CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Specimens

Tissue samples were collected from patients who underwent curative surgery for lung cancer at Maharaj Nakorn Chiang Mai Hospital, Thailand. In each case, adjacent normal mucosa was also collected. These specimens were immediately placed in vials, frozen in embedded medium to preserve cell integrity, and stored at –70 ^{o}C until analyzed. They were diagnosed by a pathologist according to pathological features of the tumors. The study processes were approved by the ethic committee of the Faculty of Medicine, Chiang Mai University.

2.1.2 Instruments

Instruments used in this study are indicated in the APPENDIX A.

2.1.3 Chemical and Reagents

All chemicals used in this study were shown in the APPENDIX B and APPENDIX C.

2.1.4 Other Materials

Other materials used in this thesis were listed as follow:

- Film development solutions (Kodak GBX, CAT. 1900984)

- Kodak medical X-ray film (Eastman Kodak Company, USA. No. REF 822 5526)
- Polyvinylidene Fluoride (PVDF) Transfer Membrane 0.45 µm (Bio Trace, USA. No. P/N 66543)

2.2 Methods

2.2.1 Preparation of cell lysates from lung tissues

Frozen tissues from lung cancer patients were thawed, diced into small pieces and weighted. The cell lysate preparation was carried out by homogenizing of 30 g-100 g of tissue in 500 µl of SDS-lysis buffer (see APPENDIX C) containing a protease inhibitor cocktail (see APPENDIX B). The homogenate was sonicated on ice by an ultrasonic sonicator (Ultrasonic Processor UP50H; Hielscher) and heated at 95 ^{o}C for 10 minutes to activate the denaturing activity of SDS. Then the mixture was centrifuged at 10,000 g, 4 ^{o}C for 15 minutes by using microcentrifuge (Eppendorf centrifuge 5417R, Eppendorf). After that, clear supernatant was carefully transferred into a clean microcentrifuge tube. Cell lysate was stored at -20 ^{o}C until used. Protein concentration in the obtained cell lysate was measured using BCA (bicinchoninic acid) protein assay kit (see APPENDIX B) utilizing chemical principle as shown below:

$$Cu^{2+} + \text{peptide bonds} \xrightarrow{OH} \text{tetradentate} - Cu^{1+} \text{ complex}$$

$$\text{tetradentate} - Cu^{1+} \text{ complex} + 2BCA \longrightarrow BCA\text{-}Cu^{1+}\text{-complex}$$

(violet color)

The intensity of violet color product was measured at 562 nm and protein concentration was calculated from bovine serum albumin (BSA) standard curve.

2.2.2 Identification of the protein which is reacted to CM5pAb

2.2.2.1 Purification of protein by immunoprecipitation

Immunoprecipitation (IP) is a method that uses the antigen-antibody reaction principle to identify a protein that reacts specifically with an antibody from mixture of proteins so that its quantity or physical characteristics can be examined. Cell lysates from tumor tissues homogenized in SDS lysis buffer containing 2% SDS were diluted with phosphate buffered saline (PBS), pH 7.5 in order to obtain 1 mg/ml of protein and less than 0.1% (w/v) of SDS final concentration. The diluted cell lysate was subjected to a preclearing step by incubating with suspended protein G coated agarose (see APPENDIX B) at 4°C for 45 minutes. The pre-clearing step helps reducing non-specific binding of proteins to agarose beads. Subsequently, the precleared cell lysate was incubated with anti-mouse p53 CM5pAb at 4°C for 4 hours before adding precleared beads into the reaction tube and continued incubation at 4°C overnight. After extensive washing, the immunoprecipitated proteins were eluted from the bead particles by adding SDS lysis buffer containing 2-mercaptothanol and heated at 95°C for 5 minutes. The eluted protein was then resolved through SDS-PAGE (10% gel) (see section 2.2.2.3) and stained with commassie blue.

2.2.2.2 Protein identification by LC-MS/MS (Liquid chromatography – tandem mass spectrometry)

After SDS-PAGE analysis of the immunoprecipitated CM5pAb reactive protein, the protein band migrated at 95 kDa was excised and subjected to protein identification by mass spectrometry at the Genome Institute of Thailand (BIOTEC). The excised protein was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) machine model Finningan LTQ Linear Ion Trap Mass Spectrometer using a Finnigan SurveyorTM MS pump with a flow splitter HPLC system (Thrmo Scentific) according to the the protocol previously described by Mitprasat et al [65].

2.2.2.3 Protein determination by Western blot analysis

(1) Separation of protein according to their molecular weight by SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their apparent sizes via the mechanism of the anionic detergent SDS making the surface charge uniformly negative. When applied the protein extract onto a gel matrix and placed in an electrical field, the negatively charged protein molecules move toward the positively charged electrode at rates dependent upon their molecular weight. A small protein molecule can move through the gel easily and hence migrates faster than a larger one. The size of a protein can be estimated by comparison of its migration distance with that of a known molecular weight standard protein marker.

