CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study were shown in Appendix A and Appendix B.

2.2 Adenoviral plasmids and recombinant adenovirus expressing scFv-M61B9ER gene

Adenoviral plasmids, pAdT-scFv-M61B9ER and pAdE-scFv-M61B9ER, and recombinant adenovirus, Ad5-scFv-M61B9ER, were kindly provided from Asst. Prof. Khajornsak Trakoolpua (Tragoolpua *et al*, 2008). The scFv-M61B9ER gene composed of the V_L and V_H chains tagged with HA sequence at the 3'-end was flank with leader sequence at upstream and KDEL sequence at downstream.

2.3 Construction of pAdTrack-CMV vector expressing scFv-M61B9cyt gene

2.3.1 Primer design

In order to amplify a part of scFv-M61B9 from pAdE-scFv-M61B9ER (Tragoolpua *et al*, 2008), a pair of primers SFvM6cyt (5'-GAG GAG GAG GTG TCG ACA TGG TGA TGA CCC AGA CTC C-3') and ScSVV3_RCy (5'-GAG GAG GAG CTG CGG CCG CTT AAG CGT AGT CCG GAA CGT C-3') were synthesized. These primers carried *Sal*I and *Not*I restriction sites, respectively.

2.3.2 Amplification of scFv-M61B9cyt gene

Six nanograms of DNA template, pAdE-scFv-M61B9ER (Tragoolpua *et al*, 2008), were amplified with 0.4 µM of each primer in 50 µl of reaction mixture containing 0.2 mM dNTPs, 1 mM $MgCl_2$, 1X PCR buffer and 2.5 U KOD HIFI DNA polymerase (Novagen). The titrated amplification condition was initiated with jump-start at 85°C for 15 sec followed by 25 rounds of 3 steps amplification: denaturation at 98°C for 15 sec, annealing at 70°C for 2 sec and extension at 72°C for 20 sec. Finally, the mixture was incubated at 72°C for 1 min. The amplified product was checked for the correct molecular size (800 bp) by 1% agarose gel electrophoresis and purified by a QIAquick PCR Purification Kit (QIAGEN).

2.3.3 Purification of PCR product by QIAquick PCR Purification Kit

Five volumes of the PB buffer were mixed together with 1 volume of PCR product and transferred to a QIAquick spin column which placed on a provided 2-ml collection tube. To bind the DNA, the QIAquick column was subsequently centrifuged at 10,000 g for 60 sec. the flow-through solution was discarded. Then, the QIAquick spin column was washed by 0.75 ml of PE buffer and centrifuged 2 times for eliminating the flow-through solution at 10,000 g for 60 sec. Finally, the DNA-binding column was replaced in a clean 1.5 ml microcentrifuge tube and eluted by loading 30 µl of distilled water and centrifuge at 10,000 g for 1 min. The size of DNA was checked by 1% agarose gel electrophoresis.

2.3.4 Ligation of the scFv-M61B9cyt gene with pAdTrack-CMV vector

Seven hundred nanograms of purified PCR product were treated with 70 U of *Sal*I and 30 U of *Not*I (Fermentas) at 37°C for 16-18 hr, whereas 500 ng of pAdtrack-CMV vector was treated with 30 U of *Sal*I and *Not*I at 37°C for 16-18 hr. Both treated DNA fragments were further purified by the QIAquick PCR Purification Kit. One hundred nanograms of treated PCR product and 50 ng of treated pAdTrack-CMV vector were added to ligation mixture in a total volume of 20 µl containing 5 U of T4 DNA ligase enzyme (Fermentas). The ligation mixture was subsequently incubated at 4°C for 16 hr.

2.3.5 Transformation of the recombinant pAdTrack-CMV vector in *E. coli* Novablue

Following the ligation steps, the ligation mixture containing ligated DNA was used for transformation into *E. coli* Novablue. Four microliters of ligation mixture were co-incubated with 100 µl of cold-thawed $CaCl_2$ competent cell on ice for 1 hr. the transforming mixture was transferred into cooled screw cap tube and subsequently incubated at 42°C for 1.5 min, then abruptly chilled on ice for 1 min. Three milliliter of non-antibiotic LB broth was added and bacteria were further cultured with shaking (120 rpm) at 37°C for 3 hr. The transformed bacteria were centrifuged (2,500 rpm) at RT for 10 min and plated on kanamycin-containing LB agar (70 µg/ml). The plates were then incubated at 37°C for overnight (14-16 hr).

