III. METERIALS AND METHODS

1. Tissue collection and storage

All tissues in this study were obtained from Thai population who had undergone surgical resections for primary stomach or colorectal cancer at Maharaj Nakorn Chiang Mai Hospital during April 2002 to June 2003. Of these, 20 and 40 specimens were stomach and colorectal cancer tissues, respectively. In each case, adjacent normal mucosa was collected for comparison. These specimens were immediately placed in vials, frozen in embedded medium for the preservation of cell integrity, and stored at –80 °C until analyzed. They were diagnosed by a pathologist according to pathological features of the tumors, which included tumor size in maximal diameter, depth of invasion, venous invasion, lymphatic invasion, perineural invasion, histological grading, lymph node metastasis, distant metastasis, and tumor staging (the AJCC TNM classification). Such patients' data were searched from the outpatient department (OPD) card at the administration and clerical section, Maharaj Nakorn Chiang Mai Hospital. The study processes were thoroughly accepted by the ethical committee of the faculty of medicine, Chiang Mai University according to document number 56/2545.

2. Preparation of tissue homogenate

Frozen tumors and paired normal tissues were thawed at room temperature (RT). Then, the tissue samples were weighed and cut into small pieces. Approximate weights (0.08-0.3 g) were lysed in 0.069M SDS lysis buffer (see Appendix), which was supplemented with protease inhibitors in 1/100 ratio by volume of SDS lysis buffer. The sample was homogenized using a bench-top homogenizer until became homogeneous and immediately heated for 10 min at 95 °C to activate the denaturing activity of SDS. After that, the samples were centrifuged at 15,000 x g for 15 min at 4 °C to remove the cell debris (Lim *et al.*, 2000), and then the supernatant was removed and total protein concentration in each tissue homogenate sample was measured using protein assay kit. If not assay on the same day, the sample was frozen at –80 °C until used.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

3. Estimation of protein concentration of tissue homogenate

Protein concentration was estimated using BCA protein assay kit purchased from PIERCE, which based on the method developed by Smith in 1985 (Smith *et al.*, 1985). The principle of this method is based on reduction of cupric cation (Cu^{2+}) to cuprous cation (Cu^{1+}) by protein (peptide bonds) in an alkaline medium (the biuret reaction) with the highly sensitivity and selective colorimetric detection of the cuprous cation using a unique reagent containing BCA. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a broad working range of 0.02-2 μg/μl.

3.1 Preparation of BSA (bovine serum albumin) standard

The 2.0 mg/ml BSA stock standard protein was diluted with dH_2O to various concentrations for preparing protein standard curve, which described below in order to determine the protein concentration of unknown samples.

Volume of stock standard (μl)	Volume of dH_2O (μl)	Final concentration (mg/ml)
400	0	2
240	160	1.2
200	200	1.0
160	240	0.8
120	280	0.6
80	320	0.4
40	360	0.2
0	400	0

3.2 Preparation of BCA working reagent

Prior to the assay, fresh working reagent was prepared by mixing 50 parts of reagent A with 1 part of reagent B. An appropriate amount for 96-well plate detection was 20 ml of reagent A combined with 0.4 ml reagent B. This was stable at RT for at least one day.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

3.3 Protein measurement by using microplate

The tissue homogenate was diluted (normally 1:5 to 1:10) with water if necessary to achieve concentration within the linear range of standard curve. Ten microlitres of each sample was added in quadruplicate to a 96-well plate together with BSA standard protein ranging from 0.2-2 mg/ml. Then, 190 μl of the working reagent was added to each well. The plate was covered with cling film and incubated at RT for 30 min. After incubation, the absorbance was measured at or near 562 nm (550 nm) on a microplate reader (Bio Kinetics Reader EL340, USA). The average absorbance of all samples was determined and a standard curve was plotted using Microsoft excel. The protein concentration for each unknown sample was calculated by using the formula as showed below.

$$Cu = (Au / As) \times Cs$$

Where:

