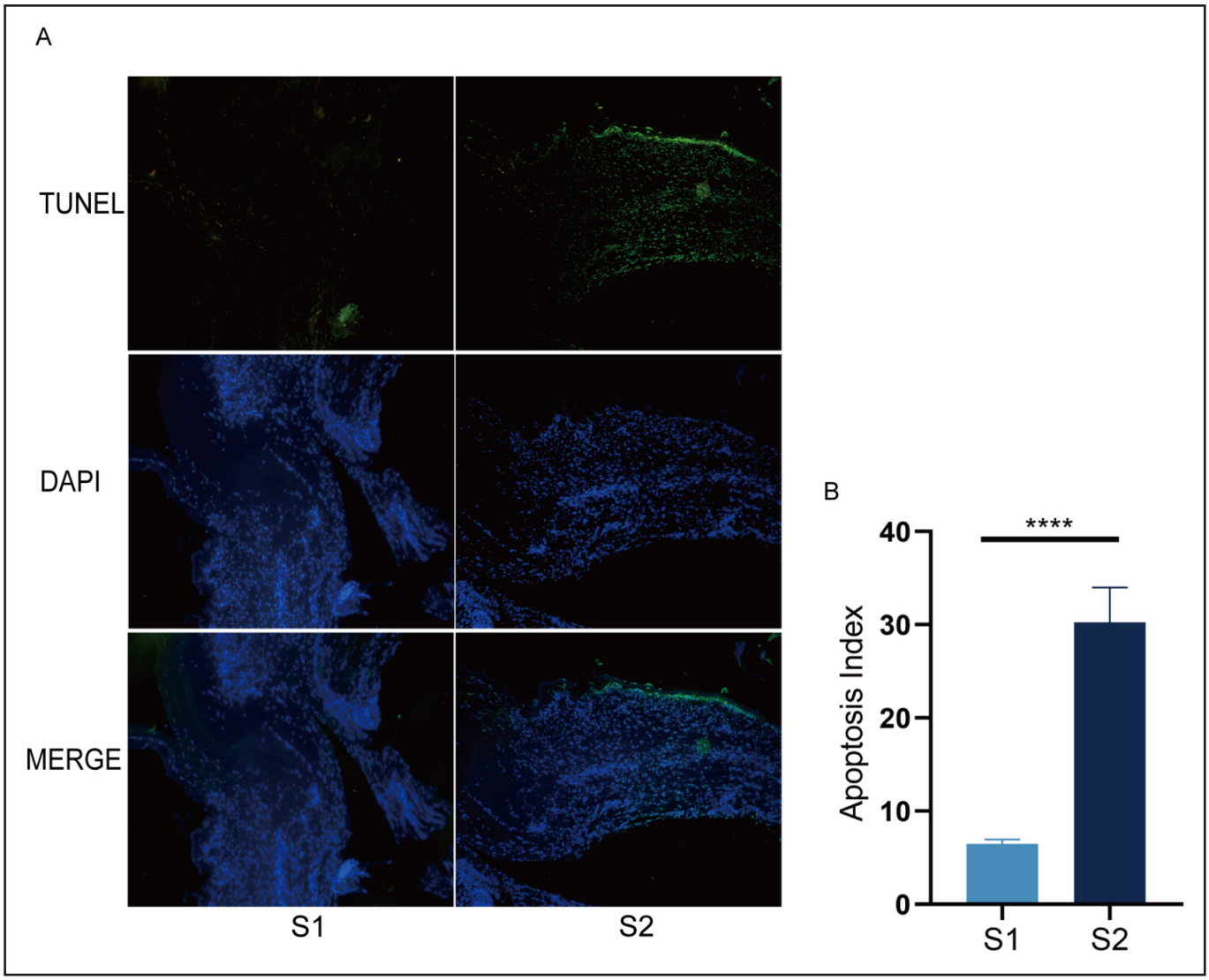

Figure 7. Apoptosis among groups. Uncalcified (S1) and calcified (S2) outer cyst wall tissue were detected by the TUNEL method (A; ×400), and the apoptotic index was calculated (B).

2021). The current study found mRNA transcription of *ASAH1* to be significantly downregulated in calcified tissue, which was consistent with previous findings that *ASAH1* is expressed at low levels in the calcified outer cyst wall. Therefore, *ASAH1* might be involved in regulation of calcification. Its regulatory mechanism in spontaneous calcification of the outer capsule wall needs further investigation (Yin *et al*., 2016). A study on vascular smooth-muscle calcification and osteoblastic differentiation found that *CP* possessed ferroxidase activity that inhibited calcification (Zarjou *et al*., 2009), which was in agreement with our results.

