# VGLL4 Promotes EV71 Virus Proliferation and Accelerates the Apoptosis of Infected RD Cells

**Shengyu Wang** ( sywang2012@126.com )
Zunyi Medical University https://orcid.org/0000-0002-9774-8319

**Xin Sun**
Department of Microbiology, College of Basic Medicine, Zunyi Medical University

**Junhua Qiao**
Department of Microbiology, College of Basic Medicine, Zunyi Medical University

**Cancan He**
Fifth Affiliated Hospital of Zunyi Medical University

**Langfei Tian**
Key Laboratory of Infectious Disease & Biosafety, Provincial Department of Education, Guizhou, Institute of Life Sciences, College of Basic Medicine, Zunyi Medical University

**Yaping Chen**
Department of Microbiology, College of Basic Medicine, Zunyi Medical University

**Research Article**

**Keywords:** EV71, VGLL4, Virus replication, Apoptosis

**DOI:** https://doi.org/10.21203/rs.3.rs-272535/v1

# Abstract

Enterovirus 71 (EV71) is one of the major pathogens causing hand, foot and mouth disease (HFMD) which affects public health increasingly. Apoptosis plays an important role in EV71 infection, but the molecular mechanism involved in EV71 induced apoptosis is not completely clear. VGLL4 is a multifunctional protein in host cells, which has been studied in tumor and cell apoptosis, but has not been reported in pathogen. In this study, the mammalian eukaryotic expression plasmid of VGLL4 fused with HA tag (HA-VGLL4) and the model of overexpression VGLL4 RD cells were successfully constructed. The effect of VGLL4 on the proliferation of EV71 was detected by western-blot assay, fluorescence quantitative PCR and cytotoxicity assay (CCK assay), and the mechanism of its effect on the proliferation of the virus was researched. The experimental results showed that VGLL4 may promote the replication of EV71 by promoting the apoptosis of infected cells. VGLL4 can be an important target for prevention and treatment of EV71 infection.


# Introduction

Hand foot and mouth disease (HFMD) causes serious health problems in the Asia Pacific region, including China. The epidemiological survey results showed that 1,500 out of 3 million children die of severe illness[10, 21]. EV71, a new enterovirus, is one of the main pathogens causing HFMD[18]. The symptoms of EV71 infection are mostly self-limited which are usually characterized by fever, maculopapules and herpes in the mouth and extremities; but a few show neurological symptoms and even death in infants and young children[23]. Therefore, the study of EV71 is of great significance.

EV71 virus belongs to the family of small RNA viruses, enteroviruses and is a single plus-stranded RNA virus. The life cycle of the virus mainly includes virus infection, replication and translation of the viral genome and assembly and release of the progeny virus. Studies have shown that EV71 can interact with host proteins such as annexinII, PSGL1, SCARB2 and nucleolin to initiate their infection process [2, 11, 15, 22]. The replication, transcription and translation of EV71 virus also require the participation of multiple host proteins, such as hnRNP K and APOBEC3G, which affect the virus replication by interacting with the virus 5'UTR[9, 17]. Reticulon3 is involved in endoplasmic reticulum transcription and translation of virus[6]; SIRT1 can affect the replication, transcription and translation of viruses[1]. Therefore, the interaction between EV71 and the host has been a hot topic in research.

EV71 is nonenveloped virus, traditional view is that the virus is released by cytolytic manner which inevitably leads to cell death. And the apoptosis stage induced by virus is very important for virus proliferation. Studies have reported that EV71 can promote cell apoptosis by inducing ROS production, and the virus can also induce cell apoptosis through the interaction between their own proteins and apoptosis proteins[8, 19]. Despite extensive research, the mechanism between viral proliferation and apoptosis remains unclear.

