APPENDIX

APPENDIX A

CHEMICALS AND MATERIALS

List of the chemicals and materials were used in this study. They were analytical grade unless otherwise stated.

Chemicals/Substances	Source
Acrylamide gel (ultra pure)	National diagnostics, USA
Ammonium persulfate	Amresco®, St. Louis, MO, USA
Anti-human Igs conjugate FITC	Dakopatts, Glostrup, Denmark
Avidin	Sigma, St. Louis, MO, USA
Boric acid	Sigma, St. Louis, MO, USA
Bovine serum albumin	Sigma, St. Louis, MO, USA
Bromophenol blue	Matheson Coleman&Bell, East Rutherford
Calcium chloride	Fisher Scientific, Chicago, Ill., USA
Chloroform	Labscan, Ireland
Developer	Eastman Kodak Company, Rochester, NY, USA
Diethy pyrocarbonate (DEPC)	Sigma, St. Louis, MO, USA
Disodium hydrogen phosphate	Merck, Darmstadt, Germany
Di-sodium hydrogen orthophosphate-anhydrous	Fisher Scientific, Leicestershire, UK

Enhanced Chemiluminescence (ECL)	Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Bio Basic Inc., Toronto, Canada
Ethylenediamine tetra acetic acid (EDTA) disodium salt	Fluka Chemika, Buchs, Switzerland
Fixer	Eastman Kodak Company, Rochester, NY, USA
Glycine	Research Organics Inc., California USA
Glycerol (ultra pure)	Bio Basic Inc., Toronto, Canada
Hydrochloric acid	Merck, Darmstadt , Germany
Isopropyl alcohol	Merck, Darmstadt , Germany
MagnaBind ™ Streptavidin beads	Pierce, Rockford, IL, USA
2-mercaptoehanol (2-ME)	BDH biochemicals, Poole, England
Methanol	Merck, Darmstadt, Germany
Mouse survivin monoclonal IgG2a antibody (D8)	Santa Cruz Biotechnology Inc., California, USA
Nonidet P40 (NP 40)	Sigma, St. Louis, MO, USA
Paraformaldehyde	Fluka Chemika, Buchs, Switzerland
Polyvinylidene fluoride (PVDF) membrane	Pall, Pensacola & Amersham, Sweden
Polyoxyethylenes orbitan monolaurate (Tween 20)	Sigma, St. Louis, MO, USA
Potassium chloride	Merck, Darmstadt , Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany

Rabbit anti-human Igs conjugate HRP	Dakopatts, Glostrup, Denmark
Skimmed milk	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt,Germany
Sodium carbonate anhydrous	Merck, Damstadt, Germany
Sodium hydroxide	Merck, Darmstadt,Germany
SuperBlock® blocking buffer	Pierce, Rockford, IL, USA
Sodium azide	Reidel-DE Haen AG Sellze-Handnover
Sodium dodecyl sulfate (SDS)	Fisher Scientific, Leicestershire UK
SUPERSCRIPT™ one-step RT-PCR kit	Invitrogen, Grand Island, New York, USA
3,3',5,5'-Tetramethybenzidine (TMB) substrate	Zymed, San Francisco, USA
Tetramethylene ethylenediamine (TEMED)	Bio Basic Inc., Toronto, Canada
Tris (hydroxymethyl) aminomethane	Research Organics Inc., St. Cleveland, OH, USA
TRIZOL reagent	Invitrogen, Grand Island, New York, USA
UltraPure ™ Agarose	Invitrogen, Grand Island, New York, USA

APPENDIX B

INSTRUMENTS

List of instruments were used in the study.

Instruments	**Source**
Adjustable automatic pipette	
Labmate p10, p20, p200 & Bio-rad p1000, USA	High Tech Lab, Poland
Analytical balance	Ohaus, USA
Autoclave, Tomy SX-500	Tomy Tech Inc., USA
Bench-top homogenizer, Con-Totque	Eberbach Corporation, USA
Electrophoresis power supply, EPS 301	Amersham, USA
Electrophoresis apparatus, BIO 101	Krackeler Scientific Inc., Albany, NY, New York, USA
Flow cytometer	Becton Dickinson FACSort, Edison Biotechnology Institute, Athens
Gel Documentation	Bio-rad, Italy
Hypercassette™	Amersham, UK
Heating Block, DB-101	General Enterprises Marketing, Thailand
Magnetic stirrer, Pyro-Magnestir	LAB-LINE, USA
MiniVE vertical electrophoresis system	Amersham,USA

