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Original Article

Prokaryotic expression of partial E gene of DENV strain and its effect on HUVEC permeability

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Abstract: Dengue virus (DENV) has become the one of the major public health problems worldwide, especially in Tropical and subtropical regions. E protein is the biggest structural proteins and the major envelope protein of the virion, which can induce protective immune response and subsequently immune pathological damage. The pathology of DENV infection is characterized as increased vascular permeability. However, the effect of Protein E on the vascular permeability is not well understood. We isolated DENV2 (M strain) from Aedes albopictus in 2002 and sequenced 1-476 bp of its E gene (GenBank No. AY278226). Here we prepared fusion protein of E gene 1-476 sequence of DENV-2 M strain and NGC strain, named M476 and N476 respectively, and their respective polyclonal antibody. In co-culture system of human umbilical vein endothelial cell (HUVEC) and recombinant protein, indirect immunofluorescence staining showed that the recombinant protein of M and NGC can bind to HUVEC. Through detecting FITC fluorescence change, it was found that M476 protein had significant effect on the permeability of HUVEC during the period of 0.5~24 h and 32~48 h, 12 h at the peak. As for N476 protein, it functioned at 0.5~48 h, 3 h at the peak. Compared with untreated cells, the morphology of HUVEC cells treated with M476 or N476 protein showed shrinking cell, wider width between cells and disrupted cell connection. Taken together, our work strongly confirmed the effect of protein E of DENV-2 on vascular permeability, which might help understanding the pathogenesis of DENV in depth.

Keywords: DENV strain, HUVEC, prokaryotic expression

Introduction

Dengue virus (DENV), a member of flaviviridae Flavivirus, has four serotypes (DENV1-4). DENV distributes widely in tropical and subtropical regions. The gene encoding DENV is about 11 kb. DENV is single-strand RNA virus wrapped by membrane virion, with a diameter of about 50 nm, constituted by capsid protein C, membrane protein M and envelope protein E [1-4]. After DENV infection there comes out a series of clinical manifestations. The patients may be asymptomatic at the early stage, and then develop dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). In severe cases, the symptoms could be plasma leakage and even life-threatening shock. The harm of DENV on human health is getting worse in the past 10 years [5], and it is the most crucial disease influencing humans among mosquito-borne, with 50~100 million people infected with DENV [6]. DENV has become the one of the major public health problems that the world is facing [7, 8].

There are three kinds of structural proteins (C, M/PrM and E) and seven kinds of non-structural proteins (Nsl, NS2a, Ns2b, NS3, NS4a, NS4b and Ns5) encodes by Dengue virus [7]. As the biggest structural proteins and the major envelope protein of the virion, protein E, constituting the projection of the virus particle surface and determining the tissue tropism of the virus, can cause a protective immune response and immune pathological damage. The abilities of mediated adhesion and penetration of viral particle to the host cell [9], result in strong immune response of the body then accompanied with neutralizing antibodies produced by the body to inhibit the adhesion of virus [10]. It was suggested that protein E participated in the interaction between virus and specific receptors on targeted cell surface and thus mediated virus infection directly; actually, protein E was the envelope protein of DENV [11-16].

The main pathology of DENV related diseases is increased vascular permeability induced sys-

Table 1. Prokaryotic expression primers sequences of M and NGC strains

Prime	Sequence (5'-3')	PCR produce (bp)
Upstream (M strain)	5'-cggcatatgcgttgcataggaatatcaaat-3'	492
Downstream (M strain)	5'-gggtcgacttaccatgtttttcctgtgtca-3'	492
Upstream (NGC strain)	5'-cggcatatgcgttgcataggaatatcaaat-3'	492
Downstream (NGC strain)	5'-aggtcgacttagccatgtttttcctgtgtca-3'	492

temic vascular damage [17]. However, the effect of protein E on the vascular permeability has never been explored.

We isolated the virus (M strain, the same below) from Aedes albopictus in the nature of Ma Wei town, Dushan County, Guizhou Province of Aedes albopictus in 2002. The virus was identified as DENV-2 by indirect immunofluorescence with anti DENV1-4 monoclonal antibodies and digested genotyping of RT-PCR products. Compared with DENV-2 NGC strain, there are a base insertion and five mutations in the virus sequence. The virus sequence was recorded by GenBank, and the number is AY278226 [11, 18]. The sequence studied in the present work is 1-476 bp of DENV-2 E gene, a core area formed by folded monomer [19]. Our targeted sequence located in E protein EDI domain, an important domain involved in by protein E mediating membrane fusion process [15, 20].

