IV. RESULTS

1. Quality controls of the study

1.1 Controls for protein quantification

Tumor tissues, which obtained from Thai patients with stomach cancer or colorectal 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 curve of protein by BCA assay was linear up to the concentration of 2 mg/ml (R^2 = 0.9793). This control homogenate was assayed together with tissue samples and the observed values were plotted on quality control chart or Levey Jennings control chart. Patterns of distribution of OCV (Optimal Condition of Variances) and RCV-K (Routine Condition of Variances-Known) are illustrated in Figure 6. It was found that %CV of OCV was 3.2% (Mean = 12.31 mg/ml, SD = 0.4 mg/ml), while %CV of RCV-K was 5.2% (Mean = 12.64 mg/ml, SD = 0.66 mg/ml), which was less than two times of the OCV. The distributions of control values in both charts were observed inside the control limits of acceptability. Thus, control data indicated that the analytical method was performed properly leading to the reliable results of protein assay.

1.2 Control for protein loading

Western blot analysis was used to examine the expression of COX-2 and COX-1 protein in the tumor tissues compared with the corresponding normal tissue. In order to examine the equality of protein loaded in each lane, several studies had used the β-actin protein which was classified as one of the house keeping gene as an internal loading control (Molina *et al*, 1999; Konturek *et al*., 2001). In the present study, the detected membrane for COX-2 and COX-1 protein expression was finally reprobed with a monoclonal anti-actin antibody and the immunoreactive protein was visualized with the ECL detection. The β-actin bands of representative tumor and normal tissues were shown in Figure 8, which were similarly expressed in these tissues.

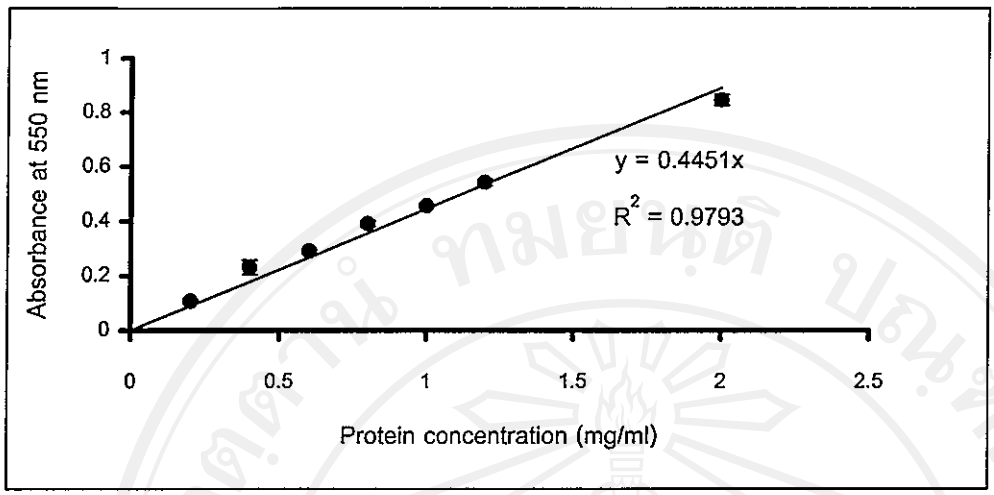


Figure 5. An example of standard curve obtained from diluting BSA to various concentrations and detected by BCA protein assay, so that protein concentration of the unknown homogenate could be calculated using this graph.

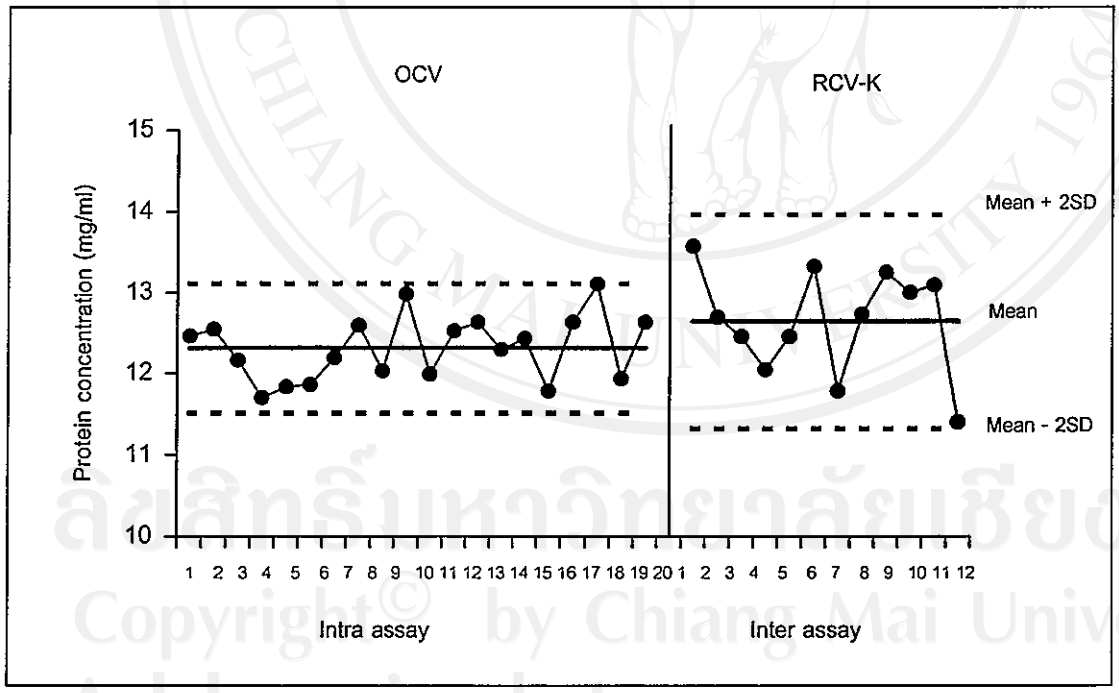


Figure 6. The quality control chart of protein concentration of the control homogenate determined by BCA assay. The chart demonstrates the variation from both Intra-assay (mean = 12.31 mg/ml, SD = 0.4 mg/ml, CV = 3.2%) and Inter-assay (mean = 12.64 mg/ml, SD = 0.66 mg/ml, CV = 5.2%).

1.3 Controls for antibody specificity

The interested protein that had been immobilized on solid supports by was analyzed using technique of immunodetection. Although COX-1 and COX-2 are the protein products encoded from different genes, they share 60% of amino acid homology (Otto and Smith, 1996; Spencer *et al*, 1999), which means that it is likely for the antibodies to cross-react to one another. Therefore, the recombinant protein of COX-1 and COX-2 purchased from Cayman Chemical Company (USA) were used in order to evaluate the specificity of the antibodies used in this study. Primary antibodies, mouse anti-COX-2 monoclonal antibody and anti-COX-1 monoclonal antibody, were used at a final dilution of 1:1,000 to probe for specific antigens that were COX-2 and COX-1, respectively. Then, the secondary HRP-conjugated goat anti-mouse IgG antibody used at a dilution of 1:1,000 was added to bind to the primary antibody and detected by the ECL detection. To assess selectivity of the antibodies, both COX-2 (1.05 ng/μl) and COX-1 (0.74 ng/μl) protein standards were similarly loaded on two gels. After that, the first one was probed with the COX-2 antibody and another one was performed with the COX-1 antibody. The results of antibody specificity testing were shown in Figure 7. Under the conditions used in this study, the anti-COX antibodies demonstrated no cross-reactivity with the alternate COX isoform.