Mini Tank Electroblotter, 77.1010-TB	Gibthai, Bangkok, Thailand
Multi-channel automatic pipette 20-200 µL	Multimate, High Tech Lab, Poland
Microplate reader, EL340	Bio-TEK Instrument, USA
PCR amplifier (Thermal cycler),AG 22331	Eppendorf ®, Germany
pH meter (Cyberscan 510)	Eutech Instrument, Singapore
Power supply, ESP 500/400	Pharmacia Fine Chemical, Sweden
Refrigerated centrifuge, 5417R	Eppendorf®, Germany
Roller mixer, SRT 1	Stuart scienctific, UK
Shaker, VRN-200	Gemmy Industrial Corporation,
Timer	Bio-rad, China
UV-Spectrophotometer	Shimadzu Corporation, Japan
Vortex mixer, VM-300	Gemmy Industrial Corporation, Germany
Water bath, WB 22	Memmert, Germany
96-well microtiter plate, 3660	Costar®, USA

APPENDIX C

REAGENTS PREPARATION

List of solutions and buffers were used in the study.

1C. Solutions and buffers for RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

1. Diethyl pyrocarbonate (DEPC)-treated water

Diethy pyrocarbonate (DEPC)	200	µl
Distilled water	100	ml
Autoclaved		

2. 85% Ethanol in DEPC-treated water

Ethanol	85	ml
DEPC-treated water	15	ml

3. Tris-Borate-EDTA (TBE) buffer

Tris (hydroxymethyl) aminomethane)	108	g
Boric acid	55	g
0.5 mM EDTA	40	ml
Add distilled water to	1,000	ml

4. 1.5% Agarose gel

Agarose gel	1.5	g
Tris Boric Acid (TBE) buffer	100	ml

Boil agarose in microwave and stand at room temperature for 15-20 minutes before pouring it on the tray plate. Wait until polymerization is complete.

5. **6X Loading dye**

Bromophenol blue	0.025	g
Sucrose	4	g

Dissolve in 10 ml distilled water and then filter through the filter membrane. Stored at 4 °C.

2C. Solutions and buffers for avidin capture ELISA

1. **Carbonate-bicarbonate buffer pH 9.6 (Coating buffer)**

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g

Dissolve in ~ 800 ml distilled water, then adjust pH to 9.6 with HCl. Add distilled water to 1000 ml. Stored at 4°C.

2. **10X Phosphate buffer saline pH 7.2 (PBS 10X, stock solution)**

NaCl	80	g
Na_2HPO_4	11.5	g
KCl	2	g
KH_2PO_4	2	g

Dissolve in ~ 800 ml distilled water, then adjust pH to 7.2 with 1N NaOH. Add distilled water to 1000 ml. Stored at room temperature.

3. **1X PBS pH 7.2 (Working buffer)**

To make 1 liter of 1X PBS pH 7.2, diluted 100 ml of 10X PBS (pH 7.2) with 900 ml distilled water. Stored at room temperature.

4. PBS + 0.5 mM $CaCl_2$ + 0.5% Nonidet P40(NP40)+ 0.05% Tween 20 (Non Protein Blocking buffer)

Solution A:

$CaCl_2$	0.055	g
Dissolve in 100 ml of distilled water		

Solution B:

10X PBS	100	ml
Distilled water	800	ml

Working solution

Solution A	100	ml
Solution B	900	ml
Tween 20	500	µl
Nonidet P40 (NP40), pre-warm at 60°C	5	ml
Mix thoroughly, freshly prepared before use.		

5. 2 % skimmed milk

Skimmed milk	0.2	g
Dissolve in (1X) PBS 10 ml		

6. 1% Bovine serum albumin

Bovine serum albumin	0.1	g
Dissolve in (1X) PBS 10 ml		

7. 1N Hydrochloric acid (stop solution)

Hydrochloric acid	41.4	ml
Distilled water	458.6	ml

3C. Solutions and buffers for flow cytometry

1. **1% BSA-PBS-NaN_3**

Bovine serum albumin (BSA)	10	g

   Dissolve and make up to 1 L with (1X) PBS. Add 2 ml of 10 % sodium azide and mix well. Stored at 4 °C.

2. **10 % NaN_3-PBS (10% Azide)**

NaN_3	1	g

   Dissolve in 10 ml of (1X) PBS. Stability about 2 months when stored at room temperature.

3. **1% Paraformaldehyde**

Paraformaldehyde	1	g

   Dissolve in 100 ml of (1X) PBS. Warmed at 56 °C and filled with 0.22 µm filter membrane.

