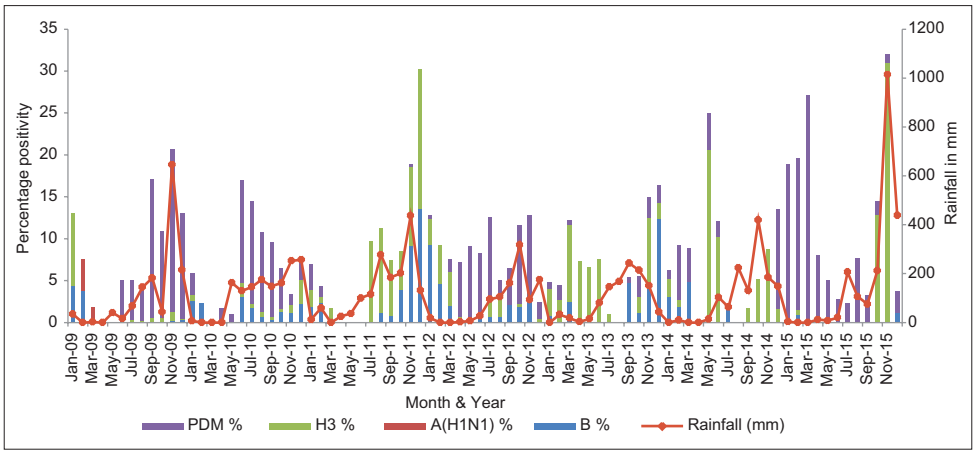
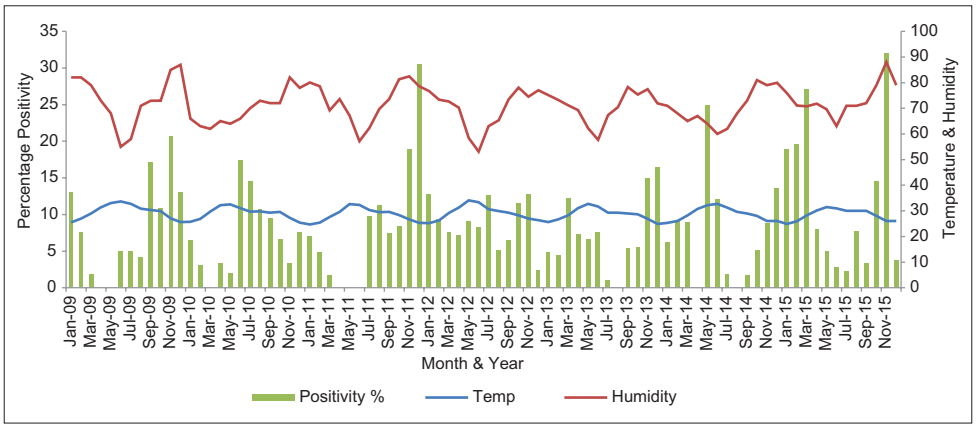


Graph 2: Impact of rainfall on influenza strains - Monthly trends (2009–2015)



Graph 3: Influence of temperature and humidity on influenza positivity (2009–2015)

in comparison with other tropical countries such as Vietnam and Taiwan where it was established the fact that winter proved to be favourable for influenza.[21,22]

Hence, in Chennai with high levels (70%–82% maximum) of humidity throughout the year [Graph 3], the virus remains relatively stable, especially during the monsoon and post-monsoon months. Influenza viruses are more stable in the cold favouring robust and highly efficient transmission at 30°C that may be due to an increase in virus half-life at lower temperatures.[23,24] In Chennai, where it is hot and humid, the graph shows marginal fall in temperature in the months of September to January and with most of the outbreaks or positivity peaks occur during these months, probably lowering of temperature along with the increase in rainfall aids transmission. The increased shedding may be due to the effect of cold conditions in the host. Alternatively, virus may be more stable within the nasal passages when the cold ambient air cools the epithelial surface.[25] Increased virion stability at lower temperatures is likely due in part to decreased activity of proteases, hence augmented transmission. Transmission would be mainly affected by decrease in temperature and low humidity.[26]

As far as vaccination is concerned, initiation of vaccination before monsoon will be very effective in Tamil Nadu, hence the month of July – August can be the preferred month considering the districts bordering Western ghats which starts receiving monsoon earlier when compared to central districts and east coast of Tamil Nadu.[27]

As suggested in few articles, as a National policy, the latitude 30 can be taken to consideration and vaccination can be initiated accordingly,[28] with the region below 30 receiving vaccination by May–June.

Conclusion

The pattern of seasonality influencing influenza in different geographical regions remains unresolved. A thorough understanding of the meteorological factors affecting the virus is of major concern. Further research is particularly essential in tropical and subtropical areas, where the understanding of seasonality is negligible and requires a combination of experimental and observational studies. Moreover, understanding of the environmental factors that drive influenza circulation also may be useful to predict how dynamics will be affected at regional levels by global climate change. The National health authorities can plan vaccination against Influenza before the occurrence of Influenza with respect to the pattern of occurrence in different states.

The study proves the fact that influenza activity is more during monsoon and post-monsoon months with a tendency to cause outbreaks in waves within a span of 2 or 3 years. The public health authorities can also take cue from this analysis to initiate Institutional Ethical Committee (IEC) activities and other containment activities during the pre-monsoon period annually, with special educative programs conducted in schools, colleges and areas of congregation.

