รายงานวิจัยฉบับสมบูรณ์

โครงการ

การผลิตแอนติบอดีสายเดี่ยวของมนุษย์ที่จำเพาะต่อโปรตีนเอ็นเอส-วันของไวรัสเดงกี่

โดย

ดร. อรณัชชา พ่วงแพ

พฤษภาคม 2555

สัญญาเลขที่ TRG5480006

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การผลิตแอนติบอดีสายเดี่ยวของมนุษย์ ที่จำเพาะต่อโปรตีนเอ็นเอส-วันของไวรัสเดงกี่

ดร. อรณัชชา พ่วงแพ   มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ ศาสตราจารย์ ดร. เพทาย เย็นจิตโสมนัส นักวิจัยที่ปรึกษา สำหรับคำแนะนำ กำลังใจ และการสนับสนุนการทำวิจัยในโครงการนี้ในทุก ๆด้าน

ขอขอบคุณ ศาสตราจารย์ ดร. วันเพ็ญ ชัยคำภา ที่ปรึกษาโครงการวิจัย ที่กรุณาอนุเคราะห์ Human antibody phage display library ซึ่งเป็นส่วนสำคัญของโครงการวิจัยนี้ อีกทั้งยังให้คำแนะนำ กำลังใจและการสนับสนุนในทุก ๆด้านเป็นอย่างดียิ่ง

ขอขอบคุณอาจารย์ นักวิจัย นักวิทยาศาสตร์ ผู้ช่วยวิจัย นักศึกษา และเพื่อนร่วมงานในหน่วยอณูเวชศาสตร์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ทุกท่านในการสนับสนุนการปฏิบัติงานของผู้วิจัยทั้งทางตรงและทางอ้อม

ท้ายนี้ ผู้วิจัยขอขอบคุณ หน่วยอณูเวชศาสตร์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล สำหรับการสนับสนุนสถานที่ อุปกรณ์-เครื่องมือวิจัย และสารเคมีพื้นฐานต่าง ๆ ซึ่งเป็นส่วนสำคัญยิ่งต่องานวิจัยนี้ และสำนักงานกองทุนสนับสนุนการวิจัยและเจ้าหน้าที่ผู้เกี่ยวข้องที่เอื้อให้การดำเนินงานวิจัยนี้สำเร็จลุล่วงตามเป้าหมาย ผู้วิจัยหวังเป็นอย่างยิ่งว่า องค์ความรู้และผลผลิตที่ได้จากโครงการวิจัยนี้จะเป็นพื้นฐานในการศึกษาพัฒนาต่อยอดผลิตภัณฑ์สู่การนำไปใช้รักษาโรคในมนุษย์ได้ในอนาคต

ดร. อรณัชชา พ่วงแพ
พฤษภาคม 2556

ABSTRACT

Project Code : TRG5480006

Project Title : Production of human single chain variable fragment antibody (HuScFv) specific to NS1 protein of dengue virus

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Dengue hemorrhagic fever caused by dengue virus infection is the major health problem in tropical countries including Thailand. Even the disease may be fatal; there is no protective vaccine or effective anti-viral agent available. Level of non-structural protein 1 (NS1) secreted from dengue virus-infected cells highly associates with disease severity. Thus, it is postulated that neutralizing or interfering of NS1 function would be the therapeutic strategy for relieving the disease severity. In this study, we generated the human single-chain variable fragment (HuScFv) specific to dengue NS1 and determined the HuScFv inhibitory effect to dengue virus infection in cell culture. The recombinant NS1 protein was produced and purified for using as the target antigen in selection of NS1-specific HuScFv from the human antibody phage display library by phage display technology. Phage-transformed E. coli clones were induced for expression of monoclonal HuScFv which were further tested for the binding activity to native NS1 secreted from dengue virus-infected cells by indirect ELISA. From 19 HuScFv tested, all clones showed higher ELISA signal than negative control and 2 of them with the highest signal; i.e. HuScFv11 and HuScFv13, were selected for further large-scale production and purification. Immunofluorescence study demonstrated that HuScFv11 and HuScFv13 could interact with cytoplasmic NS1 in dengue virus-infected cells but no interaction was observed in MOCK-infected cells. HuScFv supplemented in culture medium of dengue virus-infected cells also significantly reduced viral released into the culture supernatant. Studies of NS1-HuScFv interaction by mimotope searching and molecular docking concordantly revealed that HuScFv11 bound to N-terminal of NS1 while HuScFv13 bound to C-terminal of NS1 molecule with low-binding energy. Therefore, HuScFv11 and HuScFv13 worth further development to be therapeutic antibody for dengue virus-infected patient in the near future.

Keywords : Human single-chain variable fragment (HuScFv), non-structural protein-1 (NS1), dengue virus, phage display, therapeutic antibody

บทคัดย่อ

รหัสโครงการ : TRG5480006

ชื่อโครงการ : การผลิตแอนติบอดีสายเดี่ยวของมนุษย์ที่จำเพาะต่อโปรตีนเอ็นเอส-วันของไวรัสเดงกี่

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โรคไข้เลือดออกเป็นปัญหาสาธารณสุขที่สำคัญของประเทศในเขตร้อนรวมทั้งประเทศไทยซึ่งมีสาเหตุมาจากการติดเชื้อไวรัสเดงกี่ ผู้ติดเชื้ออาจมีอาการของโรคอย่างรุนแรงถึงขั้นเสียชีวิตได้ แต่ในปัจจุบันยังไม่มีวัคซีนป้องกันโรค หรือยาต้านไวรัสเดงกี่ที่มีประสิทธิภาพ โปรตีนเอ็นเอส-วันที่ถูกสร้างและหลั่งจากเซลล์ติดเชื้อมีความสัมพันธ์โดยตรงกับความรุนแรงของโรคในผู้ป่วย ดังนั้นการลบล้างหรือยับยั้งการทำงานของโปรตีนเอ็นเอส-วันจึงเป็นแนวทางในการรักษาเพื่อลดความรุนแรงของการติดเชื้อได้ ในการวิจัยนี้ ผู้วิจัยมีวัตถุประสงค์จะสร้างแอนติบอดีสายเดี่ยวของมนุษย์โดยสมบูรณ์ที่มีความจำเพาะต่อโปรตีนเอ็นเอส-วันของไวรัสเดงกี่ และศึกษาผลของแอนติบอดีดังกล่าวในการลดการติดเชื้อไวรัสเดงกี่ในเซลล์เพาะเลี้ยง โดยการผลิตโปรตีนลูกผสมเอ็นเอส-วันและทำให้บริสุทธิ์เพื่อใช้เป็นโปรตีนเป้าหมายในการคัดเลือกฟาจที่แสดงแอนติบอดีที่จับจำเพาะกับโปรตีนเอ็นเอส-วันจากคลังแอนติบอดีมนุษย์โดยเทคโนโลยีฟาจดิสเพลย์ และเหนี่ยวนำให้เชื้อแบคทีเรียอีโคไลที่ติดเชื้อฟาจเหล่านั้นสร้างโมโนโคลนาลแอนติบอดีสายเดี่ยวเพื่อใช้ทดสอบการจับกันของแอนติบอดีสายเดี่ยวกับโปรตีนเอ็นเอส-วันที่หลั่งจากเซลล์ติดเชื้อไวรัสเดงกี่โดยวิธีอินไดเรคท์อิไลซ่า พบว่าแอนติบอดีสายเดี่ยวที่ทดสอบจำนวน ๑๙ โคลนให้ผลบวกสูงกว่าการทดลองควบคุมทั้งหมด และได้คัดเลือก ๒ โคลนที่ให้ผลอิไลซ่าสูงสุด ได้แก่ แอนติบอดีสายเดี่ยวหมายเลข ๑๑ และ ๑๓ เพื่อนำไปผลิตในระบบใหม่เพื่อให้ได้ปริมาณและความบริสุทธิ์สูงขึ้นเหมาะสมกับการศึกษาในขั้นตอนต่อไป โดยการย้อมอิมมูโนฟลูออเรสเซนท์พบว่า แอนติบอดีสายเดี่ยวหมายเลข ๑๑ และ ๑๓ สามารถจับกับโปรตีนเอ็นเอส-วันในเซลล์ติดเชื้อไวรัสเดงกี่ได้ และไม่จับกับโปรตีนอื่นใดในเซลล์ไม่ติดเชื้อ อีกทั้งยังพบว่าเมื่อเติมแอนติบอดีดังกล่าวลงในอาหารเลี้ยงเซลล์ติดเชื้อไวรัสเดงกี่ มีผลลดการสร้างไวรัสออกมาในอาหารเลี้ยงเซลล์อย่างมีนัยสำคัญเมื่อเทียบกับการทดลองควบคุม จากผลการศึกษาปฏิสัมพันธ์ของแอนติบอดีสายเดี่ยวทั้ง ๒ โคลนกับโปรตีนเอ็นเอส-วัน โดยการหาเปปไทด์มิโมโทปและการสร้างแบบจำลองโมเลกุลทางคอมพิวเตอร์ พบว่า แอนติบอดีสายเดี่ยวหมายเลข ๑๑ จับกับบริเวณปลายด้านเอ็นเทอร์มินอล ในขณะที่แอนติบอดีสายเดี่ยวหมายเลข ๑๓ จับกับกรดอมิโนทางด้านซีเทอร์มินอลของโปรตีนเอ็นเอส-วันด้วยระดับพลังงานที่สามารถเกิดขึ้นจริงได้ตามธรรมชาติ ดังนั้น แอนติบอดีสายเดี่ยวหมายเลข ๑๑ และ ๑๓ เหมาะสมในการพัฒนาต่อไปเพื่อใช้เป็นแอนติบอดีเพื่อการรักษาผู้ป่วยติดเชื้อไวรัสเดงกี่ในอนาคต

