

# Bioinformatics analysis of key micro-RNAs and mRNAs under hand, foot, and mouth disease virus infection

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## Research article

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# Abstract

To dig the clinical significance of micro-RNA and mRNA changes under hand, foot, and mouth disease (HFMD) virus infection, we conducted this bioinformatics analysis to clarify crucial differentially expressed genes (DEGs), functional and pathway enrichment, and the relative regulatory network, using four published datasets.

Following datasets from GEO database were used for analysis: GSE85829, GSE94551, GSE52780 and GSE45589. After screening of common differentially expressed miRNAs (DE-miRNAs), five key miRNAs were acquired: miR-100-3p, miR-125a-3p, miR-1273g-3p, miR-5585-3p, and miR-671-5p. Based on above five key miRNAs, GO functional and KEGG pathway enrichment were performed using the miRPath V3 database. there were three common enriched GO terms between miRNA-derived prediction and mRNA-derived (dataset GSE45589) analysis: biosynthetic process, cytosol, and nucleoplasm. And one common KEGG pathway, cell cycle, was shared between miRNA-based and mRNA-based enrichment. Using TarBase V8 in DIANA tools, we acquired 1520 potential targets (mRNA) from the 5 key DE-miRNAs, among which 11 DEGs were also included by the 159 DE-mRNAs.

These common DEGs showed a PPI network mainly connected by SMC1A, SMARCC1, SF3B3, LIG1 and BRMS1L. Together, changes in 5 key miRNAs and 11 key mRNAs may play crucial roles in HFMD progression. Our results shed light on the crucial roles of these DEGs and several functions/pathways in HFMD. Combination of these roles may benefit the early diagnose and treatment of HFMD.

## Introduction

Hand, foot, and mouth disease (HFMD) is a common endemic childhood disease worldwide and particular in Asia. Generally, it is triggered by two major causative agents: enterovirus 71 (EV71) and coxsackievirus A16 (CA16) [1, 2], followed by coxsackie virus A6 (CA6) and coxsackie virus A10 (CA10) [3]. This disease majorly affects children under five years old [4]. The common symptoms include fever, rashes on the volar regions of the hands and feet, herpangina and difficulties in eating and drinking. Severe circumstances exhibited potentially fatal complications, including nervous (e.g. brain stem encephalitis) or cardiopulmonary systems (e.g. pulmonary edema). The severe cases may show drastic progression and die from complicated symptoms.

There have been limited tools for effective diagnosis of HFMD. Although enterovirus infection triggers various pathological responses, the mechanisms underlying HFMD development remains largely unknown. For example, what regulatory roles does EV71/CA16 infection play towards endothelial cells and neural system are unclear. Previous studies have explored the disordered signals in different aspect, such as inflammatory profiles in cytokine expression [5, 6], long non-coding RNA (lncRNA) profiles [7], and immune cell changes [8]. It is conceivable and has been supported that HFMD patients may exhibit changes in expression profiles of microRNAs and mRNAs, especially derived from blood samples [9–19]. In addition, comprehensive understanding of HFMD-related expression profiles and identification key

markers may provide huge diagnostic and prognostic values. To dig the clinical significance of micro-RNA and mRNA changes under HFMD virus infection, we conducted this bioinformatics analysis to clarify crucial differentially expressed genes (DEGs), functional and pathway enrichment, and the relative regulatory network, using four published datasets. This analysis may provide deeper insight into the mechanism of HFMD pathological development and novel strategies to prevent HFMD outbreaks.

## Methods

### Microarray Data

The key words “hand-foot-and-mouth disease” or “HFMD” were used to search relevant gene expression profile data on the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). The inclusion criteria were as follows: (1) The experiment was designed for analysis of RNA expression (either microRNA or mRNA) in response to EV71 and CA16 infection. (2) All samples were human derived (human cell lines or human exosomes). Four datasets were found informative for this analysis. All the datasets we included are as follow.

GSE85829: Using the Platform GPL11154 Illumina HiSeq 2000 (Homo sapiens). Summary: To compare microRNA expression in 16HBE (human bronchial epithelial cell) infected with EV71 and CA16. It includes 6 samples according to the experimental groups: EV71-0h, EV71-6h, EV71-12h, CA16-0h, CA16-6h and CA16-12h.

GSE94551: Using the Platform GPL11154 Illumina HiSeq 2000 (Homo sapiens). Summary: The miRNA profiling in EV71- and CA16-infected human umbilical vein endothelial cells (HUVECs) at multiple time points. It contains 6 samples according to different experimental groups: EV71-0h, EV71-72h, EV71-96h, CA16-0h CA16-72h, CA16-96h.

GSE52780: Using the GPL16730 Agilent-039659. Summary: It observed miRNAs of exosome in HFMD serum samples and distinguished between extremely severe HFMD and mild HFMD. It contains 3 samples: control, mild and extremely severe.

