IV. RESULTS

1. Quality controls of the study

1.1 Controls for protein quantification

Tumor tissue, which obtained from Thai patients with colorectal, liver, and lung cancer were homogenized and pooled in order to prepare a control tissue homogenate for the quality control of the protein assay. The protein concentration of the control homogenate was estimated from standard curve of BSA as shown in Figure 5. The standard graph of protein by BCA assay was linear up to the concentration of 2 mg/ml (R^2 = 0.9829). This control homogenate was assayed together with tissue samples and the observed values were plotted on quality control chart or Levey Jenning control chart. Patterns of distribution of OCV (Optimal Condition Variance) and RCV-K (Routine Condition Variances Known value) are illustrated in Figure 6. It was found that %CV of OCV was 3.17% (Mean = 13.89 mg/ml, SD = 0.44 mg/ml), while %CV of RCV-K was 4.5% (Mean = 13.32 mg/ml, SD = 0.6 mg/ml), which was less than twice as much as those the OCV. The distributions of control values in both charts were within the limits of acceptability. Thus, control data indicated that the analytical method was performed properly leading to the reliable results of the protein assay.

1.2 Control for protein loading

Western blot analysis was used to examine the expression of VEGF protein in the tumor compared with the corresponding normal tissue. In order to examine the equality of protein load in each lane, several studies generally use glyceraldehydes-3-phosphate dehydrogenase (GAPDH) or actin which are classified as house keeping genes. However, accumulated evidences suggested that the use of GAPDH should be avoided in experimental hypoxia, cell proliferation or carcinogenesis (Sumner *et al.*, 2003; Baskurt *et al.*, 2004) and the total actin content can vary with development, pathologically and potentially between cells within tissues. We also experienced this problem ourselves (as shown in Figure 7). Therefore, in this thesis the amount of total protein loaded into each lane was examined by staining the polyacrylamide gel with coomassie blue.

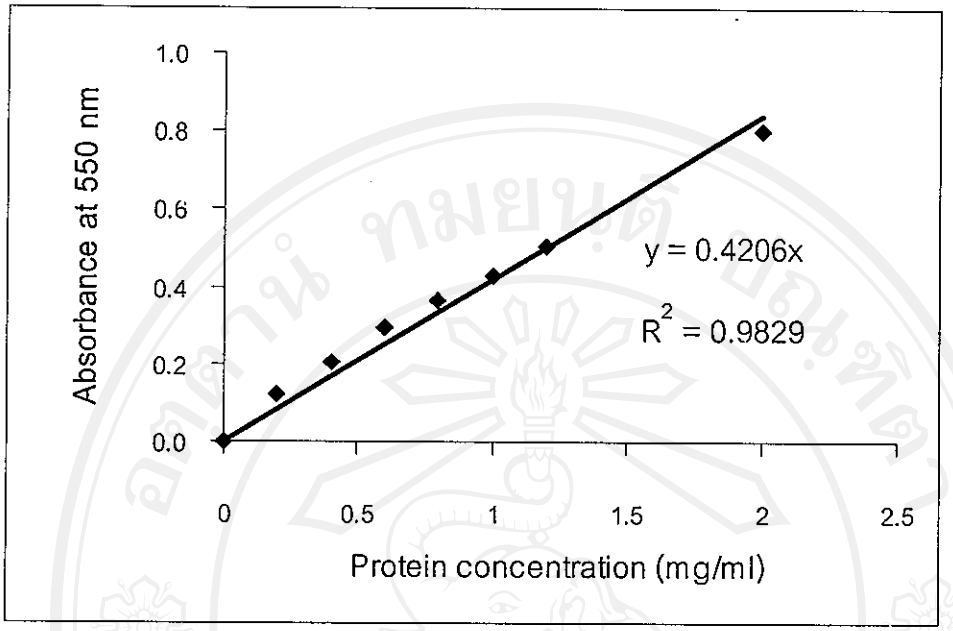

Figure 5. An example of standard curve obtained from diluting BSA to various concentrations and detected by BCA protein assay, so that protein concentration of the unknown homogenate could be calculated using this graph.

