2009 年臺灣國際科學展覽會

優勝作品專輯

編號： **090010**

作品名稱

Natural sugar as receptor and inhibitor of enterovirus 71 infection.

得獎獎項

醫學與健康科學科大會獎第二名

紐西蘭正選代表: 2009 年紐西蘭科技展覽會

學校名稱： 高雄美國學校

作者姓名： 楊雅涵

指導老師： **Jeremy W. Me　莊　好**

關 鍵 字： **Enterovirus 71、Sialic acid-linked galactose 、Receptor**

作者簡介

我是楊雅涵，就讀高雄美國學校十一年級。我從小就受到父親、母親和姊姊喜好運動和生科研究背景的影響:喜好運動、愛問問題、閱讀自然書籍與雜誌。國中時期就開始參加生科研究，對幹細胞應用和感染症防治很有興趣。目前的研究也因為喜好游泳而起，針對可能汙染游泳池的兒童夏日殺手“腸病毒”防治，提出初期研究成果與未來願景。

摘要

人類許多致命性感染症是利用辨識特殊醣基侵入人類細胞。禽流感與一般流感因辨識不同醣基為接受器而對不同宿主造成感染。經常造成兒童致命的腸病毒71 型(EV71)感染的接受器仍不清楚。本研究發現唾液酸酶處理過的腸細胞(DLD-1)可降低 20 倍以上的 EV71 感染，證實 EV71 是經由唾液酸醣基為接受器感染腸細胞。接著發現母奶內的α2,3 唾液酸半乳醣(sialic acid-α2,3 galactose)，和α2.6 唾液酸半乳醣，都可以抑制 EV71 感染。這是文獻上首次發現唾液酸半乳醣基可做為 EV71 感染的接受器，而且來自母奶的唾液酸半乳醣可抑制 EV71 病毒感染。未來將進一步研究製造一種聯結唾液酸半乳醣到抗病毒奈米分子的口香糖或皮膚噴劑，進行具有“一刀兩刃的雙重策略”來預防 EV71 感染。

Abstract

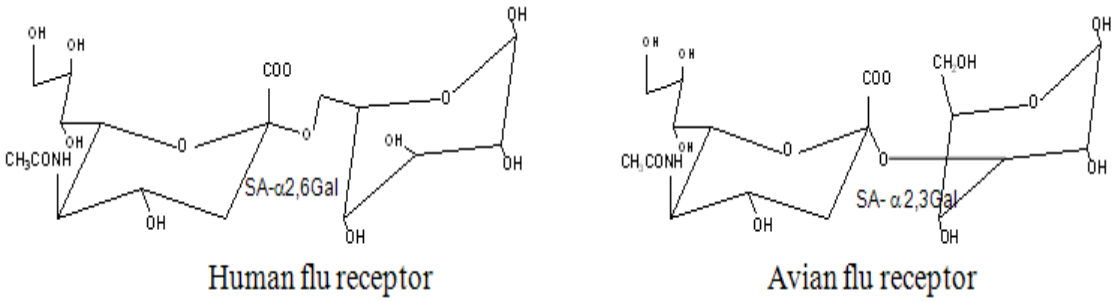
Many life-threatening microbial infections are mediated by recognition of sugar receptors on human epithelial cells. Avian flu and human flu have different ranges of susceptible hosts because of their recognizing different sugar receptors. The receptor for enterovirus 71 (EV71) that frequently causes fatal encephalitis in summer in Asia remains unclear. This study demonstrated that EV71 recognized sialic acid (SA)-linked sugar as a receptor for infection, because depletion of SA by sialidase treatment reduced more than 20-fold EV71 infection of DLD-1 intestinal cells. Further studies showed that purified SA-α2,3 galactose (SA-α2,3Gal) and SA-α2,6Gal from human milk significantly inhibited EV71 infection of DLD-1 cells. Taken together, this is the first in the literature to demonstrate EV71 uses SA-linked residues as a receptor for infection and natural food with SA-linked glycans can protect intestinal cells from EV71 infection. Future studies will try to link SA-based glycans to antivirus nanoparticles for making a chewable gum or skin spray with “double-edge sword” tactics for prevention of EV71 infections.

Natural Sugar as Receptor and Inhibitor of Enterovirus 71 Infection

一、Introduction, motivation and objective

Many viruses recognize specific sugar residue as the infection receptor. Avian influenza virus and human influenza virus use different sugar residues as their receptors, resulting in different host range of infections (1). Enterovirus 71 (EV71) prevails almost every summer season in Asia including Taiwan, causes hand-foot-mouth disease and is frequently complicated with fatal encephalitis (2,3). The transmission route of the EV71 is fecal-oral and/or droplet-aerosol route (3). My younger brother and I love to go swimming in summer, but we are afraid of EV71 infection from swimming pools.

