

*IFN/Peg-IFN + ribavirin combination therapy. In general, follow the therapy protocol in treatment-naive patients for retreatment with combination therapy including ribavirin in patients previously administered IFN or Peg-IFN monotherapy.*

- 2 *Retreatment in elderly patients with genotype 1 and high viral load: for relapsers and partial responders to previous therapy, retreatment should be with Peg-IFN + ribavirin combination therapy in general, although telaprevir + Peg-IFN + ribavirin combination therapy should be considered if it can be tolerated.*
- 3 *Retreatment in elderly patients with genotype 1 and high viral load: for null responders to previous therapy, as an adequate antiviral effect cannot be expected, careful follow-up should be considered. If ALT levels are abnormal, low-dose long-term Peg-IFN/IFN therapy or supportive therapy should be administered.*
- 4 *Retreatment in non-elderly patients with genotype 1 and high viral load: for relapsers and partial responders to previous therapy, the treatment of first choice is telaprevir + Peg-IFN + ribavirin combination therapy. If triple therapy cannot be tolerated, retreatment with Peg-IFN + ribavirin combination therapy should be considered in patients with advanced fibrosis, although waiting for the next generation DAAs is an option in patients with mild fibrosis.*
- 5 *Retreatment in non-elderly patients with genotype 1 and high viral load: for null responders to previous therapy, telaprevir + Peg-IFN + ribavirin triple therapy should be considered in patients with advanced fibrosis, if tolerated, but for patients with mild fibrosis, we should wait for the next generation DAAs.*

### 3.7 Retreatment—Genotype 1 with low viral load, and Genotype 2

#### Genotype 1, low viral load

If the previous treatment was IFN or Peg-IFN monotherapy, as a general rule, retreatment should be with Peg-IFN + ribavirin combination therapy. When Peg-IFN- $\alpha$  is contraindicated due to depression or depressive symptoms, IFN- $\beta$  can be used instead of Peg-IFN.<sup>26</sup> When the previous therapy included ribavirin, telaprevir + Peg-IFN + ribavirin combination therapy should be used. If triple therapy cannot be tolerated, then consider retreatment with Peg-IFN + ribavirin combination therapy, although there is no clear evidence regarding the efficacy of this therapy as retreatment.

#### Genotype 2, high viral load

If the previous treatment was IFN or Peg-IFN monotherapy, retreatment should be with Peg-IFN + ribavirin combination therapy (24 weeks). When the previous therapy included ribavirin, treatment with Peg-IFN + ribavirin therapy (24–48 weeks) should be considered. SVR rates  $\geq 50\%$  have been reported.<sup>141,148</sup> When Peg-IFN- $\alpha$  is contraindicated due to depression or depressive symptoms, IFN- $\beta$  can be used instead of Peg-IFN.<sup>26</sup>

#### Genotype 2, low viral load

If the previous treatment was IFN or Peg-IFN monotherapy, retreatment should be with Peg-IFN + ribavirin combination therapy (24 weeks). When the previous therapy included ribavirin, treatment with Peg-IFN + ribavirin therapy (24–48 weeks) should be considered. High SVR rates comparable to those achieved with initial treatment have been reported.<sup>85,136</sup> As with patients with genotype 2 and a high viral load, if Peg-IFN- $\alpha$  is contraindicated due to depression or depressive symptoms, IFN- $\beta$  can be used instead of Peg-IFN.<sup>26</sup>

#### Recommendations:

- 1 *In patients with genotype 1 and a low viral load, if the previous treatment was IFN or Peg-IFN monotherapy, as a general rule, retreatment should be with Peg-IFN + ribavirin combination therapy. When the previous therapy included ribavirin, telaprevir + Peg-IFN + ribavirin combination therapy should be used. If triple therapy cannot be tolerated, then consider retreatment with Peg-IFN + ribavirin combination therapy in patients with advanced fibrosis.*
- 2 *In patients with genotype 2, regardless of the viral load, if the previous treatment was IFN or Peg-IFN monotherapy, retreatment should be with Peg-IFN + ribavirin combination therapy (24 weeks). When the previous therapy included ribavirin, treatment with Peg-IFN + ribavirin therapy (24–48 weeks) should be considered.*
- 3 *In any patient group, in patients unable to tolerate Peg-IFN- $\alpha$  due to depression or depressive symptoms, IFN- $\beta$  + ribavirin combination therapy should be administered for 28–48 weeks.*

### 3.8 Treatment of patients with liver cirrhosis

#### IFN therapy for compensated cirrhosis

The state in which the hepatic functional reserve is preserved, and there is no evidence of liver failure such as jaundice, ascites, hepatoencephalopathy or esophageal varices (Child–Pugh class A) is called compensated

cirrhosis, and when there is evidence of liver failure (Child–Pugh class B, C), it is called decompensated cirrhosis. Patients with liver cirrhosis accompanying severe fibrosis are a high-risk group for hepatocellular carcinogenesis. Even if they avoid developing HCC, the prognosis is poor if they develop liver failure. Accordingly, the objective of treatment for liver cirrhosis is to prevent both HCC and liver failure, and aggressive antiviral therapy is highly necessary in patients with compensated cirrhosis. Viral eradication through IFN therapy in patients with compensated cirrhosis can be expected to reduce the risk of HCC and liver failure.<sup>8</sup> However, patients with advanced hepatic fibrosis are IFN-resistant, and pancytopenia associated with hypersplenism complicating liver cirrhosis impedes IFN therapy.<sup>78,79</sup> When a virological response is not achieved with IFN therapy, a changeover to low-dose long-term IFN therapy should be made with the improving ALT levels and inhibiting hepatocellular carcinogenesis. The safety of telaprevir + Peg-IFN + ribavirin triple therapy has not been established in patients with cirrhosis, and is not approved by national medical insurance for this patient group.

