CHAPTER IV

DISSCUSSIONS AND CONCLUSIONS

4.1 Discussions

4.1.1 Elevation of GM2AP level in lung cancer patients

The proteome is the entire set of proteins expressed by a genome, cell, tissue and organism at any given time. The proteome of an organism, tissue or even a single type of cells is much more complex than its corresponding genome. This is mostly due to alternate splicing, post-translational processing and different patterns of protein modification which affect virtually all proteins. Due to proteomes complexity, their analysis is extremely challenging. Therefore, it can potentially overcome some limitations of other approaches to identify new marker molecules for many diseases. Moreover, the proteins secreted from tumor cells are potential biomarkers for disease diagnosis and prognosis. Tantipaiboonwong et al. [59], a former researcher in our group, first used 2-DE and MALDI-TOF/MS in search of urinary biomarkers of lung cancer, and reported six up-regulated protein spots and three down-regulated protein spots in lung cancer urine samples compared to the healthy controls. Up-regulated proteins detected were plasma RBP, GM2AP, Ig lambda light chain and Ig kappa chain C regions. The down-regulated proteins were CD59 glycoprotein precursor, activator of cAMP-responsive element modulator and transthyretin (TTR). Among the up-regulated spots, GM2AP was present at 2.5-4.0 fold higher than the level found in healthy controls, hence was the protein of interest. Our objective was not to draw up a list of differentially expressed spots, but to identify proteins that would be relevant to the prediction for biomarker of lung cancer. To reach this objective, first, we used 2-DE to confirm the up-regulation of GM2AP in urine samples from lung cancer patients. The expression levels of GM2AP in both of the pooled and individual samples with different subtypes of lung cancer patients were significantly increased compared to that of healthy controls. We then excised GM2AP spot from 2-DE gel and digested with

trypsin and chymotrypsin. Due to length of the glycopeptide chain of GM2AP and the presence of amino acids that are very hydrophobic, the combination of those two enzymes seems suitable. Trypsin and chymotrypsin cleaved at C-termial side of GM2AP peptides chain at specific sites after lysine (K), arginine (R), leucine (L), tyrosine (Y), tryptophan (W) and phenylalanine (F). Subsequently, the peptide samples were analyzed by NanoLC-MS/MS. We found that the peptides were SWDNCDEGKDPAVIR and GCIKIAASLK, which were identified as GM2AP with mascot score of 1247 and 66% of sequence coverage. Then, 2-DE Western blotting was used to confirm MS identification of GM2AP on the 2-DE region using polyclonal antibodies against GM2AP. The urinary proteins were electrophoresed by denaturing SDS-PAGE. Thus, polyclonal antibodies are often the preferred choice for detection of denatured proteins. Multiple epitopes generally provide more robust detection [60]. We found that the antibodies could recognize GM2AP in lung cancer patients with higher intensity than that in healthy controls, consistent with the increase of GM2AP intensity observed on 2-DE gel. Therefore, we focused on the study of GM2AP for biomarker of lung cancer.

GM2AP is a small monomeric protein containing a single site for Asn linked glycosylation [61]. It is first synthesized as a precursor which is then glycosylated, modified and cleaved at ^{32}Ser to be in the mature form. GM2AP acts as an essential cofactor that contains at least three functional features including a hydrophobic pocket called the β-cup structure, an oligosaccharide binding site, and an area that interacts with β-hexosaminidase A (Hex A) for the *in vivo* degradation of ganglioside GM2 to GM3 [31, 32]. However, only one-third of the synthesized GM2AP is secreted [32, 62]. Cells can recapture the GM2AP via a carbohydrate-independent mechanism by various cells such as epidermal keratinocytes and fibroblast cells [32]. A lack of the functional GM2AP is a cause of the abnormal accumulation of GM2 ganglioside in tissues of patients with the AB variant of GM2 gangliosidosis disease [63]. The inherited deficiency of GM2AP was also related to the changing level of ganglioside and tumor associated gangliosides involving in cancer progression. Tumor-associated gangliosides are a result of initial oncogenic transformation and play a role in the induction of invasion and metastasis [35, 64]. Moreover, gangliosides are known to exhibit

