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A metabolomic approach to lung cancer

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ABSTRACT

Lung cancer is one of the most common cancers in the world, but no good clinical markers that can be used to diagnose the disease at an early stage and predict its prognosis have been found. Therefore, the discovery of novel clinical markers is required. In this study, metabolomic analysis of lung cancer patients was performed using gas chromatography mass spectrometry. Serum samples from 29 healthy volunteers and 33 lung cancer patients with adenocarcinoma ($n = 12$), squamous cell carcinoma ($n = 11$), or small cell carcinoma ($n = 10$) ranging from stage I to stage IV disease and lung tissue samples from 7 lung cancer patients including the tumor tissue and its surrounding normal tissue were used. A total of 58 metabolites (57 individual metabolites) were detected in serum, and 71 metabolites were detected in the lung tissue. The levels of 23 of the 58 serum metabolites were significantly changed in all lung cancer patients compared with healthy volunteers, and the levels of 48 of the 71 metabolites were significantly changed in the tumor tissue compared with the non-tumor tissue. Partial least squares discriminant analysis, which is a form of multiple classification analysis, was performed using the serum sample data, and metabolites that had characteristic alterations in each histological subtype and disease stage were determined. Our results demonstrate that changes in metabolite pattern are useful for assessing the clinical characteristics of lung cancer. Our results will hopefully lead to the establishment of novel diagnostic tools.

1. Introduction

Lung cancer is one of the most common cancers in the world and many lung cancer patients continue to have a poor prognosis. The high mortality and poor prognosis of lung cancer are mainly due to the difficulty of early diagnosis. The current diagnostic approaches to lung cancer are dependent on clinical conditions, chest X-rays, computed tomography, sputum cytology, conventional tumor markers and so on, but they are not suitable for screening study. Several oncogenes and anti-oncogenes for lung cancer, such as point mutation of K-ras and the deletion of chromosome 3p, have been found to be candidate biomarkers for lung cancer [1,2]. In addition, certain lung cancer-specific proteins made it possible to distinguish lung cancer from benign diseases [3] or to classify the histological characteristics of lung cancer [4]. However, they are not suitable for its early detection. Thus, useful clinical markers including conventional tumor markers that can be used to diagnose lung cancer at an early stage have not been discovered. Recently, metabolomics has developed rapidly. Metabolomics can be used to obtain information on the cellular processes of an organism. Therefore, among genes, proteins and metabolites, metabolites may be able to reflect physiological functions and pathological characteristics in most detail because the metabolome is the endpoint of the omics cascade. Actually, metabolomic technologies based on nuclear magnetic resonance analysis (NMR), gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and capillary electrophoresis/mass spectrometry (CE/MS) have been well-documented in the literature, and attempts have been made to apply them to various research fields. In our previous study, metabolite profiling of pancreatic cancer was performed using GC/MS, and alterations in various low molecular weight metabolites were observed [5]. In addition, metabolome analysis using LC/MS and CE/MS revealed alterations in the levels

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of various metabolites in gastric cancer, colon cancer and prostate cancer [6,7].

Cancer cells need to gain a considerable amount of energy for their proliferation, and the system for producing energy in many cancer cells is different from that in normal cells [8]. Cancer cells use large amounts of glucose and glutamine as energy sources and usually rely on glycolysis rather than oxidative phosphorylation for energy production, even in the presence of a sufficient supply of oxygen [8]. Otto Warburg first reported this phenomenon in 1956, and this phenomenon is now termed "the Warburg Effect" [9]. These results suggest that the presence of a tumor leads to alterations in the levels of low molecular weight metabolites including glycolysis and TCA cycle intermediates. Here, we carried out GC/MS-based metabolite profiling of lung cancer using lung tissue and serum obtained from lung cancer patients, and it was investigated whether alterations in the levels of low molecular weight metabolites are useful for diagnosing lung cancer.

2. Materials and methods

2.1. Sample collection

Serum samples were collected from 29 healthy volunteers without serious medical illness and 33 lung cancer patients without a previous history of other cancers at Kobe University Hospital from July 2009 to March 2010. Some patients have been diagnosed with lung cancer at the different institute, transferred to Kobe University Hospital, and then the serum sample was obtained at Kobe University Hospital. The sera were similarly collected from healthy volunteers and lung cancer patients in the morning fasting. Samples of tumor tissue and the surrounding non-tumor tissue were obtained from 7 lung cancer patients that had been treated with surgical excision. Pathological diagnosis was performed in all lung cancer patients. In this study, all lung cancer patients and healthy volunteers were informed of the purpose of this study and signed a written informed consent form. No anti-cancer agents were given to the enrolled patients.

2.2. Serum collection and preparation

Each whole blood sample was collected in a clean tube and was immediately centrifuged at 3000 × *g* for 10 min at 4 °C. The resultant serum was transferred into a clean tube and stored at −80 °C until use. The extraction of low molecular weight metabolites was performed according to our previous report [5].

2.3. Evaluation of the intra-day and inter-day variances for serum metabolites

To evaluate the intra-day variance for serum metabolites, whole blood samples of healthy volunteers ($n = 16$) were collected at 8:00 a.m.–9:00 a.m. before breakfast, 0:00 p.m.–1:00 p.m. before lunch, and 6:00 p.m.–7:00 p.m. before dinner. To evaluate the inter-day variance, whole blood samples ($n = 16$) were collected at 8:00 a.m.–9:00 a.m. before breakfast once a day for a total of 3 days. The serum was obtained by centrifugation at 3000 × *g* for 10 min at 4 °C, and then the extraction of low molecular weight metabolites was performed according to our previous report [5].

