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Original Article

Identification of genes involved in enterovirus 71 infected SK-N-SH cells

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Abstract: The enterovirus 71 (EV71) is the major pathogen of hand-foot-and-mouth disease (HFMD) and has been associated with severe neurological disease in children under 5 years of age. The molecular mechanisms underlying the response of human neural cells to EV71 infection still remain unclear. In this study, the genome microarray was employed to perform transcriptome profiling analysis in human neuroblastoma SK-N-SH cells infected by EV71. The results indicated that EV71 infection lead to altered expression of 87 human mRNA. The up-regulated gene mainly include the cytokine and chemokine, ubiquitin mediated proteolysis, Toll-like receptor signaling pathway, p53 signaling pathway, apoptosis, leukocyte transendothelial migration, MAPK signaling pathway and Jak-STAT signaling pathway, etc. Finally, the microarray results were validated using real-time RT-PCR and ELISA in the RNA and protein level, respectively. Our results suggested that the high fatality rate of EV71 infection probably derived from a severe immune response caused by cytokines and chemokines. The findings will help to better understand the host responses to EV71 infection and provide the potential strategy for prevention and control of EV71 infection.

Keywords: EV71, SK-N-SH, chemokine, gene microarray, HFMD

Introduction

The enterovirus 71 (EV71) is one of the major pathogens responsible for human cases of hand, foot and mouth disease (HFMD) [1]. Children under 5 years of age are particularly susceptible to severe forms of EV71-associated neurological diseases, including aseptic meningitis, brainstem and/or cerebellar encephalitis and acute flaccid paralysis [2]. However, the molecular mechanisms underlying the response of human neural cells to EV71 infection still remain unclear. With the development of microarray technology, the response of cell to virus can be monitored by examining the gene expression changes [3]. By the use of DNA microarrays [4, 5], it is possible to define changes in gene expression that underlie the host response to EV71 infection and to gain specific insights into the molecular mechanism of the host pathways participated in the EV71 pathogenesis.

In this report, the genome microarray was employed to perform transcriptome profiling analysis in human neuroblastoma SK-N-SH cells infected by EV71. Compared to un-infected control, EV71 infection changes the amount of many mRNA transcripts in SK-N-SH cells. The up-regulated gene mainly include the cytokine and chemokine, ubiquitin mediated proteolysis, Toll-like receptor signaling pathway, p53 signaling pathway, apoptosis, leukocyte transendothelial migration, MAPK signaling pathway and Jak-STAT signaling pathway, etc. And then, we used the real-time RT-PCR and enzyme linked immunosorbent assay (ELISA) to detect the cytokines and chemokines in EV71-infected SK-N-SH cells in the RNA and protein level, respectively. We found it is possible that the high fatality rate of EV71 infection results from a severe immune response caused by cytokines and chemokines. Previous data have implicated several chemokines and cytokines in the systemic and CNS inflammation that accompanies EV-71 infection [6, 7]. The knowledge of the host genes that are differently regulated with different kinetics during EV71 infection may be important for predicting cellular response to viral infection. Our findings will help to better understand the host responses to EV71 infection and provide the potential strat-

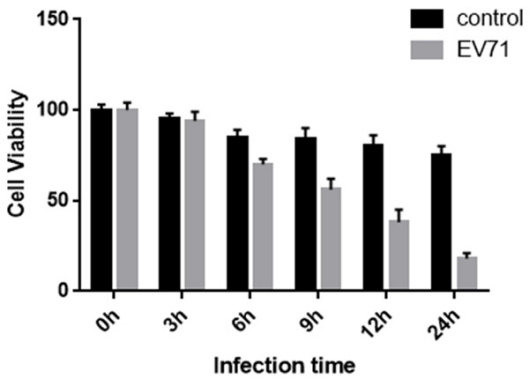

Figure 1. Growth kinetics of EV71-infected SK-N-SH cells. SK-N-SH cells were inoculated with the EV71 virus and mock control. The cells were collected at 3, 6, 9, 12 and 24 hours post-infection and cell viability were determined using MTT methods. Data was shown as mean ± SD from 3 independent experiments.

egy for prevention and control of EV71 infection.

Materials and methods

Cell and virus culture

Human rhabdomyosarcoma (RD; ATCC, CCL-136) and neuroblastoma (SK-N-SH; ATCC, HTB-11) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) plus streptomycin (200 U/ml), and cultured at 37°C in a 5% CO_2 incubator. Virus was propagated in RD cells. Briefly, 80% confluent monolayers of RD cells were inoculated with the virus, once 90% of the cells showed cytopathic effect (CPE), the culture medium and cell debris were harvested and were repeated freezing and thawing three times, cell debris was removed by centrifugation at 1,000 g for 10 minutes. The supernatants were filtered through a 0.22 μM membrane (Milipore) and stored at -80°C before use. The virus titer was determined by TCID50.