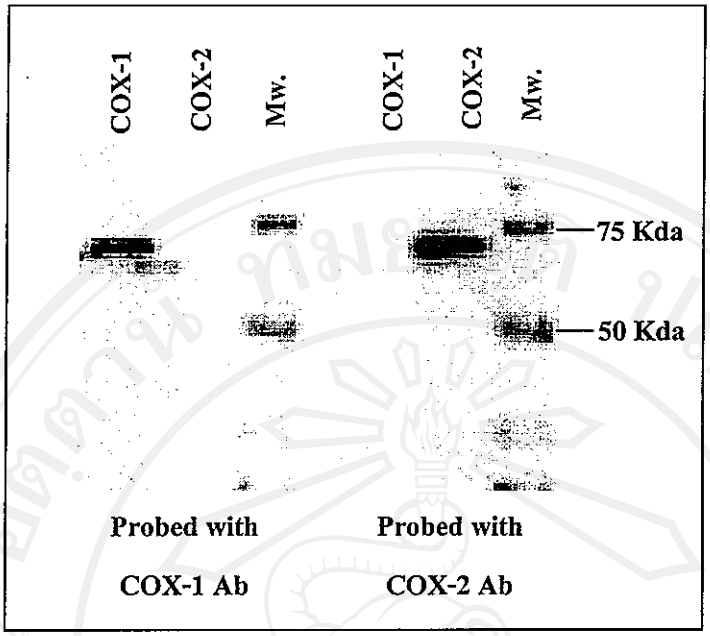


Figure 7. The specificity and cross-reactivity testing of COX-2 and COX-1 antibodies. The Blots show that there is COX-1 Ab- reacted band (around 70 Kda) only in the lane that was loaded with recombinant COX-1 protein but not with COX-2 protein and *vice versa*. Mw. represents protein molecular weight marker.

2. Study population of the patients

Sixty-four tissue samples from Thai patients undergoing surgery for primary stomach cancer or colorectal cancer at Maharaj Nakorn Chiang Mai Hospital during April 2002 to June 2003 were recruited for this study. Paired sample of gastric or colorectal tumors and accompanying normal tissues were obtained from each surgical specimen of the same patient. Of these, 24 were male and 40 were female. The mean of age was 58.1 years, which ranged between 22 and 90 years. Among them, 5 patients died of cancer. Seventeen tumors were located in the proximal colon (cecum, ascending, up to transverse colon) and 27 were in the distal colon (descending, sigmoid colon, down to rectum). Clinical characteristics of the patients are summarized in Table 3.

Table 3. Clinical characteristics of the cancer patients.

Parameters	Colorectal cancer	Stomach cancer
	Value	Value
No. of patients (cases)	44	20
Sex (cases)		
Male	15	9
Female	29	11
Age (years)		
Mean	60.5 ± 15.7	52.8 ± 18.8
Range	22-89	23-90
No. of death (cases)	3	2

3. Pathological characteristics of colorectal and stomach cancers

Representative sections from each paraffin-embedded block of 44 colorectal cancer tissues and stomach cancer tissues were stained with hematoxylin and eosin for morphological examination by a pathologist who was unaware of the results of Western blot results. Pathologic features of the tissue were listed in Table 4. Of 44 patients with colorectal cancers, 22 had metasted to lymph nodes. Of five patients with distant metastases, three had liver tumors, one had lung and liver tumors, and another had ovarian tumor. Tumor sizes of colorectal specimen varied from 2.5 to 9 cm in the maximum diameter, with a mean value of 4.9 ± 1.7 cm. In stomach cancer, 11 of the 20 patients exhibited lymph node metastasis. Of two patients with distant metastasis one had jejunum and ileum tumors, another had liver tumor. Tumor sizes of stomach specimens were 6.3 ± 3.5cm and ranges between 2.5 and 18 cm.

Table 4. Pathological characteristics of the cancer patients

Parameters	Colorectal cancer	Stomach cancer
	Value	Value
Tumor size (maximum diameter; cm)	4.9 ± 1.7	6.3 ± 3.5[a]
(Small:Large)	24:20	5:15
Histological differentiation		
(WD:MD:PD)	21:16:7	3:4:13
Depth of invasion		
(M:SM:MP:SS:SE)	0:1:2:28:13	1:0:1:9:9
Lymph node metastasis (Present:Absent)	22:22	11:9
Distant metastasis (Present:Absent)	5:39	2:18
Lymphatic invasion (Present:Absent)	39:5	15:5
Venous invasion (Present:Absent)	14:30	7:13
Perineural invasion (Present:Absent)	3:41	5:15
Stage grouping of tumor		
(I:II:III:IV)	2:17:20:5	-
(IA:IB:II:IIIA:IIIB:IV)	-	1:2:4:7:1:5

[a]Mean ± SD. (WD, well differentiation; MD, moderate differentiation; PD, poor differentiation; M, mucosa; SM, submucosa; MP, muscular propria, SS, subserosa, SE, serosa)

4. Expression of COX proteins in colorectal and stomach cancers by Western blotting

Briefly, protein complex mixtures were separated according to their molecular weight through a SDS-PAGE. Samples for analysis were boiled in the sample buffer containing 2-ME and SDS. The mercaptoethanol was used in order to reduce any disulfide bonds that hold together the protein tertiary structure, while SDS is an anionic detergent that binds strongly to, and denatures, the protein. Equal amounts of the protein solution were loaded into a gel lane and individual proteins were separated electrophoretically. After that, proteins were electro-transferred from the gels to nitrocellulose membranes. Following electroblotting, the

nitrocellulose membranes were blocked with blocking buffer to bind all protein-binding sites with nonreactive protein. Then, it was incubated with specific primary antibody against COX-2 protein. The blot was washed to eliminate excessive antibody and incubated with secondary antibody conjugated with enzyme against primary antibody.

The COX-2 expression was identified by chemiluminescent visualization using Amersham enhanced chemiluminescence (ECL) Western blotting detection kit. The locations of the antigen-antibody complex bounded to the membrane were revealed as bands on X-ray film using autoradiographic method and the intensity of the detected bands was quantified using a densitometer. After the detection of COX-2 protein, the primary and secondary antibodies were removed from the membrane using stripping buffer (see Appendix) and this membrane was subjected to detect COX-1 and β-actin protein, respectively. Steps of the protein detection were similar to COX-2 protein detection as described above, but specific primary antibody was different. Detection of β-actin was used to confirm the equality of protein loaded in each lane.

Representative immunoblots of colorectal samples using a specific anti-COX-2 antibody were shown in Figure 8a. On the same membrane, it was stripped and reprobed in order to assess the expression of COX-1 (Fig. 8b) and β-actin (Fig. 8c), respectively. COX-2 protein was detected in 13 out of 44 tumor tissues (29.5%), whereas no COX-2 protein was detected in any of the normal colorectal tissue samples (Fig. 9a and Table 5). While the level of COX-2 expression in normal tissues was below the detection limit of Western blot analysis used in this study, COX-1 expression was detectable in both tumor and normal tissues. Interestingly, it was found that there was an alteration of COX-1 expression level in tumor tissues compare with normal tissue. Therefore, COX-1 expression levels were expressed as an expression ratio between the band density ratio of COX-1/ β-actin of tumor tissue and normal tissue. To ensure that the alterations of COX-1 protein detected by Western blotting is due to the actual changes of COX-1 expression in tumor cells, not due to an experimental error, only more than two-fold alterations is considered significant change as this was done before in previous studies (Dimberg *et al.*, 1999). Therefore when the ratio was within the range of 0.5-2, it was assigned to a group which COX-1 expression was not changed. If the ratio was lesser than 0.5 or higher than 2, it would be assigned to a group of decreasing or increasing of COX-1 expression, respectively. The results were shown in Table 5.