*Peg-IFN + ribavirin combination therapy.* For some time now, outside of Japan, the standard treatment for patients with compensated cirrhosis has been the same as for chronic hepatitis C, Peg-IFN + ribavirin combination therapy.<sup>149,150</sup> In a trial comparing Peg-IFN- $\alpha$ -2b (1.0  $\mu$ g/kg per week) monotherapy and combination therapy including ribavirin (800 mg/day), mainly in patients with compensated cirrhosis, higher efficacy was seen with the latter (SVR rates, 9.8% vs 21.6%,  $P=0.06$ ). The SVR rate was 67% in patients with genotypes 2 and 3, significantly higher than that of 11% in patients with genotypes 1 and 4 ( $P=0.001$ ). Progression towards liver failure was significantly less in patients achieving SVR than in non-responders (6.2% vs 38.3%,  $P=0.03$ ).<sup>151</sup> In a clinical trial of Peg-IFN- $\alpha$ -2a 180  $\mu$ g/kg/week and ribavirin 600–1200 mg/day combination therapy, solely with patients with compensated cirrhosis, a significantly higher SVR rate was seen with genotypes 2 and 3 than with genotypes 1 and 4 (32% vs 58%,  $P=0.04$ ).<sup>150</sup> In 2011, Peg-IFN- $\alpha$ -2b or Peg-IFN- $\alpha$ -2a + ribavirin combination therapy has been approved in Japan by national medical insurance for the treatment of patients with compensated cirrhosis, irrespective of viral load or genotype. In a Japanese clinical trial of Peg-IFN- $\alpha$ -2b 1.0  $\mu$ g/kg/week in combination with ribavirin for 48 weeks in patients with compensated HCV cirrhosis, the SVR rate was 22% (15/69) in patients with

genotype 1 and a high viral load, and 79% (26/33) in other patients, indicating high efficacy in all groups other than genotype 1 with a high viral load. In a study of 48 weeks of a combination of Peg-IFN- $\alpha$ -2a in two doses, 90 and 180  $\mu$ g/week, with ribavirin, the SVR rate was 28% (17/61) in the 90- $\mu$ g group, and 27% (17/63) in the 180- $\mu$ g group, with no difference seen between groups. In the 90- $\mu$ g group, the SVR was 21% (10/48) in patients with genotype 1 and 50% (6/12) in those with genotype 2, showing high efficacy against the latter.<sup>152</sup>

In patients with compensated cirrhosis, where the doses of Peg-IFN- $\alpha$  and ribavirin are limited by the high degree of fibrosis, extended courses of combination therapy are required to achieve SVR. The HCV RNA dynamics following commencement of Peg-IFN + ribavirin combination therapy are also a good indicator of SVR in patients with compensated cirrhosis.<sup>153,154</sup> Accordingly, as with chronic hepatitis C, response-guided therapy altering the duration of treatment in accordance with the response to Peg-IFN + ribavirin therapy is useful. If HCV RNA does not become undetectable by treatment week 12 and viral clearance cannot be achieved, as with chronic hepatitis C, consideration should be given to a changeover to low-dose long-term Peg-IFN therapy with the aim of inhibiting hepatocellular carcinogenesis. Adverse reactions to Peg-IFN + ribavirin combination therapy in patients with compensated cirrhosis such as influenza-like-syndrome, depression, lethargy and cytopenia are common, but there are no great differences with chronic hepatitis in terms of safety and tolerability.<sup>149,150</sup> However, pancytopenia associated with hypersplenism may be present in the background, so reduction in the dose of both agents is often required due to severe cytopenias, including anemia, neutropenia and thrombocytopenia.<sup>151,153</sup>

The standard dose for Peg-IFN- $\alpha$ -2b in the treatment of patients with compensated cirrhosis is 1.0  $\mu$ g/kg/week, and the criteria for dose reduction and discontinuation during treatment are as follows: halve the dose in the case of a neutrophil count  $<750/\mu$ L or platelet count  $<50\,000/\mu$ L; and cease both Peg-IFN- $\alpha$ -2b and ribavirin in the case of a neutrophil count  $<500/\mu$ L, platelet count  $<35\,000/\mu$ L or Hb  $<8.5$  g/dL.<sup>155</sup> When the pretreatment Hb is  $\geq 14$  g/dL, the daily dose of ribavirin is 600 mg for patients weighing  $\leq 60$  kg, 800 mg for 61–80 kg and 1000 mg for  $>80$  kg. If the pretreatment Hb is  $<14$  g/dL, the starting dose of ribavirin is reduced by 200 mg, irrespective of weight.

The criteria for ribavirin dose reduction or discontinuation when a decline in Hb occurs during treatment are:

reduce the daily dose by 200 mg (400 mg if started at 1000 mg) for Hb <10 g/dL; and discontinue if Hb is <8.5 g/dL.<sup>81</sup>

The standard dose for Peg-IFN- $\alpha$ -2a in the treatment of patients with compensated cirrhosis is 90  $\mu$ g/kg/week. The criteria for dose reduction and discontinuation during treatment are as follows: reduce the dose to 45  $\mu$ g/mL in the case of a neutrophil count <1000/ $\mu$ L, and to 22.5  $\mu$ g/mL in the case of a neutrophil count <750/ $\mu$ L; and cease both Peg-IFN- $\alpha$ -2a and ribavirin in the case of a neutrophil count <500/ $\mu$ L, platelet count <50 000/ $\mu$ L or Hb <8.5 g/dL.<sup>156</sup> The starting doses for ribavirin are as for co-administration with Peg-IFN- $\alpha$ -2b. The criteria for ribavirin dose reduction or discontinuation when a decline in Hb occurs during treatment are: reduce the daily dose by 400 mg (600 mg if started at 1000 mg) for Hb <11 g/dL during treatment weeks 1–4, or Hb <10 g/dL during treatment weeks 5–48. For patients with heart conditions or a history of the same, in addition to the above criteria, if a decline in Hb  $\geq$ 2 g/dL in comparison to the pretreatment level persists for 4 weeks, reduce the daily dose by 400 mg (600 mg if started at 1000 mg). If Hb remains <12 g/dL 4 weeks after the dose reduction, cease ribavirin.<sup>80</sup>