Several studies have shown *miR-223* to have regulatory potential (Taïbi *et al*., 2014; Jung *et al*., 2022; Sikora *et al*., 2022; Tanigawa *et al*., 2022). Furthermore, *miR-223-3p* was found to be highly expressed in active-phase *E. granulosus* tissue (Mariconti *et al*., 2019). This is consistent with our result that *miR-223-3p* was expressed at a low level in calcified cyst wall tissue, suggesting an inactivated state of the parasitic tissue. Interestingly, *miR-223-3p* also was found to inhibit osteogenic transformation of vascular smooth-muscle cells (VSMCs) by blocking the Jak-STAT signaling pathway; the formation of calcification is closely related to the biological activity of osteogenesis (Han *et al*., 2021). We also enriched the Jak-STAT pathway, and the result might suggest its regulatory relationship with miR-223-3p in the process of exocyst wall calcification. The intracellular signal transduction pathway PI3K-AKT contributes to regulation of cell differentiation, mineralization, proliferation, and bone formation (Takeno *et al*., 2019; Tong *et al*., 2021; Huang *et al*., 2022). In addition, *MiR-223* levels have been shown to be higher in the serum of patients with chronic hepatitis B and hepatocellular carcinoma, while healthy populations demonstrate lower expression thereof. The researchers concluded that *miR-223* could be used as an indicator of patient health (Riazalhosseini *et al*., 2017). We also found DEmiRNAs to be significantly enriched in the PI3K-AKT signaling pathway, which has been shown to be involved in regulation of apoptosis in a variety of cells (Zhu *et al*., 2019). Mice with CE can secrete thioredoxin peroxidase antigen to induce selective macrophagic differentiation by triggering Akt and upregulating upstream PI3K expression, thereby limiting the inflammatory response to promote immune escape (Wang *et al*., 2019). The above-mentioned studies suggest that PI3K-AKT signaling might be involved in immune antagonism between host and CE and that it mediates immunogenic parasite apoptosis. Calcification is often considered the end point of tissue necrosis in non-osteogenic biological processes, and it is a process in which calcium salts are deposited in the foci of necrosis, limiting and stabilizing the lesion. As a defensive response of the body to the lesion, it facilitates the removal of necrotic tissue and the regression of inflammation (Buckler *et al*., 2022). Moreover, recent studies have shown that apoptosis also serves as an important regulatory target for drug inhibition of CE growth (Alyousif *et al*., 2021; Xu *et al*., 2021). Therefore, we conclude that apoptosis as a common mechanism of programmed cell death is involved in the development of external-cyst wall calcification in CE.

CONCLUSION

In this study, we discovered genes that were differentially expressed between uncalcified and calcified cyst wall tissue in cystic echinococcosis. Our results also suggested that *ASAH1* and *CP* could be used as biological indicators to predict the occurrence of calcification in the outer cyst wall of *E. granulosus*. We also enriched the Jak-STAT and PI3K-AKT signaling pathways using GO and KEGG analysis and initially explored their mutual regulation of *miR-223*-3p. Finally, we confirmed the correlation between calcified cyst wall and apoptosis via H&E staining and TUNEL assay. In summary, our study explored the molecular-biological mechanism of outer cyst wall calcification and provided a new avenue for the treatment of CE.

Conflict of interest statement

This study was supported by the National Natural Science Foundation of China (grant nos. 31760270). The authors declare that they have no conflict of interests.

REFERENCES

Alyousif, M.S., Al-Abodi, H.R., Almohammed, H., Alanazi, A.D., Mahmoudvand, H., Shalamzari, M.H. & Salimikia, I. (2021). Chemical composition, apoptotic activity, and antiparasitic effects of Ferula macrecolea essential oil against Echinococcus granulosus Protoscoleces. *Molecules* **26**. https://doi.org/10.3390/molecules26040888

Bai, Y., Zhang, Z., Jin, L., Kang, H., Zhu, Y., Zhang, L., Li, X., Ma, F., Zhao, L., Shi, B. *et al.* (2014). Genome-wide sequencing of small RNAs reveals a tissue-specific loss of conserved microRNA families in Echinococcus granulosus. *Bmc Genomics* **15**: 736. https://doi.org/10.1186/1471-2164-15-736

Bhat, O.M., Li, G., Yuan, X., Huang, D., Gulbins, E., Kukreja, R.C. & Li, P.L. (2020). Arterial medial calcification through enhanced small extracellular vesicle release in smooth muscle-specific Asah1 Gene knockout mice. *Scientific Reports* **10**: 1645. https://doi.org/10.1038/s41598-020-58568-5

