CHAPTER IV

MATERIALS AND METHODS

1. Detection of expression level of livin and survivin in tumor tissues and normal tissues with Western blot Analysis

1.1 Population of the study

Five mililitres of blood sample was collected from lung cancer patients admitted to Maharaj Nakorn Chiang Mai hospital during September 2006 to May 2008 (n=250). All of the cases were newly diagnosed, previously untreated (chemotherapy or radiotherapy), and histologically confirmed. Blood samples were also collected from the control group which were healthy blood donors. The Human subject protocol for this study was approved by the Human Experimental Committee for the Faculty of Associated Medical Sciences and the Human Experimental Committee for Faculty of Medicine, Chiang Mai University, Thailand.

1.2 Preparation of cell lysate

In the first step, tissues were weighted and diced into very small pieces using a clean razor blade. Frozen tissue should be sliced very thinly and thawed in SDS lysis buffer. Three millilitres of ice cold SDS lysis buffer was used per gram of tissue. Next, cells were disrupted and homogenized with a tissue grinder and a sonicator, while maintaining temperature at 4 ^{o}C throughout all procedures. After that, lysate mixture was transfered into microcentrifuge tubes, and centrifuged at

10000 g for 10 minutes at 4^{o} C. The obtained supernatant was removed and stored at -20 ^{o}C for further experiment.

1.3 Protein assay

The BCA^{TM} Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the Biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/ml). The BCA^{TM} method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow large numbers of samples to be assayed together.

Ten microlitres of standard (Bovine Serum Albumin; BSA) or unknown sample were triplicately pipetted into a microplate well. After that, 190 µl of the reagent mixture containing reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and B (4% cupric sulfate) in ratio 50:1 were added to each well and mixed thoroughly on a plate shaker for 30 seconds. Reaction plate was incubated at room temperature for 30 minutes. The absorbance at or near 550 nm was measured using a microplate reader. A standard curve between the average Blank-corrected 550 nm

measurements for each BSA standard versus its concentration in µg/ml was generated and used to determine the protein concentration of each unknown sample.

1.4 Separation of protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A 15% polyacrylamide separating gel (prepared by combining 7.5 ml of 30% acrylamide solution, 3.75 ml of 1.5M Tris-HCl buffer pH 8.8, 75 µl of 10% SDS, 5 µl of 10% ammonium persulfate, 5 µl of TEMED and 3.45 ml of deionized water) topping with 4% polyacrylamide stacking gel (prepared by combining 665 µl of 30% acrylamide solution, 1.25 ml of 0.5M Tris-HCl buffer pH 6.8, 50 µl of 10% SDS, 25 µl of 10% ammonium persulfate, 5 µl of TEMED and 3 ml of deionized water) was used to separate proteins from cell lysate. Separating gel ingredients were mixed and filled into gel sandwich until reaching the desired level, the gel was allowed to polymerize for 40 minutes at room temperature. After that stacking gel solution was mixed and filled on top of the separating gel. Then the comb was insert into stacking gel and allowed gel to polymerize for 30 minutes.

After obtaining the concentration of each cell lysate, loading mixture was prepared by combining 30 µg protein cell lysate with 2X loading buffer (containing 0.5 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 2% (v/v) 2-ME and 0.005% (w/v) bromphenol blue) in ratio 1:1 and loaded to each well of the gel. Separation of protein in cell lysate on SDS-PAGE was performed at a constant voltage of 180 voltages for 1.5 hours. Electrophoresis was terminated when the position of tracking dye which monitors the progress of the run reached the bottom of the gel.

1.5 Electrotransfer of separated proteins from the gel into the membrane

While the gel was running, PVDF (PALL, USA) membrane was prepared for blotting by soaking in methanol for 1 minute, in deionized water for 5 minutes and transferring buffer for at least 15 minutes. After that, two pieces of fiber pad, two filter papers were soaked in transfer buffer. After the electrophoresis was terminated, the gel was removed for transferring. The following items were assembled in order to start blotting, from the black side of the blotting cassette: fiber pad, filter paper, gel, PVDF membrane, filter paper, fiber pad and the red cassette clamp, respectively. The cassette was placed in the transfer tank, the black side was cloded to the negative electrode and the transferring buffer (25 mM Tris-base, 200 mM glycerine and 20% (v/v) methanol, and 0.037% SDS) was filled until reaching the top of cassette. Electroblotting was performed by applying 30 voltages overnight.