The role of environmental factors in the occurrence of Influenza in context to changing climatic and environmental scenario will give an insight to the perception on influenza which has profound value and of public health concern.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

VP1-Binding Protein Glucose-regulated protein 78 as an important mediator for Enterovirus 71 infecting Human Brain Microvascular Endothelial Cells

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Abstract

Purpose: Enterovirus 71 (EV71) is one of the main pathogens causing hand, foot and mouth disease, which could even induce severe brain damage in some patients. As the underlying mechanism of the invasion and replication process still remains largely unknown, we investigated the role of candidate proteins expressed during EV71 invasion in human brain microvascular endothelial cells (HBMECs) to delineate the pathophysiological mechanism of EV-71 infection. **Materials and Methods:** Ninety-one candidate EV71-associated proteins which could bind the major capsid protein (viral protein 1 [VP1]) of EV71 on the HBMEC were identified by applying an analysis of glutathione-S-transferase pull-down coupling with liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS). Seventy-eight kDa glucose-regulated protein 78 (GRP78) binding to the VP1 protein was further validated by co-immunoprecipitation, immunofluorescence and western blot analysis. To explore the role of GRP78 in EV71 infection, GRP78 was knocked down and overexpressed in HBMEC and was verified by TCID50 assay. **Results:** LC-ESI-MS/MS-identified 91 proteins were subjected to gene ontology analysis, and on molecular and biological function analysis revealed GRP78 act as an important binding protein in mediating EV71 infection. In addition, immunofluorescence demonstrated the co-localisation of GRP78 and VP1 in cytoplasm of the infected HBMEC. The TCID50 assay showed that knockdown of GRP78 could attenuate the replication capacity of EV71 in HBMEC, and the overexpression could increase the virus titre in HBEMC at 24 h post-infection suggesting that GRP78 was associated with the replication capacity of EV71 in HBMEC. **Conclusion:** These findings provided evidence that GRP78 plays an important role during the progression of EV71 infection as a mediator in HBMEC.

Keywords: Brain damage, enterovirus 71, glucose-regulated protein 78 , human brain microvascular endothelial cells

Introduction

Enterovirus 71 (EV71) is a single-stranded positive-sense RNA virus with a genome size of about 7.4 kb, which has been recognised as one of the causative factors of hand, foot and mouth disease (HFMD).[1,2] EV71 infection may cause severe diseases and can even lead to complications involving central nervous system (CNS) in children.[1,3] As for enterovirus, two possible routes by which EV71 reaches the CNS have been suggested: the EV71 either enters the CNS from the blood across the blood–brain barrier (BBB) or is transmitted to the CNS through peripheral nerves through retrograde axonal transport.[4] However, the underlying mechanism that leads to transmigration of EV71 virus particles across BBB still remains largely unclear.

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Viral protein 1 (VP1) is a structural protein exposed on the surface of EV71, which is one of the key virulence factors in the pathogenesis of EV71 during the recognition process of the virus entering into host cells.[5,6] Our previous study showed that EV71 could infect and replicate in human brain microvascular endothelial cells (HBMEC),[7] which is the prime component of BBB.[8] In this study, glutathione-S-transferase (GST) pull-down coupling with liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) method identified EV71-associated proteins in HBMEC. Among these proteins, glucose-regulated protein 78 (GRP78) was further verified as a potential VP1-binding protein, which was knocked down and overexpressed in HBMEC, and the invasion ability of EV71 in HBMEC was measured. In this study, we investigated the major virulence factors expressed on the surface of the virus and associated proteins that could play a significant role in viral invasion and further replication of the viral particles inside the host cells.

Materials and Methods

Materials

HBMEC was a generous gift from professor Huang (University of Southern California, USA), and human embryonic rhabdomyosarcoma (RD) cells were obtained from the Centre for Disease Control and Prevention of Guangdong Province, respectively. EV71 (KC122766) isolated from a severe HFMD patient was previously conserved in our laboratory. The ProteoExtract® Subcellular Proteome Extraction Kit was purchased from MerckMillipore (Billerica, MA, USA). PCMV-HA tag vector and PCMV-Flag tag vector were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-human EV71 Monoclonal Antibody, Pierce GST Protein Interaction Pull-Down Kit, Pierce BCA Protein Assay Kit, Pierce C18 Spin Columns and Halt™ Protease and Phosphatase Inhibitor Cocktail (100×) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). EV71 VP1 recombinant protein was purchased from Abnova (Taipei, Taiwan). The sequence grade trypsin protease was purchased from Promega (Madison, WI, USA). Dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Trifluoroacetic acid, formic acid, acetonitrile and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Dulbecco's Modified Eagle Medium and foetal bovine serum were purchased from GIBCO (Grand Island, NY, USA).

Cell culture and treatment

The HBMECs were cultured and treated with EV71 particles as described previously.[9] The cells exhibited the typical characteristics of brain endothelial cells with tight junctions and apical-to-basal polarity.[10] EV71 virus was amplified using RD cells, and the viral titres were estimated as described previously.[9,11] In all experiments, HBMECs were infected with the respective EV71 virus at a multiplicity of infection (MOI) of 5 PFU/cell.