Bhat, O.M., Yuan, X., Kukreja, R.C. & Li, P.L. (2021). Regulatory role of mammalian target of rapamycin signaling in exosome secretion and osteogenic changes in smooth muscle cells lacking acid ceramidase gene. *Faseb Journal* **35**: e21732. https://doi.org/10.1096/fj.202100385R

Buckler, A.J., Gotto, A.J., Rajeev, A., Nicolaou, A., Sakamoto, A., St, P.S., Phillips, M., Virmani, R. & Villines, T.C. (2022). Atherosclerosis risk classification with computed tomography angiography: A radiologic-pathologic validation study. *Atherosclerosis* **367**: 42-48. https://doi.org/10.1016/j.atherosclerosis.2022.11.013

Budke, C.M., Carabin, H., Ndimubanzi, P.C., Nguyen, H., Rainwater, E., Dickey, M., Bhattarai, R., Zeziulin, O. & Qian, M.B. (2013). A systematic review of the literature on cystic echinococcosis frequency worldwide and its associated clinical manifestations. *American Journal of Tropical Medicine and Hygiene* **88**: 1011-1027. https://doi.org/10.4269/ajtmh.12-0692

Conchedda, M., Caddori, A., Caredda, A., Capra, S. & Bortoletti, G. (2018). Degree of calcification and cyst activity in hepatic cystic echinococcosis in humans. *Acta Tropica* **182**: 135-143. https://doi.org/10.1016/j.actatropica.2018.02.027

Cong, P. (2012). Degenerative necrosis and calcification of hepatic cycstic echinococcosis. *China Medical Abstracts (Surgery)* **21**: 54.

Craig, P.S. & Larrieu, E. (2006). Control of cystic echinococcosis/hydatidosis: 1863-2002. *Advances in Parasitology* **61**: 443-508. https://doi.org/10.1016/S0065-308X(05)61011-1

Cucher, M., Prada, L., Mourglia-Ettlin, G., Dematteis, S., Camicia, F., Asurmendi, S. & Rosenzvit, M. (2011). Identification of Echinococcus granulosus microRNAs and their expression in different life cycle stages and parasite genotypes. *International Journal for Parasitology* **41**: 439-448. https://doi.org/10.1016/j.ijpara.2010.11.010

Czermak, B.V., Akhan, O., Hiemetzberger, R., Zelger, B., Vogel, W., Jaschke, W., Rieger, M., Kim, S.Y. & Lim, J.H. (2008). Echinococcosis of the liver. *Abdom Imaging* **33**: 133-143. https://doi.org/10.1007/s00261-007-9331-0

Gao, J., Zhou, X., Liu, L., Lv, G., Hou, Q., Zhang, X. & Shen, Y. (2022). Bioinformatics analysis and experimental verification of Notch signalling pathway-related miRNA-mRNA subnetwork in extracellular vesicles during Echinococcus granulosus encystation. *Parasites & Vectors* **15**: 272. https://doi.org/10.1186/s13071-022-05391-8

Govindaraj, S., Bhadra, V., Prakash, C. & Govindaraj, S. (2021). Unusual case of disappearing hepatic hydatid cyst: COVID-19 times. *BMJ Case Reports* **14**. https://doi.org/10.1136/bcr-2021-243533

Han, Y., Zhang, J., Huang, S., Cheng, N., Zhang, C., Li, Y., Wang, X., Liu, J., You, B. & Du, J. (2021). MicroRNA-223-3p inhibits vascular calcification and the osteogenic switch of vascular smooth muscle cells. *Journal of Biological Chemistry* **296**: 100483. https://doi.org/10.1016/j.jbc.2021.100483

Huang, H., Wang, X., Liao, H., Ma, L., Jiang, C., Yao, S., Liu, H. & Cao, Z. (2022). Expression profile analysis of long noncoding RNA and messenger RNA during mouse cementoblast mineralization. *Journal of Periodontal Research* **57**: 1159-1168. https://doi.org/10.1111/jre.13053

Ito, A. & Budke, C.M. (2017). The echinococcoses in Asia: The present situation. *Acta Tropica* **176**: 11-21. https://doi.org/10.1016/j.actatropica.2017.07.013

Jung, D., Shin, S., Kang, S.M., Jung, I., Ryu, S., Noh, S., Choi, S.J., Jeong, J., Lee, B.Y., Kim, K.S. *et al.* (2022). Reprogramming of T cell-derived small extracellular vesicles using IL2 surface engineering induces potent anti-cancer effects through miRNA delivery. *Journal of Extracellular Vesicles* **11**: e12287. https://doi.org/10.1002/jev2.12287

