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(Citation)

Metabolomics, 6(4):518-528

(Issue Date)

2010-06

(Resource Type)

journal article

(Version)

Accepted Manuscript

(URL)

<https://hdl.handle.net/20.500.14094/90001489>



Serum metabolomics as a novel diagnostic approach for pancreatic cancer

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An abbreviated title: Serum metabolomics for pancreatic cancer

Financial support: This study was supported by grants from the Research Fellows of the Global COE Program "Global Center of Excellence for Education and Research on Signal Transduction Medicine in the Coming Generation" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan [T.Y., N.H., T.A. and M.Y.]. and from the Education Program for Specialized Clinician in the Support Program for Improving Graduate School Education from the Ministry of Education, Culture, Sports, Science and Technology of Japan [A.I.].

Abstract

Pancreatic cancer is one of the leading causes of cancer-related death, and there is currently little hope of a cure because there are no effective biomarkers for its early detection. Therefore, the search for novel biomarkers that would allow the early detection of pancreatic cancer is ongoing. In this study, the differences between the metabolomes of pancreatic cancer patients with Stage III, Stage IVa, or Stage IVb disease (n=20) and healthy volunteers (n=9) were evaluated by metabolomics, which is the endpoint of the Omics cascade and therefore the last step in the cascade before the phenotype. In our experimental conditions using gas chromatography mass spectrometry (GC/MS), a total of 60 metabolites were detected in serum, and the levels of 18 of the 60 metabolites were significantly changed in pancreatic cancer patients compared with those in healthy volunteers. Then, Principal Component Analysis (PCA), which is a basic form of Multiple Classification Analysis, was performed, and the PCA scores plots based on the 60 metabolites highlighted the metabolomic differences between the pancreatic cancer patients and healthy volunteers. The differences between different stages of pancreatic cancer were also assessed by Partial Least Squares Discriminant Analysis (PLS-DA), which is one of Multiple Classification Analysis, and we found that it was possible to discriminate among the Stage III, Stage IVa, and Stage IVb groups. In addition, values of the 9 metabolites in 1 Stage I pancreatic cancer patient were similar to those obtained from the Stage III, Stage IVa, and Stage IVb pancreatic cancer patients. Our findings will aid the discovery of novel biomarkers that allow the early detection of pancreatic cancer by metabolomic approaches.

Keywords: pancreatic cancer; metabolomics; GC/MS; PCA; PLS-DA.

1. Introduction

Pancreatic cancer is one of the leading causes of cancer-related death, and its 5-year survival rate is the lowest among all cancers (less than 5%) (Jemal et al., 2009). Pancreatic cancer also has a median survival of less than one year with over 96% of cases being incurable at the time of diagnosis (Parkin et al., 2001). One of reasons for its high mortality is the lack of effective biomarkers for the early detection of pancreatic cancer. The commonly used biomarkers for pancreatic cancer are carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), but they are unsuitable for the early detection of pancreatic cancer (Rosty and Goggins, 2002). Although peanut agglutinin (PNA)-binding glycoproteins (Ching and Rhodes, 1989), *hTert* (telomerase catalytic subunit) (Uehara et al., 1999), and matrix metalloproteinase-2 (MMP-2) (Yokoyama et al., 2002) were also reported as potential biomarkers, they were subsequently found to lack clinical efficacy. Therefore, many studies have attempted to find novel biomarkers for the early detection of pancreatic cancer.

Metabolomics (metabolome analysis) may be a useful tool for the identification of novel biomarkers capable of acting as an early diagnostic tool. Metabolomics, which is the endpoint of the Omics cascade and therefore the last step in the cascade before the phenotype, is the comprehensive study of low molecular weight metabolites, and the metabolome represents the metabolite profiles of the cellular processes in a cell, tissue, organ, or organism. Metabolomic technologies have recently developed rapidly. These technologies, which are typically based on nuclear magnetic resonance analysis (NMR), gas chromatography mass spectrometry (GC/MS), liquid chromatography mass spectrometry (LC/MS), and capillary electrophoresis mass spectrometry (CE/MS), have been well-documented in the literature and have been applied to various research fields including the medical field. For example, in the medical

research field, CE/MS-based metabolomics were used to evaluate the metabolic states of colon and stomach cancer tissues and compare them with those of normal tissue from the same organs, and it was revealed that the nutritional conditions in the cancer microenvironment were quite different from the normal microenvironment during energy metabolism (Hirayama et al., 2009). The potential roles of sarcosine in prostate cancer progression were also demonstrated by metabolomics using GC/MS and LC/MS (Sreekumar et al., 2009).

Metabolomics, or metabolome analysis, involves two approaches, metabolite profiling and metabolic fingerprinting (Dettmer et al., 2007). In metabolite profiling, the selected metabolites in a particular environment are identified, and then, a quantitative or semi-quantitative assessment is performed. This approach is useful for understanding known metabolic pathways and biological alterations. On the other hand, metabolic fingerprinting is used to initially examine how metabolite patterns change in response to various stimuli, for example, diseases, toxic exposure, or environmental changes. This approach is carried out using statistical tools such as Multiple Classification Analysis, and aims to clarify which metabolites are important in particular environments. In this study, the differences in metabolomes between pancreatic cancer patients and healthy volunteers were evaluated by metabolite profiling based on a commercially available GC/MS Metabolite Mass Spectral Database, and then we investigated whether it is possible to discriminate among pancreatic cancer stages using metabolomic variations.

