発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., Oda, Y., Kakazu, Y., Kusano, M., Tohge, T., Matsuda, F., Sawada, Y., Horai, Y. M., Nakanishi, H., Ikeda, K., Akimoto, N., Maoka, T., Takahashi, H., Ara, T., Sakurai, N., Suzuki, H., Shibata, D., Neumann, S., Iida, T., Tanaka, K., Funatsu, K., Matsuura, F., **Soga, T.**, Taguchi, R., Saito, K., Nishioka, T.	"MassBank: A Public Repository for Sharing Mass Spectral Data for Life Sciences"	J. Mass Spectro.	45	703-714	2010
曽我朋義	CE-MS法によるメタボローム解析と医薬への応用	遺伝子医学MOOK	19号	66-70	2011
曽我朋義	CE-MSメタボロミクスの最新動向	ぶんせき	Vol461, No. 11,	585-590,	2010

Serum Metabolomics Reveals γ-Glutamyl Dipeptides as Biomarkers for Discrimination among Different Forms of Liver Disease

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This manuscript includes: 5881 words, 2 tables and 5 figures.

Abbreviations: HCC, hepatocellular carcinoma; AST, aspartate transaminase; ALT, alanine transaminase; γ-GTP, γ-glutamyl transpeptidase; CT, computed tomography; NAFLD, nonalcoholic fatty liver disease; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; CE-TOFMS, capillary electrophoresis time-of-flight mass spectrometry; GSH, reduced glutathione; GC, gastric cancer; GCS, γ-glutamylcysteine synthetase; C, healthy control; DI, drug-induced liver injury; AHB, asymptomatic hepatitis B virus infection; CHB, chronic hepatitis B; CNALT, hepatitis C with persistently normal alanine transaminase; CHC, chronic hepatitis C; CIR, cirrhosis type C; HBs, hepatitis B surface; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonist; LC-MS/MS, liquid chromatography electrospray tandem mass spectrometry; MLR, multiple logistic regression; APAP, acetaminophen; GS, glutathione synthetase; BSO, buthionine sulfoximine; DEM, diethylmaleate; ROS, reactive oxygen species.

Financial disclosure: This work was supported by Health and Labour Sciences Research Grants "Research on Biological Markers for New Drug Development" (T.S.) and "Research on Risk of Chemical Substances" (T.S.). Additional support was obtained through grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for a Global COE Program entitled "Human Metabolomic Systems Biology" in Life Sciences (T.S., M.T. and M.S.) and the ERATO Gas Biology Project (M.S.), as well as research funds from the Yamagata Prefectural Government and City of Tsuruoka.

ABSTRACT

Background & Aims: To discover noninvasive and reliable biomarkers for rapid screening diagnosis of liver diseases, a metabolome profiling approach was applied to serum samples obtained from patients with different liver diseases.

Methods: Using capillary electrophoresis and liquid chromatography mass spectrometry, we analyzed the low molecular weight metabolites in a total of 248 serum samples obtained from patients with nine types of liver disease and healthy controls.

Results: We found that γ-glutamyl dipeptides, which were biosynthesized through a reaction with γ-glutamylcysteine synthetase, were indicative of the production of reduced glutathione and that measurement of their levels could distinguish among different liver diseases. Multiple logistic regression models facilitated the discrimination between specific and other liver diseases and yielded high areas under receiver-operating characteristic curves. The area under the curve values in training and independent validation data were 0.952 and 0.967 in healthy controls, 0.817 and 0.849 in drug-induced liver injury, 0.754 and 0.763 in asymptomatic hepatitis B virus infection, 0.820 and 0.762 in chronic hepatitis B, 0.972 and 0.895 in hepatitis C with persistently normal alanine transaminase, 0.917 and 0.707 in chronic hepatitis C, 0.803 and 0.993 in cirrhosis type C, and 0.762 and 0.803 in hepatocellular carcinoma, respectively. Several γ-glutamyl dipeptides also manifested potential for differentiating between nonalcoholic steatohepatitis and simple steatosis.