Mariconti, M., Vola, A., Manciulli, T., Genco, F., Lissandrin, R., Meroni, V., Rosenzvit, M., Tamarozzi, F. & Brunetti, E. (2019). Role of microRNAs in host defense against Echinococcus granulosus infection: a preliminary assessment. *Immunologic Research* **67**: 93-97. https://doi.org/10.1007/s12026-018-9041-4

McManus, D.P., Gray, D.J., Zhang, W. & Yang, Y. (2012). Diagnosis, treatment, and management of echinococcosis. *BMJ* **344**: e3866. https://doi.org/10.1136/bmj.e3866

Peng, X.Y., Wu, X.W., Zhang, X.J. & Niu, J.H. (2005). Rerecognition of the pathological structure of fibrous cyst wall around hepatic hydatid cyst. *World Chinese Journal of Digestion* **3**: 10-13.

Riazalhosseini, B., Mohamed, R., Apalasamy, Y.D., Langmia, I.M. & Mohamed, Z. (2017). Circulating microRNA as a marker for predicting liver disease progression in patients with chronic hepatitis B. *Revista Da Sociedade Brasileira De Medicina Tropical* **50**: 161-166. https://doi.org/10.1590/0037-8682-0416-2016

Ritler, D., Rufener, R., Li, J.V., Kämpfer, U., Müller, J., Bühr, C., Schürch, S. & Lundström-Stadelmann, B. (2019). In vitro metabolomicfootprint of the Echinococcus multilocularismetacestode. *Scientific Reports* **9**: 19438. https://doi.org/10.1038/s41598-019-56073-y

Sanei, B., Nasr, E.F., Abbasivand, J.A. & Andalib, M.M. (2021). Effective factors on the disappearance of residual cavity following conservative surgery of liver hydatid cysts. *Advanced Biomedical Research* **10**: 41. https://doi.org/10.4103/abr.abr_198_20

Santosh, B., Varshney, A. & Yadava, P.K. (2015). Non-coding RNAs: biological functions and applications. *Cell Biochemistry and Function* **33**: 14-22. https://doi.org/10.1002/cbf.3079

Si, X., Ma, J., Zhang, X., Wang, H., Sun, X., Ma, X., Wang, W., Liu, Y., Liu, J. & Han, D. (2022). Preliminary study of differentially expressed proteins in mice with secondary echinococcosis based on independent full scan acquisition proteomics technology. *Chinese Journal of Schistosomiasis Control* **34**: 52-58. https://doi.org/10.16250/j.32.1374.2021221

Sikora, M., Krajewska, K., Marcinkowska, K., Raciborska, A., Wiglusz, R.J. & mieszek, A. (2022). Comparison of selected non-coding RNAs and gene expression profiles between common osteosarcoma cell lines. *Cancers* **14**: 4533. https://doi.org/10.3390/cancers14184533

Taïbi, F., Metzinger-Le Meuth, V., Massy, Z.A. & Metzinger, L. (2014). miR-223: An inflammatory oncomiR enters the cardiovascular field. *Biochimica et Biophysica Acta-Molecular Basis of Disease* **1842**: 1001-1009. https://doi.org/10.1016/j.bbadis.2014.03.005

Takeno, A., Kanazawa, I., Tanaka, K.I., Notsu, M. & Sugimoto, T. (2019). Phloretin Suppresses bone morphogenetic protein-2-induced osteoblastogenesis and mineralization via inhibition of phosphatidylinositol 3-kinases/Akt pathway. *International Journal of Molecular Sciences* **20**: 2481. https://doi.org/10.3390/ijms20102481

Tanigawa, K., Misono, S., Mizuno, K., Asai, S., Suetsugu, T., Uchida, A., Kawano, M., Inoue, H. & Seki, N. (2022). MicroRNA signature of small-cell lung cancer after treatment failure: impact on oncogenic targets by miR-30a-3p control. *Molecular Oncology* **17**: 328-343. https://doi.org/10.1002/1878-0261.13339

Tong, W., Li, J., Feng, X., Wang, C., Xu, Y., He, C. & Xu, W. (2021). Kaiso regulates osteoblast differentiation and mineralization via the Itga10/PI3K/AKT signaling pathway. *International Journal of Molecular Medicine* **47**. https://doi.org/10.3892/ijmm.2021.4874