regulatory roles in cell growth, adhesion, cell-cell interactions and signal transduction [65]. Tumor cells are synthesized and shed gangliosides into their microenvironments leading to elevated levels of tumor-associated gangliosides in the serum [66, 67]. Therefore, validation of GM2AP in lung cancer specimens is necessary prior to biomarker development, especially in early detection of cancer.

4.1.2 Identification GM2AP as biomarker for lung cancer

In the second objective, we need to identify a potential of GM2AP as biomarker of lung cancer. Forty-eight urine samples from lung cancer patients were used to confirm the presence and elevated level of GM2AP. Those patients received anticancer treatments, without the disorder of renal function as revealed by normal urine creatinine and blood urine nitrogen. It has been reported that GM2AP has been found in hemodialysis fluid obtained from male patients with acute renal failure following a coronary artery bypass surgery [68]. An increase in urinary GM2AP has also been found after gentamicin-induced acute renal failure in rat [69]. We hypothesized that the expressions of urinary GM2AP in lung cancer patients who received anticancer treatments may be caused by renal tubular dysfunction to retain proteins in the circulatory system. Therefore, the kidney function in lung cancer patients need to be investigated by Western blotting using polyclonal antibodies against kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL). KIM-1 and NGAL have been reported as the biomarkers of kidney failure. The result found that KIM-1 and NGAL were not present in urine samples of lung cancer patients. This result confirmed that the increase of urinary GM2AP was not the result of renal failure. Then, we used Western blot analysis to confirm the expression of GM2AP level in 48 lung cancer patients from Thailand samples to support our results. The finding revealed the expression level of GM2AP was increased in each subtype of lung cancer patients compared to that of the healthy controls, consistent with the increase in protein spot intensity observed in the 2-DE gel. Western blot is a semi-quantitative method, hence ELISA was additionally used to quantify the expression of GM2AP level in healthy controls and lung cancer patients. It was found that the expression levels of urinary GM2AP were significantly correlated with only histology cancer types. It was also

revealed that the urinary GM2AP level was significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma and small cell lung cancer, whereas gender and pathologic stage were not correlated in our study. The areas under ROC curve demonstrated that the urinary GM2AP can be potential diagnostic marker for lung cancer. The urinary GM2AP possessed an AUC value at 0.95 (95% CI, 0.91-0.99) with sensitivity of 90.91% and a specificity of 91.67%, respectively.

Since urine is produced by the kidneys and allows the human body to eliminate waste substances through a filtration of the blood [70]. We hypothesized that the increased levels of urinary GM2AP may come from distant organs via plasma that obtained through glomerular filtration. However, until now no study has been carried out to investigate the levels of GM2AP and its clinical roles in large number of urine or biological samples besides urine from lung cancer patients. We then have further confirmed the expression of GM2AP in a large group of lung cancer patients using urine and serum specimens from Taiwan samples. One hundred and thirty-three of urine and serum samples were collected from lung cancer age- and gender- matched individuals without the history of kidney function and radiation therapy or chemotherapy before enrollment. We found that both of the urinary and serum GM2AP levels were much higher in patients with lung cancer than in healthy controls. The urinary GM2AP possessed an AUC value at 0.89 (95% CI, 0.83-0.95) with sensitivity of 88.46% and a specificity of 85.71%, and serum GM2AP showed an AUC value at 0.90 (95% CI, 0.85-0.95) with sensitivity of 100% and a specificity of 82.71%, respectively. We concluded that anticancer treatments had no effect on the expression of GM2AP. Because GM2AP level was still increased in lung cancer patients with high sensitivity and specificity when compared to healthy controls, especially in early stage. To confirm the potential of GM2AP as specific biomarker for lung cancer, the serum of patients with other types of cancers, including colon cancer and liver cancer were examined. Our results supported that the expression level of GM2AP was highly increased in lung cancer patients, whereas the GM2AP level in colon cancer and liver cancer were found at the same protein level as those in the healthy controls. This result showed that the high expression of GM2AP was associated with only lung cancer, whereas other types of cancers did not show this characteristic. In this study, we found

