Introduction

Several minimally-invasive, easy-to-use cancer diagnostic methods using peripheral blood or urine samples have recently been developed to ease the physical burden on patients and to reduce the costs and time involved [1,2,3,4,5,6,7,8]. Rapid advances have been made in cancer diagnosis and prognosis methods based on metabolome analysis [3,9,10,11,12,13,14], which frequently involves the use of multivariate analysis techniques, such as computer-aided, machine-learning systems for data mining.

Although metabolome analysis is a promising approach in screening for diseases such as cancer, some practical limitations remain. These include the necessity to measure a huge number of metabolites [15,16,17], data-redundancy problems, including the false-discovery rate (FDR) and overfitting, and cost constraints. One approach to overcoming these problems is "focused metabolomics", which limits the objects of the analysis to those that play roles in general metabolism and share physical similarities.

Amino acids are among the most suitable candidates for focused metabolomics as they are either ingested or synthesized endogenously and play essential physiological roles both as basic metabolites and metabolic regulators. To measure amino acids, plasma free amino acids (PFAAs), which abundantly circulate as a medium linking all organ systems, would be the most favorable target because their profiles have been known to be influenced by metabolic variations in specific organ systems induced by specific diseases [18,19,20,21]. Additionally, plasma samples can be collected easily from patients.

Several investigators have also reported changes in PFAA profiles in cancer patients [22,23,24,25,26,27,28]. However, despite evidence of a relationship between PFAA profiles and some types of cancer, few studies have explored the use of PFAA profiles for diagnosis because, although PFAA profiles differ significantly between patients, the differences in individual amino acids do not always provide sufficient discrimination abilities by themselves [24,29,30]. To address this issue, we previously constructed and tested a diagnostic index based on PFAA concentrations, known as the "AminoIndex technology" [29,30,31,32,33], to compress multidimensional information from PFAA profiles into single dimension and maximize the differences between patients and controls (Figure 1). We obtained preliminary data on the efficacy of the "AminoIndex technology" for the early detection of colorectal, breast, and lung cancers in approximately 150 samples from a single medical institute [29,30].

Moreover, technologies have recently been developed to analyze amino acids with high accuracy. For example, we developed a method to measure PFAA profiles using high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)–mass spectrometry (MS) [34,35,36].

The present study aimed to determine the possibility of PFAA profiling for cancer diagnosis using a large number of samples from multiple medical institutes. We measured the PFAA profiles of approximately 200 cancer patients from three different institutes each with one of the following five types of cancer: lung, gastric, colorectal (CRC), breast, or prostate cancer. Patients were compared to five times sizes of gender- and age-matched controls also used in this study. We then compared the alterations in the

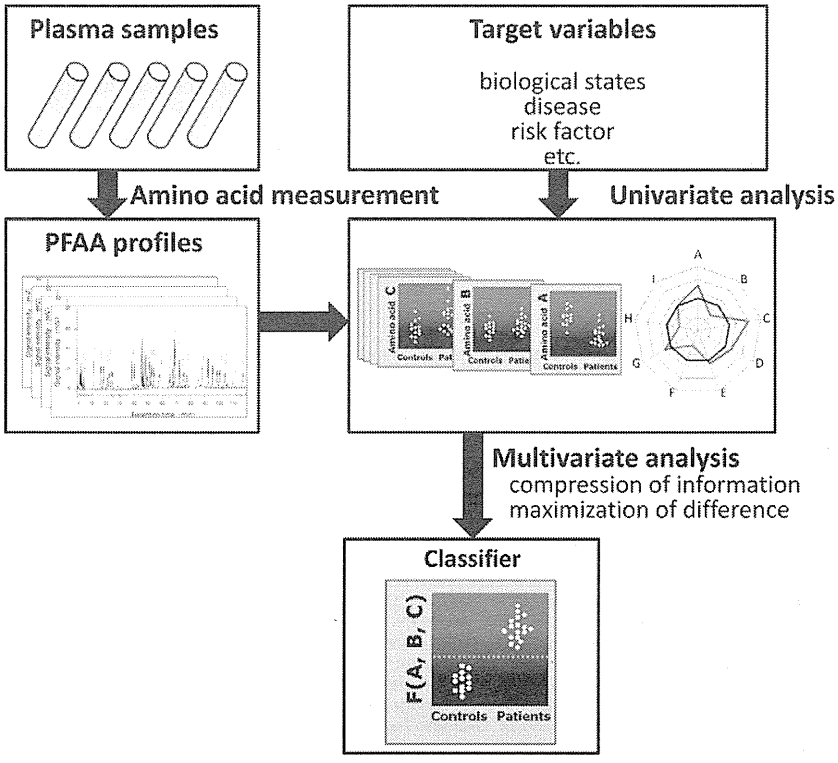

Figure 1. Concept of the generation of "AminoIndex technology". At the top of the diagram, PFAA concentrations are measured for each subject. In the middle, target variables and univariate analysis of PFAA profiles are represented. At the bottom, an estimation of the classifier with optimized discriminating power using multivariate analysis is presented.
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PFAA profiles between the cancer patients and the controls using univariate and multivariate analyses. As a result, significant alterations in PFAA profiles were observed in cancer patients in comparison to control subjects. We demonstrated two types of alterations in PFAA profiles in cancer patients: some differences reflected the metabolic changes common to many cancers, while others were specific to each type of cancer. We also found that both common and cancer type-specific alterations in PFAA profiles were observed even in the patients with early stage cancer. Furthermore, using a large number of samples allowed us to verify the robustness of PFAA profiling for the early detection of various cancers.

Materials and Methods

Ethics

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethics committees of the Kanagawa Cancer Center, the Osaka Medical Center for Cancer and Cardiovascular Diseases, the Okayama University Hospital, the Yokohama City University Medical Center, the Gunma Prefectural Cancer Center, the Shizuoka Prefectural Cancer Center, the Chiba Prefectural Cancer Center, the Yokohama Municipal Citizen's Hospital, the Yokohama Minami Kyosai Hospital, the Kanagawa Health Service Association, the Kameda Medical Center Makuhari, and the Mitsui Memorial Hospital. All subjects gave their written informed consent for inclusion before they participated in the study. All data were analyzed anonymously throughout the study.

Subjects

Data from Japanese patients with lung cancer (LC), gastric cancer (GC), colorectal cancer (CRC), breast cancer (BC), and prostate cancer (PC) were analyzed in this study. The patients had been histologically diagnosed with primary cancer at various Japanese medical institutes between 2006 and 2009. The LC patients were recruited from the Osaka Medical Center for Cancer and Cardiovascular Diseases, the Chiba Prefectural Cancer Center, the Kanagawa Cancer Center, and the Gunma Prefectural Cancer Center. The GC patients were recruited from the Okayama University Hospital, the Gunma Prefectural Cancer Center, and the Shizuoka Prefectural Cancer Center. The CRC patients were recruited from the Kanagawa Cancer Center, the Shizuoka Prefectural Cancer Center, and the Gunma Prefectural Cancer Center. The BC patients were recruited from the Yokohama City University Medical Center, the Kanagawa Cancer Center, and the Gunma Prefectural Cancer Center. The PC patients were recruited from the Kanagawa Cancer Center, the Yokohama Municipal Citizen's Hospital, the Yokohama Minami Kyosai Hospital, and the Gunma Prefectural Cancer Center. Control subjects with no apparent cancer were chosen from among those undergoing comprehensive medical examinations at three different Japanese medical institutes (the Center for Multiphasic Health Testing and Services of the Mitsui Memorial Hospital, the Kameda Medical Center Makuhari, and the Kanagawa Health Service Association) between 2008 and 2009.

Colonic polyp patients were recruited from among those undergoing endoscopic polypectomy at the Kameda Medical Center Makuhari between 2006 and 2008.

For the purposes of data analysis, the patients were assigned to five groups based on their primary cancer diagnoses (~140–200 patients per group), and five age- and gender-matched control groups were also established (Table 1). Data sets for all of the cancer patients and controls, as well as all cancer patients stratified by gender, were also analyzed.

PFAA measurement

Blood samples were collected from the controls and the patients prior to any medical treatment. Blood samples (5 ml) were collected from forearm veins after overnight fasting in tubes containing ethylenediaminetetraacetic acid (EDTA; Termo, Tokyo, Japan) and were immediately placed on ice. Plasma was prepared by centrifugation at 3,000 rpm at 4°C for 15 min and then stored at −80°C until analysis. After the plasma collection, all samples were stored and processed at the Institute for Innovation of the Ajinomoto Co., Inc. (Kawasaki, Japan). To reduce any bias

Table 1. Demographic and clinical characteristics of subjects.

Data set		LC		GC		CRC		BC		PC	
		Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls
Size	Total	200	996	199	985	199	995	196	976	134	666
	M/F	125/75	635/371	126/73	626/359	114/85	570/425	0/196	0/976	134/0	666/0
Age	Mean	65.0[a]	63.2	64.8[a]	62.9	63.7	62.4	55.3	54.5	69.4[c]	65.8
	(SD)	(10.0)	(9.2)	(10.8)	(9.7)	(9.5)	(9.5)	(12.6)	(11.1)	(6.7)	(6.1)
BMI	Mean	22.5	22.9	22.7	22.8	23.0	22.8	22.4	22.0	23.4	23.4
	(SD)	(3.8)	(3.0)	(3.2)	(3.0)	(3.7)	(3.0)	(3.4)	(3.5)	(2.7)	(2.5)
Stage	0	-		-		8		26	-	-	
	I(A)	29		120		63		75		0	
	II(B)	16		29		48		73		95	
	III(C)	54		26		59		13		19	
	IV(D)	28		24		19		0		15	
	Uncharacterized	1		0		2		9		5	

[a]$p<0.05$,
[c]$p<0.001$.
For LC, GC, CRC, and BC, cancer stages were determined according to the International Union Against Cancer TNM Classification of Malignant Tumors, 6th edition [38], and for PC, cancer stages were determined according to Jewett staging system [39].
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introduced prior to analysis, samples were analyzed in random order. The plasma samples were deproteinized using acetonitrile at a final concentration of 80% before measurement. The amino-acid concentrations in the plasma were measured by HPLC–ESI–MS, followed by precolumn derivatization. The analytical methods used were as described previously [34,35,36].

