years; genotype 1/2, 550/247; mean observation period, 36.2 ± 16.5 months), in the group with platelet counts ≥150 000/μL ($n = 586$) 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/μL ($n = 323$), 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 ($P < 0.001$).[172] The efficacy of Peg-IFN + ribavirin combination therapy is similar in patients with normal and elevated ALT levels.[173,174]

Accordingly, antiviral therapy should be considered even in patients with ALT levels ≤30 IU/mL if their platelet count is <150 000/μL. On the other hand, antiviral therapy does not need to be commenced immediately in patients with an ALT level ≤30 IU/mL and a platelet count ≥150 000/μL, 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, ≤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/μL.

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.[175]

Improvement of liver function is seen from UDCA doses of 150 mg/day.[176,177] In a Japanese nationwide multicenter double-blind trial, significantly greater improvement was seen in AST, ALT and γ-glutamyl transpeptidase levels in the groups administered 600 and 900 mg/day than in those given 150 mg/day.[176] 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.[178]

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.[179,180] 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.[181,182] In another study, long-term administration of SNMC significantly inhibited progression to liver cirrhosis in comparison with the control group.[183] 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.[183,184] SNMC therapy has also been found to significantly reduce the incidence of HCC in non-responders to IFN therapy.[185,186]

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.[187] 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 PHLEBOTOMY

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.[188] A Japanese multicenter RCT also confirmed improvement in ALT levels with therapeutic phlebotomy.[189] Other studies have reported a 50% decrease in ALT levels in 80% of patients, and normalization of ALT levels in 40–70% of patients.[190,191] Histological studies have reported inhibition of progression,[192] and even improvement,[193] of histological changes. Long-term therapeutic phlebotomy has been reported to significantly inhibit hepatocellular carcinogenesis.[190]

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 ≤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.[194] In patients on SNMC therapy, further reduction in ALT levels was seen with the addition of small volume phlebotomies.[195] 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.

REFERENCES

1 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359–62.
2 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671–5.
3 Hoofnagle JH, Mullen KD, Jones DB *et al.* Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. *N Engl J Med* 1986; **315**: 1575–8.
4 Hagiwara H, Hayashi N, Mita E *et al.* Detection of hepatitis C virus RNA in serum of patients with chronic hepatitis C treated with interferon-alpha. *Hepatology* 1992; **15**: 37–41.
5 Cardoso AC, Moucari R, Figueiredo-Mendes C *et al.* Impact of peginterferon and ribavirin therapy on hepatocellular carcinoma: incidence and survival in hepatitis C patients with advanced fibrosis. *J Hepatol* 2010; **52**: 652–7.
6 Ikeda K, Saitoh S, Arase Y *et al.* Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999; **29**: 1124–30.
7 Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; **27**: 1394–402.
8 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncir-

rhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999; **131**: 174–81.
9 Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. *J Viral Hepat* 2012; **19**: e134–e142.
10 Hezode C, Forestier N, Dusheiko G *et al.* Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009; **360**: 1839–50.
11 Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. *J Hepatol* 2012; **56**: 78–84.
12 McHutchison JG, Everson GT, Gordon SC *et al.* Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009; **360**: 1827–38.
13 McHutchison JG, Manns MP, Muir AJ *et al.* Telaprevir for previously treated chronic HCV infection. *N Engl J Med* 2010; **362**: 1292–303.
14 Hayashi N, Komada Y, Goto S. Primary analysis of TMC435 plus PegIFN/RBV in treatment-naive patients infected with HCV genotype 1 (DRAGON Study). *Kanzo* 2011; **52**: A592.
15 Hayashi N, Mobashery N. Efficacy and safety of MK-7009 in combination with Peg-IFN and ribavirin therapy in the retreatment of patients with chronic hepatitis C genotype 1 with a high viral load. *J Jpn Soc Gastroenterol* 2011; **108**: A930.
16 Chayama K, Takahashi S, Toyota J *et al.* Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 2012; **55**: 742–8.
17 Asahina Y, Tsuchiya K, Tamaki N *et al.* Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. *Hepatology* 2010; **52**: 518–27.
18 Arase Y, Ikeda K, Suzuki F *et al.* Prolonged-interferon therapy reduces hepatocarcinogenesis in aged-patients with chronic hepatitis C. *J Med Virol* 2007; **79**: 1095–102.
19 Izumi N. Inhibition of hepatocellular carcinoma by PegIFNα-2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study. *J Gastroenterol* 2012 Aug 9. [Epub]
20 Wills RJ. Clinical pharmacokinetics of interferons. *Clin Pharmacokinet* 1990; **19**: 390–9.
21 Bocci V. Administration of interferon at night may increase its therapeutic index. *Cancer Drug Deliv* 1985; **2**: 313–8.
22 Morgano A, Puppo F, Criscuolo D. Evening administration of alpha interferon: relationship with the circadian rhythm of cortisol. *Med Sci Res* 1984; **15**: 615–6.
23 Ito T, Hara A, Kodama H *et al.* Night-time administration of interferon to patients with chronic hepatitis C – influence on QOL. Tama Symposium. *J Gastroenterol* 1995; **9**: 46–9.
24 Zeuzem S, Welsch C, Herrmann E. Pharmacokinetics of peginterferons. *Semin Liver Dis* 2003; **23** (Suppl 1): 23–8.
25 Arase Y, Suzuki F, Akuta N *et al.* Efficacy and safety of combination therapy of natural human interferon beta and ribavirin in chronic hepatitis C patients with genotype 1b and high virus load. *Intern Med* 2010; **49**: 957–63.
26 Arase Y, Suzuki Y, Suzuki F *et al.* Efficacy and safety of combination therapy of natural human interferon beta and ribavirin in chronic hepatitis C patients. *Intern Med* 2011; **50**: 2083–8.
27 Katamura Y, Suzuki F, Akuta N *et al.* Natural human interferon beta plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus and a high viral load. *Intern Med* 2008; **47**: 1827–34.
28 Nomura H, Miyagi Y, Tanimoto H, Yamashita N, Oohashi S, Nishiura S. Occurrence of clinical depression during combination therapy with pegylated interferon alpha or natural human interferon beta plus ribavirin. *Hepatol Res* 2012; **42**: 241–7.
29 Matsuda F, Torii Y, Enomoto H *et al.* Anti-interferon-α neutralizing antibody is strongly associated with non-response to pegylated interferon-α plus ribavirin in chronic hepatitis C including patients with interferon-responsive IL28B-type. *Hepatology* 2010; **52** (Suppl): 767A.
30 Asahina Y, Izumi N, Uchihara M *et al.* A potent antiviral effect on hepatitis C viral dynamics in serum and peripheral blood mononuclear cells during combination therapy with high-dose daily interferon alfa plus ribavirin and intravenous twice-daily treatment with interferon beta. *Hepatology* 2001; **34**: 377–84.
31 Okushin H, Morii K, Uesaka K, Yuasa S. Twenty four-week peginterferon plus ribavirin after interferon-beta induction for genotype 1b chronic hepatitis C. *World J Hepatol* 2010; **2**: 226–32.
32 Haller O, Kochs G, Weber F. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* 2006; **344**: 119–30.
33 Sen GC. Viruses and interferons. *Annu Rev Microbiol* 2001; **55**: 255–81.
34 Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998; **67**: 227–64.
35 Soza A, Everhart JE, Ghany MG *et al.* Neutropenia during combination therapy of interferon alfa and ribavirin for chronic hepatitis C. *Hepatology* 2002; **36**: 1273–9.
36 Raison CL, Demetrashvili M, Capuron L, Miller AH. Neuropsychiatric adverse effects of interferon-alpha: recognition and management. *CNS Drugs* 2005; **19**: 105–23.

