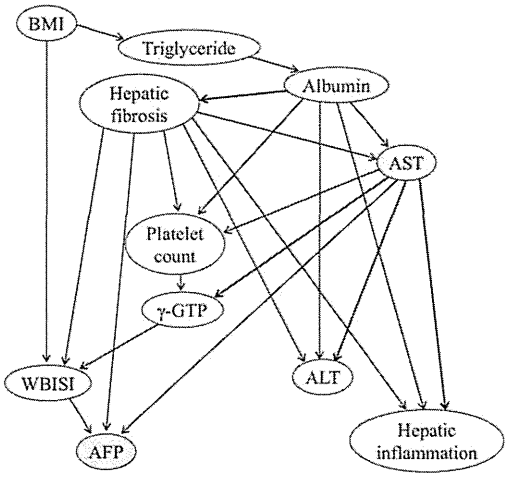

Figure 2. **Bayesian Network analysis of the associations between an elevated serum α-fetoprotein level and the clinical factors. AFP: α-fetoprotein, WBISI: whole-body insulin sensitivity index, BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ-glutamyl transpeptidase**

Table 3. **Bayesian Network Analysis of the Incidence of Elevated Serum α-fetoprotein**

Parameters			Probability
WBISI ≥ 5	AST < 50	F0–2	0.042
WBISI ≥ 5	AST < 50	F3–4	0.333
WBISI ≥ 5	AST ≥ 50	F0–2	0.219
WBISI ≥ 5	AST ≥ 50	F3–4	0.286
WBISI < 5	AST < 50	F0–2	0.169
WBISI < 5	AST < 50	F3–4	0.400
WBISI < 5	AST ≥ 50	F0–2	0.356
WBISI < 5	AST ≥ 50	F3–4	0.846

WBISI: whole-body insulin sensitivity index, AST: aspartate aminotransferase

Discussion

In the present series of studies, we first demonstrated that systemic IR directly influences the elevation of the AFP level in patients with chronic HCV infection based on the results of multivariate and causal-relationship analyses.

The multiple logistic regression analysis showed that a decreased platelet count, increased serum AST and γ-GTP levels, whole-body IR and advanced hepatic fibrosis were independently associated with an elevated AFP level. Several studies have shown that an elevated AFP level is associated with a decreased platelet count, an increased AST level and advanced fibrosis in CHC patients without HCC (5-7), consistent with our findings. Furthermore, the Bayesian Network analysis, which has the ability to assess causal relationships based on conditional probabilities, revealed that an elevated AST level, whole-body IR and advanced fibrosis were directly associated with an elevated AFP level.

In terms of markers of glucose metabolism, WBISI, an index of whole-body IR, was selected as a feasible marker for AFP elevation, whereas HOMA-IR, an index of hepatic IR, was not selected. These results suggest that IR associated with an increased AFP level may be induced by an HCV-infected liver as well as obesity or other metabolic conditions, as systemic IR develops simultaneously in multiple organs, including the liver, skeletal muscle and adipose tissue (30). In fact, the Bayesian Network analysis did not reveal a relationship between an elevated AFP level and the HOMA-IR (data not shown).

Several researchers have reported that IR in HCV-infected patients is closely associated with hepatic fibrosis (12, 16-18). Our Bayesian Network analysis also identified a direct relationship between whole-body IR and hepatic fibrosis. However, both systemic IR and advanced fibrosis were independently and directly associated with an elevated AFP level. These results suggest that it may be possible to decrease the serum AFP level by improving IR, even in patients with advanced hepatic fibrosis.

Although no reports have described an association between the serum AFP and γ-GTP levels, it is well known that γ-GTP plays important roles in the generation of oxidative stress (31) and is correlated with IR (32). The Bayesian Network analysis also showed that the γ-GTP level influences the serum AFP level via the effects of whole-body IR.

In the second part of this study, we showed that prospective lifestyle modification can improve metabolic factors, including systemic IR and the serum AFP level. We found that the leptin/adiponectin ratio, a useful marker of metabolic syndrome in the general population that is correlated with IR in individuals with or without diabetes (33-35), decreased after the intervention. Therefore, we presumed that the reduction in visceral fat achieved with the lifestyle intervention caused a decrease in the leptin/adiponectin ratio, which then improved IR. However, we found no changes in the sTNFR2 levels, a marker for tumor necrosis factor, a key cytokine involved in HCV-associated IR and obesity-associated IR (20, 35-38), despite the reduction in body weight observed in this study. We previously reported that eradication of HCV by IFN decreases the serum sTNFR2 level and improves whole-body IR (20). Therefore, these results suggest that IR and elevated AFP levels in HCV-infected patients may be inhibited by the eradication of HCV with antiviral therapy.

Unexpectedly, the platelet count, which was negatively correlated with an elevated AFP level in the retrospective study, decreased after the lifestyle intervention. We assume that the reduction of the platelet count reflects an improvement in systemic inflammation, a key feature of obesity and DM (39). The adiponectin levels, which are inversely related to adiposity, decreased slightly after the lifestyle intervention, although the changes were not statistically significant. Because the serum adiponectin levels are affected by hepatic fibrosis, regardless of the cause of liver disease (40), the effects of lifestyle intervention on the serum adiponectin levels in HCV-infected patients may differ from those observed

Table 4. Patient Characteristics and Effects of the Lifestyle Intervention on Clinical Characteristics

	Baseline	After	p value
Males/females	11/9	–	–
Age (years)	60 (37–71)	–	–
BMI (kg/m^2)	25.9 (18.9–30.5)	25.0 (17.8–29.2)	< 0.001
Alcohol intake, none/occasionally/regularly	13/6/1	–	–
History of IFN, yes/no	9/11	–	–
Platelet count (× 10^4/μL)	15.1 (10.5–23.7)	14.3 (8.1–20.6)	0.026
AST (IU/L)	45 (20–155)	42 (18–202)	0.251
ALT (IU/L)	54 (18–227)	44 (15–266)	0.173
γ-GTP (IU/L)	43 (11–137)	35 (11–110)	0.040
Total cholesterol (mg/dL)	172 (121–221)	163 (117–209)	0.042
Triglyceride (mg/dL)	108 (48–230)	87 (33–238)	0.008
HDL-C (mg/dL)	42 (24–73)	40 (29–75)	0.419
Uric acid (mg/dL)	5.8 (2.9–8.9)	5.8 (3.0–8.5)	0.337
Creatinine (mg/dL)	0.77 (0.46–0.98)	0.75 (0.40–0.93)	0.025
Total protein (g/dL)	7.5 (6.8–8.6)	7.3 (6.3–8.8)	0.006
Albumin (g/dL)	4.2 (3.5–4.9)	4.1 (3.2–4.5)	0.004
FPG (mg/dL)	101 (85–110)	89 (75–107)	< 0.001
2-h glucose (mg/dL)	140 (89–305)	120 (78–202)	0.130
FSI (μU/mL)	13 (9–18)	9 (6–21)	0.001
2-h insulin (μU/mL)	88 (31–227)	66 (18–189)	0.057
HOMA-IR	2.8 (2.3–4.7)	1.9 (1.2–4.8)	< 0.001
WBISI	3.0 (1.5–4.2)	4.2 (1.4–7.8)	< 0.001
HOMA-β	135 (75–256)	127 (58–299)	0.737
Glucose tolerance, NGT/IGT/DM	10/8/2	15/4/1	0.153
AFP (ng/mL)	7.5 (3.0–47.0)	7.0 (2.0–30.5)	0.002
Adiponectin (μg/mL)	9.6 (1.9–20.7)[a]	8.6 (2.3–25.1)[b]	0.463
Leptin (ng/mL)	9.0 (2.1–16.2)[a]	5.2 (1.3–14.6)[b]	0.039
Leptin/adiponectin ratio	1.1 (0.2–4.9)[a]	1.5 (0.4–12.0)[b]	0.028
sTNFR2 (pg/mL)	3170 (2010–5000)[a]	3050 (1600–5000)[b]	0.938
VFA (cm^2)	96 (31–220)	68 (12–159)	0.001
Liver histology			
Inflammation: A0/A1/A2/A3	0/12/7/1	–	–
Fibrosis: F0/F1/F2/F3/F4	0/8/9/3/0	–	–
Steatosis (%): < 5/5–30/≥ 30	10/8/2	–	–

Values are medians (range) or number of patients. BMI: body mass index, IFN: interferon, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ-glutamyl transpeptidase, HDL-C: high-density lipoprotein cholesterol, FPG: fasting plasma glucose, FSI: fasting serum insulin, HOMA-IR: homeostasis model assessment for insulin resistance, WBISI: whole-body insulin sensitivity index, HOMA-β: homeostasis model assessment for β cell function, NGT: normal glucose tolerance, IGT: impaired glucose tolerance, DM: diabetes mellitus, AFP: α-fetoprotein, sTNFR2: soluble tumor necrosis factor receptor 2, VFA: visceral fat area. [a]n=19, [b]n=18.

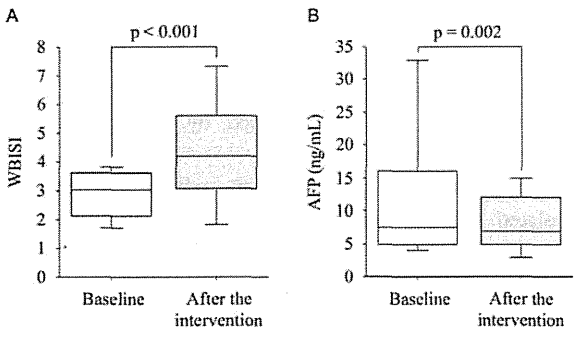

Figure 3. Effects of the lifestyle intervention on the (A) whole-body insulin sensitivity index and (B) serum α-fetoprotein level. AFP: α-fetoprotein, WBISI: whole-body insulin sensitivity index

in healthy subjects.

Several reports have shown that reductions in the serum AFP levels following IFN treatment in patients with CHC can help to prevent the development of HCC, irrespective of viral eradication (10, 11, 41). However, a large, randomized controlled trial recently showed that long-term maintenance peg-IFN therapy in patients with advanced CHC does not prevent liver-related deaths and actually increases the overall mortality, primarily due to non-liver-related causes (42). Therefore, the long-term administration of IFN to prevent HCC is not recommended in patients with advanced hepatic fibrosis. In HCV-infected patients, we previously reported that an increased BMI is associated with an increased risk of HCC at a younger age (43) and that the occurrence of hyperglycemia after a glucose load is a significant risk factor for the development of HCC (44). Taken together, it is likely that improvements in systemic IR and/or glucose me-

tabolism via appropriate lifestyle modification can help to safely prevent hepatocarcinogenesis, even in patients with advanced CHC.

