Expression profiles of differentially expressed circular RNAs and circRNA/miRNA/mRNA network in SH-SY5Y cells infected with Coxsackievirus B5

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Research Article

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Abstract

Coxsackievirus B5 (CV-B5) is the causative agent of hand, foot and mouth disease (HFMD) that can cause neurological complications and fatalities. Circular RNA (circRNA) has been shown to play an important role in regulating pathogenic process. However, their functions of circRNA in response to CV-B5 infection remain unclear. In our research, RNA-seq was employed to analyze the expression profiles of circRNAs in SH-SY5Y cells with or without CV-B5 infection. Out of 5,665 circRNAs identified to be expressed in SH-SY5Y cells, 163 circRNAs were found to be differentially expressed significantly. Moreover, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed that the differentially expressed circRNAs were mainly involved in ubiquitin-mediated proteolysis and signaling pathways during CV-B5 infection. Additionally, RT-qPCR was used to validate the RNA-seq data and a circRNA/miRNA/mRNA interaction network was constructed based on two circRNAs. Two circRNAs, such as: hsa_circ_0008378 and novel_circ_0014617, were associated with the regulation of innate immune response in host cells. Additionally, we confirmed the two circRANs up-regulated the key factors in the IFN signaling pathway, hampering viral replication. Our data provide a new perspective that facilitates further understanding on the virus-host mechanism.

1. Introduction

Hand, foot and mouth disease (HFMD) is an acute infectious disease, that mainly affects infants under five years old. The main manifestations of HFMD are fever, and vesicular rashes on hands, feet and buttocks. Previously, HFMD was mainly caused by enterovirus A71 (EV-A71) that posed serious threats in the infants' health. With the recent development of EV-A71 vaccine, Coxsackievirus B5 (CV-B5) has become one of the main pathogens of HFMD outbreaks globally[1, 2]. As one of the members of family *Picornaviridae*, CV-B5 has a positive-sense single-stranded RNA. HFMD caused by CV-B5 has also been found to be associated with neurological complications, including aseptic meningitis, viral encephalitis, and acute flaccid paralysis. To date, there is no effective treatment for HFMD caused by CV-B5. There are also very few studies that elucidated the pathogenesis mechanism caused by CV-B5 infection.

Circular RNA (circRNA) is a non-coding and single-stranded RNA transcript that can form covalently circular-closed structures without 5' end caps or 3' Poly (A) tails. High-throughput RNA-seq studies have detected a large number of different types of circRNAs with varing lengths. Several biological processes, including gene regulation, protein assembly and trafficking, and cell division are tightly regulated by circRNAs. In additional, circRNAs have been shown to be widely expressed from humans to viruses as potential regulators of microRNAs (miRNAs) and RNA-binding proteins (RPBs)[3, 4]. A recent study has showed that the expression profiles of circRNA were altered as a result of viral infection from both DNA and RNA viruses, such as herpes virus, Avian Leukosis Virus and Human Papillomavirus [5]. Moreover, many studies have confirmed that differentially expressed circRNAs were associated with the occurrence and development of other human diseases, such as circLMP2_E8_E2 and circ_0009910 have been shown play a role in acute myeloid leukemia by sponging miRNA[6]. Although circRNAs have gained much

research attention, the roles of circRNAs and their mechanism of action during CV-B5 infection remains unclear.

In our research, in order to better understand the association between CV-B5 infection and circRNA expression, RNA-seq was employed to obtain the circRNA expression profile in SH-SY5Y cells with or without CV-B5 infection. Furthermore, comprehensive bioinformatics analyses were conducted to explore and probe the functions of the differentially expressed circRNAs. Our results revealed the potential function on circRNA in host-virus interaction, aiming to the potential roles of circRNA in disease causing by CV-B5.

2. Materials And Methods

2.1 Cell Culture and CV-B5 Infection

SH-SY5Y, a neuron-specific human cell line, was grown in Dulbecco's modified Eagle's medium (DMEM) (SH30243.01, HyClone) supplemented with 10% fetal bovine serum (FBS) (FBSSA500-S, AusGeneX) and maintained in a humidified atmosphere with 5% CO_2 at 37°C. CV-B5 strain was isolated in Kunming, Yunnan province in 2014 and preserved at -80℃. At 70–80% confluency, SH-SY5Y cells were mock-infected or infected with CV-B5 at a 1 multiplicity of infection (MOI). Cytopathic effect in SH-SY5Y cells was evaluated at 24 h post infection. Three biological replicates were performed in each treatment group.

