Deep Insight Into Long Non-Coding RNA and mRNA Transcriptome Profiling in HepG2 Cells Expressing Genotype IV Swine Hepatitis E Virus ORF3

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Abstract

Background: Swine hepatitis E (SHE) is a new type of zoonotic infectious disease caused by swine hepatitis E virus (SHEV). Open reading frame 3 (ORF3) is an important virulent protein of SHEV but its function still is mainly unclear.

Methods: In this study we generated adenoviruses ADV4-ORF3 and ADV4-NC which successfully mediated overexpression of EGFP-ORF3 and EGFP, respectively, in HepG2 cells. High throughput sequencing was used to screen for differentially expressed long non-coding RNAs (lncRNAs) and mRNAs. The *cis*-target genes of lncRNAs were predicted, functional enrichment (Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)) was performed, and 12 lncRNAs with statistically significant different expression (p value ≤ 0.05 and q value ≤ 1) were selected for further quantitative real-time reverse transcription (qRT-PCR) validation.

Results: In HepG2 cells, we identified 62 significantly differential expression genes (DEGs) (6,564 transcripts), and 319 lncRNAs (124 known lncRNAs and 195 novel lncRNAs) that were affected by ORF3, which were involved in systemic lupus erythematosus, staphylococcus aureus infection, signaling pathways regulating pluripotency of stem cells, the PPAR signaling pathway, and platinum drug resistance pathways. *Cis*-target gene prediction identified 45 lncRNAs corresponding to candidate mRNAs, among which 8 were validated by qRT-PCR: *LINC02476* (two transcripts), *RAP2C-AS1*, *AC016526*, *AL139099*, and *ZNF337-AS1* (3 transcripts).

Conclusions: Our results revealed the lncRNA profile in host cells affected by ORF3, which will help to determine the function of ORF3, and the infection mechanism and treatment of SHE.

Background

Hepatitis E (HE) caused by hepatitis E virus (HEV) is a new type of zoonotic infectious disease. HEV is mainly transmitted through the fecal-oral route, can cause acute hepatitis in humans, and it is prevalent worldwide [1–3]. About 20 million people are infected with HEV every year. In addition to the clinical symptoms of hepatitis, it also causes nerve, blood, and kidney diseases, neuralgia muscular atrophy, encephalitis, myelitis, and other diseases [4]. Studies have shown that the continuous glycine motif in the β chain of HEV specific T cell receptors (TCRs) pathway might be the target for TCR binding heterozygosity recognition, thus promoting cross recognition, which might develop into a candidate for T-cell therapy for chronic HE [5].

Swine hepatitis E (SHE) is caused by swine hepatitis E virus (SHEV). In recent years, outbreaks of SHE have appeared globally and have become a public health issue in various countries [6, 7]. SHEV belongs to the family Hepeviridae [8]. Genotype IV is mainly prevalent in China. Its genome comprises about 7.6 kb, with a poly A tail structure at the 3' end and three overlapping open reading frames (ORF): ORF1, ORF2, and ORF3 [9, 10].

The ORF3 protein (hereafter referred to as ORF3) is a small and poorly characterized protein that is involved in the secretion of virus particles and other functions. Palmitoylation determines the subcellular location, membrane topology, and function of ORF3 in the life cycle of HEV [11]. ORF3 forms polymer complexes related to endoplasmic reticulum (ER)-derived membranes through affinity interaction, which might be an attractive target for the development of antiviral drugs [12]. ORF3 significantly inhibits the activity of the nuclear factor kappa-B (NF- κ B) signaling pathway, mediated by pattern-recognition receptors (PRRs), thus providing a breakthrough point for clarifying the function of ORF3 in chronic HEV infection and cirrhosis [13]. ORF3 mediates the regulation of endosomal sorting complexes required for transport (ESCRT) through the "PSAP" motif (viral late domain) [14].

Although the function of HEV ORF3 has been studied, the function of SHEV ORF3 remains unclear. In the present study, we used adenovirus mediated overexpression of genotype IV SHEV ORF3 in HepG2 cells, and performed transcriptome sequencing to identify significantly differentially expressed long non-coding RNAs (lncRNAs) and their target genes affected by ORF3, which form a basis to reveal the function of ORF3, explain the interaction mechanism between SHEV and target cells, and provide a scientific basis for the prevention and treatment of SHE.

Methods

Cell Lines

HepG2, 293A, and 293T cells were purchased from the Shanghai cell bank of the Chinese Academy of Science, and cultured at 37 °C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Life technology, Carlsbad, CA, USA) containing 10% fetal bovine serum (Life technology) supplemented with 10% penicillin (100 U/mL) and streptomycin (100 μ g/mL) (both Life technology).