GSE45589: Using the GPL16765 Human 70-mer oligonucleotide microarray. Summary: It employed the human whole genome microarray to analyze the transcriptome profiling in human neuroblastoma cells SH-SY5Y infected with EV71.

### DEG Identification

DEG analysis of the dataset was performed according to the following standards. For all the miRNA samples were the single ones in each group, DEG was regarded as a gene with a fold-change (FC)  $\geq 4$  or  $\leq 0.25$  (any treatment group vs control group). For those datasets with three time points, DEGs should be acquired at all time points, and it was selected if the expression at any of the later time point with a fold-change  $\geq 4$  or  $\leq 0.25$  vs 0 h. For dataset GSE45589 (mRNA expression), there were two independent samples. Each sample presented the degree of FC after SH-SY5Y cells were infected with EV71 strain.

And those genes with FC ratios  $\geq 2$  or  $\leq 0.5$  and  $p < 0.05$  were selected as DEGs. Heatmaps were produced to present DEGs. Those differentially expressed miRNAs (DE-miRNAs) which appeared for three times among different datasets were defined as key miRNAs in HFMD.

## Functional and Pathway Enrichments

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>) and miRPath v.3 (<http://www.microna.gr/miRPathv3/>) were used to for Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. For all DEGs, the up-regulated and down-regulated DEGs were respectively conducted with GO and KEGG pathway enrichment analysis. Any term with a p-value  $< 0.05$  and enriched genes  $\geq 2$  was selected as a differential term. Enrichment was analyzed by two methods: (1) The indirect evaluation: using the DIANA Tool miRPath V3, the targets of the Key miRNAs were selected to perform the enrichment analysis; (2) The direct method: using the GSE45589 dataset, all up-regulated and down-regulated DEGs were analyzed.

## MicroRNA-mRNA Regulatory and Protein-protein Interaction (PPI) Network

Using TarBase V8 in the DIANA tools (<http://carolina.imis.athena-innovation.gr>), the validated miRNA-mRNA pairs were obtained. The miRNAs and mRNAs among the screened-out DEGs were selected to construct the microRNA-mRNA regulatory network. The common mRNAs between those targeted by key miRNA and differentially expressed in the GSE45589 dataset were screened to construct the PPI network. This step was performed based on the DEG nodes using the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://www.string-db.org>), which provides experimental and predicted protein interaction information. The criteria of the combined score was set  $\geq 0.4$ . The PPI network was visualized using the STRING online tools.

# Results

## Differentially expressed miRNAs after HFMD virus infection

We first analyzed the GSE85829 and GSE94551 datasets and found some common differentially expressed miRNAs (DE-miRNAs). In 16HBE cells based on the GSE85829 dataset, EV71 infection induced 25 DE-miRNAs and CA16 infection induced 13 DE-miRNAs (Fig. 1 A and B), among which 7 common DE-miRNAs were found. In the HUVEC line according to the GSE94551 dataset, 39 DE-miRNAs were observed after EV71 infection and 99 DE-miRNAs were found after CA16 infection (Fig. 2 A and B), and they also shared 7 common DE-miRNAs (Fig. 2B). However, no intersection was found between two groups of 7 common DE-miRNAs. The common DE-miRNAs among four groups of cells were presented by a Venn diagram in Fig. 2C. Similarly, miRNAs in serum exosomes were analyzed based on the GSE52780 dataset, and a total of 258 DE-miRNAs were found (Fig. 3A), including 85 up-regulated and 173 down-regulated ones; the top 10 DE-miRNAs were listed in Fig. 3B. Afterwards, we screened those DE-miRNAs which appeared three times in the above samples (cell lines or exosomes) in different datasets and regarded

them as key miRNAs in HFMD. Ultimately, five key miRNAs were acquired: miR-100-3p (appeared 4 times), miR-125a-3p (appeared 3 times), miR-1273g-3p (appeared 3 times), miR-5585-3p (appeared 3 times), and miR-671-5p (appeared 3 times).