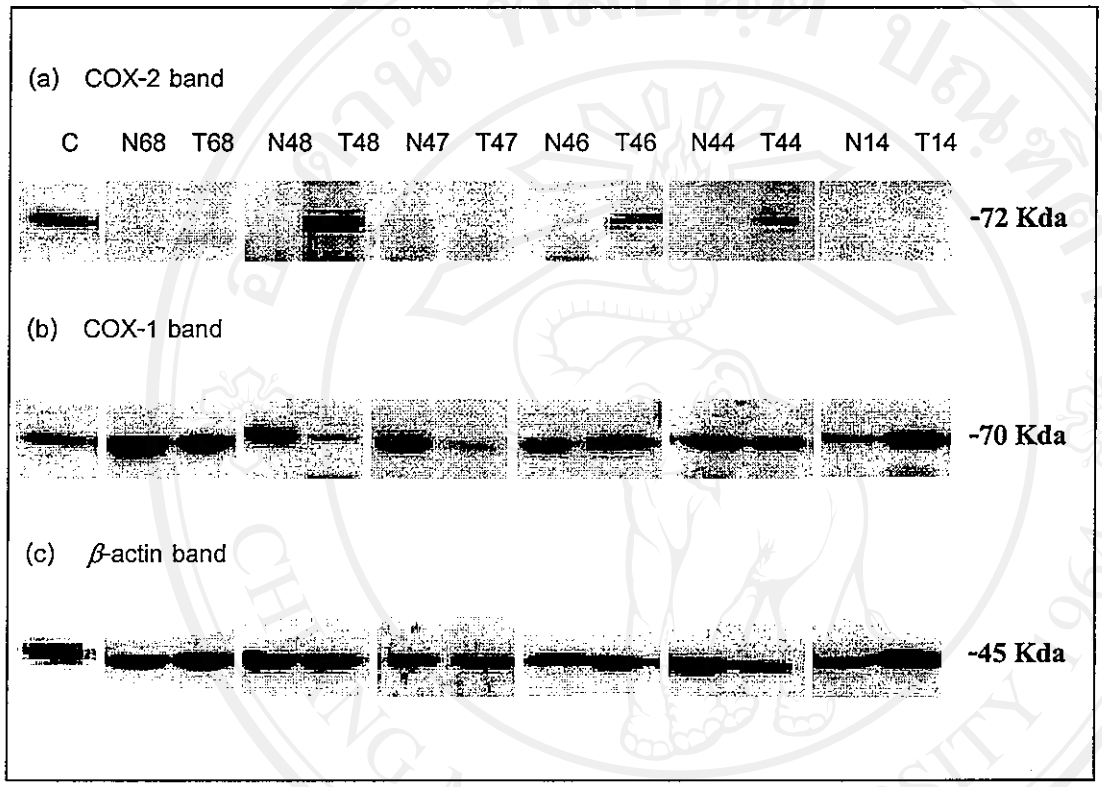


Figure 8. Protein expression of COX-2, COX-1, and β-actin assessed by Western blotting in colorectal cancer tissues and the corresponding adjacent normal tissues. T, tumor; N, normal tissue; number represents sample number. As shown in this figure, induction of COX-2 expression is observed only in tumor tissues, but not in any of the normal tissues. This figure also demonstrates that level of COX-1 expression was altered in some of the tumor tissues in comparison to normal tissues

In the same way, Figure 9 was a representative of immunoblot demonstrating COX-2 (Fig. 9a), COX-1 (Fig. 9b), and β-actin (Fig. 9c) protein expression in patients with stomach cancer examined in this study. In total, COX-2 protein was detected in 1 tumor tissue out of 20 (5%) patients examined. The density of each lane indicating COX-1 and β-actin was semi-quantified using the densitometer and expressed as a COX-1/ β-actin ratio. It was found that the levels of COX-1 expression were either decrease or not change in stomach tumors compare to normal tissues, but none was found to have an induction of COX-1 expression, as summarized in Table 5.

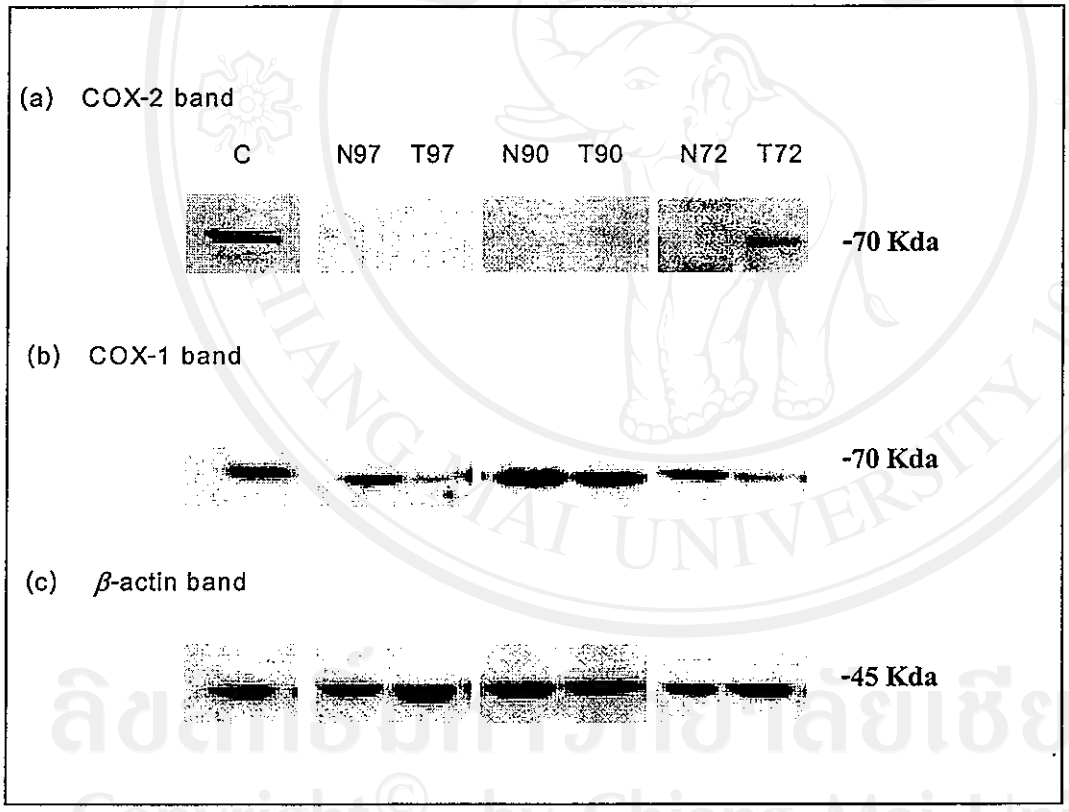


Figure 9. Protein expression for COX-2, COX-1, and β-actin as assessed by Western blotting in stomach cancer tissues and the corresponding adjacent normal tissues. T, tumor; N, normal tissue; number represents sample number.

Table 5. Comparison of COX expression in tumor with adjacent normal tissues of the cancer patients.

	Colorectal cancer No. of patients (Total 44 cases)	Stomach cancer No. of patients (Total 20 cases)
COX-2 overexpression	13*	1
COX-1 expression		
Decrease	21	16
Not change	13	4
Increase	10	0

*P<0.05, according to Chi-square test.

5. COX protein expression in relation to classification of pathological features

Pathological parameters included tumor sizes in maximum diameter, depth of invasion lymph node metastasis, distant metastasis, lymphatic invasion, venous invasion, perineural invasion, and histological differentiation. Additionally, Stage grouping of the cancers was evaluated according to the TNM classification (Table 1 and 2). In colorectal cancer, the relationship between overexpression of COX-2 and various expressions of COX-1 protein to each pathological parameter of the patients was demonstrated in Figure 10-18.