2.4. Tissue collection and preparation

The tumor and non-tumor tissues were cut into small pieces from the excised lung specimen, washed with saline to remove any blood, transferred to a clean tube, immediately frozen in liquid nitrogen, and stored at −80 °C until use. Non-tumor tissues were cut from areas that were sufficiently far away from the tumor region. Twenty milligrams of the lung tissue were transferred to a clean tube and homogenized in 1000 μl of a solvent mixture ($MeOH:H_2O:CHCl_3 = 2.5:1:1$). Then, 6 μl of 1.0 mg/ml 2-isopropylmalic acid (Sigma–Aldrich, Tokyo, Japan) dissolved in distilled water were added to each tube, and the mixture was mixed well. The mixture was subsequently shaken at 1200 × *g* for 30 min at 37 °C, before being centrifuged at 16,000 × g for 3 min at 4 °C. Nine hundred micro liters of the obtained supernatant were separately collected into a clean tube. Four hundred micro liters of distilled water were added to the collected supernatant, before being centrifuged at 16,000 × g for 3 min at 4 °C, and 800 μl of the resultant supernatant were collected into a clean tube. The collected supernatant was lyophilized using a freeze dryer before oximation and derivatization. Oximation and the subsequent derivatization were performed according to our previous report [5], and the resultant solution was subjected to GC/MS measurement.

2.5. GC/MS analysis

GC/MS analysis was performed using GCMS-QP2010plus (Shimadzu Co., Kyoto, Japan), and a DB-5 column (30 m × 0.25 mm i.d.; film thickness: 1.00 μm) (J&W Scientific, Folsom, CA) according to our previous report [5].

2.6. Data processing

The chromatogram acquisition, detection of mass spectral peaks and their waveform processing were performed using Shimadzu GCMSsolution software Version 2.53 (Shimadzu Co.), and the identification of low molecular weight metabolites was performed using a commercially available GC/MS Metabolite Mass Spectral Database (Shimadzu Co.) and a NIST Mass Spectral Library (NIST 08) according to our previous report [5]. The peaks were assigned on the condition of possessing a similarity index of more than 70. To perform a semi-quantitative assessment, the peak intensity of each quantified ion was calculated and normalized to the peak intensity of 2-isopropylmalic acid as an internal standard. The peak intensity of each quantified ion obtained from the results for the lung tissue was normalized to the tissue weight to evaluate the amount of metabolite contained per gram of each sample.

2.7. Statistics

The dataset for the multiple classification analysis was compiled from the metabolite profiling results, and we constructed a three-dimensional matrix using the sample names (observations), metabolite numbers (variable indices), and normalized peak intensities (variables). Partial least squares discriminant analysis (PLS-DA) was performed using commercially available SIMCA-P+ Software Version 12.0.1 (Umetrics, Umeå, Sweden). In chemometric analysis, UV scaling was applied to the data processing. Data are expressed as the mean ± S.D. Statistical significance was analyzed using the Student's *t*-test, and a level of probability of 0.05 was used as the criterion for significance.

3. Results

3.1. Subject characteristics

In this study, sera from 33 lung cancer patients and 29 healthy volunteers, and the lung tissue from 7 lung cancer patients were subjected to measurement by GC/MS (Table 1). Pathological diagnosis and the clinical (c) or pathological (p) stage were based on General Rule for Clinical and Pathological Record of Lung Cancer,

Table 1
Clinical characteristics of healthy volunteers and lung cancer patients.

	Healthy volunteers (serum)	Lung cancer patients (serum)	Lung cancer patients (tissue)
N (male/female)	29 (23/6)	33 (26/7)	7 (6/1)
Age (median/range)	64/34–78	65/55–81	61/53–82
Smoker/non-smoker/unknown	6/16/7	30/2/1	6/1/0
Smoking Index (median/range)	126 (0–1000)	800 (0–1800)	720 (0–1800)
Pathological diagnosis (Ad/Sq/SCLC)		12/11/10	4/3/0
c or p stages (I–II/III–IV)		11/22	6/1

Smoking Index: number of cigarettes smoked per day × years. Ad, adenocarcinoma; Sq, squamous cell carcinoma; SCLC, small cell lung carcinoma. c stage (clinical stage) and p stage (pathological stage) were based on the TNM classification.

2003, the 6th Edition. In this study, c or p stages I and II were defined as early stage disease, and c or p stages III and IV were classified as advanced stage disease.

3.2. Metabolite profiling of lung cancer

In our experimental conditions, a total of the 58 metabolites (57 individual metabolites) were detected in serum (Table 2 and Supplemental Table 1), and 71 metabolites were detected in the lung tissue (Table 3 and Supplemental Table 2). As shown in Table 2 and Supplemental Table 1, the levels of 23 of the 58 serum metabolites were significantly changed in all lung cancer patients including early and advanced disease stages compared with the healthy volunteers ($p < 0.05$). In the early stage lung cancer patients, significant alterations in the levels of 13 metabolites were observed, and the levels of 17 metabolites were significantly changed in sera of the advanced stage lung cancer patients compared with the healthy volunteers. In the lung tissue, the levels of 48 of the 71 metabolites were significantly changed in the tumor tissue compared with the non-tumor tissue ($p < 0.05$) (Table 3 and Supplemental Table 2).

3.3. Alterations in the levels of metabolites involved in the TCA cycle

Regarding the metabolites involved in the TCA cycle, 6 metabolites; i.e., citric acid, isocitric acid, succinic acid, fumaric acid, malic acid and lactic acid, were observed in sera of the lung cancer patients and healthy volunteers, and the levels of the following metabolites were significantly changed (fold induction): the levels of lactic acid (×1.51), fumaric acid (×1.76) and malic acid (×1.40) were significantly increased in the lung cancer patients compared with the healthy volunteers. In the lung tissue, 7 TCA cycle-related metabolites i.e., citric acid, isocitric acid, 2-oxoglutaric acid, succinic acid, fumaric acid, malic acid and lactic acid, were found, and the levels of lactic acid (×1.22), succinic acid (×1.84), fumaric acid (×1.86) and malic acid (×1.84) were significantly increased in the tumor tissue compared with the non-tumor tissue.