## **GO and KEGG enrichment**

Based on above five key miRNAs, GO functional and KEGG pathway enrichment were performed using the miRPath V3 database. First, GO and KEGG enrichment were acquired by the intersection targets of the five key miRNAs (only four miRNAs were included in this database) (Fig. 4A and B) Further, this was verified by the union of key miRNA related GO terms or KEGG pathways, and heatmaps were shown in Fig. 4 C and D. Overall, this result was consistent with Fig. 4 A and B. Parallely, we applied the mRNA profiles in SH-SY5Y cells infected by EV71 from the GSE45589 dataset and performed the enrichment analysis. The enriched GO functions of up-regulated and down-regulated genes were listed in Fig. 4 E and F, respectively. A total of 29 up-regulated GO functional terms were identified (meiotic nuclear division, response to radiation and regulation of microtubule-based process, etc.), accomplished by 43 down-regulated terms (positive regulation of transcription, DNA-templated, negative regulation of apoptotic process, and anatomical structure morphogenesis, etc.). Ten enriched KEGG pathways were shown in Fig. 4 G, including 2 up-regulated ones (cell cycle and spliceosome), and 8 down-regulated pathways (cytokine-cytokine receptor interaction, hematopoietic cell lineage, and intestinal immune network for IgA production, etc.). In comparison, there were three common enriched GO terms between miRNA-derived prediction and mRNA-derived (dataset GSE45589) analysis: biosynthetic process, cytosol, and nucleoplasm. And one common KEGG pathway, namely cell cycle, was shared between miRNA-based and mRNA-based enrichment.

## **Common differential mRNAs and PPI network**

Using TarBase V8 in DIANA tools, we acquired 1520 potential targets (mRNA) from the 5 key DE-miRNAs, among which 11 DEGs were also included by the 159 DE-mRNAs in the GSE45589 dataset: MACF1, MARS, SF3B3, SMARCC1, BRMS1L, SMC1A, SPHK2, LIG1, CSF3, CYR61 and FGFR1OP (Fig. 5A). Theoretically, these genes were the most likely ones influenced by HFMD virus infection. GO functional analysis showed three terms might be enriched according to these DEGs: positive regulation of cell proliferation, anatomical structure morphogenesis, and ATP binding (Fig. 5B). These common DEGs showed a PPI network mainly connected by SMC1A, SMARCC1, SF3B3, LIG1 and BRMS1L (Fig. 5C), and this network locates at a core place in the PPI network constructed by the 159 DE-mRNAs in the GSE45589 dataset (Fig. 5D, the isolated nodes were removed). Together, changes in 5 key miRNAs and 11 key mRNAs may play crucial roles in HFMD virus induced pathological changes and count be used as diagnostic markers for the HFMD.

## **Discussion**

In this study, we used five datasets to identify key RNA members in HFMD development. After filtering progressively, we were interested in 5 key miRNAs, 11 mRNAs and several important GO and KEGG

enrichment. Our results might provide some theoretical perspective about HFMD development and also a potential strategy in its early warning.

At the miRNA level, several potentially useful markers have been proposed in clinical diagnosis. A survey in Singapore reported an 6-miRNA scoring model which predicts HFMD with an overall accuracy of 85.11% in the training set and 92.86% in the blinded test set; and circulating Salivary miRNA hsa-miR-221 (downregulated in that work) was regarded as a highly validated marker [12]. Song et al have applied rhesus monkey peripheral blood mononuclear cells to search DE-miRNAs, and they identified 13 novel DE-miRNAs with 2501 targets [10]. Zhu et al performed the microarray examination and noticed 27 DE-miRNAs (15 upregulated and 12 downregulated) associated with CA16 and EV71 infection [16]. There were some other important findings about specific miRNAs. MiR-1303 was known to promote CNS lesions following CA16 infections by targeting MMP9 [11]. EV71 can evade the immune surveillance system to proliferate by activating miR-21 [20], antagonize the antiviral activity of host STAT3 and IL-6R through miR-124 [21], and induce autophagy by regulating miR-30a to promote viral replication [22]. So far, there have hardly been direct reports about the relationship between the five key miRNAs and HFMD. Only one study had surveyed the miRNA expression profile in exosome of HFMD patients [9], and it revealed that the expression level of three miRNAs (miR-671-5p, miR-16-5p, and miR-150-3p) were significantly different between mild HFMD, extremely severe SHFMD and the healthy controls. We also noticed that miR-671-5p was among the key miRNA in HFMD.

The PPI network implied that five targets SMC1A, SMARCC1, SF3B3, LIG1 and BRMS1L may play most crucial roles during HFMD progression. However, none of them has been paid enough attention to date, and they are worth more concerns in further researches. Taken different datasets together, we found three common enriched GO terms between miRNA-derived prediction and mRNA-derived analysis: biosynthetic process, cytosol, and nucleoplasm; and a common KEGG pathway, cell cycle, was noticed (Fig. 4). These functions and pathways suggest that HFMD viruses strongly drive the host proliferation. This could be also support by the GO functional enrichment constructed based on the 11 key mRNAs (Fig. 5B), which exhibited that Positive regulation of cell proliferation was the most enriched functional term. This phenomenon maybe contributes to virus amplification but also can be a homeostasis response to fight against virus invasion, particularly for epithelial cells. But the definite mechanism needs more evidences to unravel.