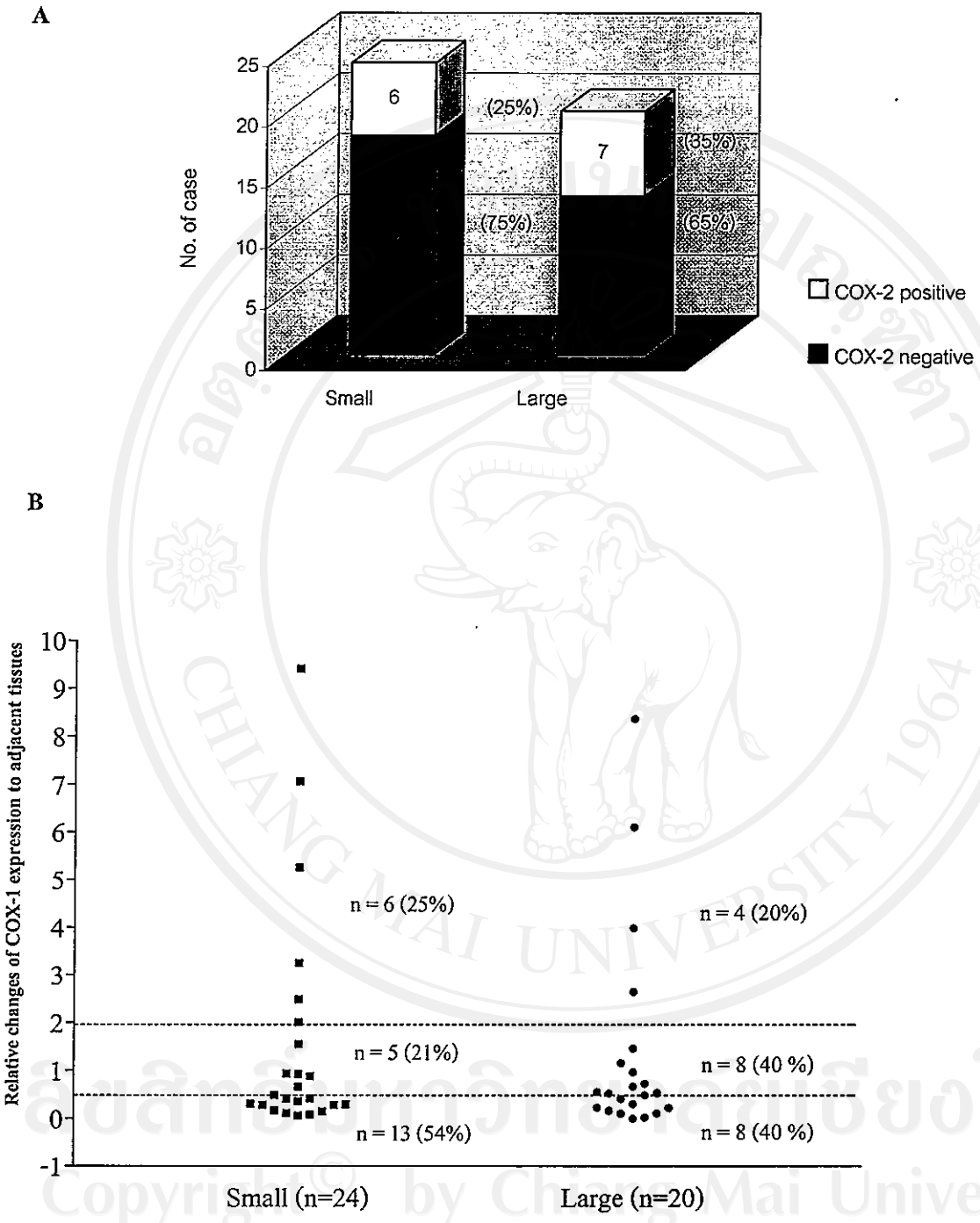
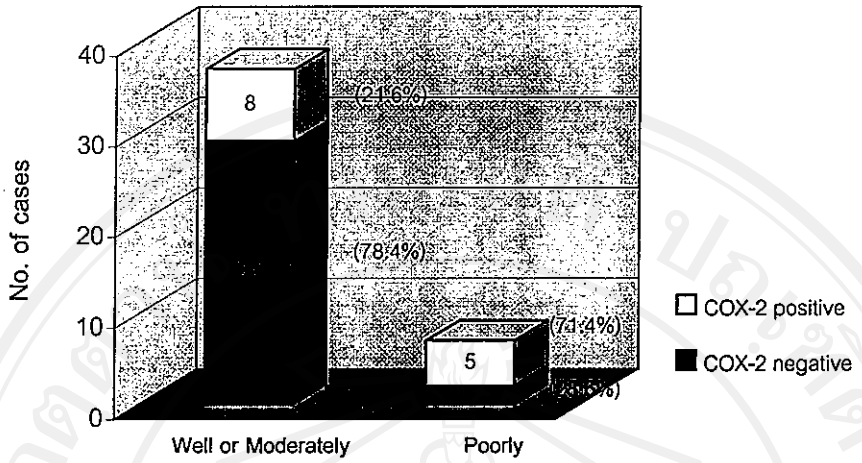


Figure 10. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to sizes of the primary colorectal tumors. Overexpressions of COX-2 were observed more often in large tumors (35%) than that in small tumors (25%); small (defined as a tumor with diameter less than 5cm), and large (defined as a tumor with diameter more than 5cm); n indicates the number of tumors in each group.

A



B

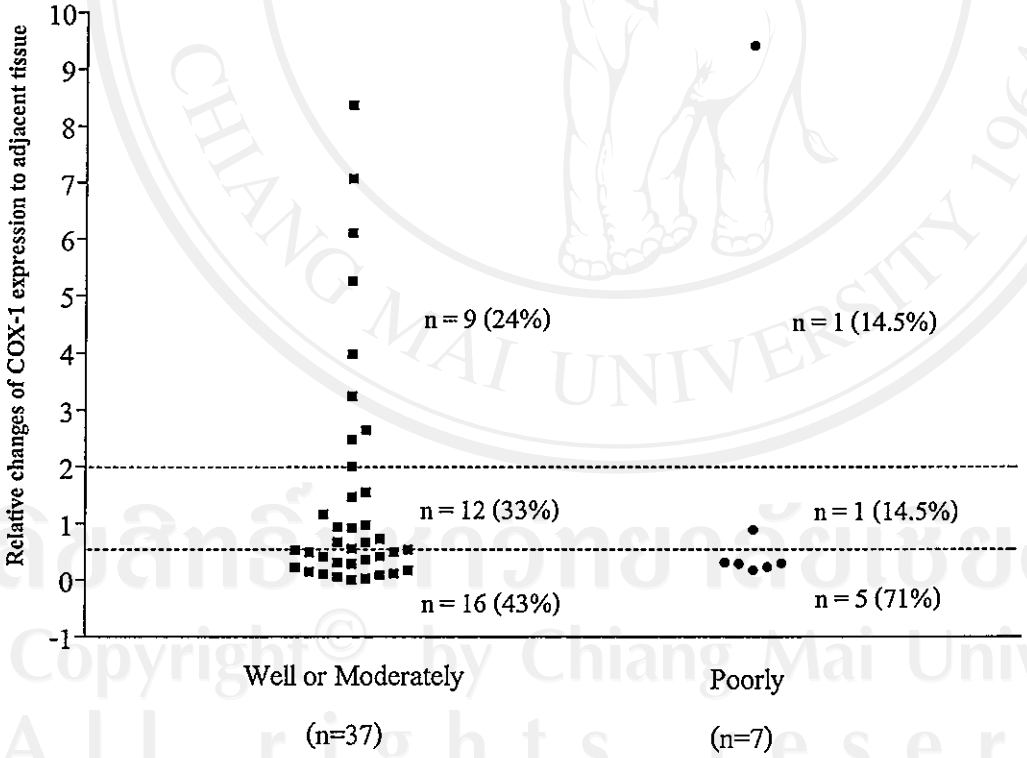


Figure 11. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to histological grading of the colorectal tumors. Overexpression of COX-2 is markedly seen more often in poorly differentiate group compare to well and moderately differentiate group ($p<0.05$); n indicates the number of tumors in each group.

