Changes of liver metabolites following hepatectomy with ischemia reperfusion towards liver regeneration

Yu Saito1 | Yuji Morine1 | Shuichi Iwahashi1 | Tetsuya Ikemoto1 | Satoru Imura1 | Hisami Yamanaka-Okumura2 | Akiyoshi Hirayama3 | Tomoyoshi Soga3 | Masaru Tomita3 | Mitsuo Shimada1

1Department of Surgery, Tokushima University, Tokushima, Japan
2Department of Clinical Nutrition and Food Management, Tokushima University, Tokushima, Japan
3Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan

Correspondence
Yu Saito, Department of Surgery, Tokushima University, Tokushima, Japan.
Email: saito.yu.1001@tokushima-u.ac.jp

Abstract
Background: Metabolome analysis is one of the omics which investigates the final product of a central dogma. Changes of liver metabolites during liver regeneration following hepatectomy (Hx) continue to remain unclear. The aim of the present study was to investigate the changes of liver metabolites following Hx with ischemia reperfusion (I/R) towards liver regeneration.

Methods: Twenty-three patients who underwent Hx were enrolled in this study. Non-tumor tissues were sampled immediately before and after Hx and a comparison was made between the liver samples taken before and after Hx using capillary electrophoresis—time-of-flight mass spectrometry (TOFMS) as metabolome analysis.

Results: The metabolic pathway showed that there was a significant increase in "lactate" following Hx. There was a significant decrease in metabolites only in the first half of the tricarboxylic acid cycle (TCA) cycle, and adenosine triphosphate (ATP) by anaerobic glycolysis did not occur in time for energy consumption of the Hx. Principal component analysis revealed remarkably different component profiles between the samples taken before and after Hx. One hundred and three metabolites were selected as critical metabolites for separating components. Valine and tryptophan increased significantly after Hx and they were regulated by resected liver volume, ischemic time and liver function.

Conclusion: The liver metabolites changed remarkably between before and after Hx. Especially, liver valine and tryptophan were increased.

KEYWORDS
ischemia reperfusion, liver regeneration, metabolome analysis, tryptophan, valine

1 INTRODUCTION

Recently, focus has been on metabolome analysis as a method of post-genomic analysis. Metabolome analysis is one of the omics, which investigates the final product of a central dogma. Metabolome analysis is defined as the comprehensive analysis of small molecules (typically <1.5 kDa) such as amino acids, organic acids, sugars, lipids, inorganic ions and so on. The number of human metabolites is estimated to range from 2500 to 8000. Currently, three major analytical methods have been widely used in metabolome analysis including gas chromatography/mass spectrometry (GC/MS), liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass
spectrumetry (CE-MS), and each method is able to analyze a different class of metabolites.3,4

It has been well established that the liver can regenerate after hepatectomy (Hx).5 The authors have previously reported that liver regeneration is a fundamental mechanism by which the liver responds to various injuries such as ischemia reperfusion (I/R) injury.6–8 Although the molecular and cellular mechanism of liver regeneration after Hx is well understood, postoperative liver failure after Hx is still one of the critical problems in clinical settings.

There have been no reports about liver metabolites during liver regeneration using metabolome analysis. It was hypothesized that some key metabolites immediately after Hx could regulate or stimulate future liver regeneration. The aim of the present study was to investigate changes of liver metabolites following Hx with I/R towards liver regeneration using human Hx samples.

2 | MATERIALS AND METHODS

2.1 | Patients

Twenty-three patients who underwent Hx between April 2014 and March 2015 were enrolled in this study. Inclusion criteria for this study were: (i) primary Hx; (ii) <15% of indocyanine green retention test (ICG R15); (iii) subsegmentectomy or more extended Hx; (iv) no biliary reconstruction and lymph node dissection; and (v) no preoperative chemotherapy. Backgrounds of the patients are shown in Table 1. The study was approved by the Tokushima University Hospital Ethics Committee and the corresponding regulatory agencies and all the experiments were carried out in accordance with the approved guidelines. Meanwhile, all the patients involved in the study signed the informed consent form and agreed to participate (To CMS ID; 1815).

2.2 | Sample collection

Non-tumor tissues were sampled immediately before and after Hx. The collected samples were quickly frozen at −80°C until sample preparation was completed.

