New Diagnostic Method for Pancreatic Cancer by Profiling of Metabolites in Serum Using GC/MS

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Abstract

Analysis of the metabolites in the serum of pancreatic cancer patients and healthy volunteers was conducted using GC/MS. A metabolite database enabled the identification of the 60 metabolites detected. SIMCA-P+ multivariate analysis software (Umetrics) was used for additional analysis. Using this software, the data could be differentiated between pancreatic cancer patients and healthy volunteers.

Keywords: GC/MS, metabolomics, organic acids, amino acids, pancreatic cancer, multivariate analysis

Introduction

Rates of cancer-related deaths continue to rise, with cancer now accounting for about one-third of all deaths in Japan. In the cases of pancreatic cancer, for which early stage detection is difficult and treatment is often ineffective, there is hope for the development of early-stage markers for this disease. Metabolome analysis (metabolomics), a method which focuses on the in-depth analysis of metabolites, has been recently introduced into the field of medical treatment. In particular, the search is on for novel markers that can be used for disease diagnosis and provide indications of drug efficacy as well as toxicity.
Metabolomics is conducted using systems consisting of various type of chromatographs interfaced to a mass spectrometer. Clinical specimens such as blood contain many types and large numbers of metabolites. For this reason, it is critical that the chromatograph used for analysis provide excellent resolution, quantitative analysis functionality and repeatability, and be equipped with a database capable of metabolite identification. For these reasons, we selected GC/MS (gas chromatograph / mass spectrometer) for conducting metabolite analysis of the clinical specimens used in this investigation. To verify the effectiveness of metabolomics by GC/MS, we conducted profiling of the metabolites in the blood of pancreatic cancer patients and healthy volunteers and compared the results using a GCMS-QP2010 Plus with SIMCA-P+ multivariate analysis software (Umetrics).

Experiment

Reagents

2-Isopropylmalic acid was dissolved in Milli-Q water, and the concentration was adjusted to 1.0 mg/mL for use as the internal standard. The methoxyamine solution used for oxymation was prepared by dissolving methoxyamine hydrochloride (Wako Pure Chemical Industries, Ltd. (Osaka)) in pyridine, and adjusting the concentration to 20 mg/mL. MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamide) used for trimethylsilyl (TMS) derivatization was obtained from GL Sciences Inc. (Tokyo).

Pretreatment

After receiving Ethics Committee approval from Kobe University Hospital and other related hospitals, serum was received from patients and healthy volunteers with their permission, and these serum specimens were used in the study. The serum was processed according to the pretreatment procedure reported previously. First, starting with 50 µL of serum, 5 µL of 2-isopropylmalic acid solution was added to the serum as an internal standard to correct for the loss of analyte during sample preparation. This synthetic compound is not detected in human serum. To this, 250 µL of methanol / water / chloroform (2.5:1:1) was added to remove the protein, and vortexing was conducted until the solution became turbid. The contents were then shaken at 1,200 rpm for 30 minutes at 37°C. Centrifugation was then conducted at 16,000 x g for 5 minutes at 4°C, 225 µL of the supernatant was transferred to a new tube, and 200 µL distilled water was added to this. After vortexing well to turbidity, centrifugation was then conducted at 16,000 x g for 5 minutes at 4°C, and 250 µL of this supernatant was again transferred to a new tube. After conducting speed vacuum concentration for 20 minutes at ambient temperature, the remaining liquid was placed in a deep freezer at -80°C for 10 minutes, after which the frozen sample was placed in a freeze dryer overnight for dehydration. The resulting residue was dissolved in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine solution, and after mixing well in a vortex mixer, shaking was conducted at 1,200 rpm for 90 minutes at 30°C. Subsequently, trimethylsilylation was conducted using 20 µL of MSTFA for derivatization, and shaking was conducted again at 1,200 rpm for 30 minutes at 37°C.