4C. Solutions and buffers for Western blot analysis

1. **Separating and stacking gel**

   - 15% separating gel

30% polyacrylamide solution	3.5	ml
1M Tris-HCl buffer pH 8.8	2.63	ml
20% SDS	35	µl
10% ammonium persulfate,	70	µl
TEMED	3	µL
Deionized water	0.755	ml

   Swirl gently to mix and pour the solution into the gel cassette.

- 4% stacking gel

30% polyacrylamide solution	0.68	ml
1M Tris-HCl buffer pH 6.8	0.5	ml
20% SDS	20	µl
10% ammonium persulfate	40	µl
TEMED	4	µL
Deionized water	2.74	ml

Swirl gently to mix and fill the top of the cassette with this mixture.

2. 10X Running buffer

Tris-base (0.25 M)	30.3	g
Glycine (1.92 M)	144	g
SDS (= 1%)	10	g

Make up to 1L with deionized water and stored at room temperature.

3. 3X Sample loading dye

1 M Tris pH 6.8	2.4	ml
20% SDS	3	ml
100% Glycerol	3	ml
Bromophenol blue	0.006	g
Deionized water	1.6	ml

Stored at room temperature

4. Protein staining solution (0.025% Coomassive brilliant blue R250)

Coomassive brilliant blue R250	0.125	g
Methanol	200	ml

Acetic acid	35	ml

Adjusted volume to 500 ml by dH_2O. Stored at room temperature

5. **Destaining gel solution I (40% methanol, 7% acetic acid)**

Methanol	400	ml
Acetic acid	70	ml

Adjusted volume to 1000 ml by dH_2O

Stored at room temperature

6. **Destaining gel solution II (5% methanol, 7% acetic acid)**

Methanol	50	ml
Acetic acid	70	ml

Adjusted volume to 1000 ml by dH_2O

Stored at room temperature

7. **Amido black 10B**

Amido black 10B (Naphthal blue)	0.1	g
Methanol	45	ml
Acetic acid	10	ml

Adjust volume to 100 ml by dH_20

Stored at room temperature

8. **10X Transfer buffer (blotting buffer)**

Trizma base (0.25 M)	30.3	g
Glycine (1.92 M)	144	g

pH should be 8.3, do not adjust and stored at room temperature.

9. **1X Transfer buffer (To make 2 L)**

10X Transfer buffer	200	ml

Methanol	400	ml
Deionized distrilled water	1400	ml

10. 10X TBS buffer pH 7.6

Trizma HCl	24.23	g
Nacl	80.06	g

Mix in 800 ml of deionized water, adjust pH to 7.6 with conc. HCl and stored at room temperature.

11. 1X TBS-tween 20 (washed buffer)

10X TBS	100	ml
Deionized water	900	ml
Tween 20	1	ml

Keep TBS-tween 20 at 4^0C..

12. 5% skimmed milk in TBS-Tween

Skimmed milk	2.5	g

Dissolve and make up to 50 ml with TBS-Tween, pH 7.6.

13. Developing solution

Stock developer	100	ml
Distilled water	400	ml

Mix thoroughly and stored at room temperature.

14. Fixing solution

Stock fixer	100	ml
Distilled water	400	ml

Mix thoroughly and stored at room temperature.

APPENDIX D

STAGING OF CANCER

Staging of colorectal, liver and lung cancer

The stage system for colorectal, liver and lung cancer used in the United Stages is the international TNM system developed by American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (Fleming *et al.*, 1997). The characteristics that form the basic of the staging system are based on the assessment of three components including T stands for the extent of the primary tumor, N stands for the absence or presence and extent of regional lymph node metastasis, and M is for the absence or presence of distant metastasis. The use of numerical subsets of the TNM component indicates the progressive extent of the malignant disease. In TNM staging, information about the tumor, lymph node, and metastasis is compound in a process called stage grouping. The stage is described in Roman numerals from I to IV. The each stage may be subdivided, (A, B, C…), if it is useful for treatment recommendations and reporting. In general, stage I implies the tumor is confined to its source of origin and stage IV implies distant metastasis or systemic disease.

Additionally, the main staging systems, which except the TNM, for colorectal cancer are the Dukes system (Dukes, 1932; Zinkin, 1983). The Dukes pathologic staging system separates colorectal malignancies into five groups. Lesions confined to the bowel wall and not penetrating the muscularis are designated A, lesions

penetrating the muscularis into surrounding fat or adventitia are designated B, lesion with positive lymph node involvement are designated C, and D stage for patients with metastasis. The details of these systems for colorectal, lung and liver cancer were showed in Table D1, D2 and D3.