2.3.6 Purification of plasmid vector by using alkaline lysis method

A kanamycin resistant colony was picked and grown in 3 ml of kanamycin-containing LB broth (70 µg/ml) with vigorous shaking (180 rpm) at 37°C for 6 hr. The 1.5 ml of culture was centrifuged 10,000 g at 4°C for 5 min. The supernatant was discarded and the cell wall of bacterial pellet was lysed by 100 µl of 1X glucomix-lysozyme and vertexed vigorously. Two hundred microliters of freshly prepared NaOH/SDS was added and mixed by inverting. Then, 150 µl of potassium acetate was added and gently mixed by vertex. The solution was centrifuged 10,000 g at 4°C for 5 min for collecting the clear supernatant. The nine hundred microliters of analytical trade absolute ethanol was added and kept on ice for 2 min. The DNA was spun down at 10,000 g at 4°C for 5 min and the supernatant was discarded. The DNA pellet was reconstituted by 100 µl of sterile DW and followed by adding 50 µl of 7.5 M ammonium acetate and incubated at -70ºC for 10 min. The supernatant was collected by centrifugation at 10,000 g at 4ºC for 5 min. Three hundred microliters of absolute ethanol was added to the saved supernatant and incubated at -70ºC for 10 min. The solution was spun down to harvest the pellet. The pellet was cleaned up with 1 ml of 70 % ethanol by centrifugation at 10,000 g at 4ºC for 5 min. The DNA pellet was dried at 37ºC about 30 min and reconstituted with 30 µl of sterile DW and stored at -20ºC.

2.3.7 Analytical *Sal*I and *Not*I digestion of pAdTrack-CMV vector expressing scFv-M61B9cyt

Three hundred nanograms of the purified vectors were digested with 5 U of *Sal*I and *Not*I at 37°C for 3 hr. The digested DNA fragments were checked by fractionating on 1% agarose gel electrophoresis to identify the band of correct insert.

2.3.8 Proof of pAdT-scFv-M61B9cyt by using polymerase chain reaction (PCR)

Twenty five nanograms of the purified vectors were amplified with 125 ng of each primer in a 20 µl of PCR mixture containing 2.5 U Taq polymerase (Eppendorf). The amplification condition was initiated with jump start at 85°C for 4 min followed by 35 rounds of 3 steps amplification: denaturation at 95°C for 50 sec, annealing at 70°C for 50 sec and extension at 72°C for 1.5 min. Finally, the mixture was incubated at 72°C for 10 min. The amplified product was checked for the correct molecular size by 1% agarose gel electrophoresis.

2.4 Construction of pAdTrack-CMV vector expressing scFv-M61B9sec gene

2.4.1 Primer design

Mutagenic primers introduce specific experimental mutation. The mutagenic oligonucleotide primers must be designed individually according to the desired mutation. Both the mutagenic primers, FMutKDEL (5'-CCG GAC TAC GCT TCT TGA GAT GAG CTC TGA GAA TTC G-3') and RMutKDEL (5'-CGA ATT CTC AGA GCT CAT CTC AAG AAG CGT AGT CCG G-3'), were designed to contain the desired mutation (stop codon) as show by underline letter and anneal to the same

sequence on opposite strands of the plasmid. The melting temperature (T_m) of the primers should be greater than or equal to 78°C following specific formula (Tm = 81.5 + 0.41(%GC) – 675/primer length in base pairs - %mismatch).

2.4.2 Site-directed mutagenesis

An amount of 100 ng of pAdT-scFv-M61B9ER template (Tragoolpua *et al.* 2008) was mixed with 125 ng of mutation primer FMutKDEL and RMutKDEL. *Pfu* Turbo DNA polymerase of 2.5 U was added to the mixture for cycle amplification. The reaction started with one round of 95ºC for 30 sec. Then, it was followed by 16 rounds consisting of 95°C for 30 sec, 55°C for 1 min and 68°C for 20 min. The reaction tube was subsequently placed on ice for 2 min. In order to destroy the template strands, 10 U of *Dpn*I restriction enzyme were added to the amplification reaction and incubated for 1 hr at 37°C. This synthesized product (pAdT-scFv-M61B9sec) was further used to transform into *E. coli* Novablue and purified using alkaline lysis method. The mutated sequence of pAdT-scFv-M61B9sec was analyzed by DNA sequencing method (First BASE Laboratories Sdn Bhd).