คำหลัก : แอนติบอดีสายเดี่ยวของมนุษย์ โปรตีนเอ็นเอส-วัน ไวรัสเดงกี่ ฟาจดิสเพลย์ แอนติบอดีเพื่อการรักษา

สารบัญ

เนื้อหางานวิจัย

INTRODUCTION

Dengue hemorrhagic fever (DHF) is the major health problem worldwide, especially in tropical and sub-tropical areas. More than 2,500 million people in more than 100 countries are in the endemic areas with the incidence of infection approximately 50 million cases annually. Most patients are at school ages and also adults. In Thailand, first dengue case was reported in year 1950 with the first epidemic in Bangkok in year 1958. Since then, DHF is observed as the seasonal outbreak annually especially in the community areas. Dengue virus infection is transmitted to human via the biting of female *Aedes* mosquitoes harboring dengue virus in their salivary gland; thus, the transmission of the disease vector indicates the distribution of DHF. The clinical manifestations of infected individuals vary from mild febrile illness (dengue fever, DF), to the more severe form of disease characterized by bleeding in internal organs (DHF), and the fatal form when patient going into shock (dengue shock syndrome, DSS). The severe form of disease is usually found in patient with the secondary infection with the dengue virus in heterologous subtype from previous infection. The more severe clinical features are postulated to be due to the effect of virus-specific antibodies at sub-neutralizing level that could enhance the binding of virus to host cell Fc receptor and facilitate viral entry in the following infection. Moreover, those antibodies with effecter sites (Fc) affect signaling pathway of the target cells and also stimulate the formation of complement attach complex resulting host cell lysis.

The causative agents of the disease, dengue viruses, are in family *Flaviviridae* which are serologically categorized into 4 subtypes. The viral positive-sense single stranded RNA genome encodes 10 proteins. Among dengue proteins, non-structural protein 1 (NS1) is tremendously produced since the early stage of infection as it is employed as an indicator for dengue virus infection diagnosis. NS1 is massively present in both infected cells and patient serum. Secretory NS1 in serum has been reported for its association with the disease severity. Those include the interaction with complement proteins which supports the formation of membrane attach complex (MAC). NS1 could also promote complement fixation on endothelial cells by binding to complement regulatory proteins. All of them are finally resulting in the lysis of endothelial cells and vascular leakage, the hallmark of the severe form of dengue infection. Thus, it is speculated that the inhibition of NS1 interaction with host proteins both in serum and on infected cell membrane could diminish the pathological effect exerted by dengue virus.

Nevertheless, a vaccine and anti-viral agent with protective efficacy against dengue infection have not been available and the disease management is only for supportiveness. Conventional antibody with Fc region specific to NS1 and other dengue proteins is concerned for the enhancement of infection known as antibody-dependent enhancement (ADE). Accordingly, antibody format lacking of Fc portion i.e. human single chain variable fragment (HuScFv) could serve as the ultimate therapeutic scheme for neutralization of NS1 in serum of dengue virus infected individual without unfavorable effect.

In this study, the recombinant dengue NS1 protein will be produced and used as target antigen for selection of NS1-specific HuScFv from human antibody phage display library. Monoclonal NS1-specific HuScFv will be produced and verified for their reactivity with NS1 protein from dengue virus. Ultimately, NS1-specific HuScFv will be demonstrated for their NS1-neutralization activity which sequentially reduces the pathogenesis of dengue virus infection.

OBJECTIVES

1. To produce and purify recombinant dengue NS1 protein
2. To select phage clone(s) expressing NS1-specific HuScFv from human antibody phage display library
3. To produce and purify NS1-specific HuScFv and verify for the binding activity and neutralizing activity to dengue virus NS1 protein

RESEARCH METHODOLOGY

In this study, the recombinant NS1 (rNS1) protein was produced and purified by molecular biology techniques. It was used as the target antigen in bio-panning step for selection of phage clones displaying NS1-specific HuScFv from the human antibody phage display library. Monoclonal HuScFv was produced from phagemid-transformed *E. coli* clones and screened for their reactivity to native NS1 by indirect ELISA. HuScFv clones with high binding activities were selected for further experiments including immunofluorescence assay and neutralization test in dengue virus-infected cell culture. The interaction of these HuScFv clones with dengue NS1 was also elaborated by mimotope searching and molecular docking approaches.

1. Amplification of dengue NS1-coding sequence

Dengue virus serotype 2 (strain 16681; DENV-2) was propagated in *Aedes albopictus* C6/36 cells and the culture supernatant containing the virus was extracted for viral genome by using QIAamp MinElute Virus Spin Kit. Viral RNA was reverse-transcribed to cDNA by using SuperScript^{TM}III First-Strand Synthesis System for RT-PCR and primer NS1-R (5'- CCG CTC GAG AGC TGT GAC CAA GGA GTT GAC CAA A-3'). Full length NS1-coding sequence was amplified by Platinum$^{®}$ *Pfx* DNA polymerase and primers NS1-F (5'-CCC GGA TCC GAT AGT GGT TGC GTT GTG AGC TGG A-3') and NS1-R. The endonuclease restriction sites of *Bam*HI and *Xho*I (underlined) were incorporated into the sequences for cloning purpose. The PCR amplicon was detected by 1% agarose gel electrophoresis and ethidium bromide staining.

2. Cloning of NS1-coding sequence into bacterial vector

The NS1 amplicon was digested at 5' and 3' ends by restriction endonuleases *Bam*HI and *Xho*I, respectively. The digested DNA was verified by agarose electrophoresis and DNA band at the size of NS1 was extracted from the gel. The DNA sequence was ligated to pET21b+ bacterial vector harboring 5' and 3' sticky ends of the same restriction endonucleases. The ligated plasmid was introduced into chemically competent BL21 (DE3) *E. coli* by heat-shock transformation. Antibiotic-resistant transformants were selected on LB agar containing ampicillin (100 ug/ml; LB-A agar).

3. Verification of *E. coli* transformants harboring recombinant plasmid

Ten transformants were randomly picked from the selective agar plate and grown on another new LB-A agar plate. Each clone was individually verified for the presence of NS1 sequence in the recombinant plasmid by PCR using primers NS1-F and NS1-R. The recombinant plasmid from PCR-positive transformant was extracted from representative *E. coli* clone and subjected to DNA sequencing. The amplified NS1 sequence was compared to NS1 sequence of dengue virus serotype 2 previously reported in the NCBI database (Accession no. U87411.1).

4. Induction and purification of recombinant NS1 (rNS1)

E. coli transformant harboring NS1 sequence was induced for the expression of C-terminal 6xHis-tagged rNS1 protein. The bacteria were grown in LB-A broth at 37°C with 250 rpm aeration until mid-log phase (OD_{600nm} = 0.4-0.6), then, IPTG was added to the culture at the final concentration of 0.5 mM. The culture was incubated at the same condition for 4 hours further before the bacterial cells were pelleted by centrifugation. The bacterial cells were resuspended in PBS and lysed for soluble protein extraction by ultrasonic sonication. The inclusion bodies containing insoluble protein was extracted by denaturing lysis buffer containing 8 M urea. The presence of rNS1 protein in both portions was analyzed by SDS-PAGE and Western blot analysis using anti-6xHis antibody. The rNS1-containing lysate was further subjected to protein purification by affinity chromatography using Co^{2+} resin according to the manufacturer's instruction. The purity of purified rNS1 fractions was determined by SDS-PAGE and Coomassie Brilliant Blue (CB) staining and measured the concentration by Bradford's assay. The purified rNS1 was prepared in 1xPBS and stored at -20ºC until use.