1.6 Immunoblotting process

After transferring separated proteins from polyacrylamide gel onto PVDF membrane, the membrane was blocked with TBS-T (20 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.05% tween-20) containing 5% skimmed milk at room temperature for 1 hour. Then, the membrane was incubated with a 1:100, 1:250 dilution (v/v) of the monoclonal antibody livin or survivin in dilution buffer (5% skimmed milk in TBS-T) to room temperature for 1 hour. After the membrane was subjected to 3 rounds of 10 minute-wash with TBS-T, 1:1,000 dilution (v/v) of horserashdish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin antibody (DakoCytomation) in dilution buffer was added.

Finally, after extensive washing, the targeted protein band was detected by ECL (Amersham ECL Plus Western Blotting Detection Reagents) from storage at 2-8 ^{o} C and allowed to equilibrate to room temperature before opening. And mix detection solutions A (ECL Plus substrate solution containing tris buffer) and B (Stock Acridan solution in Dioxane and Ethanol) in a ratio of 40:1 (for example, 2 ml solution A + 50 µl solution B). The final volume of detection reagent required is 0.1 ml/cm^2. Drain the excess wash buffer from the washed membranes and place protein side up on a plastic wrap, in an x-ray film cassette. Pipette the mixed detection reagent on to the membrane and incubate for 5 minutes at room temperature. In addition, drain off excess detection reagent and gently smooth out any air bubbles. Next, place a sheet of autoradiography film on top of the membrane. Close the cassette and expose for 15 seconds. Moreover, remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately, and on the basis of its appearance estimate how long to continue the exposure of the second piece of film. Second exposures can vary from 1 minute to 1 hour.

2. Detection of autoantibodies against livin and survivin in lung cancer patients

2.1 Production of recombinant livin and survivin tumor antigens

2.1.1 Construction of expression vector

In principle, DNA cloning is a technique for reproducing DNA fragments. A vector is required to carry the DNA fragment of interest into the host cell. Creating recombinant DNA, a plasmid vector is digested with restriction enzymes to produce two sticky ends and desired DNA insert is also digested with the same

restriction enzymes to produce pieces with the compatible sticky ends. The two samples are mixed and allowed to hybridize and then some molecules will form with pieces of interested DNA inserted into the plasmid vector at the restriction enzyme sites. DNA ligase is used to covalently link the fragments. The recombinant vector must also contain an antibiotic-resistance gene. The recombinant DNA enters into the host cell and proliferates. It is called "transformation" because the function of the host cell may be altered. Normal *Escherichia coli (E.coli)* cells are difficult to take up plasmid DNA from the medium. If they are treated with $CaCl_2$, the transformation efficiency can be significantly enhanced. Even so, only one cell in about 10,000 cells may take up a plasmid DNA molecule. A specific antibiotic is added to kill *E.coli* without any protection. The transformed *E.coli* is protected by the antibiotic resistance gene whose product can inactivate the specific antibiotic. The numbers of vectors in each *E.coli* cells are not the same, because they may also reproduce independently.

pET system is the system developed for the cloning and expression of recombinant protein in *E.coli*. In this system, fusion sequences which are small in size and can be used for affinity purification was tagged with targeted protein to facilitate detection and purification. Histidine-tag, most commonly known as "His•tag", is the most used tag world wide for tagging recombinant proteins. $(His)_6$ generally refers to 6 histidine residues present in a protein (usually fusion protein). These hexahistidine allow the purification of "$(His)_6$" recombinant protein to be purified using a Nickel column or Nickel resin etc. (the histidine binds the Nickel and then can be washed and eluted with specific buffers). $(His)_6$ is mostly used for facilitation of the purification of expressed recombinant proteins, but also for

detection purposes. As an example, Yagihashi A. and colleagues produced $(His)_6$ survivin to establish ELISA for anti-survivin antibodies (28).

