significant difference in body weight between the transgenic and control mice as already shown in Figure 1*A*. After the age of 3 months, the core gene transgenic mice developed hepatic steatosis, which is known to be one of the causes of insulin resistance in humans.[19] However, in 1-month-old mouse livers that were used in the analysis of insulin resistance, no hepatic steatosis was noted. No difference was observed in the levels of free fatty acids in the sera between the transgenic and control mice (0.56 ± 0.33 vs. 0.50 ± 0.21 mmol/L, n = 7 in each group, P = 0.65).

Then, we explored the role of the liver in pathogenesis of insulin resistance in the core gene transgenic mice. To directly measure HGP, the hyperinsulinemic-euglycemic clamp technique was conducted as described in Materials and Methods. The core gene transgenic mice showed a normal or slightly lower rate of HGP during the basal period as compared with control mice (Figure 5*A*). Although insulin infusion during the clamp suppressed HGP by 60% in the control mice, insulin induced little effect on HGP of the core gene mice (Figure 5*A*). This is consistent with the notion that insulin resistance in the core gene transgenic mice is chiefly depending on the shortage of insulin action on the liver.

To study the involvement of muscles in the development of insulin resistance in the core gene transgenic mice, we then examined whether or not insulin-stimulated glucose uptake is impaired in the skeletal muscles. The extensor digitorum longus muscle (EDL) from 2-month-old core gene transgenic and control mice were excised and exposed to insulin at the intermediate (0.30 nmol/L) and maximal (10.0 nmol/L) concentrations. There was no significant difference in 2-deoxyglucose uptake in the EDL muscle between the core gene transgenic mice and control mice at either insulin concentration (Figure 5*B*, at 0.30 nmol/L, P = 0.23 and at 10.0 nmol/L, P = 0.76). As another representative muscle that differs from EDL in metabolic properties, the soleus muscle was examined in the same manner as EDL. 2-Deoxyglucose uptake by the soleus muscle was not significantly different between the core gene transgenic and control mice (Figure 5*C*, at 0.30 nmol/L, P = 0.49 and at 10.0 nmol/L, P = 0.49). Thus, in the core gene transgenic mice, contribution of the peripheral skeletal muscle in the development of insulin resistance is negligible. This is in agreement with the observation that the core protein was exclusively present in the liver as detected by Western blotting,[20] which was confirmed by a sensitive enzyme immunoassay (Tsutsumi T. et al., unpublished data, December 2002).[21]

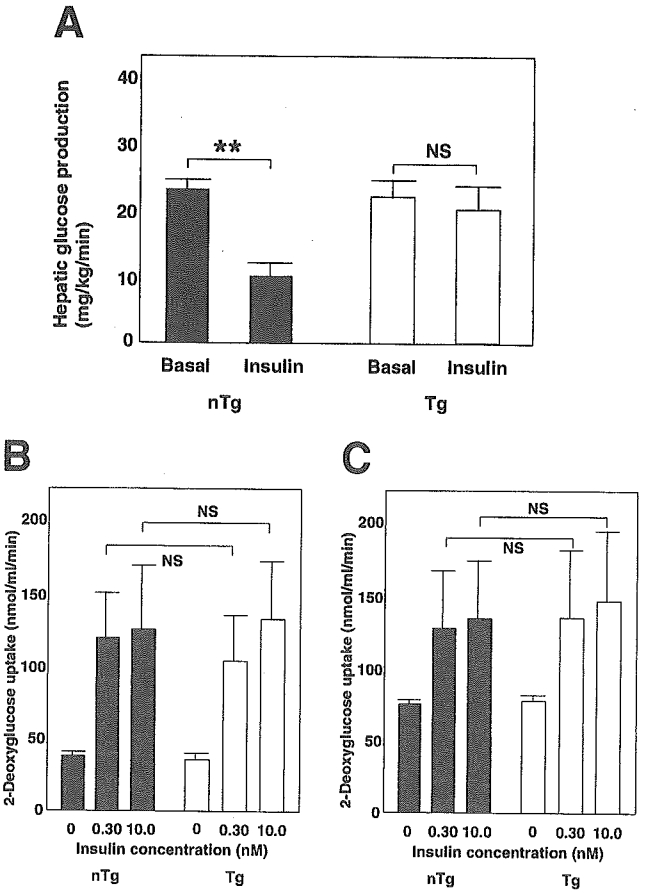

Figure 5. Characterization of glucose metabolism in the core gene transgenic mice. (*A*) Hyperinsulinemic-euglycemic clamp. Hepatic glucose production was calculated using hyperinsulinemic-euglycemic clamp. There was a failure of insulin to suppress hepatic glucose production in the core gene transgenic mice (n = 5 in each group). (*B* and *C*) Glucose uptake by the muscle after insulin stimulation. The extensor digitorum longus muscle (*A*) or soleus muscle (*B*) of 2-month-old mice were excised and exposed to insulin at intermediate (0.30 nmol/L) and maximal (10.0 nmol/L) concentrations. 2-Deoxyglucose uptake was determined as described in the Materials and Methods section (n = 8 in each group). Values are mean ± standard error; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

Elevated TNF-α Level and Altered Tyrosine Phosphorylation of Insulin Receptor Substrate-1 in the Liver and Insulin Resistance

We have noted an increase in TNF-α levels in the liver of HCV core gene transgenic mice,[13] which has also been documented in the sera of human hepatitis C patients.[22–25] On the other hand, TNF-α has been shown to induce insulin resistance in experimental animals and cultured cells.[26–29] Therefore, we next determined the protein expression level of TNF-α by ELISA in the liver of these mice that were used in the current study. The TNF-α levels in the liver of 2-month-old transgenic mice were 702.2 ± 283.3 pg/mg protein and 313.5 ±

113.6 pg/mg protein in that of 2-month-old control mice (n = 10 in each group, $P < 0.001$). Thus, the levels of TNF-α exhibited a more than 2-fold increase in the HCV core gene transgenic mice compared with the control mice, which may be associated with insulin resistance.

Suppression of tyrosine phosphorylation of IRS-1 and -2 is one of the mechanisms by which a high level of TNF-α causes insulin resistance.[29–31] We, therefore, examined the suppression of tyrosine phosphorylation of IRS-1 in response to insulin action in the core gene transgenic mice. Twenty minutes after the administration of human insulin (1 U/kg body weight), when the plasma glucose levels decreased (Figure 2*B*), IRS-1 in the liver of control mice exhibited a marked phosphorylation of its tyrosine. In contrast, phosphorylation level of tyrosine in IRS-1 in the liver of core gene transgenic mice manifested apparently no increase compared with the basal level after the administration of insulin (Figure 6). In contrast, there was no difference in the time course of tyrosine phosphorylation of IRS-2 between the core gene transgenic and control mice (data not shown). These results indicate that a suppression of tyrosine phosphorylation of IRS-1, that is, a suppression of the insulin action in the liver, is at least one of the mechanisms of insulin resistance in HCV transgenic mice, whereas pathways other than IRS-1 may also be involved.

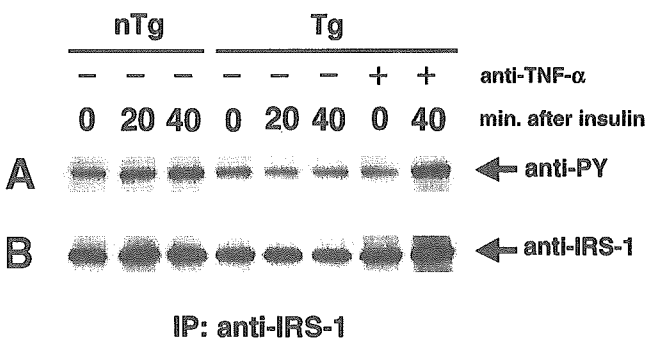

Figure 6. Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice and core gene transgenic mice with or without anti–TNF-α antibody treatment were analyzed before and 20 and 40 minutes after insulin administration. The samples were subjected to immunoprecipitation with anti-IRS-1 antibody and subsequently immunoblotted with antibodies as indicated. Experiments were performed in triplicate, and a representative picture is shown. (*A*) Immunoblot with antiphosphotyrosine antibody. There was no augmentation of phosphorylation of tyrosine in IRS-1 after insulin stimulation in the core gene transgenic mice, whereas tyrosine phosphorylation was markedly enhanced in control mice. Insulin-stimulated tyrosine phosphorylation was restored 40 minutes after anti–TNF-α antibody treatment. (*B*) Immunoblotting with anti-IRS-1 antibody as a control of IRS-1 load. nTg, nontransgenic mice; Tg, transgenic mice; anti-PY, antiphosphotyrosine antibody; anti-PS, antiphosphoserine antibody. IP, immunoprecipitation.