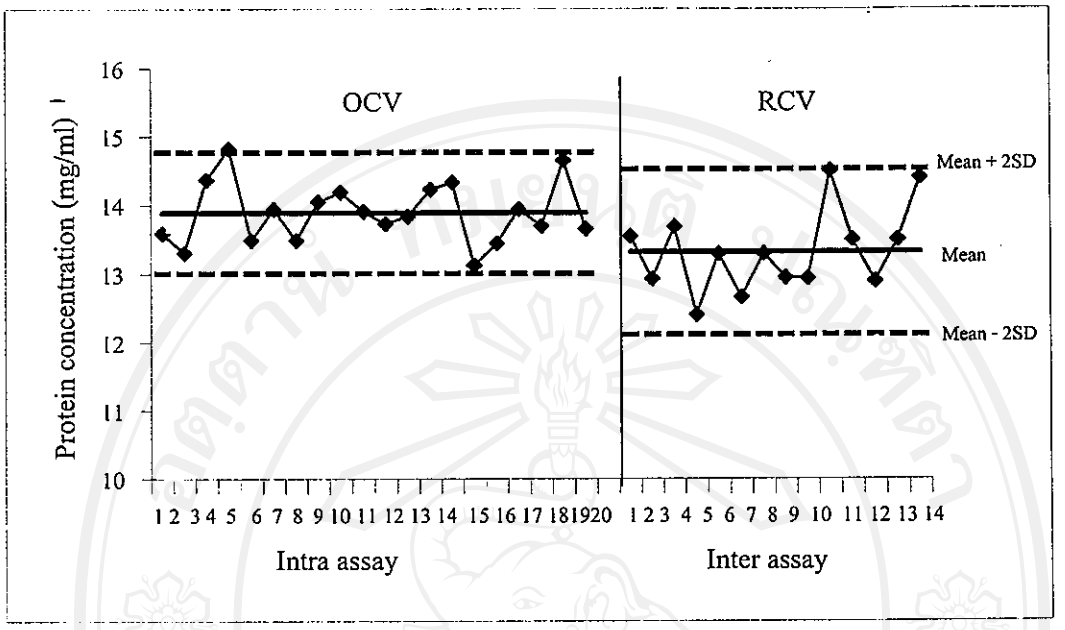

Figure 6. The quality control chart of protein concentration of the control homogenate determined by BCA assay.

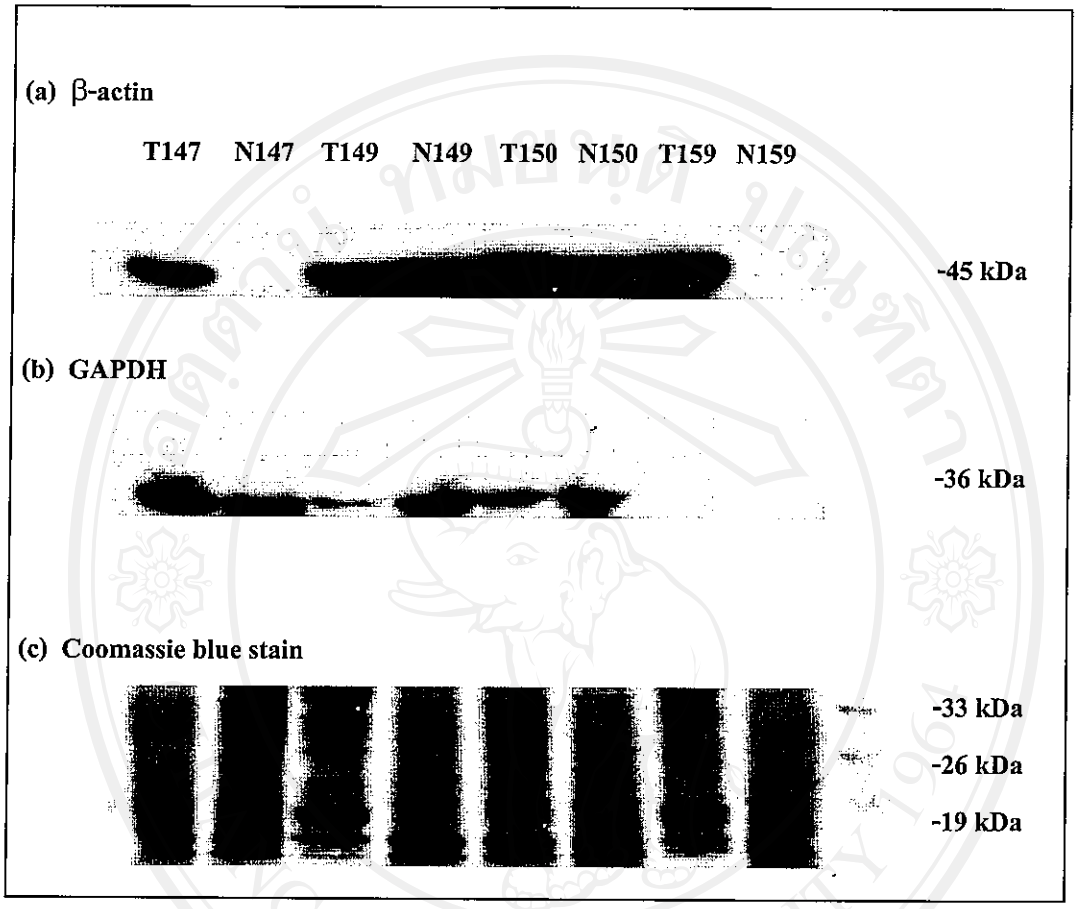

Figure 7. Western blots show unequality of β-actin protein and GAPDH protein in tumor and normal tissues, although about the same amount of protein were loaded into each lane as determined by coomassie blue staining (T, Tumor tissue; N, Normal tissue; number represents sample number)

1.3 Control of VEGF protein

As a positive control for VEGF determination by Western blotting and ELISA, recombinant human $VEGF_{165}$ purchased from R&D System was analysed along with the tissue and serum samples in every assay.

1.4 Control of total VEGF determination by ELISA

For the measurement of total VEGF Capture ELISA, the concentration of VEGF in unknown sample was calculated from the standard graph obtained from diluting recombinant human $VEGF_{165}$ to various concentrations, which was done along with the unknown sample every assays. An example of standard graph is shown in Figure 8. Serum control was assayed along with the unknown tissue samples and serum, observed values were plotted on quality control chart or Levey Jenning control chart. Patterns of distribution of OCV (Optimal Condition Variance) and RCV-K (Routine Condition Variance-Known value) are illustrated in Figure 9. It was found that % CV of OCV was 4.6% (Mean = 859 pg/ml, SD = 39.4 pg/ml), while %CV of RCV-K was 8.9% (Mean = 903.8 pg/ml, SD = 80.8 pg/ml), which was less than twice as much as those in the OCV. The distributions of control values in both charts were within the limits of acceptability.

