NOTE

Principal Component Analysis of Direct Matrix-Assisted Laser Desorption/Ionization Mass Spectrometric Data Related to Metabolites of Fatty Liver

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Abstract: Non-alcoholic steatohepatitis (NASH) is a common liver disease. NASH is characterized by fatty liver, along with inflammation. Most people with NASH are not aware of their condition, even though NASH can lead to hepatic cirrhosis. Several approaches have been tested to clarify the pathology of NASH. However, the mechanism of onset of NASH was not well-defined. In this study, a supervised multivariate analysis (principal component analysis) approach using direct matrix-assisted laser desorption/ionization mass spectrometry (dMALDI-MS) was applied to the analysis of metabolites in starvation-induced fatty liver tissue sections. This approach does not require complex pretreatments. We investigated the characteristic dynamics of metabolites in fatty liver. This approach can be applied to the analysis of human biopsy specimens of fatty liver in future studies.

Key words: direct MALDI-MS, fatty liver, principal component analysis, phosphatidylcholine, biomarker discovery

1 INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a form of metabolic liver disease characterized by histological features similar to those of alcoholic hepatitis¹⁾. A two-hit model can be used for evaluating the pathogenesis of NASH. The first stage is the occurrence of non-alcoholic fatty liver disease (NAFLD), and the second stage is characterized by inflammation, liver injury, and hepatic fibrosis (steatohepatitis). NAFLD is closely related to metabolic syndrome^{2,3)}; many nutritional approaches have been applied to prevent the accumulation of triacylglycerol in the liver^{4,5)}. However, the number of patients with NASH is increasing because no therapy has been proven effective for treating it. The mechanism of the transition from the first stage (hepatic steatosis) to the second stage (steatohepatitis) is an important question about the onset of

NASH that has not yet been answered. In order to clarify the pathogenesis of NASH, it is important to analyze the metabolites in the fatty liver. Accordingly, the metabolites in fatty liver were analyzed by conventional mass spectrometric approaches such as high-performance liquid chromatography, mass spectrometry, or gas chromatography/mass spectrometry⁶⁻⁸⁾. These conventional approaches are essential for the analysis of metabolites and for determining metabolic profiles^{9,10)}. However, before performing these methods, the metabolites need to be subjected to pretreatments such as extraction, purification, and derivatization^{11,12)}. These processes may lead to loss of metabolites that are available in limited quantities or are insoluble in buffers. Therefore a method that does not involve complicated pretreatment procedures may help determine a new profile of fatty liver. Direct matrix-assisted laser desorp-

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tion/ionization mass spectrometry (dMALDI-MS) is an attractive new approach for the non-targeting screening of metabolites in fatty liver. This method can reveal metabolic dynamics even with a trace amount of biopsy specimen without the need for pretreatment procedures such as extraction, derivatization, or purification. dMALDI-MS is being increasingly used for the analysis of biological information¹³⁻¹⁵.

In this study, we performed an unsupervised multivariate analysis—principal component analysis (PCA)—for the non-targeting screening of the starvation-induced metabolites in the fatty liver tissue section to determine whether the analysis using dMALDI-MS can help determine a new profile of fatty liver. PCA is a statistical method that can be used to identify new, meaningful, hidden differences between clusters¹⁶⁻¹⁸. We were successful in identifying several differences between the metabolites found in normal liver and fatty liver. This approach can be applied to the analysis of trace amounts of tissues, including those obtained by human biopsy.

2 EXPERIMENTAL

2.1 Chemicals

Acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Kanto Chemical (Tokyo, Japan). 2,5-dihydroxybenzoic acid (2,5-DHB) was purchased from Bruker Daltonics (Bremen, Germany). All the chemicals used in this study were of the highest purity available.

2.2 Animals

Animal care and the experiments with animals were performed in accordance with the institutional guidelines and the guidelines of the National Institute of Health and the Animal Care and Use Committee (Mitsubishi Kagaku Institute of Life Science). Male C57BL/6 mice (12 weeks old) were used. The mice were fed a standard lab chow (MF; Oriental Yeast corporation, Tokyo, Japan).

2.3 Sample preparation

We isolated the fatty livers from 16-h-fasted mice and normal livers from mice that were fed *ad libitum*. The mice were anesthetized with Nembutal (40-50 mg/kg), and their livers were perfused with saline. The isolated livers were immediately frozen in liquid nitrogen, and stored at -80°C without any fixation. The dMALDI-MS samples were prepared as described previously, with a slight modification¹⁹⁻²³. Briefly, the tissue was sliced into 10- μm -thick sections using a cryostat and mounted onto an indium tin oxide (ITO)-coated slide glass (Bruker Daltonics). A thin matrix layer was applied to the surface of the slide by using an airbrush with a 0.2-mm nozzle and DHB solution (50 mg/mL DHB, 20 mM sodium acetate, 70% methanol,

0.1% TFA) was sprayed.