37 Capuron L, Gumnick JF, Musselman DL *et al.* Neurobehavioral effects of interferon-alpha in cancer patients: phenomenology and paroxetine responsiveness of symptom dimensions. *Neuropsychopharmacology* 2002; **26**: 643–52.
38 Cotler SJ, Wartelle CF, Larson AM, Gretch DR, Jensen DM, Carithers RL, Jr. Pretreatment symptoms and dosing regimen predict side-effects of interferon therapy for hepatitis C. *J Viral Hepat* 2000; **7**: 211–7.
39 Raison CL, Miller AH. The neuroimmunology of stress and depression. *Semin Clin Neuropsychiatry* 2001; **6**: 277–94.
40 Sakai T, Omata M, Iino S *et al.* A Phase II clinical trial of Ro25-8310 (interferon-β-2a) in patients with chronic hepatitis C. *Jpn J Med Pharm Sci* 2003; **50**: 655–72.
41 McHutchison JG, Lawitz EJ, Shiffman ML *et al.* Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 2009; **361**: 580–93.
42 Ascione A, De Luca M, Tartaglione MT *et al.* Peginterferon alfa-2a plus ribavirin is more effective than peginterferon alfa-2b plus ribavirin for treating chronic hepatitis C virus infection. *Gastroenterology* 2010; **138**: 116–22.
43 Rumi MG, Aghemo A, Prati GM *et al.* Randomized study of peginterferon-alpha2a plus ribavirin vs peginterferon-alpha2b plus ribavirin in chronic hepatitis C. *Gastroenterology* 2010; **138**: 108–15.
44 Awad T, Thorlund K, Hauser G, Stimac D, Mabrouk M, Gluud C. Peginterferon alpha-2a is associated with higher sustained virological response than peginterferon alfa-2b in chronic hepatitis C: systematic review of randomized trials. *Hepatology* 2010; **51**: 1176–84.
45 Imai Y, Kawata S, Tamura S *et al.* Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. *Ann Intern Med* 1998; **129**: 94–9.
46 Okanoue T, Itoh Y, Minami M *et al.* Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. Viral Hepatitis Therapy Study Group. *J Hepatol* 1999; **30**: 653–9.
47 Nishiguchi S, Kuroki T, Nakatani S *et al.* Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995; **346**: 1051–5.
48 Di Bisceglie AM, Shiffman ML, Everson GT *et al.* Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. *N Engl J Med* 2008; **359**: 2429–41.
49 Lok AS, Seeff LB, Morgan TR *et al.* Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. *Gastroenterology* 2009; **136**: 138–48.
50 Bruix J, Poynard T, Colombo M *et al.* Maintenance therapy with peginterferon alfa-2b does not prevent hepatocellular carcinoma in cirrhotic patients with chronic hepatitis C. *Gastroenterology* 2011; **140**: 1990–9.
51 Lok AS, Everhart JE, Wright EC *et al.* Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. *Gastroenterology* 2011; **140**: 840–9. quiz e812.
52 Kajiwara E, Ooho A, Yamashita N. Effectiveness of biweekly low-dosage peginterferon treatment on the improvement of serum alanine aminotransferase and alpha-fetoprotein levels. *Hepatol Res* 2012; **42**: 254–63.
53 Sumida Y, Nakamura T, Kobata T *et al.* Low dose peginterferon-α-2a therapy lowers ALT and ASt levels significantly more than a glycyrrhizin formulation in patients with chronic hepatitis C. *Kanzo* 2011; **52**: 644–51.
54 Di Bisceglie AM, Stoddard AM, Dienstag JL *et al.* Excess mortality in patients with advanced chronic hepatitis C treated with long-term peginterferon. *Hepatology* 2011; **53**: 1100–8.
55 Nomura H, Kashiwagi Y, Hirano R *et al.* Efficacy of low dose long-term interferon monotherapy in aged patients with chronic hepatitis C genotype 1 and its relation to alpha-fetoprotein: a pilot study. *Hepatol Res* 2007; **37**: 490–7.
56 Shiratori Y, Shiina S, Teratani T *et al.* Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003; **138**: 299–306.
57 Kudo M, Sakaguchi Y, Chung H *et al.* Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case-control study. *Oncology* 2007; **72** (Suppl 1): 132–8.
58 Sakaguchi Y, Kudo M, Fukunaga T, Minami Y, Chung H, Kawasaki T. Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; **48**: 64–70.
59 Hung CH, Lee CM, Wang JH, Tung HD, Chen CH, Lu SN. Antiviral therapy after non-surgical tumor ablation in patients with hepatocellular carcinoma associated with hepatitis C virus. *J Gastroenterol Hepatol* 2005; **20**: 1553–9.
60 George SL, Bacon BR, Brunt EM, Mihindukulasuriya KL, Hoffmann J, Di Bisceglie AM. Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. *Hepatology* 2009; **49**: 729–38.
61 Morgan TR, Ghany MG, Kim HY *et al.* Outcome of sustained virological responders with histologically advanced chronic hepatitis C. *Hepatology* 2010; **52**: 833–44.

