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Prokaryotic expression and identification of scavenger receptor B2

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Summary. – There is still no effective clinical antiviral drug against human enterovirus 71 (EV71) infection, which causes hand, foot and mouth disease (HFMD) in children. Scavenger receptor class B member 2 (SCARB2) is an important receptor of EV71 as it plays a vital role in the early steps of viral infection. In this study, recombinant SCARB2 protein was expressed and purified in a prokaryotic expression system, and was identified by western blot with a monoclonal antibody and mass spectrometry analysis. Detection of the sera from mice immunized with the recombinant SCARB2 protein using ELISA and western blot showed good immunogenicity of the recombinant protein. Furthermore, in the neutralization test cytopathic effect was significantly decreased when EV71 was incubated with the immune sera before infection. In summary, the SCARB2 protein was expressed successfully, and the immune sera showed obvious antiviral effect against EV71. This study provides useful information about the interaction mechanism between SCARB2 and EV71, and is also helpful for further clinical treatment research of HFMD.

Keywords: scavenger receptor class B member 2; enterovirus 71; prokaryotic expression; immunogenicity; neutralization test

Introduction

Hand, foot, and mouth disease is a common febrile disease occurring mainly in infants and children under 5 years (Chatproedprai *et al*., 2015; Huang *et al*., 2009; Li *et al*., 2015). HFMD is usually mild and self-limited. However, various neurological symptoms may occur in some infants, such as brainstem encephalitis, aseptic meningitis, acute flaccid paralysis and fatal neurogenic pulmonary oedema (Chatproedprai *et al*., 2010; Chen *et al*., 2012; Ortner *et al*., 2009; Tu *et al*., 2007). In recent years, epidemic or sporadic outbreaks of neurovirulent EV71 infections have been reported mainly in Southeast Asia, including Taiwan, Malaysia, Singapore, Japan, and China(Lin *et al*., 2012; Sarma, 2013; Xing *et al*., 2014; Zhang *et al*., 2012). Enterovirus 71 (EV71) belongs to the *Enterovirus* genus within the *Picornaviridae* family. It is one of the main pathogens of HFMD which cause fatal neurological diseases (Lee and Chi, 2014; Man-Li *et al*., 2012).

Determining the specific cell receptor is essential to clarify the pathogenic mechanism in the early steps of EV71 infection (Wang *et al*., 1999; Yamayoshi and Koike, 2011). Currently, numbers of cellular receptors and host factors participating in EV71 infection have been identified, such as SCARB2 (Yamayoshi and Koike, 2011), PSGL-1- (Yang *et al*., 2009), vimentin (Du *et al*., 2014) and others. A variety of evidences suggest that SCARB2 plays a critical role in EV71 infection (Yamayoshi *et al*., 2009). SCARB2, a specific glucose cerebrosides esterase binding ligand, is a high-density lipoprotein. It is a type II transmembrane glycoprotein sialic acid, with the relative molecular mass of 85 kDa. SCARB2 is one of the most abundant proteins in the lysosomal membrane and takes part in membrane transport. It is pivotal in the recognition of the lysosomal compartment (Kuronita *et al*., 2002) and works as a receptor for the mannose-6-phosphate-independent transportation from glucocerebrosidase to lysosome (Blanz *et al*., 2010). To this

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Abbreviations: EV71 = enterovirus 71; HFMD = hand, foot and mouth disease; SCARB2 = scavenger receptor class B member 2

day, there is still no effective clinical antiviral drug against EV71 infection. Research and development of safe and effective vaccine, early diagnostic kits and effective antiviral drugs is pressing. In this study, we expressed recombinant SCARB2 protein and proved its immunogenicity. This study has an important benefit for improvement of prevention and therapy of HFMD.

Materials and Methods

Cells, virus, animals. RD cells (human rhabdomyosarcoma cell line) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences. Enterovirus 71 (EV71) C4 genotype (GenBank Acc. No. FJ360545.1) used in this study was isolated and preserved in Guangzhou Women and Children's Medical Center. Six weeks old female BABL/c mice were obtained from Animal Experiment Center of Guangdong Province and performed according to the protocols and guidelines of the Ethics Committee of Animal Experiment Center of Guangdong Province (permit number: SCXK 2013–0002).

