Verification of GM2 Activator Protein for Potential Application as Lung Cancer Biomarker

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***Abstract*—Lung cancer is the leading cause of cancer–related deaths worldwide. Finding effective biomarkers for early diagnosis would benefit available treatments. By 2-DE analysis, GM2 activator protein (GM2AP) was increased in urine samples of lung cancer patients. An increase of GM2AP level in urine samples of lung cancer patients were verified by Western blot analysis, using healthy donors as controls. Levels of urinary GM2AP in samples from the patients were significantly increased with 23-fold higher than that found in healthy controls. The increased level of GM2AP was also confirmed in serum samples of lung cancer patients. The levels of GM2AP were found with 14-fold in lung cancer patients compared to those from healthy controls. Multiple reaction monitoring (MRM) assay revealed that the concentration of urinary GM2AP in lung cancer patients were at 1.960, 1.334 and 9.303 μM/mL, whereas in the healthy controls was found at 1.082, 1.177 and 1.028 μM/mL. The concentration of the serum samples of lung cancer patients and healthy controls were found at 2.113, 1.284 and 1.375 μM/mL, whereas in healthy controls were found at 0.995, 0.953 and 0.768 μM/mL, respectively. Therefore, we suggest that GM2AP might serve as preliminary candidate biomarker of lung cancer.**

***Index Terms*—GM2 activator protein, lung cancer, biomarker, 2-DE analysis, Western blot analysis, multiple reaction monitoring**

I. Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide [1]. In terms of mortality rate, lung cancer is the major cause of death among cancers with incidence of 86,930 (28%) cases in males and 72,330 (26%) cases in females in 2014 [2]. Lung cancer can be divided into two histological groups: non-small cell lung cancer (NSCLC) about 80% occurrence rate and small cell lung cancer (SCLC) about 20% occurrence rate. NSCLC consists mainly of adenocarcinoma, squamous cell and large cell carcinoma, whereas SCLC is slightly more common and all known cases are due to cigarette smoking [3]. However, the main problem of lung cancer disease is a lack of early-diagnosis tools, resulting in more than 60% of patients diagnosed with advanced or metastatic disease. As a result the overall five year survival rate of patients is less than 15% [4], [5]. Therefore, the development of noninvasive diagnostic tools for discovery of novel lung cancer specific biomarker is emerging as preferred components of a

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strategy for discovery of diagnostic, prognostic and therapeutic protein biomarkers.

Proteomic technology is emerging as a powerful tool for the identification and relative quantitative measurement of complex protein mixtures. Two-dimensional gel electrophoresis (2-DE) is a commonly used tool to compare protein spot intensity between samples, while mass spectrometry (MS) can be used to characterize those proteins [6], [7]. However, a number of potentially interesting proteins might not be detected in all sample sets analyzed due in part to sample complexity with low protein concentration. Immunochemistry-based methods, such as Western blot and ELISA assays have been used to obtain relative quantitative information. But when a proper antibody for target proteins is unavailable, these methods are not adequate or feasible [8]. The use of LC-MS/MS and synthetic peptides labeled with stable isotopes, an internal standard have been reported recently as an ideal mean for the quantitative mass spectrometric analysis of their corresponding endogenous human peptides that are derived from native protein as they have the same chemical structure [9], [10].

In a previous report, the protein expression pattern of urine samples from lung cancer patients and healthy controls were compared using 2-DE. GM2AP in lung cancer patients was significantly higher than that found in healthy controls [11]. Hence, GM2AP became the protein of our focus. This protein was the choice of this study because the inherited deficiency of GM2AP has also been related to the changing level of ganglioside and tumor associated gangliosides involved in cancer progression. Tumor-associated gangliosides are a result of initial oncogenic transformation and play a role in the induction of invasion and metastasis [12], [13]. Tumor cells are synthesized and shed gangliosides into their microenvironments leading to elevated levels of tumor-associated gangliosides in the serum [14], [15]. Therefore, validation of GM2AP in lung cancer specimens is necessary prior to employment of this protein as biomarker, especially in the early detection of cancer.

