Viral replication probably promoted by EV71 VP4 protein via activating the PI3K/AKT and MAPK pathways in RD cell

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Research Article

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Abstract

Enterovirus 71 (EV71) internal capsid protein (VP4) conserves in *picornaviridae* family and plays critical role in viral infection and replication by unknown mechanisms. In this study, mRNA sequencing was performed to analyze Differentially Expressional Genes (DEGs) in VP4 protein overexpressing Human Rhabdomyosarcoma Cells. It had been showed that Focal Adhesion, PI3K/AKT and MAPK pathways were mainly activated by the significant DEGs. Co-immunoprecipitation (Co-IP) assay indicated that ITGA5 of DEGs interacted with VP4. After knockdown of the ITGA5, WB showed that PI3K/AKT and MAPK pathways were activated, RT-qPCR experiments confirmed that viral copies were increasing. Thus, it is indicated that viral replication probably can be promoted by VP4 protein with activating the PI3K/AKT and MAPK pathways and a subsequent interaction with ITGA5.

Introduction

Enterovirus 71 (EV71), which is the latest found virus that mainly causes hand-foot-mouth disease (HFMD) [1–3] and viral angina in children under 3 years old[4,5]. Severely, it would cause myocarditis, pulmonary edema, encephalitis or even death[6]. During the recent decades, HFMD has become a globally epidemic disease[7]. As a consequence, it is necessary to clarify the pathogenesis of EV71 infection. Because it will promotes the development of novel anti-EV71 strategies and improvement of clinical prevention and treatment.

EV71 is a single strand RNA (ssRNA) virus, which are composed of structural proteins (capsid) and non-structural proteins[8,9]. EV71 VP4, as an capsid protein[10], is highly conserved in Enterovirus genus of *Picornaviridae* family, including the Coxsackievirus A16 (CAV16) [11,12], human rhinovirus (HRV) [13,14], and other Enteroviruses[15–19]. As a result, The study on the functional mechanism of EV71 VP4 protein has a certain universality and provides a useful reference for finding common antiviral prevention and treatment strategies of *Picornaviridae* family.

EV71 VP4 protein, which showed the vital functions in the neutralizing antibody production [11,20], viral replication [12,21,22] and viral entry into host cells [10,23]. While the molecular mechanisms of VP4 in RD cell about EV71 infection and replication was unknown. Thus, we researched on the molecular mechanism of VP4 protein after mRNA sequencing.

Materials And Methods

2.1 Cell lines and virus

Human Rhabdomyosarcoma Cells (RD cells), RD cells with vector (RD-Vector cells) and RD cells overexpression of VP4 protein (RD-VP4 cells) were kept in our lab. RD cells were seeded in a 10 cm dish with 3×10⁶ cells and grown in DMEM containing 10% FBS and 1% PS (Penicillin and streptomycin) at 37°C in a 95% air and 5% carbon dioxide (CO₂) incubator. RD-VP4 and RD-Vector cell lines were cultured
in RD cells growth medium couple with 250 µg/mL G418 at 37°C in a 95% air and 5% CO₂ incubator. Cells were collected at 90% of confluence.