In vitro virus infection

For in vitro virus infection, 3×10^6 SK-N-SH cells were seeded onto 10 cm cell culture dishes (Corning) and incubated overnight. The cells were infected with EV71 at 10 MOI or control (the same growth medium but without virus) at 37°C in a 5% CO_2 incubator for different time point. The cells were then collected by centrifugation at 2000 g for 10 min.

Microarrays

The cDNA microarrays were made by Beijing Boao Biotechnology Co., Ltd. The used chips were Agilent G4112F Design ID 014850, 4×44 K format. They contain probes for 10,692 human expressed sequence tags (ESTs/cDNA elements) corresponding to known genes in the GenBank database, printed on nylon membrane. All the EST clones have been sequence verified.

The data analysis

Analysis software was Agilent G4450AA Feature Extraction software 10.0, Agilent Scan Control software, version A. 7.0 or later (includes XDR functionality) and Agilent GeneSpring software GX11. Genes were selected for this analysis if their expression levels in SK-N-SH cells following EV71 infection were different from that in uninfected cells control. Differences in expression levels of genes that increased or decreased by a factor of 0.5 at a minimum at two different time points were considered as significant.

MTT analysis

The cells were seeded into 96-well plates and incubated for overnight, 10 μl MTT was added into each well and incubated for 4 hours. The absorbance was then measured using Epoch Microplate Spectrophotometer (Bio-Tek Instruments, Inc.) at 490 nm.

Real-time RT-PCR

Two-step real-time RT-PCR was performed on selected genes to confirm the differential expression results obtained by microarray experiments. The reverse transcription was used for MMLV (Promega). The PCR reaction was performed on Stepone real-time PCR systems (ABI company) using a SYBR Green PCR kit (TOYOBO). Each RT-PCR experiment was performed three times.

ELISA

ELISA was performed to detect the expression difference in SK-N-SH cells following EV71 infection were different from that in uninfected cells control. The ELISA operation is carried out in accordance with the kit instructions (Boster).

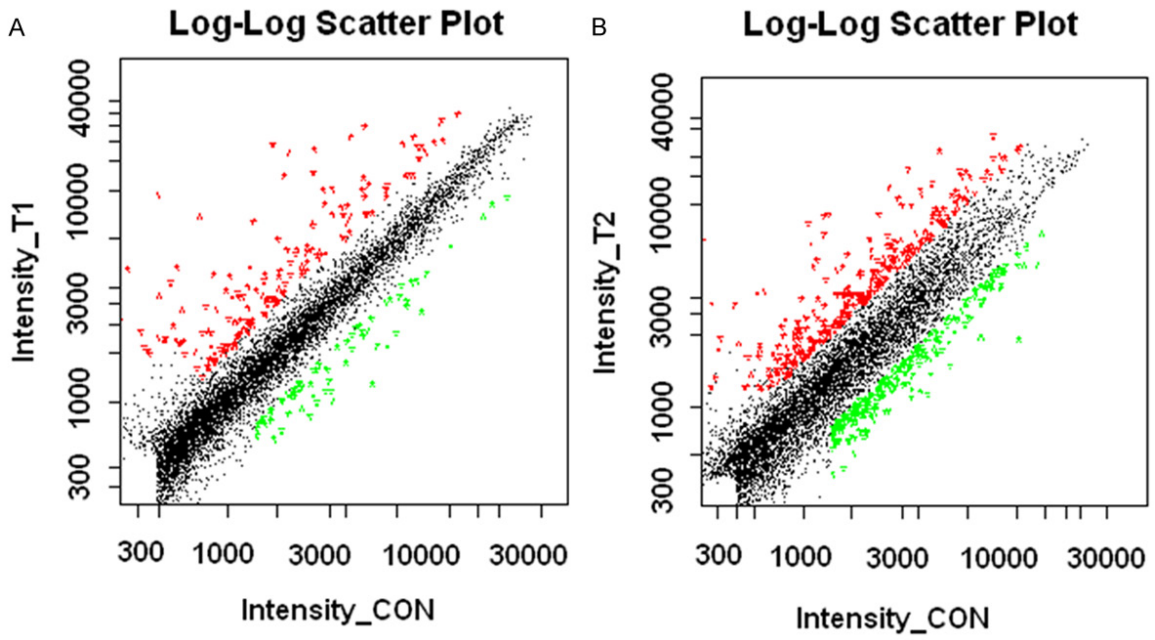

Figure 2. Heat map of the microarray gene expression profile of SK-N-SH cells infected with EV71. A. The gene profile of SK-N-SH cells infected by EV71 at 9 hours. B. The gene profile of SK-N-SH cells infected by EV71 at 12 hours. Red represents mRNAs with increased expression, and green represents mRNAs with decreased expression.