In this study, we also confirmed the GM2AP expression in urine samples. The intensity of urinary GM2AP spot in lung cancer patients were found at higher levels than that analyzed for healthy controls. GM2AP levels in urine and also in serum samples of lung cancer patients and in healthy controls were verified and compared by Western blot analysis. Levels of GM2AP were higher in lung cancer patients than in the healthy controls, consistent with observation on 2-DE gel.

Moreover, the concentration of GM2AP in urine and lung cancer patients were quantified using ^{15}N-labled with synthetic peptide (SEFVVPDLELPSELTTGNYR) coupling with a triple quadrupole mass by multiple-reaction monitoring (MRM). Quantification of GM2AP levels in urine and serum samples were determined by measuring the ratio of the endogenous tryptic peptide of GM2AP against ^{15}N-labled with synthetic peptide standard. MRM successfully detected low levels of GM2AP both in urine and serum samples in lung cancer patients and healthy controls.

II. Methodology

A. Study Population

This study was approved by the research ethics committee, Faculty of Medicine, Chiang Mai University for research purpose only (No. 412/2011). A total of 15 urine samples (n = 7 for adenocarcinoma, n = 3 for small cell lung cancer, n = 3 for squamous cell carcinoma and n = 2 other type of carcinoma) and 15 serum samples (n = 9 for adenocarcinoma, n = 2 for small cell lung cancer and n = 4 for squamous cell carcinoma) from lung cancer patients enrolled at Maharaj Nakron Chiang Mai Hospital, Chiang Mai, Thailand from November 2011 to January 2012. The patients included 8 males and 7 females. The mean age of patients was 67 (range 30-70 years). Tumor stage was defined according to the American Joint Committee on Caner/International Union against Cancer Tumor-node-metastasis (TNM) classification system [16]. The control subjects were collected from healthy donors (n=15) for urine samples and healthy donors (n=15) for serum samples with no history of cancer, recruited from Associated Medical Science (AMS) Clinical Service Centre, Chiang Mai University, Chiang Mai, Thailand.

B. Sample Preparation

Approximately 200 mL of first morning urine was collected in a sterile tube. Subsequently, the urine samples were centrifuged at 12000 ×g for 30 min at 4 ℃ to remove cellular contamination and debris. The samples were loaded into the centricon tube (MW cut off at 3 kDa) (Millipore) and centrifuged at 5000 ×g at 4 ℃ for 1 h within 4 h. Distilled water was added to the centricon tube to partially desalt and elute out some interference. The supernatant containing proteins was collected, lyophilized and stored at -80 ℃ until further analysis. For the serum samples, blood specimens were obtained and allowed to clot for 30 min at room temperature before centrifugation at 3000 ×g for 10 min. The supernatants of serum samples were collected, divided into a small number of aliquots, and stored at -80 ℃ until further analysis. The concentrated urine and serum proteins were determined using the BCA protein assay (Bio-Rad).

C. 2-Dimensional Electrophoresis Analysis

Three hundred micrograms of total urinary proteins 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 applied onto IPG strip of pH 4-7 (18 cm, Amersham GE Healthcare, Uppsala, Sweden) for 2-DE analysis. The IPG strips were subsequently rehydrated on the IPGphor IEF system (Amersham GE Healthcare, Uppsala, Sweden) at 20 ℃ 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) for the first dimension. After that, 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). For the second dimension, 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℃ 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 GE Healthcare, Uppsala, Sweden) at a wavelength of 610 nm.

D. Identification of Protein by NanoLC-MS/MS

First, the protein spots were excised from the gel, destained, excised and washed twice with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate/ACN (1:1) and dried in 100% ACN. Proteins in the spots were reduced with 10 mM DTT in 25 mM ammonium bicarbonate at 56℃ 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/ACN (1:1) and dried in 100% ACN. Then, the spots were soaked in 50 mM ammonium bicarbonate containing trypsin (Promega, sequencing-grade modified), and incubated at 37℃ for at least 16 h. subsequently extracted three times with 50% ACN in 1% trifluoroacetic acid (TFA). 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. The eluted samples were frozen and lyophilized on a speed vacuum.

Second, the 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) 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. x 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. x 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. Survey full-scan MS conditions were at mass range (m/z) of 320-2000 and resolution of 30,000 at m/z 400. The database searches were performed against Swiss-Prot database using Mascot software.