They were seeded in 6-well plate respectively with 1×10⁵ cells per well for PCR and WB assay to validate the cell lines. For VP4 gene transcript detection, total RNA was extracted from the RD-Vector cells and RD-VP4 cells respectively, the RNAs was synthesised to cDNA by a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Japan), and usual PCR application was carried out with VP4 gene primers (Table 1) and Taq PCR enzymes (TAKARA, Japan). To detect the VP4 protein, after SDS-PAGE, mouse anit-VP4 polyclonal antibody (pAb, 1:500) was used to incubate at 4 °C for overnight, and Horseradish Peroxidase (HRP) linked Goat anti mouse IgG (1:3000) as the second antibody at room temperature (1 h).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caveolin</td>
<td>TCCGGAGTTTTTCCATCCAGCC GCCGTGAAACTGTGTGTTCC</td>
</tr>
<tr>
<td>ITGA4</td>
<td>CAGGTTTAAGCATGGCCACA CTGGTTGGGAATGCTGTGTTTTGT</td>
</tr>
<tr>
<td>ITGA5</td>
<td>CGGGGGCTTCAACTTAGACG ATTCAATGGGGGTGCACGTG</td>
</tr>
<tr>
<td>ITGB1</td>
<td>GACGCCGCGCGGAAAG TCTGGAGGGCAACCTCTTTT</td>
</tr>
<tr>
<td>ITGA7</td>
<td>ATGGCCGGGGGCTCGAGGAGCCTCCAG ACGGCAAGCGGAAGGCTCCAG</td>
</tr>
<tr>
<td>FPR3</td>
<td>GACTGATTCGCTCTTTGCCCAC TCTCCTCAGAGGTGAAGCAGA</td>
</tr>
<tr>
<td>VEGF</td>
<td>CGGGAACCAGATCTCTCAC CGGCCCGGTGTGCTTA</td>
</tr>
<tr>
<td>EGF</td>
<td>TAGGCATTAGCCATGGTCG TCAAGTCATCTCCCCATCACC</td>
</tr>
<tr>
<td>FGF</td>
<td>CCCATCGGGTAAAGATGCTCC CGAGCGGCAGAGTTAGAA</td>
</tr>
<tr>
<td>PDGF</td>
<td>TGCTAAGGAGGAAGAAGG CGATGCTGAGCCCTCTCA</td>
</tr>
<tr>
<td>KCNA4</td>
<td>CCATTGTGCTGGGCTCTCTGAG AGCACTCAGTGCCAGCAGA</td>
</tr>
<tr>
<td>PRSS21</td>
<td>TATGCGGAGTGGAGCCTCGTCA AGGATGGCATGGGAAGTCAGCTG</td>
</tr>
<tr>
<td>SNURF</td>
<td>AGAAGGACTGGCTACTGAGCA ACCACCTTTGCTGCTGAGCT</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>CGACGACCCATTCCGAACGTCT CTCTCCGAATCGAACCCTGA</td>
</tr>
<tr>
<td>VP1</td>
<td>atgtgattgagagctctatag CGCTGTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>VP4</td>
<td>ATGGGCTCCGGTGCT CTCTTAAGGCGGCTGCC</td>
</tr>
</tbody>
</table>

Table 1
Primer List
The Human EV71 (BrCr strain) was purchased from the American Type Culture Collection (ATCC), and successfully preserved in our laboratory. For the virus application, RD cells were seeded into 10 cm dish at a density of $3 \times 10^6$ cells per dish. Then EV71 was inoculated in RD cells with one multiplicity of infection ($MOI = 1$). 48 h post infection, virus were harvested.

2.2 Transcriptome sequencing

2.2.1 Sample collection and transcriptome sequencing.

3 mL of RNAlater solution (TAKARA, Jap) was added to each dish with the cell density of $1 \times 10^7$ cells/mL. The mixture was transferred to a 15 mL centrifuge tube with RNase free, packaged in a box containing dry ice and sent to the company (LC Sciences, China) for total RNA extraction and transcriptome sequencing.

2.2.2 Bioinformatics analyses.

All the clean data from the RD-VP4 and RD-Vec groups were deposited in the BIG Sub database (accession number PRJCA012210). Sequencing data were first subjected to the analysis of Transcriptome assembly, Gene structure and reads distribution on the chromosome by previous articles $^{[24-26]}$, differential expressed genes (DEGs) analysis by RSEM software (v1.2.15). DEGs were screened according to the $|\log_{2}\text{fold change}| > 1$ and p-value ($< 0.05$), ggplot2 package in R was used to hierarchical clustering analysis of DEGs. In addition, DEGs were subjected to Gene ontology (GO) analysis (http://www.geneontology.org/) for categorizing the cellular component (CC), molecular function (MF) and biological process (BP). Statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was analyzed on line (https://www.kegg.jp/).

2.3 Reverse Transcriptional-quantitative Pcr (Rt-qpcr).

To validate the sequencing result, 13 DEGs were selected, including 8 up-regulated and 5 down-regulated. mRNA transcripts of the selected genes were determined by RT-qPCR with the 18s rRNA as a reference gene. Total mRNA was extracted from RD-VP4 and RD-Vec cells respectively with Trizol regent (Takara, Japan), and the RNA was synthesized to cDNA by a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Japan). qPCR experiment was performed using SYBR Premix (Invitrogen, USA) on an Applied Biosystems instrument (Biorad, USA). The experiment was designed three times biological repeats. The primers are show in Table 1.