5. Selection of specific HuScFv from the human antibody phage display library by bio-panning

Purified rNS1 was used as the target antigen for selection of phage clones displaying NS1-specific HuScFv from the human antibody phage display library (kindly provided by Professor Wanpen Chaicumpa) (Kulkeaw *et al.*, 2009). Briefly, 100 ul rNS1 in coating buffer (carbonate-bicarbonate buffer, pH 9.6) was coated into a well of EIA/RIA strip and incubate at 37ºC for 16 hours. Excess protein was washed away and the well was block with 3% skim milk in PBS. The library (containing ~10^{11} pfu) was added into the well and incubated at 37ºC for 2 hours. After extensive washing of unbound phages, 100 ul of log phase HB2151 *E. coli* was added into the well containing rNS1-bound phages to allow phage transduction. The phagemid-transformed *E. coli* clones were selected onto LB-A agar containing 2% glucose (LB-AG). *E. coli* transformants were randomly picked and screened for the presence of HuScFv-coding sequence (*huscfv*) by PCR using phagemid-specific primers, i.e., R1 (5'- CCA TGA TTA CGC CAA GCT TTG GAG CC-3') and R2 (5'- CGA TCT AAA GTT TTG TCG TCT TTC C-3').

The *huscfv*-positive *E. coli* clones were induced for monoclonal HuScFv production by 1 mM IPTG similar to induction of rNS1 production. Peptide E-tagged HuScFv produced in bacterial lysate were detected by SDS-PAGE and Western blot analysis using rabbit anti-E tag antibody followed by swine anti-rabbit IgG-HRP-conjugate and Super Signal® West Pico chemiluminescent substrate. Protein concentration of each HuScFv-containing lysate was measured at OD_{280nm} by spectrophotometer.

6. Screening of binding activity of HuScFv to recombinant and native DENV NS1

Binding activity of monoclonal HuScFv to native DENV NS1 was determined by indirect ELISA. For preparing native DENV NS1, culture supernatant of DENV-2-infected Vero cells containing secreted NS1 was precipitated with 50% saturation of ammonium sulfate and dialyzed against PBS. Ten microgram of either the native NS1 preparation (antigen) and culture medium of the non-infected Vero cells (negative antigen control) in 100 ul coating buffer was added to ELISA plate and incubated at 37ºC for 16 hours. The plate was blocked and HuScFv-containing preparations (1 mg in 100 ul) were individually added to the wells of negative antigen control. After incubation at 37ºC for 1 hour, the fluids were transferred to the wells of native NS1 preparation and incubated further for 2 hours. The unbound protein was washed extensively and the NS1-bound HuScFv was detected by rabbit anti-E tag followed by swine anti-rabbit IgG-HRP conjugate. The colorimetric reaction was developed by adding 3, 3', 5, 5;-tetramethylbenzidine (TMB) substrate and 3 M H_2SO_4. Optical density at 450 nm (OD_{450nm}) of the content in each well was measured by ELISA reader. Non HuScFv-producing lysate and mouse polyclonal anti-NS1 antibody served as negative and positive antibody controls, respectively.

7. Subcloning of *huscfv* to new bacterial expression vector

For large scale production of purified HuScFv for further experiments, *huscfv* sequence from two *E. coli* clones with the highest binding signals to native NS1 were subcloned into modified pET23b+ bacterial vector as previously described (Poungpair *et al.*, 2010). The recombinant plasmid was introduced into BL21 (DE3) *E. coli*. The transformants grew on the selective LB-A agar plate was verified for the presence of *huscfv* by PCR and induced for the production of 6xHis-tagged HuScFv by culture with 0.5 mM IPTG for 4 hours. The 6xHis-tagged HuScFv was detected in bacterial lysate by Western blot analysis using anti-6xHis antibody. The lysate of HuScFv-positive *E. coli* clones were subjected to affinity purification by using Co^{2+} resin according to the manufacturer's instruction. The purity of HuScFv fractions was determined by SDS-PAGE and CB staining. The purified HuScFv was prepared in cell culture medium by dropwise dialysis and measured for the concentration by Bradford's assay and stored at -20ºC until use.

8. Determination of binding activity of HuScFv to NS1 in DENV-infected cells

Human hepatocellular carcinoma (Huh-7) cells were grown on glass cover slip in wells of 24-well culture plate for 24 hours before they were infected with DENV-2 at MOI = 0.1. After 3 days, DENV-infected cells and MOCK-infected cells were fixed with 3.6% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 3% BSA in PBS. Both cell preparations were incubated with purified HuScFv (2.5 uM) at 37ºC for 2 hours in a humidified chamber. Thereafter, the cells were rinsed and incubated with a mixture of rabbit polyclonal antibody to human single chain antibody fragments and mouse anti-NS1 antibody at 37ºC for 2 hours. After washing, the cells were further incubated with a mixture of Cy^{TM}3-conjugated AffiniPure donkey anti-rabbit Ig, Alexa Fluor 488-conjugated goat anti-mouse Ig, and anti-nuclear staining reagent (Hoechst) to localize HuScFv, NS1, and nuclear DNA, respectively. The mounted cells were observed by using a laser scanning confocal microscope.

9. Determination of DENV infection inhibitory effect mediated by NS1-specific HuScFv

Huh-7 cells were grown in wells of 96-well culture plate for 24 hours before infection. The cells were rinsed with plain medium and added with DENV2 at MOI = 0.1 and 1 for 2 hours, then, the virus solution was removed and the RPMI medium was added. After 12 hours of infection, the cells were rinsed, the culture supernatant was replaced by HuScFv-supplemented RPMI medium (2.5 uM) and grown further under the same condition. Culture supernatants at 24 and 36 hours after treatment were collected and titrated for number of infectious virus particles released from the infected cells by foci formation assay. Infected cells in the wells were subjected to cell ELISA for semi-quantification of intracellular DENV envelope (E) antigen. Irrelevant HuScFv and non-HuScFv treatments served as negative antibody controls in the experiment.

10. Determination of NS1-specific HuScFv mimotopes and identification HuScFv epitopes on NS1 molecule

HuScFv mimotopes were identified by selecting phage clones displaying 12-mer peptide that bound to NS1-specific HuScFv by using Ph.D.-12^{TM} Peptide Library (New England Biolab). Purified HuScFv11 or huScFv13 (10 ug) was coated into a microtiter well. After blocking with 10% skim milk, 100 ul of phage library (containing 10^{11} pfu) was added to the well and incubated at 25ºC for 1 hour. Unbound phages were removed by extensive washing, after that, mid log-phase ER2738 *E. coli* was added to the well and incubated at 25ºC for 20 minutes.

Phage-transfected E. coli were grown further in LB broth at 37ºC for 4.5 hours. Propagated phages in bacterial culture supernatant containing were precipitated by PEG-NaCl and subjected to the next panning round. Bio-panning was repeated for 2 more times and the phages collected from the third round were plated appropriately onto agarose-topped LB plate containing X-gal and IPTG. Blue plaques of phage-transfected *E. coli* were randomly picked up and extracted for phage DNA. The DNA was sequenced and deduced for 12-mer peptide inserts. Concensus mimotope sequences were obtained by multiple alignment of the deduced peptide sequences by EMBOSS ClustalW2. The mimotope concensus sequences were aligned with the NS1 amino acid sequence (accession no. U87411.1) by EMBOSS Needle. The NS1 peptides matched with the mimotope concensus sequences (HuScFv epitopes) were determined.

Phage mimotope searching result was verified by peptide competitive ELISA. Commercially synthesized peptided matched with phage mimotope concensus sequences were used as a competitor of the HuScFv-NS1 binding. Various concentrations of the synthesized peptides were mixed with purified 6xHis-tagged HuScFv (5 uM) and incubated at 37ºC for 2 hours prior to adding the mixture into wells coated with native NS1 (10 ug/well). After incubating at 37ºC for 2 hours, the amount of NS1-bound HuScFv was detected by using mouse anti-6xHis antibody followed by rabbit anti-mouse Ig-HRP-conjugate. The colorimetric substrate (TMB) and 3 M H_2SO_4 were added before OD_{450nm} was measured. The OD_{450nm} in NS1-coated wells added with the respective HuScFv without pre-incubation with peptides were used as 100% binding controls. Percent binding of NS1 in the presence of peptides was calculated.

11. *In silico* analysis of NS1-HuScFv interaction

The amino acid sequences of HuScFv and NS1 were subjected to protein homology modeling using Discovery Studio 2.5 program (Accelrys Inc.) while ITASSER server was used for threading method. All models were assessed by program PROCHECK v. 3.4. The protein-protein docking was conducted by using ZDOCK program. The NS1 was a receptor while the HuScFv was a ligand. The ligand was rotated through 15 degrees. The high Z dock score docking poses which correlated with mimotope/epitope results were selected for further analysis. These docked poses were refined by using RDOCK module embedded in Discovery Studio 2.5. PyMol v. 0.99 (DeLano Scientific) was used for all protein models.