3.4. Alterations in the levels of amino acids and their derivatives

Sixteen amino acids and their derivatives were detected in sera of the lung cancer patients and healthy volunteers; i.e., L-alanine, L-glycine, sarcosine, L-valine, L-leucine, L-isoleucine, L-proline,

Table 2
The list of significantly changed metabolites ($p < 0.05$) in serum of lung cancer patients and healthy volunteers.

Metabolites	Lung cancer, early stage		Lung cancer, advanced stage		Lung cancer, all	
	p value	Fold induction	*p* value	Fold induction	*p* value	Fold induction
Lactic acid	<0.001[a]	1.42	<0.0001[a]	1.56	<0.0001[a]	1.51
2-Hydroxyisobutyric acid	0.0012[a]	1.29	<0.0001[a]	1.39	<0.0001[a]	1.35
L-Glycine	0.645	1.08	0.013[a]	1.40	0.052	1.29
Sarcosine	0.866	1.02	0.0001[a]	1.24	0.0027[a]	1.17
3-Hydroxybutyric acid	0.0038[a]	2.41	0.104	2.09	0.0029[a]	2.20
2-Hydroxyisovaleric acid	0.114	1.40	0.019[a]	1.62	0.0091[a]	1.47
Malonic acid	0.069	1.27	0.014[a]	1.22	0.0036[a]	1.31
Benzoic acid	0.012[a]	1.21	<0.0001[a]	1.48	<0.0001[a]	1.39
Octanoic acid	0.039[a]	1.23	<0.0001[a]	1.74	<0.0001[a]	1.58
Glycerol	0.092	0.74	0.208	0.79	0.031[a]	0.77
Phosphoric acid	0.068	0.86	0.106	0.87	0.013[a]	0.87
L-Proline	0.0042[a]	1.41	0.101	1.35	0.0015[a]	1.34
Glyceric acid	0.0025[a]	0.57	<0.0001[a]	0.42	<0.0001[a]	0.47
Fumaric acid	<0.0001[a]	1.61	<0.0001[a]	1.84	<0.0001[a]	1.76
L-Threonine	0.248	1.16	0.052	1.27	0.040[a]	1.24
Malic acid	0.0036[a]	1.35	0.033[a]	1.43	0.0002[a]	1.40
2-Hexenedioic acid	0.975	1.01	0.0008[a]	1.16	0.022[a]	1.11
Aspartic acid	0.0032[a]	0.58	0.051	0.68	0.0003[a]	0.65
5-Oxoproline	0.114	0.89	0.0062[a]	0.83	0.0019[a]	0.85
4-Hydroxyproline	0.056	1.68	0.414	1.31	0.029[a]	1.43
2-Propyl-glutaric acid	0.097	1.09	0.024[a]	1.16	0.018[a]	1.13
L-Glutamic acid	0.371	0.83	0.031[a]	0.67	0.026[a]	0.72
Lauric acid	0.0089[a]	0.64	0.507	0.83	0.035[a]	0.77
Aconitic acid	0.082[a]	1.49	0.471	1.02	0.327	1.17
4-Hydroxyphenyllactic acid	0.099	0.73	0.042[a]	1.48	0.402	1.23
Indol-3-acetic acid	0.434	1.12	0.015[a]	0.80	0.301	0.91
Uric acid	0.0007[a]	2.84	0.180	2.08	0.0007[a]	2.33

Values are represented as the fold-induction of peak intensity of lung cancer patients with early stage disease ($n = 11$), lung cancer patients with advanced stage disease ($n = 22$), or all lung cancer patients ($n = 33$) against that of the healthy volunteers ($n = 29$). *p* values were calculated according to the Student's *t*-test, and superscript letters (a) indicate *p* values lower than 0.05. All data were represented in Supplemental table* 1, and the significantly changed metabolites in serum of lung cancer patients were showed in Table 2.

Table 3
The list of significantly changed metabolites ($p < 0.05$) in tumor and non-tumor lung tissues.

Metabolites	p value	Fold induction
Lactic acid	0.017[a]	1.22
Propanoic acid	0.004[a]	1.20
Glycolic acid	0.012[a]	1.33
L-Alanine	0.007[a]	1.70
Oxalic acid	0.047[a]	1.41
2-Hydroxybutyric acid	0.022[a]	1.64
Butanoic acid	0.040[a]	1.82
Ethanimidic acid	0.007[a]	1.60
Malonic acid	0.049[a]	1.54
L-Valine	0.019[a]	1.88
L-Leucine	0.021[a]	1.74
Phosphoric acid	0.001[a]	1.18
L-Isoleucine	0.007[a]	2.07
L-Proline	0.008[a]	2.02
Succinic acid	0.006[a]	1.84
Glycine	0.006[a]	1.77
Pyrimidine	0.007[a]	1.92
Fumaric acid	0.002[a]	1.86
Uracil	0.011[a]	1.86
L-Serine	0.029[a]	1.82
Aminomalonic acid	0.014[a]	2.32
L-Threonine	0.023[a]	1.89
Malic acid	0.0003[a]	1.84
Aspartic acid	0.016[a]	1.59
Methionine	0.013[a]	2.05
5-Oxoproline	0.005[a]	2.06
L-Cysteine	0.019[a]	3.26
thiodiglycolic acid	0.009[a]	1.97
2-Hydroxyglutaric acid	0.002[a]	2.29
L-Glutamic acid	0.003[a]	2.31
L-Phenylalanine	0.016[a]	2.21
4-Hydroxyphenylacetic acid	0.024[a]	2.10
Lauric acid	0.001[a]	0.39
N-Acetylaspartic acid	0.009[a]	2.01
D-Ribose	0.005[a]	2.66
α-Aminoadipic acid	0.010[a]	2.36
L-Glutamine	0.019[a]	2.54
9H-purine	0.005[a]	2.18
DL-Ornithine	0.045[a]	1.59
Myristic acid	0.024[a]	0.69
4-Hydroxyphenyllactic acid	0.004[a]	2.95
L-Tyrosine	0.012[a]	2.43
D-Gluconic acid	0.005[a]	1.73
Stearic acid	0.033[a]	1.07
L-Tryptophan	0.019[a]	4.43
Inosine	0.001[a]	1.97
Adenosine	0.037[a]	1.69
Guanine	0.013[a]	2.02