Conclusions: γ-Glutamyl dipeptides are novel biomarkers for liver diseases, and varying levels of individual or groups of these peptides have the power to discriminate among different forms of hepatic disease.

Keywords: γ-glutamyl dipeptides, metabolomics, biomarker, capillary electrophoresis mass spectrometry, oxidative stress, glutathione, hepatocellular carcinoma, nonalcoholic steatohepatitis, hepatitis C virus

Introduction

Acute or chronic viral hepatitis affects populations around the world, and the disease often progresses from chronic hepatitis and cirrhosis to hepatocellular carcinoma (HCC) [1]. Accurate diagnosis at earlier stages is necessary for improved therapeutic outcomes. However, the diagnostic procedures are laborious and not risk-free. Patients with suspected liver damage are initially subjected to liver function tests that include assessment of the serum levels of aspartate transaminase (AST), alanine transaminase (ALT) and γ-glutamyl transpeptidase (γ-GTP). If these levels are abnormal, the patients are subjected to diagnostic imaging such as ultrasound and computed tomography (CT) and assays of hepatitis virus antibodies. To evaluate the severity of inflammation or fibrosis and to confirm the indications for antiviral therapy, a liver biopsy may be recommended.

Recently, nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in western countries, and it encompasses a wide spectrum of conditions associated with over-accumulation of fat in the liver ranging from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH) and cirrhosis [2]. Although SS typically follows a benign nonprogressive clinical course, NASH may eventually develop into cirrhosis and HCC . To date, a liver biopsy remains the gold standard for the diagnosis of NASH [3]. However, since the biopsy procedures carry the risk of mortality [4-5], noninvasive identification of biomarkers that can provide reliable differential diagnoses for the characterization of liver diseases is desirable.

Metabolomics, which can be defined as measurement of the levels of all cellular metabolites, has emerged as a powerful new tool for discovering new low molecular weight biomarkers and its utility has been demonstrated by the identification of new biomarkers for prostate cancer [6], Parkinson's disease [7], type 2 diabetes mellitus [8], acute myocardial ischemia [9] and preeclampsia [10].

Recently, we developed new metabolomic profiling approaches based on capillary electrophoresis mass spectrometry [11] and capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) [12-14]. The

efficacy of CE-TOFMS was demonstrated by the discovery of ophthalmate (γ-glutamyl-2-aminobutyrylglycine) as a biomarker; in mice, reduced glutathione (GSH) depletion produced acetaminophen-induced hepatotoxicity [12, 14]. In this study, to discover new noninvasive biomarkers for human liver diseases, we comprehensively analyzed the serum metabolites in a total of 248 samples from patients with nine types of liver disease or gastric cancer (GC) and from normal individuals using our metabolomic approaches, and found increased levels of γ-glutamyl dipeptides in most of the liver diseases. We further found that γ-glutamyl dipeptides were synthesized via the ligation of glutamate by various amino acids and amines with γ-glutamylcysteine synthetase (GCS), an enzyme that is feedback-inhibited by GSH, and that the levels of γ-glutamyl dipeptides were indicative of the amount of GSH production. The concentrations of serum γ-glutamyl dipeptides varied with the types of liver disease and disease stage and can therefore act as new biomarkers for liver diseases. Here, we report that a highly specific set of γ-glutamyl dipeptides alone or in combination with transaminases and methionine sulfoxide can effectively distinguish specific liver diseases from other hepatic injuries and healthy control samples.

Materials and Methods

Serum samples. A total of 248 serum samples were obtained from three institutes, Yamagata University Hospital (YUH; Yamagata, Japan), University of Tokyo Hospital (UTH; Tokyo, Japan) and Shonai Hospital (SH; Tsuruoka, Japan). The 162 YUH cases comprised 53 healthy controls (C) and patients with drug-induced liver injury (DI; n=10), asymptomatic hepatitis B virus infection (AHB; n=9), chronic hepatitis B (CHB; n=7), hepatitis C with persistently normal alanine transaminase (CNALT; n=10), chronic hepatitis C (CHC; n=24), cirrhosis type C (CIR; n=10), HCC (n=19), SS (n=9) and NASH (n=11). The 75 UTH cases comprised 4 controls and patients with DI (n=17), AHB (n=7), CHB (n=7), CNALT (n=8), CHC (n=11), CIR (n=8) and HCC (n=13). The 11 SH cases were all GC patients. Written informed consent was obtained from all the participants and the

study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review boards of YUH, UTH and SH. The study subjects were patients with viral liver diseases, drug-induced hepatotoxicity or NAFLD who were referred to the Department of Gastroenterology and Hepatology at YUH, UTH or SH.

Clinical diagnosis. All the healthy controls were confirmed to have normal liver function and no viral hepatitis infection, and none were alcoholics. The AHB and CNALT patients were confirmed to have normal liver function and to be positive for hepatitis B surface (HBs) antigen and hepatitis B virus (HBV) DNA or for anti-hepatitis C virus (HCV) antibodies and HCV RNA, respectively. DI was diagnosed based on abnormal values on biochemical tests, absence of other hepatic diseases and a history of treatment with drugs suspected of being probable causes of DI. The suspected medications were different, and the biochemical test results in each patient normalized after their withdrawal.

CHC and CIR were diagnosed on the basis of physical examination, biochemical tests, and ultrasonography and CT findings. Some patients with chronic hepatitis provided informed consent for a liver biopsy, and the procedure was performed to confirm the accuracy of the diagnosis. The diagnosis of CHB and CHC was based on increased ALT levels (above the upper limit of the normal range) in at least two blood samples assayed over a 6-month period and the presence of detectable HBs antigen and HBV DNA or detectable anti-HCV antibodies and HCV RNA, respectively. HCV infection was causative in all cirrhosis patients, and they manifested symptoms of portal hypertension such as splenomegaly, esophageal varices, encephalopathy or ascites.

The diagnosis of HCC was based on ultrasonography, CT and MRI findings that revealed features typical of HCC. HCV was causative in all case, and the α-fetoprotein (AFP) and protein induced by vitamin K antagonist

(PIVKA)-II levels were assayed in all HCC patients.

All of the SS and NASH patients underwent a liver biopsy. The tissue samples were stained with hematoxylin-eosin, reticulin and Masson trichrome, and examined by the same experienced pathologist who was blinded to the clinical data. The histological criterion for the diagnosis of NAFLD was the presence of fatty changes in hepatocytes. When hepatocytes exhibited macrovesicular steatosis, the differential diagnosis was SS or NASH. The criteria for a diagnosis of steatohepatitis were the presence of lobular inflammation and either ballooning cells or perisinusoidal/pericellular fibrosis, in addition to steatosis in the liver specimen. No patients with autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease or alcoholic liver injury were included. All patients with GC were diagnosed by pathologic studies of biopsy tissues.

Analytical and statistical technologies for biomarker discovery. Using a total of 237 samples from YUH (training cohort, n=162) and UTH (validation cohort, n=75) (**Table 1**), we performed CE-TOFMS for a comprehensive analysis of the metabolite changes to discover new biomarkers in the diagnosis of human liver diseases. To facilitate peak identification and quantification, we analyzed 162 metabolic standards listed in the KEGG LIGAND database [15] before analyzing the samples. Global mass scanning over a 50–1,000 *m/z* range was applied in the CE-TOFMS mode [12]. To focus on γ-glutamyl peptides, we employed a highly sensitive method using liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring for analyses of the patient serum samples. The Kruskal–Wallis test and Dunn's post test were used to assess the statistical significance of differences among C, DI, AHB, CHB, CNALT, CHC, CIR and HCC. The Mann–Whitney test was used to evaluate the statistical significance of differences between SS and NASH. The algorithm of the feature selection for the multiple logistic regression (MLR) models is described in the

Supplementary Information.

Results

Discovery of γ-glutamyl dipeptides in serum by metabolomic profiling. The CE-TOFMS analyses quantified the levels of 49 metabolites in the serum samples (**Supplementary Tables 1 and 2**) and revealed increases in many compounds in most liver diseases. We identified these compounds as γ-glutamyl dipeptides (e.g., γ-Glu-Gly, γ-Glu-Ala, γ-Glu-Ser, γ-Glu-Val, γ-Glu-Thr, γ-Glu-Taurine, γ-Glu-Leu, γ-Glu-Gln, γ-Glu-Lys, γ-Glu-Glu, γ-Glu-Met, γ-Glu-His, γ-Glu-Phe, γ-Glu-Arg, γ-Glu-Citrulline, γ-Glu-Tyr and γ-Glu-Trp) by comparing their migration times and exact molecular weights with those of the standards. Significant differences were observed among controls and liver diseases (p<0.0001; Kruskal–Wallis test) except for γ-Glu-Met in the validation data **(Supplementary Tables 1 and 2)**. Correlational cluster analyses of 67 compounds showed that all the γ-glutamyl dipeptides except for γ-Glu-Tyr and γ-Glu-Trp were clustered with AST, ALT and metabolites involved in oxidative stress responses, namely glucosamine [16] and methionine sulfoxide [17-19] (**Fig. 1**).

Statistical analysis and validation for biomarker discovery. From the serum samples obtained at YUH, we selected 89 liver disease patients including DI, AHB, CHB, CNALT, CHC, CIR and HCC patients, and 53 healthy controls with no significant differences in the age distribution between the training and validation cohorts (**Table 1**). As shown in the whisker box plots for the training cohort (**Fig. 2**), the levels of γ-glutamyl dipeptides and of AST and ALT, as commonly used hepatocyte biomarkers, were increased in different patterns in comparison with C. For example, the AST and ALT levels were significantly increased in patients with DI, CHB, CHC, CIR and HCC (p<0.05; Dunn's post test), but not in those with AHB and CNALT (**Fig. 2**). On the other hand, significant increases were observed in the levels of γ-Glu-Ser, γ-Glu-Val, γ-Glu-Thr, γ-Glu-Leu and γ-Glu-

Phe (p<0.05; Dunn's post test) in AHB and in the levels of all the γ-glutamyl derivatives of amino acids (p<0.05; Dunn's post test) except for ophthalmate, γ-Glu-Thr and γ-Glu-Trp in CNALT (**Fig. 2 and Supplementary Table 1**). Oxidative metabolites, methionine sulfoxide and glucosamine were significantly increased in all diseases (p<0.05; Dunn's post test) and in CHB, CNALT and CHC (p<0.0001; Dunn's post test), respectively (**Fig. 2**).

To assess their abilities to discriminate specific liver diseases from other liver diseases, we developed MLR models using combinations of several components of the γ-glutamyl dipeptides, transaminases and oxidative metabolites using the training dataset. For example, an MLR model incorporating four selected biomarkers (γ-Glu-Ala, γ-Glu-Citrulline, γ-Glu-Thr and γ-Glu-Phe) was able to differentiate HCC from the other groups (C, DI, AHB, CHB, CNALT, CHC and CIR) with an area under the receiver-operating characteristic (ROC) curve (AUC) value of 0.762 (95% CI 0.647–0.877, p=0.00025). The probability (p) of HCC is calculated by $\log(p/(1-p)) = -1.87 - 1.13 \times$ γ-Glu-Ala $+ 3.51 \times$ γ-Glu-Citrulline $- 1.65 \times$ γ-Glu-Thr $+ 6.99 \times$ γ-Glu-Phe (**Table 2**). When the concentration of γ-Glu-Ala, γ-Glu-Citrulline, γ-Glu-Thr, and γ-Glu-Phe are 1.7, 0.84, 0.54, and 0.34 μM, respectively, the probability of HCC is 65.5%. All the MLR models achieved high AUC values at statistically significant levels (between 0.754 and 0.972, p<0.011) (**Fig. 3, Table 2 and Supplementary Table 3**).