62 Camma C, Di Marco V, Lo Iacono O *et al.* Long-term course of interferon-treated chronic hepatitis C. *J Hepatol* 1998; **28**: 531–7.
63 Marcellin P, Boyer N, Gervais A *et al.* Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. *Ann Intern Med* 1997; **127**: 875–81.
64 Pradat P, Tillmann HL, Sauleda S *et al.* Long-term follow-up of the hepatitis C HENCORE cohort: response to therapy and occurrence of liver-related complications. *J Viral Hepat* 2007; **14**: 556–63.
65 Reichard O, Glaumann H, Fryden A, Norkrans G, Wejstal R, Weiland O. Long-term follow-up of chronic hepatitis C patients with sustained virological response to alpha-interferon. *J Hepatol* 1999; **30**: 783–7.
66 Saracco G, Rosina F, Abate ML *et al.* Long-term follow-up of patients with chronic hepatitis C treated with different doses of interferon-alpha 2b. *Hepatology* 1993; **18**: 1300–5.
67 Enokimura N, Shiraki K, Kawakita T *et al.* Hepatocellular carcinoma development in sustained viral responders to interferon therapy in patients with chronic hepatitis C. *Anticancer Res* 2003; **23**: 593–6.
68 Iwasaki Y, Takaguchi K, Ikeda H *et al.* Risk factors for hepatocellular carcinoma in Hepatitis C patients with sustained virologic response to interferon therapy. *Liver Int* 2004; **24**: 603–10.
69 Shindo M, Hamada K, Oda Y, Okuno T. Long-term follow-up study of sustained biochemical responders with interferon therapy. *Hepatology* 2001; **33**: 1299–302.
70 Takimoto M, Ohkoshi S, Ichida T *et al.* Interferon inhibits progression of liver fibrosis and reduces the risk of hepatocarcinogenesis in patients with chronic hepatitis C: a retrospective multicenter analysis of 652 patients. *Dig Dis Sci* 2002; **47**: 170–6.
71 Tanaka H, Tsukuma H, Kasahara A *et al.* Effect of interferon therapy on the incidence of hepatocellular carcinoma and mortality of patients with chronic hepatitis C: a retrospective cohort study of 738 patients. *Int J Cancer* 2000; **87**: 741–9.
72 Witkowski JT, Robins RK, Sidwell RW, Simon LN. Design, synthesis, and broad spectrum antiviral activity of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J Med Chem* 1972; **15**: 1150–4.
73 Lau JY, Tam RC, Liang TJ, Hong Z. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. *Hepatology* 2002; **35**: 1002–9.
74 Bodenheimer HC, Jr, Lindsay KL, Davis GL, Lewis JH, Thung SN, Seeff LB. Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. *Hepatology* 1997; **26**: 473–7.
75 Dusheiko G, Main J, Thomas H *et al.* Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. *J Hepatol* 1996; **25**: 591–8.
76 Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. *Lancet* 1991; **337**: 1058–61.
77 Schvarcz R, Ando Y, Sonnerborg A, Weiland O. Combination treatment with interferon alfa-2b and ribavirin for chronic hepatitis C in patients who have failed to achieve sustained response to interferon alone: Swedish experience. *J Hepatol* 1995; **23** (Suppl 2): 17–21.
78 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
79 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.
80 Chugai Pharmaceutical. Antiviral agent "Copegus" tablets package insert. 2011.
81 MSD. Antiviral agent "Rebetol" capsules package insert. 2011.
82 Yamada G, Iino S, Okuno T *et al.* Virological response in patients with hepatitis C virus genotype 1b and a high viral load: impact of peginterferon-alpha-2a plus ribavirin dose reductions and host-related factors. *Clin Drug Investig* 2008; **28**: 9–16.
83 Iino S, Okita K, Omata M, Kumada H, Hayashi N, Tanikawa K. Efficacy of 48 weeks' peginterferon-α-2b plus ribavirin combination therapy in patients with chronic hepatitis C genotype 1 and a high viral load – retrospective comparison with 6 months' interferon-α-2b plus ribavirin combination therapy. *Kan-Tan-Sui* 2004; **49**: 1099–121.
84 Kuboki M, Iino S, Okuno T *et al.* Peginterferon alpha-2a (40 KD) plus ribavirin for the treatment of chronic hepatitis C in Japanese patients. *J Gastroenterol Hepatol* 2007; **22**: 645–52.
85 Kumda H, Toyota J, Goto K *et al.* Efficacy of 24 weeks' peginterferon-α-2b plus ribavirin combination therapy in patients with chronic hepatitis C genotype 1 and a low viral load – retrospective comparison with 24 weeks' interferon-α-2b plus ribavirin combination therapy. *Kan-Tan-Sui* 2006; **52**: 645–63.
86 Hiramatsu N, Kurashige N, Oze T *et al.* Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. *Hepatol Res* 2008; **38**: 52–9.
87 Fellay J, Thompson AJ, Ge D *et al.* ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature* 2010; **464**: 405–8.
88 Ochi H, Maekawa T, Abe H *et al.* ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy

– a genome-wide study of Japanese HCV virus patients. *Gastroenterology* 2010; **139**: 1190–7.
89 Azakami T, Hayes CN, Sezaki H *et al.* Common genetic polymorphism of ITPA gene affects ribavirin-induced anemia and effect of peg-interferon plus ribavirin therapy. *J Med Virol* 2011; **83**: 1048–57.
90 Lin C, Lin K, Luong YP *et al.* In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem* 2004; **279**: 17508–14.
91 Lin C, Kwong AD, Perni RB. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. *Infect Disord Drug Targets* 2006; **6**: 3–16.
92 Torii H. All about hepatitis C – skin reactions to telaprevir and countermeasures. *Kan-Tan-Sui* 2011; **63**: 1188–93.
93 Thompson AJ, Fellay J, Patel K *et al.* Variants in the ITPA gene protect against ribavirin-induced hemolytic anemia and decrease the need for ribavirin dose reduction. *Gastroenterology* 2010; **139**: 1181–9.
94 Suzuki F, Suzuki Y, Akuta N *et al.* Influence of ITPA polymorphisms on decreases of hemoglobin during treatment with pegylated interferon, ribavirin, and telaprevir. *Hepatology* 2011; **53**: 415–21.
95 Mitsubishi Tanabe Pharma. Antiviral agent "Telavic 250 mg tablets" package insert. 2011.
96 Ozeki I, Akaike J, Karino Y *et al.* Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients. *J Gastroenterol* 2011; **46**: 929–37.
97 Sarrazin C, Kieffer TL, Bartels D *et al.* Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007; **132**: 1767–77.
98 Yamada I, Suzuki F, Kamiya N *et al.* Safety, pharmacokinetics and resistant variants of telaprevir alone for 12 weeks in hepatitis C virus genotype 1b infection. *J Viral Hepat* 2012; **19**: e112–119.
99 Jacobson IM, McHutchison JG, Dusheiko G *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; **364**: 2405–16.
100 Zeuzem S, Andreone P, Pol S *et al.* Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; **364**: 2417–28.
101 Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB, American Association for Study of Liver D. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; **54**: 1433–44.
102 Hayes CN, Kobayashi M, Akuta N *et al.* HCV substitutions and IL28B polymorphisms on outcome of peginterferon plus ribavirin combination therapy. *Gut* 2011; **60**: 261–7.
103 Kurosaki M, Tanaka Y, Nishida N *et al.* Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. *J Hepatol* 2011; **54**: 439–48.
104 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105–9.
105 Oze T, Hiramatsu N, Yakushijin T *et al.* Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy. *J Hepatol* 2011; **54**: 604–11.
106 Kogure T, Ueno Y, Fukushima K *et al.* Pegylated interferon plus ribavirin for genotype Ib chronic hepatitis C in Japan. *World J Gastroenterol* 2008; **14**: 7225–30.
107 Sezaki H, Suzuki F, Kawamura Y *et al.* Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. *Dig Dis Sci* 2009; **54**: 1317–24.
108 Akuta N, Suzuki F, Kawamura Y *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; **46**: 403–10.
109 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; **48**: 372–80.
110 Enomoto N, Sakuma I, Asahina Y *et al.* Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* 1995; **96**: 224–30.
111 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77–81.
112 Shirakawa H, Matsumoto A, Joshita S *et al.* Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* 2008; **48**: 1753–60.
113 El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 2008; **48**: 38–47.
114 Oze T, Hiramatsu N, Yakushijin T *et al.* Viral suppression at week 4 exceeds the IL28B SNP for predicting SVR in pegylated interferon plus ribavirin combination therapy of genotype 1 infected patients with chronic hepatitis C. *Hepatology* 2011; **54**: 852A.

115 Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645–52.
116 Ghany MG, Strader DB, Thomas DL, Seeff LB. American Association for the Study of Liver D. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335–74.
117 Berg T, von Wagner M, Nasser S *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; **130**: 1086–97.
118 Oze T, Hiramatsu N, Yakushijin T *et al.* The efficacy of extended treatment with pegylated interferon plus ribavirin in patients with HCV genotype 1 and slow virologic response in Japan. *J Gastroenterol* 2011; **46**: 944–52.
119 Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. *Hepatology* 2007; **46**: 1688–94.
120 Watanabe S, Enomoto N, Koike K *et al.* Prolonged treatment with pegylated interferon alpha 2b plus ribavirin improves sustained virological response in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan. *Hepatol Res* 2010; **40**: 135–44.
121 Akuta N, Suzuki F, Hirakawa M *et al.* A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. *J Med Virol* 2009; **81**: 452–8.
122 Research Group for Standardisation of Latest Treatment Methods for Viral Hepatitis. Guidelines for the Management of Chronic Hepatitis C. 2012.
123 Sezaki H, Suzuki F, Kawamura Y *et al.* Evaluation of long-term biochemical responses to combination therapy of interferon plus ribavirin in those infected with hepatitis C virus genotype 1b and high baseline viral load. *Hepatol Res* 2007; **37**: 787–92.
124 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061–9.
125 Reddy KR, Shiffman ML, Morgan TR *et al.* Impact of ribavirin dose reductions in hepatitis C virus genotype 1 patients completing peginterferon alfa-2a/ribavirin treatment. *Clin Gastroenterol Hepatol* 2007; **5**: 124–9.
126 Shiffman ML, Ghany MG, Morgan TR *et al.* Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. *Gastroenterology* 2007; **132**: 103–12.
127 Shiffman ML, Salvatore J, Hubbard S *et al.* Treatment of chronic hepatitis C virus genotype 1 with peginterferon, ribavirin, and epoetin alpha. *Hepatology* 2007; **46**: 371–9.
128 Oze T, Hiramatsu N, Yakushijin T *et al.* Pegylated interferon alpha-2b (Peg-IFN alpha-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN alpha-2b plus ribavirin. *J Viral Hepat* 2009; **16**: 578–85.
129 Hiramatsu N, Oze T, Yakushijin T *et al.* Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin. *J Viral Hepat* 2009; **16**: 586–94.
130 Akuta N, Suzuki F, Hirakawa M *et al.* Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology* 2010; **52**: 421–9.
131 Nasu A, Marusawa H, Ueda Y *et al.* Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. *PLoS ONE* 2011; **6**: e24907.
132 Sherman KE, Flamm SL, Afdhal NH *et al.* Response-guided telaprevir combination treatment for hepatitis C virus infection. *N Engl J Med* 2011; **365**: 1014–24.
133 Toray. Natural beta interferon formulation "Feron injectable" package insert. 2011.
134 Iwasaki Y, Shiratori Y, Hige S *et al.* A randomized trial of 24 versus 48 weeks of peginterferon alpha-2a in patients infected with chronic hepatitis C virus genotype 2 or low viral load genotype 1: a multicenter national study in Japan. *Hepatol Int* 2009; **3**: 468–79.
135 Zeuzem S, Feinman S, Rasenack J *et al.* Evaluation of the safety and efficacy of once-weekly Peg/interferon alfa-2A (PegASYS™) for chronic hepatitis C. A multinational, randomized study. *J Hepatol* 2000; **32**(Suppl. 1): 29.
136 Arase Y, Suzuki F, Akuta N *et al.* Combination therapy of peginterferon and ribavirin for chronic hepatitis C patients with genotype 1b and low-virus load. *Intern Med* 2009; **48**: 253–8.
137 Irishio K, Imai Y, Mita E *et al.* Efficacy of Peg-IFN-α-2a monotherapy in patients with chronic hepatitis C serotype 2. *Kanzo* 2011; **52**: 236–43.
138 Sato Y, Tokuue H, Kawamura N *et al.* Short-term interferon therapy for chronic hepatitis C patients with low viral load. *Hepatogastroenterology* 2004; **51**: 968–72.
139 Inoue Y, Hiramatsu N, Oze T *et al.* Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses. *J Viral Hepat* 2010; **17**: 336–44.
140 Jensen DM, Marcellin P, Freilich B *et al.* Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. *Ann Intern Med* 2009; **150**: 528–40.
141 Oze T, Hiramatsu N, Yakushijin T *et al.* Efficacy of re-treatment with pegylated interferon plus ribavirin