In order to release my concern of EV71 infection and protect my younger brother from EV71 infection during swimming season, I navigated Google website to search for preventive strategies. Currently, there is neither vaccine available for prevention of EV71, nor antiviral treatment for EV71 infection. Before development of effective antiviral agents or specific vaccine available to control epidemics of EV71, identification of the receptor(s) for EV71 and block of the receptor(s) may be a good regimen for prevention of EV71 infection. Sialic acid (SA) also known as neuraminic acid is usually linked to galactose (Figure 1) or other sugar residues as an antenna of blood group antigens, tumor antigens or viral receptors (4). Evidence has shown that different influenza viruses such as human influenza viruses use sialic acid-α2,6 galactose (SA-α2,6Gal) residue as their receptors, and avian influenza viruses use sialic acid- α 2,3galactose (SA- α 2,3Gal) as their receptors (1).



Human flu receptor　　Avian flu receptor

Figure 1. Sialic acid linked to galactose at 2,6 position (SA-a2,6Gal) or SA--a2,3Gal is a receptor for human or avian influenza virus, respectively.

The receptor for EV71 is currently unclear (3). Gastrointestinal and respiratory epithelial cells expressed abundant SA-containing glycoproteins and SA-containing glycolipids (1,5,6). It was, therefore, postulated that EV71 might use the SA-linked sugar residue on intestinal epithelial cells as a receptor, and natural SA-linked sugars may prevent human intestinal cells from EV71 infection.

This study was conducted to investigate whether SA-linked sugar residue on DLD-1 intestinal cells could be a receptor for enterovirus 71, and administration of certain natural food with SA-linked sugars from human milk could block EV71 infections. If this is the case, I may be able to use natural food containing SA-linked sugars to make chewable gum or skin spray formula for prevention of EV71 infections at home, swimming pool and school.

二、Materials and Methods

1. Study design: This study included 3 kinds of designs to reach 3 specific aims. First is to demonstrate that EV71 could infect DLD-1 intestinal cells, but not K562 myeloid cells; second is to demonstrate SA-linked glycan responsible for the entry of EV71 infection by showing that sialidase depletion of SA residues on DLD-1 cells protects DLD-1 cells from EV71 infection; and third is to identify a

potentially specific SA-linked glycan as a receptor for EV71 by showing that food with SA-linked glycan significantly blocked EV71 infection of DLD-1 cells.

2. Preparation of EV71: A clinical isolate of EV71 was obtained from the Laboratory of Virology, Department of Pathology, Chang Gung Memorial Hospital, Kaohsiung. EV71 were cultured and harvested from Vero cells. Vero cells at $5x10^5$ cells/ml were cultured in 75 cm^2 culture flasks for over night, and inoculated with EV71 at multiplicity of infection (MOI)=2 for 6 days. When more than 60% Vero cells revealed cytopathic effect (CPE), the total cell pellet was freeze and thaw for 3 times before the virus harvested by centrifugation at 1500 g for 10 minutes to separate viruses from cell debris. The virus titer was adjusted to $2x10^7$ copies/ml based on RT-PCR quantification of EV71 virus copies. A large amount of EV71 viruses were aliquoted and stored at -80℃before studies.

3. Human intestine epithelial cell line: We used DLD-1 intestinal epithelial cells as a target of EV71 infection, and K562 myeloid leukemia cells were used as control cells for comparison. The DLD-1 and K562 cell lines were obtained from Food Industrial Research Institute, Hsin-chu, Taiwan. The reason to use intestinal cell line is because it can express different levels of SA-linked glycans resembling neonatal intestine (6). DLD-1 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) medium with 10% fetal bovine serum, and harvested into $2x\ 10^6$ cells/ml for testing whether depletion of SA-linked glycans on DLD-1 cells by sialidase treatment reduced EV71 infection. Experiments were also performed to differentiate whether different natural SA-linked glycans such as SA-α2,3Gal or SA-α2,6Gal can protect against EV71 infections.

4. Sources of sialidase and natural SA-linked glycans: The sialidase (α2-3, 6-sialidase, *Clostridium perfringens*) that can cleave SA from SA-α2,3Gal and/or SA-α2,6Gal compounds was purchased from Calbiochem Inc., Darmstadt, Germany. For experiments, DLD-1 cells were pretreated with 2, 10 or 50 mU/ml sialidase for 2 hours before subjected to EV71 infections at MOI=10. Human milk SA-α2,3Gal (97% purity) and SA-α2,6Gal (98% purity) were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and tested for inhibition of EV71 infections at 0.25 mg/ml and 0.05 mg/ml.

