Table 6 Comparison of the surgical outcome of studies of patients undergoing repeat pulmonary resections for metastatic CRC

Author	Year	Total number of patients	Number of patients who underwent repeat pulmonary resection	#1	#2	Prognostic factor for second pulmonary resection	#3
Saito	2002	165	23	54.6	52.1	NA	NA
Pfannschmidt	2003	167	24	24.5*	NA	NA	NA
Vogelsang	2004	75	18	NA	NA	NA	NA
Ogata	2005	76	14	NA	23	Extrathoracic recurrence before second pulmonary metastasectomy, mediastinal lymph node metastasis is associated with poor prognosis	7/11
Welter	2007	169	33	53.8	37.1	Number of metastases by a multivariate analysis	NA
Lee	2007	59	13	NA	NA	NA	NA
Kim	2008	69	28	29	NA	NA	NA
Park	2009	202	48	79.3	NA	Elevated preoperative serum CEA level by univariate analysis	28/NA
Our series	2010	156	25	64	42.1	Lymph node involvement by a univariate analysis	10/13

NA = not available.
*Curative resection only.
#1: Five-year survival rate of patients who underwent repeat pulmonary resections after the first pulmonary resection.
#2: Five-year survival rate of patients who underwent repeat pulmonary resections after the second pulmonary resection.
#3: Total number of patients who experienced recurrence after second pulmonary resections.

reflect different patterns of recurrence after pulmonary resection. The proportion of pulmonary metastasis of patients who experienced recurrence after repeat resection was higher than after the first resection in the present study. Pulmonary metastasis after the second pulmonary resection was found in 10 patients (77%) out of 13 patients who experienced recurrence and in 39 of 93 patients (42%) who experienced recurrence after the first pulmonary resection in the present study. This higher proportion of pulmonary metastasis of patients who experienced recurrence after repeat resection is also observed in Ogata's series[13] in which the proportions was 64% (7/11) and 33% (25/75). It was speculated that patients who underwent repeat pulmonary resection are highly selected patients whose disease tended to metastasize to the lung without extrathoracic lesions after the first pulmonary resection.

Based on the data of the present study, it is speculated that repeat pulmonary resection for metastatic CRC patients with lymph node involvement should be avoided. Therefore, the preoperative assessment of lymph node involvement is important for repeat pulmonary resection. In our hospital, patients with apparent mediastinal lymph node metastases as determined by preoperative radiologic examinations were excluded as candidates for pulmonary metastasectomy. Before 2006, lymph node involvement was generally assessed by a CT scan. In 2006, FDG-PET/CT was introduced in our hospital for hilar and mediastinal lymph node staging in patients with metastatic lung tumors. One hundred thirty patients of 156 patients underwent a first pulmonary resection during this period in the present study. Fourteen of 130 patients (11%) in the CT era had lymph node involvement, whereas only 1 of 26 patients (4%) in the FDG-PET/CT era had lymph node involvement. Although the superior diagnostic performance of FDG-PET/CT compared with CT could not be shown because of the retrospective nature of the present study, we recommend the use of FDG-PET/CT for the preoperative assessment of patients with metastatic CRC.

The present study had several limitations. The analyses were performed on a large number of patients treated over several decades with changing chemotherapeutic regimens, and it is possible that reported outcomes were partly influenced by the medical treatments. Patients with recurrence after pulmonary resection who received more recently developed treatments, such as antiangiogenic therapy with bevacizumab combined with oxaliplatin-based chemotherapy,[3] may survive for a longer period than those who received an older regimen of chemotherapy regardless of the other factors that were analyzed. However, the significance and influence of chemotherapy on patient outcomes remains difficult to establish in the present study because of its retrospective nature.

Conclusions

Repeat pulmonary resection for metastatic CRC is a safe procedure that provides satisfactory outcomes. The prognostic factors for the first and repeat pulmonary resection are different. Prethoracotomy serum CEA levels and the histological type of primary CRC affect survival after the first pulmonary resection but do not affect survival after repeat pulmonary resection. Hilar or mediastinal lymph node involvement is consistently associated with a poor prognosis after the first and repeat pulmonary resections.

The preoperative assessment of lymph node involvement is important for both the first and repeat pulmonary resection. Aggressive repeat resection is justified for carefully selected patients.

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Case Report

Surgical Resection of Pulmonary Metastases from Meningioma: Report of a Case

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Abstract
Meningiomas rarely metastasize, and little information on pulmonary metastasectomy from meningioma has been documented. We herein report a case of a potentially curative resection for meningioma that metastasized to the lung. A 67-year-old woman was admitted to our hospital because of two masses in the right lung. In 1993, when the patient was 52 years old, she underwent a craniotomy for an atypical meningioma. The meningioma recurred once in the local site and was re-excised in 1997. In 2008, a screening chest X-ray detected two lung nodules in the right lung field. A computed tomographic scan demonstrated round masses with sharp borders, in the right S2 (2.2 cm in diameter) and S4 (1.1 cm in diameter) regions. A whole-body [^{18}F]2-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography/CT examination revealed intense focal FDG uptake (maximum standard uptake value [SUV_{max}] = 6.9) in the larger mass, and weak FDG uptake (SUV_{max} = 2.3) in the smaller mass. A wedge resection of S2 and a middle lobectomy of the right lung were performed, and the final diagnosis was pulmonary metastases from an intracranial meningioma. The patient is presently doing well 20 months after the surgery without any signs of recurrence. Our case demonstrates that surgery should be considered when pulmonary metastases are deemed completely resectable by a preoperative radiological examination, and that a good clinical outcome can be achieved.

Key words Meningioma · Metastasis · Lung

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Introduction

A surgical resection of pulmonary metastases from extrathoracic malignancies is now an established treatment modality for patients with various types of diseases. Meningiomas rarely metastasize, and little information on pulmonary metastasectomy from meningiomas has been documented. We herein report a case of a potentially curative resection for a metastatic meningioma of the lung, which was followed up for 20 months without any evidence of recurrence.