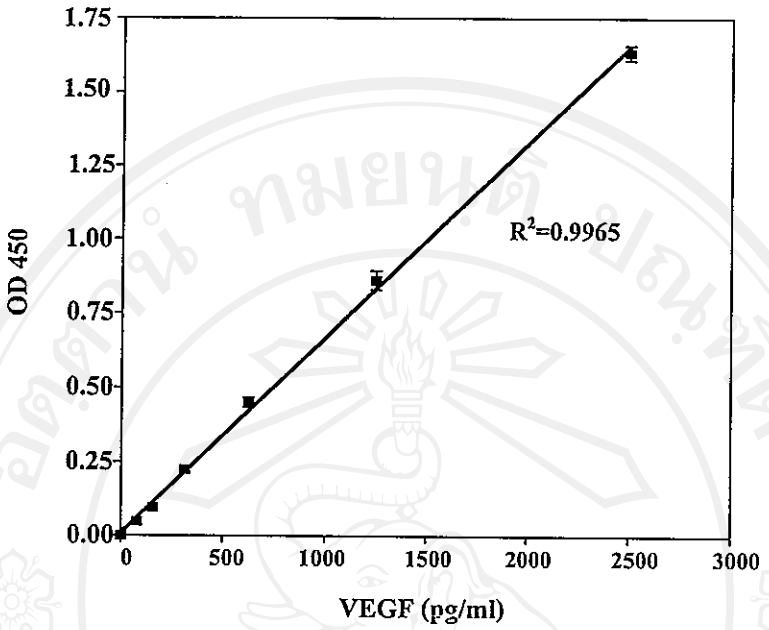

Figure 8. An example of standard graph obtained from diluting recombinant VEGF to various concentrations and detected by ELISA assay, so that VEGF of the unknown homogenate and serum could be calculated using this graph.

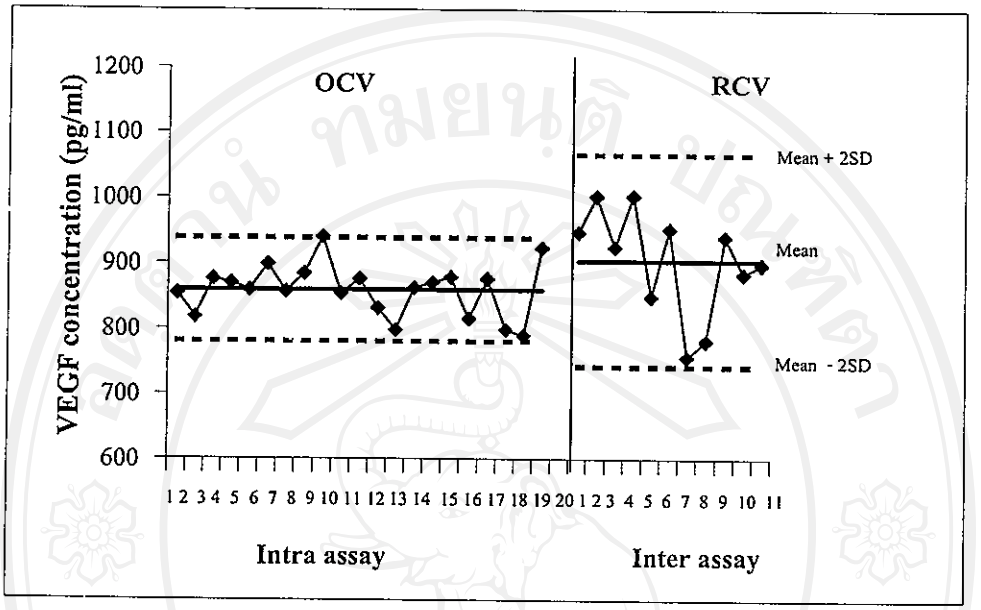

Figure 9. The quality control chart of VEGF concentration of the control serum determined by Capture ELISA assay.

2. Study population of the patients

One hundred and fourteen tissue samples from Thai patients who underwent curative surgery for cancer at Maharaj Nakorn Chiang Mai Hospital during April 2003 to June 2004 were recruited for this study, which included 76 colorectal tumors, 20 liver tumors and 18 lung tumors. In each case accompanying normal tissues were obtained to be subjected for comparison. Of these, 55 were male and 59 were female. The mean of age was 55 years, which ranged from 10 to 89 years. Clinical characteristics of patients recruited in this study are summarized in Table 5.

Table 5. Clinical characteristics of the cancer patients.

