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Identification and Verification of Hub mRNA-miRNA-lncRNA Network on Psoriasis by Integrated Bioinformatics Analysis

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Key words
Psoriasis, lncRNA-miRNA-mRNA network, Bioinformatics, LncRNA MALAT1, LncRNA XIST

ABSTRACT

Psoriasis is an immune-mediated skin disease with a high incidence, it has been demonstrated that particular long non-coding RNAs (lncRNAs) are vital in psoriasis. However, their exact functions and regulatory mechanisms in psoriasis remain unclear. In this study, GSE142582 and GSE54456 datasets from GEO database were used to screen differentially expressed microRNAs (DEmiRNAs) and mRNAs (DEmRNAs) between psoriatic lesions and normal skin. Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and protein-protein interaction (PPI) network analysis were performed to determine the enrichment pathway and hub mRNA targets. miRWalk database was used to predict the corresponding mRNA to DEmiRNAs, and StarBase database was utilized to predict LncRNA to DEmiRNAs. Hub lncRNA-miRNA-mRNA networks were integrated and constructed, and visualized using Cytoscape software. A psoriasis rat model was subsequently established to verify the hub lncRNA-miRNA-mRNA networks in psoriasis using RT-qPCR. The results demonstrated that, 813 DEmRNAs and 299 DEmiRNAs were identified. GO and KEGG analyses showed that up-regulated DEmRNAs were enriched for immune responses, inflammatory responses, and keratinization, while down-regulated DEmRNAs were enriched for intermediate filament organization and tight junctions. RT-qPCR analysis showed that the expression of lncRNA NEAT1, miR-135b-5p were significantly increased (p <0.05) compared to the normal group, while the levels of lncRNA MALAT1, lncRNA XIST, miR-125b-5p, miR-125b-2-3p, KIF2C, *MYLK, ACTG2* and *MYH11* were significantly decreased (p <0.01 and 0.05). In conclusion, the present study suggested that, over-activated immune and inflammatory systems, hyperkeratosis, and cell junction destruction were the main biological reactions driving the development of psoriasis. The lncRNA (NEAT1)-miRNA (miR-125b-5p, miR-125b-2-3p)-mRNA (KIF2C) axis, and lncRNA (MALAT1, XIST)-miRNA (miR-135b-5p)-mRNA (*MYLK*, *ACTG2*, and *MYH11*) axis play vital roles in the pathogenesis of psoriasis, which may be the important mechanisms to explain the happen and progression of psoriasis.

INTRODUCTION

Psoriasis is a chronic autoimmune inflammatory skin disease with a relatively common etiology, with a prevalence of 2% to 3% globally (Boehncke, 2015; Parisi *et al*., 2013). The disease is characterized by abnormal proliferation and division of keratinocytes, lymphocyte and neutrophil infiltration, including T cells and dendritic cells, as well as the release of inflammatory cytokines (Rendon and Schäkel, 2019). Although there are several treatment options available for psoriasis, the curative effect is limited by severe side effects and high rates of recurrence, making it a worldwide medical issue. For instance, methotrexate can inhibit the proliferation of keratin cells and activate T lymphocytes to achieve certain therapeutic purposes, but it may cause adverse reactions of gastrointestinal reactions, hematopoietic system issues, and liver dysfunction (Flytström *et al*., 2008). In addition,

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psoriasis is also prone to relapse after withdrawal of steroid hormones, retinoic acid, etc. Thus, it is essential to identify novel biomarkers and pathways involved in psoriasis to discover new insights into disease treatment.

Although researchers have increasingly focused on biological intervention RNA in treating psoriasis, the exact mechanisms of action remain not fully elucidated. MicroRNAs (miRNAs) and long noncoding RNAs (lncRNAs) regulate gene expression in many biological processes, including psoriasis. Recent studies have indicated that lncRNAs play crucial roles in psoriasis development by regulating key protein expression involved in chronic inflammatory processes, immune infiltration, and hyperproliferation (Zhou *et al*., 2019). For example, lncRNA PRINS mediates abnormal psoriatic keratinocyte proliferation by regulating G1P3 expression (Szegedi *et al*., 2010). The microRNA (miRNA)-31 inhibits protein phosphatase 6 to promote keratinocyte proliferation (Yan *et al*., 2015), and lncRNA MSX2P1 inhibits miR-6731-5p and activates S100A7 to promote keratinocyte growth and proliferation (Qiao *et al*., 2018). Proliferation and inflammation of HaCaT can be inhibited by lowing miR-221-3p expression, providing potential therapeutic intervention against psoriasis (Meng *et al*., 2021). Furthermore, lncRNA NEAT1 (nuclear enriched abundant transcript 1) stimulates inflammasome activation in macrophage, thus the activated caspase-1 promoting IL-1β production and pyroptosis, which are elevated in psoriatic samples. Thus, it is great sense to elucidate the function of lncRNA in the treatment of psoriasis. Non-coding RNAs do not exist in isolation, but appear to form complex regulatory networks implicated in disease regulation, including psoriasis. They are important competing endogenous RNAs (ceRNAs) that inhibit miRNA-mediated target repression by competing for miRNA binding sites in RNAs (Zhou *et al*., 2019). Therefore, elucidating non-coding RNA mechanisms, particularly lncRNA-miRNA-mRNA networks, in psoriasis can improve understanding of the pathogenesis and facilitate drug development.

In this study, GSE142582 and GSE54456 datasets from GEO database were used to screen differentially expressed microRNAs (DEmiRNAs) and mRNAs (DEmRNAs) between psoriatic lesions and normal skin. Then bioinformatics analysis was employed to integrate and construct hub lncRNA-miRNA-mRNA networks in psoriasis. Furthermore, the psoriasis rat model was established, and RT-qPCR was employed to verify the hub networks in psoriasis (Supplementary Fig. S1). The aim was to investigate novel and significant mechanisms of psoriasis associated dysregulation of lncRNA-miRNA-mRNA networks, which may contribute to the progression of psoriasis and provide potential therapeutic targets for psoriasis treatments.

MATERIALS AND METHODS

Collection of RNA-seq datasets

The GEO database (https://www.ncbi.nlm.nih.gov/geo) was applied to get datasets for evaluating mRNA and miRNA expression in skin tissue samples from psoriasis patients and healthy individuals. Two RNA sequencing datasets were obtained. The GSE54456 dataset contains 92 psoriasis and 82 health control samples based on the GPL9052 Illumina Genome Analyzer (*H. sapiens*). The GSE142582 dataset contains five psoriasis and five health control samples based on GPL20301 Illumina HiSeq 4000 (*H. sapiens*).

Differential expression analysis of mRNA and miRNA

The R package 3.6.3 (https://cran.r-project.org/) was used for RNA-seq expression profile analysis to identify significant DEmRNAs and DEmiRNAs. The screening criteria for significant differential expression between psoriasis and health control samples were |log2 (fold-change)| > 1 and adjusted P value < 0.05. Heat maps and volcano plots about DEmRNAs and DEmiRNAs were drawn using the Omic Studio tools (https://www.omicstudio.cn/index).

GO and KEGG analysis of DEmRNAs

The database for annotation, visualization and integrated discovery (DAVID) database (https://david.ncifcrf.gov/) was utilized for gene ontology (GO) functional enrichment analyses. After uploading DEmRNAs, enriched biological process (BP), molecular function (MF), and cellular component (CC) pathways were identified. The Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses were performed by KOBAS database (http://kobas.cbi.pku.edu.cn/index.php). A P-value of <0.05 was considered statistically significant. Bubble charts, showing GO and KEGG analyses for significant DEmRNAs in the GSE54456 dataset, were generated using ChiPlot (https://www.chiplot.online/).

Construction of protein-protein interaction (PPI) network

The screened DEmRNAs were input into the STRING database (https://cn.string-db.org/) to construct a PPI network. The regulatory relationships between genes were visualized using Cytoscape v3.7.2 (https://cytoscape.org/). Hub genes in the regulatory network were analyzed using the CytoHubba plug-in with maximal clique centrality (MCC) algorithm.

Construction of miRNA-mRNA network

The miRWalk 2.0 database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) was utilized to predict mRNA targets of the DEmiRNAs. The intersecting mRNAs between the upregulated DEmiRNAs corresponding to mRNAs and down regulated DEmRNAs, and the intersecting mRNAs between the down regulated DEmiRNAs corresponding to mRNAs and up regulated DEmRNAs, were identified. These intersecting mRNAs and their corresponding miRNAs were arranged using Excel software, and then visualized using Cytoscape v3.7.2.

Construction of lncRNA-miRNA network

StarBase v2.0 (http://starbase.sysu.edu.cn) is a profundity online tool for system recognition of RNA-RNA and protein-RNA interaction networks. In this study, down- and up-regulated DEmiRNAs were assembled to predict the target lncRNAs (filter criteria, "clip ExpNum" ≥2), then the lncRNA-miRNA interactions were generated.

