表 11-12　C 型慢性肝炎に対する初回治療ガイドライン

	genotype 1	genotype 2
高ウイルス量 1 Meq/mL 5.0 log IU/mL 300 fmol/L 以上	Peg-IFNα-2b ＋リバビリン（48～72 週間） Peg-IFNα-2a ＋リバビリン（48～72 週間）	Peg-IFNα-2b ＋リバビリン（24 週間）
低ウイルス量 1 Meq/mL 5.0 log IU/mL 300 fmol/L 未満	IFN（24 週間） Peg-IFNα-2a（24～48 週間）	IFN（8～24 週間） Peg-IFNα-2a（24～48 週間）

C 型慢性肝炎の治療（ガイドラインの補足）

1. genotype1，高ウイルス症例への Peg-IFN ＋リバビリン併用療法の投与期間延長（72 週間投与）の基準：投与開始 12 週後に HCV-RNA 量が前値の 1/100 以下に低下するが HCV-RNA が陽性（real time PCR 法）で，36 週までに陰性化した症例では，プラス 24 週（トータル 72 週間）の投与期間を延長する．
2. genotype1，高ウイルス症例への Peg-IFN ＋リバビリン併用療法で，投与開始 36 週後に HCV-RNA が陽性（real time PCR 法）でも ALT 値が正常化例は，48 週まで継続治療を行い，治療終了後の長期 ALT 値正常化維持を目指す．
3. Peg-IFN ＋リバビリン非適応例・無反応例に対する IFN 単独長期療法は，最初の 2 週間は通常量の連日または週 3 回間欠投与とし，最大 8 週間で HCV-RNA が陰性化しない症例は通常量の半分量を長期投与する．

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以下に低下するが HCV-RNA が陽性（real time PCR 法）で，36 週までに陰性化した例では 72 週間投与が可能である．

処方例

〔体重 60 kg の場合〕
Peg-IFNα-2b（ペグイントロン皮下注）
80 μg　週 1 回　皮下注
リバビリン（レベトールカプセル 200 mg）　3C（カプセル）　分 2　朝 1C・夕 2C　連日

ペグイントロンは通常 1.5 μg/kg を週 1 回皮下投与する．投与量の目安は体重 35～45 kg では 60 μg，46～60 kg で 80 μg，61～75 kg で 100 μg，76～90 kg で 120 μg，91～120 kg では 150 μg である．レベトールは 60 kg 以下では 3C（朝 1C・夕 2C），61 kg を超え 80 kg 以下では 4C（朝 2C・夕 2C），80 kg を超えるものでは 5C（朝 2C・夕 3C）である．

処方例

Peg-IFNα-2a（ペガシス皮下注）
180 μg　週 1 回　皮下注

b）**再治療**：初回 IFN 無効例の再治療にあたっては，初回治療の無効の要因を検討することが重要である．すなわち，初回治療時に HCV がいったん陰性化し再燃した例や，治療期間や薬剤投与量が不十分であった症例では再治療によりウイルス排除が可能であり，最も強力な Peg-IFN ＋ RBV 治療を行うことが基本である．RBV 併用療法を施行する場合には治療効果に寄与する因子（年齢，性別，肝疾患進行度，

表 11-13　C 型慢性肝炎に対する再治療ガイドライン

C 型慢性肝炎に対するインターフェロン(IFN)の再治療は初回治療での無効の要因を検討し，治癒目的の治療か，進展予防(発癌予防)を目指した ALT 値と AFP 値の正常化，あるいは安定化のための治療法を選択すべきである

