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Human Urinary GM2-activator Protein as a Potential Biomarker for Lung Cancer

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Abstract

Human urine can be obtained from most patients by noninvasive ways of collection. It is a rich source of biomarkers for diagnostic tests, therapeutic guidance and prognostic information for patients and clinicians. Recently, the protein expression pattern of urine samples from lung cancer patients and healthy controls were compared using two-dimensional gel electrophoresis (2-DE). GM2 activator protein (GM2AP) in lung cancer patients with 2.5-4.0 folds higher than that found in healthy controls was the protein of interest, and this protein was focused because it is related to tumor-associated gangliosides found in cancer progression. The aim of this study was to investigate the urinary biomarkers associated with lung cancer. First, we applied proteomic approach to confirm the increase level of GM2AP in urine samples from lung cancer patients and healthy controls. Then, the expression level of urinary GM2AP was quantified using Western blotting and Enzyme-Linked Immunosorbent Assay (ELISA). The results show that the expression level of GM2AP significantly increased in each subtype of lung cancer patients (P<0.05). In addition, nano liquid chromatography coupled online with tandem mass spectrometry (NanoLC-MS/MS) provided evidence of an increase of GM2AP with the glycan structure identification from doubly charge at m/z 1202.1025 corresponding to the structure of $(Hex)_3(HexNAc)_2(Fuc)_1$ linked with peptide (PIIVOGNVTLSVVG). Overall data suggested that the GM2AP may be associated with lung cancer and may be useful as a marker to monitor lung cancer prognosis.

Keywords: Biomarker; Lung cancer; GM2 activator protein; Human urine; Glycan structure

Introduction

Lung cancer is the most common cause of cancer–related mortality worldwide in male and female, accounting for 28% of all male cancer deaths and 26% of all female cancer deaths in the United States in 2013 [1]. Many patients with lung cancer are in the advanced stages of the disease at the time of diagnosis [2]. However, the poor early detection of lung cancer and ineffective treatments for advanced disease are responsible for the low overall 5-year survival rate of only 14% [3]. Therefore, the development of noninvasive diagnostic tools for discovery of novel lung cancer specific biomarker is emerging as an important to identify early stage of lung cancer and therapeutic applications. Proteomic techniques including two-dimensional gel electrophoresis (2-DE) and differential gel electrophoresis coupled with MS technology are useful for the analysis of tumor markers [4-8]. Recently, different proteomic approaches have been used for biomarker discovery in urine samples of lung cancer patients [9-12]. All of these studies have provided interesting data that contributed to enrich the urinary proteomic map of lung cancer patients. Tantipaiboonwong et al. [12] studied urinary proteome changes between lung cancer patients and healthy controls by 2-DE. Several differentially expressed proteins were identified as potential biomarkers.

The objective of this study was to identify urinary biomarker for lung cancer. Urinary proteome analysis of lung cancer patients compared to healthy controls showed an increase of GM2AP, which was confirmed by Western blot analysis. We decided to focus our study on this protein due to reports of this protein being an essential cofactor for the degradation of ganglioside GM2 to GM3 by lysosomal β-hexminidase A (Hex A) [13,14]. This protein is also associated with the changing level of ganglioside and tumor-associated gangliosides found in cancer progression [15-17]. The aberrant of gangliosides in tumor cell induced tumor angiogenesis through regulation of growth factor signaling [18,19]. Quantification of GM2AP in urine of lung cancer patients and healthy controls by ELISA confirmed a significant increase of GM2AP in lung cancer cases. However, the structure and function information available for this activator are still not well understood. Investigation of alterations in glycan structures of GM2AP in cancer patients may lead to better understanding of how glycosylation modulates the biological activities of glycoproteins in lung cancer, which could be useful as a marker for the diagnosis and prognosis of lung cancer.

