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Abstract

Enhanced detoxification contributes to beneficial effects of dietary restriction in rat as revealed by systems biology

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Seoul National University

Dietary restriction (DR) has many beneficial effects, but the detailed metabolic mechanism remains largely unresolved. For this, systems biology approach involving metabolomics and genomics was applied to identify molecular interaction networks related to effects of DR on multiple tissues. First, the metabolic profiles of urines from control and DR animals were investigated using NMR and LC-MS metabolomics approaches. Multivariate analysis presented distinctive metabolic profiles and marker signals from glucuronide and glycine conjugation pathways in the DR group. Broad profiling of the urine phase II metabolites with neutral loss scanning showed that glucuronide and glycine conjugation metabolites were generally higher in the DR group. The up-regulation
of the phase II detoxifications in the DR group were confirmed by mRNA and protein expression levels of the UGT and GLYAT in actual liver tissue. In addition, the Nrf-2 signaling pathway was shown to be up-regulated, providing a mechanistic clue for the enhanced phase II detoxification in liver tissue. Next, metabolic and genomic profiles of multiple tissues from the control and DR rat were investigated using metabolomics and cDNA microarray. Multi-organ metabolomics markers indicated higher phase II detoxification and distinct energy metabolism in the DR group. Microarray analysis with gene, pathway enrichment, and gene ontology terms provided consistent results for the mechanism revealed by multi-organ metabolomics. In addition, pathway enrichment and gene ontology analysis gave evidence of enhanced phase I detoxification in the DR group as the beneficial effect of DR. Several other markers were also found related to age-associated diseases, such as diabetes, cancers, cardiovascular, neurodegenerative, hepatic, and renal diseases. These were consistent with the histopathology and serum biochemistry results that showed actual beneficial effects of DR in current experimental system. Taken together, metabolomics, microarray, and biochemical studies provide a possible metabolic and genomic perspective in understanding the complex mechanism of the beneficial effects of DR.

Keywords: dietary restriction, metabolomics, microarray, Phase I detoxification, Phase II detoxification, UGT, GLYAT, P450.

Student number: 2012-30772
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This appendix includes the reprint of six published papers. Among them, I was involved as a first or a co-first author in one paper or five papers, respectively. These works were done during my doctor course in Seoul National University under my supervisor Professor Sunghyouk Park.
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General introduction

Dietary restriction (DR), also termed caloric restriction, refers to reduction in the consumption of food, without malnutrition [1]. The earliest study investigating the benefits of DR dates to the 1930s, when McCay and co-workers demonstrate that DR can extend lifespan and delay the onset of age-related diseases in rodents [2]. However, it was not recognized until the 1980s, the DR could be used as a good model to study the mechanism of aging or the inhibitory factors that promote longevity [3]. Since then, several studies have showed that the numerous species including yeasts [4], nematodes [5], fruit flies [6], and rodents [7] benefit from DR. Most notably, a recent 20-year-long study showed that monkeys, the species most closely related to human, similarly benefit from DR [8]. Although there has not been (or could not have been) a systematic study on the effects of DR on human lifespan, several longitudinal studies strongly suggest that DR can affect the lifespan and/or decrease the risk of age-related diseases, such as diabetes, cancers, and neurodegenerative diseases [9-13].

The above reverse correlation between the dietary intake and long term health strongly indicates that the DR’s effects should involve energy metabolism, and that DR induces modification of metabolic pathways. Despite that the relationship between diet and metabolism is straightforward, the mechanisms underlying the beneficial effects of DR are anything but simple. Indeed, after decades of intensive research efforts to understand the mechanisms of DR, several genes have been explored as the key factors for the DR pathway, such as mTOR, IGF-1, AMPK and
SIRT1 (For review, see [14]). Until now, most of them involved in early nutrient sensing steps and cellular growth signaling pathway, and specific metabolic pathways, especially those at the final steps actually responsible for the effects of DR, are largely unknown.

Systems biology seeks to integrate data from genomics, transcriptomics, proteomics, and metabolomics in order to ascertain functional linkages within a biomolecular framework (see Figure 1). By applying visualization tools for analysis, the systems biology approach allows for the construction of a “network of networks”, thereby providing a novel prospective by which biological functions can be evaluated and redefined, at the systems level. [15]. Metabolomics and genomics are the most routinely employed in systems biology due to their advanced high throughput screening technologies. Metabolomics utilize NMR and LC-MS, which are often useful for generating an integrated view of the biochemistry and physiology in complex organisms. Genomics utilize cDNA microarray to permit the simultaneous analysis and linkage of thousands of genes. With technological support, systems biology is an influential approach to understand the principal mechanisms in biology.
**Figure 1** Systems biology overview. Systems biology approaches with related tools were presented. The approaches and tools highlighted with bold were employed in current study.
Part I

Multi-platform urine metabolomics analysis revealed the contribution of phase-II detoxifications for beneficial effect of dietary restriction

I. Introduction

Previous studies have mostly focused on genomic or proteomic changes by DR, instead of directly looking at the alteration of metabolism or metabolites. Metabolomics, which has attained much interest in recent years [16-18], may be a good approach to investigate the effect of DR, with directly obtaining metabolic changes elicited by environmental factors. Compared with genomics or proteomics, which often used extracted DNA or proteins from particular tissues, metabolomics employs body fluids, i.e. urine or blood, which contained the end of products of cellular processes to more systemically reflect metabolic statuses of multiple organs. In particular, urine has been used extensively to investigate the mechanism of external stimuli, i.e, drugs or toxic responses, at most major target organs, such as lung, kidney, liver, or heart [19-25]. Still, metabolomics studies on the DR effects have been very limited. Some few previous ones reported the phenomenological urine metabolic markers changed by DR, without identification and/or validation of specific metabolic pathways reflected at the actual tissue or enzyme levels, leaving them insufficient for understanding the metabolic mechanism of DR [26,27]. In addition, those studies employed either NMR or LC-
MS approach without validation across the two analytical platforms.

Among the metabolic pathways that can directly affect the integrity of multiple organs, hence the long term health of an individual, are phase II detoxification pathways [28]. The phase II detoxification systems are conjugation reactions that attach hydrophilic moieties to reactive metabolites, thus facilitating the elimination of the harmful metabolites from body, ultimately reducing their toxicities (see Figure 2) [29]. The enzymes involved in these processes include sulfotransferase (SULT), glutathione-S-transferase (GST), glycine-N-acyltransferase (GLYAT), and uridine 5'-diphospho-glucuronosyltransferase (UGT) [30]. The beneficial effects of phase II reactions have been particularly studied in relation to the mechanism of healthful dietary ingredients. It is well-believed that many of such foods can prevent cancers, hence the term chemoprevention, by inducing phase II detoxification systems [31-33]. Although DR also substantially reduces the incidence of cancers, the exact mechanism remains elusive.

Here, multi-platform metabolomics were employed to study underlying the mechanism of beneficial effects of DR on rats. Urine metabolomics markers suggest that DR enhances phase II detoxification pathway, which was confirmed by conjugation metabolite profiling and changes in mRNA/protein expression levels of phase II enzymes in actual liver tissue. A possible molecular mechanism was also characterized by showing Nrf-2 pathway activation upon DR. The current study provides new metabolic insights on the beneficial effects of DR as well as a workflow for studying DR’s effects in metabolic perspective.
Figure 2 Mechanism of xenobiotic metabolism.
II. Materials and methods

A. Materials

1. Chemicals and reagents

HPLC-grade acetonitrile and water were purchased from Burdick & Jackson (Morristown, NJ, U.S.A.). Chemicals for NMR and LC-MS analysis were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Vendors for biological reagents are indicated in the corresponding sections.
B. Methods

1. Animal and diets

Male Sprague-Dawley (SD) rats approximately 9-weeks-old were purchased from Orient Bio (Sungnam, Seoul, Korea). The animals were housed under standard laboratory conditions with regulated temperature of 22±2 °C, humidity of 50±5% under a 12 h light/dark cycle. Animal care and all experimental procedures were conducted in accordance with the guide for animal experiments edited by the Korea Academy of Medical Science. Prior to the animal experiment, the rat were allowed to acclimatize for one week within the laboratory. After adaptation, the rats were randomly separated into two groups: control (n = 8) and DR (n = 13). The daily food consumption for the control group was calculated by counting out the remained food from the initially offered food. The DR practice was performed following the established protocols [34]. The DR group received 60% amount of food intake of the control group. The food was offered every Monday, Wednesday, and Friday. The amount of food offered on Friday was one and a half times of that offered on Monday.

2. Urine, blood, and tissue collection

For urine collection, the rats were housed into the metabolic cage once in every two weeks. The metabolic cage was specially designed to avoid fetal contamination. Ice-cold jar with sodium azide was used for urine collection to prevent urine samples from bacteria growth and spoiling of chemical components. The collected
urine samples were frozen and stored at –80 °C for analysis. Three months after dietary restriction practice, all rats were sacrificed under isoflurane anesthesia and liver, kidney, brain, and muscle tissues were collected. Blood was collected using heart puncture at the end of the experiment before sacrifice. One milliliter of collected blood was incubated at room temperature for 60 min, and then centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatants of clear yellow fluid were moved to new centrifuge tube. The serum and the same small parts of the relative tissues of each sacrificed rat were snap-frozen with liquid nitrogen, stored at –80 °C and thawed just before analysis. The rest of the tissues were prepared into paraffin blocks with routine procedures, and were used for histopathological examination.

3. Urine samples preparation for NMR

Urine samples were thawed at room temperature and centrifuged at 13,000 rpm for 10 min to remove insoluble parts. Five hundred microliter of urine was mixed with 50 μL of potassium phosphate buffer (pH = 7.4). After centrifugation at 13,000 rpm for 10 min, 450 μL of supernatants were mixed with 50 μL D_{2}O containing sodium-3-trimethylsilyl-[2,2,3,3-^{2}H_{4}]-1-propionate (TSP, 0.025%, w/v) as internal standard, then placed into a 5 mm NMR tube.

4. NMR Spectroscopic analysis of urine

All one-dimensional spectra of the urine samples were measured on a 500 MHz Bruker Avance spectrometer equipped with a cryogenic triple resonance prove
(Ochang, Korea). 500 MHz machine (VNMRS500) at Varian Inc. Korea’s facility was used for metabolite identification. The acquisition parameters were essentially the same as those previously reported [35-37]. The metabolites were identified using Chenomx (Spectral database; Edmonton, Alberta, Canada) by fitting the experimental spectra to those in the database and comparison with standard compounds.

5. LC-MS analysis of urine

For LC-MS analysis, the thawed urine samples were centrifuged at 15,000 rpm for 10 min to remove the insoluble materials, and then the supernatants were injected with an injection volume of 5 μL. The LC-MS methods utilizes an HPLC system (Agilent 1100 Series) using a Kinetex C18 analytical column (100 × 4.6 mm, 2.6 μm, Phenomenex, CA, U.S.A.) in conjugation with additional a binary pump, an auto-sampler, and a degasser (Agilent, CO, U.S.A.). During the separation, the temperature of column and auto-sampler sets at 35 °C and 4 °C, respectively. The mobile phase was a mixture of 0.1% (v/v) formic acid in distilled water (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). With a binary mobile phase, the injected samples were delivered at a flow rate of 0.35 mL/min and the entire eluent was carried into a mass spectrometer. The gradient of phase B was as follows: 0% B at 0 min, 25% B at 14 min, 100% B at 23 min, 100% B at 28.50 min, 0% B at 29 min, and 0% B at 35 min.

For MS analysis part, an LTQ XL high performance linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) equipped with
an electrospray (ESI) source operated in positive ion mode was utilized. The operating conditions of the mass spectrometer were as follows: an ion spray voltage of 5 kV, a heated capillary temperature of 275 °C, and a high purity nitrogen (> 99%) dry gas was used for the sheath gas, auxiliary gas, and sweep gas, and the relative pressures set at 35, 10, and 2 (arbitrary units), respectively. Full scanning analyses were performed in the range of m/z 75-1000, and 35 V normalized collision energy was used for MS/MS (MS²). For neutral loss scanning, data-dependent scanning was started when the neutral loss of glucuronide and glycine were detected as a decrease in m/z ratio of 176 (glucuronide) and 57 (glycine) Da. The chromatographic and mass spectral functions were controlled by the Xcalibur software (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Identification of metabolites were established using m/z values and MS² fragmentation patterns which were compared to those in the HMDB (www.hmdb.ca), METLIN (metlin.scripps.edu) and Massbank (www.massbank.jp) databases. These identifications were further confirmed using standard compounds for all but two metabolites (phenylalanylhydroxy-proline and hydroxymethoxy-indole-glucuronide). For these two, ultra-high resolution MS spectra with 15T FT-ICR (with the resolution of 3,000,000 and accuracy of 0.2 ppm) were obtained. The experiment gave molecular formula for each measured m/z value (340.10269, C₁₅H₁₅NO₈ for hydroxymethoxy-indole-glucuronide and 279.13393, C₁₄H₁₉N₂O₄ for phenylalanylhydroxy-proline), which matched nicely with the calculated monoisotopic masses of the metabolites, 340.102693 and 279.133933, respectively.
6. Multivariate data and statistical analysis

All the obtained time domain NMR data were Fourier transformed, phase corrected, and baseline corrected manually. The region (0.4-10.0 ppm) was used with the exclusion of water (4.6-5.0 ppm) and urea (5.6-6.0 ppm). One dimensional NMR chemical shifts were normalized against total integration values and 0.025% TSP, and then binned at a 0.044 ppm interval to reduce the complexity of the NMR data for pattern recognition. The MS raw data were processed using the 2.2 version of the MZmine software [38]. The peak detection was achieved consecutively using the chromatogram builder and peak deconvolution functions. After performing peak detection, peak list of individual samples were aligned using the RANSAC aligner method. Retention time, m/z ratio, and peak height of the resulting peak list were then exported as a csv file. The signals were, and then converted to an ascii text file. The binning, normalization, and conversion were done using a Perl software written in-house. The resultant data sets were then imported into SIMCA-P version 11.0 (Umetrics, Umeå, Sweden) and mean-centered with Pareto scaling for multivariate statistical analysis. All experiments were repeated at least three times. Data are presented as the means ± SD for the indicated number of independently performed experiments. Statistical significance (p < 0.05) was assessed by student’s t-test and Mann-Whitney U-test.

7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from liver tissue by easy-spin™ Total RNA Extraction Kit (Intron, Seoul, Korea). The first-strand cDNA was synthesized by High
Capacity cDNA Reverse Transcription Kit (Applied Biosystem, U.S.A.) following the manufacturer’s instruction. The primer sequences of UGT1A, UGT2B, and GLYAT were as follows: UGT1A sense: 5’-ACACCGGAACTAGACCATCG-3’; antisense: 3’-TGGAACCCCATTGCATATT-5’; UGT2B sense: 5’-ATGCGCCACAAGGGGC-3’; antisense: 3’-GCAGGAATCCACACATCCAGAGTG-5’; GLYAT sense: 5’-CCATGGAAACCCATTCAATC-3’; antisense: 3’-GTGGGACTGGGACTTTGAA-5’. The predicted sizes were 153 bp, 91 bp, and 223 bp, respectively. Beta-actin was used as control, the sense and antisense primers of which were 5’-AGCCATGTACGTAGCCATCC-3’ and 3’-CTCTCAGCTGTGGTGTAAGTG-5’, respectively. The predicted size was 228 bp. The PCR mixtures contained 1 μL of cDNA synthesized from 2 μg of total RNA, 3.2 μL of dNTP, 2 μL of 10 × ExTaq buffer, 0.1 μL of ExTaq DNA polymerase (Takara, Japan), and each 20 pmol of sense and antisense primers from UGT1A, UGT2B, GLYAT, and beta-actin in total volume of 20 μL. The PCR was performed with the following steps: Initial denaturation at 95 °C for 5 min, followed by 33 cycles (denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min) and final extension at 72 °C for 10 min. The PCR products were separated on a 1% agarose gel by electrophoresis.

8. Western blot

For western blot, 60 mg of liver tissue was measured and ground into powder using mortar under liquid nitrogen. The powders of liver tissue were suspended in 1 mL of RIPA buffer containing protease inhibitor (2 μg/mL of Aprotinin, 1 μg/mL
of Pepstatin and 1 mM of PMSF), and put on ice for 30 min. After centrifugation at 13,000 rpm for 30 min, the supernatant containing the total protein released from liver tissue was quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, U.S.A.). Twenty five micrograms of total protein was subjected to 10% SDS/PAGE, and the resolved proteins were transferred to nitrocellulose membranes (Bio-Rad, CA, U.S.A.). The membranes were blocked with blocking buffer (5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST)) for 1 h at room temperature. After washing three times with PBST, the membrane was blotted with antibodies against UGT1A, UGT2B, multidrug resistance-associated protein 3 (MRP-3), and NAD(P)H dehydrogenase 1 (NQO-1) (1:1,000; Santa Cruz Biotechnology, CA, U.S.A.), Nrf-2 (1:100; Santa Cruz Biotechnology, CA, U.S.A.), heme oxygenase-1 (HO-1) (1:1,000; Enzo Life Sciences, MI, U.S.A.), followed by treatment with anti-mouse, anti-goat, or anti-rabbit (1:10,000; Santa Cruz Biotechnology, CA, U.S.A.) secondary antibodies conjugated with HRP at RT for 1 h. Blots were analyzed by LAS 3000 (Fuji Film Corp., Tokyo, Japan).

9. Liver tissue histopathology and serum biochemistry

After fixation for 48 h, liver tissues were embedded in paraffin according to routine procedures. Four-micrometer thick sections were cut and stained with hematoxylin-eosin (H&E) and periodic acid-schiff (PAS) for histopathological evaluation. An expert pathologist at Inha University Hospital blindly analyzed the tissue slices. Serum ALT, AST, ALP, TG, LDL, and insulin levels were measured using commercial kits at Inha University hospital (Incheon, Korea).
III. Results

A. General assessment of DR effects

To confirm whether the dietary restriction (DR) procedure is in corrective progress, the general parameters, such as body weight, low density lipoprotein (LDL), and triglyceride (TG), were measured. As shown in Figure 3, the body weight was time dependently reduced in the DR group. In addition, levels of LDL and TG were also significantly decreased after three months’ of DR. As LDL and TG are two important risk factors for age-associated disease, ensuring that DR procedure induced an obvious difference between the control and DR groups, indicating that current conditions worked well as expected.
Figure 3 General assessment of DR effects. Weekly body weight changes were measured with a scale in the animal care facility (control: black filled square; DR: black filled circle). Low density lipoprotein (LDL) and triglyceride (TG) levels were measured at Inha University hospital (Incheon, Korea).
B. NMR and LC-MS analysis of urine samples

Dietary restriction is one style of diet, and in addition, the diet directly influences metabolic pathways. So it can be supposed that DR may mediate the metabolism directly. Therefore, the metabolomics is just the right approach to characterize metabolic alteration and investigate the metabolism. Because the metabolomics is concerned with the comprehensive analysis of metabolites as a whole in a given sample [39]. In addition, NMR and LC-MS are used as two canonical detection tools for metabolomics profiling, and each one has its own merits. NMR has advantages of being non-destructive and applicable to determination of molecular structures, even in mixture samples [40], but has the disadvantage of low sensitivity [41]. In contrast, LC-MS is a highly sensitive detection technique that ionizes the sample components, with the function of separating the resulting ions in vacuum based on their mass-to-charge ratios and measuring the intensity of each ion [42]. Therefore, an approach combining NMR and LC-MS is a relatively complemented method.

To characterize the effect of DR, urine metabolic profiling of the control and DR groups were measured using NMR and LC-MS-based metabolomics analysis. Urine samples can well represent the systemic effects and successfully applicable in several organism metabolomics studies [20,24,25,43,44]. Representative \(^1\)H NMR spectra with identified metabolites (Figure 4A and 4B) and an LC-MS chromatogram (Figure. 4C and 4D) are shown. A number of compounds were identified in the urine as a preliminary step in finding contribution signals. For
NMR, the assignments were established using the spectra of the standard compounds and Chenomx database (Edmonton, Alberta, Canada) (see Table 1). For LC-MS, identification of metabolites were established using m/z values and MS\(^2\) fragmentation patterns which were compared to those in the HMDB (www.hmdb.ca), METLIN (metlin.scripps.edu), and Massbank (www.massbank.jp) databases (see both Table 1 and Table 2). As it is not possible to point out the differences in metabolic profile between the control and DR groups using simple visual inspection, a multivariate statistical analysis was performed to analyze in a more holistic way and to identify the contribution signals for group classification.
Figure 4 Representative $^1$H NMR and LC-MS spectra of urine collected from the control and DR groups. The NMR spectra were taken for urine samples containing 150 mM phosphate buffer (pH 7.4) and 0.025% TSP as an internal standard for the control (A) and DR (B) groups. The LC-MS experiments were performed with injection of 5 μL urine from the control (C) and DR (D) groups. The numbers on the spectra indicate assigned peaks corresponding to the metabolites listed in Table 1. For NMR, the assignments were established using the spectra of the standard compounds and Chenomx database (Edmonton, Alberta, Canada).
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Structural identifiers</th>
<th>p-value</th>
<th>Fold change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1-Methylhistidine†</td>
<td>3.20(m), 3.25(m), 7.06(s)</td>
<td>4.85E-4</td>
<td>83.46</td>
</tr>
<tr>
<td>2 α-ketoglutarate</td>
<td>2.45(t), 3.02(t)</td>
<td>0.045</td>
<td>-22.60</td>
</tr>
<tr>
<td>3 3-indoxylsulfate</td>
<td>7.20(t), 7.28(t), 7.37(s), 7.51(d), 7.70(d)</td>
<td>0.054</td>
<td>49.86</td>
</tr>
<tr>
<td>4 3-Methylglutarate</td>
<td>0.93(d), 2.01(d), 2.26(m)</td>
<td>0.943</td>
<td>-0.52</td>
</tr>
<tr>
<td>5 Acetate</td>
<td>1.93(s)</td>
<td>0.750</td>
<td>7.01</td>
</tr>
<tr>
<td>6 Acetoacetate</td>
<td>2.30(s), 3.52(s)</td>
<td>0.012</td>
<td>31.23</td>
</tr>
<tr>
<td>7 Adenine†</td>
<td>8.32(s), 8.38(s)</td>
<td>0.710</td>
<td>16.23</td>
</tr>
<tr>
<td>8 Alanine†</td>
<td>1.49(d), 3.80(m)</td>
<td>0.063</td>
<td>-12.44</td>
</tr>
<tr>
<td>9 Allantoin†</td>
<td>5.38(s), 6.07(s)</td>
<td>0.129</td>
<td>23.92</td>
</tr>
<tr>
<td>10 Arginine†</td>
<td>1.68(m), 1.93(m)</td>
<td>0.036</td>
<td>-9.42</td>
</tr>
<tr>
<td>11 Citrate†</td>
<td>2.55(d), 2.69(d)</td>
<td>0.078</td>
<td>-47.57</td>
</tr>
<tr>
<td>12 Dimethylamine</td>
<td>2.73(s)</td>
<td>0.273</td>
<td>-8.13</td>
</tr>
<tr>
<td>13 Ethanol</td>
<td>1.17(t), 3.63(q)</td>
<td>0.140</td>
<td>14.60</td>
</tr>
<tr>
<td>14 Formate</td>
<td>8.47(s)</td>
<td>0.292</td>
<td>-19.42</td>
</tr>
<tr>
<td>15 Fucose</td>
<td>1.21(d), 1.24(d)</td>
<td>0.798</td>
<td>-1.68</td>
</tr>
<tr>
<td>16 Glycine</td>
<td>3.60(s)</td>
<td>0.704</td>
<td>-22.13</td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td>1H (ppm)</td>
<td>31P (ppm)</td>
</tr>
<tr>
<td>---</td>
<td>------------------------</td>
<td>-------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>17</td>
<td>Guanidoacetate†</td>
<td>3.80(s)</td>
<td>0.228</td>
</tr>
<tr>
<td>18</td>
<td>Hippurate†</td>
<td>3.97(d), 7.55(t), 7.64(t), 7.84(d), 8.54(s)</td>
<td>0.856</td>
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<tr>
<td>19</td>
<td>Indole-3-acetate†</td>
<td>7.13 – 7.30(m), 7.51(d)</td>
<td>0.093</td>
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<tr>
<td>20</td>
<td>Lactate†</td>
<td>1.35(d), 4.13(m)</td>
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<tr>
<td>21</td>
<td>N,N-Dimethylglycine†</td>
<td>2.93(s), 3.73(s)</td>
<td>0.018</td>
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<tr>
<td>22</td>
<td>N-Acetylaspartate</td>
<td>2.04(s), 2.53(m), 2.70(m), 4.40(m)</td>
<td>0.251</td>
</tr>
<tr>
<td>23</td>
<td>Pyridoxine†</td>
<td>2.41(s), 7.68(s)</td>
<td>0.243</td>
</tr>
<tr>
<td>24</td>
<td>Pyruvate</td>
<td>2.38(s)</td>
<td>0.714</td>
</tr>
<tr>
<td>25</td>
<td>Succinate†</td>
<td>2.41(s)</td>
<td>0.243</td>
</tr>
<tr>
<td>26</td>
<td>Taurine†</td>
<td>3.28(t), 3.43(t)</td>
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</tr>
<tr>
<td>27</td>
<td>Trigonelline†</td>
<td>4.44(s), 8.84(t), 9.13(s)</td>
<td>0.869</td>
</tr>
<tr>
<td>28</td>
<td>Tyrosine†</td>
<td>6.87(d), 7.19(d)</td>
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<tr>
<td>29</td>
<td>Uracil†‡</td>
<td>5.72(s)</td>
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<tr>
<td>30</td>
<td>Urea‡</td>
<td>5.81(s)</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>p-Cresol</td>
<td>2.25(s), 6.87(d)</td>
<td>0.393</td>
</tr>
<tr>
<td>32</td>
<td>Trans-Aconitate</td>
<td>3.49(s), 6.53(s)</td>
<td>0.531</td>
</tr>
<tr>
<td>33</td>
<td>Methylhippurate</td>
<td>249.20 17.55 249, 119, 3.03E-5</td>
<td>138.81</td>
</tr>
<tr>
<td>34</td>
<td>Phenylacetylglucine†</td>
<td>194.17 16.44 176, 76, 1.32E-4</td>
<td>86.78</td>
</tr>
<tr>
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</tr>
</tbody>
</table>

★ indicates significant peaks; † indicates metabolites; ‡ indicates stable isotopes.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mass (m/z)</th>
<th>Ret. time</th>
<th>Daughter ions</th>
<th>Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine†</td>
<td>114.19</td>
<td>2.91</td>
<td>114, 86</td>
<td>1.44E-3</td>
<td>18.90</td>
</tr>
<tr>
<td></td>
<td>3.05(s), 4.05(s)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hydroxymethoxy-indole-glucuronide</td>
<td>340.22</td>
<td>16.0</td>
<td>164, 146, 122, 118</td>
<td>5.13E-4</td>
<td>103.78</td>
</tr>
<tr>
<td>Methoxytyrosine</td>
<td>212.16</td>
<td>3.947</td>
<td>194, 166, 153, 109</td>
<td>8.03E-4</td>
<td>-11.15</td>
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<tr>
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<td></td>
</tr>
</tbody>
</table>

* The associated signal numbers (see Figure 4), structural identifiers (NMR: ppm and J values; MS: m/z, Ret. time and daughter ions), and Mann-Whitney U-test (a star with $p < 0.05$) are presented. The fold change values are from area-normalized peak intensities and represent percent changes of the DR group with respect to the control values, with negative values for the decrease and positive for the increase. All of the NMR-identified metabolites and LC-MS-identified metabolites with significant changes are included. Additional metabolites identified with LC-MS without significant changes are listed in the Table 2. All the metabolites were confirmed with standard compounds and MS/MS analysis except for those two that were confirmed with 15T FT-ICR (see the method section for details).

† They were detected both in the NMR and LC-MS.

‡ These were not used in the normalization process.
Table 2 Additional metabolites identified with LC-MS analysis*.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>RT (min)</th>
<th>m/z</th>
<th>MW</th>
<th>Chemical Formula</th>
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</thead>
<tbody>
<tr>
<td>1-Methyladenosine</td>
<td>6.17</td>
<td>282.19</td>
<td>281.1241</td>
<td>$\text{C}<em>{11}\text{H}</em>{15}\text{N}<em>{5}\text{O}</em>{4}$</td>
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<tr>
<td>1-Methylnicotinamide</td>
<td>2.85</td>
<td>137.10</td>
<td>136.06366</td>
<td>$\text{C}<em>{7}\text{H}</em>{8}\text{N}_{2}\text{O}$</td>
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<tr>
<td>7-Methylguanidine</td>
<td>6.23</td>
<td>166.15</td>
<td>165.0651</td>
<td>$\text{C}<em>{6}\text{H}</em>{7}\text{N}_{3}\text{O}$</td>
</tr>
<tr>
<td>Betaine</td>
<td>2.90</td>
<td>118.14</td>
<td>117.07898</td>
<td>$\text{C}<em>{5}\text{H}</em>{11}\text{NO}$</td>
</tr>
<tr>
<td>Choline</td>
<td>2.80</td>
<td>104.17</td>
<td>103.09971</td>
<td>$\text{C}<em>{5}\text{H}</em>{13}\text{NO}$</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>4.80</td>
<td>228.17</td>
<td>227.09060</td>
<td>$\text{C}<em>{9}\text{H}</em>{13}\text{N}<em>{3}\text{O}</em>{4}$</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>2.90</td>
<td>106.17</td>
<td>105.07898</td>
<td>$\text{C}<em>{4}\text{H}</em>{13}\text{NO}_{2}$</td>
</tr>
<tr>
<td>Homoarginine</td>
<td>3.32</td>
<td>189.17</td>
<td>188.12733</td>
<td>$\text{C}<em>{7}\text{H}</em>{16}\text{N}<em>{4}\text{O}</em>{2}$</td>
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<tr>
<td>Hypoxanthine</td>
<td>2.85</td>
<td>137.15</td>
<td>136.03851</td>
<td>$\text{C}<em>{5}\text{H}</em>{4}\text{N}<em>{2}\text{O}</em>{4}$</td>
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<tr>
<td>L-Carnitine</td>
<td>2.86</td>
<td>162.15</td>
<td>161.10519</td>
<td>$\text{C}<em>{7}\text{H}</em>{13}\text{NO}_{3}$</td>
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<tr>
<td>L-Citrulline</td>
<td>21.12</td>
<td>176.14</td>
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<td>$\text{C}<em>{6}\text{H}</em>{15}\text{N}<em>{3}\text{O}</em>{3}$</td>
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<tr>
<td>L-Leucine</td>
<td>6.49</td>
<td>132.14</td>
<td>131.09463</td>
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<tr>
<td>L-Methionine</td>
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<td>149.05105</td>
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<tr>
<td>L-Phenylalanine</td>
<td>9.65</td>
<td>166.16</td>
<td>165.07898</td>
<td>$\text{C}<em>{9}\text{H}</em>{11}\text{NO}_{2}$</td>
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<tr>
<td>L-Tryptophan</td>
<td>6.49</td>
<td>205.04</td>
<td>204.08988</td>
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</tr>
<tr>
<td>L-Valine</td>
<td>2.90</td>
<td>118.11</td>
<td>117.07898</td>
<td>$\text{C}<em>{5}\text{H}</em>{11}\text{NO}_{2}$</td>
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<tr>
<td>N-Acetylglutamine</td>
<td>4.32</td>
<td>189.18</td>
<td>188.07971</td>
<td>$\text{C}<em>{7}\text{H}</em>{15}\text{N}<em>{2}\text{O}</em>{4}$</td>
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<tr>
<td>N8-Acetylspermidine</td>
<td>2.73</td>
<td>188.26</td>
<td>187.16853</td>
<td>$\text{C}<em>{9}\text{H}</em>{21}\text{N}_{3}\text{O}$</td>
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<tr>
<td>Phenylalanylhydroxy-</td>
<td>16.14</td>
<td>279.27</td>
<td>278.12672</td>
<td>$\text{C}<em>{14}\text{H}</em>{18}\text{N}<em>{2}\text{O}</em>{4}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retention Time (RT)</td>
<td>m/z</td>
<td>Molecular Weight (MW)</td>
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<tr>
<td>---</td>
<td>-------------------------</td>
<td>---------------------</td>
<td>---------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>20</td>
<td>Pyroglutamic acid</td>
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<td>130.0479</td>
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<tr>
<td>21</td>
<td>Riboflavin</td>
<td>15.83</td>
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<td>376.13827</td>
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<tr>
<td>22</td>
<td>Tyrosine</td>
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<tr>
<td>23</td>
<td>Tyramine</td>
<td>3.19</td>
<td>138.07</td>
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<tr>
<td>24</td>
<td>Uric acid</td>
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<td>168.02834</td>
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<tr>
<td>25</td>
<td>Xanthurenic acid</td>
<td>13.24</td>
<td>206.12</td>
<td>205.0375</td>
</tr>
</tbody>
</table>

* The retention time (RT), m/z, molecular weight (MW), and chemical formula are indicated.
C. OPLS-DA multivariate analysis

To perform group separation and identify the contribution signals, the orthogonal projections to latent structure-discrimination analysis (OPLSDA) multivariate analysis were carried out using entire NMR and LC-MS data. The OPLS-DA model with NMR and LC-MS data were constructed, respectively (see Figure 5A and 5B). Each profile of samples is represented by one symbol, filled circle for control and open triangle for DR samples. In constructing this plot, the entire NMR or LC-MS data are reduced into just two or three variables by matrix rotation, and each dot tells the characteristics of the metabolic profile of each sample. Therefore, these so-called score plots can show how well these groups can be separated. Commonly, the OPLS-DA model applied for classification according to group differences. Because the between group variation can be supervised through x axis. As OPLS-DA is supervised multivariate analysis, it can classify groups in the presence of large structured noise and intra-group variation. As shown in Figure 5A and 5B, the discrimination model for both NMR and LC-MS data can be separated clearly between the control and DR groups without any overlaps with the following statistical characteristics. For NMR, the model had one predictive and three orthogonal components with $Q^2(Y) = 0.637$, $R^2(Y) = 0.936$, and total $R^2(X) = 0.659$, with 0.112 being predictive and 0.547 being orthogonal. For LC-MS, there were one predictive and two orthogonal components with $Q^2(Y) = 0.809$, $R^2(Y) = 0.968$, and total $R^2(X) = 0.508$, with 0.205 being predictive and 0.303 orthogonal. These results indicate that OPLS-DA approach is a proper and efficient method for
differentiation of the control and DR groups, and current model is trustworthy according to high values of goodness-of-fit.
Figure 5 Differentiation of the control and DR groups using multivariate analysis. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) score plot of the control and DR groups from NMR (A) and LC-MS (B). Filled circles: control group; Open triangles: DR group. The models were established using one predictive and three orthogonal components for NMR and one predictive and two orthogonal components for LC-MS, respectively.
D. Metabolites related to DR

After clear separation between the control and DR groups, identification of the metabolites contributing to the differentiation was tried. The S-plot presenting the modeled correlation ($p_{\text{corr}}$) and covariation ($p$) in a single figure was constructed, allowing us to select significant markers more easily among noisy signals (Figure 6A and 6B). As the contributing signals had higher values for both correlation and covariation, 7.42, 7.36, 7.06, 2.45, and 2.93 ppm for NMR and m/z value of 194.17, 114.19, and 340.22 for LC-MS were picked up. Based on identification results of NMR and LC-MS, these contributing signals belong to phenylacetylglycine (7.42, 7.36 ppm and m/z = 194.17), 1-methylhistidine (7.06 ppm), α-ketoglutarate (2.45 ppm), N,N-dimethylglycine (2.93 ppm), creatinine (m/z = 114.19), and hydroxymethoxy-indole-glucuronide (m/z = 340.22) (see Table 1).