One limitation of our study is that we did not measure the fucosylated fraction of AFP (AFP-L3), an accepted specific marker for HCC (45). Therefore, future studies should determine which fraction of AFP is decreased by lifestyle interventions. Another limitation is that we did not evaluate the changes in alcohol intake after the lifestyle intervention. Changes in alcohol intake may affect IR and the serum AFP level.

In conclusion, this study showed that whole-body IR, an elevated AST level and advanced fibrosis are independently and directly correlated with an elevated AFP level in patients with CHC. We also found that lifestyle modification can reduce the AFP level and whole-body IR. To our knowledge, this is the first report to examine the relationship between the serum AFP level and systemic IR and to show that lifestyle modification can reduce the serum AFP level. Further prospective studies are needed to confirm whether the reduction in the serum AFP level achieved via lifestyle modification can prevent hepatocarcinogenesis in HCV-infected patients.

The authors state that they have no Conflict of Interest (COI).

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Impairment of health-related quality of life in patients with chronic hepatitis C is associated with insulin resistance

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Abstract
Background Although it has been reported that hepatitis C virus (HCV) infection is associated with a significant decline in health-related quality of life (HRQOL), the underlying causes and mechanisms are still unknown. Insulin resistance (IR) is recognized as a distinct aspect of chronic HCV infection. Therefore, we attempted to identify the factors including IR indices that are related to the HRQOL of patients with chronic hepatitis C (CHC).
Methods One hundred and seventy-five CHC patients (91 female, 84 male, mean age, 56.4 years) not using antidiabetic agents were included and underwent a 75-g oral glucose tolerance test (OGTT) and completed a self-administered HRQOL questionnaire, the Short Form 36 (SF-36), which is a well-validated questionnaire for assessing general QOL. Scale scores were standardized and summarized into physical and mental component summary (PCS and MCS). We investigated which clinical parameters, including homeostasis model assessment of insulin resistance (HOMA-IR), were associated with decline in PCS and MCS scores in CHC patients.
Results There were no significant differences in clinical parameters between high and low MCS, but there were significant differences in age, sex, hemoglobin, liver fibrosis, OGTT pattern, and HOMA-IR between high and low PCS. Multivariate analysis showed that HOMA-IR >2 was independently associated with lower PCS (OR 2.92, $p < 0.01$).
Conclusions Our results suggest that impairment of HRQOL, especially physical domains, in CHC patients is associated with IR.

Keywords Chronic hepatitis C · Health-related quality of life · Insulin resistance

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Introduction

Chronic hepatitis C virus (HCV) infection is currently a major health problem that is estimated to affect 170 million people worldwide [1]. In a significant proportion of patients, infection leads to liver cirrhosis with potential complications, including impaired liver function, portal hypertension, and/or hepatocellular carcinoma. In addition to these serious complications, several studies have indicated that chronic HCV infection might be associated with considerable impairment of health-related quality of life (HRQOL) regardless of the disease stage [2–6]. However, the precise mechanism of decreased HRQOL in chronic hepatitis C (CHC) patients has not been elucidated, although there are some reports indicating an association with the degree of fibrosis, sex or age [7, 8].

Insulin resistance (IR) in CHC patients is often established early in the course of infection, and related to hepatic steatosis and fibrosis [9–11]. Regarding the mechanism of IR associated with HCV infection, Kawaguchi et al. [12] have reported that HCV core region can directly evoke downregulation of hepatic insulin receptor substrate (IRS)-1 and IRS-2. Moreover, Shintani et al. [13] have demonstrated using HCV core gene transgenic mice that tumor necrosis factor (TNF)-α induced by HCV infection can suppress insulin-induced tyrosine phosphorylation of

IRS-1. Recent estimates have indicated that 30–70 % of patients with CHC display some evidence of IR [14, 15].

Therefore, the aim of this study was to investigate the relationship between HRQOL in CHC patients and clinical parameters focused on IR using the 36-item Short Form Health Survey (SF-36) [16].

SF-36 is a widely used self-administered questionnaire designed for use in clinical practice and research, health policy evaluations, and general population surveys. It has demonstrated consistently high reliability and validity in a variety of patient populations including CHC [2–4, 6–8, 16–20]. Although there are several questionnaires, such as Chronic Liver Disease Questionnaire (CLDQ) [21], Liver Disease Symptom Index (LDSI) [22], and Liver Disease Quality of Life (LDQOL) [23] for evaluation of QOL in chronic liver disease patients, we used SF-36 as a non-specific questionnaire to compare HRQOL in CHC patients with that in the general population.

Patients and methods

Study population

We included 190 consecutive patients with CHC diagnosed by HCV antibody test and polymerase chain reaction, who attended Saga Medical School Hospital between January 2005 and December 2008. They were not taking, or had not recently (within 6 months) taken, any antiviral medication. Patients were excluded if they fulfilled the following criteria: (1) hepatitis B virus surface antigen positivity; (2) autoimmune liver disease, alcoholic liver disease (20 g/day alcohol), and/or medication-associated liver damage; (3) taking insulin-sensitizing or antidiabetic medication; and (4) extremity disorders, and/or other significant problems including chronic renal and heart failure. These criteria led to exclusion of 15 patients, which left 175 who were eligible for inclusion. Liver biopsy was performed in 162 of the 175 patients (within 12 months of the study). All 175 patients gave informed consent, and the study was approved by the ethics committee of Saga Medical School in accordance with the Declaration of Helsinki (approved ID number: 2004-04-04 and 2007-04-02).

Assessment of HRQOL

HRQOL of CHC patients was assessed using the SF-36, a 36-item self-administered questionnaire encompassing eight physical and mental health domains and two physical and mental summary scales [24]. We used a Japanese-validated version of SF-36, and the data of the present study were compared to the Japanese normative sample score of SF-36 in 2966 individuals [25]. The eight subscales: physical functioning (PF), role-physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-emotional (RE) and mental health (MH), were calculated from the questionnaires as described previously [26–28]. Patients completed SF-36, and the resulting scores were transformed into a scale of 0 (worst possible score) to 100 (best possible score), as recommended by the questionnaire's originators. Physical and mental summary measures were obtained from the sum scores of the corresponding subscales, that is, PF, RP, BP and GH for the physical component summary (PCS) and VT, SF, RE and MH for the mental component summary (MCS). According to the recommendations given in the manual, subscale scores were calculated if at least half of the items on the respective scale were answered. Transformation of the raw scores was performed using Microsoft Excel (Redmond, WA, USA).

Clinical and laboratory assessments

All venous blood samples were taken after a 12-h overnight fast. For the oral glucose tolerance test (OGTT), patients ingested a solution containing 75 g glucose, and venous blood samples were collected at 0, 30, 60, 90 and 120 min for the measurement of plasma glucose and serum insulin concentrations. Glucose was determined by a glucokinase method and insulin was measured using a chemiluminescent enzyme immunoassay kit (Abbott Japan, Tokyo, Japan). Glucose tolerance was categorized into 3 groups according to the criteria of the World Health Organization [29] as follows: (1) normal glucose tolerance (NGT; fasting plasma glucose level <110 and 2-h plasma glucose level <140 mg/dL); (2) impaired glucose tolerance (IGT; fasting glucose level ≤ 126 mg/dL and 2-h glucose level ≥ 140 and ≤ 200 mg/dL); and (3) diabetes mellitus (DM; fasting glucose level ≥ 126 mg/dL or 2-h glucose level ≥ 200 mg/dL).

IR was evaluated by the homeostasis model assessment of insulin resistance (HOMA-IR) method, which was calculated as follows: HOMA-IR = fasting plasma glucose $\times$ fasting serum insulin/405. Body mass index (BMI) was calculated as kg/m^2. Serum HCV-RNA levels were identified by 2 different quantitative polymerase chain reaction (PCR) assays: one was Amplicore HCV Monitor version 2.0 and the other was COBAS Taq-Man HCV Monitor Test (both Roche Diagnostics, Tokyo, Japan). High viral load was defined as ≥ 100 kIU/mL by Amplicore method and ≥ 5.0 logIU/mL by Taq-Man method. The HCV genotype was determined on the basis of the sequence of the core region [30].

Liver histology

Percutaneous liver biopsy was performed under ultrasound imaging within 12 months before SF-36. Histological

hepatic fibrosis and inflammation were scored using the METAVIR scoring system [31]. Grade of fibrosis was classified from F0 to F4, with varying degrees of fibrosis as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Based on the degree of lymphocyte infiltration and hepatocyte necrosis, activity was classified from A0 to A3, with higher scores indicating more severe inflammation.

Statistical analysis

Comparisons between groups were made using the Mann–Whitney U test for continuous variables and the χ^2 or Fisher's exact probability test for categorical data. Multivariate analysis was performed using logistic regression analysis. Relationships between continuous variables were analyzed by Pearson's correlation coefficient test. Data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS software (Cary, NC, USA).

Results

Patient characteristics

Clinical, biochemical, virological and histological characteristics of all 175 CHC patients are summarized in Table 1. Forty-four (25 %) patients were considered obese, with BMI >25 kg/m^2, and 64 (37 %) patients were evaluated for IR, with HOMA-IR >2. About one-third of the patients showed abnormal glucose tolerance; with respect to degrees of glucose tolerance, 67, 21 and 12 % of patients showed NGT, IGT and DM, respectively. A large majority of HCV was genotype 1b (76 %). Liver biopsy samples were obtained from 162 patients, and 152 (94:4 %) indicated minimal to moderate necroinflammatory activity, although 10 patients had severe activity. Regarding fibrosis in the biopsy samples, F1, F2, F3 and cirrhosis (F4) were seen in 83 (51 %), 51 (31 %), 22 (14 %) and 6 (4 %) patients, respectively. Liver biopsy was not performed in the remaining 13 of the 175 patients because of refusal.

One woman had a low serum albumin level of 2.7 g/dL, but her other hepatic function tests were well preserved, such as 80 % of prothrombin activity and 1.5 mg/dL of total bilirubin level. Therefore, we included her in this study as compensated liver cirrhosis. One woman showed a high serum level of total bilirubin of 2.7 mg/dL. However, her liver histology showed a METAVIR fibrosis stage of F2. Her indirect bilirubin level was 2.2 mg/dL; therefore, we assumed that her bilirubinemia was constitutional jaundice, such as Gilbert disease. Therefore, we included her in the F2 group.