Case Report

A 67-year-old female patient was admitted to our hospital because of two masses in the right lung. In 1993, when the patient was 52 years old, she underwent a craniotomy for a convexity tumor in another hospital. Histological studies revealed that the tumor invaded the dura mater and the bone marrow of the skull, but brain invasion was not observed. The tumor was composed of dense, short, spindle-shaped cells, and 8 mitoses were present per 10 high-power fields. No necrosis was evident. The tumor was immunoreactive for vimentin, S-100 protein, and was weakly positive for epithelial membrane antigen. Staining for keratin and smooth muscle actin was negative. Based on these findings, the final pathological diagnosis was atypical meningioma. The meningioma recurred once in the local site and was re-excised in 1997. In 2008, a screening chest X-ray detected two lung nodules in the right lung field. The patient had never smoked. A computed tomography (CT) scan demonstrated round masses with sharp borders in the right S2 (2.2 cm in diameter) and S4 (1.1 cm in diameter) regions (Fig. 1). A whole-body [^{18}F]2-fluoro-2-deoxy-D-glucose positron emission tomography/CT (FDG-PET/CT) examination revealed intense focal FDG uptake (maximum standard uptake

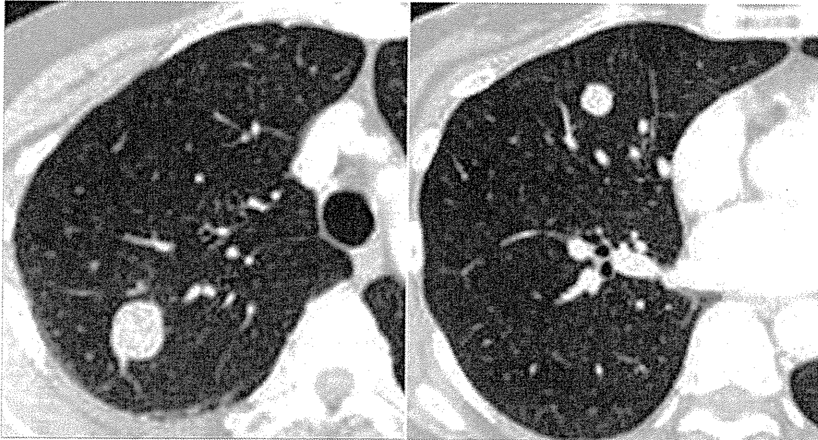

Fig. 1. Computed tomography demonstrated round-shaped masses with sharp borders in the right S2 (2.2 cm in diameter) and S4 (1.1 cm in diameter) regions

value [SUV_{max}] = 6.9) in the larger mass, and weak FDG uptake (SUV_{max} = 2.3) in the smaller mass. No other abnormal FDG uptake was observed. No serum tumor markers were elevated. Bronchoscopy was performed for the S2 lesion, but failed to yield a definitive diagnosis. Thereafter, a thoracotomy was performed. The patient was placed in the left lateral decubitus position under general anesthesia and single-lung ventilation. Through a lateral thoracotomy via the right fifth intercostal space, a wedge resection of S2 and a middle lobectomy of the right lung were performed. Macroscopically, the two lesions had well-defined borders, a whitish color, and a firm consistency. An intraoperative diagnosis of frozen tumor sections was a pulmonary metastasis from an intracranial meningioma, because the microscopic features of the convexity tumor specimen excised in 1993 and pulmonary specimen appeared to be homologous in nature. Histological studies of permanent sections revealed that the two tumors had the same histological characteristics, including immunohistochemical characteristics, as those of the convexity meningioma that was resected in 1993, and exhibited vascular invasion without lymphatic invasion (Fig. 2). The patient had an uneventful postoperative recovery and was given no postoperative therapy. To date, 20 months after the operation, the patient has had no recurrence of the tumor.

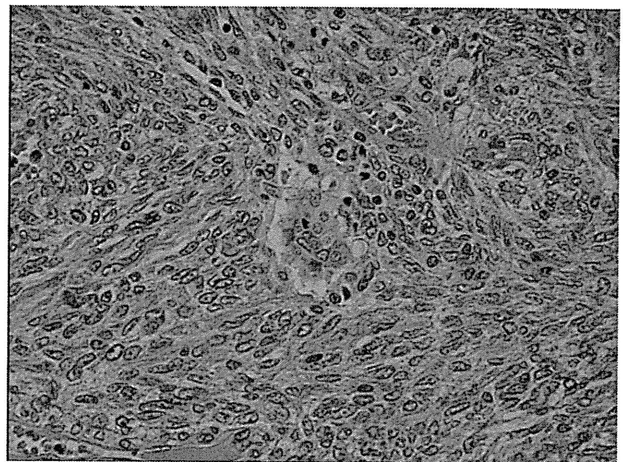

Fig. 2. Histological studies of permanent sections revealed that two tumors were composed of dense short spindle-shaped to polygonal-shaped cells, which had the same histological characteristics as those of the convexity meningioma that was resected in 1993 (hematoxylin–eosin stain, ×40)

Discussion

Meningiomas comprise 15% of intracranial neoplasms, and usually occur between the ages of 20 and 60 years, with a female-to-male ratio of 2:1.[1] The incidence of malignancy in meningioma is reported to range from 2% to 10% because of its varied definition.[2] Extracranial metastases from primary meningiomas are extremely rare, occurring in fewer than 1 in 1000 cases (0.1%).[3,4] The rarity of the extracranial metastases is explained by the tight cohesion of tumor cells, the absence of intracranial lymphatics, and a presumed lack of "fertile soil" at distant sites.[5] The lung is the most frequent site of meningioma metastases (61% of 113 cases reported by Stoller et al.[6]) followed by the liver, lymph nodes, and bone. The pathway for extracranial metastases is via the bloodstream, because intracranial lymphatics are absent. However, whether dural venous sinus invasion of the intracranial meningioma increases extracranial metastasis remains controversial. In the present case, the tumor invaded the dura mater and the bone marrow of the skull, as histologically observed at the time of the first operation, and vascular invasion was

observed in the resected specimen of the lung. These findings strongly indicate that cancer cells were spreading through the bloodstream.

Previously the definition of malignant meningioma has been controversial. The rarity of malignancy in meningiomas made it difficult to study large series, and the value of histological grading was not apparent. In this context, histological classification of meningiomas based on histological grading was proposed.[7] The World Health Organization (WHO) classification of tumors of the nervous system lists 15 histopathological variants of meningioma. The WHO grade II clear cell and choroid variants, and the WHO grade III rhabdoid and papillary variants are reported to be prone to recurrence or spread. Of these, the papillary variant carries the highest risk of late distant metastases, which are reported to metastasize in 20% of cases.[8] The tumor of the present patient was diagnosed as a grade II atypical meningioma (because of its high mitotic index (e.g., >4 mitoses per 10 high-power fields or >2.5/mm^2) which have a moderate risk of recurrence, i.e., higher than that of grade I tumors and lower than that of grade III tumors.

