CHAPTER III

RESULTS AND DISCUSSIONS

3.1. Patients and specimens

Tissue samples were obtained from fifty-four patients undergone surgical resection for cancer treatment at Maharaj Nakorn Chiang Mai Hospital during April 2005 to July 2006. These included 22 colorectal, 10 liver, and 22 lung cancer cases. Paired samples of the tumor and accompanying normal tissues were collected from the same patients. There were 32 males and 22 females, age ranged between 16 and 88 years with the median age of 59. Clinical characteristics of the patients are summarized in Table 3.1.

Table 3.1. Clinical characteristics of cancer patients

Parameters	Colorectal cancer	Liver cancer	Lung cancer	Total
No. of patients (cases)	22	10	22	54
Sex (cases)				
Male	11	9	12	32
Female	11	1	10	22
Age (years)				
Median	68	45	61.5	59
Range	30-88	16-60	21-78	16-88

3.2. Total RNA extraction

TRIZOL® reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. Total RNA isolated by TRIZOL® reagent is free of protein and DNA contamination. In addition, it maintains the integrity of the RNA while disrupting cells and dissolving cell components. However, in this study, we found the degraded RNA in 3 of 54 tumor tissues and 5 of 54 adjacent normal tissues. In these cases, they could be possibly occurred either before specimen collection or during specimen processing. Normally, the integrity of RNA may be assessed by electrophoresis on a denaturing agarose gel. This will also give some information about RNA yield. A denaturing gel system is suggested because most RNA forms extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to its size (Ambion, 2006). By this method, the degraded RNA displayed smear pattern instead of the 28s and 18s rRNA bands as expected from the intact RNA. In our study, the presence of GAPDH amplified products by single step RT-PCR could guarantee the integrity of RNA samples. In addition, the optical density measuring at 260 and 280 nm which indicated the purity of total RNA exhibited the ratio between of 1.6 to 1.8.

3.3. Clinicopathological characteristics of the tumor tissues in different types of cancer

Fresh frozen tissues were collected as representative tumor sections from 54 patients. The tissue sections were stained with hematoxylin and eosin for morphological examination by a pathologist who was unaware of the results of survivin mRNA expression by RT-PCR. Clinicopathological features of the tumor tissues were shown in Table 3.2. Among 22 specimens of colorectal cancers, 10 had metastases to the lymph nodes and 2 patients had distant metastases to the liver. According to the TNM staging, 1 of them were classified in TNM stage I, 11 in stage II, 8 in stage III and 2 in stage IV. Tumor sizes of colorectal specimens varied from 2 to 10 cm in the maximum diameter, with a mean value of 5.3 ± 1.9 cm. Colorectal tumor tissues were all adenocarcinomas. In liver cancer, five tumor tissues were hepatocellular carcinoma and the rest was cholangiocarcinoma. Two of the ten specimens had metastases to lymph nodes. One specimen was identified with distant metastasis to an adrenal gland. Three tumor tissues were classified in TNM stage II, 4 in stage IIIA, 1 in stage IIIB, 1 in stage IVA and 1 in stage IVB. Tumor sizes of liver specimens varied from 2 to 11 cm in the maximum diameter, with a mean value of 6 ± 2.8 cm. In lung cancer, 6 of 22 specimens exhibited lymph node metastasis. Two specimens were identified with distant metastasis to bone. Two specimens were classified in TNM stage IA, 13 in stage IB, 2 in stage IIB, 1 in stage IIIA and 4 in stage IV. Tumor sizes of lung specimens varied from 2 to 14 cm in the maximum diameter, with a mean value of 5.4 ± 2.7 cm. Among all tumor tissues, 7 were squamous cell carcinomas, 11 were adenocarcinomas and 4 were large-cell carcinomas.

Table 3.2. Clinicopathological characteristics of the tumor tissues

Parameters	Colorectal cancer (22 cases)	HCC (10 cases)	Lung cancer (22 cases)
Tumor size (maximum diameter;cm)	5.3 ± 1.9	6 ± 2.8	5.4 ± 2.7
(small:large)	13:9	4:6	14:8
Histological differentiation			
(WD:MD:PD)	14:7:1	3:6:1	12:5:3
Lymph node metastasis			
(Present : Absent)	10:12	2:8	6:16
Distant metastasis			
(Present : Absent)	2:20	1:9	2:20
Lymphatic invasion			
(Present : Absent)	21:1	5:5	18:4
Venous invasion			
(Present : Absent)	7:15	6:4	3:19
Perineural invasion			
(Present : Absent)	4:18	3:7	7:15
Tumor stage grouping			
I:II:III:IV	1:11:8:2	-	-
I:II:IIIA:IIIB:IVA:IVB	-	0:3:4:1:1:1	-
IA:IB:IIA:IIB:IIIA:IIIB:IV	-	-	2:13:0:2:1:0:4

Mean ± SD (WD, well differentiation; MD, moderate differentiation; PD, poor differentiation)

3.4. Semiquantitative determination of survivin mRNA in tumor tissues by RT-PCR

Since survivin was discovered in 1997 by hybridization screening of a human genomic library (Ambrosini *et al.*, 1997), the expression of survivin has been extensively studied in neoplastic and non-neoplastic tissues. Reverse Transcription-Polymerase Chain Rection (RT-PCR) is highly sensitive and specific for detection of expression level of RNA transcripts, especially in the limited amount of sample such as in the tissue biopsy (Carding *et al.*, 1992). Although real-time PCR has been used to detect the exact number of RNA molecules, in many cases the increase or decrease in expression level provides sufficient data. In addition, real-time PCR machine is not common in standard laboratory.

In this study, the semiquantitative RT-PCR was therefore used to determine the expression of survivin mRNA in tumor tissues. A house keeping gene, GAPDH, was used as an internal control to normalize the results. The expression of survivin mRNA in tissues was designated as the band intensity ratio of Survivin/GAPDH, which the intensity of each band was semi-quantified using Scion image® software. A single step RT-PCR permits reverse transcription of RNA transcripts into cDNA and immediately amplify the cDNA target by PCR. The method does not only reduce the variations due to reverse transcription reaction when several transcripts need to be analysed, but it is also practical when encountering a large number of samples with single target (Pfeffer *et al.*, 1995). Platinum® Tag DNA polymerase is designed such that a molecule is blocked by the antibody until its activity is restored after the denaturation step of PCR cycle. $MgSO_4$ is also a critical factor for function of Tag polymerase. Efficiency of amplification with specific primers is strictly sequence-

dependent. A 2X reaction mix buffer already containing 3.2 mM $MgSO_4$ was not sufficient for survivin amplification. The survivin amplified product could be seen only when 1 mM $MgSO_4$ was added up into the reaction mixture.

After the single step RT-PCR and 1.5% agarose gel electrophoresis, the amplified products of survivin cDNA and GAPDH cDNA at 431 and 306 bp, respectively, were observed (Figure 3.1). A multidrug resistant human cervical carcinoma cell line, KB-V1, as a positive control, strongly expressed survivin mRNA whereas a negative control in which the RNA template was absent showed neither of the survivin band nor GAPDH band as well as the nonspecific bands. The integrity of RNA could be verified by the presence of GAPDH amplified products in all specimens (Figure 3.1). Survivin mRNA was significantly overexpressed in colorectal ($p<0.001$), liver ($p=0.009$) and lung ($p<0.001$) tissues as compared to their corresponding normal tissues (Figure 3.2). Expression of survivin mRNA was detected in 13 of 22 (59.1%) colorectal cancer, 7 of 10 (70%) liver cancer and 14 of 22 (63.6%) lung cancer (Table 3.3). Moreover, survivin was differentially expressed in tumor tissues as the ratio between survivin and GAPDH band intensity was varied from 0.007 to 0.804. Despite undetectable survivin in normal tissues of colorectal and lung cancer (52 of 54; 96.3%), a certain amount was found in some normal liver tissues (2 of 54; 3.7 %) (Figure 3.2). Re-extraction of total RNA from these tissues and RT-PCR to confirm the results were carried out again. The results remained the same. The expression of survivin in normal tissue rather than in tumor tissues and in both tissues should have been re-examined by the pathologist. The former was to rule out the mistake in specimen collection and the latter was to reassure the early stage of tumor transformation which may be underscored by the conventional staining.

Recently, a common polymorphism at the survivin gene promoter (G/C at nt -31) that correlated with survivin gene expression in cancer cell lines was found not to be different among cervical cancer patients and control populations (Borbely *et al.*, 2006). In case of survivin mRNA negative patients, the cause of tumorigenesis may be explained by other mechanisms involving such as oncogenes and tumor suppressor genes i.e. p53 (McConkey *et al.*, 1996).

In this study, GAPDH, a key enzyme in glycolysis and also one of the abundant RNA species, was chosen as an internal control RNA. The amplification profiles of GAPDH as well as actin reach a plateau much earlier than the relatively low abundant RNA. Therefore, the condition was optimized so that the GAPDH amplification did not override the target RNA when use in combination. This housekeeping gene is constitutively expressed in many tissues. However, wide variations in GAPDH expression levels have been observed in tissues at different developmental stages (Oikarinen *et al.*, 1991), in cell treated with insulin (Nasrin *et al.*, 1990), mitogens (Rao *et al.*, 1990), as well as in virally-transformed or oncogene-transfected fibroblasts (Bhatia *et al.*, 1994). In addition, human lung cancer tissues as compared with paired normal lung tissues express much higher levels of GAPDH mRNA (Tokunaga *et al.*, 1987). Moreover, GAPDH mRNA levels were up-regulated in the presence of hypoxia in every cell line tested. The transcription of GAPDH was induced by hypoxia-inducible factor 1 (HIF-1). Thus, factors which increase HIF-1 protein expression may activate GAPDH transcription and account for high levels of GAPDH mRNA expression in cancers. (Bhatia *et al.*, 1994; Tokunaga *et al.*, 1987). According to the results, there was a difference in GAPDH expression between the

normal and tumor tissues. Level of GAPDH RNA tended to be lower in most of the normal tissues than in the tumor tissues.