1. 初回 IFN 無効例への再投与は IFN ＋リバビリン併用療法が，治療の基本である
2. リバビリン併用療法の非適応例あるいはリバビリン併用療法で無反応例では，IFN の長期投与が望ましい．なお，IFNα製剤(Peg 製剤を除く)は，在宅自己注射が可能
3. IFN 非適応例および IFN で ALT 値，AFP 値の改善が得られない症例は肝庇護剤(SNMC，UDCA)，瀉血療法を単独あるいは組み合わせて治療する
4. 進展予防(発癌予防)を目指した治療の ALT 目標値は Stage 1(F1)では，持続的に基準値の 1.5 倍以下にコントロールする．Stage 2～3(F2～F3)では，極力正常値 ALT ≦ 30 IU/L にコントロールする．
5. リバビリン併用療法を行う場合には治療効果に寄与する因子である．年齢，性別，肝疾患進行度，HCV ウイルスの遺伝子変異(コア領域 70，91 の置換，ISDR 変異)，real time PCR 法によるウイルス量などを参考にし，治療法を選択することが望ましい．

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ISDR やコア領域の HCV の遺伝子変異)などを考慮し治療法を選択することが必要である(表 11-13)．この際，注意を要するのは，Peg-IFNα-2a + RBV 療法は，Peg-IFNα-2b + RBV 療法で無効ないしは再燃例には適応がないことである．一方，RBV 併用療法の非推奨例や無反応例では IFN を単独で 2 週間連日ないしは間欠投与し，8 週以内に HCV が陰性化した場合は完全治癒をめざし 2 年間の長期投与を行うが，8 週時点で HCV が陰性化しない場合には肝発癌予防を目的とし，IFN 単独少量長期療法により肝機能(ALT)の正常化をはかることが必要である．注意を要するのは，Peg-IFNα-2a + RBV 療法は，Peg-IFNα-2b + RBV 療法で無効ないしは再燃例には適応がないことである．

c) **肝発癌抑止を目的とした IFN 療法**：HCV の排除が困難な場合でも，IFN 投与によって，肝機能(ALT)の正常化をはかり，肝発癌抑止効果が期待できる．これは「発癌抑制を目指した血清 ALT 正常 C 型肝炎症例の抗ウイルス治療ガイドライン」〔表 11-14〕にまとめられているが，これによれば血小板数が肝線維化の程度と相関することから，血小板数 15 万/μL 以下の肝線維化進展例では，たとえ ALT が 30 IU/L 以下であっても，肝発癌抑止のためには抗ウイルス療法を考慮することが明記されている．また，最近は IFNα製剤 α-2b 製剤では自己注射も可能となり，治療法の選択肢は広がっている．しかし，IFN は副作用も多いことから慎重な判断が求められる．

処方例

IFNα(HLBI)(スミフェロン DS)　600 万単位　週 3 回　筋注または皮下注

❷肝庇護療法：IFN の治療効果が高まったとはいえ，1/3 の患者ではウイルス排除は困難である．また，高齢者・肝線維化進展例や合併症のために IFN 投与が困難な症例も存在し，IFN 治療によっても ALT が正常化しない例も存在する．この場合，一般に用いられるのは，ウルソデオキシコール酸(UDCA)あるいはグルチルリチン製剤による肝庇護療法である．これ

表 11-14　発癌抑制を目指した血清 ALT 正常 C 型肝炎例への抗ウイルス治療ガイドライン

ALT 値	血小板数	
	≧ 15 × 10^4/μL	< 15 × 10^4/μL
≦ 30 IU/L	2〜4 か月ごとに血清 ALT 値フォロー ALT 異常を呈した時点で完治の可能性，発癌リスクを評価し，抗ウイルス療法を考慮	線維化進展例がかなり存在する 可能なら肝生検を施行し F2A2 以上の例に抗ウイルス療法を考慮 肝生検非施行例は 2〜4 か月ごとに血清 ALT 値を測定し，異常を示した時点で抗ウイルス療法を考慮
31〜40 IU/L	65 歳以下は抗ウイルス療法の考慮	慢性肝炎治療に準じる*

*遺伝子型，ウイルス量，年齢などを考慮し，通常の C 型慢性肝炎治療に準じて，治療法を選択する．
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らには抗ウイルス効果はみられないものの大きな副作用がなく，一定の効果が認められる．