5. Determination of EV71 infection by indirect immunofluorescence and RT-PCR analysis: Infection of EV71 was assessed by an indirect immunofluorescent staining with an EV71-specific monoclonal antibody (Chemicon Inc. CA). DLD-1 cells ($2x10^5$ cells) with and without sialidase pre-treatment for 2 hours were subjected to EV71 infection. The EV71 infected DLD-1 cells were harvested in one day for staining with mouse anti-EV71 monoclonal antibody or nonspecific antibody (5 drops for each slide) after cold acetone fixation, followed by FITC-labeled goat anti-mouse immunoglobulin antibody for fluorescent visualization. Cells were also harvested in 3 days for quantification of EV71 replication by a real time quantitative RT-PCR (qRT-PCR) analysis as previously described (7). The primers used to detect EV71 RNA copies by SYBR Green fluorescent RT-PCR were forward: 5'-CCCCTGAATGCGGCTAATC-3' and reverse: 5'-CCATATAGCTATTGGATTGGCCA-3'. The copies of virus titers were calculated based on a standard curve made by a series of well-known RNA copies of EV71.

6. Determination of SA-linked sugar residue as a receptor and inhibitor of EV 71 infection by qRT-PCR: To assess whether SA-linked sugar residues were receptors

for EV71 infection, EV71 (2x 10^6 copies/ml) were co-incubated with SA-α2,3Gal (0.05 or 0.25 mg/ml) or SA-α2,6Gal (0.05 or 0.25 mg/ml) 15 minutes before added to infect DLD-1 cells ($2x10^5$ cells) for 3 days. Replication of EV71 in DLD-1 cells was determined by qRT-PCR detection as described above.

7. Differentiation between the SA-containing glycoprotein and glycolipid responsible for EV71 infection: Sugar residues on cell surface are usually linked to protein, called glycoprotein, or linked to lipid, called glycolipid. Employing inhibitors of protein glycosylation and lipid glycosylation synthesis, we investigated if the SA-based residue responsible for EV71 infection stems from glycoprotein or glycolipid. To test whether N-linked or O-linked sialylglycoprotein on DLD-1 cells is the receptor for EV71, DLD-1 cells ($1x10^6$ cells/ml) were respectively incubated with 3mM benzyl N-acetyl-α-D-galactosaminide (Sigma-Aldrich Inc.) for 48 hours or with 0.2 mg/ml tunicamycin (Sigma-Aldrich Inc.) for 24 hours before subjected to EV71 binding assay at MOI=10. To test whether sialylglycolipid on DLD-1 cells was the receptor for EV71, cells ($1x10^6$ cells/50 μl) were incubated with 50 μl phosphotidylinositol-specific phospholipase (5U/ml) purchased from Sigma-Aldrich Inc. for 90 minutes before subjected to the test of EV71 binding assay. The EV71 binding assay was performed within one hour after washing out the treatment of specific inhibitor because DLD-1 cells in the inhibitor-free condition could re-express glycoprotein or glycolipid that might interfere the experimental interpretation.

8. Data management and statistics: Chemical structures of the SA-linked compounds were drawn by the software of Chemwindow 6.0 (Bio-Rad Inc., Hercules, CA). Specific infection of EV71 to DLD-1 cells was compared to the control cell line, K562, myeloid leukemia cells. Binding of EV71 to SA-linked sugars was

validated by depletion of SA by sialidase treatment. Inhibition of EV71 by different SA-linked sugar residues was analyzed by non-parametric analysis of Mann-Whitney U test.

三、Results and Discussion

1. Culture and quantification of EV71 titers

EV71 were initially cultured in two flasks of Vero cells at MOI = 2. The cell culture was maintained for 6 days until more than 60% Vero cells revealed cytopathic effect (CPE) (Figure2A.). The total EV71 were harvested by freeze and thaw for 3 times, followed by separation of viruses from cell debris by centrifugation. For measurement of total RNA copies in the EV71 stock solution, we made a 20-fold dilution before subjected to qRT-PCR analysis of RNA copies of EV71 (Figure2B.). Based on a standard curve made by a series of well-known EV71 copies (Figure2C.), the virus copies in the stock sample were calculated by an interpolation (Figure2D.).