2. Materials and Methods

2.1 Subjects

This study was approved by the ethics committee at Kobe University Graduate School of Medicine. The human samples were used in accordance with the guidelines of Kobe University Hospital, and written informed consent was obtained from all subjects. Our study included 21 patients with pancreatic cancer and 9 healthy volunteers, and information about the participants is summarized in Table 1 and Table 3. The blood of the pancreatic cancer patients and healthy volunteers was collected at Kobe University Hospital and its affiliated hospital.

2.2 Serum collection and preparation

The collected blood was immediately centrifuged at 3,000 x g for 10 min at 4°C, and the serum was transferred to a clean tube and stored at -80°C until use. To extract low molecular weight metabolites, 50 µl of serum were mixed with 250 µl of a solvent mixture (MeOH:H₂O:CHCl₃ = 2.5:1:1) containing 6 µl of 1.0 mg/ml 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) dissolved in distilled water, and then the solution was shaken at 1,200 x g for 30 min at 37°C before being centrifuged at 16,000 x g for 5 min at 4°C. Two hundred and twenty-five µl of the supernatant obtained were transferred to a clean tube, and 200 µl of distilled water were added to the tube. After being mixed, the solution was centrifuged at 16,000 x g for 5 min at 4°C, and 250 µl of the resultant supernatant were transferred to a clean tube, before being lyophilized using a freeze dryer. For oximation, 40 µl of 20 mg/ml methoxyamine hydrochloride (Sigma-Aldrich) dissolved in pyridine were mixed with a lyophilized sample, before being shaken at 1,200 x g for 90 min at 30°C. Next, 20 µl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (GL Science, Tokyo, Japan) were

added for derivatization, and the mixture was incubated at 1,200 x g for 30 min at 37°C. The mixture was then centrifuged at 16,000 x g for 5 min at 20°C, and the resultant supernatant was subjected to GC/MS measurement.

2.3 GC/MS analysis

GC/MS analysis was performed using a GCMS-QP2010 (Shimadzu Co., Kyoto, Japan), and a DB-5 column (30 m x 0.25 mm i.d.; film thickness 1.00 µm) (J&W Scientific, Folsom, CA) was included in the gas chromatograph system. The GC column temperature was programmed to rise from 100°C to 320°C at a rate of 4°C per minute, and the total GC run time was 60 min. The inlet temperature was kept at 280°C, and helium was used as a carrier gas at a constant flow rate of 39.0 cm per second. A sample of 1.0 µl was injected at a splitless mode, and the mass conditions were as follows: ionization voltage: 70 eV; ion source temperature: 200°C; full scan mode in the m/z range 35 – 600 with a 0.20 s/scan velocity.

2.4 Data processing

The chromatogram acquisition, detection of mass spectral peaks, and their waveform processing were performed using Shimadzu GCMSsolution software Version 2.53 (Shimadzu Co.), and the retention time correction of peaks was carried out based on the retention time of a standard alkane series mixture (C-10 to C-40) using the Automatic Adjustment of Retention Time (AART) function of the Shimadzu GCMSsolution software. The identification of low molecular weight metabolites was performed using a commercially available GC/MS Metabolite Mass Spectral Database (Shimadzu Co.), which contained a mass spectral library; method files that specified the above-described analytical conditions; and the data analysis parameters for 178 compounds, such as amino acids, fatty acids, and organic acids. A similarity index was calculated on the basis of retention time, confirmed ion and

fragmentation pattern observed in the mass spectrum of the low molecular weight metabolites. These peaks were assigned on the condition of possessing a similarity index of more than 80, and peaks with a similarity index of less than 80 were processed as unknown molecules. In addition to automatic assessment, the identified metabolites, of which a similarity index was more than 80, were manually checked. To perform the semi-quantitative assessment, the peak height of each quantified ion was calculated and normalized using the peak height of 2-isopropylmalic acid as an internal standard.

2.5 Multiple Classification Analysis

The dataset for the Multiple Classification Analysis was compiled from the metabolite profiling results, and we constructed a three-dimensional matrix using the data regarding sample names (observations), metabolite numbers (variable indices), and normalized peak intensities (variables). Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were performed using commercially available SIMCA-P+ Software Version 12.0.1 (Umetrics, Umeå, Sweden). In chemometric analysis, *pareto* scaling was applied to the data processing. The scores plots of the first two or three principal components were used to assess the similarities and differences among various groups, and the corresponding loadings plots were used to determine the metabolites responsible for the separation seen in the PCA and PLS-DA scores plots.

2.6 Statistics

Data are expressed as the mean $\pm$ standard error (SE). Statistical significance was analyzed using the Student's t-test, and a level of probability of 0.05 was used as the criterion of significance.

3. Results and Discussion

The discovery of novel biomarkers for pancreatic cancer is required for its early detection. In this study, metabolome analysis of serum was performed as a novel diagnostic approach for pancreatic cancer. The sera from 20 pancreatic cancer patients with Stage III, Stage IVa, or Stage IVb disease, and 9 healthy volunteers were subjected to measurement by GC/MS. The clinical characteristics of 20 pancreatic cancer patients are summarized in Table 1. As listed in Table 1, the pancreatic cancer patients had Stage III, IVa, or IVb disease, and their pancreatic tumors were located in various sites, such as the pancreatic head, body, tail, and uncinate process. Only four of all pancreatic cancer patients used in this study was treated with a therapeutic agent, Gemzar.