2.3 | Metabolome analysis

Frozen tissue (c.a. 40 mg) was added to methanol (500 µL) containing internal standards (20 µmol L⁻¹ each of methionine sulfone and D-camphor-10-sulfonic acid) and homogenized using a beads beater (TOMY Micro Smash MS-100R; Tomy Digital Biology, Tokyo, Japan) at 3000 rpm for 60 seconds. Then, both chloroform (500 µL) and Milli-Q water (200 µL) were added to the homogenate. The solution was thoroughly mixed, then centrifuged at 4600 g for 15 minutes at 4°C, and the aqueous fraction was centrifugally filtered through a 5-kDa-cut-off ultra-centrifugal filter unit (Ultrafree-MC-PLHCC-HMT; Human Metabolome Technologies Inc., Tsuruoka, Japan) to remove proteins. The filtrate was dried using an evacuated centrifuge and dissolved in Milli-Q water (50 µL) containing 200 µmol L⁻¹ reference compounds (3-aminopyrrolidine and trimesic acid) prior to CE-MS analysis. CE-MS-based metabolomic profiling and data analysis were carried out essentially as described.9–13

2.4 | Definitions of clinical parameters

Preoperative, postoperative and changes between pre- and postoperative valine and tryptophan were compared in terms of the following clinical parameters (resected liver volume, regeneration rate, ischemic time, FIB-4 index, and sarcopenia).

1. Resected liver volume: subsegmentectomy was grouped for the minor Hx group, and segmentectomy and lobectomy were grouped for the major Hx group.
2. Regeneration rate: liver volumes before Hx and 1 week after Hx were measured using a 3D simulation imaging system. Regeneration rate was defined as the volume increase of the remnant liver as compared with the preoperative volume. Calculations were made using the following equation: regeneration rate = [(postoperative liver volume) − (preoperative liver volume)]/[preoperative liver volume] × 100 (%). The regeneration rate was divided into low and high regeneration groups by median value.
3. Ischemic time: the cut-off value of Pringle time was set at 30 minutes.
4. FIB-4 index: the cut-off index value was set at 1.50.
5. Sarcopenia: this was defined as both low grip strength and low muscular mass.

Low grip strength was defined as <26 kg (male) or <18 kg (female).14 Muscular mass was examined with InBody 770® (Kotoku, Tokyo, Japan). Low muscular mass was defined as <90% of the standard (ranges from 90% to 110% of the standard) obtained by the InBody 770®.15

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**TABLE 1. Backgrounds of 23 patients who underwent Hx between April 2014 and March 2015**

| Gender              | 20/3 |
|---------------------|------|
| Age y (range)       | 65 (36-81) |
| Hepatitis virus     | HBV/HCV/nBnC 7/7/9 |
| Primary disease     | HCC/CCC/CRLM/Others 19/1/1/2 |
| Operative procedures| HrS/Hr1/Hr2 7/4/12 |
| Resected liver volume (%/total liver volume)| HrS/Hr1/Hr2 9 (6-12)/23 (20-28)/52 (44-61) |
| Operative time min (range) | 314 (223-452) |
| Ischemic time min (range) | 42 (25-81) |
| Blood loss ml (range) | 175 (40-644) |

CCC, cholangiocellular carcinoma; CRLM, colorectal liver metastases; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HrS, hepatic subsegmentectomy; Hr1, hepatic segmentectomy; Hr2, hepatic lobectomy; Hx, hepatectomy; nBnC, non B non C hepatitis.
2.5 Statistical analysis

All data are expressed as median (range). Statistical analysis was carried out using Prism 6.07 for Windows (GraphPad Software Inc., La Jolla, CA, USA). P-values <.05 were considered to indicate statistically significant differences. Values between before and after Hx were compared using the Wilcoxon matched-pairs signed-rank test. Heat maps of metabolite levels were generated using hierarchical clustering based on Pearson correlation coefficients using the MultiExperiment Viewer (MeV) software (Institute for Genomic Research, Rockville, MD, USA). The data were exported and analyzed by principal components analysis (PCA) using SIMCA-P software 12.0.1 (Umetrics AB, Umeå, Sweden) to visualize the metabolic changes between preoperative and postoperative patients after mean centering and unit variance scaling.

