Long non-coding RNA ENST00000469812 promotes *Enterovirus* type 71 replication via targeting the miR-4443/NUPR1 axis in rhabdomyosarcoma cells

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Abstract
Hand, foot, and mouth disease (HFMD) caused by *Enterovirus* type 71 (EV71) is a serious threat to children’s health. However, the pathogenic mechanism of EV71 is still unclear. Long non-coding RNAs (lncRNAs), some of which bind to miRNA as competitive endogenous RNAs (ceRNA) and weaken the silencing effect on the mRNA of downstream target genes, play a key role in regulating the viral infection process. In this study, through experimental verification, we found miR-4443 to be downregulated in cells infected with EV71. Next, by predicting lncRNAs that potentially regulate miR-4443, we found that EV71 infection induced upregulation of lncRNA ENST00000469812 and then further downregulated miR-4443 expression by direct interaction. We also demonstrated that nuclear protein 1 (NUPR1) is one of the target genes of miR-4443 and is involved in the ENST00000469812/miR-4443/NUPR1 regulatory axis. Finally, the ENST00000469812/miR-4443/NUPR1 regulatory axis exhibited a positive effect on EV71 replication. Here, we lay a foundation for exploring the pathogenic mechanism of EV71 and identify potential targets for HFMD treatment.

Keywords *Enterovirus* type 71 · Long non-coding RNA · microRNA · Nuclear protein 1 · Viral replication

Introduction
Hand, foot, and mouth disease (HFMD) is an acute viral infectious disease to which children under 5 years are susceptible. Since its discovery in California in 1969, there have been several outbreaks of HFMD worldwide [1]. In recent decades, HFMD has occurred mainly in China, Singapore [2], Malaysia [3], Thailand [4], Japan [5], South Korea [6], and other Western Pacific countries [7]. Between 2010 and 2018, the child mortality rate of HFMD in China was 1.79 per 10,000 patients [8]. Although the prevention and control of COVID-19 have reduced the incidence of HFMD in the spring and summer [9, 10], its incidence in the winter has continued to increase significantly [11]. Therefore, HFMD remains a serious threat to children’s health.

*Enterovirus* type 71 (EV71), a single-stranded positive-sense RNA virus, is the main pathogen causing severe HFMD with aseptic meningitis, encephalomyelitis, acute flaccid paralysis, or even death [12]. The pathogenesis of EV71 infection, however, has not been fully elucidated. For decades, non-coding RNAs (ncRNA) including microRNA (miRNA), long non-coding RNA (lncRNA), and small nucleolar RNA, have emerged as key regulators mediating the pathogenesis of virus infection [13]. As one class of non-coding RNAs longer than 200 nucleotides, some lncRNAs function as competitive endogenous RNAs (ceRNA) that bind to miRNAs, which silence downstream target mRNA genes, thus forming a lncRNA/miRNA/mRNA regulatory axis in viral infectious diseases [13, 14], such as those caused by influenza virus [15], HIV [16], hepatitis C virus [17], and SARS-CoV-2 [18]. A large number of lncRNAs and miRNAs have been found to be differentially expressed
in host cells during EV71 infection [19–24], suggesting that lncRNAs and miRNA may function together to regulate the pathogenetic process of EV71 infection. A previous study showed that lncRNA IRAK3-3 was significantly downregulated in EV71-infected rhabdomyosarcoma (RD) cells and upregulated miR-891b via the ceRNA mechanism, which itself ultimately downregulated DNA damage-inducing gene 45 β (GADD45β) and decreased apoptosis [25]. This suggests that the ceRNA mechanism mediates the EV71 infection process. However, the involvement of the ceRNA mechanism in the regulation of EV71 replication is poorly understood.

Here, we identified key lncRNAs, miRNAs, and mRNAs that regulate the pathogenesis of EV71 and examined the effects of EV71 infection on their expression. Then, the three RNA elements forming a ceRNA axis were identified, and their affect on EV71 replication was assessed. This study will provide a basis for elucidating the regulation mechanism of EV71 replication and lay a theoretical foundation for exploring therapeutic targets for HFMD.