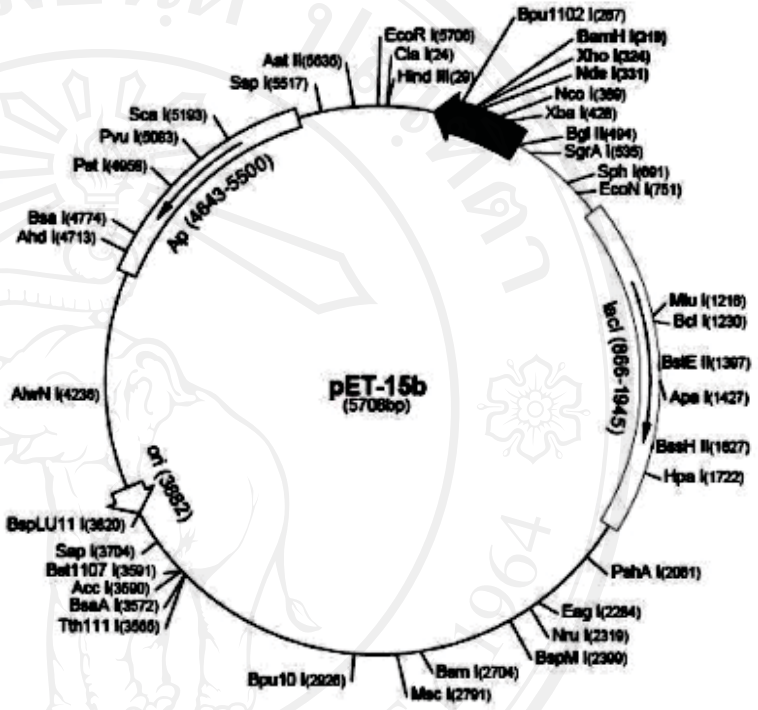
The pET-15b vector carries an N-terminal His•Tag sequence followed by a thrombin site and three cloning sites containing *NdeI*, *XhoI* and *BamHI* restriction sites, respectively. The vector carries an ampicillin resistance gene. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown in Figure 1.

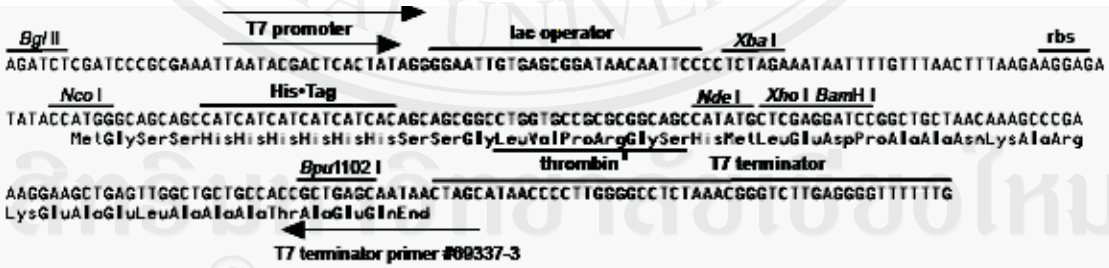
pET-15b Code: PEC 0

pET-15b Vector

pET-15b sequence landmarks

T7 promoter	463-479
T7 transcription start	452
His• Tag coding sequence	362-380
Multiple cloning sites	
(*Nde* I - *Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500





pET-15b cloning/expression region

2.1.1.1 Preparation of livin and survivin encoding DNA for insertion into pET-15b vector

Total RNA extracted from HeLa cells was changed to large cDNA by RT-PCR and then was generated to livin encoding DNA and survivin encoding DNA by PCR. Then, livin and survivin targeted gene were amplified in a PCR reaction and the incorporation of appropriate restriction enzyme target sequences on the PCR primers are required for production of sticky-ended DNA molecules available for the insertion into vector .

NdeIlivin (forward primer) and livinBamHI (reverse primer) were used to generate NdeI-livin-BamHI PCR product, as indicated in figure 2 :

