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Dynamics of serum metabolites in patients with chronic hepatitis C receiving pegylated interferon plus ribavirin: A metabolomics analysis

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ABSTRACT

Objectives. Serum samples from patients with chronic hepatitis C were subjected to metabolomics analysis to clarify the pretreatment characteristics of their metabolites and also changes in specific metabolites resulting from antiviral therapy with pegylated interferon plus ribavirin (PegIFN/RBV).

Materials/Methods. The serum levels of low-molecular-weight metabolites in the twenty patients before and 24 weeks after completion of PegIFN/RBV therapy were analyzed using capillary electrophoresis and liquid chromatography–mass spectrometry.

Results. Ten patients showed a non-virological response (NVR) and 10 achieved a sustained virological response (SVR) with eradication of viremia. The pretreatment levels of tryptophan were significantly higher in the patients of SVR than in those of NVR ($p = 0.010$). The area under the curve (AUC) value of tryptophan calculated from the receiver operating characteristic (ROC) curve for discriminating SVR from NVR was 0.84 (95% confidential interval, 0.66–1.02, $p = 0.010$). The ROC curve of multiple logistic regression model incorporating the pretreatment levels of tryptophan and γ-glutamate-arginine showed that the AUC value was highly significant (AUC = 0.92, 95% confidential interval, 0.79–1.05, $p = 0.002$). Twenty four weeks after completion of treatment, the levels of γ-glutamyl dipeptides, glutamic acid, 5-oxoproline, glucosamine and methionine sulfoxide were decreased, whereas those of 5-methoxy-3-indoleacetate, glutamine, kynurenine and lysine were increased significantly ($p < 0.05$) in both the NVR and SVR patients.

Conclusions. The pretreatment serum levels of certain metabolites including tryptophan are associated with the response to PegIFN/RBV therapy. PegIFN/RBV therapy can ameliorate the oxidative stress responsible for glutathione metabolism.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PegIFN/RBV, pegylated interferon plus ribavirin; SVR, sustained virological response; NVR, non-virological response; ALT, alanine aminotransferase; ROS, reactive oxygen species; CE-TOFMS, capillary electrophoresis–time-of-flight mass spectrometry; IL, interleukin; MRM, multiple reaction monitoring; PC, principal component; ROC, receiver operating characteristic; MLR, multiple logistic regression; AUC, area under the curve; IDO, indoleamine 2,3-dioxygenase.

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1. Introduction

Chronic hepatitis C virus (HCV) infection causes progressive liver inflammation, which predisposes patients to possible liver cirrhosis and, finally hepatocellular carcinoma (HCC) [1,2]. Antiviral therapy for chronic HCV infection comprises interferon (IFN), pegylated interferon plus ribavirin (PegIFN/RBV), or a combination of PegIFN/RBV and a HCV-related protease inhibitor. Patients who achieve a sustained virological response (SVR) with successful eradication of HCV, or even those with a non-virological response (NVR) but showing improvement of liver inflammation or the serum alanine aminotransferase (ALT) level have a reduced risk of progression to HCC [3–6]. A meta-analysis of randomized controlled trials suggests that IFN therapy can efficiently reduce HCC development in patients with HCV-related cirrhosis [7].

The oxidative stress induced by reactive oxygen species (ROS) has a close association with the inflammatory process in hepatitis [8,9]. Healthy individuals have protective mechanisms against oxidative stress, i.e., induction of anti-oxidative substrates such as glutathione, thioredoxin, vitamin A and vitamin E, or enzymes for removing ROS such as superoxide dismutase, catalase and glutathione peroxidase. However, in patients of chronic hepatitis C, these protective mechanisms are impaired, and the resulting long-term exposure to oxidative stress during viral infection leads to progressive hepatitis accompanied by a risk of HCC [10–12].

Metabolome analysis has emerged as a powerful technique for detecting low-molecular-weight metabolites in cells. Metabolome profiling approaches based on capillary electrophoresis–time-of-flight mass spectrometry (CE-TOFMS) have led to the discovery of ophthalmate (γ-glutamyl-2-aminobutyrylglycine) as a biomarker of reduced glutathione depletion in mice with acetaminophen-induced hepatotoxicity [13,14]. Recently, the serum metabolites in a total of 248 samples from patients with nine types of liver disease were analyzed comprehensively using this approach, and increased levels of γ-glutamyl dipeptides in the majority were reported [15]. That study demonstrated that γ-glutamyl dipeptides are synthesized via ligation of glutamine with various amino acids and amines by γ-glutamylcysteine synthetase, which is under feedback inhibition by glutathione, and that the level of γ-glutamyl dipeptides represents the degree of glutathione production. Therefore, γ-glutamyl dipeptides are likely to be key metabolites reflecting the extent of liver tissue injury due to oxidative stress, suggesting that monitoring of their levels in serum may be useful for predicting the course of liver disease in patients with HCV infection. In addition, γ-glutamyl transferase (GGT) is the enzyme responsible for the extracellular catabolism of glutathione, and a recognized source of γ-glutamyl dipeptides. Thus GGT can be used as a marker to indicate the amelioration of oxidative stress [16].

The metabolism of human liver cells under conditions of HCV-related hepatitis has not been extensively investigated. In addition, the changes in serum metabolite levels in patients with chronic hepatitis C treated with PegIFN/RBV remain unknown. Such analysis would yield a considerable amount of useful information on the metabolism of these patients, and might lead to the discovery of new biomarkers of chronic hepatitis C that could be useful in clinical practice. In the present study, we used CE-TOFMS to analyze serum samples collected from patients with chronic HCV infection before and after PegIFN/RBV therapy.

2. Materials and methods

2.1. *Patients and details of PegIFN/RBV therapy*

Twenty patients who received PegIFN/RBV combination therapy for chronic hepatitis C were enrolled. These patients comprised 6 men and 14 women, with an age range of 38 to 70 years (52.8 ± 9.6 years, mean ± standard deviation). All of the patients had HCV genotype 1b infection with a high viral load exceeding 5 logIU/ml. Patients with alcoholic liver injury, autoimmune liver disease, and those positive for hepatitis B surface antigen were excluded. All patients were treated with a combination of PegIFN-alpha 2b (Pegintron; MSD K.K., Tokyo, Japan) and RBV (Rebetol; MSD K.K.) in accordance with the Japanese standard prescription information supplied by the Japanese Ministry of Health, Labour and Welfare. Briefly, PegIFN was administered subcutaneously once a week and RBV was given orally twice a day to achieve the total dose. The dosages of pegIFN and RBV were determined on the basis of body weight. Patients with body weights of 35–45, 46–60, 61–75, and 76–90 kg were given PegIFN at doses of 60, 80, 100, and 120 μg, respectively, and those with body weights of <60, 60–80, and >80 kg were given RBV at doses of 600, 800, and 1000 mg, respectively. Virological responses were evaluated at 24 weeks after completion of treatment, and the clinical outcome was classified as either an SVR with HCV eradication, or an NVR without HCV eradication. Patients were considered to have achieved an SVR if monitoring at four-week intervals confirmed negativity for HCV RNA for 24 weeks after completion of the therapy. Virological responses were assayed on the basis of serum HCV RNA using a real-time PCR assay kit (COBAS TaqMan HCV Auto, Roche Diagnostics). Single nucleotide polymorphism of interleukin (IL) 28B (rs8099917) was determined by direct sequencing of genomic DNA from patients, and classified into two types: the major homozygote (T/T: homozygosity for the major allele), and the minor heterozygote or homozygote (T/G or G/G: heterozygosity or homozygosity for the minor allele).

2.2. *CE-TOFMS technique for profiling of serum metabolites*

In all CE-TOFMS experiments, we used an Agilent CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), an Agilent G3250AA LC/MSD TOF system (Agilent Technologies, Palo Alto, CA), an Agilent 1100 series binary HPLC pump, a G1603A Agilent CE-MS adapter and a G1607A Agilent CE-ESI-MS sprayer kit. Data were acquired with the G2201AA Agilent ChemStation software for CE and Analyst QS in the Agilent TOFMS software.

The metabolites were separated in a fused silica capillary (50 μm i.d. × 100 cm) filled with 1 mol/L formic acid as the electrolyte [13]. A sample solution was injected at 50 mbar for

3 s (3 nL) and a voltage of 30 kV was applied. The capillary temperature and sample tray were set at 20 °C and below 5 °C, respectively. Methanol/water (50% v/v) containing 0.1 μmol/L hexakis(2,2-difluoroethoxy)phosphazene was delivered as the sheath liquid at 10 μl/min. ESI-TOFMS was performed in the positive ion mode, and the capillary voltage was set at 4 kV. The flow rate of heated dry nitrogen gas (heater temperature, 300 °C) was maintained at 10 psig. For TOFMS, the fragmenter, skimmer and Oct RFV voltages were set at 75, 50 and 125 V, respectively. Automatic recalibration of each acquired spectrum was achieved using the masses of reference standards ([^{13}C isotopic ion of a protonated methanol dimer (2MeOH + H)]$^+$, *m/z* 66.0632 and [hexakis(2,2-difluoroethoxy) phosphazene + H]$^+$, *m/z* 622.0290). Exact mass data were acquired at a rate of 1.5 spectra/s over a 50–1000 *m/z* range. To facilitate peak identification and quantification, we analyzed 162 commercially available metabolic standards before analyzing the samples. The raw data were processed using our proprietary software (MasterHands) [17].

2.3. *Liquid chromatography tandem mass spectrometry (LC–MS/MS) technique for analysis of serum γ-glutamyl peptide*

LC–MS/MS was carried out using an Agilent 1100 series HPLC system (Agilent Technologies) and an API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA). System control and data acquisition and analyses were performed with the Applied Biosystems Analyst QS software.

The targeted γ-glutamyl peptides were separated on a Develosil RPAQUEOUS-AR-3 column (2 mm i.d. × 100 mm, 3 μm; Nomura Chemical, Seto, Japan) that was maintained at 30 °C. The mobile phase consisted of 0.5% formic acid/water as solution A and acetonitrile as solution B. The gradient was increased from 0% B at 0 min to 1% at 5 min, 10% at 15 min and 99% at 17 min and then retained at 99% until 19 min. The flow rate was 0.2 ml/min and the injection volume was 1 μl. The MS conditions for positive ions were: mode, multiple reaction monitoring (MRM); ion spray voltage, 5.5 kV; nebulizer gas, 12 psi; curtain gas, 8 psi; collision gas, 8 U; nitrogen gas temperature, 550 °C. The MRM parameters, i.e. Q1 (protonated precursor ion), Q3 (production), declustering potential, focusing potential, collision energy and collision cell exit potential for γ-glutamyl peptides, were optimized using the Analyst software.

2.4. *Principal component analysis and heat map visualization*

Principal component analysis, which is a type of unsupervised statistical analysis used widely as a statistical tool in metabolomics studies, was applied prior to the detailed data analysis [18]. This facilitates visual inspection of the distributed samples in principal component (PC) space using score plots [19], and the distance between individual samples in score plots reflects the degree of systematic variation in metabolite profiles among samples. Principal component analysis converts high-dimensional data into fewer dimensions, by projecting the data into a reduced dimensional subspace, while maintaining as much variance from the original data as possible [20]. In this study, the procedure was repeated until the datasets were presented within three dimensions. We also visualized the observed metabolomic profile as a heat map representation, and performed hierarchical clustering analysis. The metabolite concentrations were averaged in each group, and the colors on the heat map were determined by subtracting the mean over four groups after log2 transformation. Euclidean distance was used for clustering metabolites.

Table 1 – Patient characteristics according to the virological response.

	Virological response		
	non-virological responder	sustained virological responder	*p* value
Number of patient	10	10	
Sex (Male/Female)	2/8	4/6	NS[a]
Age	55.3 ± 9.0	50.2 ± 9.9	NS[b]
Height	158.5 ± 9.1	160.5 ± 9.8	NS[b]
Weight	52.0 ± 11.8	58.7 ± 11.3	NS[b]
HCV genotype 1b	10	10	
HCV-RNA > 5 logIU/ml	10	10	
AST	51.9 ± 18.7	51.8 ± 27.5	NS[b]
ALT	64.3 ± 34.2	79.0 ± 52.9	NS[b]
GGT	66.3 ± 42.7	43.1 ± 36.6	NS[b]
Il28B SNP (Ma/Mi)	4/6	10/0	0.011[a]

IL28B SNP: Ma, major homozygote (T/T), Mi, heterozygote or minor homozygote (T/G or G/G).
Data were expressed as mean ± standard deviation. NS, not significant.
[a] Fisher exact test.
[b] Mann–Whitney *U*-test.

2.5. *Statistical analyses*

The Mann–Whitney *U*-test, Wilcoxon matched-pairs signed rank test, and Fisher exact test were used to assess the statistical significance of differences at a significance level of $p < 0.05$. Receiver operating characteristic (ROC) curve analysis was used for assessing the discrimination ability of individual metabolites. To assess the ability to discriminate SVR from NVR using multiple metabolites determined before treatment, we developed a multiple logistic regression (MLR) model. Metabolites for the MLR model were selected by the forward and backward feature selection method using a threshold of $p < 0.2$ for adding and one of $p > 0.2$ for elimination of metabolites. Bootstrap analysis was conducted to obtain unbiased estimates of the developed model. We used the bootstrapping technique to obtain relatively unbiased estimates; 200 repetitions were generated by random selection of individuals allowing redundancy. We used JMP version 9.0.2 (SAS Institute, Cary, NC) for principal component analysis and development of MLR, Weka version 3.6.4 (The University of Waikato, Hamilton, New Zealand) for bootstrap analysis, Mev TM4 software version 4.8.1 (Dana-Farber Cancer Institute, Boston, MA) for

heatmap visualization, and GraphPad Prism version 6.0.1 (Intuitive Software for Science, San Diego, CA) for ROC curve analysis and box-plot visualization.

3. Results

3.1. *Patient characteristics and virological responses*

The characteristics of patients who showed NVR and SVR are shown in Table 1. All of the patients had HCV genotype 1b infection with a high viral load exceeding 5 logIU/ml. Ten patients showed NVR and 10 achieved SVR. There were no significant differences in sex, age, height, weight, aspartate aminotransferase, ALT or GGT between the two patient groups. The only factor that differed significantly between the NVR and SVR patients was the prevalence of single nucleotide polymorphism of IL28B ($p = 0.011$); in the NVR group, a higher proportion of patients (6/10, 60%) carried the IL28B heterozygote or homozygote with minor alleles of rs8099917 than in the SVR group (0/10, 0%).

3.2. *Principal component analysis of patients before and after pegIFN/RBV therapy*

Principal component analysis demonstrated no significant differences in the pretreatment metabolomics profiles among patients with chronic hepatitis C examined before the start of PegIFN/RBV therapy, even between those who subsequently showed NVR and SVR (Fig. 1). When principal component (PC) analysis was applied to compare the changes in the metabolomics profiles before pegIFN/RBV therapy with those after the therapy, significant differences were evident, the first PC being significantly decreased and the third PC significantly increased in both the NVR and SVR patients (Fig. 2). The change in the distribution area of samples reflected these results, becoming narrower after treatment than before treatment, particularly in SVR cases (Fig. 3). This indicated a

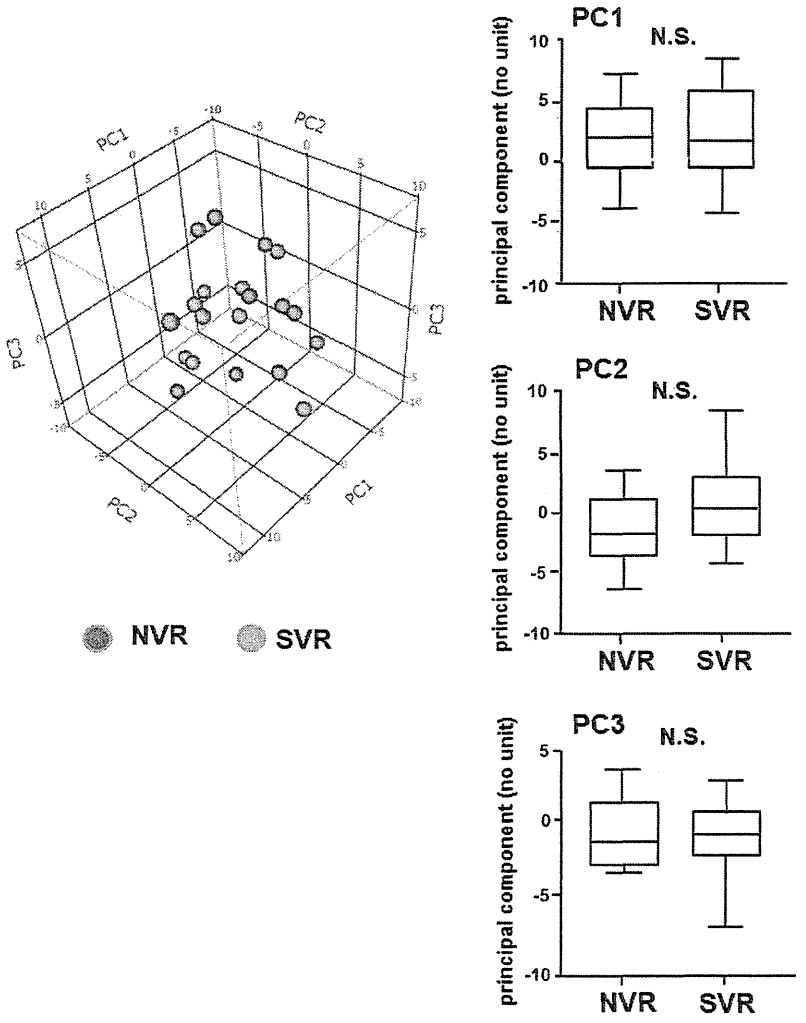

Fig. 1 – Principal component analysis of serum metabolites in the enrolled patients. No significant differences in the pretreatment metabolomics profiles were found between patients who subsequently showed a non-virological response (NVR) and those who achieved a sustained virological response (SVR). Mann–Whitney *U*-test.

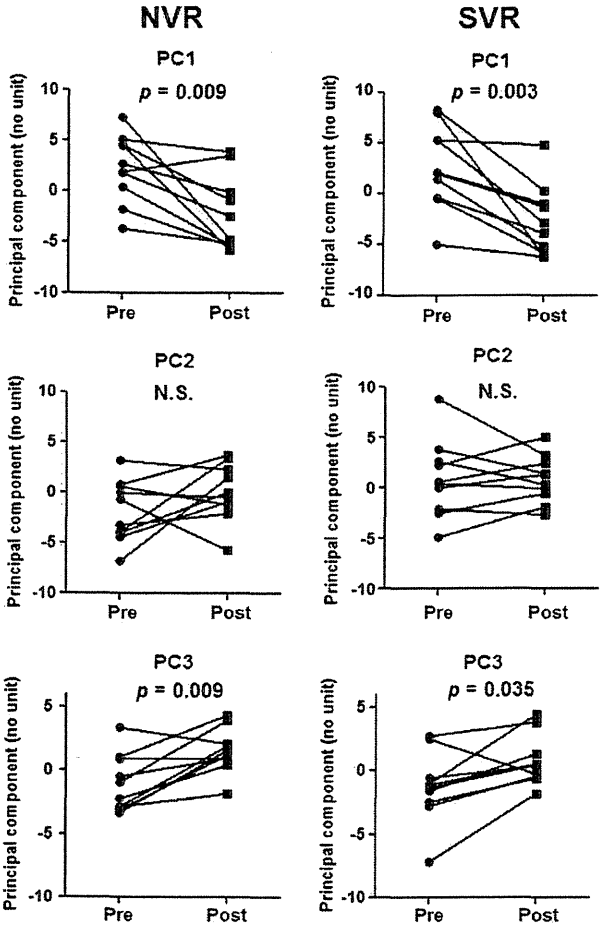

Fig. 2 – Changes in metabolomics profiles 24 weeks after pegIFN/RBV therapy compared with those before therapy. Pre and post indicate pretreatment and post-treatment, respectively. Principal component (PC) 1 was significantly decreased and PC 3 was significantly increased in both the NVR and SVR patients. Wilcoxon matched-pairs signed rank test.

loss of variation in cell metabolism among sustained virological responders, reflecting the fact that cell function had reached a state close to equilibrium as a result of successful HCV eradication.

3.3. *Comparison of metabolite levels in pretreatment serum between non-virological responders and sustained virological responders*

The pretreatment serum level of each metabolite in patients who received PegIFN/RBV therapy was compared between the NVR and SVR groups. We conducted ROC curve analysis to calculate the area under the ROC curve (AUC) values for all metabolites, and only four metabolites – tryptophan, glycine, γ-butyrobetaine and guanidoacetate – showed significant ($p < 0.05$) discrimination abilities (Supplementary Figure 1). Serum concentrations of all four of these metabolites were significantly higher in sustained virological responders than in non-virological responders ($p < 0.05$) (trypytophan: 39.7 ± 4.3 vs. 46.4 ± 6.4, p = 0.010; glycine: 168.7 ± 34.8 vs. 232.6 ± 77.0, p = 0.018; γ-butyrobetaine: 1.5 ± 0.2 vs. 1.8 ± 0.4, p = 0.049; guanidoacetate: 1.7 ± 0.5 vs. 2.1 ± 0.6, p = 0.049, NVR vs. SVR, mean ± standard deviation) (Fig. 4). The AUC values of the four metabolites for discriminating SVR from NVR were 0.84 (95% confidential interval (CI), 0.66–1.02, p = 0.010) for tryptophan, 0.78 (95% CI, 0.57–0.99, p = 0.034) for glycine, 0.76 (95% CI, 0.53–0.99, p = 0.049) for γ-butyrobetaine and 0.76 (95% CI, 0.55–0.98, p = 0.049) for guanidoacetate, all being significant (Fig. 5). We also evaluated the discrimination ability of combinations of multiple serum metabolites using MLR analysis. Among all metabolites, tryptophan and γ-glutamate-arginine were selected for the MLR model by stepwise feature selection methods. The ROC curve for the MLR model incorporating the pretreatment levels of these two metabolites for discriminating SVR from NVR showed a high and significant AUC value (AUC = 0.92, 95% CI, 0.79–1.05, p = 0.002) (Fig. 6). The mean AUC values obtained by bootstrap analysis remained high (AUC = 0.94, 95% CI, 0.93–0.95), indicating that the MLR model showed better accuracy for discriminating SVR from NVR in patients receiving PegIFN/RBV therapy for chronic hepatitis C.

3.4. *Comparison of serum levels of tryptophan between non-virological responders and sustained virological responders carrying an IL28B homozygote for major alleles of rs8099917*

The IL28B genotype is a strong host factor influencing the virological response of HCV to PegIFN/RBV therapy [21,22]. Patients carrying an IL28B homozygote for the major alleles of rs8099917 show a greater propensity to achieve SVR than those carrying an IL28B heterozygote or homozygote for its minor allele. To investigate whether the pretreatment serum level of tryptophan, which was the factor found to differ most significantly between NVR and SVR patients by analyses, was influenced by IL28B genotype, the pretreatment levels of tryptophan in patients with the IL28B homozygote for the major alleles of rs8099917 were compared between four of the 10 non-virological responders and all of the 10 sustained virological responders. The serum levels of tryptophan were significantly higher in the sustained virological responders harboring the major homozygote than in non-virological responders harboring the major homozygote (NVR vs. SVR, 37.8 ± 5.0 vs. 46.4 ± 6.4, p = 0.023) (Fig. 7). Thus the pretreatment level of tryptophan was not influenced by the IL28B genotype.

3.5. *Changes in levels of metabolites influencing the viral response after PegIFN/RBV therapy*

Changes in the serum levels of four metabolites – tryptophan, glycine, γ-butyrobetaine and guanidoacetate – whose pretreatment levels had been shown to differ significantly between the NVR and SVR groups, were examined at 24 weeks after completion of PegIFN/RBV therapy. The serum levels of all four metabolites after PegIFN/RBV therapy did not differ significantly from those before therapy in either NVR or SVR patients. Notably, there was little difference in the

serum level of tryptophan before and after the treatment (SVR: 46.4 ± 6.4 vs. 48.3 ± 12.0, p = 0.88, NVR: 39.7 ± 4.3 vs. 39.9 ± 8.1, p = 0.71, pretreatment vs. post-treatment). This suggests that the pretreatment levels of these metabolites, particularly tryptophan, may help to predict the therapeutic effect of this therapy.

3.6. *Dynamics of serum metabolite levels in patients with chronic hepatitis C before and after PegIFN/RBV therapy*

The dynamics of serum metabolite profile in patients with chronic hepatitis C before and after PegIFN/RBV therapy are shown as a heatmap in Fig. 8. Specific metabolites that showed changes in their serum levels as a result of PegIFN/RBV therapy were determined. The serum levels of four γ-glutamyl dipeptides (γ-Glu-His, γ-Glu-Lys, γ-Glu-Phe, γ-Glu-Val), glutamic acid, 5-oxoproline, glucosamine and methionine sulfoxide were decreased significantly ($p < 0.05$) at 24 weeks after completion of PegIFN/RBV therapy in both non-virological responders and sustained virological responders (Supplementary Figure 2). The serum levels of GGT were decreased significantly at 24 weeks after completion of PegIFN/RBV therapy in sustained virological responders (pretreatment vs. post-treatment: 43.1 ± 31.6 vs. 23.4 ± 8.0, p = 0.028). On the other hand, those of 5-methoxy-3-indoleacetate, glutamine, kynurenine and lysine were increased significantly ($p < 0.05$) in both groups at the same time point (Supplementary Figure 3).

4. Discussion

In this study, we demonstrated that PegIFN/RBV therapy for chronic hepatitis C altered the metabolism of cells in the liver of treated patients. These changes were confirmed by both principal component analysis of the overall metabolome and the dynamics of specific metabolites in serum associated with oxidative stress.

Principal component analysis of metabolites has been shown to be a powerful bioinformatics tool for providing an overall picture of cell metabolic status in individual patients [18–20]. Three-dimensional principle component analysis of metabolites demonstrated no differences in such profiling among the patients enrolled in this study. Twenty four weeks after completion of PegIFN/RBV therapy, principal component analysis demonstrated convergence of these metabolites in the score plots, suggesting that cell function became more homogeneous after completion of the therapy in comparison with the situation before therapy. In particular, the score plot area of the metabolites became narrower in patients who achieved successful HCV eradication. These data suggest that PegIFN/RBV therapy is able to modify the cell function of such patients, so that it approximates the normal function seen in healthy individuals.

The effect of PegIFN/RBV therapy on the viremia in patients with chronic hepatitis C is affected by various host factors. Therefore we considered that it would be informative to

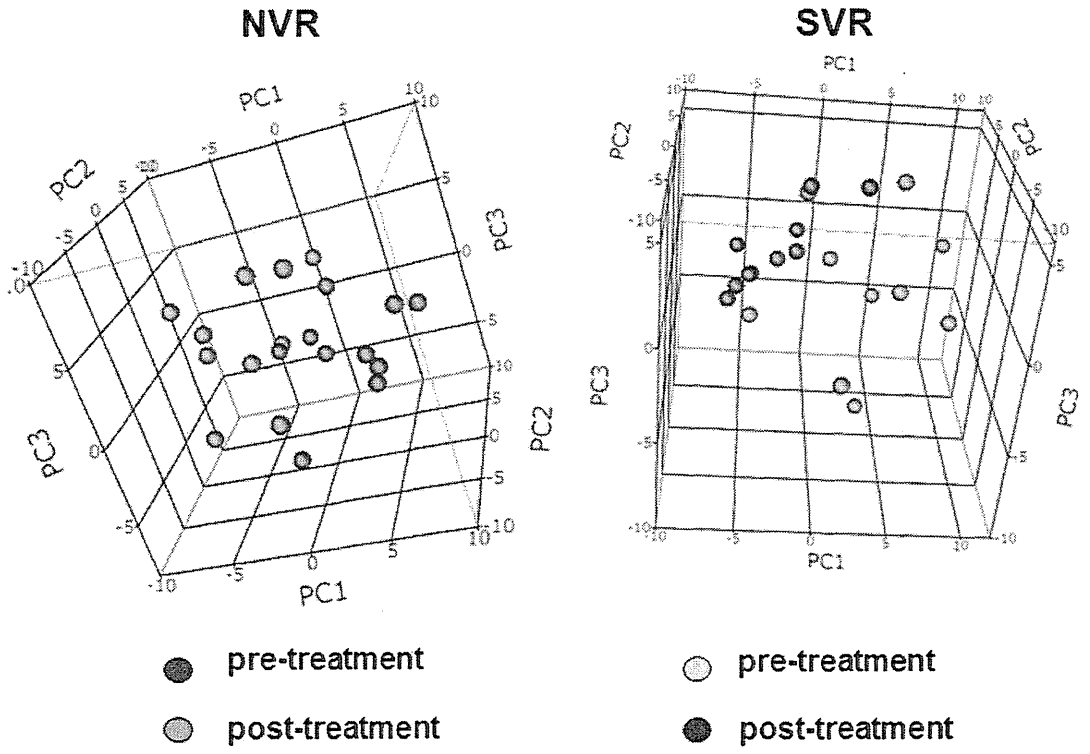

Fig. 3 – Principal component analysis of changes in serum metabolites 24 weeks after pegIFN/RBV therapy (post-treatment) compared with those before therapy (pretreatment). The distribution area of samples became narrower after therapy relative to that before therapy, and this change was particularly obvious in SVR cases.

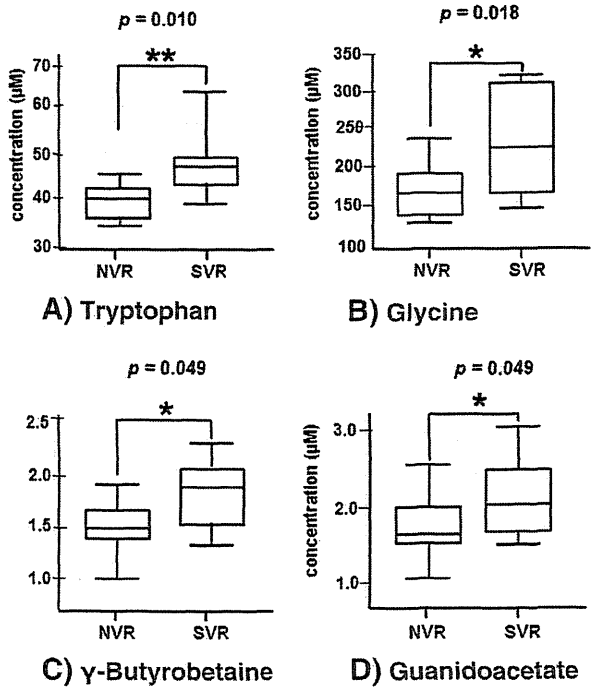

Fig. 4 – Comparison of metabolite levels in serum before treatment between the NVR and SVR patients. The serum levels of tryptophan, glycine, γ-butyrobetaine and guanidoacetate were significantly higher in SVR patients than in NVR patients ($p < 0.05$). Mann–Whitney U-test.

investigate whether the cell metabolic status of patients before the start of treatment would affect the viral response to PegIFN/RBV therapy. The serum levels of several specific metabolites, including tryptophan, glycine, γ-butyrobetaine and guanidoacetate, were significantly higher in the sustained virological responders than in non-virological responders,

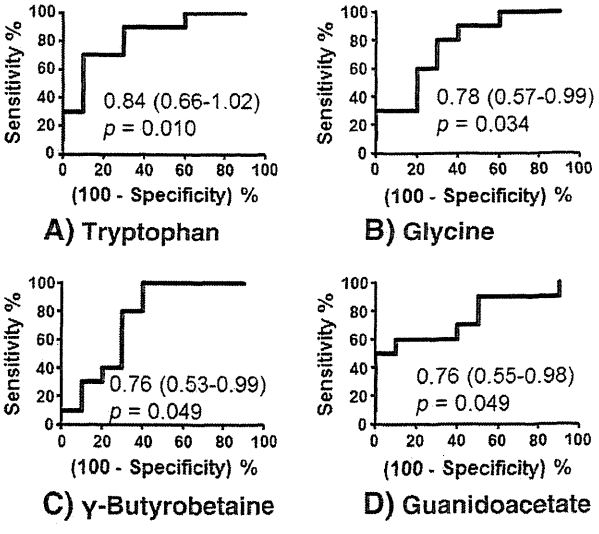

Fig. 5 – ROC curve analyses of four metabolites that were able to discriminate SVR from NVR.

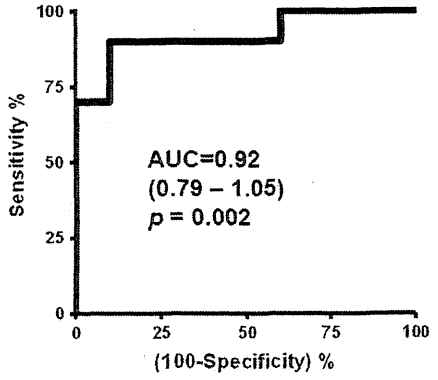

Fig. 6 – ROC curve of multiple logistic regression model incorporating the pretreatment levels of tryptophan and γ-glutamate-arginine for discriminating SVR from NVR.

although principal component analysis revealed no significant difference in the total metabolite profile between them. Our MLR analysis selected two metabolites – tryptophan and γ-glutamate-arginine – that were able to predict the viral response with some degree of accuracy. Although a further study with a larger cohort will be needed in order to confirm whether the serum levels of these metabolites are indeed associated with antiviral effect of PegIFN/RBV therapy, some of them, particularly tryptophan, have already been suggested to have such a relationship.

Tryptophan is a source of kynurenine derivatives, and the pathway responsible is dependent on indoleamine 2,3-dioxygenase (IDO) [23,24]. IDO is inducible in a large variety of cells by inflammatory cytokines such as IFN-γ, and therefore tryptophan degradation is accelerated by infection or malignant diseases that are accompanied by cellular immune activation [25,26]. IDO plays an important role in suppression of the cellular immune response [27,28], and can inhibit T-cell responses, thereby inducing immunological tolerance [29]. Genetic variants affecting serum metabolite levels may play a functional role in the liver [30]. Although no previous study

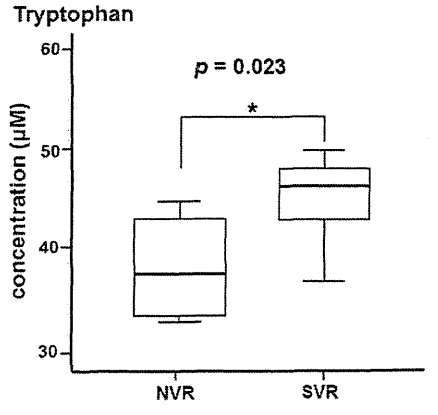

Fig. 7 – Comparison of the serum levels of tryptophan between NVR and SVR patients carrying IL28B homozygotes for the major alleles of rs8099917. Mann–Whitney U-test.

has investigated genetic differences associated with the degree of IDO induction, non-virological responders with low tryptophan levels due to active tryptophan degradation may be in a state of down-regulation of the immune response in the presence of an increased amount of inducible IDO, which in turn may be partly associated with low responsiveness to IFN-based antiviral therapy. The difference in the serum levels of tryptophan between the NVR and SVR groups in the present study was not related to IL28B genotype. These data suggest that the metabolic status of liver cells represented by differences in the serum metabolite profiles between NVR and SVR may predict the therapeutic effect of PegIFN/RBV on chronic hepatitis C.

This study identified for the first time the specific metabolites in serum whose levels were altered in patients with chronic hepatitis C receiving PegIFN/RBV therapy. We found that PegIFN/RBV therapy reduced the serum levels of four γ-glutamyl dipeptides (γ-Glu-His, γ-Glu-Lys, γ-Glu-Phe, γ-Glu-Val), glutamic acid, 5-oxoproline, glucosamine, and methionine sulfoxide. The γ-glutamyl dipeptides are formed by binding of glutamic acid to various amino acids catalyzed by γ-glutamylcysteine synthetase, and are produced as a by-product of glutathione, which has a protective effect against oxidative stress [15,31,32]. The γ-glutamyl cycle is activated by glutathione production in patients with liver diseases such as hepatitis, the glutathione being consumed to neutralize generated ROS, in turn leading to activation of γ-glutamylcysteine synthetase, and resulting in the biosynthesis of glutathione together with γ-glutamyl dipeptides [15]. This would suggest that PegIFN/RBV therapy removes oxidative stress and alters cell metabolism towards a more normal range as a result of reduced glutathione production, as was shown by principal component analysis in the present study. In addition, the serum level of GGT has been widely used as a marker of liver dysfunction, alcohol intake, or metabolic syndrome, and it can be also used as an indicator of amelioration of oxidative stress [16]. To investigate the γ-glutamyl dipeptide biosynthetic pathway, we previously performed trace analyses of γ-Glu-X and γ-Glu-X-Gly peptides by intraperitoneal injection of labeled threonine and acetaminophen [15]. The rapid decrease of labeled threonine and the gradual increasing of the labeled γ-Glu-X-Gly/γ-Glu-X ratio indicated that γ-glutamyl peptides were synthesized in the order γ-Glu-X > γ-Glu-X-Gly [15], suggesting that γ-Glu-X was synthesized rather than being a product of γ-Glu-X-Gly catalysis by GGT. Previous studies have reported that an improvement of the serum GGT level leads to a decrease of free radical production, and that the baseline level of GGT is associated with the response to IFN-based antiviral therapy in chronic hepatitis C [33–35]. In the present study, the pretreatment serum levels of GGT in non-virological responders had a tendency to be higher than those of sustained virological responders, and they were significantly decreased after successful treatment. These results suggest that this therapy helps remove oxidative stress.

On the other hand, the serum levels of 5-methoxy-3-indoleacetate, glutamine, kynurenine and lysine were increased significantly 24 weeks after completion of PegIFN/RBV therapy in both non-virological responders and sustained virological responders. These changes may have resulted from the improvement of cell metabolism by the therapy. The removal of the oxidative stress reflected production of glutamine and lysine. Improvement of tryptophan metabolism through the anti-inflammatory effect of PegIFN/RBV therapy is thought to normalize the production of kynurenine from tryptophan [23–26]. Also, the improvement of liver steatosis as a result of reduced expression of HCV core protein may generate an increase of 5-methoxy-3-indoleacetate, which binds to, and activates, peroxisome proliferator-activated receptor-γ [36,37]. Oxidative stress plays an important role in the progression of liver inflammation and hepatocarcinogenesis [8,38], including that associated with HCV infection [10–12]. The results of the present study suggest that PegIFN/RBV therapy can slow the progression of liver disease in patients with chronic HCV infection through reduction of oxidative stress.

In conclusion, the present study has shown that the pretreatment serum levels of low-molecular-weight metabolites, including tryptophan, are associated with the virological response to PegIFN/RBV therapy, and that such therapy can reduce the level of oxidative stress in patients with chronic HCV infection, as well as modifying the state of cell metabolism. However, further studies are needed to validate the present findings in a larger cohort of patients.

Author contributions

Study design, data analysis and interpretation, writing manuscript: T Saito and Sugimoto equally contributed to this work as the lead author of this manuscript; Study conduct: Soga; Study design and interpretation: M Tomita, Ueno; analysis and interpretation: Igarashi, K Saito, Shao; data collection: Katsumi, K Tomita, Sato, Okumoto, Nishise, Watanabe.

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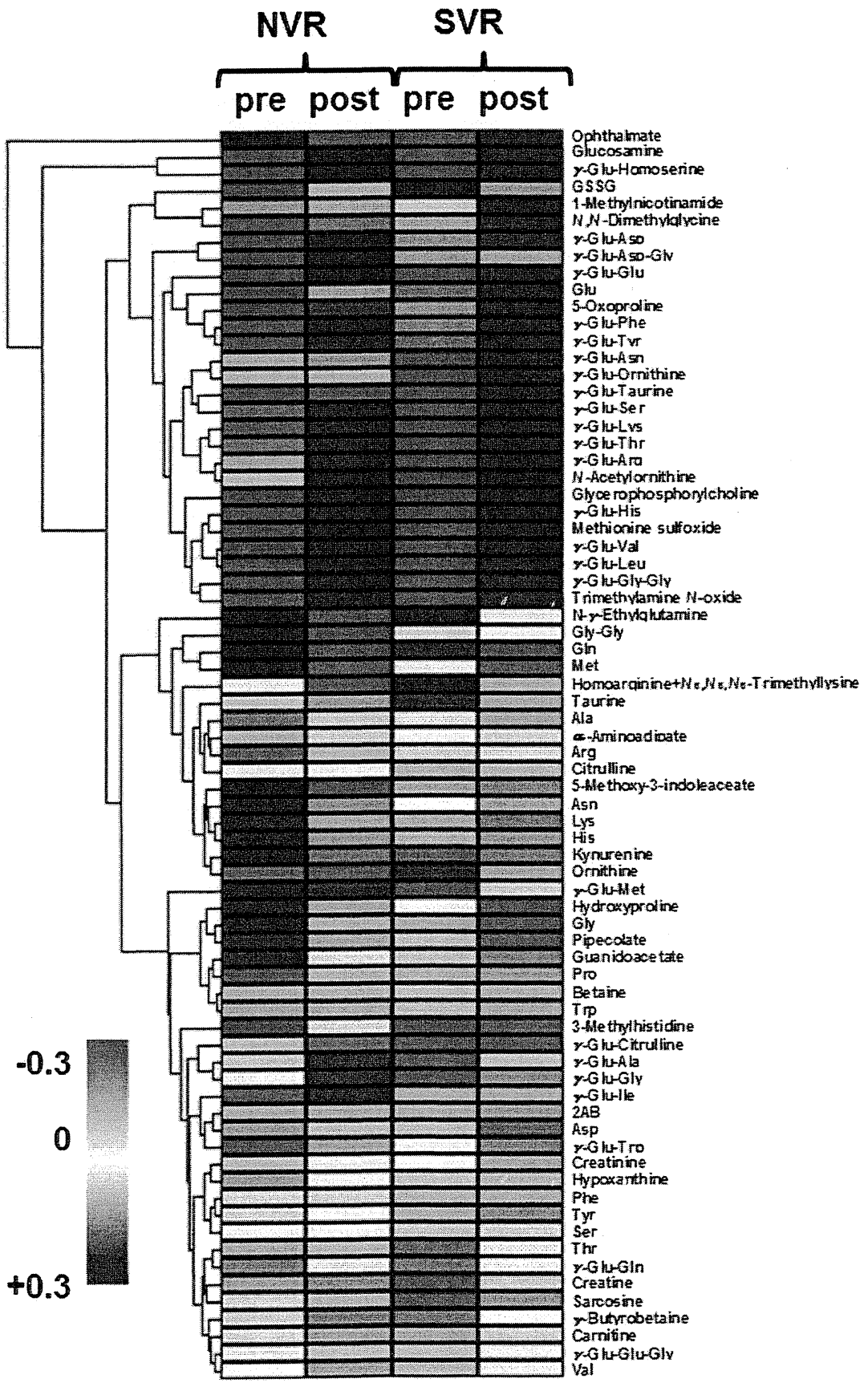
Fig. 8 – Heat map of observed metabolomic profiles. Pre and post indicate pretreatment and post-treatment, respectively. Red color indicates metabolite concentrations that were higher than average, while blue color indicates those that were lower than average. The order of the metabolites was arranged on the basis of clustering analysis.


NVR
SVR
pre
post
pre
post
Ophthalmate
Glucosamine
γ-Glu-Homoserine
GSSG
1-Methylnicotinamide
N,N-Dimethylglycine
γ-Glu-Asp
γ-Glu-Asp-Gly
γ-Glu-Glu
Glu
5-Oxoproline
γ-Glu-Phe
γ-Glu-Tyr
γ-Glu-Asn
γ-Glu-Ornithine
γ-Glu-Taurine
γ-Glu-Ser
γ-Glu-Lys
γ-Glu-Thr
γ-Glu-Arg
N-Acetylornithine
Glycerophosphorylcholine
γ-Glu-His
Methionine sulfoxide
γ-Glu-Val
γ-Glu-Leu
γ-Glu-Gly-Gly
Trimethylamine N-oxide
N-γ-Ethylglutamine
Gly-Gly
Gln
Met
Homoarginine+Nε,Nε,Nε-Trimethyllysine
Taurine
Ala
α-Aminoadipate
Arg
Citrulline
5-Methoxy-3-indoleacetate
Asn
Lys
His
Kynurenine
Ornithine
γ-Glu-Met
Hydroxyproline
Gly
Pipecolate
Guanidoacetate
Pro
Betaine
Trp
3-Methylhistidine
γ-Glu-Citrulline
γ-Glu-Ala
γ-Glu-Gly
γ-Glu-Ile
2AB
Asp
γ-Glu-Tyr
Creatinine
Hypoxanthine
Phe
Tyr
Ser
Thr
γ-Glu-Gln
Creatine
Sarcosine
γ-Butyrobetaine
Carnitine
γ-Glu-Glu-Gly
Val
-0.3
0
+0.3

Conflict of interest

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2013.07.002.

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RESEARCH ARTICLE Open Access

Impaired mitochondrial β-oxidation in patients with chronic hepatitis C: relation with viral load and insulin resistance

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Abstract

Background: Hepatic steatosis is often seen in patients with chronic hepatitis C (CH-C). It is still unclear whether these patients have an impaired mitochondrial β-oxidation. In this study we assessed mitochondrial β-oxidation in CH-C patients by investigating ketogenesis during fasting.

Methods: This study consisted of thirty patients with CH-C. Serum levels of insulin and hepatitis C virus (HCV) core protein were measured by chemiluminescence enzyme immunoassay. The subjects were then fasted, and venous blood samples were drawn 12 h and 15 h after the start of fasting. The levels of blood ketone bodies were measured by an enzymatic cycling method. The rate of change in total ketone body concentration was compared with that in eight healthy volunteers.

Results: The rate of change in total ketone body concentration between 12 h and 15 h after the start of fasting was significantly lower in CH-C patients than in healthy volunteers (129.9% (8.5-577.3%) vs. 321.6% (139.6-405.4%); P <0.01). The rate of change in total ketone body concentration in patients with a serum level of HCV core protein of 10000 fmol/L or higher was significantly lower than in patients with a level of less than 10000 fmol/L (54.8% (8.5-304.3%) vs. 153.6% (17.1-577.3%); P <0.05). The rate of change in total ketone body concentration in patients with a homeostasis model assessment of insulin resistance (HOMA-IR) of 2.5 or higher was significantly lower than in patients with a HOMA-IR of less than 2.5 (56.7% (8.5-186.7%) vs. 156.4% (33.3-577.3%); P <0.01).

Conclusions: These results suggest that mitochondrial β-oxidation is impaired, possibly due to HCV infection in patients with CH-C.

Keywords: Ketogenesis, Fasting test, Hepatic steatosis, HCV

Background

Hepatitis C virus (HCV) infection is a major cause of chronic liver injury. Hepatic steatosis is one of histologic features of chronic HCV infection with a risk of progression of liver diseases [1]. Hepatic steatosis is caused by some mechanisms, which include an increase of fatty acids uptake and synthesis, a decrease of fatty acids β-oxidation, or low level of secretion of very-low density lipoprotein. HCV core protein-transgenic mice develop hepatic steatosis due to impaired β-oxidation caused by mitochondrial damage [2]. However, there has been no evidence of impaired β-oxidation in patients with chronic hepatitis C (CH-C) *in vivo*.

During starvation, mitochondria produces acetyl CoA, which is converted into ketone bodies by fatty acids β-oxidation. In patients with impaired hepatic mitochondrial β-oxidation, ketogenesis is expected to be inadequate. Adult-onset type 2 citrullinemia (CTLN2) has been demonstrated to present as non-alcoholic fatty liver disease (NAFLD) [3]. CTLN 2 is associated with mutations in the *SLC25A13 gene encoding citrin, which is a component of* the mitochondrial malate-aspartate shuttle. Functional defectiveness of citrin impairs not only transport of aspartate from mitochondria but that of NADH into mitochondria.

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This induces activation of the citrate-malate shuttle with compensatory production of acetyl CoA, which in turn stimulates fatty acid synthesis. In addition, mitochondrial accumulation of malonyl CoA in a high $NADH/NAD^+$ environment suppresses fatty acid oxidation. These circumstances lead to hepatic steatosis in patients with CTLN 2 [4]. Inui et al. have demonstrated that suppression of fatty acid oxidation is accompanied by impaired ketogenesis in such patients [5].

Based on this background, we measured the concentration of blood ketone bodies during fasting in order to evaluate mitochondrial β-oxidation in patients with CH-C, and thus to investigate a mechanism of steatosis associated with HCV infection. Here, we report for the first time that mitochondrial β-oxidation is impaired in patients with CH-C.

Methods

Patients

Thirty patients (14 male and 16 female, the mean age 54.2, ranging from 22 to 74 years old) with CH-C were studied. The patients were admitted to Yamagata University Hospital for treatment between March 2006 and May 2009. All of the patients had been positive for both serum anti-HCV and HCV RNA for more than 6 months, and had elevated levels of serum alanine aminotransferase (ALT). They were all negative for hepatocellular carcinoma, hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, heart failure, renal insufficiency, a history of diabetes mellitus, excess alcohol intake (daily ethanol consumption >20 g) or drug abuse. None of the patients fulfilled the criteria for Metabolic Syndrome in Japan [6,7], i.e. the presence of at least two of the following three abnormalities in addition to visceral obesity (waist circumference: 85 cm or more in men, 90 cm or more in women): 1) triglycerides ≥150 mg/dl and/or HDL-cholesterol <40 mg/dl, or receiving treatment for this type of dyslipidemia; 2) systolic blood pressure ≥130 and/or diastolic blood pressure ≥85, or receiving treatment for hypertension; 3) fasting glucose ≥110 mg/dl or receiving treatment for diabetes. As a control group, eight volunteers (4 male and 4 female) were included (the mean age 30.5, ranging from 26 to 39 years old). All of them were healthy, with a BMI of <25 kg/m^2, without medication or severe disease. Written informed consent to participate was obtained from all subjects, and the study protocol was approved by The Yamagata University Hospital Ethics Committee.

Methods

Clinical and laboratory data

Body height, weight and waist circumference were measured at the time of admission. Body mass index (BMI) was calculated as: BMI = body weight (kg)/body height $(m)^2$. Venous blood samples were collected after a 12-h overnight fast for standard biochemical testing and determination of serum insulin levels, HCV genotype and HCV core protein. Serum insulin was determined by chemiluminescence enzyme immunoassay (Lumipulseprestoinsulin®, Fujirebio Inc., Tokyo, Japan). Insulin resistance was determined based on the homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR was calculated using the formula: [fasting glucose (mg/dl) × fasting insulin (μU/ml)] / 405 [8]. Insulin resistance was defined as HOMA-IR >2.5. The amount of HCV core antigen and HCV RNA in serum were measured by a chemiluminescence enzyme immunoassay (Lumispot Eiken HCV antigen®, Eiken Chemical Co., Ltd., Tokyo, Japan) and an amplicor HCV RNA detection kit (Amplicor HCV v2.0®, Roche Diagnostics, Tokyo, Japan) or a real-time PCR assay (COBAS® TaqMan® HCV Test, Roche Diagnostics, Tokyo, Japan), respectively.

Fasting test

In general, ketone bodies are not detected during periods of feeding, but after the onset of fasting, glycogen is gradually consumed and ketone bodies are produced rapidly after about 12 hours of fasting. The rate of change in ketone body production between 12 and 15 hours represented the initial increase, and was interpreted as the initial velocity of ketogenesis.

Fasting tests were performed in both subjects and volunteers. They were permitted to drink water after their last meal, and blood samples were drawn to measure the proportion of ketone bodies, glucose, insulin, free fatty acid and triglyceride levels at 12 and 15 h after the last meal. Carnitine fractionation was also measured at 12 after the last meal, using an enzymatic cycling method (Total Carnitine Kainos®, Free Carnitine Kainos®, Kainos Laboratories, Inc., Tokyo, Japan). During fasting, urine organic acids were measured by gas chromatograph-mass spectrometry and then analyzed using software to determine the presence of disorders of organic acid metabolism. Serum ketone bodies were measured by an enzymatic cycling method (3-hydroxybutyrate Kainos®, Total ketone body Kainos®, ketone body standard reagent 2®, Kainos Laboratories, Inc. Tokyo, Japan). These measurements were performed by SRL Inc. (Tokyo, Japan). The rate of change in total ketone body concentration from 12 h to 15 h was calculated using the equation: (total ketone bodies at 15 h − total ketone bodies at 12 h)/ total ketone bodies at 12 h × 100.

Histological assessment

Liver biopsies were performed under sonographic guidance in 25 patients who provided informed consent. The liver tissues were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin and silver stain. Liver histology was graded and staged by a pathologist based on the international classification [9]. The grade of steatosis was modified as follows: grade 0 = no steatosis and between 0%

and 5% of hepatocytes containing visible macrovesicular steatosis, grade 1 = between 5% and 33%, grade 2 = between 33% and 66%, and grade 3 = more than 66% according to the non-alcoholic fatty liver disease Activity Score [10].

Statistical analysis

Results are expressed as the mean ± standard deviation (SD) or the median and range (in parenthesis). Student's *t* test was used for normally distributed non-paired continuous variables. The rate of change in total ketone body concentration was assessed as a parametric value because the value was distributed parametrically after logarithmic transformation. Wilcoxon's signed-ranks test was used for paired continuous variables. Comparisons between more than two groups were made by one-way analysis of variance. All P-values were based on a two-sided test of statistical significance. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Subject characteristics

The characteristics of thirty patients with chronic hepatitis C were shown in Table 1. The mean level of BMI was less than 25 kg/m^2, and that of fasting plasma glucose was within normal range. The mean level of ALT was greater than the upper limit of normal range. Nine patients had a status of insulin resistance in whom HOMA-IR showed a level of 2.5 or greater. All patients were positive for HCV RNA in whom the mean level of serum HCV core antigen showed a 6505 fmol/L. Of the 30 patients, 20 (66.7%) were infected with the HCV genotype 1b, 6 (20.0%) with genotype 2a, and 4 (13.3%) with genotype 2b. Fifteen (60%) had a liver fibrosis grade of F1, 7 (28%) had F2, and 3 (12%) had F3. Six (24%) had a liver steatosis grade of 0, 18 (72%) had grade 1, 1 (4%) had grade 2, and none had grade 3.

Table 1 Patient characteristics

		Patients	Reference value
Age (yr) [b]		54.2 ± 10.6	
Male/Female (ratio)		14/16 (0.47)	
BMI (kg/m^2) [b]		23.7 ± 2.7	18.5 - 25
ALT (IU/L) [a]		47.5 (17–167)	8 - 42
γGTP (IU/L) [a]		41 (13–152)	10 - 47
ChE (IU/L) [b]		312.9 ± 88.2	185 - 431
Fasting plasma glucose (mg/dL) [b]		95.5 ± 9.7	70 - 109
Insulin (μIU/mL) [a]		8.7 (4.5 - 20.4)	1.84 - 12.2
HOMA-IR [a]		2.0 (1.2 - 5.1)	
HOMA-IR > 2.5 (%)		9 (30)	
Triglyceride (mg/dL) [b]		91.4 ± 31.1	30 - 149
Total cholesterol (mg/dL) [b]		175.2 ± 25.4	129 - 219
Acylcarnitine (μmol/L) [a]		9.25 (5.7 - 21.1)	6 - 23
HCV RNA (LogIU/mL) [b]		6.1 ± 0.9	
HCV core antigen (fmol/L) [a]		6505 (<20–23200)	
HCV Genotype	1b / 2a / 2b	20 / 6 / 4	
Inflammation	A0 / A1 / A2 / A3	0 / 15 / 10 / 0	
Fibrosis	F0 / F1 / F2 / F3 / F4	0 / 15 / 7 / 3 / 0	
Steatosis	G0 / G1 / G2 / G3	6 /18 / 1 / 0	

BMI, body mass index; ALT, alanine aminotransferase; γGTP, gamma-glutamyltranspeptidase; ChE, cholinesterase; HOMA-IR, homeostasis model assessment of insulin resistance.
Data are [a]medians (min-max), [b]the means ± standard deviation.

Free fatty acids concentration during fasting in CH-C patients and healthy volunteers

The concentration of free fatty acids increased by fasting in CH-C patients and healthy volunteers. The rate of change in free fatty acids concentration between 12 h and 15 h was similar in both groups (CH-C patients 48.6% ± 45.0 vs. healthy volunteers 70.3% ± 95.2; ns), as shown in Figure 1.

Ketone body concentration during fasting in CH-C patients and healthy volunteers

The levels of total blood ketone bodies were elevated in both CH-C patients and healthy volunteers 15 h after the start of fasting, but the rate of change between 12 h and 15 h after fasting was significantly different between them. The rate of change in total ketone body concentration between 12 h and 15 h was shown in Figure 2. It was significantly lower in CH-C patients than in healthy volunteers (129.9% (8.5-577.3%) vs. 321.6% (139.6-405.4%); $P < 0.01$). The rates of change in both acetoacetate (Figure 3A) and 3-hydroxybutyrate (Figure 3B) between 12 h and 15 h after fasting were also significantly lower in CH-C patients than in healthy volunteers (acetoacetate : 109.5% (−5.8-514.3%) vs. 254.8% (145.5-341.7%) , 3-hydroxybutyrate : 130.8% (−3.8-606.7%) vs. 337% (135.5-495%) ; $P < 0.01$).

There was a significant positive correlation between the concentration of total ketone body and the levels of acylcarnitine (rs 0.56, $P < 0.01$) at 12 h after fasting, as shown in Figure 4A, similar to the pattern of free fatty acids (rs 0.54, $P < 0.01$, Figure 4B). The level of acylcarnitine was significantly lower in CH-C patients than in healthy volunteers (9.25 μmol/L (5.7-21.1 μmol/L) vs. 11.65 μmol/L (9.3-17 μmol/L); $P < 0.05$).

Relationship between the rate of change in total ketone body concentration and clinical parameters in CH-C patients

We stratified CH-C patients into two groups based on the clinical parameters. The rate of change in total ketone body

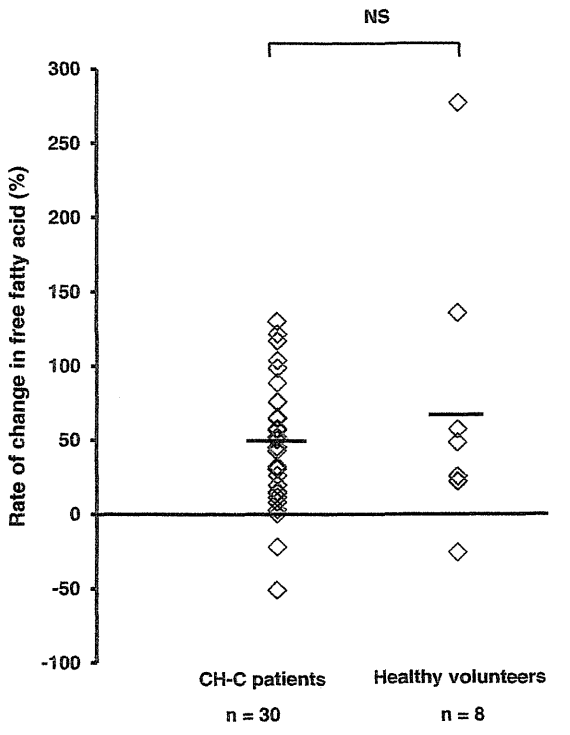

Figure 1 Free fatty acids concentration during fasting in patients with chronic hepatitis C and healthy volunteers. The rate of change in free fatty acid concentration between 12 h and 15 h after fasting was similar in both groups. The line represents a mean value. Welch's *t*-test.

concentration between 12 h and 15 h in patients with a serum HCV core protein level of 10000 fmol/L or higher was significantly lower than that in patients with a level of less than 10000 fmol/L (54.8% (8.5-304.3%) vs. 153.6% (17.1-577.3%); P <0.05) (Figure 5). In addition, the rate of change in total ketone body concentration in patients with a higher HOMA-IR value (2.5 or greater) was significantly lower than that in patients with a value of less than 2.5 (56.7% (8.5-186.7%) vs. 156.4% (33.3-577.3%); P <0.01) (Figure 6). The patients with biopsy-proven steatosis had a relatively low rate of change in total ketone body concentration between 12 h and 15 h in comparison with those without steatosis, although the rate was not significantly different between them (Figure 7). There was no significant difference in the rate of change in total ketone body concentration among the HCV genotypes (1b 120.2% (8.5-577.3%), 2a 129.9% (91.7-304.3%), 2b 135.8% (56.7-253.3%)). No significant difference in the rate of change in total ketone body concentration was demonstrated among the stages of fibrosis (F1 91.7% (17.1-436%), F2 133% (8.5-283.3%), F3 88.6% (34.2-577.3%)).

Discussion

Hepatitis C virus (HCV) is the leading cause of chronic hepatitis, subsequent liver cirrhosis and hepatocellular carcinoma. Hepatic steatosis is commonly seen in patients with chronic HCV infection having a high viral load, and it is in part associated with the development of insulin resistance [1], hepatic fibrosis [11] and hepatocarcinogenesis [12] during infection. Steatosis is also associated with a lower rate of sustained response to anti-viral therapy [13], and shows improvement after successful eradication of HCV by anti-viral therapy [14].

In general, fat accumulation in hepatocytes can result from several causes; increase of fatty acid uptake by hepatocyte, increase of fatty acid synthesis in hepatocyte, decrease of hepatic fatty acid oxidation, decrease of very-low density lipoprotein secretion. The mechanisms of steatosis in HCV infection are not fully understood. In the previous study using liver biopsy specimens of patients with HCV infection, it is shown that expression of peroxisome proliferator-activated receptor (PPAR)-α is impaired, which is an important factor in the regulation of mitochondrial β-oxidation [15]. Therefore, impaired mitochondrial β-oxidation is

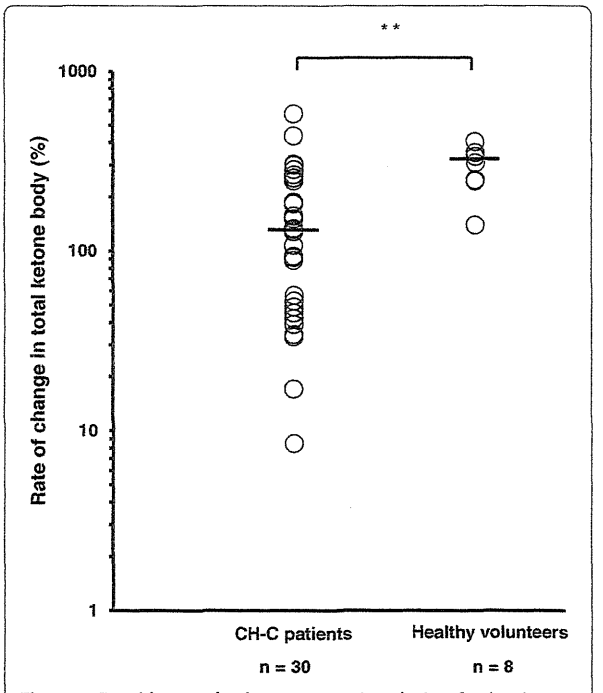

Figure 2 Total ketone body concentration during fasting in patients with chronic hepatitis C and healthy volunteers. The rate of change in total ketone body concentration between 12 h and 15 h after fasting was significantly lower in patients than in healthy volunteers (** denotes P <0.01). The line represents a median value. Log transformation was performed. Student's t test.

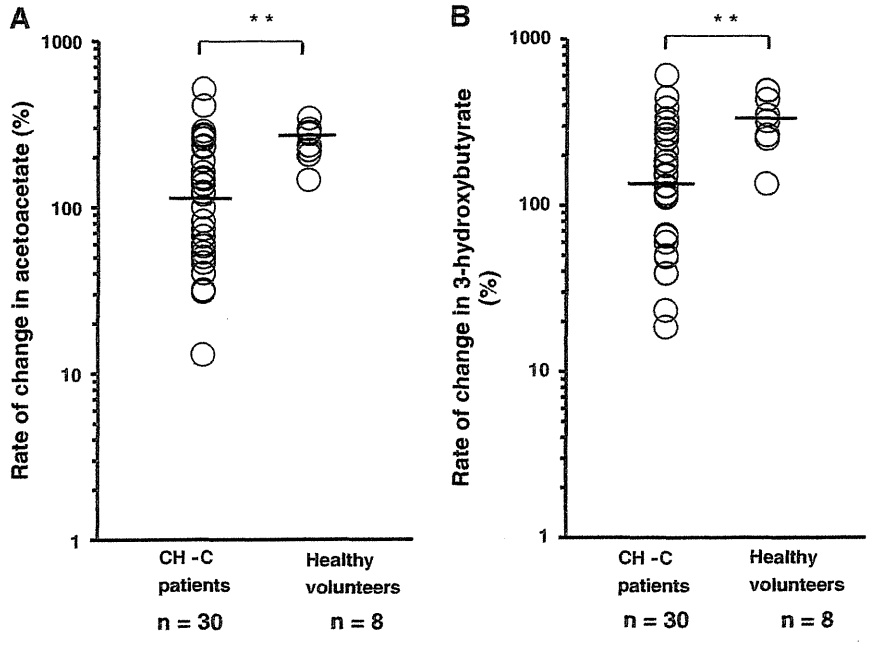

Figure 3 Ketone body fraction concentration during fasting in patients with chronic hepatitis C and healthy volunteers. The rates of change in both acetoacetate **(A)** and 3-hydroxybutyrate **(B)** between 12 h and 15 h after fasting were significantly lower in patients than in healthy volunteers (** denotes P <0.01). The line represents a median value. Log transformation was performed. Student's t test.

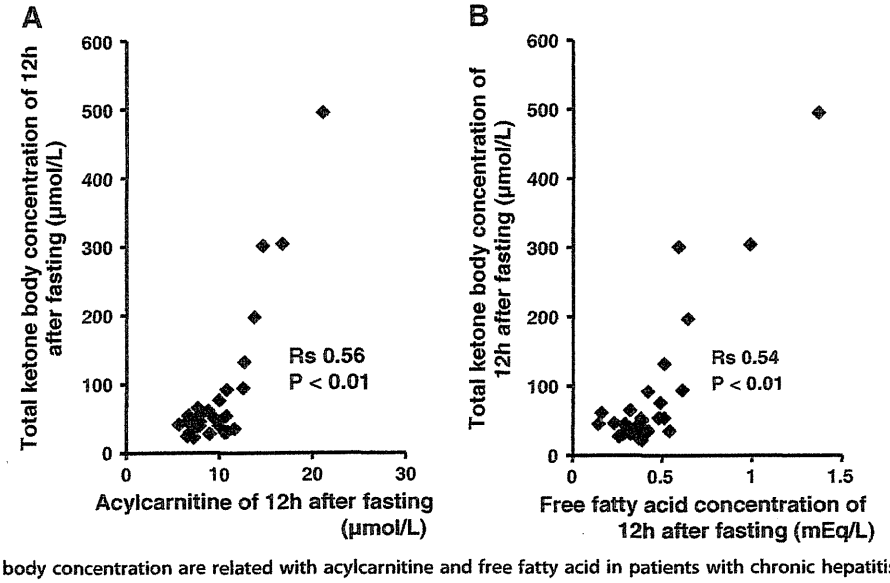

Figure 4 Ketone body concentration are related with acylcarnitine and free fatty acid in patients with chronic hepatitis C. There is a significant positive correlation between the concentration of total ketone body and the levels of acylcarnitine (rs 0.56, P <0.01),**(A)**, as well as free fatty acids (rs 0.54, P <0.01), **(B)**.

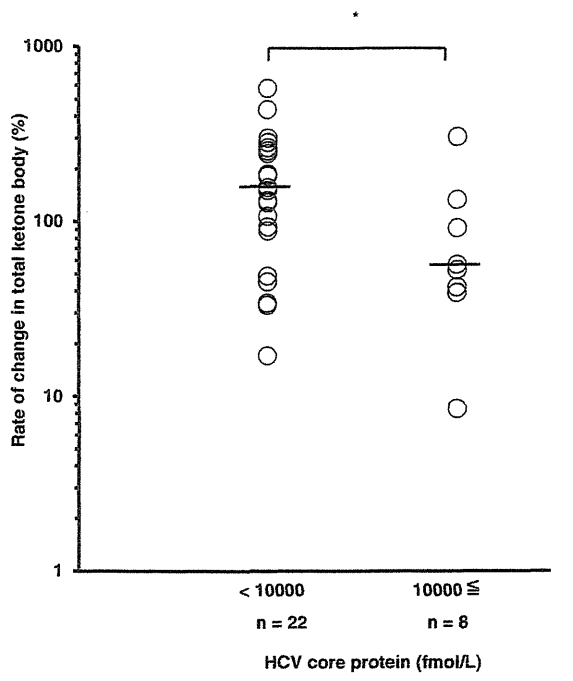

Figure 5 HCV core protein and the change in total ketone body concentration during fasting. The rate of change in total ketone body concentration between 12 h and 15 h after fasting in patients with a serum HCV core protein level of 10000 fmol/L or higher was significantly lower than that in patients with a level of less than 10000 fmol/L (* denotes P <0.05). The line represents a median value. Log transformation was performed. Student's t test.

supposed to be a mechanism of hepatic steatosis observed in the state of HCV infection.

However, there is no previous study which investigated whether mitochondrial β-oxidation is impaired in patients with CH-C *in vivo*. In the present study, therefore, we focused on the mechanism of ketogenesis in humans by investigating ketogenic capacity during fasting. The rate of change in total ketone body concentration between 12 h and 15 h after the start of fasting was significantly lower in CH-C patients than in healthy volunteers, while the rate of change in free fatty acids concentration was similar in both groups. Therefore there is a possibility that steps from acetyl-CoA to ketone bodies are impaired in patients with CH-C. In addition, Hoppel et al. reported that acylcarnitine increased during fasting and ketone bodies correlated with short-chain acylcarnitines. It is speculated that the increase in short-chain acylcarnitines may be a by-product of fatty acid β-oxidation [16]. In our patients, the level of acylcarnitine was significantly lower in CH-C patients than in healthy volunteers. Thus, these support that mitochondrial β-oxidation is impaired in patients with CH-C. Further studies are needed to assess which step is involved in the impairment of ketone bodies formation in HCV infection.

During starvation, ketone bodies increase in the body under conditions of normal mitochondrial β-oxidation. Since insulin secretion decreases during fasting, synthesis of triglyceride from acyl CoA is suppressed. Therefore, acyl CoA is β-oxidized to acetyl CoA in mitochondria. Oxaloacetate is used for gluconeogenesis during fasting. Under this condition, acetyl CoA cannot conjugate oxaloacetate, and the tricarboxylic acid (TCA) cycle is inhibited. Inhibition of the TCA cycle also occurs through consumption of nicotinamide adenine dinucleotide (NAD^+) and the production of reduced nicotinamide adenine dinucleotide (NADH) via β-oxidation. Consequently, acetyl CoA shifts towards ketogenesis. Acetyl CoA enters the TCA cycle and is used as fuel in muscle. Thus, the liver is the only organ that produces ketone bodies and secretes them into blood. In individuals with impaired hepatic mitochondrial β-oxidation, it is expected that ketogenesis would not be adequate. This is a reason why measurement of blood ketone body concentration in a fasting state facilitates assessment of mitochondrial β-oxidation *in vivo* [5].

In the present study, the rate of change in total ketone body concentration in patients with a serum level of

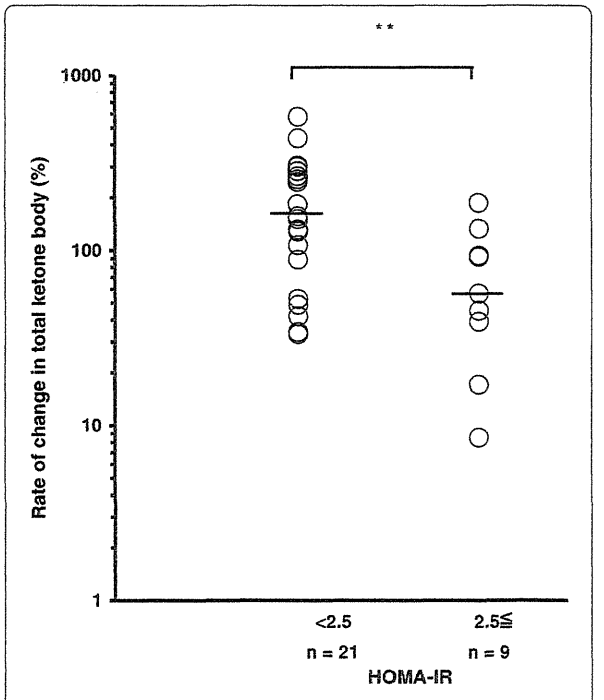

Figure 6 Insulin resistance and the change in total ketone body concentration during fasting. The rate of change in total ketone body concentration in patients with a higher HOMA-IR value (2.5 or greater) was significantly lower than that in patients with a value of less than 2.5 (** denotes P <0.01). The line represents a median value. Log transformation was performed. Student's t test.

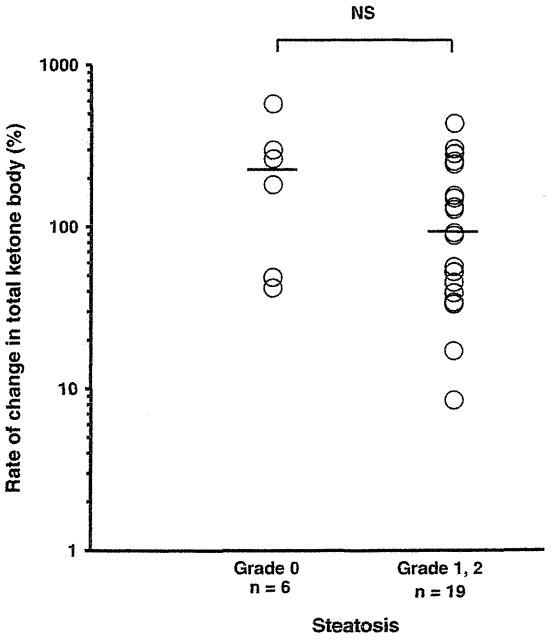

Figure 7 Hepatic steatosis and the change in total ketone body concentration during fasting. The patients with steatosis had a relatively low rate of change in total ketone body concentration between 12 h and 15 h after fasting in comparison with those without steatosis, although it was not significant. The line represents a median value. Log transformation was performed. Student's t test.

HCV core protein of 10,000 fmol/L or higher was significantly lower than in patients with a level of less than 10,000 fmol/L, showing that patients with a higher level of serum HCV core protein had lower ketogenic capacity. HCV core protein induces hepatic steatosis with disappearance of the double structure of mitochondrial membranes in HCV core transgenic mice [2]. HCV core protein is largely associated with mitochondrial dysfunction [17]. Moreover, recent studies have reported that HCV core protein downregulates the expression of PPAR-α, which is abundant in hepatocytes and is an important factor in the regulation of mitochondrial β-oxidation [15,18]. Our data suggest an impairment of mitochondrial β-oxidation by HCV infection.

Although no significant relationship between fatty acid oxidation and the grade of steatosis was demonstrated in this study (Figure 7), this issue would be worth investigating in a larger cohort of patients. HCV infection induces mitochondrial dysfunction as a result of oxidative stress, which is closely related to liver inflammation and hepatocarcinogenesis [19]. Oxidative stress is associated with impairment of fatty acid oxidation, and thus impaired ketogenesis seems to represent the increased oxidative stress in CH-C patients.

Insulin resistance in patients with CH-C has been reported [20]. At this study, insulin resistance, HOMA-IR >2.5, was observed in 9 of 30 patients. In this study, a significant positive correlation was evident between the concentration of total ketone bodies and that of free fatty acids. However, in some patients with insulin resistance, the concentrations of both free fatty acids and ketone bodies were not so high. The rate of change in the concentrations of total ketone bodies was significantly lower in patients with a higher HOMA-IR value (2.5 or greater) than in those with a value of less than 2.5. Many other factors may influence the level of fatty acid. Further studies are needed to elucidate the mechanism of insulin resistance in CH-C patients.

Our CH-C patients were significantly older than the healthy volunteers. However, we did not observe any significant correlation between the age of our subjects and the rate of change in total ketone body concentration within the age range investigated (data not shown). Elderly people in good health have a similar capacity to produce ketones to middle-aged or young adults [21].

Conclusions

The results of our study suggest that mitochondrial β-oxidation is impaired, possibly due to HCV infection. Further studies are needed to elucidate the detailed pathophysiology of impaired fatty acid metabolism in CH-C and its clinical significance.

Abbreviations

CH-C: Chronic hepatitis C; HCV: Hepatitis C virus; HOMA-IR: Homeostasis model assessment of insulin resistance; CTLN2: Adult-onset type 2 citrullinemia; NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; BMI: Body mass index; SD: Standard deviation; PPAR: Peroxisome proliferator-activated receptor; TCA: Tricarboxylic acid; NAD: Nicotinamide adenine dinucleotide; NADH: Reduced nicotinamide adenine dinucleotide; SREBP: Sterol regulatory element-binding protein.

Competing interests

No financial interests to disclosure that related to this study.

Authors' contributions

SC, MK, KT, TK, IR, HH, OK and NY contributed to data collection and data analysis. SC, ST, WH and UY contributed to data interpretation and manuscript writing. KS contributed to the design and conduct of the study. All authors read and approved the final manuscript.

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Journal of Medical Virology 85:1199–1205 (2013)

Serum Prolactin Levels and Prolactin mRNA Expression in Peripheral Blood Mononuclear Cells in Hepatitis C Virus Infection

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Prolactin is not only a pituitary hormone but an immunoregulatory hormone secreted from lymphocytes. Prolactin induction in relation to hepatitis C virus (HCV) infection has not been elucidated. The serum levels of prolactin were examined in 232 HCV-infected subjects positive for anti-HCV antibody and 65 healthy controls negative for it, who were recruited in the cohort study. The *prolactin* mRNAs were measured in peripheral blood mononuclear cells (PBMCs) of eleven healthy volunteers including five men and six women before and after stimulation by HCV in vitro. The serum level of prolactin and *prolactin* mRNA in PBMCs were measured by chemiluminescence immunoassay and real-time PCR, respectively. The serum levels of prolactin were significantly higher in the HCV-infected subjects (median: 7.5, IQR: 5.7–10.9 ng/ml) than in the controls (median: 5.6, IQR: 4.4–8.3 ng/ml) ($P < 0.01$). They were significantly higher in HCV-infected males (median: 8.0, IQR: 5.9–11.8 ng/ml) than in the controls (median: 4.8, IQR: 4.2–5.9 ng/ml) ($P < 0.001$), however, the difference was not significant between HCV-infected females (median: 7.3, IQR: 5.6–10.5 ng/ml) and the controls (median: 6.4, IQR: 5.3–9.8 ng/ml). The mRNA expression of *prolactin* was induced in PBMCs of all males, but it was induced in PBMCs of the two of six females examined in vitro. These results suggest that the serum level of prolactin is higher in HCV-infected males than in healthy males, and that HCV infection induces the mRNA expression of *prolactin* in PBMCs that is more apparent in male than in females. ***J. Med. Virol. 85:1199–1205, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: prolactin; HCV; immunity; pituitary hormone; cohort

INTRODUCTION

Hepatitis C virus (HCV) is a human pathogen that is a major threat to public health. About 170 million people are estimated to be infected worldwide with a potential risk of progression to cirrhosis and hepatocellular carcinoma [Kiyosawa et al., 1990; Cohen, 1999]. HCV-specific humoral and cellular immune responses are detectable in most infected individuals in both early and chronic phase of infection [Di Bisceglie, 2000].

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