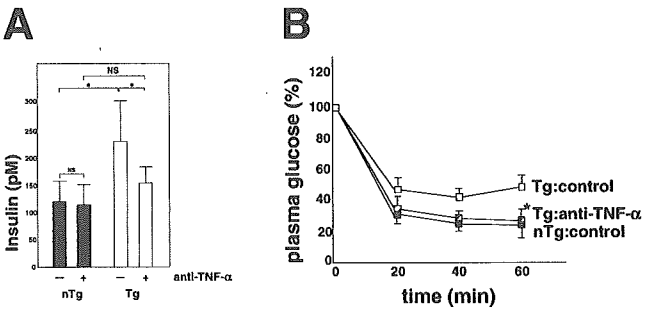

Figure 7. Serum insulin levels and insulin tolerance test after anti–TNF-α antibody treatment. (*A*) Serum insulin levels were determined in the fasting state with or without anti–TNF-α antibody treatment as described in the Materials and Methods section. Insulin levels decreased significantly after anti–TNF-α antibody treatment in the core gene transgenic mice (n = 5 in each group). (*B*) Insulin tolerance test (n = 5 in each group). Human insulin was administered by IP injection to fasted conscious mice and glucose concentrations were determined 24 hours after the second administration of anti–TNF-α antibody. As control, mice were injected with hamster IgG instead of anti–TNF-α antibody. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± standard error; $*P < 0.05$ when compared with Tg control; nTg, nontransgenic mice; Tg, transgenic mice.

The c-Jun N-terminal kinase (JNK) pathway has been shown to mediate the inhibitory effect of TNF-α on insulin action through the phosphorylation of serine in IRS-1.[32,33] Because an activation of the JNK pathway was observed in the liver of core gene transgenic mice,[13] phosphorylation of serine residues in IRS-1 was examined using antiphosphorylated serine monoclonal antibodies (Ser^{307} and Ser^{612}). However, there was no difference in the time course of serine phosphorylation after insulin stimulation between the core gene transgenic and control mice (data not shown).

Blockade of TNF-α Action Restores Insulin Sensitivity

Then the anti–TNF-α antibody was administered to block the in vivo activity of TNF-α in mice as described in the Materials and Methods section.[17] Twenty-four hours after the second administration of the anti–TNF-α antibody (200 μg/mouse), serum insulin levels in transgenic mice became significantly lower than the baseline (Figure 7*A*, 230.8 ± 70.7 vs. 153.6 ± 17.4 pmol/L, $P < 0.05$). Serum insulin levels in control mice also decreased, but there was no significant difference from the baseline (123.3 ± 36.1 vs. 112.0 ± 39.7 pmol/L, $P = 0.25$). Levels of FPG also decreased, but the difference from the baseline was not statistically significant. The insulin tolerance test conducted 24 hours after the second administration of anti–TNF-α antibody is shown in Figure 7*B*. As expected from serum insulin levels, anti–TNF-α antibody treatment restored the sen-

sitivity of the core gene transgenic mice to insulin activity. At this time point, phosphorylation of tyrosine in IRS-1 in the liver of transgenic mice in response to insulin action was restored to a similar level to that in control mice (Figure 6*A*, 40 minutes after insulin administration). These results strongly support the notion that the increased level of TNF-α is one of the bases for insulin resistance in the HCV core gene transgenic mice.

Taken together, these data indicate that the presence of the HCV core protein in the liver, at a level similar to that in human chronic hepatitis C patients,[21] confers insulin resistance to the mice by affecting the liver, by disturbing the insulin-induced suppression of hepatic glucose production.[34,35]

Discussion

Since Allison et al.[4] reported an association between HCV infection and diabetes, evidence has been accumulating connecting these 2 conditions. In such studies, HCV infection has a significantly stronger association with diabetes than hepatitis B viral infection.[4–7] The variables other than HCV infection that are associated with diabetes are cirrhosis, male sex,[5] and aging.[6] In addition to these clinic-based, case-control studies, Mehta et al.[7] have reported the result of investigation at population level. In this cross-sectional national survey, persons 40 years or older with HCV infection were more than 3 times more likely to have type 2 diabetes than those without HCV infection. Thus, the association of HCV infection with diabetes has become closer as shown by epidemiological studies. However, there are some difficulties in establishing a definite relationship between HCV infection and diabetes on the basis of epidemiological studies; in patients, there are other numerous factors perturbing the verification of the definite relationship, such as obesity, aging, or particularly advanced liver injuries. Moreover, the biological mechanism underlying diabetes or insulin resistance in HCV infection is unknown. In vitro or cultured cell studies have a very limited utility for the study of insulin resistance or diabetes because insulin resistance is a condition that involves multiple organs, such as the skeletal muscles and liver. Thus, the use of good experimental animal model systems may be useful both in establishing a definite relationship between diabetes and HCV infection and in elucidating the role of HCV in the development of insulin resistance.

In the current study, the HCV core gene transgenic mice exhibited insulin resistance as early as 1-month old, despite an apparent absence of glucose intolerance. Development of insulin resistance without any liver injury[10,11] or excessive body weight gain, as shown in the current study, clearly indicates that infection of HCV per se is a cause of the development of insulin resistance. Although only the core protein is expressed in these mice instead of HCV replication in humans, the fact that the intrahepatic core protein levels are similar between the core gene transgenic mice and chronic hepatitis C patients[20] warrants extrapolating the result into hepatitis C patients. Certainly, dispersion in the intrahepatic core protein levels in human chronic hepatitis C patients compared with the constant amount of the core protein must be taken into account. The occurrence of insulin resistance in the core gene transgenic mice as early as 1-month old also excluded the possibility that aging is a cause of insulin resistance. Nonetheless, aging could be an aggravating factor for insulin resistance. Thus, the current analysis shows a definite causal relationship between HCV infection and the development of insulin resistance.

Our earlier studies have shown the development of hepatic steatosis in these HCV core gene transgenic mice after the age of 3 months.[11] However, insulin resistance invariably preceded the occurrence of hepatic steatosis, indicating that insulin resistance is not a consequence of hepatic steatosis in these mice. Certainly, it is possible that insulin resistance in the core gene transgenic mice may be affected or aggravated after the occurrence of hepatic steatosis. On the other hand, insulin resistance may be one of the factors that cause hepatic steatosis,[19] whereas the impairment of very-low-density lipoprotein (VLDL) secretion from the liver and hypo-β-oxidation of fatty acids are considered to be the bases of development of hepatic steatosis in the core gene transgenic mice.[21,36]

The general mechanism underlying insulin resistance is not precisely understood and is considered to be multifactorial.[8,9,37,38] Chiefly, it involves glucose consumption by the skeletal muscle and glucose production in the liver. Our current analysis revealed a failure of insulin in the suppression of HPG in the liver and an absence of suppression of glucose uptake by the muscles in the core gene transgenic mice. Combined, these results indicate the insulin resistance in the core gene transgenic mice is chiefly due to hepatic insulin resistance. An elevated intrahepatic TNF-α level plays one of the roles in causing insulin resistance through suppressing insulin-induced tyrosine phosphorylation of IRS-1. It should be noted that TNF-α levels are invariably elevated in the sera of patients with HCV infection.[22] Moreover, restoration of insulin sensitivity after anti–TNF-α antibody administration strongly supports the notion that TNF-α

is, at least in this animal model, a major factor for the development of insulin resistance in HCV infection. Taken together, insulin resistance in the core gene transgenic mice mainly depends on suppression of the inhibitory effect of insulin on hepatic glucose production. This is consistent with the observation that the core protein is present only in the liver but absent in the skeletal muscle of the core gene transgenic mice (Tsutsumi T., unpublished data, December 2002).[21] Impairment in other undetermined pathways may also be responsible for the development of insulin resistance in HCV infection.

Insulin resistance alone does not always lead to the development of overt diabetes in humans or murine models. Particularly, in the models with the C57/BL6 strain,[18] hyperplasia of the islets of Langerhans in the pancreas compensates for insulin resistance by secreting higher amounts of insulin. Along with a gain in body weight by being fed a high-calorie diet, the core gene transgenic mice but no control mice developed overt diabetes, showing that obesity is a risk factor for diabetes as observed in patients or as shown in animal models for diabetes unrelated to HCV infection.[37,38] This observation would suggest that HCV infection confers insulin resistance and additional factors such as obesity, aging, or possibly inflammation may contribute to the complete development of overt diabetes. The effect of high-fat diet on control C57BL/6 mice may be milder in the current study compared with a previous study.[39] However, there was a substantial increase in FPG levels in high-fat-diet-fed control mice compared with normal-diet-fed control mice (Figures 1*B* and 4*B*). In addition, at fed-state, serum insulin levels in high-fat-diet-fed control mice were significantly increased compared with those in normal-diet-fed control mice (Figures 1*B* and 4*B*). It is unclear why plasma glucose levels were not very high at fed-state in control mice, but one possible explanation is the lower calorie content in the current study than those in the previous report: 4.70 kcal/g for our high-fat diet vs. 5.55 kcal/g for high-calorie diet in the previous study. A shorter duration of high-fat diet than the previous study (2 months vs. 6 months) may be another possible explanation.[39] Such a mild elevation in plasma glucose levels in high-fat-diet-fed C57BL/6 mice as the one observed in our study has also been described in previous studies.[40]

In conclusion, the HCV core protein induces insulin resistance in transgenic mice without gain in body weight at young age. These results indicate a direct involvement of HCV per se in the pathogenesis of diabetes in patients with HCV infection and provide a molecular basis for insulin resistance in such a condition.

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Y.S. and H.F. contributed equally to this work.