Materials and methods

Bioinformatics analysis and prediction

In investigating the lncRNAs regulating miR-4443, we used the DIANA (http://www.microrna.gr/LncBase) online software to predict lncRNAs interacting with miR-4443. Then, we searched “EV71 lncRNA” in PubMed and selected the literature PMID23220233 with the most differentially expressed lncRNAs in RD cells after EV71 infection [20]. Where we found an intersection with the upregulated lncRNAs in PMID23220233 and in DIANA, we selected 30 candidate lncRNAs potentially regulating miR-4443. Finally, we selected lncRNAs with prediction scores over 0.8, and those associated with over 2.85-fold upregulation were selected as final candidate lncRNAs.

The target genes of miR-4443 were predicted using the online software TargetScan (http://www.targetscan.org), miRWalk (http://mirwalk.umm.uni-heidelberg.de), miRanda (http://miranda.org.uk/downloads), and DIANA (https://diana.e-ce.uth.gr/home).

miRNAs, siRNAs, and plasmids

TheyR-4443mimic(5’-UUGGAGGGGCAGGGUUUU-3’), the miR-4443 inhibitor (5’-AAAACCCACGCUC-CAA-3’), siNUPR1, siENST00000469812, the respective negative controls (NC), the ENST00000469812 overexpressing plasmid (pcDNA-ENST00000469812), the dual luciferase reporter plasmids for ENST00000469812 (pGL3-ENST00000469812), the ENST00000469812 mutant (pGL3-ENST00000469812mut), 3’ UTR NUPR1 (pGL3-NUPR1WT), and the 3’ UTR NUPR1 mutant (pGL3-NUPR1mut) were synthesized by Sangon Biotech (Shanghai, China). The pcDNA3.1, pGL3-basic, and pRL-TK-Renilla plasmids were stored in our lab beforehand. The NUPR1 overexpressing plasmids were constructed using a molecular cloning technique. Briefly, the NUPR1 gene (NM_012385) was amplified from cDNA of RD cells, using the following primers: forward, 5’-CGCGAGATCCATGGCCACCTTCCCACCAGCAAC-3’; reverse, 5’-TGCTCTAGATCGGCCTAGGGCCCTCCTCG-3’. Gene fragments were then cloned into pcDNA3.1 after digestion with BamHI and XbaI.

Cell culture and transfection

Human rhabdomyosarcoma (RD) cells and human embryonic kidney 293 (HEK293) cells were obtained from ATCC. They were all cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO₂. Transfection of cells with miRNAs, siRNAs, or plasmids was performed using Lipofectamine 2000 reagent (Invitrogen, CA) according to the manufacturer’s protocol.

Virus infection and TCID₅₀ assay

The EV71 virus strain (87-2008 Xi’an Shaanxi (GenBank accession number HM003207.1) [26] was kindly donated by Dr. Wei Ye from the Department of Microbiology and Pathogen Biology, Air Force Medical University. RD cells were seeded into 24-well plates at 1×10⁴ cells/well for virus infection. The next day, the cells were infected with EV71 in DMEM at the multiplicity of infection (MOI) indicated in the text or figure legend for 1 h for virus attachment. Then, the virus supernatants were removed and replaced with DMEM containing 2% FBS. For mock treatment, RD cells were treated with medium without virus. The RNA, whole-cell proteins, and supernatants were collected at the indicated time points. EV71 virus titers in supernatants of RD cells were determined by 50% tissue culture infectious dose (TCID₅₀) assay using the Reed-Muench endpoint calculation method in RD cells. At least three independent experiments were performed for each treatment.