Values are represented as the fold-induction of peak intensity of the lung tumor tissue against that of the non-tumor tissue ($n = 7$). p values were calculated according to the Student's t-test, and superscript letters (a) indicate p values lower than 0.05. All data were represented in Supplemental table* 2, and the significantly changed metabolites in the lung tissue of lung cancer patients were showed in Table 3.

isovalerylglycine, L-methionine, aspartic acid, 5-oxoproline, L-serine, L-threonine, 4-hydroxyproline, L-glutamic acid, and L-phenylalanine. A significant increase in the serum level of sarcosine (×1.17), L-proline (×1.33), L-threonine (×1.24) and 4-hydroxyproline (×1.43) was observed in the lung cancer patients compared with the healthy volunteers. On the contrary, the levels of aspartic acid (×0.64), 5-oxoproline (×0.85) and L-glutamic acid (×0.72) were significantly decreased. Among their metabolites, in the early stage lung cancer patients, the levels of L-proline (×1.41) and aspartic acid (×0.58) were significantly changed. Regarding the advanced stage lung cancer patients, the levels of L-glycine (×1.40), sarcosine (×1.24) and 5-oxoproline (×0.83) were significantly changed. In the lung tissue, the levels of the following metabolites were significantly increased in the tumor tissue compared with the non-tumor tissue: L-alanine (×1.70), L-valine (×1.88), L-leucine (×1.74), L-isoleucine (×2.07), L-proline (×2.02), glycine (×1.77), L-serine (×1.82), L-threonine (×1.89), aspartic acid (×1.59), methionine (×2.05), 5-oxoproline (×2.06), L-cycteine (×3.26), L-glutamic acid (×2.31), L-phenylalanine (×2.21), N-acetylaspartic acid (×2.01), L-glutamine (×2.54), DL-ornithine (×1.59), L-tyrosine (×2.43) and L-tryptophan (×4.43).

3.5. Alterations in the levels of metabolites of fatty acids, nucleotides and others

In sera from the lung cancer patients, the levels of 2-hydroxyisobutyric acid (×1.35), 3-hydroxybutyric acid (×2.20), 2-hydroxyisovaleric acid (×1.47), malonic acid (×1.31), benzoic acid (×1.39), octanoic acid (×1.58), glycerol (×0.77), phosphoric acid (×0.87), glyceric acid (×0.47), 2-hexenedioic acid (×1.11), 2-propyl-glutaric acid (×1.13), lauric acid (×0.77) and uric acid (×2.33) were significantly changed in comparison to those in the healthy volunteers. In the lung tissue, the levels of propanoic acid (×1.20), glycolic acid (×1.33), oxalic acid (×1.41), 2-hydroxybutylic acid (×1.64), butanoic acid (×1.82), ethanimidic acid (×1.60), malonic acid (×1.54), phosphoric acid (×1.18), aminomalonic acid (×2.32) thiodiglycolic acid (×1.97), 2-hydroxyglutaric acid (×2.29), 4-hydroxyphenylacetic acid (×2.10), α-aminoadipic acid (×2.36), 4-hydroxyphenyllactic acid (×2.95), D-gluconic acid (×1.73) and stearic acid (×1.07) in the tumor tissue were significantly increased in comparison to those of the non-tumor tissue, and the levels of lauric acid (×0.39) and myristic acid (×0.69) were significantly decreased. Regarding nucleotides, the levels of inosine (×1.97), adenosine (×1.69), guanine (×2.02), pyrimidine (×1.92), uracil (×1.86) and 9H-purine (×2.18) in the tumor tissue were significantly increased in comparison to those in the non-tumor tissue, although no nucleotides were observed in serum.

3.6. Serum metabolites characterizing disease stage

The differences in the levels of serum metabolites among the lung cancer patients with early or advanced stage disease and the healthy volunteers were assessed using PLS-DA. The PLS-DA scores plots showed discrimination among the early stage lung cancer patients, advanced stage lung cancer patients and healthy volunteers, and the early stage patient group lied between the healthy volunteer and advanced stage patient groups (Fig. 1A). In the corresponding PLS-DA loadings plots (Fig. 1B), lactic acid, 2-hydroxyisobutyric acid, L-glycine, sarcosine, benzoic acid, octanoic acid, fumaric acid, malic acid, 2-hexenedioic acid and 2-propyl-glutaric acid contributed to the clustering of the lung cancer patients with advanced stage disease, and their levels were increased in the advanced stage patient group with or without a significant difference (Supplemental Fig. 1A). On the contrary, glyceric acid, aspartic acid, 5-oxoproline, phosphoric acid and L-glutamic acid contributed to the clustering of the healthy volunteer group, and the early stage patient group was characterized by 3-hydroxybutyric acid, L-proline, 4-hydroxyproline and uric acid (Supplemental Fig. 1A).