Statistical analysis

Differences of gene expression compared to control were analyzed by Student's t test.

Results

Growth kinetics of EV71-infected SK-N-SH cells

In order to explore the time point when EV71 infected SK-N-SH cells, we analyzed the changes of SK-N-SH cells at different time post infection. The uninfected control and EV71 strain were added into SK-N-SH cells. The cell growth kinetics and cytopathic effect (CPE) was observed, respectively. As shown in **Figure 1**, the decrease in viable cell population was apparent in EV71-infected cells at 9 hours after infection. Visible CPE was also observed in the infected cell culture at the same time point (data not shown). There was no visible effect in the cells subjected to the mock control. The decrease in number of cells and CPE was first observed at 9 h post infection and progressed to moderate and severe at 12 and 24 h respectively. Thus, we collected the SK-N-SH cells infected by EV71 at the 9 and 12 hours to perform the microarray analysis.

Microarray data reveal a set of differentially expressed related gene

Labeled cDNA was prepared from the different RNA samples and then hybridized to cDNA microarrays as instruction of Beijing Boao Company. The area of a gene filter is displayed in **Figure 2** and showing several genes were either increased or decreased in abundance following the EV71 infection. Genes were selected for this analysis if their expression levels in SK-N-SH cells following EV71 infection were different from those in un-infected control cells by at least a factor of 0.5 at a minimum at 2 different time points. **Table 1** lists 87 representative transcripts that were significantly altered at 9 h and 12 h after infection according to their known functions. According the data analysis, 28 highly expressed protein molecules were fed into the STRING online database to construct protein-protein interaction networks. The networks figure was shown in **Figure 3**.

Confirmation of altered gene expression of microarray by real-time RT-PCR and ELISA

The RNA samples from EV71- or uninfected SK-N-SH cells were extracted at 9 h and 12 h

Table 1. The differentially expressed related gene of the SK-N-SH cells infected by EV71

Pathway	Gene name	Fold change (9 hour)	Fold change (12 hour)
Cytokine-cytokine receptor interaction	Chemokine (C-X-C motif) ligand 2 (CXCL2)	+3.222	+11.382
	Interleukin 8 (IL8)	+11.22	+14.56
	Tumor necrosis factor receptor superfamily 9 (TNFRSF9)	+3.33	+7.16
	Cloning stimulating factor 2 (CSF2)	+7.47	+8.66
	Chemokine (C-C Motif) Ligand 20 (CCL20)	+15.24	+20.16
	Interleukin 6 (IL6)	+30.62	+37.28
	Chemokine (C-C Motif) Ligand 2 (CCL2)	+10.92	+20.16
	Chemokine (C-X3-C) ligand 1 (CX3CL1)	+3.18	+4.72
	Chemokine (C-X-C motif) ligand 10 (CXCL10)	+2.8	+59.76
	Ectodysplasin A2 receptor (EDA2R)	-0.977	-0.556
	Interleukin 7 (IL7)	-0.774	-0.552
	Bone morphogenetic protein receptor, type 1A (BMPR1A)	-0.596	-0.336
Ubiquitin mediated proteolysis	Transcription elongation factor B (SIII), polypeptide 2 (TCEB2)	+1.51	+2.13
	Baculoviral IAP repeat containing 3 (BIRC3)	+2.76	+3.78
	Anaphase promoting complex subunit 1 (ANAPC1)	-0.92	-0.57
	Tripartite motif containing 37 (TRIM37)	-0.98	-0.45
	Anaphase promoting complex subunit 5 (ANAPC5)	-0.89	-0.59
	HECT and RLD domain containing E3 ubiquitin protein ligase 3 (HERC3)	-0.74	-0.46
Toll-like receptor signaling pathway	Interleukin 8 (IL8)	+11.22	+14.56
	Interleukin 6 (IL6)	+30.62	+37.28
	Interferon regulatory factor 7 (IRF7)	+2.51	+3.17
	Chemokine (C-X-C motif) ligand 10 (CXCL10)	+2.8	+59.76
	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (IKBKE)	+1.42	+3.18
	MAP3K7IP1	+1.11	+2.15
p53 signaling pathway	BAX	+1.20	+2.04
	CD82	+2.04	+3.45
	BID	+1.42	+2.28
	CCNG1	-0.52	-0.47
	EI24	-0.82	-0.58
Apoptosis	BAD	+1.09	+1.81
	BAX	+1.20	+2.04
	BID	+1.42	+2.28
	BIRC3	+2.76	+3.78
	PRKAR1A	-0.66	-0.46
Leukocyte transendothelial migration	ICAM1	+11.32	+13.38
	RASSF5	+5.56	+8.23
	JAM2	+1.41	+1.82
	CTNNB1	-0.92	-0.47
	ROCK1	-1.02	-0.52
ECM-receptor interaction	ITGA6	-0.44	-0.45
	ITGAV	-0.55	-0.54
	ITGA6	-0.32	-0.36
	LAMB1	-0.88	-0.45
Wnt signaling pathway	FZD3	-0.82	-0.54
	CAMK2D	-0.78	-0.45
	PLCB1	-0.62	-0.48
	CTNNB1	-0.92	-0.47
	ROCK1	-1.02	-0.52
Starch and sucrose metabolism	PGM2	-0.66	-0.52
	PGM2L1	-0.83	-0.51
Biosynthesis of unsaturated fatty acids	HSD17B12	-0.88	-0.66
	ELOVL5	-0.92	-0.55