GC/MS Analytical Conditions

The GCMS-QP2010 Plus system was used with a DB-5 capillary column (30 m × 0.25 mm ID × 1.00 µm; J&W Scientific). The GC oven was programmed from 100°C to 320°C at a rate of 4°C/min. The injection port temperature was 280°C, and helium was used as the carrier gas at a constant linear velocity of 39 cm/sec. A 1 µL sample aliquot was injected in splitless mode. The electron ionization energy was 70 eV, the ion source temperature was 200°C, and the scan range was *m/z* 35 – 600.

Data Analysis

The total ion chromatograms (TICs) of the water-soluble metabolites in the serum specimens obtained from the pancreatic cancer patients and the healthy volunteers using the above conditions are shown in Fig. 1. Mass spectral library searches of the major chromatographic peaks were conducted using the GCMSsolution data analysis software and the GC/MS metabolite database (Shimadzu). This database was constructed using the metabolites found in human urine, and includes retention indices for standard chromatographic conditions and EI spectral data for 178 compounds, including amino acids, lipids and other organic acids. Sixty types of metabolites were identified based on a mass spectral similarity of 80 or greater, and these compounds were chosen as the target metabolites for metabolome data of the 9-member healthy group and 20-member pancreatic cancer (stage III – IV) group (Table 1). The retention times predicted from the retention indices, in addition to ion abandance ratios of the quantitation and confirmation ions (more than two ions) were used for identification of each metabolite.