処方例

強力ネオミノファーゲンシー　40〜60 mL　週 2〜3 回　静注
かつ または
ウルソデオキシコール酸（ウルソ錠 100 mg）　6 錠　分 3　毎食後

③瀉血療法：C 型慢性肝炎では肝に過剰な鉄が沈着し，鉄蓄積と肝線維化の程度が相関することや，肝臓の鉄濃度と肝機能成績が相関することから，瀉血により肝の除鉄を行うことの有用性が示されており，特に鉄制限食を併用することが重要と考えられている．IFN が無効な症例には選択肢の 1 つとして，2006 年から保険適用されている．実際には週 1 回 200 g の瀉血を Hb 11.0 g/dL 未満ないしは血清フェリチン値 10 ng/mL 未満となるまで，最長 16 週間繰り返し，その後は Hb 10.5〜10.9 g/dL，血清フェリチン値 10 ng/mL を維持するよう 4 週間ごとに 200〜400 g の瀉血を 1〜3 か月間繰り返す．

〔合併症・偶発症〕

IFN にはさまざまな副作用がみられる．Peg-IFN の副作用も基本的には IFN と同様に注意深い経過観察が必要である．頻度の高い副作用としては，治療開始早期に出現するものとして発熱・頭痛などのインフルエンザ症状，食思不振・嘔気・味覚障害・心窩部痛・便通異常などの消化器症状，皮疹・掻痒感などの皮膚症状が，治療 2〜3 か月後に出現するものとして，脱毛や抑うつ症状などがある．また，血球成分の減少も知られ，最大の効果発揮と副作用の軽減のためには，血球検査の結果によって投与量を調整することも必要である．特に RBV 併用では溶血性貧血が問題となる．一方，稀には甲状腺機能異常，眼底出血，耐糖能異常，間質性肺炎，脳出血，心筋症などの重篤な副作用も報告されている．特に 65 歳以上で，高血圧，糖尿病を合併しているものでは脳出血の危険性が高まるとされ注意が必要である．

〔患者説明のポイント〕

IFN 療法にはさまざまな副作用が存在することから，起こりうる副作用と対処法について十分な説明が必要である．特に，普段と異なる症状が出現した場合には主治医に連絡・受診するように説明することが重要である．また RBV には催奇形性が存在することから，投与中と投与終了 6 か月間は避妊の必要を十分説明しておく必要が

ある．

【医療スタッフへの指示】

IFNの投与中にみられる副作用に抑うつ症状がある．多くは医師の診察時に診断できるが，注射の際の表情の変化や言動により医療スタッフが気づくことがある．この際には医師に連絡するよう指導する．

院内感染予防対策

prevention of hospital-acquired hepatitis B and C viral infection

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【概念】

肝炎ウイルスによる院内感染は，①輸血や血液製剤などによる患者への感染のほか，②病室内や血液透析などの医療行為を介して患者間または患者・医療従事者間で発生することがある．これら肝炎ウイルスによる院内感染は集団発生したり，重篤な肝炎を発症することがあるので，医療機関では院内感染に対する特別な対策が必要である．医療従事者は針刺し事故など観血的処置に伴い受傷することが多く対応を要する．

【頻度】

輸血後肝炎の頻度は0.1%以下であり，1999年の核酸増幅検査(NAT)の導入により血液製剤の安全性はさらに高まった．しかし，感染初期のウインドウ期間の献血液による感染は，きわめて低頻度であるものの完璧な阻止は困難である．ウインドウ期間は，HBs抗原が約59日，HBV-NATが約34日，HCV抗体が約84日，HCV-NATが約23日である．

HBV汚染血液の針事故による肝炎発生の頻度は全体で6～30%である．汚染源がHBe抗原陽性の場合は陰性に比しリスクが高いとされ，肝炎発生率はHBe抗原陽性血液では22～31%，HBe抗原陰性では1～6%で，汚染源のウイルス量(HBV-DNA)が多いほど感染のリスクが高くなる．特に注意すべきは，全体の2～3%は重症化・劇症化することがあり，これらはHBe抗体陽性の汚染源，すなわちHBV precore変異またはcore promoter変異株による感染で発症することが多い．