Construction of lncRNA-miRNA-mRNA network

To better comprehend the roles of lncRNAs, miRNAs, and mRNAs in psoriasis, a lncRNA-miRNA-mRNA regulatory network was constructed using the top ten lncRNAs, DEmiRNAs, and hub genes. The resulting network was visualized using Cytoscape 3.7.2 software.

Establishment of psoriasis rat model and hub lncRNA-miRNA-mRNA regulatory network verification

Male specific pathogen free SD rats were obtained from Shanghai Shrek experimental animal Co., Ltd. (Shanghai, China), and were treated in conform to the Guide for the Care and Use of Laboratory Animals. A total of 12 rats were housed at 25 ± 2 °C temperature with a 12-h light/dark cycle. The study was approved by the Bioethics Committee of Zhejiang Academy of Traditional Chinese Medicine (Approval No. KTSB2022018). After a seven-day adaptive feeding, the rats were shaved on their back (size 3 × 3 cm^2), and imiquimod (62.5 mg) was gently applied to the shaved skin areas for 7 days to establish psoriasis rats model (n = 6). Normal control rats were coated with an equal amount of vaseline on their back (n = 6). All rats were given standard diet and water.

At the end of experiment, skin samples from the psoriasis and control rats were collected and fixed in 10% neutral buffer formalin for histological studies. The skin samples were stained with hematoxylin-eosin (H&E) staining, and the images were captured using a digital camera attached to a light microscopy (Olympus, Tokyo, Japan) at 40× and 400× magnification. Epidermal thicknesses were measured using ImageJ software.

The remaining skins were promptly frozen in liquid nitrogen, and total RNA was extracted utilizing the SPARKeasy tissues/cell RNA Rapid Extraction Kit (#AC0202, Shandong Sikejie Biotechnology Co., Ltd., Shandong, China). The cDNA was synthesized using the SPARK script II RT Plus kit (#AG0304-B, Shandong Sikejie Biotechnology Co., Ltd., Supplementary Table SI). All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and are presented in Table I. Real-Time PCR was performed using the 2× SYBR Green qPCR Mix (#AH0104-B, Shandong Sikejie Biotechnology Co., Ltd.), with the following conditions: 2 min at 94 °C, and then 40 cycles of 94 °C for 10 s, 60 °C for 35 s. *β-actin* was used as a normalization control, and the fold change for each target gene was counted by the $2^{-\Delta\Delta Ct}$ method.

Table I. The primer sequences used in the study.

Gene	Gene ID	Forward (5′ → 3′)	Reverse (5′ → 3′)
LncRNA NEAT1	66961	CAAGGCTGCGGACTGTCATCATAG	CTGCTGAAGGTGGTGGTGTGAG
LncRNA MALAT1	72289	CATACGGATGTGGTGGTGAAGC	AATGCCTGCTCGCCTCCTC
LncRNA XIST	100911498	AGGCTGCGGACTGTCATCATAG	TGCTGAAGGTGGTGGTGTGAG
miR-125b-5p	498130	AAGCTGAGTCCCTGAGACCCTAA	ATCCAGTGCAGGGTCCGAGG
miR-125b-2-3p	498140	ACCACCGACAAGTCAGGCTCT	ATCCAGTGCAGGGTCCGAGG
miR-142-3p	25205	TGTCCGCCTGTAGTGTTTCCTAC	ATCCAGTGCAGGGTCCGAGG
miR-135b-5p	64832	AAGCGACCTATGGCTTTTCATTCC	ATCCAGTGCAGGGTCCGAGG
MYLK	288057	CGTGAGGAGACAAGAAGGCATCG	GCTCTCGGCAGACACAGGTAATG
MYH11	24582	ACCTCACTCCTCAATGCCTCCTC	CCACAATGCGGTCCACATCCTTC
ACTG2	25365	GCGAGTAGCACCAGAAGAGCAC	GAATGGCAACATACATGGCAGGAAC
KIF2C	171529	CGTTCCACTCGCATATCCACTGTC	GCTCCACCTCCATTTCATTCTCCTG
miR-125b-5p RT primer		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAA	
miR-125b-2-3p RT primer		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGTCC	
miR-142-3p RT primer		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCATA	
miR-135-5p RT primer		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAT	

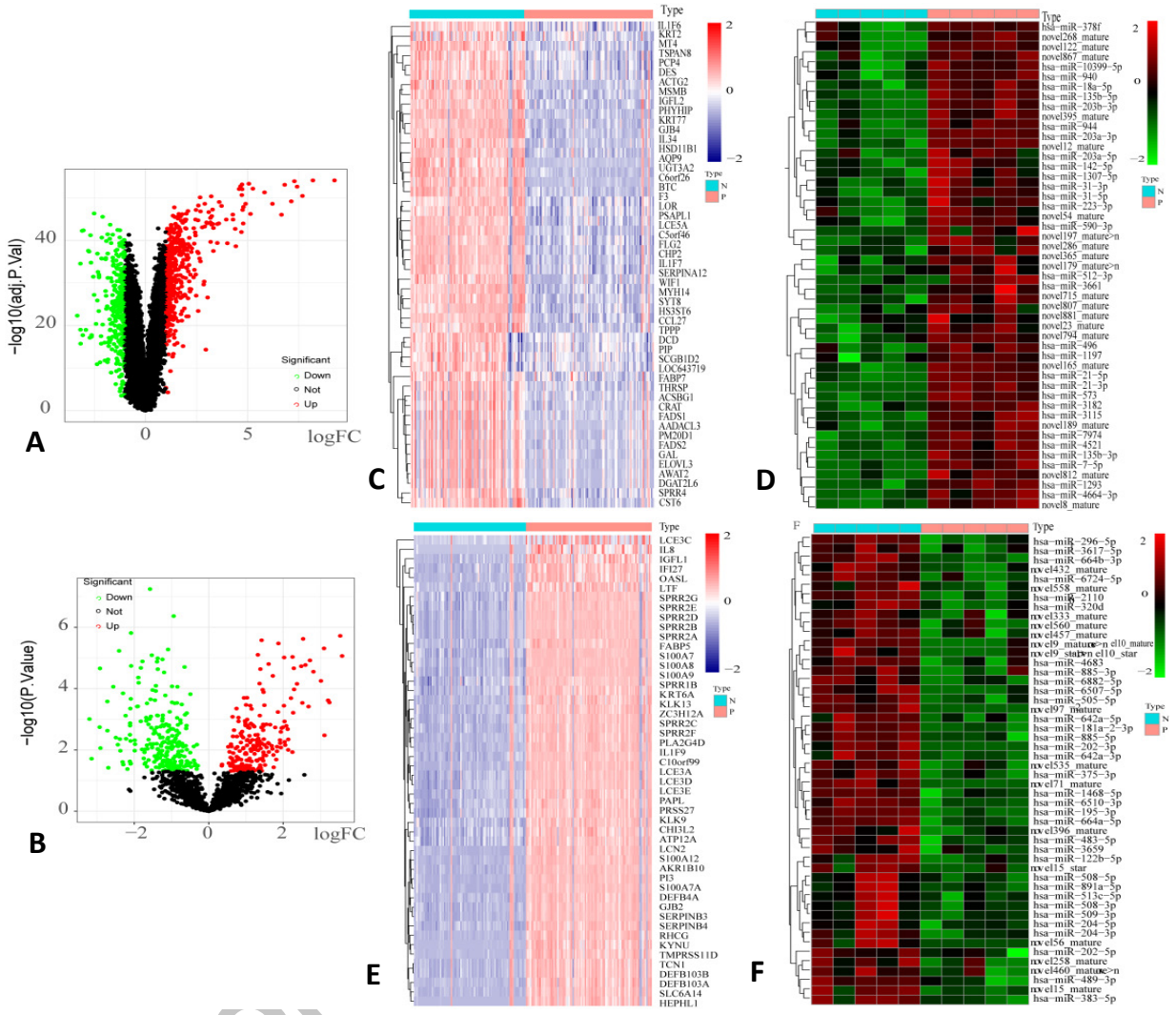

Fig. 1. DEmRNAs and DEmiRNAs between skin lesion samples and normal controls. The volcano plots show significant DEmRNAs in GSE54456 (A) and significant DEmiRNAs in GSE142582 (B). Heat maps show significant up-regulated DEmRNAs (C), down-regulated DEmiRNAs (D), down-regulated DEmRNAs (E) and up-regulated DEmiRNAs (F). Type of N indicates normal controls, and type of P indicates skin lesion samples. In panels (C, E), blue represents down-regulated DEmRNA and pink represents up-regulated DEmRNA; in panels (D,F), green represents down-regulated DEmiRNA and red represents up-regulated DEmiRNA.