Based on the work described as above, we prepared fusion protein of E gene 1-476 sequence of DENV-2 M strain and NGC strain, named M476 and N476 respectively. We used the prokaryotic expression system pET28a+ to express protein, purified the protein with the affinity arm of the 6xHis-tag at the N or C-terminus of the target protein. To explore the effect of DENV E recombinant protein on the permeability of cultured human umbilical vein endothelial cell (HUVEC), polyclonal antibody of M-476 and N-476 were prepared. Our probe of the effect of protein E on the vascular permeability would benefit the understanding of the mechanism of DHF/DSS development.

Materials and methods

Reagents, cells and animals

E gene 1-476 fragment of DENV M strain and NGC strain, E-coli DH5-alpha (α) were kept in our lab. HUVEC line was provided also by our lab and cultured in L-DMEM medium (Gibco) supplemented with 10% FBS (Evergreen, Hangzhou, China) at 37°C in a 5% CO_2. Specific pathogen-free, female C3H and BALB/c mice of 6-7 wk old were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences.

Prokaryotic expression vector pET28a+ was purchased from Generay (Shanghai, China). BL21 (DE3) was purchased from TIANGEN (Beijing, China). Restriction endonucleases Nde I and Sal I, T4 DNA ligase were purchased from Fermentas (Canada).

DNA gel extraction kit, FITC labeled dextran 4000 Da were purchased from Sigma. Biowest Agarose, ethidium bromide (EB), HEPS were the products of the Sino-American Biotechnology Company. NC membrane was from the Takara Bio. Glycine, Acr, Bis, SDS, TEMED, Triton-100, EDTA and Tris were the products from Amresco (USA). Prime STAR Hot Start DNA Polymerase, IPTG, Kan, HiS-Bind Purification Kit, DTT, and BSA were the products of Merck. Pierce and BCA Protein Assay Kit were purchased from Thermo Electron Corporation. Reap Miniprep Kit, anti-His tag monoclonal antibody, goat anti-mouse IgG antibody-HRP, DAB staining kit and blocking buffer was purchased from TIANGEN (Germany). FITC-conjugated goat anti-mouse IgG secondary antibody was purchased from Zhongshan Golden Bridge (Beijing, China). Enhanced Chemiluminescence was from Santa Cruz (USA).

Preparation of recombinant fusion protein M476, N476

The primers for E gene 1-476 fragment of DENV-2 M strain and NGC strain were synthesized by Takara (Dalian, China), which contained the NdeI, SalI restriction point. The primer sequences are listed in **Table 1**.

Target gene fragment of M476 and N476 were respectively cloned into prokaryotic expression vector pET28a(+). The plasmid then was used to transfect E.coli BL21 to generate M476 and

N476-expressed strain. His-tag M476 and N476 fusion protein were expressed after IPTG induction. Western Blot was performed to identify fusion protein with anti-His tag mouse monoclonal antibody as primary antibody and goat anti-mouse IgG antibody-HRP antibody as the second antibody. Purification of His-tag M476 and N476 fusion protein were performed by His Bind Purification Kit and the purity was assayed by SDS-PAGE. Because the fusion protein was expressed by inclusion bodies, refolding of the protein was performed using gradient dialysis.

Preparation and identification of polyclonal antibody of M476 and N476 fusion protein

SDS-PAGE was used to isolate the M476 and N476 fusion protein. The target bands containing M476 and N476 fusion protein were cut under sterile conditions and put mortar to dissolve it. 22 BALB/c mice of 6-8 wk old were divided into M group, NGC group and control group. There were 10 mice in M group and NGC group, respectively, and two in control group. The prepared M476 and N476 antigen were inoculated subcutaneously at multiple points of the mice in M and NGC group respectively. Mice in control group were given PBS alternatively. The amount of the initial antigen immunization was 50 μg each mouse. One week later, 25 μg of antigen was appended to each mouse once a week for 4 times. Seven days after the last immunization, blood serum was collected and separated to aliquots to store at -80°C. All studies were performed according to internationally recognized guidelines for animal care.