RESULTS

1. Amplification of dengue NS1-coding sequence

NS1-coding sequence was amplified from dengue virus particle released into culture supernantant of DENV-2-infected C6/36 cells. The PCR product was determined by agarose gel electrophoresis and ethidium bromide staining and the amplicon was approximately 1.2 kb in size (Figure 1, lane 1).

2. Cloning of NS1-coding sequence into bacterial vector

After digestion NS1 amplicon with restriction endonucleases, the NS1 sequence was observed as the linearized DNA at size around 1.2 kb (Figure 1, lane 2) and extracted from the gel. NS1 DNA was ligated to bacterial expression vector and introduced into *E. coli* expression host cells. Numbers of *E. coli* transformants were observed on selective agar plate after overnight incubation (data not shown).

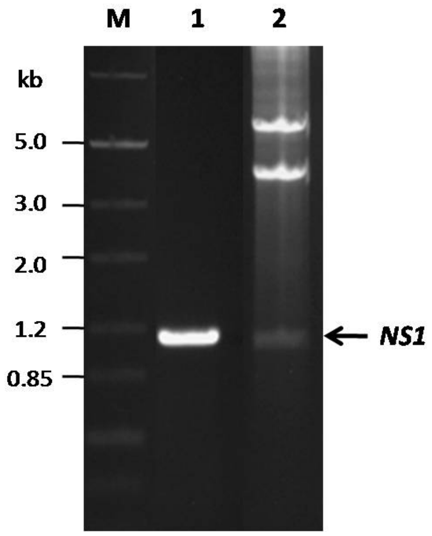
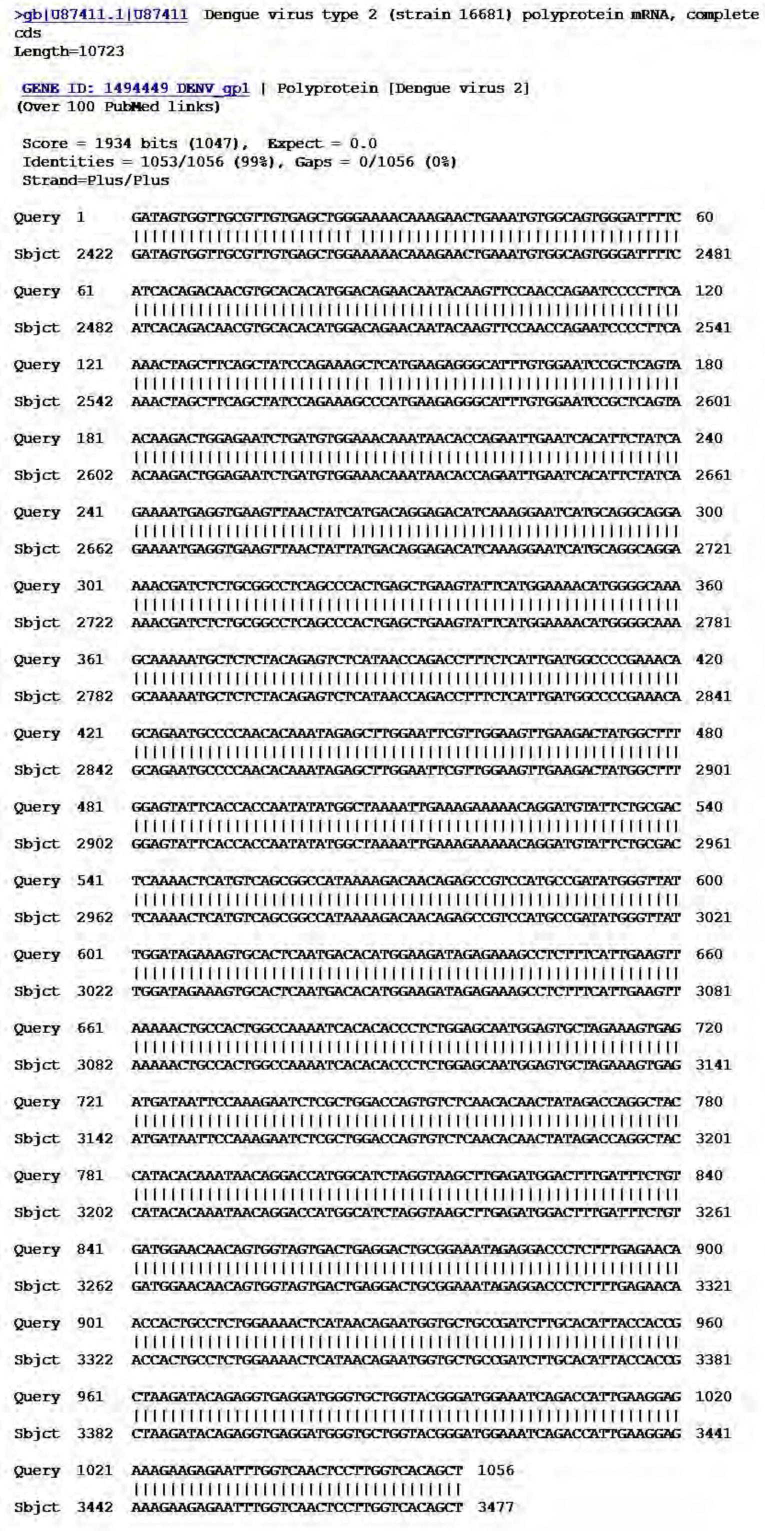


Figure 1 Amplicon of cDNA of gene coding for full-length NS1 of DENV-2, strain 16681 (~1.2 kb, arrow) (lane 1) and restriction endonuclease-digested recombinant plasmid (lane 2). ZipRuler™ Express DNA Ladder 1 (lane M). The numbers at the left are DNA sizes in kilobase pairs (kb)

3. Verification of *E. coli* transformants harboring recombinant plasmid

Recombinant plasmid-transformed *E. coli* clones were testified for the presence of NS1 sequence by PCR and yielded amplicon at size around 1.2 kb (data not shown) indicating that the selected *E. coli* clones harbored NS1-coding sequence in the recombinant plasmid. The amplified NS1 sequence was deposited to NCBI (GenBank) database (Accession no. JN692493) and compared to the previously deposited sequence (Figure 2). It was found that both sequences had 99% similarity and 3 mismatched nucleotides, *i.e.*, 25G>A, 148T>C, and 271C>T.



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>gb|U87411.1|U87411  Dengue virus type 2 (strain 16681) polyprotein mRNA, complete
cds
Length=10723

 GENE ID: 1494449 DENV_gp1 | Polyprotein [Dengue virus 2]
(Over 100 PubMed links)

 Score = 1934 bits (1047),  Expect = 0.0
 Identities = 1053/1056 (99%), Gaps = 0/1056 (0%)
 Strand=Plus/Plus

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Sbjct  3442  AAAGAAGAGAATTTGGTCAACTCCTTGGTCACAGCT  3477
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Figure 2 Sequence homology between the nucleotide sequence of the cloned cDNA of gene coding for NS1 (accession no. JN692493) and the NS1-coding sequence of DENV of the NCBI database (accession no. U87411.1).

4. Induction and purification of recombinant NS1 (rNS1)

After induction of *E. coli* transformant with IPTG, soluble and insoluble bacterial extracts were analyzed by SDS-PAGE and Western blot analysis using anti-6xHis antibody. Recombinant NS1 at size around 43 kDa was observed in insoluble portion of bacterial lysate (Figure 3, lane 1). Bacterial lysate containing rNS1 was subjected to purification step and the purified fraction was observed as the single protein band at the size of rNS1 (43 kDa) (Figure 3, lane 2).

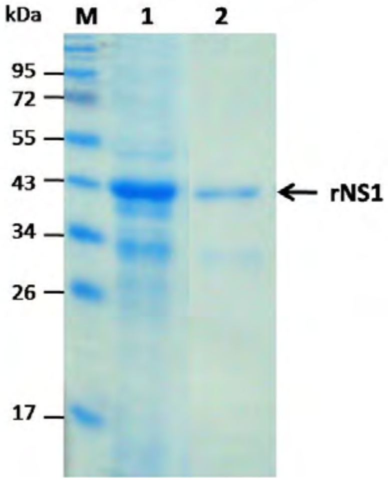

Figure 3 SDS-PAGE and Coomassie Brilliant Blue-stained homogenate of transformed *E. coli* carrying NS1-coding sequence grown under IPTG induction (lane 1) and affinity-purified rNS1 (~43 kDa, arrow) (lane 2). PageRuler^{TM} Prestained Protein Ladder (lane M). The numbers at the left are protein masses in kDa.