A review of the literature identified 11 additional patients since 1985 who underwent a potentially curative resection of meningiomas that had metastasized to the lung (Table 1).[3,6,9–16] We performed a literature search using PubMed for reports published between 1985 and 2008, and also used citation references of recent case reports. The ages of these 11 patients (5 males and 6 females) ranged from 17 to 71 (median: 61) years. The histological types varied among these 11 patients, and the clinical behavior of meningiomas does not always correlate with histological features, i.e., even histologically benign (grade I) meningioma can metastasize.[9,16] One patient with the papillary subtype had poor prognosis.[3] The interval from the time of detection of the primary tumor to detection of the pulmonary metastasis can vary widely, and ranged from 0 (detection of pulmonary metastasis of intracranial meningioma and primary meningioma simultaneously at the initial patient presentation) to 19 years in the published literature. No correlation was found in these 11 patients between the intervals from the time of primary tumor detection to detection of the pulmonary metastasis, and the prognosis. There is little information on long-term patient outcome after pulmonary metastasectomy of meningioma, due to its rarity. Adlakha et al.[3] reported a patient with the longest follow-up period among these 11 patients who died of another disease 84 months after thoracotomy, without recurrence of meningioma. Among patients with atypical meningioma, which is the same histological type as that in the present patient, D'Aiuto et al.[15] reported a successful case in which 2-year disease-free survival was achieved after the resection of multiple pulmonary metastases.

Table 1. Patients who underwent a potentially curative resection of meningioma that had metastasized to the lung

First author, year[Ref]	Age (years)/ Sex	Interval[a] (years)	No. of tumors	Size (mm)	Laterality	Histology	Outcome
Miller, 1985[9]	61/M	0[b]	1	45	Left	Transitional	NA
Stoller, 1987[6]	63/F	18	1	13	Left	Fibroblastic	NA
LeMay, 1989[10]	56/F	10	Multiple	NA	Right	Benign meningioma	Died of disease 3 years after thoracotomy
Fukushima, 1989[11]	50/M	10	9	NA	Bilateral	Papillary	NA
Kodama, 1991[12]	61/F	19	4	41	Bilateral	Hemangiopericytoma	Alive with disease 44 months after thoracotomy
Murrah, 1996[13]	53/F	10	9	45	Bilateral	Meningioma	NA
Adlakha, 1999[3]	17/F	6	1	50	Left	Papillary	Died of disease 15 months after thoracotomy
Adlakha, 1999[3]	70/F	0[b]	1	NA	Right	Psammomatous	Died of another disease 84 months after thoracotomy without recurrence of meningioma
Teague, 2005[14]	64/M	2	3	45	Bilateral	Atypical transitional	NA
D'Aiuto, 2005[15]	71/M	13	37	60	Bilateral	Atypical	No evidence of disease 24 months after thoracotomy
Fulkerson, 2008[16]	54/M	0[b]	1	20	Right	Meningioma	No evidence of disease 12 months after thoracotomy
Present case, 2010	67/F	15	2	22	Right	Atypical	No evidence of disease 20 months after thoracotomy

NA, not available
[a]Interval from the time of primary tumor detection to detection of the pulmonary metastasis
[b]The pulmonary metastasis of the intracranial meningioma and the primary meningioma were simultaneously detected at the time of the initial presentation

To the best of our knowledge, there have been only a few reports on FDG-PET/CT findings of pulmonary metastasis of meningioma. In a report by D'Aiuto et al.[15] that described a patient with multiple lung metastases from meningioma, only the nodules larger than 2 cm presented abnormal uptake (SUV_{max} was not documented) on FDG-PET.[15] Hutchins et al.[17] also reported a case with multiple lung metastases from meningioma, and demonstrated that only the largest nodule (2.1 × 2 cm) exhibited abnormal uptake (SUV_{max} = 4.4) on FDG-PET. In the present case, the larger tumor (2.2 cm) had abnormal uptake (SUV_{max} = 6.9), but the smaller tumor also had weak uptake (SUV_{max} = 2.3). Based on these findings, it is speculated that when an SUV_{max} of 2.5 is adopted as the threshold to distinguish malignant from benign processes,[18] a size of 2.0 cm or greater is required to diagnose metastatic meningiomas to the lung. The evaluation of the diagnostic power for such lesions by FDG-PET will be necessary in the future.

The present patient has had 20 months of disease-free survival after pulmonary metastasectomy, which is considered to be a satisfying result. We believe that surgery should be considered when pulmonary metastases are deemed completely resectable by a preoperative radiological examination. A long follow-up period is essential to confirm the patient's outcome because of the slow-growing nature of meningioma.

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Decreased expression of LMO7 and its clinicopathological significance in human lung adenocarcinoma

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Abstract. LIM-domain only protein 7 (LMO7) has been suggested to act as a tumor suppressor for murine lung adenocarcinoma, while its splice variant p100 LMO7/#16 is associated with invasion and metastasis of rat AH130W1 cells. However, the importance of LMO7 in human lung cancer is unknown. We investigated LMO7 protein expression by immunohistochemistry in tumor tissues obtained from 57 patients with adenocarcinoma of the lung using a rabbit anti-LMO7 antibody. Signals for LMO7 were localized to the apical surface of the bronchial epithelium and to the cell membranes of pneumocytes in non-cancerous pulmonary tissues, but were noted circumferentially around the plasma membrane of cancer cells in all 57 patients with adenocarcinoma. The LMO7-positive group (24 patients, 42%) showed equivocal to strong expression of LMO7 in more than 50% cancer cells, while the remaining 33 patients (58%) showed LMO7 expression in less than 50% of their cancer cells. The latter group had significantly more advanced disease than the LMO7-positive group with regard to T factor (p=0.011), nodal involvement (p=0.026) and p-stage (p=0.010; χ^2 test). Multivariate analysis using a logistic regression model showed that LMO7 expression was independently associated with the T factor (p=0.041). Kaplan-Meier analysis showed that a poor prognosis was associated with low expression of LMO7 (p=0.036; log-rank test). Our findings are consistent with earlier observations and demonstrate that LMO7 is inversely correlated with the development and prognosis of human lung adenocarcinoma.

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Abbreviations: LMO7, LIM-domain only protein 7; PCD1, pancreatic cancer derived 1; TGF-β, transforming growth factor-β; GST, glutathione S-transferase

Key words: LIM-domain only protein 7, immunohistochemistry, human lung adenocarcinoma

Introduction

Lung cancer is the leading cause of cancer-related death in the world (1). Despite various advances in anticancer therapy, survival rates have not improved during the last decade, and long-term survival remains very poor (2-4). Local relapse may occur after surgical removal of the primary tumor, and distant metastasis is not unusual, arising from micrometastases that are undetectable when the primary tumor is diagnosed. Thus, exploring factors that are useful for predicting the progression and outcome of lung cancer is critical.

