

## CHAPTER II

### MATERIALS AND METHODS

#### 2.1 Chemicals and materials

Name of chemicals and reagents, equipments and instruments are shown in Appendix A and B, respectively, whereas the details of the reagents and buffer used in this study are shown in Appendix C.

#### 2.2 Methods

##### 2.2.1 Sample preparation

##### 2.2.1.1 Sample information

Human urine samples from 48 lung cancer patients, after receiving any anticancer treatments were consecutively collected at Maharaj Nakron Chiang Mai Hospital, Chiang Mai, Thailand, from September 2011 to March, 2012. The study population included 33 (69%) male and 15 (31%) female patients, with a mean age of 53.3 (range 28-74 years), who had been diagnosed with lung cancer at advanced stages (7 patients in stage III and 41 in stage IV). The histology type included 25 adenocarcinoma, 15 small cell lung cancers, 4 squamous cell carcinoma and 4 other types of carcinoma. The control group was collected from Clinical Chemistry Laboratory, Maharaj Nakron Chiang Mai Hospital, 44 healthy controls donated urine samples. The study was approved by the research ethics committee, Faculty of Medicine, Chiang Mai University for research purpose only (No. 412/2011).

In addition, urine and serum samples from 133 lung cancer patients, before receiving any anticancer treatment were consecutively collected at National Taiwan University Hospital, Taipei, Taiwan from January 2012 to June 2013. The study population included 61 (46%) male and 72 (54%) female patients, with a mean age of 62 (range 30-81 years). Forty-eight were smokers and 85 were never smokers. The histology type included 109 adenocarcinoma, 4 small cell lung cancer, 11 squamous cell

carcinoma and 9 other types of carcinoma. There were 18 patients in stage I, 6 in stage II, 25 in stage III and 84 in stage IV. Sixty-six patients had tumors with activating mutations in epidermal growth factor receptor (EGFR), 58 patients had tumors with wild-type EGFR, and unknown EGFR mutation status in 9 patients. The control group was collected from health screen department, 26 healthy controls donated urine samples with a mean age of 60 (range 27-78 years) and 30 healthy controls donated serum samples with a mean age of 64 (range 30-73 years).

Paraffin-embedded pathologic tissue specimens from 143 NSCLC patients were obtained from the archives of the Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan. The cases selected were based on a distinctive pathologic diagnosis of NSCLC for patients who underwent surgical resection for tumor with curative intent without preoperative chemotherapy or radiotherapy from January, 2002 to December, 2006. The clinicopathological features, such as age, gender, cancer stage, date of recurrence, date of decease were collected retrospectively by medical chart record and cancer registry system. There were 79 (55%) male and 64 (45%) female patients with a mean age of 65.3 (range 33-82 years). There were 60 patients in stage I, 61 in stage II, 20 in stage III and 2 in stage IV. Lung tissues from unaffected parts of the patients with lung cancer were used as controls. Tumor stage was defined according to the American Joint Committee on Cancer/International Union against Cancer Tumor-node-metastasis (TNM) classification system [58]. The study was approved by the institute research ethic committee of National Taiwan University Hospital (NTUH REC no. 201103074RC). The clinical information of lung cancer patients are shown in Appendix D.

### **2.2.1.2 Sample collection**

Urine samples were collected in early morning (the first urination after waking up), and centrifuged at 12,000 ×g for 30 min at 4°C to remove cellular contamination and debris. The supernatants were loaded onto a centricon tube (MW cut off at 3 kDa) and centrifuged at 5000 ×g for 1 h at 4°C. Distilled water was added to the centricon tube to partially desalt and elute out some interference. Urine samples were then passed through a PD-10 desalting column and eluted with 10 mM phosphate buffer, pH 7.5.

The fraction containing proteins were collected and lyophilized. Serum samples were obtained from blood specimens that had been allowed to clot for 30 min at room temperature before centrifugation at 3,000 ×g for 10 min. The supernatants of the serum samples were collected and divided into a small number of aliquots. Tissue specimens were obtained within 30 min of surgical excision or biopsy. All of the specimens were stored at -80°C until further analysis.

### **2.2.1.3 Protein assay**

The concentrated urine and serum samples were determined for their protein contents using protein assay reagent (Bio-Rad) on 96-well microtiter plate platform. Bovine serum albumin was used as a standard protein (0.025 – 0.500 mg/mL) for preparing a standard curve. Triplicate samples were performed, standard derivation lower than 10% were accepted. Firstly, 10 µL of each standard and sample solution were carefully pipetted into individual wells. Secondly, 200 µL of 5-fold diluted dye reagent was added by avoiding presence of bubble. After incubation for 5 min, the absorbance at 595 nm was measured by microplate reader. The quantified sample was stored at -80°C until further analysis.

