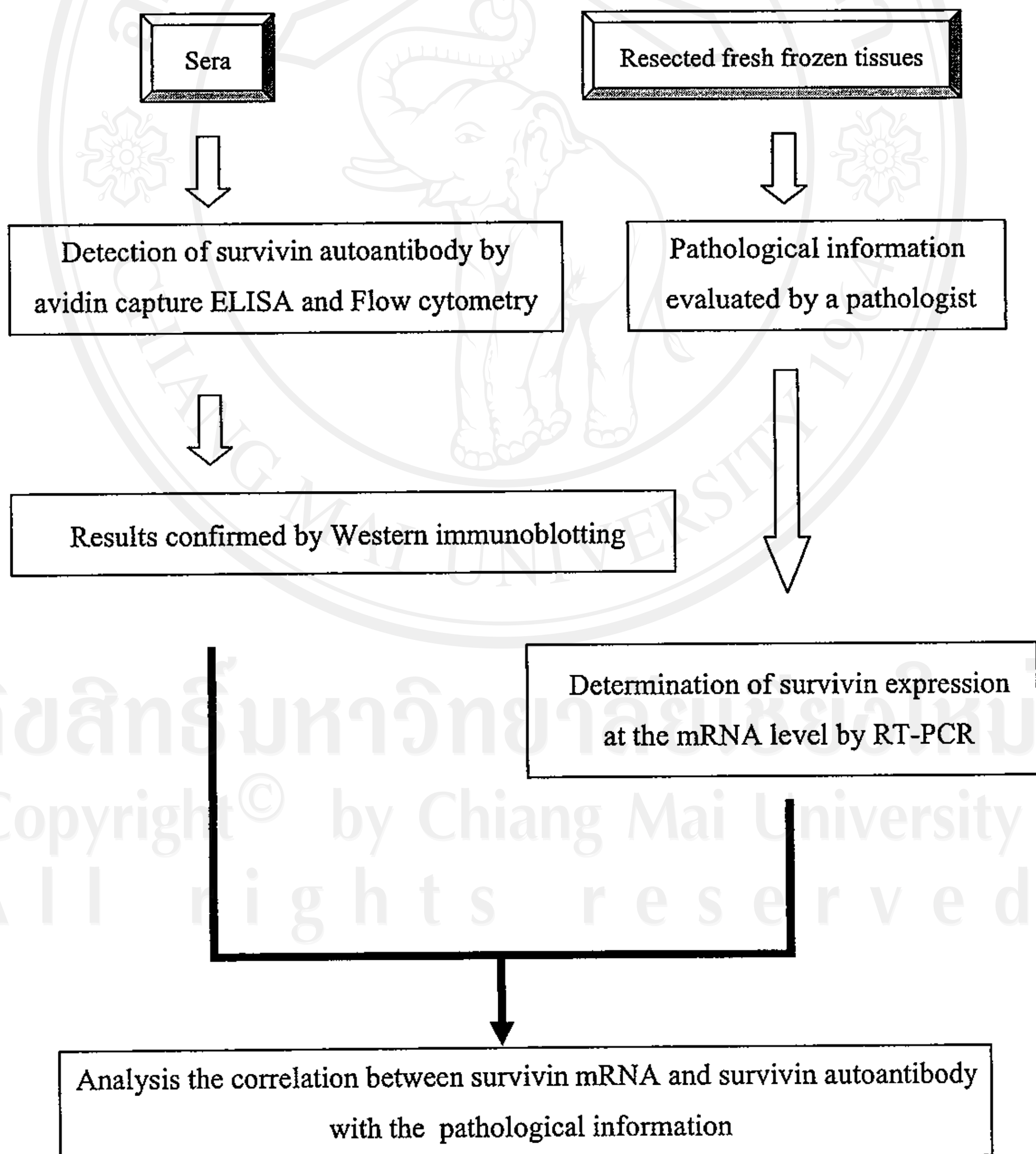
CHAPTER II

MATERIALS AND METHODS

I. Research design



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II. Materials and Methods

2.1. Tissues and serum collection

All tissue samples in this study were obtained from the patients who had undergone surgical resection for cancer treatment at Maharaj Nakorn Chiang Mai hospital during April 2005 to July 2006. Among these, 22, 11 and 22 specimens were colorectal, liver and lung, respectively. There were 32 males and 22 females, with a median age of 59 (aged between 16-88 years). In each case, the corresponding normal tissues were also collected for analysis. These specimens were placed in vial, frozen in tissue-embedding medium for preservation of cell integrity and stored at -70 °C until analyzed. They were diagnosed by the pathologist with respect to the pathological features of tumors, including tumor size, depth of invasion, lymphatic invasion, venous invasion, perineural invasion, histological grading, lymph node metastasis, distant metastasis and tumor staging (the AJCC TNM classification; see Appendix D). The patients' data were reviewed from the pathological records and outpatients department (OPD) card at the administration and clerical section, Maharaj Nakorn Chiang Mai hospital.