Materials and Methods

Sample collection

Human urine samples from forty-four healthy individuals and

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forty-eight lung cancer patients (n=25 for adenocarcinoma, n=15 for small cell lung cancer, n=4 for squamous cell carcinoma, n=4 for other types of carcinoma) were provided by Maharaj Nakron Chiang Mai Hospital, Chiang Mai, Thailand. The collections of urine samples were carried out with permission from the donors and approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University for research purpose only. Urine samples were obtained from lung cancer patients aged 28–74 years who had been diagnosed with lung cancer at advanced stages (stage III and stage IV for non-small cell lung cancers, limited and extensive stage for small cell lung cancer). Healthy donor urine samples were obtained from healthy volunteers aged 25–74 years. The tumor stage of the lung cancer was determined according to the International System for Staging Lung Cancer [20]. The clinical information for lung cancer and information of healthy controls urine samples are shown in Supporting Information Table S1 and Table S2, respectively. All urine samples were collected in early morning (the first urination after waking up) in a sterile tube. Then, the urine samples were subsequently centrifuged at 12000×g for 30 min at 4°C to remove cellular contamination and debris. The supernatants were lyophilized and stored at -80°C until further analysis.

Two-dimensional gel electrophoresis (2-DE)

Urine samples were loaded into the centricon tube (MW cut off at 3 kDa) (Millipore Corpore Corporate, MA, USA) and centrifuged at 5000×g at 4°C for 1 h. 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 (Amersham Biosciences, UK) and eluted with 10 mM phosphate buffer, pH 7.5. The fraction containing proteins were collected and lyophilized. The concentration of urine protein was determined by spectrophotometry using Bio-Rad protein microassay based on the method of Bradford (Bio-Rad Laboratories, CA, USA). Two hundred micrograms of urine samples were dissolved in IEF buffer that contained 7 M urea, 2 M thiouria, 4% CHAPS, 1% dithiothreitol (DTT) and 0.5% carrier ampholytes and 0.002% bromophenol blue. The samples were sonicated, centrifuged and then applied onto IPG strip of pH 4-7 (18 cm, Amersham Biosciences, Uppsala, Sweden) 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, Amersham Biosciences, Uppsala, Sweden) for preparative analysis. The IPG strips were subsequently rehydrated on the IPGphor IEF system (Amersham Biosciences, UK) 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). After IEF, the proteins on the strip were initially equilibrated for 15 min in equilibration buffer I, containing 6 M urea, 30% glycerol, 2% SDS, 2% w/v DTT, bromophenol blue, and 50 mM Tris-HCl, pH 8.8 and for additional 15 min in equilibration buffer II (equivalent to equilibration buffer I but containing 2.5% w/v iodoacetamide (IAA) instead of DTT). The IPG strip was placed on top of the 15% polyacrylamide gel (18×18 cm, 1.5 mm) and covered with 0.5% agarose. The 2-DE separation was electrophoresed at 45 mA per gel at 4°C until the bromophenol blue dye front reached the bottom of the gel. After electrophoresis, the protein in the gels were strained with SYPRO® Ruby and scanned using a Typhoon 9200 image scanner (Amersham Biosciences, Uppsala, Sweden) at a wavelength of 610 nm. After scanning, the gel images were analyzed by the Image Master™ 2D Platinum software version 5.0 (Amersham Biosciences, Uppsala, Sweden).

In-gel enzymatic digestion

Protein spots were manually excised from the gels, washed twice with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate, pH 8.0 for 15 min each and dried in 100% ACN. The proteins in the spots were reduced with 10 mM DTT in 25 mM ammonium bicarbonate at 56°C for 45 min, and alkylated with 55 mM IAA at room temperature for 30 min in the dark. The gel pieces were washed twice with 50% ACN in 25 mM ammonium bicarbonate buffer for 15 min each and dried in 100% ACN. 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 three times with 50% ACN in 1% trifluoroacetic acid (TFA). The extracted solutions were combined and dried using a SpeedVac concentrator (Labogene Aps, Lynge, Denmark). The digested peptides were desalted with a C18 ZipTip (Millipore, Bedford, MA, USA). Bound peptides were eluted from the ZipTip with 50% ACN in 0.1% TFA.