Preparation of Recombinant Adenovirus ADV4-ORF3 and ADV4-NC

Sangon Biotech (Shanghai, China) Co., Ltd synthesized the ORF3 gene (345 bp) of genotype 4 swine hepatitis E virus (SHEV). Adenoviruses ADV4-ORF3 and ADV4-NC were generated by Shanghai GenePharma Co, Ltd (Shanghai, China). The EGFP protein was fused to the N-terminus of ORF3. Both of them were sequenced using Sanger sequencing and no mutations compared with the published sequence of ORF3 and the rest of the plasmid were found. Briefly, *EcoRI* and *BamHI* were added to the upstream and downstream primers of the target gene respectively. SHEV-ORF3 was cloned into vector ADV4 (CMV/IRES-GFP) (Fig.S1), this vector, with a molecular weight of 7,500 bp, comprises a CMV promoter, a multiple cloning site, GFP, IRES, an ampicillin resistant gene, and the Ad5 skeleton. Highly pure and non-toxic recombinant expression shuttle plasmid ADV4-ORF3 and framework plasmid pGP-Ad-Pac were prepared. The shuttle plasmids ADV4ORF3, ADV4-negative control (ADV4-NC), and framework plasmid pGP-Ad-Pac (molecular weight of 35,000 bp, comprising of Ori promoter, pA, Ampicillin resistance gene, dE3 and Ad5 skeleton (Fig. S2)), were co-transfected into 293A cells with the RNAi mate

transfection reagent (GenePharma, Shanghai, China). Six hours after transfection, the culture medium was replaced with complete medium, and fresh medium was supplemented every seven days. Then, the cells and supernatant were collected and placed in a centrifuge tube, frozen and thawed three times, centrifuged at $800 \times g$ for five minutes, and the supernatant was regarded as the primary adenovirus solution. After three successive generations of repeated amplification and collection of adenovirus, a large amount of adenovirus was obtained, which was then purified and concentrated to obtain a high titer of recombinant adenovirus concentrate.

The ADV4-ORF3 and ADV4-NC adenovirus titers were determined in 293T cells. Briefly, 293T cells were added to a 96-well plate at 10^4 cells per well. The amount of medium added was 200 μ l. The plate was incubated in incubator for 24 h, and the adenovirus to be tested was diluted to 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} with complete medium. After aspirating off the supernatant, the adenovirus solutions at different dilutions were added at 200 μ l per well. Samples were repopulated, and culture medium without adenovirus was set as a control. The plates were incubated at 37 °C, 5% CO₂. The culture continued for 36–48 h under CO₂ conditions. The cytopathic effect (CPE) was observed using microscopy, and the virus titer was calculated according to a formula supplied by Baxter Healthcare Corporation (Round Lake, IL, USA).

Infection Assay of Recombinant Adenovirus ADV4-ORF3 and ADV4-NC

HepG2 cells were seeded into a 12-well plate (1×10^6 cells/well), infected with ADV4-ORF3 and ADV4-NC at 12 h postseeding at a multiplicity of infection (MOI) of 5:1. The cells were harvested after infection for 24 h. Then, 0.8 μ L of polybrene was added to each well to improve the transfection efficiency. Triplicate infection experiments were performed. QRT-PCR was used to confirm ORF3 overexpression. The sequences of the primers were as follows:

ORF3: forward primer: 5'-GCTCCTCCTGCTTTTGCCTA-3', reverse primer: 5'-GCTGAGAATCAACCCGGTCA-3',

GAPDH: forward primer: 5'-GCTCTCTGCTCCTCCTGTTC-3', reverse primer: 5'-CCAAATCCGTTGACTCCGAC-3'.

The sample of ADV4-ORF3 infected cells was named Ad_ORF3, and the ADV4-NC infected cells were named Ad_GFP.

RNA Sample Preparation and Transcriptome Sequencing

Total RNA was extracted and purified using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. The RNA amount and purity of Ad_GFP_1, Ad_GFP_2, Ad_GFP_3, Ad_ORF3_1, Ad_ORF3_2, and Ad_ORF3_3 samples were quantified using a NanoDrop ND-1000 instrument (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed using an Agilent 2100 with an RNA integrity number (RIN) > 7.0. Approximately 5 μ g of total RNA was used to deplete ribosomal RNA

according to the protocol of the Ribo-Zero™ rRNA Removal Kit (Illumina, San Diego, CA, USA). After removing ribosomal RNAs, the remaining RNAs were fragmented into small pieces using divalent cations under high temperature. Then, the cleaved RNA fragments were reverse-transcribed to create the cDNA using ProtoScript II Reverse Transcriptase and First Strand Synthesis Mix (Ambion, Austin, TX, USA), which were next used to synthesize U-labeled second-stranded DNAs with *E. coli* DNA polymerase I, RNase H, and dUTP. An A-base was then added to the blunt ends of each strand, preparing them for ligation to the indexed adapters. Each adapter contained a T-base overhang for ligating to the A-tailed fragmented DNA. Single- or dual-index adapters were ligated to the fragments, and size selection was performed using AMPureXP beads. After heat-labile UDG enzyme treatment of the U-labeled second-stranded DNAs, the ligated products are amplified by PCR using the following conditions: Initial denaturation at 95 °C for 3 min; eight cycles of denaturation at 98 °C for 15 sec, annealing at 60 °C for 15 sec, and extension at 72 °C for 30 sec; and then final extension at 72 °C for 5 min. The average insert size for the final cDNA library was 300 bp ($\pm$ 50 bp). Finally, we performed paired-end sequencing on an Illumina HiSeq 4000 sequencer (LC Bio, Hangzhou, China).