To validate the significance of these marker signals, a Mann-Whitney U-test on the levels of these metabolites were carried out (Figure 6C-J). All of these signals were significantly altered in the DR group compared to those of the control group. These results indicated that these metabolites are statistically meaningful and reliable contributors for group differentiation.
Figure 6 Marker signals contributing to the differentiation between the control and DR groups. S-plot analysis to identify the contributing signals for the control and DR groups from NMR (A) and LC-MS (B). The p represents modeled covariation, and p(corr) represents modeled correlation. Potential marker signals that are significantly biased across the two groups are enclosed in boxes. The levels of the signals identified by the analysis were compared by Mann-Whitney U-test, and the resulting p-values are indicated. 7.36 ppm (C), 7.42 ppm (D) and 7.06 ppm (E) from NMR, m/z = 114.19 (F), m/z = 194.17 (G), and m/z = 340.22 (H) from
LC-MS significantly increased in the DR group; 2.45 ppm (I) and 2.93 ppm (J) from NMR decreased in the DR group. The solid boxes represent the 25 and 75 percentile values with the median value inside it. The whiskers represent outliers with coefficient value 1.5.
E. Profiling of general glucuronide and glycine conjugation via neutral loss scanning

Among these contributors, phenylacetylglucose and hydroxymethoxy-indole-glucuronide are significantly increased in the DR group. In addition, these two metabolites are common phase II detoxification products, assembled by the mechanism of glycine and glucuronide conjugation, respectively. Therefore, the phenomena that many other metabolites created by phase II detoxification reaction are universal distributed in the DR group could be speculated. To validate this speculation, the general metabolites created by glycine and glucuronide conjugation were measured. As a method termed neutral loss scanning of LC-MS is usable to detect these metabolites, LC-MS to scan the overall profiles of compounds with 176 (glucuronide) and 57 (glycine) were applied. As shown in Figure 7A-7D, the numbers and the intensities of the peaks are much larger in the DR group. To confirm the statistical validity, the integrations of peak area are compared between the control and DR groups using student’s t-test. As shown in Figure 7E and 7F, the peak areas for both glucuronide and glycine are significantly increased in the DR group with the p-values of 0.017 or 0.011, respectively, indicating that the general metabolites created by the mechanism of glycine and glucuronide conjugation were enhanced in the DR group. These results confirmed that the DR group performed higher activity in phase II detoxification pathways.
Figure 7 Neutral loss scanning for general assessment of glucuronide and glycine conjugation reactions. Glucuronide (A & B) and glycine (C & D) conjugation profiling based on neutral loss scanning of m/z 176 (glucuronide) and 57 (glycine) in the control (A & C) and DR (B & D) groups. The MS² step was carried out using 35 V normalized collision energy. The neutral fragment plots show the retention times and intensities of compounds which experience the loss of a specified common neutral (m/z 176 and 57) fragment. The numbers on the peaks indicate tentative assignments of conjugated metabolites (1: Tyramine glucuronide; 2: Indole acetylglycine). The peak area obtained from the result of neutral loss scanning of glucuronide (E) and glycine (F) in the control and DR groups were calculated. The levels were compared with student’s t-test and the resulting p-values are indicated.
F. Assessment of phase II detoxification pathways in liver tissue

As the urine metabolite profile showed that the general metabolites created by glycine and glucuronide conjugation reactions were enhanced in the DR group, whether this phenomenon is observed the same in the actual tissues was another question. To confirm the levels of the two key markers, hydroxymethoxy-indole-glucuronide and phenylacetylglycine were decided to be measured in liver tissue, as liver tissue is the main organ for the phase II detoxification pathways. The LC-MS measurement of the levels of these metabolites showed that both of two markers were elevated in liver tissue from the DR group (Figure 8), consistent with the result from urine, indicating that the urine metabolomics results adequately reflected the metabolic changes in liver tissue. For more detailed characterization of the pathway, the enhanced conjugation reactions at the metabolic enzyme level were investigated. The mRNA levels of the phase II detoxification primary enzymes for glucuronide and glycine conjugation reaction were compared, such as Uridine 5'-diphospho-glucuronosyltransferase 1A (UGT1A), 2B (UGT2B), and Glycine-N-acyltransferase (GLYAT), respectively. As shown in Figure 9A and 9C, all the enzymes had significantly enhanced mRNA expression in the DR group. Subsequently, the protein levels of these enzymes were measured via western blot. Both UGT1A and UGT2B showed similarly enhanced protein levels in the DR group (Figure 9B and 9D), consistent with the mRNA level. Although the GLYAT enzyme level could not be directly measured because of the failure of all available
antibodies (data not shown), the increased mRNA level should reflect the protein level, because phase II enzymes are generally transcriptionally regulated [31], as shown for UGT1A and UGT2B. These data confirm that phase II detoxification pathways, particularly glucuronide and glycine conjugation, in the relevant organ (the liver) were up-regulated in the DR group.
Figure 8 Levels of the two marker products of phase II detoxification reactions in liver tissue. Phenylacetylglycine (PAG) and hydroxymethoxy-indole-glucuronide (HMI-glucuronide) were measured in liver tissue using LC-MS. The levels of the signals identified by the analysis were compared with student’s t-test and the resulting p-values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control: n = 8 and DR: n = 13).
Figure 9 Levels of the phase II detoxification enzymes for glucuronide and glycine conjugation in liver tissue. Reverse transcription-PCR (A) and western blot (B) represent the mRNA and the expressed protein levels of the phase II detoxification enzymes for glucuronide and glycine conjugation, such as Uridine 5’-diphospho-glucuronosyltransferase 1A (UGT1A), 2B (UGT2B) and Glycine-N-acyltransferase (GLYAT) in liver tissue. Beta-actin (Actb) was used as control. Bar charts represent the comparison of the mean band intensities for the levels of phase II enzymes in terms of mRNA (C) and protein (D) normalized to that of the Actb. Statistical analysis was performed using student’s t-test, and the resulting p-values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control: n = 8 and DR: n = 13). Western blot result for GLYAT could not be obtained, as none of the available antibodies reacted with rat GLYAT.
G. Biochemical and histopathological changes in liver tissue

To characterize the effects of DR, phenomenon on the integrity of liver tissue was next investigated via blood biochemistry and direct tissue histopathological staining. Alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) levels were measured in blood, which are generally employed to estimate the functional integrity of liver cells (Figure 10A–10C). ALP and AST levels were not much different in both groups, but ALT level was significantly decreased in the DR group. As ALT is commonly measured clinically as a part of a diagnostic evaluation of hepatocellular injury to determine liver health, this finding suggested that liver cells in the DR group were somewhat healthier in a biochemical sense. The histopathological stainings were also carried out to perform the changes in the actual tissue. Hematoxylin-eosin (H&E) staining showed no gross pathophysiological differences between the control and DR groups (Figure 10D and 10E), but the DR group exhibited noticeably denser cytoplasm, which might have been due to a decrease in glycogen granules. To confirm this, periodic acid-Schiff (PAS) staining was performed, and the DR group showed glycogen depletion relative to the control group (Figure 10F and 10G). This might well be due to reduced insulin level, which is one of the well-documented beneficial effects of DR [45-47]. To test this, insulin level was measured in blood samples. Consistent with the speculation, the insulin level is indeed significantly decreased in the DR group (Figure 11). Overall, DR induced glycogen depletion in liver tissue due to lower secretion of insulin, which promotes the absorption of glucose.
from the blood to actual tissue. These results indicate that DR induces beneficial biochemical and metabolic changes in liver tissue.
Figure 10 Blood biochemistry and tissue histopathological staining. Serum alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) activities were measured using commercial kits employing spectrophotometric assays. Average values of ALP (A), AST (B), and ALT (C) levels of each group are plotted along with their standard deviations. Student’s t-test was also performed and the resulting p-values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control: n = 8 and DR: n = 13). H&E staining and PAS staining of liver tissues were performed on paraffin block of the samples: H&E staining control (D) and DR (E) groups (200X); PAS staining control (F) and DR (G) groups (100X).
Figure 11 Absolute concentration of insulin in rat serum from the control and DR groups. Absolute concentration of insulin in rat serum was measured using commercially available kit following factory instruction. The concentration of the insulin was compared with student’s t-test and the resulting p-value is indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control: n = 8 and DR: n = 13).
H. Up-regulation of Nrf-2 signaling pathway

As transcriptional control of the expression of phase II enzymes is mediated by transcriptional factor Nrf-2, Nrf-2 signaling pathways is important to activate phase II detoxification metabolism [48,49]. Nrf-2 is also important in protecting cells from oxidative stress [50]. When cell is activated by extracellular stimulation, Nrf-2 translocates into the nucleus and regulates its downstream target enzymes, such as Heme oxygenase 1 (HO-1), Multidrug resistance-associated proteins 3 (MRP-3), and NAD(P)H quinone oxidoreductase 1 (NQO-1), also including phase II enzymes. As phase II enzymes are enhanced in the DR group, Nrf-2 pathway was decided to be investigated more in detail. To compare the activation status of the Nrf-2 pathway in the control and DR groups, the expression levels of Nrf-2 and its downstream targets were measured in liver tissue. As shown in Figure 12, Nrf-2 and its downstream targets (except NQO-1), are elevated in the DR group, performing that the Nrf-2 signaling pathway was up-regulated in the DR group. These results suggest that enhanced phase II detoxification in liver tissue occur in the DR group, at least in part, through activation of the Nrf-2 signaling pathway.
Figure 12 Up-regulation of the Nrf-2 signaling pathway. Western blots of Nrf-2 (A), HO-1 (B), MRP-3 (C), and NQO-1 (D) from the liver homogenates of the control and DR groups. Beta-actin (Actb) was used as loading control. Bar charts below the blots represent the comparison of the mean band intensities for the levels of the proteins normalized to that of the Actb. Statistical analysis was performed using student’s t-test, and the resulting p-values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control: n = 8 and DR: n = 13).
IV. Discussion

To identify the marker metabolites for DR, NMR and LC-MS analysis were carried out, most often used independently in metabolomics studies. NMR has merits in quantitation and reproducibility, and LC-MS in terms of sensitivity [51], and use of both platforms should be complementing for each other at one time. In this study, the use of both approaches expanded the coverage of the markers, and gave more reliability to them. For instance, hydroxymethoxy-indole-glucuronide, an important marker metabolite involved in glucuronidation, was detected by LC-MS, and phenylacetylglycine, that gave the idea of enhanced glycine conjugation, was detected with both techniques. Creatinine was also detected by both methods, and 1-methylhistidine was observed in NMR. Although new metabolites were not found out, use of both platforms for metabolomics will perform great potential for finding new markers and give reliable results. Hence, it is desirable to see more scientists utilize these complementary techniques for metabolomics studies.

The creatinine level was also increased in the DR group, which is consistent with previous results [27,52].Measurement of urine creatinine has been applied as a criterion for kidney functions, but the increase in this study seems to be according to increase in muscle protein degradation in the DR condition rather than kidney impairment. This is due to the fact that the amounts of collected urine, which often decreases in conditions with decreased urinary creatinine, are similar in both groups. Moreover, the level of 1-methylhistidine, which is a degradation product of muscular actin and myosin and known to be increasing with muscle break-down,
was higher in the DR group. Therefore, these two metabolic markers seem to show the phenomenon of DR, and may not contradict with the beneficial effects of DR.

In the current study, the enhancement of phase II metabolism and Nrf-2 pathway in liver tissue of the DR group were performed, initiated by metabolomics identification of the increased two marker metabolites, such as hydroxymethoxy-indole-glucuronide and phenylacetylglycine, in urines of the DR group. Prior to the non-targeted metabolomics study, non-speculation was generated on the DR’s effects, in contrast, a hypothesis of increased phase II reactions was inferred according to the identified two markers with larger number of animals (n = 8 for control and n = 13 for DR). In addition, the hypothesis of general enhancement in glucuronide and glycine conjugation reaction was proven by global profiling of the conjugated metabolites with LC-MS-based neutral loss scanning. Still, the increased levels of the above two marker metabolites and the essential phase II detoxification enzymes, such as UGT and GLYAT, were performed in liver tissue. Equally importantly, the mechanism of DR’s effects was observed by finding that the Nrf-2 pathway is up-regulated in the DR group, leading to the phase II enhancement. Integration of these results makes good sense that enhanced urinary elimination of toxic metabolites contributes to the beneficial effects of DR. In this experimental frame, the decreased serum ALT obtained from serum biochemistry may well reflect the role of protection on liver cells by the enhanced phase II detoxification.

The beneficial roles of phase II detoxification pathway can be also involved in cancer prevention [31-33]. For instance, limonene and sobrerol, monocyclic
monoterpenoid compounds, have chemoprevention activity at the initiation stage of carcinogenesis in a rat breast cancer model by inducing the phase II enzyme activities [53], and garden cress ingredients prevented the preneoplastic lesion in a rat xenograft model through enhanced UGT activities [54]. Therefore, the enhanced phase II detoxification pathway seems to be involved in reduction of cancer occurrence rate in another years-long experiment, although it was not directly measured in the current months-long experiment.

Very interesting genetic variability and disorders support the importance of phase II metabolism, especially glucuronidation, in maintaining healthy life for living organisms that experience constant exposure to xenobiotics through lifetime. Cats are very susceptible to drugs at the low levels, while it shows non-toxic response to other species at the same level [55,56], because of the uncommon low activity of UGT in cats [57,58]. Additionally, acetaminophen is quite toxic to a minor population of humans though generally safe to humans, indicating that the variability in glucuronidation of the drug among individuals induced above result [59,60]. Considering the above, enhanced phase II detoxification could have a role in the mean lifespan of a population.

For C. elegans, enhanced phase II detoxification, to which UGT is a major contributor [30], was actually recommended to be conscientious for anti-aging and lifespan extension [61]. It has been also suggested that a causation of aging is molecular damage caused by toxicants, and that enzymes involved in detoxification systems contribute to longevity assurance [62]. The detoxification enzymes responsible for longevity assurance has been also proposed by cDNA microarray
analysis on daf-2(-) worms [63]. In addition, Nrf-2 ortholog SKN-1 modulate the expression of candidate phase II genes in C. elegans [64], and is required for lifespan extension by DR [65].

For higher organisms, the roles of phase II detoxification in the DR’s beneficial effects are much less studied. In addition, the relationships are more complex between Nrf-2 pathway, its downstream target genes and DR’s effect. For example, Pearson and co-workers suggested that Nrf-2 pathway prevents carcinogen-induced tumor formation in the DR condition but not requires for the life extension [66]. Moreover, the study shows that Nrf-2 downstream target genes, such as NQO-1, HO-1, GCLC, and GST are not uniformly increased in the DR condition. Therefore, specific downstream target genes of Nrf-2 signaling involved in DR’s effect should be evaluated. In current observation, NQO-1 presented insignificant alteration, in contrast, Nrf-2 and its targets HO-1 and MRP-3 were significantly increased, indicating that the latter are the specific signature of the DR-related Nrf-2 pathway activation in rat.

Based on serum biochemistry, several effects of DR related with liver functions were performed (lower serum LDL and TG level, lower glycogen, reduced serum insulin and ALT level) that can ultimately contribute to long term health. The metabolomics and biochemical results show that the enhanced phase II detoxification in liver tissue of the DR group correlates well with the up-regulation of the Nrf-2 signaling pathway by DR. The activation of Nrf-2 pathway was performed by measuring the protein level of Nrf-2 itself and its downstream target gene HO-1 and MRP-3. Specific correlation was also established by directly
measuring the UGT and GLYAT levels from liver tissue. Overall, DR increases the Nrf-2 level and its downstream target gene that turns up phase II detoxification genes in liver tissue, which contributes to the beneficial effects of DR (Figure 13).

As the complexity of the mechanism of DR, the phase II detoxification pathway does not interpret all of the DR’s beneficial effects, and other important metabolic pathways should be exist, which could not be performed by just observing urine metabolic profiles. As a further work, metabolic profiling of blood, multi-organ extracts, or intact tissues, could be next tier to investigate more systems level for understanding the mechanism of DR.
Figure 13 Overall pathways for the DR’s beneficial inferred from the metabolomics study. The DR procedure up-regulates phase II detoxification enzymes through Nrf-2 pathway, and the products of these phase II metabolism were detected using multi-platform metabolomics approach. The enhanced phase II metabolism could be responsible for various beneficial effects of DR such as lower ALT level, reduced glycogen granule, reduced cancer incidence [67], and lower xenobiotic toxicity [49], reported here and previously.
Part II
Characterization of global metabolic and genomic alteration induced by dietary restriction using multi-organ metabolomics and cDNA microarray in rat

I. Introduction

Metabolomics quantifies the global metabolic profiling in biological tissue samples in order to investigate the variation induced by exogenous stimuli or genetic modulation. Therefore, this approach is often applied when one seeks to identify the metabolic changes and/or biomarkers that are associated with diseases, drug administration, or toxicities [16,51,68,69]. Metabolomics profiling in multiple tissues has also been successfully applied in understanding the metabolic aspects of pathology caused by inducible factors. For example, a comparison of NMR-based metabolic profiles from brain, liver, and sera of uninfected and cerebral malaria infected mice proposed that these tissues exhibit unique metabolic fingerprints [70]. Mice with cerebral malaria infections showed increased levels of triglycerides and VLDL-cholesterol only in sera and decreased levels of glutamine in both sera and brain, indicating the perturbation in ammonia detoxification and lipid and choline metabolism in cerebral malaria infected mice. As another example, an LC-MS-based metabolomics study of samples from multiple tissues, including plasma, hair, liver, and kidney, from mouse model of diabetic mellitus revealed that an endogenous compound, N-acetyl-L-leucine, is found in all of the analyzed
biological specimens, and that this metabolite appears to be a potential biomarker for diabetes [71]. Therefore, these works showed the examples that application of metabolomics in multiple tissues is feasible for systemic biological investigation.

High-density cDNA microarray facilitates the simultaneous analysis of the expression levels of thousands of genes. This profiling approach can be extended to monitor the gene expression status in tissues on a global basis and provide information on genes’ function and functional mechanism involved in environmental stimuli, diseases, and cancer [72,73]. A study used tissue microarray as a screening tool to successfully diagnose neuroendocrine carcinoma of the breast with higher specificity and sensitivity, as compared to whole-mount sections analysis [74]. In addition, a comprehensive microarray analysis has been established to identify a molecular signature for the early regenerative response genes activated in regeneration-competent tissues [75]. Therefore, tissue microarray analysis provides a reliable approach to probe the underlying mechanism of biological function. Recently, a combination technology of genomics and metabolomics is being developed, paving the way for an improved understanding of the molecular pathways [76].

Among alterations correlated with aging is a decline in glucose tolerance. Disturbances in glucose/insulin metabolism lead to a host of chronic disorders associated with aging [77]. With insulin and glucose response strongly correlated with diet ingredients [78,79], several studies have reported that insulin and glucose metabolisms are modulated by DR [10,80,81]. Despite the well-established insulin’s mechanism and effects on serum glucose tolerance, the particular
metabolic pathways activated in response to DR remain unknown.

Among the metabolic pathways that are thought to sustain healthy lifespan, hence longevity, are phase I and phase II detoxification systems (see Figure 2). Phase I detoxification is involved in rendering lipophilic xenobiotics more polar and providing sites for a conjugation reaction. As many products of the phase I reaction are non-polar in nature, phase II enzymes conjugate charged moieties to metabolites produced by phase I enzymes, promoting enhanced excretion. Through both phase I and phase II detoxification, harmful xenobiotics are eliminated to reduce the accumulation of toxicants [29]. The most well-known phase I and phase II detoxification enzymes include cytochrome P450, glycine-N-acyltransferase (GLYAT), glutathione-S-transferase (GST), uridine 5'-diphospho-glucuronosyltransferase (UGT), and sulfotransferase (SULT) [82]. These detoxification systems are known to be involved in cancer prevention [83-85], however, the connection to DR, another preventive measure for cancer, remains unresolved.

In this work, a combined multi-organ metabolomics and cDNA microarray approach was carried out to extend the understanding of the beneficial effects of DR. Multi-organ metabolomics demonstrated that DR enhances phase II detoxification and alters energy metabolism, which were supported by serum biochemistry and cDNA microarray data combined with gene, pathway enrichment, and gene ontology (GO) terms analysis. In addition, the pathway enrichment and GO terms analysis revealed that the beneficial effects of DR are attributed to increased phase I detoxification. An evaluation of metabolic and genomic markers
suggested that DR can reduce the incidence of age-related diseases. Overall, the use of multi-organ metabolomics and cDNA microarray approaches identified several metabolic and genomic markers to extend the understanding of the beneficial effects of DR and presented more reliable evidence with validations across these two approaches.
II. Methods

1. Tissues sample preparation for NMR and LC-MS

One hundred milligram tissues were sliced into several pieces and grinded using mortar under liquid nitrogen. The metabolites were extracted using double phase methanol-chloroform extraction methods as previously described [86]. Briefly, the powdered tissues were re-suspended with the mixture of 400 µL methanol and 200 µL chloroform. Three cycles of the following steps were then repeated: dipping into liquid nitrogen for 60 sec, thawing at room temperature for 2 min, and sonication for 5 min. After additionally adding a mixture of 200 µL chloroform and 200 µL distilled water, the samples were centrifuged at 15,000 g for 20 min at 4°C. The upper water phase was collected and dried with a centrifugal vacuum evaporator (Vision, Seoul, Korea). The pellets were dissolved with 500 µL buffer composed of 2 mM Na₂HPO₄ and 5 mM NaH₂PO₄ in D₂O with sodium-3-trimethylsilyl-[2,2,3,3-²H₄]-1-propionate (TSP, 0.025%, w/v) as an internal standard for NMR or with 30 µL mixture of HPLC-grade acetonitrile and water (1:1, v/v) for LC-MS.

2. NMR spectroscopic analysis of tissues

All one-dimensional spectra of the tissue extraction samples were measured on a 500 MHz Bruker Avance spectrometer equipped with a cryogenic triple resonance prove (Ochang, Korea). The acquisition parameters were essentially the same as those previously reported [25,35,36]. The metabolites were identified using
Chenomx (Spectral database; Edmonton, Alberta, Canada) by fitting the experimental spectra to those in the database and comparison with standard compounds.

3. LC-MS spectroscopy

For LC-MS analysis, the extracted metabolites were injected with an injection volume of 5 μL. HPLC was performed on an Agilent 1100 Series liquid chromatography system equipped with a degasser, an auto-sampler, and a binary pump (Agilent, Santa Clara, CA, U.S.A.). The chromatographic separation was performed on a ZIC-pHILIC Polymeric Beads Peek Column (150 × 2.1 mm, 5 μm, Merck kGaA, Darmstadt, Germany) at 35 °C, and the temperature of auto-sampler was set at 4 °C. For the solvent system, mobile phase A and B were distilled water with 10 mM ammonium carbonate (pH = 9.1) and acetonitrile, respectively. The mobile phase was delivered at a flow-rate of 0.15 mL/min and the entire eluent was carried into a mass spectrometer. The linear gradient was as follows: 80 % B at 0 min, 35% B at 10 min, 5% B at 12 min, 5% B at 25 min, 80% B at 25.1 min, and 80% B at 35 min. API 2000 Mass Spectrometer controlled by the Analyst 1.6 Software (AB/SCIEX, Framingham, MA, U.S.A.) and equipped with an electrospray ionization (ESI) source was used in negative ion mode for multiple reaction monitoring (MRM). The operating conditions of the mass spectrometer were as follows: -4.5 kV of ion spray voltage, the temperature of the heater (turbo) gas at 300°C, and curtain gas (nitrogen), ion source gas 1 (nitrogen), and ion source gas 2 (nitrogen) pressures at 30, 40, and 80 psi, respectively. For the detection,
MRM was performed with the m/z value of the precursor and fragment ions as indicated on the Table 5 which were established with the standard compounds.

4. Multivariate data analysis

All the obtained time domain NMR data were Fourier transformed, phase corrected, and baseline corrected manually using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). The region (0.4-10.0 ppm) was used with the exclusion of water (4.6-5.2 ppm) for liver, kidney, and muscle tissues and that of water (4.6-5.8 ppm) for brain tissue, respectively. One dimensional NMR chemical shifts were normalized against total integration values and 0.025% TSP, and then binned at a 0.02 ppm interval to reduce the complexity of the NMR data for pattern recognition. The binning, normalization, and conversion were done using a Perl software written in-house. The resultant data sets were then imported into SIMCA-P version 11.0 (Umetrics, Umeå, Sweden) and mean-centered with Pareto scaling for multivariate statistical analysis. Orthogonal projections to latent structure-discrimination analysis (OPLS-DA) multivariate analysis was carried out, which is a supervised method and gives segregation between two classes along the predictive components. OPLS-DA was performed with one predictive and one orthogonal component for kidney tissue, one predictive and two orthogonal components for liver and brain tissues, and one predictive and three orthogonal components for muscle tissue, respectively. Statistical significance ($p < 0.05$) was assessed by student’s $t$-test.
5. Kidney tissue histopathology and serum biochemistry

After fixation for 48 h, kidney tissue was embedded in paraffin according to routine procedures. Four-micrometer thick sections were cut and stained with periodic acid-schiff (PAS) for histopathological evaluation. An expert pathologist at Inha University Hospital blindly analyzed the tissue slices. Additionally, Serum GLU, CK, TP, ALB, GLO, CRE, BUN, Fe, HDL, AMYL, and LDH levels were measured using commercial kits at Inha University hospital (Incheon, Korea).

6. Microarray data processing

1) RNA quality check

For the quality control, RNA purity and integrity were evaluated by OD 260/280 ratio, and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, U.S.A.).

2) Sample labeling and purification

RNA labeling and hybridization were performed by using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). Briefly, 200 ng of total RNA extracted from 32 samples of kidney, liver, muscle, and brain tissues (control: n = 4 and DR: n = 4 for each tissue) was linearly amplified and labeled with Cy3-dCTP. The labeled cRNAs were purified by RNAeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000 (NanoDrop, Wilmington, U.S.A.).
3) Hybridization and scan

Six hundred nanogram of each labeled cRNA was fragmented by adding 5 µL 10 x blocking agent and 1 µL of 25 x fragmentation buffers, and then heated at 60 °C for 30 min. Finally 25 µL 2 x GE hybridization buffer was added to dilute the labeled cRNA. 50 µL of hybridization solution was dispensed into the gasket slide and assembled to the SurePrint G3 Rat Microarray, 8×60K (Agilent®). The slides were incubated for 17 h at 65 °C in an Agilent hybridization oven, and then washed at room temperature by using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). The hybridized array was immediately scanned with an Agilent Microarray Scanner (Agilent, Santa Clara, CA, U.S.A.).

4) Raw data preparation and statistical analysis

Raw data were extracted using the software provided by Agilent Feature Extraction Software (v11.0.1.1). The raw data for same gene was then summarized automatically in Agilent feature extraction protocol to generate raw data text file, providing expression data for each gene probed on the array. Array probes that have Flag A in samples were filtered out. Selected gProcessed Signal value was transformed by logarithm and normalized by quantile method. Statistical significance of the expression data was determined using fold change and independent t-test in which the null hypothesis had no difference among the two groups by each factor (Group, Time). Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene
Enrichment and Functional Annotation analysis for significant probe list was performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp). All data analysis and visualization of differentially expressed genes was conducted using R 3.0.2 (www.r-project.org).

7. Microarray data analysis

1) Pathway enrichment analysis

Pathway enrichment analysis was conducted using PathVisio 3.1 (www.pathvisio.org) [87,88]. Custom plug-ins for calculating Z-scores were added to PathVisio. The rat gene database of July 2013 was used. Pathways were considered to be down or up-regulated when meeting the following criteria: \( p \)-value < 0.05 and fold change (FC) \( \leq -1.5 \) or \( \geq 1.5 \), respectively. In the statistical pathway ranking test, significantly changed pathways were identified by Z-score, which is obtained by counting the number of genes on each pathway suitable for the defined criteria and subtracting this number from the total number of genes that meet the criteria. Based on ranking of the regulated pathway, the false discovery rate (FDR) was also calculated. Among the down or up-regulated pathways, those meet the criterion (Z-score \( \geq 1.77 \) and FDR < 0.05) were selected as pathway enrichment signals.

2) Functional enrichment analysis

ClueGO 1.4, a cytoscape (www.cytoscape.org) plug-in, was used for gene function enrichment analysis. ClueGO facilitates the visualization of functionally
related genes by displaying them as a clustered one. The cluster analysis type was performed with comparison of increased and decreased genes. Genes were interpreted to be decreased or increased when meeting the following criteria: log$_2$ scaled fold change (FC) ≤ −2 or ≥ 2 and $p$-value < 0.05 for kidney, liver, and brain tissues; and a $p$-value < 0.05 and fold change (FC) ≤ −1.5 or ≥ 1.5 for muscle tissue, respectively.

The combination of the gene ontology (GO), which is composed of four types of Biological process, Cellular Component, Immune System, and Molecular Function (update on May, 2014) and the KEGG pathway (update on October, 2008), were used in ClueGO analysis. The statistical test used for both the enrichment and depletion was based on two-sided hypergeometric option with a Bonferroni correction and kappa score of 0.3.
III. Results

A. Serum clinical biochemistry

Based on the results of the decreased body weight and insulin levels, and the reduced levels of low density lipoprotein (LDL) and serum triglyceride (TG), two important risk factors for age-associated diseases, dietary restriction (DR) seems to induce an obvious difference between the control and DR groups (see Figure 3 and Figure 11). To assess the effects of DR treatment, additional basic serum parameters were monitored. DR caused significant elevations in serum creatinine (CRE) and blood urea nitrogen (BUN) levels, and these changes were accompanied by decreases in serum total protein (TP), globulin (GLO), and Fe. No significant changes were observed in the levels of glucose (GLU), creatine kinase (CK), albumin (ALB), high density lipoprotein (HDL), amylase (AMYL), and lactate dehydrogenase (LDH) in the DR group (Table 3). CK, an established biomarker for muscle function and status, confirmed that the muscle tissue was healthy during DR. In addition, the reduction in TP and GLO in the DR group has historically been associated with liver dysfunction. However, insignificant changes in other serum proteins levels, such as ALB and LDH, suggested that the liver abnormality and destruction are unlikely. Moreover, ALT, which is an established diagnostic evaluation of hepatocellular injury and liver health, was actually decreased significantly in the DR group (see Figure 10C). These results indicated that liver tissue in the DR group was healthy. While BUN and CRE levels were meaningfully
increased in the DR group, their levels were within a normal range (BUN: 15-25 mg/dL; CRE: 0.2-0.8 mg/dL, reference from Exotic Companion Medicine Handbook). Results also showed that glycogen granules decreased in kidney tissue in the DR group (see Figure 14), indicating that kidney health was maintained during DR. Despite the DR procedure, the serum glucose levels were maintained at a normal level, suggesting a proper glucose-homeostasis.

Overall, all of the four tissues, kidney, liver, brain, and muscle, were determined to be healthy, in a biochemical sense, in the DR group. Therefore, multi-organ investigation suitably reflected the beneficial effects of DR and can be utilized for discovery of potential biomarkers and altered metabolic pathways mediated by DR.
Table 3 Summary of the items expressed in rat serum from the control and DR groups*.