Identification of antiserum was performed by Western blot. Briefly, M476 and N476 protein used for immunization was isolated by SDS-PAGE and then transferred onto PVDF membrane. The membranes were incubated with mouse antiserum (1:100) for 1 h. Then the PVDF membranes were incubated with the horse-radish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (1:100) for 1 hour at room temperature. The proteins bands were visualized using DAB staining kit according to the instruction of the manufacturer.

Binding M476 and N476 fusion protein to HUVEC

HUVEC cells were seeded onto sterile coverslips placed in wells of six-well plates and cultured. Cell seeded was coincubated M476 and N476 protein (10 μg/well) respectively for 12 hours. After that, removed cell medium, washed three times with PBS, and then fixed with 1 ml 4% paraformaldehyde for 10 min. Indirect immunofluorescence staining was used to determine the binding of M-476 and N-476 protein to HUVEC. In brief, washed the wells with PBST 3 times, 5 min/time, dried coldly followed by adding 2% BSA was added into each well, to block for 30 min. aspirated the residual blocking solution 30 min later, then M-476 and N-476 antiserum was respectively added (diluted 1:100, 200 μl/well) onto the coverslip slowly and incubated in a moist chamber at 37°C for 30 min. Then FITC-labeled goat anti-mouse IgG secondary antibody (diluted 1:1000, 100 μl/well) was added, and incubated in dark for 30 min. After washing with PBS, coverclips were observed under fluorescence microscope.

The effect of M476 and N476 protein on the permeability HUVEC monolayer

The Transwell was placed into 24-well plates, 600 μl of L-DMEM working solution was added into the lower chamber. 200 μl of HUVEC cell was seeded in the upper chamber at a density of 10^5/ml. After about 12 hours, cells were grown into monolayer.

M476 and N476 protein was added to each transwell of the upper chamber with the final concentration of 10 μg/ml. The permeability was detected after 0.5, 1, 3, 6, 12, 24, 30, 36, 42, 48 and 72 h of incubation, respectively. 10 μl/well of FITC-labeled dextran was added into the upper chamber of transwell in dark. After 15 min of culture, fluorescence value was detected by full wavelength microplate reader. LPS and PBS were used as control. Meanwhile, the morphology of monolayer was observed under inverted microscope and ultra-structure was observed under Transmission electron microscope.

Results

Identification of the M476 and N476 prokaryotic expression product by SDS-PAGE and Western blot

The recombinant plasmids pET28a of M476 and N476 were transformed to E.coli BL21. After IPTG induction, total protein was collected from cell lysate. The supernatant and precipita-

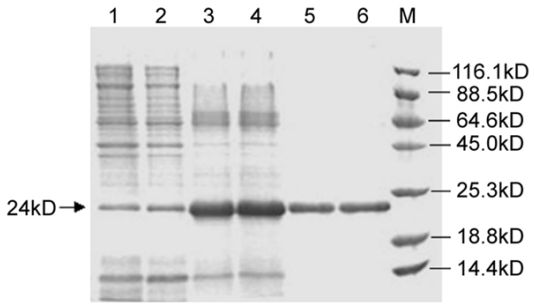

Figure 1. SDS-PAGE analysis of M476 and N476 fusion protein. Lane 1. Supernatant of pET28a(+)-M476-BL21 induced by IPTG; lane 2. Supernatant of pET28a(+)-N476-BL21 induced by IPTG; lane 3. Precipitation of pET28a(+)-M476 -BL21 induced by IPTG; lane 4. Precipitation of pET28a(+)-N476-BL21 induced by IPTG; lane 5. Purified fusion protein of pET28a(+)-M476; lane 6. Purified fusion protein of pET28a(+)-N476; M. Protein Marker.

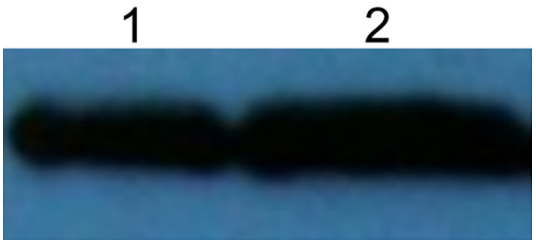

Figure 2. Western blot of His-M476 and His-N476 fusion protein lane 1. Western blot of His-M476 fusion protein; lane 2. Western blot of His-N476 fusion protein.