5. Selection of specific HuScFv from the human antibody phage display library by bio-panning

By using purified rNS1 as antigen in the phage bio-panning, 162 phagemid-transformed HB2151 *E. coli* were obtained. Among them, 136 clones (84%) carried *huscfv* inserts at size ~1,000 bp (Figure 4). From 40 randomly selected *huscfv*-positive clones, 28 clones (70%) could produce soluble monoclonal HuScFv at size around 26-34 kDa as detected by Western blot analysis using rabbit anti-E tag antibody and 19 clones of them were selected for further experiment (Figure 5).

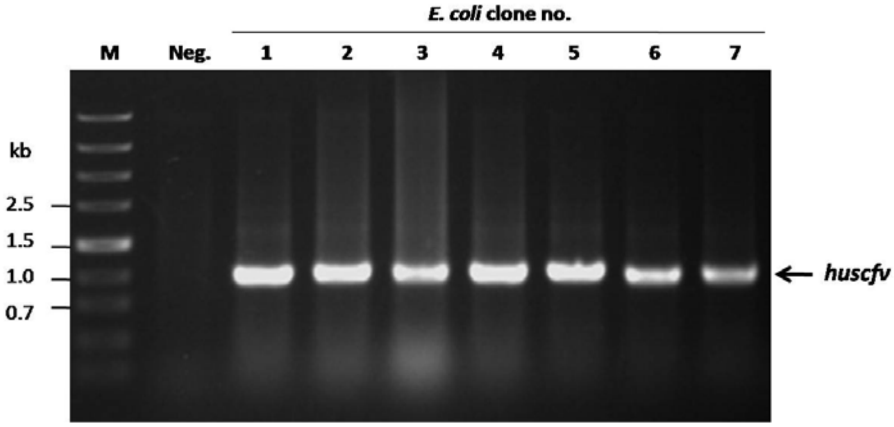

Figure 4 Agarose gel electrophoresis showing *huscfv* amplicons (~1 kb, arrow) amplified from seven representative clones of transformed HB2151 *E. coli* (lanes 1-7) derived from phage bio-panning with rNS1. ZipRuler^{TM} Express DNA Ladder 2 (lane M). Neg, original HB2151 *E. coli* which served as negative *huscfv* control. The numbers at the left are DNA sizes in kb.

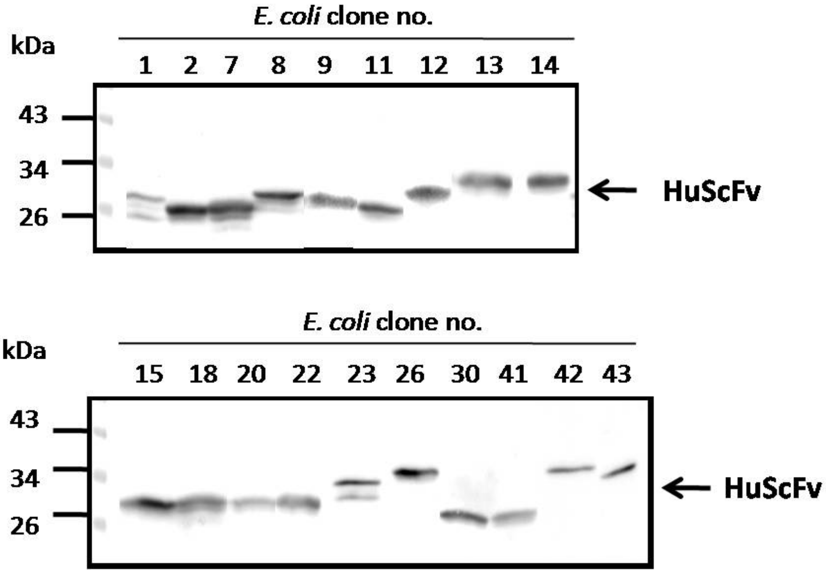

Figure 5 Western blot patterns of HuScFv (~26-34 kDa; arrows) in lysates of 19 *huscfv*-phagemid transformed HB2151 *E. coli* clones (no. 1, 2, 7, 8, 9, 11-15, 18, 20, 22, 23, 26, 30 and 41-43) using mouse anti-E tag antibody for detection. The numbers at the left of both blocks are protein masses in kDa.

6. Screening of binding activity of HuScFv to recombinant and native DENV NS1

Indirect ELISA was performed for determining the binding activities of 19 HuScFv clones to native DENV NS1. The result revealed that HuScFv of all clones gave varied ELISA signals but all were significantly higher than the signal from the negative HuScFv control (Figure 6). Among all clones, HuScFv of *E. coli* clones no. 11 and 13 (HuScFv11 and HuScFv13, respectively) exhibited the highest ELISA signals to native NS1.

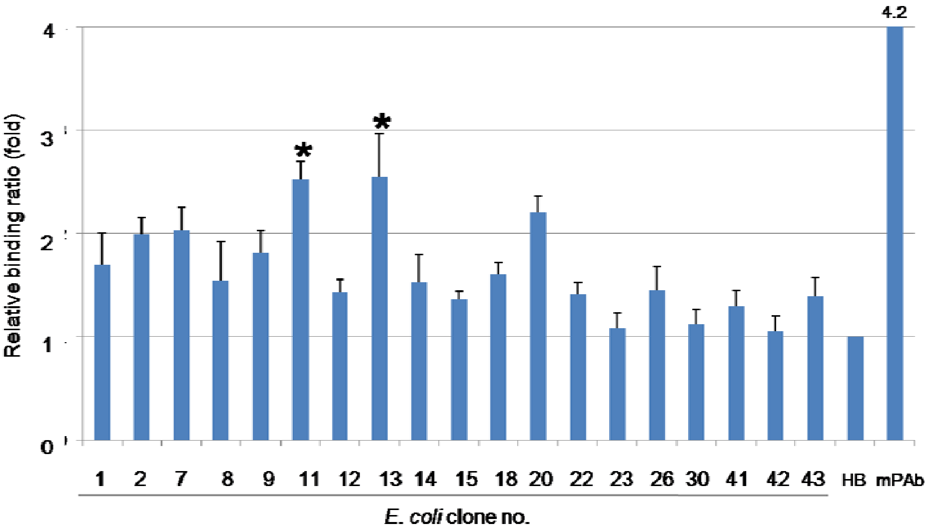

Figure 6 Indirect ELISA results for detecting the binding of HuScFv in lysates of 19 representative *huscfv*-phagemid transformed *E. coli* clones to native NS1. Clones no. 11 and 13 (asterisks) were selected for further use as the HuScFv in lysates of these clones gave the highest ELISA signal to the antigen compared with the signal from negative HuScFv control (HB). Mouse anti-NS1 polyclonal antibody (mPAb) was used as positive control. Results are shown as mean + SEM of three independent experiments.

7. Subcloning of *huscfv* to new bacterial expression vector

From indirect ELISA result, HuScFv11 and HuScFv13 yielded the highest binding signals to native NS1 and were selected for further experiments. The *huscfv* sequences of both clones were individually subcloned to modified pET23b+ vector for benefits of HuScFv production and affinity purification. The HuScFv expression in pET23b+ was higher than from previous expression system (pCANTAB5E phagemid vector). 6xHis tag was also incorporated at the C-terminus of the HuScFv for affinity purification by Co^{2+} resin. The purity of purified HuScFv was revealed in SDS-PAGE and CB staining as the single protein band at their original size (Figure 7).

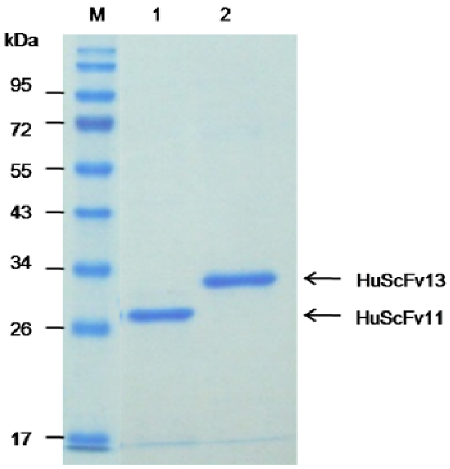

Figure 7 Patterns of purified HuScFv11 and HuScFv13 after SDS-PAGE and CBB staining (lanes 1 and 2, respectively).

8. Determination of binding activity of HuScFv to NS1 in DENV-infected cells

Native NS1 protein in DENV-infected cells was used as the target antigen to determine the binding activity of HuScFv in immunofluorescence assay. The green fluorescence indicating the location of NS1 protein was observed all over cytoplasm, especially in the perinuclear region, of DENV-infected cells which was not detected in MOCK-infected cells. Some red fluorescence of HuScFv was also co-localized the green fluorescence in the cytoplasm of DENV-infected cells as seen in yellow fluorescence after merging. No fluorescence signal except blue fluorescence of nuclear DNA was observed in MOCK-infected cells treated with same reagents (Figure 8).