Parameters	Colorectal cancer	Liver cancer	Lung cancer
No. of patients (cases)	76	20	18
Sex (cases)			
Male	30	17	8
Female	46	3	10
Age (years)			
Mean	59	49.5	56
Range	22-89	10-76	17-79

3. Expression pattern of VEGF isoform

3.1 Expression pattern of VEGF isoform in tumor tissue

The expression pattern of VEGF isoform in tumor tissues in comparison to normal tissues determined by western blot analysis are shown in Figure 10. Three major protein band were predominated detected in tumor samples with an apparent molecular mass under reducing conditions approximately 18 kDa, 23 kDa, and 26 kDa of colorectal cancer, liver cancer, and lung cancer (Figure 10a, 10b, and 10c, respectively). In colorectal and lung cancer, it was found that the 18 kDa VEGF was predominately expressed in normal, whereas the molecular weight of 23 and 26 kDa was only detected or detected at higher level in tumor tissues. Expression of the 23 kDa VEGF isoform was observed in 55.3% (42 of 76 patients) of colorectal tumors, 80% (16 of 20 patients) of liver tumor and 88.9% (16 of 18 patients) of lung tumor tissues. Whereas, expression of 26 kDa VEGF isoform was detected in 69.7% (53 of 76 patients), 50% (10 of 20 patients) and 88.9% (16 of 18 patients) of colorectal, lung and liver tumor tissues, respectively (Table 6).

Interestingly, we found that unlike colorectal and lung, normal liver tissues expressed higher level of VEGF isoform in comparison to tumor tissues (Figure 10b).

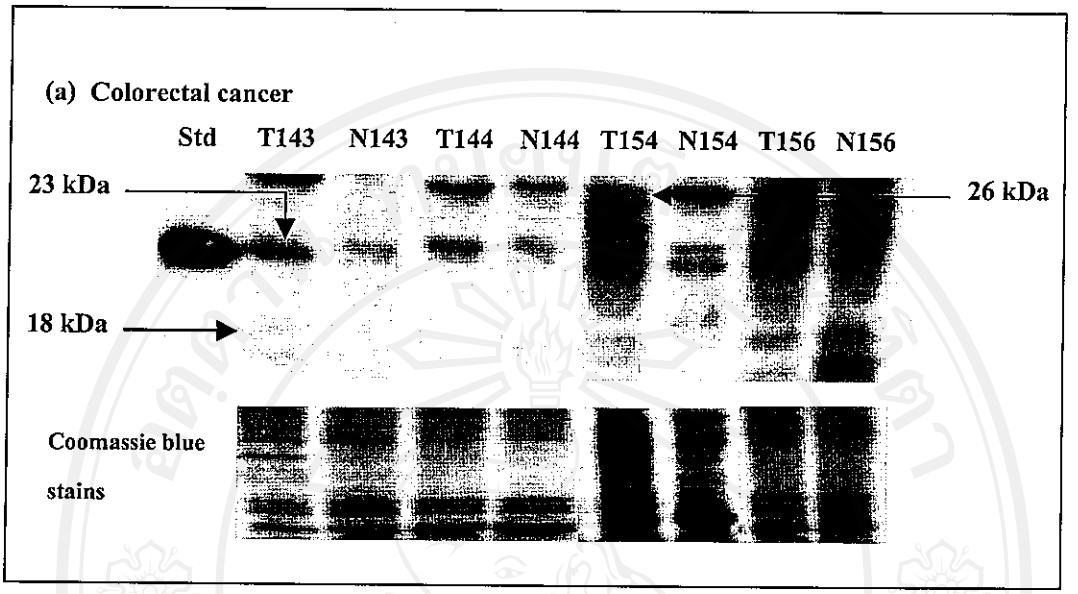

Figure 10a. Protein expression VEGF isoform in patients with colorectal cancer assessed by Western blotting in tumor tissues and corresponding adjacent normal tissues (Std, recombinant human $VEGF_{165}$ protein standard; T, Tumor tissue; N, Normal tissue and number represent sample number)

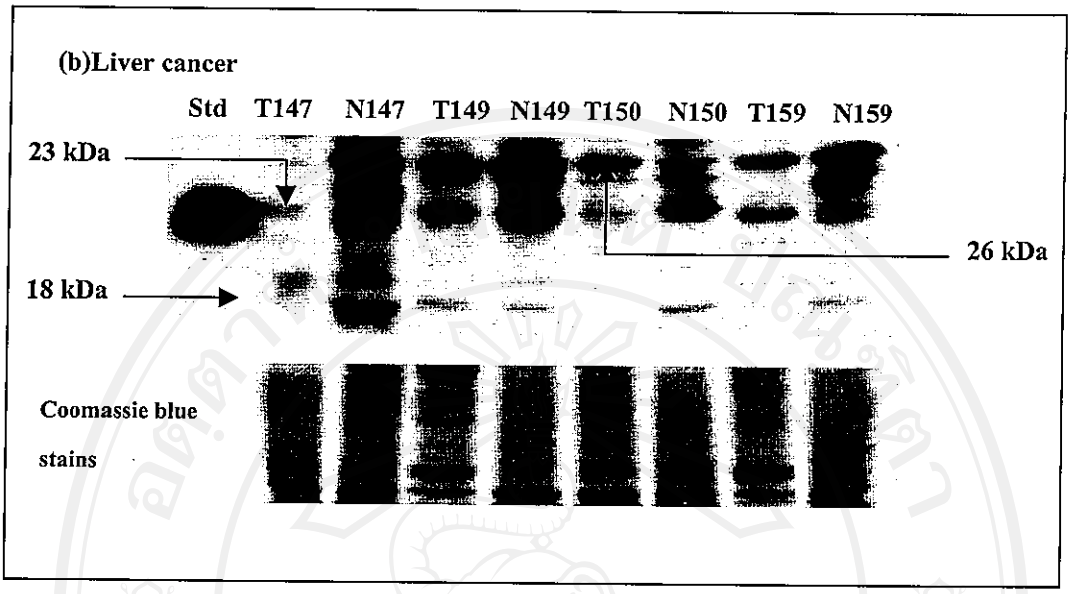

Figure 10b. Protein expression VEGF isoform in patients with liver cancer assessed by Western blotting in tumor tissues and corresponding adjacent normal tissues (Std, recombinant human $VEGF_{165}$ protein standard; T, Tumor tissue; N, Normal tissue and number represent sample number)