Statistical analysis

Results were presented as the mean±standard deviation (SD). SPSS 15.0 software (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. The differences between groups were compared by student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of DEmRNAs and DEmiRNAs

The microarray datasets of GSE54456 and GSE142582 were normalized and analyzed (Fig. 1), and 813 significant DEmRNAs were identified in the GSE54456 dataset between normal and lesion tissue, with 480 up-regulated and 333 down-regulated mRNAs (Fig. 1A, C, E). Additionally, 299 significant DEmiRNAs were identified in the GSE142582 dataset, with 140 up-regulated and 159 down-regulated miRNAs (Fig. 1B, D, F).

GO and KEGG pathway enrichment analyses for the DEmRNAs

To perform functional enrichment analyses, the identified 813 DEmRNAs were conducted GO enrichment analysis and KEGG pathway analysis using DAVID

and KOBAS respectively. The GO analysis on BP of up-regulated DEmRNAs were most enriched in innate immune response, followed by inflammatory response, immune response, defense response to virus (Fig. 2A). Down-regulated DEmRNAs were most enriched in lipid metabolic process, cell adhesion, and positive regulation of gene expression, muscle contraction, aging, intermediate filament organization, and fatty acid metabolic process (Fig. 2B).

KEGG pathway enrichment analyses revealed that up-regulated DEmRNAs were most enriched in metabolic pathways, and followed by proteoglycans in cancer, pathways in cancer PPAR signaling pathway, cell adhesion molecules (CAMs), and tight junction (Fig. 2C). In addition, down-regulated DEmRNAs were most enriched in metabolic pathways, followed by cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway, influenza A, chemokine signaling pathway and pathways in cancer (Fig. 2D).

PPI network construction and hub genes analyses

There were 813 DEmRNAs, including 480 up-regulated and 333 down-regulated, loaded into STRING online database to construct PPI network and the hub genes were identified (Fig. 3). According to the MCC algorithm, the top ten up-regulated hub genes were *CCNB2*, *KIF20A*, *CCNA2*, *KIF2C*, *TOP2A*, *UBE2C*, *BUB1*, *CDK1*, *MELK*, and *PLK1*. The top ten down-regulated hub genes were *MYH11*, *MYL9*, *ACTA2*, *TPM1*, *TPM2*, *ACTG2*, *MYLK*, *TNNT2*, *TAGLN*, and *CNN1*.

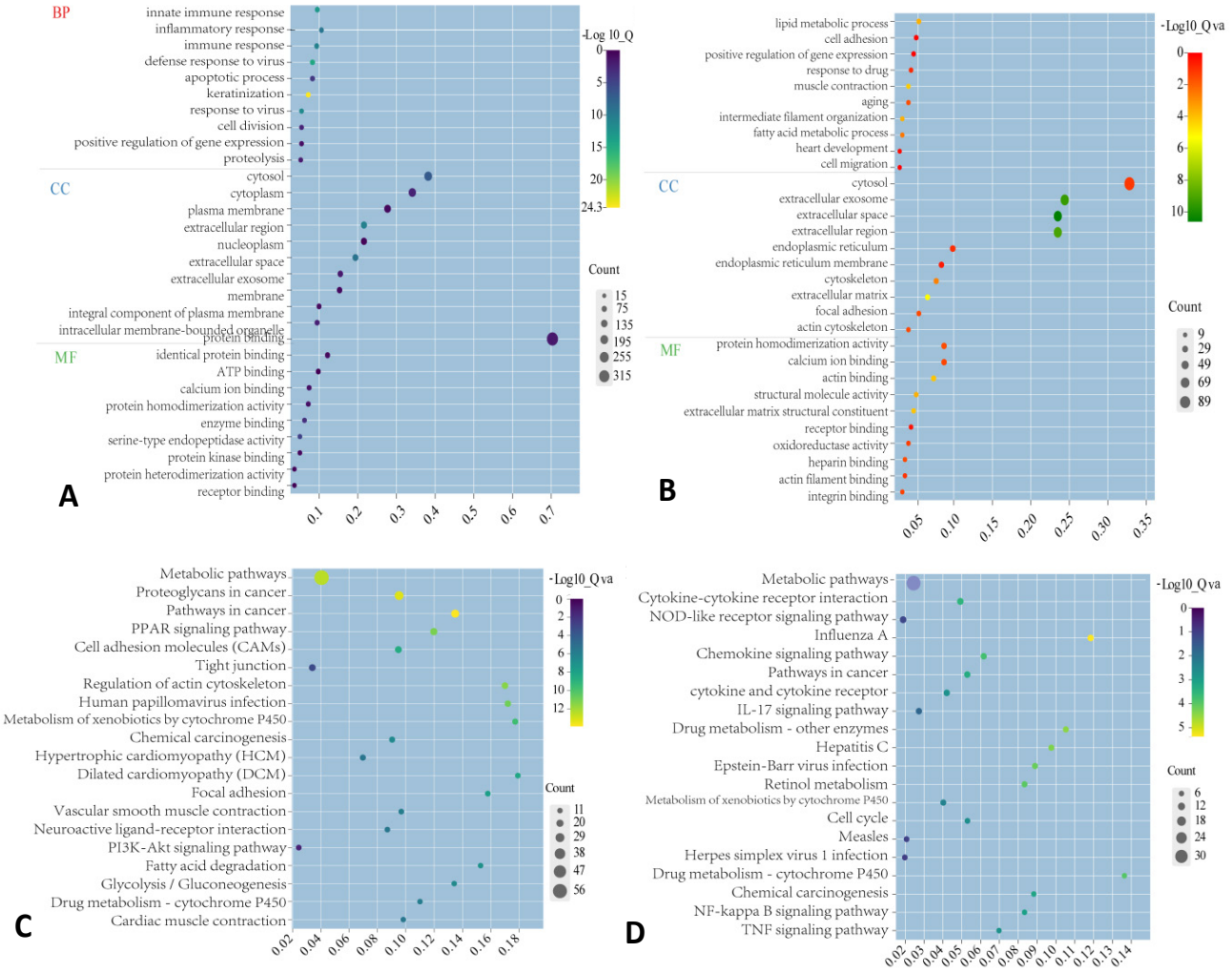

Fig. 2. GO and KEGG analyses for significant DEmRNAs in GSE54456. The bubble charts show GO analysis results of up-regulated (A) and down-regulated DEmRNAs (B), and KEGG analysis results of up-regulated (C) and down-regulated DEmRNAs (D).

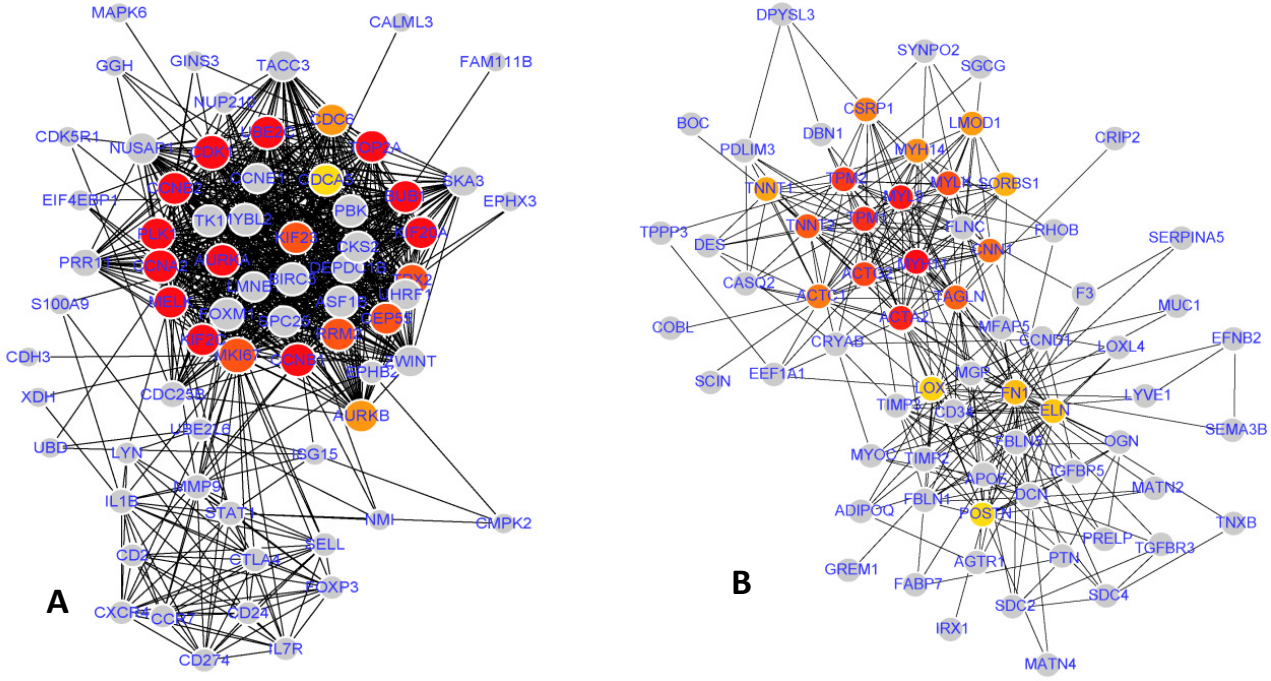

Fig. 3. Protein-protein interaction (PPI) network. (A) up-regulated hub genes network. (B) down-regulated hub genes network. Gray nodes represent hub genes, while lines illustrate the relationship between nodes. Red nodes indicate the top ten hub genes ranked by degree.