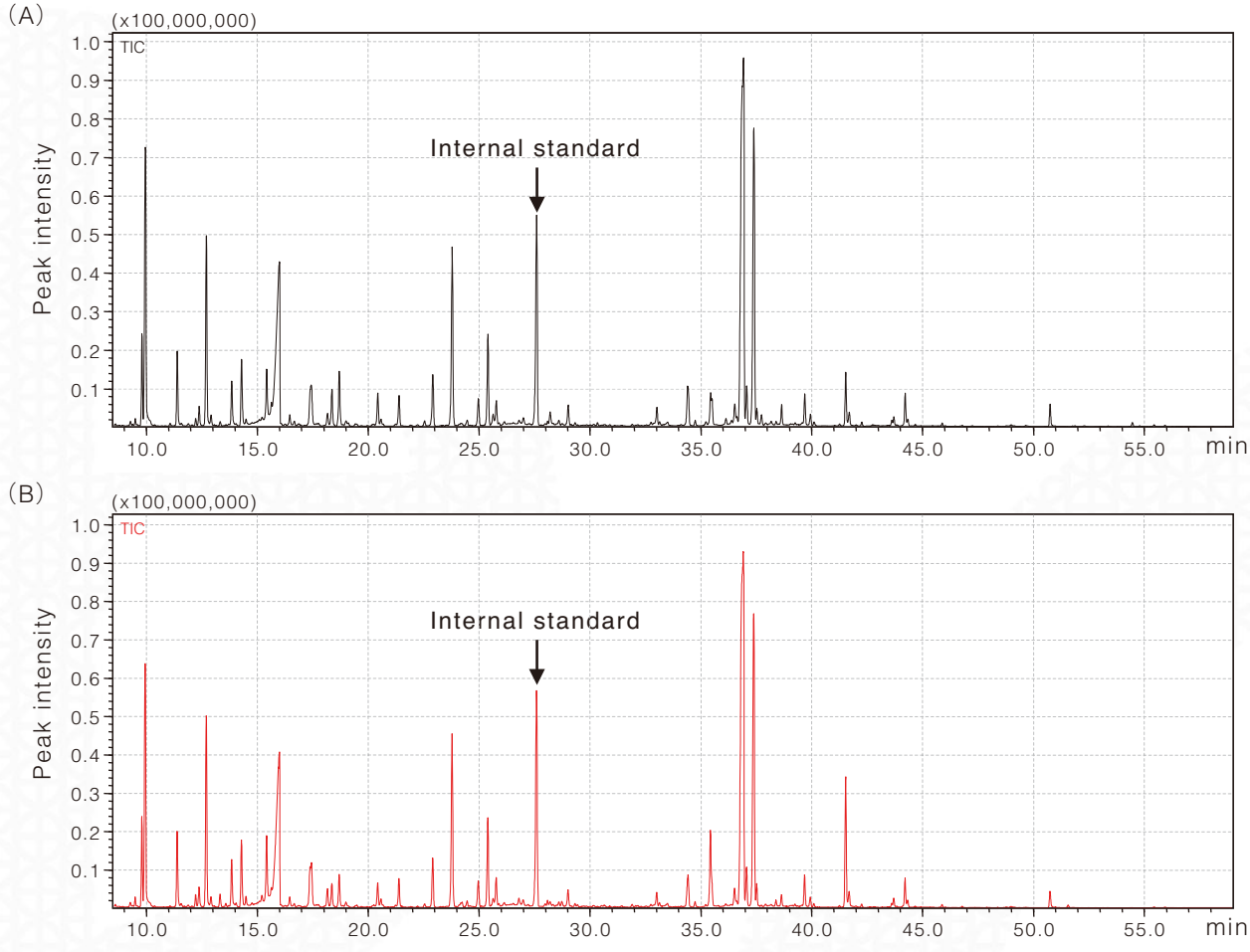

Fig. 1 TICs of Water-Soluble Metabolites Derived from Serum of Pancreatic Cancer Patients and Healthy Volunteers
The Y-axis shows peak intensity, and the X-axis shows retention time. Correction among samples was conducted using an internal standard (↓ in figure).
(A) Pancreatic cancer patients
(B) Healthy volunteers

Table 1 List of Water-Soluble Metabolites Detected in Serum of Pancreatic Cancer Patients and Healthy Volunteers