3.7. Serum metabolites characterizing histological type

The differences in the levels of serum metabolites among various histological types; i.e., adenocarcinoma (Ad), squamous cell carcinoma (Sq), and small cell lung carcinoma (SCLC), were assessed using PLS-DA. The PLS-DA scores plots exhibited discrimination among the lung cancer patient groups with each histological type and the healthy volunteer group (Fig. 2A). Corresponding PLS-DA loadings plots demonstrated that certain metabolites contributed to the clustering of various histological types as follows (Fig. 2B): the Ad group was characterized by glycine, 2-hydroxyisovaleric acid, methionine and phenylalanine (Supplemental Fig. 1B). The

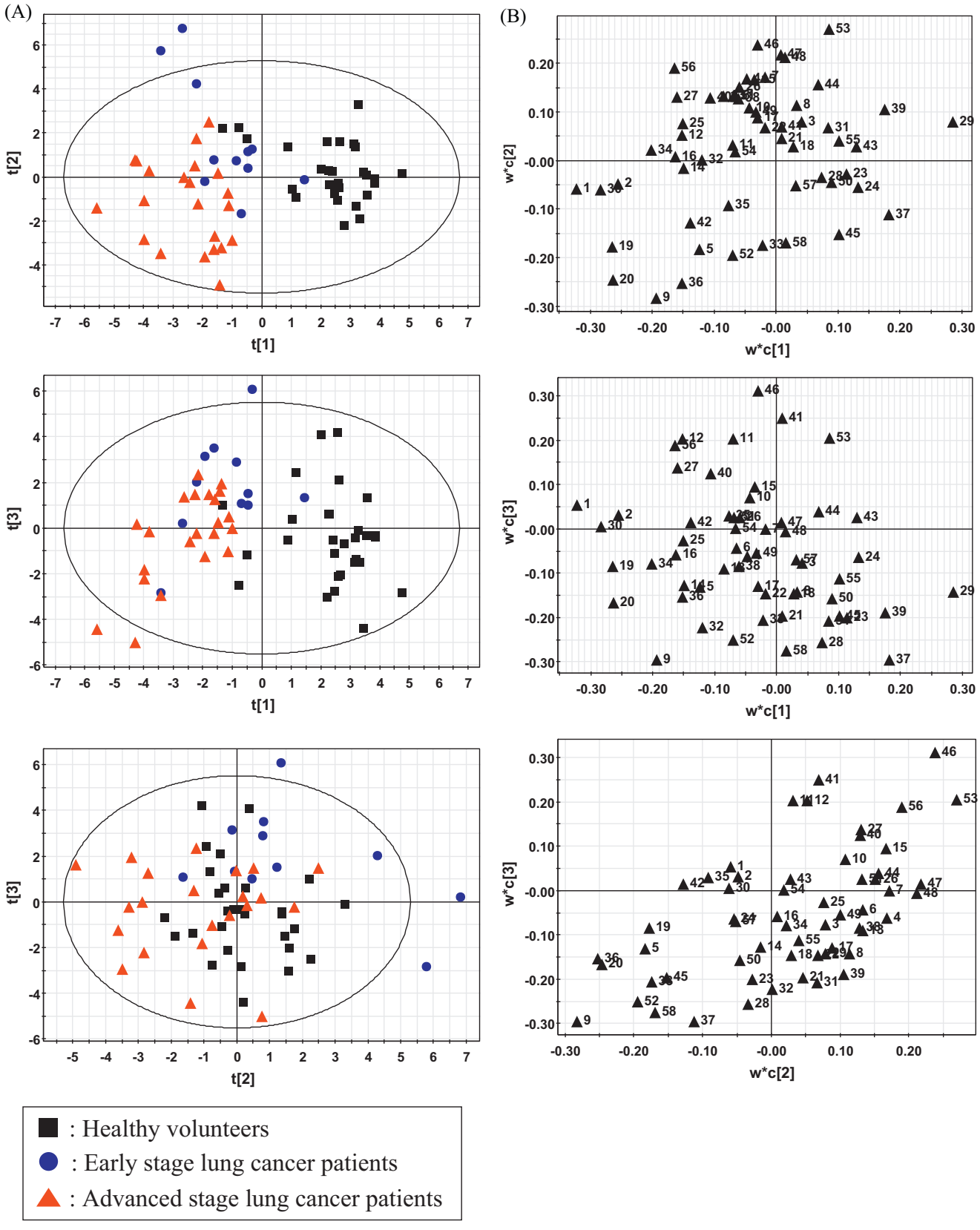

Fig. 1. PLS-DA discriminating among stages based on the metabolite profiling data. (A) PLS-DA scores plots discriminating among early stage lung cancer patients, advanced stage lung cancer patients and healthy volunteers. The black squares, blue circles and red triangles indicate healthy volunteers ($n = 29$), early stage lung cancer patients ($n = 11$) and advanced stage lung cancer patients ($n = 22$), respectively. The principal components PC1 ($t[1]$), PC2 ($t[2]$) and PC3 ($t[3]$) described 13.3, 10.5 and 11.7% of the variation, respectively ($A = 3$, $R_2X = 0.355$). The ellipse indicates Hotelling T2 (0.95) for this model. (B) PLS-DA loadings plots discriminating among early stage lung cancer patients, advanced stage lung cancer patients and healthy volunteers. The PLS-DA loadings plots were calculated on the basis of (A). Each metabolite is indicated by the corresponding number shown in Supplemental table* 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