Table II. Integrated analysis and top ten hub up-regulated and down-regulated DEmiRNAs.

Integrated analysis of DEmiRNAs	Overlapping mRNA	Related up-/ down-regulated DEmiRNAs	Top ten hub
140 up-regulated miRNAs predicted mRNA and GEO dataset had identified down-regulated DEmRNAs	172	24 up-regulated	DEmiRNAs were hsa-miR-1293, hsa-miR-1185-1-3p, hsa-miR-1285-5p, hsa-miR-141-5p, hsa-miR-1276, hsa-miR-135b-3p, hsa-miR-135b-5p, hsa-miR-106b-5p, hsa-miR-10399-3p, and hsa-miR-10399-5p
159 down-regulated miRNAs predicted mRNA and GEO dataset had identified up-regulated DEmRNAs	281	16 down-regulated	hsa-miR-125a-3p, hsa-miR-1275, hsa-miR-1468-5p, hsa-let-7e-3p, hsa-miR-149-5p, hsa-miR-193a-5p, hsa-miR-125b-2-3p, hsa-miR-125b-5p, hsa-miR-125a-5p, and hsa-miR-10a-5p

mRNA-miRNA interaction network

Subsequently, the total significant DEmiRNAs were used to predict target mRNAs via miRWalk 2.0. An integrated analysis was conducted using the predicted 140 up-regulated DEmiRNAs target mRNAs and the identified down-regulated DEmRNAs, and the results showed that there were 172 overlapping mRNA and 24 corresponding up-regulated DEmiRNAs (Supplementary Fig. S2, Table II). The top ten hub up-regulated DEmiRNAs were listed in Table II.

The integrated analysis of predicted 159 down-regulated DEmiRNAs target mRNA and the identified up-regulated DEmRNAs were performed, there were 281 overlapping mRNA and 16 related down-regulated DEmiRNAs (Supplementary Fig. S2, Table II). According to the degree of miRNA, the top ten hub down-regulated DEmiRNAs were listed in Table II.

lncRNA-miRNA interaction network

The selected 24 up-regulated DEmiRNAs and 16 down-regulated DEmiRNAs were used to predict target lncRNAs via StarBase online. As filter criteria of the clip

ExpNum was set ≥2, 5 up-regulated DEmiRNAs had no predicted lncRNAs. Finally, 11 up-regulated DEmiRNAs were found to be correlated with 221 DElncRNAs, they were hsa-miR-142-3p, hsa-miR-106b-5p, hsa-miR-142-5p, hsa-miR-135a-5p, hsa-miR-135b-5p, hsa-miR-1276, hsa-miR-144-5p, hsa-miR-1197, hsa-miR-154-3p, hsa-miR-1307-3p, and hsa-miR-1307-5p. Then the potential ceRNA regulatory network of psoriasis vulgaris was constructed, which was included 329 DEmiRNA-lncRNA interactions (Supplementary Fig. S3A). The top ten lncRNAs, which were ranked based on the number of associated miRNAs, were NEAT1, XIST, OIP5-AS1, KCNQ1OT1, MALAT1, ALO35425.3, PSMA3-AS1, GAS5, RPARP-AS1, and NORAD.

Similarly, a total of 8 down-regulated DEmiRNAs, which were hsa-miR-100-5p, hsa-miR-10a-5p, hsa-miR-122-5p, hsa-miR-125a-5p, hsa-miR-125b-5p, hsa-miR-130a-3p, hsa-miR-149-5p, and hsa-miR-193a-5p, as well as the predicted 174 lncRNAs were used to construct the potential ceRNA regulatory network of vulgaris psoriasis, which included 239 DEmiRNA-lncRNA interactions (Supplementary Fig. S3B). The top ten lncRNAs were KCNQ1OT1, XIST, NEAT1, AL049840.4, AC104581.4, LINC00943, LINC00667, AC022167.2, AL137782.1, and H19.

lncRNA-miRNA-mRNA interaction network

To better understand the roles of lncRNAs, miRNAs, and mRNAs in psoriasis, the top ten regulated lncRNAs, corresponding DEmiRNAs, and top ten regulated hub genes were used to establish a lncRNA-miRNA-mRNA regulatory network. As depicted in Figure 4A, the network consisted of nine lncRNAs, five down-regulated miRNAs, and three up-regulated hub genes. There were multiple connections between lncRNAs and miRNAs, as well as between miRNAs and mRNAs, such as lncRNA (XIST/NEAT1)-miRNA (hsa-miR-10a-5p, hsa-miR-125b-5p)-mRNA (*KIF2C*).

Similarly, Figure 4B depicted a network comprising eight lncRNAs, four up-regulated miRNAs, and three down-regulated hub genes, featuring interactions such as lncRNA (NEAT1)-miRNA (hsa-miR-106a-5p, hsa-miR-142-3p)-mRNA (*MYH11*), lncRNA (XIST)-miRNA (hsa-miR-106a-5p, hsa-miR-1276, hsa-miR-135b-5p)-mRNA (*MYH11*, *ACTG2*, *MYLK*), and lncRNA (MALAT1)-miRNA (hsa-miR-106a-5p, hsa-miR-142-3p, hsa-miR-135b-5p)-mRNA (*MYH11*, *MYLK*).

Validation of the hub lncRNA-miRNA-mRNA regulatory network

The histopathological study demonstrated that, psoriatic skin showed pathological features, such as red plaques and thickening of the epidermis, consistent with the clinical pathological change (Fig. 5A). In contrast, the skin structure of the normal control group was intact, and without hyperkeratosis or spinous layer hypertrophy. After imiquimod modeling, the number of keratinocytes increased significantly in the skin, including hyperkeratosis, hypokeratosis, spinous layer hypertrophy, superior parietal mastoid, and inflammatory cell infiltration in the dermis. In comparison to the normal group, the epidermal layer thickness of the skin remarkablely increased after imiquimod modeling (p <0.05) (Fig. 5B).

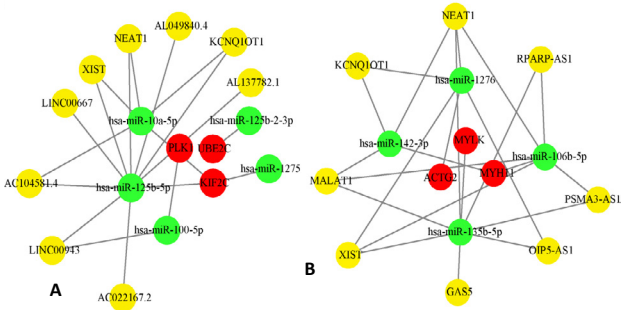

Fig. 4. The lncRNA-miRNA-mRNA regulatory network. (A) The network of lncRNAs, down-regulated miRNAs, and up-regulated hub genes. (B) The network of lncRNAs, up-regulated miRNAs, and down-regulated hub genes. Yellow nodes represent lncRNA, green nodes represent miRNA, and red nodes represent mRNAs.

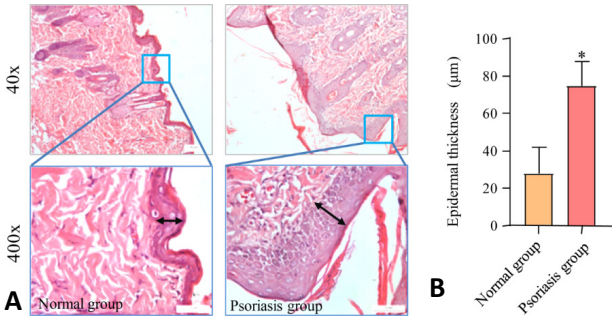

Fig. 5. Histopathology of skin tissues in psoriasis rats. (A) H&E staining of rat skin tissue (40× & 400×). (B) Statistical graph of epidermal thickness of skin. Data are presented as mean ± SD. * p <0.05, compared to Normal group.