E. Western Blot Analysis

Total of 15 μg of proteins in urine and serum samples were individually applied onto 14% SDS-PAGE. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and blocked with 5% BSA in PBS containing 0.05% Tween 20 (PBST) at 37℃ overnight. The membrane was incubated with rabbit polyclonal anti-human GM2AP primary antibody (Abcam, Cambridge, UK) at room temperature for 2 h, followed by an anti-rabbit HRP-conjugated secondary antibody (Abcam, Cambridge, UK) at room temperature for 1 h. The blot was detected with an enhanced chemiluminescence Western blotting detection system (ECL™ kit; PerkinElmer) and exposed to Fujifilm LAS-4000 Luminescent Image Analyzer (FUJIFILM Corporation, Japan). Band intensities were quantified using Image *J* program, and analyzed statistically using t-test to determine the P- value.

F. Synthetic Peptide Standard

A peptide (SEFVVPDLELPSELTTGNYR) was synthesized using Fmoc chemistry techniques employing a Prelude peptide synthesizer (Protein Technology; Tucson, AZ, USA) from Mission Biotech (Nangkang, Taipei, Taiwan). ^{15}N-labeled arginine or lysine was used at the C-terminus of the peptide. These amino acids were selected because all proteotypic peptides that result from tryptic digestion of proteins contain C-terminal arginine or lysine. The peptide purify was assessed by reversed-phase high-performance liquid chromatography (RP-HPLC), and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

G. Preparation of Urine and Serum Tryptic Digests and Addition of Internal Standard Peptides

Twenty microliters of urine and 50 μL of serum samples were denatured in 100 μL of 6 M urea, and 1.7 μL of 200 mM DTT was added to reduce disulfide bonds, followed by alkylation with 6.7 μL of 200 mM IAA in the dark at room temperature. The samples were subsequently digested with trypsin and incubated at 37℃ for overnight. 0.1% TFA was added to quench the reaction. The digested peptide mixture was applied onto a C18 ZipTip for desalting and was lyophilized on a speed vacuum. The peptides were dissolved in 0.1% formic acid in water (v/v). The final volume for MS analysis was 200 μL. Twenty microliters (2.151 μM) of the internal standard peptide of GM2AP was added to the peptide sample mixture as a relative internal standard peptide for the MRM runs.

H. MRM Assay

MRM assay was performed on a nanoLC system, which was connected to a hybrid triple quadrupole/ion trap mass spectrometer (5500 QTRAP, AB Sciex, Foster City, CA) equipped with a nanoelectrospray interface. The 5500 QTRAP was operated in positive ion MRM mode. The LC buffer system was as follows: mobile phase A, 0.1% formic acid/water and mobile phase B, 0.1% formic acid/ acetonitrile. Ten microliters of peptide was injected, separated and eluted at a flow rate of 300 μL/min on a linear gradient of mobile phase B from 2% to 40% . The gradient was ramped up to 98% B for 7 min and 2% B for 10 min to equilibrate the column for the next run. The total LC run time was 12 min. The analytical column was Agilent Poroshell 120 EC-C18 (100 mm x 2.1 mm i.d., 2.7 μm). Ion spray voltage was set to 5.5 kV, interface heater temperature of 550℃, GS1

(nebulizer gas) setting of 45 p.s.i., and curtain gas set to 20 p.s.i. Three MRM transitions per peptide were monitored and acquired at unit resolution both in the first quadrupoles (Q1) and third quadrupoles (Q3) to maximize specificity. The data analysis was performed using MRMPilot™ software (Applied Biosystems). For the concentration analysis, peak areas for transitions were extracted and normalized versus internal standard transitions (equation 1). Each normalized peak area for the individual transition was compared with the corresponding transition peaks of other runs to estimate the concentration in urine and serum samples of lung cancer patients and healthy donors.

$$Analyte\ (\mu M) = \frac{Analyte\ Area \times IS\ Concentration\ (\mu M)}{IS\ Area \times Dilution\ Factor} \quad (1)$$

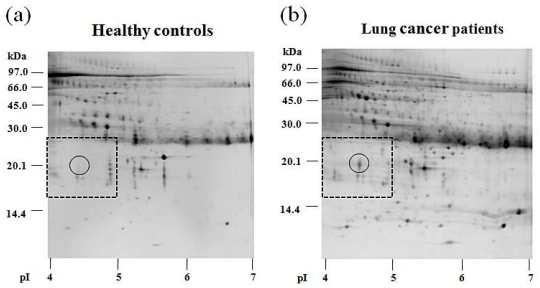
I. Statistical Analysis

Statistical analysis was carried out using t-test. A confidential level of 95% (P<0.05) for each factor was considered statistically significant.