2.2 RNA extraction, library construction, and sequencing

Total RNAs were isolated from infected as well as uninfected cells using RNAiso Plus (No.9109, TaKaRa) according to the manufacturer's instructions. The purity, concentration and integrity of extracted total RNAs were evaluated using NanoPhotomete, Qubit® 2.0 and Agilent 2100. RNA sequencing (RNA-seq) libraries were constructed using rRNA-depleted RNA. Raw sequencing data were obtained using Illumina HiSeq platform (150 bp paired-end reads). Clean data (clean reads) were subsequently retrieved by eliminating adaptor-polluted reads and low quality reads. RNA library was sequenced by Novogene Co., Ltd (Beijing, China).

2.3 Identification and analysis of differentially expressed circRNAs

Human genome sequences and gene annotations were downloaded from NCBI database (https://www.ncbi.nlm.nih.gov/projects/genome/guide/human/index.shtml). Index of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5.1b). Identification of circRNA was performed using find_circ and CIRI2. In order to lower the rate of false positive identification, we only utilized the data where the circRNA was identified in the same location by two different software [7]. The reads numbers mapped to each gene were counted using HTSeq v0.6.0. Quantification of expressed circRNAs were performed using TPM (transcript-per-million). Differential expression analysis of two groups (three biological replicates per condition) was performed using

DESeq2 R package (1.10.1). CircRNAs with *p*-values < 0.05 and fold changes (FC) > 2 were defined to be differentially expressed [8].

2.4 Enrichment analysis of differentially expressed circRNA-host gene

Gene Ontology (GO) enrichment analysis of differentially expressed circRNA-host gene and statistical enrichment in KEGG pathways were implemented by the cluster Profiler R package21[9]. CircRNAs with *p*-values < 0.05 was considered to be significantly enriched.

2.5 Real-time quantitative PCR (RT-qPCR)

Total RNAs were extracted from mock-infected and CV-B5-infected SHSY-5Y cells. Thereafter, RT-qPCR was performed using One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara, RR066A) on a 7500 real-time PCR system programmed with the following cycling conditions: 42°C, 5 min, 95°C, 10 s and 40 cycles of 95°C, 5 s and 60°C, 34 s. Primer sequences were listed in Table 1. Expression fold change relative to controls was calculated using the $2^{-\Delta\Delta Ct}$ method [10]. The expression levels of circRNAs were normalized to those of GAPDH. Six circRNAs with its fold change > 5 and *p*-value < 0.05 were selected for validation.

Table 1
Primers used for RT-qPCR analysis

circRNA	Forward primer 5'-3'	Reverse primer 5'-3'
ACTB	GCGTGACATTAAGGAGAAGC	CCACGTCACACTTCATGATGG
hsa_circ_0008650	ACAGCCTTTCCCACGACTTG	AATGGCAAAAGCAGCAAAGC
novel_circ_0002030	ACACGCAGAAGATCAGCACC	CAGTTCCCCAGTTAGCCAGC
hsa_circ_0014617	GAGTTTCCCCAGTGCCTTCG	GCCCTCGCCTATTTCTCCTTT
hsa_circ_0004299	GCACATCAAGAAGCCCATCC	CGCAGGTCACAATACGGTTAC
hsa_circ_0010796	CCAAATTTAGTGCAGAAAAGGGC	CCACACGGCTCTGGATGG
hsa_circ_0008378	TAAGAAAGCGACCCAGCCG	GCTGACTGTTGTCTGATGTCTTCC

2.6 Construction of interaction network

Putative miRNA binding sites in six identifited circRNAs were identifited by employing miRanda algorithm[11]. CircRNA/miRNA/mRNA interaction network was constructed using Cytoscape 3.4.0[12].

2.7 Analysis of the candidate circRNAs functions

Constructed the pcDNA3.1-circRAN to verify the impact on CV-B5 replication. Also analyzed the expression levels of all miRNAs and the key factors according to the circRNA/miRNA/mRNA interaction network.

3. Results

3.1 General profiles of circRNA in SH-SY5Y cells with CV-B5 infection

Total RNAs without contaminating rRNAs and linear RNAs were extracted from CV-B5-infected or mock-infected cell samples, and subsequently used as the template for RNA-seq. Following the removal of adapter sequences and low quality sequences, all clean reads generated from the samples were mapped to the reference genome. A total of 5,665 circRNAs were identified in all samples using find_circ and CIRI2 (Supplementary Table 1). Among them, 2,366 circRNAs were observed to be commonly expressed in both treatment groups. Meanwhile, 1,195 and 2,104 circRNAs were identified to be exclusively expressed in CV-B5-infected cells and mock-infected cells, respectively (Supplementary Table 1, Fig. 1A). These results indicated that circRNAs were differentially expressed in SH-SY5Y cells infected with CV-B5 compared to those in uninfected cells.