一方，HCV汚染血液の針刺し事故による肝炎発生の頻度は約1.8%であり，HBVに比し低率であるが，発症すると無治療の場合慢性化する頻度が高い．

【予防策】

医療行為の際，手袋などの標準予防策をとることが基本である．血液・体液を大量に浴びる可能性が高い場合は，必要に応じてマスク，ゴーグル，フェイスシールド，ガウンなどを着用する．針刺し事故の予防の3原則は，①針のリキャップの禁止，②適切な耐貫通性の廃棄容器の管理，③安全・防御装置のついた器材の導入である．

一方，透析ユニットや手術室などの観血的処置が行われる処置室では，患者間の感染を防止するために，①医療器材や薬剤は共用としない，②ディスポーザブル器材を使用する，③適切な消毒と滅菌を行う，④医療者が媒介しない対策(手洗いほか)など基本的な院内感染防止対策を徹底する．内視鏡の洗浄については，日本消化器内視鏡学会から指針が示されているので，これを参考にして適切に実施する．

【針刺し事故後の手順】

1) 事故直後，可及的速やかに，受傷部を流水と石けんで洗浄する．

2) 直ちに受診する．この際，汚染源が特定できる場合は，その症例のHBs抗原，HCV抗体，HIV抗体，RPR，TPLA(必要があればHTLV-1抗体)の有無を検索する．これらが未検査の場合は，患者(あるいは代理人)の了解を得たうえでこれらの

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Original Article

Hepatic steatosis in chronic hepatitis C is a significant risk factor for developing hepatocellular carcinoma independent of age, sex, obesity, fibrosis stage and response to interferon therapy

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Aim: Hepatic steatosis is linked to development of hepatocellular carcinoma (HCC) in non-viral liver disease such as non-alcoholic steatohepatitis. The present study aimed to assess whether hepatic steatosis is associated with the development of HCC in chronic hepatitis C.

Methods: We studied a retrospective cohort of 1279 patients with chronic hepatitis C who received interferon (IFN) therapy between 1994 and 2005 at a single regional hospital in Japan. Of these patients, 393 had a sustained virological response (SVR) and 886 had non-SVR to IFN therapy. After IFN therapy, these patients were screened for development of HCC every 6 months. The average period of observation was 4.5 years.

Results: HCC developed in 68 patients. The annual incidence of HCC was 2.73% for patients with a steatosis grade of 10% or greater and 0.69% for patients with a steatosis grade of 0–9%. On multivariate analysis, higher grade of steatosis was a significant risk factor for HCC independent of older age, male sex, higher body mass index (BMI), advanced fibrosis stage and non-SVR to IFN therapy. The adjusted risk ratio of hepatic steatosis was 3.04 (confidence interval 1.82–5.06, $P < 0.0001$), which was higher than that of older age (1.09), male sex (2.12), non-SVR to IFN (2.43) and higher BMI (1.69).

Conclusion: Hepatic steatosis is a significant risk factor for development of HCC in chronic hepatitis C independent of other known risk factors, which suggest the possibility that amelioration of hepatic steatosis may prevent hepatocarcinogenesis.

Key words: hepatocellular carcinoma, interferon, steatosis, virological response.

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common cancers worldwide and its incidence has been increasing. This recent increase in HCC incidence may likely be attributed to the higher prevalence of non-alcoholic fatty liver disease (NAFLD) and hepatitis C virus (HCV) infection.[1]

Non-alcoholic fatty liver disease is characterized by hepatic steatosis with or without inflammation in the absence of excessive alcohol consumption. Several studies have indicated the etiological association between NAFLD and development of HCC.[2–4] Other studies have shown that obesity or diabetes, a common etiology of non-alcoholic hepatic steatosis, is associated with development of HCC.[5–7] Although the mechanism of carcinogenesis in NAFLD has not been determined, an animal model showed that obesity-related hepatic steatosis leads to the development of hepatic

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hyperplasia, suggesting the possibility that hepatic steatosis is a pre-malignant condition.[8]

Another important etiological agent for HCC is HCV infection. Because steatosis is a common pathological feature of HCV-infected patients,[9] the important question is whether steatosis influences the progression of liver disease in hepatitis C, by analogy with NAFLD. Several studies, including ours[10] indicated that hepatic steatosis promotes the progression of hepatic fibrosis.[11–15] The association between hepatic steatosis and the development of HCC in chronic hepatitis C has been proposed[16] and was confirmed in two studies[17,18] while another study failed to show such an association.[19] The present study was conducted to analyze the association between hepatic steatosis and development of HCC in a large cohort of chronic hepatitis C patients, which enabled to adjust for known risk factors for HCC.