2.4 EV71 VP4 activation the PI3K/AKT and MAPK pathway

2.4.1 VP4 interaction with ITGA5 in RD-VP4 cells by Co-IP.

$1 \times 10^7$ cells of RD-VP4 cells were collected for extracting total proteins. 2*Strep-Flag-VP4 fusion protein was purified and eluted by Flag M2 beads (Sigma, USA), and the normal IgG (Sigma, USA) served as the
negative control. The eluate added 5×Lysis buffer, boiled at 100°C for 10 min, and centrifuged at 12,000g for 10 min. The supernatant was prepared for VP4, ITGA5 or ITGB1 protein detection.

### 2.4.2 Knockdown ITGA5.

The shRNAs for knockdown ITGA5 were bought from Jima company (Shanghai, China), lentiviral packaging was the same as the above text. RD cells were infected with shITGA5 of Lentivirus, and those infected with scramble shRNA (shScramble) of Lentivirus served as negative controls. After 48 h, RD cells were collected respectively for detecting the ITGA5 protein expression by Western blot.

### 2.4.3 EV71 VP4 activation the PI3K/AKT and MAPK pathways and promote viral replication.

In RD cells, after ITGA5 lentivirus infected for 48 h and infected with EV71 (MOI = 0.1, detection by TCID50) for 24 h or 48 h, cells were collected for Western blot and RT-qPCR. In this part, proteins level of p-AKT, p-MEK1, and p-Elk1 (CST, USA) in PI3K/AKT or MAPK pathway were detected by WB. Viral RNA copy number was calculated by RT-qPCR according to previously described\(^27\), which was carried out by detecting the VP1 gene of EV71, and pMD18-VP1 plasmid was used to generation the standard curve.

## Results

### 3.1 RD-VP4 cells Validation

RD-Vector and RD-VP4 cells were saved in our Lab. To explore the function of EV71 VP4 protein. RD-Vector and RD-VP4 cells were collected to extract the total RNAs and proteins, followed by detecting VP4 levels. After reverse transcription and PCR amplification, gene transcription of VP4 was visualized in Fig. 1A. As expected, a fusion protein VP4 at 16 KD was detected by Western blot (Fig. 1B). It suggested that the constructed RD-VP4 cells could be used in the following experiments. But there were two nonspecific bands at 15 KD and 20 KD respectively, we inferred that they were caused by polyclonal antibody (pAb).

### 3.2 DEGs analysis and validation after Transcriptome sequencing

We thereafter performed transcription sequencing in RD-VP4 and the negative control RD-Vector cells, a total of 5,708 DEGs with 2-fold changes (|Log2fold change|>1 and \(p < 0.05\)) were screened out, including 3,232 upregulated and 2,476 downregulated. In Fig. 2A, the Y-axis represented the adjusted \(p\)-value (padj). A smaller ordinate value suggested a more significant difference and a larger corresponding \(-\log_{10}\) value (padj). Therefore, plots in the upper left and the upper right corner very significantly downregulated and upregulated genes, respectively.
DEGs were subjected to GO analysis (http://www.geneontology.org/). GO analysis for categorizing the cellular component (CC), molecular function (MF) and biological process (BP). The top 10 CC, MF and BP were show in Fig. 2B, and it showed that DEGs were mostly associated to protein binding functions.

DEGs were also carried out KEGG analysis (https://www.kegg.jp/). In this analysis, it showed that DEGs were in up-regulated ten pathways (Fig. 2C) and in down-regulated two pathways (Fig. 2D). It was revealed that DEGs were mainly up-regulated enriched in Focal Adhesion, PI3K/AKT and MAPK pathway and down-regulated enriched in phospholipase D signal pathway.