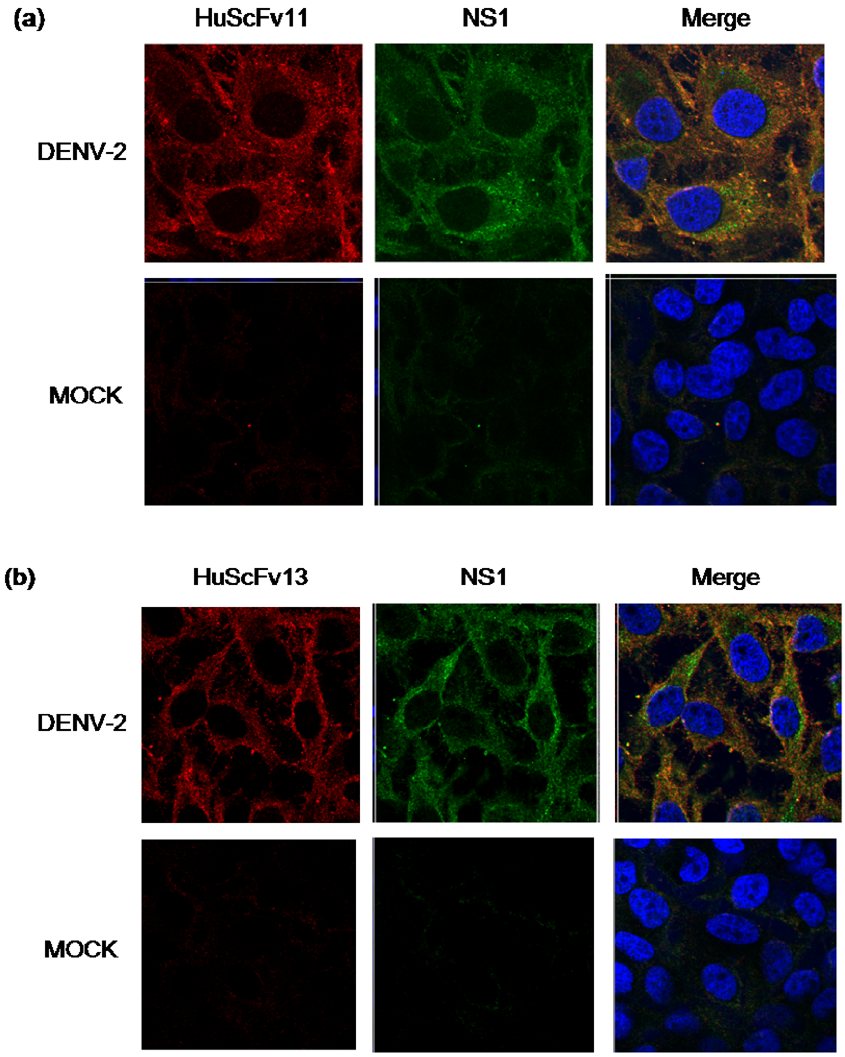

Figure 8 Locations of HuScFv11 and HuScFv13 (red fluorescence in upper left panels of a and b, respectively) and NS1 (green fluorescence in upper middle panels of a and b) in Huh-7 cells infected with DENV-2 revealed by confocal microscopy. After merging, the two proteins were found to co-localize (yellow fluorescence in upper right panels of a and b). Lower panels in a and b are respective negative controls. Huh-7 nuclei are shown in blue fluorescence.

9. Determination of DENV infection inhibitory effect mediated by NS1-specific HuScFv

Huh-7 cells were infected with DENV2 at MOI = 0.1 or 1 for 12 hours, then, the cells were rinsed and treated with either HuScFv11, HuScFv13, or controls for 24 and 36 hours further. Culture supernatants containing newly released infectious DENV2 particles were titrated by focus formation assay (Figure 9). Compared to untreated DENV2-infected cells, both HuScFv11 and HuScFv13-treated cells had significant lower DENV2 particles in the supernatant in all experimental conditions ($p < 0.01$). The highest percent reduction (60%) was observed for the cells infected with DENV2 at MOI = 0.1 and treated with HuScFv11 for 36 hours. The amounts of DENV2 released from DENV2-infected cells treated with irrelevant HuScFv control was not significantly different from those released from untreated control ($p > 0.05$).

The amounts of E antigen in DENV2-infected cells that were cultured in the medium containing HuScFv11, HuScFv13, or controls were determined by cell-ELISA. The ELISA signal of E antigen in DENV2-infected cells was not statistically different among all treatment conditions (data not shown).

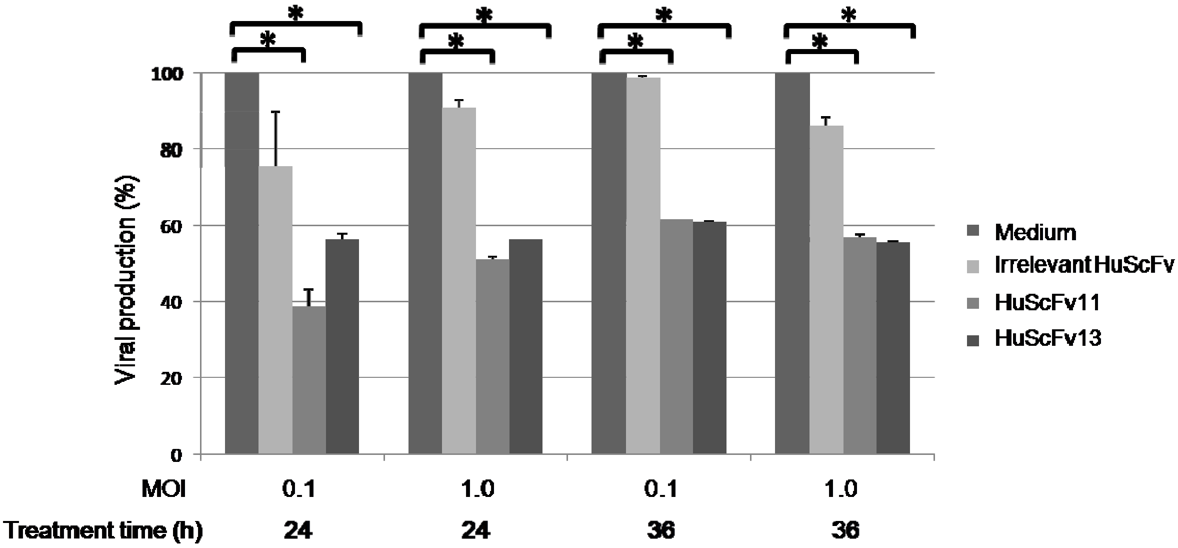

Figure 9 HuScFv mediated reduction of DENV-2 virus in infected cell culture supernatants. Data are mean + SEM of three independent experiments. *, significant difference at $p < 0.01$ by one-way ANOVA and Tukey's HSD test.

10. Determination of NS1-specific HuScFv mimotopes and identification HuScFv epitopes on NS1 molecule

DNA of phage clones derived from panning of the Ph.D.-12$^{\text{TM}}$ peptide display phages with the immobilized HuScFv11 and HuScFv13 were individually extracted and sequenced. The amino acids of these phage clones were deduced. Peptide sequences of 17 and 16 clones could be obtained from HuScFv11 and HuScFv13 panning, respectively. The phage peptide sequences reacted with the HuScFv11 could be divided into 4 mimotope types, *i.e.,* M11-1: VSVGAQRNATFT (5 clones), M11-2: VSVGAQRNATLR (4 clones) and 1 clone each of M11-3 to M11-9 (VSVGAQPECDVL, VSVGAHAECDV-, TAMMTNKHENCR, ASTYPQSSPGVT, WGNAHYRSAELN, DYFNGSSAVHAA and ASVQGWAQITQR, respectively). There was only one mimotope type for HuScFv13, *i.e.*, M13-1 (ATTTYWWTNISG). Mimotope consensus sequence of M11 mimotope types was VSVGAQRNAT--. Pairwise alignment of the HuScFv11 mimotope consensus sequence with DENV-2 NS1 peptide sequence revealed that the mimotope matched with amino acid residues 1-14 (DSGCVVSWKNKELK) of the NS1 protein (Figure 10a). The HuScFv13 mimotope (ATTTYWWTNISG) matched with amino acid residues in three regions throughout the C-terminal of the NS1, *i.e.*, 164TTNIW168, 232WSN234 and 350VTA352 (Figure 10b).

Pre-incubation of the synthetic NS1 peptides, *i.e.*, DSGCVVSWKNKELK and TTNIWWSNVTA with HuScFv11 and HuScFv13 could reduce the HuScFv binding to NS1 in a dose-dependent manner (Figure 10c).