METHODS

Patients

A TOTAL OF 1437 chronic hepatitis C patients were treated with interferon (IFN) at Musashino Red Cross Hospital between October 1994 and October 2005. Among them, 1279 patients who fulfilled the following inclusion criteria were enrolled in this study: (i) positive for HCV RNA by reverse-transcription polymerase chain reaction before IFN therapy; (ii) absence of other causes of liver disease, such as co-infection with hepatitis B virus, autoimmune hepatitis or primary biliary cirrhosis; (iii) had undergone liver biopsy within the 12 months prior to IFN treatment; (iv) were followed for more than 1 year after the completion of IFN therapy; and (v) absence of HCC during and within 1 year after the completion of therapy. A total of 158 patients were excluded: two patients who were positive for hepatitis B surface antigen, 97 patients lacking liver biopsy, 53 patients with less than 1 year's duration of follow up, and six patients who developed HCC within 1 year of the completion of IFN therapy. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee.

Patients were followed up by regular visits to our hospital every 1–3 months. Six patients died of liver-unrelated disease (two patients with gastric cancer and one patient each with lung cancer, colon cancer, pancreatic cancer and leukemia). There were 122 patients who were lost to follow up because of relocation. We included their data in the analysis, censored at the time of their last visit. The start of follow up was defined as the date of completion of first IFN therapy and the end of follow up was defined as the date of diagnosis of HCC or the date of the last visit. The average period of follow up was 4.5 years.

Clinical characteristics and laboratory data were collected at the most recent time point before liver biopsy. Diabetes mellitus was diagnosed based on a fasting plasma glucose concentration that exceeded 126 mg/dL, a casual plasma glucose concentration that exceeded 200 mg/dL, or the need for insulin or oral antihyperglycemic drugs. Information regarding alcohol consumption was obtained through an interview. Body mass index (BMI) was calculated using the following formula: weight in kilograms/height in meters squared. The baseline clinical features of patients at enrollment are summarized in Table 1.

Histological examination

Liver biopsy specimens were obtained from all patients before therapy. The median length of liver biopsy specimens was 13 mm (range 10–42 mm) and median number of portal tracts was 11 (range 4–30). Histological findings were re-evaluated recently by three independent pathologists who were blinded to the clinical details to ensure consistency over time. Fibrosis and activity were scored according to the METAVIR scoring system.[20] Fibrosis was staged on a scale of 0–4: F0 (no fibrosis); F1 (mild fibrosis: portal fibrosis without septa); F2 (moderate fibrosis: few septa); F3 (severe fibrosis: numerous septa without cirrhosis); and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of 0–3: A0 (no activity); A1 (mild activity); A2 (moderate activity); and A3 (severe activity). Percentage of steatosis was quantified by determining the average proportion of hepatocytes affected by steatosis and graded on a scale of 0%, 1–9%, 10–29% and 30% or greater as reported previously.[10] All three pathologists assigned the same scale in 85% of cases for fibrosis staging, 87% for inflammation grading and 95% for steatosis grading. If there was discordance, the scores assigned by two pathologists were used for the analysis.