*IFN monotherapy.* Apart from patients with genotype 1 and a high viral load, IFN monotherapy should be selected for patients unable to tolerate Peg-IFN + ribavirin combination therapy due to adverse reactions such as anemia or depression. At present, IFN- $\beta$  and human lymphoblastoid IFN (HLBI), an IFN- $\alpha$  formulation, are approved for national medical coverage for the treatment of patients with compensated cirrhosis with HCV genotype 1 and a low viral load, and genotype 2. They are not approved for patients with genotype 1 and a high viral load ( $\geq$ 100 kIU/mL for IFN- $\beta$ ,  $\geq$ 500 kIU/mL for HLBI). Japanese clinical trials of IFN- $\beta$  in the treatment of patients with compensated cirrhosis with genotype 1 and a low viral load, and genotype 2, yielded SVR rates in the patients administered 126 doses of 44% (8/18) in the genotype 1 low viral load group (<1 Meq/mL), 19% (3/16) in the genotype 2 high viral load group ( $\geq$ 1 Meq/mL) and 46% (6/13) in the genotype 2 low viral load group.<sup>157</sup> In a Japanese multicenter collaborative trial of HLBI in the treatment of HCV compensated cirrhosis, SVR rates in the group administered HLBI 6 MU consecutive daily for 2 weeks, then 3 MU three times weekly for 46 weeks, were 50% (1/2) in the genotype 1 low viral load group (<100 kIU/mL), 25% (3/12) in the genotype 2 high viral load group ( $\geq$ 100 kIU/mL) and 67% (4/6) in the genotype 2 low

viral load group.<sup>158</sup> In both studies, efficacy increased with increased treatment duration. Furthermore, greater efficacy was seen with genotype 2 than genotype 1, and with a low viral load than with a high viral load. The rate of discontinuation due to adverse reactions was similar to that with chronic hepatitis C, and although the incidence of influenza-like syndrome and abnormal laboratory test results was high, no cirrhosis-specific adverse events were reported. In an overseas trial of Peg-IFN monotherapy in the treatment of patients with cirrhosis, SVR rates and biochemical efficacy were both superior to standard IFN therapy. A randomized prospective study comparing standard non-pegylated-IFN- $\alpha$  and Peg-IFN- $\alpha$ -2a reported SVR rates in patients administered non-pegylated-IFN- $\alpha$ -2a 3 MU three times/week, Peg-IFN- $\alpha$ -2a 90  $\mu$ g/week and 180  $\mu$ g/week to be 8% (7/88), 15% (14/96) and 30% (26/87), respectively. No difference was seen between groups in terms of tolerability.<sup>159</sup>

HLBI therapy aiming for viral clearance comprises HLBI 6 MU weekly for 2 consecutive weeks, then 3–6 MU three times weekly. The criteria for dose reduction and discontinuation during HLBI treatment are as follows: reduce the dose or increase the interval between doses in the case of a platelet count  $\geq$ 30 000/ $\mu$ L and <50 000/ $\mu$ L, and discontinue in the case of a white blood cell counts <1500/ $\mu$ L, platelet count <30 000/ $\mu$ L or ALT level  $\geq$ 500 U/L.<sup>160</sup>

IFN- $\beta$  therapy is usually commenced at 6 MU, and is administered 3–6 MU consecutive daily until treatment week 6, then 3 MU three times a week. The criteria for dose reduction and discontinuation during IFN- $\beta$  treatment are as follows: reduce the dose or increase the interval between doses in the case of a white blood cell counts <1500/ $\mu$ L, neutrophil count <750/ $\mu$ L or platelet count <50 000/ $\mu$ L, and discontinue in the case of a white blood cell counts <1000/ $\mu$ L, neutrophil count <500/ $\mu$ L or platelet count <25 000/ $\mu$ L.<sup>133</sup> For both HLBI and IFN- $\beta$ , if HCV RNA becomes undetectable before treatment week 12, as for chronic hepatitis C, the treatment period should be extended to 48–72 weeks.

*Low-dose IFN maintenance therapy.* If HCV RNA does not become undetectable before treatment week 12 with Peg-IFN + ribavirin combination therapy or IFN monotherapy, a changeover to low-dose IFN maintenance therapy should be made with the aim of improving ALT levels and inhibiting hepatocellular carcinogenesis. Low-dose IFN or Peg-IFN maintenance therapy is useful in patients with liver cirrhosis in preventing progression

of liver disease and the development of HCC.<sup>19,47,51</sup> It is not effective in all patients, however, and discontinuation of treatment should be considered if improvement is not seen in ALT levels ( $\leq 40$  IU/L) or AFP levels ( $\leq 10$  ng/mL) within 6 months.

### IFN therapy for decompensated cirrhosis

Patients with decompensated cirrhosis are at high risk of death due to liver failure, and liver transplant is the most effective treatment in suitable cases. However, post-transplant recurrence of hepatitis C causes allograft failure in approximately 30% of recipients within 5 years, so in overseas countries, pretransplant IFN therapy is administered with the aim of HCV eradication or suppression.<sup>161,162</sup> Several studies have demonstrated the efficacy of Peg-IFN ( $\pm$  ribavirin) therapy in patients with HCV genotype 2.<sup>163–165</sup> Patients with decompensated cirrhosis are at high risk of thrombocytopenia, anemia, infections and liver decompensation, however, and treatment discontinuation due to severe cytopenias is common. Serious bacterial infections associated with IFN therapy have been reported to be more common in patients with patients with Child–Pugh C than in Child–Pugh A/B disease.<sup>166</sup>

### Treatment of patients with thrombocytopenia

In patients with marked thrombocytopenia associated with hypersplenism, it is difficult to introduce Peg-IFN or ribavirin combination therapy. Measures such as splenectomy or partial splenic embolization (PSE) are employed to increase the platelet count before commencing IFN therapy.<sup>167–169</sup> In Japan, mainly in patients with Child–Pugh A disease, Peg-IFN ( $\pm$  ribavirin) therapy is commenced following splenectomy or PSE. An increase in the platelet count is seen in almost all patients following either procedure, and high SVR rates are seen in patients with HCV genotype 2. However, postoperative complications including overwhelming post-splenectomy infection, portal vein thrombosis and hepatic dysfunction have been reported following both splenectomy and PSE.<sup>168–170</sup> The thrombopoietin receptor agonist, eltrombopag, has been developed overseas as an oral agent that increases platelet counts,<sup>171</sup> but it is not yet available for clinical use in Japan.