Amplification of SCARB2 gene. RD cells were cultured in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, Life, USA) containing 100 U/ml penicillin, 50 U/ml streptomycin and 10% FBS (Life) at 37°C with 5% CO_2 atmosphere. After RNA isolation (RNA extraction kit; Takara, Japan) from cells, the gene of SCARB2 was amplified by reverse transcription PCR (One step RT-PCR kit, Takara). cDNA was synthesized at the following conditions: 30°C for 10 min, 42°C for 30 min and 99°C for 5 min with random 9-mer primers. PCR reaction was then performed at the following conditions**:** 94°C for 5 min followed by 33 cycles at 94°C for 45 s, 56°C for 90 s, 72°C for 2 min. The sequences of primers were: forward 5' GCGGAATTCATGGGCCGATGCTGCTTC TACA 3' and reverse 5' CATCTCGAGTTAGGTTCGAATGAG GGGT 3' (Sangon Biotech,China). The products were analyzed by 1.0% agarose gel electrophoresis and were extracted using a DNA gel extraction kit (Takara).

Construction of the expression vector pET28a-SCARB2. The extracted PCR products were digested with *XhoI* and *Eco*RI and inserted into the pET28a plasmid (Takara) which was previously digested with the same enzymes, resulting in the recombinant plasmid pET28a-SCARB2. Then the recombinant pET28a-SCARB2 or pET28a purified plasmids (Plasmid purification kit; Takara) were transformed into *Escherichia coli* DH5α cells (Takara). Transformed clones were inoculated into 5 ml of Luria-Bertani (LB) medium including 100 μg/μl kanamycin at 37°C overnight, followed by extraction and digestion with *XhoI* and *Eco*RI (Takara). The recombinant plasmid was confirmed by restriction enzyme digestion and sequencing (Invitrogen, China). Finally, the verified pET28a-SCARB2 plasmid was transformed into *Escherichia coli* BL21 (DE3) (Takara) cells for protein expression.

Expression and purification of recombinant protein SCARB2. Expression and purification of recombinant protein was done according previously described methods (Manat *et al.*, 2016). The positive clones were cultivated in 5 ml LB-kanamycin medium at 200 rpm and 37°C overnight. Next day, the overnight cultures of cells carrying the pET28a-SCARB2 vectors were diluted at 1:100 in 500 ml fresh LB-kanamycin medium and left to grow in incubator at 200 rpm and 37°C for 5 h. When the optical density of the culture at 600 nm (OD_{600}) reached 0.5, isopropyl-beta-thio-galactopyranoside (IPTG; Beyotime Biotechnology, China) was added to a final concentration of 1 mmol/l. After additional incubation for 4 h at 37°C, the bacterial pellets were harvested by centrifugation at 3,000 × g for 30 min and the concentration was measured with a BCA protein detection kit (Beyotime Biotechnology, China). The expressed proteins were confirmed by SDS-PAGE. Then the SCARB2 was after staining with 0.25 mol/l KCl solution and cutting extracted from the gel. The gel was washed by distilled water and crushed. The crushed gel was after 3 cycles of freez-thawing collected by centrifugation at 12,000 × g for 2 min. The gel extract in 500 μl phosphate-buffered saline (PBS) was used for animal immunization. The concentration of the purified recombinant SCARB2 protein was determined and used for ELISA.

Identification of recombinant protein SCARB2. The SDS-PAGE gel containing the SCARB2 protein was stained with Coomassie blue (Beyotime Biotechnology). Target bands were cut and analyzed by mass spectrometry, followed by sequence analysis in NCBI database. Proteins were transferred onto PVDF membrane (Millipore, USA), and blocked with 5% non-fat milk in TBST buffer. The membrane was then incubated with mouse His antibody (1:1000) for 1 h at 37°C and HRP labeled goat anti-mouse IgG (1:3,000; Sigma, USA) for 30 min at 37°C. Finally, the bands were detected by DAB substrate (Beyotime Biotechnology).