Preparation of Polyclonal Antibodies Against ORF3 and Western-blot

Genotype IV Swine Hepatitis E Virus ORF3 gene was synthesized by Biotechnology (Shanghai) Co., Ltd, we designed the prokaryotic expression prime, forward primer: 5'-CGCTGAATTCATGGCGATGCCACCATGCG-3', reverse primer: 5'-GCCTAAGCTTTCAGCGGCGAAGCCCCAG-3', and used pET-28a (+) for construction prokaryotic vector of pET28-ORF3. The recombinant plasmid was transformed into BL21 competent cells, which were induced by 0.1 mmol/L IPTG for 6 h at 37 °C, and the cell was broken by ultrasound to collect ORF3 antigen. After SDS-PAGE electrophoresis, the target protein was cut off and used to immunize male New Zealand white rabbits. 375 µg of ORF3 protein was used to inject rabbits for the first immunization, the same method was used for the second and third injection after another two 14 days. Ten days after the last immunization, blood was collected from the heart to isolate the antiserum, and the titer of antiserum was detected by indirect ELISA.

HepG2 cells were infected with the high titer recombinant adenovirus of ADV4-NC (control) and ADV4ORF3 for 24 h. The cells were collected and lysed with RIPA containing 1 µg/ml PMSF. 40 µg of total protein was analyzed by 15% SDS-PAGE and electrically transferred to PVDF membrane for 1.5 h, which blocked with 5% skimmed milk for 2 h, washed three times with TBST, and incubated with anti-ORF3 primary antibody (1:200 dilution) at 4 °C overnight and a monoclonal mouse anti-β-actin (1:2000 dilution; Santa Cruz Biotechnology, USA), the PVDF membrane washed three times with TBST, incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:5000 dilution; Abcam, USA) and mouse-IgGk BP-HRP (1:5000 dilution; Santa Cruz Biotechnology, USA) for 2 h, and washed three times with TBST. The PVDF membrane was developed with super signal™ west pico plus chemiluminescent substrate kit (Thermo Scientific, USA), and the ORF3 and β-actin proteins were detected by CCD cameras.

Differentially Expressed lncRNAs and mRNAs Analysis

Firstly, Cutadapt [15] was used to remove the reads that contained adaptor contamination, low quality bases, and undetermined bases. The sequence quality was then verified using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). We used Bowtie2 [16] and Hisat2 [17] to map reads to the human genome. The mapped reads of each sample were assembled using StringTie [18]. Then, all the transcriptomes from Ad_GFP_1, Ad_GFP_2, Ad_GFP_3, Ad_ORF3_1, Ad_ORF3_2, and Ad_ORF3_3 samples were merged to reconstruct a comprehensive transcriptome using perl scripts. After the final transcriptome was generated, StringTie and edgeR [19] were used to estimate the expression levels of all transcripts.

Transcripts that overlapped with known mRNAs and transcripts shorter than 200 bp were discarded. Then we utilized the coding potential calculator (CPC) [20] and coding-non-coding index (CNCI) [21] to predict transcripts with coding potential. All transcripts with a CPC score < -1 and CNCI score < 0 were removed. The remaining transcripts were considered as lncRNAs. Differential expression analysis of mRNAs and lncRNAs used StringTie to perform expression level analyses for mRNAs and lncRNAs by calculating the FPKM [22]. FPKM can represent the expression of lncRNAs. In the present study we identified five class codes defining lncRNAs: Class code j indicated potentially novel isoforms (fragments), in which at least one splice junction is shared with a reference transcript; class code i indicated a transfragment falling entirely within a reference intron; class code o indicated generic exonic overlap with a reference transcript; class code u indicated an unknown, intergenic transcript; and class code x indicated exonic overlap with a reference on the opposite strand. The differentially expressed mRNAs and lncRNAs were selected using $\log_2$ (fold change) > 1 or $\log_2$ (fold change) < -1 and with statistical significance (p value < 0.05) using the R package module edgeR [19].

Target gene prediction and functional analysis of lncRNAs

To explore the function of the lncRNAs, we predicted their *cis*-target genes. In this study, coding genes in the 100 kbp regions upstream and downstream of the lncRNA were selected using a python script. Then, we performed functional analysis of the lncRNA target genes using BLAST2GO [23]. Significance was expressed as a p value < 0.05 .

Quantitative Reverse Transcription PCR (qRT-PCR) Validation

The primers were designed using NCBI Primer online software. Approximately 1 μg of total RNA was used for reverse transcription using a PrimeScript™ RT reagent kit (Takara, Shiga, Japan), and validation by qRT-PCR was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara). GAPDH was used as an internal control. The primers are listed in Table 1. The relative expression levels of lncRNAs and mRNAs were determined using the $2^{-\Delta\Delta C_t}$ method. Three independent replicates were performed. Student's t-test was used for statistical analysis. $p < 0.05$ was considered statistically significant, indicated by “*”, and $p < 0.01$ was considered highly statistically significant, indicated by “**”.