### **2.2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

In total, 15 µg of protein samples were present in sample buffer. The mixed samples were heated at 95°C for 10 min and subsequently fractionated on 15% SDS-PAGE. SDS-PAGE was performed according to Laemmli buffer system, run on Tris-glycine polyacrylamide slab gels (1.5 mm × 10 cm × 8 cm) in Bio-Rad Protein II apparatus at 60 mA for 30 min, and 120 mA for 80 min, respectively. The protein bands in the gel were visualized by staining with Coomassie blue staining or SYPRO® Ruby staining. The low-range molecular weight calibration kit was used as standard molecular weight protein marker.

### **2.2.3 Two-dimensional electrophoresis (2-DE)**

2-DE was performed with IPGphor system. IPG strip narrow range of 3-5.6 and 4-7 (18 cm length) were used in this experiment for the first dimension.

### **2.2.3.1 IEF/ the first dimension**

Two hundred micrograms of protein samples were dissolved in IEF buffer. The samples were sonicated, centrifuged and then applied onto IPG strip of pH 4-7 (18 cm length) for 2-DE analysis. To determine the glycan structure of GM2AP, 1000 µg of urine samples were loaded onto a narrow range IPG strip of 3-5.6 (18 cm length) for preparative analysis. The IPG strips were subsequently rehydrated on the IPGphor IEF system at 20°C with a gradual increase of Voltage (30 V for 14 h, 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3000 V for 1 h, 5000 V for 1 h, and focusing at 8000 V for up to 64000 Vh). The current of the IEF condition was limited at 50 µA to avoid overheating.

### **2.2.3.2 PAGE/ the second dimension**

After IEF, the strip was initially incubated in equilibration buffer with 2% (w/v) DTT for 15 min and then subsequently alkylated in the same equilibration buffer, but replacing DTT with 2.5% (w/v) IAA for 15 min in the dark. For electrophoresis in the second dimension, the strips were placed on top of the 15% polyacrylamide gel (18×18 cm, 1.5 mm) and covered with 0.5% (w/v) agarose. The second-dimensional separation was performed at 10 mA per gel for 30 min and then at 45 mA per gel, 4°C until the bromophenol blue dye front reached the bottom of the gel. After each run, the 2-DE gels were stained with SYPRO<sup>®</sup> Ruby and scanned using a Typhoon 9200 laser scanner. After scanning, the gel images were analyzed by the Image Master<sup>™</sup> 2D Platinum software version 5.0.

## **2.2.4 Protein visualization**

### **2.2.4.1 Coomassie Brilliant Blue staining**

After SDS-PAGE or 2-DE, the gels were fixed with fixative solution composed of 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min. Coomassie Brilliant Blue R-250 staining solution at 0.25% (w/v) was used to stain the gel by equilibration on an orbital shaker at room temperature for 1 h. The background and non-specific staining were washed out by immersion in the destaining solution I containing the same composition as the fixative solution, and destaining solution II that contained 10% (v/v)

methanol and 3% (v/v) acetic acid) until a protein band appeared clearly. Finally, the gel was briefly washed twice with Milli-Q water and scanned using general scanner.

#### **2.2.4.2 SYPRO® Ruby staining**

After SDS-PAGE or 2-DE, the gels were fixed with fixative solution for 30 min, rinsed twice with Milli-Q water and stained with the fresh solution of SYPRO® Ruby staining on an orbital shaker at room temperature for 1 h in darkness. The background and non-specific staining were washed out by immersion in the destaining solution II for 30 min and rinsed twice with Milli-Q water prior image scanning.