Among the 20 genetically-encoded amino acids, glutamate (Glu), aspartate (Asp), and cysteine (Cys) were excluded from the analysis because they are unstable in blood. Citrulline (Cit) and ornithine (Orn) were measured instead because they are relatively abundant in blood and are known to play important roles in metabolism. The following 19 amino acids and related molecules were therefore measured and analyzed: alanine (Ala), arginine (Arg), asparagine (Asn), Cit, glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), Orn, phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val).

Two metrics were made for each of the 19 amino acids including the absolute concentration of each amino acid, which directly reflected its availability and consumption, and the ratios associated with the specific metabolic status in each organ. The concentrations of the amino acids in the plasma were expressed in μM, and the ratios of the amino acid concentrations were expressed by the follow equation:

$$X2_{i,j} = \frac{X_{i,j}}{\sum_k X_{i,k}}$$

where $X2_{i,j}$ is ratio of the amino-acid concentration of the j-th amino acid of i-th subject, and $X_{i,j}$ is the plasma concentration (μM) of the j-th amino acid of i-th subject.

Statistical analysis

Two types of metric were used for each data set for analysis using either the amino-acid concentration or the ratio as explanatory variables.

Mean and SD. The mean amino-acid concentrations ± standard deviations (SDs) were calculated to determine summarized PFAA profiles for both patients and controls.

Mann-Whitney U-test. The Mann-Whitney *U*-test was used to assess significant differences of the plasma amino-acid concentrations between the patients and the controls.

ROC analysis. Receiver-operator characteristic (ROC) curve analyses were performed to determine the abilities of uni- and multi-variate analyses to discriminate between patients and controls. The patient labels were fixed as positive class labels. Therefore, an area under the ROC curve (AUC of ROC) value of <0.5 indicated that the amino acid level was lower in the patients than the controls, whereas an AUC of ROC value of >0.5 indicated that it was higher. The 95% confidence interval (95% CI) of AUC of ROC for the discrimination of patients based on amino acid concentrations and ratios was also estimated as described by Hanley and McNeil [37].

Two-way analysis of variance (ANOVA). The two-way ANOVA was used to evaluate the effects of gender, age, and smoking status as potential confounding factors. The presence of cancer and gender were assumed to be independent factors, age was treated as a continuous predictor rather than a categorical predictor, and the interaction term between the presence of cancer and smoking status was analyzed.

Two-class linear discrimination analysis (LDA). Linear discrimination analysis (LDA) with stepwise variable selection was performed to distinguish patients with each type of cancer from the control subjects, in which both the maximum and the minimum p-values for a term to be added or removed were set at 0.001.

Multi-class LDA for discrimination. LDA with stepwise variable selection was also performed to distinguish patients with a specific cancer from the complete data set containing all cancer patients stratified by gender (four kinds of cancer patients in each data set). Because the size of each group was smaller than that of two-class LDA, the maximum p-value for a term to be added was set at 0.05 and the minimum p-value for a term to be removed was set at 0.10. The Mahalanobis distance was used as a metric of classification. The accuracy was defined as the ratio of the correctly discriminated patients to the total number of patients with each cancer instead of AUC of ROC because ROC analysis could be applied only for two-class discrimination.

Leave one out cross—validation (LOOCV). LOOCV was performed to correct potential over-optimization for obtained LDA models. Briefly, one sample was omitted from the study data set, and the LDA model was calculated for the remaining samples to estimate coefficients for each amino acid. The function values for the left-out sample were calculated based on the model. This process was repeated until every sample in the study data set had been left out once.

Conditional logistic-regression (c-logistic) analysis. C-logistic analysis was also performed to verify the effects of age and gender, potential confounding factors, on the discriminatory abilities of obtained LDA models to differentiate patients with each type of cancer from the controls.

Subgroup analysis. To assess the effects of cancer stage, each data set was divided into a sub-data set according to disease stage and including corresponding controls, and analyzed using the ROC analysis in each data set.

Software

MATLAB (The Mathworks, Natick, MA) was used for the calculations of mean and SD, the Mann-Whitney *U*-test, ROC analysis, two-way ANOVA, LDAs, and LOOCV. GraphPad Prism (GraphPad Software, La Jolla, CA) was also used for the ROC curve analysis. LogXact (Cytel, Cambridge, MA) was used for the c-logistic analysis.

Results

Characteristics of subjects

Table 1 summarizes the characteristics of the subjects in this study. The data sets comprised 200 LC patients and 996 controls, 199 GC patients and 985 controls, 199 CRC patients and 995 controls, 198 BC patients and 976 controls, and 134 PC patients and 666 controls (Table 1). The sample size for each cancer type was greater than those in previous reports [25] and provided sufficient statistical power to test the robustness of the PFAA profiles for cancer diagnosis.

There were no significant differences in body mass index (BMI) among the data sets (Table 1). Weight loss due to malnutrition was therefore not expected to influence the results. Although significant differences in average age were observed among the data sets (LC, $p<0.05$; GC, $p<0.05$; and PC, $p<0.001$), the effects appeared to be relatively minor because the absolute values of these differences were small (Table 1).

For LC, GC, CRC, and BC, disease stages were determined according to the Sixth Edition of the International Union Against Cancer (UICC) Tumor–Node–Metastasis (TNM) Classification of Malignant Tumors [38]. For PC, the stage was determined

according to the Jewett staging system [39]. For all types of cancer, a large proportion of the patients had early-stage disease. The fractions of patients at each stage according to type of cancer were as follows: ~50% stage I, ~10% stage II, ~25% stage III, and ~15% stage IV for LC; ~60% stage I, ~15% stage II, ~13% stage III, and ~12% stage IV for GC; ~35% stages 0 and I, ~25% stage II, ~30% stage IV, and ~10% stage IV for CRC; ~5% stage 0, ~25% stage I, ~25% stage II, and ~7% stage III for BC; and ~75% stage B, ~13% stage C, and ~12% stage D for PC (Table 1).

The patients with each type of cancer could be further subdivided based on histological type (for LC, GC, CRC, and BC) or Gleason score (for PC), as is summarized in Table S1. The characteristics of 34 colonic polyp patients as well as the smoking status of patients are also summarized in Table S1.

Shared PFAA profiles among cancers

Univariate analysis was used to compare the PFAA profiles of the cancer patients and controls. The differences in the significance levels of each amino acid between the patients and the controls are shown in Figure 2A. The results of the ROC analysis are depicted in Figure 2B because the levels of significance depend on sample size. The concentrations and ratios of each amino acid profile for both patients and controls are shown in Tables S2. And the AUCs of ROC and their CIs of each amino acid are shown in Table S3 (concentration) and Table S4 (ratio), respectively.

Two-way ANOVA was used to evaluate the potential confounding effects of gender, age, and smoking status. Correcting for these factors did not greatly affect the significance levels of each amino acid, suggesting that their effects on the PFAA profiles were minor (Table S5).

The plasma concentrations of Gln, Trp, and His were significantly decreased in all of the cancers except PC, and none of the amino acids showed increased concentrations across all types of cancer ($p<0.05$). The ratios of Trp and His were significantly decreased, while those of Pro and Orn were increased, in all cancers ($p<0.05$) (Figure 2).

To further examine the shared traits among cancer patients, the PFAA profiles were compared using a pooled data set including all cancer patients and controls. Notably, the amino acids that were affected by this type of analysis had significant differences in both concentration and ratio: 11 amino acids (Asn, Gln, Cit, Val, Met, Leu, Tyr, Phe, His, Trp, and Arg) showed decreases, while four amino acids (Ser, Pro, Gly, and Orn) exhibited increases (Figure 2). Changes in Gln, Trp, His, Pro, and Orn were detected in the analysis for all types of cancer. Alterations in these amino acids might therefore reflect characteristic changes in metabolism that are common to all cancers.

Specific PFAA profiles for each cancer

In addition to the changes that were common to all of the cancers, we detected alterations in PFAA profiles that were specific to each disease type (Figure 2). Overall, the concentrations of most amino acids were decreased in GC and CRC patients, whereas no clear trends in amino acid concentrations were observed in the other groups (Figure 2). Furthermore, some of the amino acids showed opposite trends in different types of cancer. For example, the concentrations of Thr were decreased in GC and CRC patients, but increased in BC patients (Figure 2). These variations in the PFAA profiles might reflect specific characteristics of each cancer, in contrast to the limited set of amino acids that are responsible for the metabolic changes shared by all cancers.

Changes in PFAA profiles in early-stage cancers

Although alterations in the PFAA profiles of cachexic patients with advanced cancer have been well documented, few reports have considered early-stage patients. However, a large fraction of the cancer patients in the current data set were in the early stages of disease (Table 1). The differences in PFAA profiles according to disease stage were therefore examined for each cancer (Figure 3, Figure S1, Table S3, Table S4).

Notably, alterations in the PFAA profiles were detected in all patients, including those in the early stages of disease, in the current study. All amino-acid concentrations and ratios were drastically decreased in early stage disease patients, regardless of the subsequent progression. In particular, significant decreases of each amino acid concentration were observed in GC and CRC patients (Figure 3A), and changes in each ratio were notable in all of the cancer patients (Figure 3B).