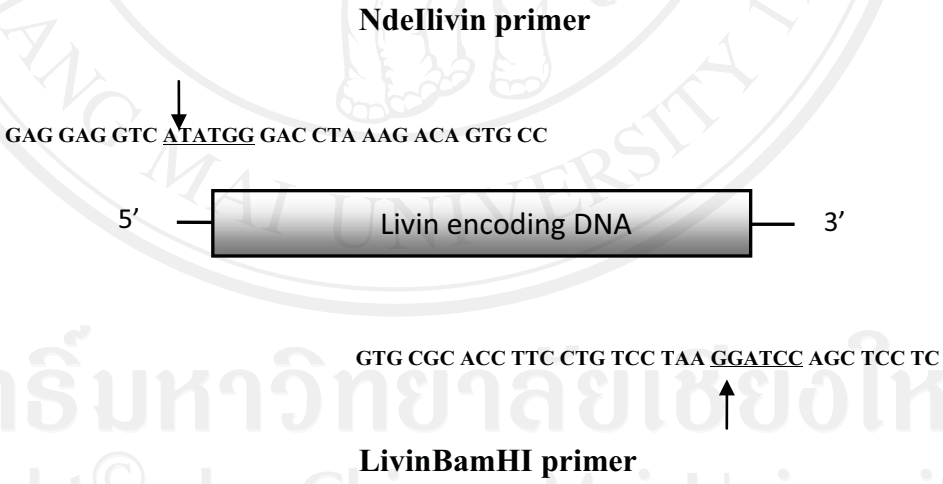


Figure 2. Representative figure illustrating restriction site of NdeIlivin (forward primer) and livinBamHI (reverse primer) for livin encoding DNA

NdeIsurvivin (forward primer) and survivinBamHI (reverse primer) were used to generate NdeI-survivin-BamHI PCR product, as indicated in figure 3:

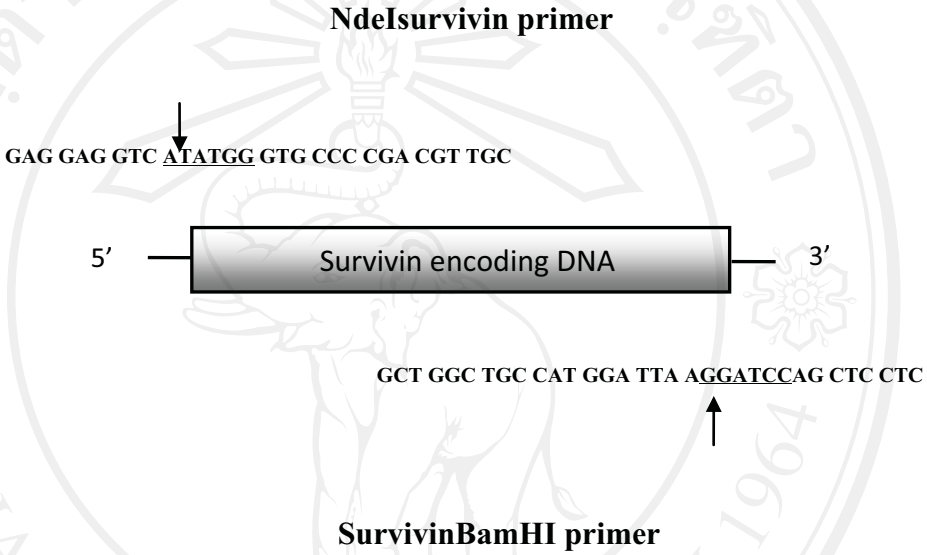


Figure 3. Representative figure illustrating restriction site of NdeIsurvivin (forward primer) and survivinBamHI (reverse primer) for survivin encoding DNA

To produce target DNA, the 25 µl reaction was performed with the mixture as described: two sets of primer specific for livin/survivin forward and livin/suvivin reverse were used at a final concentration of 10 µM. A combination of 1.0 µl of DNA template with a reaction mixture containing 2.5 µl of 10xTaq buffer, 1.0 mM of $MgCl_2$, 2mM dNTP, 5U of Taq DNA polymerase and distilled water was used in a final volume of 25 µl. And the cycling conditions included initial denaturation

step at 94 ^{o}C for 2 min, amplification was performed during 30 cycles including denaturation (94 °C for 1 min), annealing (60 °C for 50 sec) and extension (72 °C for 2 min), follow by final extension at 72 °C for 10 min.

The PCR product can be identified by its size using agarose gel electrophoresis. Agarose gel electrophoresis is a procedure that contains of administrating 5 µl of PCR product was mixed with loading buffer (Fermentas) to the ratio 6:1 into agarose gel and then applying a constant 50 voltage electric current to the gel. As a result, the smaller DNA strands move faster than the larger strands through the gel toward the positive current. The size of the PCR product can be determined by comparing its size with a 1 kb DNA ladder (Fermentas), which contain DNA fragments of known size range between 250-10000 bps, also included within the gel.