RNA extraction and real-time quantitative PCR

Viral RNA, endogenous cellular lncRNA, mRNA, and miRNA were extracted from cells using TRIzol Reagent (Takara, Japan). Then, 1 µg of total mRNAs and miRNAs were reverse transcribed to cDNAs using a PrimerScript
RT Reagent Kit (Takara, Japan) and a Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan), respectively. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using FastStart SYBR Green Master Mix (Roche, Switzerland). The PCR reaction conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 58°C (for IncRNA, mRNA and viral RNA) or 60°C (for miRNA) for 20 s, and 72°C for 20 s. Primers used in these reaction systems are listed in Supplementary Tables S1, S2, and S3. U6 primers and miRNA universal reverse primer were provided in the Mir-X miRNA First-Strand Synthesis Kit. For absolute quantification of miRNA, we performed reverse transcription (abm, Canada) on mimics of miR-4443 of 20 pmol, and the products were serially diluted for qRT-PCR. The C\text{t} values and pmol values were plotted to form a standard curve, which was used for absolute quantification of miR-4443 in RD, HEK293, and A549 cells.

**Subcellular fractionation assay**

A Nuclear and Cytoplasmic Fraction Extraction Kit (Beyotime, China) was used for separating nuclear and cytosolic RNAs. Briefly, RD cells were washed using PBS and digested with trypsin. Then, RD cells were treated with buffer A containing RNase inhibitor (Beyotime, China) and vortexed. After freezing on ice, the suspended cells were treated with buffer B and vortexed, followed by centrifugation at 16,000 \times g for 15 min at 4°C. The supernatants were collected as cytoplasmic fractions. The pellets were lysed using nuclear fraction extraction reagent, followed by vortexing and centrifugation, and the supernatants were collected as nuclear fractions. The RNAs in the cytoplasmic and nuclear fractions were extracted using TRIzol Reagent as described above.

**Antibodies and Western blot**

A mouse monoclonal antibody against EV71 VP1 (~36 kDa, Abnova, Taiwan, China), a rabbit polyclonal antibody against NUPR1 (~10 kDa, Novus, USA), and a mouse monoclonal antibody against β-actin (~42 kDa, Abcam, UK) were used as primary antibodies. Goat anti-mouse and anti-rabbit IgG conjugated with horseradish peroxidase (Zhongshanjinqiao, China) were used as secondary antibodies.

RD cells were washed with PBS and lysed in RIPA buffer containing the protease inhibitor PMSF at 4°C for 20 min. The cell lysates were obtained by centrifugation at 12,000 rpm for 5 min, and the total protein concentration was measured using the BCA method. The total proteins in 10 μg of lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Switzerland). The membranes were blocked for 1 h with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). Next, the membranes were incubated with specific primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. The blots were developed using chemiluminescent substrates (ECL, Millipore, USA).

**Luciferase reporter assay**

The luciferase reporter plasmids for ENST00000469812 (pGL3-ENST00000469812), ENST00000469812 mutant (pGL3-ENST00000469812mut), 3'UTR of NUPR1 (pGL3-NUPR1WT), and 3'UTR of NUPR1 mutant (pGL3-NUPR1mut) were synthesized as described above. HEK293 cells or RD cells were seeded into 24-well plates at 1 × 10^5 cells per well and transiently cotransfected with plasmids and miRNA mimics, including pGL3-ENST00000469812, pGL3-ENST00000469812mut, pGL3-NUPR1WT, pGL3-NUPR1mut, pRL-TK-Renilla, miR-4443 mimics, miR mimics NC, miR-4443 inhibitor, miRNA inhibitor NC, and pcDNA-NUPR1. After 48 h, the cells were collected and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, USA).

**Statistical analysis**

Statistical analysis was performed using the software GraphPad Prism 9.0. Statistical differences between two groups were analyzed by t-test, and statistical differences among multiple groups were analyzed by one-way ANOVA. A p-value less than 0.05 was considered statistically significant. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001

