phorylation within IRS1 due to a high level of tumor necrosis (TNF)-α which leads to suppression of downstream insulin signals has been shown in HCV core transgenic mice.[49]

What roles do mitochondrial ROS play in HCV-induced insulin resistance? TNF-α phosphorylates Ser^{307} of IRS1 through serine (Ser) kinases such as c-Jun N-terminal kinase (JNK), which disrupts the interaction between the catalytic domains of the insulin receptors and the phosphotyrosine-binding domain of IRS1,[54,55] even though there is a contrary report that Ser^{307} promotes insulin sensitivity in mice.[56] The intracellular redox condition is a master regulator of phosphorylation/dephosphorylation events due to the presence of redox-sensing cystein (Cys) residues within nearly all classes of protein phosphatase enzymes.[57] In general, phosphatase activity is depressed in response to an oxidative shift in the redox environment, thus leading to a concomitant increase in kinase activity either via direct oxidant-induced activation or secondary to phosphatase inactivation.[57] Inactivation of protein tyrosine phosphatases is mediated via the oxidation of a conserved redox sensitive Cys residue within their catalytic sites, which must be in the reduced state as the thiol (-SH) to form a cysteinyl-phosphate intermediate during hydrolysis.[58] Thus, research conducted over the past several years has established a role for the activation of stress sensitive Ser/threonine (Thr) kinases and their subsequent phosphorylation of inhibitory Ser/Thr residues within the insulin receptor and IRS1/2 as a potential mechanism of insulin resistance.[59] In agreement with the evidence suggesting a central role for ROS in the development of insulin resistance,[60] HCV core-induced mitochondrial ROS production is presumed to induce insulin resistance through activation of Ser/Thr kinases such as JNK1 and subsequent inhibition of tyrosine phosphorylation within IRS1 (Fig. 2). Hepatic insulin resistance induces suppressed insulin clearance as well as increased insulin secretion from pancreatic β-cells, which leads to hyperinsulinemia and represses whole-body insulin sensitivity.[61]

Hepatic steatosis

Hepatic steatosis is also one of the pathophysiological features of HCV-associated chronic liver disease.[15,16] It is characterized by the cytoplasmic accumulation of lipid droplets, mainly composed of triglyceride and cholesterol ester. The composition of triglycerides in the liver is uniquely and significantly enriched in carbon monosaturated (C18:1) fatty acids in chronic hepatitis C,[62] which is distinct from what occurs in obese patients. The mechanisms underlying HCV-related steatosis are diverse: decreased lipoprotein secretion from hepatocytes, increased synthesis of fatty acids, decreased fatty acid oxidation and increased fatty acid uptake by hepatocytes. The HCV core protein has been demonstrated to inhibit microsomal transfer protein activity[63] and to upregulate transcriptional activity of sterol regulatory element-binding protein 1, a transcription factor involved in lipid synthesis.[64] These observations underscore the importance of the core as a direct and principal regulator of HCV-associated steatosis. On the other hand, decreased fatty acid oxidation and increased fatty acid uptake are related to mitochondrial dysfunction and hyperinsulinemia, respectively. Indeed, we previously demonstrated impaired mitochondrial fatty acid oxidation concomitant with increased ROS production in iron-overloaded transgenic mice expressing the HCV polyprotein.[65] Hyperinsulinemia derived from insulin resistance inhibits lipolysis in the liver and increases fatty acid uptake by hepatocytes. As described above, mitochondrial ROS production is presumed to induce insulin resistance. Thus, inhibited fatty acid oxidation and increased fatty acid uptake are potentially related to mitochondrial ROS production induced by the core protein.

Hepatic iron accumulation

Elevated iron-related serum markers and increased hepatic iron accumulation are relatively common and correlate with the severity of hepatic inflammation and fibrosis in patients with chronic hepatitis C. Excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals.[66] This is particularly relevant for chronic hepatitis C, in which oxidative stress has been proposed as a major mechanism of liver injury. Oxidative stress and increased iron levels strongly favor DNA damage, genetic instability and tumorigenesis. Indeed, a significant correlation between 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidatively generated DNA damage,[67] and hepatic iron excess has been shown in patients with chronic hepatitis C.[68] We showed that transgenic mice expressing the HCV polyprotein fed an excess-iron diet developed HCC through hepatic accumulation of 8-OHdG.[65]

Here, we discuss the mechanisms by which hepatic iron accumulates in chronic hepatitis C, focusing on the relationship between HCV-induced ROS production and iron metabolic disorder. Systemic iron homeostasis is mainly regulated both by intestinal absorption and macrophage recycling of iron from hemoglobin because there is no efficient pathway for iron excretion.[69]

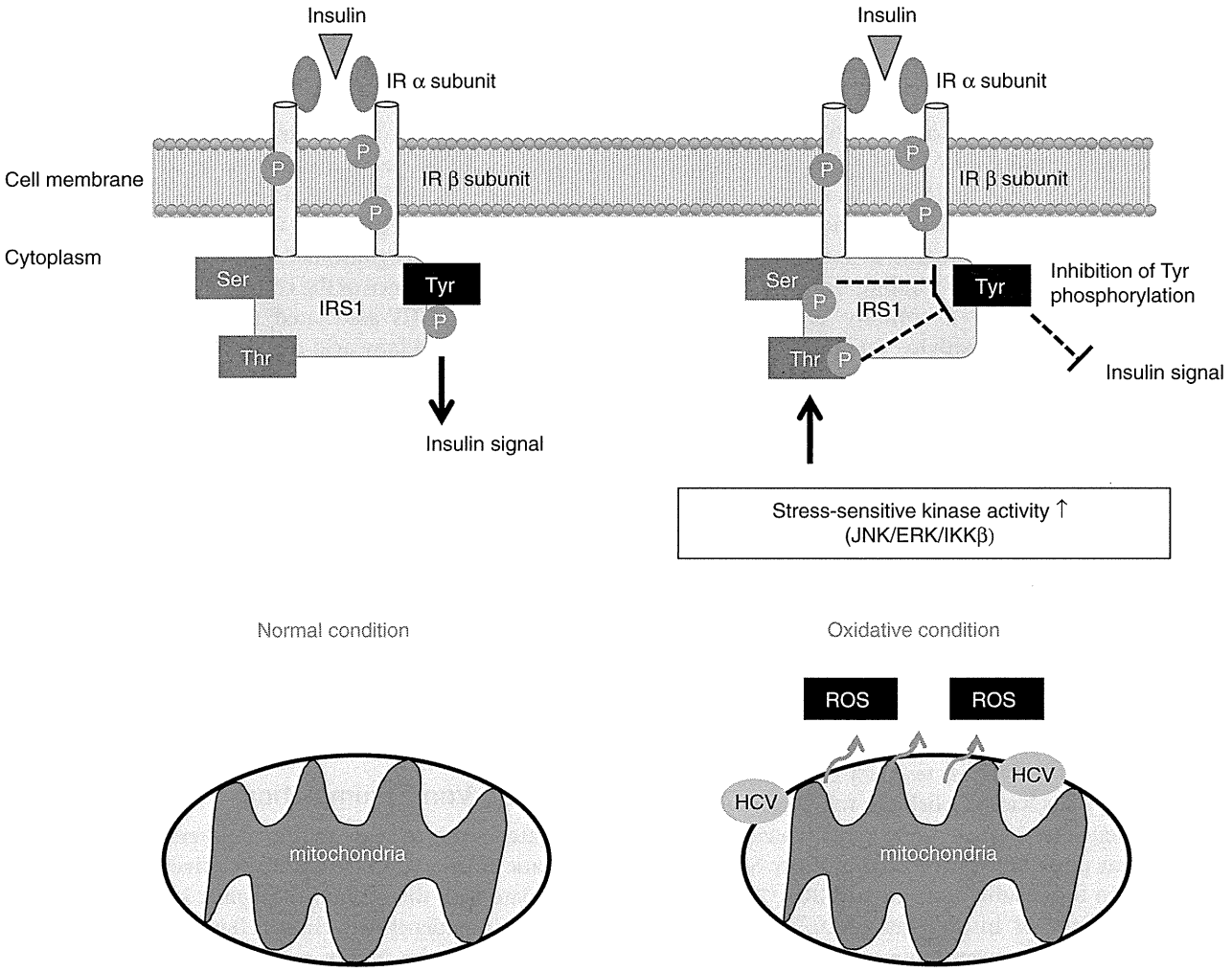

Figure 2 Schematic diagram depicting the presumed mechanism underlying the inhibitory effect on tyrosine phosphorylation within insulin receptor substrate 1 (IRS1). Hepatitis C virus (HCV) core-induced mitochondrial reactive oxygen species (ROS) production is presumed to induce insulin resistance through activation of Ser/Thr kinases such as c-Jun N-terminal kinase-1 (JNK1) and subsequent inhibition of tyrosine phosphorylation within IRS1. IR, insulin receptor; Ser, serine; Thr, threonine; Tyr, tyrosine; ERK, extracellular signal-regulated kinase; IKKβ, inhibitory-κβ kinase β.