Cu = Concentration of unknown

Cs = Concentration of standard (2 μg/μl)

Au = Absorbance of unknown

As = Absorbance of standard

Finally, these protein concentrations would be used to calculate a volume of tissue homogenate needed for gel electrophoresis (25 μg). For example, If the tissue homogenate concentration was 18 μg/μl, 5 μl of the homogenate would be used to prepare as loading mixture that was made up to 100 μl by adding 10 μl of a 1:1 (volume: volume) of bromphenol blue and 2-mercaptoethanol (2-ME) and 85 μl of SDS lysis buffer.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

4. Assessment of COX protein expression by and Western blotting

Western blot is technique for the identification and quantitation of protein. This technique depends on the electrophoretic transfer of proteins from the gels to membrane and blotted proteins on an exposed surface is blocked by blocking solution, such as BSA or skimmed milk, to prevent unoccupied protein binding site from non-specific binding of antibody. The blocked blot is then detected the target protein by antibody probing, which is two steps using primary and secondary antibody. The specific primary antibody is used to detect the protein of

interest, while the secondary antibody is directed against the primary antibody and conjugated with the enzyme that is hoseradish peroxidase (HRP). Following binding and washing, detection substrates for chemiluminescent system are applied to the blot to visualize the band detection.

The principle of chemilunescent system, enhanced chemiluminescence (ECL), bases on the detection of light emission that is achieved by performing the oxidation of luminol by the HRP in the presence of chemical enhancers such as phenols. The oxidation of luminol inalkaline conditions effects the luninol is in an excited state, which then decays to ground state via a light emitting pathway. The light produced by this ECL reaction peaks after 5-20 min and decays slowly thereafter with a half-life of approximately 60 min. The maximum light emission is wavelength at 428 nm, which can be detected by a short exposure to blue light sensitive autoradiography film. The bands are quantified using a densitometer.

4.1 Separation of protein by SDS-PAGE

SDS-PAGE is the most widely used technique for quantification of complex mixtures of proteins (Laemmli, 1970). In native form of the proteins, they fold into complex secondary, tertiary, and quaternary structures. Their surfaces may be hydrophobic or hydrophilic, with greater or lesser distribution of charge and reactive groups. The rate of migration of native proteins through a sieving medium relates to their shapes and molecular weight. SDS-PAGE is a technique that denatures the proteins to nullify structural effects on mobility by SDS, denaturant, allowing separation on a ratio of a charge to mass basis. They also separate subunits from multimeric proteins by 2-ME, which is a reducing agent to disrupt any disulfide bonds through reduction. SDS is an anionic detergent. It denatures proteins by binding to the protein chain with its negative charge molecule, dodecylsulfate group. A level of 1.4 g SDS binds to 1 g protein. This creates a charge to mass ratio that is consistent between proteins. Thus, separation on a SDS-PAGE occurs by mass alone.

The main principle of this method bases on the separation of proteins according to molecular size, as analyzing protein mixtures move through porous structure of polyacrylamide matrix. Polyacrylamide gels are formed from the polymerization of acrylamide monomer in aqueous solution in the presence of small amounts of a crosslinker, *N*, *N'*-methylenebisacrylamide (bis). Methylene group of bis is linked with two acrylamide molecules. The co-polymerization

Copyright © by Chiang Mai University
All rights reserved

of them produces a cross-linked matrix that is a mesh-like network. The polymerization proceeds via a free-radical catalysis and is initiated by the addition of ammonium persulfate and the tertiary aliphatic amine *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (TEMED). TEMED catalyzes the decomposition of the persulfate ion to produce a free radical. In addition, the pore size with polyacrylamide gel can be alternated by the total concentration and the ratio of acrylamide to bis.

Polyacrylamide gel is divided into an upper stacking gel and a lower running gel. The stacking gel has a large pore size, 2-4% acrylamide, which allows the proteins to move freely, and concentrate under the effect of the electric field. The running gel has a smaller pore, which can be altered in reproducible manner depending on size of analyzing protein. The recommended acrylamide concentrations for separation of different ranges of protein size are shown below.