3 RESULTS

3.1 Metabolic map

In the pentose phosphate pathway, there were no significant differences between before and after Hx (Figure 1A). The metabolic pathway showed that there was a significant increase in “lactate” after Hx. There was only a significant decrease in metabolites in the first half of the TCA cycle, and adenosine triphosphate (ATP) by anaerobic glycolysis did not occur in time for energy consumption of the Hx (Figure 1B). This suggested that lipid metabolism might be more dominant than glucose metabolism after Hx.

3.2 Principal components analysis

Principal components analysis showed remarkably different component profiles between before and after Hx (Figure 2A). Out of a total of 267 metabolites, 103 metabolites that had a variable importance for projection (VIP) score of more than 1.0 were selected as critical metabolites for separating components (Figure 2B). Table 2 shows all metabolites (VIP score >1.0) and Figure 2C shows hierarchical clustering of 103 metabolites. Valine (VIP score: 1.78957) and tryptophan (VIP score: 1.74943) were significantly up-regulated after Hx. However, there was a limitation of this present study, liver tissues were sampled immediately after Hx, and it was not a comprehensive analysis. Conversely, metabolome analysis is concerned with the comprehensive analysis of endogenous low-molecular-weight compounds in biological samples.

Valine, which is one of the branched-chain amino acids (BCAA), has been reported to stimulate the proliferation of hepatocytes by a dose dependent method in vitro.22 Valine was also found to be most effective in vivo among three BCAA in the Hx model. Furthermore, valine was reported to increase serum free fatty acid, or liver triglyceride, and up-regulated liver fatty acid became a source of ATP production.22 Valine, was also reported to have an antioxidative effect by down-regulating tumor necrosis factor (TNF) and up-regulating superoxide dismutase 2 (SOD2) towards human umbilical vein endothelial cells (HUVEC).23

In contrast, tryptophan was the major source of serotonin production, and platelets were major carriers of serotonin in the blood. In thrombocytopenic mice, a serotonin agonist reconstituted liver proliferation after Hx.24 Tryptophan might stimulate liver regeneration after Hx by serotonin. Furthermore, tryptophan regulated reactive oxygen species (ROS) by inducing nuclear factor (erythroid-derived 2)-like 2 (NF-E2-related factor 2 or Nrf2) in primary hepatic culture.25 In our hierarchical clustering, valine and tryptophan significantly increased after Hx, and they were regulated by resected liver volume, ischemic time and liver function. Although further investigations are necessary, these phenomena might reflect the remnant liver’s protective response for I/R injury.

It was already reported that the period immediately after Hx (0-6 hours after Hx) was critical for future liver regeneration.5,6 In the present study, liver tissues were sampled immediately after Hx, and we hypothesized that metabolites immediately after Hx would affect future liver regeneration. However, there was a limitation of this study regarding the time-point after Hx, and several time-points after Hx might be necessary.