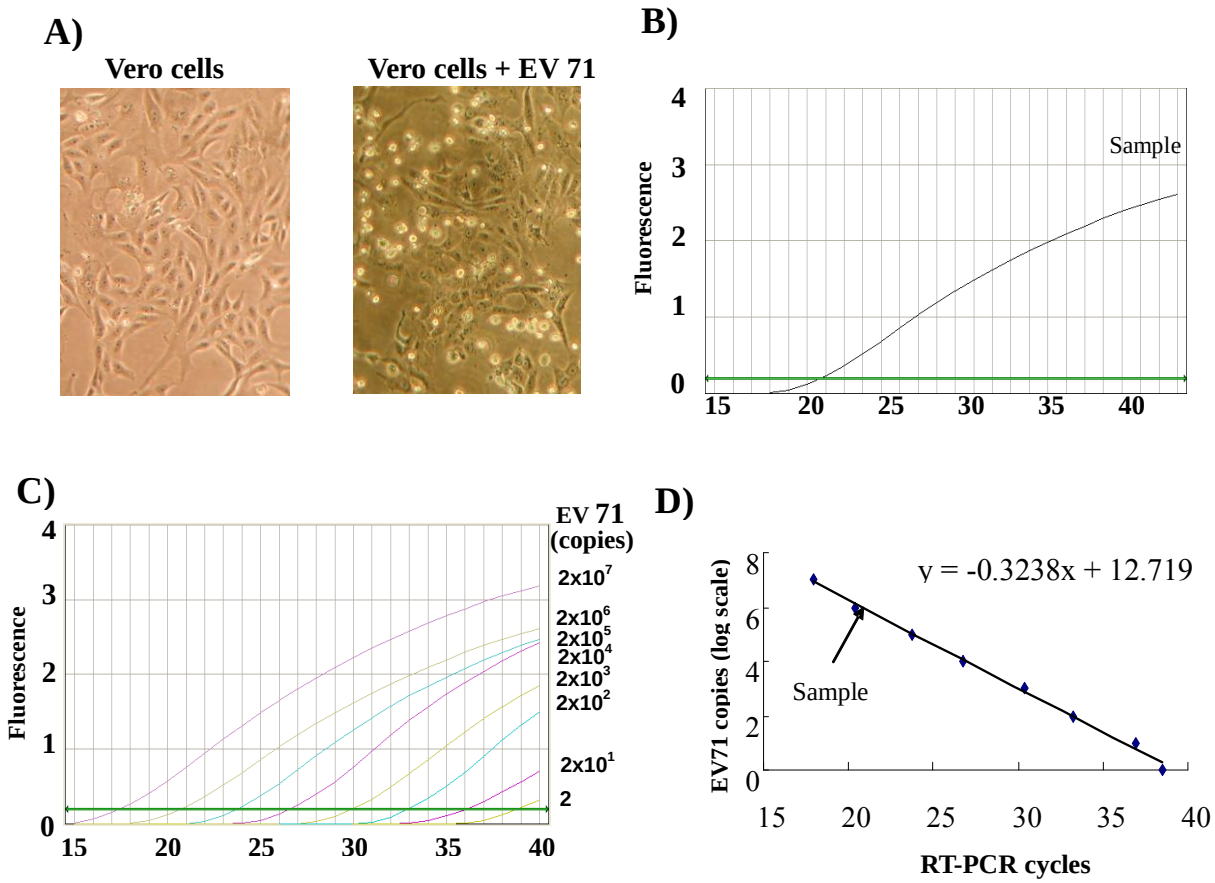


Figure 2. Culture and quantification of EV71. EV71 were cultured in Vero cells at MOI=2 until visible cytpathic effect (CPE) (A), and subjected to quantitative RT-PCR analysis of virus titer (B). Based on qRT-PCR detection of a series of well-known EV71 titers (C), the virus titers were determined by an interpolation on the standard curve (D).

Since the EV71 titers in the 20-fold dilution solution were $1x10^6$ copies/ml, the original EV71 titers in the stock solution were 2×10^7 copies/ml.

2. EV71 infection of DLD-1 intestinal cells

Experiments were next performed to observe the morphology and virus replication of EV71 infection in DLD-1 cells. It was found that EV71 did not infect K562 cells, but infected DLD-1 cells as demonstrated by indirect immunofluorescent assay in 24 hours of infection (Figure 3A, 3B). EV71 could

infect and replicate in DLD-1 cells in 4 hours, and caused a dramatic increase of replication in 24 hours of EV71 infection (Figure 3C). The virus replication titers (RNA copies of EV71) increased from 67 copies of EV71 per ml to more than 10^6 copies/ml in 2 days.