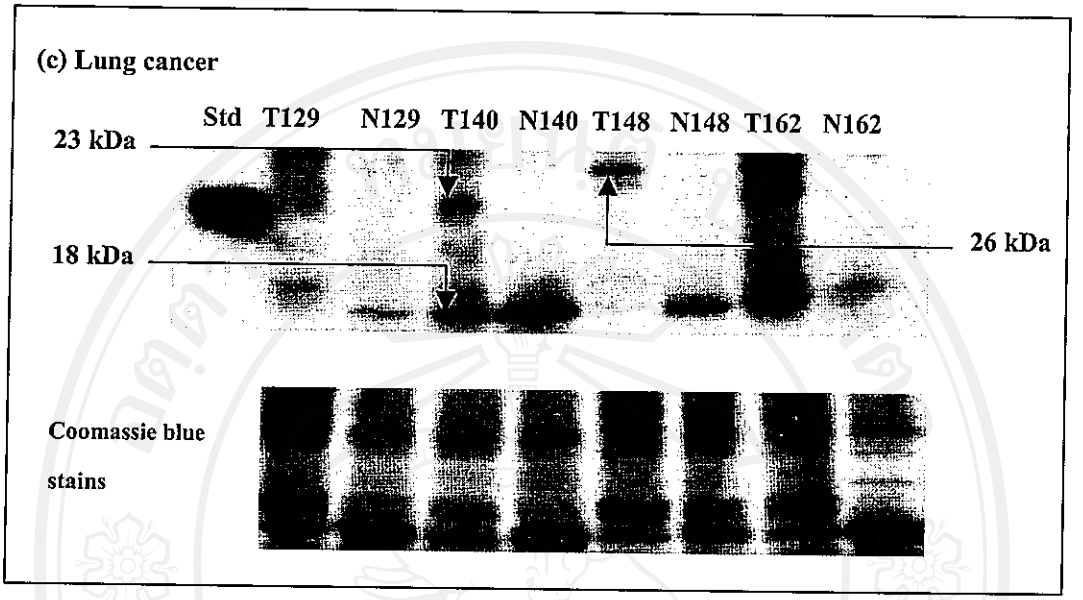

Figure 10c Protein expression VEGF isoform in patients with lung cancer assessed by Western blotting in tumor tissues and corresponding adjacent normal tissues(Std, recombinant human $VEGF_{165}$ protein standard; T, Tumor tissue; N, Normal tissue and number represent sample number).

Table 6. Summary of VEGF isoform expression in different types of tumor tissues.

Type of cancer	VEGF isoform	
	23 kDa	26 kDa
Colorectal (76 cases)	42 (55.3%)	53 (69.7%)
Liver (20 cases)	16 (80%)	10 (50%)
Lung (18 cases)	16 (88.9%)	16 (88.9%)

3.2 Expression pattern of VEGF isoform in serum of cancer patients in comparison to serum of normal healthy volunteers

Seventy-seven preoperative patient serum were collected, these included 39 cases with colorectal cancer, 20 case with liver cancer and 18 cases with lung cancer. The expression pattern of circulating VEGF were examined by western blot analysis. Only one patient serum was found to express VEGF with molecular weight of 23 kDa and 26 kDa, whereas other patient as well as normal healthy volunteers appeared to possessed a small amount of 18 kDa VEGF in their serum (Figure 11)

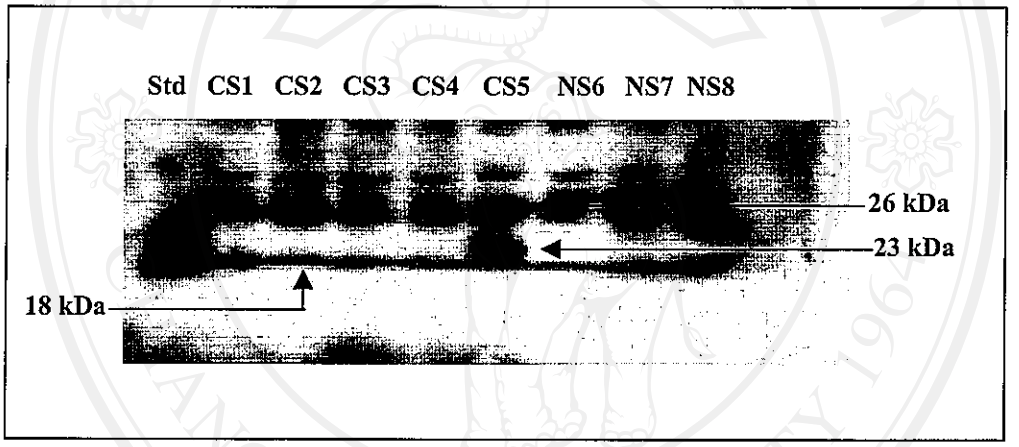

Figure 11. Western blot analysis of VEGF in serum from cancer patient compare to normal serum.. (Std, recombinant human $VEGF_{165}$ protein standard; CS, cancer serum; NS, normal serum; number represents sample number)

3.3. VEGF isoform expression in relation to classification of pathological features

The three type of cancer were classified according to the pathological features, which included tumor sizes in maximum diameter, depth of invasion, lymph node metastasis, distant metastasis and histological differentiation. Expression of the VEGF isoform in relation to the pathological features of colorectal tumors, liver tumors and lung tumors are summarized in Table 7, 8 and 9,respectively.