	Fold induction	*P* value	Similarity	Retention time (min)	Quantitation ion (*m/z*)	Confirmation ion (*m/z*)
01. Lactic acid	1.48	0.00011[a]	87	8.849	219	191
02. Glycolic acid	0.89	0.45	94	9.246	205	177
03. L-Alanine	1.02	0.83	97	10.262	190	147
04. L-Glycine	0.76	0.033[a]	95	10.768	204	176
05. Glyoxylic acid	1.16	0.53	86	11.004	233	218
06. Oxalic acid	1.01	0.87	86	11.037	190	219
07. 2-Hydroxybutyric acid	0.83	0.20	90	11.037	219	205
08. 3-Hydroxypropionic acid	1.28	0.32	81	11.576	219	177
09. Pyruvic acid	0.97	0.70	87	11.812	247	232
10. 4-Cresol	1.08	0.88	90	12.115	180	165
11. 3-Hydroxybutyric acid	2.49	0.21	96	12.149	233	191
12. 3-Hydroxyisobutyric acid	1.00	0.99	84	12.216	177	233
13. 2-Hydroxyisovaleric acid	1.36	0.24	95	12.418	247	219
14. Malonic acid	1.05	0.68	91	13.667	233	133
15. L-Valine	0.88	0.27	97	14.215	218	144
16. Urea	0.80	0.0028[a]	96	14.798	189	171
17. Octanoic acid	0.73	0.00055[a]	91	15.654	201	117
18. L-Leucine	0.94	0.60	92	16.168	232	218
19. Glycerol	0.73	0.069	90	16.203	218	205
20. Phosphoric acid	0.97	0.76	96	16.237	314	299
21. L-Isoleucine	1.05	0.71	95	16.956	232	218
22. L-Proline	1.26	0.23	98	17.157	216	147
23. Glyceric acid	0.53	0.00063[a]	87	18.261	292	189
24. Fumaric acid	1.34	0.071	85	18.462	245	217
25. Citraconic acid	1.40	0.45	93	18.796	259	184
26. L-Serine	0.99	0.92	96	19.198	306	278
27. L-Threonine	0.89	0.19	96	20.168	291	218
28. Decanoic acid	0.76	0.016[a]	81	22.129	229	117
29. Aspartic acid	1.30	0.075	96	24.393	334	306
30. L-Methionine	0.97	0.78	85	24.424	293	250
31. 5-Oxoproline	1.00	0.99	95	24.546	258	230
32. Thiodiglycolic acid	6.27	<0.0001[a]	82	24.637	294	251
33. 4-Hydroxyproline	1.29	0.12	82	24.668	332	304
34. 7-Hydroxyoctanoic acid	1.38	0.00025[a]	81	25.065	289	273
35. 2-Hydroxyglutaric acid	1.00	0.55	81	26.043	349	247
36. 3-Hydroxyphenylacetic acid	1.23	0.15	81	27.056	296	281
37. L-Glutamic acid	2.21	0.055	86	27.375	363	348
38. L-Phenylalanine	0.95	0.61	96	27.782	218	192
39. 4-Hydroxyphenylacetic acid	1.89	0.21	82	27.927	296	281
40. Lauric acid	0.76	0.047[a]	93	28.101	257	145
41. Tartaric acid	1.47	0.36	81	28.130	292	219
42. Asparagine	1.35	0.013[a]	84	28.942	348	333
43. cis-Glutaconic acid	1.11	0.60	80	30.953	346	331
44. Aconitic acid	1.54	0.030[a]	84	30.953	375	285
45. L-Glutamine	1.00	0.97	92	31.807	362	347
46. Isocitric acid	0.95	0.52	92	33.244	465	375
47. Citric acid	0.97	0.71	97	33.244	363	347
48. Glucuronic lactone	0.94	0.44	86	33.297	287	259
49. Homogentisic acid	1.26	0.031[a]	81	33.533	384	341
50. Myristic acid	0.77	0.0073[a]	95	33.533	285	129
51. Glucuronic lactone	1.14	0.45	86	33.638	230	147
52. L-Tyrosine	0.99	0.92	85	36.297	382	354
53. Palmitoleic acid	1.31	0.36	97	38.017	311	145
54. Palmitic acid	0.79	0.032[a]	97	38.447	313	145
55. N-Acetyltyrosine	1.57	0.026[a]	80	40.171	260	218
56. Uric acid	0.61	0.0029[a]	95	40.354	456	441
57. Margaric acid	0.80	0.018[a]	92	40.788	327	342
58. Indolelactic acid	1.22	0.54	93	42.198	421	292
59. Stearic acid	0.74	0.0034[a]	95	42.986	341	145
60. L-Tryptophan	0.98	0.82	95	43.139	405	377
2-Isopropylmalic acid (Internal standard)				27.620	275	147

The comparison of peak intensities of pancreatic cancer patients (20 volunteers) and healthy volunteers (9 volunteers) is indicated as "fold function." P values were calculated according to the Student's t test.

Multivariate Analysis

Multivariate analysis was conducted using SIMCA-P+ software (Ver. 12.0.0, Umetrics). First, principle component analysis, one type of multivariate analysis, was conducted (Fig. 2) using the metabolome data of the 60 metabolites identified from serum samples. These results suggested the presence of metabolites in the data that contribute to differentiation between pancreatic cancer patients and healthy volunteers. Upon conducting statistical analysis of the 60 metabolites detected in the groups of cancer patients and healthy volunteers, respectively, 18 metabolites were shown statistically to be present at significantly different levels between the two groups. Included among these were lactic acid, thiodiglycolic acid, asparagine, aconitic acid, glycine, glyceric acid, and others.