LIM-domain only protein 7 (LMO7) is a fibrous actin-binding protein that is widely expressed in adult tissues, particularly at the apical surface of lung epithelial cells (5). LMO7 is a member of a family of nine proteins containing both PDZ and LIM domains that function as protein-protein recognition modules (6,7). The PDZ and LIM families are involved in forming the Z-band of muscles through PDZ domains that bind to α-actinin or β-tropomyosin (8). LMO7 is also involved in the process of gene expression by acting as a nucleocytoplasmic shuttle protein that regulates the transcription of emerin and muscle-related genes (9). Moreover, a yeast two-hybrid study demonstrated that LMO7 binds afadin, the adaptor protein of nectins, at adherens junctions through the LIM domain (8). These observations suggest a role for LMO7 in the formation and maintenance of epithelial architecture via remodeling of the actin cytoskeleton.

On the other hand, a role of LMO7 in cancer pathology has been well documented. The P100 LMO7 splice variant (with a truncated C-terminal region) was originally identified by subtraction and differential hybridization in Yoshida hepatoma AH130W1 cells treated with transforming growth factor-β (TGF-β) (10). TGF-β induced alternative splicing of the *LMO7* gene, as well as promoted the migration of AH130W1 cells in an *in vitro* invasion assay (11,12). In addition, increased expression of LMO7 (also known as pancreatic cancer derived 1; PCD1) has been reported in cancer of the colorectal region, breast, liver, lung, pancreas, stomach and prostate, suggesting that PCD1 may play a role in cytoskeletal reorganization during carcinogenesis (13-15). Furthermore, the *LMO7* gene is located on chromosome 13q22, which is implicated in hereditary breast cancer (16,17), although it remains controversial whether LMO7 is the only gene responsible (16-18). Finally, *LMO7*-deficient

mice develop irregular and protruding epithelial lesions in the terminal and respiratory bronchioles at a young age, and these mice tend to develop lung adenocarcinoma at an older age, suggesting that LMO7 acts as a tumor-suppressor gene and that its deficiency confers a genetic predisposition to lung cancer (5). Despite these findings, the role of LMO7 in human lung carcinogenesis has yet to be studied.

We investigated the level of LMO7 protein expression and its pattern of expression by immunohistochemistry in surgically resected tissues obtained from 57 patients with primary lung adenocarcinoma. We also analyzed how LMO7 expression in tumor tissues influenced the progression of cancer and the prognosis of these patients.

Materials and methods

Surgical specimens. A total of 57 formalin-fixed samples of primary lung adenocarcinoma and adjacent normal lung tissues were obtained along with clinicopathological data from patients who underwent surgery at the Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka, Japan) between April 2000 and July 2002. All patients underwent potentially curative surgery without perioperative adjuvant therapy. Further treatment was administered only when the cancer recurred. The patients consisted of 31 men and 26 women, 42-79 years of age (mean 61.9).

Histologic classification of the tumor specimens was based on the WHO criteria. Eight patients had tumors with an acinar growth pattern, 42 had a papillary tumor pattern, 2 had bronchioloalveolar tumors and 5 had solid tumors with mucin formation (19). All tumors were staged according to the pTNM pathological classification of the International Union Against Cancer (20). There were 11 patients with p-stage IA, 18 with p-stage IB, 1 with p-stage IIA, 9 with p-stage IIB, 10 with p-stage IIIA and 8 with p-stage IIIB disease. The median postoperative follow-up period was 73 months (range 3-115).

Immunohistochemistry. To investigate LMO7 protein expression, thin sections were cut from 10% formalin-fixed and paraffin-embedded blocks of the surgical specimens, and were stained with a rabbit anti-LMO7 antibody (clone #863) as previously described (5). This antibody was specific for a recombinant protein containing amino acid residues of rat p100 #16/LMO7 (GeneBank accession no. AY609384), which was constructed as a fusion protein with glutathione S transferase (GST) using the pGEX plasmid vector (GE Healthcare UK Ltd., Buckinghamshire, UK) and was then employed as an immunogen.

Sections (4 μm) of tissues were mounted on poly-L-lysine-coated slides, air-dried and deparaffinized. Then, endogenous peroxidase activity was blocked by incubation with 5% hydrogen peroxide in 50% methanol for 20 min at room temperature. Subsequently, antigen retrieval was performed by autoclaving at 120˚C for 3 min in 10 mM citrate buffer. After blocking non-specific binding by incubation with 5% skim milk in PBS for 60 min at room temperature, the sections were incubated with polyclonal anti-LMO7 antibody overnight at 4˚C. After rinsing with PBS, the sections were incubated with biotinylated horse anti-rabbit IgG (Vector, Burlingame, CA, USA) for 30 min at room temperature, followed by washing with PBS. Immunoreactivity was detected with an avidin-biotin system (NovaRED™; Vector). In every control, sections were incubated with a 5-fold excess of GST-LMO7 fusion protein. Adjacent non-cancerous pulmonary tissues were also examined as an internal positive control for LMO7 protein expression.

Classification of immunohistochemical findings. LMO7 expression was primarily detected in the membranes of non-cancerous cells, such as pneumocytes, and some bronchial epithelial cells, as well as in cancer cells. Certain cancer cells also showed strong cytoplasmic immunostaining for LMO7. Immunopositivity for LMO7 expression was classified on the basis of the staining intensity. That is, cells with stronger immunostaining were judged to be LMO7-positive, whereas cells with weak or no immunostaining were classed as LMO7-negative.

When the percentage of LMO7-positive cancer cells in a tumor specimen was ≥50%, the tumor was classified as LMO7 'positive', while tumors with <50% positive cells were characterized as having low LMO7 expression. These semi-quantitative assessments were carried out by two independent investigators (H.N. and K.H.) without knowledge of the clinicopathological data.

Statistical analysis. Associations between LMO7 immunostaining and clinicopathological factors were assessed by the χ^2 test, except age which was assessed by the Student's t-test. Univariate and multivariate analyses of the clinicopathological factors associated with LMO7 expression were performed by the logistic regression method. Survival curves were calculated from the date of surgery to the time of death (or to final follow-up) according to the Kaplan-Meier method (21), and differences in survival among subgroups were analyzed by the log-rank test (22). Univariate and multivariate analyses of the influence of variables on overall survival were performed with the Cox proportional hazards regression model. Statistical analyses were carried out with SAS software (Cary, NC, USA), and $p<0.05$ was considered significant.