The length, exon number, chromosome distribution, distribution in gene position and the GC content of identified circRNAs were analyzed. Our results showed that the circRNAs were uniformly distributed across different chromosomes, with chromosome Y containing the smallest number of circRNAs (Fig. 1B). In addition, the genomic position distribution results showed that the circRNAs were mainly derived from exons and intron, and partly from intergenic regions (Fig. 1C). Since most of exon derived circRNAs were generated from two exons by back splicing, exons may be the preferred circRNA expression (Supplementary Table 2). Distribution analysis of circRNA sequence lengths showed that most circRNAs sequences ranged from 26 nt to 1323 nt, and that majority of circRNAs were of 100 nt to 500 nt in length (Fig. 1D). Sequence analysis showed that the circRNAs sequences have an average G + C content of 49.74% (Supplementary Table 3).

3.2 Analysis of differentially expressed circRNA

In order to screen for dysregulated circRNAs, the circRNAs expression profiles in CV-B5 infection and mock-infected cells were analyzed using DEseq 2. The abundance of each expressed circRNA was calculated based on TPM. The distribution of gene expression in all six samples was observed to be consistent (Fig. 2A). More than 50% of circRNAs had an TPM value of less than 0.1 (Supplementary Table 4). Analysis of differentially expressed circRNAs by DESeq 2 showed that out of 163 differentially expressed circRNAs upon CV-B5 infection, with 78 circRNAs were upregulated, while 85 circRNAs were downregulated (Fig. 2B). Inter-sample correlation analysis demonstrated that there was an obvious distinction in circRNA expression patterns between mock-infected cells and CV-B5-infected cells (Fig. 2C). Of these circRNAs, 52 were newly emerging circRNAs after CV-B5 infection and 65 disappeared after CV-B5 infection (Fig. 2D).

3.3 Biological function analysis for host circRNAs transcripts

In order to reveal the biological functions of differentially expressed circRNAs, GO assignments were exploited to categorize the functions of the host genes of differentially expressed circRNAs. GO functional classification was based on three different categories, namely cellular component, molecular function and biological process. The top 20 significant enrichment GO terms for CV-B5-infected cells are shown in Fig. 3A. Our results showed that most circRNA-host genes of the cellular component category were of ‘intracellular’. Meanwhile, circRNA-host genes of the molecular function category were mainly of ‘organic cyclic compound binding’ and ‘heterocyclic compound binding’. Lastly, circRNA-host genes of biological process category were mainly involved in the ‘heterocycle metabolic process’. Directed acyclic graph (DAG) was constructed to display the GO structure results. Our analysis showed that enriched GO terms in the DAG of BP, CC and MF were 6, 10, and 5, respectively (Fig. 3B-D). Additional, KEGG pathway analysis indicated that differentially expressed circRNAs may also affect important pathways, five pathways were significantly enriched, such as ubiquitin mediated proteolysis, tight junction, TNF signaling pathway, mRNA surveillance pathway and MAPK signaling pathway (Fig. 3E).

3.4 Validation of selected circRNAs

To validate the reliability of the RNA-seq results, we performed RT-qPCR to detect the expression of differentially expressed circRNAs in SH-SY5Y cells infected by CV-B5. Six typically differentially expressed circRNAs were selected for validation experiments. As shown in Fig. 4A, these results indicated that the hsa_circ_0008650, hsa_circ_0002030 and hsa_circ_0014617 were upregulated; hsa_circ_0004299, hsa_circ_0010796 and hsa_circ_0008378 were downregulated, which were consistent with our data analyzed by RNA-seq (Fig. 4B).