Screening for HCC

At enrollment, no patient had HCC or any suspicious lesion on abdominal ultrasonography or computed tomography. Patients were examined for HCC by abdominal ultrasonography or computed tomography at least every 6 months. Suspicious lesions were examined further by a triphasic contrast-enhanced computerized tomography or magnetic resonance imaging,

Table 1 Clinical characteristics of patients

Male, *n* (%)	643 (50%)
Age (years)	54.2 ± 11.9
BMI (kg/m^2)	23.4 ± 3.1
Alcohol consumption ≥20 g/day, *n* (%)	44 (3%)
Diabetes Mellitus, *n* (%)	197 (15%)
AST level (IU/L)	68.9 ± 45.3
ALT level (IU/L)	92.9 ± 75.9
GGT level (IU/L)	41.2 ± 38.2
Platelet count (×10^{10}/L)	16.4 ± 5.2
HCV genotype, *n* (%)	
1b	873 (68.2%)
2a	236 (18.4%)
2b	139 (10.9%)
3	2 (0.2%)
Not determined	29 (2.3%)
Histological findings	
Grade of activity, *n* (%)	
A0	154 (12%)
A1	574 (45%)
A2	441 (34%)
A3	110 (9%)
Stage of fibrosis, *n* (%)	
F0	24 (2%)
F1	591 (46%)
F2	378 (30%)
F3	242 (19%)
F4	44 (3%)
Grade of steatosis, *n* (%)	
0%	384 (30%)
1–9%	543 (42%)
10–29%	215 (17%)
≥30%	137 (11%)
SVR to interferon therapy, *n* (%)	393 (31%)
Development of HCC, *n* (%)	68 (5%)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, γ-glutamyltransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.

angiography or tumor biopsy to confirm the diagnosis. Diagnostic criteria of HCC on radiological findings were hyper-vascularity at angiography or hyper-attenuation at triphasic contrast-enhanced computerized tomography or magnetic resonance imaging during the hepatic arterial phase.

Statistical analysis

The SPSS software package ver. 15.0 was used for statistical analysis. Categorical data were analyzed using Fisher's exact test. Continuous variables were compared with Student's *t*-test. The time for the development of HCC was defined as the time from the completion of IFN therapy to the time of diagnosis. Annual incidence of HCC was calculated using the person-years method. Effect of hepatic steatosis on time to development of HCC was analyzed by the Kaplan–Meier method and log–rank test, after stratification by age, sex, BMI, degree of fibrosis and response to IFN therapy, as well as multivariate analysis using Cox proportional hazards regression analysis. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Background factors for steatosis

PATIENTS WITH A steatosis grade of 10% or greater were older (53.6 ± 12.6 vs 56.0 ± 9.8, $P = 0.001$), had a higher BMI (23.0 ± 3.0 vs 24.6 ± 3.3, $P < 0.0001$), higher frequency of diabetes (12% vs 24%, $P < 0.0001$), higher serum levels of aspartate aminotransferase (AST) (66 ± 46 vs 75 ± 43, $P = 0.002$), γ-glutamyltransferase (GGT) (37 ± 52 vs 52 ± 33, $P < 0.0001$), total cholesterol (173 ± 32 vs 179 ± 33, $P = 0.005$), triglycerides (123 ± 56 vs 145 ± 68, $P < 0.0001$), and a lower serum level of albumin (4.2 ± 0.3 vs 4.1 ± 0.3, $P = 0.005$) and lower platelet counts (16.6 ± 5.2 vs 15.7 ± 5.1, $P = 0.007$). Histological grade of activity (A2–3: 39% vs 54%, $P < 0.0001$), and stage of fibrosis (F3–4: 18% vs 34%, $P < 0.0001$) were higher. The proportion of non-sustained virological response (SVR) to IFN also was higher (35% vs 19%, $P < 0.0001$). These results indicate that hepatic steatosis in hepatitis C is related to metabolic factors and associated with other risk factors for the development of HCC such as older age, advanced stage of fibrosis, and non-SVR to IFN therapy.