Previous studies showed that VP4 protein band to cell membrane during virus entry [13,14,28], so we selected differential genes related to focal adhesion pathway and cell surface receptors for verification by RT-qPCR (Fig. 3). Using housekeeping gene 18s rRNA to do normalized quotient, a total of 8 genes were upregulated and 5 were downregulated. Integrin receptors ITGA4/5/7 and ITGB1, caveolin and some receptor tyrosine kinases (RTKs, EGF and FGF) were upregulated, while FPR3 and some other RTKs (VEGF and PDGF) were downregulated. These results were consistent with those in bioinformatics analysis. Then, according to reading literatures, we founded that ITGA5 and ITGB1 composited ITGA5-ITGB1 complex, which located in cell membrane[29] and could activate PI3K/AKT and MAPK pathway[30]. Thus, we selected the ITGA5 and ITGB1 to research the molecular mechanism of VP4 protein.

### 3.3 EV71 VP4 activation the PI3K/AKT and MAPK pathways and promote viral replication

VP4 protein band to cell membrane during virus entry [13,14,28], ITGA5/ITGB1 complex were up-regulated in RD-VP4 cell, and ITGA5-ITGB1 complex could activate PI3K/AKT and MAPK pathway[30], so we speculated that VP4 protein interact with the ITGA5 or ITGB1. Thus, the co-immuneprecipitated (Co-IP) assay was designed to validate the interaction between VP4 and ITGA5 or ITGB1. In RD-VP4 cells, WB result showed that both VP4 and ITGA5 existed after IP from the Fig. 4A, while there was only VP4 and did not exist ITGB1 after IP from Fig. 4B, suggesting that VP4 not interaction with ITGB1 but interaction with the ITGA5.

To explore the role of VP4-ITGA5/ITGB1 complex in the virus replication process, two ITGA5 shRNAs were used to lentiviruses packaging and knockdown the ITGA5 expression. The ITGA5 protein was downregulated effectively after shITGA5-1 and sh-ITGA5-2 lentivirus transduction RD cells (Fig. 5A). Notably, knockdown of ITGA5, phosphorylated-AKT and MEK1 proteins (p-AKT and p-MEK1) were downregulated and phosphorylated-Elk1 (p-Elk1) protein was upregulated in PI3K/AKT and MAPK pathway (Fig. 5B), suggesting that the PI3K/AKT and MAPK pathway were activated by ITGA5. Knockdown ITGA5 in RD cells, the copies of EV71, which was detected by RT-qPCR, was significantly downregulated in EV71 infection at 24 hpi and 48 hpi (Fig. 5C) respectively. This result showed that ITGA5 knockdown inhibited EV71 virus replication. Thus, we concluded that EV71 VP4 protein probably promote viral replication.
Discussion

According to bioinformatics analysis the DEGs in RD-VP4 cells which was overexpressing VP4 protein, it was found that the biological process of protein binding was significantly enriched (Fig. 2B). In addition, the Focal Adhesion, PI3K-Akt and MAPK pathways were significantly activated (Fig. 2C).

Integrin Subunit Alpha 5 (ITGA5), primarily binds to Integrin Subunit Beta 1 (ITGB1) to form a α5β1 heterodimer\(^{[29]}\). Interestingly, the ITGA5-ITGB1 complex serves as the receptor for Human Metapneumovirus\(^{[31]}\). In addition, Yan, T. et al\(^{[32]}\) proposed that ITGA5 could activate phosphatidylinositol 3-kinase (PI3K) pathway. And the activation of the PI3K/AKT pathway significantly blocks JNK-induced cell apoptosis during the EV71 infection\(^{[33]}\). It is well known that HRV and EV71 belong to the *picomaviridae*, the highly conserved VP4 protein exerts the function of membrane permeation in HRV \(^{[13, 14]}\). Therefore, we speculated that VP4 activated the downstream PI3K/AKT and MAPK pathways by interaction with the cell surface receptor ITGA5/ITGB1 complex, which also belonged to Focal Adhesion pathway.

To validate our speculation, the EV71 VP4 interaction with ITGA5-ITGB1 complex was confirmed (Fig. 4A and 4B), indicating that VP4 anchored on the cell membrane through the ITGA5. ITGA5 was silenced in RD cells by lentivirus transduction and then infected with EV71 (MOI = 0.1). Interestingly, key proteins in the PI3K/AKT and MAPK pathways were significantly changed at 48 h (Fig. 5B), and the copies of EV71 was decreased (Fig. 5C), suggestion that ITGA5 was indeed needed in EV71 infection. In conclusion, we found that VP4 activated the downstream PI3K/AKT and MAPK pathways by interaction with the cell surface receptor ITGA5, following inhibiting the apoptosis of host cells.