3.5 Construction of the circRNA/miRNA/mRNA interaction network

CircRNAs are known to offset miRNA-mediated gene regulation by acting as miRNA sponges[13]. To identify the critical roles of expressed circRNAs in CV-B5-infected SH-SY5Y cells, a circRNA/miRNA/mRNA interaction network was established. According to the KEGG pathway, we selected differentially expressed circRNAs that regulated immune system process. As shown in Fig. 5, a total of two circRNAs, namely hsa_circ_0008378 and novel_circ_0014617 were associated with 114 miRNAs, however, only 8 miRNAs have potential interactions with 112 mRNAs, while the rest (92.92% of associated miRNAs) have no mRNA binding sites (Fig. 5). Both circRNAs have putative binding sites for hsa-miR-29a-5p and hsa-miR-4535 (Supplementary Table 5). Hsa_circ_0008378 is associated with JAK-STAT signaling pathway related network involving 6 miRNAs and 62 target genes (Fig. 5A). Meanwhile, novel_circ_0014617 is involving in the RIG-I signaling pathway-related network, and is associated with 2 miRNAs and 33 target genes (Fig. 5B). All above, the circRNA/miRNA/mRNA axes may form a complex interaction network and play a role in CV-B5 infection.

3.6 Verification of circRNA hsa_circ_0008378 and novel_circ_0014617

We selected the circRNA hsa_circ_0008378 and novel_circ_0014617 from the network. Both the two circRNAs inhibited the CV-B5 replication through promoting the key factors in IFN-I signaling pathway (Fig. 6AB). Hsa_circ_0008378 was positively correlated with six targeted miRNAs, however, novel_circ_0014617 was negatively correlated with two targeted miRNAs (Fig. 6C).

4. Discussion

The prevalence of CV-B5 has been increasing every year, causing considerable threats to children under five years old. It also has been reported that adults infected with CV-B5 are at risk of developing severe clinical complications[14]. Moreover, Central nervous system (CNS) pathology and neurological sequelae often occur after CV-B5 infection, which increases risk of mortality[15, 16]. In this study, the human neuroblastoma cell line, SH-SY5Y, was adopted for studying neurological damages of CV-B5 infection. CircRNA, a special class of endogenous non-coding RNA that plays an important role in the process of immune regulation has been shown to be involved in the development of a number of diseases[17]. Therefore, studies of circRNA in virus-infected cells may provide important theoretical basis for viral disease treatment. In this study, RNA-seq was applied to investigate the circRNA expression profile of SH-SY5Y cells infected with CV-B5. The mechanisms of circRNAs identified to be involved in innate immune signaling pathway were further analyzed.

A total of 5,665 circRNAs identified to be present in SH-SY5Y cells are mainly derived from exons that are located on every chromosome except the Y chromosome. Only 3,167 circRNAs are found to be matched in the CircBase database, hence the rest are classified as newly discovered circRNAs. Moreover, 163 differentially expressed circRNAs were identified in CV-B5-infected cells. There were 26 differentially expressed circRNAs identified in both mock-infected cells and CV-B5-infected cells. We speculated that these 26 differentially expressed circRNAs may be cell-specific and unrelated to virus infection. An enrichment analysis showed that enriched GO-BP terms mainly focused on “metabolic process” and “protein modification” which is largely consistent with that of EV71 or CV-A16 virus infection virus infection. However, enrichment analysis of SH-SY5Y cells infected with EV71 or CA-16 indicates association with “gonadotropin-releasing hormone receptor pathway”, “Wnt signaling pathway”, “angiogenesis” and “p53 pathway”, which are not present in our analysis with CV-B5 infection[18, 19]. In our study, host genes that correspond to the differentially expressed circRNAs are mainly involved in “ubiquitin mediated proteolysis”, “tight junction” and the regulation of other signaling pathway, such as MAPK pathway. Ubiquitination, the most prevalent protein post-translational modification in cells, is known to play an important role in the dynamic regulation of host defense against pathogenic microbial infection[20, 21]. Ubiquitin often conjugates to lysine residues in substrate proteins, thereby inhibits the interaction of transcription factor with upper signal molecular. For example, STAT1 has linear ubiquitination which inhibits the binding of type-I interferon receptor IFNAR2, thereby restricting STAT1

phosphorylation, and resulting in type-I interferon signaling, in order to improve interferon (IFN) antiviral efficacy[22]. In our study, the key junction molecules may undergo ubiquitination modification in order to regulate the antiviral immune response, although further validation is required. Tight junction may influence virus infection by blocking virus entry into the cells[23]. MAPK signaling pathway has been shown to be involved in inflammatory response during IAV infection[24]. Hence, our results collectively indicated that the altered expression of circRNAs may be the key regulators that participate in the infectious process of CV-B5.

