ATP1B3: a virus-induced host factor against EV71 replication by up-regulating the production of type-I interferons

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Enterovirus 71 (EV71) infection can cause severe diseases, and is becoming increasingly common in children. In the current study, we carried out yeast two-hybrid assays to screen human proteins that could interact with 3A protein of EV71. Human β3 subunit of Na+/K+-ATPase (ATP1B3) protein was demonstrated to interact with the 3A protein of EV71. Although 3A protein had no effect on the expression of ATP1B3, EV71 infection resulted in elevated expression of ATP1B3 in RD cell line, both on messenger RNA (mRNA) and protein levels. Interestingly, knockdown of ATP1B3 could significantly increase the replication of EV71, whereas overexpression of ATP1B3 significantly suppressed the replication of EV71 in RD cells. Furthermore, we demonstrated that the expression of ATP1B3 could induce the production of type-I interferons. Our study demonstrated that ATP1B3 inhibit EV71 replication by enhancing the production of type-I interferons, which could act as a potential therapeutic target in EV71 infection.

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1. Introduction

Enterovirus 71 (EV71), a common pathogenic agent of hand-foot-and-mouth disease (HFMD), can cause severe complications including herpangina, aseptic meningitis, encephalitis, cardiopulmonary failure, polyomyelitis-like syndrome or even fatal disease (Chan et al., 2000; McMinn et al., 2001). EV71 predominantly affects children under 5 years old, and causes severe diseases with high morbidity and mortality (Sato et al., 2006). Since it was first isolated in 1969, EV71 outbreaks have occurred frequently in western Pacific region countries, including China (Lee et al., 2010; Samuda et al., 1987), Japan (Fujimoto et al., 2002), Malaysia (Chan et al., 2000), and Singapore (Singh et al., 2002). The World Health Organization has estimated the occurrence of one million new HFMD cases in China alone between 2011 and 2014 (World Health Organization, 2015). To date, no effective vaccines and antiviral drugs have been available to prevent or treat EV71 infection (Wu et al., 2010; Vee and Poh, 2015). Therefore, it is necessary to identify the host factors involved in the replication of EV71 to control the development and complications of HFMD.

EV71 is a non-enveloped virus with a single-stranded, positive-sense genomic RNA of approximately 7.4 kb nucleotides. Protein 3A, a nonstructural viral protein of 87 amino acids in length, is required for enterovirus RNA replication (Teoule et al., 2013). 3A proteins play important role in enterovirus replication through the formation (with the 3CD protein) of replication organelles via remodeling of the internal cell membrane. Analyses of enterovirus 3A proteins have led to the identification of several cellular partners of this protein. The 3A proteins of PV and of the related coxsackievirus B3 (CV-B3) can interact with the cellular Golgi brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1). By interacting with GBF1, 3A inhibits the cellular secretory pathway. The membrane-anchored 3A protein modulates GBF1/Arf1 activity, resulting in the preferential recruitment of PI4KI1β to sites of viral RNA replication. PI4KI1β recruitment leads to the enrichment of virus-induced membranous organelles in PI4P, which has been shown to facilitate viral RNA replication. (Belov et al., 2005; Belov and van Kuppeveld, 2012; Dorobantu et al., 2015; Greninger, 2015; Hsu et al., 2010; van der Schaar et al., 2013). However, the function of 3A in the RNA replication of EV71 has been rarely reported. So, it is necessary to detect the mechanism of protein 3A and further explore the host factors involved in EV71 infection. Thus, to identify the host factors that could interact with 3A protein of EV71 may be a potential target for the therapy of HFMD.

The Na+/K+-ATPase is a transporter for Na+ and K+ ions across the plasma membranes and widely distributed in prokaryotic and eukaryotic cells. The 43-kDa ATP1B3, a β-subunit of...
ATPase, is localized to the q22–q23 region of Chromosome (Chr) 3 (Besirli et al., 1998; Malik et al., 1998). Previous studies have demonstrated that ATP1B3 can up-regulate lymphocyte activity and promote the production of IFN-γ, IL-2, IL-4, and IL-10 (Chiampanichayakul et al., 2002) (Chruwkmalows et al., 2015). However, whether ATP1B3 can mediate the innate immune responses during EV71 infection is largely unknown. Moreover, Type I interferons (IFNs), namely IFNs-α/β, which lead to the induction of antiviral pathways within hours can be induced in virus-infected cells (Wu and Chen, 2014). Type I IFNs have clinically been used for treatment of many viruses and tumors. Thus, identifying the host factors that can induce the production of type I IFNs is extremely important in controlling viral infection.