The developed MLR models were evaluated in a blinded manner using an independent cohort (YUH) consisting of 75 individuals who were not members of the training cohort (**Supplementary Table 2**). We found that all of the MLR models also produced high AUC values at statistically significant levels (between 0.707 and 0.993, p<0.023) (**Fig. 3, Table 2 and Supplementary Table 3**). Although C, CHB and CHC were each differentiated from the other groups by a single γ-glutamyl dipeptide (γ-Glu-Phe, γ-Glu-Thr and γ-Glu-Lys, respectively), the MLR models for the other diseases required multiple biomarkers to achieve accurate

discrimination (**Table 2**). The odds ratios of ALT, AST and methionine sulfoxide were close to 1.0 compared with the odds ratios of the γ-glutamyl dipeptides, indicating their relatively lower contributions to the separation ability of the MLR models (**Table 2**). Overall, for all types of liver diseases, the MLR models mostly based on γ-glutamyl dipeptides provided complementary results, even in the second (validation) cohort.

γ-Glutamyl dipeptides as biomarkers for HCC and NAFLD. To evaluate the diagnostic potential of γ-glutamyl dipeptides for HCC, we compared their diagnostic abilities with that of AFP, an established marker for HCC (**Fig. 4**). We found that the MLR models using four γ-glutamyl dipeptides (γ-Glu-Ala, γ-Glu-Citrulline, γ-Glu-Thr, γ-Glu-Phe) (**Table 2**) were better at distinguishing HCC from CHC and CIR (AUC=0.881) than AFP (AUC=0.760) (**Fig. 4**).

We further investigated the biomarker specificities by comparing the serum γ-glutamyl dipeptide levels in GC and HCC patients (**Supplementary Fig. 2 and Supplementary Table 4**). The analyses revealed significant differences, with the exception of γ-Glu-Phe, and the levels of γ-glutamyl dipeptides was notably low in GC.

Differences in the levels of γ-glutamyl dipeptides were also observed in NAFLD. The levels of six γ-glutamyl dipeptides (γ-Glu-Val, γ-Glu-Thr, γ-Glu-Leu, γ-Glu-His, γ-Glu-Phe, and γ-Glu-Arg) were significantly higher ($p<0.05$; Mann-Whitney test) in SS than in NASH (**Supplementary Fig. 3 and Supplementary Table 5**). Although further investigations are necessary, these dipeptides can be used as noninvasive biomarkers in rapid screening for SS and NASH.

Mechanism of γ-glutamyl dipeptide biosynthesis. To confirm the γ-glutamyl dipeptide biosynthesis pathway, hepatic metabolism was investigated using a mouse model. In acetaminophen (APAP)-treated mice [12], ophthalmate, a γ-glutamyl tripeptide, was synthesized through consecutive reactions with GCS and glutathione

synthetase (GS), the same enzymes that play a role in GSH synthesis [12] (**Fig. 5**). Therefore, we investigated the alterations in the levels of hepatic amino acids, amines, γ-glutamyl dipeptides and tripeptides after administration of buthionine sulfoximine (BSO), diethylmaleate (DEM) or APAP (**Supplementary Fig. 4**). BSO treatment resulted in GCS inhibition [20] and marked reductions in most of the hepatic γ-glutamyl dipeptide and tripeptide levels (**Fig. 5 and Supplementary Fig. 4A**). In contrast, DEM treatment led to GSH depletion by oxidation of the thiol group in GSH [21], resulting in GCS activation and considerable increases in the hepatic γ-glutamyl dipeptide and tripeptide levels compared with the controls (**Fig. 5 and Supplementary Fig. 4A**). The hepatic levels of several γ-glutamyl dipeptides and tripeptides were increased with concurrent GSH depletion in APAP-treated mice (**Supplementary Fig. 4, B and C**). These results indicated that in mice, γ-glutamyl dipeptides and tripeptides were certainly synthesized via the ligation of glutamate by various amino acids through consecutive reactions with GCS and GS when GSH was depleted (**Fig. 5**). The identification details for the γ-glutamyl dipeptide biosynthetic pathway are described in the **Supplementary Information**.