Protein extraction and glutathione-S-transferase pull-down assay

Subcellular proteins of HBMEC were extracted following the manufacturer's instructions of ProteoExtract® Kit (Millipore, USA). The extracted proteins in the supernatant were kept for further GST pull-down analysis. GST pull-down assay was conducted following the instructions of pierce GST Protein Interaction Pull-Down Kit. EV71 VP1 recombinant protein (Taipei, Taiwan) was used as GST-fusion probe protein. Candidate VP1-binding proteins obtained from GST pull-down were quantified by a BCA protein quantification kit (Thermo). The VP1-binding protein was used for further study, including LC separation and MS analysis.

Liquid chromatography separation and mass spectrometry analysis

Eluted protein (100 μg) was reduced by 10 mmol/L DTT, alkylated by 20 mmol/L IAA and then digested by trypsin (Promega) at 37°C overnight. The peptides were desalted using C18 spin columns (Thermo) and were redissolved in 2% acetonitrile, 0.1% formic acid and loaded on a ChromXP C18 (3 μm, 120 Å) nanoLC trap column. The online trapping and desalting procedure were carried out at 2 μL/min for 10 min, with 0.1% formic acid in 98% acetonitrile. An elution gradient of 5%–35% acetonitrile (0.1% formic acid) in a 90 min gradient was used on an analytical column (3 μm, 100 Å, 75 μm i.d. ×15 cm, Acclaim PepMap100, C18, Dionex). MS/MS analysis was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON). Data were acquired using an ion spray. The MS was operated with TOF-MS scans. Survey scans were acquired in 250 ms, and a maximum product ion scans were collected if counts reached a threshold of 150 counts/s with a +2 to +5 charge state. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Proteins were identified by searching against the UniProt human protein database with MASCOT search engine.

Co-IP analysis

GRP78 binding to VP1 was further validated following the instructions of Co-IP kit (Waltham, MA, USA). PCMV-HA tag vector and PCMV-Flag tag vector were used for the expression and purification of VP1 and GRP78 proteins, respectively. For Co-IP, subcellular proteins of HBMEC were extracted as described above and mixed with the VP1-HA-tag recombinant protein. EV71 sample was purified and mixed with GRP78-Flag-tag recombinant protein. The mixture was incubated with antibody against VP1 or GRP78 overnight at 4°C and then added with Protein A/G agarose beads for additional gentle rocking for 2.5 h at 4°C. The IgG was used to serve as negative control. After centrifugation for five times at 1000 g for 5 min and washing with lysis buffer, the pellet was resuspended with 30 μl of sodium dodecyl sulphate (SDS) sample buffer. All the samples were analysed by SDS-PAGE and western blot (WB) analysis as described below.

Table 1: Differential proteins identified by mass spectrometry based on glutathione-S-transferase-pull-down analysis