An increasing number of studies have shown that circRNA function as works as miRNA sponges. CIRS-7, the first circRNA discovered, has been shown to play a role as a tumor-promoter by decreasing the regulation of miR-7 on central oncogenic factors via competitive interaction with miR-7[25]. Subsequently, circRNA-miRNA interaction in viral infection were put forward. During the infection of Kaposis Sarcoma-Associated Herpesvirus, hsa_CIRC_0001400 has been shown to inhibit the expression of latency-associated nuclear antigen or replication and transcription activator in order to decrease virus infectivity through miR-K12-7-5p targeting[26]. The same results have also been found in human cytomegalovirus and influenza A virus infection[5]. Thus, it is necessary to establish differentially expressed circRNAs interaction network. In order to better understand the pathogenesis of CV-B5 infection that may facilitate the development of new therapeutic target, we focused on circRNAs interaction network in IFN pathway. The host innate immune system is the first line defense against viral invasion or replication. The association between IFN signaling pathway and the viral infection have widely documented, such as HBV, HSV and IAV[27, 28].

We found that hsa_circ_0008378 mainly affects the JAK-STAT/IFN signaling pathway and related ceRNA network. A total of 62 mRNA were identified to compete for 6 miRNAs including “hsa-miR-23a-3p/hsa_circ_0008378/TNFAIP3”, “hsa-miR-23a-3p/hsa_circ_0008378/DDX5”, “hsa-miR-23c/hsa_circ_0008378/BTLA” and “hsa-miR-23b-3p/hsa_circ_0008378/JAK1” from the IFN signaling pathway. TNFAIP3 (also known as A20), which is essential for maintaining immune homeostasis, is a key regulator of inflammatory, antiviral and apoptotic signaling pathways[29]. DEAD-box polypeptide 5 (DDX5), also called p68, is an ATP-dependent RNA helicase. The roles of DDX5 in viral infection have been established. For instance, DDX5 has been shown as a host factor that exhibits antiviral activity during HBV and MYXV infection. Furthermore, Japanese encephalitis virus (JEV) and hepatitis C virus (HCV) have been found to hijack DDX5 to facilitate various steps of their replication cycles[30]. B-and T-lymphocyte attenuator (BTLA), an immune-regulatory receptor, is known to promote HSV infection in host cells by forming an interactive network of CD160/BTLA/LIGHT/HVEM[31]. Deubiquitinating enzyme HFMD virus structural protein VP3 has been shown to degrade Janus kinase 1 (JAK1) to inhibit IFN-γ signal transduction pathways[32]. Also, we found that novel_circ_0014617 is enriched in RIG-I-like receptor signaling pathway, and that 33 mRNAs competitively bind to 2 miRNAs. Surprisingly, hsa-miR-138-5p was found to interact with lncRNA-GAS5, which has been shown to reverse cardiomyocyte injury by lower CYP11B2 expression[33]. Additionally, we showed that “hsa-miR-138-5p/novel_circ_0014617/DNAJB6” and “hsa-miR-665/novel_circ_0014617/VPS4A” are enriched in the RIG-I signaling pathway. DNAJB6 has also shown to actively impact viral infection. Direct interaction in the

DNAJB6 and prion protein during viral infection has been shown to favor nuclear localization of HIV-1 pre integration complex. In addition, the delivery of primase-UL70 into cell nuclei through DNAJB6 may promote the synthesis of viral DNA during HCMV infection[34]. Although not all miRNA in the two circRNA networks selected in this study have binding with mRNA, they may be involved in CV-B5 infection, since they are the target molecules of circRNA. These miRNAs should be studied further, and the findings may provide new sights on CV-B5 infection.

In summary, our study revealed the characteristics and profiles of circRNAs in CV-B5-infected SH-SY5Y cells by RNA-seq. Differentially expressed circRNAs were identified and their potential functions were predicted by GO and KEGG. Enrichment analysis demonstrated that circRNAs associated with CV-B5 infection participate in cell metabolic process and immune response. Moreover, based on the six validated circRNAs, a circRNA/miRNA/mRNA network was constructed. Our analysis indicated that the circRNA played critical roles in the regulation of CV-B5 infection through IFN-I signaling pathway. Our results advance our current knowledge on the function of circRNAs in virus-host interactions. The findings in this study may also facilitate the identification of novel molecular targets for the prevention and treatment of CV-B5 infection.

Declarations

Interests Statement

No competing financial interests to disclose.

Authors contributions

Conceptualization and Design: Wei Chen; Methodology: Jing Li and Heng Yang; Statistical analysis: Huaran Shi, Fan Yang and Xia Ou; Investigation: Jihong Zhang and Wei Chen; Drafting of the manuscript: Jing Li; Critical revision of the manuscript: Jihong Zhang and Wei Chen. All authors approved the final version of the manuscript.