Fifty-five sera were collected from the same patients along with the tumor tissues and fifty normal sera were obtained from healthy donors. Sera were divided into aliquots and stored at -20°C until analyzed. They all were the remaining serum of the cross-matching procedure from blood bank, Maharaj Nakorn Chiang Mai hospital. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University according to the reference number 0515 (012)/2982.

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2.2. Chemicals

All chemicals used in this study were shown in Appendix A.

2.3. RNA extraction

All procedures were carried out using diethyl pyrocarbonate (DEPC)-treated distilled water. Fresh frozen tumors and paired normal tissues were weighed and cut into small pieces. According to the manufacturer's instructions, approximately 0.05-0.1 g of tissues was homogenized thoroughly by using a bench-top homogenizer in 1 ml of Trizol reagent (Invitrogen, USA), a mono-phasic solution of phenol and guanidine isothiocyanate. After that, the homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Thereafter 0.2 ml of chloroform was added into each tubes, the tubes were shaked vigorously by vertexing for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were centrifuged at 12,000 g for 15 minutes at 4°C. Following centrifugation, the mixture was separated into a lower red; phenol chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA was remained exclusively in the aqueous phase and transferred to a fresh tube. The RNA was precipitated by mixing with 0.5 ml isopropyl alcohol. After that, the samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. After that, the RNA pellet was washed twice with 85% ethanol in DEPC treated water, mixed by vortexing and centrifuged at 10,000 g for 5 minutes at 4°C. Then, the RNA was allowed to dry at

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room temperature and dissolved in 50 μl DEPC-treated water. To measure the total RNA concentration, a tiny bit of RNA sample was dissolved (2 μl of total RNA in 998 μl DEPC-treated water) and measured at wavelength 260, 270, and 280 nm using UV-1601 spectrophotometer (Shimadzu Corporation, Japan). The RNA samples were stored at -70°C until analyzed.

2.4. Calculation of total RNA concentration

The concentration of total RNA was calculated using the following formula as showed below (Hofstra, 2006).

Concentration of total RNA (μg/ml)	**= $OD_{260} \times 40 \times$ dilution factor**

Where: 1 OD (RNA) = 40 μg/ml

Dilution factor = 500 (2 μl of RNA sample in a total volume of 1000 μl)

<u>The example of calculation</u>

T5: OD_{260} = 0.394

OD_{280} = 0.240

therefore OD_{260}/OD_{280} = 1.64

The isolated RNA (T5) has an A_{260}/A_{280} ratio 1.64 indicated the purity of RNA sample.

Concentration of total RNA (μg/ml) = 0.394× 40 × 500

= 7,880 μg/ml or 7.88 μg/μl

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2.5. Semiquantitative determination of survivin mRNA in tumor tissues by reverse transcriptase polymerase chain reaction (RT-PCR)

2.5.1. Amplification of survivin mRNA by reverse transcriptase polymerase chain reaction (RT-PCR)

A half microgram of total RNA was reverse transcribted using SuperScript^{TM}III One-Step RT-PCR System with PlatinumR*Taq* DNA Polymerase (Invitrogen, USA) with specific primer (showed in Figure 2.1) in a final volume of 20 µl containing 1µM of each forward (F) and reverse (R) primers, 2X reaction buffer, 1 mM $MgSO_4$, 0.5 µM RT/PlatinumR*Taq* DNA Polymerase and DEPC-treated water. To check the integrity of the total RNA, GAPDH cDNA was amplified in the same tube. DEPC-treated water was used as a negative control and KB-V_1 cell line was used as a positive control.

The primer pairs of survivin and GAPDH cDNA were shown in the Table 2.1.