In this study we screened human ATP1B3 protein that could interact with EV71 3A protein using yeast two-hybrid system. We evaluated the effect of ATP1B3 on the inhibition of EV71 replication. Furthermore, we found that ATP1B3 can induce the production of type-I IFNs during EV71 infection. Overall, our work demonstrated that ATP1B3 might act as a potential therapeutic target in EV71 infection.

2. Materials and methods

2.1. Yeast two-hybrid screening

Full-length sequence of EV71 3A was inserted into the pGBK7 vector for expression as a fusion protein with the Gal4 DNA-binding domain (Gal4-BD). This plasmid was used to transform the AH109 yeast strain (Clontech, Palo Alto, CA), and Gal4-BD fused 3A was used as bait in a mating strategy for the screening of human homogenization cDNA library. The homogenization cDNA library was inserted into the pGADT7 vector (Clontech, Palo Alto, CA) for expression as fusions with the Gal4 activation domain (Gal4-AD) and was maintained in the Y187 strain of yeast (Clontech, Palo Alto, CA). Transformed AH109 and Y187 yeast cells were mixed together for mating. Positive clones were selected on synthetic dropout medium lacking 4 nutrients (Leu/Trp/Ade/His). The blue color was maintained in the Y187 strain of yeast (Clontech, Palo Alto, CA). Transformed AH109 yeast strain (Clontech, Palo Alto, CA), and Gal4-BD fused 3A was introduced into Escherichia coli DH5α and sequenced. The sequences were analyzed with the BLAST program in NCBI.

2.2. Confocal microscopy experiments

Confocal microscopy experiments were described previously (Choe and Kirkegaard, 2004). In brief, the transfected cells were fixed with 3% paraformaldehyde (PFA), washed with PBS and permeabilized with 0.2% Triton X-100/PBS. Anti-flag and Anti-HA antibodies were used as the primary antibodies (1:1000 dilution), Alexa Fluor 488-conjugated Donkey anti-mouse IgG and Alexa Fluor 647-conjugated Donkey anti-rabbit IgG were used as secondary antibodies (1:500 dilution; Molecular Probes, abcam). DAPI dyes (Beyotime Institute of technology, China) was used for cell nucleus stains. The cells were examined and images were captured using 100x objectives with a confocal microscope (Leica SP8). The images were refined and figures were generated using Adobe Photoshop software (Adobe Systems, San Jose, CA).

2.3. Virus, cell lines, and construction of plasmids

The subgenotype C4 strain of EV71 virus (accession no: JX986738) was isolated from a throat swab with severe clinical symptoms at the Wuhan Medical Treatment Center (also Wuhan Infectious Diseases Hospital) (Liu et al., 2014). To prepare virus stocks, viruses were propagated on 90% confluent monolayer cells in DMEM with 2% FBS as described previously (Yi et al., 2011). HEK 293T and RD cells were grown in Dulbecco’s minimal essential medium (DMEM; Lonza) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37 °C in a humidified atmosphere and 5% CO2. pCMV-flag-2B, pCMV-HA and pCMV-C1 vectors were obtained from Clontech (USA).

2.4. Knockdown of ATP1B3

The ATP1B3 small interfering RNA (siRNA) used in this study was designed at website http://sidirect2.rnai.jp/. The ATP1B3 siRNA oligonucleotide hairpins were generated with the DNA oligomers 5′-UUUUGGAACCUCAUGGUG-C3′ and 5′-CAACGAUGGCUUCAAAAUA-3′. A nonspecific siRNA 5′-UUCUCGAGCAUGUGACUCC-3′ and 5′-ACUGAGACCUUCGGAAGATT-3′ was used as negative control. All siRNAs were introduced into RD cells by the standard lipofection method using TurboFect Transfection Reagent (Thermo Scientific) following the manufacturer’s protocol. All siRNAs were purchased from gema.