Western blot analysis and lectin staining

In total, 15 µg of urine samples were present in sample buffer composed of 50 mM Tris pH 8.0, 10% glycerol, 2% SDS, beta-mercaptoethanol and 0.1% bromophenol blue. The mixed samples were heated at 95°C for 10 min and subsequently fractionated on 15% SDS-PAGE. After SDS-PAGE, proteins were transferred onto polyvinylidene fluoride (PVDF) (Millipore, Bedford, MA, USA) membranes using a semi-dry apparatus (Amersham Biosciences, USA). For 2-DE Western blot, the first and 2-DE separations were performed as described above and also transferred onto PVDF membranes. The membranes were blocked for 2 h at room temperature with 5% BSA in PBS containing 0.05% Tween 20 (PBST), 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 (FUJIFILM Corporation, Japan).

For lectin staining, the PVDF membranes were washed three times with PBST and incubated with 5% BSA in PBST for 2 h at room temperature. The membranes were washed three times with PBST, followed by staining with 10 µg/mL biotin-conjugated aleuria aurantia lectin (AAL) (Burlingame, CA, USA) 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.

ELISA assay

Human GM2AP in urine 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 µL of the diluted standard and 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 µL 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 µL of avidin conjugated HRP at 37°C for 30 min. After washing with the wash solution three times, 90 µL 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 µL of stop solution and the optical intensities were determined using absorbance at 450 nm. The urinary GM2AP concentration was calculated from a standard curve.

Electro-elution for intact protein mass determination

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) (Gene Bio-Application, Israel) 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. Protein was identified by comparison of the observed mass with the predicted mass obtained from the Swiss-Prot entries for those proteins that had been identified by MALDI-TOF peptide mass fingerprinting.

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/µL 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 (Bruker Daltonik GmbH, Bremen, Germany). Mass spectra were obtained in the range of mass to charge ratio (m/z) from 10,000 to 30,000.

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 µL) was injected onto a self packed precolumn (150 µm I.D.×30 mm, 5 µm, 200 Å) operating at a flow rate of 10 µL/min. Chromatographic separation was performed on a self packed reversed phase C18 nano-column (75 µm I.D.×200 mm, 3 µm, 200 Å) 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 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.

Statistical analysis

Statistical analysis was carried out using SPSS version 17.0. Paired samples t-test and one-way analysis of variance (ANOVA) were used to analyze the GM2AP expression in different groups. Chi-square test or Fisher exact test was used to assess the relationship between GM2AP expression and clinic pathological features. A confidential level of 95% (P<0.05) for each factor was considered statistically significant.

Results

Differential expression profiles of urine proteins on 2-DE gel in lung cancer

To search for potential biomarkers of lung cancer, we systematically analyzed urinary proteins secreted from lung cancer patients and healthy donors. First, the protein expression pattern of pooled urine samples from healthy controls and patients were separated from 2DE analysis with a narrow pH range of 4-7 (Figure 1A). One of the differentially expressed protein was identified as GM2AP marked in the 2-DE map. The urine samples from healthy individuals and patients with different subtypes of lung cancer were used for 2-DE analysis to confirm the GM2AP expression. The result revealed that GM2AP level in each subtype of lung cancer patients were greatly over-expressed compared to the mean of healthy controls (Figure 1B). This spot from 2-DE gel was excised and subjected to in-gel enzymatic digestion, followed by the nanoLC-MS/MS analysis as previously described in experimental procedure. The database searches were performed against Swiss-Prot database using Mascot software with fixed modification as carbamidomethyl (C). This protein spot was identified as GM2AP (Supporting Information Figure S1A and B).

Identification of GM2AP as a biomarker of lung cancer

To verify the cancer selective character of candidate proteins identified by mass spectrometry, the antibody-binding capacity of spot corresponding to GM2AP protein was investigated by 2-DE Western blot of healthy control and lung cancer patient to confirm the identity of the protein spot matched after 2-DE using antibodies GM2AP, which is shown in the Supporting Information Figure S2. Data suggested that GM2AP was over-expressed in lung cancer patients, consistent with the increase in protein spot intensity observed in the 2-DE gel. In addition, the expression level of GM2AP in lung cancer urine samples (n=48) were validated by Western blot analysis to compare their GM2AP levels with those of healthy controls (n=44). Figure 2A is a representative panel of urinary GM2AP profile from the four subtypes of lung cancer patients and healthy controls. The level of GM2AP was significantly increased in the each subtype of lung cancer patients when compared to the mean of healthy controls (Figure 2B, P<0.05). Western blot is a semi-quantitative method, hence ELISA was used to quantify the expression level of GM2AP in urine of healthy controls (n=44) and lung cancer patients (n=48). The patients included 33 male and 15 female individuals. The mean age of the patients was 53.3 years (range, 28-74). The mean of GM2AP level in all of lung cancer patients were measured to be 1.60 ± 1.21 ng/mL, whereas the mean of GM2AP level for healthy controls was 0.21 ± 0.14 ng/mL. There was a significantly increase in the GM2AP level for patients compared to healthy controls (P<0.05), that is about 7.62 fold increase on the median (Figure 3).