2.1.1.2 Digestion of pET-15b vector and NdeI-livin-BamHI, NdeI-survivin-BamHI PCR products with restriction enzymes

The pET-15b vector was double digested with *NdeI* and *BamHI* to linearize the supercoiled plasmid. Meanwhile, NdeI-livin-BamHI and NdeI-survivin-BamHI PCR products were also cut with *NdeI* and *BamHI* to produce stricky ends available for the insertion into vector. For digestion of pET-15b vector, a containing 10 µl mixture consist of 100 ng of pET-15b vector, 10 units of *NdeI*, 10 units of *BamHI* and 2 µl of R buffer was prepared. For digestion of NdeI-livin-BamHI and NdeI-survivin-BamHI PCR products, a reaction tube containing the 10 µl mixture consists of 50 ng of NdeI-livin-BamHI or NdeI-survivin-BamHI PCR product, 10 units of *NdeI*, 10 units of *BamHI* and 2 µl of R buffer and 6 µl of sterile distilled water was prepared. Both mixtures were incubated into the water bath overnight at 37 ^{o}C. Prior

to ligation, DNA insert was purified using NucleoSpin Extract II PCR purification kit (MACHEREY-NAGEL). Then again the linearized plasmid DNA was gel purified using the NucleoSpin Extract II gel extraction kit (MACHEREY-NAGE).

2.1.1.3 Ligation

The ligation of digested NdeI-livin-BamHI or NdeI-survivin-BamHI fragment into pET-15b vector was performed using T4 DNA ligase enzyme. The ligation mixtures consist of 50 ng of NdeI-livin-BamHI or NdeI-survivin-BamHI DNA insert, 100 ng of pET-15b vector and 2 µl of 10X ligase buffer (200 mM Tris-HCl, 100 mM $MgCl_2$ and 250 µg/ml acetylated BSA), 5 units of T4 DNA ligase was added last and gently mixed by stirring with pipette tip. The ligation reaction was incubated at 16 ^{o}C overnight.

2.1.1.4 Transformation of competent cells

After ensuring the appropriate ligation had occurred, the ligation mixture obtained from section 2.1.1.3 was then used for transformation into *E.coli* strain XL-1blue cells. Firstly, competent cell tubes was removed from the -80 ^{o}C freezers and immediately place the tube on ice. The cells were allowed to thaw on ice for ~2-5 minutes. Then 20 µl of ligation mixture was added into 200 µl competent *E.coli* strain XL-1blue and stirred gently to mix and returned the tube to the ice. After cells were incubated on ice for 1 hour, the mixture was transferred into a cold 1.5 ml microcentifuge tube. Following transferring, the cells were heat shocked by removing tubes from the ice and immediately immersing them in 42 ^{o}C water bath for 90 seconds, after which they were placed back into the ice, immediately. After the tube had sat on ice again for 1 minute, 3 ml LB broth was added to the tube and

cells were allowed to grow at 37 ^{o}C for 3 hours with 130 rpm shaking rate. After 3 hours, cells were centrifuged at 2500 rpm (22 ^{o}C) for 10 minutes to discard old medium and the pellet was resuspended in 500 µl LB broth. Then 100 µl of bacteria suspension were spreaded onto each LB plate containing 100 µg/ml ampicillin and bacteria were allowed to grow overnight at 37 ^{o}C.

2.1.1.5 Positive clones screening

Only bacteria which harbor pET-15b plasmid vector were chosen from the LB agar plate containing ampicillin. However, the resistant colonies were needed to be screened whether they have received pET-15b vector with right inserted DNA. The transformants were screened with PCR using T7 promoter and T7 terminator primers. The PCR mixture contents were the same as section 2.1.1.1. After that, pET-15b-livin/survivin plasmid from the correct transformant were purified from 10 ml overnight culture of recombinant *E.coli* XL-1 blue cell using NucleoSpin Plasmid DNA purification (MACHEREY-NAGEl) and plasmid was confirmed by DNA sequencing. After confirmation, the correct plasmid was transformed into the competent *E.coli* BL21(DE3) cells and BL21(DE3)pLysS cells, which were later used as expression host.