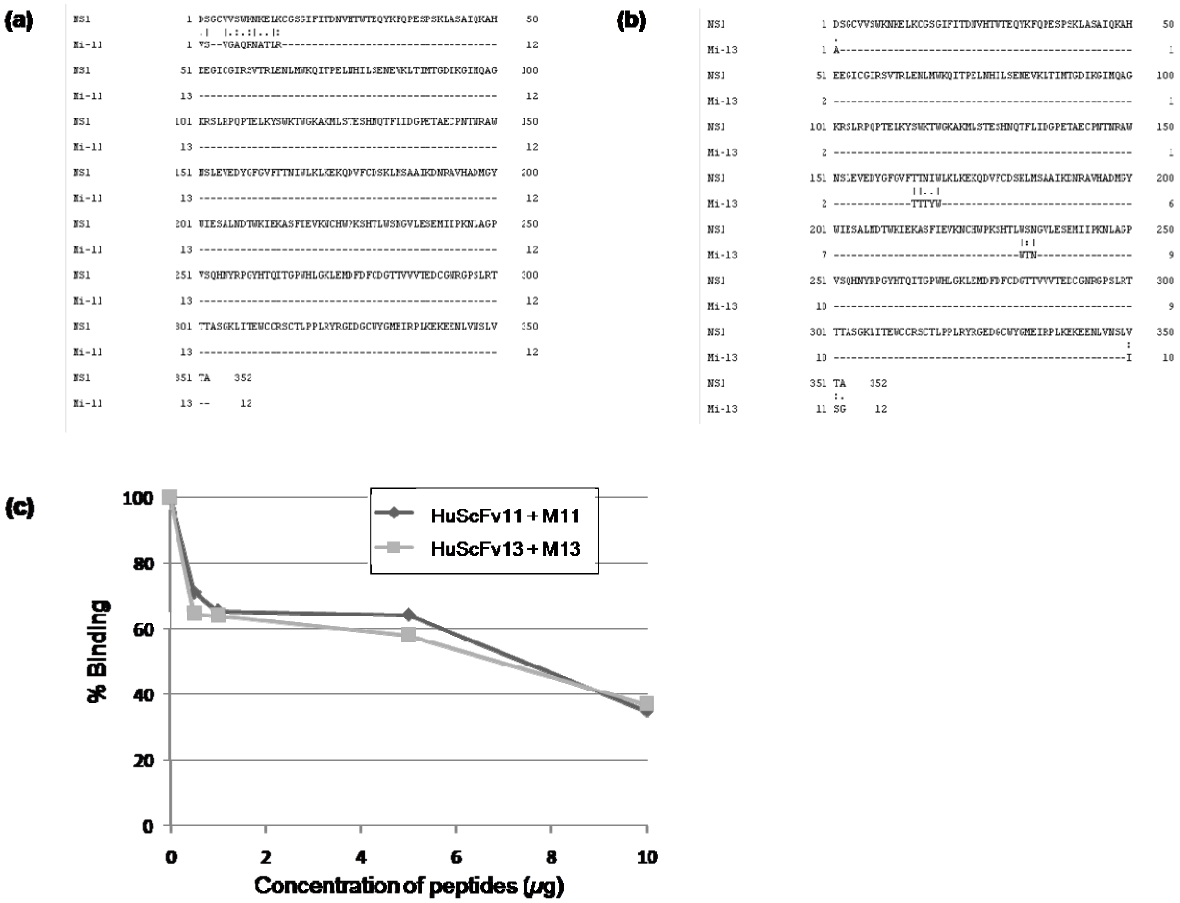

Figure 10 Alignments of the mimotope sequences of HuScFv11, *i.e.*, M11 (a) and HuScFv13, *i.e.*, M13 (b) with the linear sequence of DENV-2 NS1. The tentative epitope of HuScFv11 located at the amino acid residues 1-14 (DSGCVVSWKNKELK) while the amino acid sequence of the HuScFv13 epitope were residues 164-168 (TTNIW), 232-234 (WSN) and 350-352 (VTA). A period indicates identical amino acids; a colon indicates conserved amino

acid substitution; and an asterisk indicates semi-conserved amino acid substitution. The synthetic peptides of epitopes of HuScFv11 (DSGCVVSWKNKELK) and HuScFv13 (TTNIWWSNVTA) could block the binding of the respective HuScFv to NS1 in the competitive ELISA in dose dependent manner (c).

11. *In silico* analysis of NS1-HuScFv interaction

The C-score of NS1 model from ITASSER server was -4.16 implying that the constructed model was acceptable. For the HuScFv, PDB entry 2GHW (SARs spike protein receptor binding domain complexed with neutralizing antibody, 80R) was used as a template for homology modeling. The amino acid sequences of HuScFv11 and HuScFv13 had 67.1 and 58.3% identity and 78.3 and 69.0% similarity to the template, respectively. The Ramachandran plots of the constructed models were determined and the allowed region for both HuScFv was 98%.

Amino acid residues of HuScFv11 and HuScFv13 that complexed with the NS1 model were revealed (Figure 11a and 11b, respectively). The protein-protein docking was calculated by using ZDOCK and the dock poses were refined by using RDOCK programs. The best dock poses were selected according to the following formular: RDOCk energy = desolvation energy + (beta constant × electrostatic energy). The RDOCK energy of HuScFv11 and HuScFv13 were -4.90 and -9.77 kcal/mol, respectively. The dock poses of HuScFv11 VH and HuScFv13 VH and VL with the NS1 model were evident. Figures 11c and 11d show three dimensional structure of NS1 (blue surface) interacted with HuScFv11 (yellow ribbon) and HuScFv13 (red ribbon), respectively.

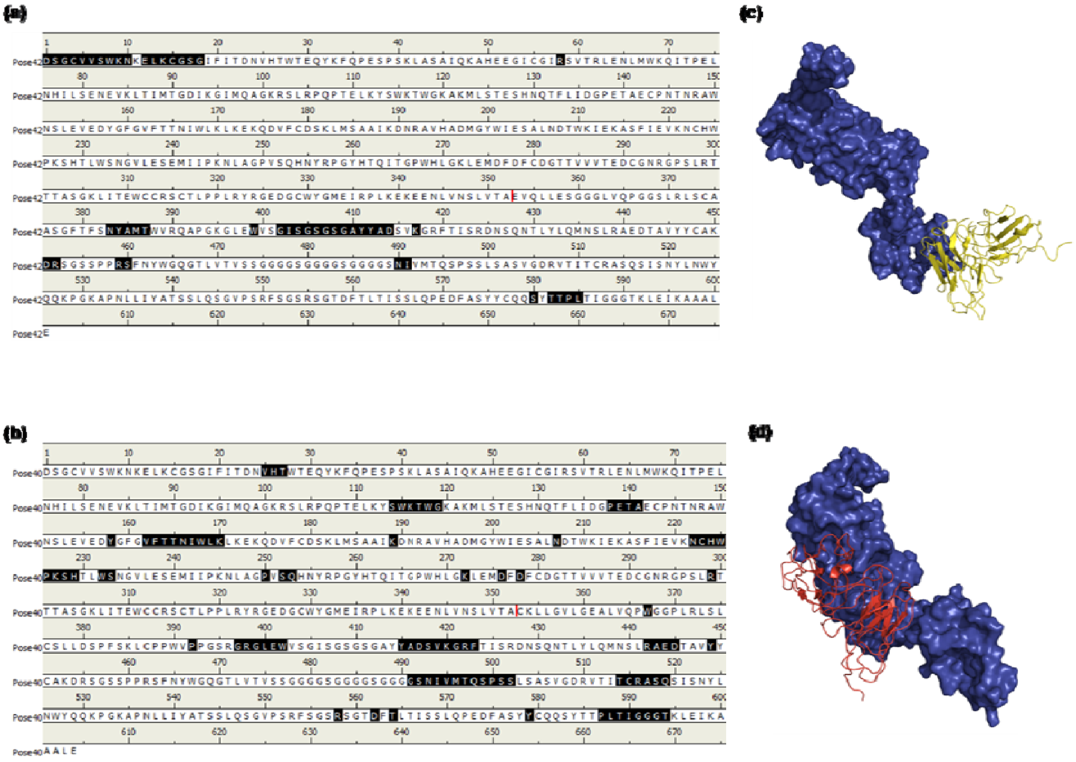

Figure 11 Results of computerized molecular docking between modeled NS1 and HuScFv11 (a) and HuScFv13 (b). Predicted interactive residues between the NS1 and the HuScFv are shown in black boxes. NS1, amino acids 1-352; HuScFv11, amino acids 353-601; HuScFv13, amino acids 353-604. (c) and (d) are three dimensional structure of NS1 (blue surface) complexed with HuScFv11 (yellow ribbon) and HuScFv13 (red ribbon), respectively.