The expression levels of the hub lncRNA-miRNA-mRNA regulatory network were investigated in the skin of psoriatic rats (Fig. 6). The results showed that the expression of lncRNA NEAT1 was significantly increased (p <0.05), while miR-125b-5p, miR-125b-2-3p, and KIF2C were significantly decreased (p <0.01), in comparison

to the normal group (Fig. 6A-D). On the other hand, the expression levels of lncRNA MALAT1 and XIST were significantly decreased (p <0.01, Fig. 6E, F), as well as a decline on *MYLK, ACTG2* and *MYH11* mRNA expression (p <0.05, Figure 6I-K). Comfortably, the expression of miR-135b-5p was notablely up-regulated in the skin of psoriatic rats, compared to the normal group (p <0.01, Fig. 6H). These findings suggested that the lncRNA (NEAT1)-miRNA (miR-125b-5p, miR-125b-2-3p)-mRNA (KIF2C) axis and the lncRNA (MALAT1, XIST)-miRNA (miR-135b-5p)-mRNA (*MYLK ACTG2* and *MYH11*) axis, were vital mechanisms involved in psoriasis.

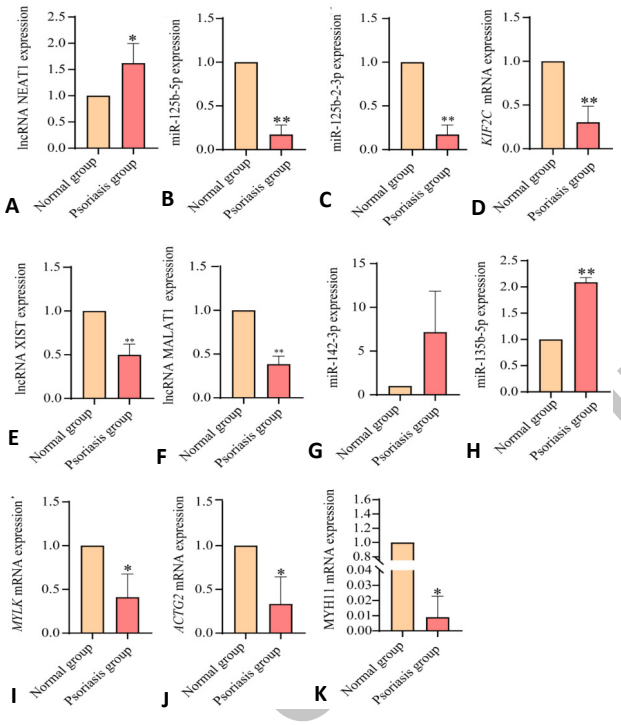

Fig. 6. Differential expression analysis of the hub lncRNA-miRNA-miRNA regulatory network. * p <0.05, ** p <0.01, vs. the normal group.

DISCUSSION

Previous studies have demonstrated the crucial role of mRNA, miRNA, and lncRNA in the development and progression of psoriasis through various signaling pathways (Zhou *et al*., 2019). However, few studies have integrated mRNA, miRNA, and lncRNA regulatory networks from diseased and non-diseased skin samples. Therefore, a comprehensive bioinformatics analysis was conducted in this study to identify their regulatory networks related to psoriasis.

Our analysis revealed that the up-regulated DEmRNAs were notably enriched in immune and inflammatory responses, while the down-regulated DEmRNAs were high enrichment in metabolic processes and cell adhesion. KEGG pathway analysis revealed that the up-regulated DEmRNAs were involved in cytokine-cytokine receptor interactions and NOD-like receptor signaling, while the down-regulated DEmRNAs were relative to CAMs, tight junctions, and act in cytoskeleton regulation. Our analysis also demonstrated a close relationship between *IL-17* and psoriasis, which aligns with previous report (Griffiths *et al*., 2021). Relationships between chemokines and inflammation have been previously highlighted and implicated in psoriasis patients (Zdanowska *et al*., 2021).

Cyclin-dependent kinase 1 (CDK1), was the up-regulated hub genes belonging to the cell cycle regulatory protein family, which is involved in cell cycle maintenance; it drives the cell cycle through chemical action on serine/threonine protein, and functions cooperatively with cyclin (Liao *et al*., 2017). Cyclin A2 (CCNA2), a key cell cycle regulator, is implicated in both DNA replication and mitotic entry and is vital in controlling the cell cycle's G1/S (initiation) and G2/M (mitosis) transitions (Cascales *et al*., 2021). Cyclin B2 (CCNB2/KIF20A) mainly involved in the cell cycle, proliferation, protein transport, and other biological processes (Shubbar *et al*., 2013).

In terms of miRNAs, we identified 299 significant DEmiRNAs in the GSE142582 dataset (140 up- and 159 down-regulated). Recent studies have highlighted the pivotal roles of miRNAs in psoriasis. For example, miR-125b was significantly decreased in psoriatic skin, targeted FGFR2 expression, and its loss may contribute to keratinocyte hyperproliferation and aberrant differentiation (Xu *et al*., 2011). miR-221-3p is a latent biomarker in some psoriasis patients, and its decreased expression inhibited HaCaT cell proliferation and inflammatory responses, making it a potential therapeutic target against psoriasis (Meng *et al*., 2021). miR-1276 positively regulated BCa cell proliferation by increasing SMAD2 expression (Zhang *et al*., 2022). miR-135b-5p inhibition inhibited SGC-7901 cell proliferation (Lu *et al*., 2018), with high miR-135b-5p levels inhibiting apoptosis (Shao *et al*., 2019). miR-135b-5p also regulated Tgfbr2/Smad4 and inhibited skeletal muscle fibrosis. Additionally, MALAT1 absorbed miR-135a-5p to promote target expression.

ceRNA network theory suggests that lncRNAs have important roles in psoriasis development and occurrence. In these networks, NEAT1, a vital par speckle component lncRNA, has an indispensable role in par speckle formation and integrity (Bu *et al*., 2020). NEAT1 promotes inflammasome activation in macrophages, while stabilizing mature caspase-1 to promote IL-1β production and pyroptosis (Zhang *et al*., 2019b). In another study,

relative to IL-1β and caspase-1 expression in normal skin biopsy samples, their expression was approximately 2.2–4.6 folds higher in psoriatic samples (Su *et al*., 2018). Thus, lncRNA NEAT1 may impact psoriasis occurrence and development via pro-inflammatory mechanisms or innate immunity.

In recent years, advances in genome and transcriptome technology have enabled the gradual revelation of lncRNA functions and mechanisms in psoriasis (Zhou *et al*., 2019). In our lncRNA-miRNA-mRNA network study, lncRNA (NEAT1)-miRNA (miR-106a-5p, miR-142-3p)-mRNA (*MYH11*), lncRNA (XIST)-miRNA (miR-106a-5p, miR-1276, miR-135b-5p)-mRNA (*MYH11*, *ACTG2*, *MYLK*), lncRNA (MALAT1)-miRNA (miR-106a-5p, miR-142-3p, miR-135b-5p)-mRNA (*MYH11*, *MYLK*), and lncRNA (XIST/NEAT1)-miRNA (miR-10a-5p, miR-125b-5p)-mRNA (*KIF2C*) axis were predicted as regulatory axis in psoriasis and further verified in psoriasis rats.

Psoriasis is manifested as abnormal epidermal proliferation (Jia *et al*., 2020) and is related to apoptosis suppression (El-Domyati *et al*., 2013). MALAT1, which promotes cell proliferation and migration in different cancers, has been reported there is an association between MALAT1 polymorphisms and psoriasis risk, but the exact effects were unclear (Xia *et al*., 2018; Mungmunpuntipantip *et al*., 2022). Studies have shown that macrophage miR-106b-5p excretion from impaired vitamin D receptor signaling induce inflammation (Oh *et al*., 2020), while Vitamin D3 analogs impact psoriasis by binding nuclear vitamin D3 receptors on genes implicated in proliferation, differentiation, and inflammation (O'neill and Feldman, 2010). MALAT1 sponges miR-106b-5p to promote colorectal cancer invasion and metastasis (Zhuang *et al*., 2019). For miR-142-3p, it reportedly disrupts MALAT1/miR-142-3p sponging to decrease cervical cancer cell invasion and migration (Xia *et al*., 2018). The mRNA myosin heavy chain 11 (MYH11), which is involved in cytoskeleton formation, cell movement, signal transduction, and muscle contraction, was found to be down-regulated in breast cancer tissue, but overexpression inhibited cancer cell proliferation and migration, and promoted the formation of intercellular substances (Xiao *et al*., 2020; Sun and Li, 2021). In our study, miR-106a-5p and miR-142-3p were significantly increased, and *MYH11* was also significantly reduced in skin lesions. Therefore, the MALAT1-miR (miR-106a-5p, miR-142-3p)-mRNA (*MYH11*) axis may regulate cell migration and promote apoptosis and thus play a critical role in psoriasis development. Additionally, our psoriasis rats model showed significantly increased expression levels of lncRNA (MALAT1, XIST) and mRNA (*MYKL*, *ACTG2* and *MYH11*), and decreased miRNA (miR-135a-5p), suggesting the lncRNA (MALAT1, XIST)-miRNA (miR-135a-5p)-mRNA (*MYKL*, *ACTG2* and *MYH11*) axis may regulate abnormal proliferation in psoriasis.