Hepcidin, which was originally isolated from human serum and urine as a peptide with antimicrobial activity,[70] is a hormone exclusively synthesized in the liver and a soluble regulator that acts to attenuate both intestinal iron absorption and iron release from reticuloendothelial macrophages.[71] Hepatic mRNA levels[72] and the 25 amino acid bioactive hepcidin levels in serum[73] are lower in chronic hepatitis C than in chronic hepatitis B or controls, despite a significant correlation between hepcidin and serum ferritin or the histological iron score. Thus, the relatively decreased synthesis of hepcidin in chronic hepatitis C contrasts with the absolute deficit or lack of hepcidin synthesis observed in hereditary hemochromatosis. The detailed mechanisms underlying the transcriptional regulation of hepcidin are discussed elsewhere. Interestingly, alcohol metabolism-mediated ROS were shown to suppress hepcidin transcription via CCAAT/enhancer-binding protein α (C/EBPα).[74] In parallel with these results, we found that hepcidin promoter activity and the DNA binding activity of C/EBPα were downregulated concomitant with increased expression of C/EBP homology protein (CHOP), an inhibitor of C/EBP DNA binding activity, and with increased levels of mitochondrial ROS in

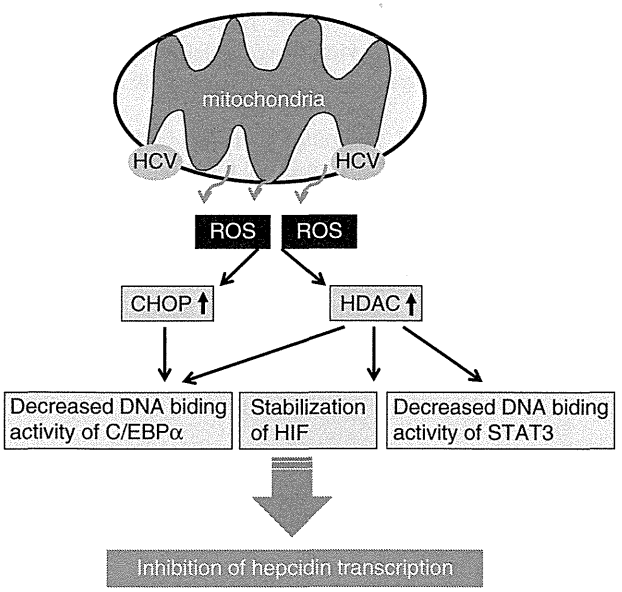

Figure 3 Schematic diagram depicting the mechanism by which mitochondrial reactive oxygen species (ROS) suppress hepcidin transcription in transgenic mice expressing hepatitis C virus (HCV) polyprotein or full-length HCV replicon cells. CHOP, CCAAT/enhancer-binding protein (C/EBP) homology protein; HDAC, histone deacetylase; HIF, hypoxia inducible factor; STAT3, signal transduction and activator of transcription 3.

transgenic mice expressing the HCV polyprotein.[75] There are several lines of evidence indicating that ROS upregulate the expression of CHOP.[76] In agreement with our observation, an *in vitro* study using hepatoma cells showed that HCV-induced ROS inhibited the binding activity of C/EBPα and signal transduction and activator of transcription 3 to the hepcidin promoter in addition to stabilization of hypoxia-inducible factor through increased histone deacetylase activity.[77] Thus, HCV core-induced mitochondrial ROS accumulate hepatic iron through the inhibition of hepcidin transcription (Fig. 3).

CONCLUSION

IN THE PRESENT review we discussed how HCV interacts with mitochondria and how subsequently occurring mitochondrial ROS production contributes to the pathophysiology of HCV-related chronic liver diseases. The mitochondrion is the key organelle that determines the cellular response to various kinds of biological stress. Therefore, it may not be surprising that HCV-induced alterations of mitochondrial functions have a critical impact on disease progression towards hepatocarcinogenesis by creating an oxidatively stressed liver microenvironment through mitochondrial ROS production. However, the molecular details underlying HCV-induced mitochondrial dysfunctions remain confusing and are still a matter of debate, which undoubtedly requires further investigation to shed light on the questions in this field.

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NUTRITION-RELATED LIVER DISORDERS: NAFLD

Iron metabolic disorder in chronic hepatitis C: Mechanisms and relevance to hepatocarcinogenesis

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Key words
hepatitis C, hepcidin, iron, oxidative stress, reactive oxygen species.

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Abstract

The liver is the major iron storage organ in the body, and therefore, iron metabolic disorder is sometimes involved in chronic liver diseases. Chronic hepatitis C is one of the liver diseases that show hepatic iron accumulation, even though its level should be recognized to be basically mild to moderate and sometimes within the normal range. The mechanisms underlying hepatic iron accumulation in chronic hepatitis C have not been fully elucidated. Reduction of the hepcidin transcription activity by hepatitis C virus (HCV)-induced reactive oxygen species may in part account for it, but the regulation of hepcidin is very complex and may depend on many variables, including the particular stage of the systemic and/or hepatic inflammatory conditions and the circulating transferrin-bound iron and intracellular iron stores. This might explain the variations in hepatic iron concentrations reported among patients with HCV-related chronic liver disease. However, even mild-to-moderate iron overload in the liver contributes to disease progression and hepatocarcinogenesis in chronic hepatitis C probably by reinforcing the HCV-induced oxidative stress through Fenton reaction. The present review highlights the current concept of hepatic iron overload status in chronic hepatitis C and discusses how iron metabolic disorder develops in this disease and the impact of hepatic iron overload on disease progression and its relevance to hepatocarcinogenesis.

Introduction

Approximately 170 million people worldwide are infected with hepatitis C virus (HCV).[1] HCV infection often remains asymptomatic but can lead to severe liver damage. However, how HCV causes liver injury and liver cancer is not fully understood. Histological examination has revealed that chronic inflammation seems to play an important role in the pathogenesis of chronic hepatitis C, and excess iron also is associated with increased morbidity and mortality.[2,3] In addition, a study using electron microscopy and X-ray microanalysis demonstrated that almost all liver specimens from patients with chronic hepatitis C had at least some lysosomal iron deposits even when no iron deposit was evident with standard optical microscopy and Prussian Blue staining.[4] Elevated iron-related serum markers and increased hepatic iron accumulation are relatively common and correlate with the severity of hepatic inflammation and fibrosis in patients with chronic hepatitis C. Excess divalent iron can be highly toxic mainly via the Fenton reaction producing hydroxyl radicals.[5] This is particularly relevant for chronic hepatitis C, in which oxidative stress has been proposed as a major mechanism of liver injury. Oxidative stress and increased iron levels strongly favor DNA damage, genetic instability, and tumorgenesis. Indeed, a significant correlation between 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidatively generated DNA damage,[6] and hepatic iron excess has been shown in patients with chronic hepatitis C.[7] Kato *et al.* reported that phlebotomy lowered the risk of progression to hepatocellular carcinoma (HCC),[8,9] which showed the critical role of iron in the development of HCC in patients with chronic hepatitis C. Thus, there is a critical interaction between HCV infection and hepatic iron overload in the progression of liver disease and the development of HCV-related HCC. However, the mechanisms underlying hepatic iron overload and its contribution to hepatocarcinogenesis in chronic hepatitis C are not fully elucidated. The present review highlights the current concept of hepatic iron overload status in chronic hepatitis C and discusses how iron metabolic disorder develops in chronic hepatitis C, the impact of hepatic iron overload on disease progression, and its relevance to hepatocarcinogenesis.