III. Results and Discussion

A. Confirmation of the Expression of Urinary GM2AP by 2-DE

Three hundred micrograms of total urinary proteins were separated on IPG strip, pH 4-7, for the first dimension. Then, the proteins in strips were separated on 15% SDS-PAGE for second dimension. After that, the 2-DE gels were fixed and stained with Sypro® Ruby staining and scanned using a Typhoon 9200 laser scanner as shown in Fig. 1(a) and Fig. 1(b). The GM2AP spot from 2-DE gels were excised, pooled and digested with trypsin. Subsequently, the peptide samples were analyzed by nanoLC-MS/MS.



(c)

Spot no.	Protein name	Swiss-Prot Accession no.	MW /pI	Queries Matched	Seq. coverage (%)	MASCOT Total (Ion) score
1	GM2 activator protein, SAP3_HUMAN	P17900	20.8 / 5.1	13	40	154

Figure 1. A total of 300 µg of urinary proteins from healthy donors (a) and lung cancer patients (b) were resolved by 2-DE, which included first dimension with immunobilizer pH 4-7 and second dimension with 15% SDS-PAGE. The gels were stained by using Sypro® Ruby. The spot indicated with black circles was identified by nanoLC-MS/MS analysis (c).

The database searches were performed against Swiss-Prot database using Mascot software, and identified as GM2AP with mascot score of 154, queries matched of 13 and 40% of sequence coverage (Fig. 1 (c), respectively.

B. Verification of Increased GM2AP Level by Western blot Analysis

Due to the significantly elevated urinary GM2AP expression level in lung cancer patients when compared with healthy controls by 2-DE analysis, it was hypothesized that GM2AP might be used as a candidate biomarker that could distinguish lung cancer from healthy donors patients. First, urine samples from lung cancer patients (n=15) verified the presence and elevated level of GM2AP. Those patients did not show presence of the disorder from renal function as revealed by normal urine creatinine (Cr) and blood urine nitrogen (BUN), and also no presence of the biomarkers of kidney failure such as kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) (data not shown). Proteins were separated using 14% SAS-PAGE, transferred to PVDF membrane and blotted with specific against GM2AP antibody. The blot was visualized by ECL system. We observed significant differential expression of GM2AP between lung cancer patients and healthy controls. Fig. 2(a) shows the blots of urinary GM2AP from lung cancer patients and healthy donors. Levels of urinary GM2AP in lung cancer patients were expressed at higher level than that of healthy controls which was expressed the lowest level.

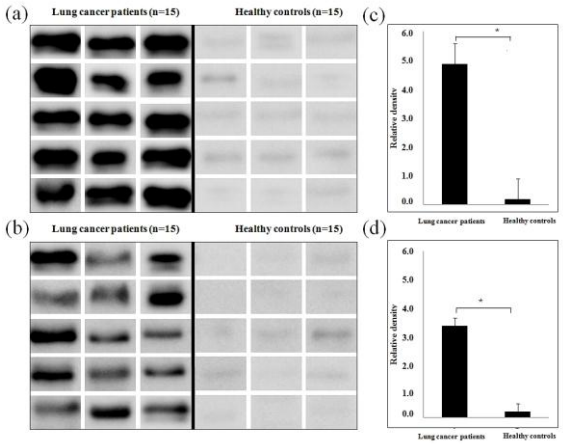


Figure 2. Western blot analysis of GM2AP. The focal areas of Western blot results visualized by ECL system are presented. Protein band intensities of GM2AP expression levels in healthy controls and lung cancer patients are shown for urine samples (a) and serum samples (b). The densitometric analysis of Western blot in urine samples (c) and serum samples (d). * P < 0.05.