□ CASE REPORT □

A Patient with *E. coli*-induced Pyelonephritis and Sepsis Who Transiently Exhibited Symptoms Associated with Primary Biliary Cirrhosis

Nobuhiko OHNO, Yasuo OTA, Syuji HATAKEYAMA, Shintaro YANAGIMOTO, Yuji MORISAWA, Kunihisa TSUKADA, Kazuhiko KOIKE and Satoshi KIMURA

Abstract

A 28-year-old woman had chief complaints of headache and a 40°C fever. At this time, findings indicative of inflammation including elevated CRP and increased WBC were observed, and *E. coli* was detected on blood and urine culture. As a result, the patient was diagnosed with pyelonephritis and sepsis. Furthermore, markedly increased hepatobiliary enzymes and elevated anti-mitochondrial antibody were confirmed. The administration of antimicrobial agents resulted in improvement of the pyelonephritis and sepsis and normalization of hepatobiliary enzyme and anti-mitochondrial antibody levels. It has been documented that the incidence of urinary tract infection is high among patients with primary biliary cirrhosis (PBC). The findings obtained from the present patient are of considerable interest in elucidating the mechanism of onset in PBC.
(Internal Medicine 42: 1144–1148, 2003)

Key words: *E. coli*, pyelonephritis, sepsis, primary biliary cirrhosis

Introduction

Primary biliary cirrhosis (PBC), the histopathology of which is characterized by chronic non-suppurative destructive cholangitis, often affects middle-aged women (1). Concerning the hematological findings, levels of hepatobiliary enzymes such as γ-GTP and ALP, are usually elevated in the absence of raised transaminases. At this point in time, the exact cause of PBC is not known. PBC is considered an autoimmune disease, which is closely correlated to the presence of anti-mitochondrial antibody (2).

It has been reported that the incidence of urinary tract infection, especially *E. coli*-induced recurrent urinary tract infection, is high among PBC patients, and that patients with recurrent urinary tract infection are more likely to produce anti-mitochondrial antibody M2 (3–6). In this report we present the patient who was diagnosed with *E. coli*-induced pyelonephritis and sepsis, and who exhibited findings indicative of PBC, including markedly increased hepatobiliary enzymes and elevated anti-mitochondrial antibody. As the pyelonephritis improved, levels of hepatobiliary enzymes and anti-mitochondrial antibody normalized. The findings obtained from the present patient are of considerable interest in elucidating the mechanism of onset in PBC.

For editorial comment, see p 1063.

Case Report

A 28-year-old woman started to develop headaches on June 10, 2001, and on around June 15, started to experience fevers sometimes reaching a temperature of 40°C and accompanied by rigors. When the patient consulted the emergency department, physical examination was unremarkable other than tenderness of the right costovertebral angle. Routine laboratory analyses showed increased CRP and WBC accompanied by shift-to-left on differential counts (WBC: 15,700/mm^3, and CRP: 19.2 mg/dl) and elevated hepatobiliary enzymes (γ-GTP: 220 IU/*l*, ALP: 642 IU/*l*) (Fig. 1). However, abdominal ultrasound was negative for cholecystitis and cholangiectasis. On June 25, the patient was admitted to our department for further investigations. The patient was apyrexial at the time of admission, but developed a fever of over 38°C the following morning.

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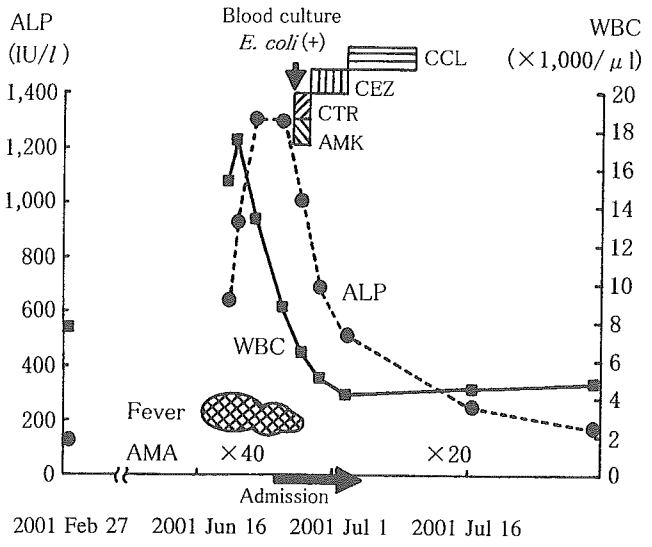

Figure 1. Clinical course of the patient. Cultures of blood and urine revealed *E. coli*. Antimicrobial therapy was initiated on June 27, resulting in the fever rapidly subsiding and the inflammation resolving. The levels of ALP decreased over time and had normalized by day 35 of the illness. WBC: white blood cell count, ALP: alkaline phosphatase, AMA: anti-mitochondrial antibody, AMK: Amikacin, CTRX: Ceftriaxone, CEZ: Cefazolin, CCL: Cefaclor.

However, no other vital signs were abnormal. Apart from the persisting tenderness of the right costovertebral angle, no other notable findings were detected on physical examination. On clinical laboratory tests on admission, findings indicative of inflammation were confirmed WBC: 8,800/mm^3 (My 3%, St 4%, and Seg 64%), CRP 4.4 mg/dl, erythrocyte sedimentation rate 100 mm/h, and urinary WBC 21–50/high power field. Moreover, although the levels of γ-GTP and ALP were elevated at 371 and 1,308 IU/*l*, respectively, no marked increases in transaminases or bilirubin were apparent (GOT: 31 IU/*l*, GPT: 47 IU/*l*, total bilirubin: 0.4 mg/dl). Furthermore, while the level of anti-mitochondrial antibody was elevated (titer: 40), the level of the M2 subtype of anti-mitochondrial antibody was not (titer: <5.0). Anti-nuclear antibody and anti-thyroglobulin antibody were negative. Cultures of blood and urine revealed *E. coli*. No abnormality was seen on the thoracoabdominal X-ray examination. Abdominal ultrasound showed mild enlargement of the left kidney, but no sign indicative of hydronephrosis was seen. Additionally, while mild splenomegaly (58×44 mm) was observed, signs indicative of hepatic, biliary or pancreatic abnormality, such as cholangiectasis, were absent.

Because *E. coli* was detected on blood and urine cultures, the patient was diagnosed with pyelonephrtis and sepsis. Antimicrobial therapy was initiated on June 27, resulting in the fever rapidly subsiding and the inflammation resolving. Regarding hepatobiliary enzymes, while the levels of γ-GTP and ALP remained elevated. Since repeat abdominal ultrasound revealed no obstruction and the level of anti-mitochondrial antibody was mildly elevated, primary biliary cirrhosis (PBC) was strongly suspected. However, levels of anti-mitochondrial antibody M2, which are a more specific indicator of PBC, were not elevated. The patient was discharged once the inflammation had ameliorated, and antimicrobial agents were administered on an outpatient basis. Levels of γ-GTP and ALP decreased over time and had normalized by day 35 of the illness. In January 2002, about six months after the onset, levels of the hepatobiliary enzymes remained normal, and the level of anti-mitochondrial antibody was confirmed to be normal (<20).

Discussion

The present patient was diagnosed with *E. coli*-induced pyelonephritis and sepsis, and exhibited findings indicative of PBC, including markedly increased hepatobiliary enzymes and elevated anti-mitochondrial antibody. As the pyelonephritis improved, levels of hepatobiliary enzymes and anti-mitochondrial antibody normalized.

It is well-known that sepsis often accompanies liver dysfunction, and the elevation of hepatobiliary enzymes, including γ-GTP and ALP, can be observed. However, it is reported in the reviews on the association between bacteremia and liver dysfunction that the elevated ALP levels induced by sepsis remain below 450 IU/*l*, if there are no other factors that worsen abnormalities of liver enzymes such as multiple organ failure (7, 8). Since the ALP level was above 1,000 IU/*l* in the present patient, we can speculate that there were some factors other than sepsis causing the elevation of ALP. In addition, we also examined the levels of hepatobiliary enzymes in 68 patients with sepsis who were admitted to our hospital (Fig. 2). The levels of mean ALP and γ-GTP in patients with sepsis were 347 IU/ml and 148 IU/ml, respectively, and the levels of ALP and γ-GTP in the present patient were considered to be extremely high. Taken together, we speculate that the elevation of hepatobiliary enzymes is probably due to PBC-like symptoms rather than cholestasis in sepsis, although the possibility that the elevation of ALP and γ-GTP in this patient is due to cholestasis in sepsis cannot be excluded completely.

PBC often affects middle-aged women, and is characterized by general malaise, pruritus, jaundice, and hepatomegaly. The condition generally advances to liver cirrhosis over time, ranging from several years to decades. Concerning hematological findings, levels of hepatobiliary enzymes such as γ-GTP and ALP, are usually elevated in the absence of raised transaminases. As PBC advances, the level of bilirubin also increases. Furthermore, the histopathology of PBC is characterized by chronic non-suppurative destructive cholangitis (1).