combination therapy for patients with chronic hepatitis C in Japan. *J Gastroenterol* 2011; **46**: 1031–7.
142 Poynard T, Colombo M, Bruix J *et al.* Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. *Gastroenterology* 2009; **136**: 1618–28, e1612.
143 Pol S, Aerssens J, Zeuzem S *et al.* Similar SVR rates in IL28B CC, CT or TT prior relapser partial- or null-responder patients treated with telaprevir/peginterferon/ribavirin: retrospective analysis of the realize study. *J Hepatol* 2011; **54**: S6–S7.
144 Muir AJ, Poordad FF, McHutchison JG *et al.* Retreatment with telaprevir combination therapy in hepatitis C patients with well-characterized prior treatment response. *Hepatology* 2011; **54**: 1538–46.
145 Taliani G, Gemignani G, Ferrari C *et al.* Pegylated interferon alfa-2b plus ribavirin in the retreatment of interferon-ribavirin nonresponder patients. *Gastroenterology* 2006; **130**: 1098–106.
146 Jacobson IM, Gonzalez SA, Ahmed F *et al.* A randomized trial of pegylated interferon alpha-2b plus ribavirin in the retreatment of chronic hepatitis C. *Am J Gastroenterol* 2005; **100**: 2453–62.
147 Zeuzem S, Andreone P, Pol S *et al.* Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; **364**: 2417–28.
148 Kanda T, Imazeki F, Azemoto R *et al.* Response to peginterferon-alfa 2b and ribavirin in Japanese patients with chronic hepatitis C genotype 2. *Dig Dis Sci* 2011; **56**: 3335–42.
149 Abergel A, Hezode C, Leroy V *et al.* Peginterferon alpha-2b plus ribavirin for treatment of chronic hepatitis C with severe fibrosis: a multicentre randomized controlled trial comparing two doses of peginterferon alpha-2b. *J Viral Hepat* 2006; **13**: 811–20.
150 Helbling B, Jochum W, Stamenic I *et al.* HCV-related advanced fibrosis/cirrhosis: randomized controlled trial of pegylated interferon alpha-2a and ribavirin. *J Viral Hepat* 2006; **13**: 762–9.
151 Di Marco V, Almasio PL, Ferraro D *et al.* Peg-interferon alone or combined with ribavirin in HCV cirrhosis with portal hypertension: a randomized controlled trial. *J Hepatol* 2007; **47**: 484–91.
152 Izumi N, Kaneko S, Nishiguchi S, Kudo M, Sata M, Omata M. Efficacy and safety of peginterferon-α-2a plus ribavirin combination therapy in the treatment of patients with chronic hepatitis C and compensated cirrhosis – a Phase II/III clinical trial. *Gastroenterology* 2011; **53**: 335–42.
153 Bruno S, Shiffman ML, Roberts SK *et al.* Efficacy and safety of peginterferon alfa-2a (40KD) plus ribavirin in hepatitis C patients with advanced fibrosis and cirrhosis. *Hepatology* 2010; **51**: 388–97.
154 Roffi L, Colloredo G, Pioltelli P *et al.* Pegylated interferon-alpha2b plus ribavirin: an efficacious and well-tolerated treatment regimen for patients with hepatitis C virus related histologically proven cirrhosis. *Antivir Ther* 2008; **13**: 663–73.
155 MSD. Peginterferon-α-2b formulation "Pegintron subcutaneous injectable" package insert. 2011.
156 Chugai Pharmaceutical. Peginterferon-α-2a formulation "Pegasys subcutaneous injectable" package insert. 2011.
157 Suzuki H, Nishigaki M, Kumada H. Interferon beta (IFN-β) therapy in patients with chronic hepatitis C and compensated cirrhosis with low viral loads, and other than serotype 1. *Jpn J Med Pharm Sci* 2006; **56**: 227–51.
158 Kumada H, Kakumu S, Okanoue T, Tsubouchi H, Hayashi N. Efficacy and safety of a natural interferon-α formulation (HLBI) in patients with chronic hepatitis C and compensated cirrhosis – a multicentre collaborative study. *Jpn J Med Pharm Sci* 2008; **59**: 599–620.
159 Heathcote EJ, Shiffman ML, Cooksley WG *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N Engl J Med* 2000; **343**: 1673–80.
160 Dainippon Sumitomo Pharma. Natural interferon-α formulation "Sumiferon injectable" package insert. 2012.
161 Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; **122**: 889–96.
162 Terrault NA, Berenguer M. Treating hepatitis C infection in liver transplant recipients. *Liver Transpl* 2006; **12**: 1192–204.
163 Annicchiarico BE, Siciliano M, Avolio AW *et al.* Treatment of chronic hepatitis C virus infection with pegylated interferon and ribavirin in cirrhotic patients awaiting liver transplantation. *Transplant Proc* 2008; **40**: 1918–20.
164 Forns X, Garcia-Retortillo M, Serrano T *et al.* Antiviral therapy of patients with decompensated cirrhosis to prevent recurrence of hepatitis C after liver transplantation. *J Hepatol* 2003; **39**: 389–96.
165 Iacobellis A, Siciliano M, Perri F *et al.* Peginterferon alfa-2b and ribavirin in patients with hepatitis C virus and decompensated cirrhosis: a controlled study. *J Hepatol* 2007; **46**: 206–12.
166 Carrion JA, Martinez-Bauer E, Crespo G *et al.* Antiviral therapy increases the risk of bacterial infections in HCV-infected cirrhotic patients awaiting liver transplantation: a retrospective study. *J Hepatol* 2009; **50**: 719–28.
167 Foruny JR, Blazquez J, Moreno A *et al.* Safe use of pegylated interferon/ribavirin in hepatitis C virus cirrhotic patients with hypersplenism after partial splenic embolization. *Eur J Gastroenterol Hepatol* 2005; **17**: 1157–64.
168 Miyake Y, Ando M, Kaji E, Toyokawa T, Nakatsu M, Hirohata M. Partial splenic embolization prior to combination therapy of interferon and ribavirin in chronic hepatitis C patients with thrombocytopenia. *Hepatol Res* 2008; **38**: 980–6.
169 Morihara D, Kobayashi M, Ikeda K *et al.* Effectiveness of combination therapy of splenectomy and long-term inter-