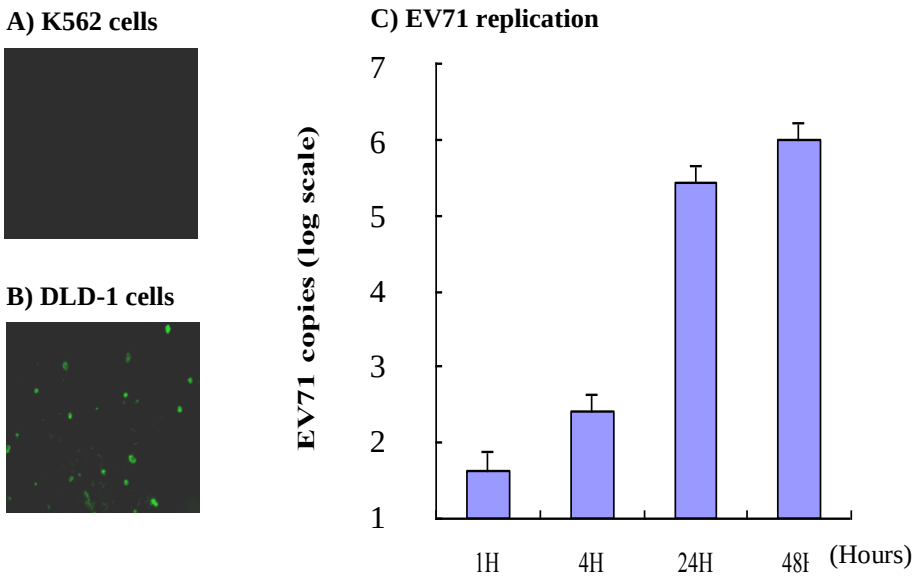


Figure3. EV71 infected and replicated in DLD-1 cells. EV71 did not infect K562 cells (A), but infected DLD-1 cells (B) in 24 hours as demonstrated by immunofluorescent assay. EV71 initiated the replication in DLD-1 cells within 4 hours (4H), and rapidly replicated in 24 hours (24H). Data presented were calculated from 4 experiments.

Results from these experiments indicated that EV71 although not infected K562 cells, infected and replicated quickly in DLD-1 intestinal cells in 24 to 48 hours.

3. Inhibition of EV71 infection in DLD-1 cells by sialidase treatment

In order to study whether SA-containing residue mediated EV71 infection of DLD-1 cells, we depleted surface SA from SA-α2,3 Gal and SA-α2,6Gal of

DLD-1 cells by preincubation of the sialidase, which cleaves SA from SA-α2,3 Gal or SA-α2,6Gal compounds, at 2, 10, and 50 mU/ml. After sialidase treatment for 2 hours, we added EV71 at MOI=10 for one hour of infection. As shown in EV71-specific immunofluorescent assay (Figure 4, upper panel), sialidase treatment decreased EV71-infected cells. Pretreatment of sialidase also significantly reduced the EV71 replication in DLD-1 cells for 3 days from 1.7 $x10^6$ copies/ml down to 7.0 x 10^4 copies/ml, with more than 20-fold reduction (Figure 4, lower panel).

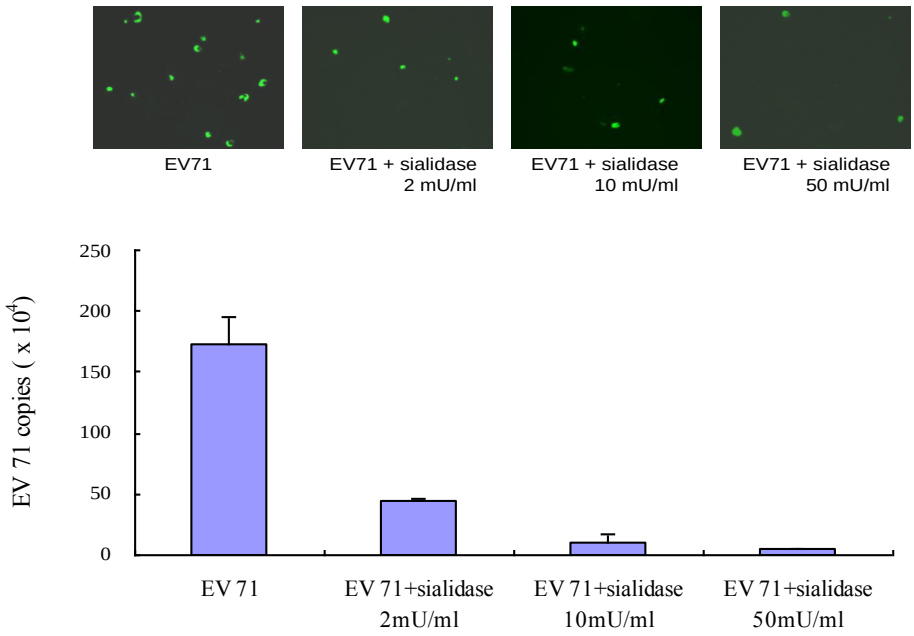


Figure 4. Sialidase treatment of DLD-1 cells decreased EV71 infection. DLD-1 cells pre-treated with different doses of sialidase significantly reduced EV71 infection in one hour under immunofluorescent assay (upper panel), and in T-PCR quantification of EV71 replication in 3 days (lower panel, data derived from 4 experiments).

This experiment suggests that SA-linked glycans on intestinal cells are responsible for the entry of EV71 infection. It was therefore hypothesized that

certain food with SA-containing glycans might block EV71 infection to DLD-1 cells. To test this hypothesis, SA- α 2,3Gal and SA- α 2,6Gal sodium salts from human milk were used in the next experiments.