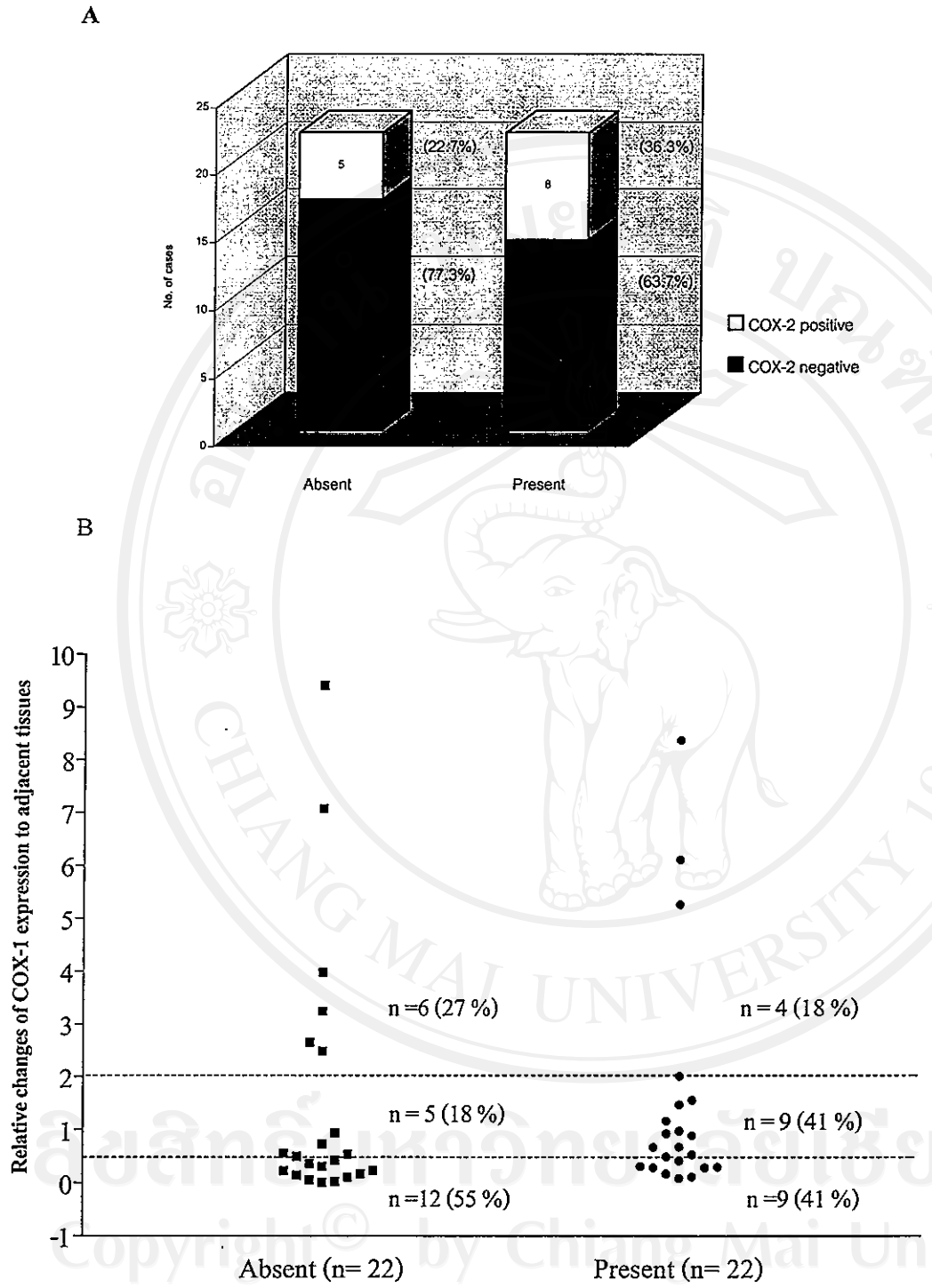
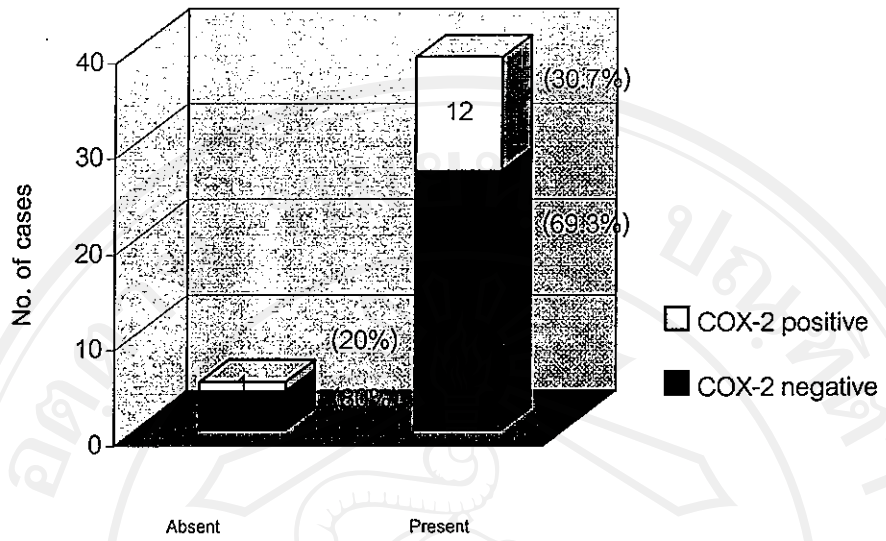


Figure 12. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to lymph node metastasis of the colorectal tumors. The figure shows that the incidence of COX-2 overexpression is higher in tumors with lymph nodes metastasis (36.3%) compare to those without (22.7%); n indicates the number of tumors in each group.

A



B

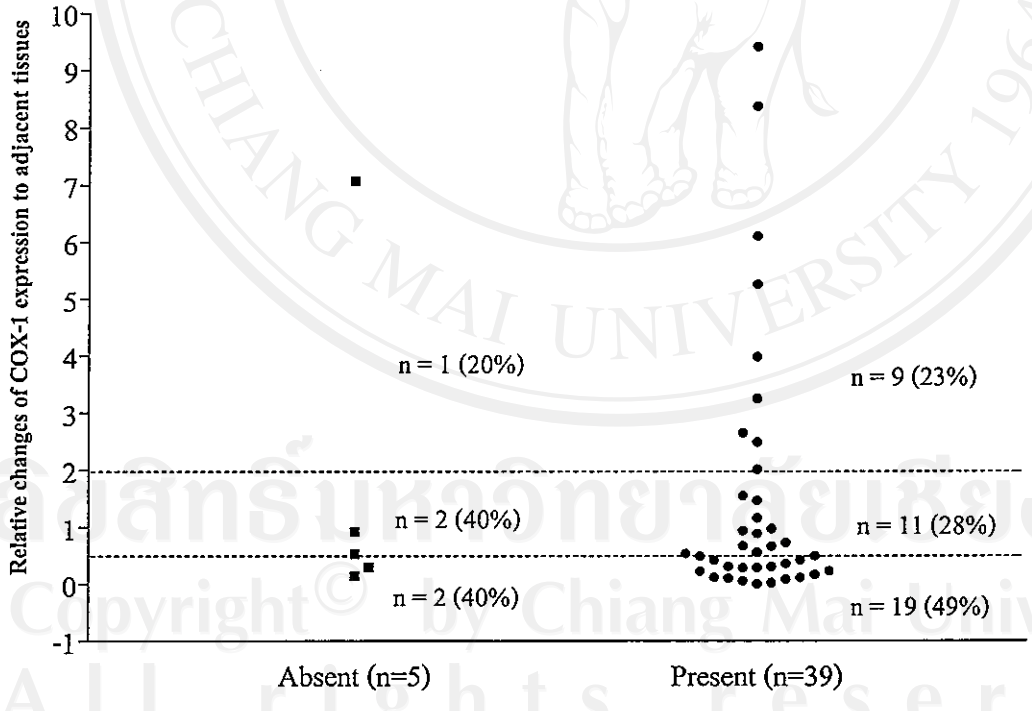


Figure 13. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to lymphatic invasion of colorectal tumors of colorectal tumors