#### Recommendations:

- 1 In patients with compensated cirrhosis (Child–Pugh class A) associated with HCV, aggressive IFN therapy should be commenced with the aims of preventing hepatocellular carcinogenesis and liver failure. This

patient group requires careful observation during treatment due to the high incidence of adverse reactions such as cytopenias.

- 2 Patients with compensated cirrhosis associated with HCV should be given Peg-IFN + ribavirin combination therapy, irrespective of genotype or viral load. The standard dose is 1.0  $\mu\text{g}/\text{kg}/\text{week}$  for Peg-IFN- $\alpha$ -2b and 90  $\mu\text{g}/\text{week}$  for Peg-IFN- $\alpha$ -2a. The usual treatment period is 48 weeks, although consideration should be given to response-guided therapy and the discontinuation criteria for chronic hepatitis C.
- 3 Patients with compensated cirrhosis associated with HCV genotype 1 and a lower viral load, or genotype 2, not suited to combination therapy with ribavirin, should be administered HLBI or IFN- $\beta$  monotherapy. HLBI therapy commences with HLBI 6 MU consecutive daily for 2 weeks, then 3–6 MU three times weekly. IFN- $\beta$  therapy is usually commenced with 6 MU daily for a week, followed by 3 MU daily for 5 weeks, then 3 MU three times a week from treatment week 7. For both HLBI and IFN- $\beta$ , if HCV RNA becomes undetectable before treatment week 12, the treatment period should be extended to 48–72 weeks.
- 4 If HCV RNA does not become undetectable before treatment week 12 with Peg-IFN + ribavirin combination therapy or IFN monotherapy in patients with compensated cirrhosis associated with HCV, long-term HLBI therapy at a dose of 3 MU three times weekly should be commenced with the aim of inhibiting hepatocellular carcinogenesis. Treatment should be discontinued if improvement is not seen in ALT levels ( $\leq 40$  IU/L) or AFP levels ( $\leq 10$  ng/mL) within 6 months.
- 5 The efficacy of IFN therapy is low in patients with decompensated cirrhosis associated with HCV (Child–Pugh class B and C). In particular, patients with Child–Pugh class C do not tolerate IFN therapy well, and serious adverse reactions such as cytopenias and infections have been reported, so IFN therapy is not recommended in this patient group.
- 6 If IFN therapy is being considered in a patient with compensated HCV cirrhosis associated with a platelet count  $\leq 50\,000/\mu\text{L}$ , one option is to perform splenectomy or PSE before commencing IFN therapy.

### 3.9 Management of patients with normal ALT levels

In a study of Peg-IFN + ribavirin combination therapy and hepatocellular carcinogenesis in 809 patients with chronic hepatitis C and normal pretreatment ALT levels (male/female, 269/540; average age,  $57 \pm 11$

years; genotype 1/2, 550/247; mean observation period,  $36.2 \pm 16.5$  months), in the group with platelet counts  $\geq 150\,000/\mu\text{L}$  ( $n = 586$ ) no significant difference was seen in the incidence of HCC according to therapeutic effect, with 1.5% of non-responders developing HCC within 3 years. In the group with platelet counts  $< 150\,000/\mu\text{L}$  ( $n = 323$ ), however, the cumulative incidence of HCC was high at 10.1% in non-responders, with no cases of HCC among the responders or relapsers. These results demonstrated that Peg-IFN + ribavirin therapy significantly inhibits hepatocellular carcinogenesis ( $P < 0.001$ ).<sup>172</sup> The efficacy of Peg-IFN + ribavirin combination therapy is similar in patients with normal and elevated ALT levels.<sup>173,174</sup>

Accordingly, antiviral therapy should be considered even in patients with ALT levels  $\leq 30$  IU/mL if their platelet count is  $< 150\,000/\mu\text{L}$ . On the other hand, antiviral therapy does not need to be commenced immediately in patients with an ALT level  $\leq 30$  IU/mL and a platelet count  $\geq 150\,000/\mu\text{L}$ , and follow-up while waiting for the next generation DAAs is a reasonable option. ALT levels may rise during the follow-up period, however, and treatment is indicated if the patient has a strong desire to commence antiviral therapy. At present, the available evidence regarding patients with normal ALT levels is mainly related to Peg-IFN + ribavirin combination therapy, although high therapeutic efficacy can also be anticipated with telaprevir + Peg-IFN + ribavirin combination therapy in this patient group.

#### **Recommendation:**

*Antiviral therapy for patients with normal ALT levels (ALT,  $\leq 30$  IU/mL) can be administered in the same way as for patients with elevated ALT levels. Aggressive therapy is particularly desirable in patients with platelet counts  $< 150\,000/\mu\text{L}$ .*

## **4. PROTECTIVE THERAPY**

THE AIM OF protective therapy is not HCV clearance, but rather to reduce inflammation and inhibit the progression of fibrotic change in the hepatic tissue. The indications for protective therapy in patients with chronic hepatitis C are: patients with abnormal ALT and AST levels unable to undergo IFN or other antiviral therapy; patients who failed to achieve viral clearance with antiviral therapy; and patients who do not wish to undergo antiviral therapy. UDCA and SNMC are the protective therapies that have been scientifically shown to be useful.