Discussion

Our analyses of 237 serum samples from patients with liver diseases and healthy controls revealed that γ-glutamyl dipeptides were increased in liver injuries and could provide specific information for different liver diseases. In APAP-induced liver injury in mice, ophthalmate, a γ-glutamyl tripeptide, was markedly increased as a byproduct of GSH synthesis [21] (**Fig. 5 and Supplementary Fig. 4B**). However, in liver diseases in humans, many γ-glutamyl dipeptides were primarily synthesized and effluxed from hepatocytes into the blood (**Figs. 1 and 5**). Although the reason for the difference is unclear, it may be attributable to species differences in the levels and activities of enzymes and transporters [22-23].

In all types of liver disease, oxidative stress resulting from a disequilibrium between the production of

reactive oxygen species (ROS) and the ability of a biological system to detoxify reactive intermediates plays a crucial role in the induction and progression of liver damage independently of its etiology [1]. In patients with hepatitis, oxidative stress is produced by inflammation induced by immunological mechanisms. Upon viral infection, NADPH oxidase produces ROS in neutrophils and macrophages, and ROS are also generated from free iron through the Fenton reaction [24-26]. ROS are further produced in hepatocytes upon the release of inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β from inflammatory cells [27]. GSH is the most abundant antioxidant in hepatocytes, and helps to protect cells against ROS. Upon depletion of GSH, ROS induce oxidative stress resulting in liver damage, and reduced GSH levels have been demonstrated in various liver diseases [28-30].

Since γ-glutamyl dipeptides are byproducts of GSH synthesis catalyzed by GCS, their levels are indirect evidence for GSH production (**Fig. 5**). Different levels of γ-glutamyl dipeptides were observed in different types of liver disease and each γ-glutamyl dipeptide showed somewhat different variation pattern among liver diseases (**Fig. 2**). This might be attributed to differences in hepatic levels of amino acids (the substrate of GCS) among liver diseases, though further study is necessary to understand the details of this observation.

In the healthy controls, the γ-glutamyl dipeptide levels were low. This arose because under reducing conditions, the level of hepatic GSH was high and a small amounts of GSH was biosynthesized (**Fig. 5A**). However, in the patients with liver diseases, GSH was consumed to neutralize the generated ROS, which in turn led to GCS activation, resulting in the biosynthesis of GSH together with γ-glutamyl dipeptides (**Fig. 5B**). Therefore, increased levels of γ-glutamyl dipeptides were observed in most liver injuries. Surprisingly, unlike AST and ALT, the levels of most γ-glutamyl dipeptides were markedly increased in asymptomatic individuals with AHB and CNALT (**Fig. 2 and Supplementary Fig. 1**), possibly because viral infection induced ROS generation followed by GSH depletion, which led to the biosynthesis of GSH and γ-glutamyl dipeptides (**Fig. 5**).

We posit that sufficiently high levels of GSH production neutralized ROS, resulting in lower incidences of AHB and CNALT.

There are relationships between liver diseases attributable to HCV infection and oxidative stress parameters such as ROS, antioxidants and inflammation. Oxidative stress increased with hepatic disease progression in HCV-infected patients [31]. Consistent with that report, among all the patients with HCV-related liver diseases, the serum levels of γ-glutamyl dipeptides, as indicators of hepatic GSH production, were markedly increased in CNALT and tended to decrease with disease progression (CNALT ≥ CHC > CIR > HCC) (**Fig. 2**). These observations led us to conclude that at the time of viral infection (CNALT), a sufficient amount of GSH production can neutralize ROS and thus weaken the pathogenesis of liver damage. However, when GSH production falls below ROS generation, oxidative stress followed by inflammation is induced, resulting in the development and progression of liver diseases. Similarly, the levels of several γ-glutamyl dipeptides were significantly lower in NASH patients than in SS patients (**Supplementary Fig. 3**), indicating low levels of GSH production in NASH patients. Based on the present observations, we suggest that NASH is susceptible to oxidative stress and progression to liver fibrosis and cirrhosis.