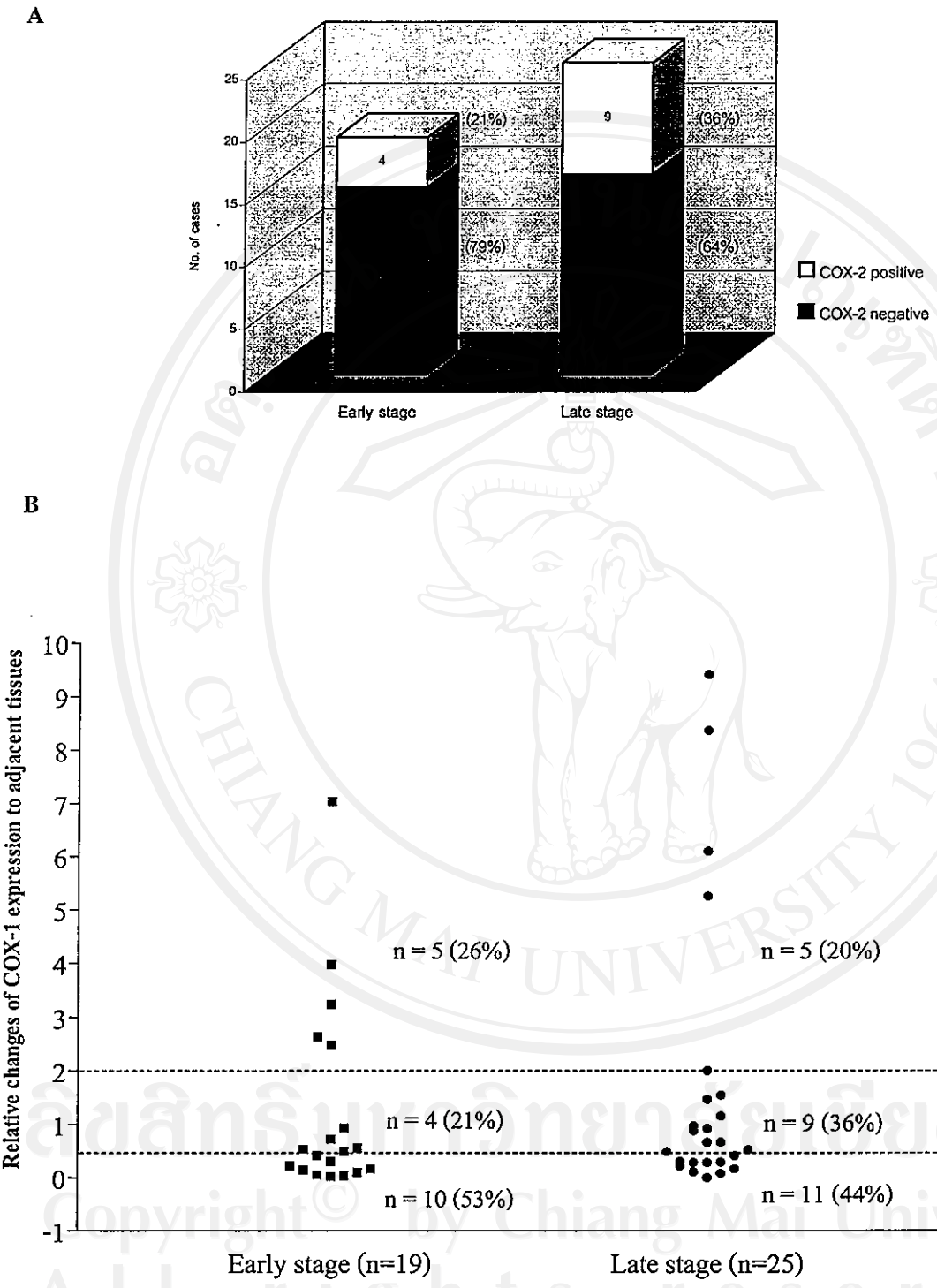
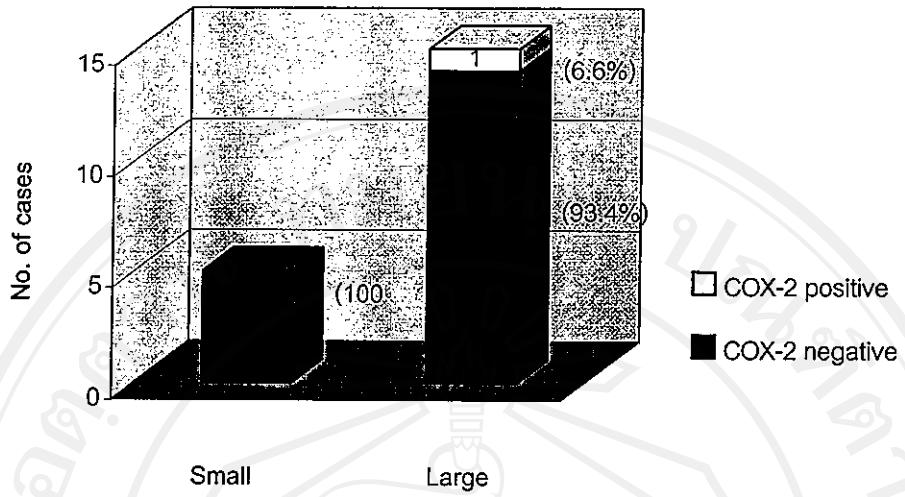


Figure 14. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to stage of the colorectal tumors; early stage is defined as tumors with T_{1-4} N_0M_0, late stage is defined as tumors with T_{1-4} N_{1-2} M_{0-1}

A



B

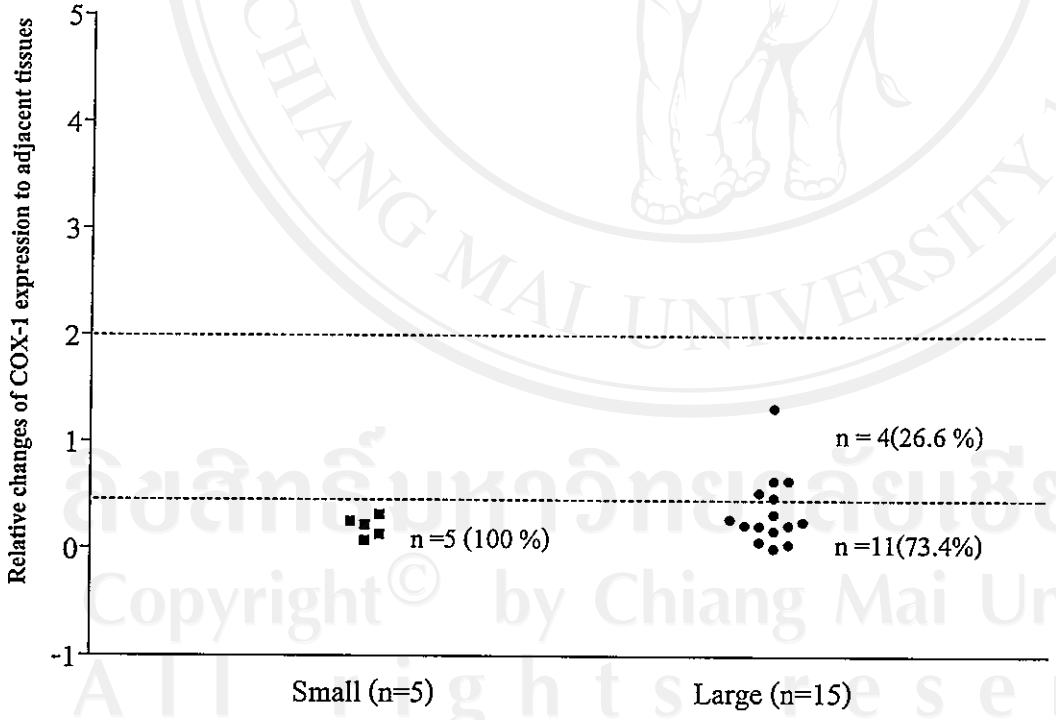


Figure15. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to sizes of the stomach tumors; small (defined as a tumor with diameter less than 2), medium (defined as a tumor with diameter between 2-5), and (defined as a tumor with diameter more than 5); n indicates the number of tumors in each group