However, this study has some limitations. First, when we probed the key roles, common DEGs were screened between EV71 and CA16 infection. However, these two viruses not necessarily have similar effects, in despite that both viruses belong to members of the genus Enterovirus. For example, Chinese scholars identified that miR-4516 presented down-regulation in EV71 infection and up-regulation in CA16 infection, and it was an important regulator of intercellular junctions by targeting PVRL1 [13]. Liu et al had analyzed microRNA profiles and acquired diverse outcomes induced by EV71 and CA16 infection [15]. The inconsistency was also shown in Fig. 2B, there were two common up-regulated miRNAs, two common down-regulated miRNAs, and three inconsistent ones (miR-502-5p, miR-503-5p and miR-542-3p). Besides, the direct regulatory relationship between 5 key miRNAs and 11 key mRNAs had not been

validated in the present study. Our further efforts would focus on the construction of diagnostic and prognostic models based on the real-world large sample using these miRNA and mRNA factors.

## Conclusions

Our results shed light on the potentially crucial roles of the 5 miRNAs, 11 coding genes and several functions and pathways in HFMD. Combination of these roles may benefit the early diagnose and treatment of HFMD.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All authors have approved the manuscript for publication.

### Competing interests

We declare no competing interests exist.

### Funding

Not applicable.

### Authors' contributions

Min Lin and Liu Yang designed this work. Sheng Lin and Liu Yang analyzed all data and wrote this manuscript. Shibiao Wang and Bin Weng helped dig the data and checked the language.

### Acknowledgements

None.

### Availability of data and materials

All the datasets we analyzed in this article were from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>).