Levels of GM2AP were significantly higher in lung cancer patients with a 23 fold increase when compared with healthy controls as shown in Fig. 2(c). However, urine is produced by kidneys and allows the human body to eliminate waste substances through filtration of the blood. The increased levels of urinary GM2AP may come from distant organs via plasma that was obtained through glomerular filtration and excreted as urine. We then used the serum samples from lung cancer patients (n=15) to investigate the presence of GM2AP level. The blots of serum GM2AP from lung cancer patients and healthy donors are shown in Fig. 2(b). It also significantly higher in lung cancer patients with 14-fold increase when compared with healthy controls (Fig. 2(d)).

C. ^{15}N-labeled Standard Peptide for Selection of Marker Peptide

The peptide was selected as representative signature peptide from isotope labeled standard peptides of GM2AP. ^{15}N-GM2AP was digested with trypsin and analyzed by nanoLC-MS/MS. The signature peptide ion of SEFVVPDLELPSELTTGNYR produced the best response at m/z 775.8 $[M+3H]^{3+}$ with molecular mass of 2324.61 Da (Fig. 3). Due to the majority of MRM approaches for protein quantification, ^{15}N stable isotope labeled synthetic peptide was used as the internal standard. Therefore, this peptide was selected, synthesized as the marker peptide for optimization and development of a MRM assay.

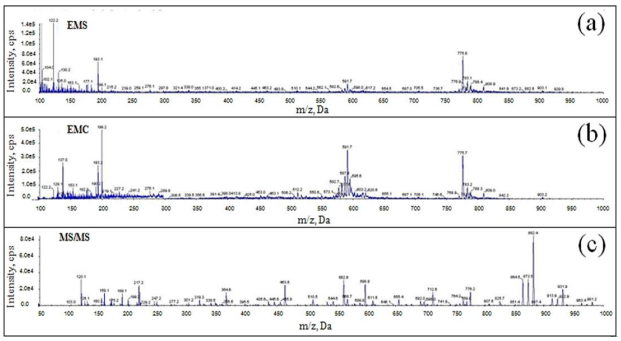


Figure 3. MRM analysis. ESM (a) scan was used to select the dominant parent ion, the most intense fragment ion were identified using the EMC (b) and MS/MS (c) fragment ion was used to select the best of fragments to extract.

D. Optimizing Transition of Internal Standard Peptide

First, we used ^{15}N-labeled GM2AP standard peptide and ^{15}N-labeled synthetic internal standard peptide of GM2AP at different concentrations of 1, 10, 50, 100, 500 and 1000 ng/mL for preliminary analysis. The samples were identified using LC-MS/MS analysis. The ion pairs of ^{15}N-labeled GM2AP standard peptide showed at the same retention time as the synthetic internal standard peptide at 7 min (data not shown). This result indicated that the chosen ion had high specificity and was a suitable internal standard for the detection of the corresponding endogenous GM2AP in urine and serum samples. Then, the transitions that corresponding collision energies (CE) of the target peptide was tuned to optimize the intensities of the fragments by direct injection of the ^{15}N-labeled synthetic peptide and GM2AP standard using a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometry equipped with a nanospray ionization source. The m/z value of the precursor was a Q1 transition, and the m/z values of its highest and second highest intensity fragment ions were Q3 transitions. The best transitions were chosen from the MS/MS spectrum according to the intensity of MS^2 peak. The best CE of every transition was selected using MRMPilot™ software. However, the ion pairs of ^{15}N-labeled GM2AP standard and synthetic internal standard peptide were difficult to discriminate because both peptides were only ^{15}N-labeled. After adjustment of the condition as described above, most significant ion pair of GM2AP standard and synthetic internal standard peptide were found at the same Q1/Q3 transition, therefore, both of them have specificity. The optimum MRM transitions of internal standard peptide as shown in Table I. The quantification was possible when the MRM assays were performed on urine and serum with complex biological samples.