2.1.1.6 Growth of the correct transformant in liquid media and storage

The corrected clone was inoculated in 5 ml LB-broth cultures (in 50 ml centrifuge tubes) and shaked overnight at 37 ^{o}C in a shaker at 180 rpm. The culture then was mixed with 1 ml steriled glycerol. Cells were dispensed into 1 ml aliquots in 1.5 ml eppendorf tubes and stored frozen -70 ^{o}C until used.

2.1.2 Expression of livin and survivin recombinant proteins in bacterial host cells

Five microlitres of frozen cell stock of *E.coli* BL21(DE3) and BL21(DE3)pLysS harboring the pET-15b- livin/survivin plasmid was inoculated into 10 ml of LB broth containing 100 µg/ml ampicillin at 37 ^{o}C, overnight with shaking at rate approximately 180 rpm. One ml of the culture was harvested aseptically and pellet was stored at -20 ^{o}C until analyzed. This fraction was considered as the non induced sample and at the same time, an aliquot of 2 ml of the bacteria culture was added into three of 125 ml flask containing 25 ml of LB broth (containing 100 µg/ml ampicillin) and allowed to grow at 37 ^{o}C until OD_{600nm} reach 0.6-0.8. Afterward, each the culture flask was activated with 25 µl of isopropyl-β-d-thiogalactopyranoside (IPTG). The culture flask was shaking (180 rpm) at 37 ^{o}C. During the first 3 hour, the induced culture was harvested. Then cells were centrifuged at 2500 rpm (25 ^{o}C) for 10 minutes to discard old medium and the pellet was stored at -20 ^{o}C overnight. Untransformed *E.coli* BL21(DE3) and BL21(DE3)pLysS were used as the negative control (Neg). For the preparation of *E.coli* lysate, the bacterial cell pellet was resuspened in 500 µl of 1X binding buffer. After that, the cell pellet was sonicated on ice for 1 hour. The cell lysate was then centrifuged at 10000 g for 30 minutes at 4 ^{o}C and the supernatant was collected into fresh centrifuge tubes and stored at -20 ^{o}C until analysis.

2.1.2.1 Determination of the level of expressed protein

Protein sample from the supernatant of cell lysate was analyzed by Western blot analysis using anti-livin monoclonal antibody (mAb) to detect for recombinant livin protein and anti-survivin monoclonal antibody (mAb) to detect for recombinant

survivin protein together with anti-histidine monoclonal antibody (mAb) (As described in section 1.6).

2.2 Optimization of ELISA

In principle of a nickel chelate microtiter plate assay for six histidine-containing proteins is the protein purification has been made significantly easier by the use of affinity tags that can be genetically engineered at either the amino- or carboxyl-terminus of recombinant proteins. One of the most widely used tags is six consecutive histidine resedues of 6His tag. These residues bind with high affinity to metal ions immobilized on chelating resins even in the presence of denaturing agents and can be mildly eluted with imidazole. A derivative of nitrilotriacetic acid (NTA), N, N-bis [carboxymethyl] lysine (BCML), was coupled to a polystyrene microtiter plate. The plate was then charged with Ni^{2+} for the capture of the 6His-tagged proteins. The plate was used in a modified enzyme-linked immunoadsorbent assay format to quantity for protein concentrations and determines the affinity of protein-ligand interactions (77).

In general a livin/survivin antigen is immobilized onto microwells. Livin/survivin specific immunoglobulin antibody is allowed to react with the antigen. The excess unbound proteins are washed-off from the microwells. An enzyme (HRP) labeled goat-antibody; specific to immunoglobulin is added to the livin/survivin-antibody complex. After washing off excess unreacted enzyme conjugate from the microwells, a substrate (TMB) is added and HRP labeled anti-human immunoglobulin antibody catalyses tetramethylbenzidin oxidation resulting in a colorimetric reaction. The color generated is measured spectrophotometrically at

450-630 nm. The intensity of the color developed gives directly the concentration of livin/survivin antibodies.