### **UDCA**

Ursodeoxycholic acid is a bile acid formulation, approved for use in doses of 600–900 mg daily by national medical insurance. The main mechanism of action of UDCA in hepatitis is a hepatocytoprotective effect. Other postulated mechanisms of action include protection of the hepatocyte cell membrane by substitution of UDCA for other cytotoxic bile acids, antioxidative stress affects, immunoregulatory effects and anti-apoptotic effects.<sup>175</sup>

Improvement of liver function is seen from UDCA doses of 150 mg/day.<sup>176,177</sup> In a Japanese nationwide multicenter double-blind trial, significantly greater improvement was seen in AST, ALT and  $\gamma$ -glutamyl transpeptidase levels in the groups administered 600 and 900 mg/day than in those given 150 mg/day.<sup>176</sup> Accordingly, the UDCA dose for the treatment of chronic hepatitis C is generally 600 or 900 mg/day. Adverse reactions are mainly gastrointestinal symptoms such as epigastric discomfort, diarrhea and constipation, but these are generally mild. A retrospective study of inhibition of hepatocellular carcinogenesis by UDCA reported that it significantly reduced the incidence of HCC.<sup>178</sup>

### **SNMC**

The main constituent of SNMC is glycyrrhizin, a compound extracted from the liquorice root. The mechanisms of action of SNMC in the treatment of hepatic dysfunction are derived from anti-inflammatory effects related to the steroid-like properties of glycyrrhizin, and hepatocyte cell membrane protective effects. These actions are considered to lead to improved ALT levels. In a Japanese double-blind trial of SNMC 40 mL daily for 1 month, significant improvement in AST and ALT levels was seen in the SNMC group in comparison with the placebo group.<sup>179,180</sup> Doses are 40–100 mL daily or alternate daily, although Japanese dosage comparison trials found significantly greater improvement in ALT levels with 100 mL than with 40 mL.<sup>181,182</sup> In another study, long-term administration of SNMC significantly inhibited progression to liver cirrhosis in comparison with the control group.<sup>183</sup> Adverse reactions to SNMC include hypokalemia and hypertension.

Studies of inhibition of hepatocellular carcinogenesis by SNMC found that the incidence of HCC was significantly lower in the treatment group than in the control group.<sup>183,184</sup> SNMC therapy has also been found to significantly reduce the incidence of HCC in non-responders to IFN therapy.<sup>185,186</sup>

### UDCA + SNMC combination therapy

An RCT comparing SNMC monotherapy and UDCA + SNMC combination therapy found significantly greater improvement in ALT levels in the combination therapy group.<sup>187</sup> This combination is useful in reducing inflammation.

#### Recommendation:

*Oral UDCA and i.v. SNMC, or both in combination, are recommended as protective therapy in patients with chronic hepatitis C.*

## 5. THERAPEUTIC PHEBOTOMY

IRON METABOLISM PLAYS an important role in patients with chronic hepatitis C. Iron is an essential metal, and a constituent of important proteins, including Hb. When iron is present in excess, however, cytotoxic hydroxyl radicals are produced, causing oxidative stress. Therapeutic phlebotomy was devised as a supportive therapy for patients with chronic hepatitis C because oxidative stress associated with iron overload is a factor in progression of liver disease. Restriction of dietary iron is also important in the management of patients undergoing iron reduction therapy. As for protective therapy, therapeutic phlebotomy is indicated in patients with chronic hepatitis C with abnormal ALT and AST levels unable to undergo IFN or other antiviral therapy, patients who failed to achieve viral clearance with antiviral therapy and patients who do not wish to undergo antiviral therapy.

In 1994, a Japanese study reported that therapeutic phlebotomy lowered ALT levels in patients with chronic hepatitis C.<sup>188</sup> A Japanese multicenter RCT also confirmed improvement in ALT levels with therapeutic phlebotomy.<sup>189</sup> Other studies have reported a 50% decrease in ALT levels in 80% of patients, and normalization of ALT levels in 40–70% of patients.<sup>190,191</sup> Histological studies have reported inhibition of progression,<sup>192</sup> and even improvement,<sup>193</sup> of histological changes. Long-term therapeutic phlebotomy has been reported to significantly inhibit hepatocellular carcinogenesis.<sup>190</sup>

In general, therapeutic phlebotomy involves removal of 200–400 mL blood at 1–2-week intervals with the aim of reducing the serum ferritin level to  $\leq 20$  ng/mL. If the Hb level drops below 9–10 g/dL, phlebotomies are discontinued to allow recovery of hematopoietic function. After the target has been reached, therapeutic phlebotomies are performed as appropriate with reference to ferritin and Hb levels. Adverse reactions are rare,

involving bradycardia and hypotension associated with the vagal reflex.

An additive effect is seen when therapeutic phlebotomy is performed in conjunction with UDCA or SNMC therapy. Greater reduction in ALT levels was seen with UDCA in combination with therapeutic phlebotomy than with UDCA monotherapy.<sup>194</sup> In patients on SNMC therapy, further reduction in ALT levels was seen with the addition of small volume phlebotomies.<sup>195</sup> The combination of therapeutic phlebotomy with another therapy with a different mode of action provides additional improvement in ALT levels.

#### Recommendations:

*Therapeutic phlebotomy is a useful therapeutic modality in patients with chronic hepatitis C. Its use in combination with a protective therapy, oral UDCA or i.v. SNMC should also be considered.*

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# Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon  $\alpha$  (PEG-IFN $\alpha$ ) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFN $\alpha$ -2b/RBV and quantified expressions of viral sensors (*RIG-I*, *MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFN $\lambda$* ). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients ( $\approx 3.3$ -fold,  $P < 0.001$ ). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold,  $P = 0.028$ ). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype ( $\approx 2.6$ -fold,  $P < 0.001$ ). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. *IPS-1* down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of *IPS-1* protein cleavage was associated with the variable treatment response. **Conclusion:** Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFN $\alpha$ /RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.<sup>1</sup> Pegylated interferon  $\alpha$  (PEG-IFN $\alpha$ ) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the

natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFN $\alpha$ /RBV combination therapy.<sup>2</sup> In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C;  $\gamma$ -GTP,  $\gamma$ -glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; *IL28*, interleukin 28; *IPS-1*, IFN $\beta$  promoter stimulator 1; *ISG15*, interferon-stimulated gene 15; *MDA5*, melanoma differentiation associated gene 5; NVR, nonvirological responders; PEG-IFN $\alpha$ , pegylated interferon $\alpha$ ; SNP, single nucleotide polymorphism; *RIG-I*, retinoic acid-inducible gene 1; RBV, ribavirin; *RNF125*, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; *USP18*, ubiquitin-specific protease 18; VR, virological responder.