Table 1
Primers for the selected significantly differentially expressed lncRNAs (p value ≤ 0.05 and q value ≤ 1) for qRT-PCR validation.

lncRNAs_name	Transcripts_name	Primer Sequence (5'-3')
PLCG1-AS19	ENST00000454626	F: CTGCCTCACAGGAGATCCAC
		R: CAGCCTTGGCCATCCTCATT
AL139099	ENST00000555043	F: CTTCTTTGTGCGCCATTGC
		R: AGCTGTAGCTGCGCTCCC
AC005062	ENST00000449573	F: TACTGGAAGATGGCGGTTCC
		R: GAGACAGAAAGCGGAGTCTCA
FP236383	ENST00000625598	F: AACGCCACTTGTCCCTCTAA
		R: ACACGGACAGGATTGACAGA
FP671120	ENST00000627981	F: AACGCCACTTGTCCCTCTAA
		R: ACACGGACAGGATTGACAGA
FP671120	ENST00000631211	F: AACGCCACTTGTCCCTCTAA
		R: ACACGGACAGGATTGACAGA
ZNF337-AS1	ENST00000428254	F: AAGCGTGGTGTCTTCCCTG
		R: CAGCATCCGCACAACAATGG
ZNF337-AS1	ENST00000455791	F: CCCGCGATCTGTCTCATTCC
		R: GGGCACAGGTAGGTGGTTAG
ZNF337-AS1	ENST00000420803	F: CAACTACCTCCCCTGTGCAA
		R: TTGGAGCCATCTTTCGAGGC
ZNF337-AS1	ENST00000421829	F: CTACCTCCCCTGTGCAAGTT
		R: GGCACAGGTAGGTGGTTAGC
ZNF337-AS1	ENST00000414393	F: CCCGCGATCTGTCTCATTCC
		R: TTGCACAGGGGAGGTAGTTG
ZNF337-AS1	ENST00000439498	F: TGGAATTCCCGCGATCTGTC
		R: GGGCACAGGTAGGTGGTTAG
AP005329	ENST00000581905	F: TCCTGTATGGTGCCTGGAGA
		R: AGGTGGACACCCTGTAGTTC

lncRNAs_name	Transcripts_name	Primer Sequence (5'-3')
AP000977	ENST00000501964	F: GTGCATGCAGGTGGCATTAG
		R: GGGACAGAGGGGGATATGGT
LINC02476	ENST00000431071	F:ACTTTCCCTGGCAAACAAAAACA
		R:GCTTCCTAGGTAGGACAGGGA
LINC02476	ENST00000426413	F:GGTGTCTGAGCTGTGAATGAGA
		R:ATGGGATGGGGCTGGGTTAT
RAP2C-AS1	ENST00000441399	F:ACTTAGCCGTGCCTGACAAA
		R:GCTCCAAAAGGCACCCTTG
RAP2C-AS1	ENST00000421483	F:CAAGGGTGCCTTTTTGGAGC
		R:AAGAGCTTGATGACTCCGGC
AC107959	ENST00000502083	F:TGCTCTGTCTGGCCAAATCC
		R:CCCATGTGAAGCATTGCCTG
AC107959	ENST00000523884	F:TCTTCACCACCACCATCAGC
		R:GCCGAGCTTTTGTGAGCATC
AC016526	ENST00000554225	F:CACACTGGCCTTAGGGTGAC
		R:ACGTCTTTGTGAAGTCGGCA

Results

Recombinant adenovirus-mediated overexpression of ORF3

HepG2 cells were infected with the high titer recombinant adenovirus of ADV4-NC (control) and ADV4ORF3 for 24 h. Enhanced green fluorescent protein (EGFP) expression was detected using fluorescence microscopy. The results showed that EGFP and the EGFP-ORF3 fusion protein were successfully expressed in HepG2 cells (Fig. 1a). Quantitative real-time reverse transcription PCR (qRT-PCR) was used to detect the relative expression of ORF3 RNA, with *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase) used as the internal control. The results demonstrated that the recombinant adenovirus successfully mediated the overexpression of ORF3 (Fig. 1b).

The indirect ELISA results showed that polyclonal antibody titer reached 1:12800. Western blot was performed on equal amounts of 40 µg protein extracted from Ad_GFP and Ad_ORF3. β-actin was used for an internal control. With rabbit polyclonal antibodies against ORF3, the western blot results indicated that

the specific band at the molecular weight about 11.7kD for cell extracts from Ad_ORF3 was found, but no ORF3 expressing in Ad_GFP was found (Fig. 1c).