Indirect ELISA of recombinant SCARB2 interaction with EV71. To analyze the interaction of recombinant SCARB2 protein with EV71 or EV71-VP1, the enzyme-linked immunosorbent assay (ELISA) was conducted as previously reported (Ding *et al.*, 2015). Briefly, the 96-well microtiter plates were coated with 2.5 μg/ml recombinant SCARB2 protein in 50 mmol/l carbonate buffer (pH 9.6) at 4°C overnight and then blocked with 5% non-fat milk in PBST for 2 h at 37°C. After being washed three times with PBST, 100 μl of EV71 (10-fold dilution in PBST) or 5 μg/ml of EV71-VP1 protein (100-fold dilution in PBST) were added and incubated at 37 °C for 1 h. Solutions were removed and 100 μl/well of VP1 monoclonal antibody (Abcam, UK) was added and incubated for 2 h at 37°C. After that, 100 μl/well of HRP labeled goat anti-mouse IgG was added and incubated for 40 min at 37°C. The plates were developed using tetramethylbenzidine and hydrogen peroxide mixture. The reaction was stopped with 50 μl of 2 mol/l of H_2SO_4, and the absorbance at 450 nm was measured on microplate reader (Bio-Rad, USA). The cutoff value was calculated as 0.1 plus mean OD value of the negative control. The values higher than 1.0 were indicated as a positive result.

Immunogenicity experiment. Immunogenicity of recombinant SCARB2 protein was detected as described previously (Ch'ng *et al.*, 2011). Six weeks old female BALB/c mice were randomly divided

into two groups of three mice. Immunization of mice was carried out at day 0, 14, 28 and 41. Purified recombinant SCARB2 protein with concentration of 200μg/ml was emulsified with equal volume of Freund's complete adjuvant (Sigma, USA). A total of 500 μl mixture was administered to the mice via intramuscular route. Sera from mice immunized with PBS were collected as negative control. Forty-two days after the primary immunization, mice were sacrificed and blood was collected for ELISA assay and western blot.

Immunoreactivity of SCARB2 recombinant protein. The sera antibody titers were detected by ELISA. Briefly, the mice serum at the dilutions of 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560, 1:5,120 and 1:10,240 in PBS were incubated with recombinant SCARB2 protein coated in 96-well microtiter plates for 1 h at 37°C. After incubation with HRP labeled goat anti-mouse IgG, the values of A_{450} were recorded. The immunoreactivity of the recombinant SCARB2 was detected by western blot. The recombinant SCARB2 protein transferred onto PVDF membrane was incubated with SCARB2 polyclonal antibody for 1 h at 37°C and HRP labeled goat anti-mouse IgG for 30 min at 37°C and developed by DAB substrate.

Neutralization assay in vitro. The 50% tissue culture infectious dose ($TCID_{50}$) of EV71 virus was detected as previously reported (Pourianfar *et al*., 2012). EV71 virus was diluted and gradient from 10^{-1} to 10^{-10} was added to RD cells in a 96-well plate. The cytopathic effect (CPE) was observed using an inverted microscope 5 days post infection. $TCID_{50}$ calculations were conducted using the Reed-Muench formula. The method of neutralization test *in vitro* was performed as previously mentioned (Li *et al*., 2014). RD cells were prepared by seeding 2×10^5 cells per well in 96-well plates. Four-fold serial dilutions (1:16, 1:64, 1:256, 1:1024) of immune sera were prepared in DMEM. Each dilution was done in quadruples and was challenged with or without equal volume of 200 $TCID_{50}$ of EV71 virus. After incubation for 1 h at 37°C, the mixtures containing serially diluted sera and EV71 were further incubated at 37°C for 5 days. The neutralizing antibody titers were defined as the highest dilution of sera that prevented the occurrence of CPE.

Results

Amplification of SCARB2 gene

The SCARB2 gene was amplified from RD cells using RT-PCR and subjected to analysis with 1.0% agarose gel electrophoresis (Fig 1). The length of the amplified product with about 1300 bp correlated with SCARB2 gene. Gene comparison by BLAST (Kaur *et al.*, 2016) showed that the amplified product was in 99% homologous with SCARB2 gene from GenBank (BT0069391) (Fig. 2).

Verification of recombinant plasmid by enzymatic digestion

To confirm the recombinant plasmid pET28a-SCARB2, plasmid isolated from transformed *E. coli* BL21 (Takara) was digested by *Xho*I and *Eco*RI. Two distinct bands obtained at about 1359 bp and 5300 bp correlated with lengths of SCARB2 gene and pET28a plasmid, respectively (Fig. 1b).