Vestigial-like Family Member 4 (VGLL4) is an important transcription cofactor. Relevant studies have shown that it can be a tumor suppressor to influence the occurrence and development of tumors by promoting apoptosis of tumor cells[4, 14, 20]. In normal cells, it can also promote apoptosis as an inhibitor of apoptosis protein (IAP)-interacting protein[3, 16]. However, the relationship between VGLL4 and viruses has not been studied. In this study, the mammalian eukaryotic expression plasmid of VGLL4 fused with HA tag (HA-VGLL4) and the model of overexpression VGLL4 RD cells were successfully constructed, which were used to studied the relationship between EV71 infection, apoptosis and host protein VGLL4.Our study found that VGLL4 can promote EV71 infected cell apoptosis, which can promote the replication of EV71.

# Materials And Methods

### Cells culture and antibody

RD cells and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM containing 10% fetal bovine serum (FBS, Gibco) and incubated in a 37℃ environment containing 5% carbon dioxide.

The EV71 VP1 antibody was purchased from Abnova, Taiwan, China; the β-tubulin antibody was purchased from Bioprimacy, Wuhan, China; the Caspase3 antibody was purchased from Proteintech, Wuhan, China; and the HA tag antibody was purchased from CST, USA.

### Virus propagation and titration

The EV71 strain BrCr (VR-1775) was purchased from ATCC and was propagated in RD cells. The cells grow to 80%, and then get infected with the virus. After 80% of the cells developed a cytopathic efect (CPE) of viral infection, the cell maintenance supernatant (DMEM containing 1% FBS and 1% 100 μg/ml penicillin-streptomycin) was collected and subjected to three freeze–thaw cycles to lyse the cells. The virus titer was determined by the 50% tissue culture infectious dose ($TCID_{50}$ assay).

### Construction of HA tag VGLL4 recombination plasmid

To construct a plasmid for VGLL4 overexpression (pHA-VGLL4), a fragment of VGLL4 was cloned into the Kpn I and Xho I sites of the HA tag fusion expression vector using the targeting sequences HA-VGLL4-F (5'-cgcggtaccCTATTTATGAAGATGGACCTGTT-3') and HA-VGLL4-R (5'-aatctcgagTTAGGAGACCACAGAGGGGGAGT-3').

### Transfection and expression of plasmids

RD cells were seeded in 6-well plate and cultured in DMEM containing 10% FBS at 37℃ in a 5% $CO_2$ environment. When the degree of monolayer cell confuence reached 60-70%, plasmids were transfected into cells by using lipofectamine 2000 (Invitrigen, USA) according to the manufacturer's instructions. The expression of plasmids were analysed by Western blot assay.

### Western blot assay

PBS-washed cell pellets were lysed in 1×RIPA buffer (CST, USA). The samples were centrifuged at 12,000 rpm for 15 min to harvest the supernatant, which were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (BioRad, USA). The membranes were blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich, Germany) and then incubated with the appropriate primary antibody overnight at 4℃. The membranes were washed with 1×PBST and incubated with a 1:5000 dilution of anti-rabbit or anti-mouse horseradish-peroxidase-conjugated antibody for 1.5 h. Following incubation, the membranes were washed extensively with 1×PBST. Immunoreactive bands detected using ECL reagents (Advansta, USA) were developed with Image Lab (Bio-Rad, USA).

### Real-time quantitative RT-PCR

The cells were lysed by TransZol up and total RNA was isolated from cells as the instruction. The RNA was reversed by TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix and analyzed with the PerfectStart$^{TM}$ Green qPCR SuperMixt. All the reagents were purchased from Transgen biotech, Beijing, China. The mRNA expression of EV71 VP1 was detected with sense primer 5'-GCAGCCCAAAAGAACTTCAC-3' and antisense primer 5'-ATTTCAGCA GCTTGGAGTGC- 3' targeting a conserved region of the VP1 gene. The GAPDH mRNA was detected using sense primers 5'-GACAACTTTGGTATCGTGGAA-3' and antisense primer 5'- CCAGGAAATGAGCTTGACA–3'. All the primers were synthesized from Sangon Biotech, Shanghai, China.