2.5. Real-time PCR

Total RNA was extracted from RD cells and the supernatants by using Trizol reagent (TAKARA). Total RNA was reverse transcribed into cDNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (promega) to detect transcript levels. SYBR green PCR master mix was used to conduct real-time qPCR to quantify the expression levels of each target gene. The real-time PCR was performed using a Lightcycler® 480 instrument (Roche, Switzerland). The PCR was set up under the following thermal cycling conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression levels were calculated using the comparative method for relative quantification after normalization to GAPDH gene expression. Primers used in RT-PCR were listed in Table 1 (Liu et al., 2012; Liu et al., 2014).

2.6. Western blot analysis and co-immunoprecipitation

Western blot and co-immunoprecipitation analyses were performed as previously described (Li et al., 2013). Briefly, after cell treatment, cells were lysed using lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl, 0.5% (vol/vol) Nonidet-P40, 1 mM EDTA, 30 mM NaF, 10% proteinase inhibitor mixture). Lysates were mixed and precipitated with antibodies or IgG and protein G-agarose beads (Roche, Basel, Switzerland) overnight at 4 °C. Beads were washed five times with lysis buffer containing 0.5 M NaCl before the bound proteins were eluted by boiling for 10 min in SDS sample lysis buffer. Protein samples were separated on polyacrylamide gels and transferred electrophoretically to polyvinylidene fluoride membranes (Millipore, Billerica, USA). Immunoblots were visualized with an Enhance Chemiluminescent Detection Kit (Pierce, Rockford, USA) by Amersham Imager 600 instrument (GE Healthcare, Fairfield, USA).

2.7. Measurement of EV71 growth in ATP1B3 overexpression RD cells after block of IFN α/β

After overexpression AT1B3, RD cells were infected with EV71 at an MOI of 1. To neutralize interferon’s activity, 2 μg of anti-human IFN-α1 (ab11408; Abcam) and anti-human IFN-β (ab6979; Abcam) were added to cell culture 3 h before EV71 infection. Twenty-four hours after EV71 infection, the viral contents were quantitated by RT-PCR using EV71 specific primers.
3. Results

3.1. Human ATP1B3 interacts with EV71 3A protein in 293T cells

Yeast two-hybrid system found several positive clones containing the coding sequence of human ATP1B3 (GenBank: BC011835.2), showing 100% nucleotide sequence identity. Data analysis in NCBI indicated that the 3A protein of EV71 could possibly interact with the N-terminal of the ATP1B3 protein. To further confirm the interaction between ATP1B3 and 3A protein, we performed immunofluorescent colocalization analysis by laser-scanning confocal microscopy. Results of confocal microscopic analysis of the subcellular localization revealed that the 3A fusion protein showed the same location with the ATP1B3 fusion protein on the membrane of 293T cells (Fig. 1A). To further confirm the interaction between EV71 3A and ATP1B3, we first proformmed exogenous Co-IP test. The RD cell line was transiently transfected with a Flag-tagged 3A expression vector and HA-tagged ATP1B3 expression vector. Following anti-Flag or anti-HA immunoprecipitation, the interaction between Flag-3A and HA-ATP1B3 was assessed by immunoblotting the precipitates for the presence of ATP1B3 or 3A. ATP1B3 or 3A protein was detected in the coimmunoprecipitation reaction from cell lysates that expressed both 3A and ATP1B3. To validate the interaction between the endogenous ATP1B3 and 3A in the context of EV71 infection, we performed immunoprecipitation experiment in RD cells infected with EV71 or mock infection using anti-3A or anti-ATP1B3.

3.2. The relationship between 3A protein levels and endogenous ATP1B3 expression in RD cells

In order to better understand the function of ATP1B3 in EV71 infection, we detected the expression levels of ATP1B3 after transfection plasmid DNA encoding enhanced green fluorescent protein (EGFP) with and without 3A into RD cells (EV71-susceptible cells). The fluorescence of EGFP was used to detect the transfection efficiency and expression levels of 3A protein (Fig. 2A). We observed that the mRNA expression levels of ATP1B3 had no significant difference after the transfection of various doses of plasmid EGFP-3A (Fig. 2B). The results showed that EV71 3A protein in RD cells cannot promote the expression of endogenous ATP1B3.