2.2.1 Finding optimal antigen concentration

At first, one hundred microlitres of 10 mM BCML in 0.1 M $NaPO_4$, pH 8, was added to each well of microtiter plate and incubated overnight at 4^{o}C. After that, washed the plate three times with 200 µl of 0.05% Tween. And then, blocked by incubating with 5% skimmed milk in TBS/Tween buffer, pH 7.5 for 1 hour at room temperature. The plate was then washed with a series of buffers: first, 50 mM Tris HCl, pH 7.5, 500 mM imidazole, 0.05% Tween; second, 0.05%Tween; third, 100 mM EDTA, pH 8.0; and last, 0.05% Tween. Next, incubated with 10 mM $NiSO_4$ for 30 minutes at room temperature. The plate was washed with 0.05% Tween and then 50 mM Tris HCl, 500 mM NaCl, pH 7.5. Plates prepared by this method can be stored at 4 ^{o}C for at least one month. Then ELISA plates were coated with 100 µl of 0, 50, 100 and 200 µg/ml of His-tag-livin/survivin fusion protein in coating buffer and incubated overnight at 4 ^{o}C. After discarding the coating solution, plate was washed with 200 µl of washing buffer were added with different concentration of Imidazol (20-80 mM Imidazol). After that, different dilutions of the commercial livin/survivin mAb (0, 1:5000 and 1:10000) mouse along with test sera (3 serum of lung cancer patient and 3 serum of normal patient) diluted in PBS-T containing 5% skimmed milk to 1:1000 were added in duplicates at 100 µl/well, and then incubated for 1 hour. After washing with high-stringency wash buffer and wash buffer, 1:3000 diluted goat anti-mouse immunoglobulin-HRP conjugate and 1:5000 diluted rabbit anti-human immunoglobulin-HRP conjugate in dilution buffer were added and incubated for 1 hour. After extensive washing with high-stringency wash buffer and

wash buffer again, 50 µl of TMB substrate solution (Zymed) was applied and the color development was stopped after 10 minutes by adding 50 µl of 1 N HCl before measuring absorbance 450-630 nm values using microplate reader.

2.2.2 Finding optimal coating buffer

In order to obtain the most suitable coating buffer for coating protein, ELISA was performed with 3 commonly used coating buffers. ELISA plates were coated with 100 and 25 µg/ml His-tag-livin and survivin fusion proteins, respectively, diluted in different coating buffer, which included PBS pH 5.2 (50 mM PBS, pH 5.2), PBS pH 7.4 (50 mM PBS, pH 7.4) and carbonate buffer pH 9.6 (50 mM carbonate, pH 9.6) and incubate overnight at 4 ^{o}C . Afterward, coated plate was used to perform ELISA with different dilutions of anti-livin/survivin mAb and test sera as described in section 2.2.1.

2.2.3 Finding optimal blocking agent

In the process of preparation of Ni^{2+}-NTA microtiter plate, one hundred microlitres of 10 mM BCML in 0.1 M $NaPO_4$, pH 8, was added to each well of microtiter plate and incubated overnight at 4^{o}C. The plate was then washed three times with 200 µl of 0.05% Tween. After that, the plate was blocked by incubating with 4 blocking agent : 0.5% skimmed milk, 5% skimmed milk, 0.5% BSA and 1% BSA 300 µl and incubated at room temperature for 1 hour. Finally, washed with a series of buffers as described in section 2.2.1.

2.2.4 Finding optimal serum dilution

6His-tag-livin/survivin fusion protein diluted in wash buffer was added to the Ni^{2+}-NTA plate and incubated overnight at 4^{o}C. Next, washed with 200 µl of washing buffer were added with different concentration of Imidazol (20-80 mM

Imidazol). And to varyserum dilution, we used 1:200, 1:1000 and 1:5000 of serum dilutions diluted in wash buffer, and then added to the plate and incubated at room temperature for 1 hour. Next, washed with high-stringency wash buffer and wash buffer, respectively. Finally, the plate was developed as described above in section 2.2.1.

2.3 Evaluation of the established ELISA

2.3.1 Reproducibility testing of the established ELISA

Optimal condition variation (OCV) or within run assay variation is a statistic value calculated from the results of 20 replicates in a single run. In this study, the optimal condition variation (OCV) of the established method for livin and survivin autoantibodies detection were calculated from 20 assay wells of ELISA performing with control serum in a single run.