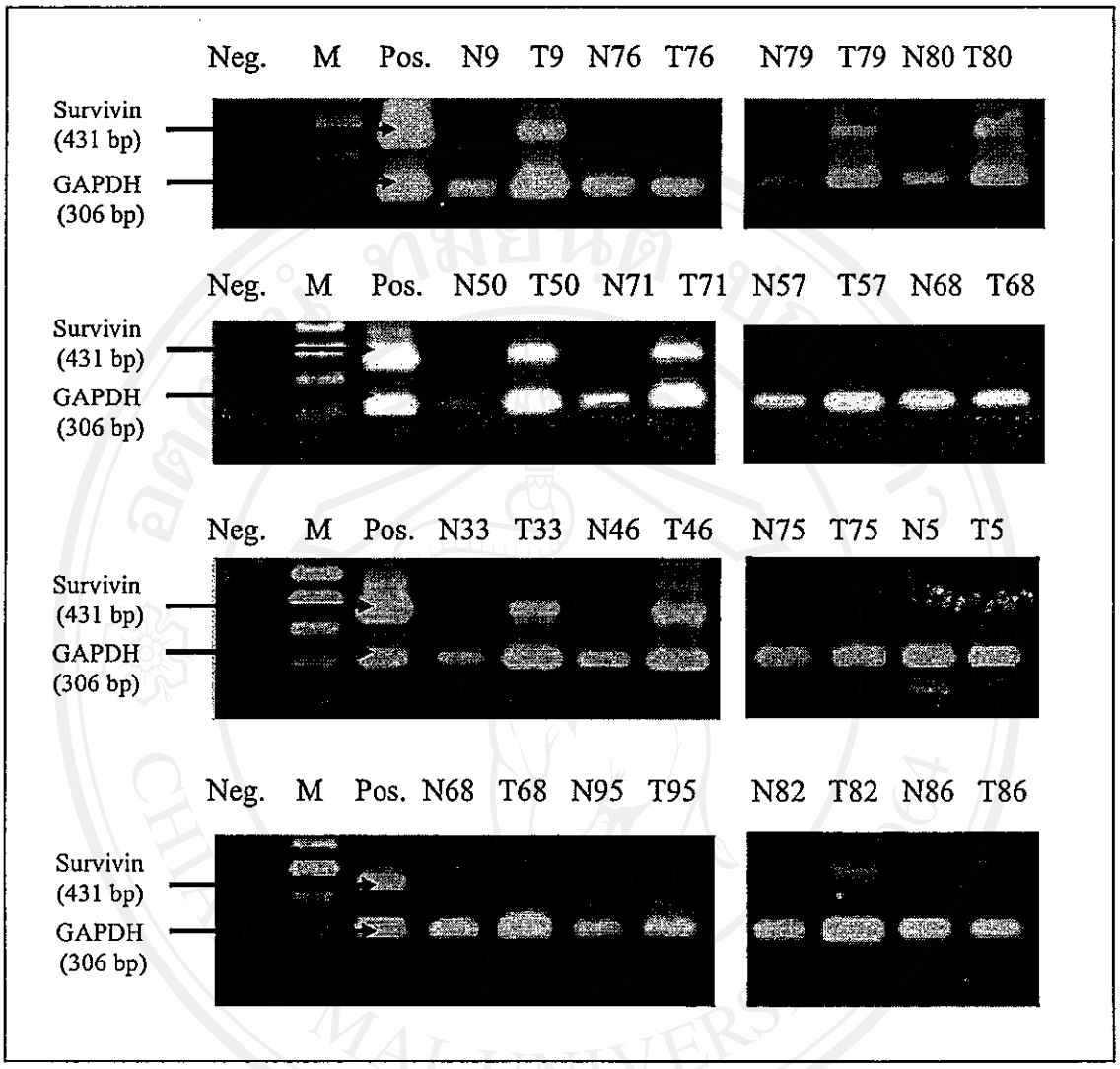

Figure 3.1. The expression of survivin in various tumor tissues was analyzed by RT-PCR. RT-PCR products of paired samples of normal and tumor tissues were demonstrated. The amplified products of survivin cDNA and GAPDH cDNA were 431 and 306 bp, respectively, as indicated. Neg. represents a negative control (no RNA template), M is a DNA molecular weight marker and Pos. is a positive control RNA from KB-V1 cervical carcinoma cell line, N = normal, T = tumor.

Table 3.3. Summary of survivin mRNA expression in different types of tumor tissues

Clinicopathological features	Survivin mRNA expression
No. of patients	
Colorectal cancer (Total 22 cases)	13 (59.1%)
Liver cancer (Total 10 cases)	7 (70%)
Lung cancer (Total 22 cases)	14 (63.6%)
Total (54 cases)	34 (63%)

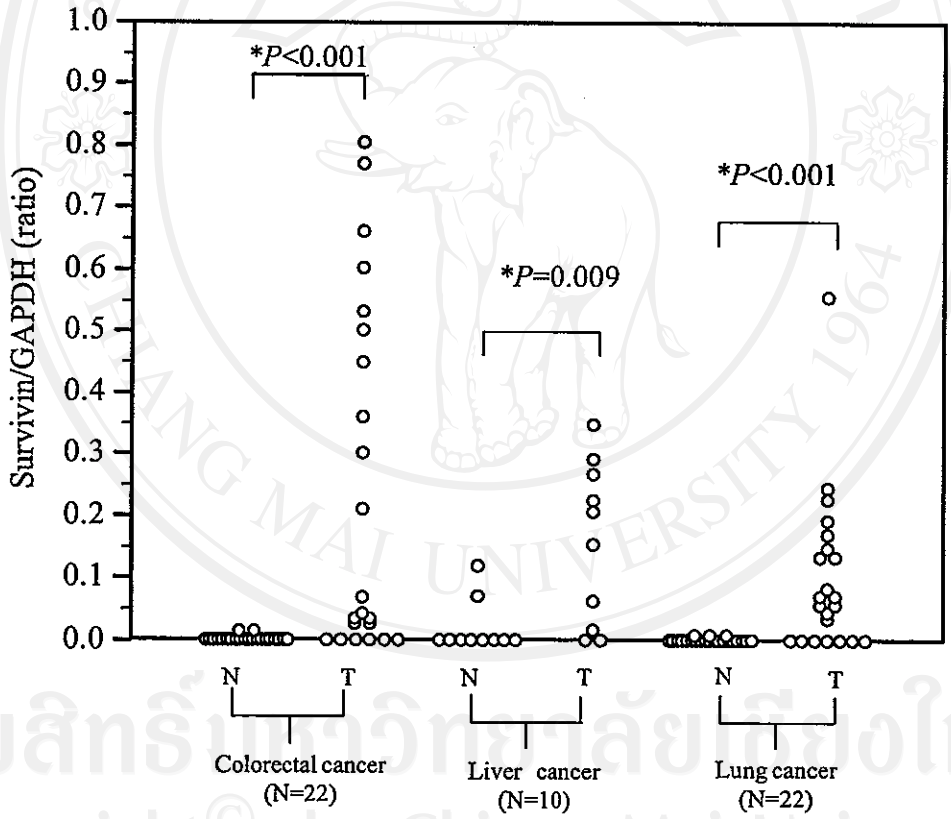

Figure 3.2. Survivin mRNA expression in various tumor tissues. Three different types of tumor tissues (T) and the corresponding normal tissues (N) from colorectal, liver and lung cancer patients were determined for the expression of survivin mRNA using RT-PCR. Level of survivin expression was semi-quantified as a ratio to the band intensity of GAPDH. *(*$P<0.05$, Mann-Whitney U test).

3.5. Optimal conditions of avidin capture ELISA for detection of survivin autoantibodies

Autoantibodies responses to tumor-associated autoantigens might be regarded as reporters in identifying aberrant cellular mechanisms in tumorigenesis. The detection of circulating survivin antibodies could potentially serve as a useful noninvasive marker and detecting them could provide a potential diagnostic or screening tool (Pizem & Cor, 2003).

In this study, prokaryotic expression system, pAK400 harboring survivin cDNA was transformed into bacteria *E. coli* strain Origami B to produce the biotinylated recombinant survivin-BCCP proteins. Without purification, a crude bacterial cell lysate was added directly to avidin-coated ELISA plate and let the selective binding occur through its biotinylated moiety.

Optimal conditions for the assay were determined by varying several parameters, including survivin-BCCP concentration, blocking solution, serum dilution and secondary antibody dilution. The optimal concentration of survivin-BCCP containing bacterial lysate was defined by varying concentrations from 12.5 to 200 µg/ml per well. Upon increasing the concentration of survivin-BCCP containing bacterial lysate, both the optical density and the background increased at the concentration-dependent manner. The 50 µg/ml of survivin-BCCP per well was chosen as a standard condition for coating (Figure 3.3). In order to block the non-specific binding on the unbound sites of the microtiter plate and enhance the signal to noise ratio, four different blocking agents including 2% skimmed milk, 1% bovine serum albumin (BSA), non-protein (NP) blocking buffer and SuperBlock® blocking buffer were tested. In the previous experiment for optimizing survivin-BCCP

containing bacterial lysate concentration, 2% skimmed milk, a blocking agents commonly used in ELISA was used in the blocking step. However, the results demonstrated that 1% BSA, NP blocking and SuperBlock® blocking buffer gave the higher signal than the 2% skimmed milk (Figure 3.4). However, NP blocking buffer is better in blocking than SuperBlock® blocking buffer because of lower background was obtained whereas 1% bovine serum albumin (BSA) is a poorly blocking solution because of its high background generated. Therefore, The NP blocking buffer was employed in all subsequent experiments. To define the amount of coating antigens with NP blocking system, various concentrations (12.5, 25, 50, and 100 μg/ml) of survivin-BCCP containing bacterial lysate was titrated. The 50 μg/ml survivin-BCCP containing bacterial lysate per well was still the concentration of choice for coating (Figure 3.5).

The next experiment was to determine the suitable serum dilution. Unfortunately, the known survivin autoantibody positive serum was unavailable to validate the assay. The serum from cancer patients with high survivin mRNA detected by RT-PCR was chosen to optimize the dilution. The results from sera of healthy individuals could not be differentiated from those sera of cancer patients in any dilution. The O.D. was increased upon decreasing serum dilutions. When the biotinylated survivin-BCCP containing bacterial lysate was omitted from the assay system (the blank well), serum in all dilutions exhibited the high O.D. as compared with the test wells of those corresponding serum samples (Figure 3.7). The high non-specific binding might be due to the presence of IgG in serum. This was in contrast to the low background of the blank well when commercial survivin monoclonal antibody (D8) was used to establish the assay conditions. The serum dilution at 1:80 showed

the highest O.D. difference between sera from healthy individuals and cancer patients and was chosen for determination of survivin autoantibody in serum samples (Figure 3.6).

Finally, horse radish peroxidase conjugated rabbit anti-human Igs antibody was also tested in various concentrations for optimization. The results shown that although the dilution at 1:4000 gave the highest O.D. but it also produced the high background. Thus, the dilution at 1:8000 which gave the lower non-specific background was used in subsequent experiments (Figure 3.8).