<table>
<thead>
<tr>
<th>Serum parameter</th>
<th>Composition</th>
<th>Group name</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>$p$-value</th>
<th>Significant change</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU blood sugar</td>
<td>C</td>
<td>158.33</td>
<td>24.94</td>
<td>0.26</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DR</td>
<td>146.92</td>
<td>13.09</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CK muscle</td>
<td>C</td>
<td>271.67</td>
<td>70.12</td>
<td>0.80</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DR</td>
<td>285.13</td>
<td>166.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP blood protein</td>
<td>C</td>
<td>6.29</td>
<td>0.33</td>
<td>0.003</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DR</td>
<td>5.79</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALB blood albumin</td>
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<td>0.12</td>
<td>0.90</td>
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<td>-</td>
</tr>
<tr>
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<td>DR</td>
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<td>0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.25</td>
<td>5.0E-4</td>
<td>↓</td>
<td></td>
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<tr>
<td>CRE kidney</td>
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<td>0.06</td>
<td>0.011</td>
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</tr>
<tr>
<td></td>
<td>DR</td>
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<td>0.08</td>
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<tr>
<td>BUN kidney</td>
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<td>2.0E-7</td>
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<tr>
<td></td>
<td>DR</td>
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<tr>
<td>Fe blood iron</td>
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<td>24.46</td>
<td>2.0E-4</td>
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<tr>
<td></td>
<td>DR</td>
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<tr>
<td>HDL lipid</td>
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<td>4.71</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>DR</td>
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</tr>
<tr>
<td>Parameter</td>
<td>Group</td>
<td>Mean</td>
<td>Standard Deviation</td>
<td>p-value</td>
<td>95% CI</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>------</td>
<td>--------------------</td>
<td>---------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>AMYL pancreas</td>
<td>C</td>
<td>1750.83</td>
<td>219.15</td>
<td>0.58</td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td>559.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH liver, heart</td>
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<td>528.33</td>
<td>255.43</td>
<td>0.85</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>552.31</td>
<td>302.14</td>
<td></td>
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</table>

*The serum parameter, composition, group name, mean and standard deviation value, and student’s t-test (p < 0.05) are presented. The symbols ‘↑’ and ‘↓’ indicate the significant increase and decrease, respectively. Serum biochemistry for total 21 serum samples (control: n = 8 and DR: n = 13) were carried out. The values of the mean, standard deviation, and p-value were calculated using standard statistical analysis, student’s t-test. The abbreviations are as follows: C, control; DR, dietary restriction; GLU, Glucose; CK, Creatine kinase; TP, Total protein; ALB, Albumin; GLO, Globulin; CRE, Creatinine; BUN, Blood urea nitrogen; HDL, High density lipoprotein; AMYL, Amylase; LDH, Lactate dehydrogenase.*
Figure 14 Kidney tissue histopathological staining. Periodic acid-Schiff (PAS) staining of kidney tissue was performed on paraffin block of the samples. PAS staining control (A) and DR (B) groups (200X).
B. NMR spectral acquisition and analysis

To investigate the effects of DR, multi-organ NMR-based metabolomics was applied. Representative $^1$H NMR spectra of kidney, liver, brain, and muscle tissues extractions from the control and DR groups are shown in Figure 15. According to the Chenomx database (Edmonton, Alberta, Canada) and comparison with standard compounds, several metabolites were identified from the extractions in kidney, liver, brain, and muscle tissues (Table 4), involved in several cellular metabolisms. Among those affected were pyrimidine metabolism (5,6-dihydrouracil, cytidine, and uridine), beta-alanine metabolism (anserine and aspartate), arginine and proline metabolism (arginine and creatine), glutathione metabolism (glycine and glutathione) and glycolysis (alanine and lactate). Of these metabolites, some were found in more than one tissues, such as alanine, aspartate, glutamine, and glutamate. On the other hand, the expression of others was limited to specific tissues, as observed for 3-aminoisobutyrate, carnitine, adenine, and cystathionine in kidney, liver, brain, and muscle tissues, respectively. For a more detailed investigation of the changes caused by DR, multivariate statistical analysis was carried out to characterize the contribution signals for group differentiation.
Figure 15 Representative $^1$H NMR spectra of tissue extractions from the control and DR groups. The NMR spectra were taken for tissue samples extracted from kidney (A), liver (B), brain (C), and muscle (D) in 500 μL of D$_2$O containing 2 mM Na$_2$HPO$_4$ and 5 mM NaH$_2$PO$_4$ (pH = 7.4) and 0.025% TSP as an internal standard. For NMR, the assignments were established using the spectra of the standard compounds and Chenomx database (Edmonton, Alberta, Canada).
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>ppm (multiplicity) for NMR</th>
<th>K</th>
<th>L</th>
<th>B</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Aminoisobutyrate</td>
<td>1.17(d)</td>
<td>-</td>
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<tr>
<td>3-Hydroxybutyrate</td>
<td>1.20(d), 2.31(q)</td>
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<td>↑</td>
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<td>3-Hydroxyisovalerate</td>
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<td>-</td>
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<tr>
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<tr>
<td>4-Pyridoxate</td>
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<td>↑</td>
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<tr>
<td>5,6-Dihydrouracil</td>
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<tr>
<td>Acetate</td>
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<tr>
<td>Adenine</td>
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</tr>
<tr>
<td>Alanine</td>
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<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Allantoin</td>
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<td></td>
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<td>↓</td>
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<tr>
<td>Anserine</td>
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<td>↑</td>
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<tr>
<td>Aspartate</td>
<td>2.66(q), 2.81(m)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>↑ ↑</td>
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<tr>
<td>Arginine</td>
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<tr>
<td>Betaine</td>
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<td></td>
<td>-</td>
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<tr>
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<td>Cystathionine</td>
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<td>Cytidine</td>
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<tr>
<td>No.</td>
<td>Substance</td>
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<td>--------------------------------</td>
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<tr>
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<td>Ethylene glycol</td>
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<td>22</td>
<td>Formate</td>
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<tr>
<td>23</td>
<td>Glucose</td>
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<tr>
<td>24</td>
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<tr>
<td>25</td>
<td>Glutamine</td>
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<tr>
<td>26</td>
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<tr>
<td>27</td>
<td>Glutathione</td>
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<tr>
<td>28</td>
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<td>3.56(s)</td>
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<tr>
<td>29</td>
<td>Glycolate</td>
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<td>Guanidoacetate</td>
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<tr>
<td>31</td>
<td>Histidine</td>
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<tr>
<td>32</td>
<td>Homoserine</td>
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<tr>
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<td>Inosine</td>
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<tr>
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<td>Isobutyrate</td>
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<td></td>
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<tr>
<td>35</td>
<td>Lactate</td>
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<td>36</td>
<td>Leucine</td>
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<td>37</td>
<td>Malonate</td>
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<tr>
<td>38</td>
<td>Methylamine</td>
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<tr>
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<td>Methylguanidine</td>
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<tr>
<td>40</td>
<td>myo-Inositol</td>
<td>3.28(t), 3.54(m), 3.63(t), 4.06(t) ↑ ↑ -</td>
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<tr>
<td>41</td>
<td>Niacinamide</td>
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<tr>
<td>42</td>
<td>Ornithine</td>
<td>3.04(t)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metabolite</td>
<td>ppm</td>
<td>K</td>
<td>L</td>
<td>B</td>
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<tr>
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<td>----------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>43</td>
<td>Oxaloacetate</td>
<td>3.56(s)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>44</td>
<td>Oxypurinol</td>
<td>8.17(s)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>45</td>
<td>Phenylacetate</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>Propylene glycol</td>
<td>1.10(d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>47</td>
<td>Pridoxine</td>
<td>2.52(s), 7.68(s)</td>
<td>-</td>
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<tr>
<td>48</td>
<td>Succinate</td>
<td>2.41(s)</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>49</td>
<td>Taurine</td>
<td>3.27(t), 3.43(t)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>50</td>
<td>Trimethylamin N-oxide</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>51</td>
<td>Tyrosine</td>
<td>6.89(d), 7.19(d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>52</td>
<td>Uridine</td>
<td>5.91(t), 7.87(d)</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>53</td>
<td>Valine</td>
<td>0.99(d), 1.04(d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>54</td>
<td>Xanthosine</td>
<td>4.44(s), 5.93(d), 7.98(s)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>NAD+</td>
<td>6.05(d), 8.21(t), 8.44(s), 8.84(d), 9.16(d), 9.35(s)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>ANP</td>
<td>6.14(d), 8.28(s), 8.58(s)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*The ppm, structural identifiers, and significant change of metabolites for the DR group comparing with the control group are indicated. The metabolites non-detected in respective tissue are remained as blank, and the significant increase or decrease confirmed with student’s t-test ($p < 0.05$) is indicated as the symbol of up or down, respectively. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissues, respectively. “s”, “d”, “t”, “q”, and “m” indicate singlet, doublet, triplet, quadruple, and multiplet, respectively.
C. OPLS-DA multivariate analysis

To further investigate the DR-induced metabolic changes reflected in multiple tissues, the NMR data was analyzed using OPLS-DA multivariate analysis. An OPLS-DA model with entire NMR data was constructed for each tissue sample (Figure 16, left panel). Dots represent specific characteristics of the metabolic profile of each sample. Differentiation models for distinguishing the control and DR groups extraction samples from kidney tissue were obtained with one predictive and one orthogonal component, that from liver and brain tissues with one predictive and two orthogonal components, that from muscle tissue with one predictive and three orthogonal components, respectively. In addition, the models had an overall goodness-of-fit, $R^2(Y)$, of 90%, 91%, 83%, and 95% and an overall cross-validation coefficient, $Q^2(Y)$, of 83%, 69%, 52%, and 30% for discrimination of the groups of kidney, liver, brain, and muscle tissues, respectively. All of the OPLS-DA models present clear differentiation without any overlap. The outstanding results of these discriminations likely arise due to the DR effects.

In order to identify the metabolites that contribute to the observed differentiation, an S-plot was established based on OPLS-DA model. Modeled covariation, $P$, and modeled correlation, $P(corr)$, offer the specific contributing signals for group separation (Figure 16, right panel). Among the signals, all of the markers with the $p$-values of less than 0.05 using student’s $t$-test were selected. Based on the tissue constituents that were identified above, the marker signals contributing to the classification are listed in Table 4.
Figure 16 $^1$H NMR-based OPLS-DA score plot and corresponding S-plot. Differentiation and contributing signals for the differentiation of the control and DR groups for kidney (A), liver (B), brain (C), and muscle (D) tissues were presented using multivariate analysis. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) score plot (left panel) of the control and DR
groups. Filled box: control group; Open circle: DR group. The models were established using one predictive and one orthogonal component for kidney tissue, one predictive and two orthogonal components for liver and brain tissues, and one predictive and three orthogonal components for muscle tissue, respectively. S-plot (right panel) analysis to identify the contributing signals for group separation between the control and DR groups.
D. Altered metabolic pathways in the DR group

In order to perform a more holistic metabolic profiling, targeted LC-MS analysis was carried out for related metabolites that are not easy to discern with NMR. Mass-to-charge ratios were established for precursor and fragment ions using standard compounds, and subsequently used for multiple reaction monitoring (MRM) parameters (Table 5). By combining NMR and LC-MS data, the altered metabolite levels were globally mapped onto intra-tissue metabolic pathways (Figure 17). Several quite different metabolites were identified in the DR group. These include 3-hydroxybutyrate (an essential ketone body) and 4-pyridoxate (a byproduct of vitamin B metabolism) in both kidney and liver tissues. Additionally, 5,6-dihydouracil (a component of pyrimidine metabolism) was found in liver tissue, acetate (a substrate to form acetyl-CoA) was found in kidney tissue, allantoin (a product of purine metabolism) was identified in liver tissue, aspartate and arginine (regulators of urea cycle) were found in muscle tissue, the nucleoside cytidine was found in brain tissue, glucose (a primary source of energy for the body's cells) was found in kidney, liver, and muscle tissues, glutamine (a key anaplerotic metabolite for TCA cycle) was found in kidney, liver, and muscle tissues, glutathione (an anti-oxidant) was identified in brain and muscle tissues, glycine and myo-inositol (an important substrate and intermediate of phase II reaction, respectively) were found in kidney and liver tissues, uridine and inosine (a basic component of RNA and an essential proper translation of the genetic code, respectively) were found in kidney and liver tissues, lactate (a key indicator of
cytosolic anaerobic metabolism) was found in liver and kidney tissues, citrate, malate, fumarate, and α-ketoglutarate (important intermediates of TCA cycle) were found in muscle tissue. For more understanding of systems biological pathways induced by DR, the genomic analysis was carried out using cDNA microarray.
Table 5 Metabolites identified with targeted LC-MS analysis*.

<table>
<thead>
<tr>
<th>Metabolites compound</th>
<th>Precursor Ion (m/z)</th>
<th>Fragment Ion (m/z)</th>
<th>Retention Time (min)</th>
<th>K</th>
<th>L</th>
<th>B</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>R5P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>229</td>
<td>97</td>
<td>7.6</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>G6P&lt;sup&gt;b&lt;/sup&gt;</td>
<td>259</td>
<td>79</td>
<td>8.6</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>F6P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>259</td>
<td>79</td>
<td>7.9</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>190.8</td>
<td>111.1</td>
<td>9.4</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
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<td>α-ketoglutarate</td>
<td>144.8</td>
<td>101</td>
<td>6.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Fumarate</td>
<td>114.8</td>
<td>71</td>
<td>7.4</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Malate</td>
<td>132.8</td>
<td>71</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>

*The m/z value of precursor ion and fragment ion, the retention time, and the change trends of the DR group compared to the control group with the \( p \)-value less than 0.05 are indicated. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissues, respectively.

<sup>a</sup>R5P, ribulose-5-phosphate.

<sup>b</sup>G6P, glucose-6-phosphate.

<sup>c</sup>F6P, fructose-6-phosphate.
Figure 17 Overall metabolic alterations in kidney, liver, brain, and muscle tissues of the DR group. The metabolic changes in the DR group compared to the control group are indicated on global intra-tissue metabolic pathways. The increased metabolites in the DR group are colored in red, those decreased in blue, those without significant changes in green, and those not detected in black. The abbreviations are as follows: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribulose-5-phosphate; NADP+/NADPH, oxidized/reduced nicotinamide
adenine dinucleotide phosphate; GSH/GSSG, reduced/oxidized glutathione; PRPP, phosphoribosyl pyrophosphate; GAA, guanidoacetic acid; CIT, citrate; AKG, α-ketoglutarate; OAA, oxaloacetate; SUC, succinate; MAL, malate; FUM, fumarate.
E. General effects on gene expression

For more holistic investigation of systems biological pathways induced by DR, a genomic analysis was applied using cDNA microarray. To evaluate the overall genomic response in the four tissues under DR condition, the differentially expressed genes were calculated using a value of $p < 0.05$, and absolute fold change (FC) greater than 1.5-fold. The number of differentially expressed genes in kidney, liver, and brain tissues were relatively comparable (No. 590, 697, and 713). By contrast, a less differential gene expression was detected in muscle tissue (No. 93).

Venn diagrams were constructed to identify the co-regulation of genes across tissues. As shown in Figure 18, the number of the differently expressed genes were identified. Specifically, 82, 46, 12, 30, 11, and 13 genes were commonly detected in kidney and liver, kidney and brain, kidney and muscle, liver and brain, liver and muscle, brain and muscle, respectively.

Surprisingly, however, in spite of the shared expression of several of the genes, no core genes were differentially expressed in all four tissues, indicating that there is no one common gene induced by DR. As consistent with previous reports, the aspects of gene responses induced by environmental stimuli are not the same in various tissues [89,90]. Therefore, the genes differentially regulated by DR in three out of four tissues were explored (Table 6) for expanded analysis. Among those, \textit{Ccng1} and \textit{Eml1} were found in kidney, brain, and muscle tissues; \textit{Ddah1}, \textit{Cdkn1a}, \textit{Dcd}
Tpd52l1, Nr4a1, Sod3, Ddc, and Pygl were simultaneously altered in kidney, liver, and brain tissues; and Hspa1b and Rt1.aa in kidney, liver, and muscle tissues.

The extent of overlap among differentially expressed genes was compared for the four tissues (Table 7). The highest overlaps of differentially expressed genes were observed as 14% between liver and kidney tissues (with respect to kidney), as well as between brain and muscle tissues (with respect to muscle). This result was highly consistent with those of the earlier results of calculation of differently expressed genes number (see Figure 18) and common changed metabolites number (Table 4).
Figure 18 Comparative analysis of differentially expressed genes using Venn diagrams. Differentially expressed genes (defined as genes with an absolute fold change (FC) ≥ 1.5-fold and \( p < 0.05 \)) were calculated among the four tissues. The number of differentially expressed genes for each comparison is indicated, including the number of overlapping genes for any pairwise or higher-order comparisons. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissues, respectively.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>K</th>
<th>L</th>
<th>B</th>
<th>M</th>
</tr>
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<tr>
<td>Hspa1b</td>
<td>heat shock 70kD protein 1B</td>
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<td>↑</td>
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<td>↑</td>
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<tr>
<td>Rt1.aa</td>
<td>MHC class I RT1.Aa alpha-chain</td>
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<td>↑</td>
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<td>↑</td>
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<td>Nr4a1</td>
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<td>↓</td>
<td>↑</td>
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<td>Ddah1</td>
<td>dimethylarginine dimethylaminohydrolase 1</td>
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<td>↑</td>
<td>↓</td>
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<tr>
<td>Pygl</td>
<td>phosphorylase, glycogen</td>
<td>↑</td>
<td>↓</td>
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<td>-</td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>cyclin-dependent kinase inhibitor 1A</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>Ddc</td>
<td>dopa decarboxylase</td>
<td>↑</td>
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<td>↓</td>
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</tr>
<tr>
<td>Tpd52l1</td>
<td>tumor protein D52-like 1</td>
<td>↓</td>
<td>↓</td>
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<td>-</td>
</tr>
<tr>
<td>RGD1311874</td>
<td>hypothetical LOC300751</td>
<td>↓</td>
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<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Ccng1</td>
<td>cyclin G1</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
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</tr>
<tr>
<td>Eml1</td>
<td>echinoderm microtubule associated protein like 1</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
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</tr>
</tbody>
</table>

*Identification and name of genes differentially regulated by DR in three out of four tissues are indicated. With a cutoff of absolute fold change (FC) ≥ 1.5-fold, \( p \)-value < 0.05, the significantly increased and decreased genes in respective tissues are indicated. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissue, respectively.
Table 7 Proportion of overlapping differently expressed genes between pairwise comparisons of tissues.

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Liver</th>
<th>Brain</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
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<td>0.14</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Liver</td>
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<td>1.00</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Brain</td>
<td>0.06</td>
<td>0.04</td>
<td>1.00</td>
<td>0.02</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.13</td>
<td>0.12</td>
<td>0.14</td>
<td>1.00</td>
</tr>
</tbody>
</table>
F. Pathway enrichment analysis

To identify the common and unique signaling pathways activated by DR, pathway enrichment analysis were performed using the program PathVisio. The results of agglomerative hierarchical clustering (Figure 19) are consistent with earlier observation of Venn diagram analysis (Figure 18) and overlapping genes (Table 7).

The pathway of fatty acid omega-oxidation was significantly up-regulated in all four tissues (Z-score > 2), and the methylation pathway was enhanced in kidney, liver, and brain tissues. In addition, kidney tissue showed up-regulation, especially, of pathways related to fatty acid metabolism, including beta-oxidation of unsaturated fatty acids, fatty acid beta-oxidation, mitochondrial LC-fatty acid beta-oxidation, synthesis and degradation of ketone bodies, glycolysis and gluconeogenesis, triacylglyceride synthesis, fatty acid biosynthesis, among others. Kidney tissue also showed up-regulated detoxification pathways, including both phase I and phase II factors (meta-pathway biotransformation, amino acid conjugation of benzoic acid, nuclear receptors in lipid metabolism and toxicity), as well as a down-regulation in pathways related to inflammatory signaling (inflammatory response pathway, prostaglandin synthesis and regulation, etc.). Liver tissue displayed a similar enrichment in up-regulated metabolic pathways for fatty acid metabolism (beta-oxidation of unsaturated fatty acids, fatty acid beta-oxidation, mitochondrial LC-fatty acid beta-oxidation, beta-oxidation meta-pathway) and detoxification reaction involving both phase I and phase II factors.
(meta-pathway biotransformation, amino acid conjugation of benzoic acid, nuclear receptors in lipid metabolism and toxicity, and glucuronidation).

In addition, both liver and kidney tissues shared up-regulated retinol metabolism, steroid biosynthesis, and tryptophan metabolism, along with a down-regulation of cholesterol metabolism. Kidney and brain, and also liver and muscle tissues shared a down-regulation of endochondral ossification, and also alanine and aspartate metabolism. Pathways involving estrogen metabolism, interactions between CFTR and other ion channels, urea cycle and metabolism of amino groups, aflatoxin B1 metabolism, polyol pathway, adipogenesis, statin pathway, spinal cord injury, and hypertrophy model were only significantly changed in kidney tissue. A distinct set of pathways related to G-protein coupled receptor organization, were down-regulated in liver tissue (class A Rhodopsin-like GPCRs, peptide GPCRs, nucleotide GPCRs, etc.), despite their up-regulation, with additional down-regulation of class C GPCRs, in brain tissue. Brain tissue displayed a down-regulation in the biological amine synthesis (biogenic amine synthesis and catecholamine synthesis), whereas these pathways were up-regulated in kidney tissue. A distinct set of pathways, related to tissue protection pathway, were found to be uniquely up-regulated in muscle tissue (hedgehog signaling pathway and NFE2L2), as well as a pathway associated with blood coagulation (blood clotting cascade and complement activation classical pathway). By contrast, these blood coagulation pathways with additional one (complement and coagulation cascades) were down-regulated in kidney tissue. Several pathways related to biosynthesis of aldosterone and cortisol, cytokines and inflammatory response, as well as the p53
signaling pathway were significantly up-regulated uniquely in liver tissue, whereas pathways related to osteoblast, PKA-HCG-Glycogen synthase, selenium micronutrient network, cholesterol biosynthesis, glutathione metabolism, type II interferon signaling, folic acid network, and especially fatty acid biosynthesis pathway were down-regulated. Brain tissue also showed down-regulation of matrix metalloproteinases, phase I biotransformations (non-P450), relationship between glutathione and NADPH, and ACE inhibitor pathway. A summary of the unique and common pathway enrichments observed in multiple tissues (FDR < 0.05) is shown via heat map in Figure 19.
Figure 19 Agglomerative hierarchical clustering analysis. Pathway enrichment analysis was determined by PathVisio. Pathways were considered to be down or up-regulated when meeting the following criterion: fold change (FC) ≤ −1.5 or ≥ 1.5 and p-value < 0.05, respectively. Among the down or up-regulated pathways, those meeting the criterion (Z-score ≥ 1.77 and FDR < 0.05) were selected as pathway enrichment signals and subjected to agglomerative hierarchical clustering. The increased pathway enrichment signals in the DR group are colored in red, decreased in blue, those without significant changed in white according to Z-score. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissues, respectively.
G. Functional enrichment analysis

To investigate the biological pathway activated by DR and their interplay in the biological network, functional enrichment analysis was carried out using gene ontology (GO). This methodology is commonly applied when searching for the relationships between the genetic terms based on the similarity of their associated genes. The significantly up and down-regulated functional activities were analyzed according to the biological processes, cellular components, immune system, and molecular function ontologies with clustering compare mode. Functionally grouped network of enriched categories were built in ClueGo (Figure 20). The degree of connectivity between terms (edges) was presented based on kappa statistics, with the size of each node reflected the statistical significance of the indicated terms.

For kidney tissue, two separate modules consisting of entirely up-regulated genes, as well as three terms without any connection, were identified. The GO terms for the two of significantly increased modules were metabolism of xenobiotics by cytochrome P450 and solute:cation symporter activity. For liver tissue, two modules consisting of increased genes or decreased genes were found with one GO term of negative regulation of intrinsic apoptotic signaling pathway in response to oxidative stress or with two GO terms of stearoyl-CoA 9-desaturase activity and fatty acid biosynthetic process, respectively. For brain tissue, one module connected with three GO terms, as well as one term without any connection, were identified. The GO term containing up-regulated genes was cellular response to alkaloid, and the two GO terms including only down-regulated
genes were odontogenesis of dentin-containing tooth and dicarboxylic acid transport. For muscle tissue, one module consisting of entirely up-regulated genes with two GO terms of elastin biosynthetic process and hindbrain morphogenesis was identified.
Figure 20 Grouping of network based on functionally enriched GO terms. Functionally grouped network of enriched terms was generated using ClueGO. GO terms were represented as nodes based on their kappa score (≥ 0.3). Functionally grouped networks of kidney (A), liver (B), brain (C), and muscle (D) tissues are linked to their biological function, where only the most significant GO term in the group is labeled. Up-regulated GO terms are in red while down-regulated in green.
IV. Discussion

For a holistic investigation of systems biological effects induced by DR, multi-organ metabolomics and cDNA microarray were carried out. Metabolomics was used to quantify a global metabolic profile and cDNA microarray was performed to create a composite genomic profile, providing a holistic report of the biological tissue systems induced by exogenous stimuli. In this study, the combination approach of multi-organ metabolomics and cDNA microarray identified several metabolic and genomic markers to extend the understanding of the beneficial effects of DR. Moreover, the combined application may be generally applicable for the evaluation of biological variations, at the systems level, to facilitate an improved understanding of molecular pathways as a whole.

With the conserved glucose or insulin/IGF-1-like pathways, such as down-regulation of antioxidant enzymes, reduced accumulation of glycogen or fat, and increase of growth and mortality, modulation of the activity of these pathways extends longevity by DR [91]. In this work, the glucose level were similar in both the DR and control groups (Table 3). This observation was made despite the fact that food intake was naturally reduced in the DR group compared to the control group. As glucose intake is limited in DR, it should be transported from the reservoir, such as liver or kidney. As expected, multi-organ metabolomics revealed that the glucose levels were significantly decreased in kidney, liver, and muscle tissues of the DR group (see Figure 17), consistent with the results of serum biochemistry, since glucose is transported from these tissues to the bloodstream. It
can be assumed that the production of glucose in kidney and liver tissues stimulates glycogenolysis, in which glycogen is converted to glucose. To confirm the glycogen state in the actual tissues, a periodic acid-Schiff (PAS) staining was also carried out for liver and kidney tissues. As shown in Figure 10 for liver tissue and Figure 14 for kidney tissue, the DR group showed glycogen depletion relative to the control group. Moreover, the glycogen synthase pathway was significantly reduced in liver tissue based on pathway enrichment analysis (Figure 19). All these results suggested that glycogen degradation is increased in kidney and liver tissues.

In addition, gluconeogenesis, another pathway for the synthesis glucose from different carbon source, should also be activated in liver tissue, and to a certain extent in kidney tissue. In gluconeogenesis, the resulting glucose produced in liver and/or kidney tissues is transported to muscle tissue. In muscle, metabolized glucose is converted to lactate by anaerobic glycolysis, which then returns to liver and/or kidney tissues, where it is used to produce glucose in the glucose-lactate cycle [92]. Similar to the glucose-lactate cycle, another pathway known as the glucose-alanine cycle exists, which permits the production of glucose [93]. Multi-organ metabolomics revealed that the main gluconeogenic precursors (lactate, alanine, and glutamine, which account for over 90% of the overall gluconeogenesis [94]) were enhanced in kidney and liver tissues in the DR group (Figure 17). Although multi-organ metabolomics revealed that alanine levels were not significantly altered in kidney, liver, and muscle tissues, it is likely that the elevated use of alanine in kidney and liver tissues are maintained by the supply from muscle tissue. A steady supplement of alanine would be likely offered from synthesis of α-
ketoglutarate from glutamate (Figure 21), and α-ketoglutarate was recovered to glutamate through conversion of arginosuccinate from aspartate. Synthesized arginosuccinate activates urea cycle, producing arginine, which can be used for glutamate synthesis as an amino acid source, and also involved in fumarate production. In addition, aspartate can be offered from oxaloacetate, an intermediate metabolite of TCA cycle. With multi-organ metabolomics showing the increase of intermediate metabolites involved in TCA cycle, such as citrate, malate, and fumarate, TCA cycle produced greater oxaloacetate for aspartate synthesis in muscle tissue (Figure 17). These results indicated that the gluconeogenic precursors, such as lactate, glutamine, and alanine, were offered sustentation by a combination process of urea and TCA cycle in muscle tissue and that gluconeogenesis was enhanced in kidney and liver tissues with application of the cycles of glucose-lactate, glucose-glutamine, and glucose-alanine (see Figure 21). In addition, pathway of glycolysis and gluconeogenesis was also elevated in kidney tissue of the DR group (see Figure 19). Taken together, multi-organ metabolomics, pathway enrichment analysis, and tissue histopathology provided evidence in supporting the activation of glycogenolysis and gluconeogenesis to satisfy the whole body’s glucose requirements in response to DR.

Multi-organ metabolomics also uncovered a significant increase in an essential ketone body, 3-hydroxybutyrate, in kidney and liver tissues of the DR group (see Figure 17). This indicated that ketone bodies were offered and used as energy sources for other tissues without the ability of glucose self-production, such as brain tissue. Interestingly, pathways related to fatty acid beta-oxidation, including
fatty acid beta-oxidation, beta-oxidation of unsaturated fatty acids, and mitochondrial LC-fatty acid beta-oxidation were significantly increased both in kidney and liver tissues. A beta-oxidation meta-pathway was similarly elevated in liver tissue under DR, and an enhanced pathway of synthesis and degradation of ketone bodies was detected in kidney tissue (Figure 19), indicating the use of alternative fuel for energy production from fatty acid beta-oxidation and ketone body synthesis during DR. Additionally, fatty acid omega-oxidation pathway was significantly enhanced in all four tissues with Z-score over two (Figure 19). Fatty acid omega-oxidation pathway, being a minor oxidation pathway, accounted for a small fraction of total fatty acid including very-long-chain fatty acids (VLCFAs, > 22 carbons), which are mainly beta-oxidized exclusively in peroxisome [95]. In peroxisomal disorder, a progressive neurodegenerative disease, elevated fatty acid omega-oxidation pathway is a rescue for reducing the accumulation of VLCFAs, followed by releasing beta-oxidized shorter-chain dicarboxylic acid into urine [96]. Here, enhanced fatty acid omega-oxidation pathway was responsible for reducing the accumulation of VLCFAs, and thereby, the incidence of neurodegenerative disease, and assisting fatty acid beta-oxidation to produce the essential energy.

Observations from pathway enrichment and GO analysis support the reduced fatty acid biosynthesis (Figures 19 and 20, respectively) in liver tissue. In contrast, biosynthesis of fatty acid and triacylglyceride were enhanced in kidney tissue (see Figure 19). These results indicated that fatty acid used for ketone synthesis is transferred from adipocytes to liver tissue and is produced by self-assembly in kidney tissue. In addition, enhanced steroid biosynthesis and reduced cholesterol
metabolism were identified both in kidney and liver tissues, whereas cholesterol biosynthesis was reduced in kidney tissue (Figure 19). Insulin, a key factor that regulates the absorption of glucose from the blood to liver tissue and glycogen synthesis, is known to decrease gluconeogenesis, and to activate lipid synthesis from fatty acid. As shown in Figure 11, insulin levels was significantly decreased in the DR group causing reverse effects, resulting in reduction of absorbed glucose level and fatty acid synthesis, along with an enhancement of glycogenolysis, gluconeogenesis, and fatty acid beta/omega-oxidation in actual tissues. Additionally, biosynthesis of aldosterone and cortisol pathway was significantly up-regulated in liver tissue of the DR group (Figure 19). As an antagonist of insulin, elevated cortisol stimulates gluconeogenesis to form glucose from lactate and certain amino acid like alanine, and inhibits the peripheral utilization of glucose by decreasing the translocation of glucose transporters (especially GLUT4) to the cell membrane [97,98].

Overall, reduced insulin resulted in enhancement of cortisol, which triggers glycogenolysis, gluconeogenesis, fatty acid beta/omega-oxidation, and ketogenesis in kidney and liver tissues to offer glucose and ketone bodies for other tissues’ use as fuel (see Figure 21). These individual results were interlinked with each other, leading to a distinctive energy metabolism as the beneficial effects of DR.
Figure 21 Overall distinctive energy metabolisms in the DR group. Altered metabolites and pathways (squared) were presented in global intra-tissue pathway. The increased metabolites and pathways in the DR group are colored in red, those decreased in blue, those without significant changes in green, and those not detected in black. The filled or dotted arrows indicate synthesis or delivery paths, respectively. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissues, respectively.
The beneficial effects of phase I and II detoxification systems, which are activated by DR, are thought to support the prevention of cancer. In this study, multi-organ metabolomics revealed that the levels of glycine and myo-inositol (an intermediate metabolite and precursor to glucuronic acid) were significantly increased in kidney and liver tissues of the DR group (Figure 17). As such, glycine and glucuronide conjugation phase II reaction should be greater in liver tissue of the DR group, as well as in kidney tissue [99]. In urine metabolomics study, the increased activity of phase II detoxification pathways in liver tissue of the DR group was confirmed by direct measurement of glycine and glucuronide conjugated metabolites, as well as by quantification of mRNA and protein levels of the essential phase II detoxification enzymes. Unfortunately, direct measurement of the glycine and glucuronide conjugated metabolites is not possible in kidney tissue by multi-organ metabolomics because of relatively lower molecular concentrations. As an alternative means, the mRNA levels of phase II detoxification enzymes, such as UGT1A, UGT2B, and GLYAT, were determined based on cDNA microarray data in kidney tissue. Indeed, the expression levels of phase II enzymes were significantly increased in kidney tissue of the DR group, as well as in liver tissue (Figure 22), consistent with the result from urine and multi-organ metabolomics studies, in which phase II detoxification reaction were elevated.

Using pathway enrichment analysis, the amino acid conjugation of benzoic acid pathway, as an initiation step of glycine conjugation phase II reaction [100], was also significantly up-regulated in both kidney and liver tissues (Figure 19). Furthermore, glucuronidation pathway, involved in a glucuronide conjugation
phase II reaction, was significantly up-regulated in liver tissue (Figure 19). These results provided the evidence supporting enhanced phase II reaction in liver and kidney tissues. Using a combination of multi-organ metabolomics and microarray, the higher activity of phase II detoxification reaction, by which attaching hydrophilic moieties to reactive metabolites and facilitating the elimination of the harmful metabolites for reducing toxicities, seems to contribute to the beneficial effects of DR.