Factors associated with the development of HCC

Hepatocellular carcinoma developed in 68 patients during follow up. An overall annual incidence of HCC development was 1.19% by person-years. The annual incidence of HCC development by person-years was higher in patients with higher grade of steatosis: 0.45% for patients without steatosis, 0.78% for patients with 1–9% of steatosis, 2.30% for patients with 10–29% of steatosis, and 3.56% for patients with 30% of steatosis. The relative risk of hepatic steatosis (grade of ≥10%) for HCC development was 4.39 (95% confidence interval 2.66–7.26, $P < 0.0001$). The difference remained significant, even after stratification for other risk factors such as IFN therapy, stage of fibrosis, age, sex and BMI (Fig. 1). When analyzed by the multivariate Cox proportional hazards regression method, a higher grade of steatosis,

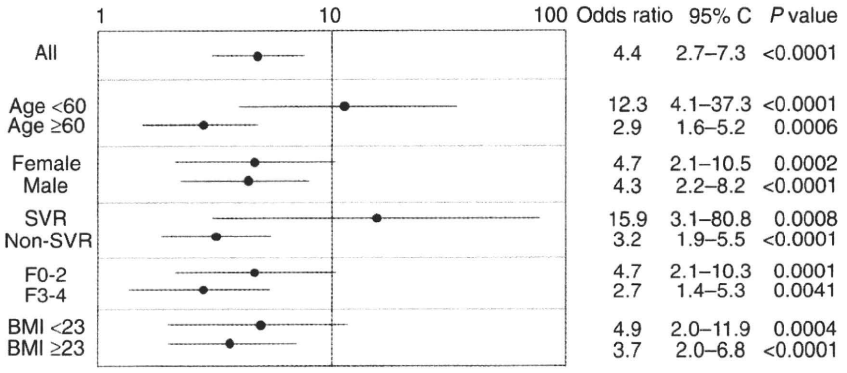


Figure 1 Relative risk differences of hepatocellular carcinoma (HCC) among patients with and without steatosis. The relative risk of hepatic steatosis (grade ≥10%) for HCC development was analyzed, after stratification for other risk factors such as interferon (IFN) therapy, stage of fibrosis, age, sex and body mass index (BMI). SVR, sustained virological response.

older age, male sex, higher BMI, an advanced stage of fibrosis and non-SVR to IFN therapy were independent risk factors associated with the development of HCC (Table 2). The adjusted risk ratio of hepatic steatosis was 3.04 (95% confidence interval 1.82–5.06, $P < 0.0001$). The presence of diabetes and consumption of ethanol were not significant. Figure 2(a) shows the Kaplan–Meier curve of the time to development of HCC in the entire cohort. The cumulative incidence of HCC was significantly higher with hepatic steatosis of 10% or greater. To adjust for other risk factors, patients were stratified according to response to IFN therapy, stage of fibrosis, age, sex and BMI. The difference remained significant, even after stratification for these confounding factors (Fig. 2b–f). Three patients died after the development of HCC. All were over 60 years old, and had significant steatosis. The impact of hepatic steatosis on the survival rate could not be analyzed due to the small number of death.

DISCUSSION

IN THIS STUDY, we have shown that the presence of significant steatosis is an independent risk factor for the development of HCC in chronic hepatitis C. Our study involved the largest number of patients, compared to previous reports, and this enabled us to adjust for other known risk factors for HCC. The impact of steatosis on HCC development remained significant even after adjusting for other risk factors such as older age, male sex, higher BMI, advanced fibrosis and non-SVR to IFN therapy. These findings indicate the need of intensive surveillance for HCC in patients with significant steatosis and provide an argument for therapeutic interventions aimed at reducing steatosis, in order to reduce the risk of HCC.

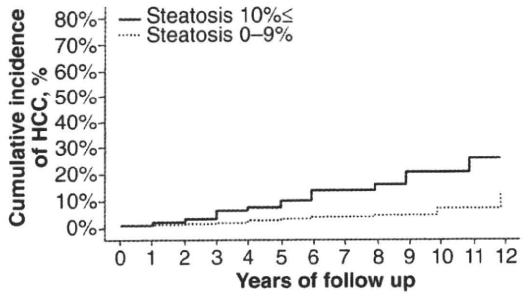
The association between hepatic steatosis and the development of HCC in chronic hepatitis C has been proposed and the possible mechanism has been discussed.[16] There are several cohort studies on this topic but their results are conflicting. The first report included 20 patients with SVR to IFN, 51 patients with non-SVR to IFN and 90 patients who did not receive IFN therapy.[17] In this cohort of 161 patients, older age, absence of IFN therapy, cirrhosis and steatosis were associated with HCC development. Another study involved 25 patients with HCC and an equal number of patients who did not develop HCC, matched for