DISCUSSION AND CONCLUSION

Number of patients with severe DENV infection has increased markedly in many tropical and subtropical areas including Southeast Asia, Africa, Middle East, Western Pacific and Americas (Wilder-Smith *et al.*, 2010). There has been extensive research in several laboratories around the world towards effective and safe DENV vaccine and anti-DENV agents. Presently, several vaccine candidates have reached phase 3 trials and a number of compounds and peptides with anti-viral potentials have been demonstrated in laboratories (Noble *et al.*, 2010; Guy *et al.*, 2011). At present, most DHF/DSS cases are given palliative treatment mainly in intensive care units.

NS1 is not only conserved across the four DENV serotypes, but also has pivotal roles in the virus infectious cycles including enhancement of viral entry into cells, replication, pathogenicity, as well as mediation of pathophysiology (Halstead *et al.*, 1973; Jacobs *et al.*, 2000; Young *et al.*, 2000; Alcon *et al.*, 2002; Alcon-LePoder *et al.*, 2005; Avirutnan *et al.*, 2006). Although, immune responses to NS1 vaccine in interferon-deficient mice was limited (Calvert *et al.*, 2006), however, protective efficacy of anti-NS1 antibody after *in vivo* dengue virus or other flavivirus challenges has been demonstrated (Falgout *et al.*, 1990; Jacobs *et al.*, 1992; Qu *et al.*, 1993; Hall *et al.*, 1996). The evidences indicated that NS1 is one of the potential vaccine targets and antibodies specific to this viral protein should be appropriate adjunctive remedy for passive immunotherapy of the diseases. However, activation of complement-mediated cytolysis by Fc-bearing anti-NS1 antibody may exacerbate the disease severity (Lin *et al.*, 2003). Thus, it was envisaged that the NS1 specific antibody for the dengue treatment should devoid of the Fc portion. Moreover, the antibody should be non-immunogenic and distributed rapidly within the recipient. Fully human single chain antibody fragments (HuScFv) (~25-35 kDa which lack Fc and are approximately 5 times smaller than conventional IgG) and bound specifically to and interfere with the functions of DENV NS1 conserved protein should be appropriate format for dengue treatment.

In this study, HuScFv which devoid of Fc fragment (could not induce ADE), bound specifically to DENV NS1 as well as inhibited the virus release from cells were produced. Monomeric recombinant NS1 (rNS1) was used as the antigen in bio-panning for selecting NS1 bound phage clones from a human ScFv phage disaply library (Kulkeaw *et al.*, 2009). The sequences coding for the rNS1 (accession no. JN692493) had 99% nucleotide similarity to the database sequence (accession no. U87411.1). There were three mismatched nucleotides between the amplified cDNA of this study and the database sequences, *i.e.*, 25G>A, 148T>C and 271C>T. Only the first mismatched position caused a change of arginine to lysine, but both are similarly polar amino acids. Thus, the rNS1 produced in this study should retain all of the bioactivities of the native NS1 (Kinney *et al*., 1997). The HuScFv phage display library has been used successfully for production of HuScFv specific to many other molecules including *Naja kaouthia* neurotoxin, and influenza virus proteins, *i.e.,* polymerases, H5 haemagglutinin and matrix protein M1 (Thathaisong *et al*., 2008; Kulkeaw *et al*., 2009; Maneewatch *et al*., 2009; Poungpair *et al*., 2009 and 2010). In this study, one round panning which was a time and reagent saving procedure was performed instead of the multiple panning rounds reported by other laboratories (Kulkeaw *et al*., 2009; Thanongsaksrikul *et al*., 2010). Among the phagemid transformed *E. coli* clones, 84% carried the recombinant *huscfv*-phagemids which conformed to the previous study showing that 85% of phages in this library carried *huscfv* (Kulkeaw *et al*., 2009). A fraction of the *huscfv*-phagemid *E. coli* clones (19 clones) were randomly selected for subsequent experiments while the other clones were kept for future requirement. All of the selected *huscfv*-positive clones derived from the panning with the monomeric rNS1 could express soluble HuScFv that bound to native DENV-2 NS1 collected from the culture supernatant of infected cells which was most likely to be hexameric forms, indicating that the HuScFv epitopes were maintained in the bacterially expressed monomeric NS1. The *huscfv* from the recombinant phagemid carried by two selected HB2151 *E. coli* transformants (clones no. 11 and 13) were subcloned into pET23b+ vector. The *huscfv*-pET23b$^+$ transformed BL21 (DE3) *E. coli* produced relatively larger amounts of the HuScFv in the bacterial lysates than the *huscfv*-phagemid transformed HB2151 *E. coli* and the HuScFv still retained the binding activity, not only to the rNS1 but also to the native NS1 in the DENV-2 infected cells. The finding that the HuScFv bound to both secreted and native NS1 led us to speculate that the HuScFv might be able to interfere with the NS1 multiple functions in the course of DENV infection including interference of the virus entry into cells and spread which are the role of secreted hexameric NS1 (Alcon-LePoder *et al*., 2005) as well as interfering with the complement modulation/activation by the membrane anchored homodimeric NS1 (Avirutnan *et al*., 2006).

The culture supernatants of DENV-2 infected Huh-7 cells grown in the medium containing either HuScFv11 or HuScFv13 for 24 and 36 h had significant reduction of the virus amounts compared with the culture fluids of the infected cells without HuScFv treatment. Less virus release into culture medium may be due to either less virus entry, replication, assembly and/or spread. The findings that the amounts of intracellular viral antigen in the HuScFv exposed infected cells were not different from the control cells indicated that the NS1 specific-HuScFv might not interfere with the virus entry and intracellular replication but inhibited the virus morphogenesis and/or release. Usually, the membrane lipid raft microdomain is a gateway for entry and release of several viruses (Bavari *et al.*, 2002). Even though the HuScFv were unable to enter the cells they might be posed at the virus exit and interfered with the virus release. It is known also that during infection, the DENV infected cells had increased membrane permeability (Dewi *et al*., 2004). Therefore the small molecular sized-HuScFv might traverse the cellular membrane and bound to intracellular NS1 which, in the effect, interfered with the virus morphogenesis. Experiments are needed to verify these speculations.

Because the crystallographic or NMR structure of DENV NS1 is not available, the protein threading method was used to construct the DENV NS1 model by using the ITASSER server. The monomeric NS1 model had C-score (-4.16) which was in an acceptable range (-5 to 2). For the HuScFv11 and HuScFv13, the model of PDB entry 2GHW of SARs spike protein receptor binding domain in complex with a neutralizing antibody was used as a template. It was found that the selected template of HuScFv was also appropriate (the Ramachandran plot allowed region was 98%). Thus both the modeled NS1 and HuScFv were suitable for molecular docking in determining the interface binding of the two proteins. The docking revealed that HuScFv11 VH and HuScFv13 VH and VL reacted with the NS1 with low RDOCK energy. HuScFv-NS1 interacting poses were conformed to the mimotope/epitope mapping results indicating that the HuScFv might use either VH or VL, or both, for target binding. Although functional significance of various NS1 regions and critical amino acid residues for the DENV infectious cycle are rarely known and required further elucidation, the data of this study indicated that the NS1 sites bound by the HuScFv11 (amino acids 1-14) and HuScFv13 (conformational epitope in the C-terminal) led to interference of the virus replication cycle by reducing DENV-2 release into the infected cell milieu.

To my knowledge, this is the first annotated report on fully human ScFv that targets NS1. The HuScFv should be safe and cause no inflammation in the recipient. They have high potential in developing further into an adjunctive, if not a sole, therapeutic agent for dengue diseases.

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OUTPUT

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จากโครงการที่ได้รับทุนจาก สกว.
   1.1 **Poungpair O**, Bangphoomi K, Chaowalit P, Sawasdee N, Saokaew N, Choowongkomon K, Chaicumpa W, Yenchitsomanus PT. NS1 specific human ScFv that interfere with dengue virus infectious cycle. (Manuscript submitted to Experimental Biology and Medicine)

2. การจดอนุสิทธิบัตร
   กำลังอยู่ในระหว่างยื่นขอจดอนุสิทธิบัตรการประดิษฐ์ เรื่อง "แอนติบอดีสายเดี่ยวของมนุษย์ที่จำเพาะต่อโปรตีนเอ็นเอสวันของไวรัสเดงกี่และมีประสิทธิภาพลดการติดเชื้อไวรัสเดงกี่ในเซลล์เพาะเลี้ยง" โดย สกว.

3. ผลงานตีพิมพ์อื่นๆในระหว่างดำเนินโครงการวิจัย

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