The surgical samples were obtained from patients after providing informed consent. This study and the use of clinical materials were approved by the relevant institutional research ethics committees. For protection of privacy, identifying information was removed from all samples before analysis in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Results

Immunohistochemistry for LMO7. We investigated the level and pattern of LMO7 protein positivity by immunohistochemistry of human lung adenocarcinoma specimens because of our previous findings regarding the expression and deficiency of LMO7 in mice (5). Normal bronchioalveolar epithelial cells showed uniformly intense LMO7 positivity. LMO7 was usually localized to the apical membranes of cells from the bronchial epithelium (Fig. 1A), while normal alveolar cells showed circumferential staining of the entire cell membrane (Fig. 1B). The antibody was confirmed to be specific for LMO7 protein because of the complete lack of immunostaining in the adjacent sections after pre-incubation with a 5-fold excess of

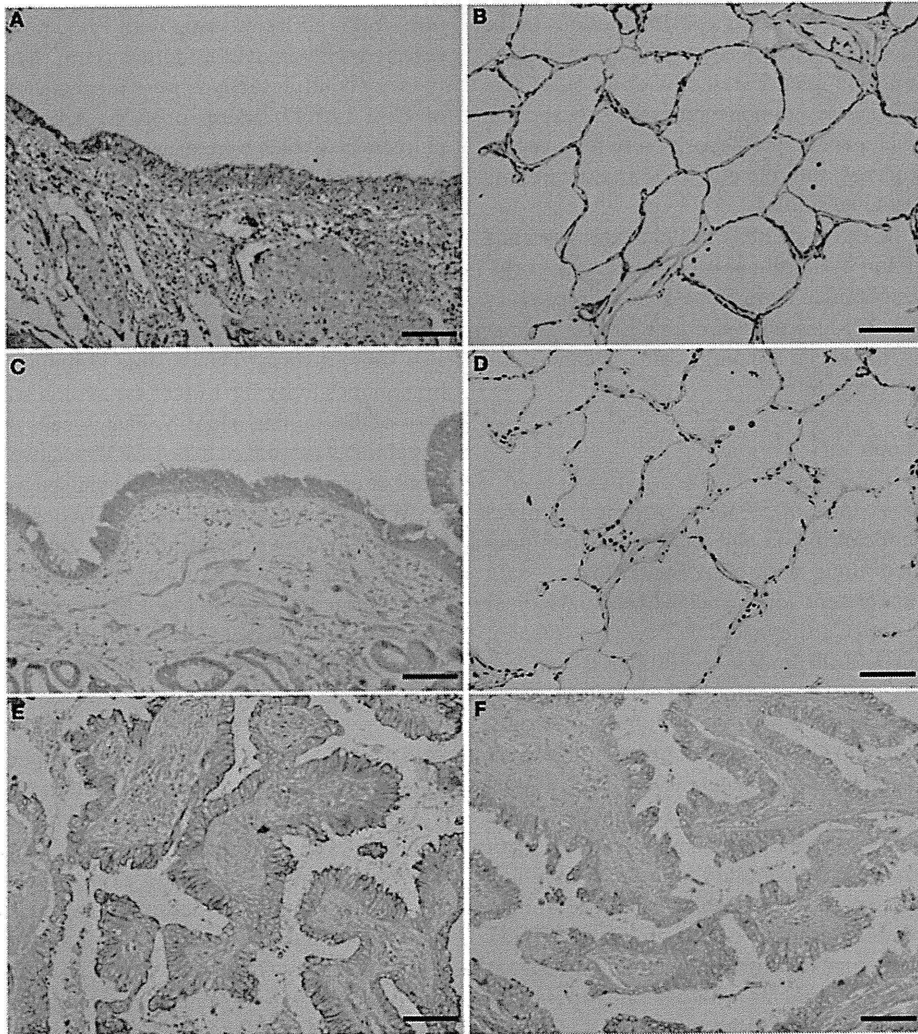

Figure 1. Immunohistochemical expression of LMO7 in primary lung adenocarcinomas and adjacent non-cancerous lung tissues. (A) Intense apical staining localized to the luminal surface of normal bronchiolar epithelial cells. The scale bar represents 50 μm. (B) Circumferential staining of the entire membrane of normal alveolar cells. The scale bar represents 100 μm. (C and D) LMO7 immunoreactivity noted in A and B was abolished after pre-incubation with the GST-LMO7 fusion protein, confirming the specificity of the anti-LMO7 antibody. The scale bars represent (C) 50 μm and (D) 100 μm. (E) Circumferential staining of the entire membrane of adenocarcinoma cells. Example of LMO7 positivity. The scale bar represents 100 μm. (F) LMO7 immunoreactivity noted in E was abolished after pre-incubation with the GST-LMO7 fusion protein. The scale bar represents 100 μm.

GST-LMO7 fusion protein (Fig. 1C and D). Certain stromal cells, including the smooth muscle cells of blood vessels and vascular endothelial cells, were also stained.

On the other hand, the adenocarcinoma cells of all 57 patients in this study showed circumferential LMO7 staining of the plasma membrane (Fig. 1E). Several patients also had tumor cells with LMO7 expression in the cytoplasm or in part of the apical region. Immunostaining for LMO7 was also abolished by pre-incubation with a 5-fold excess of GST-LMO7 fusion protein (Fig. 1F).

Correlation of decreased LMO7 expression with a poor clinicopathological outcome. To investigate the biological and clinicopathological significance of LMO7, we evaluated the relative intensity of LMO7 staining of the tumor specimens, and classified 33 patients (58%) and 24 patients (42%) into a low LMO7 group and an LMO7-positive group, respectively, as described in Materials and methods. The relationship between LMO7 expression and various clinicopathological factors is summarized in Table I. Patients in the low LMO7 group had significantly more advanced tumors than those in the LMO7-positive group with regard to T factor (p=0.011), nodal involvement (p=0.026) and p-stage (p=0.010). There was no significant relation between LMO7 expression and tumor histology (p=0.9580; χ^2 test).

Logistic regression analysis was performed with the outcome variable being the LMO7 level ('positive' vs. 'low') and the covariates being various clinicopathological factors. On univariate analysis, the T factor and nodal involvement were positively associated with the level of LMO7 expression. According to multivariate analysis, LMO7 expression was independently associated with the T factor at the time of surgery (p=0.041) (Table II). The results revealed that T1 status was linked to the LMO7-positive group, while T2-4 status was associated with lower expression of LMO7.

When postoperative overall survival curves were drawn according to the level of LMO7 expression in the 57 patients

Table I. Association between LMO7 expression and clinicopathological factors.

	LMO7 expression[a]		p-value
	Positive (n=24)	Low (n=33)	
Age (mean ± SD; years)	62.2±10.2	61.7±11.3	NS
Gender			
Male	13	18	
Female	11	15	0.977
T factor			
T1	11	5	
T2	11	19	
T3 and T4	2	9	0.011[c] (T1 vs. T2-4)
Nodal involvement			
N0	18	15	
N1	4	5	
N2 and N3	2	13	0.026[b] (N0 vs. N1-3)
Histological differentiation			
Well, moderate	21	27	
Poor	3	6	0.561
p-stage			
IA and IB	17	12	
IIA and IIB	4	6	
IIIA and IIIB	3	15	0.010[c] (I vs. II and III)

[a]positive expression, ≥50% stained cells; low expression, <50% stained cells. [b]p<0.05; [c]p<0.02. NS, not significant.

