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Biomarker discovery in biological specimens (plasma, hair, liver and kidney) of diabetic mice based upon metabolite profiling using ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry

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ABSTRACT

Background: The number of diabetic patients has recently been increasing worldwide. Diabetes is a multifactorial disorder based on environmental factors and genetic background. In many cases, diabetes is asymptomatic for a long period and the patient is not aware of the disease. Therefore, the potential biomarker(s), leading to the early detection and/or prevention of diabetes mellitus, are strongly required. However, the diagnosis of the prediabetic state in humans is a very difficult issue, because the lifestyle is variable in each person. Although the development of a diagnosis method in humans is the goal of our research, the extraction and structural identification of biomarker candidates in several biological specimens (i.e., plasma, hair, liver and kidney) of ddY strain mice, which undergo naturally occurring diabetes along with aging, were carried out based upon a metabolite profiling study.
Methods: The low-molecular-mass compounds including metabolites in the biological specimens of diabetic mice (ddY-H) and normal mice (ddY-L) were globally separated by ultra-performance liquid chromatography (UPLC) using different reversed-phase columns (i.e., T3-C18 and HS-F5) and detected by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). The biomarker candidates related to diabetes mellitus were extracted from a multivariate statistical analysis, such as an orthogonal partial least-squares-discriminant analysis (OPLS-DA), followed by a database search, such as ChemSpider, KEGG and HMDB.
Results: Many metabolites and unknown compounds in each biological specimen were detected as the biomarker candidates related to diabetic mellitus. Among them, the elucidation of the chemical structures of several possible metabolites, including more than two biological specimens, was carried out along with the comparison of the tandem MS/MS analyses using authentic compounds. One metabolite was clearly identified as N-acetyl-L-leucine based upon the MS/MS spectra and the retention time on the chromatograms.
Conclusions: N-acetyl-L-leucine is an endogenous compound included in all biological specimens (plasma, hair, liver and kidney). Therefore, this metabolite appears to be a potential biomarker candidate related to diabetes. Although the structures of other biomarker candidates have still not yet determined, the present approach based upon a metabolite profiling study using UPLC-ESI-TOF-MS could be helpful for understanding the abnormal state of various diseases.

1. Introduction

"Metabonomics" has been defined as the quantitative measurement of the dynamic multi-parametric response of a living system to pathophysiological stimuli or genetic modification [1,2]. The metabonomics approach, which is a top-down and non-hypothesis-driven analysis, was originally most widely used for the investigation of toxic mechanisms [3,4]. However, the increasing applications of metabonomics now include the study of the identification of biomarkers related to diseases [5–8]. The search for biomarker candidates, which are endogenous metabolites and/or biological molecules found in biological specimens indicating a normal or abnormal state, can be attempted by a metabolite profiling study using the appropriate analytical tools [9–18]. The significant variety and concentration of the distributed metabolites require a sophisticated analytical method to cover the extended compounds.

A number of analytical platforms are currently employed to generate global (untargeted) metabolic profiles required in a

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metabonomic study. Widely used detection techniques in metabonome studies are nuclear magnetic resonance spectroscopy (NMR) [19–22] and mass spectrometry (MS) [23–26]. The application of MS to a metabonomics study is steadily increasing, because MS makes it possible to detect and measure a variety of small biological molecules over a wide dynamic range [27–29]. Various types of MS (e.g., tandem quadrupole (TQ)-MS and time-of-flight (TOF)-MS) combined with liquid chromatography (LC) [30–33], gas chromatography (GC) [34,35] or capillary electrophoresis (CE) [36] are now commonly applied as an analytical tool for metabonome research.

Diabetes mellitus is one of the diseases of interest due to the increasing worldwide prevalence of this condition [37,38]. Diabetes in humans is a multifactorial disorder based on environmental factors and genetic background. In many cases, diabetes is asymptomatic for a long period and the patient is not aware of the disease [39,40]. The diagnosis of the prediabetic state in humans is a very difficult issue because of the variable metabolite components in plasma and urine based upon the lifestyle differences in each person. Sample collection is another difficulty in humans due to ethical considerations. Based on these considerations, animal experiments using ddY-H (spontaneous insulin resistant mice) and ddY-L (non-insulin resistant mice), which were isolated from ddY mice by selective breeding over twenty generations [41], were carried out for the identification of biomarker candidates in the plasma of prediabetic state mice [42]. The ddY-H mice undergo naturally occurring diabetes with aging [41]. The ddY-H mice are attacked by the disease at around 9–13 weeks, while the ddY-L group is healthy up to 20 weeks of testing. Since the symptoms were spontaneously induced without any loading, such as nutritional stress, the ddY-H mice seem to be a useful diabetic model species. In contrast, the ddY-L mice are normal without any diabetic symptoms and used as the control mice.