N	Unused	Total	% CI (95%)	Accession	Protein name	Peptides (95%)	Gene
1	2	2	1.595	sp|P29083|T2EA_HUMAN	Reversed general transcription factor IIE subunit 1	1	PRH1
2	4.52	4.52	6.015	sp|O43813|LANC1_HUMAN	LanC-like protein 1	2	LANCL1
3	1.9	2.02	4.127	sp|O43852|CALU_HUMAN	Calumenin	1	CALU
4	6.03	6.03	8.605	sp|P00505|AATM_HUMAN	Aspartate aminotransferase, mitochondrial	3	GOT2
5	2	2	7.801	sp|P01037|CYTN_HUMAN	Cystatin-SN	1	CST1
6	2.06	2.06	12.24	sp|P01040|CYTA_HUMAN	Cystatin-A	1	CSTA
7	5.27	5.27	5.723	sp|P02545|LMNA_HUMAN	Prelamin-A/C	3	LMNA
8	2	2	26.51	sp|P02810|PRPC_HUMAN	Salivary acidic proline-rich phosphoprotein 1/2	1	PRH1
9	2	2	39.24	sp|P02814|SMR3B_HUMAN	Submaxillary gland androgen-regulated protein 3B	1	SMR3B
10	13.85	13.85	27.16	sp|P04406|G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	8	GAPDH
11	13.69	13.69	19.18	sp|P04745|AMY1_HUMAN	Alpha-amylase 1	7	AMY1A
12	2	2	16.67	sp|P05204|HMGN2_HUMAN	Non-histone chromosomal protein HMG-17	1	HMGN2
13	6.16	6.16	8.696	sp|P06576|ATPB_HUMAN	ATP synthase subunit beta, mitochondrial	3	ATP5B
14	4.02	4.02	3.741	sp|P06748|NPM_HUMAN	Nucleophosmin=Homosapiens GN	2	NPM1
15	4.45	16.1	61.9	sp|P06899|H2B1J_HUMAN	Histone H2B type 1-J	7	HIST1H2BJ
16	4.4	4.4	5.512	sp|P07237|PDIA1_HUMAN	Protein disulphide-isomerase	2	P4HB
17	2.75	2.75	10.31	sp|P07305|H10_HUMAN	Histone H1.0	1	H1F0
18	35.47	35.47	48.08	sp|P07355|ANXA2_HUMAN	Annexin A2	18	ANXA2
19	2	2	10	sp|P07737|PROF1_HUMAN	Profilin-1	1	PFN1
20	2.02	4.02	3.591	sp|P08238|HS90B_HUMAN	Heat shock protein HSP 90-beta	2	HSP90AB1
21	6.32	6.32	8.584	sp|P08670|VIME_HUMAN	Vimentin	3	VIM
22	2	2	4.407	sp|P08865|RSSA_HUMAN	40S ribosomal protein SA	1	RPSA
23	18.9	18.9	60.48	sp|P09211|GSTP1_HUMAN	Glutathione S-transferase P	15	GSTP1
24	2	2	4.93	sp|P09493|TPM1_HUMAN	Tropomyosin alpha-1 chain	1	TPM1
25	10.13	10.13	13.96	sp|P10809|CH60_HUMAN	60 kDa heat shock protein, mitochondrial	5	HSPD1
26	34.95	34.95	31.04	sp|P11021|GRP78_HUMAN	78 kDa glucose-regulated protein	16	HSPA5
27	2.45	4.61	4.334	sp|P11142|HSP7C_HUMAN	Heat shock cognate 71 kDa protein	2	HSPA8
28	2.15	2.15	0.8969	sp|P12814|ACTN1_HUMAN	Alpha-actinin-1	1	ACTN1
29	6.37	6.37	5.271	sp|P13667|PDIA4_HUMAN	Protein disulphide-isomerase A4	3	PDIA4
30	2	2	5.069	sp|P13929|ENOB_HUMAN	Beta-enolase	1	ENO3
31	2	2	3.013	sp|P14618|KPYM_HUMAN	Pyruvate kinase PKM	1	PKM
32	16.38	16.38	11.83	sp|P14625|ENPL_HUMAN	Endoplasmin	8	HSP90B1
33	11.47	11.47	19.86	sp|P16152|CBR1_HUMAN	Carbonyl reductase [NADPH] 1	5	CBR1
34	11.36	11.36	18.55	sp|P16402|H13_HUMAN	Histone H1.3	6	HIST1H1D
35	0	11.56	14.87	sp|P19961|AMY2B_HUMAN	Alpha-amylase 2B	6	AMY2B
36	12.02	12.02	31.11	sp|P21266|GSTM3_HUMAN	Glutathione S-transferase Mu 3	6	GSTM3
37	2.6	2.6	14.47	sp|P22392|NDKB_HUMAN	Nucleoside diphosphate kinase B	2	NME2
38	7.68	7.68	19.44	sp|P23284|PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B	5	PPIB
39	2	2	6.627	sp|P23528|COF1_HUMAN	Cofilin-1	1	CFL1
40	4	4	12.44	sp|P24534|EF1B_HUMAN	Elongation factor 1-beta	2	EEF1B2
41	2	2	3.356	sp|P25311|ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	1	AZGP1
42	2.58	2.58	2.712	sp|P25705|ATPA_HUMAN	ATP synthase subunit alpha, mitochondrial	1	ATP5A1
43	8.91	8.91	11.9	sp|P26641|EF1G_HUMAN	Elongation factor 1-gamma	4	EEF1G
44	4.7	4.7	9.832	sp|P27797|CALR_HUMAN	Calreticulin	2	CALR
45	2.06	2.06	0.9549	sp|P27816|MAP4_HUMAN	Microtubule-associated protein 4	1	MAP4
46	8.03	8.03	10.1	sp|P30101|PDIA3_HUMAN	Protein disulphide-isomerase A3	4	PDIA3
47	1.31	1.31	2.016	sp|P30419|NMT1_HUMAN	Glycylpeptide N-tetradecanoyltransferase 1	1	NMT1
48	2.65	2.65	3.385	sp|P35914|HMGCL_HUMAN	Hydroxymethylglutaryl-CoAlyase, mitochondrial	1	HMGCL
49	2	2	5.528	sp|P37802|TAGL2_HUMAN	Transgelin-2	1	TAGLN2
50	4.53	4.53	3.829	sp|P38646|GRP75_HUMAN	Stress-70 protein, mitochondrial	2	HSPA9
51	1.34	1.34	10.34	sp|P39019|RS19_HUMAN	40S ribosomal protein S19	1	RPS19
52	5	5	11.92	sp|P42126|ECI1_HUMAN	Enoyl-CoA delta isomerase 1, mitochondrial	3	ECI1
53	2	2	1.726	sp|P53396|ACLY_HUMAN	ATP-citrate synthase	1	ACLY

Contd...

Table 1: Contd...