2.5 Construction and preparation of pAdEasy vector containing scFv-M61B9cyt and scFv-M61B9sec gene

2.5.1 Preparation of BJ5183-pAdEasy competent cells

The BJ5183-pAdEasy competent cells, the *E. coli* strain BJ5183 containing pAdEasy vector, were prepared by using $CaCl_2$ method. One hundred microliters of $CaCl_2$-competent BJ5183 cells were transformed with 100 ng of pAdEasy vector. The

transformed cells were propagated by spreading on ampicillin-containing LB agar (100 µg/ml). After incubation at 37°C for 18 hr, the ampicillin resistant colony were selected and further cultured at 37°C for 8 hr in 10 ml of LB broth containing 100 µg/ml ampicillin. The cultured bacteria were further pelleted at (2,500 rpm) 4°C for 10 min and reconstituted by 10 ml of 0.1 M $CaCl_2$ at 4°C. Two rounds were repeatedly performed. Finally, the suspended bacteria were pelleted and further resuspened by 2 ml of 0.1 M $CaCl_2$. The BJ5183-pAdEasy competent cells were added by 0.4 ml of 85% glycerol and stored at -70°C.

2.5.2 Transformation of pAdTrack-CMV vector containing scFv-M61B9 gene in BJ5183-pAdEasy competent cells

Four micrograms of pAdTrack-CMV vector expressing scFv-M61B9 were digested with 50 U of *Pme*I at 37°C for 16 hr. Three hundred nanograms of *Pme*I-linearized vector was heat inactivated at 65°C for 20 min and transformed into BJ5183-pAdEasy competent cells. The transformed cells were propagated by spreading on kanamycin-containing LB agar (70 µg/ml). After incubation at 37°C for 16 hr, a kanamycin resistant colony was selected for plasmid minipreps using the alkaline lysis method. The plasmid from pAdT-scFv-M61B9cyt transformant was designed as pAdE-scFv-M61B9cyt and the plasmid from pAdT-scFv-M61B9sec transformant was designed as pAdE-scFv-M61B9sec.

2.5.3 *Pac*I restriction analysis of pAdEasy vector containing scFv-M61B9 gene

Three hundred nanograms of the purified vectors were digested with 5 U of *Pac*I at 37°C for 3 hr. The digested DNA fragments were checked by fractionating on 1% agarose gel electrophoresis to identify the band of correct insert.

2.5.4 Proof of pAdE-scFv-M61B9cyt and pAdE-scFv-M61B9sec by using polymerase chain reaction (PCR)

Twenty five nanograms of the purified vectors were amplified with 125 ng of each primer in a 20 µl of PCR mixture containing 2.5 U Taq polymerase (Eppendorf). The pAdE-scFv-M61B9cyt was proved with a pair of primer SFvM6cyt and SVV3_Rcy. The pAdE-scFv-M61B9sec was proved with a pair of primer SF1B9sec and SVV3_Rcy. The amplified product was checked for the correct molecular size by 1% agarose gel electrophoresis.

2.5.5 Transformation of pAdEasy vector containing scFv-M61B9 gene in *E. coli* DH10B

One hundred microliters of the $CaCl_2$-competent *E. coli* DH10B were transformed with 100 ng of the of pAdEasy vector containing either scFv-M61B9cyt or scFv-M61B9sec gene. The transformed cells were propagated on kanamycin-containing LB agar (70 µg/ml) at 37°C for 16 hr. After incubation, a single kanamycin resistant colony was selected for vector purification by using QIAGEN Plasmid MiDi Kit.