First, serum metabolite profiling was performed by GC/MS, and representative TIC chromatograms from the pancreatic cancer patients (black) and healthy volunteers (red) were shown in Figure 1A and 1B, respectively. The overlapped TIC chromatograms of pancreatic cancer patients (black) and healthy volunteers (red) were also illustrated (Figure 1C), and it was observed that the intensity of some peaks was different between the pancreatic cancer patients (black) and healthy volunteers (red). In our experimental conditions (and using the GC/MS Metabolite Mass Spectral Database developed by Shimadzu Co.), a total of 60 metabolites (59 individual metabolites), of which a similarity index was more than 80, were detected in serum (Table 2). The intensity of each peak was measured and normalized, and then the fold induction of peak intensity was calculated as the ratio of the peak intensity of the pancreatic cancer patients to that of the healthy volunteers. As shown in Table 2, the levels of 18 of 60 metabolites were significantly changed in the pancreatic cancer patients compared with the healthy volunteers ($p < 0.05$). The significantly changed metabolites were as follows

(Fold induction): the levels of lactic acid (x1.48), thiodiglycolic acid (x6.27), 7-hydroxooctanoic acid (x1.38), asparagine (x1.35), aconitic acid (x1.54), homogentisic acid (x1.26), and *N*-acetyltyrosine (x1.57) were significantly increased in the pancreatic cancer patients, and those of L-glycine (x0.76), urea (x0.80), octanoic acid (x0.73), glyceric acid (x0.53), decanoic acid (x0.76), lauric acid (x0.76), myristic acid (x0.77), palmitic acid (x0.79), uric acid (x0.61), margaric acid (x0.80), and stearic acid (x0.74) were significantly decreased. In previous studies, it was reported that the serum levels of these metabolites were changed by other diseases. For example, a previous study suggested that lactate is a marker of energy failure in critically ill patients (Valenza et al., 2005), whereas the serum level of homogentisic acid in alkaptonuria patients was significantly increased (Bory et al., 1990). The serum urea level was significantly decreased in cirrhotic patients compared with that in healthy volunteers (Marescau et al., 1995). In addition, variations in the levels of other low molecular weight metabolites were observed in hepatocellular carcinoma patients (Xue et al., 2008) and prostate cancer patients (Lokhov et al., 2010). Thus, the levels of multiple low molecular weight metabolites are changed in various diseases, and different types of diseases seem to cause variations in the same metabolites. This may be due to inflammation, which occurs in various diseases. However, the metabolome is dynamic, and it is highly possible that certain pathological conditions lead to dynamic variations in the metabolome; i.e., variations in the levels of multiple low molecular weight metabolites. These facts raise the necessity for a multi-biomarker approach to the assessment of various pathological conditions using the levels of multiple low molecular weight metabolites. Therefore, we investigated whether the variation patterns of the levels of the 60 metabolites detected in this study, of which a similarity index was more than 80, were different between the pancreatic cancer patients and healthy volunteers. In this study, PCA, which is a basic form of Multiple Classification Analysis, was performed. The 3D- and 2D-PCA scores plots based on the 60 metabolites

exhibited differences between the pancreatic cancer patients (black) and healthy volunteers (red) (Figures 2A and 2B), although no influence on the serum metabolome by therapeutic agents was observed in the 3D and 2D-PCA scores plots (data not shown). In addition, the corresponding 2D-PCA loadings plots revealed that the variations in the levels of lactic acid, aconitic acid, thiodiglycolic acid, urea, octanoic acid, glyceric acid, uric acid, and stearic acid largely contributed to the observed separation of the pancreatic cancer patients and healthy volunteers (Figure 2C). These results indicate the statistical difference between the pancreatic cancer patients and healthy volunteers with regards to the variation patterns of the 60 metabolites detected in this study.

Next, the differences among the various pancreatic cancer stages were assessed using PLS-DA scores plots, and we found that it was possible to discriminate among the Stage III, Stage IVa, and Stage IVb groups (Figure 3A). In the corresponding PLS-DA loadings plots (Figure 3B), except the 8 metabolites (lactic acid, aconitic acid, thiodiglycolic acid, urea, octanoic acid, glyceric acid, uric acid, and stearic acid) obtained from Figure 2C, the variations in the levels of L-glycine, 3-hydroxybutyric acid, decanoic acid, 4-hydroxyproline, 7-hydroxyoctanoic acid, L-glutamic acid, 4-hydroxyphenylacetic acid, tartaric acid, asparagine, homogentisic acid, palmitoleic acid, palmitic acid, and N-acetyltyrosine were found to have contributed to the result of the PLS-DA scores plots (Figure 3A).

The metabolites characterizing the pancreatic cancer patients and furthermore each stage were selected from the results of PCA (Figure 2) and PLS-DA (Figure 3) loadings plots, and their serum levels were compared among the Stage III, Stage IVa, and Stage IVb pancreatic cancer patients and healthy volunteers (Figure 4). In Stage III pancreatic cancer, the tumor has infiltrated the surrounding pancreas regardless of its size and may have spread to nearby