N	Unused	Total	% Cov (95%)	Accession	Protein name	Peptides (95%)	Gene
54	3.6	5.71	6.886	sp|P54652|HSP72_HUMAN	Heat shock-related 70 kDa protein 2	3	HSPA2
55	0	15.14	26.93	sp|P60709|ACTB_HUMAN	Actin, cytoplasmic 1	9	ACTB
56	2	2	4.187	sp|P60842|IF4A1_HUMAN	Eukaryotic initiation factor 4A-I	1	EIF4A1
57	2.11	2.11	8.108	sp|P61626|LYSC_HUMAN	Lysozyme C	1	LYZ
58	2	2	3.456	sp|P61978|HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K	1	HNRNPK
59	2	2	10.74	sp|P62158|CALM_HUMAN	Calmodulin	1	CALM1
60	2	2	4.135	sp|P62424|RL7A_HUMAN	60S ribosomal protein L7a	1	RPL7A
61	12.18	12.18	61.17	sp|P62805|H4_HUMAN	Histone H4	7	HIST1H4A
62	5.85	5.85	16.36	sp|P62937|PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	3	PPIA
63	4.84	4.84	22.66	sp|P62987|RL40_HUMAN	Ubiquitin-60S ribosomal protein L40	2	UBA52
64	2	2	4.237	sp|P63096|GNAI1_HUMAN	Guanine nucleotide-binding protein G (i) subunit alpha-1	1	GNAI1
65	15.14	15.14	26.93	sp|P63261|ACTG_HUMAN	Actin, cytoplasmic 2	9	ACTG1
66	2	2	2.804	sp|P78371|TCPB_HUMAN	T-complex protein 1 subunit beta	1	CCT2
67	6	6	22.73	sp|P81605|DCD_HUMAN	Dermcidin	3	DCD
68	2	2	10.48	sp|P99999|CYC_HUMAN	Cytochrome c	1	CYCS
69	7.61	7.61	18.59	sp|Q06830|PRDX1_HUMAN	Peroxiredoxin-1	4	PRDX1
70	2	2	1.222	sp|Q09666|AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	1	AHNAK
71	2	2	3.245	sp|Q16658|FSCN1_HUMAN	Fascin	1	FSCN1
72	1.32	10.93	50	sp|Q16778|H2B2E_HUMAN	Histone H2B type 2-E	6	HIST2H2BE
73	2	2	6.277	sp|Q5VTE0|EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3	1	EEF1A1P5
74	2	2	2.14	sp|Q6S8J3|POTEE_HUMAN	POTE ankyrin domain family member E	1	POTEE
75	10.02	10.02	36.03	sp|Q71DI3|H32_HUMAN	Histone H3.2	6	HIST2H3A
76	36.81	36.81	10.04	sp|Q8IYH5|ZZZ3_HUMAN	ZZ-type zinc finger-containing protein 3	21	ZZZ3
77	2	2	5.291	sp|Q8NHU0|CT453_HUMAN	Cancer/testis antigen family 45 member A3	1	CT45A3
78	7.94	7.94	40.77	sp|Q93077|H2A1C_HUMAN	Histone H2A type 1-C	4	HIST1H2AC
79	4.32	4.32	11.54	sp|Q96DA0|ZG16B_HUMAN	Zymogen granule protein 16 homolog B	2	ZG16B
80	2	2	5.528	sp|Q96HU8|DIRA2_HUMAN	GTP-binding protein Di-Ras2	1	DIRAS2
81	2.18	2.18	1.923	sp|Q99798|ACON_HUMAN	Aconitate hydratase, mitochondrial	1	ACO2
82	17.14	17.14	61.9	sp|Q99877|H2B1N_HUMAN	Histone H2B type 1-N	8	HIST1H2BN
83	13.05	13.05	50	sp|Q99879|H2B1M_HUMAN	Histone H2B type 1-M	7	HIST1H2BM
84	2	2	7.143	sp|Q99880|H2B1L_HUMAN	Histone H2B type 1-L	1	HIST1H2BL
85	2.05	2.05	3.341	sp|Q9BQE3|TBA1C_HUMAN	Tubulin alpha-1C chain	1	TUBA1C
86	2	2	8.571	sp|Q9NPJ3|ACO13_HUMAN	Acyl-coenzyme A thioesterase 13	1	ACOT13
87	4	4	21.34	sp|Q9NS18|GLRX2_HUMAN	Glutaredoxin-2, mitochondrial	3	GLRX2
88	2	2	3.804	sp|Q9NV31|IMP3_HUMAN	U3 small nucleolar ribonucleoprotein protein IMP3	1	IMP3
89	2.81	2.81	10.37	sp|Q9UBQ7|GRHPR_HUMAN	Glyoxylate reductase/hydroxypyruvate reductase	2	GRHPR
90	2	2	6.962	sp|Q9UGM3|DMBT1_HUMAN	Deleted in malignant brain tumours 1 protein	1	DMBT1
91	2	2	0.5848	sp|Q9ULK4|MED23_HUMAN	Mediator of RNA polymerase II transcription subunit 23	1	MED23

N: The rank of the specified protein relative to all other detected proteins, Unused: Minimum protein threshold of 95% confidence (unused protein score >1.3), % Cov (95): The percentage of matching amino acids from identified peptides having confidence ≥95%, divided by total number of amino acids in the sequence, Accession: Accession number of detected protein; Name: Name of the detected protein, Peptides (95%): The number of distinct peptides having at least 95% confidence. Multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides. GST: Glutathione-S-transferase

Immunofluorescence assay

HBMEC (1×10^5) was placed on a coverslip and allowed for virus adhesion for 24 h. The cells were washed with Phosphate Buffered Saline (PBS) for three times and fixed with 4% paraformaldehyde for 20 min, blocked with 1% bovine serum albumin for 1 h. The treated cells were incubated with the rabbit anti-human GRP78 monoclonal antibody and rabbit anti-human EV71 monoclonal antibody overnight at 4°C. After washing, cells were stained with PE-labelled and FITC-labelled donkey anti-rabbit IgG and then washed for three times and examined under a fluorescent microscope (LeicaDMI3000B, German).

Overexpression and knockdown of glucose-regulated protein 78 in human brain microvascular endothelial cells

Three pairs of RNAi primers and overexpression vector were synthesised by Ribo Limited Co. Overexpression vector (pcnda3.1-GRP78) and the small interfering RNA (siRNA) of GRP78 were transfected, respectively, into HBMEC using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's recommendations. The interference efficiency and expression were tested by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and WB.