The lncRNA (NEAT1)-miRNA (miR-10a-5p, miR-125b-5p)-mRNA (*KIF2C*) axis was also predicted. miR-125b-5p is an important psoriasis marker that is significantly decreased in psoriasis patient serum (Zhang *et al*., 2019a) and expressed at low levels in progressive, quiescent, and stable disease phases (Gao *et al*., 2017; Zheng *et al*., 2019), and regulates keratinocyte proliferation and differentiation. Also, miR-125b-5p inhibits keratinocyte proliferation in psoriasis by targeting Akt3 (Wei *et al*., 2017). Ubiquitin-specific peptidase 2 is an underlying link between microRNA-125b and psoriasis. Pan *et al*. (2019) reported that miRNA-125b inhibited HaCaT cell proliferation by inhibiting the active BRD4/Notch signaling pathway in psoriasis. We predicted that XIST or NEAT1 regulated miR-125b-5p, consistent with previous reports (Zong *et al*., 2020). Kines in family member 2C (*KIF2C*) is a substantial mitotic regulator and promotes proliferation, which is significantly increased in skin lesions. By integrated bioinformatics analysis, lncRNA (NEAT1)-miRNA (miR-125b-5p)-mRNA (*KIF2C*) axis were predicted as vital for cell proliferation mechanisms in psoriasis. In the psoriasis rat model, the expression level of lncRNA NEAT1 was significantly increased, miR-125b-2-3p and miR-125b-5p were significantly decreased. Surprisingly, there was a decreased expression of *KIF2C* in psoriasis, which did not align with our predicted or previous reports (Zong *et al*., 2020).

CONCLUSION

In summary, in this work, we investigated a novel mRNA-miRNA-lncRNA regulatory network associated with psoriasis. Our findings suggest that this network is related to the proliferation and tight junction weakening between epidermal cells in psoriasis, which may be related to the unbalance of lncRNA (NEAT1)-miRNA (miR-125b-5p, miR-125b-2-3p)-mRNA (*KIF2C*) axis and lncRNA (XIST, MALAT1)-miRNA (miR-135b-5p)-mRNA (*MYLK*, *ACTG2* and *MYH11*) axis. This study provides new insights into the molecular mechanisms underlying the pathogenesis of psoriasis, and may contribute to the development of lncRNA or miRNA drugs for its treatment.

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IRB approval

Not applicable.

Ethical statement

This study was approved by the Bioethics Committee of Zhejiang Academy of Traditional Chinese Medicine (Approval No. KTSB2022018).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220310020325

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Boehncke, W.H., 2015. Etiology and pathogenesis of psoriasis. *Rheum. Dis. Clin.*, **41**: 665-675. https://doi.org/10.1016/j.rdc.2015.07.013

Bu, F.T., Wang, A., Zhu, Y., You, H.M., Zhang, Y.F., Meng, X.M., Huang, C. and Li, J., 2020. LncRNA *NEAT1*: Shedding light on mechanisms and opportunities in liver diseases. *Liver Int.*, **40**: 2612-2626. https://doi.org/10.1111/liv.14629

Cascales, H.S., Burdova, K., Middleton, A., Kuzin, V., Müllers, E., Stoy, H., Baranello, L., Macurek, L. and Lindqvist, A., 2021. Cyclin A2 localises in the cytoplasm at the S/G2 transition to activate PLK1. *Life Sci. Alliance*, **4**: e202000980. https://doi.org/10.26508/lsa.202000980

El-Domyati, M., Moftah, N.H., Nasif, G.A., Abdel-Wahab, H.M., Barakat, M.T. and Abdel-Aziz, R.T., 2013. Evaluation of apoptosis regulatory proteins in response to PUVA therapy for psoriasis. *Photodermatol. Photoimmunol. Photomed.*, **29**: 18-26. https://doi.org/10.1111/phpp.12012

Flytström, I., Stenberg, B., Svensson, Å. and Bergbrant, I.M., 2008. Methotrexate vs. ciclosporin in psoriasis: Effectiveness, quality of life and safety. A randomized controlled trial. *Br. J. Dermatol.*, **158**: 116-121.

Gao, Y.L., Dong, H., Dong, L.D., Li, D. and Yu, N., 2017. Expression of miR-21, miR-203a, miR-146a, miR-125b in different development stages of epidermal keratinocytes of psoriasis. *J. Ningxia Med. Univ.*, **39**: 1133-1136.

Griffiths, C.E.M., Armstrong, A.W., Gudjonsson, J.E. and Barker, J.N.W.N., 2021. Psoriasis. *Lancet*, **397**: 1301-1315. https://doi.org/10.1016/S0140-6736(20)32549-6

Jia, J., Mo, X., Liu, J., Yan, F., Wang, N., Lin, Y., Li, H., Zheng, Y. and Chen, D., 2020. Mechanism of danshensu-induced inhibition of abnormal epidermal proliferation in psoriasis. *Eur. J. Pharmacol.*, **868**: 172881. https://doi.org/10.1016/j.ejphar.2019.172881

Liao, H., Ji, F. and Ying, S., 2017. CDK1: Beyond cell cycle regulation. *Aging* (Albany NY), **9**: 2465-2466. https://doi.org/10.18632/aging.101348

Lu, M., Huang, Y., Sun, W., Li, P., Li, L. and Li, L., 2018. miR-135b-5p promotes gastric cancer progression by targeting CMTM3. *Int. J. Oncol.*, **52**: 589-598. https://doi.org/10.3892/ijo.2017.4222

Meng, Z., Qiu, J. and Zhang, H., 2021. MiR-221-3p as a potential biomarker for patients with psoriasis and its role in inflammatory responses in keratinocytes. *Skin Pharmacol. Physiol.*, **34**: 300-306. https://doi.org/10.1159/000515114

Mungmunpuntipantip, R. and Wiwanitkit, V., 2022. MALAT1 polymorphisms and psoriasis risk: Correspondence. *Int. J. Immunogenet.*, **49**: 88. https://doi.org/10.1111/iji.12570

Oh, J., Matkovich, S.J., Riek, A.E., Bindom, S.M., Shao, J.S., Head, R.D., Barve, R.A., Sands, M.S., Carmeliet, G., Osei-Owusu, P., Knutsen, R.H., Zhang, H., Blumer, K.J., Nichols, C.G., Mecham, R.P., Baldán, Á., Benitez, B.A., Sequeira-Lopez, M.L., Gomez, R.A. and Bernal-Mizrachi, C., 2020. Macrophage secretion of miR-106b-5p causes renin-dependent hypertension. *Nat. Commun.*, **11**: 4798. https://doi.org/10.1038/s41467-020-18538-x

O'neill, J.L. and Feldman, S.R., 2010. Vitamine D analogue-based therapies for psoriasis. *Drugs Today (Barc)*, **46**: 351-360. https://doi.org/10.1358/dot.2010.46.5.1473264

Pan, M., Huang, Y., Zhu, X., Lin, X. and Luo, D., 2019. miR-125b-mediated regulation of cell proliferation through the Jagged-1/Notch signaling pathway by inhibiting BRD4 expression in psoriasis. *Mol. Med. Rep.*, **19**: 5227-5236. https://doi.org/10.3892/mmr.2019.10187

Parisi, R., Symmons, D.P., Griffiths, C.E. and Ashcroft, D.M., 2013. Global epidemiology of psoriasis: A systematic review of incidence and prevalence. *J. Invest. Dermatol.*, **133**: 377-385. https://doi.org/10.1038/jid.2012.339

Qiao, M., Li, R., Zhao, X., Yan, J. and Sun, Q., 2018. Up-regulated lncRNA-MSX2P1 promotes the growth of IL-22-stimulated keratinocytes by inhibiting miR-6731-5p and activating S100A7. *Exp. Cell Res.*, **363**: 243-254. https://doi.org/10.1016/j.yexcr.2018.01.014

Rendon, A. and Schäkel, K., 2019. Psoriasis pathogenesis and treatment. *Int. J. mol. Sci.*, **20**: 1475. https://doi.org/10.3390/ijms20061475

Shao, L., Chen, Z., Soutto, M., Zhu, S., Lu, H., Romero-Gallo, J., Peek, R., Zhang, S. and El-Rifai, W., 2019. Helicobacter pylori-induced miR-135b-5p promotes cisplatin resistance in gastric cancer. *FASEB J.*, **33**: 264-274. https://doi.org/10.1096/fj.201701456RR

Shubbar, E., Kovács, A., Hajizadeh, S., Parris, T.Z., Nemes, S., Gunnarsdóttir, K., Einbeigi, Z., Karlsson, P. and Helou, K., 2013. Elevated cyclin B2 expression in invasive breast carcinoma is associated with unfavorable clinical outcome. *BMC Cancer*, **13**: 1. https://doi.org/10.1186/1471-2407-13-1

Su, F., Xia, Y., Huang, M., Zhang, L. and Chen, L., 2018. Expression of NLPR3 in psoriasis is associated with enhancement of Interleukin-1β and Caspase-1. *Med. Sci. Monit.*, **24**: 7909-7913. https://doi.org/10.12659/MSM.911347

Sun, Y. and Li, H.X., 2021. Expression of ACTG2 and MYH11 in 150 cases of incasive ductal carcinoma of the breast and its relationship with prognosis. *Chin. J. Cancer Prev. Treat.*, **27**: 1977-1983.