2.5.6 Purification of plasmid vector by using QIAGEN Plasmid MiDi Kit

A single colony of transformant was picked and inoculated with a starter culture of 2-5 ml LB medium containing kanamycin (70 µg/ml). After incubation at 37°C for 8 hr with vigorous shaking, two hundred microliters of the starter culture was further grown in 100 ml of LB medium containing kanamycin (70 µg/ml) at 37°C for 16 hr with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000 g at 4°C for 15 min. The bacterial pellets were resuspended in 4 ml of P1 buffer. The bacteria were completely resuspended leaving no cell clump. The P2 buffer of 4 ml was added and the mixture was gently mixed but thoroughly by inverting for 4-6 times. The mixture was incubated at room temperature for 5 min. Four microliters of chilled P3 buffer were added to the lysate. The mixture was mixed immediately and gently by inverting for 4-6 times and poured into the barrel of the QIAfilter cartridge. The cartridge was incubated at room themperature for 10 min. A QIAGEN-tip 100 was equilibrated by applying 10 ml of QBT buffer. The column was allowed to empty by gravity flow. The cap from the QIAfilter nozzle was removed. The plunger was gently inserted into the QIAfilter. The cell lysate was filled into the previously equilibrated QIAGEN-tip until lysate passing through the QIAfilter cartridge. Approximately 10 ml of the lysate were generally recovered after filtration. The clear lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 10 ml of QC buffer. The QC buffer was allowed to move through the QIAGEN-tip by gravity flow. The DNA was eluted with 5 ml of QF buffer. The eluate was collected in a 10 ml tube. To precipitate DNA, 3.5 ml of isopropanol was added to the eluted DNA at room temperature. After mixing and centrifuging immediately at 15,000 g for 10 min, the supernatant was carefully decanted without

disturbing the pellet. The pellet was dried for 10 min and redissolved in suitable volume of distilled water.

2.6 Production of recombinant adenoviruses expressing scFv-M61B9

2.6.1 Cell culture

The 293A, an embryonic human kidney transformed carrying sheared human adenovirus type 5 DNA, and HeLa cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10mM nonessential amino acids, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

2.6.2 *Pac*I linearization of the pAdEasy vector containing scFv-M61B9 gene

One microgram of pAdEasy vector containing either scFv-M61B9cyt or scFv-M61B9sec gene was digested with 10 U of *Pac*I at 37°C for 16 hr. The *Pac*I digestion was heat inactivated at 70°C for 10 min and stored at -20°C.

2.6.3 Transfection of 293A cells with *Pac*I linearized pAdEasy vector containing scFv-M61B9 gene

1×10^5 cells in a total volume of 500 µl growth medium (DMEM containing 10 % fetal bovine serum) were plated on a 24 well-tissue culture plate for 24 hr before transfection, by which time they reached 60-70% confluence. A transfection mixture was prepared by adding 1 µg of the *Pac*I linearized pAdEasy vector and 0.5 µl of Transfectin (Biorad) to 100 µl of serum-free DMEM. After incubation at room

temperature for 20 min, the transfection mixture was added to the cell. The cells were incubated at 37°C in 5% CO_2 for 4-6 hr and then added by 400 µl of the growth medium. The transfected cells were monitored for GFP expression under a fluorescence microscope at the following days. The culture medium containing recombinant adenovirus was harvested after 7 day post transfection, when GFP plaques had appeared.

2.7 Production of scFv-M61B9 in HeLa cell compartments.

1×10^5 HeLa cells, which were seeded on a 24 well-tissue culture plate, were transduced with 100 µl of 293A culture medium containing Ad5-scFv-M61B9ER (Tragoolpua et al. 2008), Ad5-scFv-M61B9cyt and Ad5-scFv-M61B9sec and incubated at 37°C in 5% CO_2 until a GFP signal for 100% transduction of the cells was observed.

2.8 ScFv-M61B9 extraction by FractionPREP^{TM} Cell Fractionation System

The transduced cells were collected by centrifugation at 700 g for 5 min and washed with 5-10 ml of ice-cold PBS. After centrifugation at 700 g for 5 min, the pellet was resuspended in 1 ml of ice-cold PBS and transferred to a 1.5 ml microcentrifuge tube. The resuspended cells were spun for 5 min at 700 g and removed supernatant. The pellet was resuspended in 400 µl of Cytosol Extraction Buffer-Mix and incubated sample on ice for 20 min with gentle tapping 3-4 times every 5 min. After centrifugation at 700 g for 10 min, the supernatant (Cytosolic Fraction) was collected and kept on ice. The pellet was resuspended in 400 µl of ice-cold Membrane Extraction Buffer-A Mix and mixed well by vortex for 10-15 sec.