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(*IL28B*) that encodes for type III IFN $\lambda$ 3 were shown to be strongly associated with a virological response to PEG-IFN $\alpha$ /RBV combination therapy.<sup>3-5</sup> In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFN $\alpha$ /RBV.<sup>3</sup> However, mechanisms involving resistance to PEG-IFN $\alpha$ /RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.<sup>6</sup> The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.<sup>7,8</sup> The IFN $\beta$  promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFN $\beta$ , mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in IFN $\beta$  gene activation.<sup>9-12</sup> RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,<sup>8,13</sup> a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.<sup>14</sup> Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,<sup>15</sup> and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.<sup>16,17</sup> Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.<sup>9,18</sup> Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFN $\alpha$ /RBV combination therapy.<sup>19</sup> We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFN $\alpha$ /RBV combination therapy.<sup>19</sup> However, association of gene expression involv-

ing innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFN $\alpha$ /RBV combination therapy in CH-C patients.

## Patients and Methods

**Patients.** Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.<sup>19</sup> Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

**Treatment Protocol.** The patients were administered subcutaneous injections of PEG-IFN $\alpha$ -2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5  $\mu$ g kg<sup>-1</sup> week<sup>-1</sup> for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFN $\alpha$ -2b was reduced to 0.75  $\mu$ g kg<sup>-1</sup> week<sup>-1</sup> when either neutrophil count was less than 750/mm<sup>3</sup> or platelet count was less than 80  $\times$  10<sup>3</sup>/mm<sup>3</sup>. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

**Measurement of Hepatic Gene Expression.** Liver biopsy was performed immediately before initiating

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Additional Supporting Information may be found in the online version of this article.

**Table 1. Patient Characteristics and *IL28B* Genotype**

	<i>IL28B</i> Major*	<i>IL28B</i> Minor†	P-value‡
Patients, n	54	34	
Age (SD), year	58.8 (10.0)	59.1 (10.3)	0.918§
Sex, n (%)			0.051
Male	13 (24.1)	15 (44.1)	
Female	41 (75.9)	19 (55.9)	
BMI (SD), kg/m <sup>2</sup>	22.7 (3.5)	23.5 (3.6)	0.193§
ALT (SD), IU/L	61.3 (50.7)	62.4 (44.7)	0.962§
γ-GTP (SD), IU/L	36.7 (25.9)	57.3 (52.4)	0.010§
LDL-cholesterol (SD), mg/dL	103.3 (29.8)	91.8 (26.9)	0.067§
Hemoglobin (SD), g/dL	14.1 (1.4)	14.4 (1.3)	0.186§
Platelet count (SD), ×10 <sup>3</sup> /μL	161 (6.4)	163 (4.4)	0.489§
Fibrosis stage, n (%)			0.532
F1, 2	38 (70.4)	26 (76.5)	
F3, 4	16 (29.6)	8 (23.5)	
Viral load (SD), ×10 <sup>6.3</sup> IU/mL	1.7 (1.4)	1.9 (2.0)	0.788§
%HCV core 70 & 91 a.a. double mutation¶	8.9	43.5	0.001
%ISDR wild**	43.5	51.7	0.486
Viral response, n (%)			<0.001
SVR	17 (31.5)	13 (38.2)	
TVR	26 (48.1)	3 (8.8)	
NVR	11 (20.4)	18 (52.9)	

Unless otherwise indicated, data are given as mean (SD).

\*rs8099917 TT and rs12979860 CC.

†rs8099917 TG and rs12979860 CT.

BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; SVR, sustained virological response; TVR, transient virological response; NVR, nonvirological response.

‡Comparison between *IL28B* major and minor genotypes.

§Mann-Whitney U test.

||Chi-square test.

¶HCV core mutation was determined in 68 patients.

\*\*ISDR was determined in 75 patients.

the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of the positive and negative cytoplasmic viral sensor (*RIG-I*, *MDA5*, and *LGP2*), the adaptor molecule (*IPS-1*), the related ubiquitin E3-ligase (*RNF125*), the modulators of these molecules (*ISG15* and *USP18*), and *IFNλ* (*IL28A/B*) was quantified by real-time quantitative polymerase chain reaction (PCR) using target gene-specific primers. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen reagent (Nippon Gene, Toyama, Japan) from the liver biopsy specimen, which was 0.2-0.4 cm in length and 13G in diameter. Complementary DNA (cDNA) was transcribed from 2 μg of total RNA template in a 140-μL reaction mixture using the SYBR RT-PCR Kit (Takara Bio, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio) with the SYBR RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Assays were performed in duplicate and the expression levels

of target genes were normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and hydroxymethylbilane synthase (*HMBS*), an enzyme that is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two housekeeping genes was used in the present study. Sequences of the primer sets were as follows: *RIG-I*, 5'-AAAGCATGCA TGGTGTTCAG-3', 5'-TCATTCGTGCATGCTC ACTGATAA-3'; *MDA5*, 5'-ACATAACAGCAACATG GGCAGTG-3', 5'-TTTGGTAAGGCCTGAGCTGG AG-3'; *LGP2*, 5'-ACAGCCTTGCAAACAGTACAAC CTC-3', 5'-GTCCCAAATTTCCGGCTCAAC-3'; *IPS-1*, 5'-GGTGCCATCCAAAGTGCCTACTA-3', 5'-CAGC ACGCCAGGCTTACTCA-3'; *RNF125*, 5'-AGGGCA CATATTCGGACTTGTC-3', 5'-CGGGTATTAAC GGCAAAGTGG-3'; *ISG15*, 5'-AGCGAACTCATCT TTGCCAGTACA-3', 5'-CAGCTCTGACACCGACA TGGA-3'; *USP18*, 5'-TGTTCTGCTTCAATGACT CCAATA-3', 5'-TTTGGGCATTTCCATTAGCACT C-3'; *IFNλ*: 5'-CAGCTGCAGGTGAGGGA-3', 5'-G GTGGCCTCCAGAACCTT-3'; *GAPDH*, 5'-GCACC GTCAAGGCTGAGAAC-3', 5'-ATGGTGGTGAAGA CGCCAGT-3'; *HMBS*, 5'-AAGCGGAGCCATGTCT GGTAAC-3', 5'-GTACCCACGCGAATCACTCTCA-3'.