Szegedi, K., Sonkoly, E., Nagy, N., Németh, I.B., Bata-Csörgo, Z., Kemény, L., Dobozy, A. and Széll, M., 2010. The anti-apoptotic protein G1P3 is overexpressed in psoriasis and regulated by the non-coding RNA, PRINS. *Exp. Dermatol.*, **19**: 269-278. https://doi.org/10.1111/j.1600-0625.2010.01066.x

Wei, T., Folkersen, L., Biskup, E., Xu, N., Manfe, V., Niazi, O. and Gniadecki, R., 2017. Ubiquitin-specific peptidase 2 as a potential link between microRNA-125b and psoriasis. *Br. J. Dermatol.*, **176**: 723-731. https://doi.org/10.1111/bjd.14916

Xia, C., Liang, S., He, Z., Zhu, X., Chen, R. and Chen, J., 2018. Metformin, a first-line drug for type 2 diabetes mellitus, disrupts the MALAT1/miR-142-3p sponge to decrease invasion and migration in cervical cancer cells. *Eur. J. Pharmacol.*, **830**: 59-67. https://doi.org/10.1016/j.ejphar.2018.04.027

Xiao, W., Li, X., Ji, C., Shi, J. and Pan, Y., 2020. LncRNA Sox2ot modulates the progression of thoracic aortic aneurysm by regulating miR-330-5p/Myh11. *Biosci. Rep.*, **40**: BSR20194040. https://doi.org/10.1042/BSR20194040

Xu, N., Brodin, P., Wei, T., Meisgen, F., Eidsmo, L., Nagy, N., Kemeny, L., Ståhle, M., Sonkoly, E. and Pivarcsi, A., 2011. MiR-125b, a microRNA downregulated in psoriasis, modulates keratinocyte proliferation by targeting FGFR2. *J. Invest. Dermatol.*, **131**: 1521-1529. https://doi.org/10.1038/jid.2011.55

Yan, S., Xu, Z., Lou, F., Zhang, L., Ke, F., Bai, J., Liu, Z., Liu, J., Wang, H., Zhu, H., Sun, Y., Cai, W., Gao, Y., Su, B., Li, Q., Yang, X., Yu, J., Lai, Y., Yu, X.Z., Zheng, Y., Shen, N., Chin, Y.E. and Wang, H., 2015. NF-κB-induced microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis. *Nat. Commun.*, **6**: 7652. https://doi.org/10.1038/ncomms8652

Zdanowska, N., Kasprowicz-Furmańczyk, M., Placek, W. and Owczarczyk-Saczonek, A., 2021. The role of chemokines in psoriasis. An overview. *Medicina (Kaunas)*, **57**: 754. https://doi.org/10.3390/medicina57080754

Zhang, L.Y., Shan, H., Zhu, X.Y., Sun, Y. and Chen, Y., 2019a. Clinical significance of measurement the level of miR-146a and miR-125b in psoriasis patients serum. *Zhejiang Clin. Med. J.*, **21**: 1315-1317.

Zhang, P., Cao, L., Zhou, R., Yang, X. and Wu, M., 2019b. The lncRNA Neat1 promotes activation of inflammasomes in macrophages. *Nat. Commun.*, **10**: 1495. https://doi.org/10.1038/s41467-019-09482-6

Zhang, Z., Zhao, H., Zhou, G., Han, R., Sun, Z., Zhong, M. and Jiang, X., 2022. Circ_0002623 promotes bladder cancer progression by regulating the miR-1276/SMAD2 axis. *Cancer Sci.*, **113**: 1250-1263. https://doi.org/10.1111/cas.15274

Zheng, Y., Cai, B., Li, X., Li, D. and Yin, G., 2019. MiR-125b-5p and miR-181b-5p inhibit keratinocyte proliferation in psoriasis by targeting Akt3. *Eur. J. Pharmacol.*, **862**: 172659. https://doi.org/10.1016/j.ejphar.2019.172659

Zhou, Q., Yu, Q., Gong, Y., Liu, Z., Xu, H., Wang, Y. and Shi, Y., 2019. Construction of a lncRNA-miRNA-mRNA network to determine the regulatory roles of lncRNAs in psoriasis. *Exp. Ther. Med.*, **18**: 4011-4021. https://doi.org/10.3892/etm.2019.8035

Zhuang, M., Zhao, S., Jiang, Z., Wang, S., Sun, P., Quan, J., Yan, D. and Wang, X., 2019. MALAT1 sponges miR-106b-5p to promote the invasion and metastasis of colorectal cancer via SLAIN2 enhanced microtubules mobility. *EBioMedicine*, **41**: 286-298. https://doi.org/10.1016/j.ebiom.2018.12.049

Zong, Y., Zhang, Y., Hou, D., Xu, J., Cui, F., Qin, Y. and Sun, X., 2020. The lncRNA XIST promotes the progression of breast cancer by sponging miR-125b-5p to modulate NLRC5. *Am. J. Transl. Res.*, **12**: 3501-3511.

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Supplementary Material

Identification and Verification of Hub mRNA-miRNA-lncRNA Network on Psoriasis by Integrated Bioinformatics Analysis

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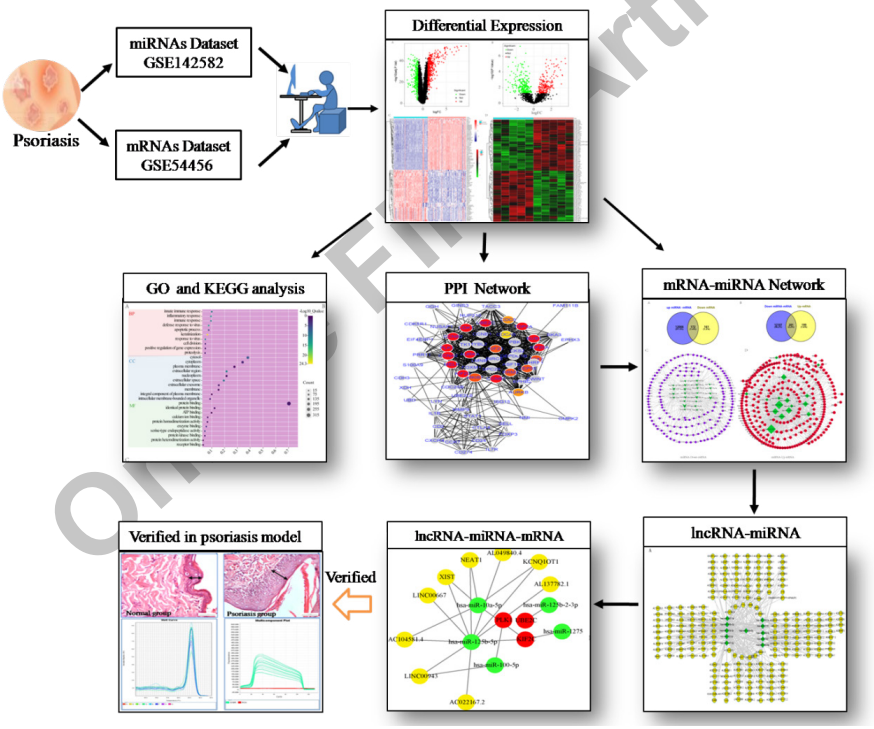

Supplementary Fig. S1. Workflow of bioinformatics analysis to research psoriasis pathogenesis.