In conclusion, the present study identified the changes of liver metabolites in Hx with I/R towards liver regeneration. Liver metabolism after Hx; (ii) liver metabolites changed remarkably between before and after Hx; and (iii) liver valine and tryptophan were remarkably increased after Hx and they were regulated by resected liver volume, ischemic time and liver function.
FIGURE 1  Metabolic map during liver regeneration. A, Pentose phosphate pathway; there were no significant differences between before and after hepatectomy (Hx). B, Glycolysis/tricarboxylic acid cycle (TCA) cycle; there was significant decrease only in metabolites in the first half of the TCA cycle, and ATP by anaerobic glycolysis did not occur in time for energy consumption of Hx. N.D., not detected.
FIGURE 2. (A) Principal components analysis (PCA); PCA shows remarkably different components between before and after hepatectomy (Hx). (B) Key 103 metabolites (VIP >1.0): 103 metabolites in a total of 267 metabolites that had a VIP score of more than 1.0 were selected as critical metabolites for separating components. C, Hierarchical clustering. VIP, variable importance for projection.
| Metabolites                      | VIP score | Metabolites                   | VIP score |
|---------------------------------|-----------|-------------------------------|-----------|
| Lactate                         | 1.97604   | UTP                           | 1.4773    |
| CDP                             | 1.96398   | 1-Methyladenosine             | 1.46181   |
| NADPH                           | 1.94671   | O-Phosphoserine               | 1.45222   |
| Tyr                             | 1.91601   | 1-Methylnicotinamide          | 1.44062   |
| UDP-glucose                     | 1.86349   | Met                           | 1.4312    |
| Ala                             | 1.85245   | 2-Hydroxybutyrate             | 1.41113   |
| ADP                             | 1.8519    | cis-Aconitate                 | 1.39913   |
| CTP                             | 1.82183   | N-Acetylg glucosamine         | 1.37152   |
| Leu                             | 1.8197    | Inosine                       | 1.36748   |
| Choline                         | 1.81496   | Carnitine                     | 1.36734   |
| Succinate                       | 1.80697   | Gluconate                     | 1.35793   |
| ATP                             | 1.80538   | 2,3-DPG                       | 1.34099   |
| beta-Ala                        | 1.80077   | Glutathione(ox)               | 1.33471   |
| Urate                           | 1.79285   | 2PG                           | 1.33419   |
| Val                             | 1.78957   | g-Glu-Arg                     | 1.3306    |
| Homovanillate                   | 1.78688   | Uridine                       | 1.29395   |
| gamma-Glu-cys                   | 1.78348   | g-Glu-Ala                     | 1.29336   |
| Citrate                         | 1.78155   | 5-Hydroxylysine               | 1.29306   |
| Ile                             | 1.77265   | g-Glu-Gly-Gly                 | 1.28145   |
| Pro                             | 1.76593   | 2-Hydroxyglutarate            | 1.25326   |
| Phe                             | 1.76262   | ADP-glucose                   | 1.24392   |
| Xanthine                        | 1.75003   | IMP                           | 1.2395    |
| Trp                             | 1.74943   | F1.6P                         | 1.2363    |
| Gly                             | 1.74604   | Creatinine                    | 1.21506   |
| Adenosine 3′,5′-diphosphate     | 1.7337    | CMP-N-acetylneuraminate       | 1.21174   |
| Kynurenine                      | 1.71002   | g-Glu-Trp                     | 1.20549   |
| UDP-glucuronate                 | 1.69116   | Sarcosine                     | 1.2001    |
| CMP                             | 1.67643   | Fumarate                      | 1.19877   |
| Uracil                          | 1.66857   | 4-Methyl-2-oxopentanoate      | 1.19797   |
| o-Acetyl carnitine              | 1.66437   | Ru5P                          | 1.17545   |
| Thiamine                        | 1.66161   | Mucate                        | 1.16441   |
| Glycerophosphate                | 1.6597    | N-Acetylhistidine             | 1.16288   |
| N-Acetylglucosamine 6-phosphate | 1.63273   | g-Glu-Phe                     | 1.1496    |
| Isocitrate                      | 1.62491   | g-Glu-Leu                     | 1.13668   |
| Glucose                         | 1.62172   | g-Glu-Val                     | 1.11575   |
| 4-Oxopentanoate                 | 1.60649   | Gly-Leu                       | 1.11075   |
| Hypoxanthine                    | 1.6039    | N,N-Dimethylglycine           | 1.10164   |
| Nicotinamide                    | 1.59889   | g-Glu-His                     | 1.09598   |
| Xanthosine                      | 1.59198   | Cysteine-glutathione disulfide - divalent | 1.09562 |
| N-Acetylglucosamine 1-phosphate | 1.58238   | 4-Pyrrolo[1,2-c]pyrimidine    | 1.08533   |
| Lys                             | 1.55611   | N-Acetylmethionine            | 1.0849    |
| GABA                            | 1.5491    | Putrescine (1,4-butanediamine) | 1.06563 |
| cAMP                            | 1.54597   | Pantothenate                  | 1.07741   |
| threo-beta-methylaspartate + Glu| 1.53009   |                               |           |

(Continues)
metabolites changed remarkably between before and after Hx. In particular, liver valine and tryptophan were increased after Hx.

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**DISCLOSURE**

Conflicts of Interest: Authors declare no conflicts of interest for this article.

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