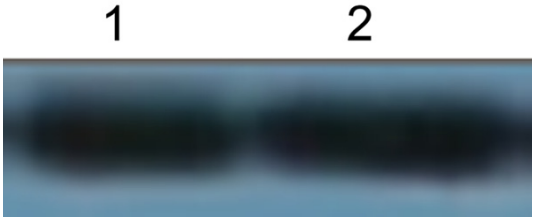

Figure 3. Western blot of M strain and NGC strain antiserum lane 1. Western blot detection of M strain antiserum; lane 2. Western blot detection of NGC strain antiserum.

tion from centrifugation were analyzed at SDS-PAGE (**Figure 1**). The molecular weight of our target protein was about 28 KDa, consistent with the theoretical value. Western blot was performed to identify the expression product. The results were as shown in **Figure 2**, two clear bands appeared, which was consistent with the expected results, suggesting the recombinant protein was the destined protein.

After purified by His-Bind Purification Kit, the recombinant protein was analyzed by SDA-PAGE and gel image instrument. It was shown that the purity of recombinant protein reached to 90%.

Identification of antiserum of M476 and N476 protein

We then prepared the antiserum of M476 and N476. Western blot showed two clear bands which was consistent with the expected results (**Figure 3**).

M476 and N476 protein bond with HUVEC

To test the effect of M476 and N476 protein on HUVEC cells, we firstly demonstrated the binding of these two recombination fusion protein with HUVEC cells. Indirect immunofluorescence staining was shown that in both of HUVEC cells cultures co-incubated with M476 or N476 protein, FITC specific fluorescence label could be found at the surface of the cell. Cell control was set in order to distinguish the nonspecific adsorption of fluorescence label, using PBS instead of protein (**Figure 4**).

The effect of the M476 and N476 protein on the permeability of HUVEC monolayer

We firstly determined the timing that M476 and N476 protein has the greatest impact on the permeability by detecting FITC fluorescence change at full range microplate reader. It was showed that, M476 protein had significant effect on the permeability of HUVEC during the period of 0.5~24 h and 32~48 h, 12 h at the peak. As for N476 protein, it functioned at 0.5~48 h, 3 h at the peak. During the period of 0.5~10 h and 24~36 h, the effect of N476 protein was stronger than M476, while during the period of 10~24 h and 36~48 h, M476 protein had the higher effect (**Table 2**; **Figure 5**).

Then we observed the morphology of HUVEC cells at the peak timing that the effect of M476 and N476 protein was most significant under inverted microscope and TEM. For M476 protein, we observed at 12 h after protein treatment. It was found that the width between cells became wider compared with untreated cells, cell connection was disrupted and cell was shrinking (**Figure 6A**). In cultures of HUVEC coincubated with N476 protein, we observed at 3 h after N476 protein treatment. The similar changes were seen and in some field, cell fusion was seen. (**Figure 6B**) At the respective

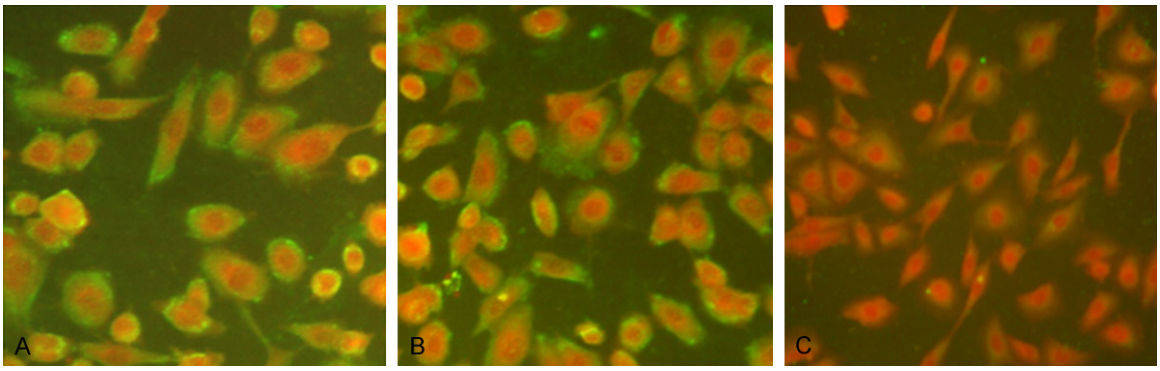

Figure 4. The image of HUVEC cells under fluorescence microscope (200×. A. The combination of HUVEC with M476 protein; B. The combination of HUVEC with N476 protein; C. HUVEC cells in control.