Separation size range (Kda)	% Acrylamide in running gel
36 – 205	5%
24 – 205	7.5%
14 – 205	10%
14 – 16	12.5%
14 – 45	15%

4.1.1 Preparation of running gel (10%)

Two clean glass plates and spacer previously washed with water and ethanol were assembled and locked onto the gel-casting stand (Scie Plus, UK). The running gel solution was prepared following the method showed in Appendix. The solution was poured carefully into the slab mold until reached a level that leave the space for the comb to be inserted and 1 cm below the bottom of the wells. To eliminate the meniscus, a thin layer of water is layered on the surface of the gel mixture before it polymerized. It was allowed to polymerize at RT for 45 min and its polymerization was from a line that became visible at the top of the gel.

Copyright © by Chiang Mai University
All rights reserved

4.1.2 Preparation of stacking gel (4%)

After polymerization, the layer of water was poured off from the top of the gel. The stacking gel solution was prepared following the method shown in Appendix. The solution was poured slowly onto the polymerized running gel until reached the top of the plates.

Then, the comb was inserted into the layer of the stacking gel solution and allowed to polymerize at RT for 45 min.

4.1.3 Preparation of the sample and electrophoresis

The set of slab clamp with a solidified gel was removed from the casting stand and placed in the gel tank. The running buffer (see Appendix) was filled into the upper and lower chamber with a necessary volume of buffer. Ensure that the sample wells were fully filled with the buffer. Then, the comb was removed by pulling straighten up slowly to avoid any damage of the well. After working out the amount of tissue homogenate needed and mixed with sample buffer as described previously, the protein mixture was heated at 95 °C for 5 min. First lane on each gel was loaded with 5 μl of molecular weight marker of protein (Amersham International plc, UK), while 25 μg protein of each sample mixture was applied onto the other lanes. Following application, the gel was run at 190 volts of constant voltage untill the bromphenol blue tracking dye reached the bottom of the running gel for 1-1.5 hr, which electrophoresis was terminated.

After electrophoresis, the running buffer was discarded and the gel apparatus was dismantled. The glass-plate sandwich was pried open to remove the gel that was placed in the transfer buffer for further the blotting step.

4.2 Transfer of proteins

While the gel was running, two pieces of fiber pad, one piece of nitrocellulose membrane (8x6 cm in size, Amersham Pharmacia Biotech, UK) and two pieces of filter paper (9x7 cm in size) were soaked in transfer buffer (see Appendix). Once the bromphenol blue had reached the bottom, the gel was removed from the electrophoresis tank. The following items were assembled in order starting from the black side of the cassette: fiber pad, filter paper, gel, membrane, filter paper, fiber pad, and white cassette clamp, respectively. The glass rod was used when necessary to smooth out any air bubble. The assembly was placed in the transfer tank with orientation of the black cassette closest to the negative electrode. Then, the transfer buffer was filled in the tank until reached the maximum filled lines. Electroblotting was performed by applying 30 volts at constant voltage for overnight and the water cooled base of the tank was

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

rinsed thoroughly with water to prevent overheating heating effects of the system. After transfer, the transfer sandwich was dismantled and then the blotted membrane was removed and placed in TBS-Tween buffer at 4 °C until detected.

4.3 Immunodetection

The blotted membrane was placed in plastic container and incubated with 20 ml of 5% BSA in TBS-Tween for 1 hr at RT on a shaker (Gemmy VRN-200, Germany) to block non-specific binding sites. Primary antibody, mouse COX-2 monoclonal antibody (Cayman Chemical, USA), was diluted (1:1,000) in 5% skimmed milk in TBS-Tween. Then, the blocking buffer was poured off and the blot was incubated with primary antibody for overnight at 4 °C on a shaker following by washing with six changes of TBS-Tween each for 20 min. Secondary anti-mouse immunoglobulins antibody conjugated with HRP (Dako Denmark) was diluted (1:1,000) in 5% skimmed milk in TBS-Tween. After washing membrane, the blot was incubated for 2 hr at RT on a shaker with 8 ml of diluted secondary antibody then washed with six changes of TBS-Tween each for 20 min.