3.3. The dynamic expression of ATP1B3 in RD cells during EV71 infection

Next we assessed the expression of ATP1B3 during EV71 infection. The RD cells were infected with EV71 at a MOI of 1 for 0, 6, 12, 24, 48 and 72 h respectively. During the infection of EV71, we found that the mRNA expression levels of ATP1B3 were elevated continuously in RD cells and had a significant difference compared with that in the uninfected cells. Consistent with the results of mRNA levels, western blot revealed that protein levels of ATP1B3 were also significantly elevated after EV71 infection (Fig. 3A). On the other hand, we infected the RD cells at different MOIs of EV71 for 72 h and detected ATP1B3 expression. We observed that both the mRNA and protein levels of ATP1B3 in RD cells were positively correlated with the infection doses of EV71 (Fig. 3B). Thus, ATP1B3 expression levels were elevated along with the increasing of infection time and doses of EV71.

3.4. ATP1B3 inhibits the replication of EV71 in RD cells

Since EV71 infection could induce the production of ATP1B3, we investigated whether ATP1B3 affected the replication cycle of EV71. ATP1B3 siRNA and its control siRNA were transfected into RD cells to knockdown ATP1B3 expression and negative control, respectively (Fig. 4A). The presence of EV71 in the cell cultures was determined by quantitative real-time PCR using EV71 specific primers. The expression of EV71 3C protein was analyzed by western blot to represent the replication of EV71 in RD cells. Our results showed that knockdown ATP1B3 could increase both the mRNA and 3C protein levels of EV71 (Fig. 4B). Furthermore, pCMV-ATP1B3 vector was transfected into RD cells and induced about a three-fold rise of ATP1B3 expression compared with the negative control.
control group (Fig. 4C). We observed that overexpression of ATP1B3 could inhibit the expression of both the mRNA and 3C protein levels of EV71 (Fig. 4D). Our results showed that ATP1B3 could inhibit the replication of EV71.

### 3.5. EV71 replication is associated with type I IFNs

The type I IFNs act as the key effectors in the innate immune response against RNA virus infection (Kuo et al., 2013; Randall and Goodbourn, 2008). To explore the mechanism of the ATP1B3-mediated anti-EV71 response, we examined the relationship between ATP1B3 expression levels and Type I IFNs production. The levels of IFNs-α/β mRNA in RD cells were detected by quantitative real-time PCR. The intracellular levels of IFNs-α/β mRNA in RD cells were significantly decreased after knockdown of ATP1B3 expression using ATP1B3 siRNA (Fig. 5A). On the contrary, IFNs-α/β mRNA levels were significantly increased when ATP1B3 was overexpressed compared with control vector (Fig. 5B). This showed that ATP1B3 promoted the production of type I IFNs. Since EV71 replication was reduced in ATP1B3 overexpression cells, it would be intriguing to determine if such reduction is associated with the elevated type I interferon expression. We infected ATP1B3 overexpression cells or vector controls with EV71 at an MOI of 1, cultured cells with anti-human interferon-α/β antibodies or isotype control IgG, and examined the viral replication using RT-PCR assay. We found that ATP1B3 overexpression-induced reduction of viral growth was increased by treatment with antiviral-α/β antibodies but not by its isotype control IgG (Fig. 5C), indicating that neutralization of type I interferon in the cell culture by anti-interferon-α/β antibodies restored EV71 growth in ATP1B3 overexpression cells.

### 3.6. Discussion and conclusions

In recent years, EV71 epidemics have occurred in various provinces in China and caused an increasing proportion of severe complications and deaths in children. According to Chinese center for disease control and prevention, from January 2015 to December 2015, 2.01 million cases of HFMD had been reported in mainland China, of which 124 were dead (National Health and Family Planning Commision of People’s Republic of China, 2016). More than 80% of the pathogens isolated from patients died from HFMD were identified as EV71. Lots of research on developing antiviral drugs and vaccines were largely unsuccessful. Intravenous immunoglobulin, ribavirin and pleconaril have been demonstrated the antiviral activity in vitro and in vivo, but these drugs cannot inhibit the cytopathic effect induced by EV71 (Yip et al., 2013). Previous studies have shown that 3A protein of
enterovirus plays a critical role in viral RNA replication and is a potential target for antiviral therapy (Deng et al., 2014; Gao et al., 2015; Sadeghipour et al., 2012). Itraconazole and enviroxime can target viral protein 3A and/or 3AB and suppress replication of EV71 in vitro (Arita et al., 2010; Gao et al., 2015). So we conducted a yeast two-hybrid screening procedure to screen human proteins that could interact with EV71 3A protein. Moreover, we confirmed human protein ATP1B3 is the target of EV71 3A protein. Thus it is important to explore the function of human ATP1B3 during EV71 infection.

Previous studies have shown that some subunits of Na⁺/K⁺-ATPase can interact with various viral proteins and affect the replication of viruses. It has been demonstrated that ATP1B1 subunit interacts with HCMV UL136 protein, M2 proteins of influenza A and B viruses and inhibit the virus replication (Cui et al., 2011; Mi et al., 2010). ATP1A1 has also been reported to inhibit entry of coronavirus into host cells by regulating the Src signaling pathway (Burkard et al., 2015). ATP1B3 is expressed separately from the α subunit, and plays a role in regulation of the immune response (Chreuwkaamlow et al., 2015). A recent published study suggested that ATP1B3 can modulate the restriction of HIV-1 production and NF-κB activation in a BST-2 dependent manner (Nishitsui et al., 2015). All of these researches suggest that ATPase subunits play an important role in innate immunity against virus infection. However, the function of ATP1B3 in EV71 infection has been largely unknown. In this manuscript, we found EV71 infection can induce increases in ATP1B3 levels. Further studies demonstrated that ATP1B3 can inhibit EV71 replication along with the induction of IFN-α/IFN-β. Therefore ATP1B3 play an important role in antiviral immune response. Type I IFNs represent an essential innate defense mechanism for

![Fig. 4.](image-url)

**Fig. 4.** The relationship between ATP1B3 expression and the replication of EV71. (A) ATP1B3 in mRNA and protein levels were shown after knockdown ATP1B3. (B) Total EV71 RNA and protein levels after knockdown ATP1B3 were determined by RT-PCR and western blot with EV71 specific primers and anti-3C antibody. (C) ATP1B3 in mRNA and protein levels were shown after overexpress ATP1B3. (D) Total EV71 RNA and protein levels after overexpress ATP1B3 were determined by RT-PCR and western blot with EV71 specific primers and anti-3C antibody. The bar graphs showed as the means ± SD of at least three independent experiments. (*P < 0.05, **P < 0.01).
controlling EV71 infection both in vitro and in vivo (Hung et al., 2011; Liu et al., 2005; Liu et al., 2012). Previous studies have shown that EV71 infection can induce IFN-β production which is dependent on the infectious dose, but the interferon is not cell type specific (Lu et al., 2012). It has also demonstrated that EV71 infection promoted the expression of son of sevenless and increased the secretion of IL-1α, IL-2, IL-6, IL-12, TNF-α, IFN-β and IFN-γ (Shi et al., 2014). In our current study, we found that the levels of type I IFNs (IFN-α/IFN-β) were negatively associated with EV71 replication (Samuel, 2001). In our manuscript we found overexpression ATP1B3 inhibited EV71 growth, and this inhibition could be effectively abolished by type I IFNs antibodies in the cell culture. Our data suggests that ATP1B3 inhibits the replication of EV71 by inducing the production of type I IFNs.

To our knowledge, this is the first time to illustrate the function of ATP1B3 in innate immunity and the life cycle of EV71 at molecular level. ATP1B3 can act as a potential new therapeutic target for EV71 infection and the related disorders. This finding will broaden our understanding of host defense mechanism and the pathogenesis of EV71 infection.

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