TABLE I. Q1/Q3 TRANSITIONS OF THE INTERNAL STANDARD PEPTIDE FOR THE FIRST AND SECOND MRM ASSAY

Analytes	Q1 mass	Q3 mass	DP (V)	EP (V)	CE (V)	CXP (V)
Internal standard peptide	775.9	217.2	171	10	51	16
		463.4	141	10	31	28

E. Quantitation of GM2AP Level in Urine and Serum Samples by MRM Aaasy

The protocol was carried out individually for each urine sample of lung cancer patients (n=3) and healthy donors (n=3). Each sample was reduced, and alkylated prior to trypsin digestion. The ^{15}N-labeled internal standard peptide was added to the sample as internal standard. The nanoLC-MRM analysis was performed under the same conditions as described above. The most intense transition ions were selected, as the MRM signature for the development of a specific LC-MRM for GM2AP quantification. The peak areas of all monitored parent to product ion transitions of the GM2AP peptide were normalized by the peak area of the corresponding MRM transitions of the SEFVVPDLELPSELTTGNYR internal standard peptide. The area ratio of target GM2AP protein in urine or serum samples and internal standard from acquirement data were analyzed using peak area of urine or serum samples (peak of analyte) versus peak area of ^{15}N labeled internal standard peptide (peak of IS). The signature of peptide was found at high GM2AP protein or peptide concentration. Then, the concentration of endogenous GM2AP was determined using formula described in the methodology. The concentrations of urinary GM2AP levels in lung cancer patients were observed at 1.960, 1.334 and 9.303 μM/mL, whereas GM2AP level in healthy donors were found at 1.082, 1.177 and 1.028 μM/mL, respectively (Table II). At the same quantitative conditions, the concentrations of the serum samples of individual lung cancer patients (n=3) were determined, 2.113, 1.284 and 1.375 μM/mL, whereas healthy donors (n=3) were observed at 0.995, 0.953 and 0.768, respectively (Table III). The concentration of GM2AP level in lung cancer patients was determined to be substantially higher than that found in those of healthy donors. However, peak area of GM2AP standard and synthetic internal standard peptide was detected at a retention time of 7 min, whereas the peak areas of urine and serum signal shift to 1 min. This shift did not seem to affect the GM2AP quantification. It should be noted that the actual concentration of GM2AP level should be higher due to some limitation of efficiency of trypsin digestion. Therefore, the relative difference in GM2AP concentration observed across samples in this study represented the utility of MRM assay in complex protein mixtures for measuring the protein marker in lung cancer. For future investigation,

larger group of lung cancer patients and healthy controls will be required for confirmation of these preliminary results and for validation of GM2AP as a biomarker of lung cancer.