Table 2.1. Primer sequences synthesized according to Wang *et al.*, 2003.

cDNA	Primer sequences	Amplifed product (bp)
Survivin	(F) 5'GCATGGGTGCCCCGACGTTG 3' (R) 3'AACTCCGGAGACCGGCCTCG 3'	431
GAPDH	(F) 5'CGAAGTCAACGGATTTGGTCGTAT 3' (R) 5'AGCCTTCTCGGTGGTGAAGAC 3'	306

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Figure 2.1. A schematic drawing of the transcripts of survivin. The arrows show the positions of the survivin PCR primer (Adapted from *http://www.molecular-cancer.com/content/4/1/11/figure/F1 accessed 5 November 2006).*

The thermal cycler was programmed as follows so that PCR amplification was initiated immediately after cDNA synthesis.

cDNA synthesis

1 cycle: 55°C for 30 minutes

Denaturation

1 cycle: 94°C for 2 minutes

PCR amplification

30 cycles: 94°C for 15 seconds (denaturation)

55°C for 30 seconds (annealing)

72°C for 1 minute (extension)

Final extension

1 cycle: 72°C for 10 minutes

After that, the PCR products were verified by electrophoresis on 1.5% agarose gels (Invitrogen, Grand Island, New York, USA), followed by ethidium bromide staining and visualized under UV transillumination.

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2.5.2. Agarose gel electrophoresis

Agarose gel was dissolved in 1X Tris-Borate-EDTA (TBE) buffer at a boiling temperature. After cooling down, it was poured into the tray and left for polymerization. The gel was put submarine in 1X TBE buffer. A 20 μl of amplified product was mixed with 4 μl of 6X loading dye was added into the sample before loading into each well. Electrophoresis was carried out at 50-100 volts until the loading dye reached the bottom of the gel. After that, the gel was stained with 0.002% ethidium bromide for 30 minutes and detected by Gel Documentation (Bio-rad, Italy). Survivin and GAPDH cDNA were detected at 431 and 306 bp, respectively. The band intensity was normalized using Scion image® software.

2.6. Detection of survivin autoantibody by avidin capture ELISA

2.6.1. Survivin-BCCP fusion protein

Recombinant protein survivin fusion with biotin carboxyl carrier protein (Survivin-BCCP) was kindly provided by Assist. Prof. Chatchai Tayapiwatana. Briefly, survivin cDNA was amplified with specific primer by PCR, and cloned into pAK400cb vector. The biotinylated fusion protein survivin-BCCP was expressed in the cytoplasm of *Escherichia coli* strain Origami B. The bacterial lysate containing biotinylated protein survivin-BCCP were lyophilized and analyzed by indirect ELISA and Western immunoblotting.

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2.6.2. Optimization of survivin-BCCP concentration

Avidin (Sigma, St. Louis, MO, USA) solution (2 mg/ml in water) was stored frozen in portions until use. For each ELISA plate, 25 μl of avidin stock was diluted in 5 ml coating buffer (see Appendix) (Cordiano *et al.*, 1995; Winkler *et al.*, 1997) and 50 μl were added per well. ELISA plates (Costar®, USA) were incubated overnight at 4°C. After that, the plate was washed with wash buffer (0.05% Tween 20-PBS) four times, and blocked with 2% skimmed milk-PBS (100 μl/well) for 2 h at room temperature (RT). After incubation, the plates were washed four times with wash buffer. Then 50 μl of various concentration (12.5, 25, 50, 100, and 200 μg/ml) of survivin-BCCP were applied and incubated at RT for 1 h. Thereafter the plates were washed with wash buffer four times. Then 50 μl of various concentration of mouse survivin monoclonal IgG2a antibody; D_8 (Santa Cruz Biotechnology Inc., California, USA) was added into each well and incubated at RT for 1 h. After incubation, the plates were washed with wash buffer four times and 50 μl of 1:3000 dilution of goat anti-mouse immunoglobulins labeled with horseradish peroxidase was added into each well and incubated at RT for 1 h. After washing four times with wash buffer, 50 μl of 3,3,5,5'-tetramethybenzidine (TMB) substrate (Zymed, San Francisco, USA) was added to each well and incubated at RT in the dark for 10 minutes. The reaction was stopped by adding 50 μl of 1 N HCL and the absorbance was measured by microplate reader (EL340, Bio-TEK Instrument, USA) at 450 nm.