**Genotyping for *IL28B* (rs8099917 and rs12979860) Polymorphism.** Genetic polymorphism in a tagged SNP located near the *IL28B* gene (rs8099917 and rs12979860) was determined by direct sequencing of PCR-amplified DNA. In brief, after extraction from whole blood samples, genomic DNA was amplified by PCR. Sequences of the primer sets were: rs8099917, 5'-ATCCTCCTCTCATCCCTCA TC-3', 5'-GGTATCAACCCACCTCAAAT-3'; rs129 79860, 5'-GGACGAGAGGGCGTTAGAG-3', 5'-AG GGACCGCTACGTAAGTCAC-3'.

Both strands of the PCR products were sequenced by the dye terminator method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Chiba, Japan); nucleotide sequences were determined by a capillary DNA sequencer ABI3730xl (Applied Biosystems). Homozygosity (rs8099917 GG and rs12979860 TT) or heterozygosity (rs8099917 TG and rs12979860 CT) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (rs8099917 TT and rs12979860 CC) was defined as having the *IL28B* major allele.

**Western Blotting.** Western blotting was performed using samples from 14 patients (six from *IL28B* major patients and eight from *IL28B* minor patients) as described.<sup>19</sup> In brief, liver biopsy specimens of



approximately 10 mg were homogenized in 100  $\mu$ L of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30  $\mu$ g of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti- $\beta$ -actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon<sup>20</sup> were used for a positive control for cleaved IPS-1.

**Definitions of Response to Therapy.** A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFN $\alpha$ -2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

**Statistical Analysis.** Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney *U* test for two groups. All tests of significance were two-tailed and  $P < 0.05$  was considered statistically significant.

## Results

**Patient Characteristics and IL28B Genotype.** Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report

of the rarity of a minor homozygote in Japanese patients.<sup>3</sup> *IL28B* minor patients were significantly associated with a higher  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

**Gene Expression Involving Innate Immunity and IFN $\lambda$  in the Liver.** Hepatic expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFN $\lambda$*  (*IL28A/B*) expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFN $\lambda$*  based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the *ISG15/USP18* system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFN $\delta$*  was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the *RIG-I/IPS-1* and *ISG15/USP18* systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

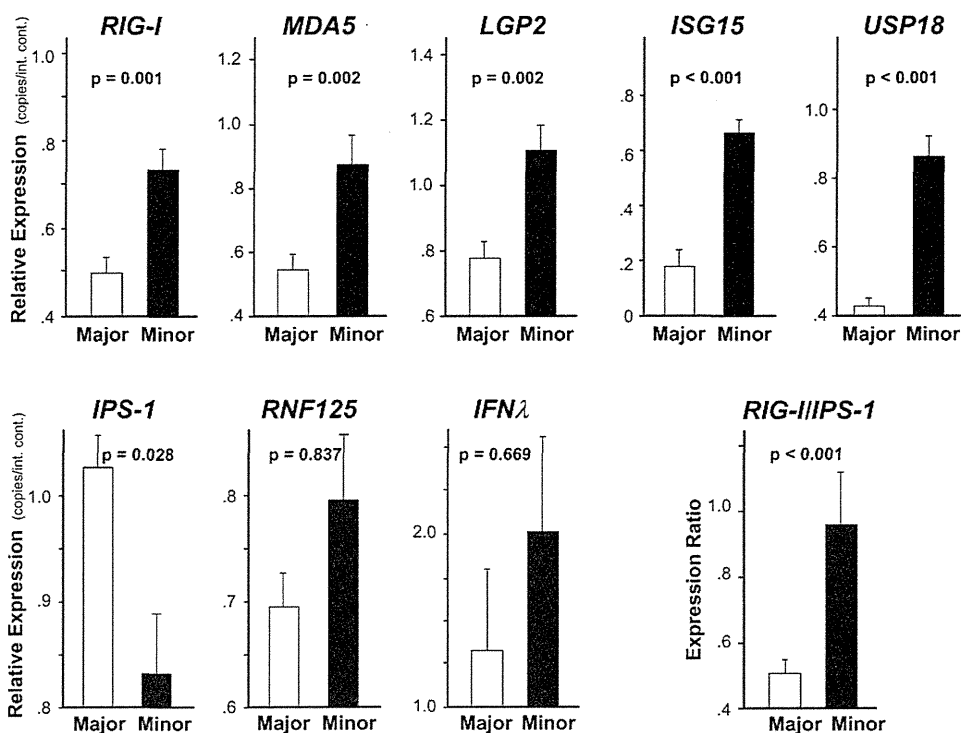


Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The P-values were determined by the Mann-Whitney U test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

**Receiver Operator Characteristic (ROC) Analysis.** To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

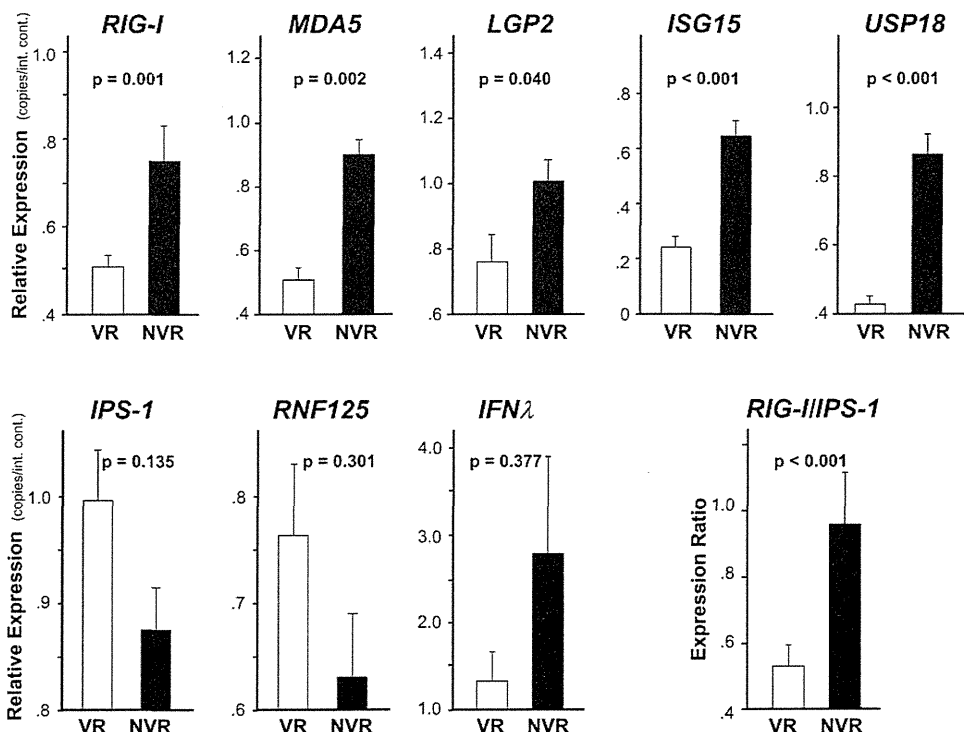


Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The P-values were determined by the Mann-Whitney U test.