#### **2.2.5 Enzyme-linked immunosorbent assay (ELISA)**

Human GM2AP in urine and samples from lung cancer patients and healthy controls were analyzed using commercially available sandwich immunoassay from Uscn Life Science Inc., according to the instruction manual provided by the manufacturer. The sensitivity limit of the GM2AP assay was 0.156-10 ng/mL. Briefly, 100  $\mu$ L of the diluted standard and 100  $\mu$ L samples were added in duplicate to the well of a microtiter plate coated with an antibody specific to GM2AP. Dilution buffer alone was added to a pair of duplicate well to serve as blank. After incubation at 37°C for 2 h, 100  $\mu$ L of biotin-conjugated antibody specific to GM2AP was added to each well and incubated at 37°C for 1 h. The plate was washed three times with the wash solution, followed by incubation with 100  $\mu$ L of avidin conjugated HRP at 37°C for 30 min. After washing with the wash solution three times, 90  $\mu$ L of substrate solution was added to each well. The plate was covered with aluminum foil to protect from light and incubated for 20 min to allow for color development. The reaction was stopped by the addition of 50  $\mu$ L of stop solution and the optical intensities were determined using absorbance at 450 nm. The urinary GM2AP concentration was calculated from a standard curve.

#### **2.2.6 Lectin staining**

After the completion of SDS-PAGE or 2-DE, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using a semi-dry apparatus. The membranes were blocked for 2 h at room temperature with 5% BSA in PBS containing

0.05% Tween 20 (PBST), followed by staining with 10 µg/mL biotin-conjugated aleuria aurantia lectin (AAL) for 2 h at room temperature and washed three times with PBST. Then, the membranes for lectin blotting were incubated with streptavidin-conjugated HRP (10 µg/mL) for 1 h and washed three times with PBST. The membranes were developed with an enhanced chemiluminescence Western blotting detection system, and exposed to Fujifilm LAS-4000 Luminescent Image Analyzer.

### **2.2.7 Western blot analysis**

Fifteen micrograms of protein eluted from urine and serum samples were applied in each lane onto 14% SDS-PAGE or 2-DE. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a semi-dry apparatus. The membranes were blocked for 2 h at room temperature with 5% BSA in PBST at 37°C for overnight, followed by incubation with rabbit polyclonal anti-human GM2AP primary antibody (1:1000 dilution, Abcam, Cambridge, UK) for 2 h at room temperature. After washing with PBST three times for 10 min each, the primary antibody was detected with an anti-rabbit HRP-conjugated secondary antibody (1:5000 dilution, Abcam, Cambridge, UK) for 1 h at room temperature, washed with PBST three times for 10 min each. The blot was developed with an enhanced chemiluminescence Western blotting detection system (ECL™ kit; PerkinElmer) and exposed to Fujifilm LAS-4000 Luminescent Image Analyzer.

### **2.2.8 Immunohistochemical staining (IHC)**

The tissue slides were deparaffinized in xylene, rehydrated through graded alcohol, immersed in 3% hydrogen peroxide at 37°C for 10 min to block endogenous peroxidase activity. Subsequently, the slides were incubated with rabbit anti-GM2AP antibody (1:1000 dilution; ab118433) at 4°C overnight. The slides were sequentially incubated with a secondary antibody at room temperature for 1 h and stained with DAB (3, 3-diaminobenzidine). Finally, the sections were counterstained with hematoxylin. Using ImageScope software morphometry was performed on entire tissue sections stained with antibodies against GM2AP. IHC detection was used to determine the GM2AP levels by adding the percentage score to the staining intensity score. The percentage of positively stained cells was scored as 0, 0%; score 1, 1-20%; score 2, 20-

100%. Intensity was scored as 0, negative staining; 1, moderate staining; and 2, strong staining. The composite IHC scoring system was: IHC score=0, for positive staining in 0% tumor cells; IHC score =1, for moderate staining or strong staining in  $\leq 20\%$  tumor cells; IHC score =2, for strong staining in  $> 20\%$  tumor cells.

## **2.2.9 Protein identification and data annotation**

### **2.2.9.1 In-gel enzymatic digestion**

Protein spots were manually excised from the gel and transferred to siliconized eppendorfs. The gels were washed twice with 50% (v/v) ACN in 25 mM ammonium bicarbonate buffer, pH 8.0 for 15 min each. The 100% (v/v) ACN was added and the gels were dried under vacuum condition. The completely dried gel pieces were swollen in 10 mM DTT in 25 mM ammonium bicarbonate buffer and incubated at 56°C for 45 min, and then the solution was discarded. Subsequently, the gel pieces were alkylated with 55 mM IAA in 25 mM ammonium bicarbonate buffer and incubated at room temperature for 30 min in darkness. The gel pieces were washed twice with 50% (v/v) ACN in 25 mM ammonium bicarbonate buffer for 15 min each, and added 100% (v/v) ACN and dried under vacuum condition. Dried gel pieces were swollen in 25 mM ammonium bicarbonate containing trypsin and chymotrypsin and incubated at 37°C for at least 16 h. Peptides were subsequently extracted twice with 50% (v/v) ACN in 1% (v/v) TFA. The extracted solutions were combined and dried using a SpeedVac concentrator.