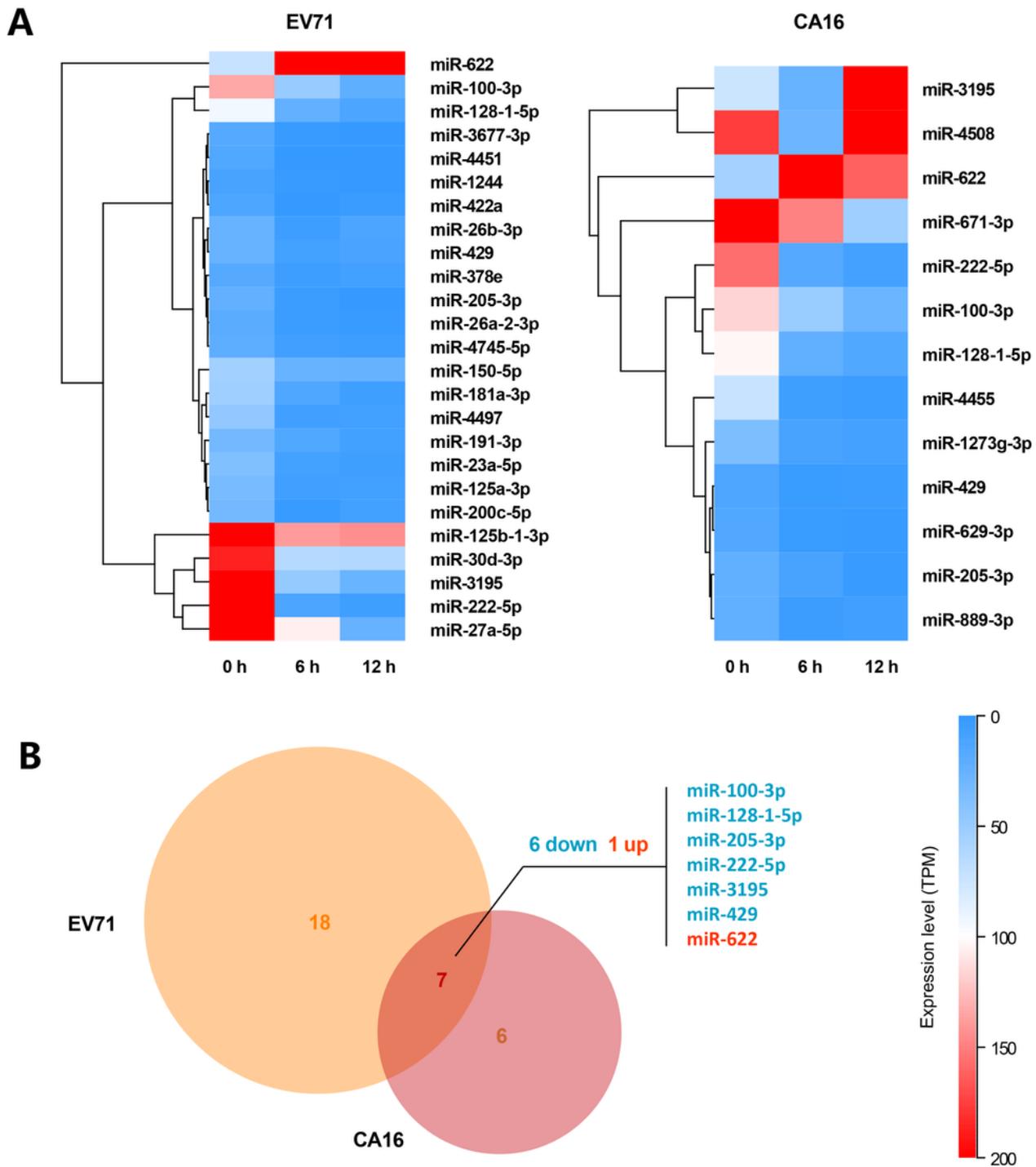
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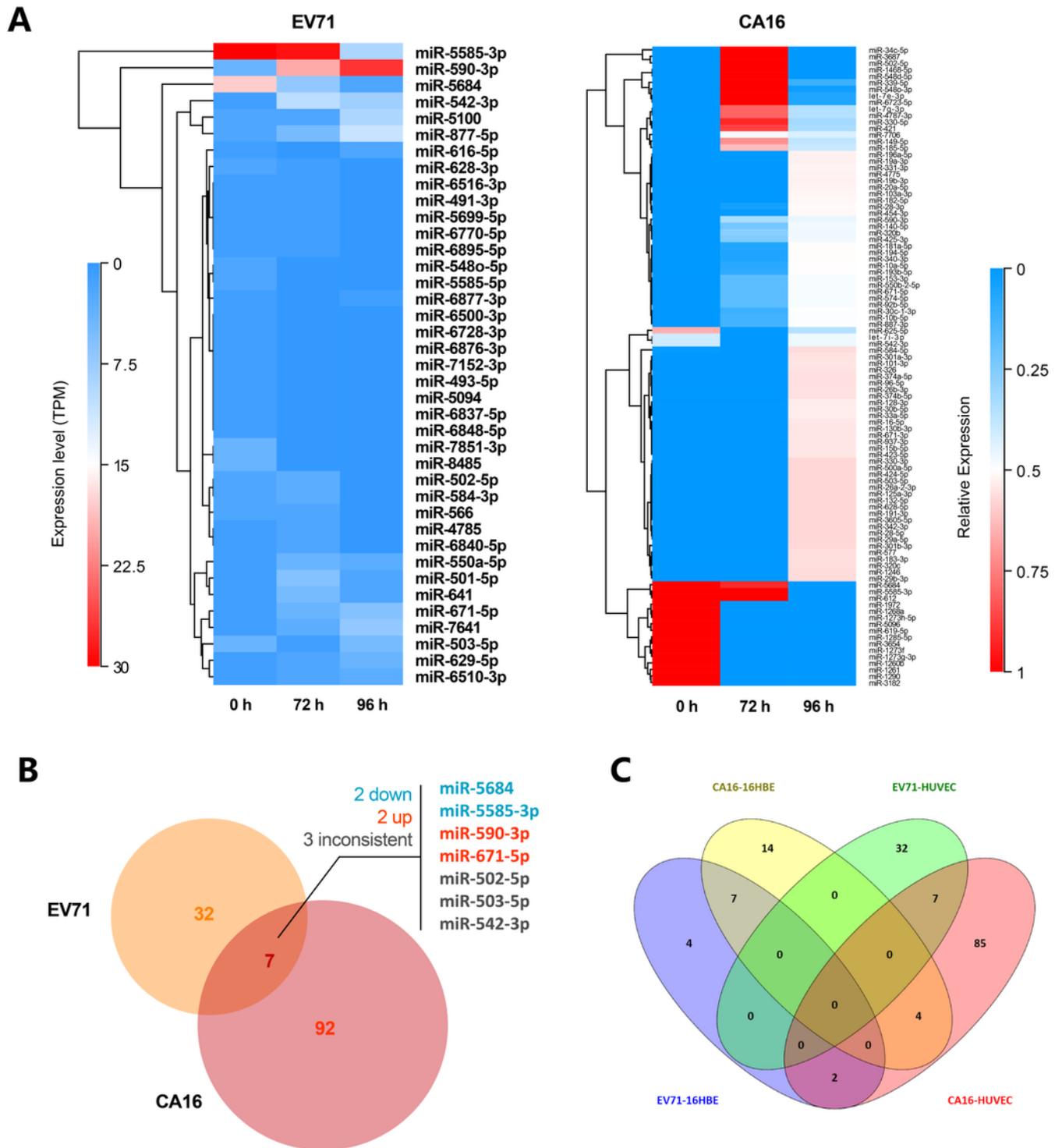
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## Figures