feron in patients with hepatitis C virus-related cirrhosis and thrombocytopenia. *Hepatol Res* 2009; **39**: 439–47.
170 Ogata T, Kage M. Re-examination of splenectomy in patients with heptic cirrhosis – changes, risks and benefits. *Kanzo* 2010; **51**: 205–18.
171 McHutchison JG, Dusheiko G, Shiffman ML *et al.* Eltrombopag for thrombocytopenia in patients with cirrhosis associated with hepatitis C. *N Engl J Med* 2007; **357**: 2227–36.
172 Harada N, Hiramatsu N, Oze T *et al.* Incidence of hepatocellular carcinoma in HCV-infected patients with normal alanine aminotransferase levels categorized by Japanese treatment guidelines. *J Gastroenterol* 2012 Sep. 14 [Epub].
173 Hiramatsu N, Inoue Y, Oze T *et al.* Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case-control study. *J Gastroenterol* 2011; **46**: 1335–43.
174 Kainuma M, Furusyo N, Azuma K *et al.* Pegylated interferon alpha-2b plus ribavirin for Japanese chronic hepatitis C patients with normal alanine aminotransferase. *Hepatol Res* 2012; **42**: 33–41.
175 Ikegami T, Matsuzaki Y. Ursodeoxycholic acid: mechanism of action and novel clinical applications. *Hepatol Res* 2008; **38**: 123–31.
176 Omata M, Yoshida H, Toyota J *et al.* A large-scale, multicentre, double-blind trial of ursodeoxycholic acid in patients with chronic hepatitis C. *Gut* 2007; **56**: 1747–53.
177 Takano S, Ito Y, Yokosuka O *et al.* A multicenter randomized controlled dose study of ursodeoxycholic acid for chronic hepatitis C. *Hepatology* 1994; **20**: 558–64.
178 Tarao K, Fujiyama S, Ohkawa S *et al.* Ursodiol use is possibly associated with lower incidence of hepatocellular carcinoma in hepatitis C virus-associated liver cirrhosis. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 164–9.
179 Suzuki F, Ohta T, Takino T, Fujisawa K, Hirayama C. Effects logic examination. Seventy-one patients in Group A of glycyrrhizin on biochemical tests in patients with chronic hepatitis. Double-blind trial. *Asian Med J* 1983; **26**: 423–38.
180 Suzuki H. Therapeutic effect of stronger neo-minophagen in patients with chronic hepatitis C – a double blind trial. *Jpn J Clin Exp Med* 1977; **102**: 562.
181 Iino S, Tango T, Matsushima T *et al.* Therapeutic effects of stronger neo-minophagen C at different doses on chronic hepatitis and liver cirrhosis. *Hepatol Res* 2001; **19**: 31–40.
182 Miyake K, Tango T, Ota Y *et al.* Efficacy of Stronger Neo-Minophagen C compared between two doses administered three times a week on patients with chronic viral hepatitis. *J Gastroenterol Hepatol* 2002; **17**: 1198–204.
183 Kumada H. Long-term treatment of chronic hepatitis C with glycyrrhizin [stronger neo-minophagen C (SNMC)] for preventing liver cirrhosis and hepatocellular carcinoma. *Oncology* 2002; **62** (Suppl 1): 94–100.
184 Arase Y, Ikeda K, Murashima N *et al.* The long term efficacy of glycyrrhizin in chronic hepatitis C patients. *Cancer* 1997; **79**: 1494–500.
185 Ikeda K. Glycyrrhizin injection therapy prevents hepatocellular carcinogenesis in patients with interferon-resistant active chronic hepatitis C. *Hepatol Res* 2007; **37** (Suppl 2): S287–293.
186 Ikeda K, Arase Y, Kobayashi M *et al.* A long-term glycyrrhizin injection therapy reduces hepatocellular carcinogenesis rate in patients with interferon-resistant active chronic hepatitis C: a cohort study of 1249 patients. *Dig Dis Sci* 2006; **51**: 603–9.
187 Tsubota A, Kumada H, Arase Y *et al.* Combined ursodeoxycholic acid and glycyrrhizin therapy for chronic hepatitis C virus infection: a randomized controlled trial in 170 patients. *Eur J Gastroenterol Hepatol* 1999; **11**: 1077–83.
188 Hayashi H, Takikawa T, Nishimura N, Yano M, Isomura T, Sakamoto N. Improvement of serum aminotransferase levels after phlebotomy in patients with chronic active hepatitis C and excess hepatic iron. *Am J Gastroenterol* 1994; **89**: 986–8.
189 Yano M, Hayashi H, Yoshioka K *et al.* A significant reduction in serum alanine aminotransferase levels after 3-month iron reduction therapy for chronic hepatitis C: a multicenter, prospective, randomized, controlled trial in Japan. *J Gastroenterol* 2004; **39**: 570–4.
190 Kato J, Miyanishi K, Kobune M *et al.* Long-term phlebotomy with low-iron diet therapy lowers risk of development of hepatocellular carcinoma from chronic hepatitis C. *J Gastroenterol* 2007; **42**: 830–6.
191 Kawamura Y, Akuta N, Sezaki H *et al.* Determinants of serum ALT normalization after phlebotomy in patients with chronic hepatitis C infection. *J Gastroenterol* 2005; **40**: 901–6.
192 Kato J, Kobune M, Nakamura T *et al.* Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. *Cancer Res* 2001; **61**: 8697–702.
193 Sartori M, Andorno S, Rossini A *et al.* A case-control histological study on the effects of phlebotomy in patients with chronic hepatitis C. *Eur J Gastroenterol Hepatol* 2011; **23**: 1178–84.
194 Wakusawa S, Ikeda R, Takikawa T, Hayashi H, Yano M, Yoshioka K. Combined phlebotomy and ursodeoxycholic acid treatment in the patients with chronic hepatitis C. *Hepatol Res* 2000; **18**: 54–62.
195 Tanaka N, Horiuchi A, Yamaura T *et al.* Efficacy and safety of addition of minor bloodletting (petit phlebotomy) in hepatitis C virus-infected patients receiving regular glycyrrhizin injections. *J Gastroenterol* 2009; **44**: 577–82.

