Elucidating the host–pathogen interaction between human colorectal cells and invading Enterovirus 71 using transcriptomics profiling

Yan Long Edmund Lui a,b,c,d,⇑, Tuan Lin Tan c, Peter Timms a,b,e, Louise Marie Hafner a,b,⇑, Kian Hwa Tan c, Eng Lee Tan d,f

a School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Queensland, Australia
b Institute of Health and Biomedical Innovation, Queensland University of Technology, Queensland, Australia
c School of Chemical and Life Sciences, Singapore Polytechnic, Singapore
d Centre for Biomedical and Life Sciences, Singapore Polytechnic, Singapore
e Faculty of Science, Health, Education & Engineering, University of the Sunshine Coast, Queensland, Australia
f Department of Paediatrics, University Children’s Medical Institute, National University Hospital, Singapore

1. Introduction

Enterovirus 71 (EV71) is a positive sense, single stranded, non-enveloped RNA virus belonging to the Picornaviridae family [1–4]. It is one of the main etiological agents for Hand, Foot and Mouth Disease (HFMD) which commonly affects infants and children. This disease is usually self-limiting, characterised by various symptoms such as fever, rashes, poor appetite and multiple ulcers in mouth [5–7]. The route of transmission of EV71 was postulated to happen via direct contact of vesicular fluid or droplet from the infected or faecal-oral route [5,8–10]. EV71 could have emerged in the human population as early as 1974 in CA (USA) after it was isolated from a case of coxsackievirus [11]. Recent molecular evolution studies have predicted that EV71 may have been circulating in the human population for decades before it emerged in 1997 and Taiwan in 1998 [15–19]. Since then, there have been various outbreaks, epidemics and pandemics that have periodically resulted in critical pathways including the immune response and the stress response. Together, data from this study provide valuable insights into the host–pathogen interaction between human colorectal cells and EV71.
Drug Administration (FDA) to prevent HFMD. There is therefore a need to gain a better understanding of the mechanism of EV71 pathogenesis. The ability to cause infection directly correlates to the interaction between the host and pathogen. In order to survive and proliferate during infection, the pathogen must be able to sense, react and adapt to the ever-changing microenvironment within the host. Such adaptations and the use of the host resources have developed highly sophisticated mechanisms not only to exploit the biosynthetic machinery for replication, but also to specifically evade the host immune system.

During infection, viruses regulate host gene expression to enhance their survival. Such activities include altering the cellular microenvironment to allow successful virus replication and evasion of the host immune system [21–23]. Elucidating the mechanism that the virus uses to bypass host defence systems and establish infections will provide us with a better understanding of the pathogenesis mechanism of the EV71 virus and will thus aid in the development of potential antiviral therapeutics for HFMD patients. In this study, transcriptomics profiling was performed using colorectal cells infected with EV71 using mRNA microarray.

2. Materials and methods

2.1. Cell culture and virus propagation

Human colorectal cell line (HT29) (ATCC® Catalog No. HTB-38™) was maintained in Roswell Park Memorial Institute medium (RPMI) (PAA Laboratories, Austria) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (PAA Laboratories, Austria) and 2% penicillin-streptomycin (PAA Laboratories, Austria) at 37 °C with 5% CO2. The EV71 strain used in this study was isolated from a fatal baby with age 60 days from a hospital in Nanning, China [20]. The virus stock was prepared by propagation of viruses using 90% confluent HT29 cells monolayer in RPMI with 10% FBS at 37 °C with 5% CO2. The virus titre was determined using 50% tissue culture infective dose (TCID50) per millilitre (mL) according to Reed and Muench method [24].

2.2. EV71 infection for mRNA profiling

HT29 cells were seeded at a concentration of 5 × 10^5 cells/mL in each well of a 6-well plate and incubated for 24 h at 37 °C with 5% CO2. Cells were washed twice with phosphate buffered saline (PBS) and infected with EV71 at multiplicity of infection (MOI) of 1 or nil respectively. The culture media were removed and replaced with 2 mL of fresh RPMI medium after 1 h of incubation at 37 °C with 5% CO2. The respective infected cells were harvested at 36 h posts infection (hpi). The total cellular RNA of HT29 cells were extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, the cells were lysed and homogenise using lyses solution provided (Qiagen, Hilden, Germany). Total RNA were purified and eluted (Qiagen, Hilden, Germany). Harvested total RNA was quantified based on 2^-ΔΔCT method using the housekeeping gene β-actin as the reference gene [25].