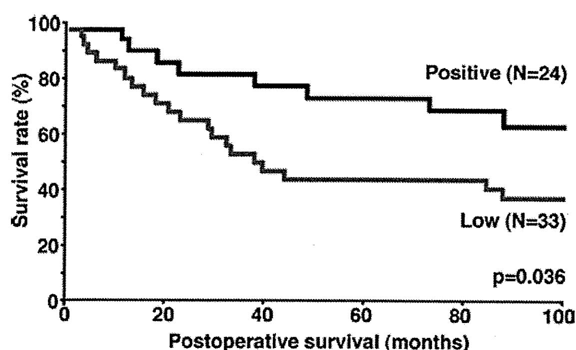

Figure 2. Comparison of Kaplan-Meier survival curves between the low LMO7 expression group and LMO7-positive group of patients with lung adenocarcinoma. The difference of survival between the two groups was statistically significant (p=0.036; log-rank test).

Table II. Univariate and multivariate analyses of prognostic indicators with the Cox proportional hazards model.

Variables	Risk ratio (95% CI)	p-value
Univariate model		
T factor (T1 vs.T2-4)	2.893 (1.116-9.855)	0.027[a]
Nodal involvement (N0 vs. N1-3)	2.276 (1.092-4.896)	0.028[a]
Histological differentiation (well, moderate vs. poor)	3.226 (1.255-7.358)	0.017[b]
LMO7 expression (positive vs. low)	2.348 (1.071-5.676)	0.033[a]
Multivariate model		
T factor (T1 vs. T2-4)	2.996 (1.085-10.642)	0.033[a]
Nodal involvement (N0 vs. N1-3)	1.509 (0.698-3.344)	0.296
Histological differentiation (well, moderate vs. poor)	3.889 (1.424-9.612)	0.010[b]
LMO7 expression (positive vs. low)	1.853 (0.813-4.629)	0.146

[a]p<0.05; [b]p<0.02.

Table III. Results of univariate and multivariate logistic regression analyses.

Model	Odds ratio (95% CI)	p-value
Univariate model		
Age	1.004 (0.956-1.056)	0.867
Gender (male vs. female)	0.985 (0.341-2.856)	0.977
T factor (T1 vs. T2-4)	0.211 (0.056-0.703)	0.011[b]
Nodal involvement (N0 vs. N1-3)	0.278 (0.083-0.845)	0.024[a]
Histological differentiation (well, moderate vs. poor)	0.643 (0.124-2.744)	0.557
Multivariate model		
T factor (T1 vs. T2-4)	0.262 (0.065-0.946)	0.041[a]
Nodal involvement (N0 vs. N1-3)	0.391 (0.107-1.333)	0.134
Histological differentiation (well, moderate vs. poor)	0.734 (0.121-3.749)	0.714

[a]p<0.05; [b]p<0.02.

with lung adenocarcinoma, patients in the low expression group had a significantly worse prognosis than those in the LMO7-positive group (p=0.036) (Fig. 2).

When the Cox proportional hazards model was employed for multivariate analysis, it showed that LMO7 expression was not an independent prognostic factor, although LMO7

was significantly associated with the prognosis according to univariate analysis (Table II). However, the T factor that was independently associated with LMO7 expression according to logistic regression analysis (Table III) was an independent prognostic indicator in the Cox proportional hazards model (Table II).

Discussion

To clarify the role of LMO7 in the pathogenesis of human lung adenocarcinoma, we examined LMO7 protein expression with an anti-LMO7 antibody that effectively detects LMO7 under a variety of experimental conditions (5). Our immunohistochemical analysis showed that LMO7 was localized circumferentially in the plasma membrane of adenocarcinoma cells, and that decreased expression of LMO7 was significantly correlated with tumor progression and a poor prognosis of patients with lung adenocarcinoma. LMO7 is mainly expressed by normal bronchiolar and alveolar epithelial cells in the lungs of humans as well as in mice, and a knockout study indicated that LMO7 acts as a tumor suppressor for murine lung adenocarcinoma (5). Therefore, our findings are not only consistent with earlier observations, but also demonstrate that down-regulation of LMO7 expression is related to the clinicopathological features of lung adenocarcinoma and to the prognosis. Of course, this study did not exclude the possibility that down-regulation of LMO7 expression could also be a useful prognostic indicator for other types of cancers, particularly hereditary human breast cancer (16-18).

The low LMO7 expression group was significantly associated with more advanced tumors in the present study. Multivariate logistic regression analysis showed that LMO7 expression was independently associated with the T factor. Furthermore, Kaplan-Meier analysis of survival revealed a significant difference between the LMO7-positive group and the low expression group in the patients with lung adenocarcinoma. These results support a potential influence of LMO7 on tumor progression and the prognosis of human lung carcinoma. Although Cox proportional hazards analysis failed to identify LMO7 expression as an independent prognostic indicator, the T factor was an independent variable.

Immunohistochemistry does not provide us with data on the molecular mechanisms underlying changes in protein levels. It is thus unclear whether LMO7 immunolabelling is correlated with the level of native LMO7 protein, splice variants or mutant protein. Although up-regulation of LMO7 has been observed by immunohistochemistry in various types of human cancers (13-15), the biological significance of these findings remains unknown. Our studies have demonstrated that overexpression of P100 LMO7, a splice variant induced by TGF-β, enhances the migration, proliferation and invasion of MDCK cells (unpublished data). Based on these oncogenic properties, up-regulation of LMO7 in various types of cancers may reflect a role in tumor progression.

In conclusion, down-regulation of LMO7 is related to the clinicopathological features of human lung adenocarcinoma and to the prognosis, but it remains unclear whether LMO7 may be a candidate for molecular-targeting therapy. Further studies are required to elucidate the role of LMO7 in the pathogenesis of human lung adenocarcinoma.

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Plasma Free Amino Acid Profiling of Five Types of Cancer Patients and Its Application for Early Detection

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Abstract

Background: Recently, rapid advances have been made in metabolomics-based, easy-to-use early cancer detection methods using blood samples. Among metabolites, profiling of plasma free amino acids (PFAAs) is a promising approach because PFAAs link all organ systems and have important roles in metabolism. Furthermore, PFAA profiles are known to be influenced by specific diseases, including cancers. Therefore, the purpose of the present study was to determine the characteristics of the PFAA profiles in cancer patients and the possibility of using this information for early detection.

Methods and Findings: Plasma samples were collected from approximately 200 patients from multiple institutes, each diagnosed with one of the following five types of cancer: lung, gastric, colorectal, breast, or prostate cancer. Patients were compared to gender- and age- matched controls also used in this study. The PFAA levels were measured using high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)–mass spectrometry (MS). Univariate analysis revealed significant differences in the PFAA profiles between the controls and the patients with any of the five types of cancer listed above, even those with asymptomatic early-stage disease. Furthermore, multivariate analysis clearly discriminated the cancer patients from the controls in terms of the area under the receiver-operator characteristics curve (AUC of ROC >0.75 for each cancer), regardless of cancer stage. Because this study was designed as case-control study, further investigations, including model construction and validation using cohorts with larger sample sizes, are necessary to determine the usefulness of PFAA profiling.

Conclusions: These findings suggest that PFAA profiling has great potential for improving cancer screening and diagnosis and understanding disease pathogenesis. PFAA profiles can also be used to determine various disease diagnoses from a single blood sample, which involves a relatively simple plasma assay and imposes a lower physical burden on subjects when compared to existing screening methods.

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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

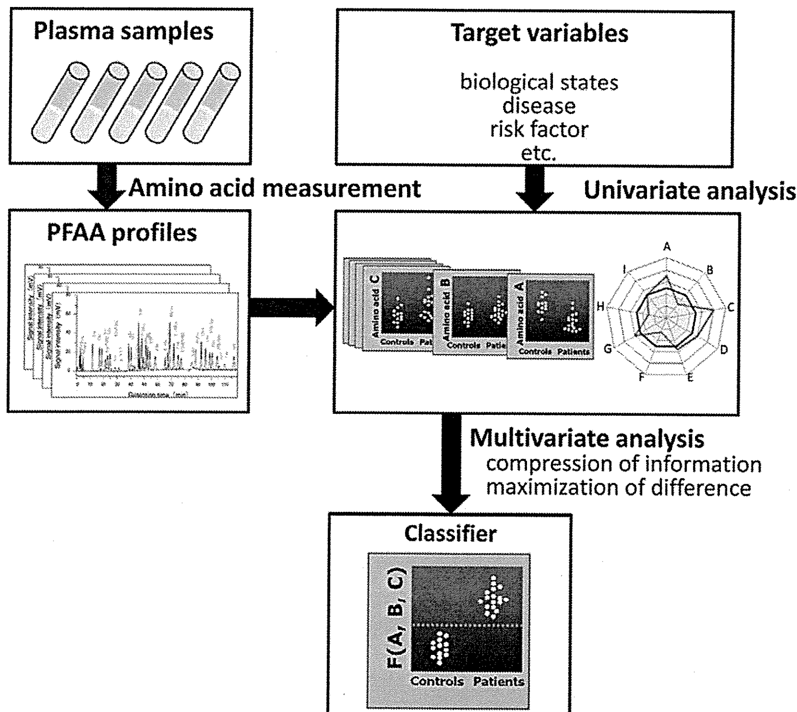

Figure 1. Concept of the generation of "AminoIndex technology". At the top of the diagram, PFAA concentrations are measured for each subject. In the middle, target variables and univariate analysis of PFAA profiles are represented. At the bottom, an estimation of the classifier with optimized discriminating 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

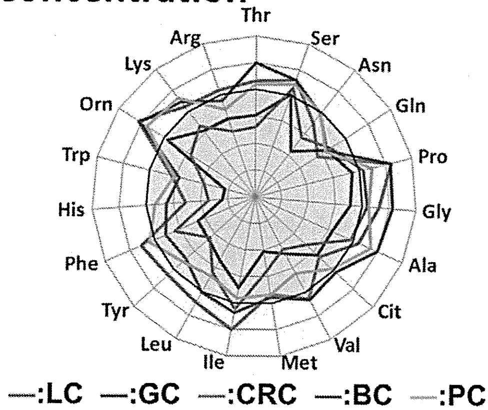
Concentration

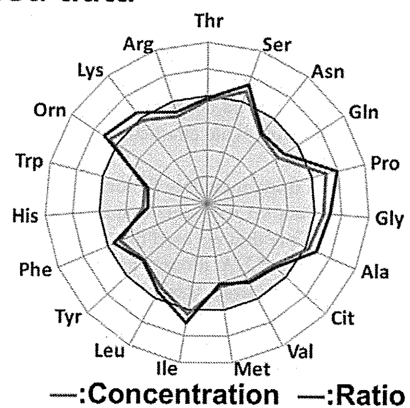
Amino acid	LC	GC	CRC	BC	PC	Pooled
Thr						
Ser						
Asn						
Gln						
Pro						
Gly						
Ala						
Cit						
Val						
Met						
Ile						
Leu						
Tyr						
Phe						
His						
Trp						
Orn						
Lys						
Arg						

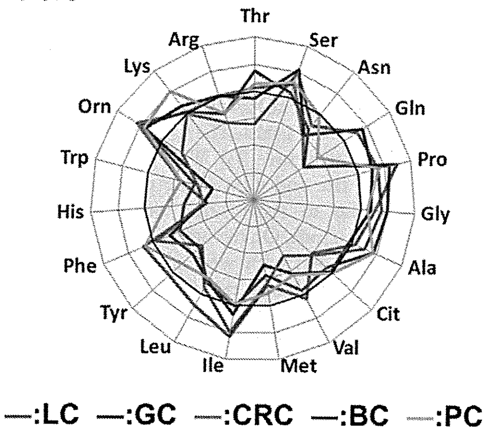
Ratio

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







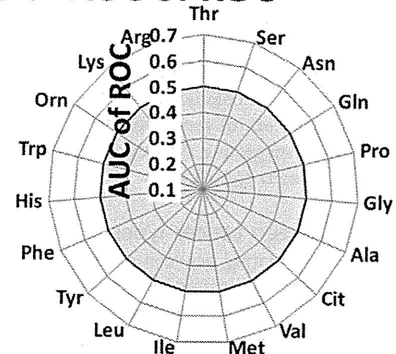


Figure 2. PFAA profiles of cancer patients. The results of the Mann-Whitney *U*-test (A) and receiver-operator characteristic (ROC) curve analysis (B) are indicated. A. Colored cells indicate that the concentration or ratio is increased in cancer patients at $p<0.001$ (red), $p<0.01$ (orange), and $p<0.05$ (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.