TCID50 assay

HBMECs were treated with siRNA-3 oligonucleotides, a universal sequence that had no significant homology to any known human mRNA in the databases, empty vector and overexpression vector targeted GRP78 for 24 h. Then, the culture medium was discarded and cultured with fresh medium for another 24 h. After the GRP78 siRNA group (HBMEC infected with GRP78 siRNA), the scramble control group (HBMEC infected with negative control), the control group (uninfected HBMEC), empty vector and overexpression vector groups were infected EV71 particles (MOI = 5) at 37°C with 5% CO_2 for 1.5 h, and the virus supernatant was discarded. HBMECs were washed with hank's solution for once, and the time of infection was calculated at this time point. Finally, the culture medium and cells among the four groups at 1 h and 24 h were collected for determination of the virus titre of EV71 by a TCID50 assay. Briefly, the viral titres were determined on RD cell monolayers by means of the TCID50 standard method and calculated using the Reed–Muench protocol.[12]

Reverse transcription-quantitative polymerase chain reaction and Western blot analysis

For quantitative RT-PCR, total mRNA of the treated HBMECs was extracted using RNAesy Kit (QIAGEN). Each sample was measured in triplicate. The primers for GRP78 (primer F: GAGAAGTTTGCTGAGGAAGA, primer R: GAAAGTTTACCTCCCAGCTT) were designed using DNASIS Max. Glyceraldehyde -3-phosphatedehydrogenase (GAPDH) was chosen as the reference gene. PCR reactions were conducted in 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The cycle conditions included an initial denaturation step at 95°C for 2 min, followed by 40 amplification cycles for 15 s at 95°C and 1 min at 60°C. Relative expression for mRNA was determined using $2^{-\Delta\Delta Ct}$ method. All reactions were performed in triplicate. Proteins of HBMEC were extracted using PhosphoSafe™ lysis reagent for WB analysis. The proteins were boiled, electrophoresed by SDS-PAGE and transferred on to Polyvinylidene Difluoride (PVDF) membranes. The membranes were incubated with antibodies against VP1, GRP78 and GAPDH (diluted by TBST at 1:1000) respectively, then washed and incubated with goat

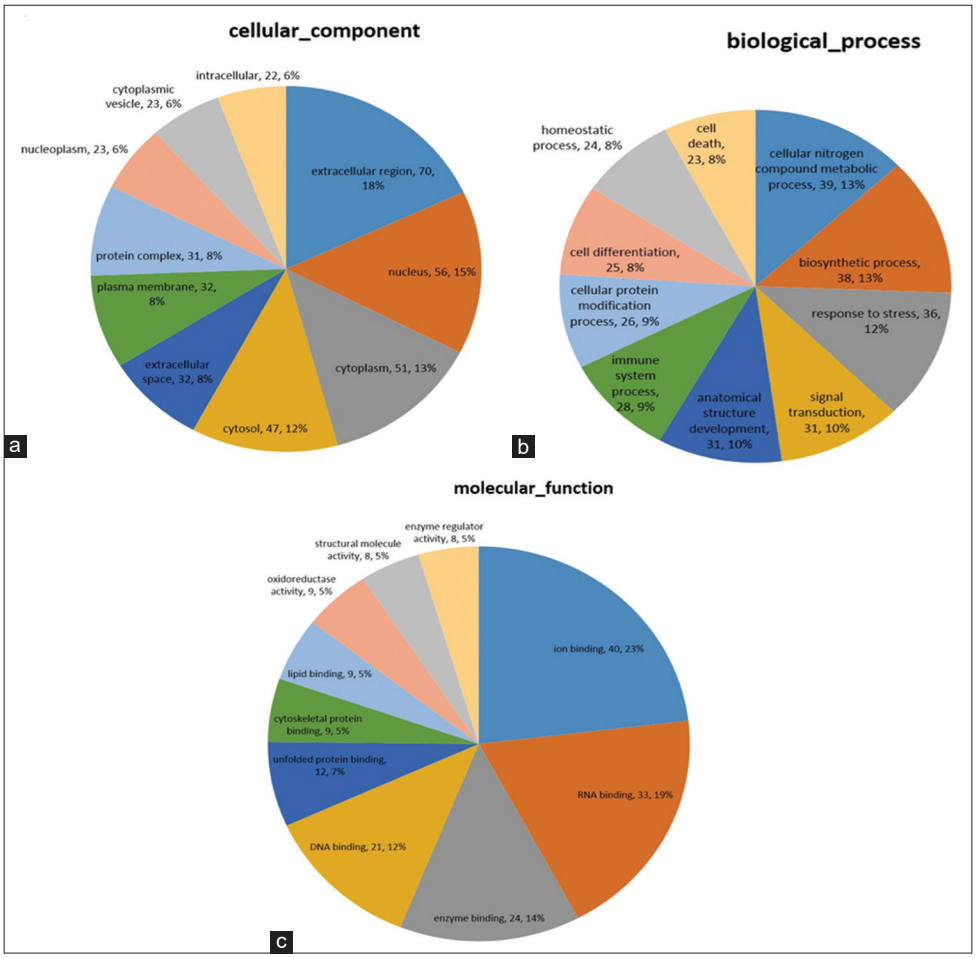

Figure 1: Gene ontology enrichment of candidate viral protein 1-binding proteins. Functional enrichment of candidate viral protein 1-binding proteins by gene ontology analysis. (a) 22% of the 91 potential viral protein 1 candidate-binding proteins were located at the extracellular or plasma membrane. (b) Viral protein 1-binding proteins were mainly enriched as anatomical structure development and biosynthesis process-related proteins. (c) Viral protein 1-binding proteins were mainly enriched as ion-binding and RNA-binding proteins

anti-mouse antibody (diluted by TBST at 1:3000). Protein bands were visualised using Emitter-coupled logic (ECL) substrate and the Image Quant RT ECL System (GE Healthcare).

