Table 5. Oncologists' opinion on strategies suggested to alleviate the burden associated with communicating discontinuation of anticancer treatment

	Necessary (%)	Absolutely necessary (%)
Inpatient hospice is readily available and patient information is exchanged smoothly among facilities	49	36
Quiet and private rooms are available for breaking bad news	56	25
After breaking bad news, a nurse, psychologist or medical social worker is available for emotional support	63	24
A reduction in the oncologist's total workload to give sufficient time for the breaking of bad news	54	23
While breaking bad news, a nurse, psychologist or medical social worker is available for emotional support	56	13
Having an opportunity to attend educational workshops about how to break bad news	51	6.8
A psychiatrist or psychologist is available for consultation if the oncologist feels overburdened	42	6.6
Before breaking bad news, having the opportunity to discuss the situation with colleagues and receive advice	54	6.1
After breaking bad news, specialists in physician–patient communication are available to give advice to the oncologist about how they should break bad news	60	5.5
Having opportunities to share experiences and feelings with the colleagues within the hospital	51	5.5
Before breaking bad news, information about what the patient and family want to know is available from nurses	65	5.0
Before breaking bad news, the oncologist receives a memo from the patient and family about what they want to know	61	3.8
After breaking bad news, the oncologist receives a questionnaire to identify what the patient and the family are feeling and thinking	65	3.3
Have an opportunity to share experiences and feelings with colleagues from other hospitals	47	3.0

their families; and (iii) developing a multidisciplinary care model with other professionals and facilities.

This study emphasizes the importance of communication skills. Previous studies suggested that communication skills training increases both patient satisfaction (27,28) and oncologists' confidence (29). However, to the best of our knowledge, existing communication skills training does not specifically address issues surrounding the discontinuation of anticancer treatments. The present study highlights the importance, under these difficult circumstances, of helping the patient maintain hope, dealing with the oncologists' fear of being blamed by the patients and their families, and strengthening patient self-control. The results indicate that a communication skills training program specifically targeting skills for communicating the discontinuation of anticancer treatment needs to be developed. This program should include strategies to deal with oncologists' concerns, such as that by breaking bad news to a patient, the oncologist will deprive the patient of hope, that the oncologist may be blamed by the patient's family and that the patient may lose self-control.

The oncologists surveyed stressed the importance of a reduction in their total workload to give them sufficient time to facilitate effective communication with patients. A previous study suggested that physicians face excessive workloads that are associated with a lower quality of patient care (30). Several studies have suggested that the perception of having insufficient time to communicate with patients is the factor most strongly associated with oncologist burnout (22,31). In Japan, according to a 2008 revision by the Ministry of Health, Labor and Welfare in Japan of the payment of fees for medical treatment, an additional fee for

outpatient care can be applied when a physician is directly involved in clinical practice for 5 min or longer. This indicates that the Ministry of Health, Labor and Welfare in Japan defines the time for consultation and implies that most physicians in Japan are too busy to spend 5 min or more on each outpatient. These results stress that a reduction in physicians' workload is vital.

Many oncologists surveyed in the present study agreed with the importance of multidisciplinary cooperation with other professionals and facilities. Two types of cooperation were considered to be particularly valuable: (i) that after breaking bad news, a nurse, psychologist or medical social worker was available to follow up with patients and their families; and (ii) the availability of other facilities, especially inpatient palliative care units. Previous studies have shown that cancer patients' participation in nurse-led interventions resulted in an improvement in depressive moods (32,33). Multiple intervention studies have indicated that practice-based interprofessional collaboration can improve patients' health-care processes and outcomes (34). Furthermore, existing literature indicates that regional palliative care programs succeed in increasing family satisfaction (35,36). These findings suggest that developing a multidisciplinary team to support oncologists, not only within a hospital, but also beyond the hospital (as a region), is of considerable importance in achieving patient and family satisfaction. As the number of palliative care units in Japan is not enough, increasing the reimbursement for inpatient hospice would be important as policy. Moreover, because oncologist burden was not measured in these previous studies, prospective observational or interventional studies are needed to determine whether a team

approach, such as in-hospital and regional palliative care programs, could alleviate oncologist burden.

The limitations of the present study include the moderate (67%) effective response rate, which may mean that the entire oncological population is not represented by the oncologists who participated in the present study. Furthermore, because this study was performed in Japan, the results are likely to be influenced by factors relating to Japanese culture and the Japanese health-care system and, as such, may not be applicable to other countries.

In conclusion, a considerable number of oncologists experienced high levels of burden in communicating the decision to discontinue anticancer treatment. To alleviate oncologist burden, potentially useful strategies include: (i) communication skills training specifically targeting discontinuation of anticancer treatment; (ii) a reduction in total workload to allow oncologists sufficient time to break bad news; and (iii) the development of a multidisciplinary model to facilitate cooperation with other professionals and facilities.

Authors' Contribution

H.O.: conception and design, provision of the study material, collection of data, data analysis and interpretation, manuscript writing and administrative support. T.M.: conception and design, provision of the study material, data analysis and interpretation, final approval of the manuscript, administrative support, and financial support. T.E., H.A., K.T., A.O. and K.S.: provision of the study material and final approval of the manuscript.

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Conflict of interest statement

None declared.

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Prognostic Impact of Microscopic Positive Margin in Gastric Cancer Patients

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Background: Complete resection with negative surgical margins has been a long-held surgical philosophy based on the concept that even minimal remaining cancer cells will develop recurrences.

Objectives: This study investigated the clinical significance of microscopic positive margin on the outcome of patients with gastric cancers.

Methods: The relationships between the margin status and other clinicopathologic factors were examined in gastric cancer patients undergoing gastrectomy, and then the prognostic impact of the margin status was evaluated by univariate and multivariate analysis.

Results: The microscopic positive margin was identified in 23 patients (2.8%) by standard H&E staining. The positive margin showed a strong correlation significantly with tumor size ($P < 0.05$). Microscopic positive margin was found to be a significant prognostic factor on univariate analysis (5-year survival rate 51.9% vs. 82.2%, $P < 0.0001$), as well as multivariate analysis (risk ratio 3.24, 95% CI: 1.24–6.50, $P < 0.01$). Detailed analysis of margin status demonstrated that patients with positive margin in a deep site and/or in multiple layers showed poor survival.

Conclusions: Microscopic positive margin was found to be an independent prognostic factor in gastric cancer patients. The status of the surgical margin might provide useful information for selecting additional treatments and performing intensive follow-up.
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INTRODUCTION

Although recent advances in diagnostic techniques have lead to the detection of an increasing number of early gastric cancers, surgical resection remains the main curative treatment for most patients with this lethal disease [1–3]. Complete resection with negative surgical margins has been accepted as the most effective treatment based on the surgical philosophy that even minimal remaining cancer cells will develop recurrences.

However, microscopic positive margins are sometimes diagnosed histologically even if the stomach was resected with both proximally and distally sufficient margins on intraoperative findings in gastric cancer patients. Previous studies advocated that re-operation should be considered in such patients because of the poor survival shown by patients with positive surgical margin. However, some authors demonstrated that aggressive tumor biology might be one of the factors contributing to microscopic positive margins after gastrectomy, and therefore these patients are frequently accompanied by hematological and/or peritoneal metastases even if locoregional recurrence develops [3–6].

This study was designed to investigate the prognostic significance of microscopic positive margins in gastric cancer patients undergoing gastrectomy with Japanese standard lymph node dissection. The relationship between the detailed margin status and prognosis was also evaluated.

PATIENTS AND METHODS

A total of 1,205 patients with gastric cancer underwent gastrectomy at Kyoto Prefectural University of Medicine from January 1997 to June 2009. Of these patients, 122 patients, who died of other disease during their follow-up period, 146 patients, who were lost to follow-up, and 113 patients who were diagnosed as M1 (including R1 for positive peritoneal cytology), were excluded from this retrospective study.

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The gastrectomy and lymphadenectomy was performed according to the general rules for gastric cancer study in surgery and pathology by the Japanese research society for gastric cancer. All of the patients who underwent macroscopic curative resection (R0 and R1) were accompanied with D2 or modified D2 lymphadenectomies (at least dissection of all perigastric lymph nodes in the relevant part of the stomach plus the left gastric artery, the common hepatic artery, and the celiac artery nodes). After removing the stomach from the surrounding tissues, the stomach was resected with macroscopically sufficient margins both proximally and distally. In general, we have determined the resection line at least 2 cm away from the macroscopic margin in patients with early gastric cancers, and 3 cm away in localized-type advanced gastric cancers, and 5 cm away in diffuse-type advanced gastric cancers. On the other hand, the duodenum is usually dissected 2 cm apart from pyloric ring without findings of macroscopic duodenal invasion. The clinicopathologic findings of these patients were determined retrospectively based on their hospital records. Histological classification of gastric cancer was based on the 14th Japanese Classification of Gastric Carcinomas (JCGC), in which the T- and N-categories are the same as those in the 7th TNM classification. The resected stomach was sectioned in principle on and along the lesser curvature. This section line was used as a reference line. When tumor invasion of the proximal or distal cut ends was suspected, additional sections parallel to the reference line from the entire margin areas of tumors were taken.

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These sections were assessed systematically by the pathologists, and categorized the patients according to the methods described below.

There were 25 patients who underwent apparently curative resection in intraoperative findings but showed a microscopic positive surgical margin. However, we excluded two patients who underwent a second operation from the present analyses, because they died of other diseases during the follow-up period. The remaining 801 patients who underwent curative gastrectomy with lymphadenectomy (R0) were classified into control group. The relationships between margin status and other clinicopathologic factors were examined, and then the prognostic impact of the margin status was evaluated by univariate and multivariate analysis. We also analyzed patients stratified according to T-category.

The 23 patients were divided into subgroups according to the status of positive margin as follows: a single layer versus multiple layers, and a superficial site versus a deep site, and investigated the relationship between the margin status and prognosis of the patients. Patients with cancer cells infiltrating in a single layer at the histological margin were classified into the "single layer group," and the remaining patients with cancer cells infiltrating in more than two layers into the "multiple layers group." On the other hand, patients with cancer cells infiltrating only the mucosal layer at the histological margin were classified into the "superficial layer group," and the remaining patients with cancer cell infiltrating deeper than the submucosal layer were classified into the "deep site group."

The entire study population was followed until death or the final date of observation (March 31, 2010), by outpatient clinic consultation, and/or communication with patients via telephone or letter. Median and mean follow-up period were 35.9 and 36.5 months (range 1–60 months), respectively.

All statistical analyses and graphics were performed with JMP 7.0.1 package (SAS Institute, Inc., Tokyo). Values for all continuous data are expressed as the median and as the mean depending on their distributions. Chi-square test and t-test (or Mann–Whitney test) were used for univariate comparisons. Overall survival rates were calculated by the Kaplan–Meier method, with the date of gastrectomy as the starting point. Differences in survival were examined by log-rank test. Multivariate analysis of prognostic factors related to survival was performed using the Cox proportional hazard model. We conducted multivariate analysis using only variables that were significant prognostic factors on univariate KM analysis. For all analyses, the significance of differences was accepted at $P < 0.05$.

RESULTS

Microscopic positive margins were identified in 23 patients by standard H&E staining in the present study. All of these patients demonstrated viable cancer cells infiltration at either or both gastrointestinal resection lines at the esophageal, gastric, or duodenal interface between the invasion site and surrounding tissues. The mean number of harvested LNs were 36. Some patients underwent gastrectomy along with additional organ resection, splenectomy and pancreatectomy. Of the 23 patients, 9 (39%) showed distal margin involvement, 12 (52%) showed proximal margin involvement, and 2 (9%) showed involvement of both margins. Among 23 patients with positive margins, postoperative chemotherapy was performed in 10 patients, and intensive follow-up in 13 patients, but none of the patients underwent irradiation therapy. Our postoperative chemotherapeutic regimens consisted of S-1 + CDDP (three patients), S-1 (two patients), UFTE (two patients), 5FU + l-LV (one patient), MTX + 5FU + CDDP (one patient) and DTX + CPT11 (one patient). None of the patients in the present study received NAC.

Clinicopathologic features of patients with positive margins and control groups are listed in Table I. As shown, in the table, there was

a significant difference in tumor size ($P < 0.05$) but not in other factors including histological grade.

Although 14 of the 23 patients developed the recurrent diseases, there were no patients who developed only at the surgical stump. The patterns of recurrences were peritoneal metastases in six patients, liver metastases in five patients, and lymph node metastases in three patients.

The microscopic positive margin was a significant prognostic factor on univariate analysis (5 years overall survival rates (5YSR): 52% vs. 82%, $P < 0.0001$). Besides the margin status, univariate analysis also demonstrated that age, tumor size, location, resection type, lymphatic and/or blood vessels invasion (LBVI), pT stage, pN stage, and pM stage were significant prognostic factors in the present study. Margin status, tumor size, blood vessel invasion, pT stage, pN stage, and pM stage were independent prognostic factors on multivariate analysis (Table II).

TABLE I. Clinicopathological Features of the Patients With Negative and Positive Margin

	Negative margin (%)	Positive margin (%)	P
Sex			
Male	514 (67.1)	18 (78.3)	0.244
Female	252 (32.9)	5 (21.7)	
Unknown	35 (4.4)	0 (0.0)	
Age (years)[a]	63.66 ± 0.41	68.09 ± 2.22	0.0655
Tumor size (mm)[a]	44.8 ± 1.17	64.73 ± 9.67	0.0041
Location			
L	227 (30.6)	6 (26.1)	0.1953
M	323 (43.6)	7 (30.4)	
U	162 (21.9)	7 (30.4)	
LMU	29 (3.9)	3 (13.0)	
Unknown	60 (7.5)	0 (0.0)	
Macro-type			
Local	500 (65.4)	16 (69.6)	0.6729
Diffuse	265 (34.6)	7 (30.4)	
Unknown	36 (4.5)	0 (0.0)	
ly			
Negative	442 (58.4)	11 (47.8)	0.3154
Positive	315 (41.6)	12 (52.2)	
Unknown	44 (5.5)	0 (0.0)	
v			
Negative	569 (72.3)	16 (69.6)	0.5423
Positive	187 (24.7)	7 (30.4)	
Unknown	45 (5.6)	0 (0.0)	
Histologic grade			
Diff.	373 (48.9)	11 (47.8)	0.9179
Undiff.	390 (51.1)	11 (47.8)	
Unknown	38 (4.7)	1 (4.3)	
pT stage			
T1	400 (52.2)	10 (43.5)	0.2256
T2	83 (10.8)	1 (4.4)	
T3	122 (15.9)	3 (13.0)	
T4	161 (21.0)	9 (39.1)	
Unknown	35 (4.4)	0 (0.0)	
pN stage			
pN0	488 (64.5)	11 (47.8)	0.1724
pN1	84 (11.1)	2 (9.1)	
pN2	87 (11.5)	3 (13.6)	
pN3	98 (13.0)	7 (31.8)	
Unknown	44 (5.5)	0 (0.0)	
pM stage			
M0	738 (97.4)	22 (95.6)	0.6453
M1	20 (2.6)	1 (4.4)	
Unknown	43 (5.4)	0 (0.0)	

ly, lymphatic vessel invasion; v, blood vessel invasion.
[a]Two-tailed t-test of mean ± standard deviation (SD).

TABLE II. Univariate and Multivariate Analysis of Prognostic Factors in Patients With Positive Margin

	Univariate analysis			Multivariate analysis		
	n	5-YSR	P	RR	95% CI	P
Sex						
Male	558	81.0	0.4195			
Female	266	84.1				
Unknown	0					
Age						
≤65	449	84.9	0.0207	1		0.2043
≥66	375	78.3		1.31	0.86–2.00	
Unknown	0					
Tumor size						
≤40	420	95.3	<0.0001	1		0.0071
>40	332	61.2		2.2	1.20–4.04	
Unknown	72					
Location						
LMU	33	35.4	<0.0001	1		0.1237
L	237	80.4		1.12	0.47–2.69	
M	335	90.7		0.58	0.27–1.23	
U	172	73.8		0.82	0.42–1.60	
Unknown	47					
Resection type						
TG	206	65.1	<0.0001	1		0.6066
PG	63	85.6		1.11	0.43–2.82	
DG	532	88.4		0.7	0.34–1.45	
Unknown	23					
Macro						
Diffuse	523	82.2	0.7573			
Localized	276	79.8				
Unknown	25					
Histologic type						
Diff.	390	82.4	0.5216			
Undiff.	405	80.5				
Unknown	29					
ly						
Absent	454	94.9	<0.0001	1		0.131
Present	327	62.5		1.68	0.84–3.38	
Unknown	43					
v						
Absent	586	89.3	<0.0001	1		0.0035
Present	194	56.6		1.93	1.24–3.03	
Unknown	44					
pT						
T1	417	96.7	<0.0001	1		0.0227
T2	85	95.7		0.46	0.36–0.59	
T3	127	66.3		2.01	1.23–3.30	
T4	171	48.2		1.88	0.72–4.90	
Unknown	24					
pN						
N0	502	95.1	<0.0001	1		<0.0001
N1	86	78.5		1.73	1.63–1.84	
N2	90	62.7		2.39	1.98–2.88	
N3	105	29.0		5.48	2.72–10.99	
Unknown	41					
Pm						
M0	763	83.0	<0.0001	1		0.0005
M1	21	19.1		3.55	1.80–6.66	
Unknown	40					
Margin status						
Negative	766	82.2	<0.0001	1		0.0059
Positive	23	51.9		3.24	1.45–6.50	
Unknown	35					

n, number of patients; lt, lymphatic vessel invasion; 5-YSR, 5-year overall survival rate; v, blood vessel invasion.

There was a significant difference in the T1 subgroup (5YSR: positive margin vs. control; 68.6% vs. 97.4%, $P < 0.0001$). There was also a significant difference in the T2–T4 subgroup (5YSR: positive margin vs. control; 37.6% vs. 65.3%, $P = 0.002$).

Figure 1 shows the typical microscopic findings of positive margins in the patients. Then, we analyzed the relationship between the depth of positive margin and the tumor characteristics, and found the significant correlation between the depth of positive margin and pT stage (data not shown).

Patients with positive margin in a deep site showed a poorer survival than those with a positive margin in a superficial layer, although there was no significant difference. Furthermore, patients with positive margins in multiple layers showed poorer survival than those with a positive margin in a single layer, although there was no significant difference (Fig. 2).

DISCUSSION

Complete resection with negative surgical margins has been accepted as the most effective treatment based on the surgical philosophy that even minimal remaining cancer cells will develop recurrences. We, however, have had experiences with gastric cancer patients who did not develop any postoperative recurrences despite a positive surgical margin on microscopic findings. In recent years, stapler instruments have usually been utilized for organ excisions and the stapled lines should be removed before histological examination, and therefore margins examined histologically are not exactly the same as the true surgical margins. Moreover, some previous studies demonstrated that aggressive tumor biology might be one of the factors contributing to microscopic positive margin after gastrectomy, and therefore, these patients are frequently accompanied by hematological and/or peritoneal metastases even if locoregional

recurrence develops [3–6]. These findings prompted us to investigate the prognostic significance of microscopic positive margins for gastric cancer patients underwent gastrectomy in recent series.

The microscopic positive margin was found in 2.8% of gastric cancer patients in the present study (14.5% of all patients including non-curative cases; data not shown), which is consistent with previous studies reporting the rates from 1% to 20% [7–11]. Concerning the prognostic impact of surgical margin status, previous reports demonstrated that a positive margin was associated with a worse outcome [7,12,13]. However, Cho et al. reported that a positive surgical margin was a significant prognostic factor in node-negative patients but not in node-positive patients, and therefore concluded that aggressive treatments, such as re-operation, are warranted for such patients with node-negative disease [6]. We performed a survival analysis evaluating the margin status as a candidate factor in the present study, and found that a positive margin was a significant prognostic factor on both univariate [3,7,12–14] and multivariate analyses. Our findings indicate that the histological margin status is always significant in gastric cancer patients undergoing gastrectomy. We think physicians should recommend re-operation especially for early gastric cancer patients with histologically positive margins.

However, another notable finding is that recurrences have not necessarily developed in patients with early gastric cancers diagnosed as having a positive surgical margin histologically. Indeed, some patients with microscopic positive margins showed disease-free survival for more than 5 years after gastrectomy regardless of the stage of disease. Therefore, we should inform patients that approximately 70% of patients diagnosed as having positive margins histologically did not develop recurrences. There are some possible causes of the apparent discrepancy between margin status and prognosis. The first possibility is that a difference between the true surgical margin and diagnostic margin would be responsible for the discrepancy. Recently, stapler instruments have
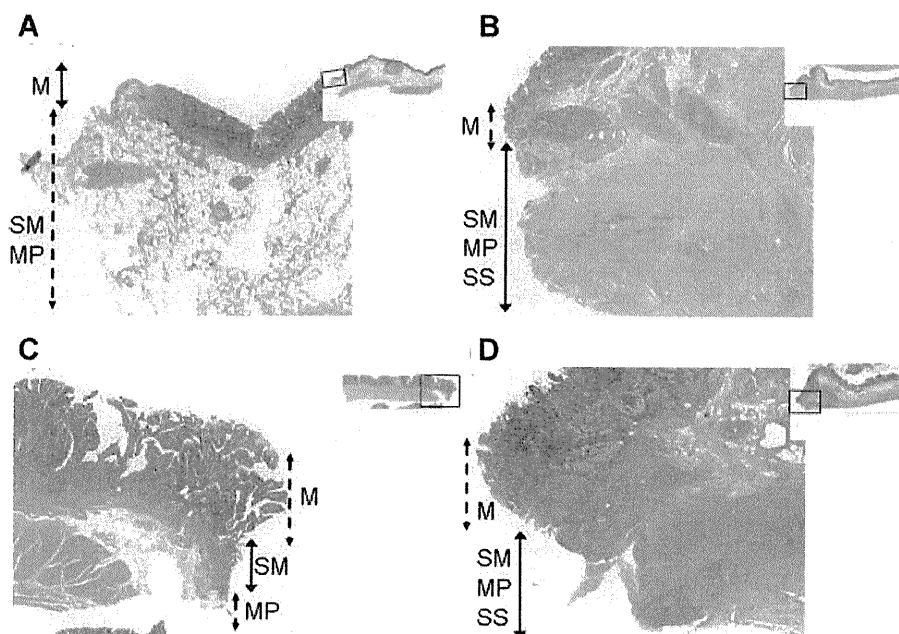

Fig. 1.   Typical microscopic findings of positive margins. Patients with positive margin were divided into subgroup according to the involved depth; a superficial site group (A) versus a deep site group (B). Meanwhile, patients were also divided into subgroup according to the involved number; a single layer group (C) versus multiple layers (D). The layers of positive margin are indicated by solid line. The layers of negative margin are indicated by a dotted line. The magnification was 250.
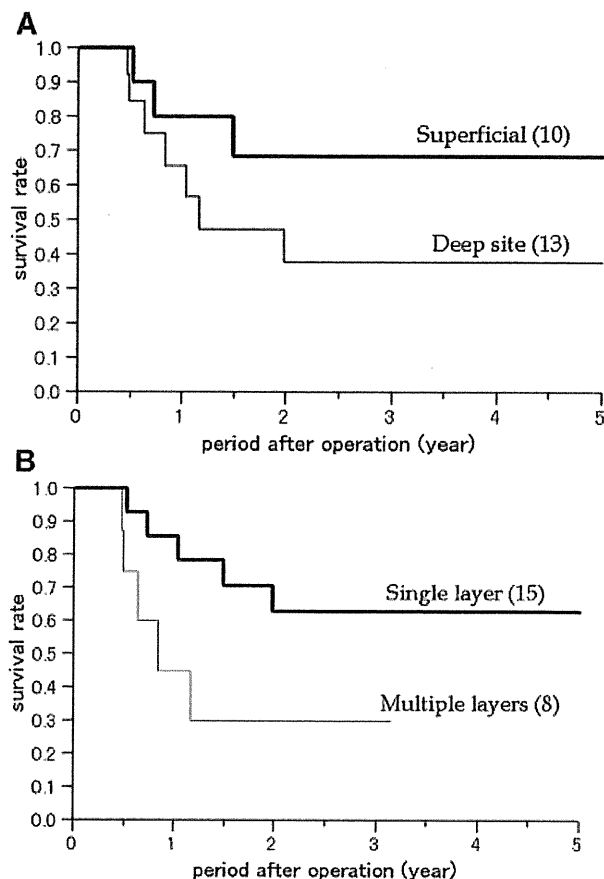
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A

B

Fig. 2. Survival curves of patients with positive surgical margins stratified according to the detailed margin status. **A:** Patients with positive margin in a deep site showed a poorer survival than those with a positive margin in a superficial layer (5-year survival rates: 38% vs. 69%, $P = 0.18$). **B:** Patients with positive margin in multiple layers showed a poorer survival than those with a positive margin in a single layer (5-year survival rates: 30% vs. 63%, $P = 0.067$).

usually been utilized for organ excisions and the stapled lines should be removed before histological examination. Therefore, the margins examined histologically are not exactly same as the true surgical margins. The second possible reason is that the minimal remaining cancer cells were successfully overcome by the patients' own immune system or postoperative non-surgical adjuvant therapies [15]. Another possibility is that the remaining cancer cells exist only within the width of the stapled line in the remnant digestive organs, such as the esophagus, stomach, and/or duodenum, and the natural disappearance of these few cancer cells would result from impaired blood supply.

In order to examine the useful findings of positive margin for predicting the patients' prognosis, we conducted a detailed analysis of microscopic margin status. Then, patients with positive margin in a deep site and/or multiple layers were associated with a worse prognostic outcome. Although there were more than 30% survival difference in the detailed analyses, the differences were not statistically significant. We consider the absence of a significant difference would be partially due to the small number of patients with positive margin in the present study. Therefore, the impact of these microscopic findings in surgical margin would become more remarkable as increasing numbers of patients are accumulated.

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It is not clear whether re-operation should be considered for such patients with histologically positive margin. Previous reports indicated that the gastric cancers that result in R1 resection with microscopic positive margin might tend to have a characteristically aggressive behavior and that distant metastasis was the most common site of recurrence in such patients [16–18]. Although, in the present study, there was no significant correlation between the positive-margin patients and clinicopathological factors indicating general aggressive behavior of tumors, the pN-stage, the degree of lymphatic invasion and also the pT-stage tended to be higher in positive-margin patients than in negative-margin patients. The small numbers of the positive-margin patients might be cause of the no statistical significance in this study. Concerning the type of relapse, maybe if patients with positive margin survived longer after the development of recurrences, some patients could develop not only peritoneal and lymphatic recurrences but also local recurrence in the surgical stump.

In conclusion, microscopic positive margin was found to be an independent prognostic factor in gastric cancer patients. The status of the surgical margin might provide useful information for selecting additional treatments and performing intensive follow-up.

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PLoS one

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.

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
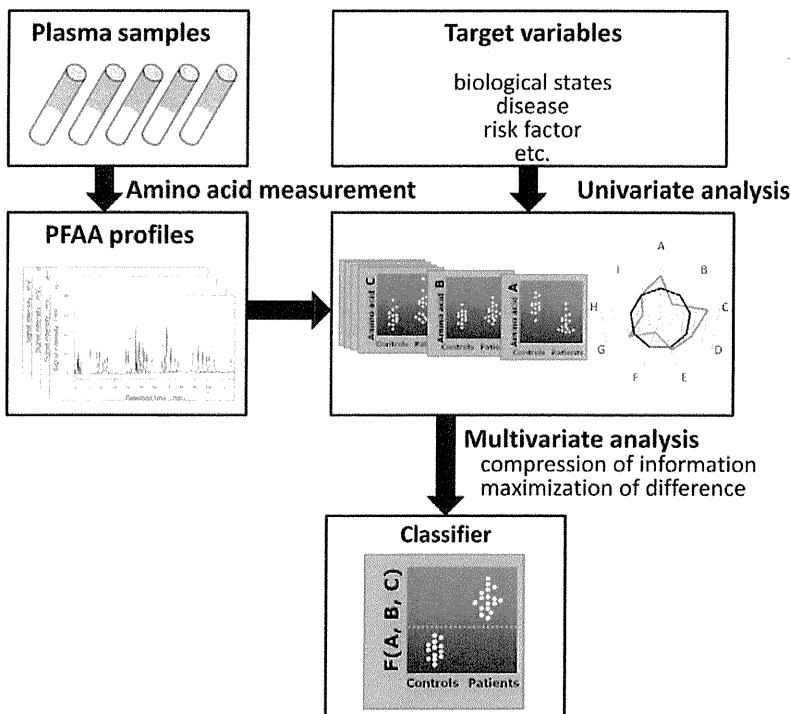

Figure 1. Concept of the generation of "AminoIndex technology". At the top of the diagram, PFAA concentrations are measured for each subject. In the middle, target variables and univariate analysis of PFAA profiles are represented. At the bottom, an estimation of the classifier with optimized discriminating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

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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 $\pm$ 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
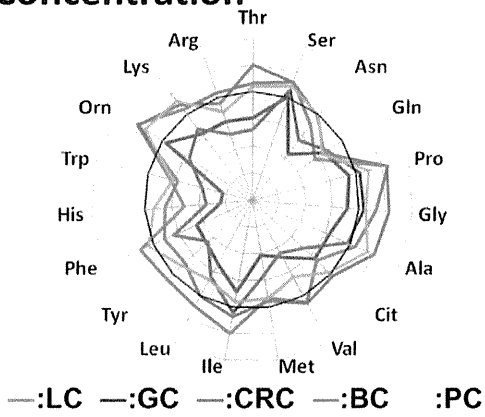
A

Concentration

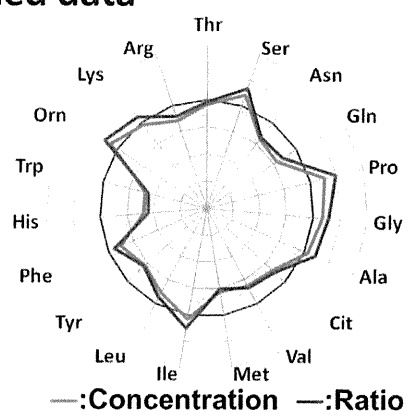| Amino acid	LC	GC	CRC	BC	PC	Pooled
Thr						
Ser						
Asn						
Gln						
Pro						
Gly						
Ala						
Cit						
Val						
Met						
Ile						
Leu						
Tyr						
Phe						
His						
Trp						
Orn						
Lys						
Arg						

Ratio

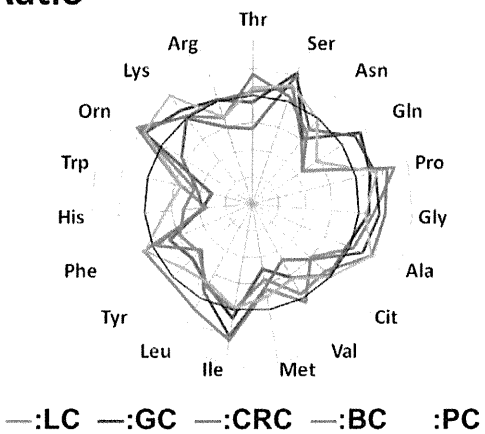| Amino acid	LC	GC	CRC	BC	PC	Pooled
Thr						
Ser						
Asn						
Gln						
Pro						
Gly						
Ala						
Cit						
Val						
Met						
Ile						
Leu						
Tyr						
Phe						
His						
Trp						
Orn						
Lys						
Arg						

B

Concentration

—:LC —:GC —:CRC —:BC :PC

Pooled data

—:Concentration —:Ratio

Ratio

—:LC —:GC —:CRC —:BC :PC
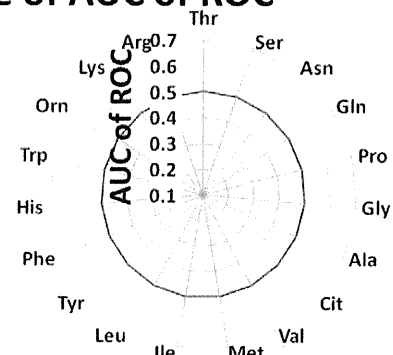
Scale of AUC of ROC

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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.
doi:10.1371/journal.pone.0024143.g002

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].
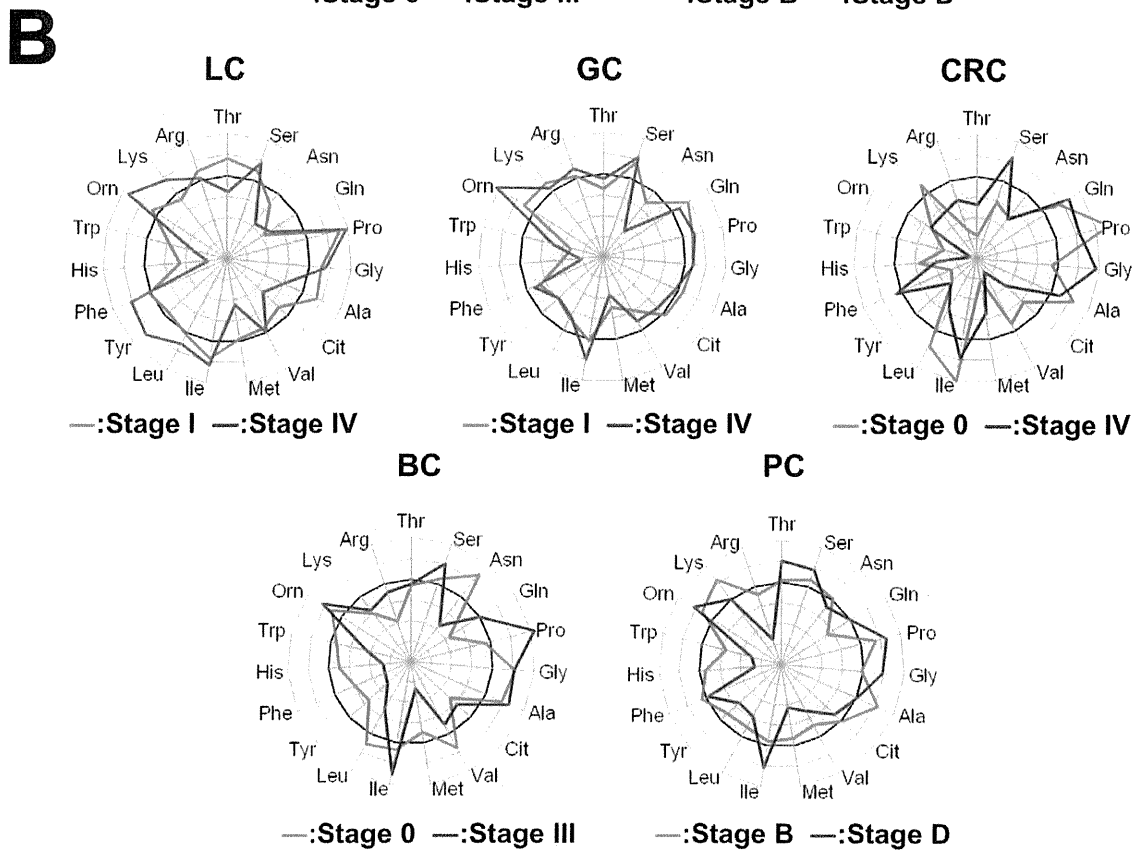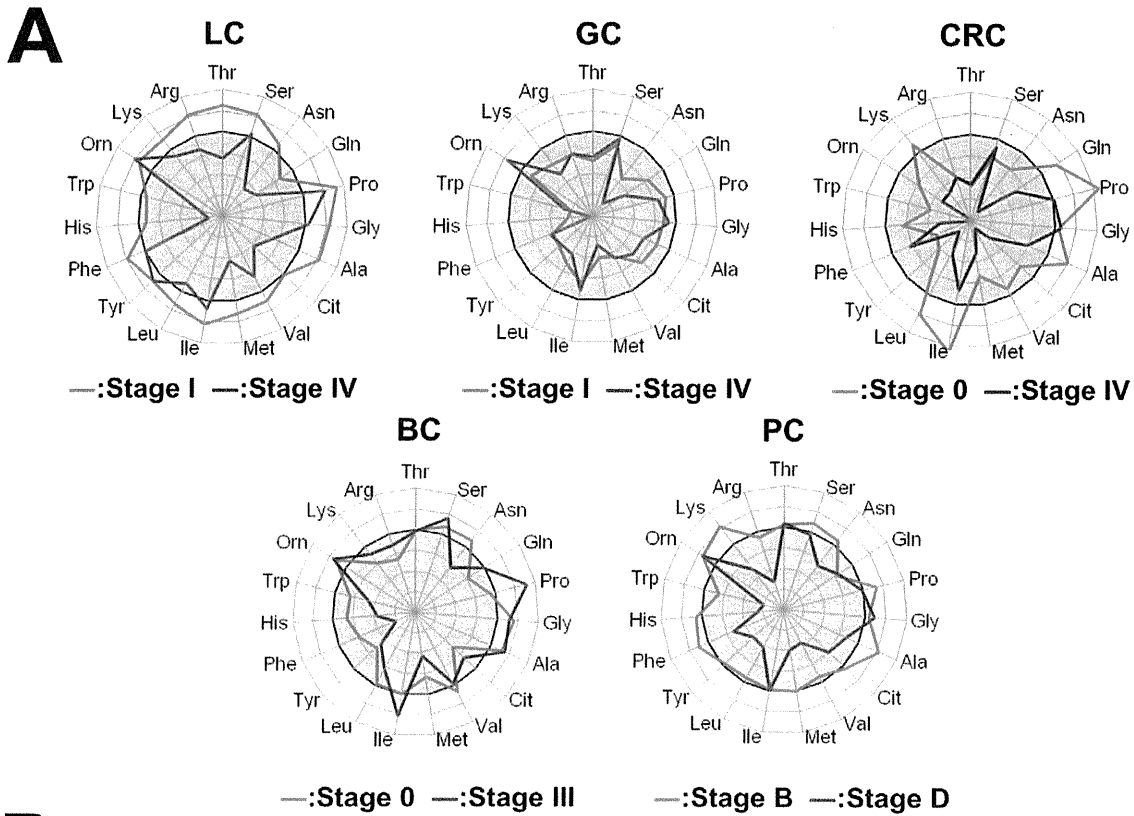
Figure 3. PFAA profiles of early- and advanced-stage cancer patients. The axes show the AUC of ROC for each amino acid for discriminating patients from controls. A. Comparison of concentrations of cancer patients and controls. B. Comparison of ratios of cancer patients and controls. Scale as described 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.
doi:10.1371/journal.pone.0024143.t002

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)

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.
doi:10.1371/journal.pone.0024143.t005

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)

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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