In addition, two pathways were significantly increased in both kidney and liver tissues, involving nuclear receptors in lipid metabolism and toxicity and meta-pathway biotransformation (Figure 19), belonging to phase I detoxification, which is mediated by P450 [101]. These results indicated that DR induces higher activity of phase I detoxification pathways. As very consistent with the result of pathway enrichment analysis, a GO term named metabolism of xenobiotics by cytochrome P450 was significantly increased in kidney tissue of the DR group. The kidney tissue is involved in important physiological functions, including maintenance of water and electrolyte balance, metabolism and secretion of hormones, and excretion of the waste products. Although liver is generally considered the major organ responsible for drug metabolism, kidney is also now recognized to play an important role in metabolism of drugs, hormones, and xenobiotics [102]. Recently, cytochrome P450 protein isoforms, which play a dominant role in xenobiotic metabolism [103], have been found in kidney tissue [104]. As an example, aristolochic acid, a commonly-used traditional Chinese herbal medicine, performs slower clearance and higher accumulation in kidney and liver tissues of
cytochrome P450 reductase-null mice [105]. Moreover, nephrotoxicity in response to a calcineurin inhibitor, a therapy of transplantation, is reduced in a time-dependent manner by enhancing the levels of cytochrome P450 [106]. Studies show that dietary restriction recovers P450 gene expression levels in old age mice as much as the level of younger age group [107]. Thus, evaluated pathways and GO term related to phase I detoxification and its responsibility for higher elimination of toxic metabolites contributed to the beneficial effects of DR.

With the combination of pathway enrichment and GO analysis, enhanced phase I detoxification in the DR group was revealed as a novel pathway compared with urine metabolomics only discovering phase II detoxification. As phase I reaction are solely based on chemical modification such as oxidation and reduction, the most products of phase I detoxification remain rather non-polar. Given that non-polar metabolites are difficult to eliminate, they are usually phase II enzyme substrates. Phase II enzymes then promote the conjugation of charged derivatives, facilitating excretion in urine (see Figure 2) [108]. Therefore, it is not surprising that urine metabolomics was failed to discover phase I detoxification reaction up-regulated by DR, because of its role for modification and its lower production in urine.

In addition, renal organic cation/anion transport systems regulate the homeostasis of various positively/negatively charged organic solutes, including xenobiotics and endogenous substances, by mediating proximal tubular secretion and reabsorption [109]. To keep the clearance of the internal environment from potentially dangerous of xenobiotics and endogenous substances, renal transport
systems are effective in facilitating the flux of drug molecules for excretion [110]. Therefore, evaluated GO term of solute:cation symporter activity performed the effectiveness of excretion and provided evidence in support of enhanced phase I and II detoxification in the DR group.

Taken together, the beneficial effects of DR are thought to derive from the enhancement of phase I and II detoxification, as is summarized in Figure 23. Considering that use of multi-organ metabolomics and cDNA microarray uncovers particular pathways for DR’s beneficial effects through phase I and phase II detoxification, the combination approach is powerful for system biological analysis.
Figure 22 Levels of the phase II detoxification enzymes for glucuronide and glycine conjugation in liver and kidney tissues. Based on cDNA microarray data, the expression levels of the phase II detoxification enzymes for glucuronide and glycine conjugation, such as Uridine 5'-diphospho-glucuronosyltransferase 1A (UGT1A), 2B (UGT2B) and Glycine-N-acyltransferase (GLYAT) in liver (A) and kidney (B) tissues were examined. Statistical analysis was measured using Mann-Whitney U-test, and the resulting $p$-values are indicated. The solid boxes represent the 25 and 75 percentile values with the median value inside it.
Figure 23 Overall particular pathways for DR’s beneficial effects through phase I and phase II detoxification. The particular functional pathways involved in phase I and phase II detoxification providing the beneficial effects of DR in liver or kidney tissues are presented, respectively. The approaches for the identification of the particular pathways are shown in bold.
DR also can decrease the risk of age-related diseases and slow their progression [10,51,111]. Based on multi-organ metabolomics and cDNA microarray, several markers involved in age-related diseases and disorders were discovered. The elevation of uridine, inosine, and cytidine were identified in the DR group (see Table 4). Recently, a study showed that dietary uridine supplementation, but neither thymidine nor deoxyuridine, reduces the number of intestinal tumors and tumor burden by 40% ($p < 0.05$) relative to the control diet in Apc (Min/+ ) mouse model, a powerful tool for studying the genetic and dietary mechanisms which contribute to intestinal cancer phenotypes [112]. In addition, a clinical trial assessing the potential of the nutritional supplement inosine to treat Parkinson’s disease demonstrated that the elevated urate, an antioxidant, reduces the risk of Parkinson’s disease or slows its progression [113]. Furthermore, increased neuronal cytidine and uridine levels augment cytidine triphosphate (CTP) levels both in vitro [114] and in vivo [115]. Moreover, elevated CTP levels are important for the synthesis of major brain phospholipids, particularly phosphatidylcholine, that are associated with membrane-dependent process, such as potassium-induced striatal dopamine release [116]. Accordingly, these researches provide evidence that DR can decrease the incidence of neurodegenerative diseases and tumorigenesis.

Considering that the ammonia production is increased by elevated glucose-alanine cycling that results from high levels of gluconeogenesis in kidney and liver tissues and glycolysis in muscle tissue, ammonia toxicity could pose a problem during DR [117]. To lower the level of ammonia in these tissues, ammonia consumption pathway should be activated. Enhancements in glutamine synthesis
and urea cycle were identified, both of which can mitigate ammonia accumulation. As a result of these activities, glutamine levels in kidney, liver, and muscle tissues were increased. Urea cycle was additionally activated by increased aspartate and arginine in muscle tissue, accounting for further reduction in excess ammonia levels (see Figure 21). In addition, elevated pathways of biogenic amine synthesis and urea cycle and metabolism of amino groups in kidney tissue were evaluated (see Figure 19). These results confirmed that DR induces ammonia detoxification as its beneficial effects.

Several genes involved in preventing age-related disease were activated in the DR group. Among these was Ccng1, a transcriptional target of tumor suppressor p53, which was differently expressed in kidney, brain, and muscle tissues, as well as significantly increased in the DR group (Table 6). This gene is induced by DNA damage in a p53 dependent manner, and its expression recovers p53 accumulation in cyclin G1–/– mouse embryonic fibroblasts cells lines [119]. Elevated p53 pathway was observed in liver tissue of the DR group, supporting the observation that enhanced cyclin G1 caused the recruitment of p53. Accordingly, these results suggested that these tissues may be on a cancer prevention mode under DR condition.

Expression level of the gene Ddah1 was increased in kidney and liver tissues of the DR group as well (Table 6). Dimethylarginine dimethylaminohydrolases (DDAHs) generally play a role in metabolizing the serum asymmetric dimethylarginine (ADMA), which has been regarded as a risk factor for a number of disorders [120] and cardiovascular diseases [121]. Clinically, higher levels of
ADMA are routinely measured in patients with chronic kidney disease [122] and cirrhosis [123]. In rat model with cholestatic liver disease, DDAH activity is significantly decreased compared to the control group [124]. Accordingly, these results indicated that DR may reduce ADMA-associated diseases.

In kidney, liver, and brain tissues, the expression levels of the both the \textit{Cdkn1a} and \textit{Tpd52ll} genes were significantly reduced in the DR group (Table 6). The reduced cyclin-dependent kinase inhibitor 1A, also known as p21, which is a senescence marker [125,126], indicated that DR prevent the process of aging. Although the function remains elusive, the tumor protein D52-like 1 is very frequently overexpressed in multiple human cancers, including breast, lung, endometrial, hepatocellular, colon, pancreatic, ovarian, high-grade prostate carcinomas, and leukemia. Accordingly, its suppression by DR could have chemoprevention effects.

Given the role that the histocompatibility complex (MHC) class I antigen presentation pathway plays in the detection of virally infected cells by cytotoxic T lymphocytes [127], DR induces higher possibility to alert the immune system to virally infected cells according to the result of enhancement of gene level in kidney, liver, and muscle tissues of the DR group (Table 6).

Methylation pathway was significantly increased in kidney, liver, and brain tissues (Figure 19). DNA methylation is a well-defined epigenetic mechanism involved in regulation of gene expression. Additionally, proper methylation is important for development, and contributes to chromosomal stability. Loss of genomic DNA methylation has been noted as part of the aging process [128]. In
addition, previous studies have reported that damaged DNA fragmentation increases with age in rats, and that this phenomenon is reduced by DR [129]. Some effects of Sirt, which may play a pivotal role in the beneficial effects of DR, are also mediated through DNA methylation [130,131]. These results suggested that higher methylation induced by DR might play a protective role in age-related DNA damage.

The polyol pathway, also called sorbitol-aldose reductase pathway, generates sorbitol from unused glucose, which freely crosses kidney cell membrane. In diabetes, excessive activation of the polyol pathway results in elevated sorbitol levels and reactive oxygen species (ROS), while decreasing nitric oxide and glutathione [132]. Thus, reduction of the polyol pathway observed in kidney tissue of the DR group (see Figure 19) indicated that kidney tissue was releasing glucose into the bloodstream rather than producing sorbitol and that prevention of diabetes may be a beneficial effect of DR.

In muscle tissue, hedgehog signaling pathway and NFE2L2 were enhanced in the DR group (Figure 19). The hedgehog signaling pathway plays an important role in tissue homeostasis, modulating tissue regeneration. Its misregulation has been associated with several types of cancers [133,134]. In addition, NFE2L2, also known as Nrf-2, plays an important role in modulating the expression of several antioxidant enzymes. The significant increase of these pathways under DR condition can improve the ability of self-protection from tissue injury.

The biosynthesis of aldosterone pathway was significantly up-regulated in liver tissue of the DR group (Figure 19). An exogenous aldosterone, in remnant kidney
rat model, performed greater proteinuria, glomerulosclerosis, and hypertension, indicating its contribution to hypertension and renal injury in the remnant kidney model [135]. Thus, the enhanced biosynthesis of aldosterone in the DR group could help prevent renal deterioration.

Hypertrophy model pathway was significantly decreased in kidney tissue (Figure 19). Kidney hypertrophy due to cell arrest in the G1-phase and its enlargement result in loss of renal function. These results indicated that kidney tissue keeps healthy during DR [136].

The pathways related to inflammation, including prostaglandin synthesis and regulation and inflammatory response involving fibronectin, collagen, and laminin expression, were reduced in kidney tissue (Figure 19). Prostaglandins play a key role in the generation of the inflammatory response and their production is generally very low in uninflamed tissue, but increases immediately in acute inflammation prior to the recruitment of leukocytes and the infiltration of immune cells [137]. With interstitial inflammatory rat model induced by injection of bovine serum albumin, the expressions of fibronectin, collagen, and laminin were increased in renal tissue [138]. Accordingly, these results indicated that lower inflammatory response contributed to the beneficial effects of DR.

GPCRs are a "superfamily" that comprises a group of proteins divided into three main classes: A, B, and C. With no detectable shared sequence homology between classes, these proteins are involved in a wide variety of physiological processes that include nervous system transmission and inflammatory mediation. In brain tissue, the metabotropic glutamate/pheromone GPCRs class C was decreased and
the additional pathways related to several others’ GPCR was significantly increased (Figure 19). As metabotropic glutamate receptors included in GPCRs class C still can be divided into several groups, the subgroups have a different role in biological function. For example, agonists for group II (mGlu2/3) receptors and antagonists for group I (in particular mGlu5) receptors have shown activity in animal and/or human conditions of fear, anxiety or stress, having an influence opposite to that shown by group I or group II mGlu-receptor agonists on excitotoxic neuronal death [139,140]. The opposite regulation of GPCRs in brain tissue of the DR group is not easy to reconcile, however, the role of GPCRs should be speculated as the beneficial effects of DR in nervous system and inflammatory regulation based on the non-changed level of glutamate and increased level of MHC class I gene, and warrants further confirmation.

Oxidative stress is biological state and has been defined as an imbalance in the antioxidant organization, ultimately resulting in potential for cell damage. Several biological and pathological processes have been linked to oxidative stress, including aging [141], inflammation [142], carcinogenesis [143], and in diseases such as Parkinson’s [144] and Huntington’s [145]. Apoptosis, an essential process for the health of most multi-cellular organisms, has also been linked to these disorders and diseases [146-149], suggesting that both processes might be involved in the development of pathologies. Dietary restriction has also been shown to affect age-related phenotypes, such as enhancement of ROS level, and to increase the rate of apoptosis [150]. Thus, higher regulation of intrinsic apoptotic signaling pathway
in response to oxidative stress may well reflect the role of protection from oxidative stress on liver cells by DR (see Figure 20).

Stearoyl-CoA desaturase is a key enzyme in fatty acid synthesis, and its increased expression levels are associated with tumor malignancy [151]. Stearoyl-CoA desaturase knock-out mice showed reduced body adiposity and increased insulin sensitivity, as well as resistance to diet-induced obesity. These studies were performed in stearoyl-CoA desaturase knock-out mice, suggesting that this enzyme appears to be an important metabolic modulator, and that inhibition of its expression could benefit for the treatment of obesity, diabetes and other metabolic disorder [152]. Considering the above, the decreased GO term of stearoyl-CoA 9-desaturase activity could account for reduced incidence of cancer, diabetic disease, and metabolic disorder by DR (see Figure 20).

Cellular response to alkaloid was enhanced in brain tissue of the DR group (Figure 20). Alkaloids are a group of natural products, containing mostly basic nitrogen atoms. Over ten classes of alkaloid have been found and applied clinically. One such as alkaloid, vincamine, is used as a peripheral vasodilator to combat the effect of aging. Administration of vincamine performs approximately 50% reduction in brain of Fe concentration, whose disturbance is associated with aging related neurodegenerative disease, including Parkinson's, Alzheimer's, and Huntington's diseases [153]. Down-regulation of the channels, such as 5-hydroxytryptamine receptor 1B (HTR1B) and transient receptor potential cation channel subfamily V member 1 (Trpv1) involved in cellular response to alkaloid, induces psychiatric disorder [154]. The elevation of cellular response to alkaloid,
thus, resulted in higher availability to modulate alkaloid’s susceptibility and reduction of the incidence of neurodegenerative diseases by DR.

Elastin biosynthetic process was found to be increased in muscle tissue of the DR group (Figure 20). Elastin is a protein in connective tissue, and plays a role for resuming tissues’ shape after stretching or contracting. Impairment of elastin has been implicated in several diseases, including Hurler disease [155] and Williams syndrome [156]. The fragmentation of elastin has been observed in aortas from aged mice [52], and higher levels of elastin are present in the extracellular matrix of cells derived from the younger animals [157]. These results indicated that enhanced elastin biosynthetic process contributes, at least in part, to the beneficial effects of DR.

Overall, DR-associated several markers were identified using multi-organ metabolomics and cDNA microarray analysis. These results showed that DR induced beneficial metabolic and genomic changes in the analyzed tissues, preventing age-related diseases, including diabetes, cancer, cardiovascular, hepatic, renal, and neurodegenerative diseases. Moreover, altered gene expression by DR likely reduces age-related disorders, such as ammonia toxicity, senescence, inflammation response, and ROS (see Table 8). Notably, certain additional metabolites, genes, pathways, and GO terms were revealed in current study. However, due to some limitations, their actual functions correlate to DR were remained unresolved and should be evaluated as further works.
Table 8 Overall markers for DR’s beneficial effects*.

<table>
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<tr>
<th>Approach</th>
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*The approach, name of markers, significant change of markers for the DR group comparing with the control group, and respective function of markers are indicated. The metabolites not detected in respective tissue are remained as blank, and the significant increase or decrease confirmed with student’s t-test (p < 0.05) is indicated as the symbol of up or down, respectively. “K”, “L”, “B”, and “M” indicate kidney, liver, brain, and muscle tissues, respectively.
Conclusion

With systems biology including urine metabolomics, multi-organ metabolomics, and cDNA microarray, the beneficial effects of DR were investigated. Urine metabolomics revealed markers from glucuronide and glycine conjugation reaction in the DR group. Extensive profiling of urine samples using neutral loss scanning presented enhanced levels of phase II metabolites. The up-regulation of phase II detoxification in the DR group was confirmed by mRNA and protein expression levels of the associated enzymes, UGT and GLYAT, in the liver tissue. Additionally, the Nrf-2 signaling pathway was found to be up-regulated, giving evidence of the enhancement of phase II detoxification activity in the liver. Multi-organ metabolomics revealed higher phase II detoxification and distinct energy metabolism pathways in the DR group. Multi-organ cDNA microarray analysis coupled with gene, pathway enrichment, and gene ontology terms provided consistent results for the mechanism revealed by multi-organ metabolomics study. In addition, pathway enrichment and gene ontology analysis provided evidence of enhanced phase I detoxification in the DR group. Several other markers were also found to be related with age-associated diseases such as diabetes, cancer, and cardiovascular, neurodegenerative, hepatic, and renal diseases. This was consistent with histopathology and serum biochemistry results that showed the actual beneficial effects of DR in the current experimental system. Collectively, DR
induces higher phase I and phase II detoxification, a distinctive energy metabolism, and lower the incidence of age-related diseases to perform its beneficial effects.
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microsomes: identification of relevant acetaminophen UDP-

Kinetics of acetaminophen glucuronidation by UDP-
glucuronosyltransferases 1A1, 1A6, 1A9 and 2B15. Potential implications in


국문초록

시스템 생물학을 통한 쥐에서의 식이제한의 유익한 효과에 기여하는 해독작용의 증가 연구

문 혁
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식이제한은 많은 유익한 효과가 있지만 그에 대한 자세한 대사체학적 메커니즘은 아직 잘 알려져 있지 않다. 대사체학과 유전체학을 포함하고 있는 시스템 생물학 접근방법을 사용하여 여러 조직에서의 식이제한 효과와 연관된 분자간 상호작용을 찾고자 하였다. 먼저, 핵자기공명기기와 질량분석기를 사용하여 대조군과 식이제한군에서 얻은 소변 샘플에 대한 대사체학적 프로파일링을 진행하였다. 다변량 통계분석 결과는 식이제한 군에서 독특한 대사체학적 프로파일링을 보여주었고 glucuronide나 glycine 콘쥬게이션 경로와 연관된 마커들을 규명하였다. Neutral loss scanning을 이용한 소변샘플에서의 phase II 대사물질의 프로파일링 결과에서는 식이제한군에서 glucuronide나 glycine 콘쥬게이션 대사물질이 보편적으로 많이 존재함을 보여주었다. 간조직에서의 UGT와 GLYAT의 메신저 RNA와 단백질 발현량의 비교 분석을 통하여 식이제한군에서 phase II 해독기전이 증가됨을
확인하였다. 또한 간조직에서의 증가된 phase II 해독기전에 대한 메커니즘은 Nrf-2 신호전달과정이 증가된 결과로부터 단서를 찾았다. 다음, 대사체학과 cDNA 마이크로어레이를 사용하여 대조군과 식이제한군에서 얻은 다중장기의 대사체학적 및 유전체학적 프로파일링을 조사하였다. 다중장기 대사체학 마커들은 식이제한군에서 phase II 해독기전의 증가와 독특한 에너지 메커니즘을 보여주었다. 또한 게놈, 강화경로와 유전자 온돌로지를 결합한 마이크로어레이 분석결과는 다중장기 대사체학에서 밝힌 메커니즘과 동일하였다. 그리고 강화경로분석과 유전자 온돌로지 분석결과는 식이제한 군에서 증가된 phase I 해독기전이 식이제한의 유익한 효과 중 하나임을 제시하였다. 본 연구에서 발견된 다른 마커들은 당뇨질환, 암, 심장질환, 신경퇴행성질환, 간질환과 신장질환 등 노화 관련 질환들과 연관성을 확인하였다. 이러한 결과는 본 실험조건에서 실제 식이제한의 유익한 효과를 보여주는 조직병리학, 혈액생화학 결과들과 동일하다. 결론적으로, 대사체학, 마이크로어레이와 생화학적 연구결과는 식이제한 유익한 효과의 복합적인 메커니즘을 알아가는데 대사체학적, 유전학적 관점을 제공한다.

주요어: 식이제한, 대사체학, 마이크로어레이, Phase I 해독기전, Phase II 해독기전, UGT, GLYAT, P450.
학번: 2012-30772
This appendix includes the reprint of six published papers. Among them, I was involved as a first or a co-first author in one paper or five papers, respectively. These works were done during my doctor course in Seoul National University under my supervisor Professor Sunghyouk Park.

Appendix

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Enhanced Phase II Detoxification Contributes to Beneficial Effects of Dietary Restriction as Revealed by Multi-platform Metabolomics Studies*

He Won‡§, Hye-ji Yang¶§, Yong Jin An¶, Joon Mee Kim¶, Dae Hyun Lee**, Xing Jint†, Sung-woo Park‡‡, Kyung-Jin Min§§, and Sunghyouk Park¶¶

Dietary restriction (DR) has many beneficial effects, but the detailed metabolic mechanism remains largely unresolved. As diet is essentially related to metabolism, we investigated the metabolite profiles of urines from control and DR animals using NMR and LC/MS metabolomic approaches. Multivariate analysis presented distinctive metabolic profiles and marker signals from glucuronide and glycine conjugation pathways in the DR group. Broad profiling of the urine phase II metabolites with neutral loss scanning showed that levels of glucuronide and glycine conjugation metabolites were generally higher in the DR group. The up-regulation of phase II detoxification in the DR group was confirmed by mRNA and protein expression levels of uridine-ribosylphosphor glucuronosyltransferase and glycine-N-acetyltransferase in actual liver tissues. Histopathology and serum biochemistry showed that DR was correlated with the beneficial effects of low levels of serum alanine transaminase and glycoxygen granules in liver. In addition, the Nuclear factor (erythroid-derived 2)-like 2 signaling pathway was shown to be up-regulated, providing a mechanistic clue regarding the enhanced phase II detoxification in liver tissue. Taken together, our metabolomic and biochemical studies provide a possible metabolic perspective for understanding the complex mechanism underlying the beneficial effects of DR. Molecular & Cellular Proteomics 12: 10.1074/mcp.M111.021352, 575-586, 2013.

It has been known for more than 70 years that dietary restriction (DR) can extend the life span and delay the onset of age-related diseases, based on an early rodent study showing such effects (1). However, not until the 1960s was DR recognized as a good model for studying the mechanism of or inhibitory measures for aging (2). So far, extensive studies employing model organisms such as yeasts, nematodes, fruit flies, and rodents have shown that DR has beneficial effects in most of the species studied (for a review, see Ref. 3). Most notably, a recent 20-year-long study showed that monkeys, the species closest to humans, also benefit from DR similarly (4). Although there has not been (or could not have been) a systematic study on the effects of DR on the human life span, several longitudinal studies strongly suggest that changes in dietary intake can affect the life span and/or disease-associated marker values greatly (5-7).

This inverse correlation between dietary intake and long-term health strongly indicates that DR’s effects should involve metabolism, and that DR elicits the reorganization of metabolic pathways. It also seems quite natural that something we eat should affect the body’s metabolism. Despite this seemingly straightforward relationship between diet and metabolism, the mechanisms underlying the beneficial effects of DR are anything but simple. Intensive efforts, spanning decades, to understand the mechanisms of DR have identified several genes that might mediate the effects of DR, such as mTOR, IGF-1, AMPK, and SIRT1 (for a review, see Ref. 8). Still, most of them are involved in early nutrient-sensing steps, and specific metabolic pathways, especially those at the final steps actually responsible for the effects of DR, are largely unknown.

This might be at least partially due to the fact that previous studies have focused mostly on genomic or proteomic...
changes induced by DR, instead of looking at changes in metabolism or metabolites directly. Metabolomics, which has gained much interest in recent years (8–11), might be a good alternative for addressing the mechanistic uncertainty of DR’s effects, with the direct profiling of metabolic changes elicited by environmental factors. In contrast to genomics or proteomics, which often employ DNA or proteins extracted from particular tissues, metabolomics studies mostly employ body fluids (i.e., urine or blood), which can reflect the metabolic status of multiple organs, enabling investigations at a more systemic level. In particular, urine has been used extensively to study the mechanism of external stimuli (i.e., drugs or toxic insults) at most major target organs, such as the lung, kidney, liver, or heart (12–18). Still, metabolomics studies of DR effects have been very limited. A few previous ones reported the changes in phenomenological urine metabolic markers with DR, without identification and/or validation of specific metabolic pathways reflected at the actual tissue or enzyme level (19, 20). Therefore, those studies fell short of providing a mechanistic perspective on DR’s effects. In addition, they employed either NMR or LC/MS approaches without validation across the two analytical platforms.

Among the metabolic pathways that can directly affect the integrity of multiple organs, and hence long-term health, are phase II detoxification pathways (21). Typically, xenobiotics, endogenous metabolites are metabolized first by a phase I system, such as cytochrome P450, which modifies the compounds so that they have hydrophilic functional groups for increased solubility. In many cases, though, these modifications might increase the reactivity of the compounds, leading to cellular damage. The phase II detoxification systems involve conjugation reactions that attach charged hydrophilic molecular moieties to reactive metabolites, thus facilitating the elimination of the harmful metabolites from body, ultimately reducing their toxicity (22). These systems are thus especially important in protecting cellular macromolecules, such as DNA and proteins, from reactive electrophilic or nucleophilic metabolites. The enzymes involved in these processes include glutathione-S-transferase (GST), sulfotransferase, glycine-N-acetylsulfotransferase (GLYAT), and uridine diphospho-glucuronyltransferase (UGT), with the last enzyme being the most prevalent (23). The beneficial effects of phase II reactions have been particularly studied in relation to the mechanism of healthy dietary ingredients. It is well believed that many such foods can prevent cancers (hence the term “chemoprevention”) by inducing phase II detoxification systems (24–26). Although DR also substantially reduces the incidence of cancers, the exact mechanism remains elusive.

Here, we employed multi-platform metabolomics to obtain metabolic perspectives on the beneficial effects of DR on rats. Our results about urine metabolomic markers suggest that DR enhances the phase II detoxification pathway, which was confirmed by means of conjugation metabolic profiling and changes in mRNA/protein expression levels of phase II enzymes in actual liver tissues. A possible molecular mechanism was also addressed through the exploration of Nuclear factor erythroid-derived 2-like 2 (Nrf-2) pathway activation upon DR. We believe the current study provides new metabolic insights into DR’s beneficial effects, as well as a workflow for studying DR’s effects from a metabolic perspective.

**EXPERIMENTAL PROCEDURES**

Chemicals and Reagents—HPLC-grade acetone/titr and water were purchased from Burdick & Jackson (Muskegon, MI). Chemicals for NMR and LC/MS analyses were obtained from Sigma-Aldrich (St. Louis, MO). Vendors for both drugs and reagents are indicated in the corresponding sections.

Animal and Diet—Male Sprague-Dawley rats (8 weeks of age) were purchased from Orient Bio (Gumnam, Seoul, Korea) and housed on a 12-h light/dark cycle prior to the experiment. Animal care and all experimental procedures were conducted in accordance with the guide for animal experiments edited by the Korea Academy of Medical Science. After 7 days of acclimatization, rats were randomly assigned to two groups: control (n = 10) and DR (n = 13). Rats in the control group had ad libitum access to standard rodent chow at all times. The DR practice was performed following the established protocols (27). Specifically, rats in the DR group received 60% of the food intake of the control group at specified feeding times. The food intake of the control group was calculated by subtracting the amount of the remaining food from the initial amount of food given. The amount of remaining food was carefully measured to include all the broken chow pieces in the cages. Food was given every Monday, Wednesday, and Friday, and a factor of 1.5 was considered in the calculation of the DR group’s food allotment for Friday and Monday feeding.

**Urine, Blood, and Tissue Collection**—The pooling of urine samples was done over a 24-h period. The urine was collected in co-occluded collection jars with sodium oxide in them to prevent bacterial growth and sample deterioration. This collection was done once every 2 weeks. Fecal contamination was prevented by using commercially available metabolic cages specifically designed for that purpose. The pooled urine samples were frozen and stored at −80°C for subsequent analysis. Blood was collected from the heart upon sacrifice at the end of the experiment. In order to obtain serum, the collected blood was incubated at room temperature for clotting and then centrifuged at 1,500 rpm for 10 min at 4°C. The supernatant (clear yellow fluid) was moved to centrifuge tubes, snap-frozen with liquid nitrogen, stored at −80°C, and thawed just before analysis. For liver tissue collection, the same small parts of the liver tissue of each sacrificed rat were snap-frozen with liquid nitrogen and stored at −80°C until use. The rest of the tissues were anced in paraffin blocks using routine procedures and were used for histopathological examination.

**NMR Spectroscopic Analysis of Urine**—For NMR analysis, urine samples were thawed at room temperature, and 500 µl of urine was mixed with 55 µl of potassium phosphate buffer (pH ~ 7.4). The insuble parts were removed via centrifugation at 13,000 rpm for 10 min. A mixture of 500 µl of supernatant and 56 µl of D2O containing sodium-3-trimethylsilyle[2,2,3,3,3-H4] 1-propanol (0.02%), w/w, as an internal standard was placed in a 5-mm NMR tube. All one-dimensional spectra of the urine samples were measured with an NMR spectrometer (Avance 500, Bruker Biospin, Rheinstetten, Germany) operating at a proton NMR frequency of 500.13 MHz. The NMR experiment was performed at the NMR facility at the Korea Basic Science Institute. We also used a 500 MHz machine (Varian Inc., Palo Alto, CA) at Vanin Inc. Korea’s facility for metabolite identification. The acquisition parameters were essentially the same as those previously re-
Reverese Transcription PDR and Western Blot Analysis—Total RNA was isolated from the liver tissues using the easy-spinTM Total RNA Extraction Kit (Intron, Inc., Seou, Korea). The first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA) according to manufacturer instructions. The primer sequences of UGT1A1, UGT2B1, and GLYT were as follows: UGT1A1, sense 5'-ACAAGGCAAGACAGCATCACTG-3’, antisense 3’-TTGGAACCCCGATCTGATATT-5’, UGT2B1, sense 5’-ATGCGGACAAAGGCG-3’, antisense 3’-GGGAAATCCATCAACATC- CGGGAGGTT-5’; GLYT, sense 5’-CCGTTGAACCCGTATATT-3’, antisense 3’-GGTGGGCGCAAGTTGGA-5’. The predicted size was 229 bp.

For Western blots, 60 mg of liver tissue was measured and ground into powder using a mortar under liquid nitrogen. The powders of liver tissue were suspended in 1 ml of RIPA buffer containing protease inhibitor (0.2 μg/ml of Aprotinin, 1 μg/ml of Pepstatin, and 1 μM of PMSF) and put on ice for 30 min. After centrifugation at 13,000 rpm for 30 min, the supernatant containing the total protein released from the liver tissue was quantified using a BCA Protein Assay Kit (Pierce, Appaloa, WA). Twenty-five micrograms of total protein was subjected to 10% SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membranes (Bio-Rad). The membrane was blocked with antibodies against UGT1A1, UGT2B1, multidrug resistance-associated protein 3 (MRP-3), NADPH dehydrogenase (1NAD-1) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB (1:100; Santa Cruz Biotechnology), and heme oxygenase-1 (HO-1) (1:1000; Enro Life Sciences, Ann Arbor, MI), followed by treatment with anti-rabbit, anti-goat, or anti-rat IgG (1:10000; Santa Cruz Biotechnology) secondary antibodies conjugated with HRP at room temperature for 1 h.

Liver Histopathology and Serum Biochemistry—After fixation for 48 h, liver tissues were embedded in paraffin according to routine procedures. Four-micrometer-thick sections were cut and stained with H&E and periodic acid–Schiff stains for histopathological evaluation. An expert pathologist at Inha University Hospital blindly analyzed the tissue slices. Serum aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), and low density lipoprotein (LDL) levels were measured using commercial kits at Inha University hospital (Incheon, Korea).

RESULTS

General Assessment of DR Effects—To ensure that our DR procedure led to a difference between the control and DR groups, we measured general parameters such as body weight, serum LDL, and serum TG (supplemental Fig. S1). Body weight showed time–dependence differences between the two groups. In addition, LDL and TG levels significantly decreased after 3 months of DR. Given that LDL and TG are important risk factors for age-associated diseases, our DR
Phase II Reactions Contribute to DR’s Effects

procedure established a clear difference between the control and DR groups.

NMR and LC/MS Metabolomic Analysis of DR Effects—Dietary intake is intimately related to metabolism, but the effects of DR on specific metabolic pathways, as related to the beneficial results, have been little explored. We measured the urine metabolic profiles of the control and DR rat groups, because urine can reflect well the systemic effects of external stimuli and has been used extensively for organism-level metabolomics studies (13–15, 32, 33). We used both NMR and LC/MS to widen the metabolite coverage and cross-confirmation of detected metabolites.

Representative NMR spectra with identified metabolites (Figs. 1A and 1B) and an LC/MS chromatogram (Figs. 1C and 1D) are shown here. Although the overall signal profiles are quite similar for the two groups, there were seemingly specific signals in each group. To evaluate the statistical meaning of those signals and exclude possible confounding variables not related to the group difference, we applied the orthogonal projections to latent structure-discrimination analysis (OPLS-DA) multivariate analysis to all of the NMR and LC/MS data. The discrimination model for NMR and LC/MS could differentiate between the control and DR groups without any overlap (Figs. 2A and 2B) with the following statistical characteristics. For NMR, the model had one predictive and three orthogonal components with $Q^2(Y) = 0.637$, $R^2(Y) = 0.936$, and total $R^2(Y) = 0.659$, with 0.112 being predictive and 0.547 being orthogonal. For LC/MS, there were one predictive and two orthogonal components with $Q^2(Y) = 0.808$, $R^2(Y) = 0.986$, and total $R^2(Y) = 0.593$, with 0.305 being predictive and 0.303 orthogonal. These results indicate that the LC/MS approach performed slightly better in separating the class-specific signals from the confounding variables. Still, both models had quite high cross-validated predictability and goodness-of-fit values, meaning reliable differentiation between the groups.

Metabolites Related to DR—With the successful distinction between the control and DR groups, we tried to identify specific metabolites contributing to the difference. For this, we built the S-plot from the orthogonal projections to latent structure-discrimination analysis (OPLS-DA) model and picked the signals with high correlation and signal-to-noise ratio values. The distribution of the correlation and covariance values suggested that the DR group was represented by relatively conspicuous signals, whereas the control group was represented by the sum of weaker contributing signals (Figs. 3A and 3B). The analysis also showed that the NMR signals at 7.36, 7.42, 7.05, 2.45, and 2.93 ppm and LC/MS signals at $m/z = 114.19$, 194.17, and 340.22 were among the major contributors. The NMR spectral analysis and MS/MS analysis showed that these signals belonged to phenylacetylglucose, 1-methylhistidine, 2-oxoglutarate, N,N-dimethylglycine, creatinine, and hydroxymethoxyindole glucuronide (Table I). To confirm the significance of these metabolites found via the multivariate approach, we performed a Mann-Whitney U test on the levels of these metabolites (Figs. 3C–3J). The results reflected the statistical validity of these markers in the differentiation of the control and DR groups.