Early-stage cancer patients are generally asymptomatic. Moreover, most of the subjects in the present study did not show significant weight loss (a symptom typical of cachectic patients) (Table 1), anorexia, or decreases in serum albumin concentrations (data not shown). The changes in the PFAA profiles in cancer patients therefore appeared to be independent of any effects caused by poor nutrition resulting from tumor progression.

Discriminating cancer patients and controls by PFAA profiles

The results of the univariate analyses suggested that cancer patients and controls could be discriminated using multivariate analysis. By assuming that the presence of cancer and the concentrations or ratios of the PFAA profiles were objective and explanatory variables, respectively, LDA was able to distinguish cancer patients from the corresponding controls with variable selection. The results of variable selection are indicated in Table 2 (concentration) and Table S6 (ratio), respectively.

The discrimination abilities for each cancer patient were evaluated using the AUC of ROC of the discriminate score and were found to be >0.75 in all cases (Table 3 and Table S7). In concrete analysis, AUCs for the discrimination of patients based on the amino acid concentrations and ratios, respectively, were also estimated as follows: 0.802 (95% CI: 0.766~0.838) and 0.802 (95% CI: 0.767~0.837) for LC; 0.849 (95% CI: 0.816~0.882) and 0.816 (95% CI: 0.780~0.852) for GC; 0.874 (95% CI: 0.842~0.906) and 0.881 (95% CI: 0.851~0.910) for CRC; 0.778 (95% CI: 0.741~0.815) and 0.778 (95% CI: 0.741~0.815) for BC; and 0.783 (95% CI: 0.740~0.826) and 0.779 (95% CI: 0.740~0.819) for PC (Table 3 and Table S7). The discriminate analysis was therefore able to adequately distinguish between different types of patient cancer.

Variable selection was also performed for each cancer patient. Eight amino acids were selected in more than two of the five kinds of cancers: Gln, Ala, Val, Ile, His, Trp, Orn, and Lys for the concentrations (Table 2A); and Ser, Gln, Val, Met, His, Trp, Lys, and Arg for the ratios (Table S6). Four of the amino acids (Gln, Val, His, and Trp) among each set were selected for both explanatory variables (Table 2 and Table S6). These amino acids were similar to those associated with all types of cancer as indicated by the univariate analysis (Gln, Trp, His, Pro, and Orn).

On the other hand, some amino acids incorporated into the LDA model were not identified as significant amino acids by the univariate analysis. For example, the Val concentration did not show a significant alteration in the univariate analysis (Figure 2A), but it was incorporated into the LDA model (Table 2). Because plasma concentrations of each amino acid are metabolically connected to each other, there might be a potential correlation that cannot be

A

Concentration

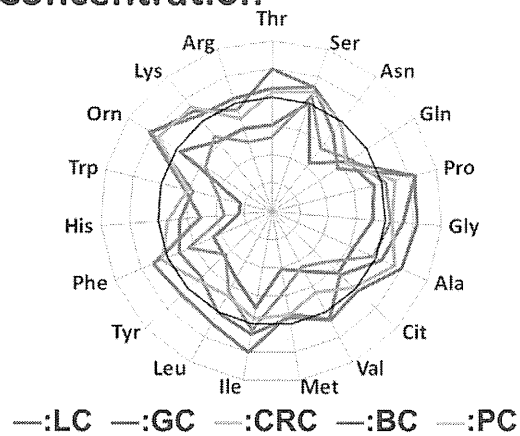
Amino acid	LC	GC	CRC	BC	PC	Pooled
Thr						
Ser						
Asn						
Gln						
Pro						
Gly						
Ala						
Cit						
Val						
Met						
Ile						
Leu						
Tyr						
Phe						
His						
Trp						
Orn						
Lys						
Arg						

Ratio

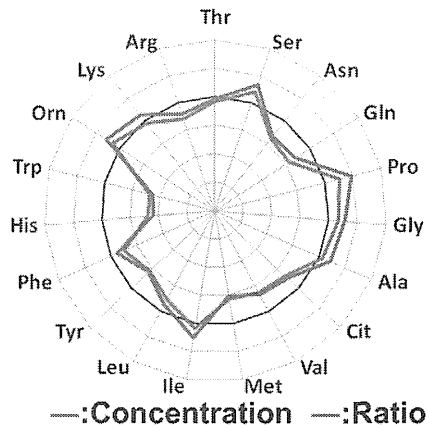
Amino acid	LC	GC	CRC	BC	PC	Pooled
Thr						
Ser						
Asn						
Gln						
Pro						
Gly						
Ala						
Cit						
Val						
Met						
Ile						
Leu						
Tyr						
Phe						
His						
Trp						
Orn						
Lys						
Arg						

B

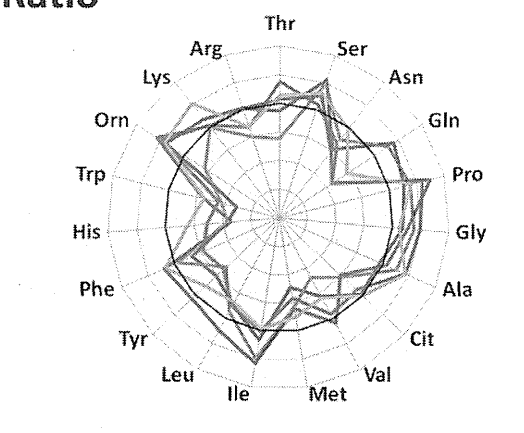
Concentration



Pooled data



Ratio



Scale of AUC of ROC

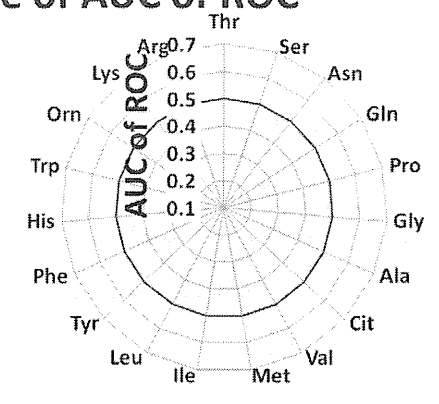


Figure 2. PFAA profiles of cancer patients. The results of the Mann-Whitney *U*-test (A) and receiver-operator characteristic (ROC) curve analysis (B) are indicated. A. Colored cells indicate that the concentration or ratio is increased in cancer patients at $p<0.001$ (red), $p<0.01$ (orange), and $p<0.05$ (pink), and decreased in cancer patients at $p<0.001$ (blue), $p<0.01$(sky blue), and $p<0.05$ (light blue), respectively. B. Axes show the AUC of ROC for each amino acid to discriminate patients from controls. Concentrations and ratios of each cancer patient and the pooled data set are indicated, respectively. Black bold lines indicate the point where the AUC of ROC = 0.5.
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detected by the univariate analysis alone. Indeed, Spearman's partial correlation coefficient between Val and cancer (or not) was −0.127 ($p<0.001$), while the correlation coefficient between these two factors was 0.035 (not significant). Therefore, this suggested that the obtained LDA model reflected the metabolic network of PFAAs, which were not apparent thorough univariate analysis.

Because the obtained results may have been over-optimized, LOOCV was carried out to generate an unbiased analysis. This produced AUCs similar to those obtained for LDA, suggesting that there was no obvious over-optimization in the obtained LDA models (Table 3 and Table S7).

Subgroup analyses of divided data sets according to cancer stage, including corresponding controls, were then performed to assess the ability of PFAA profiles to distinguish between stages of cancer for each type of disease. In any stage of each cancer, the AUC of ROC was found to be higher than 0.75, suggesting that the obtained LDA models would thus be expected to be effective in detecting early as well as advanced stage cancers (Table 3 and Table S7).

The discrimination abilities for all cancer patients were also evaluated. The AUCs of ROC for both concentrations and ratios were 0.796 (95% CI: 0.779~0.814) and 0.785 (95% CI: 0.767~0.803), respectively (Table 3 and Table S7). Notably, most of the 19 amino acids were statistically selected for these discriminations: 16 for the concentrations and 12 for the ratios. Even using a rough classification, regardless of the type of cancer, it was possible to discriminate between patients and controls with high accuracy, and the overall contributions of numerous amino acids might reflect the large-scale characteristic changes associated with cancer metabolism.

A c-logistic analysis using matching factors (gender and age) was performed for each data set to evaluate and correct for potential confounding factors. Note that we used the combinations of amino acids obtained from the LDA models as explanatory variables. Although the c-logistic analysis was performed using all of the significant variables identified by the univariate analysis, the amino acids identified in the LDA were utilized to correct for potential confounding factors more adequately (data not shown). Both the levels of significance (Table 2 and Table S6) and the discrimination abilities (Table 3 and Table S7) were not significantly altered by correcting for the potentially confounding factors, suggesting that these results were independent of gender and age effects.

To evaluate patients with non-neoplastic diseases, the PFAA profiles of colonic polyp patients were substituted into the LDA model for CRC. Most of the colonic polyp patients (31/34, 91.2%) were classified into the control group for the concentrations and ratios of both models, suggesting that the obtained models could discriminate CRC patients specifically.

Discrimination between cancer types by PFAA profiles

In addition to differentiating between patients with each type of cancer and the controls, discrimination among patients within each cancer group was also performed by separating all the cancer patients into each disease subtype according to gender. This was done because the results of the present analyses identified changes in PFAA profiles that were common to all types of cancer as well as those specific to individual cancers.

The accuracies of all discriminant analyses using amino acid concentrations as explanatory variables were close to or better than 50% both in male patients (Table 4) and female patients (Table 5) data set. The discrimination accuracy among cancer patients was less than that between patients and controls. Six amino acids (Gly, Cit, Val, Tyr, Trp, and Arg) were commonly selected in these analyses, regardless of gender (data not shown). An additional six amino acids (Gln, Met, Leu, His, Orn, and Lys) were selected in the male patient data set, and four (Thr, Ser, Ile, and Phe) were selected in the female patient data set (data not shown). Five of the 16 amino acids listed above were selected in the discrimination between patients and controls, while the remainder might have been responsible for the characteristic features of each cancer.