that the high expression of GM2AP in both urine and serum are significantly correlated with pathologic stage. In earlier stages of lung cancer, the levels of GM2AP tend to be higher. Despite tremendous works endeavored in seeking for specific cancer markers, very few have become clinically applicable. Prostate specific antigen (PSA) in prostate cancer [71] and cancer antigen 125 (CA-125) in gynecological oncology are examples [72, 73]. Recently, researchers have suggested many serum-detectable biomarkers for cancers [74-78] but few have been successfully adapted into clinical application, especially for early detection. Our results suggest that urine and serum GM2AP levels can be potential markers assisting in lung cancer diagnosis, especially for early stage.

However, it was still unclear why GM2AP were secreted into urine and serum of lung cancer patients. The expression of GM2AP may be produced by tumor cells of lung and secreted into the circulating system. Using IHC, we also demonstrated that GM2AP was expressed essentially by tumor cells. None of the surrounding non-tumor tissue expressed GM2AP. Except pathology stages, there was no correlation between the GM2AP expression and histology type, age and gender. The rate of GM2AP expression or IHC score 2 in stage I, II and III was similar, but much lower in stage IV. However, the aberrant expression of GM2AP is related to tumor associated gangliosides involving in cancer progression and plays a role in the induction of invasion and metastasis [36]. Antitumor immune response was suppressed by gangliosides synthesized by tumor cells and shed into their microenvironment [89-84] Many studies have shown that tumor-derived gangliosides inhibited the cellular immune response *in vitro,* such as natural killer cell cytotoxicity [85-87]. Recent study showed that GM2AP impairs insulin signaling [88]. It is possible that GM2AP may inhibit insulin signaling to reduce uptake of glucose in normal cell, therefore, cancer cell will have more glucose to support cell growth. On the other hand, GM2AP can hydrolyze platelet activating factor (PAF) and inhibit PAF-initiation inflammation response [89], hence, GM2AP may help cancer cell to escape from antitumor response by inhibiting inflammation. Because GM2AP expression at stage III and IV was lower than I and II, the anti-inflammatory property of GM2AP may play an important role in early stage of cancer through reducing immune response that recognizes or destroys tumor cell. However, this hypothesis needs to be confirmed.

Besides being a diagnostic marker, GM2AP can also be a prognostic marker. Because the higher expression of GM2AP (IHC score 2) was significantly associated with a reduction of both overall survival time and disease-free survival time. Moreover, the multivariate analysis suggested GM2AP score is an independent prognostic factor besides pathology stage. The results indicated that GM2AP could possibly induce tumor growth. A higher expression of GM2AP in early stages of lung cancer may imply early metastatic disease. This finding supported that GM2AP may be a pivotal modulator involved in lung cancer development because we did not find the increased GM2AP expression level in other types of cancers. Our results suggested that GM2AP may be used as a potential prognostic marker to predict NSCLC patient and the increased GM2AP expression is a possible cause for the induction of the malignant progression, especially in early stage of lung cancer. However, the prognostics of tumour markers are important predictors, since they reflect the extent and aggressiveness of the cancer. A good prognosis can often be based on the combination of patient, tumour and treatment characteristics. Solely using extent of disease as with tumor-node-metastasis (TNM) staging can often be improved by considering more predictive characteristics [90]. For example, the survival of testicular cancer patients can be predicted based on alpha-fetoprotein (AFP), human chorionic gonadotropin (HCG) and lactate dehydrogenase (LDH) levels, combined with extent of disease characteristics [91]. The prognostic classifications can be devised, which are helpful for informing patients, decision-making on treatment, and in medical research, e.g. for stratification in randomized clinical trials.