2.3. mRNA microarray analysis

Extracted total RNA were labelled with GeneChip HT 3’ IVT Express Kit following manufacturer’s instruction. Hybridization to GeneChip PrimeView Human Gene Expression Array was performed in accordance to manufacturer’s recommendations. Every chip was scanned at a high resolution by the Affymetrix GeneChip Scanner 3000 according to the GeneChip Expression Analysis Technical Manual procedures (Affymetrix, Santa Clara, CA). Briefly, total extracted RNA undergo two rounds of cDNA synthesis prior to in vitro transcription to synthesise biotin modified amplified RNA (Affymetrix, Santa Clara, CA). The amplified RNA was purified and fragment for the hybridisation onto the GeneChip PrimeView Human Gene Expression Array (Affymetrix, Santa Clara, CA). Raw data for the microarray were analysed with the Partek® Genomics Suite (Partek Incorporated, Saint Louis, USA) where p < 0.05 is considered significant.

2.4. cDNA synthesis and quantitative real time polymerase chain reaction

For the conversion of mRNA to cDNA, 1 µg of the total RNA was reverse transcribed using using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) in accordance to the manufacturer’s instructions. Briefly, 1 µg of the extracted RNA was mixed with enzyme reverse transcriptase and buffer to a volume of 20 µl and subjected to thermal profile of 25 °C for 5 min, 42 °C for 30 min followed by 85 °C for 5 min in accordance to the manufacturer’s instructions.

The quantitative real time polymerase chain reaction (qPCR) was performed using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, CA, USA) on the BioRad CFX96™ Real-Time PCR system (Bio-Rad Laboratories, CA, USA). The primers used in this study are listed in Table 1. Briefly, 1 µl of cDNA and 1 µl of the forward and the reverse primers were added to iTaq™ Universal SYBR® Green Supermix. The reaction mix was then subjected to thermal profile of denaturation at 95 °C for 30 s, followed by amplification and quantification in 40 cycles at 95 °C for 5 s followed by 60 °C for 30 s. At the end of amplification cycles, melting temperature analysis was performed by the BioRad CFX96TM Real-Time PCR system (Bio-Rad Laboratories, CA, USA). Relative gene expression was quantified based on 2^-ΔΔCT method using the housekeeping gene β-actin as the reference gene [25].

2.5. Data analysis

All statistical analysis was performed on GraphPad Prism Version 6.0c (GraphPad Software, USA). Student’s t test was used to compare two groups. p values of <0.05 were considered statistically significant.

3. Result and discussion

Systemic analyses of host response to EV71 provide critical clues to understand the molecular mechanism of EV71 pathogenesis
during infection of a human host. Regulation and alteration of the host cellular system can also be monitored by studying the host cellular transcriptomics change following enterovirus infection. Transcriptomics analysis via RNA-sequencing, mRNA microarray and PCR array have been previously used to analyse host gene expression [26–28]. However, these studies have been performed on rhabdomyosarcoma (RD) cells or SH-SY5Y cells, of muscle cells or neuronal origin respectively. In a clinical context, this may not be a good representative model during EV71 infection of a human host as various studies have demonstrated that the gastrointestinal tract was the first site for EV71 proliferation [29–31]. Considering that different cell types have varying cellular content, host–pathogen interaction may differ from cell types to cell types [1,32,33]. We have previously reported a human colorectal adenocarcinoma cell line (HT29) with epithelioid morphology as a useful in vitro model to study the pathogenesis of EV71 [33]. To this end, this study

![Fig. 1](image1.png)

**Fig. 1.** Heat map of the microarray mRNA expression profile of EV71 infected and non-infected control colorectal cells, HT29. The two-way hierarchical cluster heat map showed differential expressed mRNAs of two groups of samples. The mRNAs were chosen according to the cut off $p < 0.05$ where blue represents mRNAs with decreased expression and red represent mRNAs with increased expression. ($n = 3$, *p* values of <0.05).

![Fig. 2](image2.png)

**Fig. 2.** Pathway analysis of differentially expressed genes of EV71 infected and non-infected control colorectal cells, HT29. Pathways with enrichment score of <2 was generated using Partek® Genomics Suite (Partek Incorporated, Saint Louis, USA) ($n = 3$).
utilised HT29 as a model for the transcriptomics analysis of host response to EV71 infection.