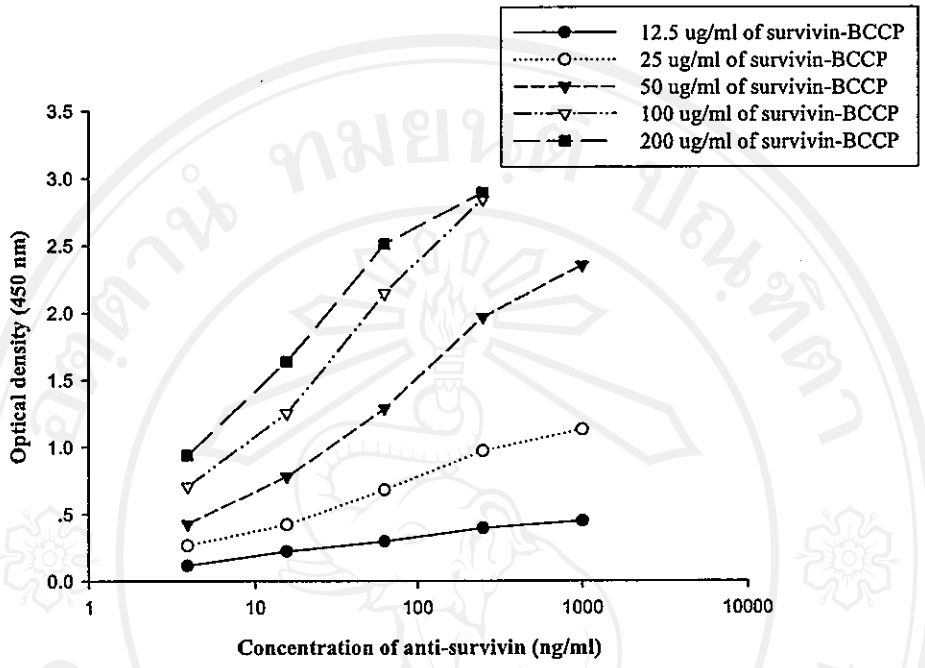

Figure 3.3. Optimization of survivin-BCCP containing bacterial lysate in avidin capture ELISA. Various concentrations of survivin-BCCP containing bacterial lysate (12.5, 25, 50, 100, and 200 μg/ml) were added into the avidin-coated microtiter plate. Different concentrations of commercial mouse monoclonal anti-survivin (D8) were tested for optimal conditions of the assay.

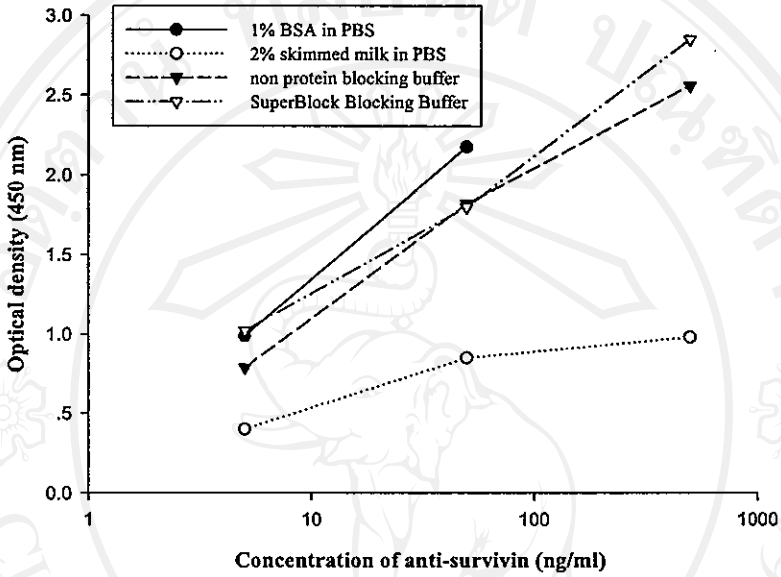

Figure 3.4. Determination of optimal blocking solution. Four differential blocking solutions including 2% skimmed milk, 1% bovine serum albumin (BSA), non-protein (NP) blocking and SuperBlock® blocking buffer were tested in blocking step of avidin capture ELISA.

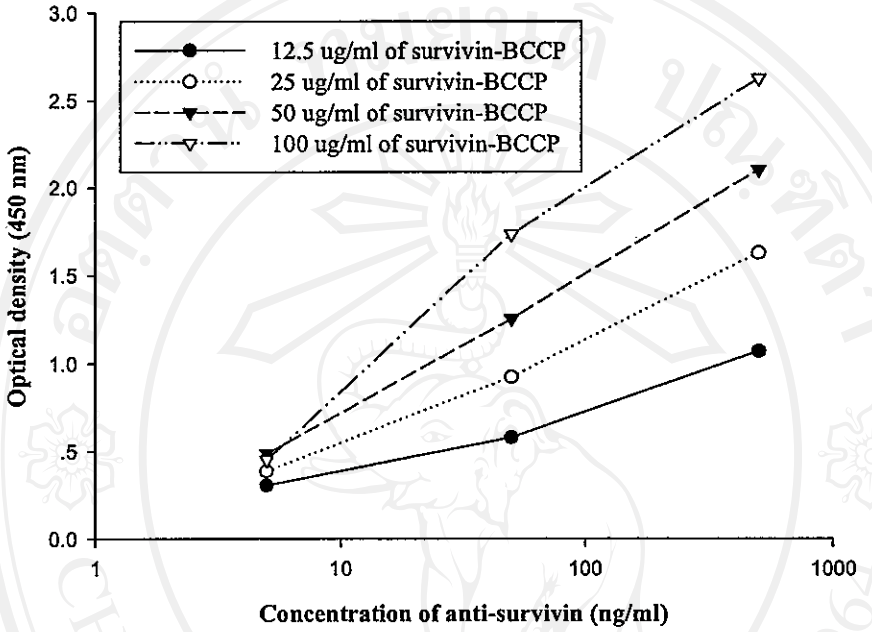

Figure 3.5. Determination of the survivin-BCCP containing bacterial lysate concentrations when using NP blocking buffer system. Various concentrations of survivin-BCCP containing bacterial lysate (12.5, 25, 50 and 100 μg/ml) were tested.

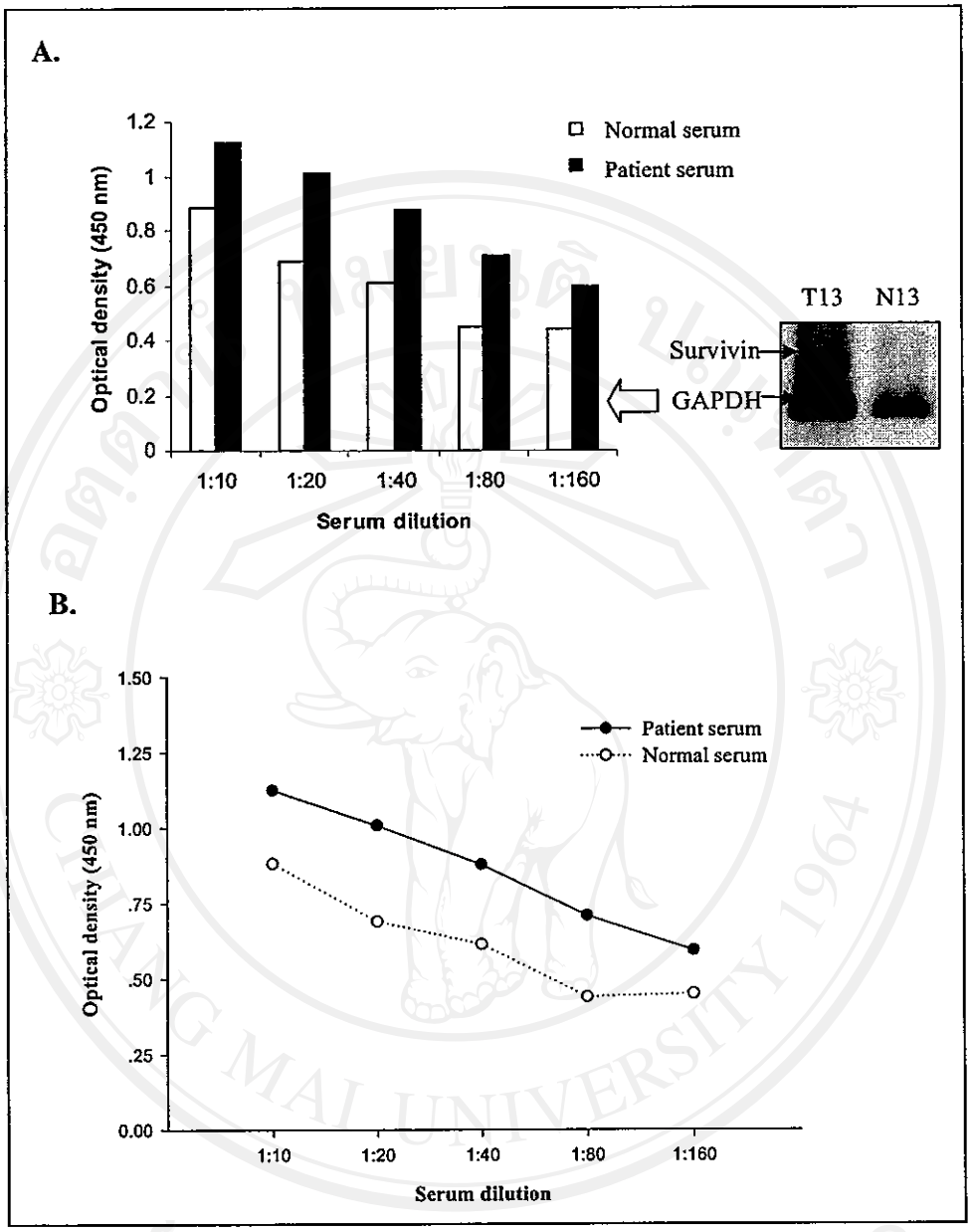

Figure 3.6. Optimization of serum dilutions in avidin capture ELISA. The serum used to optimize the assay was selected from the patient whom survivin mRNA overexpressed was shown by RT-PCR. Two-fold dilution of normal and tumor sera were varied from 1:10 to 1:160. The black and white bars (A) or line plot (B) represented the absorbance of autoantibody to survivin from serum of healthy individuals (N) and cancer patient (T), as indicated.