Profiling of General Glucuronide and Glycine Conjugation via Neutral Loss Scanning—Two metabolites with high levels in the DR group, hydroxymethoxyindole glucuronide and phenylacetylglucose, turned out to be common products of phase II detoxification reactions, created through glucuronide and glycine conjugation mechanisms, respectively. As there could be many other conjugation products from these pathways, we decided to measure general profiles of compounds that have
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Fig. 2. Differentiation of control and DR groups using multivariate analysis. OPLS-DA score plot of control and DR groups from NMR (A) and LC/MS (B). Black circles: control group; open triangles: DR group. The models were established using one predictive and three orthogonal components for NMR, and one predictive and two orthogonal components for LC/MS.

Fig. 3. Marker signals contributing to the differentiation between the control and DR groups. S-plot analysis to identify the contributing signals for the control and DR groups from NMR (A) and LC/MS (B). P represents modeled covariance, and Pcor represents modeled correlation. Potential marker signals that are significantly biased across the two groups are enclosed in boxes. The levels of the signals identified by the analyses were compared via Mann-Whitney U test, and the resulting p-values are indicated. 7.06 ppm (O), 7.42 ppm (E), and 7.06 ppm (B) from NMR, m/z = 114.19 (P), m/z = 194.17 (G), and m/z = 340.22 (H) from LC/MS significantly increased in DR group; 2.45 ppm (O) and 2.50 ppm (U) from NMR decreased in DR group. The solid boxes represent the 25th and 75th percentile values with the median value inside. The whiskers represent outliers with a coefficient value of 1.5.

conjugated glucouronide or glycin. LC/MS enables the detection of these profiles through neutral loss scanning of 176 (glucouronide) and 57 (glycin). Fig. 4 shows that the p-values for the DR group were higher in number and intensity. Statistical analysis with Student's t test on the peak area showed that the general profiles of both glucouronide and glycin conjugated metabolites were enhanced in the DR group, with p-values of 0.017 or 0.011, respectively, confirming that the DR group had higher activity in these pathways.

Assessment of Phase II Detoxification Pathways in the Liver—As the urine metabolite profile showed generally enhanced conjugation reactions, we tried to confirm that the differences were also manifested in the actual tissues. For that, we first measured the levels of the two key markers, hydroxymethoxyindole glucouronide and phenylacetylglucine, in the liver tissue, as the liver is the main organ for the phase II detoxification pathways. The LC/MS measurement of the levels of these metabolites showed that they were elevated in the livers of the DR group (supplemental Fig. S2), indicating that the urine metabolomics results adequately reflected the metabolic changes in the liver tissues. For more detailed pathway analysis, we investigated the enhanced conjugation reactions at the metabolic enzyme level. We compared the mRNA levels of UGT1A, UGT2B, and GLYAT, primary enzymes for glucouronide and glycin conjugation reactions, respectively. Figs. 5A and 5C show that all the enzymes had significantly enhanced mRNA expression in the DR group. Then, we measured the protein levels of these enzymes via Western blot. Both UGT1A and UGT2B showed similarly enhanced protein levels in the DR group (Figs. 5B and 5D), consistent with the
### Table 1
Metabolites identified via NMR and LC/MS analysis

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>ppm (multiplicity) for NMR: m/z, Ret. time (min), daughter ions for LC/MS</th>
<th>p value lower than</th>
<th>Fold change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1-methylhistidine</td>
<td>3.20(t), 3.25(t), 7.65(s)</td>
<td>4.86 &lt; 10^-4</td>
<td>83.46</td>
</tr>
<tr>
<td>2 2-carboxyglutarate</td>
<td>2.45(s), 3.02(t)</td>
<td>0.045</td>
<td>22.60</td>
</tr>
<tr>
<td>3 3-indolylsulfate</td>
<td>7.20(t), 7.20(t), 7.37(s), 7.51(d), 7.79(d)</td>
<td>0.054</td>
<td>40.86</td>
</tr>
<tr>
<td>4 3-methylthiopurine</td>
<td>0.93(t), 2.01(d), 2.28(m)</td>
<td>0.040</td>
<td>-0.52</td>
</tr>
<tr>
<td>5 Acetate</td>
<td>1.50(s)</td>
<td>0.072</td>
<td>7.01</td>
</tr>
<tr>
<td>6 Acetocarboxylic acid</td>
<td>2.30(t), 3.52(t)</td>
<td>0.012</td>
<td>31.23</td>
</tr>
<tr>
<td>7 Adamantimide</td>
<td>8.32(t), 8.36(s)</td>
<td>0.710</td>
<td>16.23</td>
</tr>
<tr>
<td>8 Alphenol</td>
<td>1.49(t), 3.60(m)</td>
<td>0.063</td>
<td>12.44</td>
</tr>
<tr>
<td>9 Albutenamide</td>
<td>5.38(t), 6.07(a)</td>
<td>0.120</td>
<td>23.92</td>
</tr>
<tr>
<td>10 Acyclic acid</td>
<td>1.86(m), 1.95(m)</td>
<td>0.038</td>
<td>-9.42</td>
</tr>
<tr>
<td>11 Citrate</td>
<td>2.55(t), 2.09(t)</td>
<td>0.076</td>
<td>-47.57</td>
</tr>
<tr>
<td>12 Dimethylsulfone (DMSO)</td>
<td>2.73(a)</td>
<td>0.273</td>
<td>-9.13</td>
</tr>
<tr>
<td>13 Ethanol</td>
<td>1.17(b), 3.63(a)</td>
<td>0.140</td>
<td>4.10</td>
</tr>
<tr>
<td>14 Formate</td>
<td>8.47(s)</td>
<td>0.028</td>
<td>-19.42</td>
</tr>
<tr>
<td>15 Fucose</td>
<td>1.21(b), 1.24(a)</td>
<td>0.798</td>
<td>-1.68</td>
</tr>
<tr>
<td>16 Glycine</td>
<td>3.00(s)</td>
<td>0.764</td>
<td>-22.13</td>
</tr>
<tr>
<td>17 4-Quinolone</td>
<td>3.80(t)</td>
<td>0.026</td>
<td>-10.42</td>
</tr>
<tr>
<td>18 Hopurinic acid</td>
<td>3.70(s), 5.20(s), 7.40(s), 7.84(d), 8.54(s)</td>
<td>0.856</td>
<td>2.82</td>
</tr>
<tr>
<td>19 Indole-3-acetate</td>
<td>7.13-7.30(m), 7.51(d)</td>
<td>0.091</td>
<td>24.79</td>
</tr>
<tr>
<td>20 Lactate</td>
<td>1.30(b), 4.10(m)</td>
<td>0.037</td>
<td>-14.89</td>
</tr>
<tr>
<td>21 N,N-dimethylglycine</td>
<td>2.05(s), 3.70(s)</td>
<td>0.018</td>
<td>-45.23</td>
</tr>
<tr>
<td>22 N-acetylaspartate</td>
<td>2.0(s), 2.53(m), 2.73(m), 4.40(m)</td>
<td>0.251</td>
<td>-9.28</td>
</tr>
<tr>
<td>23 Pyrolysin</td>
<td>2.41(t), 6.68(b)</td>
<td>0.243</td>
<td>-20.99</td>
</tr>
<tr>
<td>24 Pyruvate</td>
<td>2.38(s)</td>
<td>0.714</td>
<td>5.99</td>
</tr>
<tr>
<td>25 Saccharose</td>
<td>2.41(s)</td>
<td>0.012</td>
<td>-20.99</td>
</tr>
<tr>
<td>26 Taurine</td>
<td>3.28(r), 3.42(t)</td>
<td>0.094</td>
<td>-49.03</td>
</tr>
<tr>
<td>27 Trigonolin</td>
<td>4.41(b), 8.84(b), 0.13(a)</td>
<td>0.890</td>
<td>6.79</td>
</tr>
<tr>
<td>28 Tyrosine</td>
<td>0.87(b), 7.19(d)</td>
<td>0.245</td>
<td>-10.98</td>
</tr>
<tr>
<td>29 Uracil (uridine)</td>
<td>5.72(s)</td>
<td>0.010</td>
<td>-20.99</td>
</tr>
<tr>
<td>30 Urea</td>
<td>5.8 (w)</td>
<td>0.091</td>
<td>-5.89</td>
</tr>
<tr>
<td>31 p-cresol</td>
<td>2.05(s), 6.82(s)</td>
<td>0.393</td>
<td>-5.89</td>
</tr>
<tr>
<td>32 xanthine-acetate</td>
<td>3.49(t), 6.35(s)</td>
<td>0.037</td>
<td>-5.79</td>
</tr>
<tr>
<td>33 Methylhypoxanthine</td>
<td>2.40(t), 17.55(d), 149, 119, 105</td>
<td>3.01 &lt; 10^-5</td>
<td>138.81</td>
</tr>
<tr>
<td>34 Phosphocreatine/glycerine</td>
<td>184.17, 184.17, 176, 149, 149, 91</td>
<td>1.20 &lt; 10^-6</td>
<td>86.78</td>
</tr>
<tr>
<td>35 Creatinine</td>
<td>114.19, 3.81, 114.46, 46</td>
<td>1.44 &lt; 10^-3</td>
<td>16.90</td>
</tr>
<tr>
<td>36 Hydroxymethyloxanthine</td>
<td>340.22, 163.164, 146, 122, 118</td>
<td>5.13 &lt; 10^-4</td>
<td>103.78</td>
</tr>
<tr>
<td>37 Methionine</td>
<td>235.13, 3687-156, 156, 103, 109</td>
<td>8.03 &lt; 10^-4</td>
<td>-11.15</td>
</tr>
</tbody>
</table>

The associated signal numbers (see Fig. 1), structural identifiers (NMR: ppm and multiplicity; MS: m/z, ret. time and daughter ions), and Mann-Whitney U-test (stars with p < 0.05) are presented. The fold change values are from area-normalized peak intensities and represent percent changes in the DR group with respect to the control values, with negative values for decreases and positive values for increases. All of the NMR-identifiable metabolites and LC/MS-identified metabolites with significant changes are included. Additional metabolites identified via LC/MS without significant changes are listed in supplemental Table S1. All the metabolites were confirmed with standard compounds and LC/MS analysis, except for two that were confirmed via 1H and 13C trans-cycloketones (see "Experimental Procedures" for details).

* Detected in both NMR and LC/MS studies.

** Not used in the normalization process.

mRNA level. Although the GLYAT level could not be directly measured because of the failure of all available antibodies (data not shown), the increased mRNA level should reflect the protein level, because phase II enzymes are generally transcriptionally regulated (24), as shown for UGT1A1 and UGT2B7. These data confirm that phase II detoxification pathways, particularly glucuronide and glycine conjugation, in the relevant organ (the liver) were up-regulated in the DR group.

Biochemical and Histopathological Changes in the Liver—We next investigated the effects of DR on the integrity of the liver via blood chemistry and direct tissue staining. We measured alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) levels, which are commonly used to assess the functional integrity of liver cells (Figs. 6A–6C). ALP and AST levels were similar in both groups, but there was small, yet significant, decrease in ALT level in the DR group. As ALT is more specific to liver than the others, this finding suggested that the liver cells in the DR group were somewhat healthier in a biochemical sense. We also looked at the changes in the actual tissue via histopathological staining. H&E staining showed no gross pathophysiological differences between groups (Figs. 5D and 6D), but the DR group exhibited noticeably denser cytoplasm, which might have been due to a decrease in glycogen granules. To test this, we performed periodic acid-Schiff staining, and the DR group showed glycogen depletion relative to the control.
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Fig. 4. Neutral loss scanning for general assessment of glucuronide and glycine conjugation reactions. Glucuronide (A, B) and glycine (C, D) conjugation profiling based on neutral loss scanning of m/z 176 (glucuronide) and 57 (glycine) in control (A, C) and DR (B, D) groups. The MS/MS step was carried out using a 35-V normalized collision energy. The plots show the retention times and intensities of compounds that experience the loss of a specified common neutral m/z 176 (and 57) fragment. The numbers on the peaks indicate tentative assignments of conjugated metabolites: 1: tyramine glucuronide; 2: indole acetylglucine.

Fig. 5. Levels of the phase II detoxification enzymes for glucuronide and glycine conjugation in liver tissues. Reverse transcription PCR (A) and Western blot (B) represent the mRNA and the expressed protein levels of the phase II detoxification enzymes for glucuronide and glycine conjugation, such as uridine diphosphoglucuronosyltransferase 1A (UGT1A) and 2B (UGT2B) and glycine-N-acetyltransferase (GLYAT), in liver tissues. Beta-actin (Actb) was used as a control. Bar charts represent the comparison of the mean band intensities for the levels of phase II enzymes in terms of mRNA (C) and protein (D) normalized to that of the Actb. Statistical analysis was performed using Student’s t test, and the resulting p values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control, n = 8; DR, n = 13). A Western blot result for GLYAT could not be obtained, as none of the available antibodies reacted with rat GLYAT.

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control and DR groups, we measured the expression levels of Nrf-2 and its downstream targets in the liver tissue. Fig. 7 shows that Nrf-2, along with its downstream targets (except
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Fig. 6. Blood biochemistry and histopathological staining. Serum alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) activities were measured using commercial kits employing spectrophotometric assays. Average values of the ALP (A), AST (B), and ALT (C) levels of each group are plotted, along with their standard deviations. Student’s t-test was also performed, and the resulting p values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control, n = 8; DR, n = 13). H&E and periodic acid-Schiff staining of liver tissues were performed on paraffin blocks of the samples. H&E staining control (D) and DR (E) groups (200×); periodic acid-Schiff staining control (F) and DR (G) groups (100×).

Fig. 7. Up-regulation of the Nrf-2 signaling pathway. Western blots of Nrf-2 (A), HO-1 (B), MRP-3 (C), and NQO-1 (D) from the liver homogenates of the control and DR groups. Beta-actin (Actb) was used as a loading control. Bar charts below the blots represent the comparison of the mean band intensities for the levels of the proteins normalized to that of the Actb. Statistical analysis was performed using Student’s t-test, and the resulting p values are indicated. Error bars represent standard deviation. All the animals for which metabolomics data were obtained were tested (control, n = 8; DR, n = 13).

NQO-1, was elevated in the DR group, showing that the Nrf-2 signaling pathway was up-regulated in the DR group. These results suggest that enhanced phase II detoxification in the liver tissue occur in the DR group, at least in part, through activation of the Nrf-2 pathway.

DISCUSSION

In identifying the marker metabolites for DR, we employed NMR and LC/MS approaches, most often used independently in metabolomics studies. NMR has merits for quantitation and reproducibility, and LC/MS in terms of sensitivity (39). Here, the use of both techniques expanded the coverage of the markers and gave added reliability to them. For example, hydroxymethoxyindole glucuronide, which gave an important clue regarding glucuronidation, was detected via LC/MS, and phenylacetylglutamine, which led to the idea of enhanced glycine conjugation, was detected with both techniques. Creatinine was also detected by both methods, and 1-methylhistidine was observed in NMR studies. Through a urine metabolomics approach, we showed that the creatinine level increased in the DR group, consistent with previous results (19, 20). The urine creatinine level has been used as a marker for kidney function, but the increase in our results seems to be due to an increase in muscle protein turnover in the DR condition rather than kidney impairment, as the amounts of collected urine, which often decrease in conditions with decreased urinary creatinine, were similar for both groups. Furthermore, we found that the level of 1-methylhistidine, which is produced from muscular actin and myosin, was higher in the DR group. Therefore, these two metabolic markers do not seem to contradict the idea of a beneficial effects of DR.

From a biological perspective, an important aspect of the current study is the finding of enhanced phase II metabolism and Nrf-2 pathway activation in the liver tissue of the DR group. The initial clue came from the metabolomic identification of increased hydroxymethoxyindole glucuronide and
phenylacetylglucosamine in urine from the DR group. Although there have been isolated reports on the relationship between DR and UGT activities, those might be limited by their study of particular isotypes with the use of non-dietary chemical substrates (40, 41) or targeted investigation of DR’s effects on those UGTs for a short period of time (42). Our non-targeted metabolomics study did not set any prior assumptions about DR’s effects and generated a hypothesis of increased phase II reactions based on the identified markers in larger number of animals (n = 8 for control and 13 for DR). In addition, the hypothesis of a general increase in glucuronide and glycine conjugation was proven through global profiling of the conjugated metabolites with LC/MS-based neutral loss scanning, which is not limited to particular isotypes and products. Equally important, we addressed the mechanism of DR’s effects by finding that the Nrf-2 pathway is up-regulated by DR, leading to phase II enhancement. Phase II enzymes, such as UGT and G6PD, are essentially detoxification enzymes, and therefore it makes good sense that enhanced urinary elimination of toxic metabolites contributes to the beneficial effects of DR. In our experimental frame, the decreased serum ALT might well reflect protective effects on the liver cells by the increased phase II detoxification.

Specific life-beneficial roles of the phase II detoxification pathway can be also appreciated by its well-documented involvement in cancer prevention (24–26). It has been suggested that dietary flavonoids or unsaturated antioxidants can prevent a variety of cancers, which can be attributed to enhanced phase II metabolism. For example, limonene and sobrerol prevented the initiation of carcinogenesis in a rat breast cancer model by increasing phase II enzyme activities (43), and garden cress ingredients inhibited the formation of preneoplastic regions in chemically insulted rat colons through enhanced UGT activities (44). Therefore, the enhanced phase II pathway is likely to contribute to decreased cancer incidence in a future years-long experiment, although it was not directly measured in the current months-long experiment.

Very interesting species differences in phase II metabolism also support its importance, especially in terms of glucuronidation, in maintaining healthy life in living organisms that experience constant exposure to xenobiotics through their lifetime. Cats are very sensitive to drugs and toxins at levels not toxic to other species (45, 46). Notably, they are quite susceptible to the toxicity of acetaminophen, a widely used analgesic for humans. This species-unique toxicity of xenobiotics was found to be due to the unusually low activity of UGT in cats (47, 48). Although generally safe to humans, acetaminophen can be quite toxic to a minor population of humans, which also might be attributable to variable glucuronidation of the drug among individuals (49, 50). Considering the above and the well-known importance of the phase II pathways in detoxifying general xenobiotics, enhanced phase II detoxification could have a role in the mean life span of a population.

For the invertebrate C. elegans, enhanced phase II detoxification, to which UGT is a major contributor (23), was actually suggested to be responsible for anti-aging and life span extension (51), which are important beneficial effects of DR. It also has been suggested that an important cause of aging is molecular damage caused by toxic metabolites, and that enzymes involved in detoxification systems contribute to longevity assurance (50). The roles of enzymes in detoxification systems on longevity assurance also has been suggested by DNA microarray analysis of daf-2(r) worms (53). In addition, the Nrf-2 ortholog SKN-1, one of the longevity-assurance genes, regulates the expression of candidate phase II genes.
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In C. elegans (54) and is required for life span extension by DR (55, 59).

For higher organisms, the roles of phase II detoxification in DR’s beneficial effects have been much less studied. In addition, the relationships among the NF-2 pathway, its downstream target genes, and DR's effects are more complex. For example, one study suggested that the NF-2 pathway should be required for reducing carcinogen-induced tumour formation in the Drosophila strain, but that it should not be required for life extension or increased insulin sensitivity (56). In addition, it was shown that NF-2 downstream effectors, such as NQO-1, HO-1, the glutamate-cysteine ligase catalytic subunit, and GST are not uniformly enhanced in the Drosophila condition. Therefore, individual effector molecules might need to be evaluated in order to address a coherent pathway from DR and its beneficial effects through NF-2 signalling. Our observation of insignificant changes in NQO-1 may be understood in this sense. It seems that the increase in NQ-2 and its targets HO-1 and MRP-3 might be the specific signature of DR-related NF-2 pathway activation. In particular, the elevation of the HO-1 level can be easily related to the beneficial effects of DR, as it protects cells from a variety of diseases caused by reactive oxygen species and inflammation by producing anti-oxidant and anti-inflammatory substances such as carbon monoxide and biliverdin.

We observed many effects of DR related to liver functions (lower serum LDL and TG levels, lower chylocoen, and reduced ALT level) that can ultimately contribute to long-term health. Our metabolomics and biochemical results show that the enhanced phase II detoxification in the liver of the DR group correlates well with the up-regulation of the NF-2 signaling pathway by DR. We showed the activation of NF-2 pathway by measuring the protein level of NF-2 itself and of its downstream target genes HO-1 and MRP-3. Specific correlation was also established by directly measuring the UGT and GYAT levels of the liver tissue. Collectively, we suggest that DR up-regulates the NF-2 that turns up phase II detoxification genes in liver, which contributes to the beneficial effects of the DR (Fig. 8).

Given the complexity of the mechanism of DR, which has hindered its detailed investigation for decades, we admit that the phase II detoxification pathway may not account for all of DR’s beneficial effects, and that there should be other important metabolic pathways that could not be addressed by looking at urine metabolic profiles. For example, increased GST conjugation, suggested to be important in anti-aging because of its electrophile-scavenging role (57), was not detected in this study with significance. It might be that we did not use particular electrophile-generating compounds, or that UGT conjugation is a much more prevalent form of detoxification than others (i.e. GST conjugation) for the excretion of toxic materials (23). However, we note that GST A1 expression did not increase to a statistically significant degree in the DR group in a previous study (58), consistent with our results.

In this sense, blood, multi-organ tissue extracts, or intact tissues themselves could be the next tier of targets for studies seeking a more systems-level understanding of the mechanism of DR. As DR essentially perturbs metabolism, studies of DR’s effects with metabolomics, followed by the investigation of changes at protein and gene expression levels, as laid out in the current study, might be one efficient way to solve this complex puzzle.

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[3] This article contains supplemental material. These authors contributed equally to this work.

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REFERENCES


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A Highly Facile and Specific Assay for Cancer-Causing Isocitrate Dehydrogenase Mutant Using $^{13}C_4$-Labeled α-Ketoglutarate and Heteronuclear NMR

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Supporting Information

ABSTRACT: Isocitrate dehydrogenase mutations with neomorphic activity of converting α-ketoglutarate to 2-hydroxyglutarate have been found in many types of cancers. We report an NMR-based assay specific for the mutant using $^{13}C_4$-labeled α-ketoglutarate. It can be done in a complex mixture without extraction, gives time-dependent absolute quantitation, and is applied to enzyme inhibition studies. Its merits over conventional assays should facilitate inhibitor developments for a new class of target-oriented anticancer agents.

INTRODUCTION

Isocitrate dehydrogenase (IDH) is an enzyme in the tricarboxylic acid cycle and catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), consuming 1 equiv of NADPH. Mutation of this enzyme was first implicated in a brain tumor, when a point mutation of its isocitrate (IDH1) allele was detected in the specimen of 22 human glioblastomas multiformes in 2008 by whole-exome sequencing. Since then, more mutations have been found in as many as 10 different cancer types such as acute myeloid leukemia (AML), cartilage tumors, thyroid tumors, and cholangiocarcinoma. Currently, IDH mutations account for more than 75% of grade II and III gliomas and secondary glioblastomas. Contrary to the initial suggestion of loss of function by the mutation, the mutant enzyme was shown to have a very interesting gain of function activity ("neomorphic activity") that converts the product of the wild-type IDH (WT-IDH) enzyme (α-KG) to another metabolite, 2-hydroxyglutarate (2-HG), consuming 1 equiv of NADPH. Therefore, the level of 2-HG is much higher in cancers harboring the mutant IDH (MT-IDH) enzyme, and 2-HG has been named the first and so far the only "oncometabolite," a small molecule that can cause cancer. Now, details of carcinogenesis by this oncometabolite are being actively investigated, and modulations of the DNA epigenetic status and HIF-1α activity have been suggested as possible mechanisms.

Therefore, inhibitors of the MT-IDH enzyme are expected to be highly specific target-oriented anticancer agents and are under heavy investigation. One prerequisite of the development of such inhibitors is a specific assay method for the enzyme activity. Currently, there are two major approaches for measuring the IDH enzyme activity: one involving liquid chromatography–mass spectrometry (LC–MS) to detect the product of the enzyme reaction (2-HG) and the other measuring the levels of NADPH, as adapted in classical kits. The LC–MS approach usually employs a multiple-reaction monitoring scheme to detect only the desired reaction product. However, large shifts in the retention time of 2-HG can occur when using a complex mixture such as cell lysates, used commonly to mimic the native environment of the enzyme (see supplementary Figure 1 in the Supporting Information). These shifts are most likely due to the matrix effects from cellular metabolites, and they can compromise the credibility of the assay. In addition, the LC–MS approach requires sample extraction steps from the cell lysates to remove the protein and other molecules. The detection of NADPH in commercial enzyme kits can be used only for the WT-IDH enzyme assay, not for the mutant, because the MT-IDH enzyme generates NADP(+) instead of NADPH. More importantly, the indirect
Figure 1. Reactions of WT- and MT-IDH and optimization of the HCACO pulse sequence to detect the products of either form of the IDH enzyme. (a) IDH-mediated reactions for both WT and MT. $^{13}C_3$-labeled carbon atoms are indicated with asterisks, and the detectable H–C–CO spin systems are indicated with dotted boxes. The chemical shifts of the spin system are also presented. (b) HCACO pulse sequence with the key optimized off-resonance shaped pulses indicated with open shapes. These selective pulses had Q3 shapes with a 12.5 kHz B1 field. The center frequencies of the CA- and CO-shaped pulses were set at the indicated values in part a. Hard pulses are represented with vertical lines (90° pulse) or rectangles (180° pulse).

detection of NADPH can be affected by the presence of unrelated redox enzyme activities, giving rise to the specificity concern of the assay. Therefore, there is a great need for a convenient and specific assay that can measure the activity of both WT- and MT-IDH enzymes in complex mixtures such as cell lysates.

**EXPERIMENTAL SECTION**

**Chemicals and Reagents.** The stable isotope $^{13}C_3$-labeled α-ketoglutarate (1.2,3,4-$^{13}C_3$, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Recombinant WT- and MT-IDH (both for isotype I) were purchased from Ozyme (Rockville, MD). The MT-IDH inhibitor, AGI-5198, was bought from Xenobiot (San Diego, CA).

**Cell Lines and Culture Condition.** The human dermal fibroblast (HDF) cell was kindly provided by Professor Jin Ho Chung, College of Medicine, Seoul National University (Seoul, Korea). The cells were grown in Dulbecco’s modified Eagle medium (DMEM; HyClone, UT) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U/mL; HyClone, UT). Cells were cultured at 37°C under 5% CO$_2$ in a 90% humidified incubator.

**Sample Preparation for NMR Spectroscopy.** The reaction was run either in a buffer system (150 mM NaCl, 10 mM MgCl$_2$, 20 mM Tris, and 0.03% bovine serum albumin at pH 7.5) or in cell lysates. Cell lysates were used for demonstration of the background signals in the complex mixture. Cell lysates were prepared from cultures of 10$^5$ HDF cells. The cultured cell pellets were washed three times with a phosphate-buffered saline buffer and resuspended with 200 μL of the cell lysate buffer. The samples were sonicated with 10 cycles of 3 s hard pulse and 1 s rest on ice. The samples were centrifuged at 16,000g for 20 min at 4°C, and the supernatants were moved into new centrifuge tubes. To the cell lysates or the buffer were added a recombiant enzyme, a mixture of 0.1 μg of α-KG and 0.05 μg of NADPH, and inhibitors, if necessary. The mixtures were incubated at 37 °C overnight before NMR measurement. For α-KG measurement, the spectrum was obtained before incubation. In terms of the cost, one reaction requires 0.1 μg of $^{13}C_3$-labeled α-KG, costing $0.15 per reaction at the current price. If one tests 10 compounds in one reaction, which is commonly done for screening, the α-KG cost is $0.90 per compound.

**NMR Measurement.** All NMR spectra were measured on a 600-MHz Bruker Avance spectrometer with a cryogenic probe (NCUKEF, Seoul National University, Seoul, Korea). One-dimensional NMR spectra were obtained with the NOESY preturation pulse sequence, and HCACO spectra were obtained with a gradient-selected HCACO pulse sequence at 310 K (see Figure 1). The centers of the chemical shifts of the off-resonance-shaped pulses for carbonyl carbon and α-carbon were set at 178 and 74 ppm for detection of 2-HG and at 208 and 34 ppm for detection of α-KG, respectively. Q3-shaped pulses with 1000 points and a 12.5 kHz B1 field strength were used for these pulses. The time course measurements of 2-HG and α-KG were obtained at 310 K for 24 h.

**RESULT AND DISCUSSION**

In the current report, we designed a novel method that can detect specifically the product (2-HG) or substrate (α-KG) of the MT-IDH enzyme in complex mixtures without any purification steps. The method uses commercially available
stable isotope $^{13}C_3$-labeled α-KG at C1–C4 positions and a tailored version of a heteronuclear HCACO NMR experiment (Figure 1). The use of the HCACO experiment in an enzyme assay was reported for monooxynase, but it required a custom-synthesized substrate, and only detected the product. Here, the quadruply labeled α-KG was chosen instead of the fully labeled one because the latter has another H–C–C–O spin system involving C2 and C3 that can be detected in both the substrate and product (Figure 1a). For the NMR experiment part, we optimized the HCACO experiment such that it can specifically detect the H–C–C–O spin system in either the substrate (α-KG) or product (2-HG) (Figure 1b). This optimization exploited the difference in the chemical shift values of the H–C–C–O spin system in 13C-labeled HCACO experiment to the respective values above, we expected to selectively detect either one. Therefore, our approach should be readily applied to many other dehydrogenase reactions involving α-keto acid and α-keto acid conversion. The concept was tested by detecting α-KG or 2-HG in the complex mixtures of cell lysates. For this, we incubated recombinant MT-IDH (B132H) enzyme and the $^{13}C_3$-labeled α-KG in the HDF cell lysates. A conventional one-dimensional NMR experiment gave a complicated spectrum because of many compounds in the cell lysates, and it was impossible to observe the formation of 2-HG or decrease of α-KG (Figure 2a, left). The use of the tailored heteronuclear HCACO experiment gave a clear signal for each of them. The signal of α-KG could be observed before the enzyme reaction, and that for 2-HG was observed after the enzyme reaction (Figure 2a, middle and right). With the exceedingly low (10%) natural abundance of the H–C–C–O spin system of the background compounds, the substrate and products could be detected with virtually no ambiguity as single peaks in the complex cell lysate mixtures. Because we were interested in detecting only the activity of the MT-IDH enzyme, possibly in the presence of the WT-IDH one, we tested whether our assay can report only the activity of the MT-IDH enzyme. Figure 2b shows that only MT-IDH gave rise to the expected NMR signal and that WT-IDH or the reaction without enzyme did not give any observable peaks. The results confirm that our assay is highly specific for MT-IDH activity and that the detected peak is not from nongenetic conversion of the substrate. The assay also allowed time-dependent monitoring of the product generation or substrate consumption thanks to the nondestructive nature of the NMR approach (Figure 3a,b). In contrast, other assays such as LC–MS require destructive sample extraction, and either aliquots or multiple samples are needed. Time-dependent monitoring of the enzyme reaction is a prerequisite in any enzyme kinetic study, but it also requires absolute quantification of the product. Therefore, we developed the procedure for the absolute quantitation of 2-HG directly using the HCACO spectra. This absolute quantitation cannot be done conventionally using internal standards such as trimethylsilyl propanoic acid (TSP), because its signal is not observed in the isotopically edited HCACO experiment, or
using known amounts of labeled 2-HG, because it is not commercially available. Therefore, we used a two-step approach. First, we generated the labeled 2-HG from labeled α-KG through MT-IDH enzyme reaction in situ in the NMR tube, obtained its one-dimensional 1H NMR spectrum where both 2-HG and TSP can be detected, and quantitated the produced 2-HG based on integration of the known amount of TSP. Then, we used the same sample to make serial dilutions and obtained their HCACO spectra. The correlation between the integration values of the single 2-HG peaks on HCACO and the diluted concentrations was excellent and provided the calibration curve for the absolute quantitation (Figure 3c). This absolute quantitation of the product enabled us to obtain the mutant enzyme activity in "mole of 2-HG/min/mL" (=milliminit/mL) units generally used in enzyme kinetics (Figure 3d). The particular enzyme preparation we used turned out to have 0.03 milliminit/mL of IDH enzyme activity per reaction tube. Because the time-dependent assay can be done with a single sample without any extraction step, our NMR assay method can be applied to detailed enzyme kinetic studies under various conditions.

One of the ultimate goals of an enzymatic assay is application to the inhibitor screening. As a demonstration of the practical utility of our assay, we tested whether it can detect inhibition of the MT-IDH enzyme by a known enzyme inhibitor. As shown in Figure 4a, AGI-5198, a previously reported inhibitor of the
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REFERENCES
Integrated Systems and Technologies

An NMR Metabolomics Approach for the Diagnosis of Leptomeningeal Carcinomatosis

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Abstract

Leptomeningeal carcinomatosis (LC) is the third most common metastatic complication of the central nervous system. However, the current modalities to reliably diagnose this condition are not satisfactory. Here, we report a preclinical proof of concept for a metabolomics-based diagnostic strategy using a rat LC model incorporating glioma cells that stably express green fluorescent protein. Cytologic diagnoses gave 66.7% sensitivity for the 7-day LC group and 8% for the 3-day LC group. MRI imaging could not diagnose LC at these stages. In contrast, nuclear magnetic resonance-based metabolomics on cerebrospinal fluid detected marked differences between the normal and LC groups. Predictions based on the multivariate model provided sensitivity, specificity, and overall accuracy of 88% to 99% in both groups for LC diagnosis. Further statistical analyses identified lactate, acetate, and creatine as specific for the 7-day LC group with glucose as a specific marker of the normal groups. Overall, we showed that the metabolomics approach provided both earlier and more accurate diagnostic results than cytology and MRI imaging in current use. Cancer Res. 72(2002); 5179-87. ©2012 AACR.