It was found that expression of VEGF isoform with molecular weight 23 kDa was significantly correlated with a tumor size smaller (maximum diameter $\leq$ 5 cm, $p = 0.016$), whereas the 26 kDa VEGF isoform was significantly correlated with advanced clinical stage of the

(stage III and IV, p = 0.004). However, no significant correlation between expression of the VEGF isoform and the pathologic features were observed in liver and lung tumors (Table 8,9).

Table 7. Summary of the relationship between VEGF isoform expression and pathological features in colorectal cancers.

Pathological features	VEGF isoform			
	23 kDa	*p* value*	26 kDa	*p* value*
No. of patients				
Colorectal cancer (Total 76 cases)	42 (55.3%)		53 (69.7%)	
Tumor size				
≤ 5 cm (45 cases)	30 (71.4%)	0.016**	30 (56.6%)	0.483
> 5 cm (31 cases)	12 (28.6%)		23 (43.4%)	
Histological differentiation				
Weli (42 cases)	22 (52.4%)	0.574	28 (52.8%)	0.517
Moderate or Poor (34 cases)	20 (47.6%)		25 (47.2%)	
Tumor stage grouping				
Early stage (I or II) (34 cases)	22 (52.4%)	0.136	18 (34.0%)	0.004**
Late stage (III or IV) (42 cases)	20 (47.6%)		35 (66.0%)	

*Chi-square test

**Significant difference

Table 8. Summary of the relationship between VEGF isoform expression and pathological features in hepatocellular carcinoma (HCC)

Pathological features	VEGF isoform			
	23 kDa	*p* value*	26 kDa	*p* value*
No. of patients				
Liver cancer (Total 20 cases)	16 (80.0%)		10 (50.0%)	
Tumor size				
≤ 5 cm (8 cases)	7 (43.8%)	0.494	6 (60.0%)	0.068
> 5 cm (12 cases)	9 (56.3%)		4 (40.0%)	
Histological differentiation				
Well (7 cases)	5 (31.3%)	0.482	4 (40.0%)	0.639
Moderate or Poor (13 cases)	11 (68.8%)		6 (60.0%)	
Tumor stage grouping				
Early stage (I or II) (7 cases)	6 (37.5%)	0.639	5 (50.0%)	0.16
Lately stage (III or IV) (13 cases)	10 (62.5%)		5 (50.0%)	

*Chi-square test

Table 9. Summary of the relationship between VEGF isoform expression and pathological features in non-small cell lung cancers (NSCC)

Pathological features	VEGF isoform			
	23 kDa	*p* value*	26 kDa	*p* value*
No. of patients				
Lung cancer (Total 18 cases)	16 (88.9%)		16 (88.9%)	
Tumor size				
≤ 5 cm (7 cases)	7 (43.8%)	0.231	7 (43.8%)	0.231
> 5 cm (11 cases)	9 (56.3%)		9 (56.3%)	
Histological differentiation				
Well (5 cases)	4 (25.0%)	0.457	4 (25.0%)	0.457
Moderate or Poor (13 cases)	12 (75.0%)		12 (75.0%)	
Tumor stage grouping				
Early stage (I or II) (8 cases)	7 (43.8%)	0.867	6 (37.5%)	0.094
Lately stage (III or IV) (10 cases)	9 (56.3%)		10 (62.5%)	

*Chi-square test

4. Determination of total VEGF by ELISA

4.1 Optimization of the ELISA for determination of total VEGF

There are many parameters which influence the result obtained in an ELISA. One of the most important parameters is antibodies concentration. The best way to determine the optimal capture and detection antibody concentrations is to performs a grid experiment as described in Materials and Methods (section 5.1). To form the grid, a 96-well plate was divided into 4 quadrants. Each quadrant is a minigrid, identifying different capture antibody and standard concentration at one particular detection antibody concentration. Figure 12 shows standard curves of recombinant human $VEGF_{165}$ concentration between 0-1,000 pg/ml obtained by using mouse anti-VEGF monoclonal antibody as capture antibody and goat anti-VEGF polyclonal antibody as detection antibody.

On the other hand, standard curves of VEGF using goat anti-VEGF polyclonal antibody as capture antibody and mouse anti-VEGF monoclonal antibody as detection antibodies is shown in Figure 13. From standard graphs obtained from multiple combinations of antibody pair concentrations, it was suggested that the suitable capture antibody is goat anti-VEGF polyclonal antibody (cat. no. AF-293-NA, R&D system) at concentration 200 ng/ml and the suitable detection antibody is mouse anti-VEGF monoclonal antibody (cat. no. MAB293, R&D system, USA) at concentration as 0.5 μg/ml this gives the highest signal to noise ratio with an acceptable background.