Table 2. Dynamic level of fluorescence values in out-chamber

Time	Fluorescence values			
	M476 pro	N476 pro	LPS	Cell
0.5 h	2211	2683	2405	1193
1 h	2265	3162	3659	2226
3 h	2032	4059	2462	1879
6 h	3042	3054	2088	1740
12 h	3287	2665	3041	1186
24 h	2258	2634	2805	2522
30 h	2027	3170	3020	1897
36 h	3084	2120	4377	1774
42 h	2406	2211	4152	1603
48 h	2002	1581	2399	2280
72 h	2095	2761	2848	2772

timing, TEM found significant ultra-structural changes as well (**Figure 6C-F**).

The result from M group was shown in **Figure 6C** and **6D**. The width of part of nuclear membrane became wider, part of the mitochondrial ridge disappeared and even was of great vacuolization, microvillus dropped and envelope was not complete. The result from NGC group was shown in **Figure 6E** and **6F**. The cell membrane was broken, cell microvillus disappeared, a large amount of mitochondrial was heavily vacuolization, and mitochondrial ridge disappeared.

Discussion

Protein E contains three domains, EDI, EDII and EDIII, respectively. Among them, the antigen from EDI and EDII region can bind to the DENV specific antibody [20], and the mono-antibody which has strong neutralized ability and serum specificity mainly bind to EDIII region [16, 21-23].

The gene in our current research is at the 1-476 bp of the full length E gene of DENV-2. It mainly locate at the EDI region of protein E. EDI is a monomer state core region [14] and the fusion peptide located at EDII region form an extensive finger fold, which may be related to the dimerization and membrane fusion of protein E [15, 16]. The processes of protein E allosteric induced membrane fusion go through EDI region. Therefore it is of great significance to study this region.

The study of the effect of Protein E to the permeability of HUVEC makes us better understand the pathogenesis of DHF/DSS. Vascular endothelial cells as the initial barrier of the circulatory system could regulate cell adhesion molecules and impact the inter-cells communication then mediate the increase of the permeability and cause plasma leakage through regulating cytokines, chemokine, and cell receptors after DENV infection. Infected cells can produce monocytes to affect the growth and permeability of endothelial cells in vitro. Other immune cells, such as lymphocytes, could interact with endothelial cells to cause plasma leakage. DENV could also directly infect HUVEC to induce cell death or apoptosis to destroy vascular wall. It was considered that protein E was participated in the interaction between virus and specific receptors on targeted cell surface and then mediated virus infection directly; actually, the envelope protein of the DENV was the protein E [11].

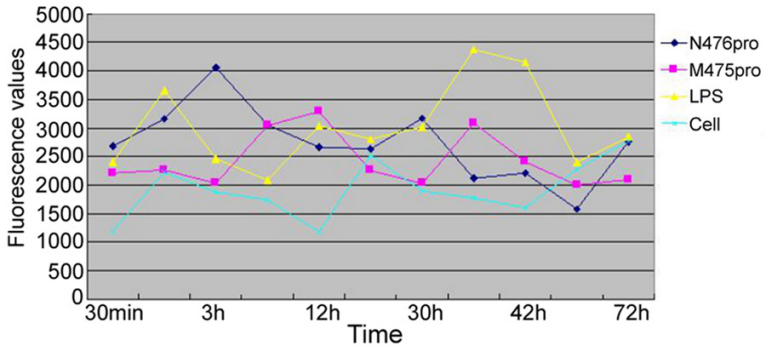

Figure 5. Dynamic level of fluorescence in values in out-chamber.