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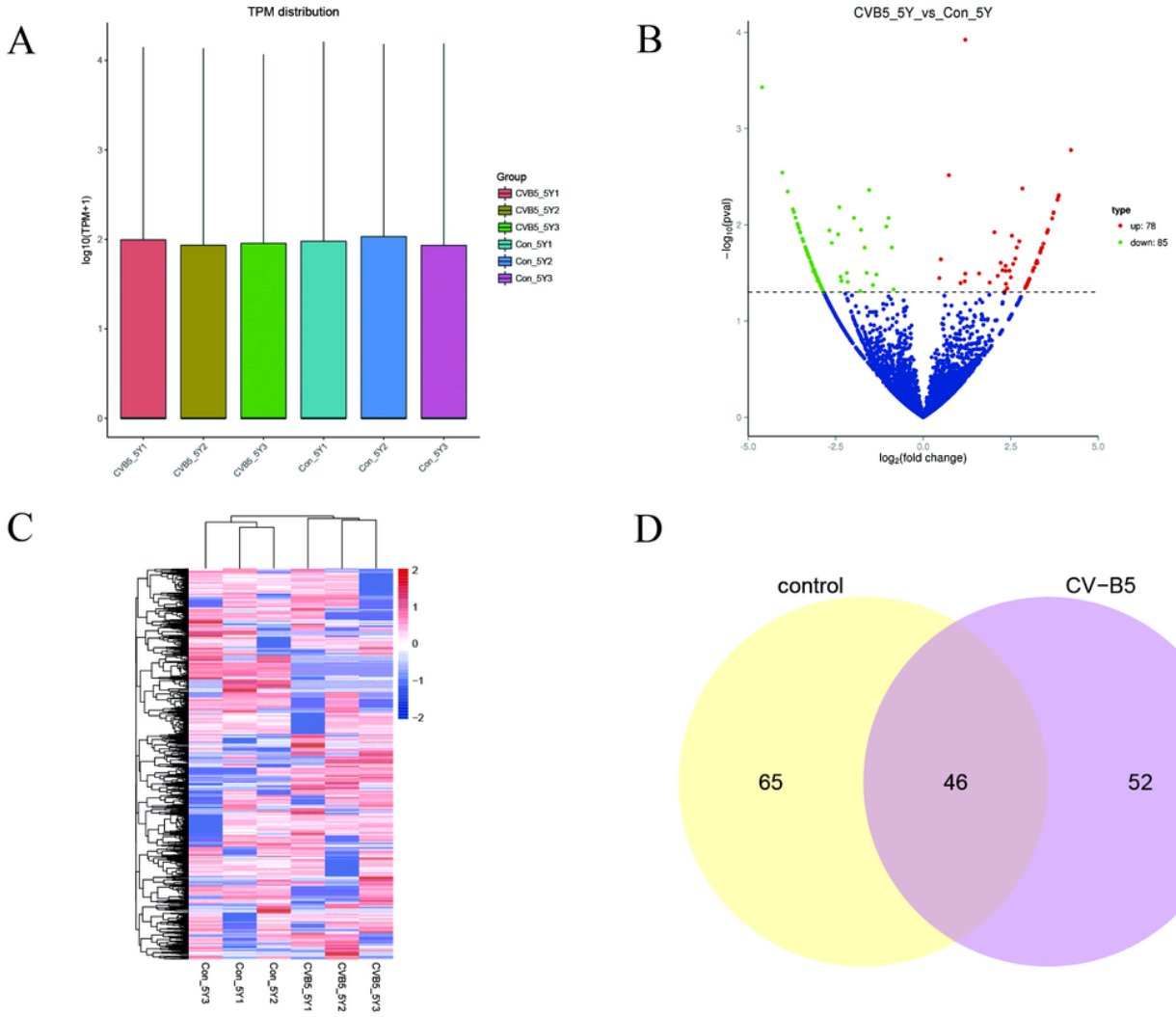
Figures

Figure 1



Genomic features of circRNAs detected in CV-B5-infected SH-SY5Y cells. (A) Venn diagram of the number of differentially expressed circRNAs in each group. A total of 2,366 circRNAs were expressed in two groups. (B) Genomic distribution of circRNAs. (C) Chromosomal distribution of circRNAs. (D) Length distribution statistics of circRNAs.