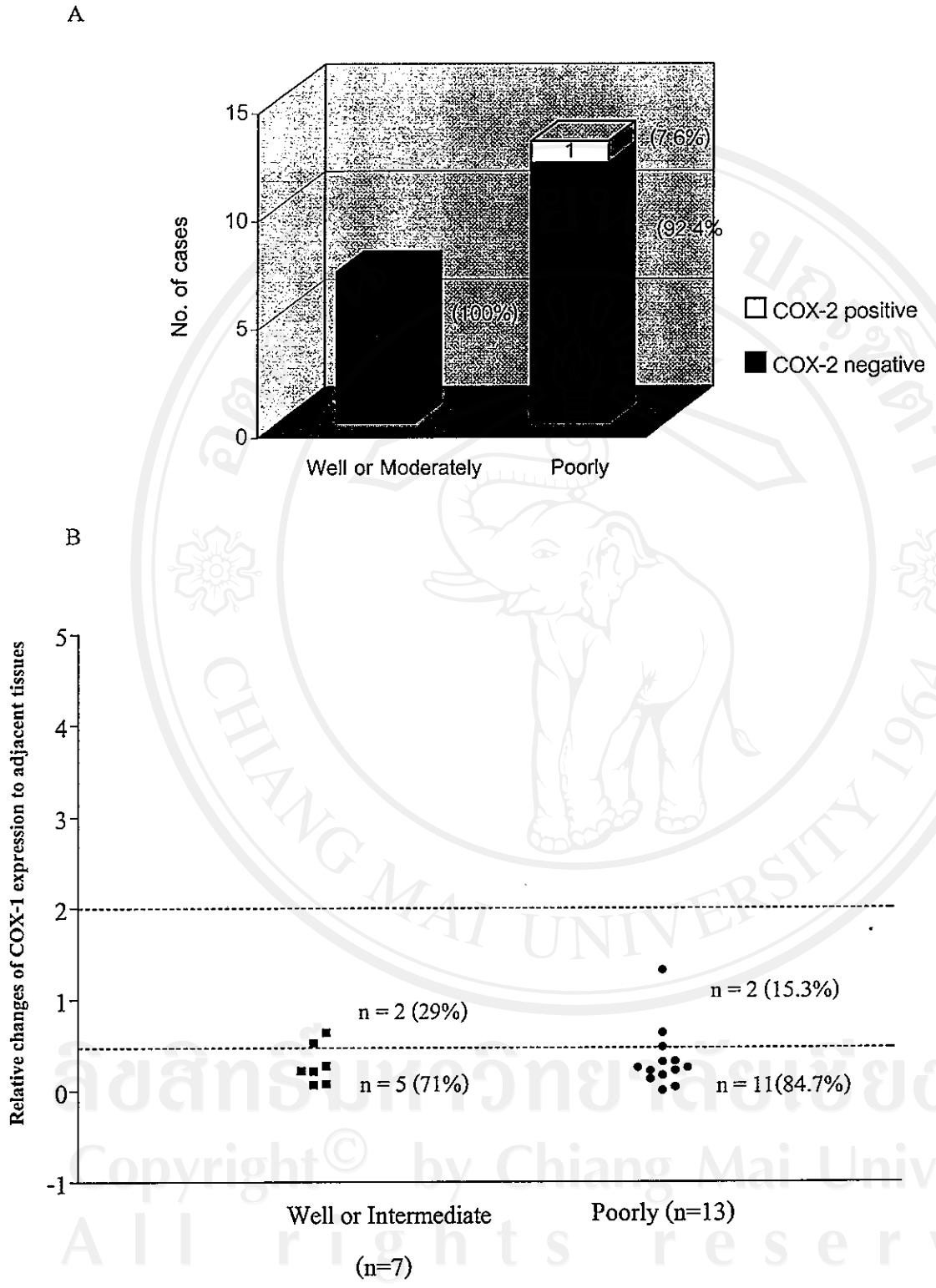


Figure 16. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to histological grading of the stomach tumors; well (defined as well differentiation), intermediate (defined as intermediate differentiation), and poorly (defined as poorly differentiation); n indicates the number of cases in each group.

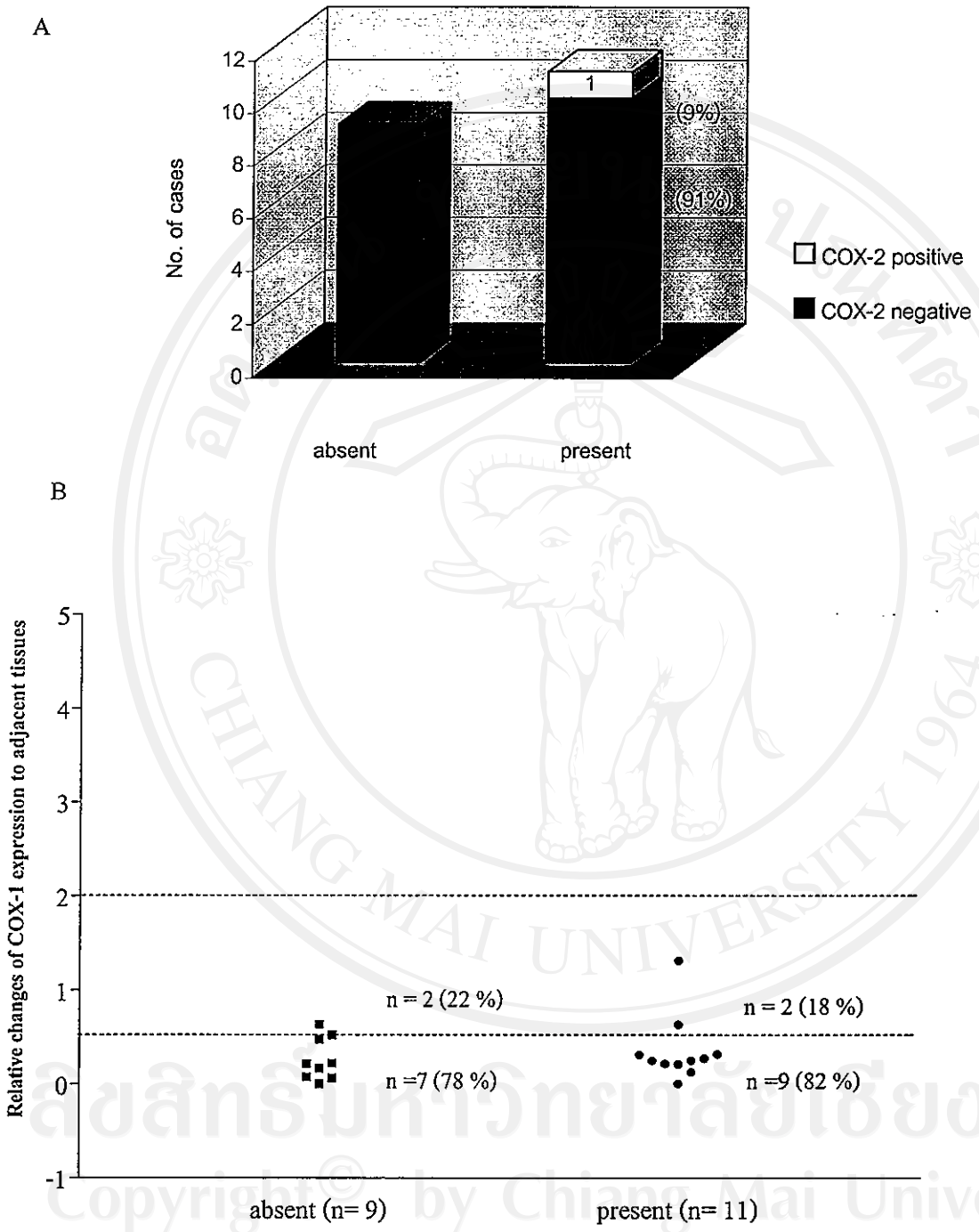


Figure 17. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to lymph node metastasis of the stomach tumors; n indicates the number of tumors in each group.