Introduction

Leptomeningeal carcinomatosis (LC) is a disorder caused by the seeding of the leptomeninges, the pia, arachnoid, and cerebrospinal fluid (CSF) within the subarachnoid space by malignant cells. This disorder is the third most common metastatic complication of the central nervous system and increasingly common in cancer patients (1, 2). Although LC most often presents in patients with widely disseminated and progressive systemic cancer (7-98%), it can present after a disease-free interval (90%) and even be the first manifestation of cancer (7-19%), occurring even in the absence of other evidence of systemic disease (3). For patients diagnosed with LC, the median survival time of untreated patients is known to be 4 to 6 weeks, which can extend to 3 to 6 months with treatment (4, 5). Treatment can also improve or stabilize a patient’s neurological status and maintain their neurological quality of life (5). Thus, an early diagnosis of LC is important to alert the oncologist to begin therapy before neurologic deterioration.

The most useful laboratory test in the diagnosis of LC has been the examination of CSF via cell counts and protein concentrations (6-8). However, abnormalities in the CSF, although suggestive of LC, are not diagnostic. The presence of malignant cells in the CSF detected in a cytologic examination is diagnostic of LC. However, the assignment to a particular tumor is often not possible, which is true for most cytologic analyses (6-8). Of the patients with final results that reveal positive CSF cytology, up to 43% will be cytologically negative upon initial examination (6,7,9,10). The yield increases to 98% with a second round of CSF examination, but there is little benefit for further rounds of examination (6,7,9,10). The low sensitivity of CSF cytology makes it difficult not only to diagnose leptomeningeal metastasis but also to assess the patient’s response to treatment (2). Biochemical markers, immunohistochemistry, and molecular biology techniques have been applied to CSF in an attempt to find reliable biomarkers of the disease. Numerous biochemical markers have been evaluated, but their use has been generally limited by poor specificity and sensitivity (3). Therefore, it is important to develop a new method that improves the diagnostic sensitivity of LC, which can enhance the evaluation of prognosis and reduce unnecessary medical procedures.

While genomic and proteomic approaches focus on upstream genetic and protein variations, metabolomics is increasingly being used to understand the global metabolic changes that occur in response to alterations in nutrition, genetics, and environment (10,11), which can complement the information obtained from genomics and proteomics. In addition, metabolomics has been used to identify metabolite-based biomarkers for a variety of diseases conditions (see ref. 12 for a
recent review) Metabolic profiles can be investigated using high-throughput analytical tools, such as nuclear magnetic resonance (1H-NMR) spectroscopy. 1H-NMR spectroscopy of biodegradable nucleic acids, proteins, and lipids can give comprehensive small-molecular profiles of metabolites that are regulated by various physiological signals involved in maintaining homeostasis. Homeostasis can be disrupted by changes in a patient’s physiological condition, resulting in the perturbation of metabolic levels. Thus, monitoring these perturbations can provide novel information about the patient’s physiological condition but also new biomarkers for disease diagnosis.

Because the metabolite composition of CSF is directly related to the biological processes of the brain, profiling CSF metabolites can provide important information for diagnosing various brain abnormalities, such as brain injury (13), Huntington’s disease (14), and Parkinson’s disease (15). Himmelreich and colleagues (16) showed a practical application of rapidly identifying the bacterial species in bacterial meningitis based on CSF metabolite profiles, which may help appropriate selection of antibiotics in a clinical setting. With the recent report by Walsh and colleagues (17) detecting approximately 700 CSF metabolites and building a public database, we expect CSF metabolomics studies to be applied to a wider variety of systems.

The purpose of our study was to develop a new NMR spectrometric method that could be used to improve the diagnosis of LC. We first designed a rat LC model with glioma cells stably expressing GFP and used NMR-based metabolomics to characterize CSF metabolites in a mouse. We were able to obtain earlier and significantly improved diagnostic results compared with currently used cytologic or radiological approaches.

Materials and Methods

This experiment was approved by the animal care committee of Seoul National University Hospital.

Rat LC model and sham operation

To produce LC rats, GFP-expressing F98 cells (Supplementary Materials and Methods and Supplementary Fig. S1) were prepared in 50 μL of serum-free Dulbecco’s Modified Eagle’s Medium and intrathecally transplanted into the parietal area of 6-week-old Fisher 344 rats (1 × 10^6 cells/rat; Supplementary Materials and Methods). For the sham operation, rats (n = 4) were intrathecally injected with 50 μL of serum-free Dulbecco’s Modified Eagle’s Medium into MRI imaging and CSF collection (Supplementary Materials and Methods) were conducted 3 (n = 8–9 day LC group) and 7 days (n = 9–7 day LC group) after cell implantation. Rats in the sham group also underwent in vivo MRI imaging 3 days after the operation.

NMR spectroscopy data acquisition

All NMR spectra were acquired at 1:2 with a 500 MHz NMR spectrometer (Agilent, Varian) equipped with an IR-MA5 pass-echo probe (Agilent). A CSF sample (40 μL) was placed in the probe with a re-needle technique and single Z-gradient capability. The receiver gain was adjusted carefully not to cause signal overflow. The sample was spun at 5 kHz by a stream of nitrogen gas with 547 (the magic angle) relative to the applied magnetic field. The spectra were acquired with a total number of complex points of 16 K, sweep width of 7290 Hz, and 4096 transients. The 90° pulse was calibrated with each sample on each sample of the water resonance. The water signal was saturated using weak power continuous wave during the recycle delay.

Data processing

The time-domain spectra were apodized with an exponential function (1 Hz), Fourier transformed, phased, and manually baseline corrected. The signals were referenced to the TSP signal at 0.00 ppm and normalized against the total integration values. Water and ethanol peaks were removed to prevent artifacts in downstream analyses. To reduce the complexity of the NMR data for pattern recognition, the spectra were binned at every 0.0005 ppm interval. The normalization and binning were conducted using an in-house built Perl program.

Statistical analysis

Statistical analysis was conducted with R (from R Project for Statistical Computing) and SIMCA-P+ 11.0 (Umetrics). Chemisn (Spectral database) and an in-house built database were used for metabolite identification. Partial least square regression and OPLS-DA were conducted to identify latent patterns and compare the overall metabolite profile. Class discrimination models were built using the cross-validated predictability value did not significantly increase to avoid overfitting of the statistical model. Diagnostic performance was obtained by prediction of one left-out sample based on the distinction model constructed with the rest of the samples. The method was repeated until all the samples were left out once, to a priori cut-off value of 0.5 was used to evaluate the prediction results (18). Signals specific for each group were identified by conducting the Wilcoxon rank-sum test on all the ppm variables using the in-house written R script.

Results

CSF cytology, MRI imaging, and histology

Among the 9 CSF samples obtained 7 days after F98 cell implantation (7-day LC; 6 6475) were found to have malignant cells in the CSF cytologic examination as evidenced by the expression of GFP (Fig. 3A). In contrast, no samples collected 3 days after F98 cell implantation (3-day LC) exhibited positive CSF cytology. MRI imaging revealed meningeal enhancement in all 77 rats that underwent glioma cell implantation (Fig. 2A and B), but it also showed the enhancement in the 4 rats from the sham group (Fig. 2C). All implanted rats (n = 17) had F98 cells infiltrating the leptomeninges as evidenced by GFP expression, which confirmed the proper establishment of LC in both 7-day (Fig. 3A) and 3-day (Fig. 3B) groups.

MRI, leptomeningeal infiltration of F98 cells was more prominent in 7-day LC rats than in 3-day LC rats, suggesting that 3-day LC group is in an earlier phase of the LC development. Overall, among the 17 histology-confirmed LC rats, 6 in the 7-day LC group could be diagnosed with cytology, and MRI imaging was not useful due to the false positive effects.
Metabolic Diagnosis of Leptomeningeal Carcinomatosis

HR-MAS MR spectra and multivariate analysis

We acquired the NMR data with a high-resolution magic angle-spinning (HR-MAS) probe because the CSF volume obtained from a rat (typically ~40 μL) is far smaller than that required for a usual 5-mm probe (~300 μL). HR-MAS MR spectra of the 26 CSF samples (9 A and 9 samples for normal, 3-day LC, and 7-day LC rats, respectively) from the animal model gave reasonable signals, even though the sample volumes were less than one-tenth of that used for a 3-mm NMR probe (Fig. 4 and Supplementary Fig. S5). Chemical shifts and scalar coupling values were used to identify a number of metabolites known to be present in CSF, such as lactate (1.35 and 4.42 ppm) and glucose (multiple signals between 3 and 4 ppm). As the intragroup variation hindered the assignment of the spectral characteristics of each group by simple visual inspection, we applied a more holistic approach of multivariate statistical analysis to the entire NMR data set. First, partial least square (PLS) analysis was conducted with all 3 groups to determine the differences with time (Fig. S3). Our results show that spectral features move toward the right side along the first component axis as time increases. The metabolite profiles of 7-day LC rat samples were quite different from those of normal rats. In comparison, the profiles of 3-day LC rats were between the normal and 7-day LC rat samples in the direction of the first component. Still, the directional changes in the second component were opposite between the 3-day and 7-day LC rat samples, suggesting that the overall metabolite profiles may not change in a simple linear fashion with time.

To maximize the difference between a given pair and obtain quantitative information about the diagnostic performance at each time point, orthogonal projections to latent structure-discriminant analysis (OPLS-DA) modeling was conducted for samples on normal versus 3-day or 7-day LC rats. Both pairs yielded differentiation models with 1 predictive and 2 orthogonal components (Fig. 3A and C). The models featured 8.965 (0.980) of goodness of fit and 8.759 (6.717) of predictive values for normal versus 3-day LC rat (normal vs. 7-day LC rat) differentiation. Both 3-day and 7-day LC rat samples were well separated from normal rat samples, suggesting that relatively early metabolic changes at day 3 may be sufficient to distinguish these samples from those of normal rats.

Diagnostic predictions

Good statistical characteristics of a differentiation model do not necessarily translate into good practical performance. Therefore, we conducted a diagnostic prediction test with the established differentiation models. We left out one CSF sample at a time, constructed a new model with the remaining samples, and predicted the disease status of the left-out sample. The procedure was repeated until all of the CSF samples were tested once, with a diagnosis based on a priori cut-off value of 0.5. The test was conducted on both 3-day (Fig. 6B) and 7-day LC samples (Fig. 6A) against normal rat samples. The prediction results showed that the models correctly predicted 16 samples out of a total of 18 for both groups. One identical normal sample (from case 3) was mispredicted as LC samples.

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and one LC sample from each of the 3-day and 7-day LC groups was mispredicted as normal. Thus, our metabolomics model showed sensitivities of 89% (7-day LC group) or 98% (3-day LC group), a specificity of 69%, and an overall accuracy of 89% in the prediction of LC. These results suggest that this method could be useful for both relatively early (day 3) and late stage (day 7) LC diagnosis.

Analysis of marker signals

To identify the metabolites that are characteristic of LC, we conducted a Wilcoxon rank sum test on all ppm variables (Fig. 7A). Although we could not identify all of the significant signals (blue dots) due to the relatively low signal-to-noise ratio and overlaps, we were able to identify glucose (3.40, 3.48, 3.73, and 3.65 ppm) as being specific for the normal group and lactate (3.38 and 4.02 ppm), acetate (1.93 ppm), and creatine (3.85 and 3.89 ppm) for the 7-day LC group by database analysis and comparisons with standard compounds (Fig. 7B; see Supplementary Information SI). We also tested a correlation of the NMR spectral markers with the thickness of the LC infiltration in the 7-day LC animals. We built a partial least squares regression model between the levels of the 4 markers as independent variables and the thickness as a dependent variable. The regression model given good correlation with R² value of 0.85 between the actual values and regression-predicted values (Supplementary Fig. S5). The number and intensity of significant signals were smaller for the analysis of normal versus 3-day LC rat samples, and reliable identification of metabolites

![Figure 2: Contrast-enhanced T2WI MRI imaging of rat brain. A normal rat (A) shows no meningeal enhancement, whereas meningeal enhancement (arrow) was observed in both rats with LC (B) and sham operations (C). Thus, meningeal enhancement was not a specific finding for the LC. CC, corpus callosum; OL, occipital lobe; CL, caudate nucleus; OB, olfactory bulb; T, thalamus.](image)

![Figure 3: Histopathological examination showed the T-RE cells that expressed GFP infiltrating the leptomeninges. Leptomeningeal infiltration indicated by arrows of T-RE cells was more prominent in 7-day LC than in 3-day LC rats. Histopathology from the brains of 7-day LC and 3-day LC rats presented the late and early LC status, respectively.](image)
was difficult for 3-day LC rat samples (Fig. 7A bottom). Therefore, the distinction between normal and 3-day LC rat samples should be due to the sum of small and measurable signals rather than several conspicuous metabolite signals. In addition, the pattern of the significant signals of 3-day and 7-day LC rat samples appeared to be different.

Discussion

Metabolomics is a global assessment of endogenous metabolites within a biological system. This assessment is gaining increased attention for its role in diagnosing cancer (14), refining preoperative differential diagnosis, detecting tumor progression, and monitoring responses to treatment (30). Magnetic resonance spectroscopy (MRS) is a method that detects metabolites within cells, tissues, or bulk fluids and has been used in medical fields along with MRI imaging. Notably, proton MRS has been used for the detection of brain, breast, and prostate cancers, which led to the discovery of choline, lactate, and amino acid peaks as biomarkers for cancer. Analogously, in vitro NMR spectroscopy of fine-needle aspiration biopsy samples of primary tumors exhibited 100% accuracy in differentiating between benign and malignant samples by the measurement of choline peaks (21, 22). With these successful applications of NMR-based metabolomics to the medical field, we hypothesized that LC could also be diagnosed using the different metabolite profiles in CSF measured by in vitro NMR spectroscopy. In the present study, we used a rat LC model using F-98 glioma cells expressing GFP to enhance the detectability of cancer cells in cytology and histopathology.
We applied the NMR spectroscopic method using an HET-MAS probe for the diagnosis of LC in a rat model. The approach showed a sensitivity and specificity of 99% and an overall accuracy of 99% for the 7-day LC model. In the 5-day LC group, we observed increases in lactate, creatine, and acetate levels and decreases in glucose levels compared with those of the normal group (P < 0.006 for all). Although we could not reliably identify marker signals in the model of an early stage (3-day LC), we were able to obtain similar diagnostic performance at day 3. This good performance constitutes the most significant results of our study because they were significantly better than those obtained using other currently available modalities on the same animals. For example, cytology, which is often used to confirm the presence of LC, failed to detect any glioma cells on day 5 and detected cancer cells in significantly less samples (66.7%) at a late stage (day 7). In addition, although in vivo MR imaging detected meningioma enhancement in LC model rats in both early and late stage groups (3-day and 7-day LC rats), enhancement in the sham operation group was also detected. Therefore, radiological enhancement is nonspecific and in vivo MR imaging has very low specificity, which may limit their use.

In many tumour tissues, more than 50% of cellular ATP can be generated from glycolysis, even in the presence of oxygen, which is essential in the term aerobic glycolysis (Warburg Effect, ref. 23). By using aerobic glycolysis, even in the presence of oxygen, and functional mitochondria, tumour cells divert a large portion of pyruvate to extramitochondrial breakdown to lactate, the typical product of anaerobic glycolysis (23). In vivo MR of the brain showed increased aerobic glycolysis in gliomas, which was measured as increases in lactate levels and correlate to the grade of gliomas (23). Thus, we believe that the observed increase in lactate levels and decrease in glucose levels can be explained by the aerobic glycolysis of disseminated cancer cells in CSF.

In terms of the elevated levels of acetate or creatine in the CSF of 7-day LC rats, similar observations have been made in...
another malignancy, which were conducted using in vitro NMR spectroscopy. Recently, Imperato and colleagues (28) reported the metabolite content of intact liver samples of advanced neuroblastomas using HR-MAS NMR spectroscopy, which exhibited higher levels of creatine, glutamine, glutamate, acetate, and glycine in neuroblastomas. They also showed that acetate and creatine are characteristic of stage IV neuroblastomas, which led to the conclusion that high levels of acetate and creatine in tumors could be used in diagnosis and staging. High acetate concentrations in tumors are known to be mainly due to the enhanced lipid synthesis in the generation of cell membranes, which reflects the high growth activity of neoplasms (27). Thus, [1-13C]acetate positron emission tomography has become a useful tool for detecting various types of malignant tumors, such as prostate cancer (28, 29), renal carcinoma (30), hepatocellular carcinoma (31), brain astrocytoma (32), and gliomas (33).

The elevation of creatine levels in the CSF of 7-day LC rats can be attributed to the changes in energy metabolism (34). A significant downregulation of creatine kinase has been reported in some cancers, such as oral squamous cell carcinoma, resulting in elevated levels of creatine (35). However, the creatine levels of the gliomas used in the present study are known to be lower than those of the cerebral cortex where creatine is highly produced. In addition, the total creatine concentration in gliomas tends to decrease according to the degree of malignancy (36), and the F-98 cell line is derived from a high-grade glioma of a Fisher 344 rat. Thus, we believe that the elevation of creatine levels in the CSF of 7-day LC rats is due to the destruction of the cerebral cortex by seeded cancer cells in the CSF space (subarachnoid space).

Contrast-enhanced (e.g., gadolinium chelate agents) MR imaging is the technique of choice used to evaluate patients that are suspected to have LC (37). Gross changes, such as peritumoral edema, can lead to similar MR imaging findings (37–41). Similar to the present study, surgically induced contrast enhancement in meningiomas is also well known (42). Thus, the detection of malignant cells in the CSF has been pursued to confirm the diagnosis of LC. However, our study further emphasizes that contrast-enhanced MR imaging may not be sufficient for the diagnosis of LC mainly due to its low specificity.

Although we obtained good diagnostic performance in both early day 3 and late day 7 stage LC models, we could not reliably identify the marker metabolites in the early stage LC group. Most of the contributing signals for 3-day LC rats were low intensity or overlapped. Therefore, the distinction at the early stage may have been a result of the sum of small contributions. Still, the overall profiles of the significant signals were different between 3-day and 7-day LC groups and changed nonlinearly with time (see Fig. 5A and 7). For example, glucose and lactate, important and conspicuous marker signals on day 7, did not exhibit significantly different levels on day 3. As we failed to detect glioma cells on day 3 by cytology, a small number of cancer cells may have generated cancer-related metabolites just enough to make the overall profile different, but not enough
to allow such metabolite identification. Future experiments with human samples with both a larger number of samples and increased sample volumes should enable metabolite detection.

As stated above, the diagnosis of LC can be conducted by MR imaging, CSF cytology, histology, and NMR-based metabolomics. Among these approaches, CSF cytology can provide a definite answer in animal models but cannot be conducted in clinical settings. From a practical perspective, NMR-based metabolomics showed the best performance among the aforementioned clinically applicable techniques. One theoretical advantage of NMR metabolomics over cytology is that NMR measures small molecules (typically those with MW < 400) which are highly diffusible, exhibiting uniform distribution regardless of the sampling positions used. In comparison, the cells observed in cytology are much less diffusible in the subarachnoid space where the CSF is collected; therefore, these cells may reflect rather local status. The significantly higher sensitivity of NMR method at day 7 (89%) over cytology (66.7%) may be due to these factors.

For a diagnostic measure, another important aspect is specificity. Although we cannot assess the specificity of LC diagnosis against all possible brain diseases, we may address the specificity against LC models using cancer cells from different tissues. It is expected that the distinction between related LC models based on different primary cancer cells should be more difficult than that between LC and other brain pathologies. In addition, it is clinically important to determine the disease stage of a particular LC case. Therefore, we established another LC model using mammary adenocarcinoma cells (13785 MTH III), measured its CSF metabolite profiles, and compared the results from the original model using brain cancer cells. For the specificity, we were able to observe a distinct group of significant marker signals (i.e., glutamate, creatine, and lactate) of the 7-day LC developed with the breast cancer cells from those with the brain cancer cells (acetate, glucose, creatine, and lactate; Supplementary Fig. S6). These results suggest that different metabolite profiles can be detected depending on the particular types of metastasizing cancer cells. We admit that this comparison of LC models is not a full resolution of the specificity concern. Still, the results are encouraging in that we obtained differential metabolite profiles even for different subtypes of single brain pathology (i.e., LC). In actual human cases, additional information is available. Differentiation with meningioma, for example, can be also helped by clinical information such as medical history (e.g., presence of the underlying cancer or not), clinical picture (e.g., fever or not), and CSF findings (e.g., protein level). Therefore, we believe that the metabolomics approach is quite promising in human cases, and that our results warrant its human application. The diagnostic specificity can be ultimately assessed using patients with diverse brain pathological conditions.

For humans, NMR techniques require lumbar puncture for CSF collection, which is somewhat invasive, whereas MR imaging is noninvasive. CSF sampling is still routinely conducted as MR imaging suffers from low specificity as described above. In addition, we only needed 0.4 ml of CSF, which can be readily provided from routine CSF samples (~ 10 ml) for cytology examinations. Thus, NMR samples can be obtained almost "for free" in terms of the reduced amount of labor of clinicians and suffering by patients. In addition, the NMR metabolomics approach does not require sample pretreatment and the data can be directly denoised, allowing easy implementation. As with other analytical approaches, there are also some difficulties in NMR metabolomics, including low sensitivity, resonance overlapping, and poor processing times. These may be addressed with better hardware and software in the near future with the availability of cryogenic microprobes, higher field magnets, and signal decomposition algorithms. Particularly for the sensitivity of quantifying low-level metabolites, the NMR metabolomics is expected to perform much better for actual human samples that have the ultimate relevance to the diagnosis. That is due to the large volume of attainable CSF volume (we above) that enables the use of standard 5-mm probes for enhanced sensitivity and/or concentration of the samples. Overall, we expect to see more samples of NMR metabolomics applied to diagnostic research with CSF as recently shown with pneumococcal or cryptococcal meningitis (10).

In conclusion, we have shown that the diagnosis of LC is possible using a metabolomics-based approach in a rat model. This approach was superior to the currently used CSF cytology and MR imaging procedures. It also enabled detection of LC at an early stage when CSF cytology failed. To the best of our knowledge, this study is the first report of a metabolic diagnostic approach for LC, and it may become a model clinical protocol that can augment or replace current diagnostic methods, if proven in human samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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A new mechanism in the binding between Homer3 EVH1 domain and inositol 1,4,5 trisphosphate receptor suppressor domain

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Abstract: The suppressor domain of inositol 1,4,5 trisphosphate receptor (IP, R) has critical roles in regulating the calcium channel by interacting with many binding partners. The residues 49–53 (IPKRK) of the suppressor domain was suggested to be a canonical Homer EVH1 domain binding site and is also the first a part of calmodulin (CaM) binding site. As CaM binding of the suppressor domain has been shown to involve large-scale conformational changes, we studied the binding characteristics of the Homer EVH1 suppressor domain with NMR spectroscopy and biochemical pull down assays for mutants. Our data show that the suppressor domain employs the IPKRK motif in a similar but subtly different way compared to previously characterized interactions, and that the suppressor domain does not undergo large-scale conformational changes. Chemical shift assignments of the Homer3 EVH1 domain found that a new set of residues, located at the opposite side of the previously reported binding site, is also involved in binding, which was confirmed by mutant binding assays. Further analysis suggests that F40 in the new binding sites may have a critical role as a conformational lock-switch in Homer-target binding. The proposed mechanism is implicated in the signaling network involving calcium channels.

Key words: Homer3 EVH1 domain, inositol 1,4,5 trisphosphate receptor, NMR.

Résumé : Le domaine de l’inhibiteur de récepteur de l’IP3 joue un rôle critique dans la régulation du canal calcique par son interaction avec plusieurs partenaires de liaison. Il a été suggéré que les résidus 49–53 (IPKRK) du domaine de l’inhibiteur de récepteur constituent un domaine cancomique de liaison de l’IP3 de Homer et sont la première partie du domaine de liaison de la calmoduline (CaM). Pour que la liaison de la CaM au domaine de l’inhibiteur implique un large spectre de changements de conformation, nous avons étudié les caractéristiques de liaison de l’IP3 à l’Homer au niveau du domaine de l’inhibiteur par spectroscopie en RMN et des données biochimiques par précipitations de mutans. Nos données montrent que le domaine de l’inhibiteur utilise le motif IPKRK de manière similaire mais subtilement différente comparativement aux interactions caractérisées précédemment, et que le domaine de l’inhibiteur ne subit pas de changements de conformation importants. Les assignations de champ chimique du domaine EVH1 de l’Homer en montrant un nouveau ensemble de résidus, localisés à l’opposé du site de liaison précédemment rapporté, sont aussi impliqués dans la liaison, qui a été confirmé par des tests de liaison de mutans. Une analyse plus approfondie suggère que le résidu F40 au nouveau site de liaison possède jouer un rôle critique comme interrupteur de blocage dans la liaison chimique de Homer. Le mécanisme proposé est impliqué dans le réseau de signalisation affectant les canaux calciques. [Traduit par la Réduction]

Mots-clés : domaine EVH1 de Homer3, récepteur de l’inositol 1,4,5 trisphosphate, RMN.

Introduction

IP, R is a large tetrameric intracellular Ca2+ release channel composed of about 2700 amino acids (Taylor et al. 2004). Its primary sequence can be functionally separated into five sub-domains: the N-terminal suppressor domain, the IP, R-binding core domain, the internal coupling domain, the transmembrane domain, and the cytoplasmic domain (Uchida et al. 2003). Among them, the suppressor domain is named for its ability to prevent IP, R binding to the IP, R-binding core domain (Yoshikawa et al. 1996). However, the suppressor domain has a key role in IP, R-induced gating, as the channel loses its ability to open when the suppressor domain is deleted (Uchida et al. 2003; Yoshikawa et al. 1996, 1999). The loss of gating happens, even though the ligand binding affinity of IP, R is significantly enhanced. In addition to its regulation of the ligand binding affinity, the suppressor domain is also involved in intramolecular interactions with IP, R-binding core domain (Bonacic et al. 2005), 54–55 region (Chan et al. 2008) and gatekeeper domain (Schug and Joseph 2006). Moreover, IP, R suppressor domain contains binding sites for a variety of molecules such as Homer, calmodulin (CaM), Gai, and RACK1 (Qin et al. 2007). Therefore, IP, R suppressor domain is a critical region for the mediation of the channel activity. Homer is an adaptor protein and has three varieties in mammals: Homer 1, Homer 2, and Homer 3. It is highly expressed in the post-synaptic density of neurons (Bergersen et al. 2008; Fugnini et al. 2002; Shirakashi-Yamaguchi and Furutachi 2007; Xiao et al. 1998) and is known to have a scaffolding role with a critical implication in Ca2+ signaling (Hayashi et al. 2006a; Worley et al. 2007). Homer can be separated into a C-terminal coiled-coil region, which
plays a critical role in self-multimerization, and an N-terminal enabled/yoladione-stimulated phosphophosphate (EpsA/VAP) homology 1 (EVIH) domain, which binds to specific preline-rich regions (Pro-Pro-x-Pro, PXXPs), referred to as a "homer binding motif" (Devesagare et al., 2005; Hayashi et al., 2006b; Kato et al., 1998; Shiraishi-Yamaguchi and Fujinori 2007; Shibire et al., 2005; Tadokoro et al., 1999; Tu et al. 1998; Xiao et al., 1998). Therefore, Homer binds targets containing these motifs that are often found in channel proteins, such as IP_{3}Rs, metabotropic glutamate receptor (mGluRs), transient receptor potential cation channel (TRPc), and many others (Matsuda et al., 2000; Shiraishi-Yamaguchi and Fujinori, 2007; Tu et al., 1998; Xiao et al., 2000). Although the atomic structures of EVIH domain and parts of the coiled-coil domains have been reported (Benceken et al., 2000; Hayashi et al., 2009; Huang et al., 2008), the structure of the entire Homer molecule is not available. Likewise, Homer-target protein interaction has been described at atomic resolution only for Homer-peptide 1 complexes (Benceken et al., 2000), and it is not known for a Homer-protein complex. Therefore, additional binding sites or mechanisms may exist in Homer-target protein interactions.

The Homer binding motif is conserved in the suppressor domain of all three subtypes of IP_{3}R. For IP_{3}R{\textsubscript{1}}, it corresponds to the residues 49–51 (IPxX) in the suppressor domain (Tu et al., 1998), Yoon et al., 2003, deletion of which leads to the loss of binding between HomR and the N-terminal part of IP_{3}R{\textsubscript{1}} (Tu et al., 1998). Interestingly, this Homer binding motif overlaps with the binding sites for Ca{\textsuperscript{2+}} (residues 48–51). Still, the Homer EVIH domain does not have β-hands that wrap around the binding targets in Ca{\textsuperscript{2+}} target binding. Therefore, there could be some similarities and differences between the interactions of the two proteins with the suppressor domain. In addition, as the Ca{\textsuperscript{2+}} suppressor domain binding triggers unusually large-scale conformational changes in the suppressor domain (Kang et al., 2011), it would be interesting to see if similar changes occur in Homer-suppressor domain interactions.

Through NMR spectroscopy, mutagenesis, and biochemical pull-down assays, we show that the binding mechanism of Homer EVIH domain is quite different from that of Ca{\textsuperscript{2+}} and that some residues on the opposite site of the canonical binding sites in the Homer EVIH domain should be involved in the interaction. By comparing our results with previously reported structures, we provide a possible mechanism for the involvement of this new site in the binding.

Materials and methods

Cloning of IP_{3}R suppressor domain and Homer3 EVIH domain

Cloning was performed using a polymerase chain reaction (PCR) based approach. The cloning of IP_{3}R suppressor domain for pET11b vector was described previously (Kang et al., 2011). For cloning into His-GST-EV (GST) vector (Kang et al., 2007), the IP_{3}R suppressor domain gene was amplified using the sense primer CAAGGCACGGCAATCAAGGAGATCTGTCGTTTTTCAACATC and antisense primer CTGCTGATCTGTGAAAGGGCTTTTTCACGGCTATGACTGT. The PCR product was double digested with Nco I and Bam I restriction enzymes and then ligated into GST vector. The Homer3 EVIH domain was cloned from Homo sapiens homer homolog 3 gene (gene bank accession number NM001453/2); the residue numbers used in the text correspond to this gene product and is offset by three residues to those reported in pET code 2PIV (Benczech et al., 2000). The sense primer had the sequence of GAACACATGACAGCTGAGACCAGGAGCAG and the antisense primer had the sequence of ATCTGCTAGACATCGAGGAGACCTG. The PCR product was purified, double digested with Nde I and 3′-Sac I enzymes and then ligated into pET11b vector. The construct was transformed into Rosetta (DE3) competent cells, and the colonies were selected by colony PCR.

The site directed mutagenesis of IP_{3}R suppressor domain and Homer3 EVIH domain were performed using Mutate-Quick Site-Directed Mutagenesis Kit (MBI Fermentas) following the manufacturer's instruction. The mutant constructs were transformed into BL21 (DE3), harboring a chaperone plasmid pG-TD (with gilT and gilS) to increase the soluble expression. The correctness of the clones was confirmed by DNA sequencing (Macrogen, Seoul, Korea).

Expression and purification of IP_{3}R suppressor and Homer3 EVIH domain

The expression, purification, and isotope labeling of IP_{3}R suppressor domain was done as reported previously (Kang et al., 2011). For the GST-IP_{3}R suppressor and Homer3 EVIH constructs, the bacteria coli was cultured in LB media containing kanamycin at 37 °C until the OD_{600} reached around 0.6. Then, a final concentration of 1 mM IPTG was added, and the induction was carried out at 35 °C. For the mutated constructs transformed into BL21 (DE3) harboring a chaperone plasmid pG-TD (with gilT and gilS) was added 30 min prior to the IPTG induction, 10% labeled and 90% double labeled Homer EVIH domains were added to the IP_{3}R suppressor domain were prepared by using M9 medium supplemented with appropriate combination of 154 ammonium chloride and 154 glucose, respectively. The Homer3 EVIH domain was purified with Ni-Ni affinity chromatography using standard procedures using the 6 x 10^{4} g/Ml codon in the pET18 vector, which was left uncleaved. The resulting protein fractions were further subjected to gel filtration with Superdex 200 75 HiLoad 90/70 (GE Healthcare, New Jersey, USA), with a buffer containing 20 mMNaPO_{4} pH 6.5; 0.1 M NaCl, pH 6.5. The GST-IP_{3}R suppressor domain was prepared using standard procedures using glutathione resin.

Pull-down assay

Resin-immobilized Homer3 EVIH domain was prepared using purified Homer3 EVIH and NHS activated sepharose 4 fast resin according to the manufacturer’s instructions (GE Healthcare). Sixty micromolar of the prepared resin was incubated with purified wild type VIT and mutated IP_{3}R suppressor domain in the same buffer used in PUL (20 mM NaPO_{4}, pH 6.5) for 10 min at 4 °C, and then extensively washed with the above buffer. IP_{3}R suppressor domain bound to the Homer3 resins were eluted by boiling the wash resin in the SDS-sample buffer. The presence of bound IP_{3}R suppressor domain was checked by 15% SDS-PAGE. Untrated Sepharose CL-4B resin (Sigma-Aldrich, Inc., Missouri, USA) was used as a negative control. For the reciprocal pull-down assay of WT and the mutated Homer3 EVIH domain, we used GST-IP_{3}R suppressor domain immobilized to GSH resin. The bound Homer3 EVIH domain was eluted along with GST-IP_{3}R with the addition of the elution buffer containing 10 mM GSH.