### $TCID_{50}$ assay

The virus was diluted from $10^{-1}$ to $10^{-8}$ and added to Vero cells in 96-well plates; the cells were cultured at 37℃ for 5 days and observed daily. The $TCID_{50}$ values were measured by counting the cytopathic effect (CPE). Further calculations were conducted using the Reed-Muench method.

### Cytotoxicity assay

The cytotoxic effect was assayed by Cell Counting Kit (CCK) (Beyotime Biotechnology, Shanghai, China).

## Results

### Transfection of HA- VGLL4 recombinant plasmid in RD cell

RD cells transfected with pHA-VGLL4 or the control vector were lysed with RIPA at 24h, and western blot assay were performed to evaluate the transfection efficiency of the plasmid in cells. The plasmid was successfully transfected and performed high transfection efficiency into RD cells (Fig 1).

### VGLL4 promotes the expression of EV71 virus protein

RD cell transfected with pHA-VGLL4 or the control vector were infected with EV71 at an MOI of $10^3$TCID$_{50}$/mL. The cells were lysed with RIPA and western blot assay were performed to detect the expression of EV71 VP1 protein at 24 h post infection. The results showed that EV71 VP1 protein were highly low expressed, which means that VGLL4 could promote EV71 proliferation in RD cells (Fig 2).

### VGLL4 facilitates the replication of EV71 genomic RNA

Because we speculated that VGLL4 might enhance EV71 replication real-time quantitative RT-PCR was performed to detect the mRNA level of the VP1 gene of EV71. RD cells were transfected with the pHA-VGLL4 or the control plasmid; at 24h post-transfection, the cells were infected with EV71 at an MOI of $10^3$TCID$_{50}$/mL and then harvested at 24h post infection. The results of real-time quantitative RT-PCR showed that the amount of VP1 mRNA in RD cells overexpressing VGLL4 was significantly increased at 24h post infection compared with the control group (Fig 3).

### VGLL4 upregulated virus titer in cell maintenance supernatant

To further confirm that VGLL4 could facilitate propagation of EV71. The above RD cell maintenance supernatant were collected and the viral titers were calculated by the TCID$_{50}$ assay. The results showed that the titer of EV71 were $10^{5.54}$TCID$_{50}$/mL in cells transfected with pHA-VGLL4 and $10^{4.83}$ TCID$_{50}$/mL in cells transfected with control vector, which means the production of viral particles were increased by VGLL4 (Fig 4).

### VGLL4 promotes EV71-induced apoptosis

Because VGLL4 is an inhibitor of apoptosis protein (IAP)-interacting protein[3], we speculated that VGLL4 might enhance EV71 replication by accelerate the cell apoptosis. RD cells were transfected with pHA-VGLL4 plasmid or the control plasmid; at 24h post transfection, the cells were infected with EV71 at an MOI of $10^3$TCID$_{50}$/mL. At 24h post infection. The cell viability was evaluated by cytotoxicity assay and the cell lysates were analyzed by western blot assay. As showed in figure, the cell viability was significant reduced in VGLL4 overexpression cells infected with EV71 (Fig 4). Cleaved caspase3 are considered to be hallmarks of apoptosis. As showed in Fig 5, the cleaved caspase3 was up-regulated in VGLL4 overexpression cells infected with EV71. Thus, VGLL4 increases EV71-induced apoptosis, which may be beneficial for the virus production.

## Discussion

EV71 infection mainly occurs in infants and children under 5 years old, which can cause a serious public health[5, 7, 12]. However, the interaction between the virus and the host remains unclear. In this paper, we identified the roles of VGLL4 in EV71 replication.

With the completion of the Human Genome Project, more and more genes have been discovered, most of which have unknown functions. Using expression system to express target genes in mammalian cells is

an important means to study gene function and its interaction. In this study, a recombinant plasmid expressing VGLL4 with HA label was successfully inserted into mammalian eukaryotic expression plasmid and was expressed in RD cells. This lays the foundation for the following functional research.