**Results**

**EV71 infection induces downregulation of miR-4443 expression**

To find miRNAs related to the EV71 infection process, we searched PubMed and found that six miRNAs, including miR-451a, miR-3960, miR-4516, miR-320c, miR-4443, and miR-3665, were significantly differentially expressed in several studies (Supplementary Table S4) [27–33]. Thus, we regarded these miRNAs as potentially involved in the EV71 infection process. Furthermore, in order to determine which miRNAs were closely related to EV71 infection, we infected RD cells with EV71 at an MOI of 0.1 and 1.
To examine the effect of lncRNAs, we performed qRT-PCR on RD cells infected with EV71. The results showed that EV71 infection at an MOI of 2 for 12 h significantly increased the expression of all three lncRNAs (Fig. 2B). The expression of ENST00000469812 was significantly upregulated at 12 h and 24 h postinfection and showed the highest level of upregulation and the strongest MOI dependence (Fig. 2C). The expression of ENST00000451940 was upregulated at 12 h and 24 h after EV71 infection, but no correlation with infection time was observed (Fig. 2D). ENST00000418747 expression was negatively correlated with MOI (Fig. 2E).

Considering that EV71 induced upregulation of ENST00000469812 (Fig. 2C) and downregulation of miR-4443 (Fig. 1H) and that ENST00000469812 had the highest prediction score (Supplementary Table S6), we inferred that ENST00000469812 negatively regulates miR-4443 expression. The gene for lncRNA ENST00000469812, also known as AC121756.1, LINC02046-201, RP11-501O2.5, and NONHSAT092602.2, is located at position 148276682–148279681 on human chromosome 3, with a total length of 378 nt. This long intergenic lncRNA is distributed mainly in the cytoplasm. To confirm this, the cytoplasmic and nuclear components of RD cells were extracted, and the cellular distribution of ENST00000469812 was analyzed by qRT-PCR. Sequence alignment was used to identify potential binding sites between ENST00000469812 and miR-4443 (Fig. 2G). A luciferase reporter assay showed that miR-4443 reduced the luciferase activity of wild-type ENST00000469812 but...
Long non-coding RNA ENST00000469812 promotes Enterovirus type 71 replication via targeting the...

EV71 infection results in upregulation of lncRNA ENST00000469812, which regulates miR-4443 expression. (A) Flow diagram of prediction of lncRNAs potentially regulating miR-4443. (B) Expression levels determined by qRT-PCR of three candidate lncRNAs 12 h after infection of RD cells with EV71. (C to E) Expression levels of ENST00000469812 (C), ENST00000451940 (D), and ENST00000418747 (E) determined by qRT-PCR in RD cells 12 h and 24 h after EV71 infection. (F) Subcellular distribution analysis of ENST00000469812 by qRT-PCR. (G) Luciferase reporter assay of the interaction between ENST00000469812 and miR-4443 in HEK293 cells. (H) Regulatory effects of ENST00000469812 overexpression and silencing on miR-4443 expression in RD cells. *, p < 0.05; **, p < 0.01; ****, p < 0.0001; hpi, hours postinfection.

Fig. 2 EV71 infection results in upregulation of lncRNA ENST00000469812, which regulates miR-4443 expression. (A) Flow diagram of prediction of lncRNAs potentially regulating miR-4443. (B) Expression levels determined by qRT-PCR of three candidate lncRNAs 12 h after infection of RD cells with EV71. (C to E) Expression levels of ENST00000469812 (C), ENST00000451940 (D), and ENST00000418747 (E) determined by qRT-PCR in RD cells 12 h and 24 h after EV71 infection. (F) Subcellular distribution analysis of ENST00000469812 by qRT-PCR. (G) Luciferase reporter assay of the interaction between ENST00000469812 and miR-4443 in HEK293 cells. (H) Regulatory effects of ENST00000469812 overexpression and silencing on miR-4443 expression in RD cells. *, p < 0.05; **, p < 0.01; ****, p < 0.0001; hpi, hours postinfection.

ENST00000469812 regulates nuclear protein 1 (NUPR1) expression by targeting miR-4443

In order to identify the target genes of miR-4443, four online programs, including TargetScan, miRWalk, miRanda, and DIANA were used for prediction. Taking the intersection of the above four sets, we identified 19 target gene candidates (Fig. 3A). Furthermore, qRT-PCR of RD cells transfected with miR-4443 mimics or inhibitors (Supplementary Fig. S3) revealed that miR-4443 mimics and inhibitors induced downregulation and upregulation of NUPR1 transcription, respectively, while the other 18 genes did not exhibit the predicted effect (Fig. 3B). Western blot assay also confirmed that miR-4443 downregulated translational expression of NUPR1 in RD cells (Fig. 3C).