Wnt5a Signaling Mediates Biliary Differentiation of Fetal Hepatic Stem/Progenitor Cells

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non-canonical Wnt signaling; Calcium/calmodulin-dependent kinase II (CaMKII); hepatic maturation; primitive bile ductal structure; hepatic nuclear factor 1β (HNF1β)

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Abbreviations:

Ab, antibody; ALB, albumin; CaMKII, calcium/calmodulin-dependent kinase II; CK, cytokeratin; CPS, carbamoyl phosphate synthetase; DAPI;

4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; E, embryonic day; EGF, Epidermal growth factor; FCS, fetal calf serum; HGF, hepatocyte growth factor; HNF, hepatic nuclear factor; MRP, multidrug resistance-associated protein; P, postnatal day; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PKC, Protein kinase C; RT, reverse transcriptase; TCF, T-cell factor; TAT, tyrosine aminotransferase; TGF, transforming growth factor; TO, tryptophan-2,3-dioxygenase.

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Abstract

The molecular mechanisms regulating differentiation of fetal hepatic stem/progenitor cells, called *hepatoblasts*, which play pivotal roles in liver development, remain obscure. Wnt signaling pathways regulate the development and differentiation of stem cells in various organs. While a β-catenin-independent non-canonical Wnt pathway is essential for cell adhesion and polarity, the physiological functions of non-canonical Wnt pathways in liver development are unknown. Here we describe a functional role for Wnt5a, a non-canonical Wnt ligand, in the differentiation of mouse hepatoblasts. Wnt5a was expressed in mesenchymal cells and other cells of wild-type mid-gestational fetal liver. We analyzed fetal liver phenotypes in Wnt5a-deficient mice using a combination of histological and molecular techniques. Expression levels of Sox9 and the number of HNF1β-positive HNF4α-negative biliary precursor cells were significantly higher in Wnt5a-deficient liver relative to wild-type liver. In Wnt5a-deficient fetal liver, *in vivo* formation of primitive bile ductal structures was significantly enhanced relative to wild-type littermates. We also investigated the function of Wnt5a protein and downstream signaling molecules using a three-dimensional culture system that included primary hepatoblasts or a hepatic progenitor cell line. *In vitro* differentiation assays showed that Wnt5a retarded the formation of bile duct-like structures in hepatoblasts, leading instead to hepatic

maturation of such cells. While Wnt5a signaling increased steady-state levels of phosphorylated Calcium/calmodulin-dependent protein kinase II (CaMKII) in fetal liver, inhibition of CaMKII activity resulted in the formation of significantly more and larger-sized bile duct-like structures *in vitro* compared with those in vehicle-supplemented controls.

Conclusions: We demonstrate that Wnt5a-mediated signaling in fetal hepatic stem/progenitor cells suppresses biliary differentiation. We also suggest that activation of CaMKII by Wnt5a signaling suppresses biliary differentiation.

Introduction

Hepatic stem cells are multipotent stem cells located within ductal plates in fetal and neonatal livers, and canals of Hering in pediatric and adult livers.[1] The extrahepatic stem cell niches are peribiliary glands within the bile ducts in humans.[2] Hepatic stem/progenitor cells, called hepatoblasts in fetal liver, proliferate actively and give rise to hepatocytes and cholangiocytes.[3, 4] Lineage commitment of such cells can be traced by several cell surface markers, including NCAM, ICAM-1, and EpCAM, in humans.[1, 5] While our group[6] and others[7] demonstrated roles for transcription factors regulating the biliary differentiation of hepatic stem/progenitor cells, the molecular mechanisms behind these events have yet to be fully elucidated.