The accuracies were similar between the analyses using ratios as explanatory variables and those using concentrations both in male patients (Table S8) and female patients (Table S9). Seven amino acids (Gln, Cit, Val, Tyr, Trp, Lys, and Arg) were commonly selected regardless of gender in these analyses (data not shown). An additional four amino acids (Ala, Met, Leu, and His) were selected in the male patient data set, and four (Thr, Ser, Ile, Orn) were selected in the female patient data set (data not shown). Five amino acids (Cit, Val, Tyr, Trp, and Arg) from each set were selected for both explanatory variables, suggesting that the changes to the respective PFAAs were specific to certain types of cancer.

LOOCV was also carried out and resulted in similar accuracies for the discrimination analyses, suggesting that there was no obvious over-optimization in the obtained models (Table 4, Table 5, Table S8 and Table S9).

Discussion

The present study demonstrated the use of PFAA profiling as a focused metabolomics approach for the early detection of patients with any of five types of cancer. Combining novel analytical techniques and both univariate and multivariate statistical analyses, previously unknown aspects of amino acid metabolism in humans have been revealed. The sample size in the present study was considerably larger than those reported previously [25,29,30], and provided sufficient statistical power to test the robustness of PFAA profiling for cancer diagnosis. We also demonstrated the possibility of detecting cancers, both specifically and broadly, using multivariate analysis to compress the PFAA profile data, even for patients with early stage cancer.

In the previous studies, the alterations in PFAA profiles in cancer patients sometimes seem inconsistent[22,23,24,25,26,27,28,29,30], and some discrepancies existed between our current study and those reported in the literature [25]. This discrepancy may be due not only to sample size and the varying predominance of early stage cancers but also to some other factors such as amino acid measurement methods. On the other hand, alterations in the PFAA profiles in our present study were consistent with the results of our previous studies, in which samples were collected from a single medical institute [29,30]. Furthermore, there are also many similarities between our results and those of previous studies. For example, decreases in His and Gln levels, which have been observed broadly in previous reports, and increases in Pro and Ala levels in BC are consistent with our findings [25].

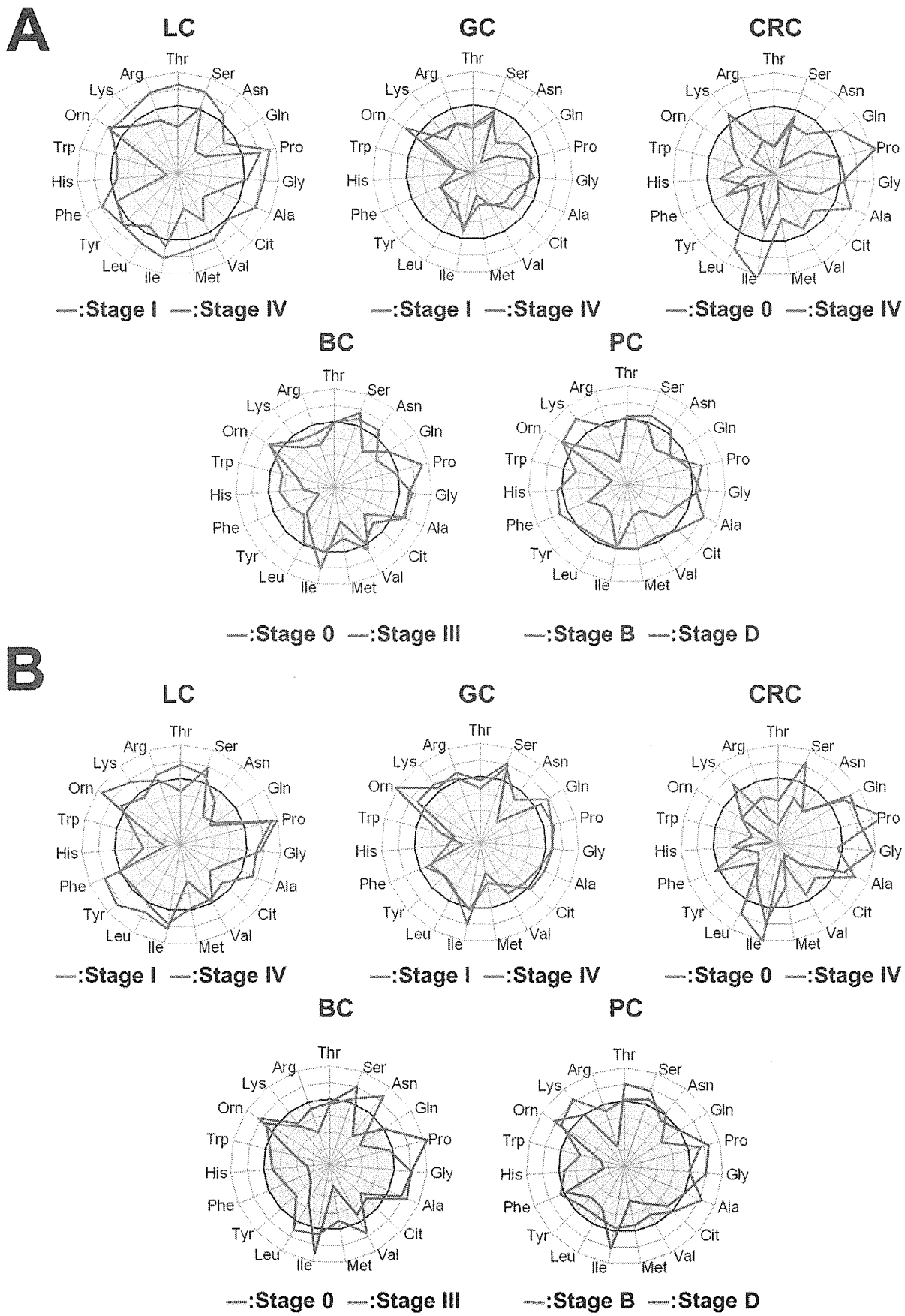
A
LC
GC
CRC
Thr
Ser
Asn
Gln
Pro
Gly
Ala
Cit
Val
Met
Ile
Leu
Tyr
Phe
His
Trp
Orn
Lys
Arg
—:Stage I —:Stage IV
—:Stage I —:Stage IV
—:Stage 0 —:Stage IV
BC
PC
—:Stage 0 —:Stage III
—:Stage B —:Stage D
B
LC
GC
CRC
—:Stage I —:Stage IV
—:Stage I —:Stage IV
—:Stage 0 —:Stage IV
BC
PC
—:Stage 0 —:Stage III
—:Stage B —:Stage D

Figure 3. PFAA profiles of early- and advanced-stage cancer patients. The axes show the AUC of ROC for each amino acid for discriminating patients from controls. A. Comparison of concentrations of cancer patients and controls. B. Comparison of ratios of cancer patients and controls. Scale as described for Figure 2. For LC, GC, CRC, and BC, cancer stages were determined according to the International Union Against Cancer TNM Classification of Malignant Tumors, 6th edition [38], and for PC, cancer stages were determined according to Jewett staging system [39].
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Cancer is expected to become the leading cause of death worldwide within a few years. Therefore, it is crucial that methods for the prevention, early detection, and treatment of cancers should be implemented to reduce mortality. Various screening methods have been established for the cancers included in our study. However, the high specificity of these methods means that subjects must undergo each screening examination separately, which can be expensive and time consuming. These examinations can also impose a physical and/or mental burden upon subjects, which can lead to avoidance. By contrast, the method described in the present study involves a relatively simple plasma assay and imposes a low physical burden on subjects. This method could also be used as versatile health assessment as other diseases in which PFAA profiles can be altered, such as diabetes[18], hepatic failure[19], and renal failure[21], can also be evaluated.

It should be noted that the models derived from this case-control study could not be used directly to make further observations or predictions, despite providing a preliminary demonstration of the potentially high value of this method for cancer discrimination. Further investigations, including model construction and validation using cohorts with larger sample sizes, are in progress to clarify the clinical utility of this approach. Moreover, the possibility of continuous PFAA profiling as a means to determine prognosis after surgery or chemotherapy is also being investigated.

Our investigation demonstrated two types of alterations in PFAA profiles of cancer patients: those in a limited set of amino acids reflecting metabolic changes common to many cancers; and those in a larger group of amino acids representing metabolic characteristics specific to each cancer. Alterations in PFAA profiles were observed even in patients with early-stage cancer, most of whom had no apparent symptoms. This strongly suggested that the alterations in PFAA profiles identified in the current study were independent of the effects of poor nutrition caused by tumor progression.

Many previous reports have shown that metabolism, including that of amino acids, is notably altered in cancer cells [3,13,40] and that changes in PFAA profiles can also occur [22,24,25,26,27,28,29,30], especially in cachexic patients with advanced cancer [23,25]. Among whole metabolites, amino acids have been frequently identified as having associations with cancer in other studies [10,13,41,42,43]. The current study demonstrated that mechanisms other than malnutrition can drive the changes in PFAA profiles.

Besides cancer-dependent malnutrition, significant decreases in PFAA concentrations and various indicators of nutritional status such as BMI and serum albumin levels are observed in cancer-independent cachexia [44,45,46]. In the present study, no apparent decreases in those indicators were observed, strongly suggesting that alterations in PFAA were also independent of nutritional status mediated by factors not related to cancer.

Table 2. Variables incorporated into LDA and c-logistic models using concentrations as explanatory variables.