NMR spectroscopy

The NMR binding experiments were done with 1H-15N HSQC approach on either Bruker 600 MHz NMR spectrometer (Korea Basic Science Institute, Ochang, Korea) or Varian 900 MHz NMR spectrometer (Korea Basic Science Institute, Ochang, Korea).
spectrometer (Korea Institute of Science and Technology, Korea). Both instruments are equipped with cryogenic probes. To monitor changes in the IP$_R$ suppressor domain, a spectrum of the 7-N$_2$H$_5$ labeled IP$_R$ suppressor domain was measured with the addition of unlabeled Homer3-EVII domain in NMR sample buffer (500 mM Na$_2$PO$_4$, pH 6.5). To observe changes in Homer3-EVII domain, a spectrum of 7-N$_2$H$_5$ labeled Homer3-EVII domain was recorded with the addition of unlabeled IP$_R$ suppressor domain in NMR sample buffer (500 mM Na$_2$PO$_4$, pH 6.5). Resonance assignments for the Homer3-EVII domain were obtained in double and triple resonance experiments, such as HSQ, HNCAC, HNCO, HNCOCA, HN(CO)NH, HB(CO)NH, HCC(CO)NH, and HCC(NH)COH (Reuzet and Wüthrich 2000). This study made use of the NMR facility at Korea Basic Science Institute, which is supported by BioMB Research Program of the Korean Ministry of Science and Technology (2020760). Access to a 900 MHz NMR machine (Varian) was also kindly provided by the Korea Institute of Science and Technology 900 NMR facility supported by KIST (Grant No. 2910206).

**Results**

NMR binding study of IP$_R$ suppressor domain

To study the binding between the IP$_R$ suppressor domain and the Homer3-EVII domain, and to compare it with that between the suppressor domain and CaM, we carried out NMR spectroscopy that gives conformation-sensitive binding information. The HSQC spectrum of the 7-N$_2$H$_5$ labeled IP$_R$ suppressor domain alone was used as a control, and increasing amounts of unlabeled Homer3-EVII domain were added for titration experiments. The overall features of the spectrum did not change much during the titration up to the molar ratio of 1:7 (Fig. 1A). Nevertheless, a small set of peaks disappeared, while others showed up as the Homer3-EVII domain concentration increased (Fig. 1A). These changes were minimal at the ratio of 1:3, but readily visible at the ratio of 1:7 with about 22 new, 5 disappearing, and 12 intensity-changed peaks (supplementary Fig. S3). These results suggest that the binding between Homer3-EVII domain and the suppressor domain does not involve general conformational changes in the suppressor domain as seen with CaM, but rather locally-restricted interactions (Kang et al. 2011).

To test if the suggested PPKRF motif in the suppressor domain is actually involved in the local interaction, we identified the peaks for K51, K52, and F33 of the suppressor domain using the resonance assignment we reported previously (Kang et al. 2011). The peaks for K51 almost disappeared in the 1:7 titration, whereas those for K52 and F33 showed slight decreases in intensities (arrows in Fig. 1A). The results confirm the involvement of the PPKRF motif in the binding interaction.

Pull-down assays with proline mutants

At NMR experiments cannot test the involvement of the two important proline residues in the PPKRF motif because of the lack of amide protons in these residues, we also performed pull-down assays with site-directed mutants of the proline residues. We generated PPKRF mutants, with the prolines replaced with histidine, and tested the binding with reimmobilized Homer3-EVII domain (Figs. 2A and 2B). For a possible mutant of PPKKE, we could not get soluble protein and could not test the binding. The PPKRF mutant, with the first proline replaced with histidine, showed similar binding affinity with the WT, but KKKKF without any proline showed significantly decreased binding to Homer3-EVII domain. The data indicates that the first proline is crucial in the binding and that at least one proline is required for the binding.

Fig. 2. Pull-down assay for interaction between Homer3-EVII domain and WT or mutated IP$_R$ suppressor domain. WT, PPKKF, and KKKKF indicate wild type and two of the mutated IP$_R$ suppressor domain, respectively. (A) SDS-PAGE result for the pull-down assay. Immobilized Homer3-EVII domain or Sepharose CL-4B resin (control) was incubated with protein (WT and mutated IP$_R$ suppressor domains). After extensive washing, the bound proteins were eluted and analyzed using SDS-PAGE. The input lane represents the purified WT IP$_R$ suppressor domain sample, and the control lane the negative control. (B) The bar chart of the band intensities for the bound proteins and the mutated proteins of the IP$_R$ suppressor domains from pull-down assay. The heights and the error bars represent mean and standard deviation of the band intensities from three independent experiments. The peaks for PPKRF and KKKKF compared to WT are 0.044 and 0.039, respectively. The band intensities were obtained using ImageJ software.

Binding site characterization of Homer3-EVII domain

After the study on the binding site in the suppressor domain, we looked at the interaction site on the Homer3-EVII domain side. As a prerequisite for the binding site identification on the Homer3-EVII domain, we obtained the HSQC spectrum of the Homer3-EVII domain and tried to obtain assignments of the peaks. We used triple resonance spectra (HNCAC, HNCA, HNCO, HN(CO)CA, HN(CO)NH, HB(CO)NH, HCC(NH)CO, and HCC(NH)COH) on 15N and 13C double-labeled Homer3-EVII domain, and obtained the assignment of all the visible backbone resonances (Fig. 3A). Then, we carried out the NMR binding assay by adding unlabeled IP$_R$ suppressor domain to 7-N$_2$H$_5$ labeled Homer3-EVII domain. We observed decreased peak intensities for II peaks from Homer3-EVII domain, compared to those obtained without the suppressor domain (Fig. 3B). By referring to the above-established assignment, the changed peaks were identified as belonging to K24, I28, Y59, F60, S52, T75, G78, Y99, C96, Q52, and I93 residues (Fig. 3B).

After the identification of the binding sites, we mapped the residues on the reported three-dimensional structure of the Homer3-EVII domain (Fig. 4) (PURB ID:2P4V; Bensen et al. 2006) and their exposure on the surface (supplementary Fig. S2). The Homer3-EVII domain possesses two half-moon shaped beta sheet

*Supplementary data are available with the article through the journal Web site at http://mc.manuscriptcentral.com/bcb-2013-0030.

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Fig. 3. 1H-15N correlation spectrum of the 15N-labeled Homer3 EVH1 domain. (A) The backbone assignment of Homer3 EVH1 domain obtained using triple-resonance NMR spectral analysis. (B) Spectral changes of Homer3 EVH1 domain after binding to IP3, K suppressor domain. The spectra of Homer3 EVH1 domain (300 μmol/L) in the absence (black) and presence (red) of IP3, K suppressor domain at ratio of 1:2 are overlaid. The identities of the perturbed residues, either disappearing or shifting, are indicated on the spectra. (Please see the online version for colour.)
regions, facing opposite directions. Eight of the disappeared residues (K24, D28, T73, G18, Y95, G196, G92, and F93) were found in the larger half-moon region, and the other three (Y39, F40, and S52) in the smaller region. The larger region corresponds to the canonical binding site (CBS) of the Homer EVH1 domain (Peterson and Volkman 2007). The other area seems to be a new binding site (NBS) that has not been characterized.

**Binding activities of Homer3 EVH1 domain mutants**

As our NMR binding assay found a group of new residues affected by the binding, as well as previously known ones, we tried to confirm them by biochemical binding assays using mutants. We built mutants for both CBS and NBS. All the mutants had alanine in the place of the original amino acid residues: K24A, D28A, Y95A, F40A, S52A, T73A, G18A, F93A, and Y39A. We recombinantly expressed and purified all the mutants and tested their binding to GST-IPR suppressor domain immobilized to GSH resin. Compared to the WT, all but one of the mutants, including those with mutations in the CBS, showed decreased binding affinity (Figs. 5A and 5B). The only exception was S52A mutant that has a mutation in the NBS. The pull-down data confirm the NMR results, showing the involvement of the two separate sites in the binding.

**Discussion**

We started the present study on the recognition that the PPKRF motif overlaps with the CαM binding motif of the suppressor domain. Actually, it is the first five residues of the 49–81 region. As the CαM-suppressor domain binding causes dramatic spectral changes attributable to global conformational changes (Kang et al. 2011), we wanted to see if the same phenomenon is observed with the Homer-suppressor domain case. Our NMR data exhibited changes in a small set of peaks in both molecules, which excludes large-scale conformational changes. This difference between the Homer and CαM binding, despite binding site overlap, may be due to the environmental difference of the binding regions. The PPKRF motif in the suppressor domain for Homer binding is much shorter than the 49–81 regions. In addition, although the PPKRF constitutes the very first part of the CαM binding sites, most of it is in an apex region in the three-dimensional structure of the suppressor domain (Supplementary data Fig. S2). In comparison, the bulk of the CαM binding sites (49–81) constitute an almost “armpit”-like region spanning the beta and alpha domain with many of the residues buried deep inside, which requires global dynamic conformational changes for binding (Kang et al. 2011).

The different degrees of conformational changes in the target may be also related to the different degrees of wrapping of the targets. CαM has the well-known “wrap-around” binding mechanism, where the two lobes of CαM nearly completely bury the circumference of the target peptides (Ikura et al. 1992; Kang et al. 2007). In comparison, EVH1 domain and target interaction seems to involve a general target-binding pocket interaction that does not cause large conformational changes. Still, this lack of large conformational changes does not mean a rigid-body interaction, and limited local changes may occur (see below). This type of Homer-suppressor domain interaction seems consistent with the mechanism of Homer in relation to calcium signaling. It has been proposed that Homer’s role in calcium regulation is to connect TRPC and IP3R, and that the entry of calcium through TRPC is modulated by competition between different forms of Homer isoforms for the proline-rich sites in these proteins (Yuan et al. 2003). Therefore, CαM seems to regulate IP3R through conformational changes of the calcium channel, whereas Homer is likely to do it through enabling selective binding to other regulatory proteins.

Functional coupling between Homer and IP3R has been demonstrated (Tu et al. 1998; Yuan et al. 2003). However, very little has been known for the structural aspects of the interaction, and most are inferred from the study involving nGluR-Homer interaction (Benceken et al. 2009). Still, there are some important points that should be noted before applying the analogy to the suppressor
domain-Homer interaction. In the mGluR1-Homer complex structure, the most intimate interactions from the mGluR side is provided by the second F and the last F in the PSPI motif of mGluR1 corresponding to PSO and F53 in the suppressor domain (Benescen et al. 2006). These mGluR1 residues have critical contacts with C92 and I77 in the CBS of Homer. In the suppressor domain, however, the corresponding PSO and F53 are largely buried (only 9.3% and 14% exposed for all atoms, respectively). Therefore, the peptide motif conformation in the suppressor domain should be different than that in the mGluR1 peptide. This difference in the detailed binding mechanism is consistent with our pull-down assays using IP-8 suppressor domain mutants. The mutations of the first F into K in the suppressor domain did not affect the binding with the Homer3 EVH1 domain, whereas the mutation of the corresponding F to K resulted in the loss of binding between mGluR1 and Homer (Ts et al. 1998). There is also an example for this type of different contributions for particular residues in the EVH1 binding motif. For Mena EVH1-peptide complex, it is the first rather than the second proline residue that is inserted deep into the CBS in the EVH1 domain (Prehoda et al. 1999). How these detailed differences for the residues in the PXXP motif might occur is still an open question, but could be explained as follows. One possibility is the local conformational change of the PPRKK motif in the suppressor domain to expose PSO and F53 to enable similar close contacts seen in mGluR1-Homer interaction. The conformational changes, if any, may occur relatively easily, as the motif is in the loop region, but may not propagate to other region of the suppressor domain, judging from the spectral changes. Also possible is the involvement of F223 in the binding, instead of F53, which is just next to F53 and is much more exposed (57%). The confirmation of these should await the elucidation of the full complex structure.

In the NMR experiment with the 15N-labeled IP-8 suppressor domain and the Homer3 EVH1 domain, we found that the residues in CBS, mostly on the external surface of the beta-sandwich formed by residues R13–F29 and F70–A94, are affected by the binding. Interestingly, residues in CBS, almost on the opposite face formed by the other beta-sheets in the beta sandwich structure, were also affected. Our biochemical pull-down experiments also support the involvement of the NBS residues in the binding. As mutations of the residues decreased the binding affinity. To the best of our knowledge, the NBS is a new region involved in Homer-target binding. We expect that it may be also important in binding to other proteins. Neither Homer nor the suppressor domain underwent large scale conformational changes that could have allowed the far-apart CBS and NBS to contact the suppressor domain at the same time. At this point, direct contact itself with the suppressor domain could happen at either CBS or NBS. The involvement of these two sites suggests that the Homer-suppressor domain interaction may not be explained with conventional binding mechanisms. Here, we propose a new binding mechanism whereby the residues in NBS might function as a conformational lock-switch that should be turned on for efficient binding. In this model, there are small but necessary conformational changes in NBS, F46 in particular, occurring during the binding interaction. Actually, this insight in the conformational lock-switch was obtained when we compared the NBS regions of the structures of a rat Homer EVH1 domain in the presence and absence of a ligand and human Homer EVH1 domain (PDB ID: 4DDW, JDDW, and ZP4 respectively) (Beneschen et al. 2000; Huang et al. 2008). The overlay of these structures showed very high overall similarity among all three structures of the Homer EVH1 domain (Fig. 4). However, there was an important difference between the bound and unbound structures of the rat Homer EVH1 domain: the conformation of F46 in NBS in the two states differed by almost 90°. It should be noted that the two unbound structures with the same conformation for F46 are from different species (rat vs. human), and that the bound and unbound structure comparison was done for EVH1 domains from a single species (rat). Actually, F46 turned out to be the only residue having the same orientation between the unbound states of rat and human EVH1 domains and different orientations (75.5 Å RMSD) between the bound and unbound rat EVH1 domains. We showed that the suppressor domain mutant F46A has reduced binding affinity, and that may be due to the F48A mutant’s inability to assume the required conformation of F46 as present in the bound state of the rat EVH1 (4DDW). The decreased conformational affinity for Y39A mutant may be also explained by the closeness of Y39 to F40.

The conformational lock-switch mechanism may also explain how the conformational change in F40 can affect the binding at the CBS on the other side of the beta sandwich in the case of 4DDW. It may be explained by a long continuous beta strand corresponding to S6–D6 in human EVH1 domain that is a part of both CBS and NBS, as it runs in both faces of the beta sandwich (see Fig. 4). It may be through this strand that F40’s conformational change in NBS is linked to the binding event in CBS; this strand is interesting to note that the internal part of the beta strand (corresponding to S2–S5 in human EVH1 domain), the immediate neighbor of F40, loses electron density upon binding to the ligand...
(1D0V). In a sense, this lock-switch mechanism is similar to the common effects of autocrine and paracrine messengers on enzyme-substrate interactions.

The presence of NBS in Homer suppressor domain interaction is reminiscent of yet another noncanonical binding site described for Homer-NFAT interaction (Huang et al., 2008). There, a region involving residues A30, G31, I33, C54, and A66 of the EVH domain, not the CBS, was shown responsible for NFAT-Homer binding. This five amino acid region forms a distinct promiscuous pocket to the binding pocket formed by the NBS residues (see Fig. 6). Overall, Homer may be able to use these multiple binding sites to interact with other proteins depending on cellular conditions. The existence of multiple binding regulatory sites and the proposed conformational lock-switch seems consistent with Homer’s role as an adaptor protein linking diverse molecules. So far, Homer has been described as a dumbbell-like tetramer with a pair of EVH domains at both ends interacting with other proteins (Hayashi et al., 2006a, 2006b; Peterson and Volkmann, 2009). Based on our previous results, it can be speculated that one EVH domain may be able to bind different proteins, transferring signals from one to another protein.

Acknowledgements

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Metabolomic comparison between cells over-expressing isocitrate dehydrogenase 1 and 2 mutants and the effects of an inhibitor on the metabolism

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Abstract
The R132H and R172K mutations of isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) have neomorphic activity of generating 2-hydroxyglutarate (2-HG) which has been implicated in the oncogenesis. Although similarities in structure and enzyme activity for the two isoforms have been suggested, the difference in their cellular localization and biochemical properties suggests differential effects on the metabolic oncogenesis. Using U87 cells transfected with either wild-type (WT) and mutant (MT) IDH genes, the MT-IDH1 and MT-IDH2 cells were compared with NMR-based metabolomics. When normalized with the respective WT-IDH cells, the general metabolic shifts of MT-IDH1 and IDH2 were almost opposite. Subsequent analysis with LC-MS and metabolic pathway mapping showed that key metabolites in the pentose phosphate pathway and tricarboxylic acid cycle are disproportionately altered in the two mutants, suggesting different activities in the key metabolic pathways. Notably, lactate level was lower in MT-IDH2 cells when produced more 2-HG than MT-IDH1 cells, indicating that the Warburg effects can be overridden by the production of 2-HG. We also found that the effect of a mutant enzyme inhibitor is mainly reduction of the 2-HG level rather than general metabolic normalization. Overall, the metabolic alterations in the MT-IDH1 and 2-HG can be different and seem to be commensurate with the degree of 2-HG production.

Keywords: isocitrate dehydrogenase, 2-hydroxyglutarate, inhibitor, metabolomics.


Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to a-ketoglutarate (a-KG), with concomitant reduction of nicotinamide dinucleotide coenzymes (Reitman et al. 2010). Depending on the isotypes, the localization and the produced coenzymes are different, with cytosolic IDH1 generating NADPH, and mitochondrial IDH2 and IDH3 generating NADPH and NADH, respectively. Recently, mutations of IDH1 and IDH2 were strongly implicated in a variety of cancers, with R132H of IDH1 and homologous R172K of IDH2 being the most prevalent (Parsons et al. 2008; Yan et al. 2009; Yang et al. 2012). These mutations are different from generally found

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cancer-causing mutations, in that they have a neomorphic activity of converting α-KG, the product of the wild-type (WT) enzyme, to 2-hydroxyglutarate (2-HG) with the consumption of NADPH (Dang et al. 2009). This change in metabolism and the increase in 2-HG level itself have been suggested at the core of the tumorigenesis mechanism of the mutations (Figueroa et al. 2010; Koivunen et al. 2012; Lu et al. 2012; Yang et al. 2012; Losman et al. 2013). 2-HG has also been shown to directly inhibit enzymes involved in chromatin methylation, such as TET (ten-eleven translocation) methyl cytosine hydroxylase and Junonji histone demethylase, affecting the epigenetic control of cell differentiation (Chowdhary et al. 2011; Xu et al. 2011).

Since IDH is a core metabolic enzyme in the tricarboxylic acid (TCA) cycle, metabolic alterations caused by the IDH mutations have been investigated. However, there have been discrepancies in some of the most important metabolic changes caused by the mutations. For example, the levels of citrate, α-KG, fumarate, or malate were reported to be similar in WT and mutant (MT) IDH1 cells in some studies (Dang et al. 2009; Gross et al. 2010), but much affected by IDH2 mutations in others (Zhao et al. 2009; Reitman et al. 2011). The levels of these intermediates are important in the tumorigenic mechanism of MT-IDH, since α-KG directly affects the activities of α-KG-dependent prolyl hydroxylases and dioxygenases (Xu et al. 2011; Koivunen et al. 2012; Yang et al. 2011). These enzymes are critical in regulating hypoxia-induced factor α and chromatin methylation status, important in cancer cell survival and differentiation. Other metabolites were also analyzed using LC-MS, and over half of the metabolites that existed at different levels in WT- and MT-IDH1 cells did not change much upon 2-HG treatment (Reitman et al. 2011). For IDH2, a previous study reported general similarity on the metabolic profile of the MT-IDH1 and IDH2 cells (Reitman et al. 2011). However, recent studies began to find biochemical and activity differences among these mutant enzymes (Jin et al. 2011; Wurd et al. 2013). Therefore, key metabolic features of the IDH mutations and any possible differences in the metabolic profile caused by IDH1 and IDH2 mutations are yet to be firmly established.

The strong relationship between IDH mutations and cancers led to a search for inhibitors of MT-IDH as possible anticancer agents, and recent studies have yielded the proof-of-concept discovery of such inhibitors (Popovic-Muller et al. 2012; Zheng et al. 2013). One of them, AGI-5108, specifically inhibited IDH1 mutant enzyme, particularly R132H, with significant effects on WT and IDH2 mutants (Rohle et al. 2013). It also inhibited the growth of MT-IDH1 glioma xenografts in mice, up-regulated genes involved in cell differentiation. Interestingly, the inhibitor did not affect the genome-wide DNA methylation status, although it dose-dependently reduced the level of histone methylation, suggesting new aspects of the inhibitor mechanism. Despite such detailed study of the effects of the inhibitor, its effects on the levels of cellular metabolites, important phenotypes of IDH mutations, have not been reported.

To address the changes in metabolic profile and compare the differences between the IDH1 and IDH2 mutations, we performed NMR-based metabolomics studies on glioma cell lines transfected with the mutated IDHs. We also studied the metabolic changes of WT- and MT-IDH1 caused by IDH1-specific inhibitor. Our results suggest that IDH1 and IDH2 mutations can have very different metabolic profiles in terms of 2-HG and other TCA intermediates. For the inhibitor, the change in 2-HG, rather than general metabolic changes, should be the biggest contributor to its effects on the metabolism of cells with MT-IDH1.

Materials and methods

Chemicals and reagents

HPLC-grade acetonitrile and water were purchased from Burdick & Jackson (Morristown, NJ, USA). Chemicals for NMR and LC-MS analysis were obtained from Sigma-Aldrich (St. Louis, MO, USA). The MT-IDH inhibitor, AGI-5108, was bought from Xcessbio (San Diego, CA, USA).

Lentiviral vector construction and preparation

The genes for human IDH (GenBank accession number NM_003590) and IDH2 (GenBank accession number NM_002168) were obtained from Origene (Rockville, MD, USA), PCR amplified, and cloned into lentiviral vector CDS26A.1 (System Bioscience, Mountain View, CA, USA) with Xbal/EcoRI restriction sites to construct pCDH-EF1α-FLAG-IDH. Primers designed for FLAG-tagged IDH1 and IDH2 cDNA cloning are IDH1-F, 5′-CTAGCTGCTTAGAATGCCAAAAAAATCAGTGG-3′; and IDH1-R, 5′-CGGGGAATCTTCTTCTGCCCCATGCATCCTTTCGATATATCTTG-3′; IDH2-F, 5′-CTAGCTGCTTAATGAGGCCCCTCCTATCCGCGG-3′; IDH2-R, 5′-CCGGGAATCTTCAACTTGTGTCGTCATCCGATCATCTGAGATAG-3′. FLAG-tagged IDH1-R132H and IDH2-R172K were amplified with the above clones as templates using standard site-directed mutagenesis. The cloned inserts were expressed under enhanced constitutive EF-1α promoter. The sequences of the cloned genes were confirmed by ABI BigDye® Terminator Cycle Sequencing Kit (Foster city, CA, USA).

The recombinant lentivirus was produced by SeoulBio Biosciences Institute (Daejeon, Korea). Expression of lentiviral particles were produced in 293TN cells cultured in Dulbecco’s modified Eagle’s medium and collected after 48 h. Then, the virus was filtered through 0.45-μm filter membrane (Millipore, Billerica, MA, USA), and immediately stored at −70°C. Titer was determined by TCID50 method and the concentrated titer was 2 × 10^5 IFU/mL.

Cell lines and culture condition

Human U87 MG glioma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI medium with 10% fetal bovine serum (FBS) at 37°C. To express WT-IDH1 and WT-IDH2, or MT-IDH1 and MT-IDH2 transgenes in cells, U87 MG cells were transfected with lentivirus for 24 h in the presence of 4-8 μg/mL polybrene. Transfected cells were passed for no more than 10 passages. These
cells were maintained in RPMI 1640 including 10% fetal bovine serum (FBS) and penicillin (Hyicycle, Logan, UT, USA). The cells were cultured at 37°C and 5% CO₂ in a 90% humidified incubator.

**Western blot analysis**

The protein levels of WT- and MT-IDH were evaluated by western blot analysis. Cells were lysed in ice-cold lysis buffer composed of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, and protease inhibitor cocktail (Sigma, MO, USA), and the concentration of lysate protein was evaluated with the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). Approximately 50 μg of protein was loaded in each lane of a polyacrylamide denaturing gel for electrophoresis. After electrophoresis, the protein was transferred to nitrocellulose membranes for blotting. We used a rat monoclonal antibody to IDH1 (Dia nouva, Hamburg, Germany), a mouse monoclonal antibody to IDH1-R132H (Dia nouva), a FLAG antibody for FLAG tagged IDH1 (Sigma), a rabbit polyclonal antibody to IDH2 (Proteintech, Chicago, IL, USA), and a mouse monoclonal antibody to IDH2-R172K (NewEast Biosciences, King of Prussia, PA, USA), and a rabbit polyclonal antibody to β-actin (Abcam, Cambridge, UK) Primary antibodies were detected by horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Paso Robles, CA, USA).

**Immunocytochemistry**

WT- and MT-IDH cells were cultured on six-well chamber slides. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min at 37°C and blocked with 3% bovine serum albumin containing 0.1% Triton X-100 in phosphate-buffered saline. The cells were stained with the following primary antibodies: IDH1 (Dia nouva), RH1-R132H (Dia nouva), IDH2 (Proteintech), IDH2-R172K (NewEast Biosciences), FLAG (Sigma), and β-actin (Abcam). In addition, the stained cells were visualized on a confocal laser scanning microscope (Leica SP2, Bannockburn, IL, USA) using Alexa 594- and Alexa 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA).

**Sample preparation for NMR and LC-MS spectroscopy**

Metabolites extraction was performed on WT- and MT-IDH cells (3 x 10⁵ cells). The counted cell pellets were resuspended with 200 μL of mixture composed of acetonitrile, methanol, and distilled water (5 : 5 : 2). The samples were centrifuged at 15,000 g for 20 min at 4°C. The supernatant was collected, divided into two at a ratio of 1 : 1 for LC-MS and NMR, respectively, and then dried with a vacuum centrifuge (Vision, Seoul, Korea). The pellets for LC-MS were dissolved with 30 μL of mixture of HPLC-grade acetonitrile and water (1 : 1, v/v), and those for NMR with 500 μL buffer composed of 2 mM Na₂HPO₄ and 5 mM Na₂HPO₄ in D₂O with 0.025% TSP (trimethylsilylpropionic acid sodium salt-d₄) as an internal standard.

**NMR and LC-MS measurement**

Untargeted metabolomic profiling was performed using NMR, and targeted profiling by LC-MS multiple reaction monitoring was performed for the metabolites that are not readily differentiable with NMR. For the overlapping metabolites, such as succinic and 2-HG, both methods gave consistent results. One-dimensional NMR spectra were measured on a 500-MHz Bruker Avance spectrometer (Bruker, Billerica, MA, USA) equipped with a cryogenic triple resonance probe (KBSL, Ochang, Korea). The acquisition parameters were essentially the same as those reported previously (Wen et al. 2010). Kwon et al. 2011). The metabolites were identified using Chenomx spectral database (Edmonton, Alberta, Canada) by fitting the experimental spectra to those in the database. Both chemical shifts and J-coupling patterns were fitted for all the detectable signals of given metabolites. Identified compounds were also confirmed by comparison with standard compounds. LC-MS data were obtained with an API 2000 mass spectrometer (AB/SciEx, Framingham, MA, USA) connected with an HPLC (Agilent 1100 Series, Agilent, CO, USA). Mobile phases were 10 mM Ammonium carbonate (pH 9.1) in distilled water (A) and acetonitrile (B), and the flow rate was 0.15 ml/min. The gradient scheme was as follows: 80% B at 0 min, 35% B at 10 min, 5% B at 12 min, 5% B at 25 min, 80% B at 25.0 min, and 80% B at 35 min. The Zic-pHlic Polymeric Beads Peak Column (150 x 2.1 mm, 5 μm, Merck, Barmstadt, Germany) was used at 35°C, and the autosampler temperature was set at 4°C. Other parameters were set as previously reported (An et al. 2012).

**Multivariate data analysis**

The time domain NMR data were Fourier transformed, phase corrected, and baseline corrected manually using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). The processed NMR data were exported to an ascii file and binned at a 0.003 ppm interval to reduce the complexity of the NMR data for pattern recognition. Moreover, the signals were normalized against total integration values and 0.025% TSP. The region corresponding to water (4.6-3.9 ppm) was removed from the spectra. The binning and normalization were performed using Perl software (www.perl.org) written in-house. The results were then imported into SIMCA-P version 11.0 (Umetrics, Umeå, Sweden).

**Results**

Establishment of cell lines for WT- and MT-IDH expression

To characterize the effects of MT-IDH on the metabolism of glioma cells, we infected U87 glioma cell line with lentiviral vectors harboring MT- and WT-IDH genes. We constructed the vectors for MT-IDH1 (R132H), MT-IDH2 (R172K), WT-IDH1, and WT-IDH2 with FLAG tags in the open reading frame. The IDH2 constructs also contained the original mitochondrial targeting sequence. The expression of the intended enzymes were tested by western blot with antibodies specific for both WT- and MT-IDH and those only for MT-IDH, as well as that for the FLAG tag (Fig. 1a). All the transfected cells expressed the expected proteins, as detected by the FLAG and isotype-specific antibodies. The use of FLAG antibody for all proteins showed that each mutant enzymes was expressed in comparable levels. In addition, the mutant-specific antibody confirmed the mutant protein expression. The site of the over-expression was also tested with immunocytochemistry (Fig. 1b). IDH1 was expressed in broad cellular regions, whereas IDH2 was expressed mainly in the cytosolic region (including mitochondria), consistent with its mitochondrial localization.
The metabolic feature of the cell lines detected by NMR
Given the successful establishment of the cell lines expressing the desired mutant IDH enzymes, we tried to compare the metabolic changes induced by the IDH mutations. For this, we profiled the metabolites using NMR spectroscopy (Fig. 2a) which has been used extensively in untargeted metabolite profiling. The NMR spectra of the extracted metabolites from the cell lines, especially the one expressing MT-IDH2, gave visually different profile (Fig. 2b). The difference was most prominent in the regions spanning 1.8–2.4 ppm and 3.8–4.2 ppm, where many of organic acids, including 2-HG, appear. As a prerequisite for the investigation of the metabolites contributing to this difference, we identified a number of metabolites on the NMR spectra based on the chemical shift and J-coupling patterns (Table 1).

Multivariate analysis and the metabolic difference between mutant isotypes
Then, to compare the metabolic changes in a more systematic way and to consider the intragroup variations, we performed multivariate statistical analysis using partial least squares discriminant analysis (PLS-DA) on the entire NMR data (Fig. 3a). The differentiation model was built with six components, yielding very high goodness-of-fit and predictability values ($R^2 = 0.912$ and $Q^2 = 0.992$). Accordingly, its score plot shows that the four groups can be cleanly separated. The biggest difference was observed between WT- and MT-IDH2 along the first PLS component, consistent with the visual difference of the NMR spectrum ($R^2_X$ of PLS1 = 0.35). The difference between the WT- and MT-IDH1 was also observed along the second PLS component with smaller magnitude ($R^2_X$ of PLS2 = 0.146). To characterize these difference in more detail, we identified metabolites contributing most to the differentiation with the loading plot of scaled correlation (Fig. 3b) in reference to Table 1. The analysis shows that both MT-IDH1 and MT-IDH2 have increased level of 2-HG (4.02, 1.84, 2.27, 2.00 ppm), and decreased levels of alanine (1.48 ppm) and leucine (1.71 ppm) compared with their respective wild types. In addition, MT-IDH1 exhibited higher levels of glutamate (2.35 ppm) and glutathione (2.16 ppm) and lower levels of betaine (3.87 ppm), N-Acetylglutamine (3.68 ppm), and Trimethylamine N-oxide (3.24 ppm) than MT-IDH2. We also constructed a heat map of the identified metabolites.
Fig. 2 Representative NMR spectra of the extracted metabolites from WT- and MT-IDH1,2 cell lines. The NMR spectra were taken for the cell extracts in 500 μL 2H2O containing 2 mM Na3HPO4, 5 mM NaN3PO4, and 0.025% TSP as an internal standard. The entire region of the spectra (a) and the zoomed region for 2-hydroxyglutarate (2-HG) (arrows) (b) are shown. Specific metabolite peaks were assigned using Chenomx database and standard compounds. The numbers in (a) correspond to the metabolites listed in Table 1.

with their relative fold-change to further characterize the metabolic difference between MT-IDH1 and MT-IDH2 as normalized to their respective wild types (Fig. S3). The results demonstrate that the metabolic shift of MT-IDH1 and MT-IDH2 from their respective WT isotypes is almost opposite to each other.

Altered metabolic pathways in the IDH mutants
In addition to the NMR-based untargeted metabolomic profiling, we characterized some of the key metabolites involved in TCA cycle and related energy metabolism with LC-MS (Table S1). This should be helpful for IDH-associated metabolism, because the WT- and MT-IDH use isocitrate and α-KG, key intermediates of the TCA cycle, as the substrates. With the combination of the NMR and LC-MS data, we mapped the altered metabolic levels onto the metabolic pathways (Fig. 4a and b). The analysis revealed that the levels of key energy metabolites are quite different between the cells expressing MT-IDH1 and MT-IDH2, except the high production of 2-HG. These include ribulose-5-phosphate, the substrate of the committed step of the pentose phosphate pathway (PPP); lactate, a key indicator of cytosolic anaerobic metabolism; oxaloacetate and succinate, key intermediates in TCA cycle; glutamate, a key anaplerotic metabolite for TCA cycle; and others.

Characterization of the effects of MT-IDH1 inhibitor on metabolism
Recently, AGI-5189, an MT-IDH1 (R132H) specific inhibitor, was introduced (Rohle et al. 2013). Although its efficacy on decreasing the 2-HG levels and inhibition of the tumor growth was demonstrated (Rohle et al. 2013), its
effects on the metabolism has not been studied yet. Therefore, we tried to study if the inhibitor can reverse the metabolic phenotypes caused by the mutation. Unfortunately, a specific inhibitor for the MT-IDH2 (R172K) is not yet available, and we only studied the effects of the IDH1 inhibitor in this study. The NMR metabolomics analysis after treating the inhibitor to both WT- and MT-IDH1 cells shows that the inhibitor moves the global metabolic profile of the MT-IDH1 about three-fifths toward that of the WT-IDH1 along the PLS1 direction (Fig 5a). In comparison, the treatment did not alter the metabolic profile of WT-IDH1 in any appreciable degree. The deviation of the inhibitor-treated MT-IDH1 along the PLS2 direction may suggest that the inhibitor causes secondary metabolic changes, but that it is not as significant as the primary changes (PLS1 R²X: 0.231, PLS2 R²X: 0.161). When the metabolic effects of the inhibitor treatment were compared after the normalization with the WT-IDH1 cells, the overall metabolic profile with and without the inhibitor was largely similar, except for the biggest difference of 2-HG level (Fig. 5b). The 2-HG level decreased by the inhibitor up to that of the WT cells, and its change was by far the largest among the measured metabolite levels.