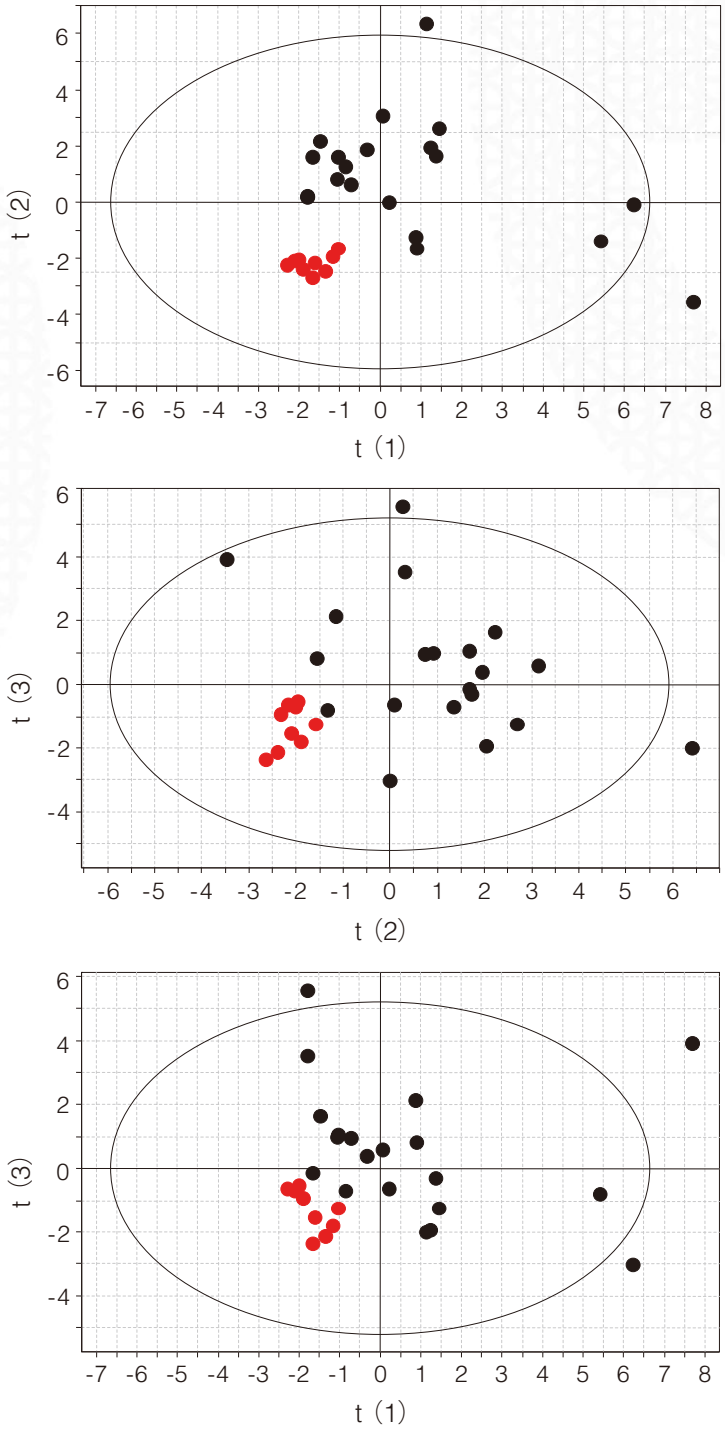

Fig. 2 Principle Component Analysis Score Plots of Metabolome Data from Pancreatic Cancer Patients and Healthy Volunteers
It was possible to clearly distinguish between groups of cancer patients (•) and healthy volunteers (•).

Conclusion

This investigation suggested the possibility that profiling of metabolites in serum by GC/MS can be used as a new diagnostic method for pancreatic cancer screening. There is also the possibility that this technique can be applied to diseases other than pancreatic cancer as long as a metabolome profile specific to that disease can be obtained. It is evident from this investigation that using GC/MS to conduct metabolome analysis can help to clarify profiles specific to various diseases, and it is anticipated that screening and early diagnosis of many diseases may one day be possible from a single drop of blood.

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Data presented here were not acquired using instruments approved under Japan's Pharmaceutical Affairs Law.

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The methods and libraries contain metabolite-related information for amino acids, fatty acids and other organic acids, including 261 electron ionization spectra and 50 chemical ionization spectra.

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