Statistical analysis

Data were analysed by one-way ANOVA tests. SPSS software (version 13.0) SPSS: Statistical Package for Social Sciences, IBM was used for statistical analysis and $P < 0.05$ was considered to be statistically significant.

Results

Identification of viral protein 1-binding proteins

GST-tagged VP1 protein was used as a bait protein to capture the proteins extracted from HBMEC. LC-ESI-MS/MS analysis identified 91 common proteins in two biological replicates [Table 1]. The gene ontology analysis of 91 proteins is shown in Figure 1a-c, which demonstrated their specific location after cellular component analysis, binding efficiency to various components by molecular function analysis and their specific cellular and biological functions by biological process analysis.

Identification of glucose-regulated protein 78 as an enterovirus 71-binding protein

The interaction between 78 kDa GRP78 and VP1 was further verified using GST-Pull down and WB analysis. After incubating the extracted protein with VP1, the VP1-binding proteins were analysed by WB using anti-GRP78 antibody, which could be observed as an obvious band in the second lane of immunoblot [Figure 2a]. GRP78 as a VP1-binding protein was further verified by Co-IP coupled with WB analysis. IgG was used as negative control. As shown in Figure 2b, after interacting with VP1-HA-tag protein which was used as a bait protein, western blotting performed with anti-GRP78 antibody showed positive expression results in the input lane and GRP78 lane. Similarly, when using the GRP78-Flag-tag protein as a bait protein, positive expression results were observed in the input lane and VP1 lane, whereas the IgG lane showed a negative result [Figure 2c]. In addition, immunofluorescence demonstrated the co-localisation of GRP78 and VP1 as shown in Figure 2d. On the basis of the above results, it could be suggested that GRP78 was a binding protein of EV71 VP1.

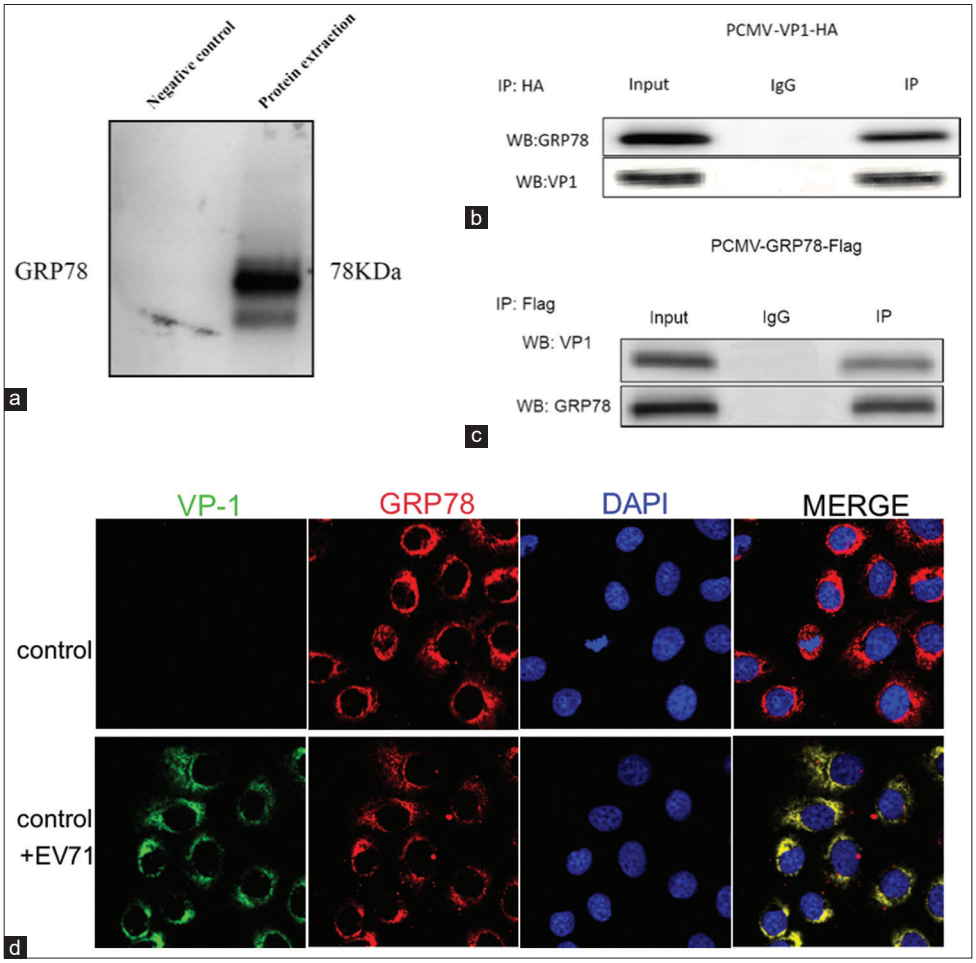

Figure 2: Glucose-regulated protein 78 was verified as viral protein 1-binding protein by Co-IP assay combine with immunofluorescence analysis (a) glutathione-S-transferase pull-down assay confirmed that glucose-regulated protein 78 is a viral protein 1-binding protein. (b and c) Validation of glucose-regulated protein 78 as a viral protein 1-binding protein by Co-IP coupled with western blot analysis. IgG was used as negative control. (d) Immunofluorescence experiments confirmed the co-localisation of glucose-regulated protein 78 and viral protein 1