3.1. Identification of deregulated mRNAs during EV71 infection

To identify the differentially transcribed mRNAs in HT29 during EV71 infection, we performed mRNAs profiling using GeneChip PrimeView Human Gene Expression Array. Comparative mRNAs expression between EV71 infected cells and control non-infected cells were performed. Microarray hybridisation identified 699 genes being differentially expressed significantly during EV71 infection \( (p < 0.05) \) (Fig. 1 and Supplementary Table 1). Using Partek\textsuperscript{®} Genomics Suite, 699 differentially transcribed genes between EV71 infected and control non-infected HT29 were further analyses to generate pathways with enrichment score of \( < 2 \) (Fig. 2). These differentially transcribed genes were involved in critical pathways including the immune response, the stress response, metabolism, cytotoxicity, cytokines and the cytoskeleton network (Fig. 2).

In response to viral infection, cells were known to induce an immunological response to combat against invading pathogens. In particular, immune response cytokines IL29/interferon (IFN)-\( \lambda_1 \) and IFN-\( \beta_1 \) were significantly up-regulated in EV71 infected cells (Supplementary Table 1). The microarray results were validated using qPCR. In line with the microarray findings, qPCR revealed that both IL29 and IFN-\( \beta_1 \) were significantly up-regulated by approximately 20 fold and 3 fold in EV71 infected cells respectively (Fig. 3a and b). This is not surprising as both IL29 and IFN-\( \beta_1 \) are cytokines which play important roles in host antiviral response [34–36]. In addition, IL29 has been previously reported to be up regulated during viral infection such as influenza A, hepatitis C virus and Sendai viruses [34–36]. A key characteristic of cytokines is the ability to interact with one another to establish coordinated defence mechanism against invading pathogens. IL29 functions by binding to a unique receptor through a pathway identical to IFN-\( \beta_1 \) and IFN-\( \gamma_1 \) receptors inducing cellular antiviral activity to inhibit virus replication [34–36]. Synergetic effect between IL29, IFN-\( \beta_1 \) and IFN-\( \gamma_1 \) were shown to promote cellular

**Fig. 3.** Expression level of types I, II and III IFN signalling pathways in response to EV71 infection. Confluent HT29 cells were infected with or without EV71 (MOI of 1). Total intracellular RNA were harvested 36 hpi, converted to cDNA and measured by quantitative real time polymerase chain reaction (qPCR). (A) Expression of IL29/IFN-\( \lambda_1 \); (B) expression of IFN-\( \beta_1 \); (C) expression of IFN-\( \gamma_1 \) \((n = 3, \ p \ values \ of \ <0.05)\).

**Fig. 4.** Expression level of interferon stimulated genes in response to EV71 infection. Confluent HT29 cells were infected with or without EV71 (MOI of 1). Total intracellular RNA were harvested 36 hpi, converted to cDNA and measured by quantitative real time polymerase chain reaction (qPCR). (A) Expression of ISG54; (B) expression of ISG56 \((n = 3, \ p \ values \ of \ <0.05)\).
antiviral response [34–36]. Indeed, the use of IL29, IFN-β1 and IFN-γ1 as antiviral treatment has been shown to inhibit various viruses including herpes simplex-1 virus, mouse hepatitis virus type 2, human T-lymphotropic virus-1, cytomegalovirus and hepatitis C virus [37–43]. As such during EV71 infection, HT29 may up regulate cytokines such as IL29, IFN-β1 and IFN-γ1 (types I, II and III IFN) to induce interferon stimulated genes (ISG) as part of cellular antiviral response to slow down virus replication.

To further verify this reasoning, we performed qPCR on IFN-gamma1 receptor, ISG54 and ISG56 to investigate if these genes have been up regulated during EV71 infection. In agreement with our hypothesis, our qPCR results show significant up regulation of these cytokines in response to EV71 infection (Figs. 3c and 4a, b). This support notion that the host colorectal cells up-regulate cytokines such as IL29, IFN-β1 and IFN-γ1 (types I, II and III IFN) possibly by a series of JAK-STAT signalling pathways to induce ISG such as ISG54 and ISG56 (Fig. 5). However, Lei et al. (2010, 2011, 2013) previously reported that EV71 3C protein suppressed the host immune system, for example type I interferon, during infection in RD cells (muscle cells) [44–46]. As such, the mechanism whereby EV71 mediate host immune system may differ from cell type to cell types. To support this hypothesis, Chi et al. (2013) have compared the expression of IFN-signalling pathway between intestinal epithelial cells and muscle cells, which demonstrates that intestinal cells have indeed a significant higher expression of cytokines response in comparison with muscle cells [47]. This may explain the fact it takes three times longer for the virus to kill HT29 cells in comparison with RD cells [32,33]. In addition, IFN signalling pathways such as type I interferon was previously demonstrated to play an important role during EV71 pathogenesis [48].