A luciferase reporter assay performed in HEK293 cells demonstrated that miR-4443 reduced luciferase activity of wild-type NUPR1-3’UTR, but not that of NUPR1-3’UTR with a “GCCUCCCAA” mutation at nt position 4743–4750 of NUPR1 mRNA (Fig. 3D). Likewise, the miR-4443 inhibitor
Y. Lu et al. explore whether EV71 influenced NUPR1 expression through ENST00000469812 and miR-4443. The results of qRT-PCR and Western blot assays confirmed that the transcriptional and translational levels of NUPR1 were significantly increased in RD cells 12 and 24 h after EV71 infection (Fig. 3E and F). Using a luciferase reporter assay in HEK293 cells, we confirmed that ENST00000469812 overexpression significantly weakened the inhibitory effect of miR-4443 on NUPR1 luciferase activity (Supplementary Fig. S4A), indicating that NUPR1 is one of the target genes of miR-4443. In addition, endogenous miR-4443 affected the NUPR1 luciferase activity significantly (Supplementary Fig. S4B), suggesting that low levels of miR-4443 are sufficient to reduce the NUPR1 luciferase activity.

Since EV71 infection induced regulation of miR-4443 expression by ENST00000469812, it was of interest to explore whether EV71 influenced NUPR1 expression through ENST00000469812 and miR-4443. The results of qRT-PCR and Western blot assays confirmed that the transcriptional and translational levels of NUPR1 were significantly increased in RD cells 12 and 24 h after EV71 infection (Fig. 3E and F). Using a luciferase reporter assay in HEK293 cells, we confirmed that ENST00000469812 overexpression significantly weakened the inhibitory effect of miR-4443 on NUPR1-3'UTR (Fig. 3G), suggesting an
Long non-coding RNA ENST00000469812 promotes Enterovirus type 71 replication via targeting the...
Discussion

LncRNA is an important nucleic acid element that regulates the pathogenic process of viruses [13], the cellular immune response, metabolic processes, and other functions. Li et al. [19] performed transcriptomic analysis to find 23 dysregulated lncRNAs in RD cells and 104 dysregulated lncRNAs in infant mice infected with EV71. Studies using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes cluster analysis of the lncRNA–mRNA coexpression network showed the differentially expressed lncRNAs to be closely associated with pathogenic process of EV71 infection. Yin et al. [20] found that over 4800 lncRNAs were differentially expressed in RD cells infected with EV71. Meng et al. [21] reported that, in peripheral blood mononuclear cells (PBMCs) of children with HFMD, there were 8541 differentially expressed lncRNAs, all of which were related to the host’s immune and inflammatory response. In this study, we investigated the role of lncRNA ENST00000469812 in the EV71 infection process. An earlier study showed that a homologous lncRNA, RP11-501O2.5, was highly expressed in endometrial cancer and might promote the occurrence and development of tumors [38]. However, the detailed function of ENST00000469812 has not yet been clarified. In this study, EV71 infection upregulated the expression of ENST00000469812, which further promoted EV71 replication, suggesting a role of ENST00000469812 in EV71 infection through an as yet undiscovered mechanism. Our data suggest that overexpression of an ENST00000469812 mutant might have an opposite effect from ENSTW00000469812, but the mechanisms still need to be explored.