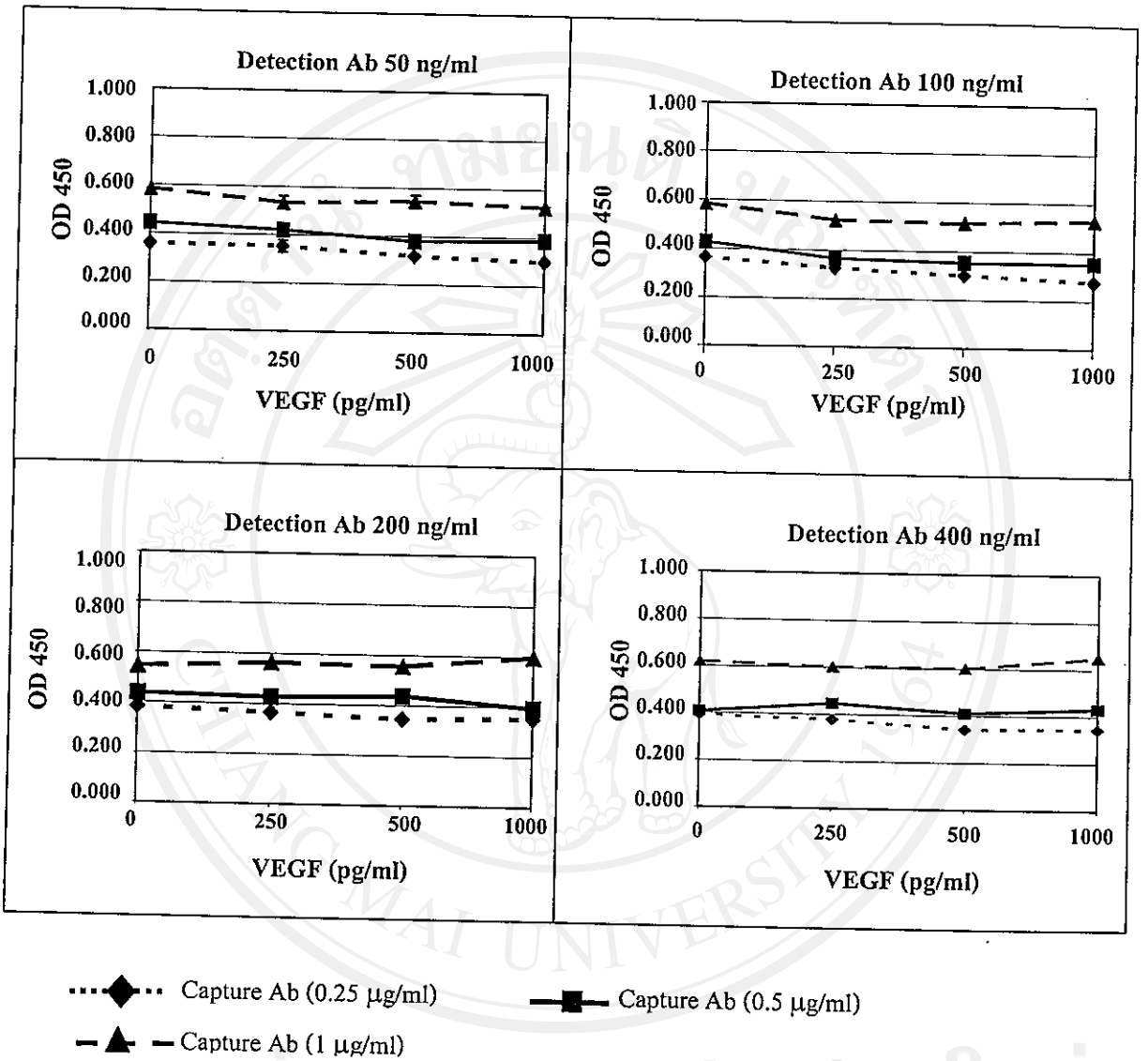

Figure 12. Optimization of capture ELISA useing mouse anti-VEGF monoclonal antibody as capture antibody and goat anti-VEGF polyclonal antibody as detection antibody.