Effectively, the intestinal epithelial immune system, being the initial site of infection, plays a critical role in the disease progression and clinical prognosis. Impaired or immature immunity has previously been associated with increased morbidity and mortality in EV71 of young children and immune deficient adults [48]. The ability to eliminate viral pathogen such as EV71 at the initial site of infection is critical as a weaker immune response results in a higher viral titre leading to a systemic spread with more serve complications [47]. One of the key antiviral responses that the intestinal epithelial immune system utilises may be the types I, II and III IFN to eliminate invading EV71 (Fig. 5).

4. Conclusion

In conclusion, this study utilised HT29 as an in vitro model to investigate on the transcriptomic changes in human colorectal cells in response to EV71 infection. Human whole genome microarray was employed to monitor changes in profiles between EV71 infected cells and uninfected control cells. The result reveals altered expression of human mRNAs in selected pathways including the immune response, the stress response, metabolism, cytotoxicity, cytokines and the cytoskeleton network. Interestingly, our results shows significant up regulation of types I, II and III IFN which suggests that during EV71 infection, this may be the key antiviral response HT29 cells undertake to eliminate invading EV71. Taken together, data from this study provide valuable fundamental information toward understanding of host–pathogen interaction between human colorectal cells and invading EV71. Elucidating the mechanism that the virus uses to bypass host defence systems and establish infections will provide us with a better understanding of the pathogenesis mechanism of the EV71 virus and will thus aid in the development of potential antiviral therapeutics for HFMD patients.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Y.L.E.L. perform the work, evaluate the result and wrote the paper. Y.L.E.L., T.L.T., L.M.H., P.T., K.H.T., E.L.T. provide research idea, design the work and evaluate manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Mrs. Phoon Meng Chee from the Department of Microbiology, National University of Singapore for providing the EV71 strain 5865/sin/000009. This research was supported by Singapore Polytechnic. Yan Long Edmund Lui was...
References

[1] Lui, Y.L.E., Lin, Z., Lee, J.J., Chow, V.T.K., Poh, C.L. and Tan, E.L. (2013) Beta-actin variant is necessary for enterovirus 71 replication. Biochem. Biophys. Res. Commun. 433, 607–610.

[2] McMinn, P.C. (2002) An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol. Rev. 26, 91–107.

[3] McMinn, P.C. (2012) Recent advances in the molecular epidemiology and control of human enterovirus 71 infection. Curr. Opin. Virol. 2, 199–205.

[4] Ooi, M.H., Wong, S.C., Lewthwaite, P., Cardosa, M.J. and Solomon, T. (2010) Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. 9, 1097–1105.

[5] Wong, S.S., Yip, C.C., Lau, S.K. and Yuen, K.Y. (2010) Human enterovirus 71 and hand, foot, and mouth disease. Epidemiol. Infect. 138, 1071–1089.

[6] Ho, B.C., Yu, S.L., Chen, J.J., Chang, S.Y., Yan, B.S., Hong, Q.S., Singh, S., Kao, C.L., Chou, Y.L., Su, K.Y., Li, K.C., Cheng, C.L., Cheng, H.W., Lee, J.Y., Lee, C.N. and Yang, P.C. (2011) Enterovirus-induced miR-141 contributes to shutdown of host protein translation by targeting the translation initiation factor eIF4E. Cell Host Microbes 9, 58–69.

[7] Huang, H.I., Wu, K.F. and Shih, S.R. (2012) Viral and host factors that contribute to pathogenicity of enterovirus 71. Future Microbiol. 7, 467–479.

[8] Lee, T.C., Guo, H.R., Su, H.J., Yang, Y.C., Chang, H.L. and Chen, K.T. (2009) Diseases caused by enterovirus 71 infection. Pediatr. Infect. Dis. J. 28, 904–910.

[9] Liu, C.C., Tseng, H.W., Wang, S.M., Wang, J.R. and Su, I.J. (2000) An outbreak of enterovirus 71 infection in Taiwan, 1998: epidemiologic and clinical manifestations. J. Clin. Virol. 17, 23–30.

[10] Brown, B.A., Oberste, M.S., Alexander Jr, J.P., Kennett, M.L. and Pallansch, M.A. (1999) Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J. Virol. 73, 9969–9975.