Amino acid	LC		GC		CRC		BC		PC		Pooled	
	LDA	C-logit	LDA	C-logit	LDA	C-logit	LDA	C-logit	LDA	C-logit	LDA	C-logit
Thr							+++	+++			+++	+++
Ser	+++	+++			+++	+++					+++	+++
Asn												
Gln	−−−	−−−					−−−	−−−	−−−	−−−	−−−	−−−
Pro	+++	+++									+++	+++
Gly							+++	++				
Ala					+++	+++	+++	+++	+++	+++	+++	+++
Cit	−−−	−−−	−−−	−							−−−	
Val	−−−	−	−−−	−−	−−−	−−−			−−−	−−−	−−−	−−−
Met											−−−	−−−
Ile	+++	+++	+++	+	+++	+++			+++	++	+++	+++
Leu					+++	+++					+++	++
Tyr					−−−	−−−	−−−	−−				
Phe	+++	+++									+++	+++
His	−−−	−−−	−−−	−−−	−−−	−−−					−−−	−−−
Trp	−−−	−−−	−−−	−−−	−−−	−−	−−−	−−−	−−−	−−−	−−−	−−−
Orn	+++	+++					+++	+++	+++	+++	+++	+++
Lys			+++	+++	+++	+++			+++	+++	+++	+++
Arg					−−−	−−−			−−−	−−−	−−−	−−−

+, ++, +++: positive coefficients in the model.
−, −−, −−−: negative coefficients in the model.
+,−: $p<0.05$, ++,−−: $p<0.01$, +++,−−−: $p<0.001$.
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Table 3. Discrimination performance of LDA and c-logistic models using concentrations as explanatory variables.

Model	Subjects		LC	GC	CRC	BC	PC	Pooled
LDA	All	AUC	0.802	0.849	0.874	0.778	0.783	0.796
		CI	(0.766~0.836)	(0.816~0.882)	(0.842~0.906)	(0.741~0.815)	(0.740~0.826)	(0.779~0.814)
	LOOCV	AUC	0.792	0.845	0.868	0.769	0.767	0.793
	Stage 0 patients	AUC	-	-	0.903	0.813		
		CI			(0.807~1.00)	(0.726~0.900)		
	Stage I patients	AUC	0.752	0.859	0.859	0.754		
		CI	(0.698~0.805)	(0.820~0.898)	(0.800~0.918)	(0.692~0.817)		
	Stage II(B) patients	AUC	0.870	0.829	0.921	0.786	0.764	
		CI	(0.772~0.969)	(0.726~0.933)	(0.877~0.954)	(0.727~0.847)	(0.710~0.819)	
	Stage III(C) patients	AUC	0.844	0.834	0.817	0.755	0.777	
		CI	(0.780~0.908)	(0.748~0.920)	(0.743~0.892)	(0.621~0.889)	(0.669~0.885)	
	Stage IV(D) patients	AUC	0.901	0.843	0.950	-	0.873	
		CI	(0.837~0.966)	(0.734~0.951)	(0.895~1.00)		(0.771~0.974)	
C-logit	All	AUC	0.806	0.850	0.876	0.776	0.786	0.798
		CI	(0.771~0.841)	(0.816~0.883)	(0.845~0.907)	(0.739~0.812)	(0.743~0.829)	(0.780~0.815)

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Nevertheless, it remains unclear how the metabolic changes occurring in cancer patients affect the PFAA profile of the whole body, even in patients with early-stage tumors. To clarify the relationship between carcinogenesis and changes in PFAA profiles, we are further investigating the contribution of local effects caused by cancer cell metabolism and the systemic responses of the immune system against tumors or factors released by cancer cells.

Changes in metabolism can be detected in cancer cells even in early-stage patients. Hirayama *et al.* reported no significant correlation between the levels of cancer cell metabolites, including several amino acids, and the tumor stage [13]. The metabolism of Trp is of particular interest because it was identified as one of the most important amino acids in relation to cancer progression in our study. Overexpression of indoleamine-2,3- dioxygenase (IDO), the first enzyme in the kynurenine Trp metabolism pathway in humans, has been reported in cancer cells [47]. IDO is induced in many different tumors and has been suggested to play a role in cancer-mediated evasion of the immune system [47,48,49,50].

Arg, Orn, Cit, and Pro are known to be closely related to immune function. For example, Qiu *et al.* reported an association between the urea cycle and metabolic alterations in CRC patients and found no correlation between the metabolite profile and cancer progression [43]. Cancer cells also release factors that can alter general physical conditions. For example, the transcriptional regulatory molecule high-mobility group B1 (HMGB1) was recently shown to regulate cancer-cell tumorigenesis, expansion, and invasion [51,52,53].

Further elucidation of these mechanisms might allow for the development of both static and dynamic models of carcinogenesis through system analysis [31]. Recently, computer-aided studies have been reported that integrate hierarchical 'omics' datasets for the systemic understanding of metabolic phenotypes to reconstruct the regulatory network from physiological data by means of system analysis. System analysis of cancer patients based on whole body amino acid metabolism could reveal information concerning the nature of a disease and help to establish strategies for its prevention, early detection, prognosis, monitoring, and treatment.

In contrast to many similar efforts to detect biomarkers of disease as single specific molecules (DNA, microRNA, proteins, peptides, or metabolites) in peripheral blood, our approach was to focus on the metabolic status, which is indicative of multivariate function, using non-specific metabolites. Therefore, we believe that our method is superior to those used in other studies, both in versatility and efficiency, because only one amino acid measurement can be applied for detection of various disease states (i.e., renal failure, hepatic failure, and nutritional status).

Table 4. Multiclass discriminant analyses of male cancer patients using concentrations as explanatory variables.

		Patients with:			
		LC	GC	CRC	PC
Discriminated as:	LC	**72(69)**	*19(22)*	*12(13)*	*26(26)*
	GC	*18(19)*	**58(52)**	*16(17)*	*25(25)*
	CRC	*13(14)*	*25(28)*	**71(69)**	*16(17)*
	PC	*22(23)*	*24(24)*	*15(15)*	**67(66)**
	Total	125	126	114	134
	Accuracy	**57.6%(55.2%)**	**46.0%(41.3%)**	**62.3%(60.5%)**	**50.0%(49.3%)**

The numbers in the blanket indicate the results of LOOCV.

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Table 5. Multiclass discriminant analyses of female cancer patients using concentrations as explanatory variables.

		Patients with:			
		LC	**GC**	**CRC**	**BC**
Discriminated as:	LC	**41(37)**	*4(6)*	*8(11)*	*43(44)*
	GC	*13(14)*	**40(38)**	*15(16)*	*30(30)*
	CRC	*6(8)*	*13(13)*	**52(47)**	*17(17)*
	BC	*15(16)*	*16(16)*	*10(11)*	**106(105)**
	Total	75	73	85	196
	Accuracy	**54.7%(49.3%)**	**54.8%(52.1%)**	**61.2%(55.2%)**	**54.1(53.6%)**

The numbers in the blanket indicate the results of LOOCV.
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Supporting Information

Figure S1 PFAA profiles of cancer patients stratified by progression stage. The axes show the AUC of ROC for each amino acid for discriminating patients from controls. A. Comparison of concentrations of cancer patients and controls. B. Comparison of ratios of cancer patients and controls. Scale as described for Figure 2. For LC, GC, CRC, and BC, cancer stages were determined according to the International Union Against Cancer TNM Classification of Malignant Tumors, 6th edition [38], and for PC, cancer stages were determined according to Jewett staging system [39].
(TIF)

Table S1 Detailed demographic and clinical characteristics of subjects. a: $p<0.05$, c: $p<0.001$ *: For LC, GC, CRC, and BC, cancer stages were determined according to the International Union Against Cancer TNM Classification of Malignant Tumors, 6th edition [38], and for PC, cancer stages were determined according to Jewett staging system [39].
(XLS)

Table S2 PFAA profiles of cancer patients and controls.
(XLS)

Table S3 AUCs of ROC of each amino acid concentration for discrimination for cancer patients from controls.
(XLS)

Table S4 AUCs of ROC of each amino acid ratio for discrimination for cancer patients from controls. AUCs were calculated using all patients and controls, and patiens and matched controls stratified by cancer stage.
(XLS)

Table S5 Significance values for PFAA profiles for each data set by two-way ANOVA for the effects of cancer existence and other parameters. Column headings indicate Mann-Whitney U-test of cancer existence (None), two-way ANOVA for the effects of cancer existence and gender (Gender), cancer existence and age (Age), and cancer existence and smoking status (Smoking).
(XLS)

Table S6 Variables incorporated into LDA and c-logistic models using ratios as explanatory variables. +, ++, +++: positive coefficients in the model −, −−, −−−: negative coefficients in the model +,−: $p<0.05$, ++,−−: $p<0.01$, +++,−−−: $p<0.001$.
(XLS)

Table S7 Discrimination performance of LDA and c-logistic models using ratios as explanatory variables.
(XLS)

Table S8 Multiclass discriminant analyses of male cancer patients using ratios as explanatory variables. The numbers in the blanket indicate the results of LOOCV.
(XLS)

Table S9 Multiclass discriminant analyses of female cancer patients using ratios as explanatory variables. The numbers in the blanket indicate the results of LOOCV.
(XLS)

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Author Contributions

Conceived and designed the experiments: YM HY MY NO. Performed the experiments: MH AG MA TI T. Miura NS EB HK FI MM II AC FO HM OT T. Mitsushima MY NO. Analyzed the data: YM AI KH. Contributed reagents/materials/analysis tools: HM. Wrote the paper: YM AI KH.