Table 1 Patient characteristics ($n = 175$)

	n or mean ± SD (range)
Age (years)	56.4 ± 9.9 (24–74)
Male/female	84/91
Body weight (kg)	59.1 ± 9.5 (39.4–91.5)
BMI (kg/m^2)	23.0 ± 2.9 (15.6–30.3)
>25/<25	44/131
WBC (/μL)	5,031 ± 1,622 (2,400–15,900)
Hemoglobin (g/dL)	13.8 ± 1.5 (9.4–17.8)
Platelets (×10^4/μL)	15.9 ± 5.1 (6.0–30.5)
Albumin (g/dL)	4.1 ± 0.3 (2.7–5.2)
Total bilirubin (mg/dL)	1.0 ± 0.3 (0.4–2.8)
AST (IU/L)	52 ± 34 (15–233)
ALT (IU/L)	63 ± 52 (9–395)
γ-GT (IU/L)	52 ± 52 (11–341)
Total cholesterol (mg/dL)	173 ± 31 (94–263)
Fasting glucose (mg/dL)	88.8 ± 14.0 (68–220)
Fasting insulin (μU/mL)	8.7 ± 5.3 (1.6–33.3)
HOMA-IR	1.9 ± 1.2 (0.3–6.8)
>2/<2	64/111
OGTT pattern: NGT/IGT/DM	118/37/20
Viral load: high/low	165/8
HCV genotype: 1/2/unknown	133/38/4
Histology ($n = 162$)	
Necroinflammatory: A1/A2/A3	93/59/10
Fibrosis: F1/F2/F3/F4	83/51/22/6

Data are expressed as mean ± SD (range). Histological necroinflammation grade and fibrosis stage were scored according to the METAVIR scoring system

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *BMI* body mass index, *DM* diabetes mellitus, *γ-GT* gamma-glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment of insulin resistance, *IGT* impaired glucose tolerance, *NGT* normal glucose tolerance, *OGTT* oral glucose tolerance test, *WBC* white blood cell

Assessment of HRQOL

We compared the HRQOL scores in CHC patients in our study with those in the Japanese normative sample, and there was no difference except for evaluation of GH (Fig. 1).

We evaluated factors associated with HRQOL scores in CHC patients. Dividing PCS and MCS score into 2 groups by each median score (PCS 57, MCS 46), univariate and multivariate analysis were performed. Univariate analysis showed that older age, female sex, low hemoglobin, high fasting insulin level, high HOMA-IR value, and impaired glucose tolerance were significant factors associated with lower PCS. In contrast, there was no significant factor

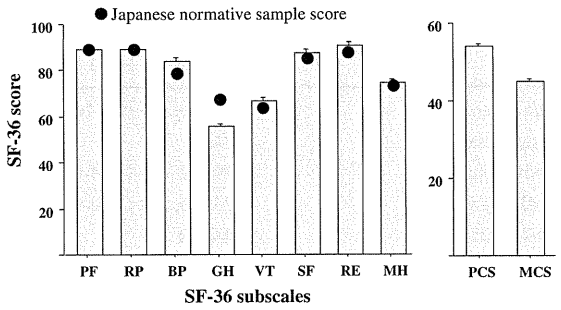

Fig. 1 SF-36 mean subscale scores in CHC patients. *Dots* are Japanese normative sample mean scores. In physical and mental component summary (PCS and MCS), mean score was 50, calculated from Japanese normative sample scores. *BP* bodily pain, *GH* general health, *MH* mental health, *PF* physical functioning, *RE* role-emotional, *RP* role-physical, *SF* social functioning, *VT* vitality

associated with MCS (Table 2). Multivariate analysis after adding fibrosis factor to the significant factors by univariate analysis indicated that high HOMA-IR value was the only significant factor associated with lower PCS (OR 2.92, $p < 0.01$, 95 % CI 1.37–6.18) (Table 3).

Correlations between SF-36 subscale, PCS and MCS scores and HOMA-IR

The correlation coefficients between eight subscales of SF-36 and HOMA-IR are shown in Table 4. RP and GH scores associated with PCS were strongly and negatively correlated with HOMA-IR. Relationships between PCS, MCS and HOMA-IR are shown by scatter plots (Fig. 2). PCS had a negative correlation with HOMA-IR value ($r = -0.234$, $p = 0.018$) (Fig. 2a); meanwhile, MCS was not associated with HOMA-IR ($r = 0.09$, $p = 0.48$) (Fig. 2b). Similar results were obtained for analysis of patients ($n = 152$) excluding F4 stage fibrosis or DM pattern in 75 g OGTT, which might have influenced their HRQOL or insulin resistance (Fig. 2c for PCS: $r = -0.224$, $p = 0.02$ and Fig. 2d for MCS: $r = 0.084$, $p = 0.58$).

Discussion

This cross-sectional study regarding HRQOL of Japanese CHC patients indicated that the impairment of the physical aspect of HRQOL was significantly related to IR evaluated by HOMA-IR, but was not associated with the demographic factors, inflammatory activity, fibrosis stage or viral factors.

IR or abnormal glucose metabolism are known to be clinical characteristics of HCV-infected patients [9–13]. Previous reports have indicated that eradication of HCV by interferon (IFN) therapy improves IR [32] and HRQOL scores [33, 34]. We have previously shown that sustained viral disappearance induced by IFN treatment improves systemic as well as hepatic insulin sensitivity, and decreases serum levels of soluble TNF receptor [35]. Lecube et al. [36] have demonstrated that serum levels of proinflammatory cytokines in CHC patients are higher than those in patients with chronic liver disorder due to other causes. Moreover, it has also been reported that the levels of circulating inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8 and TNF-α are related to fatigue in patients with acute myelogenous leukemia and myelodysplastic syndrome [37]. Judging from these reports, we assume that HCV infection increases systemic IR and production of inflammatory cytokines, which lead to impairment of HRQOL.

However, the limitation of this study was that there was no comparison of the relationship of QOL with IR between CHC and other chronic liver disease patients, including hepatitis B or fatty liver disease, and no data regarding inflammatory cytokines in the present study. A previous study has indicated that there was a significant association between declined physical functioning and elevated HOMA-IR in the general elderly population, not related to HCV or liver disease [38]. Therefore, we cannot conclude whether the association of HRQOL and IR is characteristic of HCV-infected patients, and whether it is mediated via cytokines. It should be clarified whether this association is dependent on disease etiology.

Our study indicated that IR was only associated with the physical component of HRQOL and not with the mental component. Meanwhile, Tillman et al. [39] have reported that MCS of SF-36, but not PCS, in CHC patients was significantly lower than that in patients with non-HCV liver disease such as chronic hepatitis B, primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis. Although that study had some differences in age, race and the setting of the control group from our study, these are not enough to explain the discrepancies in the results. Bonkovsky et al. [7] have reported that there is a significant difference in BMI between high and low scores of PCS but not MCS in CHC patients. Moreover, a systematic review has indicated that PCS in CHC patients with sustained virological response by IFN treatment was improved [40]. These reports support our results. At present, however, it is controversial which component, mental and/or physical, in QOL is impaired by HCV infection.

The causal relationship between HRQOL and IR remains largely speculative because our study was cross-sectional. Although it has been reported that glucose intolerance or high plasma glucose level might cause weak muscle strength and impair physical function [41], it is possible that impairment of physical aspects influences IR.

Table 2 Univariate analysis of factors associated with PCS and MCS

	PCS			MCS		
	<57 ($n = 89$)	>57 ($n = 86$)	p value	<46 ($n = 89$)	>46 ($n = 86$)	p value
Age (years)	58.1 ± 9.3	54.6 ± 10.3	0.02	55.6 ± 9.5	57.2 ± 10.1	0.26
Male/female	35/54	49/37	0.02	41/48	43/43	0.60
Body weight (kg)	59.3 ± 9.5	58.9 ± 9.6	0.83	58.8 ± 9.2	59.7 ± 9.8	0.47
BMI (kg/m^2)	23.3 ± 3.0	22.7 ± 2.8	0.16	22.8 ± 2.9	23.1 ± 2.9	0.45
WBC (/μL)	5,124 ± 1,990	4,932 ± 1,100	0.74	4,998 ± 1,881	5,065 ± 1,322	0.20
Hemoglobin (g/dL)	13.6 ± 2.2	14.0 ± 2.1	0.04	13.8 ± 2.0	13.8 ± 1.4	0.67
Platelets (×10^4/μL)	15.6 ± 5.2	16.2 ± 5.0	0.45	16.4 ± 5.2	15.4 ± 5.0	0.17
Albumin (g/dL)	4.1 ± 0.36	4.1 ± 0.4	0.43	4.1 ± 0.37	4.1 ± 0.39	0.07
Total bilirubin (mg/dL)	0.9 ± 0.30	0.9 ± 0.35	0.47	0.9 ± 0.35	1.0 ± 0.29	0.05
AST (IU/L)	53 ± 33	52 ± 35	0.84	51 ± 31	54 ± 37	0.53
ALT (IU/L)	61 ± 43	65 ± 61	0.62	62 ± 56	64 ± 49	0.86
γ-GT (IU/L)	50 ± 52	54 ± 54	0.61	48 ± 50	56 ± 55	0.32
Total cholesterol (mg/dL)	175 ± 31	172 ± 31	0.54	177 ± 31	170 ± 30	0.19
Fasting glucose (mg/dL)	89.8 ± 16.8	87.7 ± 10.3	0.36	87.4 ± 9.4	90.1 ± 17.5	0.50
Fasting insulin (μU/mL)	9.7 ± 6.1	7.7 ± 4.0	0.009	9.3 ± 6.2	8.1 ± 4.1	0.50
HOMA-IR	2.1 ± 1.3	1.6 ± 1.0	0.001	1.9 ± 1.3	1.8 ± 1.0	0.37
OGTT pattern: NGT/IGT + DM	53/36	65/21	0.02	59/30	59/27	0.74
Viral load: high/low	83/5	82/3	0.50	83/6	82/2	0.16
HCV genotype: 1/2	66/21	67/17	0.79	64/23	69/15	0.26
Histology						
A1/A2,3	41/36	52/30	0.19	47/32	46/34	0.80
F1,2/F3,4	62/18	72/10	0.08	68/13	66/15	0.67