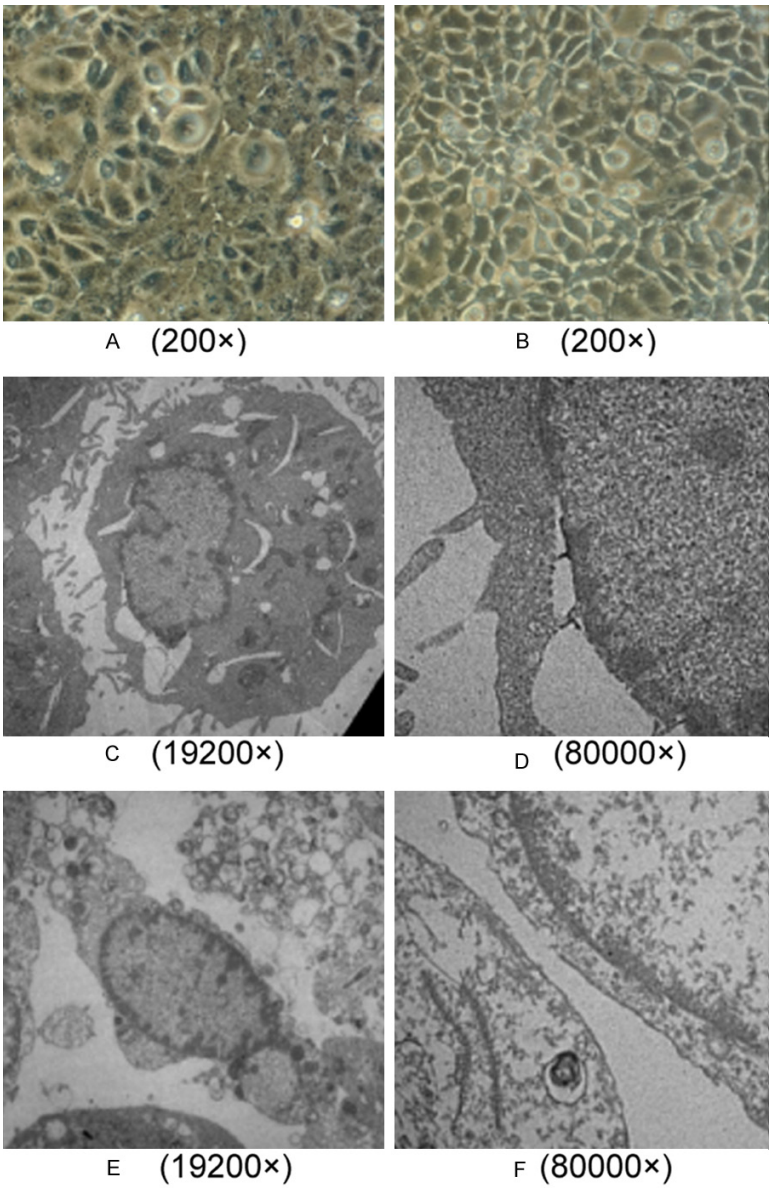

Figure 6. Transmission electron microscope of HUVEC. A. HUVEC inoculate with M476 protein (200×); B. HUVEC inoculate with N476 protein (200×); C and D. Transmission electron microscope of HUVEC inoculate with M476 protein for 12 h; E and F. Transmission electron microscope of HUVEC inoculate with N476 protein for 3 h.

DENV can infect cells in two ways, one is binding with the Fc receptor of sensitive cell through the IgG then to infect cell, which is called ADE effect; the other one is directly interact with the sensitive cells but not the Fc receptor to infect cells. Protein E as an envelope protein is believed to involve in this process [21, 24]. In current study, the binding of the fragment of 1-476 in gene E with HUVEC was similar with the second mechanism, which means it directly binds with the HUVEC but not Fc receptor. We also approved HUVEC is the sensitive cell type to DENV-2. More importantly, this protein significantly increases the permeability of single layer HUVEC and obviously affects the cell shape and ultra-structure in our study.

Cell membrane plays a pivotal role for cell function. Microvillus increases the surface of the cell, which is good for cell uptake. Mitochondrial where oxidation reaction takes place is the energy factory, which could produce energy for cell [20]. The cell in physiological condition interacts with other cell or the basal membrane through cytoskeleton. The change of cytoskeleton function can alter cell shape and the status of cell-cell contact and the cell to basal membrane connection. Meanwhile, the change of the status of cell-cell contact and the cell to basal membrane connection can cause the rearrangement of cytoskeleton protein through cell signal transduction then finally change the permeability of endothelial cells. The change in the constringency of endothelial cells was thought to be the final common pathway resulted from the

alteration of permeability induced from different signal and mechanisms.

The interaction of virus protein with receptor is the initial step of virus infection. Protein E of DENV binding with the receptor in the membrane of target cell is of great importance for the DENV infection to cell. This study pave the road for the better understand the pathogenesis and prevention of DENV infection. For instance design some drug to block the binding site for the virus in the surface of target cell or use some protein containing similar pattern with the virus protein to block the receptor in the surface of sensitive cell. This study will provide some hint to the development of new antivirus method.

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Disclosure of conflict of interest

None.

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