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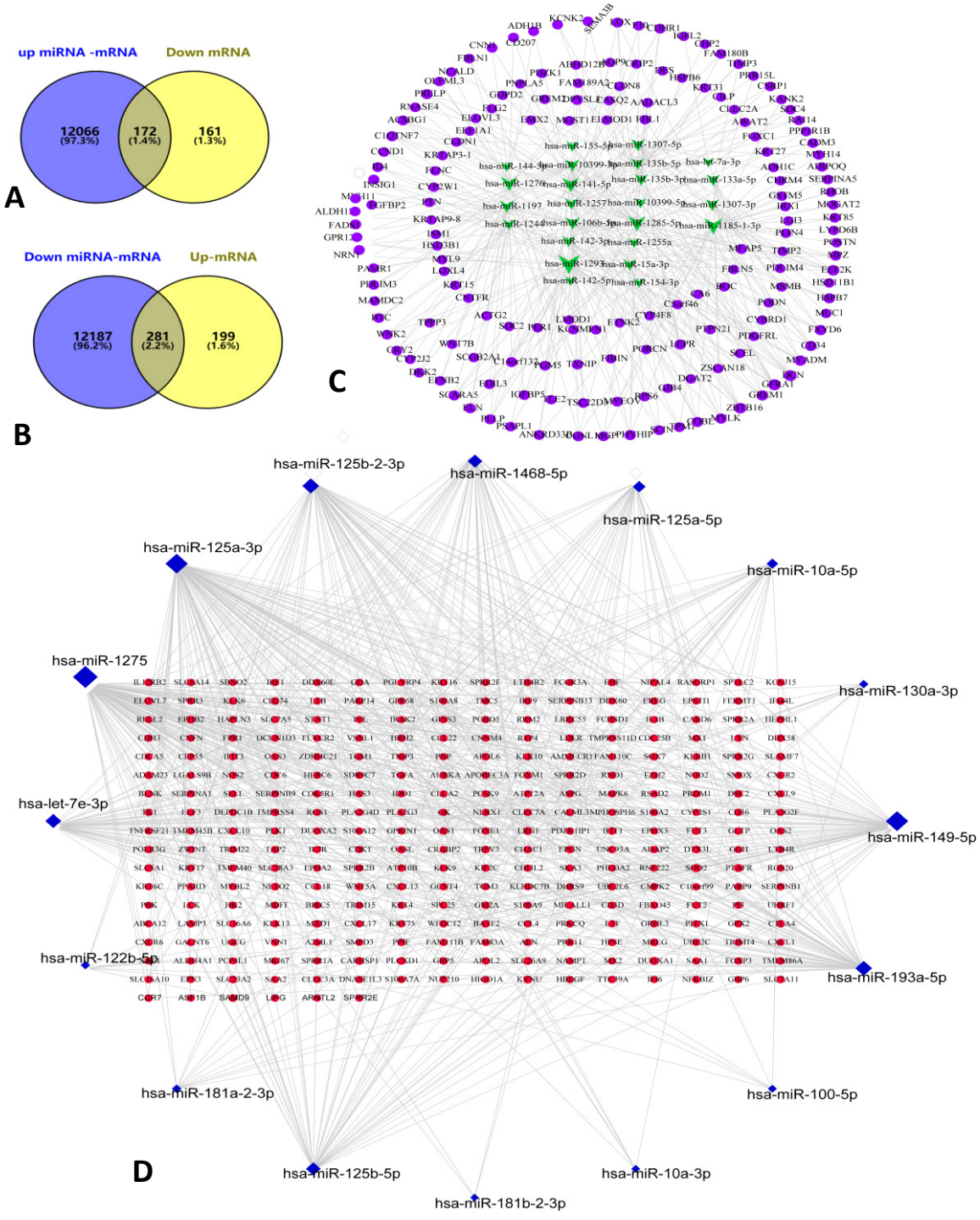
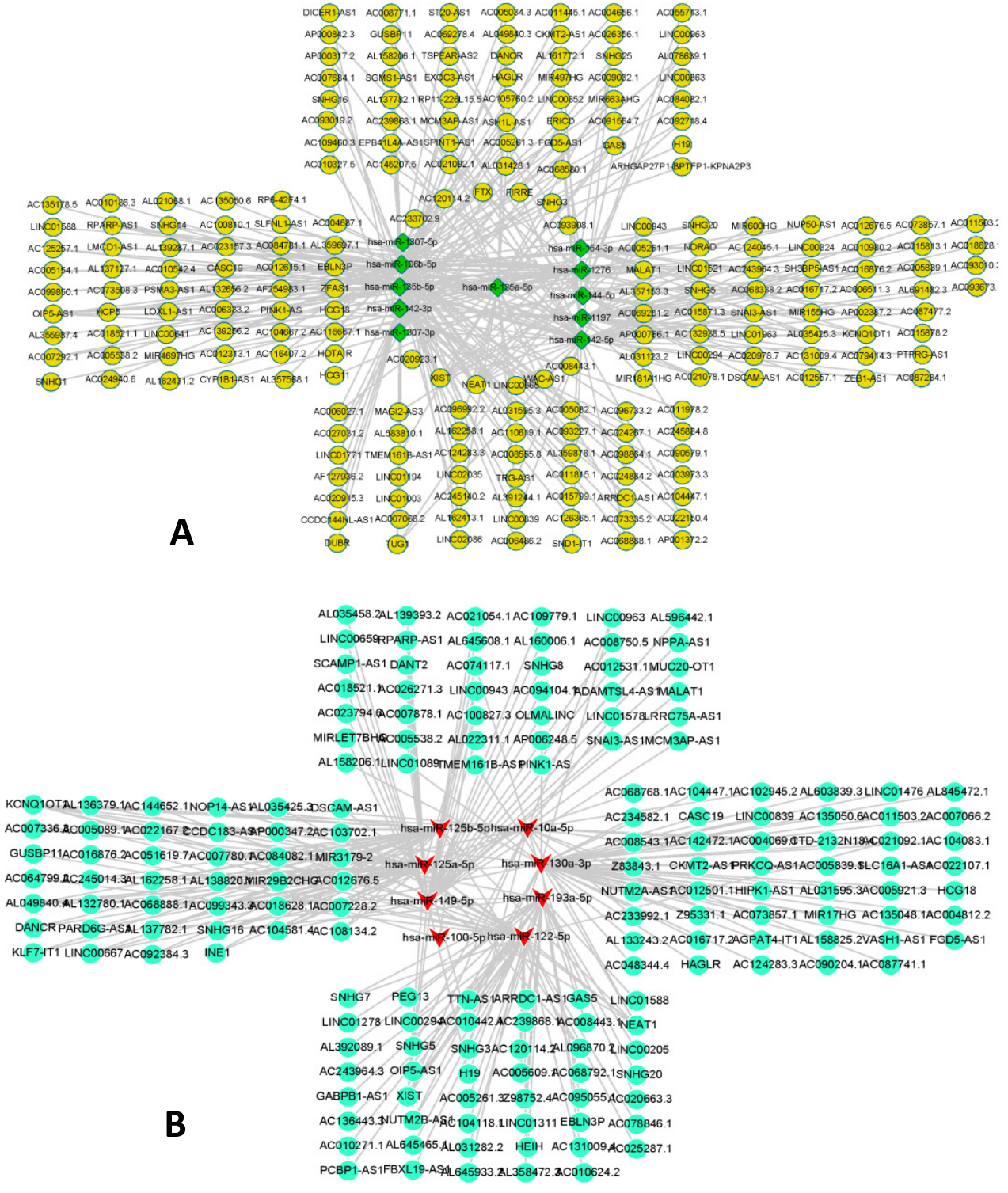
0030-9923/2023/0001-0001 $ 9.00/0



Supplementary Fig. S2. (A) Venn diagram comparison of the up regulated DEmiRNAs predicted target mRNAs and the down regulated DEmRNAs. (B) Venn diagram comparison of down regulated DEmiRNAs predicted target mRNAs and the up regulated DEmRNAs. (C) The network of up-regulated DEmiRNAs predict target mRNA and the down-regulated DEmRNAs. Green nodes represent up-regulated DEmiRNAs and purple circles represent mRNA. (D) The network of down-regulated DEmiRNAs predict target mRNA and the up-regulated DEmRNAs. Blue diamonds represent down-regulated DEmiRNAs and red circles represents mRNA.



Supplementary Fig. S3. The lncRNA-miRNA regulatory network. (A) The network of up-regulated DEmiRNAs and predicted target lncRNAs. Yellow nodes represent lncRNA, while green nodes represent miRNA. (B) The network of down-regulated DEmiRNAs and the predicted target lncRNAs. Blue nodes represent lncRNA, while red nodes represent miRNAs. Interactions between DEmiRNA and lncRNA were expressed as the grey lines.

Supplementary Table SI. The reaction system and process of cDNA synthase.

Components	Volume (μL)
Total RNA	≤ 9
gDNA eraser	1
RNase free H_2O	Up to 10
42 °C for 5 min	
2× SPARKscript II RT plus master mix	10
50 °C for 15 min and 85 °C for 5 min	