At this point in time, the exact cause of PBC is not known. There are some reports that infectious agents themselves are related to the pathogenesis of PBC and their DNAs are detected by PCR in the liver of some patients, but

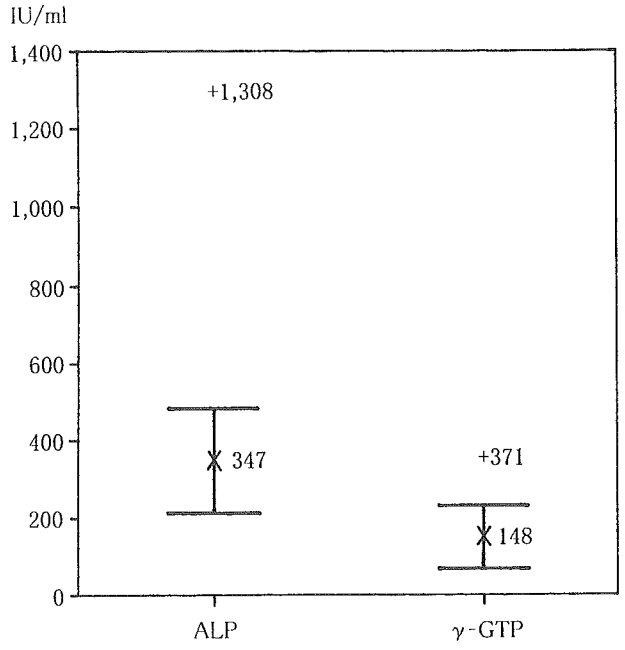

Figure 2. Serum ALP and γ-GTP levels in 68 patients with sepsis who were admitted to the University of Tokyo Hospital in April and May 2003. Serum samples were collected when examination of blood cultures were performed. +: patient's data, X : mean. Bars denote 99% confidence interval.

the results are contraversial (9, 10). On the other hand, PBC is considered to be an autoimmune disease, which is closely correlated to the presence of anti-mitochondrial antibody, since the sensitivity and specificity of anti-mitochondrial antibody are high at 95% and 98%, respectively (2). Several subtypes of anti-mitochondrial antibody have been identified: M1 to M9 (11). Of these, anti-mitochondrial antibody M2, which recognizes components of the mitochondrial inner membrane (M2), is most closely correlated to PBC (12). In fact, one study compared the positive rates of anti-M2 antibody among healthy individuals, patients with PBC and patients with other chronic hepatopathies, and reported a specificity of 100% for PBC. On the other hand, the sensitivity of the previous assay was only 54%, and that of the new assay using porcine myocardium is 78%. It has also been reported that sensitivity is even lower when the anti-mitochondrial antibody titer is low (13).

In the present patient, while increased bilirubin was not seen, markedly increased hepatobiliary enzymes were confirmed. Also, since an abdominal ultrasound failed to demonstrate any abnormality, including dilatation of biliary ducts, the patient's symptoms appeared to be associated with PBC. Furthermore, increased anti-mitochondrial antibody strongly supported this also. Consideration of these factors suggested that symptoms were associated with PBC. However, the patient tested negative to anti-M2 antibody. This could represent a false-negative result, due to the low sensitivity, as discussed previously. Further studies such as immunoblotting for the possible antigens for M2 might be required to exclude the possibility that anti-M2 antibody is negative for this patient, and histological examination is also important to diagnose the present patient with PBC.

Furthermore, in the present patient, the levels of hepatobiliary enzymes increased during episodes of pyelonephritis, then normalized as the pyelonephritis was alleviated, suggesting that the renal inflammation was involved in the onset of the PBC-like symptoms in the present patient. Interestingly, a study found that the incidence of urinary tract infection among patients with PBC, with other chronic hepatopathies, and with rheumatoid arthritis (RA) was 19%, 7% and 8%, respectively (3). Moreover, in another study, the incidence of urinary tract infection in healthy women, age-matched with PBC patients, was 5% (4). These findings suggest that the incidence of urinary tract infection among PBC patients is high. In addition, *E. coli* accounts for about 70% of urinary tract infections accompanying PBC (3). One study found that urinary tract infection was recurrent in about 60% of PBC patients, and that recurrence was due to the same organism, generally *E. coli* (5). Another study documented that about half of women, who did not have hepatopathy but were taking preventative antimicrobial therapy due to a past history of recurrent urinary tract infection, showed increased anti-mitochondrial antibody M2 production (6). These findings suggest that the incidence of increased anti-mitochondrial antibody M2 in women with a past history of recurrent urinary tract infection is markedly higher than that in patients with other chronic hepatopathies, or in healthy individuals. The above findings imply that the incidence of urinary tract infection, especially recurrent urinary tract infection induced by *E. coli*, is high among PBC patients, and that patients with recurrent urinary tract infection are more likely to produce anti-mitochondrial antibody M2. In other words, a possible mechanism of onset in PBC can be suggested by these epidemiological findings; that *E. coli*-induced recurrent urinary tract infection induces the production of anti-mitochondrial antibody, ultimately contributing to the development of causing PBC. As it has been reported that some kinds of autoantibodies, like rheumatoid factors and antinuclear antibodies, can be detected frequently in the serum of patients with infectious diseases including urinary tract infections (14), the relationship between infections and autoantibodies should be interpreted very cautiously. However, taking those epidemiological findings into account, the mechanism by which *E. coli*-induced recurrent urinary tract infection causes the production of anti-mitochondrial antibody, ultimately contributing to the development of causing PBC, cannot be excluded.

Another factor related to the involvement of *E. coli* in the onset of PBC is the molecular homology between *E. coli* and humans. Molecular homology is believed to play an important role in the activation of autoreactive T cells (15, 16), and this activation could actually be the cause of several autoimmune diseases (17, 18). It has been reported that, in

PBC patients, the antigens that trigger production of anti-mitochondrial antibody are two mitochondrial proteins (70 and 45 kDa proteins) (19). The 70 kDa protein is believed to be the pyruvate dehydrogenase complex (PDC), which is one of the constituents of the 2-oxo acid dehydrogenase complex (E2) (20). Several studies have reported the expression of the pyruvate dehydrogenase complex in the E2 component (PDC-E2) or molecules that cross-react with PDC-E2 to be elevated in the biliary epithelial cells of PBC patients (21, 22). Furthermore, the level of T cells reactive to PDC-E2 in the peripheral blood of PBC patients has been reported to be higher than that of healthy individuals (23), and T cells reactive to the 163–176 peptides of human PDC-E2 have been found in the liver and porta hepatis lymph nodes of PBC patients (24). These findings suggest that PDC-E2, particularly the 163–176 peptide region, is an important antigen for the onset of PBC. Furthermore, peptides originating from *E. coli* PDC-E2 activated T cells are reactive to human PDC-E2 163–176 peptides (25), and as a result, the molecular homology between *E. coli* and human PDC-E2 has been hypothesized (24). According to this theory, cross-reactivity between these two complexes could lead to the onset of PBC (24). These findings suggest that antibodies produced by recurrent *E. coli* infection, which should only react with *E. coli* PDC-E2, cross-react with human PDC-E2. In other words, these antibodies have been called "anti-mitochondrial antibodies" associated with PBC, and ultimately cause PBC.

However, it has been reported that the production of anti-mitochondrial antibody alone is not sufficient to induce PBC. In other words, even when the production of anti-mitochondrial antibody is induced by the (repeated) administration of a recombinant E2 component, chronic non-suppurative destructive cholangitis, which is thought to be typical of PBC, is not induced in a sustained manner (26). On the other hand, chronic non-suppurative destructive cholangitis has been reported to be induced by the following stimuli: inoculation of a PDC-E2/BCKD-E2 hybrid molecule; administration of lipopolysaccharide and adjuvant to mice that had undergone a thyroidectomy soon after birth; or immunization using PDC or PDC-E2/E3 BP (27, 28). As a result, continuous non-suppurative destructive cholangitis can be induced only when production of anti-mitochondrial antibody is combined with some type of immunological dysregulation. Moreover, several studies have reported that the incidence of PBC among those with a family history of PBC is several hundred times higher when compared to the general public (29, 30), thus suggesting the involvement of genetic factors in the onset of PBC. It is highly possible that susceptibility to immunological dysregulation could be genetically determined.

Regarding the mechanism of onset of the transient PBC-like symptoms during an episode of *E. coli*-induced pyelonephritis in the present patient, we can advance the hypothesis below. Infection with *E. coli* induced the production of antibodies against *E. coli* PDC and other bacterial components, and anti-mitochondrial antibody reactive to PDC in biliary epithelial cells was produced due to cross-reactivity. This lead to the onset of clinical symptoms associated with cholangitis and increased the levels of hepatobiliary enzymes. However, as the patient did not have the immunological dysregulation, anti-mitochondrial antibody was not continuously produced, and cholangitis improved and levels of the hepatobiliary enzymes normalized in parallel with resolution of the bacterial infection.

At present, numerous studies are being conducted to ascertain the mechanism of onset of autoimmune diseases, but many points remain unknown. The results obtained from the present case are of considerable interest in ascertaining the mechanism of onset of PBC, which is considered an autoimmune disease. A further point of note is that, as levels of hepatobiliary enzymes are not usually measured in patients with urinary tract infection, these enzymes may be latently high in many other patients with urinary tract infection. Patients with clinical features similar to those seen in the case presented should form the subjects of future investigations in order to elucidate the mechanism of onset of PBC and to develop effective therapy for this disease.

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□ CASE REPORT □

Acute Hepatitis E with Elevated Creatine Phosphokinase

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Abstract

Acute hepatitis E is caused by infection with hepatitis E virus, which is endemic in developing countries. Recently, the number of cases with acute hepatitis E is increasing in Japan due to increased travel to the endemic areas. This paper reports a case of a Japanese man with acute hepatitis E who had a history of traveling to south China. Serum creatine phosphokinase was elevated on admission without symptoms of muscle damage (isoenzyme MM 100%), and normalized in parallel with resolution of hepatitis, raising the possibility of an association between elevation of creatine phosphokinase and acute hepatitis E. However, we need to investigate further the incidence of elevation of serum creatine phosphokinase in many cases with acute viral hepatitis including hepatitis A, B, and C to determine whether muscle disorder is characteristic of acute hepatitis E.
(Internal Medicine 42: 899–902, 2003)

Key words: acute hepatitis E, hepatitis E virus, creatine phosphokinase

Introduction

Acute hepatitis E is caused by infection with the hepatitis E virus (HEV). HEV has a wide endemicity in tropical and subtropical areas and several outbreaks have been reported in many developing countries in Asia, America, and Africa (1). In contrast, few cases occur in developed countries where imported cases of this disease tend to occur sporadically. In Japan, the number of acute hepatitis E cases is increasing in accordance with the rising incidence in both travelers to endemic areas and people from these regions visiting Japan. There have also been recent reports of some cases of fulminant hepatitis due to HEV that had been acquired in Japan (2). Attention has thus been drawn to the investigation of routes of transmission and elucidation of the pathogenesis of HEV infection.

This paper describes a case of acute hepatitis E occurring after a trip to south China. This patient had the interesting finding of an elevated serum creatine phosphokinase (CK) level that normalized with improvement of the hepatitis. Although the cause of the elevated CK level was not identified, it is suggested to be associated with hepatitis E.

Case Report

A 59-year-old Japanese man was admitted to the University of Tokyo Hospital with jaundice on the December 22, 1999. He had no history of liver dysfunction and liver function tests at a routine checkup 10 months previously had been almost normal [aspartate aminotransferase (AST) 46 IU/*l*, alanine aminotransferase (ALT) 26 IU/*l*, γ-glutamyl transpeptidase (γ-GTP) 73 IU/*l*, total bilirubin (T.Bil) 0.5 mg/dl]. Alcohol intake was reported at 500 ml beer on a daily basis for forty years. The patient had taken a four-day business trip to south China (Suzhou and Shanghai) in late October 1999, during which he had experienced no symptoms. During this visit, no unboiled water or uncooked food was consumed and the patient denied having had sexual intercourse. However, he used unboiled city water when he brushed his teeth. At the beginning of December he noticed brown urine and itching of the skin; however, the clinical course did not include fever or diarrhea. Investigations at the previous day of admission revealed abnormal liver function tests (AST 695 IU/*l*, ALT 1,225 IU/*l*, γ-GTP 87 IU/*l*, T.Bil 15.2 mg/dl).

Physical examination on admission revealed the following findings: blood pressure 166/100 mmHg, heart rate 72/min, temperature 36.8°C, weight 51.7 kg, and height 1.69 m. The skin appeared jaundiced but there were no eruptions or vascular spiders. The liver edge was palpable 2 cm below the costal margin and had a smooth, soft consistency. Superficial lymph nodes were not palpable. Consciousness was clear and neurological findings were grossly normal.

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Table 1. Laboratory Data on Admission

Hematology		**Blood Chemistry**		**Viral markers**	
WBC	5,300 /μl	TP	6.5 g/dl	IgM anti-HAV-Ab	–
Neu	74.5%	Alb	3.3 g/dl	anti-HAV-Ab	–
Baso	5%	ChE	137 IU/*l*	HBs-Ag	–
Eos	0.5%	LDH	409 IU/*l*	anti-HBs-Ab	–
Mono	5%	AST	499 IU/*l*	HBe-Ag	–
Lym	33%	ALT	973 IU/*l*	anti-HBc-Ab	–
RBC	443×10^4/μl	γ-GTP	131 IU/*l*	IgM anti-HBc-Ab	–
Hb	14.0 g/dl	Al-P	441 IU/*l*	anti-HCV-Ab	–
Ht	41.50%	T.Bil	14.6 mg/dl	IgG anti-VCA-Ab	160X+
Plt	20.2×10^4/μ*l*	D.Bil	10.5 mg/dl	IgM anti-VCA-Ab	–
		Amy	193 IU/*l*	anti-EBNA-Ab	160X+
Coagulation tests		CK	1,217 IU/*l*	IgG anti-CMV-Ab	38.2
PT	12.8 s	CK-MM	100%	IgM anti-CMV-Ab	1.76
PT activity	73%	CRP	0.3 mg/dl	CMV-antigenemia	–
aPTT	32.4 s			CMV-DNA	–
Fbg	273 mg/dl				
				ANA	–

WBC: white blood cell, Neu: neutrophil, Baso: basophil, Eos: eosinophil, Mono: monocyte, Lym: lymphocyte, RBC: red blood cell, Hb: hemoglobin, Ht: hematocrit, Plt: platelet, PT: prothrombin time, aPTT: activated partial thromboplastin time, Fbg: fibrinogen, TP: total protein, Alb: albumin, ChE: cholinesterase, LDH: lactate dehydrogenase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, Al-P: alkaline phosphatase, γ-GTP: γ-glutamyl transpeptidase, T.Bil: total bilirubin, D.Bil: direct bilirubin, Amy: amylase; CK: creatine phosphokinase, CRP: C- reactive protein, anti-HAV-Ab: anti-hepatitis A virus-antibody, HBs-Ag: hepatitis B virus (HBV) surface antigen, HBe: HBV envelope, HCV: hepatitis C virus, VCA: viral capsid antigen, EBNA: Epstein-Barr virus nuclear antigen, CMV: cytomegalovirus, ANA: anti-nuclear antibody.

Laboratory tests on admission are shown in Table 1. Leukocyte, erythrocyte and thrombocyte counts were within normal ranges. The liver enzyme profiles showed transaminase levels to be markedly elevated with an increase in ALT exceeding that seen for AST. Bilirubin levels, both direct and indirect, were also markedly elevated, serum albumin was slightly low and prothrombin activity was at the lower limit of normal. In addition to liver inflammation, the CK level was also elevated with quantitation of CK isoenzymes showing 100% CK-MM, indicating skeletal muscle damage. Hepatitis viral markers, hepatitis B virus (HBV) surface antigen (HBs-Ag), anti-hepatitis C virus-antibody (anti-HCV-Ab), anti-hepatitis A virus (HAV)-Ab, IgM anti-HAV-Ab were all negative. Serological tests for Epstein-Barr virus (EBV) indicated past infection. IgM anti-cytomegalovirus (CMV)-Ab was mildly positive but CMV-DNA was not detected. Anti-nuclear antibody was also negative. Ultrasonography revealed mild hepatomegaly. No obstruction of the biliary system was apparent and no abnormality in the gall bladder was identified. Mild splenomegaly was present. These findings were compatible with acute hepatitis.

Clinical course

Acute hepatitis was suspected from the clinical course prior to admission and laboratory results. Acute hepatitis A, B, and C were all ruled out on serological tests and autoimmune hepatitis was also excluded as autoantibodies such as anti-nuclear antibody were negative. Regarding other viral causes of hepatitis, the EBV profile was suggestive of EBV infection at some undetermined time in the past and although IgM anti-CMV-Ab was positive, the low blood and serum CMV-DNA load shown by real time polymerase chain reaction (RT-PCR) suggested this to be an unlikely cause of hepatitis.

The possibility of HEV infection was therefore considered and frozen serum kept on the day of admission was sent to the National Institute of Infectious Diseases (Tokyo, Japan) for serological testing for HEV. Enzyme-linked immunosorbent assay (ELISA), subsequently revealed high-titers of anti-HEV antibodies (IgM 1: 6,400, IgG 1: 51,200) diagnostic of acute hepatitis E.

We considered the patient to be in the convalescent stage on admission for the following reasons; elevation of ALT exceeded that of AST, symptoms were improving, and coagulation tests were normal. After admission, management was conservative, consisting of rest and observation, during which AST had decreased to 57 IU/*l* by January 12, 2000 (Fig. 1). The patient was subsequently discharged and followed up in the outpatient clinic, with liver function tests having normalized by January 17.

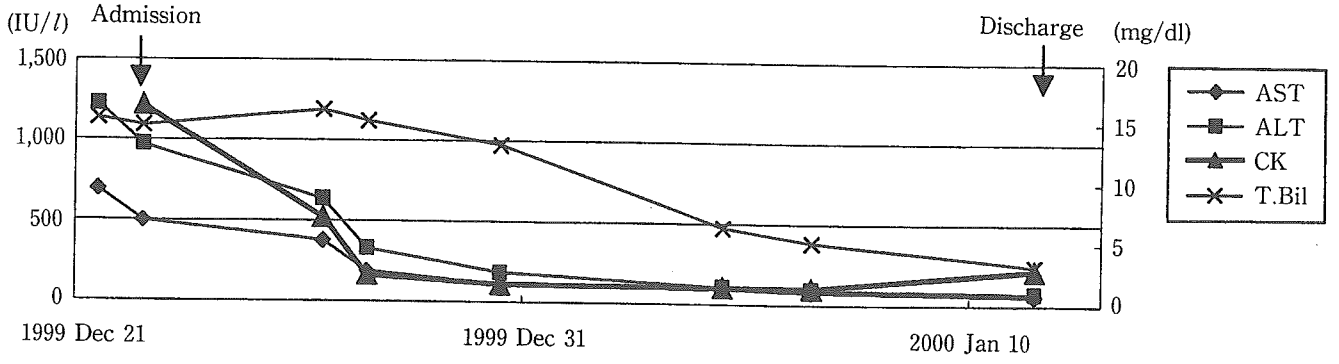

Figure 1. Clinical course. AST : aspartate aminotransferase, ALT : alanine aminotransferase, CK : creatine phosphokinase, T.Bil : total bilirubin.

The elevated CK level on admission (1,217 IU/*l*, CK-MM 100%) showed a myogenic pattern, suggesting it to be caused by skeletal muscle damage. However the cause of muscle damage was not identified, as there were no findings of muscle atrophy, muscle weakness, or myalgia and no history of excessive exercise, or intramuscular injection. During the period of observation, CK normalized in parallel with the resolution of hepatitis (Fig. 1) and remained normal one year after the onset of symptoms. Anti-HEV antibody (IgM) was proved to be negative in February 2001.

Discussion

HEV is a major causative agent of acute sporadic or endemic hepatitis in developing areas such as southeast Asia, China, the Middle East, and Africa (3–5), with several outbreaks having been reported in these endemic areas. The virus was cloned as a 7.6 kb RNA virus by Reyes et al in 1990 (6) and thereafter termed HEV. Transmission occurs via the feco-oral route through contaminated water and the incubation period from infection to onset of hepatitis is generally 2–7 weeks. The clinical manifestations of acute hepatitis E are similar to that of acute hepatitis A, and consist of fever, general fatigue, and gastrointestinal symptoms such as loss of appetite and vomiting. However, differences between these two forms of viral hepatitis exist; the incubation period of acute hepatitis E, averaging of 6 weeks, is longer than that of acute hepatitis A, acute hepatitis E has its peak prevalence in young adults and a lower incidence of secondary infection (7). Furthermore, reports from endemic areas describe a high rate (10–20%) of fulminant hepatitis E in pregnant women, especially in the third trimester (8, 9) although the mechanism by which this occurs has not been clarified.

The present patient had a history of travel to south China 7 weeks before the onset of hepatitis. From the history of travel abroad and in the absence of evidence of acute hepatitis A, B, or C, a diagnosis of acute hepatitis E was suspected. This was confirmed by conducting an ELISA, which showed a high-titer of anti-HEV antibodies, diagnostic of acute hepatitis E. IgM anti-HEV-Ab usually appears in the serum of infected patients with the onset of symptoms and remains for about 3–4 months. IgG anti-HEV-Ab can be detected promptly after the IgM response in the acute phase and is boosted in the early-convalescent phase and remains positive for several years (1, 10). In a study of a nonendemic area, the specificities of IgG and IgM anti-HEV-Ab for diagnosing acute hepatitis E were 92.1% and 98.6%, respectively (11). In this case, high titers of both IgG and IgM antibodies were recorded about 2 weeks after the onset of symptoms. Symptoms and biochemical liver dysfunction resolved gradually with rest, with the patient making an uneventful recovery.

This case was characteristic in that serum CK-MM levels were elevated, indicating skeletal muscle damage. The causes of elevated CK include exercise, trauma, hyperthermia, alcohol and other toxins, drugs, and burns. The present patient had no symptoms of muscle atrophy, muscle weakness, or myalgia and no history of vigorous exercise or intramuscular injection, so the cause of the elevated CK was not identified. It is known that infection with some microorganisms can cause myositis. Bacteria reported to cause myositis include group A *Streptococci*, *Clostridium* and *Legionella*, while viruses that produce muscle inflammation can include coxsackievirus, echovirus, and EBV although influenza virus is the most common agent. In contrast, to our knowledge there have been no reports of acute hepatitis E accompanied by myositis or muscle dysfunction. Regarding the occurrence of myositis or muscle dysfunction in other viral hepatitis infections, a few cases of HAV, HBV or HCV infection with elevation of CK have been reported in the literature (12–14). All 12 patients with acute hepatitis A, B, or C who were admitted to our department over a ten-year period showed the normal range of CK. However, many viral infections, apart from influenza virus, are asymptomatic and could hence be overlooked as a cause of myositis. In the case presented here, the CK level normalized in parallel with the resolution of hepatitis and remained normal one year after the onset of symptoms. From these findings it cannot be

denied that HEV infection was transiently complicated with myositis leading to the elevation of CK, although this association between HEV infection and an elevated CK level may not be causally related. Further cases require analysis in order to examine this association.

A recent survey showed that the seroprevalence of HEV in the Japanese population was 4–19% and that most seropositive individuals had no history of travel to endemic areas or hepatitis (15). Moreover, cases in which infection probably occurred in Japan have been reported (16, 17). The use of PCR showed that unidentified cases previously reported as non-A non-B non-C hepatitis had included cases of acute hepatitis E (2). Although the causative agent is not identified in 20–30% cases of sporadic acute hepatitis, HEV markers have not been examined in most of them and it is therefore necessary to keep HEV infection in mind in a case of non-A non-B non-C hepatitis.

It is also reported that HEV infection can be zoonotic; in the US and Taiwan new HEV strains were isolated from patients with non-A non-B non-C hepatitis and phylogenetic analyses showed that these were closely related to HEV strains isolated from pigs in the same areas (18–21). In the present case, the genotype of HEV, which would have been acquired in south China, was not analyzed. The circulation of HEV among animals and the transmission from these to humans in Japan, as has been suggested to occur in the US and Taiwan, requires investigation in the future. In conclusion, we reported a case of acute hepatitis E with a history of travel to south China. In this case elevation of CK occurred in parallel with the course of hepatitis.

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Methylation status of suppressor of cytokine signaling-1 gene in hepatocellular carcinoma

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Background. Silencing of the suppressor of cytokine signaling (*SOCS-1*) by aberrant methylation at the CpG island in the coding region gene has been reported in hepatocellular carcinoma (HCC). However, principally, it is methylation in the 5′-noncoding region but not that in the coding region which determines the regulation of gene expression. ***Methods.*** Methylation-specific PCR was performed for the analysis of methylation status both in the 5′-noncoding region and the CpG island of *SOCS-1* from 22 HCC tissue samples with adjacent non-HCC tissue samples and from two cell lines. ***Results.*** Using primers in the CpG island, 9 of 22 HCC samples exhibited aberrant methylation of *SOCS-1*, while only 1 of 22 adjacent non-HCC samples did so. The unmethylation pattern was detected in 1 of 22 HCC and in 5 of 22 non-HCC samples. Thus, aberrant methylation of SOCS-1 was significantly associated with HCC (P = 0.0076 by Fisher's exact test). Using primers in the 5′-noncoding region, aberrant methylation was observed in 12 of 22 HCC and in 2 non-HCC samples. The unmethylated pattern was observed in 5 of 22 HCC and in 10 of 22 non-HCC samples (P = 0.0042). There was no significant correlation between the methylation status of *SOCS-1* and clinicopathological findings, such as the presence or absence of cirrhosis or the histological grade of HCC. ***Conclusions.*** Aberrant methylation of the *SOCS-1* had a significant correlation with HCC. The rate of aberrant methylation was similar in the 5′-noncoding region and in the CpG island. Aberrant methylation of *SOCS-1* may be associated with hepatocarcinogenesis, although further studies are necessary.

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Introduction

The majority of cases of hepatocellular carcinoma (HCC) are associated with hepatitis B or C viral infection.[1,2] Despite the absence of an appropriate in vitro replication system or a practical infectious animal model system, the mechanism underlying hepatocarcinogenesis in human hepatitis viral infection is on a stable path to elucidation, albeit slowly. Both the direct and indirect effects of hepatitis viruses on HCC development have been shown.[3–6] Accumulation of gene aberrations, such as inactivation of tumor suppressor genes or activation of oncogenes, which may be induced through inflammation-mediated continuous death of hepatocytes followed by regeneration, is considered to be one of the mechanisms underlying hepatocarcinogenesis.[3,4] On the other hand, viral gene products are suggested to contribute to HCC development by their direct effects on hepatocytes.[5–8] Such direct effects have been demonstrated by the use of model systems including mice.[5–7]

In contrast, gene alterations that play pivotal roles in hepatocarcinogenesis in the majority of HCC tissues have not been identified yet. To date, the genes for the APC-axin-GSK-3β complex may be only one of such candidate genes.[9,10] Such gene alterations include not only mutations in the genes per se but also epigenetic changes, which lead to either suppression or augmentation of gene expression. A change in the methylation state of the gene is one of the epigenetic changes that are associated with carcinogenesis. A possible role of methylation of genes in HCC development has been reported[11] for a tumor suppressor gene, $p16^{INK4}$: $p16^{INK4}$ expression was downregulated by methylation of the

control region. Expression of some other cancer-related genes may also be inhibited by methylation.

Silencing of the suppressor of cytokine signaling-1 (SOCS-1; also known as SSI-1 or JAB) is a member of the SOCS protein family. It switches off cytokine signaling by directly interacting with Janus kinase (JAK) proteins; its expression renders cells unresponsive to interleukin-6 stimulation.[12] The SH2 domain of SOCS-1 binds to a JH1 domain of JAK2 and inhibits its phosphorylation, downregulating the JAK/STAT pathway.[12,13] SOCS-1 inhibits the biological effects of cytokines in vivo; its forced expression interrupts macrophage differentiation induced by IL-6 and suppresses CD23 expression induced by IL-4.[12,13] Thus, SOCS-1 modulates the immune system through interacting with the cytokine network.