In a previous study [42], the identification of biomarker candidate(s) related to the prediabetes in mice plasma was performed based upon a metabolite profiling study. The low-molecular-mass metabolites in the plasma of the ddY-H and ddY-L mice at fixed weekly intervals were determined by UPLC separation and ESI-TOF-MS detection. Several biomarker candidates extracted from the differential analysis of the multivariate statistical methods were identified through a database search. Some of them were interesting compounds. Many unknown compounds were also detected as candidates of the prediabetic biomarker. As a series of our study for biomarker discovery related to diseases, we further tried to detect the potential biomarker(s) in diabetes mellitus. The aim of the present research is the identification and comparison of biomarker candidate(s) in several biological specimens, i.e., plasma, hair, liver and kidney, of ddY-H mice. The discovery of the potential biomarker(s) detected in all specimens for the early diagnosis of diabetes mellitus is the goal of this study.

2. Experimental section

2.1. Materials and chemicals

Leucine-enkephalin (a reference of *m/z* value) was purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (CH_3CN), methanol, sodium dodecyl sulfate (SDS) and formic acid (HCOOH) were of LC-MS grade (Wako Pure Chemicals, Osaka, Japan). All other chemicals, such as the metabolites, were of special reagent grade. Deionized and distilled water (H_2O) was used throughout the study (Aquarius PWU-200 automatic water distillation apparatus, Advantec, Tokyo, Japan).

2.2. Mice (ddY-H and ddY-L)

Our own colony of ddY-H (spontaneous insulin resistant mice) and ddY-L (non-insulin resistant mice) [41], isolated from the ddY mice by inbreeding, were used in this study. The mice were maintained on 12 h light/dark cycles with free access to the standard chow pellets (MF diet, Oriental Yeast Co., Ltd.) and water *ad lib*. The animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at the University of Shizuoka.

2.3. Determination of glucose and triglyceride

Twenty ml of blood was obtained from the caudal vein for the determination of the glucose and triglyceride (TG). The serum glucose and TG were measured by the Glucose CII Test-Wako (Wako Pure Chemical Industries, Ltd.) and Triglyceride E Test-Wako (Wako Pure Chemical Industries, Ltd.), respectively.

2.4. Sample collection

Biological specimens (i.e., blood, hair, liver and kidney) were obtained from the mice at 5, 9, 13 and 20 weeks without fasting. The body weight of each mouse was initially measured, and then the back hairs were cut at the root with an electric razor. All blood samples

Table 1
UPLC-ESI-TOF-MS conditions.

UPLC (separation conditions)		
Column	ACQUITY UPLC™ HSS T3-C18 column (1.8 μm, 150 mm × 2.1 mm i.d., Waters)	Discovery® HS-F5 HPLC Column (3 μm, 150 mm × 2.1 mm i.d, SUPELCO)
Mobile phase A	0.1% HCOOH in H_2O	
Mobile phase B	0.1% HCOOH in CH_3CN	
Gradient elution	B% = 0% maintained (0–1 min), 100% linearly increased (1–11 min) and maintained (11–13 min), 0% maintained (13–18 min)	B% = 0% maintained (0–10 min), 100% linearly increased (10–25 min) and maintained (25–30 min), 0% maintained (30 min–40 min)
Column temperature	40 °C	
Flow rate	0.4 ml/min	0.3 ml/min
Injection volume	10 μL	
TOF-MS (LCT Premier XE conditions)		
Polarity	ESI positive (W mode)	
Capillary voltage	3000 V	
Sample cone voltage	10 V	
Desolvation gas flow	700 L/h	
Cone gas flow	50 L/h	
Source temperature	120 °C	
Desolvation temperature	350 °C	
MS range	*m/z* 50–650	