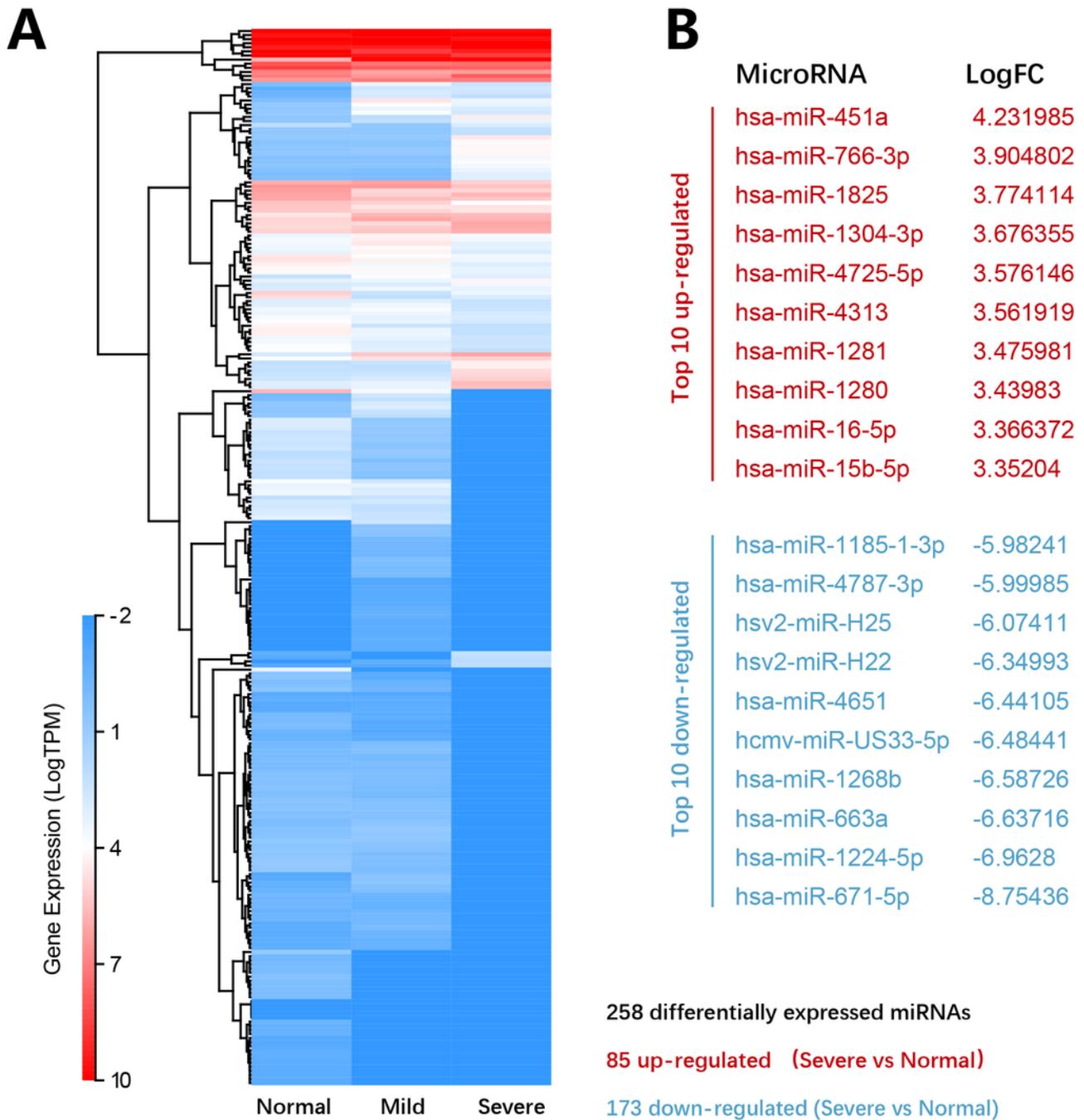
**Figure 1**

Differentially expressed miRNAs after HFMD virus infection in 16HBE cells based on the GSE85829 dataset. (A) Left: EV71 infection induced 25 DE-miRNAs; Right: CA16 infection induced 13 DE-miRNAs. (B) The Venn diagram of 7 common DE-miRNAs between EV71 and CA16 infection.