The Wnt-family secreted ligands and the corresponding Frizzled-family cell surface receptors play a crucial role in the differentiation, proliferation, and self-renewal of stem cells in various organs.[8] Wnt signaling pathways involve interactions between a complex set of molecular cognates that includes 19 different Wnt ligands and 10 Frizzled (Fzd) receptors in humans and mice (reviewed at http://www.stanford.edu/~rnusse/wntwindow.html). Upon binding to Fzd receptors on the surface of a target cell, Wnt proteins activate one of two classes of downstream pathways distinguishable by their dependency on β-catenin. Examples of canonical β-catenin-dependent pathways include β-catenin-dependent activation of T-cell factor

(TCF) by either Wnt1 or Wnt3.[8] In contrast, Wnt4 and Wnt5a activate non-canonical β-catenin independent pathways that include downstream molecules such as Calcium/calmodulin-dependent protein kinase II (CaMKII), Rho-kinase, Rac1, Calcineurin, and Protein kinase C (PKC).[9]

In liver development, β-catenin is known to regulate the maturation, expansion, and survival of hepatoblasts, and its deletion results in increased apoptosis of hepatoblasts in mid-gestational fetal livers.[10] While the function of non-canonical Wnt signaling in liver development is currently unknown, β-catenin-independent Wnt pathways have been shown to function predominantly as regulators of cell polarity and mobility in other organs.[9] In systemic Wnt5a-deficient (KO) mice, the size of caudal structures, lung morphogenesis, and intestinal elongation are also abnormal.[11-13]

Recent reports demonstrate that Wnt5a regulates hematopoietic, mesenchymal, and neural stem cell functions.[14-16] Wnt5a has been shown to increase the repopulation of short- and long-term hematopoietic stem cells by maintaining these cells in a quiescent G0 state.[14] Wnt5a maintains mesenchymal stem cells and promotes osteoblastogenesis in preference to adipogenesis in bone marrow,[15] and also improves the differentiation and functional integration of stem cell-derived dopamine neurons.[16] In healthy adult mouse liver, Wnt5a is expressed in mature hepatocytes and cholangiocytes.[17] Nonetheless, the physiological functions of Wnt5a and the signaling

cascades that it initiates during liver development and in hepatic stem/progenitor cells are unknown.

In this study, we investigated the function of Wnt5a and its downstream targets in the development of murine fetal hepatic stem/progenitor cells. Analysis on Wnt5a KO mice demonstrated that loss of Wnt5a abnormally promotes the formation of bile ductal structures in fetal liver *in vivo*. Wnt5a-supplementation not only retarded the formation of bile-duct like structures, but also promoted hepatic maturation of hepatic stem/progenitor cells *in vitro*. CaMKII activity, which showed Wnt5a-dependence in fetal liver, suppressed the formation of bile-duct like structures. These data indicate that Wnt5a-mediated CaMKII signaling plays an essential role in the differentiation of murine fetal hepatic stem/progenitor cells.

Materials and Methods

Animals

Systemic Wnt5a KO mice in C57BL/6 background were originally generated by Yamaguchi et al.[11] Wnt5a KO mice and wild type (WT) littermates were produced by crossbreeding Wnt5a heterozygous mice. All animals were treated based on the guidelines of the Institute of Medical Science, University of Tokyo and those of Tokyo Medical and Dental University.

In vitro bile duct-like differentiation assay of primary hepatoblasts

Bile duct-like differentiation assays were performed as previously described[6] with some modifications. Fetal hepatic cells of E14.5 liver were dissociated with collagenase[4] and Dlk^+ cells were isolated from the resulting population using a magnetic cell sorter (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and then cultured in collagen gel (Nitta Gelatin, Osaka, Japan). After 30 minutes of incubation at 37°C on basal-layer collagen, 1 or 2×10^4 cells were suspended in 1 ml DMEM/F12, mixed with 1 ml collagen gel solution, and plated onto basal-layer collagen in 6-well culture dishes. Plated cells were cultured for 7 days with an additional 2 ml DMEM supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO), 1×insulin/transferrin/selenium, 20 ng/ml epidermal growth factor (EGF, PeproTech,

Rocky Hill, NJ), 20 ng/ml hepatocyte growth factor (HGF, PeproTech) and 25 ng/ml tumor necrosis factor α (PeproTech).

In vitro bile duct-like differentiation assay of hepatic progenitor cell line

The HPPL liver progenitor cell line has been reported to exhibit characteristics of differentiated cholangiocytes in three-dimensional culture.[18, 19] As in the previous report, we maintained HPPL cells in DMEM/F-12 containing 10% FCS, 1× insulin/transferrin/selenium, 10 mM nicotinamide, 10^{-7} M Dex, and 5 ng/ml HGF and EGF and suspended cells in a mixture of type I collagen and Engelbreth-Holm-Swarm sarcoma gel (EHS gel, Becton Dickinson, Bedford, MA) at a density of 4×10^4 cells/ml. Cell suspension was added to each cell culture insert (Millipore, Billerica, MA) and after incubation at 37°C for 2 h, 500 μl of DMEM/F-12 with growth factors was added above and below the insert and the cells were cultured for seven days. To test the effects of inhibitors of CaMKII, Rho-kinase, Rac1, Calcineurin, and PKC on HPPL differentiation, KN93, KN92, KN62, Y-27632, NSC23766, Cyclosporin A, and Go6976 (see *Materials* in Supplementary file) were added individually to the culture medium when each three-dimensional culture was initiated. Independent analyses were performed in triplicate and 5 fields were randomly selected for counting the cysts that indicate bile duct-like differentiation of cells.

In vitro hepatic maturation assay of primary hepatoblasts

To induce hepatic differentiation, primary hepatoblasts from WT E14.5 mice were cultured as previously described.[6] Briefly, 2.5×10^5 MACS-isolated Dlk^+ cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1× non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-7} M Dex in each well of a 6-well gelatin-coated dish. After 5 days, the resulting cells were supplemented with medium containing 20% EHS gel for an additional 2 days prior to analysis.

Materials and methods providing details of materials, cell isolation, Hematoxylin-eosin (HE) staining, RT-PCR analysis, immunostaining, immunoblot analysis, Wnt5a-blocking experiments, microarray analysis, and statistics procedures are described in the Supplementary file.