4.1.3 *N*-linked glycan structure of GM2AP in lung cancer

GM2AP is a glycoprotein that was significantly increased in lung cancer specimens and is one of the key factors involved in the developmental process of lung cancer. The modifications of glycan structure are known to affect the glycan-related properties of glycoproteins. The cancer-associated modifications of glycans on the glycoproteins may provide opportunities to discover new biomarkers for cancer diagnosis and treatment [92-93]. However, the glycan structure of urinary GM2AP in lung cancer patient has not been reported. Therefore, analysis of GM2AP by mass

spectrometry is necessary prior to biomarker development, especially for the detection of its PTM, most notably glycosylation. First, we used MALDI-TOF/MS analysis to confirm the intact protein mass of GM2AP in lung cancer urine samples. The molecular mass of GM2AP was higher than that of the predicted protein mass from data base, which was a revealed marker ion characteristic of glycosylation modification. The glycosylation reactions were catalyzed by the action of glycosyltransferases, which add sugar chains to various complex carbohydrates such as glycoproteins and glycolipids. The changes in carbohydrate structure (glycan) were associated with many physiological and pathological events, including cell adhesion, migration, cell growth, cell differentiation and tumor invasion [94]. Therefore, the glycosylation of GM2AP in lung cancer patients may be catalyzed by glycosyltransferases which adds sugar residues in addition to the common *N*-glycan of GM2AP. Increased of sugar residues will enhance the intact protein mass observed by MALDI-TOF/MS. Then, the glycan structure of urinary GM2AP in lung cancer was identified by nanoLC-MS/MS. The glycopeptide ions of GM2AP were be fragmented efficiently by the HCD feature of a linear LTQ Orbitrap hybrid mass spectrometer. An attractive aspect of this dissociation option is the generation of distinct Y1 ions (peptide plus one HexNAc), thus allowing unequivocal assignment of *N*-glycosylation sites of glycoproteins. As a result, the common glycan oxonium ions in addition to the Y1 ion were also detected. This high resolution mass spectrum at the circled peak was also coreresponded to the glycopeptide of interest. The glycosylation was found with one glycosylation site at asparagine residues N63 of GM2AP. There were increased in monosaccharide sugar residues as found in glycopeptide data. The sugar increase helps to identify the *N*-linked glycan present on the glycopeptide as a fucosylated *N*-linked glycan core $(Hex)_3(HexNAc)_2(Fuc)_1$, where Hex = hexose, HexNAc = *N*-acetylhexosamine, and Fuc = fucose). *N*-linked glycan structure of GM2AP in lung cancer patient was found to possess a fucose residue on the common *N*-linked glycoprotein, whereas the typical *N*-linked glycoprotein, exhibiting the common pentasaccharide core of *N*-acetylglucosamine (GlcNAc) and mannose (Man) monosaccharide units linked via GlcNAc to the amide side chain of asparagines [95, 96]. We then examined the expression level of fucosylation on the 2-DE western blotting using AAL lectin