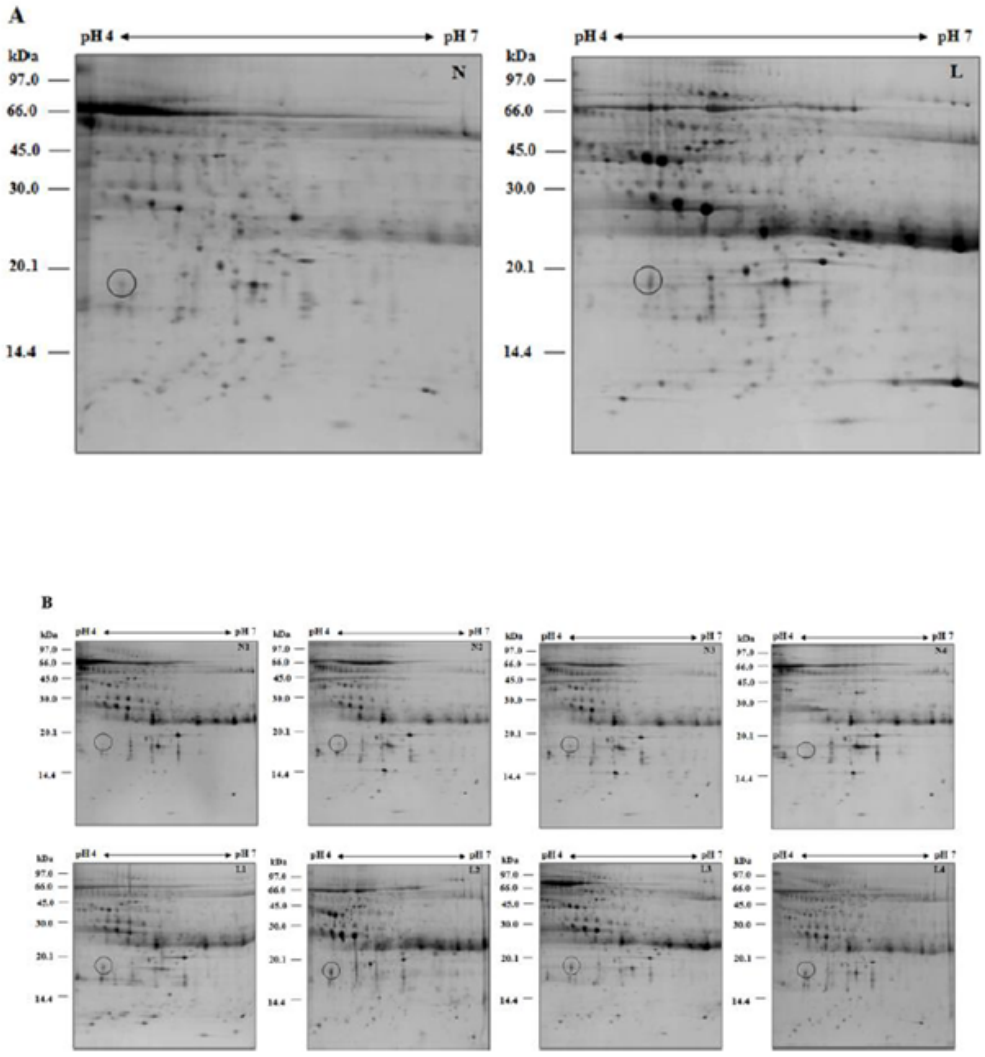

Figure 1: 2-DE analysis images of healthy control and lung cancer patient urine samples. A total of 200 µg of urine samples from healthy donors and lung cancer patients were applied to a pI 4-7 IPG strip for the first dimension and separated on 15% SDS-PAGE gel for the second dimension in the 2-DE. The gels were strained by using Sypro Ruby. (A) Pooled urine samples of healthy controls and lung cancer patients. (B) Urine samples of healthy donor and patients with different subtypes of lung cancer, N: Healthy control. L: Lung cancer patient (L1: Adenocarcinoma, L2: Small cell lung cancer, L3: Squamous cell carcioma, L4: Other types of carcinoma). The black circles indicate the position of GM2AP. They are overexpressed in each subtype of lung cancer patients when compared with that of healthy donors.

The urinary GM2AP level measured in the male patients (1.16 ± 1.07 ng/mL) was higher than that measured in the female patients (1.13 ± 1.05 ng/mL). According to histologic type, the urinary GM2AP level measured in patients with adenocarcinoma, small cell lung cancer and squamous cell carcinoma were 1.25 ± 1.12, 1.48 ± 1.35 and 2.27 ± 2.20 ng/mL, respectively. The urinary GM2AP level was measured to be 1.69 ± 1.54 and 0.63 ± 0.38 ng/mL in patients with stage III and IV, respectively. The expression levels of GM2AP of all the patients were included in the statistical analysis and significant correlation (P<0.05) was found with histology cancer type, whereas gender and pathologic stage were not correlated (Table 1).

Detection of intact protein mass of GM2AP in lung cancer

To confirm the molecular mass of GM2AP in lung cancer patient, the GM2AP spot in 2-DE gel were excised and applied to electro-elution for MALDI-TOF/MS as described in experimental procedure. The protein mass spectrum of GM2AP appeared as one major peak at 18663 Da for urine samples obtained from lung cancer patients. This was significantly greater than the predicted GM2AP mass of 17589 Da (data not shown), thus suggesting post-translational modifications (PTM).

The PTM of GM2AP has been identified as glycosylation. We then investigated the difference of GM2AP glycan moiety in healthy control and cancerous urine with lectin staining, which was performed on the 2-DE Western blotting. AAL lectin signaling was increased fucosylated urinary GM2AP in lung cancer patient compared to that of healthy control (Supporting Information Figure S3).

Determination the glycan structure of GM2AP in lung cancer

In order to determine the glycan structure of GM2AP, the GM2AP spots from 2-DE were in-gel digested with trypsin and chymotrypsin. The resulted peptides were further analyzed by nanoLC-MS/MS. The