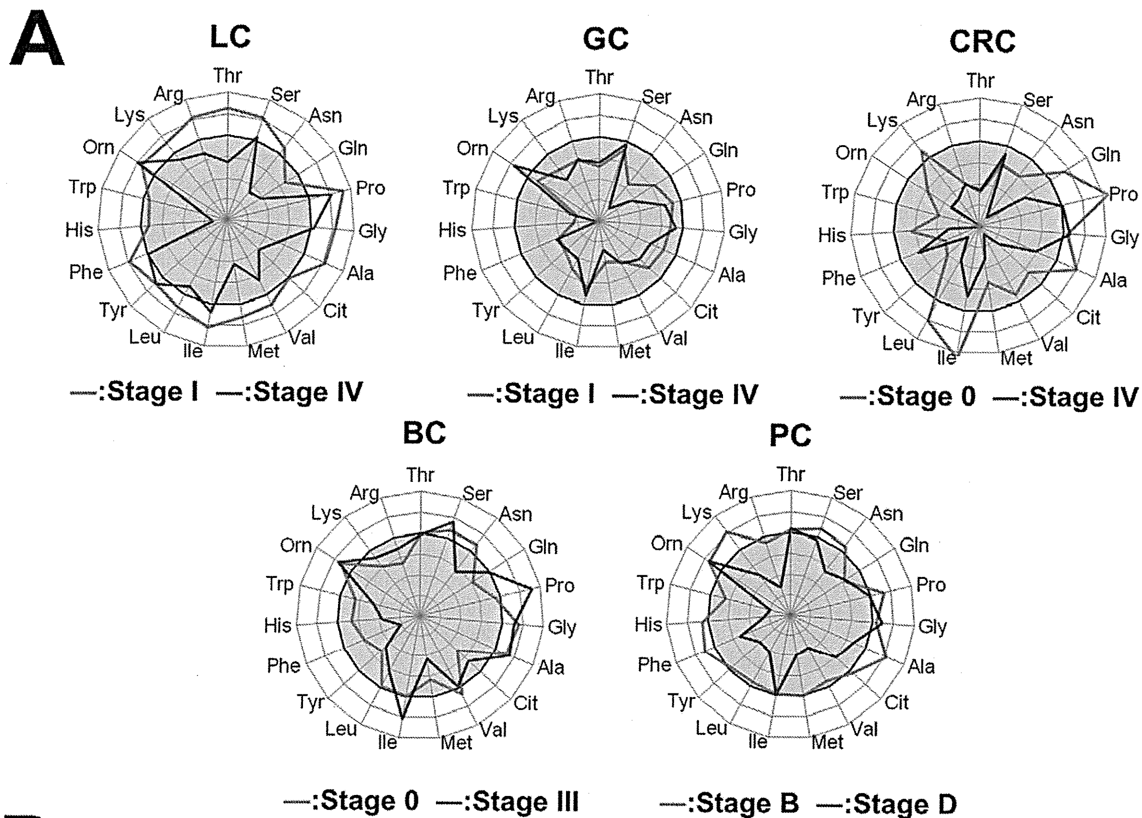
The accuracies were similar between the analyses using ratios as explanatory variables and those using concentrations both in male patients (Table S8) and female patients (Table S9). Seven amino acids (Gln, Cit, Val, Tyr, Trp, Lys, and Arg) were commonly selected regardless of gender in these analyses (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].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His
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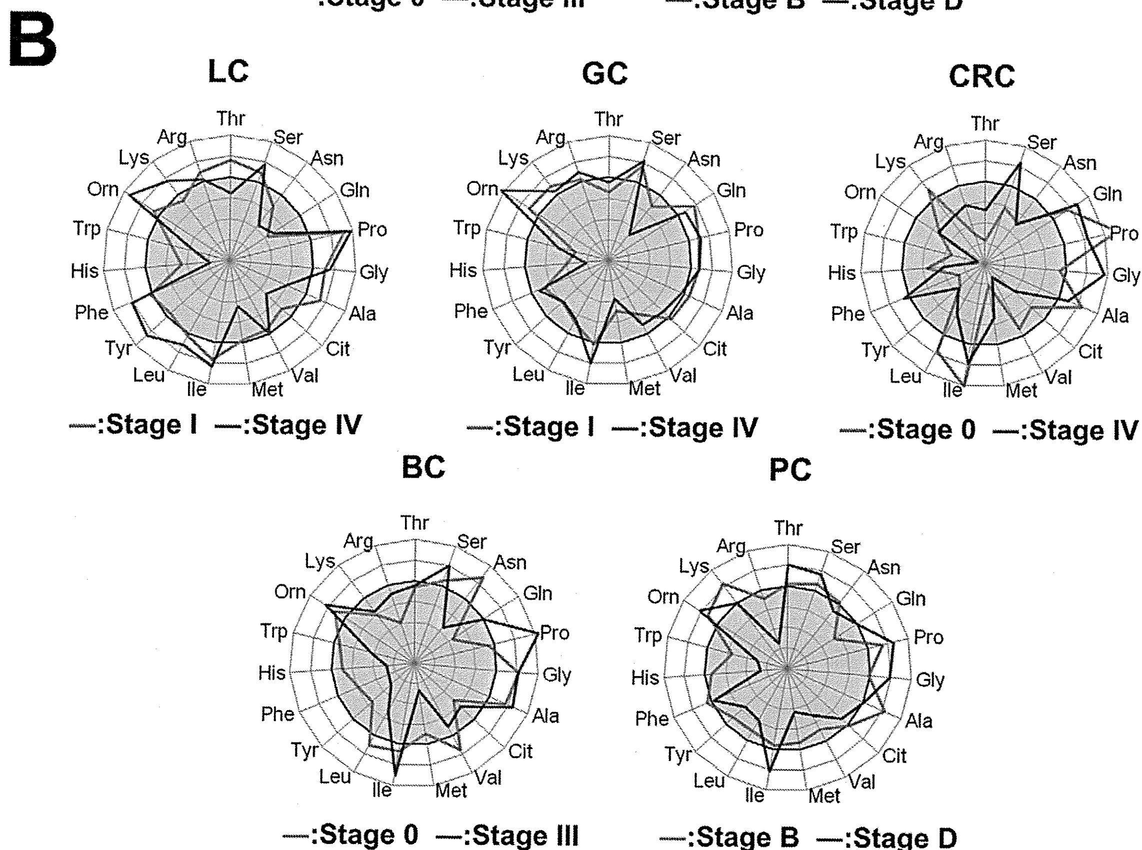
—:Stage I —:Stage IV
—:Stage 0 —:Stage IV
—:Stage 0 —:Stage III
—:Stage B —:Stage D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Tyr
Phe
His
Trp
Orn
Lys
Arg
—:Stage I —:Stage IV
—:Stage I —:Stage IV
—:Stage 0 —:Stage IV
—:Stage 0 —:Stage III
—:Stage B —:Stage D

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].
doi:10.1371/journal.pone.0024143.g003

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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