Expression of recombinant SCARB2 protein

The pET28a-SCARB2 plasmid was transformed into *E. coli* BL21 cells. Positive clones were induced by IPTG to produce recombinant protein. To verify the recombinant SCARB2 protein, the *E. coli* cells were lysed, subjected to 10% SDS-PAGE and stained with Coomassie blue. Molecular weight of recombinant protein was 55 kDa (Fig. 3a).

Identification of recombinant protein SCARB2

The recombinant SCARB2 protein was identified by western blot with His monoclonal antibody. The results indicate that the molecular weight of recombinant SCARB2 protein

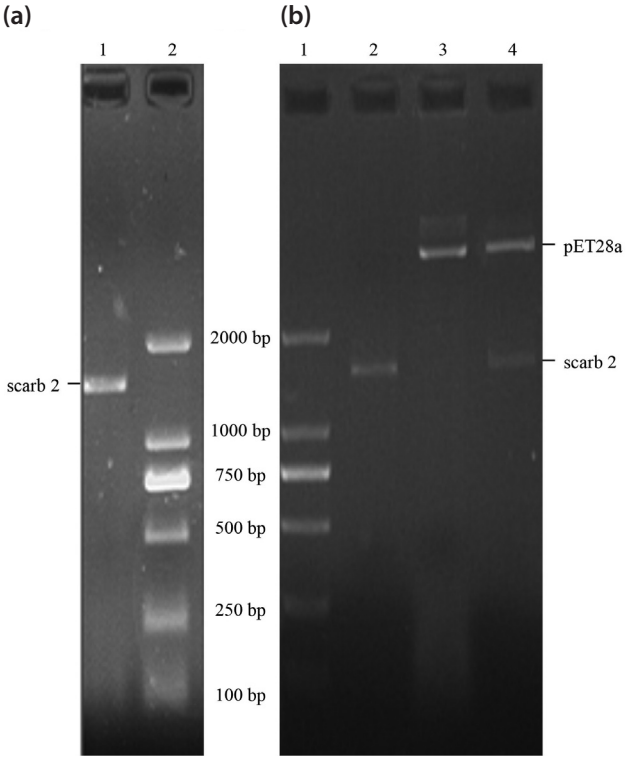


Fig. 1

Amplification of SCARB2 gene and construction of the recombinant plasmid

(a) Agarose gel electrophoresis of SCARB2 gene. Lane 1, amplified SCARB2 gene; lane 2, DL2000 DNA molecular marker. **(b)** Identification of recombinant plasmid by enzymatic digestion. Lane 1, DL2000 DNA molecular marker; lane 2, amplified SCARB2 gene; lane 3, pET28a plasmid; lane 4, recombinant pET28a-SCARB2 plasmid digested by *Eco*RI and *Xho*I.

Fig. 2

Sequence analysis of SCARB2

The results of BLAST analysis show the highest homology in GenBank database SCARB2. Homo sapiens scavenger receptor class B member 2 (SCARB2), transcript variant 1, mRNA Sequence ID: ref NM 005506.3; length: 4780 bp; number of matches: 1; score: 2499; bits 1353; identities: 1358/1360 (99%); gaps: 1/1360 (0%); strand: plus/minus.

with tag was 55 kDa (Fig. 3b). It also showed that His monoclonal antibody reacted with the lysate of induced *E. coli*, but did not react with the uninduced expression bacteria.

The obtained SCARB2 bands were cut from the Coomassie stained gel and analyzed by mass spectrometry. Protein sequence was acquired from NCBI, and protein score was