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2.6.3. Optimization of protein solution for blocking

For blocking step, the blocking solution including, 2% skimmed milk, 1% bovine serum albumin (BSA) in PBS, non-protein blocking buffer containing 0.05 M $CaCl_2$, 0.5% Tween 20, 0.5% Nonidet P40 (NP40) in PBS and SuperBlock® blocking buffer (Pierce, USA) were tested. The efficiency of each blocking solution were compared by using mouse survivin monoclonal IgG2a antibody (D_8) as a standard according to the method described previously.

2.6.4. Optimization of serum dilution

The dilution of serum was optimized according to the protocol described previously in 2.6.2. Two-fold dilutions of normal and patient sera in 50 µl volume were varied from 1:10 to 1: 160. In addition, 50 µl of diluted (1:8000) rabbit anti-human immunoglobulins labeled with horseradish peroxidase; HRP (recommended by data sheet of Dako, Denmark) was added instead of HRP-conjugate goat anti-mouse immunoglobulins.

2.6.5. Optimization of secondary antibody

Secondary antibody (rabbit anti-human Igs-HRP) was also tested for optimal dilution. Fifty µl of various dilutions (1:4000, 1:8000 and 1:16,000) of rabbit anti-human immunoglobulins labeled with horseradish peroxidase were carried out with respect to the standard protocol described earlier.

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2.6.6. Determination of survivin autoantibody in serum by avidin capture ELISA

Survivin autoantibody were determined from fifty-five patient sera and fifty normal sera with the optimal condition for avidin capture ELISA according to the method described previously. The mouse survivin monoclonal IgG2a antibody (D_8) was included for each run of ELISA. Each sample was done in duplicates.

2.7. Detection of survivin autoantibody by flow cytometry (indirect immunofluorescence)

2.7.1. Titration for optimal concentration of Streptavidin beads for biotinylated survivin coating

$5x10^7$ beads/100 µl of MagnaBind ™ Streptavidin beads (Pierce, USA) were washed three times with 1 ml phosphate buffered saline (PBS) by gently agitation. After each wash, the beads were separated by side of the tube using magnet and the supernatant was aspirated. The beads were then resuspended with 100 µl of PBS and 20 µl of various concentrations (0.5, 1, 2, 5 and 10 mg/ml) of the biotinylated survivin was added. The tubes were gently agitated and shaken at room temperature for 30 minutes. After that, the survivin coated beads were washed four times with 1 ml PBS and resuspended with 100 µl of 1%BSA-NaN_3. Then, 2.5 µl of survivin coated beads were mixed with 22.5 µl of 1% BSA-PBS–NaN_3. Twenty-five µl of mouse survivin monoclonal IgG2a antibody (10 µg/ml) was added and mixed gently. After 30 minute-shaking at RT, the beads were washed with 1 ml of 1% BSA-PBS–NaN_3 three times and resuspended with 25 µl of 1% BSA-PBS–NaN_3. Then 25 µl of anti-mouse

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Igs-FITC (1:50) was added and incubated at RT in the dark for 30 minutes and mixed every 5 minutes. The beads were subsequently washed three times with 1 ml of 1% BSA-PBS–NaN_3 and fixed with 500 µl of 1% paraformaldehyde in PBS. The resulting beads were analyzed by flow cytometer (Becton Dickinson; FACSort, Athens).

2.7.2. Optimization of serum dilution

The various dilution (1:20 and 1:80) of serum dilution from normal and patient sera were chosen and determined according to the method described previously.

2.7.3. Determination of survivin autoantibody in serum by flow cytometry

Survivin autoantibody was determined from fifty patient and normal sera with the optimal conditions for indirect immunofluorescence flow cytometry according to the method described previously. In each run, the mouse survivin monoclonal IgG2a antibody (D_8) and anti-BCCP as a positive control and CD-147 mAb as a negative control were included.

2.8. Western blot analysis

Western blot is a technique for identification and quantitation of protein. The technique consists of four step procedures including, separation of polypeptides by SDS-PAGE, electrotransfer of separated proteins from the gel into blotting membrane, labeling of the transferred proteins by antibodies conjugated with the enzyme and

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detecting of the labeling enzyme signal (Page & Thorpe, 2002). The details of procedure used in this experiment were as follows.

2.8.1. Separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.8.1.1. Preparation of stacking and separating gel

A 15% separating gel and 4% stacking gel were used (see Appendix). The separating gel was allowed to polymerize for 45 minutes at room temperature with the overlayer of 1% SDS. The stacking gel was then poured on top of the separating gel. The combs were inserted into stacking gel and the gel was left for polymerization.