To detect VGLL4 influence on EV71 proliferation, VGLL4 was overexpressed and EV71 was infected in RD cells. Western blot assay was to test the EV71 VP1 expression and real-time quantitative RT-PCR was to detect the EV71 RNA replication. At the same time, $TCID_{50}$ assay was performed to detect the virus titer in RD cells maintain supernatant. All above experiments fully proved that VGLL4 can promote the proliferation of EV71.

Apoptosis is closely related to EV71 replication. The effect of VGLL4 on the cellular viability of EV71 infected cells were evaluated by cytotoxicity assay. The results showed that VGLL4 can inhibit the cellular viability of EV71 infected cells. Therefore, VGLL4 may play an important role in EV71 inducing apoptosis. Since cleaved-caspase-3 is hallmark of apoptosis and cleaved-caspase-3 has been reported to promote the proliferation of EV71[13]. The effect of VGLL4 on EV71 virus induced cleaved caspase3 was detected by western blot assay. The results showed that cleaved caspase3 levels were significantly increased in VGLL4 overexpressing cells.

In conclusion, evidence is provided to demonstrate that VGLL4 can promote EV71 infected cell apoptosis, which can promote the replication of EV71. Thus, VGLL4 may be a potential therapeutic target for anti-EV71.

# Declarations


### Funding

This work was funded by the Zunyi Medical University & Science and Technology Bureau of Zunyi City Collaborative Fund (Zunshi Kehe HZ Zi [2019] No. 18), the Doctor's Research Initiation Fund of Zunyi Medical University (F-986) and the Zunyi Medical University & Science and Technology Bureau of Zunyi City Collaborative Fund (Zunshi Kehe HZ Zi [2020] No. 16).


### Conflict of interests

The authors declare that they have no conflicting interests.

### Availability of data and material

Not applicable.

### Code availability

Not applicable.

### Author contributions

Sengyu Wang performed and designed the main experiments and wrote the paper; Xin Sun participated in writing and editing the paper; Junhua Qiao, Cancan He,Langfei Tian and Yaping Chen was involved in executing the study.

### Ethical approval

This article does not contain any studies with animals performed by any of the authors.

### Consent to participate

The authors declare that they agree to participate.

### Consent for publication

The authors declare that they agree to publish.

# References


1. Han Y, Wang L, Cui J, Song Y, Luo Z, Chen J, Xiong Y, Zhang Q, Liu F, Ho W, Liu Y, Wu K, Wu J (2016) SIRT1 inhibits EV71 genome replication and RNA translation by interfering with the viral polymerase and 5'UTR RNA. J Cell Sci 129:4534-4547
2. Imura A, Sudaka Y, Takashino A, Tamura K, Kobayashi K, Nagata N, Nishimura H, Mizuta K, Koike S (2020) Development of an Enterovirus 71 Vaccine Efficacy Test Using Human Scavenger Receptor B2 Transgenic Mice. J Virol 94
3. Jin HS, Park HS, Shin JH, Kim DH, Jun SH, Lee CJ, Lee TH (2011) A novel inhibitor of apoptosis protein (IAP)-interacting protein, Vestigial-like (Vgl)-4, counteracts apoptosis-inhibitory function of IAPs by nuclear sequestration. Biochem Biophys Res Commun 412:454-459
4. Kim JY, Kim EK, Lee WM, Hong YO, Lee H (2020) VGLL4 with low YAP expression is associated with favorable prognosis in colorectal cancer. APMIS
5. Kou Z, Jia J, Liu X, Luo T, Xin X, Gong J, Zhang J, Sun D, Jiang F, Gao R (2020) Epidemiological characteristics and spatial-temporal clusters of hand, foot, and mouth disease in Qingdao City, China, 2013-2018. PLoS One 15:e0233914
6. Kumamaru E, Kuo CH, Fujimoto T, Kohama K, Zeng LH, Taira E, Tanaka H, Toyoda T, Miki N (2004) Reticulon3 expression in rat optic and olfactory systems. Neurosci Lett 356:17-20
7. Lee JT, Yen TY, Shih WL, Lu CY, Liu DP, Huang YC, Chang LY, Huang LM, Lin TY (2019) Enterovirus 71 seroepidemiology in Taiwan in 2017 and comparison of those rates in 1997, 1999 and 2007. PLoS One 14:e0224110
8. Li H, Bai Z, Li C, Sheng C, Zhao X (2020) EV71 infection induces cell apoptosis through ROS generation and SIRT1 activation. J Cell Biochem 121:4321-4331
9. Lin JY, Li ML, Huang PN, Chien KY, Horng JT, Shih SR (2008) Heterogeneous nuclear ribonuclear protein K interacts with the enterovirus 71 5' untranslated region and participates in virus replication.