Data are presented as mean ± SD. PCS was divided into 2 groups by median value: PCS <57 and PCS >57. MCS was divided into 2 groups by median value: MCS <46 and MCS >46

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *BMI* body mass index, *DM* diabetes mellitus, *γ-GT* gamma-glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment of insulin resistance, *IGT* impaired glucose tolerance, *NGT* normal glucose tolerance, *OGTT* oral glucose tolerance test, *WBC* white blood cell

Table 3 Multivariate analysis of factors associated with decline in physical component summary in CHC patients

	PCS <57		
	OR	p value	95 % CI
Age >60 years	1.87	0.07	0.93–3.78
Sex: female	1.53	0.33	0.64–3.68
Liver fibrosis: F3–4	1.22	0.68	0.46–3.25
IGT + DM	1.78	0.13	0.82–3.82
Hemoglobin <14	1.71	0.23	0.70–4.14
HOMA-IR >2	2.92	<0.01	1.37–6.18

DM diabetes mellitus, *HOMA-IR* homeostasis model assessment of insulin resistance, *IGT* impaired glucose tolerance

Table 4 Correlation coefficient between eight subscale scores of SF-36 (PF, RP, GH, VT, SF, RE, MH) and HOMA-IR

	Correlation coefficient	p value
PF	−0.143	0.06
RP	−0.252	0.0007
BP	−0.093	0.2
GH	−0.258	0.0005
VT	−0.110	0.1
SF	−0.158	0.04
RE	−0.181	0.02
MH	−0.076	0.3

BP bodily pain, *GH* general health, *MH* mental health, *PF* physical functioning, *RE* role-emotional, *RP* role-physical, *SF* social functioning, *VT* vitality

Longitudinal studies are needed to verify the detailed mechanism of this relationship.

The present study failed to demonstrate the difference in HRQOL defined by SF-36 scores between healthy individuals and CHC patients, except for general health score. This result was different from previous studies that have indicated that CHC patients have a diminished HRQOL compared with healthy controls across all SF-36 scores [2–6]. The reason for this discrepancy is outlined below. First, almost all the patients were willing to visit our tertiary

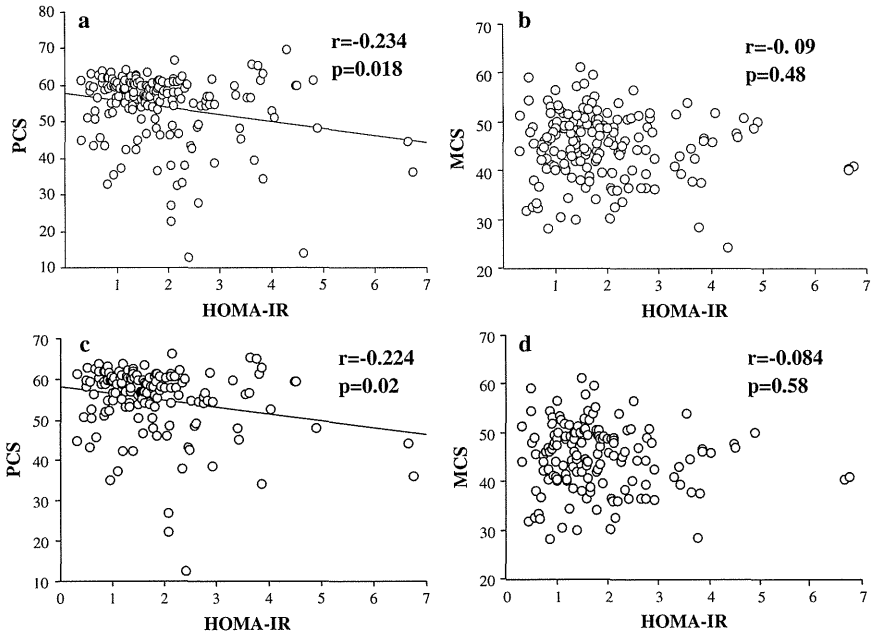

Fig. 2 Correlations between physical component summary (PCS) score and homeostasis model assessment of insulin resistance (HOMA-IR) (**a**, **c**). Correlations between mental component summary (MCS) score and HOMA-IR (**b**, **d**). PCS was associated with HOMA-IR in all patients (**a** $r = -0.234$, $p = 0.018$ by Pearson's correlation coefficient test) and in those excluding F4 stage fibrosis or diabetes mellitus (DM) pattern in 75 g OGTT (**c**, $r = -0.224$, $p = 0.02$). MCS was not associated with HOMA-IR in all patients (**b** $r = 0.09$, $p = 0.48$) and in those excluding F4 stage fibrosis or DM pattern in 75 g OGTT (**d** $r = 0.084$, $p = 0.58$)

hospital for IFN treatment in the future, so their QOL might be better than that in the general HCV-infected patients. Second, a previous large cross-sectional survey of unselected HCV-positive patients contained many with low household income, untreated diabetes, or a history of intravenous drug use, which were shown to be independent predictors of reduced HRQOL [42]. Our study did not include such patients.

Previous reports suggest that advanced liver fibrosis, especially cirrhosis, is strongly associated with decline of QOL [7, 8]. In the present study, we could not find a significant difference in HRQOL score between mild (F0–F2) and severe (F3, F4) fibrosis. However, we cannot deny the association between QOL and liver fibrosis, because our result might have been due to the small number of cases of liver cirrhosis ($n = 6$). Liver fibrosis evokes IR, therefore, further studies are necessary to elucidate the relationship between fibrosis and IR and QOL.

In conclusion, this study shows that diminished HRQOL, especially physical domains, in CHC patients is associated with IR. Improvement in IR due to weight reduction by diet and/or exercise, or using insulin sensitizers, might improve HRQOL in CHC patients, following good adherence to IFN treatment, although the relationship between IR and HRQOL warrants further exploration.

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Conflict of interest The authors declare that they have no conflict of interest.

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Control of a tumor suppressor PDCD4: Degradation mechanisms of the protein in hepatocellular carcinoma cells

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ABSTRACT

In this study, we demonstrate that EGF inhibits the TGF-β1-induced apoptosis of Huh7 cells. TGF-β1 up-regulates the expression of PDCD4 causing apoptosis, by stimulating the synthesis of PDCD4 mRNA via the Smad signaling pathway. TGF-β1 also inhibits the activation of S6 kinase 1 which phosphorylates the serine 67 residue of PDCD4 and leads to the phosphorylation of serine 71 and serine 76 in the β-TRCP binding sequence. This phosphorylation sequence causes the protein to be degraded in the ubiquitin–proteasome system. EGF activates S6 kinase 1 via the PI3K–Akt–mTOR signaling pathway and stimulates the degradation of PDCD4. EGF also suppresses PDCD4 mRNA levels. As the mTOR inhibitor rapamycin up-regulated PDCD4 mRNA levels, the PI3K–Akt–mTOR signaling pathway may control the transcription of the PDCD4 gene as well as the degradation of the protein. TPA also inhibited the TGF-β1-induced apoptosis of Huh7 cells, stimulating the degradation of the PDCD4-protein. Analyses using PDCD4 mutants with changes of serines 67, 71 and 76 to alanine revealed that the phosphorylation of serine 67 is not essential for the TPA-induced suppression of the protein. The mitogens could not suppress the PDCD4-mutant proteins with changes of serine 71 and/or serine 76 to alanine, however, indicating that phosphorylations at these residues are necessary for the proteasome-mediated degradation of PDCD4. The phosphor-mimic S71/D and S76/D mutants were able to be degraded in the ubiquitin–proteasome system unlike the mutants with changes of serine to alanine. The expression of S71/D mutant was suppressed with EGF but that of S76/D mutant was not indicating that at least partly the phosphorylation of both sites was mediated by different enzymes.

1. Introduction

It is widely known that transforming growth factor-β1 (TGF-β1) induces apoptosis in hepatocytes and in some hepatoma cell lines. Epidermal growth factor (EGF) is a potent growth factor for hepatocytes that inhibits the apoptosis induced by TGF-β1 [1–3]. We previously demonstrated that the expression of the programmed cell death 4 (PDCD4) gene increases in cultured hepatoma cells exposed to TGF-β1 and that ectopic PDCD4 over-expression induces such cells to undergo apoptosis [4]. These observations suggest that the induction of apoptosis by TGF-β1 is mediated by PDCD4. We observed both cellular changes and the localization of PDCD4 in the cytoplasm and nucleus when it is introduced to a hepatoma cell line. We further demonstrated that PDCD4 expression in the nucleus, associated with nuclear fragmentation, DNA ladders and apoptosis. These results confirmed that PDCD4 induces apoptosis in hepatoma cells [4].

The PDCD4 gene is so named because its expression is increased when apoptosis is induced [4,5]. Currently, this gene is considered a tumor suppressor gene that has the ability to inhibit carcinogenesis [6,7]. PDCD4 has a nuclear localization signal (NLS) at both its N and C termini. In the central region of the protein, two MA3 domains exist that are homologous to the M1 domain of eIF4G protein synthesis initiation factor [8,9]. At the molecular level, PDCD4 has been reported to inhibit cap-dependent translation by binding its MA3 domain to eIF4A, another translation initiation factor. PDCD4 also inhibits the transcription of certain genes inhibiting the activation of Ap-1, a transcription factor that promotes cell proliferation [10,11]. PDCD4 also suppresses invasion and metastasis [12–14]. Regarding apoptosis, PDCD4 is known to activate the caspase cascade by activating Bax and to release cytochrome C from the mitochondria, although the pathway of Bax activation has yet to be elucidated [4]. Recently, it has been reported that PDCD4 knockdown also induces apoptosis [15].

We have investigated the expression levels and significance of PDCD4 in hepatoma cells. Measurements of PDCD4-protein amounts

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were made in human hepatoma specimens using the Western blot technique. In all 10 of the subjects examined, the results showed decreased PDCD4 expression in cancer cells compared with non-cancer cells [4]. PDCD4 expression was also shown to be decreased in many other tumor tissues [16,17] via several different control mechanisms: the methylation of CpG islands suppresses its transcription [18], miRNAs (particularly miRNA 21) inhibit its translation [19] and mitogens stimulate the degradation of PDCD4-protein [20–22].