4. Blockade of EV71 infection by SA-derived glycans from milk

Purified SA- α 2,3Gal (molecular weight 633) and SA- α 2,6Gal (molecular weight 655) from human milk (0.05 mg/ml or 0.25 mg/ml) were used to inhibit EV71 infection of DLD-1 cells. As showed in Figure 5, incubation of SA- α 2,3Gal and SA- α 2,6Gal from human milk with EV71 before infection significantly inhibited EV71 infection of DLD-1 cells.

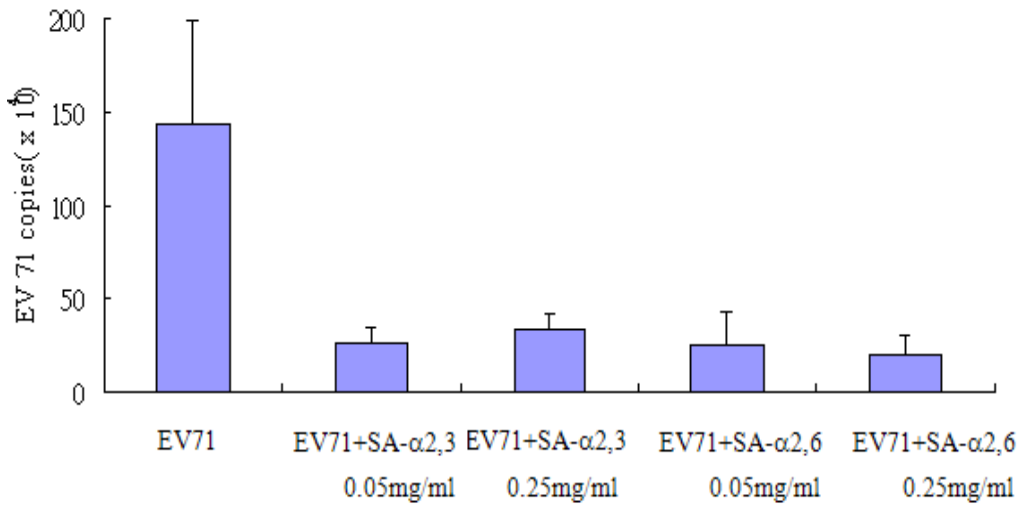


Figure 5. Incubation of EV71 with SA-□ 2,3Gal and SA-□ 2,6Gal decreased EV71 infection of DLD-1 cells. DLD-1 cells co-incubated with SA-linked galactose (SA-□ 2,3Gal or SA-□ 2,6Gal) significantly reduced EV71 replication for 3 days as quantified by RT-PCR analysis. Data presented were calculated from 3 duplicate studies.

This result suggests that EV71 recognizes not only SA–α2,3Gal but also SA-α2,6Gal epitopes as receptors to infect DLD-1 intestinal cells.

5. SA-linked O-glycan and glycolipid but not N-glycan responsible for EV71 infection

To differentiate whether SA-containing glycoprotein or SA-containing glycolipid responsible for EV71 infection, we used benzyl N-acetyl-α-D-galactosaminide (3mM), tunicamycin (0.2mg/ml) and phosphotidylinositol-specific phospholipase (5U/ml) to respectively deplete O-linked glycan, N-linked glycan and glycolipid of DLD-1 cells. It was found that depletion of O-linked glycan or glycolipid, but not N-linked glycan, significantly decreased EV71 infection of DLD-1 cells (Figure 6). Particularly, O-linked glycan was the major entry of EV71 infection because depletion of O-glycans by benzyl N-acetyl-α-D-galactosaminide (3mM) reduced the most EV71 infection.

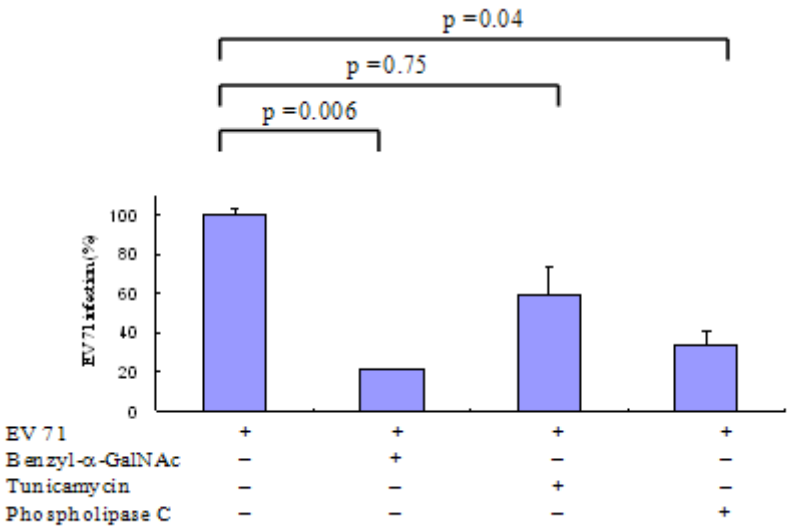


Figure 6. O-glycan and glycolipid but not N-glycan responsible for the EV71 infection. O-glycan synthesis inhibitor, benzyl N-acetyl-α-D-galactosaminide and glycolipid anchorage inhibitor, phosphotidylinositol- specific phospholipase, but not N-glycan synthesis inhibitor, tunicamycin, significantly inhibited EV71 infections. Data presented were calculated from 2 experiments, and p values were tested by Mann-Whitney U test.

The fact that depletion of O-linked glycan or glycolipid inhibited EV71 infection to DLD-1 cells suggests that SA-linked sugar residues are more important than proteins on intestinal cells responsible for EV71 infection. Future studies will focus on how to use or modulate sugar residues for prevention of EV71 infections.

四、Summary of the Study and Further Studies:

1. Natural SA-containing glycans in human milk could inhibit EV71 infections, suggesting a chewable gum or skin spray formula made with SA-linked sugars may be used to prevent EV71 infection at home and school.

2. It is well known that human influenza viruses recognize SA-α2,6Gal as a receptor, but avian influenza viruses recognize SA-α2,3Gal as a receptor (1). This discrepancy has limited avian flu outbreak in humans. In this study, it was found that pretreatment of sialidase directed against SA-α2,3Gal and SA-α2,6Gal reduced 20-fold EV71 infection, and both SA-α2,3Gal and SA-α2,6Gal could inhibit EV71 infections, suggesting EV71 may cause disseminated infections to gastrointestinal tract, respiratory tract and brain where possess abundant SA-α2,3Gal and/or SA-α2,6Gal expressing cells.

3. Further studies on a design for “double-edge sword” to bind EV71 and kill EV71 simultaneously. Interruption of sugar-lectin interactions for antiviral treatment has been recently described (8). This strategy provides only one target for antiviral treatment. Currently, there is no vaccine or antiviral agent available for EV71 treatment. Based on a strategy to link a SA-derived sugar residue, which can compete EV71 invasion receptor, to an antiviral agent such as cationic chitosan or lactoferrin, which can kill EV71 by targeting viral

envelope (9), we may be able to use the SA-linked antiviral agent as a "double-edge sword" to specifically bind EV71 and kill EV71 simultaneously, as shown in Figure 7.

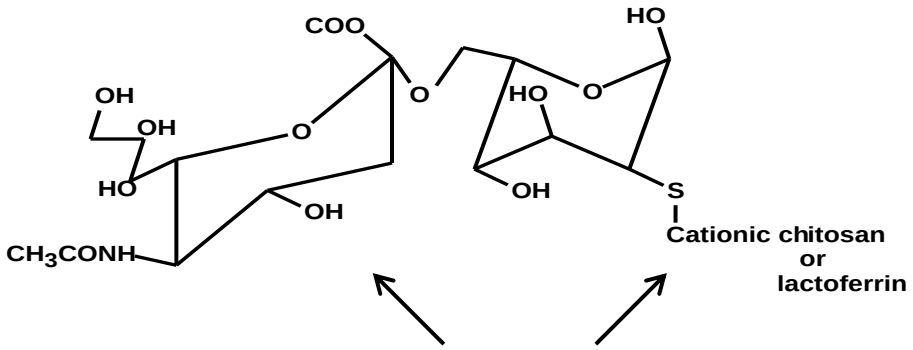


Figure 7. SA-based "double-edge sword" on blocking and killing of EV71 infections. SA-linked galactose can block EV71 infection by competition of sugar receptor, and the galactose can be linked with cationic compounds (10), such as chitosan or lactoferrin, for virus killing.

Based on this hypothesis, I have begun to do some pilot experiments on whether cationic chitosan could inhibit EV71 infection. As shown in Figure 8, it was found that chitosan (m.w. 100-130 Kd, water soluble) obtained from Dr. JY Wang, National Cheng Kung University, at 0.1 mM could reduce 2^5 times of EV71 infection, and pretreatment of sialidase at 2mU/ml could reduce 2^7 times of virus infection in DLD-1 cells.