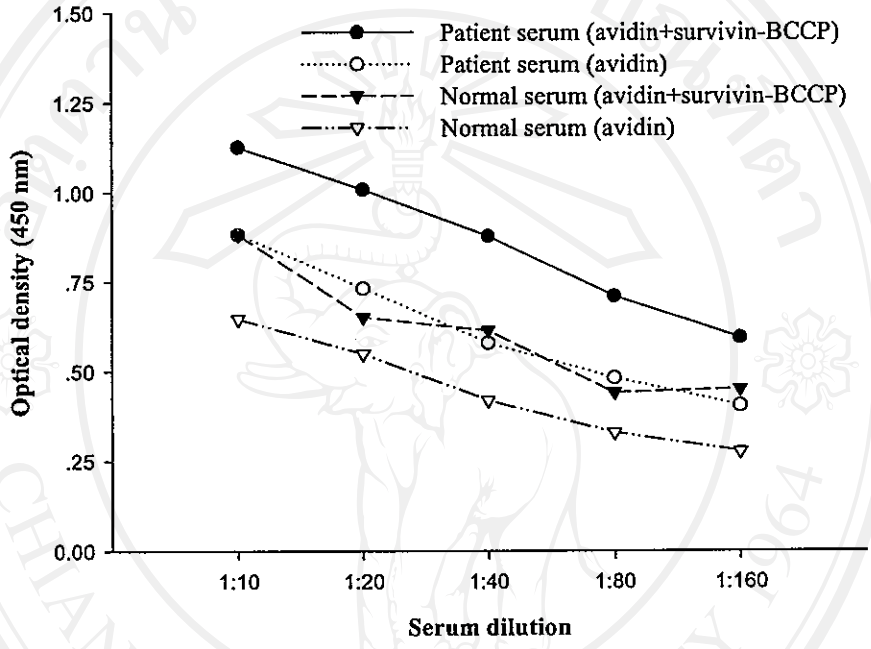

Figure 3.7. Reactivity of serum from healthy individuals (N) and cancer patients (T) in the avidin capture ELISA. Serum in all dilutions (1:10-1:160) were applied to microtiter wells previously coated with avidin in the presence (test wells) or absence (blank wells) of biotinylated survivin-BCCP containing bacterial lysates. Serum in all dilution exhibited the high O.D. in blank wells as compared with the test wells of those corresponding serum.

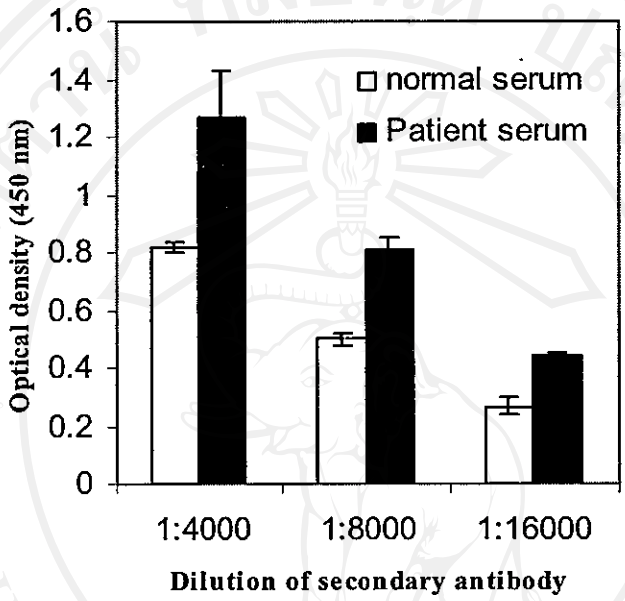

Figure 3.8. Optimization of secondary antibody dilution. Two-fold dilution of secondary antibody (HRP conjugated rabbit anti-human Igs) was assessed at 1:4000, 1:8000, and 1:160000. The black and white bars represented the absorbance of autoantibody against survivin from serum of healthy individuals (N) and cancer patient (T), as indicated.

3.6. Survivin autoantibody in sera by avidin capture ELISA

The avidin capture ELISA system for survivin autoantibody detection was optimized. The assay range detecting commercial survivin monoclonal antibody (D8) in a 2-fold dilution ranged from 3.9-500 ng/ml was as shown in Figure 3.9. In this study, CD147-BCCP, the irrelevant fusion protein which was constructed by the same methods as survivin-BCCP, was included as a negative control antigen in binding to anti-survivin autoantibody. The O.D. in the well of this negative control antigen was 0.119 ± 0.008. H-ras mAb, the mouse immunoglobulin, which was used as negative control antibody in binding to survivin-BCCP also gave low signal (0.122 ± 0.023).

Fifty-five patients with different types of cancer including colorectal, liver and lung and 50 normal individuals were examined for the presence of autoantibodies to survivin. The commercial survivin monoclonal antibody (D8) was included as a control along with the serum samples in each assay so that the results were comparable between different sets of experiments. Nevertheless, there was no significant difference of survivin autoantibody between serum of healthy individuals and cancer patients ($P = 0.538$, Mann-Whitney U test) (Figure 3.10). The O.D range of the former was 0.392-1.008 and $\overline{X}$ ± S.D was 0.695±0.153 whereas the O.D. range was 0.451-1.114 and $\overline{X}$ ±S.D was 0.723±0.182 in the latter. In the absence of survivin-BCCP containing bacterial lysate in the avidin capture ELISA system, high background O.D. of the test wells seemed to be the inevitable problem which could not be eliminated from the serum (Table 3.4). None of the blocking buffer was able to reduce this background.

According to Winkler *et al.* (1997), they also could not reduce the non-specific binding of serum to streptavidin captured wells in the detection of antibodies to feline

foamy virus (FeFV). Antibodies against other bacterial proteins that co-purified with the recombinant FeFV fusion protein (such as biotin carboxyl carrier protein), antibody against avidin, anti-biotin or with specificity for components of the plastic in the plates might also be responsible for these non-specific reactions (Dale *et al.*, 1994; Winkler *et al.*, 1997). In addition, alternative means was carried out by pre-absorption of serum with survivin-BCCP containing bacterial lysate before being tested in the avidin capture ELISA. The result showed decreased absorbance when using the pre-incubation step. However, no difference was observed between serum from healthy individuals and cancer patients (Figure 3.11).

Megliorino *et al.* (2005) used purified recombinant survivin protein tagged with histidine as coating antigen in ELISA for the detection of anti-survivin antibody in sera from 1137 patients with 11 different types of cancer. Of a total of 1137 cancer sera analyzed, 8.4% (96/1137) were shown to have autoantibodies against survivin Among these 1,137 samples, the sera from 11 of 51 lung cancer patients (21.6%) and sera from 4 of 49 colorectal cancer patients (8.2%) were reactive with recombinant his-tagged survivin in ELISA (Megliorino *et al.*, 2005). In addition, the sera from 20 of 189 lung cancer patients (10.6%), sera from 10 of 151 colorectal cancer patients (6.6%) as well as sera from 15 of 160 hepatocellular carcinoma patients (9.4%) were positive for survivin autoantibody (Rohayem *et al.*, 2000). It must be noted that the number of serum from healthy subject group was small. For examples, Yagihashi *et al.* (2005) reported 18 of 31 (58.1%) lung cancer patients positive for survivin autoantibody in comparison with only 7 healthy serum control group (Yagihashi *et al.*, 2005). Similarly, 11 of 46 (23.9%) breast cancer patients were positive using a cutoff

value for positivity determined above the mean absorbance + 2SD of only 10 healthy control samples (Yagihashi *et al.*, 2005).

As mentioned earlier, although the method described here possesses several advantages with its biotinylation site at BCCP, the size of BCCP itself may confer a limitation. Smith *et al.* (1998) described when using the large size of the biotinylated domains, e.g. 75-105 amino acids in length that intracellular proteolysis was problematical and the extent of biotinylation was <100% (Smith *et al.*, 1998). The size of biotinylated domains which used in our study was large (87 amino acid). Western blot analysis of the survivin-BCCP containing bacterial lysate displayed the band of approximately 30-35 kDa and a lesser extent of 15 kDa which was thought to be a degradation product of survivin-BCCP fusion protein (Figure 3.17; 1A). It may either interfere the protein interaction between antigen and antibody or contribute to the high background O.D. affecting the sensitivity and specificity of the test. Instead of using the whole BCCP domain as a biotin acceptor, one could alternatively tag the recombinant protein with a short (13 amino acids) biotin acceptor peptide. A short 'biotinylation peptide' also serves as an in *vivo* substrate mimic for *E. coli* biotin holoenzyme synthetase (BirA), an enzyme which usually performs highly selective biotinylation of *E. coli* biotin carboxyl carrier protein (BCCP) (Schatz, 1993).

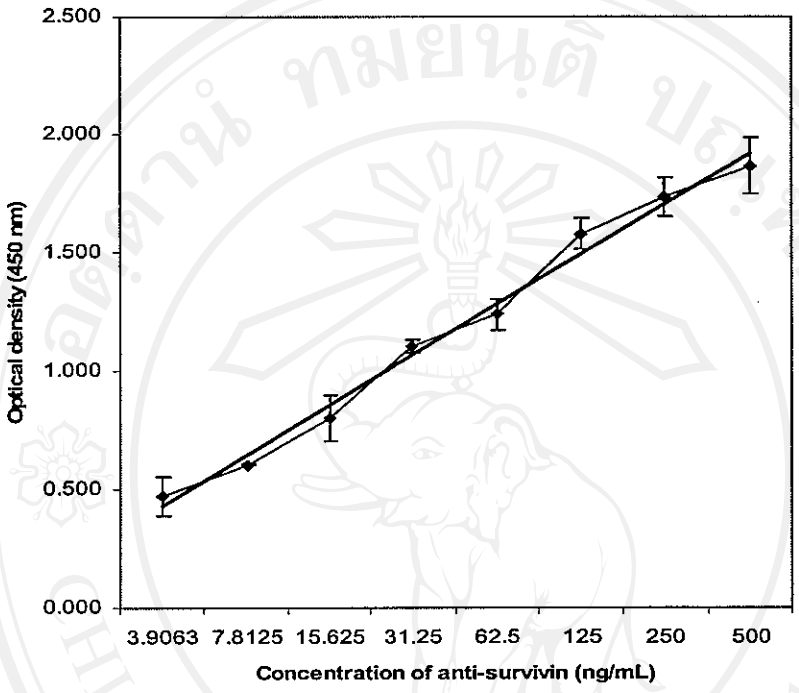

Figure 3.9. Systemic control of the avidin capture ELISA system. When the ELISA was performed with sera, the survivin mAb (D8) was used as the systemic control for analysis and comparison the absorbance of all data. The line plot represented the mean absorbance of survivin mAb (D8) with minimal error bar.