### **2.2.9.2 Peptide clean-up using C18 ZipTip®**

The digested peptides were desalted with a C18 ZipTip. The tip was washed and equilibrated with  $2 \times 10 \mu\text{L}$  of ACN and  $3 \times 10 \mu\text{L}$  of 50% (v/v) ACN in 0.1% (v/v) TFA, respectively. Then, the peptide solution (dissolved in 10% (v/v) ACN in 0.1% (v/v) TFA) was drawn and released for 10-15 times. The tip bound peptide was washed with  $10 \mu\text{L}$  of 10% (v/v) ACN in 0.1% (v/v) TFA before elution by drawing and releasing with  $10 \mu\text{L}$  of 50% (v/v) ACN in 0.1% (v/v) TFA for 10-15 times. The eluted solution was collected and dried in vacuum.

### **2.2.9.3 Electro-elution for intact protein mass**

The GM2AP spots of lung cancer were excised from 2-DE gels and extracted by electro-elution using Midi GeBAflex-tube (MW cut off at 3.5 kDa) according to instruction manual provided by the manufacturer. Following electroelution, salts, SDS and dye were removed by dialysis using the same electroelution tubes. The proteins were then concentrated in a vacuum centrifuge for subsequent MALDI-TOF/MS analysis to determine precise molecular mass of GM2AP.

### **2.2.9.4 MALDI-TOF mass analysis**

For mass spectrum analysis, one micro-liter of each protein sample were mixed with equal volume of matrix solution consisting of 2,5-dihydroxybenzoic acid (50 nmol/ $\mu$ L in 50% ACN). One micro-liter of the resulting mixture was spotted onto a 384-well MALDI target plate and allowed to air dry at room temperature. After crystallization, the sample was deposited manually on top of the matrix and vacuum-dried. Analysis was performed on an Ultraflex II MALDI-TOF/TOF mass spectrometer. Mass spectra were obtained in the range of mass to charge ratio (m/z) from 10,000 to 30,000.

### **2.2.9.5 NanoLC-MS/MS analysis**

High resolution and high mass accuracy nanoflow LC-MS/MS experiments were performed on a LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nanoelectrospray ion source (New Objective, Inc.), an Agilent 1100 Series binary high-performance liquid chromatography pump (Agilent Technologies), and a Famos autosampler (LC Packings). The digestion solution (6  $\mu$ L) was injected onto a self packed precolumn (150  $\mu$ m I.D. x 30 mm, 5  $\mu$ m, 200  $\text{\AA}$ ) operating at a flow rate of 10  $\mu$ L/min. Chromatographic separation was performed on a self packed reversed phase C18 nano-column (75  $\mu$ m I.D. x 200 mm, 3  $\mu$ m, 200  $\text{\AA}$ ) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% ACN as mobile phase B operated at 300 nL/min flow rate. Electro-spray voltage was maintained at 1.8 kV and capillary temperature was set at 200°C. Survey full-scan MS conditions were at mass range (m/z) of 320-2000 and the resolution of the mass