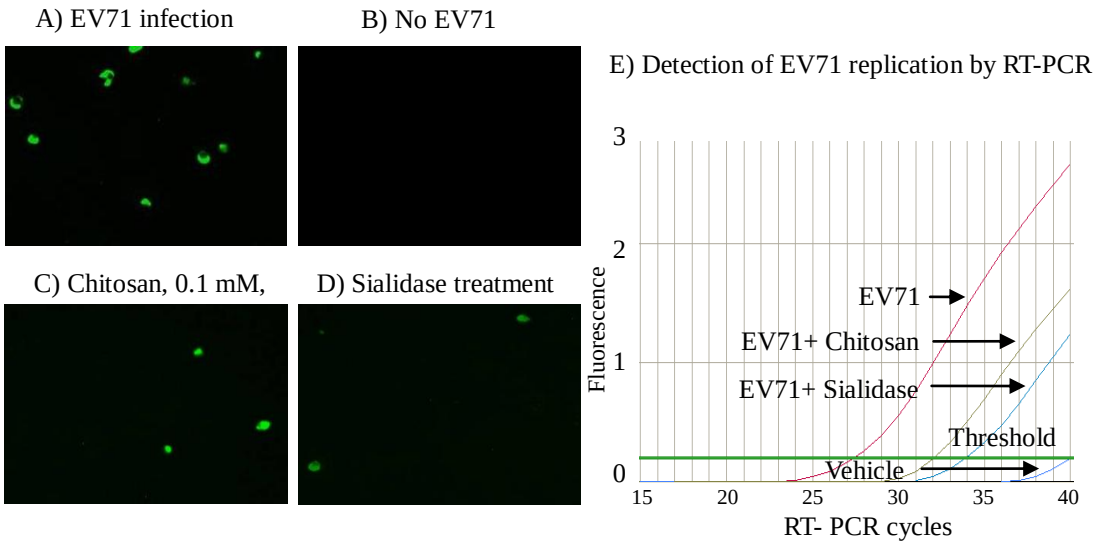


Figure 8. Chitosan and sialidase pretreatment protected DLD-1 cells from EV71 infections. EV71 at MOI=10 could infect DLD-1 cells ($2x10^5$ cells/ml) in one day as stained by anti-EV71 specific antibody (Ab) (A), but not control Ab (B). The infection could be inhibited by pretreatment of chitosan at 0.1 mM (C), and pretreatment of SAα2,3Gal sialidase at 2 mU/ml (D). The blockade of virus infection was validated by RT-PCR (repeated for 3 times), showing decrease of 2^5 to 2^7 virus replication (E).

Chitosan contains poly-(1,4)-β-D-glucosamine that reveals water-soluble and positive (cationic) charge, and is well-known to form nanoparticles for immune modulation (11). My future plan will try to make a chewable gum or skin spray formula made of sialylated glycans linked with chitosan nanoparticles for better prevention and treatment of enterovirus 71. Alternatively, another combination of sialylated glycans with cationic protein (10), such as lactoferrin that can kill EV71, for making one kind of "double-edge sword" nanoparticles to bind EV71 by SA-based glycans and kill EV71 by cationic protein, simultaneously.

五、References:

1. Ho M-W. 2008. Where's the bird flu pandemic. Retrieved from http://www.i-sis.org.uk/WTBFP.php on 21 Sep. 08.
2. Chang LY & Huang LM, *et al*. 2007. Neurodevelopment and cognition in children after enterovirus 71 infection. *N Engl J Med* 356:1226-34.
3. Chang LY. 2008. Enterovirus 71 in Taiwan. *Pediatr Neonatol* 49:103-12.
4. Varki A & Cummings R, *et al.* 2008. Essentials of Glycobiology. Retrieved from http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=glyco.chapter.988 on 15 Nov. 08.
5. Nilsson EC & Jamshidi F, *et al*. 2008. Sialic acid is a cellular receptor for coxackievirus A24 vairant, an emerging virus with pandemic potential. *J Virol* 82:3061-8.
6. Schnabl KL & Field C, *et al*. 2008. Ganglioside composition of differentiated Caco-2 cells resembles human colostrum and neonatal rat intestine *Br J Nutr* 2008;20:1-7.
7. Li CC & Yang KD, *et al.* 2002. Clinical manifestations and laboratory assessment in an enterovirus 71 outbreak in southern Taiwan. *Scan J Infect Dis* 34:104-9.
8. Balzarini J. 2007. Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. *Nat Rev Microbiol* 5:583-97.
9. Weng TY & Chen LC, *et al*. 2005. Lactoferrin inhibits enterovirus 71 infection by binding to VP1 protein and host cells. *Antivirus Res* 67:31-7.
10. Lee RT & Lee YC. 2006. Carbohydrate ligands of human C-reactive protein: binding of neoglycoproteins containing galactose-6-phosphate and galactose-terminated disaccharide. *Glycoconj J* 23:317-27.
11. Chen CL & Wang JY, et al. 2008. The effect of water-soluble chitosan on macrophage activation and the attenuation of mite allergen-induced airway inflammation. *Biomaterials* 29:2173-82.

六、Acknowledgement:

The author would like to thank her mentors from school and laboratory for their directions on her study in the past 2 years. Their suggestion and help have advanced her knowledge and made this study successful.

評語

本著作探討腸病毒 71 型，以自然醇為接受器，及其抑制劑，本研究成果對未來腸病毒 71 防制治療可作參考。

本實驗使用的致病腸病毒，如能改為不具感染性、異變性探討。其研究過程，對高中學生較有保護作用。