Table 3.4. Optical density values at 450 nm obtained by avidin capture ELISA assay using commercial survivin mAb (D8), the sera of ten healthy individuals and ten cancer patients

	Blank well (avidin)	Test well (avidin + biotinylated survivin-BCCP proteins)
Blank	0.064 ± 0.011	0.085 ± 0.007
commercial survivin mAb (D8)	0.175 ± 0.016	1.238 ± .063
N1	0.557	0.873
N2	0.603	0.656
N3	0.675	0.981
N4	0.761	0.914
N5	0.478	0.717
N6	0.756	0.890
N19	0.595	0.647
N36	0.643	0.746
N39	0.669	0.908
N50	0.598	0.834
T5	0.394	0.701
T13	0.565	0.890
T14	0.674	0.781
T17	0.658	1.057
T20	0.505	0.880
T44	0.676	0.823
T57	0.960	1.222
T76	0.806	0.828
T80	0.674	1.001
T82	0.553	0.651

N = serum from healthy individuals, T = serum from cancer patients

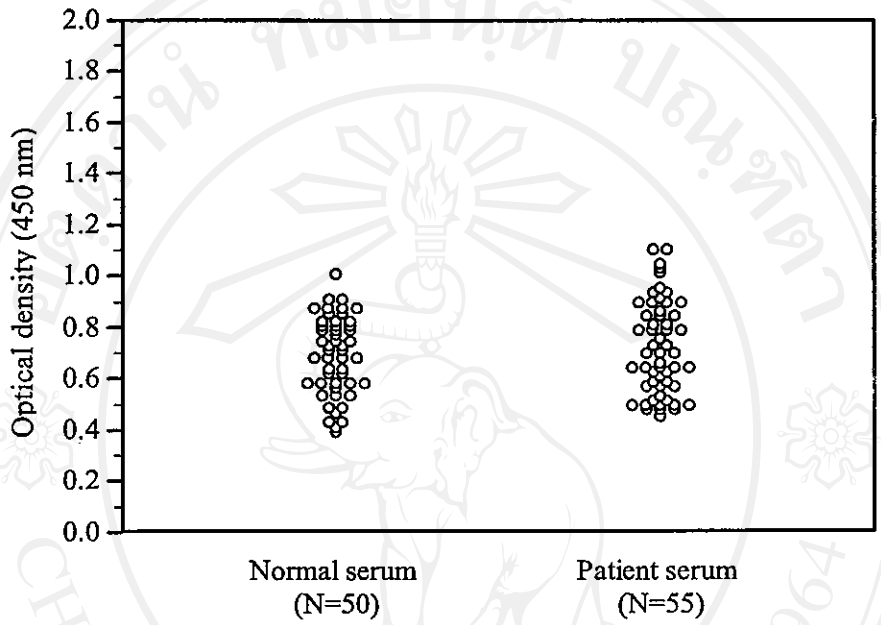

Figure 3.10. Survivin autoantibody between sera of cancer patients and healthy individuals by avidin capture ELISA. The survivin autoantibodies in patient sera (N=55) were compared with those of normal sera (N=50). There was no significant difference of survivin autoantibody between those two types of serum (P = 0.538, Mann-Whitney U test).

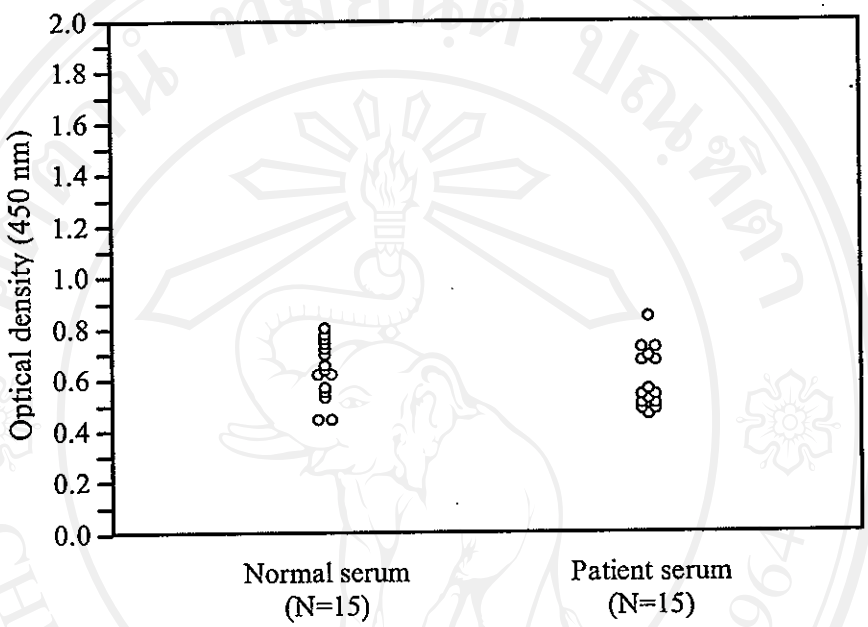

Figure 3.11. Determination of survivin autoantibody using pre-incubation method. Survivin-BCCP was pre-incubated with healthy normal or patient sera. There was no significant difference of survivin autoantibody between those two types of serum ($P = 0.285$, Mann-Whitney U test).

3.7. Optimal conditions of indirect immunofluorescence flow cytometry for detection of survivin autoantibody.

According to the nonspecific background problem of avidin capture ELISA in detection of survivin autoantibody in serum, the flow cytometry was set up as an alternative method to reduce the high background. As a control, M6-1E9 (CD-147 mAb) and FITC conjugated mouse or human monoclonal antibody were used as a negative control to determine nonspecific binding of the resulting beads. Survivin mAb (D8) and anti-BCCP were used as a positive control to set markers for distinguishing fluorescence-negative and fluorescence-positive bead populations (Figure 3.12).

MagnaBind ™ Streptavidin beads were incubated with various concentrations (0.5, 1, 2, 5 and 10 mg/ml) of survivin-BCCP containing bacterial lysate and stained with FITC conjugated mouse mAb and analyzed by a flow cytometer. The percent fluorescence intensity of positive beads of each concentration was shown in Figure 3.13. These results demonstrated the optimal concentration of biotinylated survivin for streptavidin beads coating at 10 mg/ml.

To optimize the sera dilution, the survivin coated beads reacted with 1:20 and 1:80 dilutions of normal and patient sera. The results showed difference of mean fluorescence intensity between normal and patient sera was observed when dilution of sera was used at 1:20 (Figure 3.14).

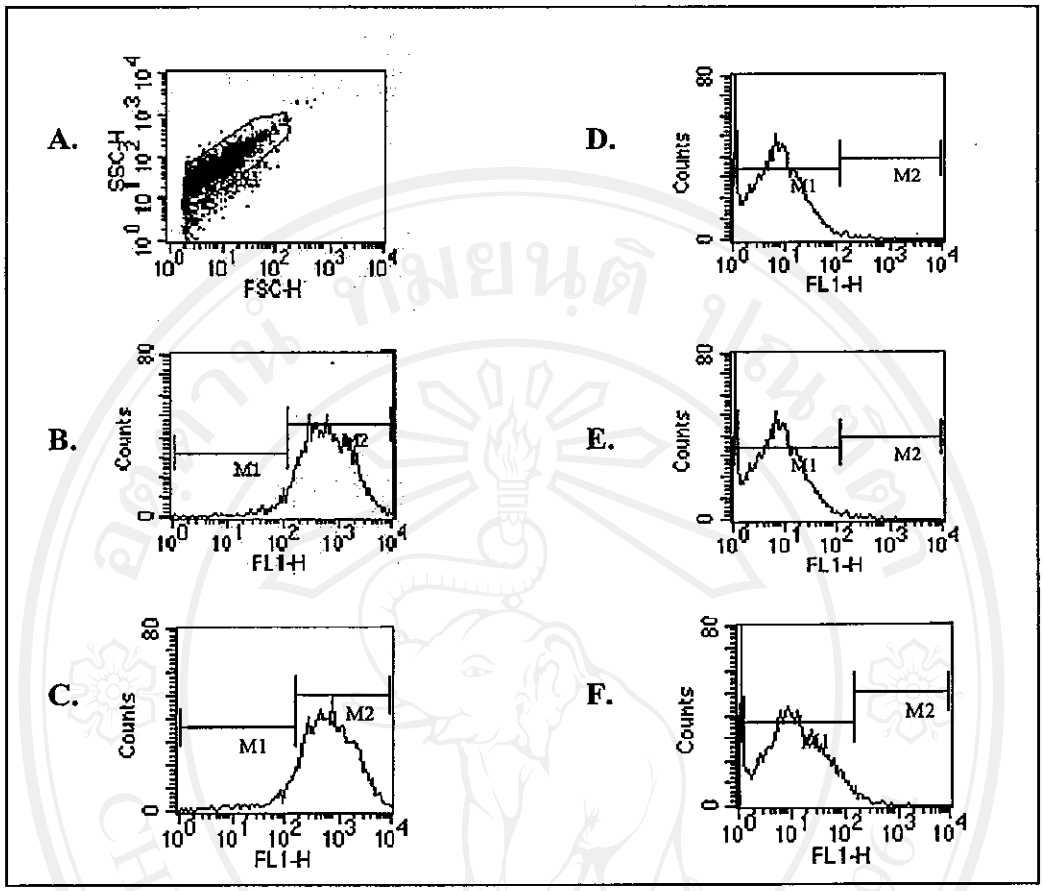

Figure 3.12. Immunofluorescence analysis of the survivin autoantibody by indirect immunofluoresence flow cytometry. Population of surivin coated beads were stained with FITC-conjugated secondary antibody (A). As a positive control, survivin coated beads were stained with survivin mAb (B) or stained with anti-BCCP (C) at dilution 1:20 as primary mAb then counterstained with FITC conjugated anti-mouse immunoglobulins at dilution of 1:50. Survivin coated beads were also stained only with conjugate (D; anti-mouse-FITC and E; anti-human-FITC) or M6-1E9 (CD147 mAb) (F) as a negative control. Horizontal line (M1) and (M2) represent the fluorescence intensity of negative control beads and positive control beads, respectively.

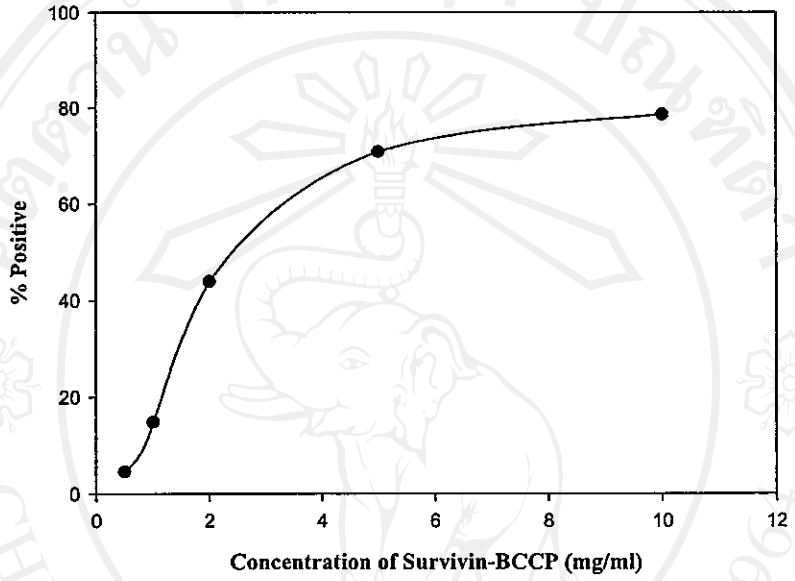

Figure 3.13. Optimization of biotinylated survivin coating onto Streptavidin Magnetic beads. A various concentration of Survivin-BCCP (0.5, 1, 2, 5 and 10 mg/ml) were coated on MagnaBind ™ Streptavidin beads and assayed by the indirect immunofluorescence flow cytometry. The line plot represented the percent positive of immunofluorescence intensity.