lymph nodes. It may also have spread to other body organs through the lymph system. On the other hand, pancreatic cancer Stage IV can be divided into two groups, i.e., Stage IVa and Stage IVb. Stage IVa involves adjacent organs or blood vessels, although the location is restricted. On the other hand, Stage IVb disease has spread to distant organs, such as the liver and lung; i.e., metastasis. Therefore, not only pancreatic tumors but also other factors are supposed to affect the serum metabolome. For example, the transfer of pancreatic tumors to other organs via the blood may have influenced the observed separation among the Stage III, Stage IVa, and Stage IVb groups. Besides their transfer via the blood, multiple differences in the pathological conditions of pancreatic cancer would be involved in the results in this study. Actually, characteristic variations of metabolites among the Stage IVb, Stage IVa, Stage III, and healthy volunteer groups were observed. The serum levels of 3-hydroxybutyric acid and tartaric acid tended to be increased in the Stage IVb group compared with the healthy volunteer group, while no variations in the Stage IVa and Stage III groups were observed (Figure 4). Regarding 4-hydroxyproline and palmitoleic acid, the significant increases were observed in the Stage IVa and Stage III groups, respectively (Figure 4). The serum level of L-glutamic acid was changed according to the following order: Stage IVb > Stage IVa > Stage III > healthy volunteers (Figure 4). The 4-hydroxyphenylacetic acid level was increased in the Stage IVb and Stage III groups, although no variations in the Stage IVa group were observed (Figure 4). These results suggest that metabolomics is capable of representing the pathological conditions of pancreatic cancer in more detail, although the relationship between alterations of metabolites and locations of tumor in pancreatic tissue was not confirmed from results of metabolite profiling in this study (data not shown).

Finally, the potential of metabolomics as an early diagnostic tool for pancreatic cancer was investigated using the serum from 1 Stage I pancreatic cancer patient. The clinical

characteristics of the Stage I pancreatic cancer patient are summarized in Table 3. As listed in Table 3, the serum concentrations of CEA and CA19-9, which are classic tumor markers, were normal levels. Regarding the 21 metabolites shown in Figure 4, their serum levels in the Stage I pancreatic cancer patient were compared with the healthy volunteers (n=9), and the fold-induction was calculated. The obtained results were as follows: Lactic acid, x1.89; aconitic acid, x1.09; thiodiglycolic acid, x8.52; urea, x0.82; octanoic acid, x0.33; glyceric acid, x0.70; uric acid, x0.95; stearic acid x1.37; L-glycine, x1.07; 3-hydroxybutyric acid, x1.02; decanoic acid, x0.55; 4-hydroxyproline, x0.76; 7-hydroxyoctanoic acid, x1.38; L-glutamic acid, x2.17; 4-hydroxyphenylacetic acid, x1.10; tartaric acid, 0.81; asparagine, x1.60; homogentisic acid, x1.35; palmitoleic acid, x4.36; palmitic acid, x2.28; and N-acetyltyrosine, x1.52. Values of lactic acid, urea, decanoic acid, thiodiglycolic acid, 7-hydroxyoctanoic acid, L-glutamic acid, asparagine, homogentisic acid, and N-acetyltyrosine were similar to those obtained from the Stage III, Stage IVa, and Stage IVb pancreatic cancer patients (Table 2). Regarding palmitic acid, the opposite variation was observed (Table 2), suggesting the possibility of effects on the serum metabolome by cancer invasion and metastasis. These results indicate that serum metabolomics may be useful for the early detection of pancreatic cancer, although larger studies are needed in the future.

Thus far, many researchers have attempted to find novel biomarkers for pancreatic cancer using proteomic and transcriptomic approaches (Hibi et al., 2009; Sitek et al., 2009; Yu et al., 2009), but their research findings have not demonstrated a practical use. Based on the findings of our current study, it was indicated that the pathogenesis of pancreatic cancer leads to variations in the serum levels of low molecular weight metabolites, and our study supports the potential of metabolomics as an early diagnostic tool for pancreatic cancer, although larger studies are needed to verify its practical utility.

4. Concluding Remarks

In serum metabolome analysis using GC/MS, it was confirmed that the levels of various metabolites were changed in pancreatic cancer patients compared with those in healthy volunteers. In addition, our results suggested that metabolomics is capable of representing the differences between the various stages of pancreatic cancer. Our findings will hopefully lead to the discovery of novel biomarkers that allow the early detection of pancreatic cancer by metabolomic approaches. The variations in the serum levels of metabolites may also provide promising markers not only for the early diagnosis of pancreatic cancer but also its prognosis.

Acknowledgements

We thank Shimadzu Co. for their technical support and helpful discussion.

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Figure legends

Fig 1. Representative TIC chromatograms from the pancreatic cancer patients and healthy volunteers.

(A) Pancreatic cancer patients (black). (B) Healthy volunteers (red). (C) The overlapped TIC chromatograms of pancreatic cancer patients (black) and healthy volunteers (red).

Fig 2. PCA based on the metabolite profile data of pancreatic cancer patients and healthy volunteers.

(A) 3D-PCA scores plots discriminating between the pancreatic cancer patients and healthy volunteers. The black and red circles indicate the results of the pancreatic cancer patients (n=20) and healthy volunteers (n=9), respectively. The principal components PC1 (t[1]), PC2 (t[2]), and PC3 (t[3]) described 20.4, 16.3, and 12.6% of the variation, respectively (A=3, $R^2X=0.493$). The sphere indicates Hotelling T² (0.95) for this model. **(B) 2D-PCA scores plots discriminating between the pancreatic cancer patients and healthy volunteers.** 2D-PCA scores plots consisting of the principal components PC1 (t[1]), PC2 (t[2]), or PC3 (t[3]) were shown. The black and red circles indicate the results of the pancreatic cancer patients (n=20) and healthy volunteers (n=9), respectively. **(C) 2D-PCA loadings plots discriminating between the pancreatic cancer patients and healthy volunteers.** The 2D-PCA loadings plots were calculated on the basis of (B). Each metabolite is indicated by the corresponding number shown in Table 2.

Fig. 3 PLS-DA based on the metabolite profile data of pancreatic cancer patients and healthy volunteers.