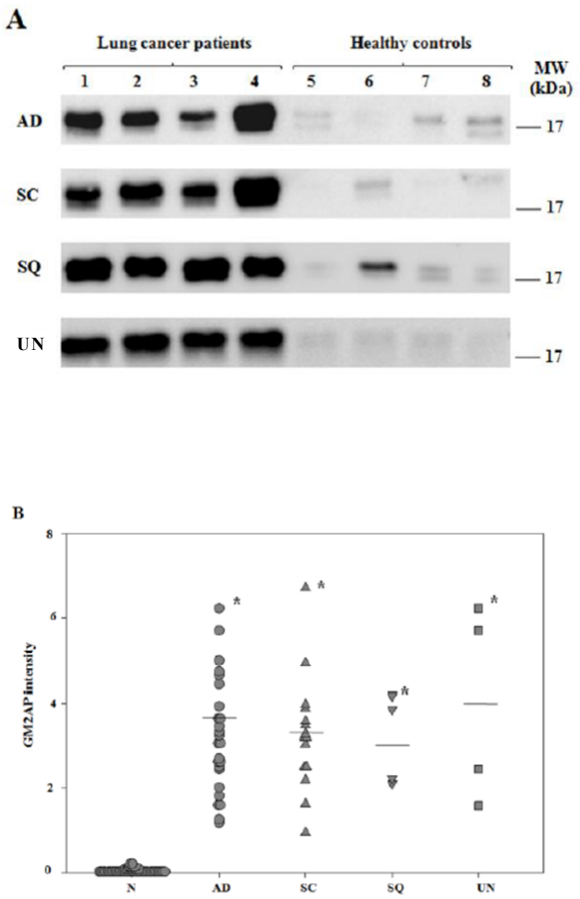

Figure 2: Quantification of the GM2AP in lung cancer patients and healthy donors are subjected to 15% SDS-PAGE and blotted with anti-GM2AP antibody. (A) Western blotting analyses of four representative lung cancer patients in four subtypes are showed (lanes 1-4); AD: Adenocarcinoma, SC: Small cell lung cancer, SQ: Squamous cell carcinoma and UN: Other types of carcinoma and from a representative of healthy donors (lanes 5-8). (B) Scatter plot of the relative intensities of GM2AP quantified in urine from healthy controls (n = 44) and different types of lung cancer patients (n = 48) in urine samples; N: Healthy control, AD: Adenocarcinoma, SC: Small cell lung cancer, SQ: Squamous cell carcinoma, UN: Other type of carcinoma. Black bars indicate the average levels for GM2AP in each subtype of lung cancer patients. *: *P* value < 0.05 compared to the value for the healthy urine samples.

mass spectra showed the oxonium ion at m/z 204.087 and Y_1 ion, peptide with a HexNac was present in HCD. The parent ion at m/z 1202.1025 and peptide molecular mass of 1363.813 Da (PIIVPGNVTLSVVG) of GM2AP was calculated; in the meantime, glycan composite $(Hex)_3(HexNAc)_2(Fuc)_1$ also been determined as shown in Figure 4.

Discussion

The human 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. [12], 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 controls. GM2AP in lung cancer patients was present with 2.5-4.0 fold higher than that 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 of lung cancer. To reach this objective, we first used 2-DE to confirm the up-regulation of GM2AP in urine samples from lung cancer patients. We found that the expression level 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 (Figure 1A and B). This result was confirmed by Western blotting in the 2-DE region suggesting that the expression level of GM2AP in lung cancer patients was higher than in healthy controls.

In this study, we then focused on the GM2AP which was significantly increased in urine of lung cancer patients. GM2AP is a small monomeric protein containing a single site for Asn linked glycosylation

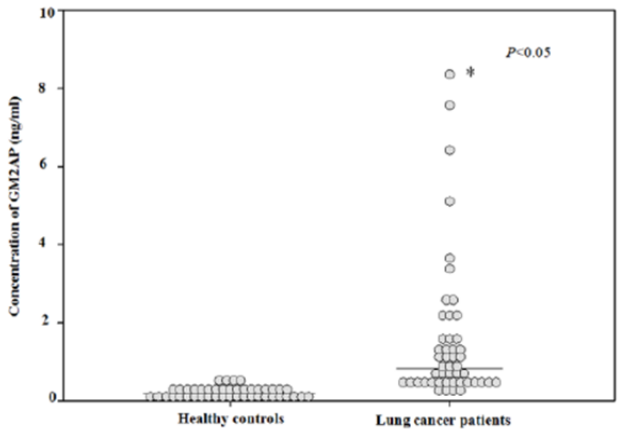

Figure 3: Quantification of the expression level of GM2AP in urine samples from lung cancer patients and healthy controls. GM2AP level was quantified by ELISA in urine of the 44 healthy controls and of the 48 lung cancer patients. The expression level of GM2AP was significantly increased from 0.21 ± 0.14 ng/mL in controls to 1.60 ± 1.21 ng/mL in patients. Black bars indicate the average levels for GM2AP in lung cancer patients. *: *P* value < 0.05 compared to the value for urine samples from healthy controls.

	n	GM2AP mean ± SD (ng/mL)	*P* value
All patients	48	1.60 ± 1.21	
Gender			
Male	33	1.16 ± 1.07	0.358
Female	15	1.13 ± 1.05	
Histology			
Adenocarcinoma	25	1.25 ± 1.22	0.009 *
Small cell lung cancer	15	1.48 ± 1.35	
Squamous cell carcinoma	4	2.27 ± 2.20	
Other type of carcinoma	4	2.99 ± 2.63	
Phatologic stage			
III	7	1.69 ± 1.54	0.312
IV	41	0.63 ± 0.38	

Table1: Quantification of the GM2AP level in lung cancer patient urine samples by ELISA.