MAPK signaling pathway	DUSP9	+1.32	+1.91
	DUSP14	+1.42	+1.91
	MAP3K7IP1	+1.14	+1.93
	RASA1	-0.68	-0.54
	GNG12	-0.64	-0.58
Regulation of actin cytoskeleton	BDKRB1	+1.82	+2.17
	ITGA6	-0.32	-0.36
	ITGAV	-0.55	-0.54
	GNG12	-0.54	-0.56
	ROCK1	-1.02	-0.52
Jak-STAT signaling pathway	CSF2	+7.47	+8.66
	Interleukin 6 (IL6)	+30.62	+37.28
	Interleukin 7 (IL7)	-0.774	-0.552
Proteasome	PSMB10	+3.36	+3.38
	PSMB3	+1.21	+1.78
Inositol phosphate metabolism	MINPP1	-0.66	-0.57
	PLCB1	-0.50	-0.51
Tight junction	JAM2	+1.41	+1.82
	CTNNB1	-0.99	-0.51
ErbB signaling pathway	BAD	+1.09	+1.81
	CAMK2D	-0.82	-0.47
TGF-beta signaling pathway	ROCK1	-1.02	-0.52
	BMPR1A	-0.63	-0.32
Cell cycle	ANAPC5	-0.83	-0.56
Regulation of autophagy	ULK2	-0.71	-0.52
PPAR signaling pathway	MMP1	+3.10	+7.21
B cell receptor signaling pathway	IFITM1	+3.45	+3.68
Phosphatidylinositol signaling system	PLCB1	-0.50	-0.51
Adherens junction	CTNNB1	-0.99	-0.51
Gap junction	PLCB1	-0.50	-0.51
Neuroactive ligand-receptor interaction	P2RY11	+1.20	+1.64
	BDKRB1	+1.72	+2.13
Olfactory transduction	CAMK2D	-0.82	-0.47

after the infection. The expression of related genes was measured by real time RT-PCR and ELISA. We firstly confirmed the higher expression gene such as Chemokine (C-X-C motif) ligand 2 (CXCL2), Interleukin 8 (IL-8), Tumor necrosis factor receptor superfamily 9 (TNFRSF9), Chemokine (C-C Motif) Ligand 20 (CCL20), Cloning stimulating factor 2 (CSF2), Interleukin 6 (IL-6), Chemokine (C-C Motif) Ligand 2 (CCL2), Chemokine (C-X3-C) ligand 1 (CX3CL1) and Chemokine (C-X-C motif) ligand 10 (CXCL10) by real-time RT PCR. As shown in **Figure 4**, the expression level of CCL2, CCL20, CXCL2, IL-8 and IL-6 in the SK-N-SH cells infected by EV71 was higher than that of in the control cells. There was no distinct difference in the level of CX3CL1, CSF2 and CX3CL1 between EV71-infected SK-N-SH cells and control cells. Meanwhile, we furtherly use the ELISA to confirm the altered gene in the protein level. As shown in **Figure 5**, the expression level of CCL2, CCL20, CXCL2, IL-8 and IL-6 in the SK-N-SH cells infected by EV71 was higher than that of in the control cells.