TABLE II. SUMMARY OF MRM QUANTITATIVE ASSAY IN URINE SAMPLES OF HEALTHY DONORS AND LUNG CANCER PATIENTS

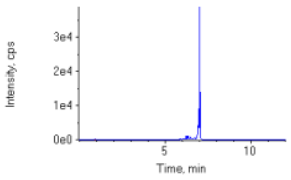
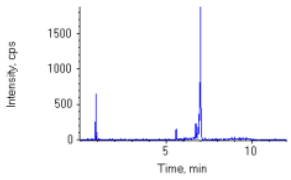
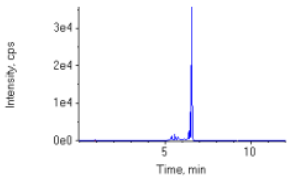
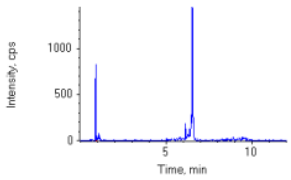
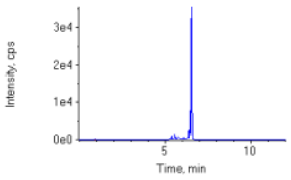
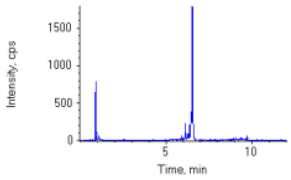
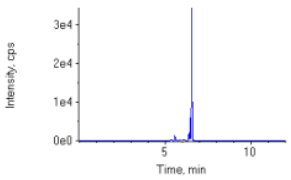
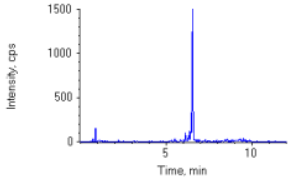
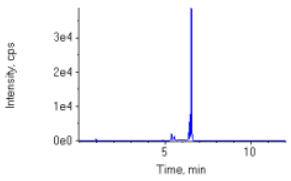
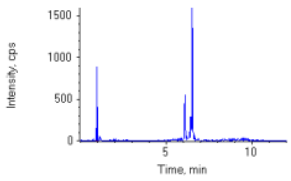
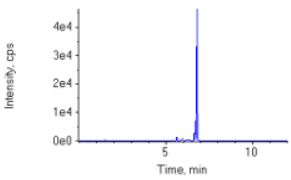
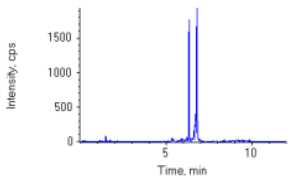
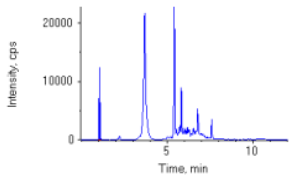
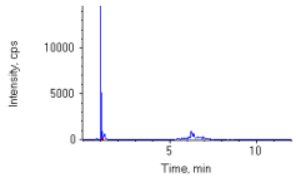
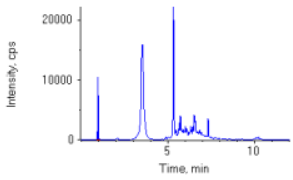
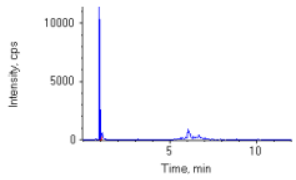
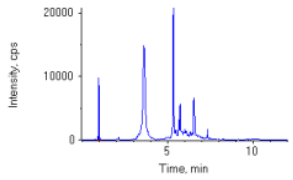
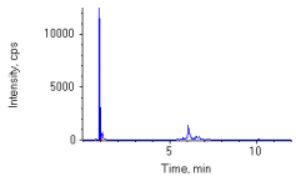
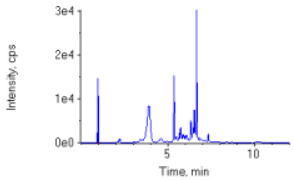
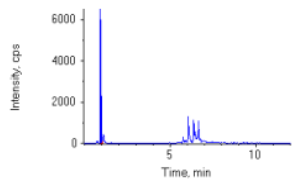
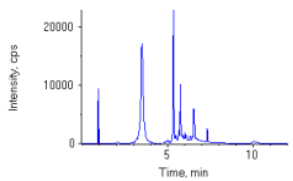
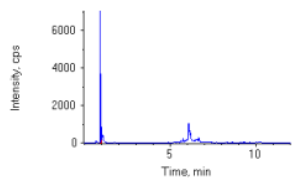
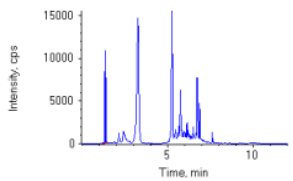
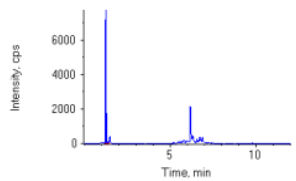
Sample Name	Peak of Analyte	Peak of IS	Analyte/IS Ratio	Dilution Factor	Concentration (μM)
Urine N1			**0.505**	**10X**	**1.082**
Urine N2			**0.547**	**10X**	**1.177**
Urine N3			**0.478**	**10X**	**1.028**
Urine P1			**0.911**	**10X**	**1.960**
Urine P2			**0.62**	**10X**	**1.334**
Urine P3			**4.325**	**10X**	**9.303**

TABLE III. SUMMARY OF MRM QUANTITATIVE ASSAY IN SERUM SAMPLES OF HEALTHY DONORS AND LUNG CANCER PATIENTS

Sample Name	Peak of Analyte	Peak of IS	Analyte/IS Ratio	Dilution Factor	Concentration (μM)
Serum N1			**1.156**	**4X**	**0.995**
Serum N2			**1.106**	**4X**	**0.953**
Serum N3			**0.893**	**4X**	**0.768**
Serum P1			**2.456**	**4X**	**2.113**
Serum P2			**1.492**	**10X**	**1.284**
Serum P3			**1.601**	**4X**	**1.378**

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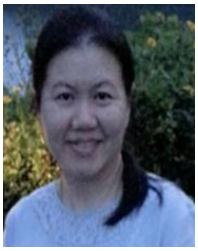

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