(A) PLS-DA scores plots discriminating between the various stages of pancreatic cancer. The blue triangles, red squares, black circles, and green diamonds indicate Stage III (n=3), Stage IVa (n=7), Stage IVb (n=10), and healthy volunteers (n=9), respectively. The principal components PC1 (t[1]), PC2 (t[2]), and PC3 (t[3]) described 17.5, 12.9, and 5.8% of the variation, respectively (A=3, $R^2X=0.362$). The ellipse indicates Hotelling T² (0.95) for this model. **(B) PLS-DA loadings plots discriminating between the various stages of pancreatic cancer.** The PLS-DA loadings plots were calculated on the basis of (A). Each metabolite is indicated by the corresponding number shown in Table 2.

Fig. 4 The serum levels of 21 targeted metabolites obtained from Fig. 2 and Fig. 3.

Values are shown as the fold-induction of the peak intensity of the pancreatic cancer patients (n=20: Stage IVb, n=10; Stage IVa, n=7; Stage III, n=3) compared with that of the healthy volunteers (n=9). The pancreatic cancer Stage IVb patients, Stage IVa patients, Stage III patients, and healthy volunteers are illustrated as black, crossed, dotted, and white bars, respectively. Data are shown as the mean $\pm$ SE, and asterisks indicate a significant difference according to the Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

Fig 1.

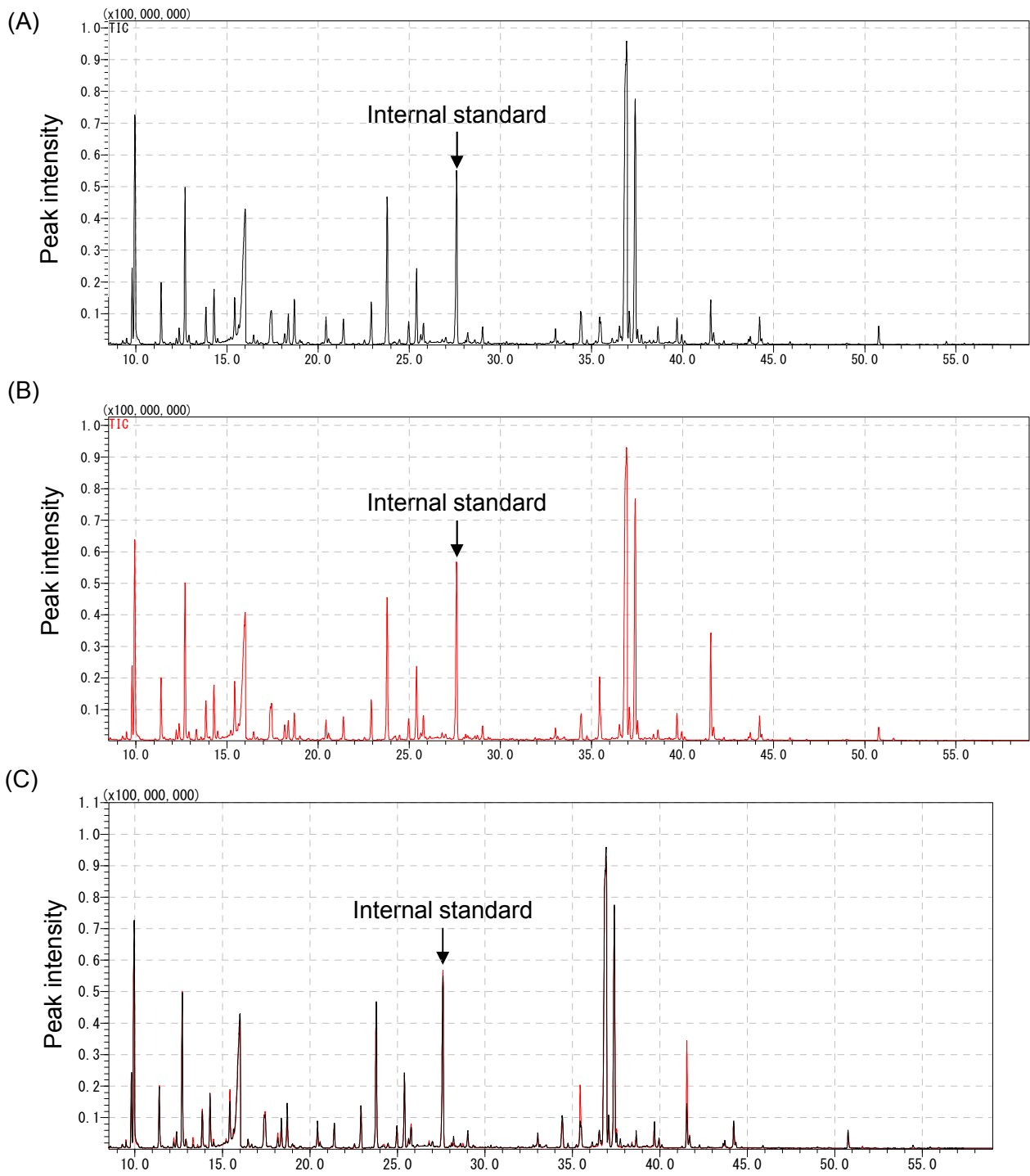
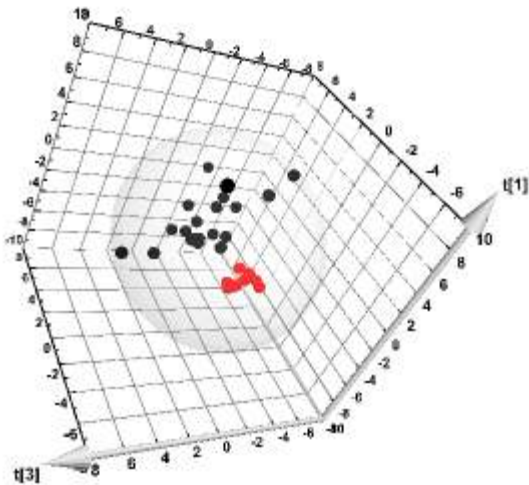
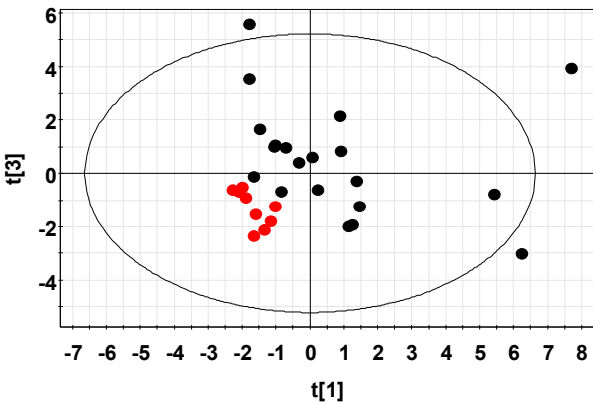
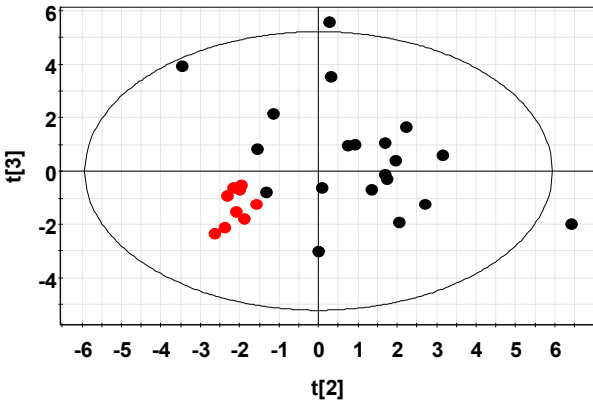
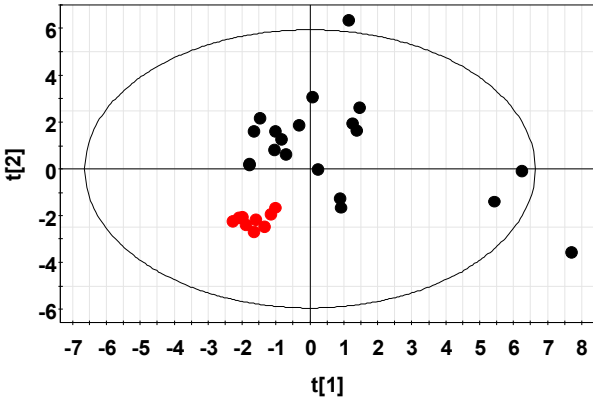