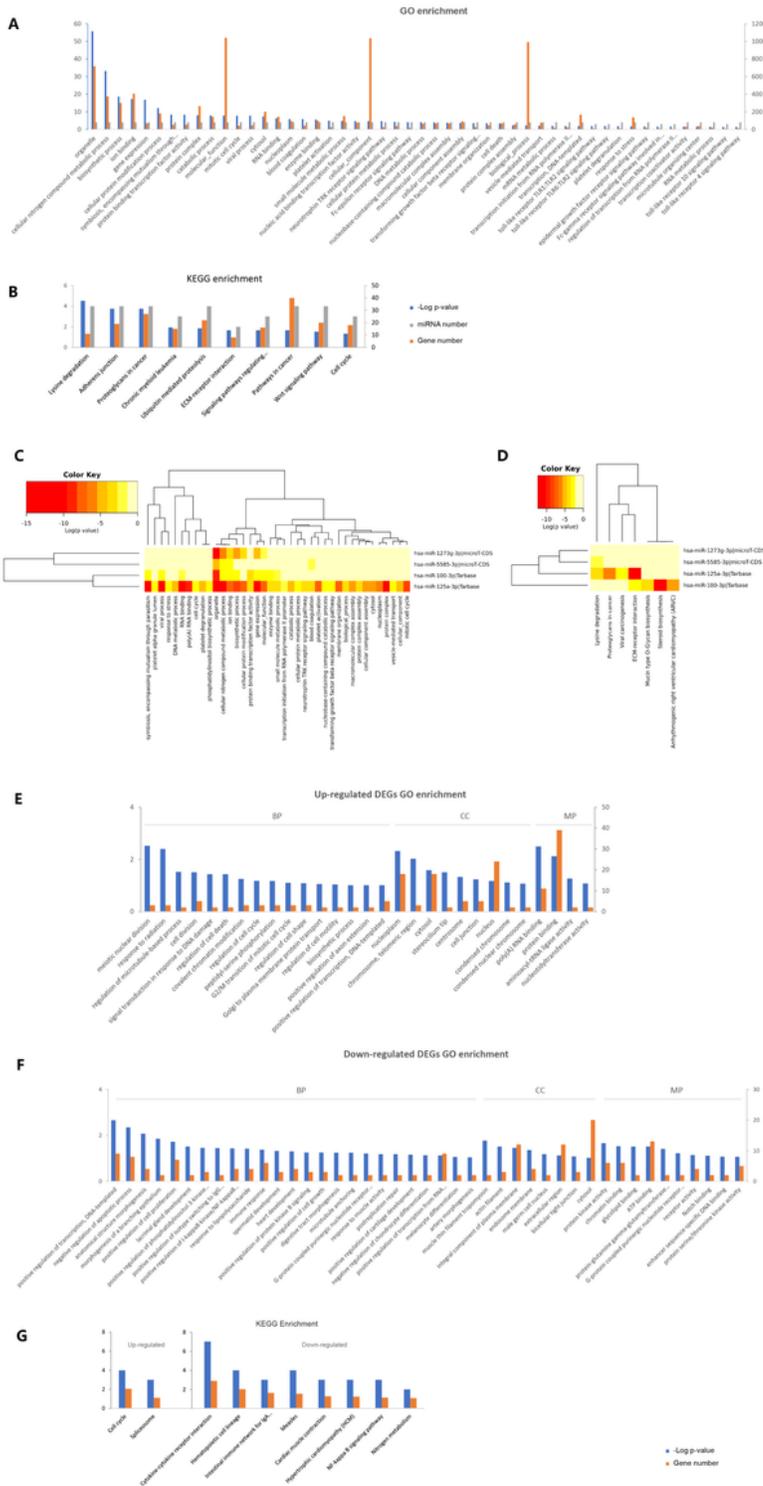
**Figure 2**

Differentially expressed miRNAs after HFMD virus infection in the HUVEC cell line based on the GSE94551 dataset. (A) Left: 39 DE-miRNAs were observed after EV71 infection; Right: 99 DE-miRNAs were found after CA16 infection. (B) The Venn diagram of 7 common DE-miRNAs between EV71 and CA16 infection. (C) The Venn diagram of common DE-miRNAs among four groups of HFMD virus infection.