Recently, *SOCS-1*-deficient mice have been shown to die within 3 weeks after birth from a myeloproliferative disorder resulting from unbridled interferon (IFN)-γ and tumor necrosis factor (TNF)-α signaling.[14] As a negative regulator of cytokine signaling, *SOCS-1* is now a candidate gene for inactivating mutations that will favor the development of malignancies; SOCS-1 may inhibit cell proliferation induced by oncogenic forms of other known SOCS-1-interacting proteins. In addition to the results in hematopoietic neoplasia, recently suppression of SOCS-1 expression has been reported in HCC, in which the CpG-rich domain in the coding region of *SOCS-1* was found to be aberrantly methylated.[15] However, in general, it is the methylation of the 5′ non-coding region, which contains the promoter, but not that of the coding region, which determines gene expression.[16,17] We therefore conducted this experiment to evaluate the methylation status of the *SOCS-1* in HCC by methylation-specific PCR (MSPCR) using primers located both in the 5′-noncoding region and in the CpG-rich domain (CpG island) of the coding region.

Patients and methods

Patients

We studied 22 patients (19 males and 3 females; median age, 63.5 years) with HCC who had underlying chronic hepatitis C with or without cirrhosis (8 without and 14 with cirrhosis), all of whom underwent hepatectomy between 1997 and 2000 at the University of Tokyo Hospital. This study was approved by the ethics review committee of the institute, and carried out in accordance with the World Medical Association Helsinki Declaration, adopted in 1964 and amended in 1996. Informed consent was obtained from each patient. All the patients were positive for anti-hepatitis C virus (HCV) confirmed by the second-generation enzyme immunoassay and HCV-RNA by reverse-transcriptase-polymerase chain reaction (RT-PCR), and none were positive for serum hepatitis B surface antigen (HBsAg). The clinicopathological features of the patients are shown in Table 1.

Tissue samples and cell lines

The cancerous (HCC) and noncancerous (non-HCC) liver tissue samples obtained from these patients were fixed in 10% formalin for hematoxylin and eosin staining, or immediately frozen and stored at −80°C until further use. The histological staging of the noncancerous tissues was performed according to the European classification for chronic hepatitis,[18] and that of cancerous tissue was based on the TNM classification.[19] All the 22 tumors were classified as advanced HCCs: 5 well-, 14 moderately, and 3 poorly differentiated HCCs (see Table 1). Human HCC cell lines PLC/PRF/5, HuH-7, and the B-cell line, BJAB, were obtained from the American Type Culture Collections. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

DNA preparation and bisulfite treatment

Genomic DNA was extracted from the frozen tissues by standard proteinase K digestion and phenol/chloroform extraction.[20] Then, bisulfite modification of genomic DNA was carried out as described previously[21] with slight modification. Briefly, DNA (1 μg) in a volume of 20 μl was denatured by NaOH at a final concentration at 0.3 M for 15 min at 37°C. Then, 113 μl 3.6 M sodium bisulfite (Sigma-Aldrich, St. Louis, MO, USA) at pH 5 and 7.2 μl 10 mM hydroquinone (Sigma-Aldrich), both freshly prepared, were added and mixed well. Then, the samples were incubated under mineral oil at 95°C for 15 min followed by incubation at 50°C for 4 h, and this cycle was repeated 15 times. Modified DNA was purified and resuspended in 50 μl water. The modification was completed by adding NaOH at a final concentration of 0.3 M for 5 min at room temperature, after which ethanol precipitation was carried out.

Genomic and methylation-specific PCR (MSPCR)

Bisulfite-modified and unmodified DNA was subjected to amplification using the PCR method. Primers used for the PCR in the current study are shown in Table 2. Amplification was carried out in a thermal cycler for a total of 35 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 30 s at 72°C in 50 μl reaction mixture containing 200 mM deoxynucleoside triphosphates (dNTPs), 1.0 mM of each primer, and 1× PCR buffer [16.6 mM

Table 1. SOCS-1 gene methylation and clinicopathological findings of 22 hepatocellular carcinoma (HCC) patients

		Methylation of SOCS-1							
		CpG island				5′-noncoding			
		M[a]		U[a]		M[a]		U[a]	
	n	HCC	nHCC	HCC	nHCC	HCC	nHCC	HCC	nHCC
Sex									
Male	19	7	1	1	4	10	2	5	9
Female	3	2	0	0	1	2	0	0	1
Cirrhosis									
−	8	2	0	0	1	3	0	2	4
+	14	7	1	1	4	9	3	3	5
Pathology of HCC[b]									
WD	5	3	0	0	2	3	0	1	3
MD	14	4	1	1	2	7	1	3	6
PD	3	2	0	0	1	2	1	1	0
Tumor size (cm)									
<2	5	2	0	0	0	2	2	2	1
≥2	17	7	1	1	5	10	0	3	9
Vascular invasion									
Absent	19	8	1	1	4	11	2	5	9
Present	3	1	0	0	1	1	0	0	1
Distant metastasis[c]									
M0	20	9	1	1	4	12	1	4	10
M1	2	0	0	0	1	0	1	1	0
Stage grouping[c]									
I	4	1	0	0	0	1	2	2	1
II	5	3	0	0	2	3	0	1	2
III	11	4	1	0	3	6	1	2	7
IV	2	1	0	1	0	2	0	0	0
Overall	22	9* (41%)	1* (5%)	1* (5%)	5* (23%)	12* (55%)	2* (9%)	5* (23%)	10* (46%)

[a] M, hypermethylated pattern; U, unmethylated pattern; not all samples were informative for methylation status
[b] WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated
[c] According to TNM classification
* $P < 0.01$ when the association of HCC and methylation was judged by Fisher's exact test for each of CpG island and 5′-noncoding region

Table 2. Polymerase chain reaction (PCR) primers used in the current study

	Sequence	Position
Forward		
HM1F	TTCGCGTGTATTTTTAGGTCGGTC	(400–423)
HM2F	GAGTATTCGCGTGTATTTTTAGG	(395–417)
UM1F	TTATGAGTATTTGTGTGTATTTTTAGGTTGGTT	(391–423)
UM2F	TGAGTATTTGTGTGTATTTTTAGG	(394–417)
UMPF-M	GTTCGGTTTCGTTTAGTTTTCGAGG	(−708–684)
UMPF-U	GTTTGGTTTTGTTTAGTTTTTGAGG	(−708–684)
Reverse		
HM1R	CGACACAACTCCTACAACGACCG	(537–559)
UM1R	CACTAACAACACAACTGGTACAACAACCA	(537–565)
UM2R	CAACACAACTCCTACAACAACCA	(543–565)
UMPR-M	ACCCCGACCGACCGCGATCTC	(−590–570)
UMPR-U	ACCCCAACCAACCACAATCTC	(−590–570)

ammonium sulfate, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.001% (w/v) gelatin] and 1.25 units of Ampli-Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The PCR products were separated in a 2.0% agarose gel and visualized by staining with ethidium bromide.

Reverse transcription (RT)-PCR

Total RNA was extracted from cells using RNAzolB (TEL-TEST, Friendswood, TX, USA). Three micrograms of total RNA were reverse transcribed by Superscript II (Gibco-BRL, Gaithersburg, MD, USA) using oligo(dT) primer and subjected to PCR. Primers for RT-PCR of SOCS-1 gene expression were as follows: forward, 5′-CACGCACTTCCGCACATTCC-3′; reverse, 5′-TCCAGCAGCTCGAAGAGGCA-3′. For the RT-PCR, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification, so that semiquantitative comparisons of the mRNA abundance between different samples were possible. RT-PCR with glyceraldehyde phosphate dihydrogenase (GAPDH) primers was done to adjust the amounts of RNA in each experiment.

Statistical analysis

Fisher's exact test was used for statistical evaluation, and P values below 0.05 were considered significant.

Results

Methylation status of SOCS-1 in cultured cell lines

First, the methylation status of the CpG island in the coding region of *SOCS-1* was analyzed in cell lines by MSPCR using the primer sets, HM1F+HM1R and UM1F+UM1R, according to the method of Yoshikawa et al.[15] MSPCR using these primers, however, could not determine the methylation status of the gene: the use of the primers resulted in dimer formation without methylation- or unmethylation-specific bands. We therefore redesigned new sets of primers located in the CpG island of *SOCS-1* (Table 2; HM2F+HM1R for detecting a methylation-specific band and UM2F+UM2R for an unmethylation-specific band). MSPCR with these sets of primers enabled successful detection of methylation- and unmethylation-specific bands in PLC/PRF/5 cells (Fig. 1). The unmethylation-specific band alone was detected in HuH-7 cells, in agreement with the previous report.[15]

Analysis using the primers located in the 5′-noncoding region (see Table 2) yielded a similar pattern, excluding that there were both methylation- and unmethylation-specific bands also in HuH-7 cells (Fig. 1). Accordingly, the primer sets HM2F+HM1R and UM2F+UM2R were used for the analysis of the methylation status of the CpG island, and UMPF-M+UMPR-M and UMPF-U+UMPR-U were used for the 5′-noncoding region, thereafter.