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REPORT

日本肺癌学会・日本呼吸器内視鏡学会・日本臨床細胞学会・3学会合同委員会報告：肺門部早期肺癌実態調査アンケート報告

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Early Hilar Type Lung Cancer in Japan: A Survey from January 2006 to December 2007

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ABSTRACT —— ***Background.*** Although sputum cytology is the only way to detect early hilar lung cancer in lung cancer screening, there are also various problems. Therefore, the subcommittees concerning sputum cytology was established in each the Japan Lung Cancer Society and the Japanese Society of Clinical Cytology, and in a joint effort with the Japan Society for Respiratory Endoscopy, the investigation of these problems were reported by the committee of the three societies. We concluded that confirming the usefulness of sputum cytology at present is the inevitable and the most important issue. ***Objective.*** We clarified the actual situation of diagnosis for early hilar lung cancer in Japan by a questionnaire. ***Subject and Methods.*** We sent questionnaires to authorized and associated institutes of the Japan Society for Respiratory Endoscopy, and respondents were questioned concerning the following items. The basic items were a) the number of bronchoscopies performed, b) the number of lung cancer resections, c) the number of diagnoses of new early hilar lung cancer, d) the modes of detection, e) histological type, f) treatment modalities that can be estimated in each institute from 2006 to 2007. Moreover, to the extent possible, we ask them to respond to g) the number of advanced hilar squamous cell carcinomas, h) the number of sputum cytology examinations that were found to be positive or suspected to be positive, i) the number of peripheral lung cancers detected by sputum cytology, j) the number of cancers in otorhinological field or esophageal cancers detected by sputum cytology. ***Results and the Estimated Number of Diagnosis in Japan.*** The questionnaires were sent to 504 authorized and associated institutes of the Japan Society for Respiratory Endoscopy and returned from 308 (61.1%) of them. These institutes, in the cases of primary lung cancer resections, covered 57.1% of the field study result of the Japanese Association of General Thoracic Surgery. A total of 150 diagnosed cases of early hilar lung cancer in a year were reported. By the reported number and the covering ratio, the number of early hilar lung cancer diagnosis was estimated between 154 and 270 cases per year. Also, 4,000 cases of hilar squamous cell carcinoma in a year in Japan were estimated. Concerning the mode of detection, sputum cytology was the most numerous, accounting for 90% of squamous cell carcinomas; however, the rate of early cancer was less than 10% of hilar squamous cell carcinoma, and moreover, there were regional differences in the detection rates. ***Conclusions.*** The national survey of hilar lung cancer suggested that there were 4,000 patients with hilar squamous cell carcinoma at present. However, the ratio of early cancer was less than 10%, and the regional differences in the ratio of early to not early hilar squamous cell carcinoma were also suggested. Based on these, there might have been more opportunities of early diagnosis of hilar lung cancer than were actually diagnosed. Further quality control and much more sputum cytologic examinations for lung cancer screening is recommended.

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KEY WORDS —— Early hilar-type lung cancer, Squamous cell carcinoma, Bronchoscopic examination, Sputum cytology, Lung cancer mass screening

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要旨 —— 背景. 喀痰細胞診は肺癌検診において，肺門部早期肺癌の発見のための唯一のスクリーニング法であるが，さまざまな問題点も存在している．このため3学会（日本肺癌学会，日本臨床細胞学会，日本呼吸器内視鏡学会）合同委員会において検討を重ね，アンケートを行った．**目的.** 全国の肺門部（早期）肺癌の確定診断の実態を明らかにする．**対象と方法.** 日本呼吸器内視鏡学会気管支鏡認定施設・関連認定施設にアンケートを送付し，2006年，2007年の気管支鏡検査件数，肺癌切除例数，新規肺門部早期癌診断例数，その発見動機，組織型，治療法を，さらに可能な範囲で肺門部進行扁平上皮癌数，喀痰細胞診陽性・疑陽性による検査件数，喀痰細胞診による末梢型肺癌例数などに関して回答を求めた．**結果.** 504施設にアンケートを送付し308施設より回答を得た．これらの施設は日本胸部外科学会全国集計の57.1％をカバーしていた．年間150例程度の肺門部早期肺癌が報告された．報告数とカバー率から肺門部早期肺癌の全国における初回診断数は年間154～270例程度と推定され，肺門部の扁平上皮癌に関しては全国で年間約4,000例の存在が推定された．しかし，早期癌の比率は肺門部扁平上皮癌全体の10％を下回っていた．さらに，その発見率には地域差が見られた．**考察および結論.** 肺門部肺癌に関しては，現在診断されているよりも，さらに多くの症例で早期診断の機会があったと推測され，肺癌検診のさらなる精度管理や喀痰細胞診の受診勧奨など，検討すべき事項が存在するものと推定された．

索引用語 —— 肺門部早期肺癌，扁平上皮癌，気管支鏡，喀痰細胞診，肺癌集団検診

はじめに

肺癌は本邦においても癌死亡死因の第一位を占めている．喫煙率の低下が報告されている現在においても肺癌死亡は増加を続けており，早期発見による治療が社会的要請となっている．そのため肺癌検診のあるべき姿に向けた議論も活発となっている．[1-4]

肺癌早期発見の手法に関しては，胸部単純X線写真，[1,5-9] 喀痰細胞診，[1,5-19] 胸部CT[1,4,8,9,12,20] などがあり，そのいずれをもってしても完璧なものはなく，併用することにより，より精度の高いスクリーニングが可能となる．[12] 一方で，費用対効果の視点から，その有用性とニーズを見極めることも重要である．[9] 喫煙率の低下が報告されている現状において，喀痰細胞診は本当に必要なのか？　全世界的に腺癌が増加している現在，喀痰細胞診に要する費用をCTによる検診に振り分けるべきではないか，という議論も見られる．

肺癌検診における喀痰細胞診に関しては，有用性を報告する施設が見られるものの，一方で発見例の減少を示唆する施設や，喀痰細胞診の併用を中止する市町村が出現するなど，さまざまな混乱や問題点が存在する．[10] これらに対応すべく，日本肺癌学会では，集団検診委員会内に喀痰細胞診による肺癌検診小委員会を，また，日本臨床細胞学会では総務委員会内に肺癌検診ワーキンググループを設置し，2つの委員会の合同委員会で検討を重ねてきた．検討を重ねる中で，喀痰細胞診による早期発見を必要とする肺門部扁平上皮癌の実態を把握することが今後の対応を決める上で最も緊急かつ重要であるとの共通認識に至った．一方，著者らが知りうる範囲においては本邦における肺門部肺癌の実態は，系統だった調査や大規模な統計などで研究・公表されたものはなかった．そこで，前述の2学会に加え，日本呼吸器内視鏡学会学術企画委員会を中心とした肺癌検診ワーキンググループとともに，肺門部早期肺癌実態調査アンケートを行い，多くの施設の多大な協力の下に回答を集計することができたので，ここに報告する．

対象と方法

日本肺癌学会集団検診委員会喀痰細胞診による肺癌検診小委員会，日本臨床細胞学会総務委員会肺癌検診ワーキンググループ，および日本呼吸器内視鏡学会学術企画委員会肺癌検診ワーキンググループの合同会議でアンケート項目の検討を行い，2006，2007年（平成18，19年）における各施設の，a）気管支鏡検査件数，b）肺癌切除例数，c）新規肺門部早期肺癌診断例数，d）その発見動機，e）組織型，f）治療法，を必須回答項目とした．さらに可能な範囲で，a）肺門部進行扁平上皮癌数，b）喀痰細胞診陽性・疑陽性による検査件数，c）喀痰細胞診による末梢型肺癌発見例数，d）喀痰細胞診による耳鼻科領域癌発見例数・食道癌発見例数，に関しても回答を求めた（Table 1）．

アンケートの配布先は，日本呼吸器内視鏡学会気管支鏡認定施設および関連認定施設（計504施設）の気管支鏡検査責任者宛とした．アンケートは2009年（平成21年）1月10日に発送し，アンケートの回収期限を2009年（平成21年）2月28日としたが，最終的にはアンケート督促状配布を2009年3月30日に行い，アンケート最終締め切りを2009年4月30日とした．

また，アンケート回収施設における原発性肺癌切除例数と日本胸部外科学会で施行している毎年の全国集

Table 1. Items of the Japanese National Survey of Early Hilar Lung Cancer

Study period: 2006-2007
1) Basic items
a) Number of bronchoscopies performed
b) Number of lung cancer resections
c) Number of diagnoses of new early hilar lung cancer
d) Modes of detection
e) Histological type
f) Treatment modalities

2) Others
a) Number of advanced hilar squamous cell carcinomas
b) Number of sputum cytology examinations found to be positive or suspected to be positive
c) Number of peripheral lung cancers detected by sputum cytology
d) Number of cancers in otorhinological field or esophageal cancers detected by sputum cytology

Table 2. Annual Numbers

Current survey results	2006	2007
Bronchoscopies	64,250	65,584
Lung cancer resections (A)	14,670	15,356
New early hilar lung cancers	155	152
The number of primary lung cancer resections in Japan surveyed by the Japanese Association for Thoracic Surgery (B)	26,531	26,092
The reported ratio (A/B) of the current questionnaire	55.3%	58.9%
Throughout 2006 and 2007	57.1%	

計[21,22]から，これらの施設の肺癌切除例数の比率を算出し，日本全国で発生する肺門部進行扁平上皮癌数と肺門部早期肺癌数を推定した．

結　果

1）アンケートの回収状況

2009年3月31日までに165施設（32.7%）より回答があった．2009年4月30日までには72施設（14.3%）から回答が追加で送られ，2009年5月18日までにはさらに71施設（14.1%）より回答があった．総計では，308施設（61.1%）よりの回答を得た．

2）回答施設における気管支鏡検査数，肺癌切除例数，新規肺門部肺癌診断数（Table 2）

回答施設における気管支鏡検査数は2006年64,250件，2007年65,584件であった．同様に原発性肺癌切除例数は2006年14,670例，2007年15,356例であった．日本胸部外科学会で施行している毎年の全国集計に占めるこれらの施設の肺癌切除例数の比率は2006年55.3%（14,670/26,531），2007年58.9%（15,356/26,092）で，2年間通年では57.1%であった．回答施設における新規肺門部早期肺癌の診断例数は2006年155例，2007年152例であった．