Fig 2.
(A)



(B)



(C)

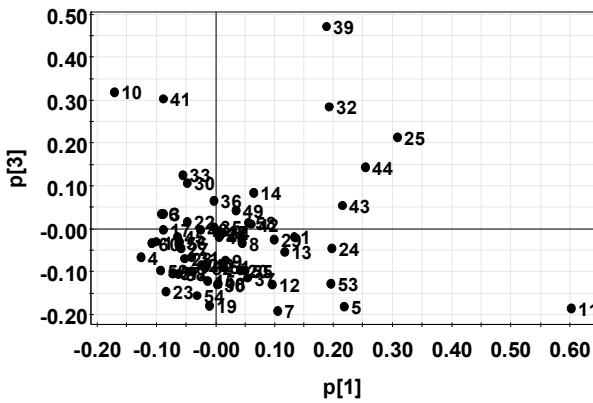
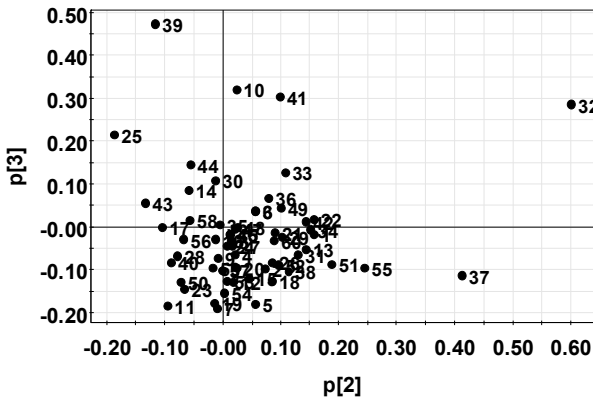
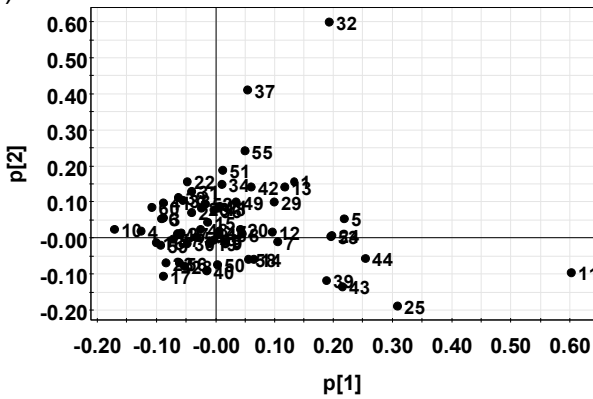
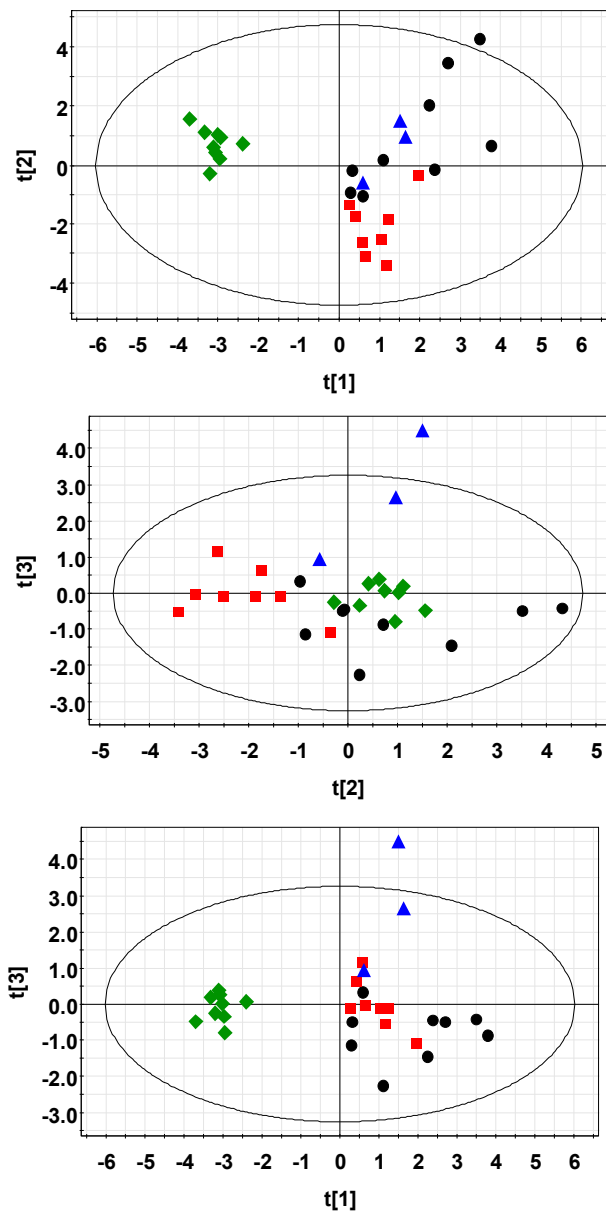