[11] Schmidt, N.J., Lennette, E.H. and Ho, H.H. (1974) An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. 129, 304–309.

[12] Lee, M.S., Tseng, F.C., Wang, J.R., Chi, C.Y., Chong, P. and Su, I.J. (2012) Challenges to licensure of enterovirus 71 vaccines. PLos Negl. Trop. Dis. 6, e1710.

[13] Lee, K.K., Lam, T.T., Chan, Y.F., Bible, J.M., Kamarulzaman, A., Tong, C.Y., Takebe, Y.L.E. Lui et al. / FEBS Open Bio 4 (2014) 426–431 and Solomon, T. (2010) Beta-actin variant is necessary for enterovirus 71 replication. Biochem. Biophys. Res. Commun. 433, 607–610.

[42] Okuse, C., Rinaudo, J.A., Farrar, K., Wells, F. and Korba, B.E. (2005) Synergistic antiviral effect of a combination of mouse interferon-alpha and interferon-gamma on early infection with HTLV-I in vitro. Int. J. Immunopharmacol. 14, 1069–1079.

[43] O’Donofrio, C., Franzese, O., Puglianiello, A., Peci, E., Lanzilli, G. and Bonmassar, E. (2011) Antiviral activity of alpha/beta interferons and interferon-29 functions cooperatively with interferon to induce antiviral gene expression and inhibit hepatitis C virus replication. J. Biol. Chem. 286, 30079–30089.

[44] Sainz Jr., B. and Halford, W.P. (2002) Alpha/beta interferon and gamma interferon synergize to inhibit the replication of herpes simplex virus type 1. J. Virol. 76, 11540–11547.

[45] Pierce, A.T., DeSalvo, J., Foster, T.P., Kosinsk, A., Weller, S.K. and Halford, W.P. (2002) Synergistic antiviral activity of alpha/beta interferon and interferon-gamma on mouse hepatitis virus. J. Med. Virol. 69, 188–194.

[46] Fuchizaki, U., Kaneko, S., Nakamoto, Y., Sugiyama, K., Kikuchi, M., Ichikawa, T. and Kobayashi, K. (2003) Synergistic antiviral effect of a combination of mouse interferon-alpha and interferon-gamma on hepatitis C virus. J. Med. Virol. 76, 11541–11550.

[47] Lui, Y., Timms, P., Hafner, L., Tan, T., Tan, K. and Su, I.J. (2013) Characterisation of enterovirus 71 replication kinetics in human colorectal cell line, HT29. SpringerPlus 2, 267.

[48] Leong, W.F. and Chow, V.T.K. (2006) Transcriptomic and proteomic analyses of oral enterovirus 71 infection model with central nervous system involvement. J. Gen. Virol. 85, 69–77.

[49] Khong, W.X., Yan, B., Yeo, H., Tan, E.L., Lee, J.J., Ng, J.K., Chow, V.T. and Alonso, S. (2012) A non-mouse-adapted enterovirus 71 (EV71) strain exhibits neurotropism, causing neurological manifestations in a novel mouse model of EV71 infection. J. Virol. 86, 2121–2131.

[50] Liu, J., He, Y.Q., Yi, L.N., Zan, H., Kung, H.F. and He, M.L. (2011) Viral kinetics of enterovirus 71 in human abdomysarcoma cells. World J. Gastroenterol. 17, 4135–4142.

[51] Lei, X., Sun, Z., Liu, X., Jin, Q., He, B. and Wang, J. (2011) Viral kinetics of human enterovirus 71 in human abdomysarcoma cells. World J. Gastroenterol. 17, 4135–4142.

[52] Lui, Y., Timms, P., Hafner, L., Tan, T., Tan, K. and E. (2013) Characterisation of enterovirus 71 replication kinetics in human colorectal cell line, HT29. Genevières et al. / FEBS Open Bio 4 (2014) 426–431.

[53] Sainz Jr., B. and Halford, W.P. (2002) Alpha/beta interferon and gamma interferon synergize to inhibit the replication of herpes simplex virus type 1. J. Virol. 76, 11540–11547.

[54] Pierce, A.T., DeSalvo, J., Foster, T.P., Kosinsk, A., Weller, S.K. and Halford, W.P. (2002) Synergistic antiviral activity of alpha/beta interferon and interferon-gamma on mouse hepatitis virus. J. Med. Virol. 69, 188–194.