HCC is one of the most common cancers in humans, and primarily develops in patients with chronic liver disease. Its early detection is important because effective treatments are available for the management of non-advanced cancers [32]. Until now, the diagnosis of HCC has relied on combinations of imaging techniques and measurements of the serum levels of AFP [33] and PIVKA-II [34]. Although they are reliable tumor markers for the diagnosis and monitoring of primary HCC, high levels of serum AFP and plasma PIVKA-II have also been observed in some gastric carcinomas [34-35]. However, the serum γ-glutamyl dipeptide levels in GC and HCC patients revealed significant differences, and the levels of several γ-glutamyl dipeptides was notably low in GC (**Supplementary Fig. 2**). We suspect that this arose through differences in the tissue activities of the glutathione

system, since GSH is mainly synthesized *de novo* in the liver, and posit that the γ-glutamyl dipeptide levels may reflect hepatic dysfunction.

Drug-induced hepatotoxicity is a frequent cause of liver injury, and the predominant clinical presentation is acute hepatitis and/or cholestasis. Overdoses of APAP, the most commonly used analgesic and antipyretic, can lead to possibly fatal hepatitis and several hundred deaths attributable to this drug occur annually in the United States. Our DI samples were from patients with so-called idiosyncratic hepatotoxicity, and the underlying mechanisms of this disease remain unclear. Interestingly, the changes in the serum levels of γ-glutamyl dipeptides were similar among the DI samples although the causative drugs differed widely and the mechanisms responsible for the development of hepatotoxicity may also be different. Our findings revealed that the amount of γ-glutamyl dipeptide production attributable to a reduction in the hepatocellular GSH concentration was a common feature in drug-induced idiosyncratic hepatotoxicity. With AUC values of 0.817 (training data) and 0.849 (validation data) (**Supplementary Table 3**), the serum levels of ALT and γ-Glu-Citrulline could be used to distinguish between DI patients on the one hand and patients with viral hepatitis infection and healthy controls on the other (**Table 2**). Therefore, we suggest that these compounds represent noninvasive biomarkers that facilitate rapid screening for DI.

In summary, our CE-TOFMS and LC-MS/MS metabolomics-based analyses of serum samples from patients with liver diseases showed quantitative differences in γ-glutamyl dipeptides in various liver diseases. Our highly specific set of γ-glutamyl dipeptides, transaminases and methionine sulfoxide enabled us to discriminate among liver diseases including DI, AHB, CHB, CNALT, CHC, CIR and HCC, indicating that they can be used as multiple biomarkers in rapid screening for different types and stages of liver disease. Furthermore, we have shown that γ-glutamyl dipeptide synthesis was catalyzed by GCS, the enzyme that is feedback-inhibited by GSH, and thus the levels of these biomarkers were indicative of hepatic GSH production. As observed in patients with

HCV-related liver diseases and NAFLD, the serum γ-glutamyl dipeptide levels tended to decrease during the course of liver disease progression, indicating an increase in oxidative stress resulting from decreased GSH production during liver disease progression. Therefore, γ-glutamyl dipeptide measurement can potentially provide valuable information about the hepatic reduction-oxidation state to gain insights into the role of oxidative stress in the pathogenesis and progression of liver diseases.

Acknowledgments

This work was supported by Health and Labour Sciences Research Grants "Research on Biological Markers for New Drug Development" (T.S.) and "Research on Risk of Chemical Substances" (T.S.). Additional support was obtained through grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for a Global COE Program entitled "Human Metabolomic Systems Biology" in Life Sciences (T.S., M.T. and M.S.) and the ERATO Gas Biology Project (M.S.), as well as from research funds from the Yamagata Prefectural Government and City of Tsuruoka.

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