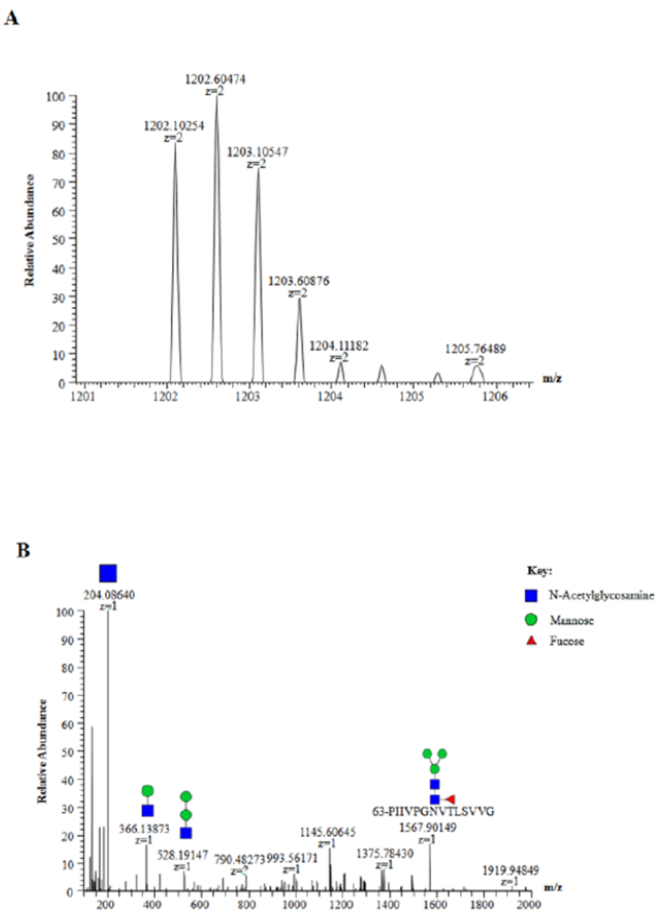

Figure 4: Mass spectra of the glycopeptides of urinary GM2AP in lung cancer patient. The parent ion at m/z 1202.1025 (A). *N*-linked glycan structure (2HexNAc3Hex1dHex) of GM2AP in lung cancer urine sample (B).

[21]. It is first synthesized as a precursor which is then glycosylated, modified and cleaved at ^{32}Ser to be in the mature form. GM2AP is acting as a cofactor essential that contains at least three functional features including a hydrophobic pocket called the ß-cup structure, an oligosaccharide binding site, and an area that interacts with Hex A for the vivo degradation of ganglioside GM2 to GM3 [22,23]. The critical *in vivo* role that is played by this activator protein is demonstrated by the occurrence of the AB variant form of GM2 gangliosidosis, a severe lysosomal storage disease cause by a lack of a functional activator protein [24]. Moreover, the inherited deficiency of GM2AP was also related to the changing level of ganglioside and tumor associated gangliosides important in cancer progression. Tumor-associated gangliosides are a result of initial oncogenic transformation and play a role in the induction of invasion and metastasis [16,17]. Tumor cells are synthesized and shed gangliosides into their microenvironments leading to elevated levels of tumor-associated gangliosides in the serum [25]. Changes in the amounts of gangliosides in serum can influence the rate of tumor growth through an undetermined mechanism [15].

In order to identify a potential of GM2AP as biomarker of lung cancer, Western blot analysis was used to confirm and support our results. In this study, the patients had no disorder of renal function as revealed by normal urine creatinine and blood urea nitrogen. The finding the expression level of GM2AP was increased in each subtype of lung cancer patients compared to healthy controls (Figure 2), consistent with the increase in protein spot intensity observed in the 2-DE gel. Moreover, using an ELISA for the quantification of GM2AP in urine samples, we then found that the expression level of GM2AP was significantly correlated with only histology cancer types (Table 1). An overexpression of GM2AP level was observed in 54.2% of patients with adenocarcinoma, 29.2% of patients with small cell lung cancer, 8.3% of patients with squamous cell carcinoma, and 8.3% of patients with other carcinoma. The present study also showed the urinary GM2AP level to be significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma and small cell lung cancer, whereas age, gender and pathologic stage were not correlated in our study. Therefore, the expression level of GM2AP might be a useful determinant for histology cancer type prognosis of lung cancer.