staining. AAL lectin recognize α1-3, α1-4 and α1-6 fucose to GlcNAc that present on GM2AP [97]. Western blotting of urine samples from lung cancer patient showed that a protein of approximately 18.6 kDa was highly fucosylated when compared to healthy control. This assignment is consistent with the fucosylated *N*-linked glycan core was known to be one of the most common glycoform in insect cell and in proteins expressed in insect cell lines [98], especially recombinant GM2AP in insect cells. The oligosaccharide attached to recombinant GM2AP was found to be identical and corresponded to the structure of $GlcNAc_2(Fuc)_1Man_3$ [99]. However, the very low abundance protein of urinary GM2AP in healthy donors was difficultly observed on 2-DE gel, because they were usually obscured by high-abundance proteins such as patient diseases. Some healthy donors did not found of this protein into circulating. Therefore, it cannot be passed into the circulating system and excreted as urine. As a result, the glycan structure of GM2AP presence in healthy donor was not detectable in urine samples. Some studied have demonstrated that most glycan are degraded in lysosome by highly ordered and specific pathways employing glycosidases and glycosyltransferase [100]. The loss of enzymes involved in degradation could lead to the accumulation of substances in patient tissue and appearance in urine [103]. For example, the removal of core fucose (α1-6Fuc to GlcNAc) and probably any peripheral fucose residues linked to the outer branches of the chain (α1-3Fuc to GlcNAc) appears to be the first step in degradation because patients lacking glycosidases enzyme still have intact *N*-glycan bound to asparagines [100]. It is possible that the increase amount of fucose residue on common *N*-linked glycan structure may be associated with glycosidases and/or glycosyltransferase that induce GM2AP expression in lung cancer patients. Moreover, the aberrant expressions of the enzymes in turn cause cancer cells to produce glycoproteins with specific cancer-associated aberrations in glycan structures [5, 7, 48, 101, 102]. Cancer-associated aberrations in glycan structures can be used to improve on existing cancer biomarkers, and provide a compelling rationale for the discovery of new biomarkers through the isolation and identification of glycoproteins that contain these glycan structures. Therefore, GM2AP may be useful as a potential urinary biomarker to monitor lung cancer progression. However, the secretory

mechanism and biological function of GM2AP that associated with lung cancer progression require additional work.

4.2 Practical application

To achieve practical utility, it is important to develop highly sensitive and specific assays for individual biomarkers. In this study, an ELISA was selected the choice for clinical use as this assay provided reliable, quantitative and accurate data. Using ELISA to detect the amount of GM2AP in specimens, we needed to know the optimal standard level of GM2AP in lung cancer patient with information on anti-cancer treatments and healthy donors. This approach was possible for our research. ROC curves have been used in medicine to determine a cut-off value for a clinical test. Selected cut-off points for GM2AP levels appeared to have good discriminating power to differentiate lung cancer patients and healthy donors. For example, the cut-off value of 0.38 ng/mL was determined for the GM2AP test for lung cancer. A test value below 0.38 is considered to be normal and above 0.38 to be abnormal. This method also can be used for preliminary diagnosis in normal cases. However, many methods for the clinical diagnosis of lung cancer include chest X-ray, computed tomographic (CT) scans, and other imaging techniques. Although imaging data play an important role in diagnosis, they are limited by inability to detect hidden lesions, subclinical lesions, and small metastases. There are also disadvantages such as radiation exposure, high-cost, and low specificity. In addition to CT scans, there are many invasive diagnostic methods for auxiliary diagnosis such as bronchoscopy and needle biopsy, but these methods are painful and time-consuming. Therefore, an ELISA assay of GM2AP is a good way for early diagnosis in lung cancer, because this method is highly specific as the process involves implementing a pair of antibodies against the candidate protein. Its high sensitivity also permits the quantification of proteins in human specimens at concentrations below the ng/mL range and is a noninvasive diagnostic method.

4.3 Conclusions

The identification of urinary proteomics developed in this study can serve as an ideal and efficient method to establish a panel of potential biomarkers. This study revealed that the level of GM2AP presented in urine and serum samples could be useful for the auxiliary diagnosis of lung cancer. The expression of GM2AP in lung cancer tissues could be a good prognostic factor for NSCLC, especially in early stage, in predicting both overall survival and disease-free survival. Additional study on MS provided evidence of an increase of GM2AP glycosylation. Cancer-associated aberrations in glycosylation of glycoprotein can add to the knowledge on existing cancer biomarkers. Therefore, our study demonstrates that GM2AP might serve as potential diagnostic and prognostic markers for lung cancer.