4.4 Visualization of detected protein band

Prior to the detection, an equal volume of detection solution A with detection solution B, ECL detection reagents (Amersham Biosciences, UK), was mixed allowing sufficient total volume to cover the blot (3 ml/membrane). The excess TBS-Tween was drained off from the washed membrane by touching the edge against a tissue paper and the membrane was placed on the piece of cling film, protein sided up. Then, the mixed detection solution was added to the protein side of the membrane and incubated for 1 min at RT following by draining off excess detection solution. After that, the membrane was placed on to a new piece of cling film, protein sided down, and wrapped up and gently smoothed out any air bubbles. The wrapped membrane was placed in the film cassette; protein sided up. In the dark room, a sheet of X-ray film (Eastman Kodak, USA) was placed on top of the membrane and the cassette was closed then exposed for 1, 5, 10 min or the required for optimal detection depending on the level of band signal and film background. After exposure, the film was performed as followed: It was removed from the cassette, immediately developed in developing solution for 1 min, washed in

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

dH_2O for 1 min (Eastman Kodak, USA), fixed in fixing solution (Eastman Kodak, USA) for 5 min, washed in dH_2O for 5 min, and let it dry at RT. For quantification of protein bands, the resultant film was then scanned the band intensities using a densitometer.

4.5 Stripping and reprobing membrane

The membrane detected previously was placed in plastic container and soaked in 20 ml of stripping buffer (see Appendix) in water bath at 50 °C for 30 min to remove primary and secondary antibodies from the membrane. Then, it was washed with six changes of TBS-Tween each for 20 min. The membrane was blocked with 20 ml 5% BSA in TBS-Tween for 1 hr at RT on a shaker. The immunodetection protocol was repeated, stage 4.3 to 4.4, to sequential reprobe the membrane with a variety of antibodies, which are 1:1,000 diluted mouse COX-1 antibody and 1:1,000 diluted mouse β-actin antibody, respectively. The incubation times for β-actin detection were as followed: 1 hr at RT with primary antibody, 3 x 20 min washing, 1 hr with secondary antibody, and 3 x 20 min washing, respectively.

5. Classification of pathological features

Serial section cut from each paraffin embedded block were stained with hematoxylin and eosin for morphological examination by a pathologist in a blinded fashion without knowledge of the results of Western blotting. These specimens were subjected to pathological examination, which identified tumor size (small, <5 cm; large, ≥5 cm), depth of invasion (early cancer, mucosa or submucosa; advanced cancer, muscularis propria or subserosa or serosa), lymph node metastasis (absent or present), distant metastasis (absent or present), venous invasion (absent or present), lymphatic invasion (absent or present), perineural invasion (absent or present), and histological grading (well differentiated, moderately differentiated, poorly differentiated). The pathological tumor staging and grouping were determined according to the AJCC TNM classification (as described previously in Table 1 and 2).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

6. Investigation of the relationship between COX protein expression to the pathological features

After Western blotting and detection, the expression of COX-2 in tumor tissues and the paired adjacent normal tissues was designated as the presence or absence of the COX-2 band on the film by an eyesight and then compared with the malignant potential and TNM stage grouping using statistical test. On the other hand, the expression of COX-1 in these tissues was designated as the band density ratio of COX-1/β-actin, which the intensity of each band was semi-quantified using a densitometer. The β-actin was used as internal control to confirm equal of protein loading in each lane, as is believed to be one of the house keeping gene. The band intensities were expressed as ratio of COX-1/β-actin before subjected to comparison.

7. Statistical analysis

Chi-square method was used to test significance of the difference in the correlation between COX-1 or COX-2 expression in the tumors and pathological parameters and $p<0.05$ was considered as the statistically significant value.

CHIANG MAI UNIVERSITY 1964
ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved