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Abstract. The survival times of patients with advanced colorectal cancer (CRC) have increased due to the introduction of chemotherapy involving irinotecan and cetuximab. However, further studies are required on the effective pretreatment methods for identifying patients with CRC who would respond to particular treatments. The aim of the present study was to identify biomarkers for predicting the efficacy of chemotherapy for CRC. A total of 123 serum samples were collected from 31 patients with CRC just prior to each of the first four rounds of chemotherapy. Serum metabolome analysis was performed using a multiplatform metabolomics system, and univariate Cox regression hazards analysis of the time to disease progression was conducted. Octanoic acid and 1,5-anhydro-D-glucitol were identified as biomarker candidates. In addition, the serum level of octanoic acid was indicated to be significantly associated with the time to disease progression (hazard ratio, 3.3; 95% confidence interval, 1.099-11.840; P=0.033). The serum levels of fatty acids, in particular polyunsaturated fatty acids, tended to be downregulated in the partial response group. The findings of the present study suggest that the serum level of octanoic acid may serve as a useful predictor for the prognosis of CRC.

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Introduction

In Japan, the mortality rate of patients with colorectal cancer (CRC) has been increasing. According to the vital statistics of Japan, in 2015, CRC was the leading cause of cancer-associated mortality in females and the third leading cause of cancer-associated mortality in males (1). The 5-year survival rate of patients with CRC following curative resection is ~80%, but 10-20% of cases are unresectable (2). In the last two decades, the mean survival time of patients with advanced CRC has increased from 1 year to ~2 years due to the introduction of chemotherapy drugs, including irinotecan and oxaliplatin. These therapeutic agents are used in combination with fluorouracil and leucovorin as standard regimens, FOLFILI and FOLFOX4, for advanced CRC (3-5). Recently, targeted biological therapeutic agents have proved to be useful for treating CRC (6,7). Cetuximab, a targeted biological therapeutic agent, has significantly improved the survival times of patients with CRC with metastatic lesions, particularly when the cancer is refractory to the aforementioned anticancer drugs (8). According to the BOND-1 study (9), combination therapy with cetuximab and irinotecan produced a higher response rate compared with cetuximab alone. In addition, cetuximab has the capacity to reverse drug resistance by abrogating drug efflux, restoring apoptosis and impairing DNA repair activity when administered in combination with irinotecan (8,9).

A number of randomized trials have suggested that mutations in the Kirsten rat sarcoma viral oncogene homolog (KRAS) and v-Raf murine sarcoma viral oncogene homolog B (BRAF) genes are negative prognostic predictors among patients with metastatic CRC who are treated with anti-epidermal growth factor receptor (EGFR) antibodies (10-13). The efficacy of KRAS mutations as predictive biomarkers is well established, but the effects of BRAF mutations remain unknown (14). Further investigation is required on the effective pretreatment

methods for identifying patients with CRC that would respond to particular treatments. The ability to predict the efficacy of particular treatments in each patient during the pretreatment period would allow doctors to provide a customized treatment for each patient, which may improve the quality of life and prognosis of the patient. Therefore, it is necessary to identify biomarkers that could be used to predict the efficacy of treatments for CRC.

Metabolomics is the comprehensive study of low molecular weight molecules, known as metabolites, in a particular organism or tissue. Metabolomics is a useful approach to understanding the state of a patient's body, as metabolites and their concentrations directly reflect the underlying biochemical activity and state of cells/tissues. In addition, metabolites represent the endpoint of the omics cascade and therefore can be directly linked to molecular phenotypes (15). Therefore, metabolomics is employed as a tool for discovering candidate biomarkers that may aid disease diagnosis, toxicological testing, prognosis and risk assessment (16-18). We have previously demonstrated the usefulness of metabolomics for facilitating the diagnosis and evaluation of pancreatic cancer and CRC (19-22). However, to the best of our knowledge, a limited number of studies have investigated whether metabolite biomarkers could be used to predict the response to cancer treatment. A previous study involving high-resolution magic angle spinning magnetic resonance spectroscopy indicated that a high concentration of glycine was correlated with poor progression-free survival time in patients with locally advanced rectal cancer (23). The aim of the present study was to use gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/MS (LC/MS) to identify biomarkers that could be used to predict the response to chemotherapy (cetuximab plus irinotecan or cetuximab alone) for CRC. First, biomarker candidates were searched for among serum metabolites by performing a semi-quantitative analysis without the corresponding stable isotopes. A quantitative analysis was subsequently conducted using the corresponding stable isotopes for the selected biomarker candidates and their efficacy was validated.

Materials and methods

Patients and treatment. The present study was conducted as part of a phase II trial of chemotherapy for CRC, a clinical trial of third-line or later treatment for CRC (24), involving cetuximab plus irinotecan or cetuximab alone, performed at the National Cancer Center Hospital (Tokyo, Japan). The present study was approved by the Ethics Committees of the National Cancer Center Hospital and Kobe University Graduate School of Medicine (Kobe, Japan). Written informed consent was obtained from all patients prior to participation in the present study. The patients were recruited from the National Cancer Center Hospital between December 2008 and March 2015. The inclusion criteria were as follows: A histologically confirmed diagnosis of metastatic CRC that was surgically unresectable or had recurred; having received ineffective irinotecan, oxaliplatin or fluoropyrimidine-based chemotherapy; an age of >20 years; an Eastern Cooperative Oncology Group performance status score of 0-2 (25); a life expectancy of at least 2 months; possessing the wild-type

KRAS gene; and having at least one radiologically measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 (26). The present study, as aforementioned, was conducted as an accompaniment to a phase II clinical trial of third-line or later treatment for CRC, and in the event that irinotecan alone was not able to produce sufficient therapeutic effects, cetuximab + irinotecan or cetuximab alone was expected to be effective as a third-line treatment. Therefore, 'having received ineffective irinotecan' was included in the inclusion criteria. In addition, the minimum treatment-free period between the end of the previous therapy and the day of registration was 4, 2 and 4 weeks for patients who had received prior chemotherapy, radiotherapy and major surgery, respectively. The exclusion criteria were as follows: Having received EGFR signal transduction inhibitors or anti-EGFR antibodies; having suffered double cancer within the past 5 years; and were suffering from active inflammation or severe complications, including heart disease, diabetes, and ileus or interstitial lung disease.

A total of 31 patients with CRC, including 21 males and 10 females, participated in the present study (median age, 65; range, 48-79 years). The subjects were allocated to groups A and B. Patients who satisfy the following conditions are regarded as 'Irinotecan unreliable' and were allocated in group B; having advanced peritoneal metastasis, having ascites in the pelvic cavity and upper abdomen within 14 days prior to registration, drainage of ascites is done within 14 days prior to registration. However, patients who did not satisfy the aforementioned conditions were allocated in group A. The patients in group A (n=27), consisting of 19 males and 8 females, were treated with cetuximab and irinotecan, and the patients in group B (n=4), consisting of 2 males and 2 females, were treated with cetuximab alone. The patients received cetuximab on day 1 of a 7-day treatment cycle. Cetuximab was initially administered at a dose of 400 mg/m^2 as a 2-h infusion and was subsequently administered at a weekly dose of 250 mg/m^2 as a 1-h infusion. Irinotecan was administered once every 2 weeks, starting on day 1, at a dose of 150 mg/m^2 as a 90 min infusion. Irinotecan administered consecutively with cetuximab (Fig. 1). The aforementioned treatments were continued until disease progression or unacceptable toxicities occurred. Responses were evaluated using computed tomography (CT) scans obtained every 8 weeks for each patient and measuring the lesions using the RECIST version 1.0 criteria.

Serum samples. Serum samples were collected from all patients prior to each of the first four rounds of chemotherapy (days 1, 8, 15 and 22) at the National Cancer Center Hospital (Fig. 1) and were used for the metabolome analysis. At 30 min post-blood sampling, the samples were centrifuged at 1,500 x g for 10 min at 4˚C. Next, the obtained serum was immediately transferred to a clean tube and stored at -80˚C until it was used. The serum samples were subjected to analysis by GC/MS and LC/MS.

Chemicals and isotopes. [U-^{13}C$_6$ (98%)]-labeled 1,5-Anhydro-D-glucitol, ([U-^{13}C$_6$ (98%)]-labeled 1,5-AG) and [1-^{13}C]- labeled Octanoic acid were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Octanoic acid-1-^{13}C, 2-isopropylmalic acid (as an internal
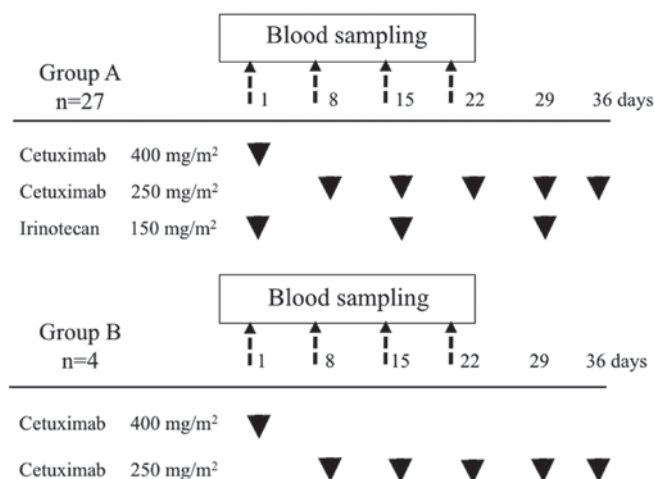
Figure 1. Chemotherapy protocols. The patients were allocated to group A or group B. The patients in group A (n=27) were treated with cetuximab and irinotecan. The patients in group B (n=4) were treated with cetuximab alone.

standard), 2-bromohypoxanthine (as an internal standard) and 10-camphorsulfonic acid (as an internal standard) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). 1,5-AG and octanoic acid were acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dilauroylphosphatidylcholine (PC12:0/12:0) (as an internal standard) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

GC/MS procedures. The GC/MS extraction and derivatization procedures were performed according to the methods described in our previous studies (20-22,27). A total of 20 μl of each sample was dispensed into a 1.5-ml Eppendorf tube. The samples were then extracted with 250 μl methanol with or without the corresponding stable isotopes, prior to being shaken in a vortex mixer. A total of 10 μl 2-isopropylmalic acid (0.5 mg/ml) was added as an internal standard. The mixture was incubated at 1,200 rpm for 30 min at 37°C. The mixture was centrifuged at 19,300 x g for 5 min at 4°C and 200 μl supernatant was transferred to a new Eppendorf tube capped with a pierced cap. Subsequent to being centrifuged for 30 min in a vacuum concentrator (Thermo SpeedVac; Thermo Fisher Scientific, Inc., Waltham, MA, USA), the mixture was freeze-dried overnight. For the derivatization, 80 μl methoxamine hydrochloride in pyrimidine (20 mg/ml) were added as the first derivatizing agent. The mixture was subsequently incubated at 1,200 rpm for 90 min at 30°C. The second derivatizing agent, 40 μl N-methyl-N-(trimethylsilyl) trifluoroacetamide, was added and the mixture was incubated at 1,200 rpm for 30 min at 37°C. The mixture was centrifuged at 19,300 x g for 5 min at 25°C, and the supernatant was transferred to a vial for analysis by GC/MS. The GC/MS analysis was performed on a GCMS-TQ8040 GC-MS/MS system (Shimadzu Corporation, Kyoto, Japan) with a fused silica capillary column (BPX5; inner diameter: 30 m x 0.25 mm; film thickness, 0.25 μm; SGE Analytical Science, Melbourne, Australia). The data processing was performed according to previously described methods (21,22,27). The MS data were exported to a personal computer, on which the GCMS solution software ver. 4.30 (Shimadzu Corporation) had been

installed, and the peaks of the targeted metabolites and the corresponding stable isotopes were detected by the software and subsequently checked manually. According to the methods described in our previous study (22), quality control plasma samples were prepared to ensure the reproducibility of the acquired metabolomic data. In the analysis of the quality control plasma samples, coefficients of variation (CV) for the ratio of the peak area value for each metabolite to that for the internal standard were calculated, following the exclusion of metabolites with a CV >30%. In the semi-quantitative analysis, the peak intensity of each metabolite was normalized to that of the internal standard. In the GC/MS-based quantitative analysis of the targeted metabolites (octanoic acid and 1,5-AG), the peak area of each metabolite was corrected using the data for the corresponding stable isotope ([U-^{13}C$_6$ (98%)]-labeled 1,5-AG and [1-^{13}C]-labeled Octanoic acid), and quantitation was conducted based on multi-point calibration curves.

LC/MS procedures. For the LC/MS-based hydrophilic metabolite analysis, 20 μl of each sample was mixed with 900 μl of a solvent mixture (H$_2$O:CHCl$_3$:methanol, 1:1:2.5) containing 1 mM 2-bromohypoxanthine and 1 mM 10-camphorsulfonic acid as internal standards. The mixture was agitated at 1,400 rpm for 30 min at 37°C. Subsequently, 630 μl of the supernatant was transferred to a new Eppendorf tube, and 280 μl distilled water was added to the tube. Subsequent to being mixed, the mixture was centrifuged at 16,000 x g for 5 min at 4°C, and 500 μl of the resultant supernatant was passed through an ultrafiltration filter, prior to being centrifuged at 14,000 x g for 60 min at 4°C. The collected solution was dried via centrifugal concentration and lyophilization, and reconstituted with 100 μl water. The resultant supernatant was subjected to analysis. For the LC/MS-based lipid analysis, 10 μl of each sample was mixed with 80 μl methanol and 10 μl 500 ppb PC12:0/12:0 dissolved in methanol as an internal standard. The mixture was put on ice for 10 min. Following centrifugation at 16,000 x g for 5 min at 4°C, 60 μl of the supernatant was transferred to a new Eppendorf tube and subjected to analysis.

According to the method described in our previous studies (20,27), the LC/MS analysis was performed using a Nexera LC system (Shimadzu Corporation) equipped with two LC-30AD autosamplers, a CTO-20AC column oven and a CBM-20A control module, coupled with an LCMS-8040 triple quadruple mass spectrometer (Shimadzu Corporation). The analytical conditions of LC/MS were as follows: Electrospray voltage: 3.5 kV, -3.5 kV; Curve desolvation line (CDL): 250°C; Heat block temperature: 400°C; Nebulizing gas (N$_2$): 3.0 l/min; Drying gas pressure: 15 l/min; CID gas (Argon) pressure: 0.23 MPa. The cationic hydrophilic metabolites were separated using a pentafluorophenyl column (Discovery HS F5; 150x2.1 μm; 3 μm; Supelco, Inc., Bellefonte, PA, USA) with a guard column (20x2.1 μm; 3 μm; GL Sciences, Inc., Tokyo, Japan), while the anionic hydrophilic metabolites were separated using an octadecylsilylated silica column (InertSustain C18; 150x2.1 μm; 3 μm; GL Sciences, Inc., Tokyo, Japan). The mobile phase used for the analysis of the cationic metabolites was composed of A: 0.1% formic acid in water and B: 100% Acetonitrile. The flow rate was 0.3 ml/min, and the column oven temperature was set at 40°C. The gradient

Table I. Patient characteristics and evaluation of the response to chemotherapy.

Variables	PR	Non-PR	P-value
Total, n	7	24	
Sex, n			0.784[a]
Male	5	16	
Female	2	8	
Median age (range), years	67.0 (48-77)	64.5 (51-79)	0.607[b]
PS, n			0.664[a]
0	2	9	
1	5	15	
BMI	21.2	21.6	0.499 [b]
Group, n[c]			0.247[a]
A	7	20	
B	0	4	
OS, days	433	336	0.028[d]
PFS, days	253	165.5	0.043[d]
TTP, days	162	102.5	0.047[d]

[a]P-values were calculated using Pearson's χ^2 test. [b]P-values were calculated using the Student's t-test. [c]Group A, cetuximab plus irinotecan; Group B, cetuximab alone. [d]P-values were calculated using the log-rank test. PR, partial response; Non-PR, non-partial response; OS, overall survival; PFS, progression-free survival; TTP, time to progression; BMI, body mass index; PS, performance status score.

program for mobile phase B was as follows: 0 min, 0%; 7 min, 0%; 20 min, 40%; 20.1 min, 100%; 25 min, 100%; 25.1 min, 0%; and 35 min, 0%. The mobile phase used for the analysis of the anionic metabolites was composed of A: Water containing 15 mM acetic acid and 10 mM tributylamine, and B: 100% Methanol. The flow rate was 0.3 ml/min, and the column oven temperature was set at 35˚C. The gradient program for mobile phase B was as follows: 0 min, 0%; 0.5 min, 0%; 20 min, 75%; 20.1 min, 98%; 24 min, 98%; 24.1 min, 0%; and 30 min, 0% (28). The m/z value and retention time for each metabolite are listed in our previous studies (28). Lipids were separated using an octadecylsilylated silica column (InertSustain C18, 100x2.1 μm; 3 μm; GL Sciences, Inc.) with a guard column (10x3 μm; 5 μm; GL Sciences). The mobile phase used for the lipid analysis consisted of A: 20 mM ammonium acetate in water and B: 100% Methanol. The flow rate was 0.4 ml/min, and the column oven temperature was set at 40˚C. The gradient program for mobile phase B was as follows: 0 min, 80%; 13 min, 98%; 30 min, 98%; 30.1 min, 80%; and 35 min, 80%. The aforementioned metabolites were mainly analyzed based on their physicochemical properties and/or spectral similarity with the molecules included in public/commercial spectral libraries (putative annotation), which are shown in our previous studies (28), as chemical reference standards could not be obtained.

Statistical analysis. Data are presented as mean ± standard deviation. Univariate analyses were performed by conducting comparisons of treatment efficacy using the Mann-Whitney U test. Pearson's χ^2 test was used to evaluate the differences between groups for categorized variables. The unpaired Student's t-test was used to measure the means and

relationships of continuous variables. Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic performance of the targeted metabolites based on their area under the curve (AUC), sensitivity and specificity values. The optimal cut-off values for the selected serum metabolites were determined from their ROC curves. The Youden index was used to determine the optimal cut-off values for each serum metabolite (29). In univariate analysis of the selected serum metabolites, the likelihood ratio test was used to evaluate P-values. The time to progression (TTP) was analyzed using Cox proportional hazards regression analysis and the Kaplan-Meier method. TTP estimates were compared using the log-rank test. The patients who were lost to follow-up were evaluated using the data at the date of the last follow-up. P<0.05 was considered to indicate a statistically significant difference. Kaplan-Meier curves were produced using the package 'survival' for R. The log-rank test was performed using the statistical software R ver. 1.37 (http://www.jichi. ac.jp/saitama-sct/SaitamaHP.files/statmedOSX.html) (27,30). The other analyses were performed using the default conditions of JMP 11 (SAS Institute, Inc., Cary, NC, USA).

Results

Patient characteristics and serum samples. A total of 31 patients with CRC participated in the present study, and all were treated with chemotherapy, namely cetuximab plus irinotecan or cetuximab alone. The median duration of the follow-up period was 781.5 days, and the median survival time was 390 days. The median TTP was 152 days. In total, 123 serum samples were collected from the patients prior to the first to fourth rounds of treatment (days 1, 8, 15 and 22)

Table II. Quantitative analysis of the targeted metabolites using gas chromatography/mass spectrometry.

Metabolite	PR (n=7)		Non-PR (n=24)		Fold-change[a]	P-value[b]
	Mean, μM	SD	Mean, μM	SD		
Octanoic acid	4.09	0.82	5.06	1.07	0.81	0.043
1,5-AG	155.78	48.42	106.78	34.25	1.46	0.026

[a]The fold-change values were calculated based on the ratio of PR to non-PR. [b]The P-values were calculated using the Mann-Whitney U-test. PR, partial response; SD, standard deviation; 1,5-AG, 1,5-anhydro-D-glucitol.

(Fig. 1). One patient succumbed prior to providing a fourth sample.

Evaluation of treatment responses GC/MS. The therapeutic responses of the subjects are summarized in Table I. The overall survival period, progression-free survival and time to progression differed significantly between the partial response (PR) and non-partial response (non-PR) groups (P=0.028, P=0.043 and P=0.047, respectively), whereas there were no significant differences observed with regard to gender, age and body mass index. None of the patients achieved a complete response. A total of 7 patients (22.6%) achieved a PR, while 14 patients (45.2%) experienced stable disease and 9 patients (29.0%) experienced progressive disease. Only 1 patient (3.2%) could not be evaluated due to mortality prior to the first CT scan. The aim of the present study was to compare the serum levels of metabolites between the PR and non-PR groups. Therefore, the patient that could not be evaluated was included in the non-PR group, and overall, a total of 24 patients (77.4%) were classed as non-PR.

In the GC/MS-based semi-quantitative analysis, a total of 111 metabolites were detected in the serum samples. The serum levels of each metabolite were compared between the PR and non-PR groups using the Mann-Whitney U test to determine their associations with the therapeutic response. The serum levels of two metabolites, including octanoic acid and 1,5-AG, exhibited significant intergroup differences [octanoic acid; P=0.030, 1,5-AG; P=0.006, data not shown] prior to the first round of treatment.

To obtain quantitative data, a targeted metabolome analysis was performed using the corresponding stable isotopes. Stable isotope labeling can be used to achieve a more accurate quantification (31). The serum levels of octanoic acid and 1,5-AG, which were analyzed, along with their respective results are indicated in Table II. Box plots of the serum levels of these two metabolites in the PR and non-PR groups are presented in Fig. 2. In the quantitative analysis, it was indicated that the PR group exhibited significantly lower serum levels of octanoic acid compared with the non-PR group (P=0.043). By contrast, the serum level of 1,5-AG was significantly higher in the PR group compared with the non-PR group (P=0.026; Table II).

To evaluate the predictive accuracy of serum metabolite levels for predicting the efficacy of treatment for CRC, the AUC, sensitivity and specificity values were calculated for octanoic acid and 1,5-AG via ROC analysis. The optimal cut-off values for each serum metabolite were determined using the Youden index. The sensitivity, specificity and AUC
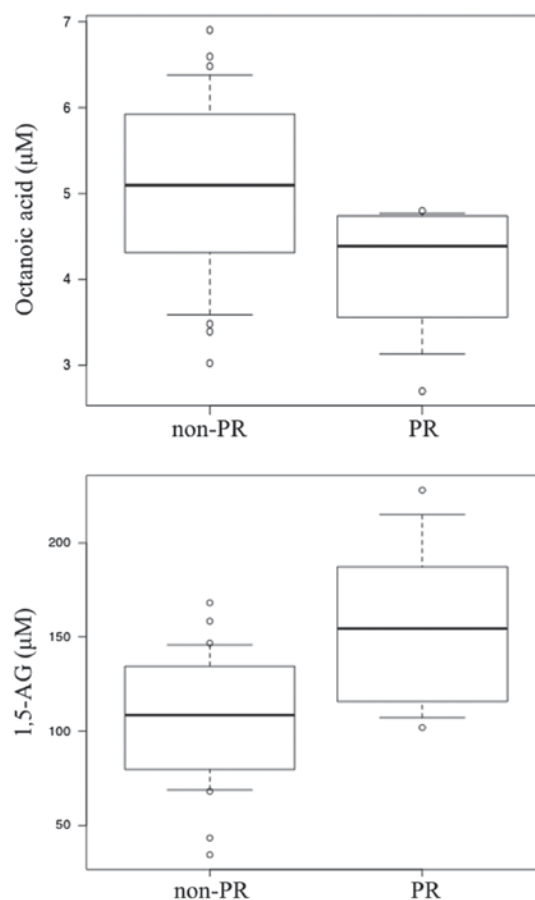

Figure 2. Box plots of the targeted metabolites. The box plots indicate the serum levels of the targeted metabolites, octanoic acid and 1,5-AG in the PR and non-PR groups. The boxes represent interquartile ranges. The horizontal bar within each box indicates the median value. The upper horizontal line of each box indicates the 75th percentile, and the lower horizontal line indicates the 25th percentile. The upper horizontal bar above the box indicates the 90th percentile, and the horizontal bar below the box indicates the 10th percentile. The circles represent data points beyond the 10th and 90th percentiles. 1,5-AG, 1,5-anhydro-D-glucitol; PR, partial response.

of octanoic acid were 100%, 62.5%, and 0.77 μM, respectively. The sensitivity, specificity and AUC of 1,5-AG were 57.1%, 91.7%, and 0.78 μM, respectively. Therefore, octanoic acid and 1,5-AG exhibited moderate predictive accuracy (Table III). TTP was subsequently analyzed to evaluate the prognosis of the subjects. Disease progression was observed in 14 of the 31 patients during the observation period, and the median TTP was 162 days in the PR group and 102.5 days in the non-PR group (Table I).

Table III. Predictive accuracy of the targeted metabolites.

Metabolite	AUC (95% CI)	Sensitivity, %	Specificity, %	Cut-off value, μM[a]
Octanoic acid	0.77 (0.59-0.95)	100.0	62.5	4.8
1,5-AG	0.78 (0.57-0.99)	57.1	91.7	154.5

[a]Cut-off values were calculated based on the highest Youden index value. AUC, area under the curve; 95% CI, 95% confidence interval; 1,5-AG, 1,5-anhydro-D-glucitol.

Table IV. Cox proportional hazards regression analysis of the associations between serum metabolite concentrations and the time to progression.

Metabolite	Cut-off value, μM	Univariate analysis		
		Hazard ratio	95% CI	P-value[a]
Octanoic acid	≤4.80/>4.80	3.3	1.10-11.84	0.0325
1,5-AG	≤154.5/>154.5	1.1	0.35-4.92	0.869

[a]P-values were calculated using the likelihood ratio test. 95% CI, 95% confidence interval; 1,5-AG, 1,5-anhydro-D-glucitol.

Cox proportional hazards regression analysis was performed using the serum levels of octanoic acid and 1,5-AG, whose optimal cut-off values were determined from their ROC curves. Only the serum level of octanoic acid was indicated to be significantly associated with the TTP in the univariate analyses [≤4.80/>4.80 μM; hazard ratio, 3.3; 95% confidence interval (CI), 1.1-12; P=0.033; Table IV). Kaplan-Meier analysis indicated that the patients with lower serum octanoic acid levels achieved better prognoses compared with those with higher serum octanoic acid levels (Fig. 3). By contrast, the serum level of 1,5-AG was not indicated to be significantly associated with the TTP in the univariate analyses (≤154.5/>154.5 μM; hazard ratio, 1.1; 95% CI, 0.35-4.92; P=0.87; Table IV).

Furthermore, the changes in the levels of the targeted metabolites in the serum samples that were obtained from the first to the fourth round of treatment are indicated in Table V. The concentration of octanoic acid remained almost the same from prior to the first round of treatment to the fourth round of treatment in the PR and non-PR groups. By contrast, the concentration of 1,5-AG was higher in the PR group compared with that in the non-PR group throughout the treatment period, although its concentration in the PR group gradually decreased. Although the serum levels of the metabolites differed significantly between the groups prior to treatment, none of the metabolites exhibited significant intergroup differences in their serum levels following treatment.

Evaluation of treatment responses: LC/MS. A total of 200, 28 and 21 metabolites were detected in the serum samples during the LC/MS-based semi-quantitative lipid, anion and cation analyses, respectively. There were a number of metabolites whose serum levels differed significantly between the PR and non-PR groups prior to the first round of therapy;

18 of these metabolites were detected in the LC/MS-based lipid analysis, and 1 metabolite, glutaconic acid, was detected in the LC/MS-based anion analysis (Table VI). By contrast, none of the metabolites detected during the LC/MS-based cation analysis exhibited significant pretreatment intergroup differences in their serum levels. A quantitative analysis of the serum levels of the 18 metabolites detected in the LC/MS-based lipid analysis or the metabolite (glutaconic acid) detected in the LC/MS-based anion analysis could not be performed, as it was difficult to obtain corresponding stable isotopes. The association between the therapeutic response and the serum levels of lipid species was examined. The resultant volcano plot of all of the lipid species detected in the present study indicated that the serum levels of fatty acids tended to be downregulated following treatment (Fig. 4), although the serum levels of a number of the other aforementioned lipid metabolites did not change significantly. This tendency was particularly pronounced for unsaturated fatty acids, polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (Table VII). In the LC/MS-based lipid analysis conducted in the present study, free PUFAs were detected in the sera, and the serum levels of unsaturated fatty acids, in particular PUFAs, were higher in the non-PR group compared with those in the PR group. In fact, greater therapeutic effects tended to be observed in the patients with lower serum PUFA levels.

Discussion

Using metabolomics, the present study aimed to identify serum biomarkers that could be used to predict the therapeutic efficacy of chemotherapy for CRC during the pretreatment period. Metabolomics is a useful technique for identifying metabolites that are directly linked to certain molecular

Table V. Concentrations of the targeted metabolites according to quantitative analysis.

A, Octanoic acid

Groups	1st round[a]	2nd round	3rd round	4th round
PR	4.09 (1.00)	4.79 (1.03)	4.44 (0.96)	5.27 (1.14)
Non-PR	5.06 (1.00)	5.23 (1.03)	5.22 (1.03)	4.92 (0.97)
P-value[b]	0.043	0.391	0.189	0.901

B, 1,5-AG

Groups	1st round[a]	2nd round	3rd round	4th round
PR	155.78 (1.00)	145.09 (0.93)	116.24 (0.75)	117.05 (0.75)
Non-PR	106.78 (1.00)	106.44 (1.00)	108.81 (1.02)	101.58 (0.95)
P-value[b]	0.026	0.084	0.678	0.469

Concentrations, in μM, were determined prior to the respective rounds of chemotherapy. [a]Numbers in parentheses are the ratios against the concentrations of the 1st round in each group. [b]P-values between PR and non-PR groups were calculated using the Mann-Whitney U-test. PR, partial response; 1,5-AG, 1,5-anhydro-D-glucitol.
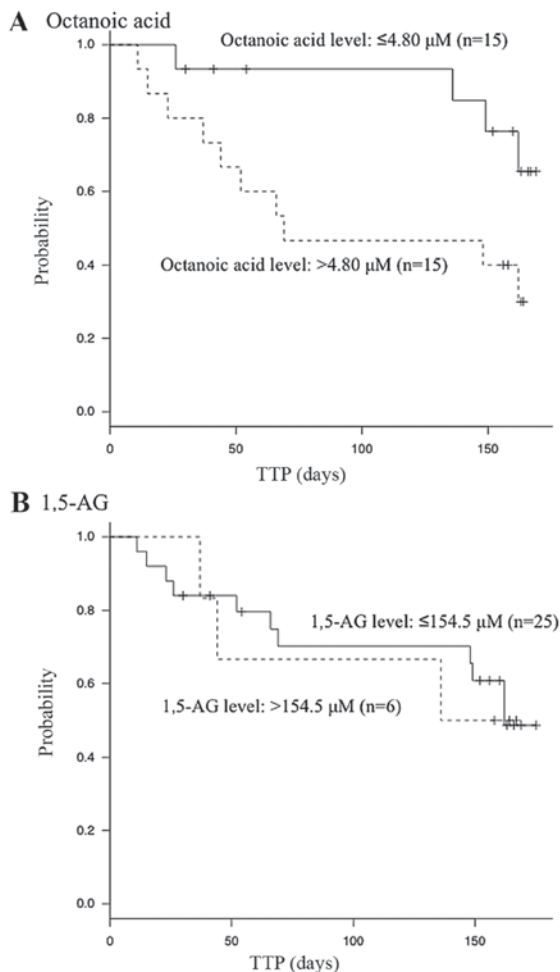

Figure 3. Kaplan-Meier curves of TTP based on the serum concentrations of the targeted metabolites. (A) Patients with lower serum octanoic acid levels, ≤4.80 μM, exhibited longer TTP compared with patients with serum octanoic acid levels of >4.80 μM. (B) The analysis of 1,5-AG did not detect a significant intergroup difference. 1,5-AG, 1,5-anhydro-D-glucitol; TTP, time to progression.

phenotypes. Therefore, the serum levels of metabolites were analyzed using GC/MS and LC/MS. In the semi-quantitative analysis, two metabolites, octanoic acid and 1,5-AG, were identified as biomarker candidates that could be useful for predicting the therapeutic efficacy of chemotherapy for CRC. In the quantitative analysis using the corresponding stable isotopes, it was indicated that the pretreatment serum levels of these metabolites differed significantly between the PR and non-PR groups. However, only the serum level of octanoic acid was significantly associated with the TTP, and the patients with lower serum octanoic acid levels exhibited good prognoses.

Narayanan *et al* (32) indicated that caprylic acid had inhibitory effects on the viability of colorectal, skin and breast cancer cells *in vitro*. In addition, the study reported that caprylic acid downregulated the expression of genes that are important for cell cycle division and progression in colon cancer cells, including cyclin-dependent kinase 2 (CDK2), CDK4, cyclin A2 (CCNA2) and CCND1. In another study, cetuximab increased the expression levels of the CDK inhibitors p21 and p27, and decreased the expression level of cyclin D1 by arresting the cell cycle in the G_1/G_0 phase (33). Based on the aforementioned findings, octanoic acid may have synergistic effects when administered in combination with cetuximab. The present study demonstrated that patients with lower serum levels of octanoic acid exhibited better prognoses compared with patients with higher serum octanoic acid levels. Yamasaki *et al* (34) indicated that 3 mM octanoic acid reduced bladder cancer cell proliferation, but did not inhibit cell migration and invasion. There have been numerous studies on biomarkers of CRC involving metabolomic analysis (35,36); however, to the best of our knowledge, there is a limited number of studies on octanoic acid as a biomarker candidate or on the association between the levels of octanoic acid and the prognosis of CRC. Uchiyama *et al* (37) performed serum metabolomic analysis using capillary electrophoresis-time-of-flight MS in order to identify biomarkers that may be used

Table VI. The significantly-altered metabolites in LC/MS-based semi-quantitative lipid and anion analysis.

Metabolomics	PR (n=7)		Non-PR (n=24)		Fold-change[a]	P-value[b]
	Mean	SD	Mean	SD		
Lipid analysis						
Fatty acids						
18:1 (n-9)_trans-elaidic acid and (n-7)_trans-vaccenic acid	0.1611	0.0354	0.1334	0.0991	1.208	0.042
18:2 (n-6)_linoleic acid	39.2849	3.5305	44.5618	6.6877	0.882	0.047
20:2 (n-6)_cis-11-14-eicosadienoic acid	0.8759	0.4409	1.8429	1.3528	0.475	0.026
20:4 (n-6)_arachidonic acid	0.0097	0.0031	0.0271	0.0206	0.356	0.021
22:4 (n-6)_docosatetraenoic acid	0.0331	0.0106	0.0846	0.0647	0.391	0.003
20:1 (n-9)_cis-11-eicosenoic acid	3.1298	1.4758	6.8503	5.5053	0.457	0.016
24:1 (n-9)_nervonic acid	0.1312	0.0615	0.2411	0.1377	0.544	0.034
26:0_cerotic acid	0.0815	0.0198	0.1228	0.0408	0.663	0.034
Phosphatidylcholines						
PC (16:0e/18:2)	0.0074	0.0058	0.0159	0.0112	0.465	0.023
PC (16:0/20:4) & PC (16:1/20:3)	7.1136	0.9062	6.4849	2.4726	1.097	0.023
PC (17:0/16:0) & PC (18:0/15:0)	3.6935	1.0740	2.6142	0.9398	1.413	0.030
PC (18:0/20:4)	0.6628	0.0808	0.5768	0.2268	1.149	0.042
PC (18:1e/18:2)	0.2680	0.0849	0.3612	0.1006	0.742	0.026
PC (18:1e/16:0) & PC (18:0e/16:1)	2.8203	0.7449	2.1057	0.7165	1.339	0.042
Phosphatidylethanolamines						
PE (16:0/20:4)	0.1779	0.0691	0.2674	0.0946	0.665	0.005
PE (18:0/20:4)	0.1160	0.0456	0.3105	0.2602	0.374	0.034
PE (20:0/18:2)	51.0289	5.6977	58.8697	8.3221	0.867	0.030
PE (20:1/18:2)	0.0185	0.0041	0.0327	0.0155	0.567	0.014
Anion analysis						
Glutaconic acid	0.1467	0.0618	0.0941	0.0460	1.559	0.047

[a]The fold-change values were calculated based on the ratio of PR to non-PR. [b]The P-values between PR and non-PR groups were calculated using the Mann-Whitney U-test. PR, partial response; Non-PR, non-partial response; SD, standard deviation; FA, fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines.
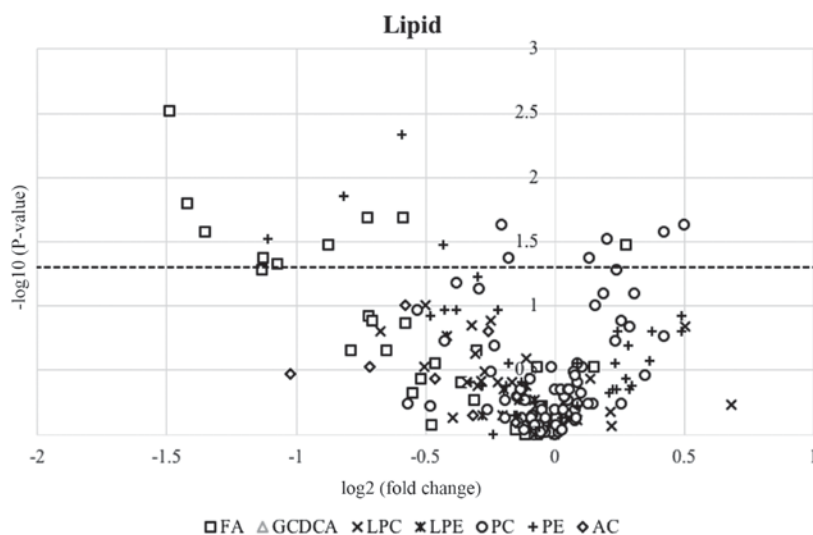

Figure 4. Volcano plot analysis of the lipid species. X axis, the PR to non-PR ratio on a log2 scale; Y axis, -log10 (P-value). The P-values were calculated using the Mann-Whitney U-test and the horizontal dashed line represents a P-value of 0.05. AC, acylcarnitines; FA, fatty acids; GCDCA, glycochenodeoxycholic acid; LPC lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PR, partial response.

Table VII. Detailed results of the LC/MS-based semi-quantitative analysis of serum fatty acids.

Serum fatty acids	PR (n=7)		Non-PR (n=24)		Fold-change[a]	P-value[b]
	Mean	SD	Mean	SD		
Saturated fatty acids						
12:0_lauric acid	0.085	0.035	0.088	0.037	0.97	0.60
14:0_myristic acid	0.223	0.084	0.277	0.148	0.81	0.54
15:0_pentadecylic acid	0.024	0.010	0.034	0.023	0.70	0.37
16:0_palmitic acid	3.736	1.627	5.473	4.126	0.68	0.48
17:0_margaric acid	0.047	0.015	0.052	0.031	0.90	0.92
18:0_stearic acid	3.294	0.816	4.238	2.306	0.78	0.39
20:0_arachidic acid	0.076	0.026	0.080	0.033	0.95	1.00
21:0_heneicosanoic acid	0.026	0.015	0.027	0.012	0.96	0.92
22:0_behenic acid	0.098	0.020	0.106	0.050	0.92	1.00
23:0_tricosanoic acid	0.061	0.023	0.055	0.038	1.11	0.30
24:0_lignoceric acid	0.209	0.037	0.219	0.140	0.95	0.30
25:0_pentacosanoic acid	0.072	0.048	0.100	0.182	0.72	0.85
26:0_cerotic acid	0.161	0.035	0.133	0.099	1.21	0.03
27:0_heptacosanoic acid	0.024	0.016	0.024	0.019	0.98	0.92
Polyunsaturated fatty acids						
18:2 (n-6)_linoleic acid	0.876	0.441	1.843	1.353	0.48	0.05
20:2 (n-6)_cis-11-14-eicosadienoic acid	0.033	0.011	0.085	0.065	0.39	0.03
20:3 (n-6)_dihomo-γ-linolenic acid and (n-9)_mead acid	0.036	0.016	0.054	0.030	0.67	0.13
20:4 (n-6)_arachidonic acid	0.178	0.069	0.267	0.095	0.67	0.02
22:4 (n-6)_docosatetraenoic acid	0.010	0.003	0.027	0.021	0.36	0.00
22:5 (n-6)_docosapentaenoic acid	0.099	0.074	0.162	0.128	0.61	0.13
18:3 (n-3)_α-linolenic acid and (n-6)_γ-linolenic acid	0.125	0.093	0.173	0.096	0.73	0.28
20:5 (n-3)_eicosapentaenoic acid	0.051	0.033	0.063	0.027	0.81	0.22
22:6 (n-3)_docosahexaenoic acid	0.122	0.066	0.203	0.093	0.60	0.02
Monounsaturated fatty acids						
14:1 (n-5)_myristoleic acid	0.032	0.020	0.054	0.036	0.61	0.12
16:1 (n-7)_palmitoleic acid	0.344	0.171	0.595	0.466	0.58	0.22
17:1 (n-7)_cis-10-heptadecanoic acid	0.085	0.045	0.134	0.096	0.64	0.22
18:1 (n-9)_cis-oleic acid and (n-7)_cis-vaccenic acid	1.305	0.603	2.867	2.314	0.46	0.05
18:1 (n-9)_trans-elaidic acid and (n-7)_trans-vaccenic acid	3.130	1.476	6.850	5.505	0.46	0.04
20:1 (n-9)_cis-11-eicosenoic acid	0.116	0.046	0.311	0.260	0.37	0.02
24:1 (n-9)_nervonic acid	0.131	0.061	0.241	0.138	0.54	0.03

[a]Fold-change values were calculated based on the ratio of PR to non-PR. [b]P-values between PR and non-PR groups were calculated using the Mann-Whitney U-test. PR, partial response; SD, standard deviation.

for the early detection of CRC. As a result, it was indicated that the serum level of octanoic acid increased with the clinical stage of CRC, but the diagnostic power of octanoic acid alone for detecting cancer did not suffice. Conversely, it was demonstrated that the serum level of octanoic acid was higher in patients with colorectal adenoma compared with that in the controls, so octanoic acid may be a useful biomarker for diagnosing adenoma. Octanoic acid is a dietary metabolite. Jansen et al (38) reported that the intake of full fat products,

which contain saturated fatty acids, including octanoic acid, increases the risk of pancreatic cancer in a dose-dependent manner. However, to the best of our knowledge, no studies examining the influence of full fat products or octanoic acid on the risk of CRC have been reported, and therefore, further investigation is required.

1,5-AG is a non-metabolizable glucose analogue that is found in plasma (39). The plasma 1,5-AG level has been proposed as a marker of glycemic control in patients with

diabetes. In addition, hyperinsulinemia and insulin resistance have been reported as risk factors for cancer, including CRC (40). In the present study, the patients with lower serum levels of 1,5-AG exhibited worse prognoses compared with the patients with higher serum 1,5-AG levels. It is unlikely that this result was affected by the frequency of pretreatment diabetes, as only 1 of the 31 patients had a medical history of diabetes. By contrast, Meyerhardt *et al* (41) suggested that a higher dietary glycemic load and a greater total carbohydrate intake had significant associations with an increased risk of recurrence and mortality in patients with stage III CRC, based on investigations of the dietary habits of patients and the calculation of glycemic index values. These results are consistent with the findings of the present study indicating that patients with lower serum levels of 1,5-AG exhibited worse prognoses. However, further studies are required in order to fully elucidate these associations.

It has been reported that n-3 PUFAs have anti-inflammatory and anticancer effects, the latter of which involve the enhancement of oxidative stress in cancer cells (42,43). In addition, n-3 PUFAs enhance the efficacy of treatment in a dose-dependent manner when combined with chemotherapy and radiotherapy (44,45). By contrast, n-6 PUFAs promote colorectal carcinogenesis via inflammation and peroxidation (46,47). In the LC/MS-based lipid analysis conducted in the present study, greater therapeutic effects were observed in the patients with lower serum PUFA levels. One possible explanation for these findings is that n-6 PUFAs, which are linked with carcinogenesis, make a greater contribution to therapeutic responses compared with n-3 PUFAs. Furthermore, Hardy *et al* (48) confirmed that the effects of free fatty acids on breast cancer (e.g., on proliferation and apoptosis) depended on the types of fatty acids and the relative frequencies of oleic acid and palmitic acid. According to the aforementioned findings, not only the concentration of each fatty acid, but also the proportions of each type of fatty acid, including saturated, monounsaturated and polyunsaturated, may influence carcinogenesis and proliferation in CRC, and hence, could affect therapeutic efficacy.

The present study presented with a number of limitations. First, the serum levels of the targeted metabolites may have been changed not only by the chemotherapy, but also by the decline in the general condition of the patients due to their underlying diseases. In the present study, the serum samples were obtained from patients with CRC for which second-line chemotherapy had failed and who therefore may have had subclinical organ disorders. Therefore, the serum levels of the targeted metabolites may have exhibited a more distinct change in the event that serum samples were used from patients who had been treated with cetuximab plus irinotecan, or cetuximab alone as a first-line chemotherapy. Secondly, it is unclear whether the aforementioned biomarkers are specific to cetuximab therapy or whether they are applicable to all chemotherapies for CRC. Thirdly, it was difficult to evaluate why the aforementioned serum metabolites appeared to be useful as biomarkers for predicting the efficacy of chemotherapy in patients with CRC due to the limited number of existing studies. Fourthly, the number of samples was low as the study was attached to a phase II trial of chemotherapy. Fifth, the diagnostic power of these metabolites was not validated using other cohorts. Therefore, a further large-scale study is required to evaluate the results of the present study.

The aim of the present study was to establish biomarkers for predicting the efficacy of chemotherapy for CRC. The serum metabolite levels were analyzed in patients with CRC who were treated with chemotherapy consisting of cetuximab plus irinotecan or cetuximab alone. In such patient cases, changes in metabolite concentrations can be induced by treatment and/or disease progression. Therefore, it may be difficult to identify such biomarkers following treatment, and therefore the study focused on serum metabolites that could be used as predictive biomarkers prior to treatment. As a result, octanoic acid and 1,5-AG were identified as biomarker candidates for predicting the efficacy of chemotherapy for CRC. In the quantitative analysis, the serum concentrations of octanoic acid and 1,5-AG differed significantly between the PR and the non-PR groups. In addition, the present study suggests that the serum concentration of octanoic acid is useful for predicting the prognosis of patients with CRC. Therefore, the findings of the present study may aid in improving the quality of life and prognosis of patients with CRC who are treated with chemotherapy.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SN, TK, TH, KK, HS, YM, KH and MY conceived the idea for the present study, designed the study and assisted in drafting the manuscript. TI and SF analyzed and interpreted the data. TH, KK, HS, YM and KH collected the specimens and patient information. TI wrote the manuscript. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committees of the National Cancer Center Hospital (Tokyo, Japan; approval no. G2008-005) and Kobe University Graduate School of Medicine (Kobe, Japan; approval no. 1833) and was performed in accordance to the principles outlined in the Declaration of Helsinki for all human and animal experiments. In addition, in the investigations involving human subjects, written informed consent was obtained from all subjects.

Patient consent for publication

Informed consent for the publication of any data/ associated images was obtained in from all participants.

Competing interests

The authors declare that they have no competing interests.

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