Figure 2

Differentially expressed circRNAs following CV-B5 infection. (A) Box plots showing the expression levels of circRNAs from each sample. (B) Volcano plots depicting differentially expressed circRNAs following CV-B5 infection. (C) Heatmap of maximally expressed circRNAs across all samples. (D) Venn diagrams of differentially expressed circRNAs identified in mock-infected and CV-B5-infected SH-SY5Y cells.

Figure 3

Enrichment analyses of all differentially expressed circRNAs. (A) Biological process (BP), cell component (CC), and molecular factor (MF) of GO terms. (B, C, D) Directed acyclic graph (DAG) of BP, CC and MF. (E) Top 20 KEGG pathways of host genes of all circular RNAs.

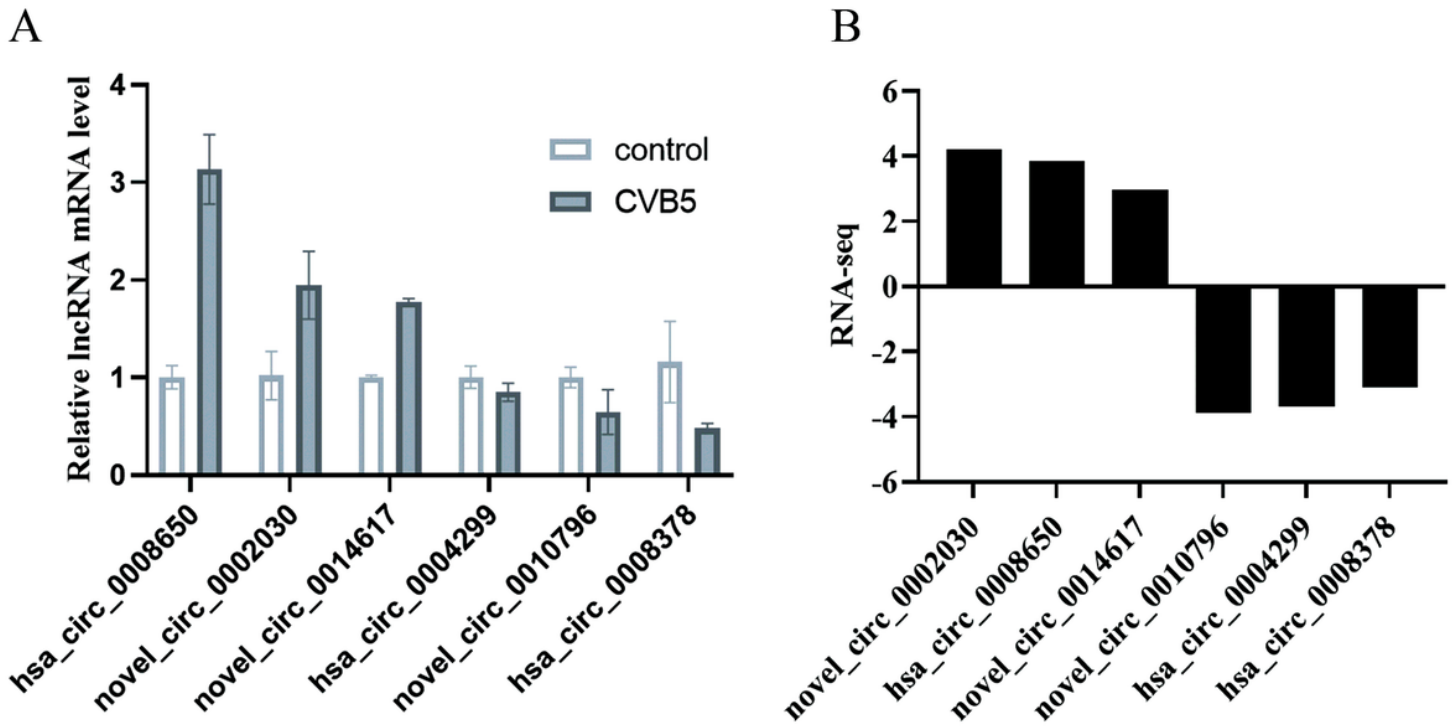


Figure 4

Validation of differentially expressed circRNAs by qRT-PCR. (A) Expression levels of six circRNAs validated by qRT-PCR. (B) Expression levels of six circRNA validated by RNA-seq.

Figure 5

Construction of circRNA/miRNA/mRNA interaction network. (A) Network of circRNA hsa_circ_0008378. (B) Network of circRNA novel_circ_0014617.