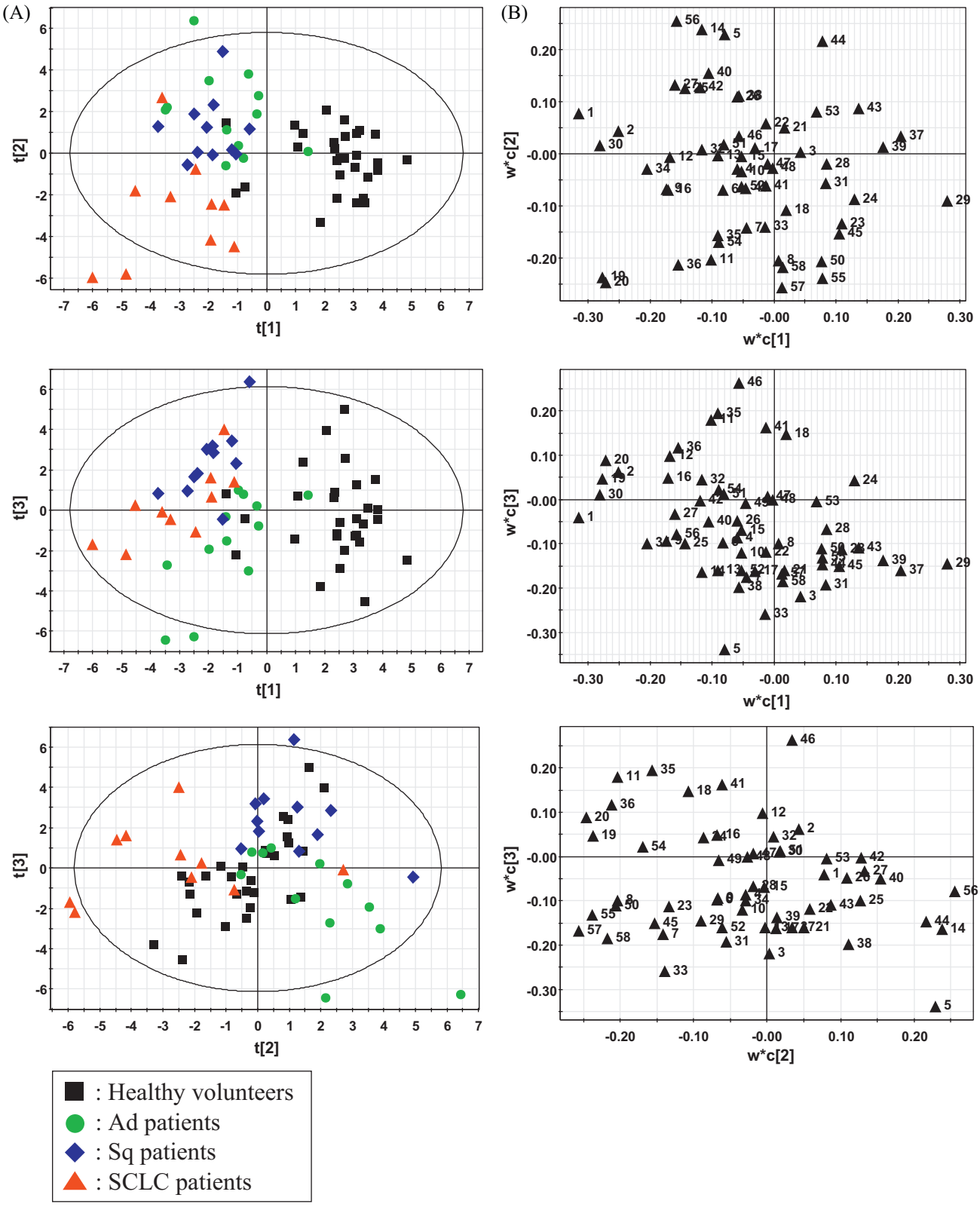

Fig. 2. PLS-DA discriminating among histological types based on the metabolite profiling data. (A) PLS-DA scores plots discriminating among various histological types of lung cancer patients. The black squares, green circles, blue diamonds, and red triangles indicate healthy volunteers ($n = 29$), Ad patients ($n = 12$), Sq patients ($n = 11$) and SCLC patients ($n = 10$), respectively. The principal components PC1 ($t[1]$), PC2 ($t[2]$) and PC3 ($t[3]$) described 13.5, 11.1 and 14.1% of the variation, respectively ($A = 3$, $R_2X = 0.387$). (B) PLS-DA loadings plots discriminating among various histological types of lung cancer patients. The PLS-DA loadings plots were calculated on the basis of (A). Each metabolite is indicated by the corresponding number shown in Supplemental table* 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Sq group was typified by aconitic acid (Supplemental Fig. 1B), and pyruvic acid, benzoic acid, octanoic acid, isovalerylglycine, 2-hexenedioic acid and palmitoleic acid were found to characterize the SCLC group (Supplemental Fig. 1B).

3.8. Sensitivities of conventionally applied tumor markers and serum metabolites

The tumor markers that are conventionally applied in lung cancer are carcinoembryonic antigen (CEA), which suggests Ad or non-small cell lung carcinoma (NSCLC); cytokeratin 19 fragment (CYFRA), which suggests Sq or NSCLC; squamous cell carcinoma related antigen (SCC), which suggests Sq; and neuron-specific enolase (NSE) and progastrin releasing peptide (pro-GRP), which suggest SCLC. The sensitivities of these tumor markers for the lung cancer patients enrolled in this study are shown in Supplemental Table 3. The standard values for each tumor marker in our hospital are as follows: CEA: 0–5.0 ng/ml, CYFRA: 0–3.5 ng/ml, and NSE: 0–10.0 ng/ml. The sensitivity of CEA for Ad was 33.3%, the sensitivity of CYFRA for Sq was 72.7%, and the sensitivity of NSE for SCLC was 90.0%. The sensitivity of CEA for Ad was not very high compared with those of CYFRA for Sq and NSE for SCLC. The sensitivities of the metabolites obtained from the metabolomic approach (Supplemental Fig. 1B) are exhibited in Table 4. In this study, the average values of the healthy volunteers and the maximum or minimum values of the healthy volunteers were used as cut-off values. In the case of the maximum or minimum values of the healthy volunteers that were used as cut-off values, the sensitivity of 2-hydroxyisovaleric acid was higher than of CEA for Ad. The sensitivity of aspartic acid was relatively high, although it was lower than the sensitivity of CYFRA for Sq. The sensitivity of octanoic acid was also the same as that of NSE for SCLC.