Analysis of differentially expressed genes (DEGs) and transcripts

After the six samples (Ad_GFP_1, Ad_GFP_2, Ad_GFP_3, Ad_ORF3_1, Ad_ORF3_2, and Ad_ORF3_3) passed the quality control test, DNA sequencing was carried out. The raw data generated by sequencing was preprocessed using Cutadapt [16] to filter out the unqualified sequences and produce valid data (clean data). The mapped reads of each sample were assembled using Cufflinks [24]. Then, all transcriptomes from 6 samples were merged to reconstruct a comprehensive transcriptome using Cuffmerge. After the final transcriptome was generated, Cuffdiff was used to estimate the expression levels of all transcripts.

The distribution statistics of genes in each sample were represented by fragments per kilobase of exon model per million mapped reads (FPKM) box charts, and the expression levels of genes were interpreted from the analyses shown in Fig. 2a. The gene expression density distribution of log₁₀ (FPKM) could be used to compare the expression trend of different samples. In this study, the gene expression density distribution of each sample showed a normal distribution, and the gene expression trend of biological repeat samples were consistent (Fig. 2b). All the DEGs were analyzed using volcano plots [25], which provided the overall distribution of the DEGs (Fig. 2c). The results indicated that there were 62 significant DEGs, including 28 upregulated genes and 34 downregulated genes. We identified 6,564 significantly differentially expressed transcripts, including 3,097 upregulated transcripts and 3,467 downregulated transcripts (Fig. 2d and Fig. 2e). Cluster analysis of the DEGs provided a visual display of the gene expression in each sample. To better reflect the cluster expression pattern, we used log₁₀ (FPKM + 1) to display gene expression. At the same time, FPKM could be displayed by the Z value. The partial DEGs are shown by a heatmap, in which red indicates upregulated DEGs and dark blue indicates downregulated DEGs (Fig. 2f).

Analysis of differentially expressed lncRNAs

The software StringTie was used to assemble the reads. Known mRNAs and transcripts less than 200 bp were removed, and then lncRNAs were predicted among the remaining transcripts. The prediction software used comprised CPC and CNCI. FPKM was used to measure the expression levels of the lncRNAs. There were five class codes of lncRNAs, including i, j, o, u, and x (Fig. 3). The proportions of these five class codes of six samples (Ad_GFP_1, Ad_GFP_2, Ad_GFP_3, Ad_ORF3_1, Ad_ORF3_2, and Ad_ORF3_3) are shown in a pie chart (Fig. 3a).

To show the distribution of lncRNA candidates in the chromosomes more intuitively, we used the software of Circos (www.circos.ca) to map the lncRNAs to the genome. This analysis was mainly divided into two parts. First, genome mapping was carried out according to the different classifications of the lncRNAs. Second, genome mapping was carried out according to the lncRNAs in the different samples. During mapping, each chromosome was analyzed every 25 mb as the basic unit. When the lncRNA-

related genome in different samples was visualized as a map, the expression of the lncRNAs in each segment was counted. When the genome of different lncRNA types was visualized, the number of lncRNAs in each segment was counted (Fig. 3b).

We identified 319 significantly differentially expressed lncRNAs, including 124 known lncRNAs and 195 novel lncRNAs. Among them, 158 lncRNAs were significantly upregulated and 161 lncRNAs were significantly downregulated (Fig. 3c and Fig. 3d). In the follow-up study, we took the 124 known lncRNAs with significantly differential expression as the research target, and the top 100 known lncRNAs were shown using a heatmap, in which red represented upregulated lncRNAs and dark blue represented downregulated lncRNAs (Fig. 3e).

Comparative analysis of the structural characteristics of differentially expressed lncRNAs and mRNAs

It has been reported that the structural characteristics (length distribution, the number of exons, and ORF length) and expression levels of lncRNA and mRNA were very different [26]. Therefore, this analysis mainly focused on the structural characteristics and expression levels of lncRNAs and mRNAs. lncRNA and mRNA length statistics and comparisons revealed the percentages of different lengths of transcripts (Fig. 4a).

Although lncRNAs do not have an obvious ORF and cannot encode protein, we compared the lncRNAs and the ORF lengths of the mRNAs to discuss the differences between them. The principle of ORF analysis was based on the six frames translation principle of nucleic acids. The distributions of the ORF lengths of the lncRNAs and mRNAs are shown in Fig. 4b and Fig. 4c. Statistics of lncRNA and mRNA exons are shown in Fig. 4d, and the statistics of lncRNA and mRNA expression levels are shown in Fig. 4e.

Analysis of the interaction between differentially expressed lncRNAs and mRNAs

There are two kinds of regulation modes of lncRNA, one is *cis* regulation, i.e., the lncRNA regulates the expression of its neighboring genes. According to the prediction positional relationships, the *cis* regulatory target genes of lncRNAs were defined as a differentially expressed lncRNA and differentially expressed mRNA within 100 kbp in either direction in the chromosome [27, 28]. The second type of regulation is *trans* regulation, i.e., the expression of lncRNA cross chromosome regulatory genes, and is difficult to verify in later stages. The target genes of lncRNAs with significantly differential expression were analyzed for functional enrichment using gene ontology GO (<http://geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.kegg.jp/kegg>). The top 20 of GO enrichment terms are shown in Fig. 5a; the top 5 GO terms were UDP-glucuronate biosynthetic process, UDP-glucose 6-dehydrogenase activity, transdifferentiation, tetracycline transport, and tetracycline transmembrane transporter activity. The top 20 KEGG enrichment pathways are shown in Fig. 5b; the top 5 pathways were

systemic lupus erythematosus, staphylococcus aureus infection, signaling pathways regulating pluripotency of stem cells, the PPAR signaling pathway, and platinum drug resistance.