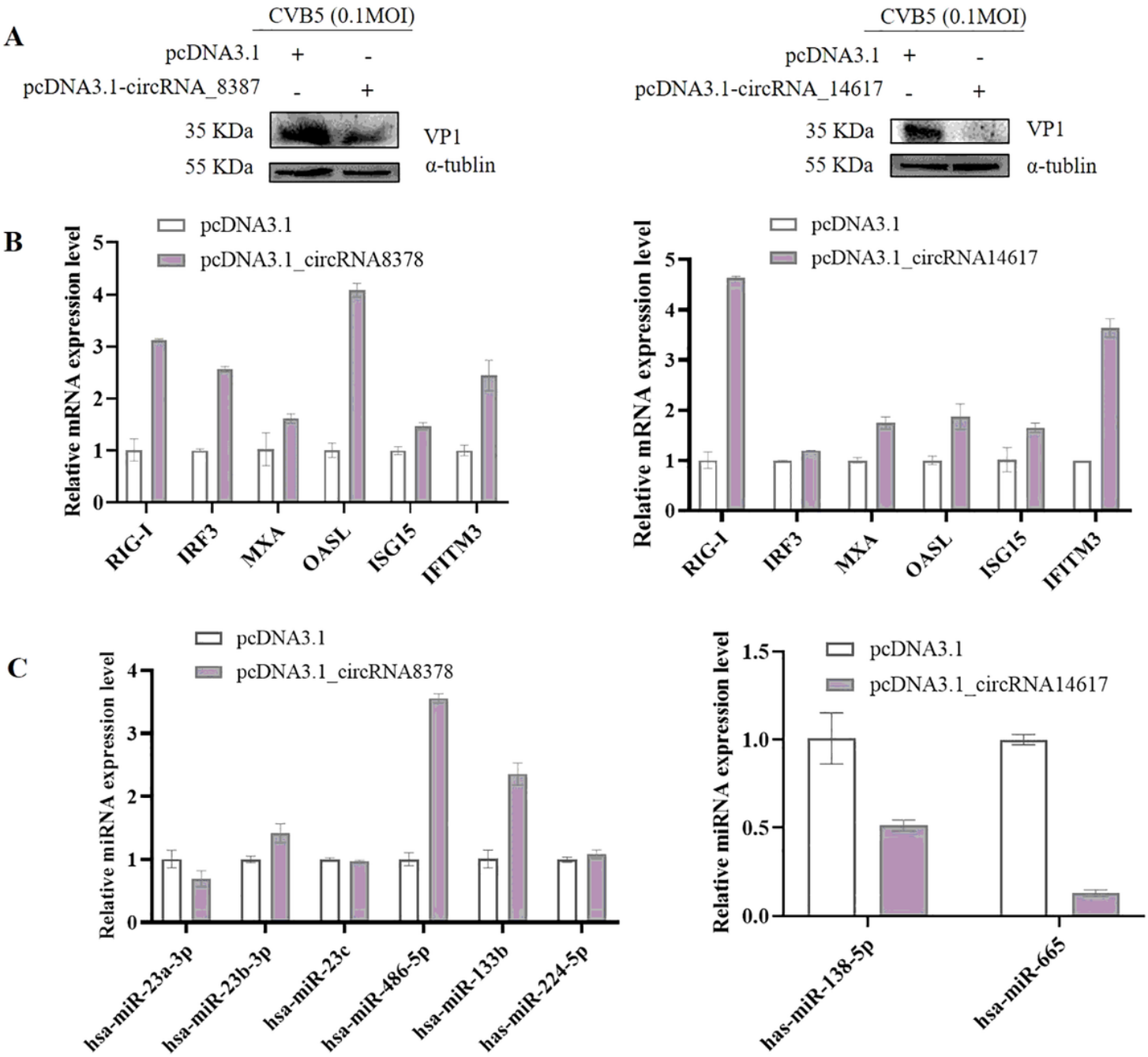


Figure 6

The verification of circRNA hsa_circ_0008378 and novel_circ_0014617. (A) Western blot analysis of CV-B5 specific protein VP1 expression transfected with pcDNA3.1-circRNAs. (B) RT-qPCR detected the expression of genes in IFN-I pathways (RIG-I, IRF3, MxA, OSAL, ISG15 and IFITM3). (C) RT-qPCR detected the expression of target miRNA according to network.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx
- SupplementaryTable4.xlsx
- SupplementaryTable5.xlsx