肺門部早期肺癌の診断例がなかった施設数は2006年238施設（77.3%），2007年238施設（77.6%）であった．

3）肺門部早期肺癌例の発見動機（Table 3）

肺門部早期肺癌例の発見動機別に見ると2006年は喀痰細胞診によるもの93例，うち検診時発見例59例，血痰26例，他疾患観察時の気管支鏡検査によるもの31例，その他22例，不明16例であった．2007年は喀痰細胞診によるもの69例，うち検診時発見例36例，血痰32例，他疾患観察時の気管支鏡検査によるもの23例，その他24例，不明10例であった．2年間を通して喀痰細胞診発見例が最多を占めていた．

4）肺門部早期肺癌例の組織型（Table 4）

扁平上皮癌が2006年140例，2007年135例と大部分を占めた．ごく少数ながら非扁平上皮癌も見られた．

Table 3. Modes of Detection

Modes of detection	2006	2007
Sputum cytology	93	69
Sputum cytology in population-based mass screening	59	36
Bloody sputum	26	32
Bronchoscopies performed for other pulmonary disorders	31	23
Others	22	24
Unknown	16	10

Table 4. Histological Type

Histological type of early hilar lung cancer	2006	2007
Squamous cell carcinoma	140	135
Adenocarcinoma	5	6
Large cell carcinoma	0	2
Small cell carcinoma	5	3
Others	1	3
Unknown	4	3

5) 肺門部早期肺癌例の初回治療法（Table 5）

肺門部早期肺癌例の主たる初回治療法は，2006年では手術[6]が49例，PDTレーザー[23,24]が66例，外照射が17例であった．腔内照射と化学療法が各3例で，無治療例が8例見られた．2007年では手術が59例，PDTレーザーが45例，外照射が26例であった．腔内照射は4例で，化学療法が13例，無治療例が5例見られた．手術，PDTレーザーが大半を占め，次いで外照射が続いた．

6) 肺門部進行肺癌例数（Table 6）

以下，Table 11まで可能な範囲で回答を寄せた施設の集計である．これらの施設における早期肺癌の定義を満たさない肺門部扁平上皮癌の診断数は2006年には1,222例，2007年には1,270例であった．

7) 喀痰細胞診陽性または疑陽性で気管支鏡検査の対象となった症例数（Table 7）

喀痰細胞診陽性で気管支鏡検査の対象となった症例数は2006年には689例，2007年672例であった．一方，疑陽性の症例数は2006年439例，2007年411例で，その合計は2006年1,128例，2007年1,083例であった．

Table 5. Initial Treatment

Initial treatment for early hilar lung cancer	2006	2007
Surgery	49	59
PDT laser	66	45
Laser apart from PDT	6	2
Brachytherapy	3	4
External irradiation	17	26
Chemotherapy	3	13
Untreated	8	5
Others	1	3
Unknown	0	1

Table 6. Cases of Progressive Hilar Squamous Cell Carcinoma

	2006	2007
Advanced hilar squamous cell carcinoma	1,222	1,270

Appendix: All cases of hilar squamous cell carcinoma not defined as early hilar lung cancer were considered to be advanced hilar squamous cell carcinoma.

Table 7. Number of Cases Which Required Bronchoscopic Examination Due to Positive or Suspected to Be Positive Findings on Sputum Cytology*

	2006	2007
Positive findings on sputum cytology	689	672
Suspected to be positive findings on sputum cytology	439	411
Total	1,128	1,083

*Including cases in which pathological changes were not seen on bronchoscopy.

8) 喀痰細胞診が発見動機となった末梢型肺癌および耳鼻科領域癌，食道癌の症例数（Table 8，9）

喀痰細胞診が発見動機[15]となった末梢型肺癌は2006年203例，2007年225例であった．また喀痰細胞診が発見動機[13]となった耳鼻科領域癌は2006年20例，2007

Table 8. Number of Cases with Peripheral Lung Cancer Detected by Sputum Cytology

	2006	2007
Peripheral type lung cancer detected by sputum cytology	203	225

Table 9. Number of Otorhinological or Esophageal Cancer Detected by Sputum Cytology

	2006	2007
Otorhinological cancers detected by sputum cytology	20	11
Esophageal cancers detected by sputum cytology	7	5

Table 10. The Ratio of Early to Advanced Hilar Squamous Cell Carcinoma

	2006	2007
Responding institutions	179 (58.1%)	181 (59.0%)
Total number of bronchoscopies performed	37,027	38,242
Early hilar squamous cell carcinoma	121 (9.1%)	99 (7.2%)
Advanced hilar squamous cell carcinoma	1,222 (90.9%)	1,270 (92.8%)

年 11 例であり，同様に喀痰細胞診が発見動機となった食道癌は 2006 年 7 例，2007 年 5 例であった．

9）報告施設における肺門部早期扁平上皮癌，非早期扁平上皮癌の比率（Table 10）

オプションとした（あるいは，回答が可能な施設での）アンケート項目である肺門部非早期癌の診断数の報告は 2006 年 179 施設（58.1%），2007 年 181 施設（59.0%）から得られた．これらの施設における早期/非早期の比率は 2006 年肺門部早期扁平上皮癌 121 例（9.1%），肺門部非早期扁平上皮癌 1,222 例（90.9%），2007 年肺門部早期扁平上皮癌 99 例（7.2%），肺門部非早期扁平上皮癌 1,270 例（92.8%）であった．

10）地域別に見た肺門部肺癌における早期癌の比率（Table 11）

地域別に見た肺門部扁平上皮癌における早期癌の比率は北海道 37/221（16.7%），東北 27/186（14.5%），関東 94/934（10.1%），東海 16/291（5.5%），甲信越 18/137（13.1%），北陸 21/93（22.6%），近畿 17/419（4.1%），中国 15/109（13.8%），四国 13/79（16.5%），九州・沖縄 17/297（5.7%）であった．2×10 のカイ 2 乗検定では，地域間格差が見られた（$p<0.0001$）．

Table 11. Ratio of Early Cancer to Total Cases of Hilar Squamous Carcinoma in Each Japanese Region

Region	Early cancer / Total hilar type squamous cell carcinoma	%
Hokkaido	37/221	16.7
Tohoku	27/186	14.5
Kanto	94/934	10.1
Tokai	16/291	5.5
Koshinetsu	18/137	13.1
Hokuriku	21/93	22.6
Kinki	17/419	4.1
Chugoku	15/109	13.8
Shikoku	13/79	16.5
Kyushu・Okinawa	17/297	5.7

$p<0.0001$.

11）日本全国における肺門部進行扁平上皮癌推定診断数と肺門部早期肺癌推定診断数（Table 12）

肺門部進行扁平上皮癌診断数についてアンケートに回答した施設における原発性肺癌切除例数と，日本胸部外科学会で施行している毎年の全国集計[21,22]から，これらの施設の肺癌切除例数の比率を算出し，日本全国で発生する肺門部進行扁平上皮癌数を推定した．肺門部進行扁平上皮癌診断数についてアンケートに回答した施設は平均 180 施設，これらの施設における非早期肺門部扁平上皮癌の診断数は平均 1,246 例であった．これらの施設の肺癌切除例における日本全体の肺癌切除例に占める割合は平均 32% であった．これらより，日本全国では年間あたり 3,894 例の非早期肺門部扁平上皮癌が診断されていると推定された．

同様に早期肺門部扁平上皮癌の診断数について推定すると，肺門部早期扁平上皮癌について回答を寄せた施設は平均で 154 施設で，これらの施設の肺癌切除例数は日本全体の 57.1% をカバーしていた．このことより年間平均で 270 例の肺門部早期扁平上皮癌の診断例の存在が推定された．しかしながら，アンケートに回答した施設が早期肺門部扁平上皮癌の診断の熱意のある施設のみである可能性も否定し得ないため，3 学会合同の委員会では，早期肺門部扁平上皮癌の推定診断数については 154～270 という幅を持たせた表記として報告することとした．

考　察

喀痰細胞診は肺癌検診において，肺門部早期肺癌の発見のための唯一のスクリーニング法であるが，さまざまな問題点も包含している．このため日本肺癌学会および

Table 12. Estimated Number of Patients with Hilar Type Squamous Carcinoma in Japan

	2006	2007	Average
Responding institutions with data about advanced hilar squamous cell carcinoma	179	181	180
Advanced hilar squamous cell carcinoma (A)	1,222	1,270	1,246
Lung cancer resections performed at responding institutions (B)	8,043	8,686	8,365
National lung cancer resections based on survey by the Japanese Association for Thoracic Surgery (C)	26,531	26,092	26,312
The covering ratio of the responding institution in Japan (D = B/C)			32%
Estimated number of patients with advanced hilar squamous cell carcinoma in Japan (E = A/D)			3,894
The responding institutions with data about early hilar squamous cell carcinoma (F)	308	308	308
Early hilar squamous cell carcinoma (G)	155	152	154
Lung cancer resections at responding institutions (H)	14,670	15,356	15,013
National lung cancer resections based on survey by the Japanese Association for Thoracic Surgery (C)	26,531	26,092	26,312
The reported ratio of the responding institutions in Japan (I = H/C)			57.1%
Maximum estimated number of early hilar lung cancer in Japan (J = G/I)			270
Estimated number of early hilar lung cancer cases in Japan			154-270

日本臨床細胞学会内に喀痰細胞診に関する小委員会が設置され，合同の委員会において検討が重ねられた．さまざまな視点からの問題点の発掘や提案の中で，喀痰細胞診による早期発見を必要とする肺門部扁平上皮癌の実態を把握することが今後の対応を決める上で最も緊急かつ重要であるとの結論に至り，本アンケートを行った．