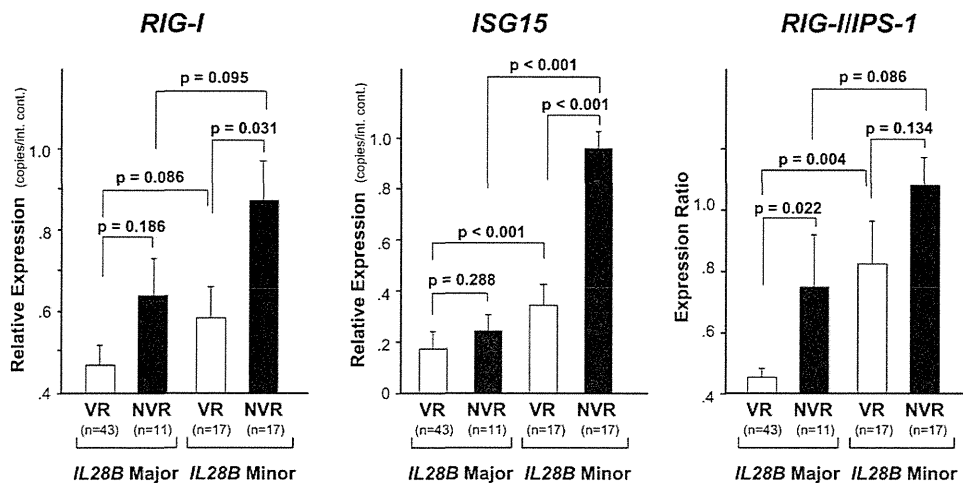


Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio, no statistically significant difference was found in

NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

**Factors Associated with NVR.** In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic expressions of *RIG-I*, *MDA5*, *LGP2*, *ISG15*, and *USP18*, and *RIG-I/IPS-1* ratio were significantly

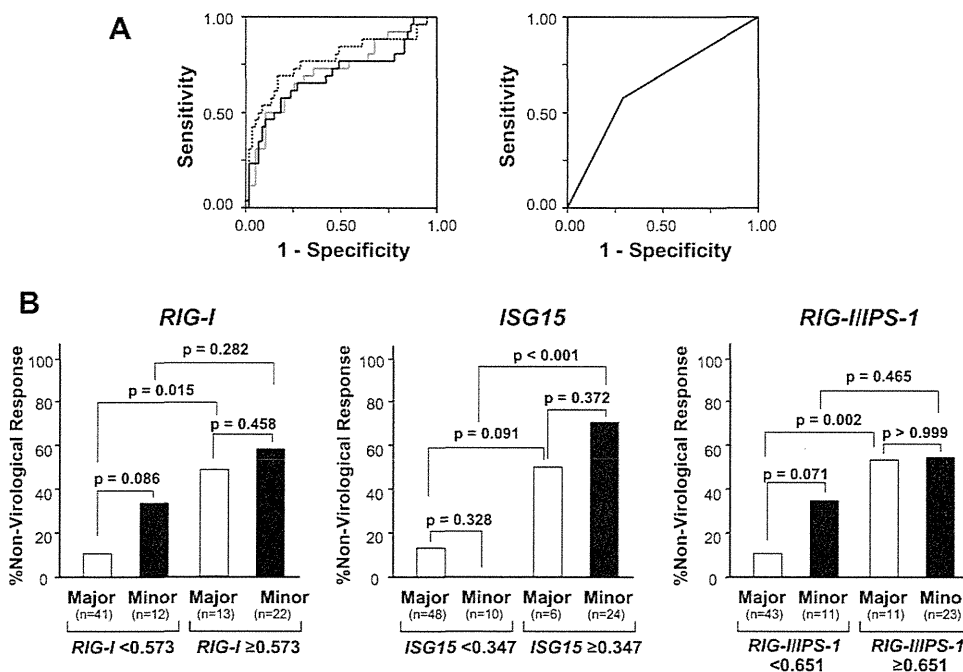


Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/ISG15* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

**Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses**

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
<i>RIG-I</i> (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
<i>ISG15</i> (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
<i>RIG-I/IPS-1</i> (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
<i>IL28B</i> genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

\*Genotype at rs8099917.

†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

***IPS-1 and RIG-I Protein Expression in the Liver.*** Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

**Table 3. Factors Associated with Nonvirological Response**

Factors	Univariate Analysis		Multivariate Analysis*	
	Risk Ratio (95% CI)	P-value	Risk Ratio (95% CI)	P-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m <sup>2</sup> )	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10 <sup>4</sup> /μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
<i>IL28B</i> genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/int. control)				
<i>RIG-I</i>	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
<i>MDA5</i>	1.53 (1.12-2.00)	0.001		
<i>LGP2</i>	1.34 (1.04-1.74)	0.026		
<i>IPS-1</i>	0.90 (0.78-1.04)	0.143		
<i>RNF125</i>	0.93 (0.83-1.04)	0.204		
<i>ISG15</i>	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
<i>USP18</i>	1.67 (1.27-2.20)	<0.001		
<i>IFNλ</i>	1.02 (0.99-1.05)	0.170		
<i>RIG-I/IPS-1</i> ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.

\*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5*, *LGP2*, *USP18*, and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.

†rs8099917 TT and rs12979860 CC.

‡rs8099917 TG and rs12979860 CT.