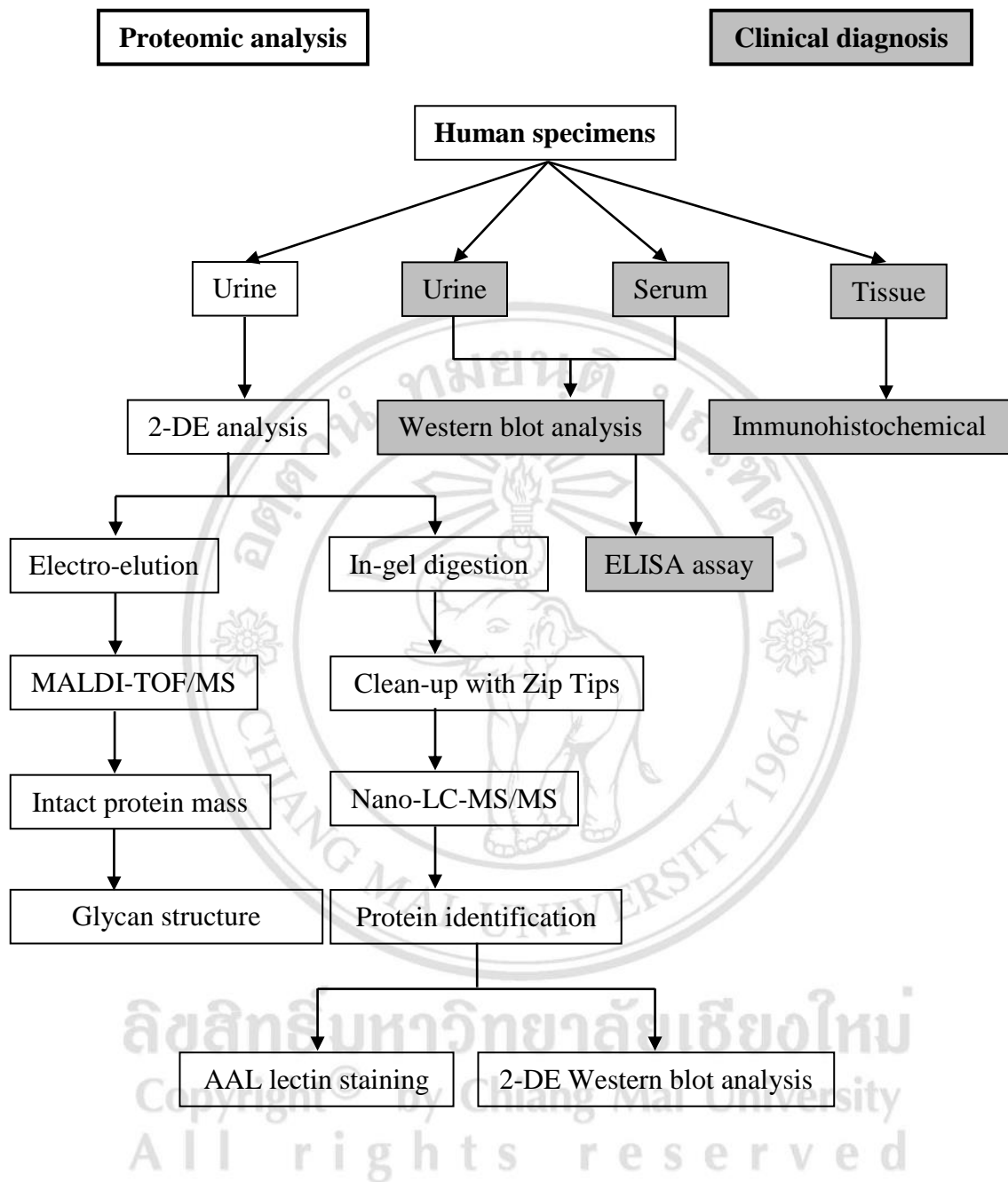
spectrometer was set to 30,000. The three most intense ions were sequentially isolated for Higher-energy C-trap Dissociation (HCD) at resolution of 7500 with normalized collision energy (NCE) 28. For protein identification, the MS and MS/MS ion data were annotated with the in-house MASCOT search engine, assuming that peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Trypsin plus chymotrypsin enzyme with 5 miss cleavages was allowed, and peptide mass tolerances of 5 ppm and 0.01 Da were used for the MS and MS/MS ions search. Each search was performed using the Swiss-Prot Human database. For the glycan analysis, HCD was easier to generate Y1 and oxonium ions. The glycopeptides were manually identified by the presence of glycan-specific oxonium ions in the HCD tandem mass spectra.

#### **2.2.9.6 Data annotation**

The MASCOT search engine was used for peptide mass fingerprinting (PMF) and MS/MS ions search (<http://www.matrixscience.com>). Search parameters were monoisotopic mass, oxidized at methionine residues and carbamidomethylated at cysteine residues. Only one missed trypsin and chymotrypsin cleavage were allowed, and peptide mass tolerances of 50 ppm were used for PMF and MS/MS ions search. Protein was identified by comparison of the observed mass with the predicted mass obtained from the Swiss-Prot database.

#### **2.2.10 Statistical analyses**

Statistical analysis was performed using the SPSS for Windows version 17.0 (SPSS Inc, Chicago, IL). Receiver operating characteristic (ROC) curve analysis was applied to determine the cut-off score for the expression of GM2AP. The correlation between GM2AP expression and clinicopathologic features of patients with lung cancer was evaluated by the Chi-square test or Fisher exact test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. Survival curves were obtained with the Kaplan-Meier method. Differences were considered statistically significant when *P* value was less than 0.05.



**Figure 2.1** Flow chat of proteomic analysis and clinical diagnosis for identify biomarker for lung cancer