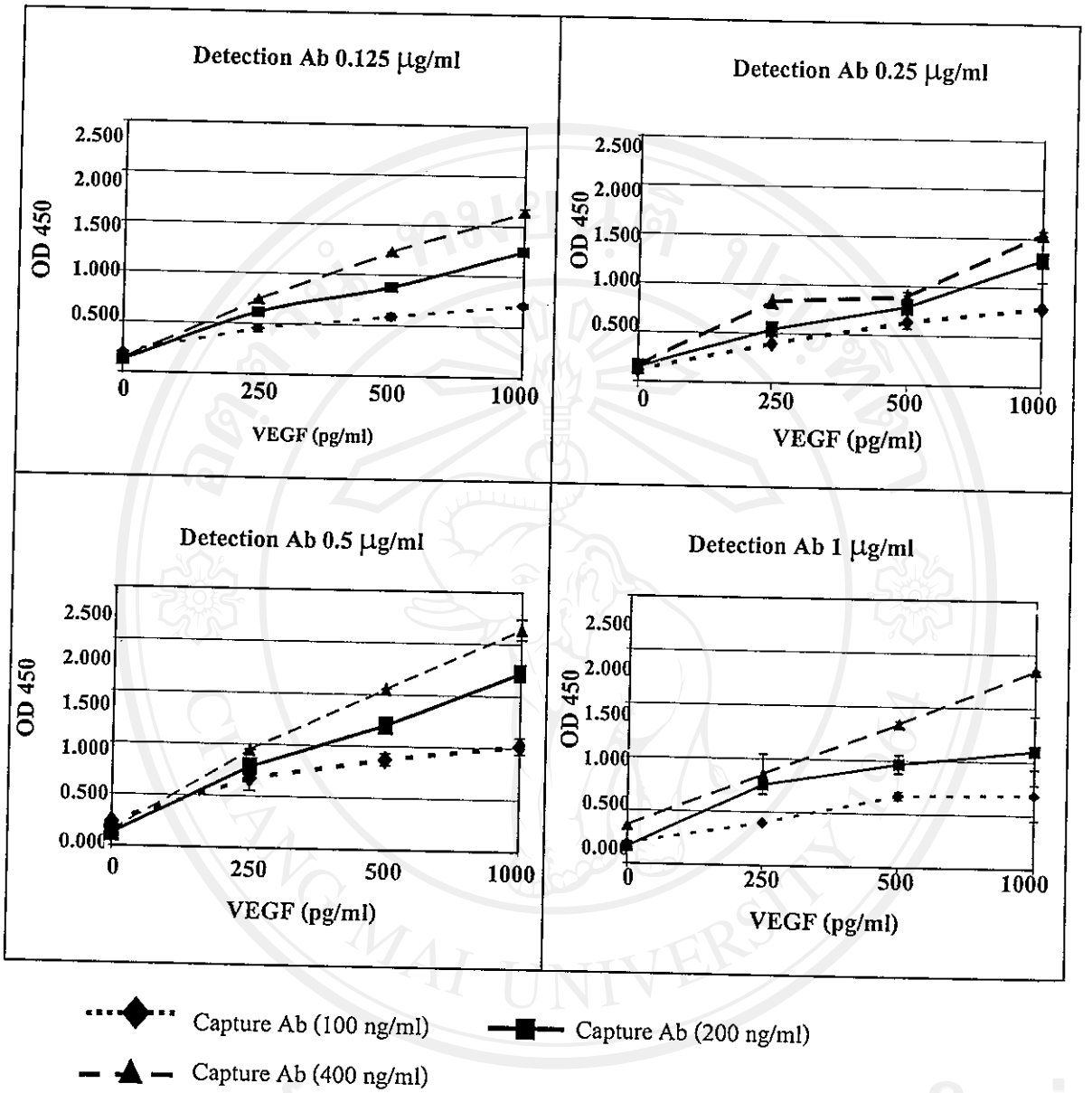

Figure 13. Optimizations of capture ELISA using goat anti-VEGF polyclonal antibody as capture antibody and mouse anti-VEGF monoclonal antibody as detection antibody.

4.2. Determination of specificity of the ELISA

4.2. Determination of specificity of the ELISA

In order to determine specificity of the ELISA for the measurement of total VEGF, recombinant $VEGF_{165}$ protein standard at various concentrations (500 and 1000 pg/ml) was added into the serum sample and subjected to ELISA. The percentages of recovery at various concentrations of VEGF calculated from the obtained measurement values are shown in table 10. The result shows that percentage of recovery rang from 75.38% to 127.32%, indicating an acceptable level of specificity of the assay.

Table 10. The percentage of thes recovery of standard VEGF from serum assessed by ELISA

Std. VEGF Conc. (pg/ml)	% Recovery			Mean (%)	SD	% Recovery range Mean ±1SD
	1	2	3			
500	92	100	74	88.7	13.32	75.38-102.02
1,000	126	113	93	110.7	16.62	94.08-127.32

4.3 Level of total VEGF protein in tumor tissues in comparison to normal tissues

Total VEGF in tissues measured by ELISA were significantly higher in tumor tissues of colorectal ($p<0.001$) and lung ($p=0.003$), but not of liver ($p=0.444$) as shown in Table 11.

Table 11. Level of total VEGF protein in tumor tissues in comparison to normal tissues

Type of cancer	N	VEGF concentration (pg/mg tissue)		*p* value *
		Normal tissues	Tumor tissues	
Colorectal	68	13.16 ± 11.14	17.56 ± 15.63	< 0.001
Lung	18	11.20 ± 2.66	14.08 ± 5.07	0.003
Liver	20	12.53 ± 3.98	13.60 ± 6.37	0.444

* Paired sample t-test

4.4 Level of total VEGF protein in serum of cancer patients in comparison to healthy volunteers

Preoperative serum was collected from 77 cancer patients; these included 39 patients with colorectal cancer, 20 with liver cancer and 18 with lung cancer. Serum from 52 healthy volunteers were also collected and subjected to comparison. The result showed that cancer patients significantly possessed higher level of circulating VEGF than those in healthy volunteers. While the level of total circulating VEGF in healthy volunteer was only 605 ± 384 pg/ml, it was1,068 ± 649 pg/ml, 1,251 ± 568 pg/ml and 836 ± 346 pg/ml in patients with colorectal, lung and liver cancer, respectively (Table 12).

Table 12. Level of VEGF protein in serum of cancer patients in comparison to healthy volunteers

Types of cancer	N	VEGF concentration (pg/ml)	*p* value*
Colorectal	39	1,068 ± 649	0.001
Lung	18	1,251 ± 568	0.001
Liver	20	836 ± 346	0.017
Healthy volunteers	52	605 ± 384	

* Independent-sample t-test