背景には，肺癌死亡の増加を止めきれていない日本の現況やCT検診への期待と不安，喫煙率の変化に伴う罹患構造の変化へどう対応するか，喀痰細胞診による肺癌発見例の減少を報告する施設がある一方で不変であるとする施設が見られるなど，さまざまな混乱や不安などがあったと思われる．一方，著者らが知りうる範囲においては本邦における肺門部肺癌の実態は，系統だった調査や大規模な統計などで研究・公表されたものはなかった．すなわち，我々は推論に基づく，あるいは根拠の希薄な個々の限られた経験に基づく主張をそれぞれの立場で繰り返していた．合同委員会では，今回，このような反省と視点に基づき，全国実態調査を企画した．

一方，アンケートでは，回答施設での負担を考慮し，必須回答を求めたものと，可能であれば協力をお願いしたものが存在した．このため，本報告では必須回答からの解析と，部分施設での回答からの解析の2通りとなっている．

今回の全国実態調査で判明した主な点を簡単にまとめると，

1）年間150例程度の肺門部早期肺癌の診断例が報告された．

2）早期癌の発見動機としては喀痰細胞診が最も多く，ついで血痰，他疾患時の気管支鏡検査時に偶然発見されたものが続いた．

3）組織型としては扁平上皮癌が90%を占めた．

4）治療法としてはレーザーと手術が大半を占めていた．

5）喀痰細胞診により発見された肺門部早期癌より多数の末梢型肺癌や，耳鼻科領域癌，食道癌なども，喀痰細胞診が契機となり発見されていた．

6）肺門部扁平上皮癌において早期の比率は10%を下回っていた．

7）肺門部扁平上皮癌における早期癌の比率には，地域別に検討すると有意差が見られた．

また，肺癌切除例数を利用して日本全国の罹患数を推定すると，

1）肺門部早期肺癌の全国における初回診断例数は年間154～270例程度と推定された．

2）全国では年間約4,000例（3,894＋（154～270））の肺門部扁平上皮癌の存在が推定された．

これらの結果から，喀痰細胞診が肺門部早期肺癌の発見動機として主要な位置を占めていることが伺えた．さらに，喀痰細胞診により発見された早期肺門部肺癌よりも多数の末梢型肺癌や，耳鼻科領域癌，食道癌なども発見されていた．喫煙率の低下が報告されている中，今なお，日本全体では年間4,000例近い肺門部扁平上皮癌が発生していることも重要な事実である．今後の集団検診における喀痰細胞診の位置付けをめぐる議論の中において，客観的数値としての意義は少なくない．今回肺門部扁平上皮癌例数の全国推計値が初めて算出されたことになるが，これが果たして，今後どのように推移していくのか，あるいは地域により罹患数が異なるのか否か，などを検討していく必要がある．

一方，肺門部扁平上皮癌において早期の比率は10％を下回っていたこと，早期癌の比率に地域間格差が存在したことは大きな問題点と考えられた．がん医療の均てん化が叫ばれる中，地域差が見られることは，改めて，その必要性・重要性を喚起しなければならない．むろん，精度管理の重要性に関しては論を待たない.[25] しかし，精度管理の重要性に関しては，すでにこの20年間，繰り返し，述べられてきた.[25-29] その上でのこの実態であることを考慮すると，喀痰細胞診に関しては，現状の精度管理は機能不全に陥っていると言わざるを得ない．従来の考え方，手法を乗り越えたものを目指す必要がある．この点に関しては，さらに議論が必要と考える．

ちなみに喀痰細胞診の感度に関する複数の検討では，喀痰細胞診の感度は少なくとも70％程度[11,14]であり，改善の余地が相当程度にあると推察されうる．

推定値の妥当性についての議論

合同委員会においては，推定値の妥当性に関する議論も行われた．まず，アンケートであるため，回答は自主的に行われた．このためのバイアスの存在も危惧された．全数調査でないことも明らかであった．

今回のアンケートでは，気管支鏡の認定施設および関連認定施設にアンケートが配布され，その61.1％にあたる308施設からの回答を得た．これらの施設における気管支鏡検査件数は年平均64,917件であった．2007年に日本呼吸器内視鏡学会安全対策委員会が施行した2006年の気管支鏡の実態調査では，アンケートに回答した375施設で74,770件の気管支鏡検査が報告[30]されており，今回の我々の数値と近似した報告となっている．また原発性肺癌の回答施設の切除例数は年平均15,013であり，日本胸部外科学会の全国集計における原発性肺癌切除例数に占める割合は平均で57.1％であった．本アンケートは全数調査ではないものの，気管支鏡検査実施施設および原発性肺癌切除例数において，少なくとも日本の約半数以上をカバーしたものである．

本報告では，日本胸部外科学会の全国集計における原発性肺癌切除例数に占める割合を，種々の推定を行う場合のカバー率として採用している．日本全体における肺癌切除例数における各施設の比率，気管支鏡検査における各施設の比率，扁平上皮癌の診断数における各施設の比率，さらには早期扁平上皮癌診断例数における各施設の比率が同等であると仮定している．前述のように切除例数における比率と気管支鏡施行例数の比率はほぼ一致しており，極端に大きな問題はないものと考えられた．米国におけるような全国的がん統計を有していない日本では，現状把握が著しく困難である．

また委員会では，集計された肺門部非早期（進行）扁平上皮癌には末梢発生も入っており，そのために真実よりも多い数値が計上されているのではないか，という疑問が出された．末梢発生のものが紛れ込む場合には，以下の3つの場合が主と考えられる．

（ア）4ないし5次気管支発生のいわゆる中間型であれば，増大すれば肺門型として計上されることは十分にあり得る．しかしながら，そのような中間型の早期癌は，定義的には「非肺門型」であっても，喀痰細胞診の対象なので，この調査の対象としては「肺門型」として扱っても問題はないと判断した．

（イ）一方，全くの末梢発生で肺門リンパ節に転移し，さらに気管支に浸潤してきた，というようなものが計上されることも否定できないが，多くは末梢に大きな陰影があるため，末梢型で肺門リンパ節転移ありと正しくカウントされる可能性が高いと思われる．

（ウ）末梢発生だが距離的には太い気管支に近接している，というようなものの場合には，増大することによって肺門と一塊になり肺門型として計上されることはあり得る．ただし，腺癌・大細胞癌などは最初から除外されているので，末梢型扁平上皮癌でそのような増大形式をとった場合ということになる．

上記（ア）（イ）（ウ），特に問題になるのは（ウ）のような場合であるが，その頻度は不明だが，著しく多いとは思えない．頻度が不明なことと他に適当な推定の材料がないことから，このような問題があることは踏まえつつ，ここでは「集計された肺門部非早期（進行）扁平上皮癌を肺門部あるいは中間部発生とする」と仮定することとした．

さらに，日本胸部外科学会の全国集計に関しての指摘も見られた．日本胸部外科学会非認定施設で切除されている分は，この推計では無視した．それに伴い，今回の推定値は若干過少推定になっている可能性がある．また，肺門部進行扁平上皮癌数を答えた180施設とそれ以外の施設における「肺門部進行扁平上皮癌数と，切除肺癌数

の割合」はおおむね同様の傾向を示すと仮定している．当該180施設における肺門部進行扁平上皮癌数の割合がそれ以外の施設よりも高い場合には，国内で発生する肺門部進行扁平上皮癌数は過大に推定され，逆にそれ以外の施設よりも低い場合には，国内で発生する肺門部進行扁平上皮癌数は過少に推定されている可能性がある．

肺門部早期扁平上皮癌数に関しても同様の可能性が考えられる．すなわち，肺門部早期扁平上皮癌の診断例はある程度専門の病院に集まる傾向があるため，切除肺癌数と同様の傾向を示さない可能性がある．したがって，最小推定値として「アンケートの集計値そのもの」，最大推定値として肺癌切除例数から求めた推定値を用いることとした．

このように推定手法に由来する推定値の巾が存在しうるものの，今回，現時点における全国の肺門部扁平上皮癌の診断実態が3学会の学会員および気管支鏡診断施設における協力により，本邦において初めて明らかになったことの意義は大きいと考えられる．

今後疾病構造の変化により疾患の罹患頻度がどのように変化していくのか，肺癌検診と精度管理の必要性が説かれて20年以上の長きにわたるにもかかわらず，早期癌の段階で発見される頻度が10% 以下であること，地域間格差が見られる可能性があることなどから，今後進めるべき精度管理のあり方など，我々が考えるべき課題は大きい．

米国よりCT検診が喫煙者に対して有効であるという報告[4]がなされているその一方で，東京から肺癌をなくす会の検討では，発見された扁平上皮癌の2/3で喀痰細胞診が陽性であり，なかんずく1/3はCTは陰性で喀痰細胞診のみが陽性であった．[12] このことはCTによるスクリーニングの限界をも示している．肺癌早期発見の手法に関しては，いずれの方法をもってしても完璧なものはなく，併用することにより，より精度を高く維持できると考えられる．非喫煙者肺癌に比べて予後が不良と言われる喫煙者肺癌に，今後我々はどのように向き合うか，も問われている．

以上，今回のアンケートにより明らかとなった肺門部扁平上皮癌の本邦における診断実態を報告した．本アンケートの結果が日本の肺癌診療の向上に寄与することを期待したい．

本論文内容に関連する著者の利益相反：平田哲士［企業の職員・法人の代表］NPO法人セルサイト，渡辺洋一［企業の職員・法人の代表］NPO法人新しい医療技術を普及させる会．馬場委員については，やむを得ざる事情を勘案し，編集委員会委員長および利益相反管理委員会委員長の判断により，特例としてCOI報告を免除した．

本報告は3学会合同委員会報告であり，それぞれの学会雑誌に各学会の小委員長名で掲載される．

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