Fig 3.

(A)



(B)

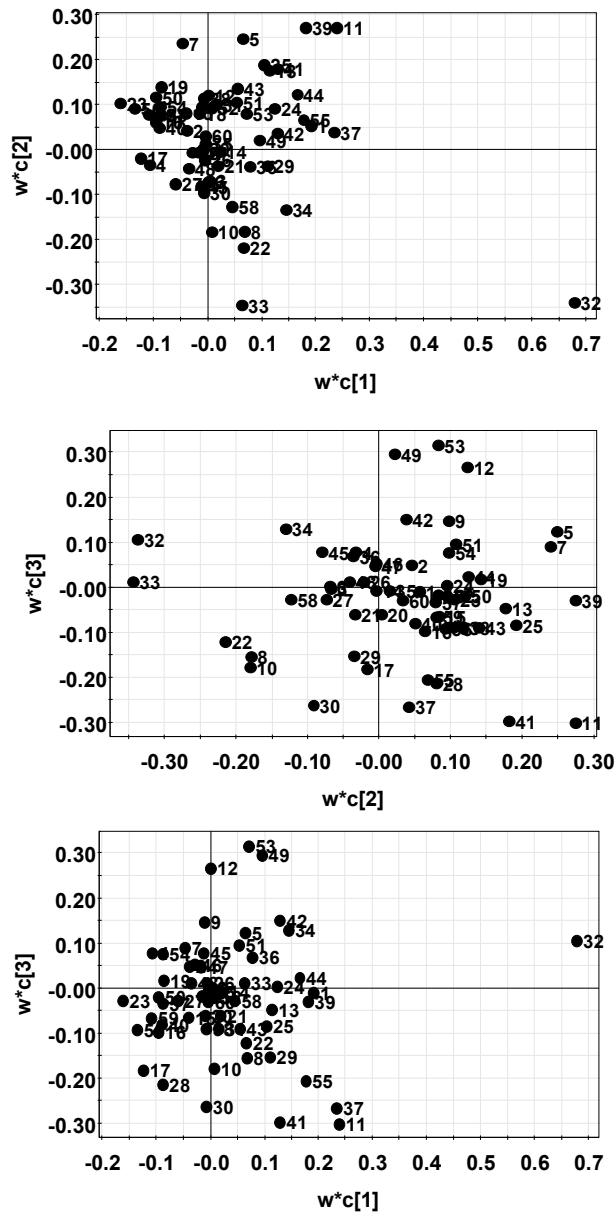


Fig 4.