J Gen Virol 89:2540-2549

10. Mao LX, Wu B, Bao WX, Han FA, Xu L, Ge QJ, Yang J, Yuan ZH, Miao CH, Huang XX, Zhang C, Xu H (2010) Epidemiology of hand, foot, and mouth disease and genotype characterization of Enterovirus 71 in Jiangsu, China. J Clin Virol 49:100-104
11. Nishimura Y, Shimojima M, Tano Y, Miyamura T, Wakita T, Shimizu H (2009) Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat Med 15:794-797
12. Qi L, Su K, Xia Y, Tang W, Shen T, Li Q (2019) Enterovirus 71 vaccine acceptance among parents of children < 5 years old and their knowledge of hand, foot and mouth disease, Chongqing, China, 2017. PLoS One 14:e0225569
13. Song F, Yu X, Zhong T, Wang Z, Meng X, Li Z, Zhang S, Huo W, Liu X, Zhang Y, Zhang W, Yu J (2018) Caspase-3 Inhibition Attenuates the Cytopathic Effects of EV71 Infection. Front Microbiol 9:817
14. Song H, Luo Q, Deng X, Ji C, Li D, Munankarmy A, Jian W, Zhao J, Fang L (2019) VGLL4 interacts with STAT3 to function as a tumor suppressor in triple-negative breast cancer. Exp Mol Med 51:1-13
15. Su PY, Wang YF, Huang SW, Lo YC, Wang YH, Wu SR, Shieh DB, Chen SH, Wang JR, Lai MD, Chang CF (2015) Cell surface nucleolin facilitates enterovirus 71 binding and infection. J Virol 89:4527-4538
16. Tajonar A, Maehr R, Hu G, Sneddon JB, Rivera-Feliciano J, Cohen DE, Elledge SJ, Melton DA (2013) Brief report: VGLL4 is a novel regulator of survival in human embryonic stem cells. Stem Cells 31:2833-2841
17. Wang H, Zhong M, Li Y, Li K, Wu S, Guo T, Cen S, Jiang J, Li Z, Li Y (2019) APOBEC3G is a restriction factor of EV71 and mediator of IMB-Z antiviral activity. Antiviral Res 165:23-33
18. Wang Q, Zhang W, Zhang Y, Yan L, Wang S, Zhang J, Sun J, Chang Z, Wang Z (2014) Clinical features of severe cases of hand, foot and mouth disease with EV71 virus infection in China. Arch Med Sci 10:510-516
19. Wen W, Qi Z, Wang J (2020) The Function and Mechanism of Enterovirus 71 (EV71) 3C Protease. Curr Microbiol 77:1968-1975
20. Yamaguchi N (2020) Multiple Roles of Vestigial-Like Family Members in Tumor Development. Front Oncol 10:1266
21. Yang F, Zhang T, Hu Y, Wang X, Du J, Li Y, Sun S, Sun X, Li Z, Jin Q (2011) Survey of enterovirus infections from hand, foot and mouth disease outbreak in China, 2009. Virol J 8:508
22. Yang SL, Chou YT, Wu CN, Ho MS (2011) Annexin II binds to capsid protein VP1 of enterovirus 71 and enhances viral infectivity. J Virol 85:11809-11820
23. Yu H, Cowling BJ (2019) Remaining challenges for prevention and control of hand, foot, and mouth disease. Lancet Child Adolesc Health 3:373-374