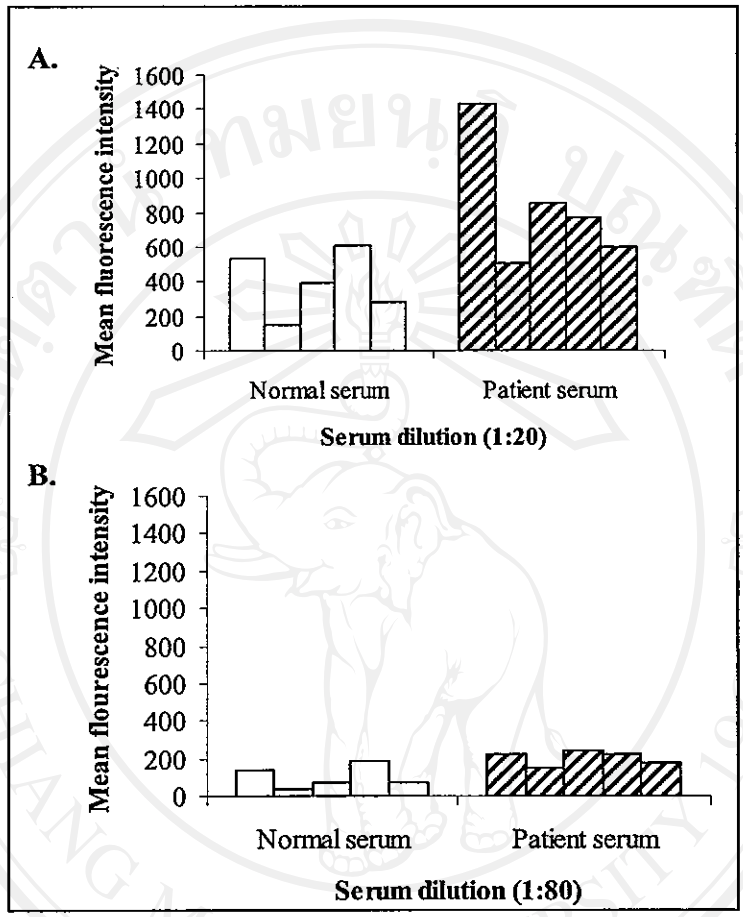

Figure 3.14. Optimization of serum dilution of the indirect immunofluorescence flow cytometry. The dilution of serum was tested at 1:20 (A) and 1:80 (B). The white and striped bars represented the mean fluorescence intensity of survivin autoantibody from sera of healthy individuals and cancer patients, respectively.

3.8. Survivin autoantibody in sera by indirect immunoflorescence flow cytometry

Detection of survivin autoantibody by this method involved streptavidin, a bacterial protein produced from *Streptomyces avidinii* similar to egg-white protein in its ability to bind biotin. It has almost no net charge at neutral pH, does not contain carbohydrate and exhibits lower non-specific binding (Stratech, 2006).

After optimization for the indirect immunoflorescence flow cytometry was successful, daily standardization was performed. This ensures the consistency and performance of the flow cytometer. Thus, the survivin mAb (D8) was included as a systemic control in each experiment of sera analysis so that the mean fluorescence intensity of all subjects could be compared (Figure 3.15).

Fifty cancer patients and 50 normal individuals' sera were examined for survivin autoantibodies. The results demonstrated no significant difference between survivin autoantibody of patient and normal sera (P=0.064, Mann-Whitney U test) (Figure 3.16).

Flow cytometer technology has a significant advantage, its ability to analyze samples in a very short time and to identify various tumor markers in the same tube. This assay could also be adapted to detect antibodies to multiple antigens by coating different antigens in parallel beads. This technology, however requires extensive training in running the instrument, troubleshooting and data analysis and costly for adaptation as a routine method in laboratories in developing countries. Unfortunately, the quality of survivin-BCCP may render the sensitivity and specificity of the assay in determination of survivin autoantibody. Moreover, the non-specific binding of serum to streptavidin-coated beads caused high background so that the method failed to differentiate between patient and normal sera. Norton *et al.* (1996) also reported non-

specific serum binding to streptavidin in a biotinylated peptide based enzyme immunoassay (Norton, 1996).

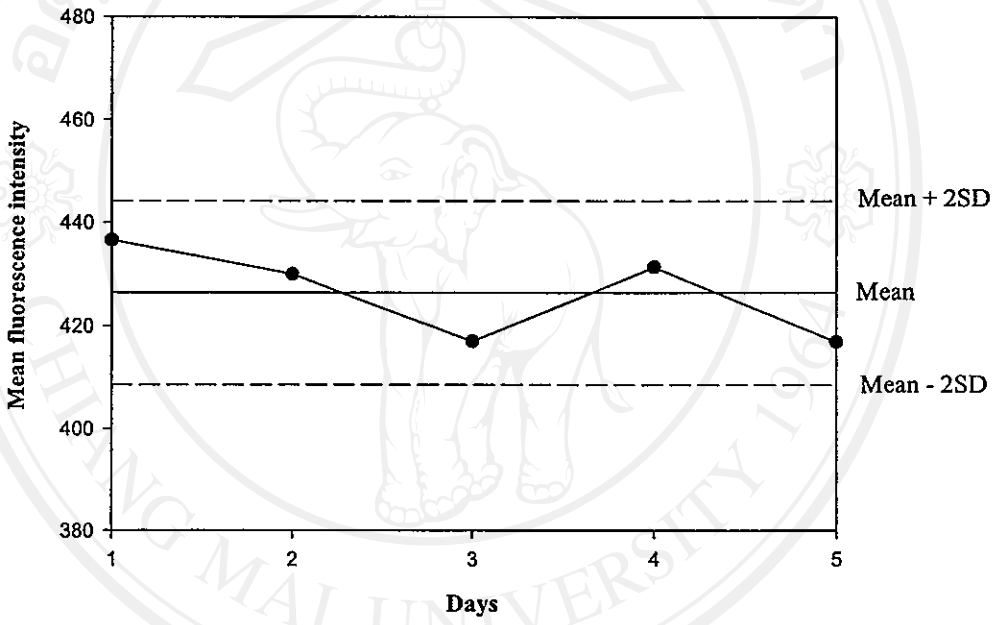

Figure 3.15. Systemic control of the indirect immunofluorescence flow cytometry system. When the flow cytometry was performed with sera, the commercial survivin mAb (D8) was used as the systemic control for analysis and comparison the mean fluorescence intensity of all data. The line plot represented the mean fluorescence intensity of commercial survivin mAb (D8) of independent experiments.

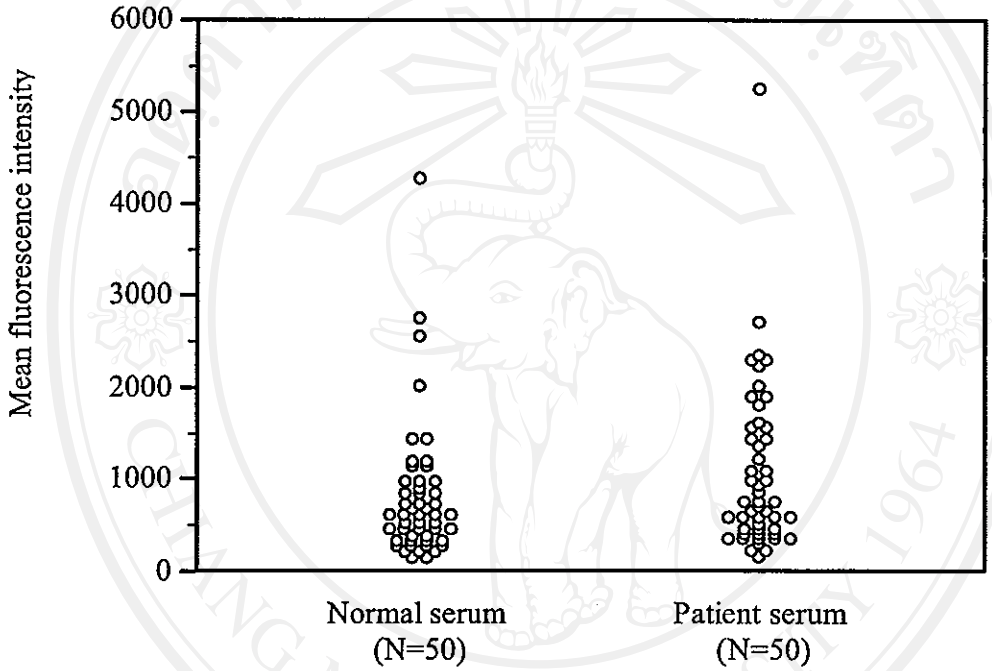

Figure 3.16. Immunofluorescence analysis of the survivin autoantibody by indirect immunofluoresence flow cytometry. The survivin autoantibodies in sera from cancer patient sera (N=50) were compared with finding in healthy individuals (N=50). There was no significant difference of survivin autoantibody between those two types of serum ($P = 0.064$, Mann-Whitney U test).

3.9. Western blot analysis

To further evaluate the results from avidin capture ELISA and flow cytometry, some of sera with high O.D. value and high fluorescence intensity were tested against recombinant denatured survivin-BCCP by immunobloting. The commercial survivin mAb (D8), anti-BCCP and avidin-HRP were recruited and expected to produce a positive band. In addition, bacterial lysate alone and the irrelevant biotinylated fusion protein, CD147-BCCP, were also included as a negative control.

The results demonstrated that commercial survivin mAb (D8) recognized survivin-BCCP at the molecular weight of ~30 kDa. Anti-BCCP and avidin-HRP also reacted with survivin-BCCP as shown in figure 3.18. The reactive band of ~30 kDa represented survivin (16.5 kDa) fused to BCCP (14 kDa). In contrast, none of the reactive bands was detected in bacterial lysate alone and CD147-BCCP containing bacterial lysate (Figure 3.17). When immunoblotting was carried out to detect survivin autoantibody in serum, some of immunoreactive non-specific bands were found in both healthy normal and patient sera (Figure 3.18 and 3.19). Moreover, bacterial lysate alone or CD147-BCCP containing bacterial lysate produced many non-specific bands as shown in figure 3.18. We also tried to detect survivin autoantibody in sera using commercial his-tagged survivin available in our lab. The results showed a reactive band of 16.5 kDa that corresponded to the size of his-tagged survivin recognized by a commercial survivin monoclonal antibody (D8) without any non-specific bands. However, no reactivity was found in the sera (Figure 3.20). As discussed previously, survivin autoantibody was positively detected in either ELISA or immunoblotting using purified recombinant his-tagged survivin protein which was produced in their own laboratories (Chang *et al.*, 2004; Koziol *et al.*, 2003; Megliorino *et al.*, 2005; Rohayem

et al., 2000; Yagihashi *et al.*, 2005; Yagihashi *et al.*, 2005; Yagihashi *et al.*, 2001; Yagihashi *et al.*, 2005; Zhang, 2004; Zhang *et al.*, 2003). The amount of purified recombinant his-tagged survivin used in these studies was 4-10 μg/lane which was much more than in ours (5 pg/lane). The increase of purified recombinant survivin was not affordable because it was supplied in total of 500 pg/vial for other research purpose.