Electrophoresis was run as follows. The glass plates were first cleaned in detergent, washed with water and ethanol and allowed to dry. They were aligned and taken to the clamp. The separating gel solution (7%, see APPENDIX C) was prepared and poured onto the glass plate quickly before the acrylamide polymerized. The monomer solution was immediately overlaid with distilled water. The gel was allowed to polymerize for 30 minutes. The overlaied solution was completely rinsed off with distilled water. Then, the staking gel solution (4%, see APPENDIX C) was prepared and poured onto the top of the separating gel. The comb was inserted into the gel solution and the gel was allowed to polymerize for 15 minutes. After polymerization was completed, the comb was taken off by pulling it straight up slowly and gently. The wells were completely washed with distilled water. After that, the gel was placed in the electrophoresis chamber. The 1X running buffer (see APPENDIX C) and filled into the reservoir until reached a level. Ensure that the sample wells were fully filled with the buffer. After working out the amount of tissue homogenate needed and mixed with 2x sample buffer (see APPENDIX C), the protein mixture was heated at 95 ^{o}C for 5 minutes. First lane on each gel was loaded with 5 µl of molecular weight marker of protein (see APPENDIX B), while 30 µg protein of each sample mixture was applied onto the other lanes. Following application, the gel was run at 125 volts of constant voltage until the bromphenol blue tracking dye reached the bottom of the running gel for 1.5-2 hours, 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.

(2) Protein transfer by electroblotting

The separated proteins on the gel were transferred to PVDF membrane. While the gel was running, two pieces of fiber pad, one piece of PVDF membrane (8.5x6 cm in size) and two pieces of filter paper (9x7 cm in size) were soaked in 1X transfer buffer (see APPENDIX C). 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, PVDF membrane, filter paper, fiber pad and red casstte 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 1X transfer buffer was filled in tank until reached the maximum filled lines. Electroblotting was performed by applying 30 volts at constant voltage for overnight at 4 ^{o}C to prevent overheating heating effects of the system.

(3) Immunodetection of the protein of interest

After blotting, the PVDF membrane was incubated sequentially with 5% skimmed milk in TBS containing 0.05% Tween-20 (TBS-Tween), blocking solution, for 1 hour with shaking at room temperature to block non-specific binding. Then the membrane was incubated with primary antibodies diluted in blocking solution for 1 hour with shaking at room temperature. List of antibodies and dilution used in this study is shown in Table 2.1. Afterward, the excess primary antibodies were removed by washing 6 times, 10 minutes each with washing buffer (see APPENDIX C). The next step, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) at a 1:1,000 dilution in blocking buffer for 1 hour with shaking at room temperature. Then they were washed with washing buffer eight

times, 8 minutes each to remove excess antibodies. After an extensive washing with TBS-Tween the bound proteins were visualized with a chemiluminescence-based procedure.

Table 2.1 List of antibodies and dilution used in this study

Antibodies	**Catalogue number** **Company**	**Working dilution**
Primary antibody		
- Anti-GAPDH antibody [6C5]	- Cat. No. ab8245 Abcam, Japan	1 : 1,000
-p53 protein (DO-7) liquid mouse monoclonal antibody	- Cat. No. NCL-L-p53-DO7 Novocastra, UK.	1 : 1,000
- Lyophilized rabbit polyclonal antibody p53 protein (CM5)	- Cat. No. NCL-p53-CM5p Novocastra, UK.	1 : 500
- Rabbit polyclonal antibody Glucosidase IIβ (H-195)	- Cat. No. sc-10774 Santacruze, inc., USA.	1 : 1,000
- Rat Monoclonal Anti-GRP78 (76-E6)	- Cat. No. sc-13539 Santa cruz, inc., USA.	1 : 200
- Mouse Monoclonal Anti-GRP94 (2H3)	-Cat. No. sc-53929 Santa cruz, inc., USA.	1 : 200
Secondary antibody		
- Polyclonal Goat Anti-Mouse Immunoglobulins/HRP	- Cat. No. P0447 DakoCytomation, Denmark.	1 : 1,000
- Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP	- Cat. No. P0448 DakoCytomation, Denmark.	1 : 1,000
- Peroxidaase-Conjugated Rabbit Anti-Rat Immunoglobulins	- Cat. No. P0450 DakoCytomation, Denmark.	1 : 1,000
- Rabbit TrueBlot: Anti-Rabbit IgG HRP	- Cat. No. 18-8816 eBioscience,inc., USA	1 : 1,000

(4) Visualization of the immunodetected protein by chemiluminescence (ECL)-based procedure

The principle of enhanced chemiluminescence (ECL) bases on the detection of light emission that is achieved by performing the oxidation of luminol by the HRP in presence of chemical enhancers such as phenols. The oxidation of luminol in alkaline conditions activates the luminol into 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 minutes and decays slowly thereafter with a half-life of approximately 60 minutes. The maximum light emission is wavelength at 428 nm, which can be detected by a short exposure to blue light sensitive autoradiography film (X-ray film). In this study, the detection reagent was added to the protein side of the membrane. The excess buffer was drained and the membrane was placed on new piece of cling film. Then, the wrapped membrane was placed into the film cassette; protein sided up. The work was carried out as quickly as possible in order to minimize the delay between incubating the membrane in the detection reagent and exposing it to the film. In the dark room, a sheet of X-ray film was placed on top of the membrane and the cassette was closed then exposed for optimal detection time depending on the level of band signal and film background. After that, the film was performed as followed: It was removed from the cassette, immediately developed in developing solution for 1 minute, washed in water for 1 minute, fixed in fixing solution for 1 minute, wash in water for 1 minute and let it dry at room temperature. Finally, the protein band was quantitated using a Quantity One program.