Table D1. The staging guidelines of the colorectal cancer

Primary Tumor (T)				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
TIS	Carcinoma in situ intra epithelial tumor or invasion of lamina propria*			
T1	Tumor invades or submucosa			
T2	Tumor invades muscularis propria			
T3	Tumor invades through the muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues			
T4	Tumor directly invades other organs or structure, and/or perforates visceral peritoneum**			
Regional Lymph nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph nodes metastasis			
N1	Metastasis in 1 to 3 regional lymph nodes			
N2	Metastasis in 4 or more regional lymph node			
Distant Metastasis (M)				
MX	Distant metastasis cannot be assessed			
M0	No distant metastasis			
M1	Distant metastasis			
Stage grouping				
AJCC/UICC				Dukes'
Stage 0	Tis	N0	M0	A
Stage I	T1	N0	M0	A
	T2	N0	M0	A
Stage II	T3	N0	M0	B
	T4	N0	M0	B
Stage III	Any T	N1	M0	C
	Any T	N2	M0	C
Stage IV	Any T	Any N	M1	D

*Note: Tis includes cancer cells confined within the grandular basement membrane (intraepithelial) or lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa.

**Note: Direct invasion in T4 includes invasion of other segments of the colorectum by way of the serosa, for example, invasion of the sigmoid colon by carcinoma of the cecum.

Table D2. The staging guidelines of the lung cancer

Primary Tumor (T)	
TX	Primary tumor cannot be assessed, or tumor proven by the presence of malignant cells in sputum or bronchial washing but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
TIS	Carcinoma in situ
T1	Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus,* (i.e., not in the main bronchus)
T2	Tumor with any of the following features of size or extent: More than 3 cm in greatest dimension Involves main bronchus, 2 cm or more distal to the carina Invades the visceral pleura Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
T3	Tumor of any size that directly invades any of the following: chest wall (including superior sulcus tumors), diaphragm, mediastinal pleura, parietal pericardium; or tumor in the main bronchus less than 2 cm distal to the carina, but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung.
T4	Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina; or separate tumor nodules in the same lobe; or tumor with a malignant pleural effusion**
Regional Lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph nodes metastasis
N1	Metastasis to ipsilateral preibronchial and/or ipsilateral hilar lymph nodes, and intrapulmonary nodes including involvement by direct extension of the primary tumor
N2	Metastasis to ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis to contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1***	Distant metastasis present

Stage grouping

Stage grouping of the TNM subsets has been revised as follows:

Occult Carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T1	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	Any T	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1

*Note: The uncommon superficial tumor of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified T1.

**Note: Most pelural effusions associated with lung cancer are due to tumor. However, there are a few patients in whom multiple cytopathologic examinations of pleural fluid are negative for tumor, In these cases, fluid is non-bloody and is not an exudates. When these elements and clinical judgment dictate that the effusion is not related to the tumor, the effusion should be excluded as a staging element and the patient should be staged T1, T2, or T3.
***Note: M1 includes separate tumor nodule(s) in a different lobe (ipsilateral or contralateral).

Table D3. The staging guidelines of the liver cancer

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Solitary tumor 2 cm or less in greatest dimension without vascular invasion
T2	Solitary tumor 2 cm or less in greatest dimension with vascular invasion, or multiple tumors limited to one lobe, none more than 2 cm in greatest dimension without vascular invasion, or a solitary tumor more than 2 cm in greatest dimension without vascular invasion
T3	Solitary tumor more than 2 cm in greatest dimension with vascular invasion, or multiple tumors limited to one lobe, none more than 2 cm in greatest dimension, with vascular invasion, or multiple tumors limited to one lobe, any more than 2 cm in greatest dimension, with or without vascular invasion
T4	Multiple tumors in more than one lobe or tumor(s) involve(s) a major branch of the portal or hepatic vein(s) or invasion of adjacent organs other than the gall bladder or perforation of the visceral peritoneum
Regional Lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage grouping

Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage IIIA	T3	N0	M0
Stage IIIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IVA	T4	Any N	M0
Stage IVB	Any T	Any N	M1

(The original source for this material is the AJCC® cancer Staging Manual, 5th edition (1997) published by Lippincott-Raven Publishers, Philadelphia, Pennsylvania.)

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Poster Presentation

- Jaimulwong T., Lertprasertsuk N., Chotpadiwetkul R. Differential Survivin mRNA expression in tumour tissues. The sixth national symposium on graduate research, Chulalongkorn University, Bangkok, Thailand. October 13-14 2006.

- Jaimulwong T., Lertprasertsuk N., Chotpadiwetkul R. Prognostic marker of Survivin expression in colorectal and lung cancer. APOCP Satellite Meeting 2006, Chiangmai Orchid Hotel, Chiangmai, Thailand. November 6-7 2006.