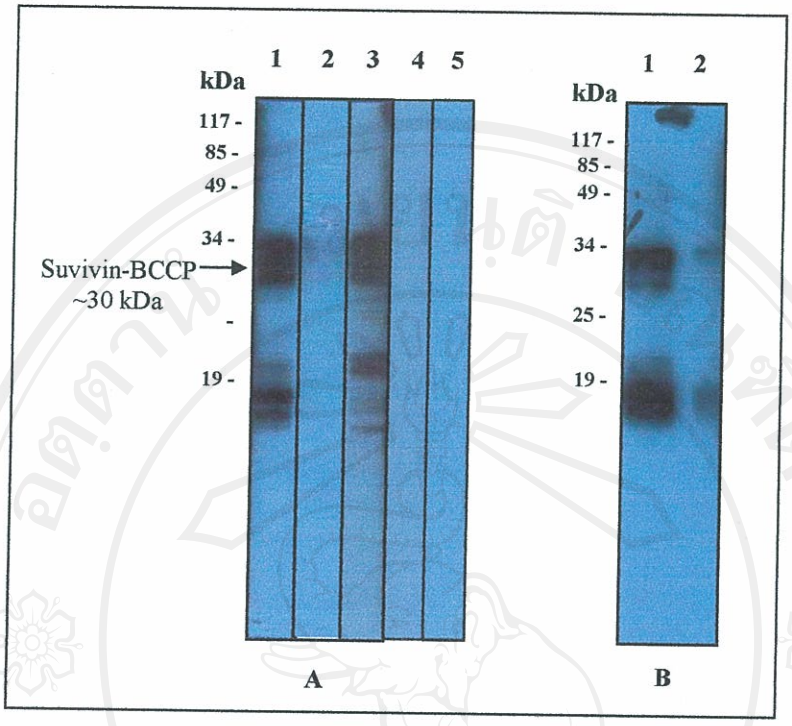

Figure 3.17 Control system of survivin autoantibody detection by Western immunoblotting. Survivin-BCCP containing bacterial lysate (lane 1-3A and 1-2B), bacterial lysate alone (lane 4A) or CD-147 containing bacterial lysate (lane 5A) was separated by 15% SDS-PAGE under reducing condition and transfer onto the PVDF membrane. The membranes were probed with commercial survivin mAb (lane 1A, 4A, 5A and 1B), avidin-HRP (lane 3A) and anti-BCCP (lane 2B). The immunoreactive bands were visualized by chemiluminescent substrate detection system. The molecular weight about 30 kDa that corresponded to the size of survivin-BCCP was detected.

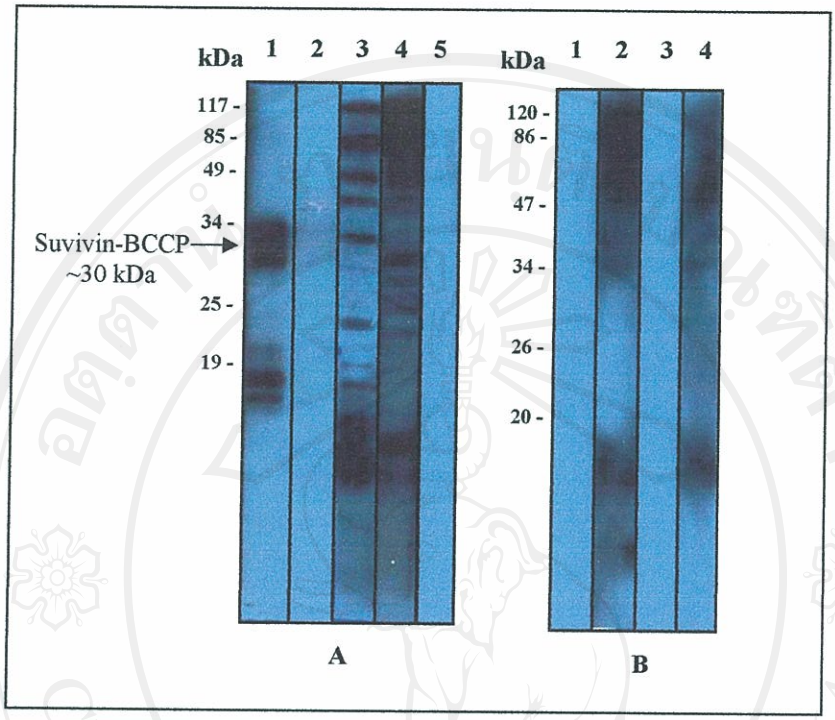

Figure 3.18 Analysis of survivin autoantibody by Western immunoblotting. Survivin-BCCP containing bacterial lysate (lane 1A-5A), bacterial lysate alone (lane 1B, 2B), and CD-147 containing bacterial lysate (lane 3B, 4B) were analyzed by Western immunoblotting. The membranes were probed with survivin mAb (lane 1A), patient serum (lane 4A, 2B, 4B) and healthy individual serum (lane 3A). The molecular weight about 30 kDa that corresponded to the size of survivin-BCCP was detected as shown.

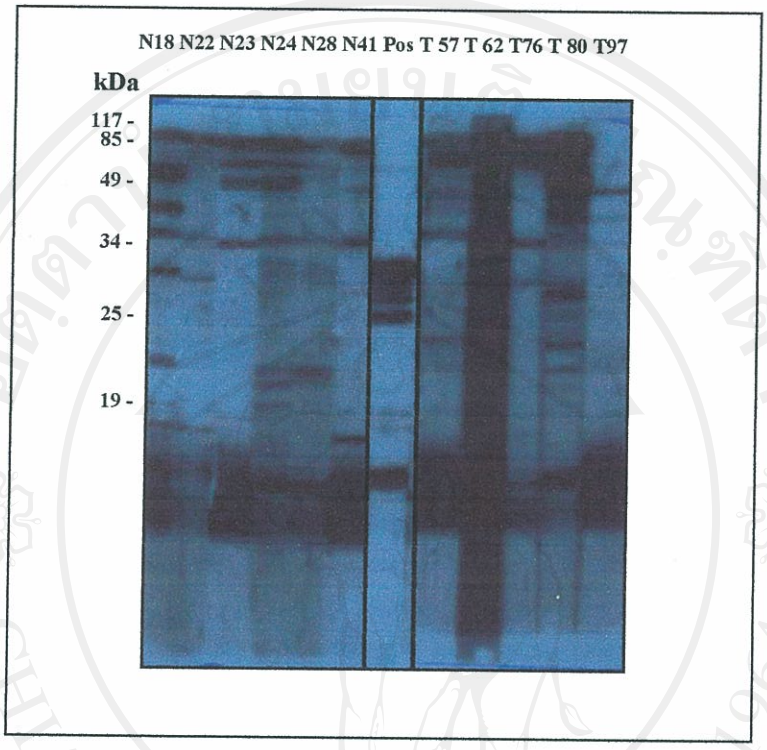

Figure 3.19. Western blot analysis of survivin autoantibodies using survivin-BCCP containing bacterial lysates. The strip membranes harboring survivin-BCCP containing bacterial lysates were incubated with commercial survivin mAb (Pos.), patient (T) and healthy normal (N) sera as indicated.

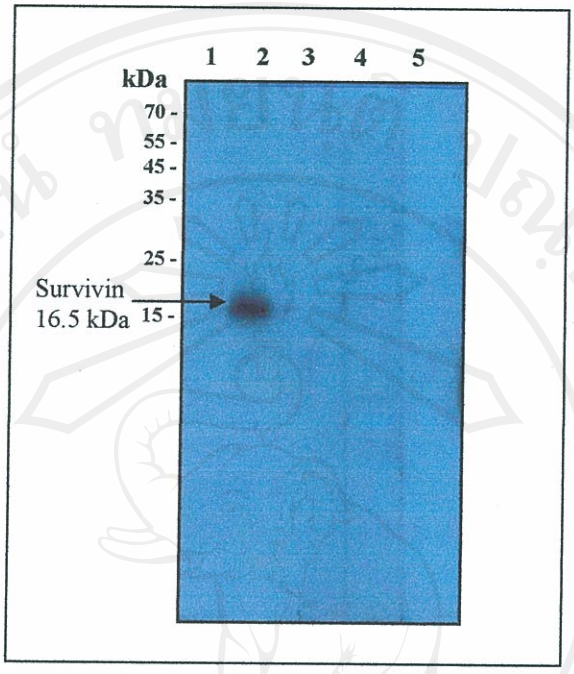

Figure 3.20. Western blot analysis of autoantibodies against survivin using purified recombinant his-tagged survivin. The purified recombinant his-tagged survivin was separated by 15% SDS-PAGE under reducing condition and transfer into PVDF membrane. The strip membranes were incubated with commercial survivin mAb (Lane 2) or normal (Lane 3) and patient serum (Lane 4). The immunoreactive bands were visualized by chemiluminescent substrate detection system. The molecular weight of that corresponded to the size of purified recombinant his-tagged survivin was detected as shown. Lane 1 = negative control (HRP conjugated goat anti-mouse Igs), Lane 5 = negative control (HRP conjugated rabbit anti-human Igs).

3.10. Survivin expression in correlation to classification of clinicopathological features

Survivin significantly expressed in various types of tumor tissues. The association between survivin mRNA and the clinicopathological parameters including age, sex, tumor size in maximum diameter, depth of invasion, lymph node metastasis, distant metastasis, lymphatic invasion, venous invasion, perineural invasion, and histological differentiation was analyzed. Additionally, stage grouping of the cancer was evaluated according to the TNM classification. Summary of clinical features and molecular characteristics of 54 patients were demonstrated in Table 3.5. The results showed no association between survivin mRNA expression and the clinicopathological parameters in all three types of cancer ($P>0.05$, Chi-square test) (Table 3.6, 3.7, and 3.8).