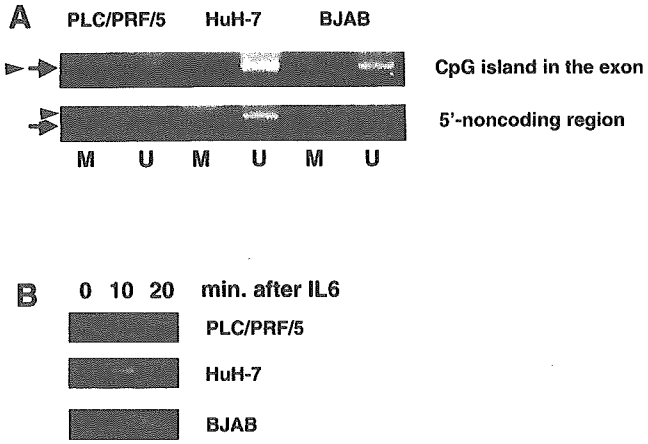

Fig. 1. Genomic and methylation-specific (MSPCR) analysis of cultured hepatoma cell lines in the CpG island and 5′-no-coding region of the *SOCS-1* gene. DNA from human hepatoma cell lines, PLC/PRF/5 and HuH-7, and a B-cell line, BJAB, was analyzed by MSPCR after bisulfite treatment as described in the Patients and methods section. **A** MSPCR with the primers in the CpG island and those in the 5′-noncoding region. **B** RT-PCR showing the expression of SOCS-1 in cell lines before and after the addition of IL-6 (10 ng/ml). The *arrow* indicates the position of the methylation-specific band; the *arrowhead* indicates the position of the unmethylation-specific band. *M*, MSPCR with methylation-specific primers; *U*, MSPCR with unmethylation-specific primers

Expression of SOCS-1 mRNA in cell lines

The expression of SOCS-1 was determined by semiquantitative RT-PCR. Although SOCS-1 expression was abundant in HuH-7 and BJAB cells in the baseline and was enhanced by the addition of IL-6 (10 ng/ml), only marginal expression and no enhancement were detected in PLC/PRF/5 cells. These results are consistent with the methylation status that was determined in the current study and with the expression status in the baseline that was observed in a previous report.[15]

Methylation status of SOCS-1 in human tumor samples

Then, DNA extracted from human HCC and non-HCC tissues was tested for the methylation status of the *SOCS-1* by MSPCR. Only 10 and 6 tissue samples were informative for determining a methylation-specific band

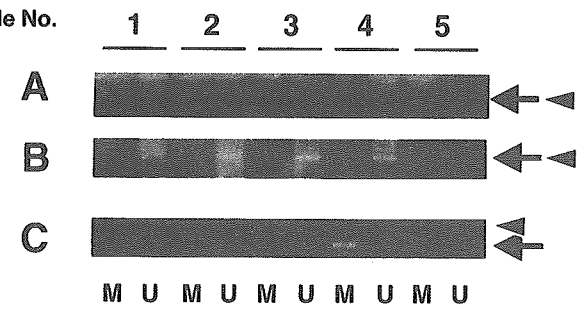

Fig. 2. MSPCR analysis of the CpG island and 5′-noncoding region of the *SOCS-1* from 22 HCC tissue samples. With the same primers used in Fig. 1, 22 pairs of HCC and non-HCC samples were analyzed by MSPCR. Representative cases are shown. Samples *1* and *2* were well-differentiated HCC, *3* and *4* were moderately differentiated HCC, and *5* was poorly differentiated HCC. Panel **B** shows their non-HCC counterparts. **A** MSPCR of DNA from HCC tissue samples with the primers in CpG island. **B** MSPCR of DNA from non-HCC tissue samples with the primers in CpG island. **C** MSPCR of DNA from HCC tissue samples with the primers in 5′-noncoding region. The *arrow* indicates the position of the methylation-specific band; the *arrowhead* indicates the position of the unmethylation-specific band HCC, hepatocellular carcinoma; M, MSPCR with methylation-specific primers; U, MSPCR with unmethylation-specific primers

and an unmethylation-specific band, respectively, when the primers in the CpG island were used. In 9 HCC tissue samples the band indicative of aberrant methylation in the CpG island was detected, while the band indicating unmethylation was detected in 1 HCC tissue sample (Fig. 2). In contrast, in the corresponding non-HCC tissue samples, only 1 exhibited the methylation pattern, whereas the unmethylation pattern was observed in 5 non-HCC tissue samples. Thus, aberrant methylation of *SOCS-1* was significantly associated with HCC rather than with non-HCC tissues ($P = 0.0076$ by Fisher's exact test).

Using primers in the 5′-noncoding promoter region, 14 and 15 HCC tissue samples were informative for a methylation-specific band and an unmethylation-specific band, respectively. Aberrant methylation was observed in 12 HCC tissue samples whereas the unmethylation pattern was detected in 5 HCC tissue samples. In contrast, only 2 non-HCC tissues exhibited aberrant methylation whereas the unmethylation pattern was detected in 10 non-HCC tissues: there was also a significant correlation between HCC and aberrant methylation of *SOCS-1* ($P = 0.0042$).

Neither a methylation-specific nor an unmethylation-specific band in 12 HCC and 16 non-HCC tissues was detected using the primers in the CpG island and in 5 HCC and 10 non-HCC tissue samples using the primers in the 5′-noncoding promoter region, suggesting that *SOCS-1* in these tissues was in a mosaic state of methylation. This suggestion was examined using a hepatoma cell line HLF: neither a methylation- nor an unmethylation-specific band was detected, but after 5-azacytidine treatment of the cell line for 3 days, which cancels methylation of the gene,[22] an unmethylation-specific band appeared, demonstrating that *SOCS-1* in the cell line is methylated in a mosaic fashion. Consequently, *SOCS-1* gene expression was turned on as determined by RT-PCR.

Correlation between SOCS-1 methylation and clinicopathological findings

The relationship between the methylation status of *SOCS-1* and clinicopathological findings is shown in Table 1. When the methylation status in HCC tissue samples was correlated with parameters such as the presence or absence of cirrhosis as the underlying liver disease, the histological degree of HCC, tumor sizes, vascular invasion, distant metastasis, or tumor stages, no significant association was noted.

Discussion

In the current study, we analyzed the methylation status of *SOCS-1*, a negative regulator of the JAK/STAT pathway, by the MSPCR method. Using the primers located in the CpG island in the coding region, aberrant methylation was observed in 9 of 22 (41%) HCC tissue samples, and 12 of 22 (54.5%) HCC tissue samples by the use of primers in the 5′-noncoding region. The former rate is almost compatible with the incidence in a previous report.[14] It is notable that a similar or higher rate of aberrant methylation was detected in the 5′-noncoding promoter region of *SOCS-1*. It is established that methylation in the promoter region is essential in the regulation of (silencing) the genes.[16,17] The frequent occurrence of aberrant methylation in the promoter region of *SOCS-1* further supports the notion that the downregulation of SOCS-1 expression is common in human HCC. Very recently, methylation in the promoter of SOCS-1 gene was reported in pancreatic tumors.[23]

In our MSPCR analysis, a substantial number of samples showed neither the methylated nor unmethylated pattern. The reason for this dual negativity is unclear. One possibility is a mosaic methylation pattern that may exist in the *SOCS-1*. If not all the susceptible cytosine residues are methylation, i.e., a gene is methylated in a mosaic fashion, one cannot determine the methylation status by MSPCR. This possibility was confirmed using a hepatoma cell line, as shown in the Results section. Neither a methylation- nor an unmethylation-specific band was detected, but after

5-azacytidine treatment of the cell line for 3 days, which cancels methylation of the gene,[22] an unmethylation-specific band appeared, demonstrating that *SOCS-1* in the cell line is methylated in a mosaic fashion.

SOCS-1 transcription is activated by signal transducer and activator of transcription (STAT) and the resultant proteins negatively regulate the JAK/STAT pathways either by directly inhibiting JAKs or by binding to receptors and blocking further association with STATs. Of the eight SOCS family members, SOCS-1 is a negative regulator of IL-6 signals. The silencing of *SOCS-1* results in constitutive activation of the JAK/STAT pathway. Without negative feedback by SOCS-1, the downstream pathways and target genes are strongly activated.[24] There are several lines of evidence supporting the idea that the JAK/STAT pathway may be involved in oncogenesis. The constitutive activation of the JAK/STAT pathway including STAT3 is observed in a number of transformed cells.[25] Thus, SOCS-1 is considered to be a tumor suppressor candidate, which chiefly has a role in the development of hematopoietic malignancies.[26] Also, an association of the SOCS-1 in hepatocarcinogenesis has recently been suggested.[15] There are a variety of gene products in the downstream of the JAK/STAT pathway, including c-*myc* or c-*fos*.[27] The activation of the pathway thus may cause an activation of oncogenes or growth-associated genes and eventually lead to oncogenesis. The precise role of SOCS-1 in hepatocarcinogenesis is currently unclarified and requires further study, but it might play an essential role in the majority of HCCs.

Our current results confirmed those of a previous study[14] and added a new piece of information on methylation of the promoter region of *SOCS-1*. However, the presence of cases negative for both methylation and unmethylation may limit the application of this technique for the analysis of hepatocarcinogenesis. In addition, recently the association between the core protein of hepatitis C virus and the JAK/STAT pathway has been reported as a potential proliferator of hepatocytes.[28] Besides aberrant methylation, association of SOCS-1 with HCV may cause a down-regulation of *SOCS-1* expression. In relation to this issue, it is interesting to note that a few patients in our series exhibited aberrant methylation of *SOCS-1* in the adjacent non-HCC tissue samples. Infection with HCV, which is present in all patients, may be associated with *SOCS-1* expression in human HCC tissues. Further studies are necessary for deciphering the complicated involvement of the SOCS-1 and JAK/STAT pathway in hepatocarcinogenesis, possibly in association with HCV infection.

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