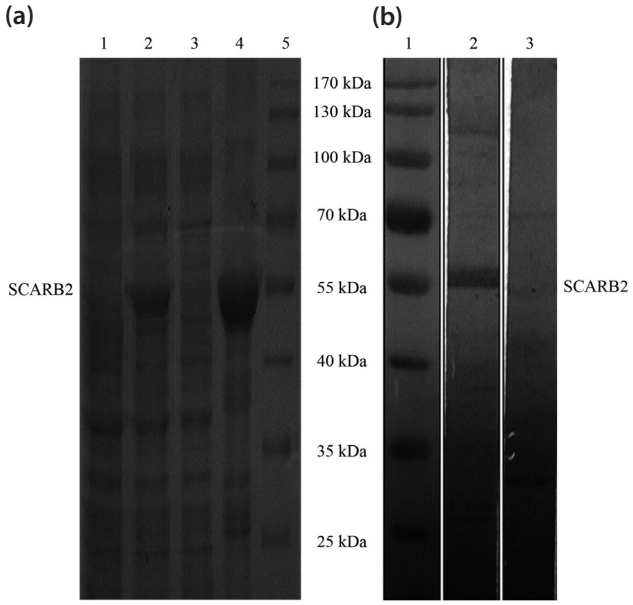
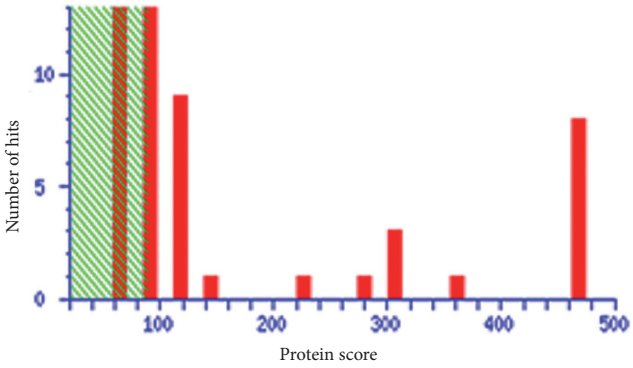


Fig. 3

SDS-PAGE and western blot analysis of recombinant SCARB2 protein **(a)** SDS-PAGE analysis of recombinant SCARB2 protein. Bacterial lysate transformed with pET28-SCARB2 plasmid: lane 1, without induction; lane 2, after induction; lane 3, soluble fraction after induction; lane 4, precipitated fraction after induction; lane 5, protein marker. **(b)** Western blot analysis of recombinant SCARB2 protein using His antibody. Lane 1, protein marker; total proteins of bacterial lysate with pET28a-SCARB2 plasmid: lane 2, after induction; lane3, before induction.



gi|30584161 Mass:54825 Score:469 Expect:4.1e-40 Matches:7

Homo sapiens scavenger receptor class B, member 2 (synthetic construct)

Observed	Mr (expt)	Mr (calc)	ppm	Start		End	Miss	Ions	Peptide
1180.7133	1179.7060	1179.6764	25.1	382	-	390	1	—	K.RFQINIYVK.K
1212.5952	1211.5880	1211.5822	4.72	83	-	92	0	11	R.VEEVGPYTYR.E
1407.7194	1406.7122	1406.7024	5.68	391	-	402	1	—	K.KLDDFVETGDIR.T
1610.8066	1609.7993	1609.7024	-6.66	83	-	95	1	—	R.VEEVGPYTYRELR.N
1646.7971	1645.7898	1645.7447	27.5	263	-	275	0	89	K.DEVLYVFPSDFCR.S
2179.1282	2178.1209	2178.0663	26.4	276	-	294	0	164	R.SVYTITFSDYESVQGLPAFR.Y
2567.3108	2566.3035	2566.2560	18.5	403	-	424	1	142	R.TMVFPVMYLNESVHIDKETASR.L

Fig. 4

Protein mass spectrometry analysis Mascot Score Histogram: Protein score is -10*Log (P), where P was the probability that the observed match is a random event. Protein scores greater than 88 were significant (P <0.05). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits.

-10*Log (P), where P-value was the probability that the observed match was a random event. Protein scores >88 were significant (P <0.05) and false discovery rate was 0.00%, showing that the protein was SCARB2 (Fig. 4).

Recombinant SCARB2 protein and EV71 virus interaction proved by ELISA

To determine the interaction between recombinant SCARB2 and EV71 or EV71-VP1 protein, the ELISA assay was used. SCARB2 reacted with EV71 and VP1 protein (Table 1). Our findings were consistent with Yamayoshi *et al.* (2012) who showed that SCARB2 was an EV71 receptor, and further confirmed that SCARB2 could bind with EV71 virus VP1 protein. This result also confirmed that the recombinant SCARB2 protein has good biological activity.