Regulation of systemic iron homeostasis

In normal adults, storage iron is deposited in hepatocytes and tissue macrophages and mobilized in response to acute need. Serum iron levels are determined both by intestinal absorption and macrophage recycling of iron from hemoglobin because there is no efficient pathway for iron excretion.[10] Regulatory effectors that modulate intestinal iron absorption probably also

modulate the release of iron from tissue macrophages and hepatocytes. Hepcidin appears to be such a regulatory effector. It is a small, cysteine-rich peptide, cleaved from a larger precursor.[11–13] Hepcidin, which was originally isolated from human serum and urine as a peptide with antimicrobial activity,[11,13] is a hormone exclusively synthesized in the liver and a soluble regulator that acts to attenuate both intestinal iron absorption and iron release from reticuloendothelial macrophages.[12,14] Increased plasma iron from macrophage recycling of aged red blood cells or from intestinal absorption of iron stimulates hepatocytes through several signaling pathways to produce more hepcidin. Ferroportin is an iron exporter on the surface of absorptive intestinal enterocytes, macrophages, hepatocytes, and placental cells, all of which release iron into plasma.[15–17] Circulating hepcidin can bind to ferroportin, cause internalization, and trap iron in hepatocytes, macrophages, and absorptive enterocytes.[18] Thus, coupling the internalization of ferroportin to hepcidin levels generates a homeostatic loop regulating the iron plasma level and the tissue distribution of iron.

Transcriptional regulation of hepcidin

Knowledge of how hepcidin transcription is regulated within hepatocytes appears to be indispensable for understanding the mechanisms underlying hepatic iron overload in chronic hepatitis C because hepcidin is the central regulator of systemic iron homeostasis. Important elements of the signaling pathway present on the hepatic plasma membrane that affect hepcidin transcription include transferrin receptor 2 (TfR2),[19] HFE,[20] which is the protein affected in the most common form of genetic hemochromatosis, and hemojuverin (HJV),[21] a member of the bone morphogenetic protein (BMP) receptor family. The mechanisms by which TfR2, HFE, and HJV are linked to changes in hepcidin transcription are incompletely understood, but the discovery of HJV revealed that the well-known sons of mothers against decapentaplegic (SMAD) signal transduction pathway was important in this process.[22] Notably, animals that lack hepatocyte SMAD4, a protein that combines with other members of the SMAD family to regulate transcription of target genes, develop significant iron overload associated with a profound reduction in hepcidin expression.[23] Interleukin 6 (IL-6) activates hepcidin transcription through a pathway that involves janus kinase-signal transducer and activator of transcription (STAT) signaling and a binding site for the transcription factor STAT3.[24,25] The transcription factor CCAAT/enhancer-binding protein α (C/EBPα) is also clearly involved in regulating hepcidin transcription.[26] C/EBPα knockout mice demonstrate decreased hepcidin expression and iron overload.[26]

The pathways described earlier activate hepcidin transcription, but only one pathway has been identified that represses hepcidin expression. The transmembrane serine protease (TMPRSS6) is part of the pathway that suppresses hepcidin expression as revealed in TMPRSS6 mutant mice.[27]

Hepatic iron accumulation in chronic hepatitis C

Based on the assumption that one-third of iron stores are normally in the liver, this would translate to a normal median hepatic iron content of 0.27 g for men and 0.13 g for women.[28] Extensive studies reported median hepatic iron concentrations of 396 (range 0–2105) and 458 (range 114–2190) μg/g dry weight liver tissue in patients with chronic hepatitis C.[29,30] These results suggest that hepatic iron content in patients with chronic hepatitis C is approximately 0.50~0.69 g, equivalent to two to five times the normal hepatic iron content if the liver weight is estimated to be 1500 g. In contrast, a hepatic iron index (μmol Fe/g liver tissue/patients age) of 1.9 or more has been reported to be typical of patients with hereditary hemochromatosis.[31] If the hepatic iron index of a patient aged 60 with hereditary hemochromatosis is 1.9, the hepatic iron concentration of this patient is assumed to be 6384 μg/g liver tissue. Thus, we should understand that hepatic iron content is much less in chronic hepatitis C than in hereditary hemochromatosis, even though it is recognized to be one of liver diseases that show hepatic iron accumulation.

There also remains uncertainty as to whether iron predominantly accumulates in hepatocytes or the reticuloendothelial system, mainly Kupffer cells, in patients with chronic hepatitis C. Some clinical studies showed that iron was mainly localized in the reticuloendothelial system,[32,33] whereas others reported its localization in hepatocytes.[34] Interestingly, Fiel *et al.* documented that even ribavirin-associated hemolysis deposited iron preferentially in hepatocytes in patients with chronic hepatitis C.[35] Hepatocytic iron accumulation may indicate potential DNA damage and genetic instability in association with HCV-induced oxidative stress, whereas iron deposition in Kupffer cells may contribute to cytokine release leading to inflammation or fibrosis. However, further investigations are needed to clarify this issue.

Mechanisms underlying hepatic iron accumulation in chronic hepatitis C

HFE is a major histocompatibility class I-like (MHC) molecule that, unlike other known classical and non-classical MHC proteins, has a regulatory role in the functions of iron metabolism in cells and the body. A homozygous mutation in the HFE protein in humans that changes cysteine at position 282 to tyrosine is responsible for iron overload and organ damage resulting in hemochromatosis.[36] The role of HFE mutations in chronic hepatitis C has been well reviewed.[37] In general, patients with chronic hepatitis C seem to have no difference in the prevalence of heterozygosity for HFE mutations as compared with a control population. It is still controversial as to whether HFE mutations are associated with hepatic iron overload in chronic hepatitis C probably because of the different methodologies used to measure hepatic iron and/or confounding variables such as demographic parameters, environmental factors, hepatic inflammatory activity, and the duration of HCV infection among the reported studies. In addition, HFE mutations are seemingly not associated with the progression of liver disease in chronic hepatitis C patients even though HFE may affect Kupffer cells or interact with immune cells.

Fujita *et al.* showed for the first time that hepatic hepcidin messenger RNA (mRNA) levels adjusted by serum ferritin values were significantly lower in patients with chronic hepatitis C than in those with chronic hepatitis B or those without hepatitis B virus (HBV) or HCV infection.[38] Of note, the relative expression of hepcidin for iron stores was lower in chronic hepatitis C than in chronic hepatitis B or chronic liver diseases without HBV or HCV infection, even though hepcidin expression levels were strongly

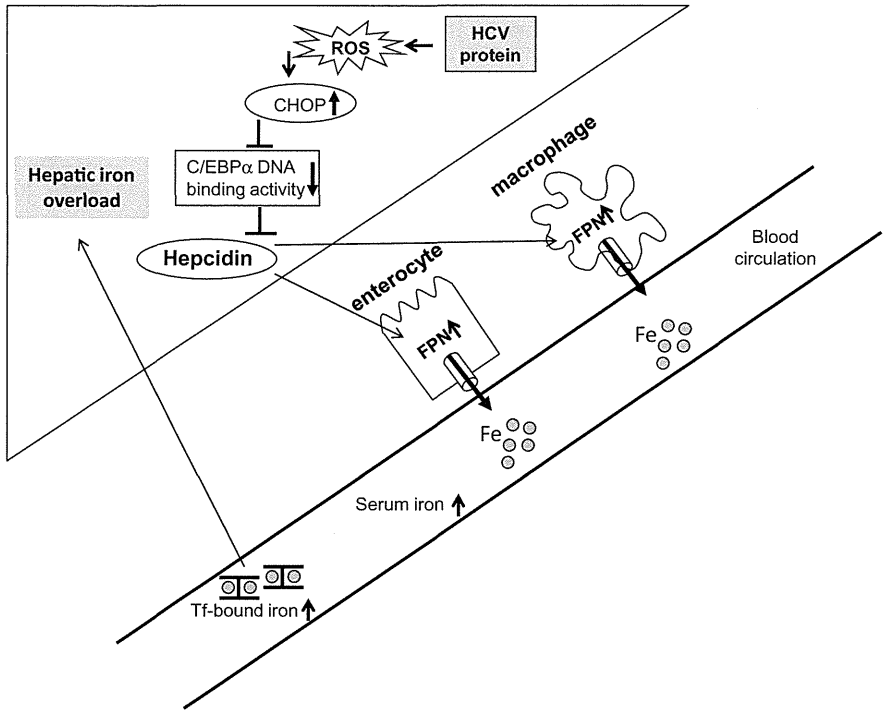

Figure 1 Schematic diagram depicting the mechanisms underlying the hepatic iron accumulation in transgenic mice expressing the hepatitis C virus (HCV) polyprotein. HCV protein-induced reactive oxygen species (ROS) increase hepatic expression of CCAAT/enhancer-binding protein (C/EBP) homology protein (CHOP) and subsequently reduce DNA binding activity of C/EBPα, which leads to reduction of hepcidin transcription. Decreased hepcidin expression increases ferroportin (FPN) expression in the enterocytes and reticuloendothelial macrophages resulting in increased duodenal iron transport and macrophage iron release, which lead to hepatic iron accumulation.

correlated with serum ferritin and the degree of hepatic iron deposition. These results suggested that hepcidin might play a pivotal role in iron overload in patients with chronic hepatitis C. A recent study using a validated immunoassay of the 25 amino acid bioactive hepcidin in serum also revealed that serum hepcidin levels were lower in patients with chronic hepatitis C than in controls despite a significant correlation between hepcidin and serum ferritin or the histological iron score in both groups.[39] Thus, the relatively decreased synthesis of hepcidin in chronic hepatitis C contrasts with the absolute deficit or lack in hepcidin synthesis observed in hereditary hemochromatosis and may account for the mild-to-moderate hepatic iron overload observed in some patients with chronic hepatitis C.

Regulation of hepcidin transcription by HCV, iron overload, and inflammation

The next question is how hepcidin transcription is suppressed in the presence of HCV infection. Which pathway for regulating hepcidin transcription is affected? Oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases.[32] The HCV core protein induces the production of reactive oxygen species (ROS) through inhibition of mitochondrial electron transport.[40] Interestingly, alcohol metabolism-mediated ROS were shown to suppress hepcidin transcription via C/EBPα.[41] Therefore, we investigated the mechanisms underlying hepcidin transcription inhibited by HCV focusing on ROS production, which plays a critical role in the pathogenesis of both alcoholic liver disease and chronic hepatitis C. Hepcidin promoter activity and the DNA binding activity of C/EBPα were downregulated concomitant with increased expression of C/EBP homology protein, an inhibitor of C/EBP DNA binding activity, and with increased levels of ROS in transgenic mice expressing the HCV polyprotein[42] (Fig. 1). Thus, the mechanisms underlying HCV-related hepatic iron overload appear to have some similarities with alcohol-induced iron overload in terms of disrupted hepcidin transcription through suppressed activity of C/EBPα. In agreement with our observation, an *in vitro* study by Miura *et al.* using hepatoma cells showed that HCV-induced ROS inhibited the binding activity of C/EBPα to the hepcidin promoter through increased histone deacetylase activity.[43]

Hepcidin is also regulated by both circulating transferrin-bound iron and intracellular iron stores. The exact mechanism is still unknown but seems to involve the BMP/SMAD pathway. As yet, there is no convincing evidence that accounts for the suppressive transcription of hepcidin through the BMP/SMAD cascade in chronic hepatitis C. Taking into account the significant correlation between hepcidin and serum ferritin, or the histological iron score, hepcidin transcription seems to be properly regulated in response to the iron concentration in chronic hepatitis C. Thus, the opposing effects of HCV-induced hepcidin-suppressive factors and iron load-induced hepcidin-stimulation factors potentially regulate hepcidin transcription in chronic hepatitis C. As suggested by Girelli *et al.*,[39] in the early phase of chronic hepatitis C hepcidin may be prominently suppressed by HCV, but as iron accumulates, the negative influence of viral factors may be masked by the positive stimulation of iron.

Inflammation also regulates hepcidin transcription. Pro-inflammatory cytokines such as IL-6 mediate this response by inducing transcription of hepcidin mRNA via STAT3, which binds to a STAT-responsive element within the hepcidin promoter.[24,25] Our transgenic mice expressing the HCV polyprotein did not show any inflammation in the liver. A possible pitfall in this experimental model was that we could not take the inflammatory effect on

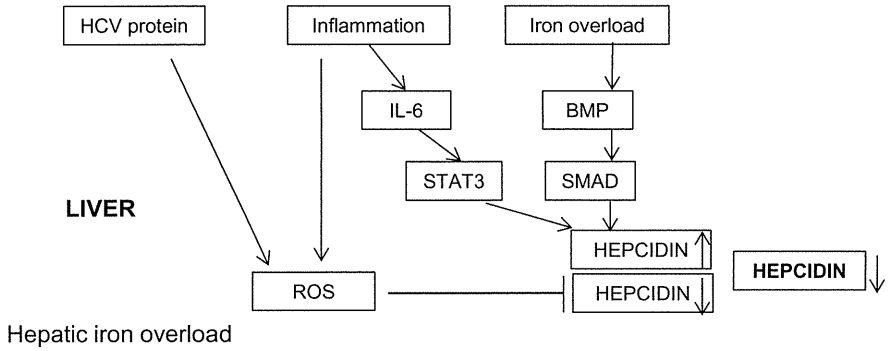

Figure 2 Schematic diagram depicting the assumed mechanisms underlying the hepatic iron accumulation in patients with chronic hepatitis C. Hepcidin transcription in chronic hepatitis C may be potentially regulated by the opposing effects of hepatitis C virus (HCV)-related reactive oxygen species (ROS)-induced hepcidin suppression and iron load-induced hepcidin stimulation. Inflammation may also have the opposing effects of stimulation and suppression of hepcidin transcription through the interleukin (IL)-6/signal transducer and activator of transcription (STAT) pathway and ROS pathway, respectively. Consequent relative suppression of hepcidin expression is potentially one of the mechanisms underlying the hepatic iron accumulation in patients with chronic hepatitis C. BMP, bone morphogenetic protein; SMAD, sons of mothers against decapentaplegic.

hepcidin regulation into account, which is different from what is observed in patients with chronic hepatitis C. Serum levels of IL-6 have been shown to be elevated in patients with HCV-related chronic liver disease,[44] which raises the possibility that IL-6 acts to stimulate hepcidin expression through the STAT3 pathway. This would be expected to counteract the decrease in hepcidin transcription caused by HCV-induced ROS. However, no significant relationship has been found between serum IL-6 and hepcidin in patients with chronic hepatitis C,[39,45] even though a paracrine effect of local IL-6 release on hepcidin transcription in the liver cannot be excluded. On the other hand, chronic inflammation with production of pro-inflammatory cytokines has the potential to deliver an additional burden of ROS, which would be expected to reinforce the decrease in hepcidin transcription. Most likely, during chronic inflammation states in vivo like chronic hepatitis C, the regulation of hepcidin is more complex and may depend on many variables, including the particular stage of systemic and/or hepatic inflammatory disease. This might explain the variations in hepatic iron concentrations reported among patients with HCV-related chronic liver disease. The schematic outline in Figure 2 depicts the assumed mechanisms underlying the hepatic iron accumulation in chronic hepatitis C.

Relevance of hepatic iron overload to hepatocarcinogenesis

Studies in HCV-infected and uninfected chimpanzees demonstrated that iron loading did exacerbate liver injury in HCV-infected chimpanzees and that HCV infection increased the susceptibility of the liver to injury following iron loading.[46] Increased hepatic iron deposition is reported to be associated with more advanced liver fibrosis in patients with chronic hepatitis C.[47] Recently, it has been prospectively shown in the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis Trial cohort that stainable iron in hepatocytes and portal tract cells predicts progression and outcomes (Child–Pugh score > 7, ascites, encephalopathy, variceal bleeding, spontaneous bacterial peritonitis, HCC, and death) in advanced chronic hepatitis C.[48] Thus, iron is a cofactor that influences the severity and progression of chronic hepatitis C.

Although the association of markedly increased iron accumulation in the liver with hepatocarcinogenesis in hereditary hemochromatosis has been well described,[49] it remains to be elucidated whether mild-to-moderate increases in hepatic iron accumulation contribute to the development of HCC in patients with HCV-associated chronic liver diseases. Nevertheless, there are several lines of evidence that suggest the association of hepatic iron overload with hepatocarcinogenesis in chronic hepatitis C. It has been reported that hepatic iron storage is strongly correlated with hepatic 8-OHdG levels and that subsequent oxidative DNA damage in the liver is associated with an increased risk of HCC development.[2] In addition, the decrease in hepatic 8-OHdG content caused by phlebotomy lowers the risk of progression to HCC, which indeed shows the critical role of the iron-overload state in the development of HCC in patients with chronic hepatitis C.[8,9]

We investigated whether mild iron overload actually induced HCC in the presence of HCV protein using transgenic mice expressing the HCV polyprotein. Transgenic mice fed an excess-iron diet showed marked hepatic steatosis, including the centrilobular microvesicular type, ultrastructural alterations of the mitochondria and decreased degradation activity of fatty acid at 6 months, as well as hepatic accumulation of lipid peroxidation products and 8-OHdG at 12 months after the initiation of feeding. Of note, hepatic tumors including HCC developed in 5 of 11 (45%) transgenic mice fed the excess-iron diet at 12 months after the initiation of feeding but did not in control mice or transgenic mice fed the control diet.[50] These results indicate the importance of oxidative stress and subsequent mitochondrial injury synergistically induced by iron loading and HCV proteins in the development of HCC. Thus, there seems to be a close relationship between the development of HCC and oxidative DNA damage synergistically induced by hepatic iron accumulation and HCV proteins. However, further investigations are needed to clarify the detailed mechanisms by which hepatic iron accumulation results in the development of HCC in chronic hepatitis C.

Acknowledgments

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Risk Factors for Survival and the Development of Hepatocellular Carcinoma in Patients with Primary Biliary Cirrhosis

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Abstract

Objective Early diagnosis of hepatocellular carcinoma (HCC) is critical in the management of patients with primary biliary cirrhosis (PBC), since the prognosis of PBC has improved. The aim of this study was to investigate whether HCC development affects the prognosis of PBC and to identify the risk factors for HCC in Japanese patients with PBC.

Methods We compared the survival rates between patients with HCC and those without and analyzed the risk factors for HCC development in 210 patients with PBC who were followed up for a median period of 8.5 years.

Results HCC developed during follow-up in 11 patients (5.2%) and was diagnosed simultaneously at the time of diagnosis of PBC in five patients (2.4%) who were excluded from the analysis. A Kaplan-Meier analysis showed a significant difference in overall survival between the patients who did and did not develop HCC ($p<0.001$). A multivariate analysis revealed age (OR: 1.08, 95% confidence interval [CI]: 1.03-1.13, $p=0.001$), the albumin level (OR: 0.24, 95% CI: 0.10-0.56, $p=0.001$), the total bilirubin level (OR: 1.60, 95% CI: 1.09-2.36, $p=0.017$) and HCC development (OR: 2.97, 95% CI: 1.24-7.15, $p=0.015$) to be significant prognostic factors and identified only an advanced histological stage (Scheuer's classification III or IV, OR: 6.27, 95% CI: 1.80-21.83, $p=0.004$) to be a risk factor associated with HCC.

Conclusion HCC development significantly affects the survival of patients with PBC, and an advanced histological stage is the only risk factor associated with HCC development. These results highlight the important role of liver fibrosis in hepatocarcinogenesis in patients with PBC.

Key words: diabetes mellitus, liver fibrosis, prognosis, survival, ursodeoxycholic acid

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Introduction

Primary biliary cirrhosis (PBC) is a progressive cholestatic liver disease characterized by the presence of highly specific antimitochondrial antibodies, portal inflammation and lymphocyte-dominated destruction of the intralobular bile ducts that eventually leads to cirrhosis (1). The incidence of PBC has increased over recent decades, possibly attributable to augmented testing of liver biochemistry rather than a rise in disease incidence. The routine use of biochemical screening has also made it possible to diagnose PBC at an earlier stage (2). In addition to early diagnosis, the high prevalence of treatment with ursodeoxycholic acid (UDCA) makes it possible for patients with PBC to live longer. However, the natural history of PBC is still debated and depends on several variables and symptoms of liver disease (3). In general, the risk of cancer development increases as humans live longer, and the development of hepatocellular carcinoma (HCC) is no exception.

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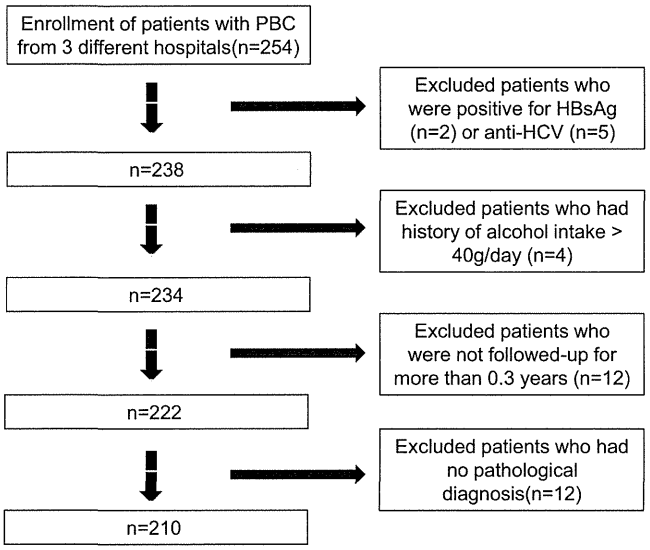

Figure 1. Numbers of patients included and excluded in the retrospective cohorts.

Patients with PBC are considered to be at low risk for the development of HCC (4), although several reports have revealed that PBC is associated with an increased risk of HCC (5-8). Additionally, whether the development of HCC affects the overall survival of patients with PBC remains controversial (5, 7, 9, 10). This matter and the identification of risk factors for HCC development are of importance for improving the prognosis of patients with PBC. There are, however, few studies reporting the risk factors for HCC among patients with PBC (5-7, 9, 11). The age at diagnosis (7, 11), male sex (5, 7, 10, 11), a history of blood transfusions (7, 11), cigarette smoking (6), portal hypertension (11), a more advanced histologic stage of disease (9, 10) and superinfection with hepatitis C virus (HCV) (6) have been reported to be risk factors for HCC in patients with PBC. Such risk factors remain a matter of debate due to recent changes in the nutritional environment and/or lifestyle. For instance, previous studies did not include obesity or diabetes mellitus as clinical parameters for assessing risk factors related to the development of HCC in patients with PBC, even though these parameters have been recognized to be risk factors for the development of HCC (12, 13).

The aim of this study was to determine whether HCC development affects the prognosis of PBC and to identify the risk factors for HCC development in Japanese patients with PBC based on recent clinical evidence.

Materials and Methods

This study was conducted among three series of patients from three different liver disease centers who were treated for a diagnosis of PBC between May 1984 and May 2010. A total of 245 patients were recruited, including 116 patients from Kawasaki Medical School affiliated hospital, 72 patients from Yamaguchi Grand Medical Center and 57 patients from Kawasaki Hospital. The diagnosis of PBC was established when the patient fulfilled at least one of the following criteria defined by the Intractable Hepato-Biliary Disease Study Group of Japan: (i) laboratory abnormalities consistent with chronic cholestatic liver disease and the presence of chronic nonsuppurative destructive cholangitis, (ii) positive antimitochondrial antibodies and a liver histology compatible with PBC, (iii) a medical history and laboratory abnormalities consistent with chronic cholestatic liver disease and positive antimitochondrial antibodies. The histological stage was classified according to Scheuer's classification (14). The following 35 patients were excluded from the analysis due to confounding risk factors for HCC or incomplete clinical parameters: hepatitis B surface antigen (HBsAg) positivity in two patients, anti-HCV antibody (anti-HCV) positivity in five patients, a history of excessive alcohol consumption (>40 g/day) in four patients, a lack of follow-up for more than 0.3 years in 12 patients and a lack of pathological diagnosis in 12 patients (Fig. 1).

The diagnosis of diabetes mellitus was made based on a history of antidiabetic medication use, including oral hypoglycemic agents and insulin, since the diagnostic criteria for diabetes mellitus proposed by the Japan Diabetes Society were not applied to all patients due to the lack of several biochemical parameters. The diagnosis of portal hypertension was made based on the presence of esophageal or gastric varices, ascites or splenomegaly. The clinical characteristics at diagnosis of PBC are shown in Table 1. The patients were regularly assessed with biochemical tests every one to four months and followed for a median period of 8.5 (range, 0.3-25.8) years. To determine the presence of HCC, abdominal ultrasonography was performed in all patients without HCC at intervals of four to 12 months. HCC was diagnosed based on the findings of abdominal ultrasonography and confirmed based on the findings of computed tomography (CT), magnetic resonance imaging, hepatic arteri-

Table 1. Clinical Characteristics of Patients at Diagnosis of PBC

Characteristic	Mean ± SD or frequency (number of analyzed patients)
Age (years)	58 ± 11 (210)
Gender (Male/Female)	31/179
Body mass index (kg/m^2)	22.4 ± 3.1 (196)
Blood transfusion (+/-/unknown)	13/192/5
Diabetes mellitus (+/-)	19/191
Portal hypertension (+/-)	39/171
Platelet count (× 10^4/μL)	21.9 ± 8.2 (210)
ALT (IU/L)	58 ± 53 (210)
AST (IU/L)	56 ± 40 (207)
Alkaline phosphatase (IU/L)	523 ± 434 (207)
γ-GT (IU/L)	230 ± 233 (207)
Total bilirubin (mg/dL)	0.9 ± 0.9 (210)
Albumin (g/dL)	3.9 ± 0.5 (209)
Prothrombin time (%)	82.9 ± 16.4 (209)
IgM (mg/dL)	410 ± 279 (203)
Anti-HBc (+/-/unknown)	13/40/157
Histological stage (I or II/III or IV)	169/41
Treatment with UDCA (+/-)	189/21

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyltransferase, Anti-HBc: anti-hepatitis B core antibody, UDCA: ursodeoxycholic acid

ography and/or a fine-needle aspiration liver biopsy.

Overall survival was defined as the period from the day of PBC diagnosis until death, liver transplantation or the last medical examination and compared between the patients who did and did not develop HCC. Laboratory parameters measured at the time of PBC diagnosis were compared to those measured at the time of HCC diagnosis in patients who developed HCC during the follow-up period. The study protocol conformed to the 1975 Declaration of Helsinki Declaration and was approved by the ethics committees of the involved institutions.

Statistical analysis

Baseline continuous variables are expressed as the mean ± SD. Comparisons between groups were made using the Mann-Whitney test for continuous variables and the χ^2 test with Yates correction or Fisher's exact test for categorical variables. Cumulative survival was calculated using the Kaplan-Meier method, and the differences among the groups were analyzed with the log-rank test. Univariate and multivariate analyses of predictors of survival were performed using the Cox proportional hazards model. A multivariate analysis of predictors for the development of HCC was performed using the logistic regression test. A p value of less than 0.05 was considered to be significant. All analyses described above were performed using the SPSS software package (version 11, SPSS Inc., Chicago, IL).

Results

Development of HCC in patients with PBC

Sixteen (7.6%) of the 210 patients with PBC developed HCC. A diagnosis of HCC was made in 11 patients (5.2%) during follow-up, whereas HCC and PBC were almost simultaneously diagnosed in the remaining five patients (2.4%) who had diabetes mellitus and advanced histological stages, except for a man aged 84 who was potentially at high risk for HCC due to his age. These five patients (three men and two women) were thereafter excluded from the analysis. The clinical and histological features of the 11 patients who developed HCC during follow-up are summarized in Table 2. The HCC incidence according to gender was 3.6% (1/28) in men and 5.6% (10/177) in women. The mean interval between the diagnosis of PBC and the development of HCC was 11.4±5.7 years. Antibodies to hepatitis B core antigens (anti-HBc) were positive in two patients (18.2%), negative in five patients (45.4%) and unknown in four patients (36.4%), respectively. Six patients (54.5%) and one patient (9.1%) had advanced histological stages (III) and diabetes mellitus at the diagnosis of PBC, respectively. It should be noted that the follow-up period until the development of HCC was significantly longer in the patients with mild histological stages (I or II) (14.8±4.1 years) than in those with advanced histological stages (III) (8.5±5.5 years), suggesting a potential risk for HCC development after a long period of time even in patients with a mild histological stage of PBC. Six patients had solitary tumors whose size was less than 30 mm, except for one. These tumors were treated with local ablation, such as percutaneous microwave coagulation therapy, percutaneous ethanol injection or radiofrequency ablation, transarterial chemoembolization (TACE), combination of local ablation and TACE or liver transplantation, in all patients, except one (case 9 in Table 2) who could not be treated due to rupture of HCC.

To determine whether HCC develops as liver damage progresses, laboratory parameters (platelet count, alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase, γ-glutamyltransferase, total bilirubin, albumin and prothrombin time) were compared between the two time points of PBC diagnosis and HCC diagnosis in the 11 patients. The serum albumin level, prothrombin activity and platelet count were significantly lower at the time of HCC diagnosis than at the time of PBC diagnosis, suggesting that the development of HCC largely depends on the progression of liver disease (Fig. 2). Patients with PBC who developed HCC had significantly lower cumulative survival rates than those who did not. The 5-, 10-, 15- and 20-year cumulative survival rates of the PBC patients with HCC and those without HCC were 91/98%, 61/87%, 51/83% and 10/80%, respectively ($p<0.001$, Fig. 3).

Factors associated with survival in patients with PBC

We next investigated the factors associated with survival in patients with PBC in order to determine whether HCC development actually affects the prognosis. In addition to the baseline characteristics of the patients at the time of PBC diagnosis, treatment with UDCA and the development of HCC were incorporated into the parameters used to ana-

Table 2. Characteristics of 11 Patients with PBC who Developed HCC

Case	Age at PBC/HCC diagnosis	Sex	BMI	DM	Anti-HBc	Histological stage at§	HCC				Cause of death
							Solitary or multiple	Maximum size (mm)‡	Vascular invasion	Therapy¶	
1	64/67	M	25.8	-	+	III	Solitary	10	-	PMCT	Sepsis
2	66/75	F	26.9	-	-	III	Multiple	45	-	PEI + TACE	Hepatic failure
3	65/74	F	23.2	-	+	III	Multiple	32	-	TACE	Hepatic failure
4	59/70	F	23.3	+	-	III	Multiple	20	-	REI + TACE	HCC
5	61/75	F	17.8	-	-	II	Multiple	30	-	TACE	Hepatic failure
6	38/55	F	19.5	-	-	I	Solitary	30	-	PEI + TACE	Hepatic failure
7	38/55	F	20.8	-	NA†	I	Multiple	15	-	TACE	Hepatic failure
8	58/76	F	23.1	-	-	I	Solitary	30	-	TACE	Hepatic failure
9	64/66	F	22.2	-	NA	III	Solitary	100	+	-	HCC rupture
10	41/49	F	27.7	-	NA	I	Solitary	10	-	Transplantation	Alive
11	48/65	F	24.6	-	NA	III	Solitary	27	-	TACE	Alive

BMI: body mass index, DM: diabetes mellitus, Anti-HBc: anti hepatitis B core antibody, †NA: unknown, §at the diagnosis of PBC, ‡tumor sizes in cases 2, 3, 4, 5 and 7 represent the largest ones among multiple tumors. ¶PMCT: percutaneous microwave coagulation therapy, PEI: percutaneous ethanol injection, TACE: transarterial chemoembolization, RFA: radiofrequency ablation

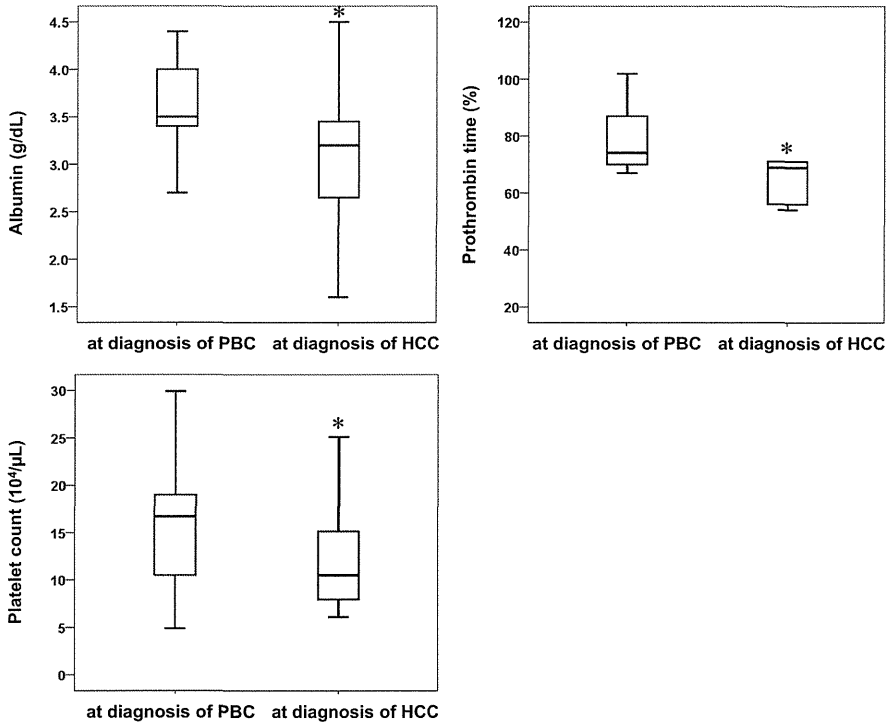

Figure 2. Serum albumin levels, platelet counts and prothrombin activity at diagnosis of primary biliary cirrhosis (PBC) and hepatocellular carcinoma (HCC) in the patients with PBC who developed HCC. The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. *: $p<0.05$

lyze the factors associated with the survival of patients with PBC. However, we did not incorporate the response to treatment with UDCA into these parameters due to a lack of established biochemical criteria for the response to UDCA allowing for prediction of the prognosis. Treatment with UDCA (600 mg daily) was started within six months from diagnosis of PBC in 186 patients (91%). The reason why UDCA was not administered in the remaining 19 patients was unknown. The duration of UDCA treatment was almost the same as the follow-up period, since almost all of the patients continued taking UDCA due to a lack of moderate to severe adverse effects.

A univariate analysis identified age, portal hypertension, platelet count, AST, the total bilirubin and albumin levels, prothrombin activity, advanced histological stage (Scheuer criteria III or IV) and HCC development to be significant predictors of survival in patients with PBC (Table 3). Among these predictors, a multivariate analysis revealed age (OR: 1.08, 95% CI: 1.03-1.13, p=0.001), the total bilirubin (OR: 1.60, 95% CI: 1.09-2.36, p=0.017) and albumin levels (OR: 0.24, 95% CI: 0.10-0.56, p=0.001) and HCC development (OR: 2.97, 95% CI: 1.24-7.15, p=0.015) to be significant factors associated with survival in patients with PBC (Table 3).

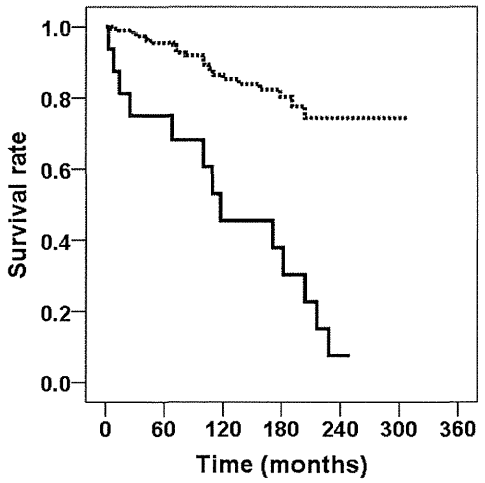

Figure 3. Cumulative survival curves for the patients with primary biliary cirrhosis who developed hepatocellular carcinoma (HCC) and those who did not. The solid and broken lines indicate patients with HCC and those without HCC, respectively. Log-rank test p<0.001

Factors associated with the development of HCC in patients with PBC

As the development of HCC has been demonstrated to be significantly associated with survival in patients with PBC, it is important to determine screening targets for HCC to improve the prognosis for patients with PBC. In this study, the patients with PBC who developed HCC had lower platelet counts (p=0.008), lower albumin levels ((p=0.02) and more advanced histological stages of disease (Scheuer criteria III or IV) (p=0.005) at the time of PBC diagnosis than those who did not (Table 4). A multivariate analysis identified only an advanced histological stage (Scheuer criteria III or IV) (OR: 6.27, 95% CI: 1.80-21.83, p=0.004) as being a predictor of the development of HCC in patients with PBC (Table 5).

Discussion

The frequency of HCC development in patients with PBC is estimated to be around 3% (0.7-3.6%) according to recent and relatively large cohort studies conducted in European countries, the United States and Japan, although this frequency increases as the histological stage progresses (5-10, 15, 16). In this study 5.2% of the patients with PBC developed HCC during follow-up and 2.4% of the patients had HCC simultaneously at the time of PBC diagnosis. The incidence of HCC was slightly higher in the present study than that reported in previous studies; however, it is unknown whether this difference is significant. The higher incidence of HCC observed in the present study may be explained by the relatively longer period of follow-up in a restricted number of institutions. Such situations potentially include less drop out patients during the follow-up period.

HCC was demonstrated to develop as liver damage progressed in the patients with PBC, as indicated by significant decreases in the serum albumin levels, prothrombin activity and platelet counts at the time of HCC development. These results are consistent with a previous observation of a Japanese study group in which all patients had progressed to an advanced histological stage (Scheuer criteria III or IV) by the time of HCC development (7). We also found that HCC development is a significant risk factor for survival in patients with PBC. Because the PBC patients had already progressed to an advanced histological stage at the time of HCC diagnosis, these results appear to be reasonable and are consistent with those of a study from Spain and Italy (9) and a recent Japanese report based on a nationwide survey (10). In fact, the six PBC patients with HCC (6/9, 67%) evaluated in the present study died of hepatic failure (Table 2). In some of the patients with HCC, it was difficult to determine whether the cause of death was hepatic failure or HCC. Therefore, deaths resulting from progressive hepatic failure related to portal venous invasion or rupture of HCC were defined as deaths caused by HCC. Deaths resulting from progressive hepatic failure during the course of treatment for HCC without portal venous invasion were defined as deaths caused by hepatic failure.

In contrast to PBC, HCC development (n=19) was not found to be a significant risk factor for survival in patients with alcoholic liver cirrhosis (n=103) who were followed during almost the same period as those followed in this study and who developed hepatic failure within a relatively short period of time unless they stopped drinking alcohol (unpublished data). The long-term clinical course of PBC may also account for the association between HCC development and a poor prognosis in patients with PBC. In addition to HCC development, age, the albumin level and the total bilirubin level at onset of PBC were selected as significant prognostic factors in patients with PBC in the present study. These results are in part consistent and in part inconsistent with those of previous studies (7, 17), most likely due to the different backgrounds of the patients studied.

That the majority (90%) of patients were treated with UDCA may account for why we did not find any differences in survival between the treated patients and the untreated patients. However, a limitation of this study is that the response to treatment with UDCA could not be incorporated into the analytic factors for survival. The response to UDCA has been reported to be associated with a better prognosis among PBC patients with moderately advanced disease compared with patients with mild disease (18), who accounted for the majority (80%) of the patients in the present study. Recent biochemical criteria for predicting the outcomes of patients with early PBC at low risk for long-term development of liver cirrhosis (19) may be useful for assessing whether the response to UDCA treatment affected the

Table 3. Univariate and Multivariate Analyses of Predictors for Survival in Patients with PBC

Variables	Univariate			Multivariate		
	Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value
Age (years)	1.09	1.04-1.14	<0.001	1.08	1.03-1.13	0.001
Male	1. 65	0.63-4.34	0.31			
Body mass index (kg/m^2)	0.92	0.81-1.05	0.24			
Blood transfusion	1.19	0.28-5.03	0.62			
Diabetes mellitus	3.70	1.56-7.14	0.009			
Portal hypertension	3.34	1.93-7.04	0.002			
Platelet count (× 10^4/μL)	0.92	0.87-0.97	0.003			
ALT (IU/L)	1.00	0.99-1.00	0.64			
AST (IU/L)	1.01	1.00-1.01	0.04			
Alkaline phosphatase (IU/L)	1.00	1.00-1.00	0.24			
γ-GT (IU/L)	1.00	1.00-1.00	0.87			
Total bilirubin (mg/dL)	2.03	1.50-2.76	<0.001	1.60	1.09-2.36	0.017
Albumin (g/dL)	0.13	0.06-0.27	<0.001	0.24	0.10-0.56	0.001
Prothrombin time (%)	0.94	0.91-0.96	<0.001			
IgM (mg/dL)	1.00	1.00-1.00	0.12			
Anti-HBc	0.83	0.23-3.00	0.27			
Histological stage III or IV	5.10	2.40-10.66	<0.001			
Treatment with UDCA	3.35	0.45-24.78	0.24			
Development of HCC	5.15	2.31-11.49	<0.001	2.97	1.24-7.15	0.015