Table 3.5. Summary of correlation between survivin mRNA expression and clinicopathological features in colorectal adenocarcinoma, liver and lung cancer

Clinicopathological features	Survivin mRNA expression		P value
	Positive	Negative	
No. of patients			
Colorectal adenocarcinoma (Total 22 cases)	13 (59.1%)	9 (40.9%)	
Liver cancer (10 cases)	7 (70%)	3 (30%)	0.836
Lung cancer (Total 22 cases)	14 (63.6%)	8 (36.4%)	
Age*			
< 60	14 (25.9%)	13 (24.1%)	0.158
≥ 60	19 (35.2%)	7 (12.9 %)	
Sex			
Male	22 (40.8%)	10 (18.5%)	0.391
Female	12 (22.2%)	10 (18.5%)	
Tumor size			
< 3 cm	1 (1.9%)	4 (7.4%)	0.076
3-5 cm	16 (29.6%)	10 (18.5%)	
> 5 cm	17 (31.5%)	6 (11.1%)	
Histological differentiation**			
Well	17 (31.5%)	12 (22.2%)	0.633
Moderate	13 (24.1%)	5 (9.3%)	
Poor	3 (5.5%)	2 (3.7%)	
Lymphatic invasion			
Present	27 (50%)	17 (31.5%)	0.728
Absent	7 (13%)	3 (5.5%)	
Venous invasion			
Present	8 (14.8%)	8 (14.8%)	0.230
Absent	26 (48.2%)	12 (22.2%)	
Perineural invasion			
Present	8 (14.8%)	6 (11.1%)	0.749
Absent	26 (48.2%)	14 (25.9%)	
Lymph node metastasis			
Present	9 (16.7%)	9 (16.7%)	0.233
Absent	25 (46.3%)	11 (20.3%)	
Distant metastasis			
Present	2 (3.7%)	3 (5.5%)	0.347
Absent	32 (59.3%)	17 (31.5%)	
Tumor stage grouping*			
I	10 (18.5%)	6 (11.1%)	0.275
II	13 (24.1%)	3 (5.5%)	
III	7 (13%)	7 (13%)	
IV	4 (7.4%)	4 (7.4%)	

P<0.05, Chi-square test, * 1 missing data, ** 2 missing data

Table 3.6. The correlation between survivin mRNA expression and clinicopathological features in colorectal adenocarcinoma

Clinicopathological features	Survivin mRNA expression		P value
	Positive	Negative	
No. of patients			
Colorectal adenocarcinoma (Total 22 cases)	13 (59.1%)	9 (40.9%)	-
Age*			
< 60	3 (13.6%)	5 (22.7%)	0.203
≥ 60	9 (40.9%)	4 (18.2%)	
Sex			
Male	6 (27.3%)	5 (22.7%)	1.000
Female	7 (31.8%)	4 (18.2%)	
Tumor size			
<3 cm	-	1 (4.5%)	0.436
3-5 cm	7 (31.8%)	5 (22.7%)	
> 5 cm	6 (27.3%)	3 (13.6%)	
Histological differentiation			
Well	7 (31.8%)	7 (31.8%)	0.137
Moderate	6 (27.3%)	1 (4.5%)	
Poor	-	1 (4.5%)	
Lymphatic invasion			
Present	13 (59.1%)	8 (36.4%)	0.409
Absent	-	1 (4.5%)	
Venous invasion			
Present	2 (9.1%)	5 (22.7%)	0.074
Absent	11 (50%)	4 (18.2%)	
Perineural invasion			
Present	2 (9.1%)	2 (9.1%)	1.000
Absent	11 (50%)	7 (31.8%)	
Depth of invasion			
Early cancer (M or SM)	-	-	-
Advanced cancer (MP or SS or SE)	13 (59.1%)	9 (40.9%)	
Lymph node metastasis			
Present	5 (22.7%)	5 (22.7%)	0.666
Absent	8 (36.4%)	4 (18.2%)	
Distant metastasis			
Present	-	2 (9.1%)	0.156
Absent	13 (59.1%)	7 (31.8%)	
Tumor stage grouping			
I	-	1 (4.5%)	0.157
II	8 (36.4%)	3 (13.6%)	
III	5 (22.7%)	3 (13.6%)	
IV	-	2 (9.1%)	

P<0.05, Chi-square test, *1 missing data

Table 3.7. The correlation between survivin mRNA expression and clinicopathological features in liver cancer

Clinicopathological features	Survivin mRNA expression		P value
	Positive	Negative	
No. of patients			
Liver cancer (Total 10 cases)	7 (70%)	3 (30%)	-
Age			
< 60	7 (70%)	2 (20%)	0.300
≥ 60	-	1(10%)	
Sex			
Male	6 (60%)	3 (30%)	1.000
Female	1 (10%)	-	
Tumor size			
<3 cm	1 (10%)	1 (10%)	0.530
3-5 cm	1 (10%)	1 (10%)	
> 5 cm	5 (50%)	1 (10%)	
Histological type			
Hepatocellular carcinoma	5 (50%)	-	0.167
Cholangiocarcinoma	2 (20%)	3 (30%)	
Histological differentiation			
Well	2 (20%)	1 (10%)	0.788
Moderate	4 (40%)	2 (20%)	
Poor	1 (10%)	-	
Lymphatic invasion			
Present	2 (20%)	3 (30%)	0.167
Absent	5 (50%)	-	
Venous invasion			
Present	5 (50%)	1 10%)	0.500
Absent	2 (20%)	2 (20%)	
Perineural invasion			
Present	2 (20%)	1 (10%)	1.000
Absent	5 (50%)	2 (20%)	
Lymph node metastasis			
Present	1 (10%)	1 (10%)	1.000
Absent	6 (60%)	2 (20%)	
Distant metastasis			
Present	1 (10%)	-	1.000
Absent	6 (60%)	3 (30%)	
Tumor stage grouping			
I	-	-	0.117
II	3 (30%)	-	
III	2 (20%)	3 (30%)	
IV	2 (30%)	-	

P<0.05, Chi-square test

Table 3.8. The correlation between survivin mRNA expression and clinicopathological features in lung cancer

Clinicopathological features	Survivin mRNA expression		P value
	Positive	Negative	
No. of patients			
Lung cancer (Total 22 cases)	14 (63.6%)	8 (36.4%)	-
Age			
< 60	4 (18.2%)	6 (27.3%)	0.074
≥ 60	10 (45.5%)	2 (9%)	
Sex			
Male	10 (45.5%)	2 (9%)	0.074
Female	4 (18.2%)	6 (27.3%)	
Tumor size			
< 3 cm	-	2 (9%)	0.136
3-5 cm	8 (36.4%)	4 (18.2%)	
> 5 cm	6 (27.3%)	2 (9%)	
Histological type			
Adenocarcinoma	6 (27.3%)	5 (22.7%)	0.335
Squamous cell carcinoma	6 (27.3%)	1 (4.5%)	
Large cell carcinoma	2 (9%)	2 (9%)	
Histological differentiation*			
Well	8 (36.4%)	4 (18.2%)	0.964
Moderate	3 (13.6%)	2 (9.1%)	
Poor	2 (9.1%)	1(4.5%)	
Lymphatic invasion			
Present	12 (54.5%)	6 (27.3%)	0.602
Absent	2 (9.1%)	2 (9.1%)	
Venous invasion			
Present	1 (4.5%)	2 (9.1%)	0.527
Absent	13 (59.1%)	6 (27.3%)	
Perineural invasion			
Present	4 (18.2%)	3 (13.6%)	1.000
Absent	10 (45.5%)	5 (22.7%)	
Lymph node metastasis			
Present	3 (13.6%)	3 (13.6%)	0.624
Absent	11 (50%)	5 (22.7%)	
Distant metastasis			
Present	1(4.5%)	1(4.5%)	1.000
Absent	13 (59.1%)	7 (31.9%)	
Tumor stage grouping			
I	10 (45.5%)	5 (22.7%)	0.351
II	2 (9.1%)	-	
III	-	1 (4.5%)	
IV	2 (9.1%)	2 (9.1%)	

P<0.05, Chi-square test, *1 missing data

Changes in gene expression are at the basis of many crucial physiologic and pathologic processes, such as differentiation or neoplastic transformation. In this study, we have investigated whether survivin transcripts is correlated to the histologic and clinical characteristics of cancer. The results showed no correlation between survivin mRNA expression in cancer patients and age, sex, tumor size in maximum diameter, depth of invasion, lymph node metastasis, distant metastasis, lymphatic invasion, venous invasion, perineural invasion, histological differentiation and tumor stage. Similarly, Chen *et al.* (2004) found no relationship between survivin mRNA expression in colorectal cancer (CRC) and sex, tumor size, histological types, lymph node metastasis, distant metastasis and Dukes' stage. However, they found the expression of survivin correlated with apoptotic index (AI) and proliferating cell nuclear antigen labeling index (PI). Survivin positive tumors had significantly lower values for AI than survivin negative tumors ($P<0.001$), and PI in survivin positive tumors was higher than in survivin negative tumors ($P\leq0.001$) (Chen *et al.*, 2004). In addition, the another study using univariate survival analysis showed that survivin expression was significantly associated with a poorer clinical outcome (failure-free survival and overall survival) for the entire group of anaplastic large-cell lymphoma patients (Schlette *et al.*, 2004).

Interestingly, in this study, survivin positive tumors were found more frequently in tumor with stage II of colorectal adenocarcinoma and liver cancer. It has been reported the presence of survivin transcripts was associated with a significantly worse outcome only in group with stage II of colorectal carcinoma (CRC) and the patients with survivin positive tumors displayed a five year survival rate lower than the patients with survivin negative tumor (p=0.001). In addition, the patients with

stage II survivin positive CRC had a hazard ratio for death due to recurrent cancer of 11.2 (95% confidence interval 1.4–86.7; p=0.02) compared with those with survivin negative tumors of the same stage (Sarela *et al.*, 2000). However, we could not be analysis of those parameters. Due to small number of patient grouping and all of tumor tissues were fresh frozen tissues that obtained from patient who had recently undergone surgical resections and follow up for cancer treatment during study. In summary, whether survivin is related to disease progression (prognostic) or treatment resistance (predictive) should be addressed in larger patient groups and long term study to allow for proper subgroup analysis.

Recently, histological examination using hematoxylin and eosin (H & E) staining has been the gold standard for cancer detection. However, H & E staining is standard for long term experience in the practice, inexpensive, but time consuming for routine practice. Moreover, conventional histological analysis and immunohistochemistry techniques have problems including sampling error and poor sensitivity (Kim *et al.*, 2005). Instead of looking for cancer cells under the microscope, survivin gene transcript amplification by single step RT-PCR in tissue specimens has been suggested because this method is highly sensitivity and rapid. By this method, the entire procedure can be completed in one day. Furthermore, survivin expression in tumor can be useful as confirmation test from histological diagnosis.