2.8.1.2. Preparation of the sample and electrophoresis

The gel cassette was placed in the gel tank. The running buffer (see Appendix) was filled into the upper and lower chamber in an appropriate volume, being ensure that the sample wells were fully filled with the buffer. Then, the comb was removed by pulling up slowly to avoid any damage of the well. The wells were flushed with the buffer before use.

A 5 μg of survivin-BCCP was mixed with 5 μl of (2X) sample loading dye. Samples were heat at 100°C for 5 minutes. Following application of the samples, the gel was electrophoresed at constant voltage of 190 volts. Electrophoresis was carried out until the tracking dye reached the bottom of the separating gel. It took about two hours.

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Upon completion, the running buffer was discarded and the gel apparatus was dismantled. The gel was ready for the blotting step.

2.8.2. Electrotransfer of separated protein from the gel into the blotting membrane

While the gel was running, polyvinylidene fluoride (PVDF) membrane (8×7 cm in size) was prepared for blotting by soaking the membrane in methanol for 1 minute and deionized water for 5 minutes. After that, PVDF membrane, two pieces of fiber pad and two blotting papers were soaked in the transfer buffer. As the electrophoresis was terminated, the gel was removed for transfer. The following items were placed in orders blotting: fiber pad, blotting paper, gel, membrane, blotting paper, filter pad, respectively. The glass rod was used to remove any air bubble between the gel and the membrane. The cassette was placed into the transfer tank, ensuring that the gel was at the cathode and the buffer was poured until reaching the maximum filled line. Electroblotting was preformed by applying at constant voltage of 30 volts overnight. The water cooled base of a tank was filled thoroughly with water to prevent overheating effect of the system. After transferring, the blotted membranes were removed and placed in TBS-Tween buffer until further detection step.

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2.8.3. Immunodetection

The blotted membrane was placed in a plastic container and incubated with 5% skimmed milk in TBS-Tween for 1 hr at RT on a shaker (Gemmy VRN-200,

Germany) to block non specific binding sites. Primary antibody, either mouse survivin monoclonal IgG2a antibody or sera were diluted (1:100) in 5% skimmed milk in TBS-Tween. Then, the blocking buffer was poured off and the membrane was cut 0.5 cm strips. Each strip was incubated with sera for 1 h at RT on a shaker, followed by washing with five changes of TBS-Tween each for 5 min. Secondary anti-mouse and anti-human immunoglobulins antibody conjugated with HRP (Dako Denmark) were diluted at 1:3000 and 1:1000 in 5% skimmed milk in TBS-Tween, respectively. After washing membrane, the blot was incubated for 1 hr at RT on a shaker with diluted secondary antibody then washed with five changes of TBS-Tween each for 5 min.

2.8.4. Detection of the survivin autoantibody signal

Prior to the detection, an equal volume of detection solution A with and detection solution B, ECL detection reagents (Amersham Biosciences, UK) was mixed together in a ratio of 1:1 before used. The final volume of detection reagent required is 0.1ml/cm^2 of the membrane. After the excess washed buffer was drained, the membrane was placed as the protein side upward on a piece of cling film. The mixed detection reagent was then spreaded onto the membrane which was further incubated at room temperature for 1 min at RT, followed by draining off excess detection solution. After that, the membrane was wrapped up and gently smoothed out any air bubbles. The membrane was exposed to the X-ray film (Eastman Kodak, USA) in the cassette for appropriate time required for optimal detection depending on the level of signal intensity. After exposure, the film was immediately put in the

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developing solution (Eastman Kodak, USA) for 1 min, washed in distilled water for 1 min, fixed in a fixing solution (Eastman Kodak, USA) for 5 min, washed in distilled water for 5 min, and finally it was left drying at RT.

2.9. Statistical analysis

All statistical analysis was performed by the SPSS 7.5 software package for windows. Differences in the numerical data between the two groups were evaluated using the Mann-Whitney U test. Chi-square (χ^2) test was used to test significance of the difference in the correlation between survivin mRNA and survivin autoantibody with the pathological parameters which included tumor size, depth of invasion; early cancer, mucosa or sub-mucosa, advanced cancer, muscularis propia or subserosa or serosa, lymp node metastasis; absent or present, distant metastasis; absent or present, venous invasion; absent or present, lymphatic invasion; absent or present, perineural invasion; absent or present and histological grading (well differentiated, moderately differentiated, poorly differentiated). The *p*-value less than 0.05 was considered statistically significance.

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