The significantly differentially expressed mRNAs, including 62 genes and 6,564 transcripts, were used to predict the target genes of the 124 known lncRNAs. In this study, only the upstream and downstream 100 kb mRNAs and known lncRNAs were predicted for *cis* regulation. Finally, the target genes prediction results identified 45 lncRNAs corresponding to candidate mRNAs (Table S1).

QRT-PCR validation of partial differentially expressed lncRNAs

We selected 12 significantly differentially expressed lncRNA (p value ≤ 0.05 and q value ≤ 1) for qRT-PCR validation, including six upregulated lncRNAs with $|\log_2 \text{fold-change}| (|\log_2 \text{FC}|) \geq 2$, and six downregulated lncRNAs with $|\log_2 \text{fold-change}| (|\log_2 \text{FC}|) \geq 1$. There were several transcripts for some lncRNAs. GAPDH was used as an internal control. The primers are listed as Table 1. These 12 lncRNAs corresponded to 21 transcripts. Among them, the upregulated lncRNAs were *PLCG1-AS1*, *AL139099*, *AC005062*, *FP236383*, *FP671120* (ENST00000627981, ENST00000631211) and *ZNF337-AS1* (ENST00000428254, ENST00000455791, ENST00000420803, ENST00000421829, ENST00000414393, ENST00000439498); the downregulated lncRNAs were *AP005329* (ENST00000581905, ENST00000578800), *AP000977*, *LINC02476* (ENST00000431071, ENST00000426413), *RAP2C-AS1* (ENST00000441399, ENST00000421483), *AC107959* (ENST00000502083, ENST00000523884), and *AC016526*. The qRT-PCR validation results showed that the expression patterns eight lncRNAs were consistent with the sequencing data, which indicated that there was a certain number of false-positives in the high-throughput sequencing data (Fig. 6 and Table S2).

Discussion

SHE is a new type of zoonotic infectious disease, which seriously threatens the development of animal husbandry and public health [29, 30]. The typical clinical symptoms of SHE are jaundice, and the liver of pigs show pathological changes characteristic of hepatitis. About one week after infection with SHEV, fecal detoxification begins to appear in pigs, which lasts until 55 days after infection. At 14–32 days after infection, symptoms of viremia appear. At 17–24 days after infection, anti-HEV IgM antibodies can be detected. At 3 months after infection, IgG is maintained at a high level. In infected animals, pathological sections show typical manifestations of acute hepatitis [31–33].

HEV ORF3 protein is a viral regulatory protein, which regulates many kinds of cell signaling pathways by targeting the regulatory subunits of p85 α that bind to tyrosine kinase Src, phosphatidylinositol 3 kinase, phospholipase C gamma, and adaptor protein GRB2. However, ORF3 mainly activates the mitogen-activated protein kinase (MAPK) pathway through Src homology 3 domains [34]. ORF3 encodes a phosphorylated protein with unclear function, which promotes the elimination of alpha (1)-microglobulin/bikunin precursor (AMBP) in hepatocytes, thus affecting their metabolism [35]. ORF3 is the main factor affecting the outflow of HEV from infected cells, and exists on the surface of the released

HEV particles, which might be regulated by lipid metabolism [36]. ORF3 is the key to infection *in vivo*, which might regulate the response of the host. The epidermal growth factor receptor (EGFR) pathway can regulate the signal transduction of endometrial growth factor, thus affecting cell survival. Signal transducer and activator of transcription 3 (STAT3) expression can also be regulated, leading to a significant reduction of the inflammatory response. These findings might provide strong support for the effect of ORF3 on virus replication [37–40]. Taken together, several functions of the ORF3 protein have already been reported. ORF3 interacts with signal transduction pathway proteins by targeting proline rich regions and Src homologous domains. Whether the EGFP-ORF3 fusion protein can still bind to these regions requires further study. LncRNAs are noncoding RNAs with a length of more than 200 nucleotides [41, 42]. LncRNAs play an important role in many life activities, such as dose compensation effect, epigenetic regulation, cell cycle regulation, and cell differentiation regulation. LncRNAs have become a hot topic in genetic research [43–47].

In this study, we used transcriptome sequencing to identify the significantly differentially expressed lncRNAs and mRNAs in HepG2 cells overexpressing SHEV ORF3. We identified 62 genes and 6,564 transcripts, including 195 novel lncRNAs and 124 known lncRNAs. Among them, we chose the 124 known lncRNAs as our study object, which would be easy to validate and functionally analyze in later research [48]. LncRNA regulation can be divided into two types: *cis* regulation [49, 50] and *trans* regulation [27, 51]. We focused on the prediction and analysis of *cis* regulated differentially expressed mRNAs and lncRNAs.