2.4 Mass spectrometry

Mass spectrometry was performed with a MALDI-TOF/TOF-type instrument: Ultraflex II TOF/TOF (Bruker Daltonics) equipped with a 355-nm Nd:YAG laser with a repetition rate of 200 Hz. Data were acquired in the positive-ion mode by using an external calibration method. The mass spectrometer parameters were set to obtain the highest sensitivity with m/z values in the range of 300-1000. All the spectra were acquired automatically using the Flex Imaging software (Bruker Daltonics). The laser was irradiated 100 times per position. The peaks were normalized to the total ion current and then compared. PCA was performed using the ClinProTools 2.1 software (Bruker Daltonics) on the m/z values for the samples of fatty liver and normal liver. MS/MS was performed on the tissue section in the positive-ion mode, and the Lipid Search (http://lipidsearch.jp/manual_search/) database was used to determine the molecular species of phosphatidylcholine (PC).

2.5 Workflow of direct MALDI-MS and multivariate analysis

The workflow of dMALDI-MS and PCA is shown in Fig. 1. Frozen tissue sections were sliced and mounted on ITO-coated slide glass (Fig. 1a) and sprayed with a matrix solution (Fig. 1b). The data for each data point were obtained (Fig. 1c), and the spectra were stored and compared by PCA (Figs. 1d and e). The 100 most intense peaks were compared by PCA. To investigate the effect of different sample-preparation conditions, we performed the same experiment using the same samples on a different day (i.e., experiments were performed on day 1 and day 2). The differences in the peaks between the normal tissue and the fatty liver tissue were identified by MS/MS and database search (Fig. 1f).

3 RESULTS AND DISCUSSION

Figure 2 shows the mass spectra from the normal liver sections and the starvation-induced fatty liver sections. We obtained about 750 spectra for both the sections having an m/z value in the range of 300-1000. There were some differences between the 2 sections in some peaks (arrow point in Fig. 2). Unsupervised multivariate analysis, i.e., PCA was performed to identify the different peaks in both the sections. PCA is a statistical method used to reduce multi-dimensional data sets to lower dimensions and to identify new, meaningful, hidden, differences between the clusters. The result of PCA is represented by 2 multi-dimensional plots: the score plot and the loading plot. The score plot is based on the PCA and shows the similarities and differ-

Fig. 1 The Workflow of dMALDI-MS and PCA. Liver sections were sliced and mounted on the plate (a), coated by DHB solution (b), and laser scanned (c). The obtained spectra were accumulated (d) and PCA was performed using the ClinProTools software (e). Some molecules in the liver tissue sections were identified by MS/MS analysis.

Fig. 2 The Mass Spectra of Normal Liver (a) and Fatty Liver (below) with the m/z Values in the Range of 300-1000.

ences among the samples; samples with similar peak patterns are clustered closely in the plot. The loading plot shows the influence of the data variables, in this case the m/z values. **Figure 3a** shows the score plot of the first and second principal component of the PCA. On the basis of these principal components (cumulative proportion rate, 92%) the data were divided into 4 groups. The first principal component contained the information about the differences in the sample preparations, indicating that the conditions of sample preparation strongly influenced the discrimination of the liver samples. This result did not compromise the intended diagnostic use of the data because the data of normal liver and fatty liver were clearly separated on the second principal component (proportion rate, 27%). The obtained results, therefore, support the fact that the spectra can be used to differentiate between pathological and normal conditions. The corresponding loading plot demonstrated the influence of the m/z value on the respective principal components (**Fig. 3b**). The loading plot showed that the differences in the normal liver and fatty liver were apparent at several m/z values (e.g., 780, 878, 438, and 390). The differences between the peaks of the normal liver and fatty liver are shown in **Table 1**. On-tissue MS/MS was used to assign peaks. The peak at m/z 780 was identified as that of PC, because the presence of the fragment ions at the m/z values 86, 147, and 184, and neutral loss of 59 and 183 under MS/MS analysis is indicative of PC (**Fig. 4**)²⁴⁻²⁶. The peak at m/z 780 can be assigned as PC (1-acyl 34:2) + Na. The major molecular species of PC (1-acyl 34:2) should be PC diacyl 16:0-18:2, considering the abundance of fatty acids in the liver. Generally, PC is