3.9. Evaluation of the intra-day and inter-day variances for serum metabolites

Finally, the intra-day and inter-day variances for serum metabolites were evaluated (Supplemental Table 4 and Supplemental Fig. 2). To evaluate the intra-day variance for serum metabolites, whole blood samples ($n = 16$) were collected at 8:00 a.m.–9:00 a.m. before breakfast (Day 1_A), 0:00 p.m.–1:00 p.m. before lunch (Day 1_B), and 6:00 p.m.–7:00 p.m. before dinner (Day 1_C). To evaluate the inter-day variance, whole blood samples ($n = 16$) were collected at 8:00 a.m.–9:00 a.m. before breakfast once a day for a total of 3 days (Day 1_A, Day 2_A and Day 3_A). The results of the serum metabolites listed in Supplemental Table 1 were shown in Supplemental Fig. 2. Some metabolites, such as L-alanine, oxalic acid, 2-hydroxybutyric acid, 3-hydroxyisobutyric acid, L-valine, octanoic acid, ethylmalonic acid, leucine, glycerol, phosphoric acid, isoleucine, decanoic acid, malic acid, methionine, 4-hydroxyproline, L-phenylalanine, 4-hydrophenyllactic acid, palmitic acid and margaric acid, exhibited the diurnal variation, and the levels of these metabolites were higher or lower at Day 1_B and/or Day 1_C compared with Day 1_A, although it was the small-scale variation. On the other hand, the number of serum metabolites with the dynamic inter-day variance was small; i.e., L-glycine, glyoxylic acid, 3-hydroxyisobutyric acid, aspartic acid, L-glutamic acid and palmitoleic acid. These results indicate that blood collection in the morning fasting is suitable for serum metabolite profiling.

4. Discussion

In this study, sera from 29 healthy volunteers and 33 lung cancer patients, and surgically resected tumor tissue and the surrounding normal tissue from 7 lung cancer patients were subjected to GC/MS-based metabolomics, and then it was investigated whether the pathogenesis of lung cancer leads to alterations in the levels of low molecular weight metabolites, and furthermore, whether the targeted metabolites are useful for diagnosing lung cancer.

The pathogenesis of lung cancer may vary the metabolites involved in the TCA cycle and its related signaling pathways. In this study, the levels of metabolites related to the TCA cycle; i.e., succinic acid, fumaric acid and malic acid, were revealed to be different between lung cancer and normal tissues (Table 3 and Supplemental Table 2). Most cancer cells depend on aerobic glycolysis rather than oxidative phophorylation for energy production [8,9]. Cancer cells also use glutamine as a major source of energy [10], and glutamine metabolism allows tumor cells to sustain TCA cycle activity during tumor proliferation. Thus, the biological systems for energy production in cancer cells are different from those in normal cells. Therefore, their characteristic metabolic pathways might result in alterations in the levels of some metabolites in tumor tissue. Actually, a previous study revealed that the levels of amino acids and their derivatives in colon and stomach cancer tissue were significantly higher than those in normal tissue [6]. In this study, higher levels of most amino acids in lung cancer tissue compared with normal tissue were also demonstrated (Table 3 and Supplemental Table 2). Interestingly, the change of the glutamine level observed in lung cancer (Table 3 and Supplemental Table 2) was different from that seen in colon and stomach cancer [6]: the glutamine level was higher in the lung tumor tissue compared with the normal tissue, whereas its level in colon and stomach tumor tissues was nearly equal to that in normal tissue. The glutamine level was expected to be decreased because a large amount of glutamine is consumed in cancer cells through glutamine metabolism. There are several reasons why the glutamine level was higher in lung tumor tissue compared with the normal tissue. In a previous study, it was reported that cigarette smoke affected metabolic conversion in human alveolar epithelial carcinoma A549 cells, and the glutamine level was particularly increased [11]. Most lung cancer patients enrolled in this study were smokers, suggesting that exposure to cigarette smoke or cell inflammation might lead to an increased glutamine level. In a previous study, it was also revealed that the levels of lactate, alanine, succinic acid, aspartic acid and citric acid but not glutamine were higher in human lung tumor tissue [12], consistent with our findings (Table 3 and Supplemental Table 2). Taken together, glycolysis, the TCA cycle and its related signaling pathways may be abnormal in various tumor tissues including lung tumor tissue.