Table 1. Indirect ELISA of recombinant SCARB2 reaction with EV71and VP1

	VP1 monoclonal antibody (1:100)		Negative mouse serum (1:100)		Control
SCARB2 (1:1000)	EV71 (1:10)	VP1 (1:100)	EV71 (1:10)	VP1 (1:100)	
OD_{450}	1.423	1.274	0.09	0.08	0.06

BALB/c mice immunization with recombinant protein SCARB2

To verify the immunoreactivity of the recombinant protein SCARB2, we have immunized BALB/c mice with the recombinant protein, and successfully obtained the mice sera containing SCARB2 polyclonal antibody. As detected by ELISA, the titer of the SCARB2 polyclonal antibody was 1:16,000. The immunoreactivity of the recombinant protein SCARB2 was confirmed by western blot. The protein at about 55 kDa specifically reacted with the mice sera while no reaction was observed with the negative mice sera (Fig. 5a). These

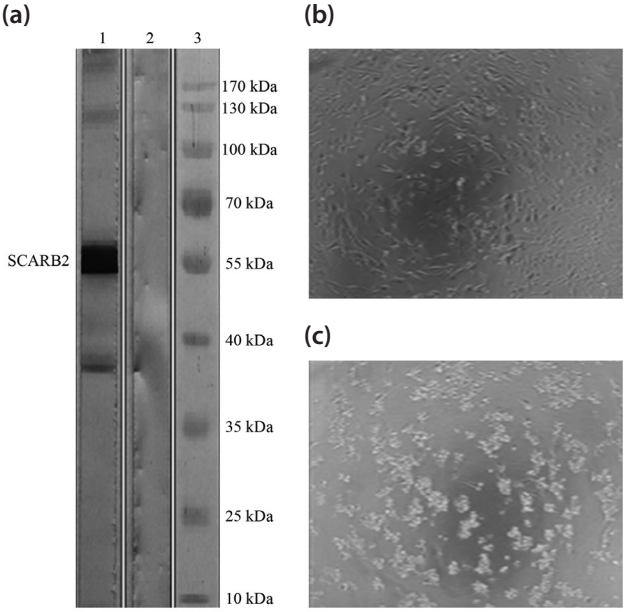


Fig. 5

Immunoreactivity and activity of recombinant SCARB2 protein
(a) Immunoreactivity of recombinant SCARB2 protein. Recombinant protein SCARB2 detected with: lane 1, polyclonal antibodies; lane 2, negative control sera; lane3, protein marker. **(b)** Non-infected RD cells. **(c)** Cytopathic effect of RD cells after EV71 infection.

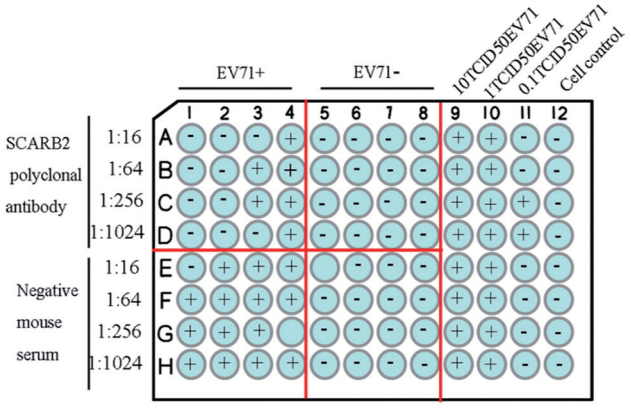


Fig. 6

Virus neutralization experiment *in vitro*
Immune and negative mouse sera at dilutions of 1:16, 1:64, 1:256, 1:1,024, were incubated with 100 $TCID_{50}$ EV71. Virus control: 10 $TCID_{50}$ EV71, 1 $TCID_{50}$ EV71, 0.1 $TCID_{50}$ EV71. Cell control: non-infected RD cells. "+" indicates positive CPE; "-" indicates negative CPE.

results suggest that polyclonal antibody against recombinant SCARB2 protein generated in mice could react with purified recombinant SCARB2 protein specifically.