The PDCD4-protein is phosphorylated at serine 67 by S6 kinase1 which is initiated by the serum-activated Akt–mTOR signaling pathway, and PDCD4 can be further phosphorylated at serine 71 and serine 76, ubiquitinated and ultimately degraded in a proteasome [21]. Previously, we demonstrated that both TPA and EGF inhibit TGF-β1-induced apoptosis, suppressing the expression of PDCD4 in Huh7 hepatoma cells. We also suggested that in TPA treatments, either PKC-δ and -ε or another member of their signaling pathway associates with and phosphorylates the PDCD4-protein, leading to its degradation via the ubiquitin–proteasome system [23]. It is not completely clear, however, which mechanisms are involved in inhibiting the TGF-β1-induced apoptosis that is mediated by mitogens.

In this study, we investigate the mechanisms involved in the inhibition of TGF-β1-induced apoptosis and the suppression of PDCD4 that is carried out by mitogens.

2. Materials and methods

2.1. Cell culture and apoptosis assay

The human hepatoma cell line Huh7 was obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. To assay the TGF-β1-induced apoptosis and the pro-apoptotic signals, 5×10^5 cells were seeded in 60 mm dishes and cultured for 48 h. Then, the culture medium was replaced with a fresh medium containing 5 ng/ml TGF-β1. The cell samples were collected at 6, 12, 24 and 48 h.

For the apoptosis assay, cells were stained with Hoechst 33342 (Wako, Osaka, Japan), and apoptotic nuclei were counted under microscopy as previously described [4].

2.2. Reagents

The growth factors used were EGF and TGF-β1 (R&D Systems, Minneapolis, MN, USA). The PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma-Aldrich. The protein kinase inhibitors, rapamycin, LY294002, wortmannin, Ro-31-8425, SB203580, PD98059 and SP600125 were the products of Calbiochem (San Diego, CA, USA). The proteasome inhibitor MG132 was also obtained from Calbiochem.

The anti-PDCD4 antibody was prepared against the N-terminal 15 amino acid sequences of the protein in a rabbit as previously described [4]. The anti-Bax antibody (P-19) and anti-cytochrome C antibody (H-104) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-caspase-3, anti-caspase-8 and anti-caspase-9 antibodies were products of Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-Akt, p-Akt (S473), S6 kinase 1 and p-S6 kinase 1 (T389) antibodies were also obtained from Cell Signaling Technology Inc.

2.3. Preparation of pTK-EGFP-N1-PDCD4 and PDCD4 mutant (pTK-EGFP-N1-PDCD4mts) plasmids

A TK-promoter sequence was amplified by PCR using a pRL-TK vector (Promega, Madison, WI, USA) as the template and primers TK-1 (ATT AAT AAA TGA GTC TTC GGA CCT CGC GGG) and TK-2 (GCT AGC TTA AGC GGG TCG CTG CAG GGT CGC). That sequence was conjugated to the pT7Blue T-vector (Merck4Biosciences, Dermstadt, Germany) to create pT7Blue-TK-promoter. Separately, a pEGFP-N1-PDCD4 plasmid was prepared by conjugating PDCD4 to the N-terminal of EGFP in the pEGFP-N1 vector (Clontech, Laboratories Inc., Palo Alto, CA, USA). Then, the TK-promoter sequence was cut from the pT7Blue-TK-promoter vector using the Ase1 and Nhe1 restriction enzymes and conjugated to the Ase1 and Nhe1 sites of pEGFP-N1-PDCD4 to create the pTK-EGFP-N1-PDCD4 plasmid which contained the TK-promoter rather than the CMV-promoter. We used this plasmid and the QuikChange site-directed mutagenesis kit (Aligent Technologies, Santa Clera, CA, USA) according to the manufacturer's protocol to create the plasmids pTK-EGFP-N1-PDCD4-mt67 (S67/A), pTK-EGFP-N1-PDCD4-mt71 (S71/A) and pTK-EGFP-N1-PDCD4-mt76 (S76/A), in which either serine 67, 71 or 76 of PDCD4 was replaced with alanine, respectively. The primers that were used for the replacement of serine 67 with alanine (S67/A) were S67A-1 (CTA AGG AAA AAC GCA TCC CGG GAC) and S67A-2 (GTC CCG GGA TGC CTT TTT CCT TAG):those used for the replacement of serine 71 with alanine (S71/A) were S71A-1 (CAT CCC GGG ACG CTG GCA GAG GCG) and S71A-2 (CGC CTC TGC CAG CGT CCC GGG ATG): and those used for the replacement of serine 76 with alanine (S76/A) were S76A-1 (GGC AGA GGC GAT GCG GTC AGC GAC) and S76A-2 (GTC GCT GAC CGC ATC GCC TCT GCC). The above methods used for the replacement of serine with alanine were also used for the preparation of the plasmids pTK-EGFP-N1-PDCD4mt67D(S67/D), pTK-EGFP-N1-PDCD4mt71D(S71/D) and pTK-EGFP-N1-PDCD4mt76D(S76/D) in which either serine 67, 71 or 76 of PDCD4 was replaced with aspartic acid (D), respectively. The primers S67D-1 (CTA AGG AAA AAC GAT TCC CGG GAC) and S67D-2 (GTC CCG GGA ATC GTT TTT CCT TAG) were used for the replacement of serine 67 with aspartic acid (S67/D), those used for the replacement of serine 71 with aspartic acid (S71/D) were S71D-1 (CCC GGG ACG ATG GCA GAG GCG) and S71D-2 (CGC CTC TGC CAT CGT CCC GGG) and those used for the replacement of serine 76 with aspartic acid (S76/D) were S76D-1 (GGC AGA GGC GAT GAT GTC AGC GAC) and S76D-2 (GTC GCT GAC ATC ATC GCC TCT GCC). For the preparation of double and triple mutants, the above procedures were repeated for each mutation.

2.4. Transfection of plasmids

Huh7 cells were cultured for 4 days and then transfected with the PDCD4 plasmids using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 20 to 24 h of transfection, the cells were divided into 2–5 dishes, cultured for another 20 to 24 h and treated with inhibitors and/or mitogens before being used for Western blot analyses.

2.5. Western blot

The cytosol fractions were obtained as described previously [4]. Briefly, the harvested cells were suspended in a hypotonic buffer (250 mM sucrose, 20 mM HEPES/KOH at pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1.8 mg/ml iodoacetamide), passed through a 25-gauge needle and fractionated by centrifugation. The supernatants of centrifugation at 100,000 *g* for 60 min at 4 °C were used as the cytosol fraction. The total cell extracts were obtained by sonication in a SDS buffer containing 2.3% SDS, 0.05 M Tris–HCl at pH 6.8 and 0.1 mM PMSF followed by centrifugation at 10,000 *g* for 10 min at 4 °C. The protein amounts were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard. Each 30 or 40 μg protein sample was mixed with NuPAGE sample buffer (Novex, San Diego, CA, USA) and separated on polyacrylamide gel (SDS-PAGE). The bands in the gels were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were then blocked by incubating with 5% skim milk in PBS containing 0.1% Tween 20 either for 1–2 h at room temperature or overnight at 4 °C, followed by an incubation with

the first antibody for 1 h at room temperature. Specific bands were visualized by further incubation with the horseradish peroxidase (HRP)-conjugated second antibody, which was followed by a chemiluminescence reaction using the ECL system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The rabbit polyclonal anti-β actin antibody (Cell Signaling Technology Inc.) was used as a control.

2.6. Real-time RT-PCR

The cellular total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan). Reverse transcription (RT) and real-time PCR were performed using the TaqMan® Gene Expression Assays (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. The primer code number used for PDCD4 was Hs00205438-m1, and that for the control GAPDH was Hs99999905-m1.

3. Results

3.1. EGF suppressed the expression of PDCD4 and the apoptosis induced by TGF-β1

EGF is known to inhibit TGF-β1-induced apoptosis, pro-apoptotic mitochondria events and caspase cascade activation [1–3]. As shown in Fig. 1A, TGF-β1 induced apoptosis in the hepatoma cell line Huh7, while EGF inhibited that apoptosis in a dose-dependent manner. It was shown previously that TGF-β1 up-regulated PDCD4 expression and activated pro-apoptotic signals [4]. To confirm this result, Huh 7 cells were cultured in the presence of TGF-β1 and various concentrations of EGF, and their cytosolic fractions were subjected to Western blot. As shown in Fig. 1B, TGF-β1 increased PDCD4 expression and activated pro-apoptotic signals. Specifically, a decrease in the Bax level, an increase in cytochrome C and the activation of caspase-8, -9 and -3 were observed in the cytosol fractions, which agreed with previous results [4]. The Western blot analysis showed that EGF treatments decreased the PDCD4 and cytochrome C levels and increased the Bax levels in the cytosol fraction, blocking the activation of caspase-8, -9 and -3, in an EGF-dose-dependent fashion (Fig. 1B). These results indicate that EGF suppresses TGF-β1-induced apoptosis by inhibiting PDCD4 expression as PDCD4 over-expression causes apoptosis [4]. To ensure that EGF specifically suppressed the TGF-β1-induced PDCD4 expression, Huh7 cells were cultured for 24 h in the presence of TGF-β1 and treated with EGF in a medium without serum. The TGF-β1-induced PDCD4 over-expression was reduced, reaching its normal level after 3 h of EGF treatment (Fig. 1C).

3.2. EGF stimulates PDCD4 degradation through the PI3K–Akt–mTOR–S6K1 signaling pathway

The cellular increase in PDCD4 levels caused by TGF-β1 treatments returned to normal a few hours after treatment with EGF (Fig. 1C). These results indicate that the degradation of PDCD4 can be controlled with an EGF-activated signaling pathway. To test this, protein kinase inhibitors were employed. The mTOR inhibitor rapamycin significantly increased PDCD4 levels regardless of whether EGF and/or TGF-β1 were present (Fig. 2A). The PI3K inhibitors, wortmannin (Fig. 2A) and LY294002 (Fig. 2B) also blocked the EGF-induced suppression of PDCD4, although the effects were weaker than those of rapamycin, while a pan-PKC inhibitor Ro-31-8425 did not affect the EGF-induced suppression of PDCD4 (Fig. 2A). The inhibitors of p38, MEK-1 and JNK were not significantly effective at increasing PDCD4 levels compared with the inhibitors of the PI3K–mTOR signaling pathway (Fig. 2B). These results indicate that in the EGF treated Huh7 cells, the PDCD4-protein levels are primarily suppressed through the PI3K–mTOR signaling pathway.

The results of this experiment suggest that EGF activates the PI3K–mTOR signaling pathway. They also indicate that the PDCD4-protein is degraded by the ubiquitin–proteasome system after being phosphorylated by S6 kinase 1 (S6K1), a phosphorylation that is activated through the PI3K–mTOR signaling pathway [21]. To confirm this, the EGF-induced phosphorylation levels of Akt and S6K1 were examined. As shown in Fig. 3, the phosphorylation of Akt and S6k1 was stimulated by EGF treatment. LY249002, an inhibitor of the Akt-upstream enzyme PI3K, inhibited the EGF-induced phosphorylation of Akt: however, rapamycin, an inhibitor of the Akt-downstream enzyme mTOR, was less effective in suppressing Akt-phosphorylation. The EGF-induced

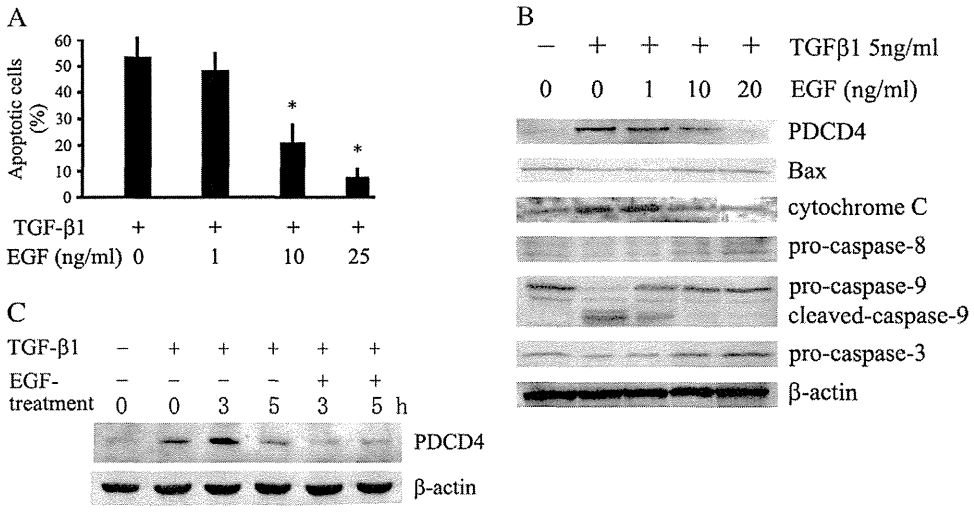

Fig. 1. EGF inhibits the TGF-β1-induced apoptosis of Huh7 cells by recovering the TGF-β1-induced PDCD4 expression. (A) Huh7 cells were cultured for 24 h with 5 ng/ml TGF-β1 in the presence of various amounts of EGF, as indicated in the figure. The cells were stained with Hoechst 33342. The numbers of apoptotic irregular cell nuclei were counted under a microscope, and the data are presented as the mean + the standard deviation for five randomly chosen visualized areas. * $P < 0.01$ when compared to cells without EGF. (B) EGF suppressed the TGF-β1-induced activation of pro-apoptotic molecules. After culturing Huh7 cells as described in (A), the cytosolic cell fractions were analyzed by Western blot. (C) EGF returned the TGF-β1-induced PDCD4 over-expression to basal levels in 3 h. Huh7 cells were cultured for 20 h in the presence of 5 ng/ml TGF-β1 and then treated with 20 ng/ml EGF in the absence of FBS for the times indicated in the figure. The cell extracts were analyzed by Western blot.

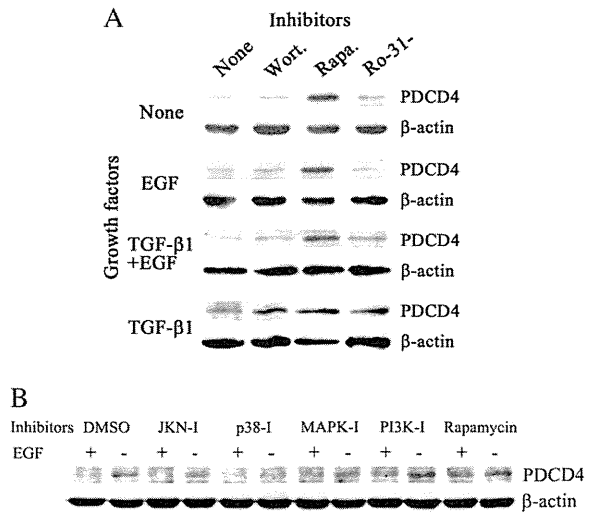

Fig. 2. Rapamycin and two PI3K inhibitors up-regulated PDCD4 expression and inhibited EGF-induced PDCD4 suppression but other kinase inhibitors had only a slight effect. (A) Huh7 cells were cultured for 20 h with 20 ng/ml EGF and/or 5 ng/ml TGF-β1 in the presence of DMSO as a control (None), 10 nM wortmannin (Wort.), 0.1 nM rapamycin (Rapa.) or 80 nM Ro-31-8425 pan-PKC inhibitor (Ro-31-). (B) Huh7 cells were cultured for 4 h without FBS in the presence of either DMSO, 100 nM SP600125 (JNK-I), 1.2 μM SB203580 (p38-I), 4 μM PD98059 (MAPK-I), 3 μM LY294002 (PI3K-I) or 0.1 nM rapamycin. The cells were then further cultured for 3 h with (+) or without (−) 20 ng/ml EGF. Cell extracts were subjected to Western blot to analyze their PDCD4 expression.

phosphorylation of S6K1 was largely inhibited by both LY249002 and rapamycin. MG132, a proteasome inhibitor, inhibited the PDCD4 suppression caused by EGF in both the presence and the absence of TGF-β1 (Fig. 4). These results indicate that EGF stimulated the activation of S6K1 and the consequent degradation of PDCD4 in proteasomes [21].

3.3. S67 Phosphorylation is necessary for EGF-induced suppression of PDCD4 but not for that induced by TPA

It was demonstrated that PDCD4 is phosphorylated at serine 67 (S67) by S6K1 which is activated by mTOR. The phosphorylation at S67 triggers further phosphorylations at serine 71 (S71) and serine 76 (S76) and is followed by the protein's degradation in the ubiquitin–proteasome system [21]. We have shown previously that the TPA-induced suppression of the protein was not inhibited by the mTOR inhibitor rapamycin. To determine whether the phosphorylations of

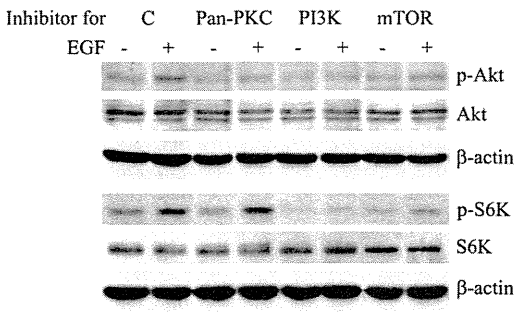

Fig. 3. The inhibition of the Akt-mTOR signaling pathway suppressed the EGF-induced activation of S6K-1. Huh7 cells were cultured for 4 h in the absence of FBS with either DMSO (C), or one of the following inhibitors: 100 nM Ro-31-8425 (Pan-PKC), 3 μM LY294002 (PI3K) or 0.1 nM rapamycin (mTOR). The cells were further cultured for 30 min after the addition of 20 ng/ml EGF, then harvested and subjected to Western blot analysis.

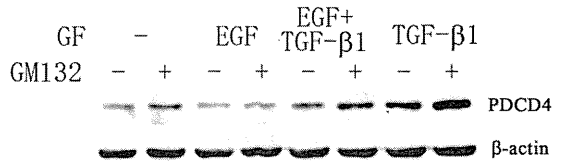

Fig. 4. MG132, a proteasome inhibitor inhibited the EGF-induced suppression of PDCD4. Huh7 cells were cultured for 20 h in the presence or absence of EGF (20 ng/ml) and/or TGF-β1 (5 ng/ml) and then treated for 4 h with 25 μM MG132. The cells were subjected to Western blot analysis.

S67, S71 and S76 are involved in the TPA- or EGF-induced suppression of PDCD4, mutant plasmids were created that replaced the protein's serine with alanine at S67 (S67/A), S71 (S71/A) and S76 (S76/A). These mutants were expressed in Huh7 cells. As shown in Fig. 5, the ectopic expression of PDCD4-GFP fusion proteins was low in the wild type- and S67/A mutant-transfected cells. However, the ectopic expression of other mutants, S71/A and S76/A, as well as their combinations S67/A–S71/A, S67/A–S76/A, S71/A–S76/A and S67/A–S71/A–S76/A, was quite high and was unaffected by the mitogen treatments (Fig. 5A and C). However, treatment with MG132, a proteasome inhibitor, up-regulated the expression of the fusion protein in both wild type- and S67/A-transfected cells, but did not alter the expression of the S71/A, S76/A or combination mutants (Fig. 5B upper panel). Rapamycin treatments also up-regulated the fusion-protein expression of wild type- and S67/A mutant-transfected cells but did not alter the protein's expression in any other mutants (Fig. 5B lower panel). The transfected cells were treated with either TPA or EGF in the presence of rapamycin: TPA down-regulated the ectopic expression of the fusion protein but EGF was less effective for down-regulating the expression levels in the wild type plasmid transfected cells (Fig. 5C and D), in the mutant-transfected cells, TPA down-regulated the ectopic expression of the fusion protein in only the S67/A mutant-transfected cells, while EGF had no effect on any cell type (Fig. 5C). The TPA-induced suppression of both the wild type- and S67A mutant-PDCD4 was inhibited by the pan-PKC inhibitor Ro-31-8425 (Fig. 5D). These results indicate that the phosphorylation of S67 by S6K1 is required for the EGF-induced degradation of PDCD4 but is not essential for the TPA-induced suppression of PDCD4, while phosphorylation of S71 and S76 is necessary for the mitogen-induced suppression of PDCD4. Furthermore, the expression of the PDCD4-GFP fusion protein with mutations at S71 and/or S76 was not suppressed by either EGF or TPA.

3.4. Serine 71 and serine 76 are phosphorylated by different enzymes

As shown in Fig. 6A, the expression levels of the phospho-mimic PDCD4 mutants S67/D, S71/D and S76/D were low but were up-regulated by the treatment with MG132 proteasome inhibitor, like the S67/A PDCD4 mutant, in Huh7 cells. Rapamycin also increased the expression of S67/D and S71/D mutants while the reagent did not increase that of the S76/D mutant (Fig. 6A). The expression of all these mutants S67/D, S71/D and S76/D was up-regulated by the treatment with Ro-31-8425 pan-PKC inhibitor as shown in Fig. 6B. When the Huh7 cells transfected with these mutants were treated with EGF or TPA in the presence of the pan-PKC inhibitor, EGF suppressed the expression of S67/D and S71/D mutants but not that of the S76/D mutant (Fig. 6B). These results indicate that all of these phosphor-mimic mutants can be degraded in the ubiquitin–proteasome system and the phosphorylation of S76 is at least partly mediated by EGF-induced signaling pathway.

3.5. The PI3K–mTOR signaling pathway controls the PDCD4 mRNA levels

As shown in Fig. 7, treating Huh7 cells with TGF-β1 up-regulated the PDCD4 mRNA levels in agreement with previous results [4]. When the

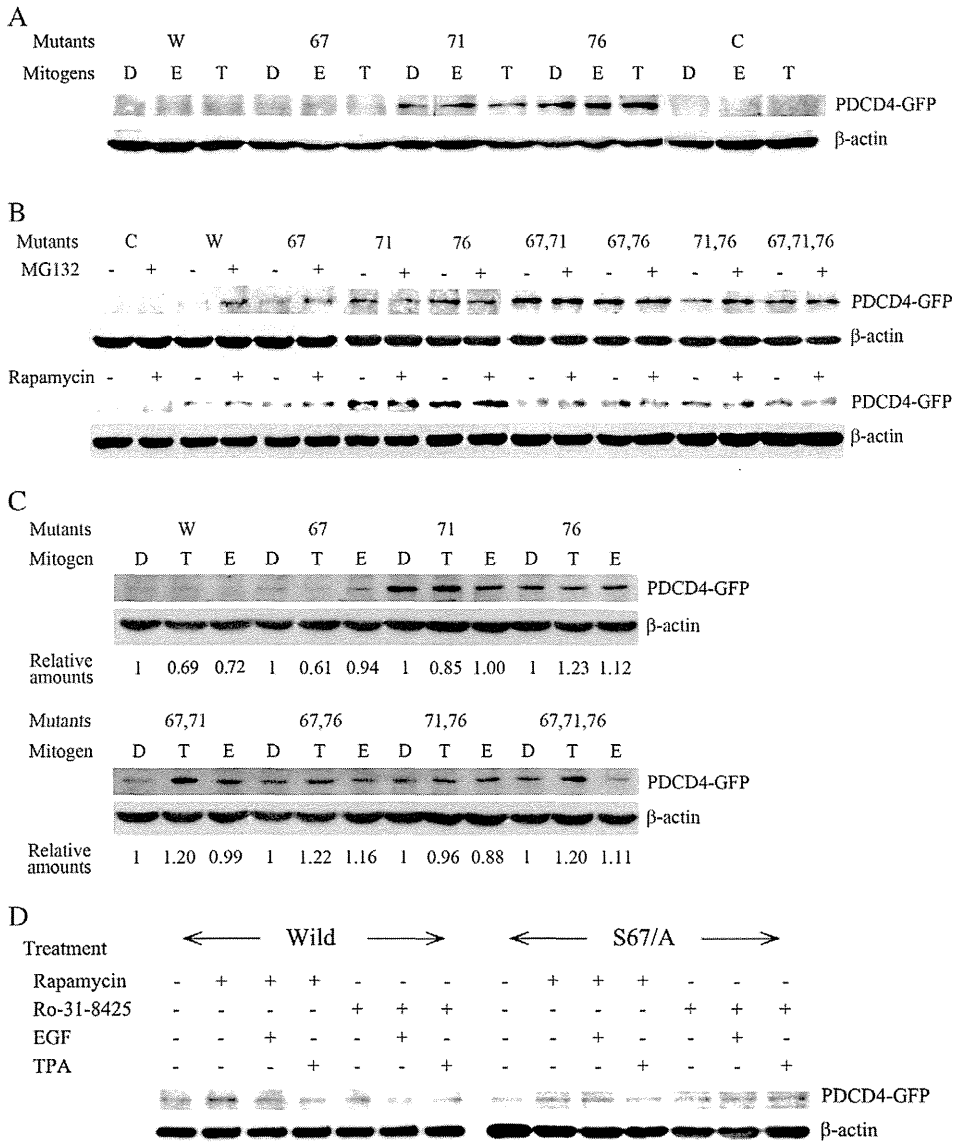

Fig. 5. S67 Phosphorylation is necessary for the suppression of PDCD4 by EGF but not by TPA. (A) The ectopic expression of wild type- and S67/A mutant-PDCD4-GFP fusion proteins was very low in the Huh7 cells. The expression of S71/A and S76/A mutants was high and was not affected by the mitogens. Huh 7 cells were transfected with the wild type PDCD4 plasmid pTK-GFP-PDCD4 (W) and the mutant PDCD4 plasmids pTK-GFP-PDCD4mts containing S67/A (67), S71/A (71) and S76/A (76) mutant PDCD4. The cells were divided into 3 dishes 24 h after transfection. The transfected cells were cultured for another 24 h and then treated for 3 h with DEMSO (D) as a control, 20 ng/ml EGF (E) or 50 nM TPA (T). The cells were subjected to Western blot analysis. Column C represents cells that were transfected with an empty vector. (B) The expression levels of wild type and S67/A mutant PDCD4 were up-regulated by treatment with MG132 or rapamycin but the expression levels of other mutants were not affected. Huh 7 cells were transfected with either pTK-GFP-PDCD4 plasmid containing wild type PDCD4 (W) or pTK-GFP-PDCD4mts containing single mutant PDCD4, S67/A (67), S71/A (71) or S76/A (76): double mutant S67/A–71S/A (67, 71), S67/A–S76/A (67, 76) or S71/A–S76/A (71, 76): or triple mutant S67/A–S71/A–S76/A (67, 71, 76). Each transfected cell culture was divided into 2 dishes 24 h after transfection. For the MG132 treatment (upper panel), the 2 divided dishes were treated for 4 h with control DMSO (−) or 20 μM MG132 (+) after 24 h of culture. For the rapamycin treatment (lower panel), the 2 divided dishes were treated for 8 h with control DMSO (−) or 0.1 nM rapamycin after 24 h of culture. The cells were then subjected to Western blot analysis. Column C represents cells that were not transfected. These experiments were repeated at least 4 times and similar results were reproducibly observed. A representative result is shown in each case. (C) The expression of S67/A mutant PDCD4 was decreased by TPA but not by EGF, while the expressions of the other mutants were not affected by either mitogens. Huh 7 cells were transfected with wild type and mutant PDCD4 plasmids, and the transfected cells were divided into 3 dishes as described in (B). The dishes were treated with 0.1 nM rapamycin for 3 h after 24 h of culture. Then the cells were treated in the absence of FBS for 3 h with either DMSO as a control (D), 50 nM TPA (T) and 20 ng/ml EGF (E) in the presence of the rapamycin. The cells were subjected to Western blot analysis. The relative amounts are averages of 6 independent experiments and the representative amount of DMSO as a control (D) was 1 for each mutant. (D) The pan-PKC inhibitor Ro-31-8425 recovers the TPA-induced down-regulation of both the wild type- and S67A mutant-PDCD4. Huh7 cells were transfected with the wild type- or S67A mutant-PDCD4-GFP plasmid, and the cells were divided into 7 dishes 24 h after transfection. After culturing another 24 h, the dishes were treated in the absence of FBS for 3 h with 20 ng/ml EGF or 50 nM TPA in the presence and absence of rapamysin (0.1 nM) or Ro-31-8425 (100 nM), as indicated in the figure.

cells were treated with EGF, the PDCD4 mRNA levels were lower than those of the EGF-free cells in both the presence and absence of TGF-β1 (Fig. 7A). When mTOR activity was inhibited by rapamycin, the PDCD4 mRNA level increased (Fig. 7B). We observed that the PI3K inhibitor LY249002 also up-regulated the mRNA level (data not shown). Rapamycin induced the phosphorylation of Smads, up-regulating the expression of target genes of the Smad signaling system [24,25]. The phosphorylation of Smad2 (S465/467) and Smad3 (S423/425) was

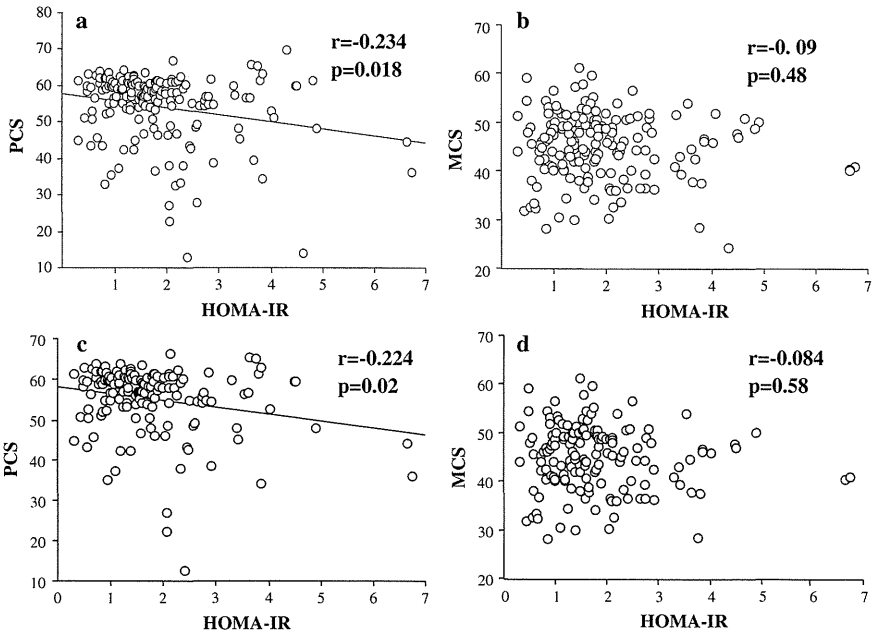

Fig. 2 Correlations between physical component summary (PCS) score and homeostasis model assessment of insulin resistance (HOMA-IR) (**a**, **c**). Correlations between mental component summary (MCS) score and HOMA-IR (**b**, **d**). PCS was associated with HOMA-IR in all patients (**a** $r = -0.234$, $p = 0.018$ by Pearson's correlation coefficient test) and in those excluding F4 stage fibrosis or diabetes mellitus (DM) pattern in 75 g OGTT (**c**, $r = -0.224$, $p = 0.02$). MCS was not associated with HOMA-IR in all patients (**b** $r = 0.09$, $p = 0.48$) and in those excluding F4 stage fibrosis or DM pattern in 75 g OGTT (**d** $r = 0.084$, $p = 0.58$)

hospital for IFN treatment in the future, so their QOL might be better than that in the general HCV-infected patients. Second, a previous large cross-sectional survey of unselected HCV-positive patients contained many with low household income, untreated diabetes, or a history of intravenous drug use, which were shown to be independent predictors of reduced HRQOL [42]. Our study did not include such patients.