Therefore, clarifying the mechanism of EV71 VP4 activated the downstream PI3K/AKT and MAPK pathways by interaction with the cell surface receptor ITGA5 is beneficial to the development of new antiviral drugs targeting ITGA5 and also provide a reference for the treatment of other enteroviruses.

Conclusion

VP4 protein probably promote viral replication by activation the PI3K/AKT and MAPK pathways by interaction with ITGA5 in RD cell.

Declarations

Availability of data and materials

Datasets used and/or analyzed in this study available from the corresponding author on reasonable request.

Ethics approval
Authors’ contributions

XHG conceived the study and designed the experiments; DL, YW, XG and YH performed the experiments; XHG wrote the paper; YX guided the experiment and helped analysis of data; XZ polished the Language of the paper. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

**RD-VP4 cells Validation.** The constructed RD-vector and RD-VP4 cells validation on mRNA level and on protein level. A. VP4 gene transcription by RT-PCR amplification. RD-Vet/1 and RD-Vet/2 Lanes were samples from RD cells transfected empty vector, RD-VP4/1 and RD-VP4/2 Lanes were samples from RD cells transfected VP4 overexpression vector (RD-VP4 cells). B. VP4 protein expression by Western blot. RD Lane is sample from RD cells transfected empty vector, RD-VP4 Lane is sample from RD-VP4 cells. After SDS-PAGE, Rabbit anit-VP4 polyclonal antibody (pAb) was used to incubate at 4 °C for overnight, and Horseradish Peroxidase (HRP) linked Goat anti Rabbit IgG as the second antibody.
Figure 2

DEGs analysis of RD-VP4 Cells VS RD-Vet Cells after Transcriptome sequencing. (A). Volcano map visualizing DEGs between RD-vector cells and RD-VP4 cells, in which red plots were upregulated DEGs, green plots were downregulated DEGs and blue plots represented similarly expressed genes. The Y-axis represented the adjusted p-value (padj). (B). The top 10 GO terms (CC, MF and BP) of DEGs in RD-VP4 cells. GO analysis for categorizing the cellular component (CC), molecular function (MF) and biological process (BP). (C). The top 10 enriched KEGG pathways in RD-VP4 cells. DEGs in up-regulated ten pathways. (D). DEGs in down-regulated two pathways.
Validation the DEG by RT-qPCR. The transcriptional level of 13 DEG was detected by RT-qPCR assay with SYBR staining. Housekeeping gene 18s rRNA as a internal control gene, and 3 biological repeats for every gene were carried out.

EV71 VP4 interaction with ITGA5. In RD-VP4 cells, Immuno-precipitants (IP) Assay with mouse rabbit anti-Flag antibody, then WB Assay detected the VP4, ITGA5 (A) and VP4, ITGB1 (B) respectively. Input was Cell
lysate. Rabbit anti-VP4 pAb, Mouse anti-ITGA5 mAb, Mouse anti-ITGB1 mAb and HRP conjected goat anti-mouse IgG were used.

**Figure 5**

**EV71 VP4 activating the PI3K/AKT and MAPK pathway and promote viral replication.** (A) Lentiviruses shScramble, shITGA5-1 and sh-ITGA5-2 infected RD cells 48 h (shScramble was a control with empty vector), Knockdown efficacy was detected by WB, Mouse anti-ITGA5 mAb, Mouse anti-GAPDH pAb and HRP conjected goat anti-mouse IgG were used. (B) after Lentiviruses infected RD cells 48 h, EV71 infection (MOI=0.1), and after 24 h and 48 h, Protein levels of p-AKT, p-MEK1 and p-Elk1 in PI3K/AKT and MAPK pathway were detected by WB. Phosphorylated Mouse anti-AKT mAb, phosphorylated Mouse anti-MEK1 mAb, and phosphorylated Mouse anti- p-Elk1 mAb, Mouse anti-GAPDH pAb and HRP conjected goat anti-mouse IgG were used. (C)The copies of EV71 were detected by RT-qPCR of VP1 gene with SYBR staining. pMD18-VP1 plasmid was used to generation the standard curve, and 3 biological repeats for every gene were carried out.