Wang, C., Yang, S., Niu, N., Tao, J., Du, X., Yang, J., Zhu, M., Wang, Y. & Zhao, W. (2021b). lncRNA028466 regulates Th1/Th2 cytokine expression and associates with Echinococcus granulosus antigen P29 immunity. *Parasites & Vectors* **14**: 295. https://doi.org/10.1186/s13071-021-04795-2

Wang, H., Zhang, C.S., Fang, B.B., Li, Z.D., Li, L., Bi, X.J., Li, W.D., Zhang, N., Lin, R.Y. & Wen, H. (2019). Thioredoxin peroxidase secreted by Echinococcus granulosus (sensu stricto) promotes the alternative activation of macrophages via PI3K/AKT/mTOR pathway. *Parasites & Vectors* **12**: 542. https://doi.org/10.1186/s13071-019-3786-z

Wang, S., Wang, S., Wang, W., Dai, Y., Qiu, Z., Ke, W., Geng, M., Li, J., Li, K., Ma, Q. *et al*. (2021a). In Vitro and In Vivo Efficacy of albendazole chitosan microspheres with intensity-modulated radiation therapy in the treatment of spinal echinococcosis. *Antimicrobial Agents and Chemotherapy* **65**: e00795-21. https://doi.org/10.1128/AAC.00795-21

Wen, H., Vuitton, L., Tuxun, T., Li, J., Vuitton, D.A., Zhang, W. & McManus, D.P. (2019). Echinococcosis: Advances in the 21st Century. *Clinical Microbiology Reviews* **32**: e00075-18. https://doi.org/10.1128/CMR.00075-18

Xiao, J., Zhu, Y., Wu, J., Bai, M., Xin, Y., Wang, Q. & Zhao, J. (2022). Expression profiling of exosomal miRNAs derived from different stages of infection in mice infected with Echinococcus granulosus protoscoleces using high-throughput sequencing. *Parasitology Research* **121**: 1993-2008. https://doi.org/10.1007/s00436-022-07536-1

Xu, W.L., Aikeremu, D., Sun, J.G., Zhang, Y.J., Xu, J.B., Zhou, W.Z., Zhao, X.B., Wang, H. & Yuan, H. (2021). Effect of intensity-modulated radiation therapy on sciatic nerve injury caused by echinococcosis. *Neural Regeneration Research* **16**: 580-586. https://doi.org/10.4103/1673-5374.293153

Yin, Y., Zhang, Q., Yang, Y., Yang, S., Wang, H., Shi, J., Wang, Z., Yang, Y., Lin, Y., Li, Z. *et al*. (2016). Study on the genes related to cystic calcification in patients with cystic hydatid disease. *Chinese Journal of Preventive Medicine* **50**: 434-438.

Yu, A., Wang, Y., Yin, J., Zhang, J., Cao, S., Cao, J. & Shen, Y. (2018). Microarray analysis of long non-coding RNA expression profiles in monocytic myeloid-derived suppressor cells in Echinococcus granulosus-infected mice. *Parasites & Vectors* **11**: 327. https://doi.org/10.1186/s13071-018-2905-6

Yu, X., Yasen, Y., Wang, C., Li, M., Fang, Z., Wang, J. & Ye, J. (2021). Construction of ceRNA regulatory network in mice with Echinococcosis-induced allergic reactions. *Acta Tropica* **224**: 106120. https://doi.org/10.1016/j.actatropica.2021.106120

Zarjou, A., Jeney, V., Arosio, P., Poli, M., Antal-Szalmas, P., Agarwal, A., Bblla, G. & Bblla, J. (2009). Ferritin prevents calcification and osteoblastic differentiation of vascular smooth muscle cells. *Journal of the American Society of Nephrology* **20**: 1254-1263. https://doi.org/10.1681/ASN.2008070788

Zhang, X.O., Dong, R., Zhang, Y., Zhang, J.L., Luo, Z., Zhang, J., Chen, L.L. & Yang, L. (2016). Diverse alternative back-splicing and alternative splicing landscape of circular RNAs. *Genome Research* **26**: 1277-1287. https://doi.org/10.1101/gr.202895.115

Zhao, R., Wang, B., Yang, X., Xiao, Y., Wang, X., Shao, C. & Tang, R. (2016). A drug-free tumor therapy strategy: cancer-cell-targeting calcification. *Angewandte Chemie International Edition* **55**: 5225-5229. https://doi.org/10.1002/anie.201601364

Zhu, R., Wang, W. & Yang, S. (2019). Cryptotanshinone inhibits hypoxia/reoxygenation-induced oxidative stress and apoptosis in renal tubular epithelial cells. *Journal of Cellular Biochemistry* **120**: 13354-13360. https://doi.org/10.1002/jcb.28609