Previous reports suggest that advanced liver fibrosis, especially cirrhosis, is strongly associated with decline of QOL [7, 8]. In the present study, we could not find a significant difference in HRQOL score between mild (F0–F2) and severe (F3, F4) fibrosis. However, we cannot deny the association between QOL and liver fibrosis, because our result might have been due to the small number of cases of liver cirrhosis ($n = 6$). Liver fibrosis evokes IR, therefore, further studies are necessary to elucidate the relationship between fibrosis and IR and QOL.

In conclusion, this study shows that diminished HRQOL, especially physical domains, in CHC patients is associated with IR. Improvement in IR due to weight reduction by diet and/or exercise, or using insulin sensitizers, might improve HRQOL in CHC patients, following good adherence to IFN treatment, although the relationship between IR and HRQOL warrants further exploration.

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Conflict of interest The authors declare that they have no conflict of interest.

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HEPATOLOGY

Severity of non-alcoholic steatohepatitis is associated with substitution of adipose tissue in skeletal muscle

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Abstract

Background and Aims: The pathogenesis of non-alcoholic fatty liver disease (NAFLD) is now focusing on its organ cross-talk with not only adipose tissue but also systemic skeletal muscle. Cross-sectional and longitudinal studies were conducted to determine the role of intramuscular adipose tissue content (IMAC) measured by computed tomography on the severity of NAFLD/non-alcoholic steatohepatitis (NASH).

Methods: Two hundred eight Japanese patients with NAFLD/NASH diagnosed by liver biopsy were enrolled into a cross-sectional study. Twenty-one patients were enrolled in a longitudinal study and received a programmed diet and exercise intervention, in some cases the combination of pharmacotherapy. We measured IMAC in the multifidus muscle and biochemical parameters, and conducted liver histology to assess NAFLD/NASH status.

Results: Histopathological stage in terms of simple steatosis and Brunt's classification was significantly correlated with IMAC ($P < 0.01$). Multivariate logistic regression analysis indicated that risk factors associated with the severity of NASH were IMAC and aging (IMAC: odds ratio = 2.444, $P < 0.05$; Age: odds ratio = 2.355, $P < 0.05$). The interventions improved histopathological changes in 11 patients with NASH as well as IMAC.

Conclusion: These results suggest that skeletal muscle fat accumulation may have been linked to the pathogenesis and severity of NASH.

Introduction

The National Cholesterol Education Program Expert Panel on the Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) and International Diabetes Federation have proposed diagnostic criteria for metabolic syndrome[1,2] that are now widely used in clinical research. Generally, studies of metabolic syndrome have shown that the prevalence of obesity has progressively increased in Japan in association with increasing adoption of Westernized lifestyles.

Visceral fat accumulation is considered to be one of the main risk factors for metabolic syndrome.[3–5] Abdominal computed tomography (CT) is commonly used to determine hepatic lipid deposition as well as visceral fat accumulation. We previously reported that visceral fat accumulation is correlated with the grade of lipid deposition in hepatocytes in patients with chronic hepatitis C and non-alcoholic fatty liver disease (NAFLD).[6–10] We have also developed a quantitative method to evaluate steatosis (intramuscular adipose tissue content [IMAC]) in the lumber multifidus muscle by abdominal CT as follows: IMAC = region of interest (ROI) of the multifidus muscle (Hounsfield units)/ROI of subcutaneous fat (Hounsfield units). Using this method, we investigated the association between visceral fat and skeletal muscle, and liver of NAFLD.[10]

We have reported that patients with NAFLD showed greater increases in IMAC compared with healthy individuals. We also found that IMAC improved significantly following improvements of insulin resistance, visceral fat accumulation, and hepatic lipid deposition achieved by diet and exercise interventions.[10]

The multifidus muscle was chosen in the present study. The reasons why this study focused on this muscle were as follows: (i) this muscle supported the trunk during extension, folding, and rotation of the upper body; (ii) this muscle was shown on CT at the umbilical level, which is used to quantify visceral fat accumulation; and (iii) it was possible to estimate the effects of exercise therapy.

Recently, it is well known that visceral fat accumulation induce peripheral organ such as skeletal muscle, liver, and myocardium as

"ectopic fat."[11–13] And, several reports have focused on the relationship between skeletal muscle fat accumulation and NAFLD/non-alcoholic steatohepatitis (NASH).[14–16] Therefore, the aims of the present study were to: (i) measure IMAC in the multifidus muscle on abdominal CT in patients with NAFLD or NASH; (ii) evaluate the relationship between IMAC and other markers, pathological severity of NASH; and (iii) determine whether therapeutic interventions (diet and exercise, or combination of medication) could improve IMAC of the multifidus muscle.

Methods

Patients. A total of 208 consecutive Japanese patients were attended Eguchi Hospital, Saga Medical School, Hiroshima University Hospital, or Nara City Hospital between January 2004 and April 2010 for the treatment of NAFLD were enrolled into the present studies. All of the patients had undergone biopsies at centers for digestive and liver diseases at each hospital. In this study, patients with evidence of excessive alcohol intake (> 20 g/day), other causes of liver diseases (e.g. viral hepatitis, autoimmune liver disease, biliary disease, liver cirrhosis, and hepatocellular carcinoma), or being treated with antihypertensive or antidiabetic agents were excluded. All of the patients underwent abdominal CT to measure IMAC as a marker for muscle steatosis.[10] All of the patients provided written informed consent, and the study protocol was approved by institutional review boards at each hospital.

Physical examination and serum biochemistry. Bodyweight and height were measured in all subjects. Body mass index (BMI) was calculated as bodyweight in kilograms divided by the square of the height in meters. Venous blood samples were taken from all subjects at around 09:00 h after a 12-h overnight fast. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), total cholesterol, triglyceride, albumin, fasting plasma glucose (FPG), and plasma insulin concentrations were determined by enzyme immunoassays.

Estimate of insulin resistance was calculated the homeostasis model of assessment-insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI). HOMA-IR reflects hepatic insulin resistance, whereas QUICKI reflects skeletal muscle insulin resistance.[17,18] The HOMA-IR was calculated using the formula: fasting plasma insulin × FPG/405, and the QUICKI was calculated using the formula: 1/(log fasting plasma insulin + log FPG). Based on a review of the literature, the following scores were calculated for each patient: FIB4 index,[19,20] NAFIC score.[21]

Liver histology. Liver biopsy specimens were fixed in 10% formalin and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin and Azan for histological evaluation. All liver biopsy specimens were reviewed by experienced hepatologists (Y.E and Y.S.) who were blinded to the patient's clinical status. Adequate liver biopsy samples were defined as samples > 1 cm long and/or ≥ 6 portal tracts. NASH was defined as steatosis with lobular inflammation and ballooning degeneration with or without Mallory–Denk bodies or fibrosis.[22,23] The histological findings of NASH were interpreted and scored by activity grade and fibrotic stage according to the classification system proposed by Brunt *et al.*[24] Hepatitis disease activity (i.e. necroinflammatory grade) was determined from the composite NAFLD activity score (NAS) as described by Kleiner *et al.*[25] NAS is the unweighted sum of the scores for steatosis, lobular inflammation, and hepatocellular ballooning, and ranges from 0 to 8. NAS < 3 is defined as "simple steatosis," NAS 3 or 4 is defined as "borderline NASH," and NAS ≥ 5 is defined as "definite NASH." If liver histology was too atypical to make a judgment, cases with an NAS of ≥ 5 were considered to have NASH. The severity of hepatic fibrosis (i.e. stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis.

Abdominal CT protocol and assessment. Unenhanced spiral acquisition of the liver was obtained during a breathhold at 5.0 mm collimation, 15.0 mm/rotation table speed (HQ mode, pitch 1:3), 120 kV (p), and auto mA (Bright Speed ELITE SD; GE Healthcare, Waukesha, WI, USA). Images were reconstructed at 10-mm intervals. All patients underwent abdominal CT in the morning after a 12-h overnight fast. On CT, ROIs of 40 mm^2 were placed along the periphery of the liver and the spleen, away from major vessels, at five points in each organ. The mean values of the five ROIs (Hounsfield units) were used to determine the liver-spleen (L/S) ratio as an index of hepatic fat accumulation.[26,27] Subcutaneous fat area (SFA; cm^2) and visceral fat area (VFA; cm^2) were measured at the umbilical level and were calculated using Fat Scan software (N2 System Co., Osaka, Japan).[28] Visceral obesity was defined as VFA ≥ 100 cm^2.[29]

CT analysis of the multifidus muscle. Subfascial muscular tissue in the multifidus muscle in an umbilical-level CT cross-sectional image was precisely traced, and CT values (in Hounsfield units) and area (cm^2) were measured using Advantage Workstation 4.1 software (GE Healthcare). CT values were measured for five 60 mm^2 ROIs on subcutaneous fat away from major vessels, and the mean values were used to determine the multifidus muscle/fat attenuation ratio.[10]

Longitudinal assessment of IMAC, histopathological changing, and other parameters. Patients with NASH diagnosed by liver biopsy received lifestyle intervention. The target of NAFLD/NASH treatment dietary energy intake was defined as standard bodyweight × 25–30 kcal, and exercise therapy was performed to achieve a target of 23 metabolic equivalent tasks (METs) × h/week (physical activity) + 4 METs × h/week (exercise).[9] After intervention, patients were divided into two groups according to whether or not IMAC was improved by diet and exercise therapy. To evaluate whether improvements in IMAC affected the histological findings, histopathological changes determined by Matteoni's and Brunt's classifications were compared between a group with the improvements in IMAC and a group with the non-improvements in IMAC.[22,24]