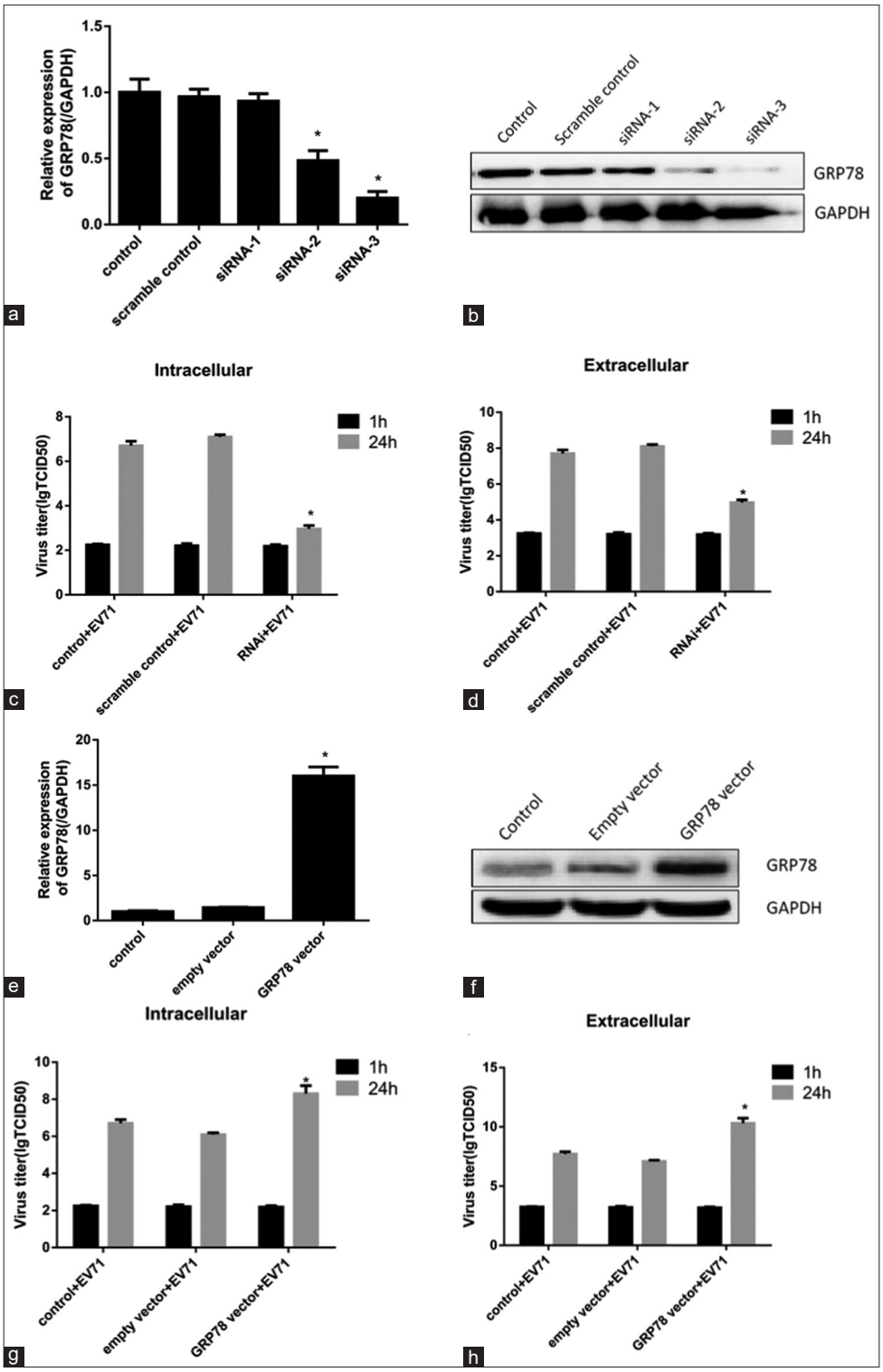

Figure 3: Influence of glucose-regulated protein 78 knockdown and overexpression on enterovirus 71 replication in human brain microvascular endothelial cell by TCID50 analysis The relative RNA (a) and protein expression (b) of glucose-regulated protein 78 treated with glucose-regulated protein 78-small interfering RNA. The enterovirus 71 titre in the intracellular (c) and extracellular (d) compartments of human brain microvascular endothelial cells treated with the glucose-regulated protein 78 small interfering RNA s detected by a TCID50 assay. The relative RNA (e) and protein expression (f) of glucose-regulated protein 78 treated with glucose-regulated protein 78 overexpression vector. The enterovirus 71 titre in the intracellular (g) and extracellular (h) compartments of human brain microvascular endothelial cells treated with the glucose-regulated protein 78 overexpression vector detected by a TCID50 assay

Knockdown of glucose-regulated protein 78 in human brain microvascular endothelial cell

HBMEC was treated with three 200 nmol/L siRNA oligonucleotides, targeted GRP78 for 24 h and cultured with fresh medium for another 24 h. To develop the optimal transfection system, the transfection efficiency of siRNA