miR-4443 is differentially expressed in a variety of tumor cells and is involved in the occurrence and development of cancers such as breast cancer [39–41], non-small-cell lung cancer [42], ovarian cancer [43], and hepatocellular carcinoma [44]. miR-4443 disables T cells and macrophages by inhibiting tumor necrosis factor receptor (TNFR)-related factor 4 (TRAF4) [45], suggesting that miR-4443 regulates the replication of EV71 by affecting immune responses. Two studies have indicated that miR-4443 is involved in regulation of EV71 infection. Xun et al. [33] first reported that miR-4443 was downregulated by EV71 infection and that 27 dysregulated miRNAs mediated changes in Wnt, MAPK, TGF-β, and mTOR signaling pathways after EV71 infection in RD cells. Zhu et al. [32] found that miR-4443 was significantly downregulated by EV71 and coxsackievirus A16 (CVA16) infection, which might activate the MAPK signaling pathway. EV71 infection has been shown to alter the expression of cytokines by activating the MAPK pathway, and this affects replication of EV71 [46, 47]. When the MAPK pathway was inhibited, cytokine release was inhibited, and the EV71 replication level was reduced [48, 49]. Thus, the MAPK pathway seems to mediate the pathogenesis of EV71 or the immune response of the host.

The role of miR-4443 is complex and depends on the downstream target genes. Through prediction and experimental verification, NUPR1 was found to be one of the target genes of miR-4443, as was already shown in a previous study [50], and this affects the proliferation, migration, and invasion of osteosarcoma cells [51]. p38 MAPK has been shown to induce the expression of NUPR1 in astrocytes and pancreatic cancer cells [52], and silencing of NUPR1 reduces the expression level of p38 MAPK [53], suggesting that NUPR1 interacts with the MAPK pathway. Our study showed that miR-4443 regulated the expression of NUPR1, thereby possibly affecting the MAPK pathway and the replication of EV71.

NUPR1, a small protein related to the stress response, is upregulated and associated with endoplasmic reticulum and oxidative stress in cells after external stimulation [54]. There is considerable evidence that stress-induced upregulation of NUPR1 maintains and enhances cell metabolism [55]. Upregulation of NUPR1 promotes autophagy, which ultimately promotes tumor cell migration and invasion [56] and also has a protective effect on cells and tissues [57]. However, other studies showed that NUPR1 had the opposite effect on autophagy [58, 59], suggesting that NUPR1 might perform different functions under different conditions. EV71 infection causes a stress response in susceptible cells. For example, EV71 infection induces endoplasmic reticulum stress, which further promotes EV71 replication [60]. The VP1 protein of EV71 has been shown to induce endoplasmic reticulum stress in cells, increase the expression of peripheral myelin protein 22 (PMP22), and promote cellular autophagy [61], which is a key factor enhancing EV71 replication [61, 62]. Thus, it was inferred that EV71 induces the activation of the ENST00000469812/miR-4443/NUPR1 axis, which further causes cellular stress and promotes other activities such as autophagy and facilitates EV71 replication.

According to the information on the Expression Atlas website (https://www.ebi.ac.uk/gxa/home), miR-4443 is mainly expressed at low levels in the testes and choroid plexus. Information in miRBase (https://www.ebi.ac.uk/) and LNCediting (http://bioinfo.life.hust.edu.cn/LNCediting/mirna/) also indicates that miR-4443 is expressed at medium or low levels in cell lines and tissues. When we performed absolute quantification of miRNA in cell lines, we also found that the expression levels of miR-4443 were low. However, we still found that miR-4443 affected the replication of EV71, which indicates that endogenous miR-4443 levels are sufficient to affect the ENST00000469812/miR-4443/NUPR1 regulatory axis.
In summary, our study showed that EV71 infection results in upregulation of lncRNA ENST00000469812, which binds to and downregulates miR-4443 and upregulates the target gene NUPR1. The ENST00000469812/miR-4443/NUPR1 regulatory axis further promotes EV71 replication. This study provides a theoretical basis for the elucidation of the pathogenic mechanism of EV71 and lays a foundation for the treatment of HFMD.

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Author contributions YL performed the experiments and analyzed the data. ML and CL helped to prepare materials for experiments. YL, HZ, and ZG wrote the manuscript. HZ, YL, and ZG conceived and designed the research. HZ and KD supervised this work. All of the authors have read the manuscript and agreed with the data presented.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals. All procedures were done in accordance with ethical standards.

Informed consent This article does not contain any informed consent because there are no human tests.

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