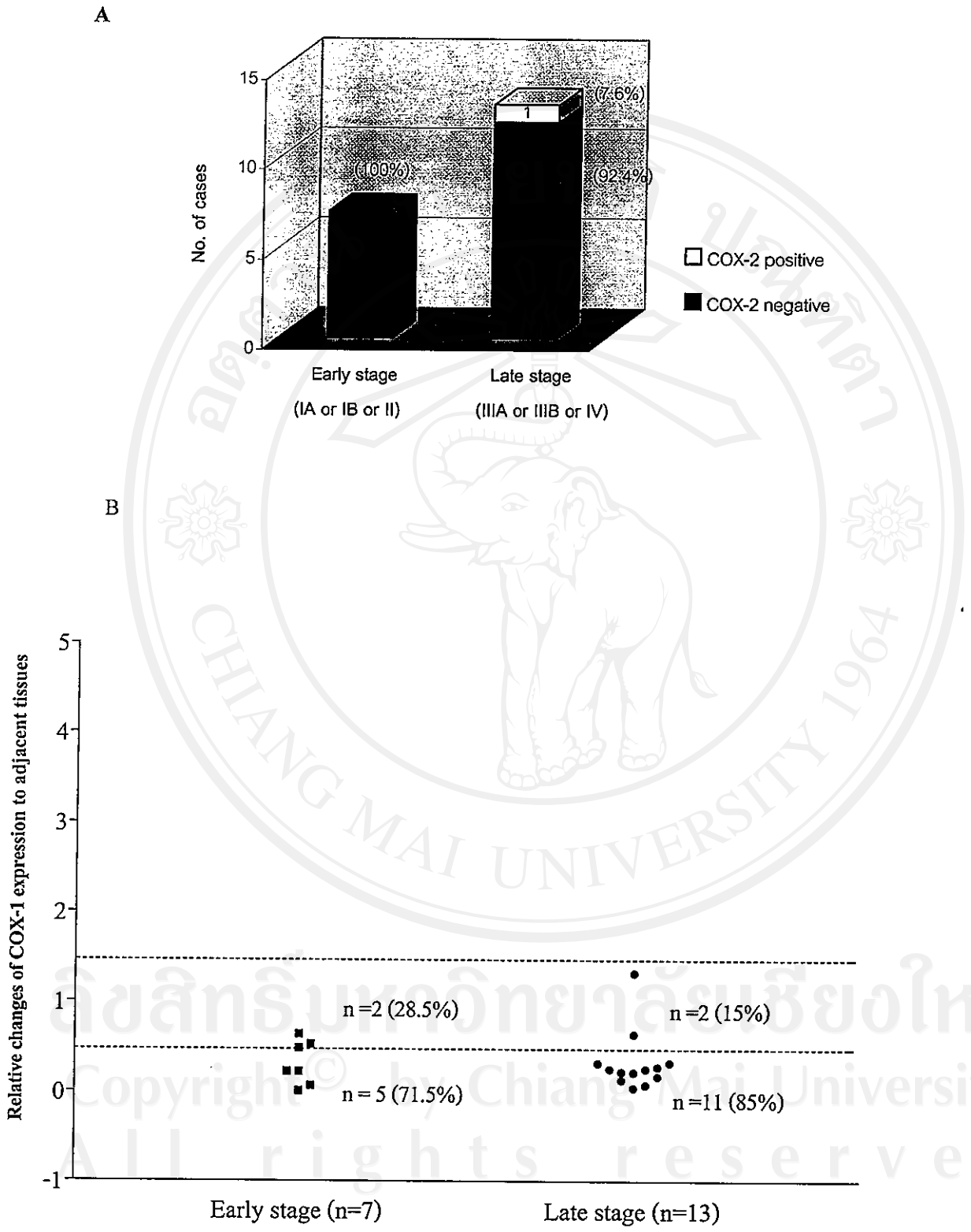


Figure 18. Distribution of COX-2 overexpression (A) and COX-1 alteration (B) in relation to stage of the stomach tumors; early stage defined as tumors stage IA-IB or II and late stage defines as tumor stage IIIA-IIIB or IV

Table 6. Summary of relationship between COX-2 expression and pathological features in colorectal and stomach cancers

Pathological features	COX-2 overexpression	
	Colorectal cancer (cases)	Stomach cancer (cases)
No. of patients		
Colorectal cancer (Total 44 cases)	13 (29.5%)	-
Stomach cancer (Total 20 cases)	-	1 (5%)
Tumor size		
<5 cm	6 (25%)	-
≥5 cm	7 (35%)	1 (6.6%)
Histological differentiation*		
Well or Moderate	8 (21.6%)	-
Poor	5 (71.4%)	1 (7.6%)
Depth of invasion		
Early cancer (M or SM)	-	-
Advanced cancer (MP or SS or SE)	13 (30.2%)	1 (5.2%)
Lymph node metastasis		
Absent	5 (22.7%)	-
Present	8 (36.3%)	1 (9%)
Distant metastasis		
Absent	11 (28.2%)	1 (5.5%)
Present	2 (40%)	-
Lymphatic invasion		
Absent	1 (20%)	1(20%)
Present	12 (30.7%)	-
Venous invasion		
Absent	12 (40%)	1 (7.6%)
Present	1 (7.1%)	-
Perineural invasion		
Absent	12 (29.2%)	1 (6.6%)
Present	1 (33.3%)	-
Tumor stage grouping		
Early stage (I or II), (IA or IB or II)	4 (19%)	-
Late stage (III or IV), (IIIA or IIIB or IV)	9 (39.1%)	1 (7.6%)

*P<0.05, according to Chi-square test.

Table 7. Summary of relationship between COX-1 expression and pathological features in colorectal and stomach cancers

Pathological features	COX-1 expression	
	Decrease:Not change:Increase	Decrease:Not change
	Colorectal cancer (cases)	Stomach cancer (cases)
No. of patients		
Colorectal cancer (Total 44 cases)	21:13:10	-
Stomach cancer (Total 20 cases)	-	16:4
Tumor size		
<5 cm	13:5:6	5:0
≥5 cm	8:8:4	11:4
Histological differentiation		
Well or Moderate	16:12:9	5:2
Poor	5:1:1	11:2
Depth of invasion		
Early cancer (M or SM)	1:0:0	1:0
Advanced cancer (MP or SS or SE)	20:13:10	15:4
Lymph node metastasis		
Absent	12:4:6	7:2
Present	9:9:4	9:2
Distant metastasis		
Absent	18:12:9	14:4
Present	3:1:1	2:0
Lymphatic invasion		
Absent	2:2:1	4:1
Present	19:11:9	12:3
Venous invasion		
Absent	15:10:5	10:3
Present	6:3:5	6:1
Perineural invasion		
Absent	18:13:9	12:3
Present	3:0:1	4:1
Tumor stage grouping		
Early stage (I or II), (IA or IB or II)	10:4:5	5:2
Late stage (III or IV), (IIIA or IIIB or IV)	11:9:5	11:2