Twenty two microliters of Membrane Extraction Buffer-B was added and then mixed by vertex for 5 sec. After incubation on ice for 1 min, the mixture was mixed by vertex for 5 sec again and centrifuged for 5 min at 1000 g. The supernatant (Membrane Fraction) was transferred immediately to a clean pre-chilled tube and kept on ice. The pellet was resuspended in 200 µl of ice-cold Nuclear Extraction Buffer Mix and mixed by vertex for 15 sec. The mixture was kept on ice for 10 min with constant vertex for 15 sec every 10 min. After centrifugation at top speed in a microcentrifuge for 10 min, the sample was transferred to a clean pre-chilled tube (Nuclear Fraction). All Fractions were stored at -70 °C for future use.

2.9 Detection of scFv-M61B9 by immunological techniques

2.9.1 Indirect ELISA

To detect scFv-M61B9 in each fraction, both the culture medium and cell lysates were used to perform ELISA. First, the culture medium in 24-well plate was collected. Then, the transduced cells were harvested by scraping cells in 200 µl of sterile PBS (phosphate buffer saline). After three cycles of freezing in a methanol/dry ice bath and rapid thawing at 37°C, cell debris was removed by centrifugation and cell lysate (supernatant) was collected. Sixty nine well plate (COSTA®) was coated with 0.5 µg of egg white avidin (Sigma) in 50 µl of carbonate/bicarbonate buffer, pH 9.6 at 4°C for 12 hr. The coated plate was blocked by adding 200 µl of 2% BSA in PBS, pH 7.2, and incubated for 1 hr at room temperature. After washing 5 times with washing buffer (0.05% Tween 20 in PBS pH 7.2), 5 µg of CD147-BCCP fusion protein or survivin-BCCP fusion protein in 50 µl of 2% BSA in PBS were added into the wells.

After incubation at room temperature for 1 hr, the plated was washed 3 times with washing buffer. Culture medium and cell lysate were added and incubated at room temperature for 1 hr. Fifty microliters of mouse anti-CD147 mAb clone M6-1B9 (10 µg/ml), mouse anti-survivin mAb (10 µg/ml), mouse anti-BCCP mAb (1:500) were used as controls. After washing, the mouse antibody was traced by HRP-conjugated goat-anti-mouse immunoglobulins antibody whereas scFv-M61B9 in culture medium or cell lysate was detected by HRP-conjugated anti-HA antibody. After 1 hr incubation and 5 times washing, 100 µl of TMB color substrate was applied to each well. The plated was allowed to stand at room temperature for a short duration for signal development. The enzymatic reaction was stopped by adding 100 µl of 1 N HCl and the absorbance at 450 nm was measured.

2.9.2 SDS-PAGE and Western immunobloting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed for analysis of scFv-M61B9. The protein in each transduced-cell fraction, whole cell lysate or culture supernatant from Ad5-scFv-M61B9ER transduced cells was separated in 12% polyacrylamide gel under reducing condition. For Western immunobloting, the separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane. Blotted membrane was blocked at 4°C for 18 hr in 5% skim milk in PBS, pH 7.2, and then incubated with the HRP-conjugated anti-HA antibody diluted in 5% skim milk in PBS, pH 7.2, for 1 hr at room temperature on a shaking platform. After washing step, the immuno-reactive bands were then visualized by chemiluminescent substrate detection system.

To verify the binding activity of the generated scFv-M61B9, the bacterial proteins containing CD147-BCCP, survivin-BCCP fusion protein and no fusion protein were separated on a 12% SDS-PAGE under reducing condition and electroblotted onto PVDF membrane. Blotted membrane was blocked and then incubated with transduced-cell lysate. Mouse anti-CD147 mAb M61B9, mouse anti-survivin mAb and mouse anti-BCCP mAb were used as controls. Following washing step, HRP-conjugated anti-HA antibody or HRP-conjugated goat-anti-mouse immunoglobulins antibody was added to the membrane and incubated for 1 hr. After washing step, the immuno-reactive bands were then visualized by chemiluminescent substrate detection system.