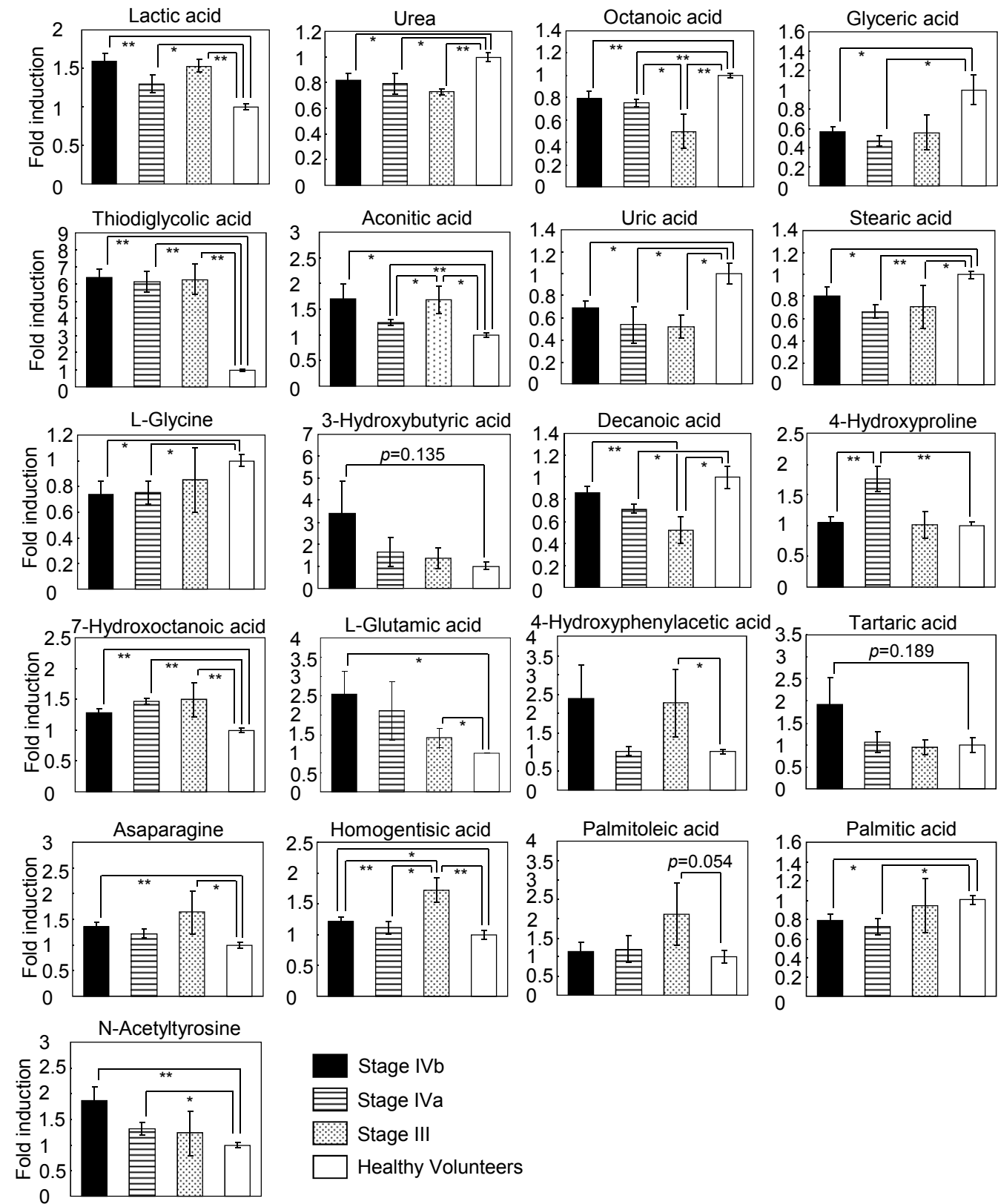


Table 1. Characteristics of pancreatic cancer patients and healthy volunteers

Pancreatic cancer patients	
Age (Range)	64.9 ± 2.13 (48-79)
<i>n</i> (Male/Female)	20(10/10)
Stage (III/IVa/IVb (Male/Female))	3(2/1)/7(3/4)/10(5/5)
Location (Head/Body/Tail/Body and tail/Uncinate process)	11/2/5/1/1
Healthy volunteers	
Age (Range)	61.6 ± 3.74 (34-72)
<i>n</i> (Male/Female)	9(3/6)

Staging of pancreatic cancer was based on TNM classification. The ages of the pancreatic cancer patients and healthy volunteers are shown as mean ± SE.

Table 2. Comparison of the serum metabolites between pancreatic cancer patients and healthy volunteers

	Fold induction	<i>p</i> value	Similarity	Retention time (min)	Quantified ion (m/z)	Confirmed ion (m/z)
1. Lactic acid	1.48	0.00011 ^a	87	8.849	219	191
2. Glycolic acid	0.89	0.45	94	9.246	205	177
3. L-Alanine	1.02	0.83	97	10.262	190	147
4. L-Glycine	0.76	0.033 ^a	95	10.768	204	176
5. Glyoxylic acid	1.16	0.53	86	11.004	233	218
6. Oxalic acid	1.01	0.87	86	11.037	190	219
7. 2-Hydroxybutyric acid	0.83	0.20	90	11.037	219	205
8. 3-Hydroxypropionic acid	1.28	0.32	81	11.576	219	177
9. 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

Table 2 (Continued)

	Fold induction	<i>P</i> value	Similarity	Retention time (min)	Quantified ion (m/z)	Confirmed ion (m/z)
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. <i>cis</i> -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

Values are represented as the fold-induction of the peak intensity of the pancreatic cancer patients (n=20) compared with that of the healthy volunteers (n=9). *P* values were calculated according to the Student's *t*-test, and superscript letters (^a) indicate lower *p* values than 0.05. The similarity index was calculated on the basis of retention time, confirmed ion and fragmentation pattern observed in the mass spectrum of the low molecular weight metabolites. The peak intensity was quantified for each quantified ion and normalized to that of 2-isopropylmalic acid as an internal standard.

Table 3. Characteristics of Stage I pancreatic cancer patient

Age (year)	53
Sex	Male
Location	Pancreatic body
CEA (ng/ml)	2.1
CA19-9 (U/ml)	8

Staging of pancreatic cancer was based on TNM classification.