**Discussion**

Despite reported similarities (Ward et al. 2010; Reitman et al. 2011), the MT-IDH1 and MT-IDH2 have different dimerization properties with their respective WT counterparts (Jin et al. 2011) and localization, which have direct implication in their ability to produce 2-HG (Ward et al. 2013). In addition, the two isotypic mutations exhibited different metabolic reactions against environmental changes (Grassian et al. 2014). In a hypoxic condition, for example, HCT116 cells with IDH1 mutation showed significant decrease in reductive carboxylation flux, whereas those with IDH2 mutations did not show such difference when compared to WT cells. The difference in the clinical

<table>
<thead>
<tr>
<th>Metabolites (ID)</th>
<th>Chemical shift (ppm) &amp; multiplicity</th>
<th>Change MT vs. WT in IDH2</th>
<th>Change MT vs. WT in IDH1</th>
<th>Change MT vs. WT in IDH1 with inhibitor</th>
</tr>
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<tbody>
<tr>
<td>1 2-Hydroxyglutarate</td>
<td>1.84(m), 2.00(m), 2.27(m), 4.02(c)</td>
<td>▲</td>
<td>▲</td>
<td>–</td>
</tr>
<tr>
<td>2 3-Hydroxyisovalerate</td>
<td>1.25(s)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3 5,6-Dihydroxycarbonyl</td>
<td>2.65(t), 3.38(t)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>4 Acetate</td>
<td>1.92(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>5 Acetacetyl</td>
<td>2.24(s), 3.42(s)</td>
<td>▲</td>
<td>▲</td>
<td>–</td>
</tr>
<tr>
<td>6 ANP</td>
<td>6.15(d), 8.27(s), 8.54(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>7 Alanine</td>
<td>1.48(d)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>8 Betaine</td>
<td>3.23(s), 3.87(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>9 Creatine</td>
<td>3.04(s), 3.93(s)</td>
<td>–</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>10 Glutamine</td>
<td>2.05(m), 2.13(m), 2.35(m), 3.76(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>11 Glutathione</td>
<td>2.10(m), 2.56(m), 2.96(m), 3.81(m)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>12 Glycine</td>
<td>3.54(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>13 Glutamine</td>
<td>0.94(t), 1.01(d)</td>
<td>▲</td>
<td>▲</td>
<td>–</td>
</tr>
<tr>
<td>14 Lactate</td>
<td>1.32(d), 4.10(l)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>15 Leucine</td>
<td>0.97(t), 1.71(m)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>16 Methion</td>
<td>5.97(s)</td>
<td>▲</td>
<td>▲</td>
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<tr>
<td>17 N-Acetylglycine</td>
<td>2.09(e), 3.69(d)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>18 NAD+</td>
<td>6.04(d), 6.08(d), 8.20(t), 8.41(s), 8.84(d), 9.16(d), 9.34(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>19 Oxaloacetate</td>
<td>3.78(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>20 Oxyurinol</td>
<td>7.96(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>21 Phenylalanine</td>
<td>7.32(d), 7.38(l), 7.43(l)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>22 Succinate</td>
<td>2.41(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>23 Taurine</td>
<td>3.26(t), 3.43(l)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>24 Threonine</td>
<td>1.32(t), 3.69(d), 4.25(t)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>25 Trimethylamine N-oxide</td>
<td>3.24(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>26 Tyramine</td>
<td>6.00(d), 7.20(d)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>27 Valine</td>
<td>0.99(t), 1.05(d)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>28 Xanithine</td>
<td>7.96(s)</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
</tbody>
</table>

*The signal number (see Fig. 2a), chemical shift and multiplicity, the change of MT group against WT group with the p-value less than 0.05 (up and down triangles) are indicated.*


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outcome by the IDH1 and IDH2 mutations has also been reported, although uncertainties do exist because of the heterogeneity in coexisting mutations and patient population. Poor and favorable prognosis were reported for Acute myeloid leukemia (AML) patients with IDH1 and IDH2 mutations, respectively (Schnittger et al. 2010; Boissel et al. 2011; Chou et al. 2011). Different leukemia-free survivals were also reported depending on the isotopic mutations for myelodysplastic syndrome (Patnaik et al. 2012). For glioma, clinical studies comparing IDH1 and IDH2 mutations are rare because of the low incidence of the IDH2 mutations in glioma. Nevertheless, it has been reported that IDH1 and IDH2 mutations occur in a mutually exclusive manner (Ahmadi et al. 2012), and that an IDH1 mutation (R132C) exhibits strong association with astrocytoma compared with IDH2 mutations occurring predominantly in oligodendrogial tumors (Hartmann et al. 2009). Therefore, the metabolomic difference between the mutant isotypes suggested in this study should not be surprising. In fact, despite the similarity described for a subset of metabolites for MT-IDH1 and IDH2 cells (Reitman et al. 2011), an interesting orthogonality in the metabolomic profiles can be found between the two isotypic MT cells. The orthogonality can be clearly seen in the principal component analysis result (Fig. 1c in Reitman et al. 2011), if one draws lines connecting the WT and MT cells of each IDH1 and IDH2. This is consistent with our results showing the metabolomic orthogonality between MT-IDH1 and IDH2 cells when normalized to their respective WT cells (see Fig. 3a and c) to consider the inherent difference between the WT-IDH1 and IDH2 cells. This “normalized” difference may not have been clear in the previous report, since direct comparison between MT-IDH1 and MT-IDH2 was mainly investigated. Therefore, the normalization against the respective WT cells constitutes an important difference between ours and the previous report by Reitman et al. As we used cell lines, the interpretation may be easier than clinical isolates, but limitations do exist. Therefore, it will be interesting to apply our normalization approach to clinical isolates to further test the translatability of our results.
Among the metabolomic differences, MT-IDH2 cells showed unique metabolic characteristics compared not only to MT-IDH1 but also to many other cancer cells. Generally, cancer cells produce high levels of lactate as the consequence of aerobic glycolysis (Warburg effect) (Warburg 1956; Hirschhaeuser et al. 2011). However, MT-IDH2 cells exhibited lower levels of lactate, whereas MT-IDH1 cells exhibited higher levels as in typical cancer cells. To correlate this with actual metabolic flux important in the Warburg effect, we measured the time-dependent changes in the glucose and lactate levels in the media (Jain et al. 2012). The glucose level decreased in time for MT-IDH2, but increased for MT-IDH1 (Figure S1a), indicating higher glucose uptake in MT-IDH2 than MT-IDH1 with respect to their respective WT cells. Furthermore, the lactate level in the media, hence its
secretion, also time-dependently decreased in MT-IDH2 only (Figure S1b). These results indicate that the increased glucose uptake in MT-IDH2 cells did not result in the increased lactate secretion and that the Warburg effect was overridden in MT-IDH2 cells. The lower intracellular lactate level in MT-IDH2 cells may be due to the consumption of NADH, required in lactate production, for the maintenance of the cellular reduction potential. As the high level 2-HG generation in MT-IDH2 cells requires an equivalent amount of NADPH, a large amount of NADH may be diverted to replenishing the reducing potential through nicotinic amide transhydrogenase, which interconverts NADPH and NADH (Hoeck and Rydström 1988). This is also consistent with the increased NAD+ level in MT-IDH2 cells, but not in MT-IDH1 cells, compared to their relative WT cells (see Fig. 3c).

For MT-IDH1 cells, 2-HG level was not as high as that in MT-IDH2 cells, although higher than that in WT-IDH1 cells, and the Warburg effect may not be overridden for the 2-HG production. The results are a bit different from a previous report showing unchanged and slightly higher lactate levels in MT-IDH1 and MT-IDH2 cells, respectively (Reitman et al. 2011). This may be due to their different 2-HG levels from those in our study, about twice more 2-HG produced by MT-IDH1 than MT-IDH2 cells, or to different cell types used (HOG cell lines in Reitman et al. (2011) vs. U87 cell lines here). As the 2-HG level was much higher in MT-IDH2 than MT-IDH1 in our results, it is speculated that the relative level of 2-HG may be inversely related to lactate production. Since the 2-HG levels can vary significantly among the cells harboring the IDH mutations (Wang et al. 2013), the lactate level may vary accordingly, explaining the apparent difference between previous results (Reitman et al. 2011) and ours. Although the increase in the lactate level through increased lactate dehydrogenase (LDH) activity is one of the common features of cancer cells (Hirschhaeuser et al. 2011), our results suggest that the cells with IDH mutations can be exceptions. This is consistent with a recent study specifically addressing the down-regulation of LDH in glioma-bearing IDH mutations (Chen et al. 2013), and a previous study focused on IDH2 in AML showing decreased LDH activity in patients with IDH2 mutation (Chou et al. 2011).

MT-IDH2 cells showed decreased levels for many of the measured metabolites, including those in the TCA cycle. One notable exception is the high level of ribulose-5-phosphate in MT-IDH2 cells. It may be speculated that MT-IDH2 cells depend on higher activity of the PPP to generate the NADPH required for the production of 2-HG. Although cytosolic NADPH produced by PPP cannot cross the mitochondrial membrane, evidence suggests that shuttle systems involving WT-IDH1/2 and isocitrate/NKG or serine one carbon metabolism should be able to transport the reduction potential of NADPH across the mitochondrial membrane (Lewis et al. 2014). When the levels of glutamine and glutamate were measured in the media of the cells according to time, the glutamine concentration decreased in MT-IDH2 cells, but not in MT-IDH1 cells, indicating a faster uptake of glutamine in the former cells (Figure S1c). This is consistent with the enhanced dependence on glutamine metabolism of the mutant cells producing a large amount of 2-HG (Seltzer et al. 2010), although it was actually shown for MT-IDH1. It seems that the amount of 2-HG production may be more important than the particular isotypic mutation in determining the glutamine uptake. Therefore, it appears that MT-IDH2 cells devote most of their metabolic resources into the production of 2-HG, although the actual flux of TCA remains to be determined. In contrast, the MT-IDH1 cells maintained generally balanced levels of many of the TCA metabolites, including NKG, even with the production of 2-HG from NKG. In addition, glutamate, an anaplerotic intermediate for NKG, actually increased, consistent with the time-dependent decrease of the glutamate level in the media, suggesting increased glutamate uptake only for MT-IDH1 (Figure S1d). The increased glutamate level in MT-IDH1, again, may be possible due to the comparably lower production of 2-HG by MT-IDH1 than MT-IDH2, both by absolute amount and normalized amount against each WT cells. The higher level of 2-HG in MT-IDH2 than MT-IDH1 is consistent with a previous report (Ward et al. 2013), but not with another (Reitman et al. 2011). The difference should not be due to the relative amount of the MT enzyme expression in our results, because similar amounts were detected using the same antibody against FLAG, when normalized with actin (see Fig. 1). It may be due, rather, to the requirement of WT-IDH for the production of 2-HG by MT-IDH1, but not by MT-IDH2, and the localization difference (Jin et al. 2011; Ward et al. 2013).

We also investigated the effects of IDH inhibitor on the metabolic profile of MT-IDH1 cells and compared it with those of WT-IDH1 cells. The inhibitor specifically affected the MT-IDH1 metabolic profile, and did not induce noticeable changes in WT-IDH1 cells. The metabolic changes represented by PLS1 was almost entirely contributed by the 2-HG decrease. The absence of general metabolic changes by the inhibitor treatment was also reported in a recent study (Gressian et al. 2014). Therefore, the previously observed favorable phenotypes of the inhibitor treatment (Rohle et al. 2013) should be largely due to the 2-HG decrease rather than general metabolic normalization. Recently, another inhibitor developed against the IDH1 mutant inhibited the colony formation of AML cells derived from patients (Chaturvedi et al. 2013). Our metabolomics approach may be applied to investigate the actual metabolic effects of these inhibitor molecules. We also tested the effects of exogenous 2-HG. The treatment of WT-IDH cells with 2-HG (30 mM as used in Reitman et al. 2011) induced metabolic shifts in the WT-IDH1 toward those of the respective mutant cells along the PLS2 dimension (Figure S2a and b). However, the profiles were significantly different.
along the PLS1 dimension, indicating large non-specific effects. The difference was more pronounced in the IDH1 than IDH2, as can be seen in the heat map analysis (Figure S2C and d). Along with our data, there are also membrane permeability concerns for 2-HG (Xu et al. 2011; Lu et al. 2012) and toxicity issues with membrane-permeable octyl-2-HG (Pusch et al. 2014). Therefore, interpretation of the results obtained using exogenous 2-HG instead of MT-IDH may require caution.

Although it is generally accepted that the IDH mutations can increase the level of 2-HG, the first oncometabolite, a recent study on a large number of AML patients found that about half (~48%) of the AML patients with increased 2-HG level did not carry IDH mutations (Wang et al. 2013). Conversely, only about 4% of the AML patients with normal 2-HG level carried IDH mutations. Here, we showed that similar levels of protein expression in isotypic MT-IDH cells can lead to different metabolomic profiles and different levels of 2-HG. Therefore, the involvement of IDH mutations and 2-HG in the oncogenesis may not always coincide with each other. Dedicated studies on specific metabolic pathways affected by isotypic IDH mutants and other cellular enzymes that might contribute to the altered 2-HG levels may provide clues to their exact metabolic roles in the oncogenesis.

Acknowledgments and conflict of interest disclosure

This study was supported by a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (1420290) and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012011362 and 200903144). The authors have no conflicts of interest to declare.

All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Comparison of the cellular exo-metabolic changes.
Figure S2. Multivariate analysis on the metabolic changes induced by 2-HG treatment.
Table S1. Metabolites identified with LC-MS analysis.

References

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Schnitzer S., Haferlach C., Ulke M., Alpermann T., Kern W. and Haferlach T. (2010) IDH1 mutations are detected in 6.6% of 1414 AML patients and are associated with intermediate risk karyotype and unfavorable prognosis in adults younger than 60 years and unmuted NPM1 status. *Blood* 116, 5486–5496.


Urinary Metabolite Profiling Combined with Computational Analysis Predicts Interstitial Cystitis-Associated Candidate Biomarkers

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ABSTRACT: Interstitial cystitis/painful bladder syndrome (IC) is a chronic syndrome of unknown etiology that presents with bladder pain, urinary frequency, and urgency. The lack of specific biomarkers and a poor understanding of underlying molecular mechanisms present challenges for disease diagnosis and therapy. The goals of this study were to identify noninvasive biomarker candidates for IC from urine specimens and to potentially gain new insight into disease mechanisms using a nuclear magnetic resonance (NMR)-based global metabolomics analysis of urine from female IC patients and controls. Principal component analysis (PCA) suggested that the urinary metabolome of IC and controls was clearly different, with 140 NMR peaks significantly altered in IC patients (FDR < 0.05) compared to that in controls. On the basis of strong correlation scores, fifteen metabolite peaks were nominated as the strongest signature of IC. Among those signals that were higher in the IC group, three peaks were annotated as tyramine, the pain-related neurotransmitter, and two peaks were annotated as 2-oxoglutarate. Levels of tyramine and 2-oxoglutarate were significantly elevated in urine specimens of IC subjects. An independent analysis using mass spectrometry also showed significantly increased levels of tyramine and 2-oxoglutarate in IC patients compared to controls. Functional studies showed that 2-oxoglutarate, but not tyramine, retarded growth of normal bladder epithelial cells. These preliminary findings suggest that analysis of urine metabolites has promise in biomarker development in the context of IC.

KEYWORDS: Metabolomics, NMR, metabolites, interstitial cystitis, bladder, biomarker

INTRODUCTION

Interstitial cystitis/painful bladder syndrome/interstitial cystitis (IC) is a chronic visceral pain syndrome of unknown etiology that presents with a constellation of symptoms, including bladder pain, urinary frequency and urgency, and small voided volumes, in the absence of other identifiable etiologies.3-12 IC is a common condition affecting approximately 1 out of 77 people, which translates into three to eight million women and one to four million men in the United States alone. Of those affected, approximately 80% of patients are female. Due to lack of consistent and effective treatments, the chronic pain from IC reduces quality of life and generates a great public health burden. IC results in more than $100 million/year in both direct healthcare expenses and indirect costs due to reduced productivity and work performance.

Diagnostic tests for IC include urine cytology, potassium sensitivity tests (considered outdated), cystoscopy with and without hydrodistention and/or bladder biopsy, and biofluid-based assays.5-8 However, cystology is nondiagnostic, and cystoscopic appearance is often normal in IC patients. Classic ulcerations of the bladder lining (Hunner’s ulcers) are found in only 5–10% of patients with IC symptoms, and bladder biopsy is also often nondiagnostic. Although hydrodistension in patients with IC demonstrates punctate bleeding (also called hemorrhages), these findings also occur with hydrodistention of normal bladders. On the contrary, assays of urine components are noninvasive and can be easily repeated. Urine is also much less complex than serum but nevertheless can contain disease biomarkers.9

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Metabolic profiling using nuclear magnetic resonance (NMR) spectroscopy can provide global chemical fingerprints of the physiology and metabolism of cells and can identify physiological and pathological states of biological samples. NMR profiles of metabolites can be interpreted through computational methods using multivariate statistical analysis. Metabolomics approaches have been used to identify biomarkers of disease using urine, plasma, saliva, fecal extract, and sputum.\textsuperscript{10-12} These sources represent noninvasive methods for disease profiling and are thus much preferred to invasive methods.

In this study, we attempted to identify IC-associated metabolites by NMR using urine specimens from IC patients and control subjects. Our findings provide preliminary evidence that metabolomics analysis of urine can potentially segregate IC patients from control subjects.

# MATERIALS AND METHODS

## Ethics Statement

This study was approved by the Ethics Committee of Inha University Hospital in South Korea. Written informed consent was obtained from all subjects. The Institutional Review Board of Inha University Hospital approved collection, curation, and analysis of all samples.

## Reagents

Cell culture medium and heat-inactivated and dialyzed fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Crystal violet solution was obtained from Promega (Madison, WI). All reagents for sample preparation for NMR and liquid chromatography–mass spectrometry (LC–MS) analysis as well as tyramine and 2-oxoglutarate were obtained from Sigma-Aldrich.

## Subjects and Urine Specimen Collection

Patients and healthy control subjects were recruited from an outpatient urology clinic at Inha University Hospital. Workup included symptom assessment, cystoscopic evaluation, physical examination, urodynamics, and/or urine culture. Patients with a history of other diseases (such as any types of cancer, inflammation, or diabetes, etc.) were excluded. All subjects were of Asian female descent living in South Korea. To avoid possible contamination with vaginal or urethral cells, first morning urine specimens were obtained using clean catch methods in a sterile environment. The deidentified specimens were centrifuged to remove cell debris, and supernatants were processed into individual aliquots of 1 mL/tube before storage at \(\text{\textdegree}80\text{ C}\) until further analysis.

## \(^1H\) NMR Analysis of Urine

\(^1H\) NMR spectroscopy-based metabolomics analysis was performed to search for soluble metabolites that segregate with the diagnosis of IC. The NMR facility at the Korea Basic Science Institute was used for this study. Sample preparation for \(^1H\) NMR was done as follows: aliquots of urine specimens (500 µL/each) were resuspended in 50 µL of D, O containing sodium 3-trimethylsilyl[\(\text{\(2,2,3,3,\text{\text{-}}\text{\text{H}}\)}\)]-propionate (TSP, 0.025%, w/v) in a 5 mm NMR tube. An NMR spectrometer (Bruker Biospin, Avance 500) operating at a proton NMR frequency of 500.13 MHz and equipped with a triple-resonance cryogenic probe was used for these experiments. All one-dimensional spectra of the urine samples were measured.

## Data Processing

The NMR data in urine samples from IC patients and healthy subjects were preprocessed as previously described.\textsuperscript{13} We excluded one subject from the IC patient group and three subjects from the control group because their spectra were outliers based on PCA analysis. Fourier transformation and phase and baseline correction of the time domain data were manually performed. The resulting frequency domain data were binned at a 0.002 ppm interval. The signals were normalized against total integration values and 0.025% TSP. The region corresponding to water (4.6-5.6 ppm) was removed from all spectra. Data pretreatment including baseline correction, chromatogram alignment, time-window setting, hierarchical multivariate curve resolution, H-MCR, and normalization were performed in MATLAB (version 7.3) using custom scripts. Identification of detected NMR spectra was performed using VNMRSS500 (Varian Inc.). The metabolites were identified by a database search based on spectra and chromatographic retention index, using Chenomx (spectral database; Edmonton, Alberta, Canada) by fitting the experimental spectra to those in the database.

## Identification of Metabolic Marker Candidates

To identify potential metabolites as marker candidates that can discriminate IC patients from healthy subjects, we applied a two-step approach. First, a nonparametric Wilcoxon rank sum test was performed to extract significant features from normalized NMR data. Second, the resultant NMR peak profiles, which contain profiles of 140 variables, were then imported into MetaboAnalyst, version 2.0.\textsuperscript{14} Mean-centering with Pareto scaling was performed prior to multivariate statistical analysis. Partial least-squares discriminant analysis (PLS-DA), important variable selection with sum of absolute regression coefficient, and model evaluation with permutation strategy were carried out according to a published protocol.\textsuperscript{15}

## Liquid Chromatography–Mass Spectrometry (LC–MS or Alternatively HPLC–MS)

For LC–MS analysis, the supernatant of centrifuged urine samples was directly injected with an injection volume of 5 µL. HPLC was performed on an Agilent 1100 series liquid chromatography (Agilent, CO). The chromatographic separation was performed on a Zic-Phile Polymers Beads Peak Column (3.5 × 2.1 mm, 5 µm, Merck KGaA, Darmstadt, Germany) at 35 °C. Mobile phases A and B were DW with 10 mM ammonium carbonate (pH 9.0) and acetonitrile, respectively. The mobile phase was delivered at a flow-rate of 0.15 mL/min. The linear gradient was as follows: 80% B at 0 min, 35% B at 10 min, 5% B at 12 min, 5% B at 25 min, 80% B at 25.1 min, and 80% B at 35 min. An API 2000 mass spectrometer controlled by the Analyst 1.6 software (AB/SCIEX, Framingham, MA) and equipped with an electrospray ionization (ESI) source was used in positive ion mode for detecting tyramine and in negative ion mode for detecting 2-oxoglutarate. For mass detection, multiple reaction monitoring (MRM) was performed with the m/z value of parent and fragment ions. The MRM transitions were 138 → 121 (tyramine) in positive ion mode and 145 > 101 (2-oxoglutarate) in negative ion mode. Two samples were excluded from the LC–MS analysis because of their abnormal detection levels.

## Cell Culture and Proliferation Assay

Immortalized normal human bladder epithelial cells, TRT-HU1, were maintained as described previously.\textsuperscript{16} TRT-HU1 cells were
seeded in 24-well culture plates at a density of 1 × 10^5 cells per well in standard growth medium. For the next 3 days, the cells were treated with varying doses of tyramine or 2-oxoglutarate. Crystal violet staining analysis was performed for determination of cell proliferation.18

**RESULTS**

**Characteristics of the Study Subjects**

The Inha Institutional Review Board approved collection and analysis of all samples (IUIH-IRB no. 10-0751). All patients and healthy control subjects were recruited for this study from an outpatient urology clinic at Inha University Hospital (South Korea). A clinical diagnosis of IC was made by two independent urologists (T.L. and S.P.) according to NIDDK criteria (e.g., frequency, urgency, bladder pain, discomfort, and the presence of glomerulations during cystoscopic hydrodistention) before any treatment or medication was given. In total, we enrolled 64 female subjects (43 IC patients and 21 normal subjects) with a mean age of around 51. Population-based, age-matched controls were recruited from one clinic using the same standard operating procedures (SOPs) during the same research period (2010–2013). The clinical and pathological features of the subjects are described in Table 1.

**Table 1. Clinical and Pathological Features of Patients with IC and Control Subjects**

<table>
<thead>
<tr>
<th>variables</th>
<th>no. of patients (%)</th>
<th>no. of controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no.</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>mean age ± SD</td>
<td>50.7 ± 10.7</td>
<td>51.4 ± 13.7</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>female</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Grade (IPSS Symptom Score)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>severe (≥20)</td>
<td>16 (37.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>moderate (9–19)</td>
<td>17 (39.5)</td>
<td>6 (28.6)</td>
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<tr>
<td>mild (0–6)</td>
<td>10 (23.5)</td>
<td>18 (71.4)</td>
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<tr>
<td>Symptoms</td>
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<tr>
<td>frequency</td>
<td>31 (72.1)</td>
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</tr>
<tr>
<td>urgency</td>
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<tr>
<td>discomfort</td>
<td>9 (20.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>pain</td>
<td>17 (39.5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

1H NMR Spectra of Urine Specimens from IC Patients and Controls

Because analysis of urine metabolites is a promising, noninvasive approach to study bladder disease, as shown with bladder cancer,17 we investigated the metabolite profile of the individual urine samples using 1H NMR spectroscopy. An NMR spectrometer equipped with a triple-resonance cryogenic probe was used for the analysis. NMR-based metabolomics requires relatively simple sample preparation and provides structural information on metabolites. Our analysis and data acquisition resulted in a total of 4501 metabolites detected. The spectra featured visually identifiable differences in the signal ranges of 6.5–7.5, 3.5–4.0, and 2.0–2.5 ppm, suggesting metabolic differences between IC patients and controls (Figure 1).

To compensate for possible outliers within samples, principal component analysis (PCA) was performed on the NMR spectral data of the urine samples from patients and controls. The Perato scaling method and division of the mean-centered data by the square root of the standard deviation were used (Figure 2A). The scores plot for partial least-squares (PLS) components showed differentiation of the IC samples from controls with good separation and dispersion (Figure 2B). We further attempted to assess how accurately our predictive model fit using the leave-one-out cross-validation method (also called rotation estimation) as well as randomized permutation. The observed statistic of this analysis using MetaboAnalyst software14 was significant (p = 0.012), suggesting that these signatures may significantly differentiate patients from healthy controls (Figure 2C).

**Identification of NMR Peaks Increased in IC Specimens**

Given the above result, we tried to identify NMR signals responsible for the difference. We sought to capture the most significantly and differentially detected NMR peaks and found that there was a significant difference in the NMR peak distribution between IC and control specimens. On the basis of multivariate statistical analysis, a total of 140 NMR peaks were significantly different between IC and controls (FDR < 0.05) (Figure 3). We then focused on the NMR peaks that most heavily contributed to the separation with respect to high correlation and signal-to-noise ratio values. We selected the top 15 NMR peaks based on the partial least-squares discriminant analysis (PLS-DA) model using MetaboAnalyst software.14 NMR signals at 3.2485, 4.3505, 3.243, 2.9068, 2.2924, 3.2304, 3.0157, 3.0212, 2.9625, 4.4422, 0.7017, 3.4533, 4.3432, 9.2718, and 3.0102 ppm are among the major factors separating the groups with high correlation and intensity of signal (Figure 4A). These key candidate metabolites contribute to the separation of patients and controls with a coefficient 0.7 or more (Figure 4A). Given that a coefficient of 0.53 or above is considered to be statistically significant (with a correlation coefficient of ≥ 0.95% or less), levels of these top 15 NMR peaks are considered to be strongly correlated to the IC group. Although the intensities of four NMR peaks at 3.4505, 4.4422, 4.3533, and 4.3432 ppm were significantly decreased in the IC group, the intensities of the other 11 peaks were increased in this group (Figure 4B). These findings suggest that these top 15 NMR peaks would be useful for further annotation and analysis.

**Identification of Differentially Expressed Metabolites in Urine of IC Patients**

Independent quantification of the metabolites that were upregulated in patients showed that 11 NMR peaks (e.g., 3.2485 and 3.243 ppm) were significantly upregulated in IC patients (p < 0.05). Annotation of the NMR peaks was performed using MeltDB, Chenomx, and MetaboAnalyst software. We were able to annotate several of the discriminant peaks, including the most significant peak at 3.2485 ppm, which was identified as tyramine, a neuro-transmodulator related to pain.19 Other NMR peaks, such as 3.243 and 2.924 ppm, were also annotated to tyramine, and peaks at 3.0157 and 3.0212 ppm were annotated to 2-oxoglutarate (Figure 5A). Figure 5B shows the structures and relative abundance of tyramine and 2-oxoglutarate. Urinary concentrations of 3.2485, 2.924, and 3.243 ppm (annotated as tyramine) and 3.0157 and 3.0212 ppm (annotated as 2-oxoglutarate) were increased in the IC patient group compared to that in controls (Figure 5C). An additional LC–MS analysis was able to confirm the NMR-based data and showed that levels of tyramine and 2-oxoglutarate were significantly increased in urine specimens from IC patients compared to that in controls (approximately 2-fold, p < 0.05) (Figure 5D-E).

The findings above suggest that bladder cells may sense higher level of metabolites, resulting in biological changes. We then
tested the effects of tyramine and 2-oxoglutarate on cell proliferation in the hTERT-immortalized urethelial cell line TRT-HU1 (Figure S5). To do this, TRT-HU1 cells were treated with varying concentrations of tyramine or 2-oxoglutarate. 2-Oxoglutarate, also known as α-ketoglutarate, is a key intermediate in the Krebs cycle, which plays a role in amino acid synthesis, nitrogen transport, and oxidation reactions. TRT-HU1 bladder cell proliferation was suppressed in the presence of 2-oxoglutarate in a dose-dependent manner. This observation is consistent with previous observations by other groups. Previous
We also found that four NMR peaks, including those at 4.3505 and 4.4422 ppm, were significantly downregulated in patients compared to normal controls (Figure 6A). Two NMR peaks, at 4.4422 and 4.444 ppm, were annotated as trigonelline. The coefficient of the 4.444 ppm peak was just below 0.7. Box plots shown in Figure 6B suggest that levels of trigonelline might be significantly lower in urine samples of IC patients compared to those of controls.

**DISCUSSION**

In this study, we report, to our knowledge, the first global analysis of metabolic patterns in urine specimens derived from IC patients and healthy subjects. NMR-based metabolomics analysis identified 140 NMR peaks, which collectively distinguished the IC patient urinary profile from that of controls. The PLS-DA model using Metabo Analyst software revealed that 15 NMR peaks are significantly changed in urine of IC patients. Levels of tyramine and 2-oxoglutarate were significantly increased in the urine specimens from IC patients compared to those from controls. These compounds may be associated with bladder pathology; however, confirmatory studies are necessary.

Our metabolomic data suggest that tyramine might be concentrated in the urine of IC patients. Tyramine is a product of tyrosine metabolism and, like other trace amines, is a neurotransmodulator. Tyramine is detectable in plasma, serum, and urine, and the measured level is significantly altered in certain disorders characterized by pain, such as common headaches, migraines, urticaria, irritable bowel syndrome, and joint pain. Thus, our findings suggest the interesting possibility that urine metabolites may elicit or reflect one’s pain perception during bladder filling and discomfort associated with IC. A specific class of tyramine receptor, the trace amine associated...
Figure 5. Upregulated metabolites that could be used to segregate IC patients from normal subjects. (A) NMR peaks at 3.0212 and 3.0157 ppm were annotated as 2-oxoglutarate, and those at 3.2483, 3.243, and 2.924 ppm were annotated as tyramine, using a 300 MHz machine (VNMR300) at Varian Inc., Korea. No annotation was available for the other three peaks using our software. (B) Chemical structures of tyramine and 2-oxoglutarate are shown. (C) NMR peaks indicating that candidate metabolites, tyramine and 2-oxoglutarate, were significantly increased in IC patients compared to those in controls. Wilcoxon rank sum test of the relative difference of the marker signals for the IC and control groups. All signals showed statistical significance with FDR < 0.05 (tyramine, 3.2483 ppm; 2.924 ppm; 3.243 ppm, 2-oxoglutarate, 3.0157 ppm; 3.0212 ppm). (D, E) LC–MS analysis showed the relative levels of two biomarker metabolites in urine from IC patients compared with those from controls. The bar graphs represent the relative peak area on LC–MS analysis for tyramine (D) and 2-oxoglutarate (E). Statistical analysis was performed using Student's t-test, and the resulting p-values are indicated. Error bars represent standard error. (F) Biological effects of 2-oxoglutarate on bladder cells. 2-Oxoglutarate treatment inhibited cell proliferation. Proliferation of TRT-HU1 cells treated with varying doses of 2-oxoglutarate (0, 1, 10, or 25 mM) was measured over time (days 0, 1, 2, and 3). Cell proliferation was determined by crystal violet assay. *, p < 0.05 (Student’s t-test).

receptor (TAAR1), is a G-protein coupled receptor (GPCR) expressed in the brain with a wide distribution in other organs. One class of GPCRs, the transient receptor potential (TRP) channel family, has been shown to regulate urothelial sensory perception and bladder function. TRPV (transient receptor potential channel subfamily V), located in the urothelium, plays a role in the bladder sensor web. Pharmacological antagonists against TRPV reduce bladder hyperactivity and urinary incontinence in mouse and rat cystitis models. In addition, anticholinergic agents, which block the neurotransmitter acetylcholine in the central and the peripheral nervous system, have been shown to improve bladder cellular architecture and provide relief from pain and urgency.

In the current study, we also found that the relative concentration of 2-oxoglutarate was increased in the urine of IC patients. 2-Oxoglutarate (also called α-ketoglutarate), which is an important player in the Krebs cycle, is known to be involved in the cellular detoxification of oxidative damage. Previous...
research has shown that 2-oxoglutarate converts to citrate during hypoxic states, resulting in cell growth and viability, suggesting the possibility of an antiproliferative and antiangiogenesis factor of 2-oxoglutarate. However, no functional role of urinary 2-oxoglutarate has been proposed in the setting of bladder wall abnormalities or bladder diseases, and no correlations have been previously described.

In summary, our findings indicate that urinary metabolites may allow the segregation of IC patients from normal individuals and may reflect the underlying biology of IC, which is still largely unknown. Further attempts to validate the clinical relevance of urinary metabolites may provide novel insights into the etiology of IC and will identify urinary metabolites as biomarkers of IC that have the potential to be employed clinically.

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Notes
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