Routine condition variation (RCV) or between run assay is a statistic value calculated from various in runs (vary in times). In this study, the RCV was determined by assaying control serum used to obtain OCV simultaneously with the experimental samples in each run. Precision test was evaluated from percentage of coefficient of variation (%CV) by using the following formula:

$$\%\,CV = \frac{SD \times 100}{Mean}$$

2.3.2 Verification of autoantibody specificity by pre-absorption experiment

2.3.2.1 Purification of $(His)_6$-survivin protein by His-Bind Resin chromatography

An overnight culture of a positive clone was harvested by centrifugation and the cell pellet was resuspended in lysis buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, 6 M urea, pH 7.9) at 10 ml lysis buffer per 40 ml cell culture, The suspension was vortexed for 1 min at room temperature until homogeneously. The cell lysate was recentrifuged at 10000 g for 10 min and the supernatant was transferred to a fresh tube to further purified by using the His-Bind Resin (Novagen). Firstly, a bottle of His-Bind Resin was gently mixed by inversion until completely suspended. After that a widemouth pipet was used to transfer 5 ml of 50% His-Bind Resin slurry to column and allow resin to pack under gravity flow. When level of storage buffer drops to top of column bed, used the following sequence of washes to charge and equilibrate column:

a) 3 volumes of sterile deionized water
b) 3 volumes of 1X Binding Buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, 6M urea, pH 7.9)

After 1X Binding Buffer was drained to top of the column bed, 80 ml of prepared cell lysate was loaded to column, the first flow through designated as lane 2. A flow rate of approximately was adjusted to 10 volumes per hour as it was recommended to be optimal for efficient purification. If flow rate is too fast, more impurities will contaminate the eluted fraction. The column was washed with 10 volumes 1X Binding Buffer and 6 volumes 1X Wash Buffer (0.5M NaCl, 80 mM imidazole, 20 mM Tris-HCl, 6 M urea, pH 7.9), washed fractions were designed as

FT1 and FT2 respectively. The recombinant protein was eluted once time with 1 volumes 1X Elute Buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and these eluted fractions were marked as E1. Finally, 6 volumes 1X Strip Buffer (0.5 M NaCl, 100 mM EDTA, 20 mM Tris-HCl, pH 7.9) was used to wash Ni^{2+} from column and used resin was kept in stripping buffer. The 15 µl of each collected fraction were mixed with 15 µl of 2X loading buffer and analyzed by SDS-PAGE for determination of the eluted $(His)_6$-survivin protein. After that, gel was subjected to Coomassie blue staining for the visualization of bands indicating the $(His)_6$-survivin protein content in the gel. Coomassie (also known as Brilliant Blue, Brilliant Blue G, Acid Blue 90, C.I. 42655, or Brilliant Blue G 250) is a blue dye commonly used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was soaked in dye for 30 minutes and then destained for 30 minutes or more with Destain I, before the gel was destained with Destain II until the background of gel was clear. This treatment allows the visualization of bands indicating the protein content of the gel. The visualization on the gel usually contains a set of molecular weight marker so that protein MW can be determined in an unknown solution.

2.3.2.1.1 Dialysis of protein

Ten millilitres of the purified $(His)_6$-protein fraction was dialyzed using Dialysis tubing cellulose membrane (MW 10000) at 4 ^oC against 1 L PBS buffer pH 7.4 (10 mM phosphate buffer, 150 mM NaCl and 0.1% sodium azide, pH 7.4). Changed twice the first 6 hours and continued overnight. After that, the purified $(His)_6$-survivin protein was dispensed into 1 ml aliquots in 1.5 ml eppendorf tubes and stored frozen at -20 ^oC until used.

2.3.2.2 Pre-absorption of samples with recombinant survivin

Serum samples (100 µl of a 1:1000 dilution) were incubate with 0, 25, 50 and 100 µg/ml of recombinant survivin antigen for 1 hour at 37°C and then subjected to the anti-survivin ELISA described above.

2.4 Detection of livin and survivin specific autoantibodies in lung cancer patient serum using the generated $(His)_6$-livin and survivin proteins

After obtaining the optimal ELISA condition, the reactivity of antibodies from lung cancer patient sera with the produced $(His)_6$-livin and $(His)_6$-survivin proteins were examined by this condition. Two hundred and fifty of lung cancer patient sera from Maharaj Nakorn Chiang Mai Hospital were analyzed to investigate their reactivity to the coated $(His)_6$-livin and $(His)_6$-survivin antigens by ELISA.

2.5 Statistical analysis

The chi-square tests were used to test the association between the finding of autoantibodies specific to livin and survivin and their expression levels in tumor tissues as well as the tumor tissue pathological features. In addition, the statistical analyses was performed using SPSS version (version 14.0) software (SPSS Inc., USA).