Discussion

Increasing evidence suggests that both inflammatory cytokines may play a central role in HFMD derived from EV71 infection [8-10]. The inflammatory cytokines are the mediators of the innate and immune response and play key roles in the pathophysiology of infection. The persistent hyper-inflammatory cytokine levels might result in progression to multiple organ damage [11]. Previous studies have indicated that the serum levels of inflammatory cytokines and chemokines such as interleukin (IL)-1β, IL-6, and CXCL1 in EV71 patients with serious encephalitis were significantly higher than those of the uncomplicated patients [12].

In the study, we use microarray to analyze the gene changes of SK-N-SH cells infected by

Figure 3. The protein-protein interaction networks of highly expressed genes. 28 highly expressed protein molecules were fed into the STRING online database to construct protein-protein interaction networks.

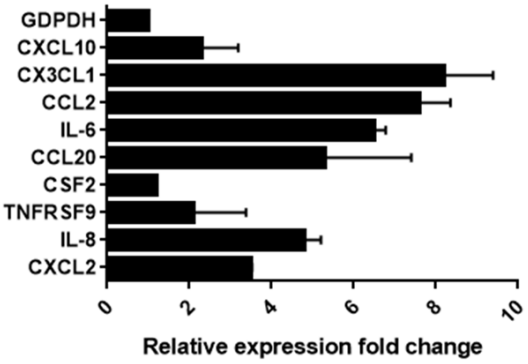

Figure 4. Valuation differential expression selected genes by real-time RT-PCR. 9 expression levels of selected genes from the microarray assay were validated by real-time RT-PCR at 12 hours after the EV71 infection. The relative fold change was calculated based on endogenous control normalization and repeated three times independently.

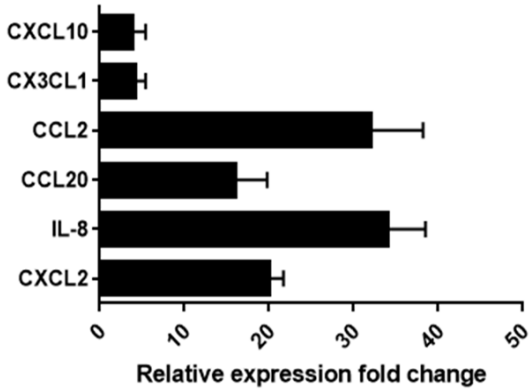

Figure 5. Valuation differential expression selected genes by ELISA. 6 expression levels of selected genes from the microarray assay were validated by ELISA at 12 hours after the EV71 infection. The relative fold change was calculated based on control and repeated three times independently.

EV71. The up-regulation genes were validated by real-time PCR and ELISA. Our results showed the cytokine and chemokine participate the EV71 infection to SK-N-SH cell. Chemokines, a group of small (8-12 kd) proteins, are key regulators of leukocyte migration and play important roles in many physiological and pathological immune and inflammatory contexts. Chemokines are characterized by the presence of 3 to 4 conserved cysteine residues and can be subdivided into 4 families based on the positioning of the N-terminal cysteine residues [13, 14].

CCL2 (chemokine (C-C motif) ligand 2; monocyte chemoattractant protein-1, (MCP-1)) is a CC chemokine that attracts monocytes, memory T lymphocytes, and basophils. CCL2 and its receptor CCR2 are involved in inflammatory reactions, including monocyte/macrophage migration, Th2 cell polarization, and the production of TGF-β and procollagen in fibroblast cells [15, 16]. IL-8 was identified as a neutrophil-specific chemotactic factor and later classified as a member of the CXC chemokine family. The major effector functions of IL-8 are activation and recruitment of neutrophils to the site of infection or injury [17, 18]. Patients with EV71 encephalitis had a significantly higher frequency of IL-8-251TT genotype and CCL2-2510GG genotypes than patients with EV71-related HFMD without encephalitis [19, 20].

Wang et al demonstrated that the plasma levels of interferon (IFN)-induced protein (IP-10), MCP-1 and IL-8 were significantly higher in patients with pulmonary edema (PE) than in those with uncomplicated brainstem encephalitis (BE). Overexpression of the chemokine cascade in the central nervous system compartment appears to play an important role in the elicitation of the immune response to EV71 [12]. Systemic inflammation caused by EV71 infection exacerbated the deterioration of the disease, and resulted in the disease progression to the critical illness stage.

Since the cause of cytokine/chemokine release during EV71 infection is still unclear, further investigation is needed to explore the sources for each of these cytokines and chemokines as well as the underlying mechanisms for how their presence is associated with infection and inflammation in this disease and is regulated. Answering these unknowns may help to translate these findings from bench to bedside.

Acknowledgements

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Disclosure of conflict of interest

None.

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