Alterations in the levels of small molecular weight metabolites in lung tumor tissue reflect the variations in the levels of serum metabolites in lung cancer patients. Some metabolites exhibited similar changes in serum and tumor tissue, whereas some metabolites demonstrated opposite changes. For example, the levels of fumaric acid, malic acid, lactic acid, 2-hydroxyisobutyric acid and L-proline were elevated in serum as well as lung tumor tissue (Tables 2 and 3 and Supplemental Tables 1 and 2). However, the changes in the levels of glyceric acid, aspartic acid and 5-oxoproline differed between serum and tumor tissue (Tables 2 and 3 and Supplemental Tables 1 and 2). These results suggest that when a tissue turns cancerous, the levels of some small molecular weight metabolites are elevated and that these metabolites move from the tissue into the blood, resulting in increases in their blood levels. Regarding certain metabolites, when a tissue turns cancerous, the metabolites move from the blood into the tissue to promote cancer cell proliferation, resulting in decreases in their blood levels. We also analyzed serum metabolites based on disease progression and histological subtypes. The levels of some metabolites, such as lactic acid, benzoic acid and fumaric acid, increased with disease progression (Supplemental Fig. 1A), suggesting the alteration of these metabolites through metastasis to other organs or increased inflammation. On the contrary, the levels of some metabolites, for

Table 4
Sensitivities of the examined metabolites.

Characterized metabolites	Ad		Sq		SCLC	
	Average values as cut-off	Max or Min values as cut-off	Average values as cut-off	Max or Min values as cut-off	Average values as cut-off	Max or Min values as cut-off
All lung cancer						
Glycerol	91.7	0	81.8	0	60.0	10.0
Phosphoric acid	75.0	16.7	81.8	18.2	60.0	20.0
Glyceric acid	91.7	8.3	100	9.1	100	0.0
Aspartic acid	91.7	0	90.9	45.5	100	20.0
5-Oxoproline	66.7	0	81.8	0	90.0	10.0
L-Glutamic acid	75.0	0	90.9	0	100	10.0
Lauric acid	83.3	0.0	100	18.2	80.0	20.0
Ad						
Glycine	83.3	8.3				
2-Hydroxyisovaleric acid	91.7	41.7				
Methionine	66.7	25.0				
Phenylalanine	50.0	8.3				
Sq						
3-Hydroxybutyric acid			45.5	27.3		
Aconitic acid			72.7	18.2		
SCLC						
Pyruvic acid					80.0	20.0
Benzoic acid					100	70.0
Octanoic acid					100	90.0
Isovalerylglycine					90.0	40.0
2-Hexenedioic acid					100	70.0
Palmitoleic acid					60.0	20.0

Each sensitivity is presented as a percentage of patients with values higher than the cut-off value for each metabolite. Cut-off values were estimated using two values; i.e., the mean value for healthy volunteers (left column) and the maximum (Max) or minimum (Min) values for healthy volunteers (right columns). The metabolites characterizing all lung cancer and each histological type on the basis of the results obtained from Figs. 1B and 2B are listed in the left column. Ad, adenocarcinoma; Sq, squamous cell carcinoma; SCLC, small cell lung carcinoma.

example glyceric acid and L-glutamic acid, decreased with disease progression (Supplemental Fig. 1A), suggesting the transfer of these metabolites from the blood into the lung tissue and their subsequent consumption in the tissue. In our study, it was observed that differences between histological types are reflected in the serum metabolite levels (Supplemental Fig. 1B). Borkzuk et al. revealed gene expression profiles associated with NSCLC histological subtype in human lung carcinoma tissue [13]. In another report by Seike et al., proteomic analysis outlined the protein profiles associated with each histological type of lung cancer [4]. No report has evaluated the relationship between the changes in the metabolite levels and lung cancer histological type, and our metabolomic approach is the first report to propose differences in the metabolite profile among the various histological subtypes of lung cancer, and moreover, it raises the possibility that alterations in the serum metabolite levels of lung cancer patients represent the influence of cancer as well as inflammation. Taken together, metabolomics may be able to demonstrate the characteristics of lung cancer in detail.

For discovery of serum metabolites as biomarker candidates, understanding of the intra-day and inter-day variances is important. Our study exerted that some metabolites exhibit the diurnal variation, although it was the small-scale variation (Supplemental Fig. 2). The diurnal variation may be due to the regular diet and activities of daily living, because there were few serum metabolites with the dynamic inter-day variance in the case of blood collection in the morning fasting (Supplemental Fig. 2). These results indicate that blood collection in the morning fasting is suitable for the serum metabolite profiling, and our data from the lung cancer patients and healthy volunteers (Tables 2 and 3 and Supplemental Tables 1 and 2) were acquired using blood collected in the morning fasting.

The tumor markers conventionally applied in lung cancer are CEA (for NSCLC and Ad), CYFRA (for NSCLC and Sq), SCC (for Sq), NSE (for SCLC), and pro-GRP (for SCLC). In our hospital, CEA, CYFRA and NSE are applied as a blood screening test. In the study by the Hamburger Group for the Standardization of Tumor Markers, the sensitivity of each tumor marker was defined as follows [14,15]: the sensitivity of CEA for Ad, 58.0%; the sensitivity of CYFRA for Sq, 66.4%; and the sensitivity of NSE for SCLC, 58.6%. These sensitivities are not so high, and similar results were exhibited in our study (Supplemental Table 3). As shown in Table 4, some serum metabolites had higher sensitivities than the conventionally used tumor markers. Each cellular process induces alterations in the levels of multiple metabolites, and our metabolomic approach can measure the levels of a variety of metabolites. Therefore, understanding the changes in the levels of multiple metabolites may lead to the discovery of markers for the early detection of lung cancer.

5. Conclusion

In GC/MS-based metabolome analysis of lung cancer, it was revealed that the levels of various metabolites in serum and lung tissue were changed by the pathogenesis of lung cancer. In addition, alterations in the serum metabolite levels may represent the differences between disease stages or histological subtypes in lung cancer. Our results propose a novel approach to developing diagnostic tools for lung cancer and suggest that knowing the pattern of alterations in the levels of small molecular weight metabolites is useful for understanding diseases. Further experiments using the larger number of samples are required to pursue these possibilities, and the independent test cohort for analysis of serum metabolites is also needed as the next research.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2011.02.008.

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