A previous report used HepG2 and EGFP-expressing HepG2 as controls, and in response to EGFP-ORF3, transcriptome sequencing showed that *CLDN6*, *YLPM1*, *APOC3*, *NLRP1*, *SCARA3*, *FGA*, *FGG*, *FGB*, and *FREM1* were upregulated, and *SLC2A3*, *DKK1*, *BPIFB2*, and *PTGR1* were downregulated [52]. We examined our raw data from the high-throughput sequencing, and examined the 13 previously reported genes one by one. The results showed that *CLDN6*, *YLPM1*, *APOC3*, *NLRP1*, *SCARA3*, *FGB*, *FREM1*, *SLC2A3*, *DKK1*, *BPIFB2*, and *PTGR1* were present in our raw data (Table S3); however, they did not show significantly differential expression. *FGA* and *FGG* was not present in our raw data. The possible reasons for these discrepancies were analyzed and summarized. In our study, the latest software, StringTie, was used to assemble and quantify the reads, and edgeR was used to determine difference statistics and perform visual mapping. The highlight of our project is that we analyze the data from the gene and transcript levels, respectively. EdgeR was used for differential analysis ($|\log_2 \text{fold change}| \geq 1$ (multiple of difference > 2 times), $p < 0.05$). At the same time, we provided further definitions or suggestions for the difference threshold according to the initial operation results. Another very important reason is that the expression systems are different. Xu et al. used lentivirus-mediated overexpression of ORF3 in HepG2 cells, screened positive clones using G418, and constructed stable cell lines. However, we used adenovirus directly to mediate high-level overexpression of ORF3 in HepG2 cells. The expression level of ORF3 might affect the differentially expressed genes and their fold change in the target cells. We used adenovirus to mediate the overexpression of ORF3 in HepG2 cells. Although ORF3 is fused with EGFP, whether the function of the EGFP-ORF3 fusion protein is identical to that of native ORF3 requires further experimental verification. As reported before, transcriptome sequencing using HepG2 cells and EGFP expressing-HepG2 cells as negative controls indicated that the expression of EGFP did not interfere with

the study of ORF3 protein function. The function of lncRNAs can be predicted by the function of their target gene; therefore, the top 20 pathways were predicted by KEGG analysis and comprised systemic lupus erythematosus, staphylococcus aureus infection, signaling pathways regulating pluripotency of stem cells, the PPAR signaling pathway, platinum drug resistance, pentose and glucuronate interconversions, pancreatic cancer, N-Glycan biosynthesis, homologous recombination, herpes simplex virus 1 infection, fat digestion and absorption, the Fanconi anemia pathway, complement and coagulation cascades, central carbon metabolism in cancer, cardiac muscle contraction, carbohydrate digestion and absorption, cAMP signaling pathway, bile secretion, base excision repair, and ascorbate and aldarate metabolism. Among them, we are very interested in the bile secretion pathway, because jaundice is the typical clinical symptom caused by SHEV, and jaundice can only be caused if bile cannot be excreted [52, 53–56]. Bilirubin is an orange-yellow bile pigment, which is the main metabolite of iron porphyrin compounds *in vivo* and is an important basis for judging jaundice in the clinic. In the smooth endoplasmic reticulum (ER), bilirubin and UDP-glucuronic acid are esterified under the action of glucuronosyl transferase to generate glucuronosyl bilirubin, which is then excluded from the liver together with bile under the action of Golgi bodies and lysosomes. However, bilirubin metabolism disorder leads to jaundice, and the typical clinical symptom of SHE is jaundice [57–59]. Therefore, SHEV-ORF3 might affect the pentose and glucuronate interconversions of the above top 20 pathways and mediate the occurrence of jaundice. The obstruction of the bile excretion channel leads to increased internal pressure in the bile duct and capillaries, which leads to the reverse flow of glucuronide bilirubin into the blood and the increase of serum bilirubin concentration, leading to obstructive jaundice [60–62]. Therefore, SHEV-ORF3 might also mediate the formation of obstructive jaundice by influencing the bile secretion of the above top 20 pathways. These two pathways will be studied in our follow-up experiments, and might represent a new direction to elucidate the pathogenesis of SHEV.

In the present study, we identified the lncRNA and mRNA differential expression profiles in HepG2 cells overexpressing SHEV ORF3. The structures of the lncRNAs were compared with those of the mRNAs, and the functional enrichment of target genes was analyzed using GO and KEGG, which revealed the possible functions of the lncRNAs. The qRT-PCR validation results demonstrated that the sequencing data was reliable. In the future, we will investigate the pathogenesis of SHEV by studying the significantly differentially expressed of lncRNAs and their regulatory target genes.