SCARB2 polyclonal antibody neutralization test in vitro

EV71 virus $TCID_{50}$ estimation was implemented in a 96-well plate, by the cumulative cytopathic effect (Table 2). $TCID_{50}$ value of EV71 virus used in this study was 10^{-7}/ml, meaning that 100 µl of this EV71 virus diluted 10^{7} times could infect 50% of RD cells in one well. In order to verify the neutralizing activity of the SCARB2 polyclonal antibody (SCARB2 pAb) in mice sera, we identified a series of concentrations of SCARB2 pAb that may prevent RD cells from EV71 infection. *In vitro* neutralizing antibody titer against EV71 in the mice sera exceeded 1:256 (Fig. 6). According to these results, the immune mice serum is a good candidate for viral diagnostics.

Discussion

The pivotal event in the EV71 infection proceeding is the interaction of virus and specific receptor on the host cells

Table 2. $TCID_{50}$ calculation (inoculation dose 50 µl)

	Inoculation	CPE	No CPE	Total		CPE rate	Percentage
				CPE	No CPE		
10^{-1}	8	8	0	52	0	52/52	100
10^{-2}	8	8	0	44	0	44/44	100
10^{-3}	8	8	0	36	0	36/36	100
10^{-4}	8	8	0	28	0	28/28	100
10^{-5}	8	8	0	20	0	20/20	100
10^{-6}	8	8	0	12	0	12/12	100
10^{-7}	8	4	4	4	4	4/8	50
10^{-8}	8	0	8	0	12	0/12	0
10^{-9}	8	0	8	0	20	0/20	0
10^{-10}	8	0	8	0	28	0/28	0

surface (Lin and Shih, 2014). The interaction between the virus and host cell receptor plays an extremely important role in early steps of viral infection. Therefore, it is very important to identify and describe receptors. Two different membrane proteins, human P-selection glycoprotein ligand-1 (Frenette *et al.*, 2000) and human SCARB2 protein (Yamayoshi *et al.*, 2009) have been identified as cellular receptor of EV71. SCARB2 protein is widely expressed and directly involved in systemic infection of human body. The replacement of exon 4 plays an important role in the interaction with EV71 and SCARB2 protein (Yamayoshi and Koike, 2011). This shows the widespread use of SCARB2 protein as an EV71 receptor (Yamayoshi *et al.*, 2012).

As far as we know, most of receptors are glycoproteins and lipoproteins in cell membranes; however the number of the receptor molecules is too low to be isolated and purified. In this study, we successfully amplified SCARB2 gene and constructed the pET28a-SCARB2 vector. SCARB2 protein was expressed in an *E. coli* system. Purified recombinant SCARB2 protein was identified by western blot using His monoclonal antibody and mass spectrometry analysis. Expressed SCARB2 protein could interact with EV71-VP1 protein. This result is consistent with Yamayoshi's conclusion that SCARB2 is receptor of EV71virus (Yamayoshi *et al.*, 2012). But the SCARB2 protein used in Yamayoshi's experiment was expressed by eukaryotic expression system, while our results show that the recombinant protein expressed in prokaryotic system also had good biological activity. We have successfully immunized BALB/c mice with SCARB2 protein, and further confirmed that the recombinant SCARB2 protein had good immunoreactivity. In order to verify the neutralizing activity of the anti SCARB2 sera, we performed neutralization test *in vitro.* The results suggested that the mice sera immunized with SCARB2 can not only neutralize EV71 virus but also decrease the CPE of RD cells infected with EV71 virus. The antibody neutralizing titer was 1:256. These results provide an experimental basis of EV71 infection and pathogenesis and help to understand the precise interaction between EV71 and SCARB2 receptor. Also the study provides options for the development of EV71 vaccine and antiviral drugs research. Furthermore, further study of the SCARB2 monoclonal antibody will provide further theoretical basis for studies of antiviral drugs.

In conclusion, SCARB2 protein was successfully expressed in a prokaryotic expression system and was effectively expressed in *E. coli* BL21 for the first time. Purified recombinant SCARB2 protein had a strong immunoreactivity as well as good biological activity. Sera from mice immunized with the protein could neutralize EV71 and prevent EV71 infection of RD cells. This study provides a reference for interacting mechanism between EV71 and SCARB2 receptor, and for development of prophylactic treatment against EV71 infection.

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