**Figure 3**

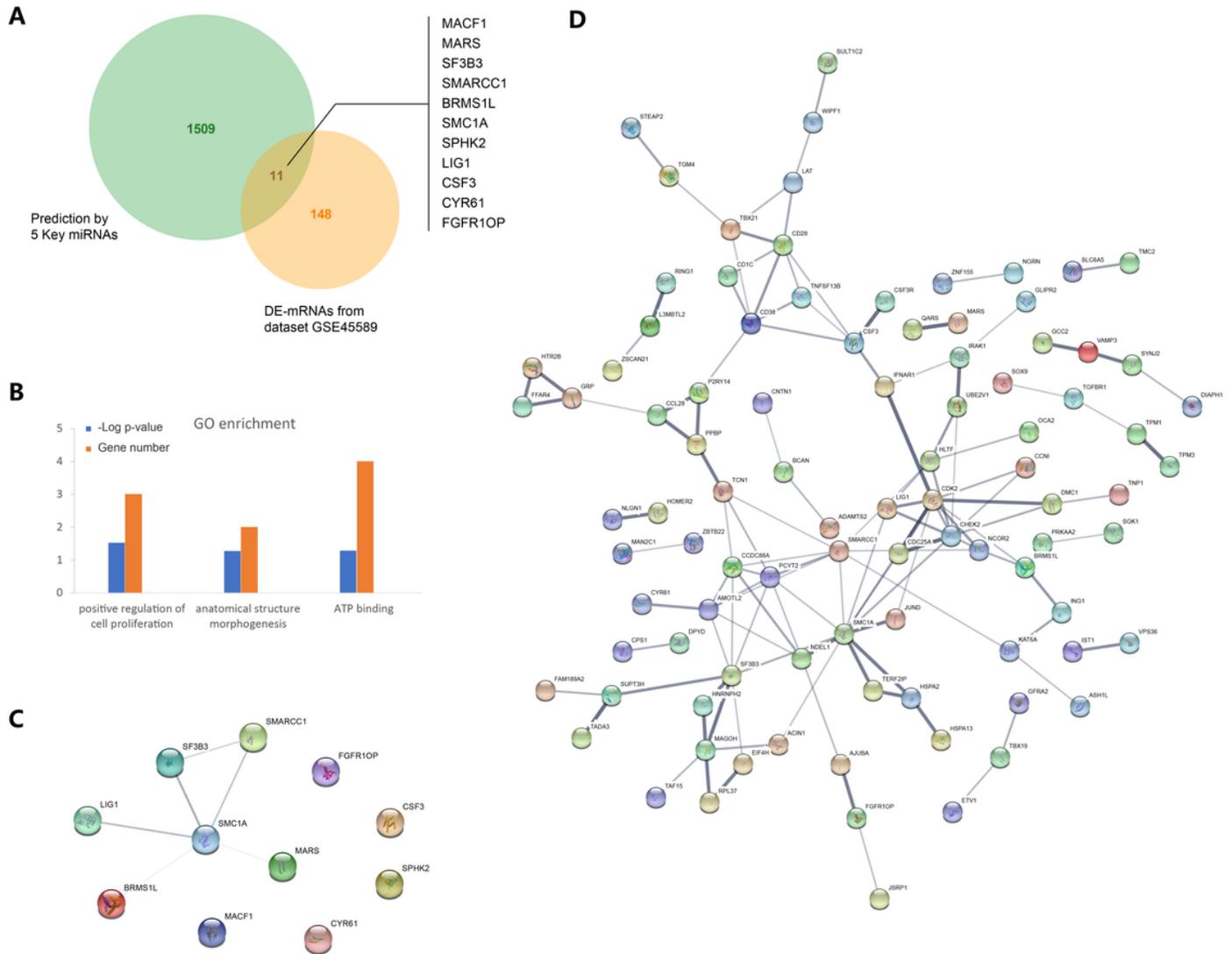
MicroRNAs in serum exosomes from HFMD patients were analyzed based on the GSE52780 dataset. (A) Heatmap of 258 DE-miRNAs were found, including 85 up-regulated and 173 down-regulated ones. (B) The top 10 up-regulated and down-regulated DE-miRNAs.



**Figure 4**

GO and KEGG enrichment of key miRNAs and DE-mRNAs. Based on above five key miRNAs, GO functional and KEGG pathway enrichment were performed using the miRPath V3 database. (A) GO enrichment based on the by the intersection targets of the five key miRNAs (only four miRNAs were included in miRPath database). (B) and KEGG enrichment based on the by the intersection targets of the four key miRNAs. (C) GO enrichment verification through the union of key miRNA related GO terms,

presented as a heatmap. (D) Verification of KEGG pathway through the union of key miRNA related KEGG pathways, presented as a heatmap. (E) The enriched GO functions of up-regulated DEGs in SH-SY5Y cells infected by EV71 from the GSE45589 dataset. (F) The enriched GO functions of down-regulated DEGs in SH-SY5Y cells infected by EV71 from the GSE45589 dataset. (G) Enriched KEGG pathways in SH-SY5Y cells infected by EV71 from the GSE45589 dataset.



**Figure 5**

Common differential mRNAs and PPI network. (A) Using the TarBase V8 tool, we acquired 1520 potential targets (mRNA) from the 5 key DE-miRNAs, among which 11 DEGs were also included by the 159 DE-mRNAs in the GSE45589 dataset: MACF1, MARS, SF3B3, SMARCC1, BRMS1L, SMC1A, SPHK2, LIG1, CSF3, CYR61 and FGFR10P. (B) GO functional analysis showed three terms might be enriched according to these DEG. (C) These common DEGs showed a PPI network mainly connected by SMC1A, SMARCC1, SF3B3, LIG1 and BRMS1L. (D) The PPI network constructed by the 159 DE-mRNAs in the GSE45589 dataset (the isolated nodes were removed).