Conclusions

SHEV-ORF3 might affect the pentose and glucuronate interconversions and mediate the formation of obstructive jaundice by influencing the bile secretion. Our findings are of great significance in revealing the function of SHEV ORF3 and explaining the molecular mechanism of SHEV pathogenesis.

Abbreviations

SHEV: Swine hepatitis E virus; ORF3: open reading frame; lncRNA: long non-coding RNA; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; NC: negative control; FPKM: fragments per

kilobase of exon model per million mapped reads; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

HJ, QH, JC, LG and XL contributed to conception and design of the study. HJ, XS and YL contributed to data acquisition and data analysis. ZZ, YZ and WL contributed to data interpretation. HJ, BL and GG drafted the manuscript. HJ and LW revised the manuscript. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

The sequencing data of all experimental samples in the fastq format have been submitted to the Sequence Read Archive of NCBI under the accession number GSE147129.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

Adenovirus of ADV4-ORF3 and ADV4-NC mediated overexpression of ORF3 in HepG2 cells. a Fluorescence microscope observation of HepG2 cells infected by adenovirus of ADV4-ORF3 and ADV4-NC for 24 h. b qRT-PCR validation for the relative expression level of ORF3. c Western-blot analysis of HepG2 cells infected by adenovirus of ADV4-ORF3 and ADV4-NC for 24 h with rabbit polyclonal antibody against ORF3. β -actin was used for an internal control.

Figure 2

Analysis of differentially expressed genes (DEGs) and transcripts. a Distribution of gene expression values for each sample. The x-axis is the sample name and the y-axis is log10 (fragments per kilobase of exon model per million mapped reads (FPKM)). The box graph of each region corresponds to five statistics (the maximum, the upper quartile, the median, the lower quartile, and the minimum respectively from the top to the bottom). b Gene expression density distribution. The x-axis is the log10 (FPKM) and

the y-axis shows the gene expression density. c Volcano analysis of differential expression levels of genes; the x-axis is $\log_2$ (fold change), which represents the variation of the differential expression of multiple genes in different samples, and the Y-axis shows the $-\log_{10}$ (p value), which represents the statistical significance of the change in the gene expression levels; red indicates upregulated DEGs and dark blue indicates downregulated DEGs. d The statistics of the frequency of up and downregulation of genes with significantly differential expression. Red represents upregulated genes and dark blue represents downregulated genes. e The statistics of the frequency of up and downregulated transcripts with significant differential expression. Red represents upregulated transcripts and dark blue represents downregulated transcripts. f Heatmap of partial DEGs; red indicates upregulated DEGs and dark blue indicates downregulated DEGs.

Figure 3

Analysis of differentially expressed long noncoding RNAs (lncRNAs). a Pie chart of different class code proportions of the lncRNAs in each sample. There are five class code of lncRNA: J indicates a potentially novel isoform (fragment); i indicates a transfragment falling entirely within a reference intron; o indicates generic exonic overlap with a reference transcript; u indicates an unknown, intergenic transcript; and x indicates exonic overlap with the reference on the opposite strand. b Visualization results of lncRNAs

from different samples, showing the distribution of lncRNA candidates in the chromosome more intuitively. c Volcano analysis of differentially expressed lncRNA levels; the x-axis is log2 (fold change) and the y-axis was -log10 (p value). Red represents upregulated lncRNAs and dark blue represents down-regulated lncRNAs. d Statistics of the frequency of up and downregulated lncRNAs with significant differential expression. Red represents upregulated lncRNAs and dark blue represent down-regulated lncRNAs. e Heat map of differentially expressed lncRNAs; red represents up-regulated lncRNAs and dark blue represents downregulated lncRNAs.

Figure 4

Analysis of the structural characteristics of differentially expressed long noncoding RNAs (lncRNAs) and mRNAs. a lncRNA and mRNA length statistics and comparisons; the x-axis represents the transcript length, and the y-axis represents the proportion. b Distribution of the ORF lengths of lncRNAs. c Distribution of the ORF lengths of mRNAs. d Statistics of lncRNA and mRNA exons. The x-axis represents the exon number of the lncRNA and mRNA, and the y-axis represents the proportion. e lncRNA and mRNA expression levels were statistically analyzed from two levels of log10 (fragments per kilobase of exon model per million mapped reads (FPKM)) and number.

Figure 5

Analysis of the functional enrichment of target genes of the significantly differentially expressed long noncoding RNAs (lncRNAs). a Gene ontology (GO) functional enrichment analysis of the predicted target genes of the differentially expressed lncRNAs. y-axis: GO terms; x-axis: Rich factor. The color of each bubble represents the p value, and the bubble size represents the gene number. b Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the predicted target genes of the differentially expressed lncRNAs. y-axis: Pathway name; x-axis: Rich factor. The color of each bubble represents the p value, and the bubble size represents the gene number.

Figure 6

Quantitative real-time reverse transcription PCR (qRT-PCR) validation for the eight selected significantly differentially expressed long noncoding RNAs (lncRNAs). “*” represents a p-value < 0.05, “**” represents a p-value < 0.01.

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