Interestingly, GM2AP is a glycoprotein that was significantly increased in lung cancer urine is one of the key factors involved in the developmental process of lung cancer. The glycosylation change in the glycan moieties of glycoproteins may provides opportunities to discover new biomarkers for cancer diagnosis and treatment [26]. The discovery of the cancer-associated modifications of glycans on the glycoproteins may also improve on the specificity of existing cancer biomarkers [27,28]. However, the glycan structure of urinary GM2AP in lung cancer patient has not been reported. In this study, 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, which was a revealed marker ion characteristic of glycosylation modification. *N*-linked glycosylation is the most common form of glycosylation that found in eukaryotes. It is occurs when glycans are attached to asparagine residues on the core protein. Therefore, the *N*-linked glycan structure of urinary GM2AP in lung cancer patients was determined using nanoLC-MS/MS analysis. The glycopeptides ions of GM2AP can 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 Y_1 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 Y_1 ion were also detected. We found that the *N*-glycan structure of GM2AP was corresponded to the structure of $(Hex)_3(HexNAc)_2(Fuc)_1$ linked with peptide (PIIVOGNVTLSVVG) (Figure 4). In general, *N*-glycosylated proteins comprise a conserved core structure consisting of two *N*-acetylglycosamine residues linked to asparagine followed by three branched mannosyl residues [21]. In this study, the *N*-linked glycan structure of GM2AP in lung cancer patient was found fucose residue on the common core protein. This fucose residue was confirmed on the 2-DE Western blotting and followed by AAL lectin staining. Because AAL lectin is a commercially available lectin that preferentially to fucose linked (α -1,6) or (α -1,3) to acetylglycosamine that present on glycoprotein. The strong AAL lectin signal in lung cancer patient showed increased fucosylated GM2AP when compared to healthy control (Supporting Information Figure S3). Wendeler et al. [29] studied the glycosylation of recombinant GM2AP in insect cells. The purified GM2AP was first subjected to endoglycosidase digestion and analyzed by ESI-mass spectrometry. The glycosylation was found to be identical and corresponded to the structure $(GlcNAc)_2Fuc(Man)_3$, consistent with the *N*-linked glycan structure of GM2AP in our finding in lung cancer. However, the glycan structure of urinary GM2AP in healthy control was not detected, because the concentration of GM2AP is too low and some healthy

control have no this protein that excrete to urine. Some studies have demonstrated that most glycans are degraded in lysosomes by highly ordered and specific pathways employing endo- and exoglycosidases, sometimes aided by glycoproteins. Thus, the loss of enzyme and/or GM2AP degradation could lead to the accumulation of substances in patient tissue and the appearance in urine [29]. For example, the removal of core fucose (Fucα-1,6GlcNAc) and probably any peripheral fucose residues linked to the outer branches of the chain (e.g., Fucα-1,3GlcNAc) appears to be the first step in degradation because patients lacking this enzyme still have intact *N*-glycan bound to asparagines [30]. Moreover, many reports suggest that structural changes in cell surface carbohydrates may promote tumor transformation. Dennis et al. [26] reported the α-1,6 linked fucosylation of complex-type glycans may be an important feature of tumor progression related to increased metastasis [31]. Therefore, these preliminary findings suggest that the GM2AP may be useful as a potential urinary marker to monitor lung cancer progression. However, the secretory mechanism and biological function of GM2AP that associated with lung cancer progression needs further work.

Conclusion

The identification of urinary proteomics developed in this study can serve as an ideal efficient method to establish a panel of potential biomarkers. This study revealed that the urinary GM2AP was increased in lung cancer patients compared to healthy controls. The expression level of GM2AP was validated by Western blot and quantified using ELISA to be significantly higher in lung cancer patients. 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, we suggest that the urinary GM2AP may be useful as a marker for lung cancer diagnosis.

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