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyltransferase, Anti-HBc: anti hepatitis B core antibody, UDCA: ursodeoxycholic acid, HCC: hepatocellular carcinoma

Table 4. Comparison of Clinical Characteristics at Diagnosis of PBC between Patients who Developed HCC and Those who Did Not

Variables	Patients with HCC	Patients without HCC	p value
Number of patients	11	194	
Age (years)	58 ± 11	58 ± 10	0.91
Gender (Male/Female)	1/10	27/167	0.54
Body mass index (kg/m^2)	23.6 ± 2.7	22.3 ± 3.1	0.10
Blood transfusion (+/-)	1/10	11/178	0.50
Diabetes mellitus (+/-)	1/10	14/180	0.58
Portal hypertension (+/-)	4/7	33/161	0.12
Platelet count (× 10^4/μL)	16.0 ± 7.5	22.5 ± 8.0	0.008
ALT (IU/L)	47 ± 29	59 ± 55	0.66
AST (IU/L)	60 ± 36	55 ± 40	0.40
Alkaline phosphatase (IU/L)	468 ± 338	518 ± 432	0.72
γ-GT (IU/L)	127 ± 86	236 ± 238	0.08
Total bilirubin (mg/dL)	1.6 ± 1.7	0.9 ± 0.6	0.11
Albumin (g/dL)	3.6 ± 0.6	4.0 ± 0.5	0.02
Prothrombin time (%)	76.8 ± 14.0	83.6 ± 16.5	0.30
IgM (mg/dL)	479 ± 393	405 ± 273	0.91
Anti-HBc (+/-/unknown)	2/6/3	11/30/153	0.65
Histological stage (I or II/III or IV)	5/6	163/31	0.005
Treatment with UDCA (+/-)	11/0	175/19	0.33

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyltransferase, Anti-HBc: anti hepatitis B core antibody, UDCA: ursodeoxycholic acid

Table 5. Multivariate Analysis of Predictors for Development of HCC in Patients with PBC

Variables	Odds ratio	95% CI	p value
Histological stage III or IV	6.27	1.80-21.83	0.004

survival of patients with PBC in this study.

Diabetes mellitus was demonstrated to increase the risk of HCC in a large cohort of patients without concomitant liver disease (12) and in a cohort of patients with hepatitis C, hepatitis B or alcoholic cirrhosis (20, 21). However, these previous studies did not include diabetes mellitus as a clinical parameter for assessing risk factors related to the development of HCC in patients with PBC (5-7, 9-11). We did not find any associations between diabetes mellitus and the development of HCC, although the proportion of patients with diabetes mellitus (9%) in this study was relatively small. Although a link between insulin resistance and metabolic hepatocarcinogenesis has been reported (22), the contribution of diabetes mellitus to the development of HCC in patients with chronic liver diseases may vary according to the etiology of liver disease.

We did not identify male sex to be a significant factor associated with HCC development in patients with PBC, which is inconsistent with the results of some previous studies (5, 7, 10, 11). In particular, the discrepancy between the present study and a recent Japanese nationwide study (10) may be explained by at least three factors. First, the patient sample size was much smaller in this study than in the nationwide survey. Second, the present study included more patients with an advanced histological stage of disease (Scheuer criteria III or IV) (18%) than the nationwide survey (12%), although other clinical parameters, such as age, gender and the frequency of treatment with UDCA, were similar between the two studies. Third, the exclusion criterion regarding a history of excessive alcohol consumption (>40 g/day) employed in this study may be associated with this discrepancy since the nationwide survey did not clearly

exclude patients with a history of excessive alcohol consumption. As shown in a previous study (9), we also found only an advanced histological stage (Scheuer's classification III or IV) to be a risk factor associated with the development of HCC in patients with PBC. Considering that cholangiocytes, not hepatocytes, are primarily affected and liver fibrosis progresses as the histological stage advances in PBC, these results indeed highlight the important role of liver fibrosis in hepatocarcinogenesis in patients with PBC. In addition, it should be noted that the follow-up period until the development of HCC was significantly longer in the patients with a mild histological stage (I or II) than in those with an advanced histological stage (III or IV). These results suggest a potential risk for HCC development after a long period of time, even in PBC patients with a mild histological stage of disease at the time of diagnosis.

In conclusion, the development of HCC has been demonstrated to affect the survival of Japanese patients with PBC. Considering that the prognosis of PBC has improved in general as a result of early diagnosis and the use of UDCA, the early diagnosis of HCC is also crucial for obtaining a better prognosis for patients with PBC. Therefore, strict surveillance for HCC is necessary among patients with PBC who are in the advanced stage of disease.

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

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Original Article

Clinical usefulness of non-protein respiratory quotient measurement in non-alcoholic fatty liver disease

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Aim: Little is known about the effects of non-alcoholic fatty liver disease (NAFLD) on energy metabolism, although this disease is associated with metabolic syndrome. We measured non-protein respiratory quotient (npRQ) using indirect calorimetry, which reflects glucose oxidation, and compared this value with histological disease severity in NAFLD patients.

Methods: Subjects were 32 patients who were diagnosed with NAFLD histopathologically. Subjects underwent body composition analysis and indirect calorimetry, and npRQ was calculated. An oral glucose tolerance test was performed, and plasma glucose area under the curve (AUC glucose) was calculated.

Results: There were no differences in body mass index, body fat percentage or visceral fat area among fibrosis stage groups. As fibrosis progressed, npRQ significantly decreased (stage 0, 0.895 ± 0.068; stage 1, 0.869 ± 0.067; stage 2, 0.808 ± 0.046; stage 3, 0.798 ± 0.026; $P < 0.005$). Glucose intolerance worsened and insulin resistance increased with fibrosis stage. npRQ was negatively correlated with AUC glucose ($R = -0.6308$, $P < 0.001$), Homeostasis Model of Assessment – Insulin Resistance ($R = -0.5045$, $P < 0.005$), fasting glucose ($R = -0.4585$, $P < 0.01$) and insulin levels ($R = -0.4431$, $P < 0.05$), suggesting that decreased npRQ may reflect impaired glucose tolerance due to insulin resistance, which was associated with fibrosis progression. Estimation of fibrosis stage using npRQ was as accurate as several previously established scoring systems using receiver–operator curve analysis.

Conclusion: npRQ was significantly decreased in patients with advanced NAFLD. Our data suggest that measurement of npRQ is useful for the estimation of disease severity in NAFLD patients.

Key words: glucose intolerance, NAFLD, npRQ

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the most common chronic liver diseases. NAFLD is associated with metabolic syndrome and insulin resistance and often involves abnormal glucose and lipid metabolism.[1–6] Based on this, the NAFLD Asia–Pacific Working Party has recommended screening for metabolic syndrome and body composition in all NAFLD patients.[6] NAFLD treatment consists of diet and exercise interventions for weight loss,[4–6] and nutritional guidance and management are essential. As a part of a nutritional guidance and management program, our institution performs anthropometric measurement of NAFLD patients using a body composition analyzer, evaluation of glucose metabolism using a 75-g oral glucose tolerance test (OGTT), and evaluation of energy metabolism using indirect calorimetry. These basic tests are performed in routine practice.

Indirect calorimetry is a method used in physiological testing and enables easy and non-invasive evaluation of energy metabolism in real time.[7] The non-protein respiratory quotient (npRQ) calculated from indirect calorimetry data represents the ratio of carbohydrate to fat oxidation, and its value is said to be an indicator of prognosis in liver cirrhosis.[8] In addition, although it has been reported that NAFLD disease progression is

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