2.2.3 Characterization the CM5pAb reactive protein

2.2.3.1 Cell and culture condition

Characterization of the CM5pAb reactive protein in response to stress signals was carried out utilizing a human lung adenocarcinoma epithelial cell line (A549) as a cell model. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) medium with 110 mg/ml pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and supplemented with 10% fetal bovine serum. The cell line was grown at 37 ^{o}C under 5% CO_2 atmosphere.

2.2.3.2 Investigation of genotoxic stress response

In order to induce DNA damage and genotoxic stress, cutured cells were irradiated with UV (Ultraviolet) ray. In order to get an optimal dose of UV irradiation, 5 x 10^5 A549 cells were seeded into 100 mm dish containing 10 ml of DMEM medium and cultured at 37^{o}C in a humidified incubator with 5% CO_2 overnight. The following morning, after cells reaching 80% confluent, culture media was removed and cells were irradiated with various doses of UV 287 nm (0, 8, 16, 32, 48, 64 J/m^2) in order to induce DNA damage. After irradiation, fresh culture media was immediately added. Culture was continued at 37°C, 5% CO_2 and the whole cell lysate was prepared 3 hrs thereafter by adding 200 µl of SDS lysis buffer (see APPENDIX C). The irradiated cells were scraped into a microcentrifuge tube and sonicated with an ultrasonic sonicator (Ultrasonic Processor UP50H, Hielscher) on ice and heated at 95 ^{o}C for 10 minutes. Cell lysate was then centrifuged for 10 minutes at 4 ^{o}C and 14,800 g (Eppendorf centrifuge 5417R, Eppendorf) and the obtained

supernatant was subjected to Western blot analysis in order to determine protein expression level of p53.

Afterward A459 cells were irradiated with the optimal dose and cell lysate was prepared at different time points (3, 6, 24, 36 hours) in the same manner as previously described and subjected to Western blot analysis. Blots were probed with specific antibodies against human p53, glucosidase II, GRP (glucose regulated protein)-78 and GRP-94 to characterize changes in the level of these proteins. Blots were also probed for GAPDH to check for equal loading of protein.

2.2.3.3 Investigation of ER stress response

In order to induce ER stress, tunicamycin was used to block the synthesis of all N-linked glycoproteins (N-glycans) thus causing ER stress[66]. To get the optimal dose of tunicamycin, A459 cells were cultured in DMEM media containing various concentration of tunicamycin (1, 3, 5 μg/ml) and the whole cell lysate was prepared 3 hrs thereafter with the same procedure as previous described in section 2.2.3.1. Afterward A459 cells were cultured with the optimal dose of tunicamycin and cell lysate was prepared at different time points (3, 6, 24, 36 hours) in the same manner as previously described and subjected to Western blot analysis. Blots were probed with specific antibodies against human p53, glucosidase II, GRP (glucose regulated protein)-78 and GRP-94 to characterize changes in the level of these proteins. Blots were also probed for GAPDH to check for equal loading of protein.

2.2.3.4 Investigation of protein sub-cellular localization in response to stress signal

In order to investigate the sub-cellular localization of the CM5pAb reactive protein and p53 protein in response to genotoxic stress and ER stress, A459 cells were cultured and treated with optimal concentration of UV irradiation or tunicamycin as described in section 2.2.3.2 and 2.2.3.3, respectively. Sub-cellular fractionation of cell lysate was prepared using nuclear isolation buffer (NIB; see APPENDIX C) to separate the nuclear and cytoplasmic fractionations. Cell pellets obtained 100 mm-culture dish was lysed with 1 ml of cold-NIB and incubated on ice for 10 minutes. The mixture was then centrifuged at 1,000g for 5 minutes (Eppendorf centrifuge 5417R, Eppendorf). During this step, the nuclear fraction was pelleted at the bottom of the tube, whereas the cytosolic fraction was suspended in the supernatant. The supernatant was transferred to a separate tube and 100 µl of SDS lysis buffer (see APPENDIX C) was added to the nuclear pellet. The cytosol contained in the supernatant was then concentrated by precipitating with cold acetone. Five milliliters of cold acetone was added to the supernatant and incubated at -20°C for 10 minutes. The protein precipitates were then pelleted by centrifugation at 4,000 rpm (Eppendorf centrifuge 5417R, Eppendorf) for 30 minutes. The cytosolic fraction was resuspended in 100 µl of SDS lysis buffer. Both the nuclear and cytosolic fraction were then boiled at 95°C for 10 minutes and subjected for Western blot analysis.