detected as $[M+Na]^+$, and $[M+K]^+$ ions under normal conditions; thus, the peak intensity at m/z 796 (1-acyl 16:0-18:2+K) was compared with that of the peak intensity observed for the normal liver section; we confirmed that the peak at m/z 796 was increased by $123 \pm 14.6\%$. The peaks at m/z 808 and 802 were also assigned as PC (data not shown); however, the molecular species could not be identified because no neutral loss of carboxylic acids was observed. It was interesting to note that the dynamics of the peak at m/z 802 were different from those of the peak at m/z 780 and the peak at m/z 808. It is speculated that persistent fatty liver may change the ratio of the PC species in the tissue section, leading to the disruption of homeostasis in the liver. The change in PC ratio noted in this study was consistent with that observed in the previous LC/MS study²⁷. Ginneken et al. also reported that PC (1-acyl 34:2) was the most increased PC specie, suggesting PC (1-acyl 34:2) was important role in evolution of fatty liver²⁷. The effect of PC ratio on the onset of NASH should be evaluated further. The other peaks can be considered as the biomarkers of fatty liver (**Table 1**). The molecules that correspond to the m/z values of 422, 438, 390, 567, and 613 were not reported in the past studies⁶⁻⁸. Hence, our data suggested that dMALDI-MS was successful in identifying a new profile of fatty liver, although the new molecules could not be identified in this study. The identification of low-molecular-weight components by dMALDI-MS needs further investigation. Further studies are needed to identify the unknown molecules.

Fig. 3 (a) Score Plot of the First and Second Principal Components Resulting from the PCA of the MALDI Spectra of Normal and Fatty Livers. (b) Loading Plot of the First and Second Principal Components.

The numbered components (No.1-15) are summarized in **Table 1**.

Table 1 Summary Table of Different Peak between Normal and Fatty Liver.

No	m/z	Normal liver (Mean $\pm$ SD)	Fatty Liver (Mean $\pm$ SD)	P -value	Change(%) (vs Normal)	Molecule
1	780	181.6 $\pm$ 4.0	211.9 $\pm$ 6.6	0.024*	117	PC(1-acyl 34:2) + Na ⁺
2	878	79.3 $\pm$ 1.6	107.0 $\pm$ 4.3	0.005**	135	
3	880	133.2 $\pm$ 5.6	151.3 $\pm$ 2.6	0.073*	114	
4	854	40.2 $\pm$ 1.3	71.0 $\pm$ 2.8	0.001**	177	
5	808	132.0 $\pm$ 1.1	144.2 $\pm$ 1.0	0.002**	109	PC(1-acyl 36:2) + Na ⁺
6	880	88.7 $\pm$ 4.1	103.0 $\pm$ 5.6	0.235*	116	
7	856	54.6 $\pm$ 3.2	62.5 $\pm$ 2.9	0.328*	115	
8	422	62.1 $\pm$ 4.7	65.1 $\pm$ 3.2	0.416	105	
9	438	21.8 $\pm$ 0.6	13.0 $\pm$ 1.5	0.007**	60	PC(1-acyl 36:5) + Na ⁺
10	391	93.8 $\pm$ 7.2	53.2 $\pm$ 4.7	0.012*	57	
11	802	30.2 $\pm$ 1.1	17.4 $\pm$ 1.0	0.001**	58	
12	567	76.3 $\pm$ 4.9	44.3 $\pm$ 3.2	0.001**	58	
13	844	31.1 $\pm$ 2.5	20.9 $\pm$ 2.6	0.081*	67	
14	613	13.8 $\pm$ 0.5	10.2 $\pm$ 0.6	0.014**	74	
15	824	25.3 $\pm$ 1.5	19.4 $\pm$ 2.9	0.358*	77	

* denotes significant difference $P < 0.05$ and ** denotes significant difference $P < 0.01$.

Fig. 4 The Direct MS/MS Spectrum of the Precursor Ion at m/z 780.
The ions of the diagnostic fragments suggest that the ion at m/z 780 is that of PC (1-acyl 34:2).

4 CONCLUSION

In this study, we analyzed the dMALDI-MS data related to fatty liver by PCA. We were successful in detecting hitherto an unreported dynamics of some metabolites in the fatty liver sections and have successfully shown the feasibility of PCA using dMALDI-MS data as a tool for analysis of metabolites, or biomarker discovery. This approach can be used in the analysis of human biopsy specimens of fatty liver in future studies.

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