Table 2 Multivariate analysis of risk factors for hepatocellular carcinoma

Predictor		Odds ratio (95% CI)	*P*-value
Age	By every 10 years	1.09 (1.05–1.13)	<0.0001
Sex	Male vs female	2.12 (1.28–3.51)	0.004
Stage of fibrosis	F3–4 vs F0–2	4.30 (2.59–7.14)	<0.0001
Grade of steatosis	≥10% vs <10%	3.04 (1.82–5.06)	<0.0001
Response to IFN	Non-SVR vs SVR	2.43 (1.13–5.23)	0.023
Diabetes	Present vs absent	0.75 (0.42–1.33)	0.319
Ethanol consumption (g/day)	≥20 vs <20	0.50 (0.07–3.60)	0.478
BMI (kg/m2)	≥23 vs <23	1.69 (1.02–2.86)	0.043

BMI, body mass index; CI, confidence interval; IFN, interferon; SVR, sustained virological response.

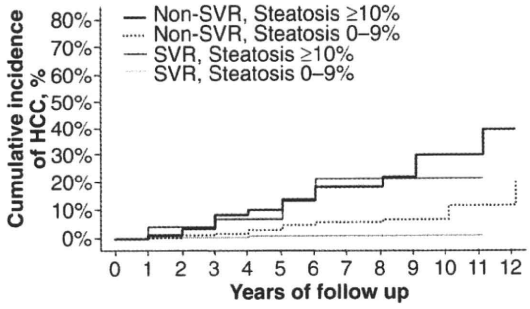
(a)



Number of patients at risk

Steatosis 0–9%	927	824	620	503	320	227	161	117	77	49	27	10
Steatosis ≥10%	352	271	207	157	113	83	54	48	32	17	9	1

(b)



Number of patients at risk

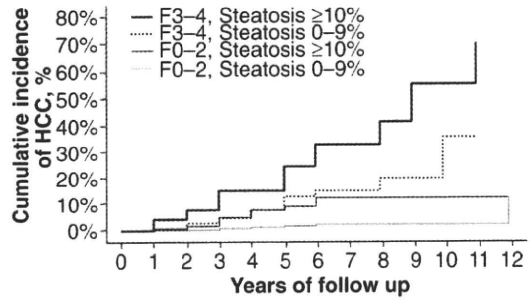
SVR

Steatosis 0–9%	326	254	204	153	81	55	33	21	15	10	5	0
Steatosis ≥10%	67	50	34	22	14	10	4	4	4	2	2	0

Non-SVR

Steatosis 0–9%	601	507	416	350	239	172	128	96	62	39	22	10
Steatosis ≥10%	285	221	173	135	99	73	50	44	28	15	7	1

(c)



Number of patients at risk

F0–2

Steatosis 0–9%	759	623	509	415	266	188	137	99	64	39	25	10
Steatosis ≥10%	234	190	146	107	77	55	37	32	19	11	6	1

F3–4

Steatosis 0–9%	118	81	61	50	36	28	17	16	13	6	3	0
Steatosis ≥10%	168	138	111	88	54	39	23	18	13	10	2	0

(d)

Number of patients at risk

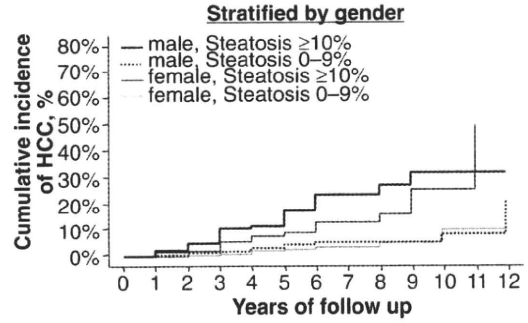
Age <60

Steatosis 0–9%	549	457	367	298	188	148	111	83	53	33	19	7
Steatosis ≥10%	193	154	111	83	61	48	34	31	23	12	6	1

Age ≥60

Steatosis 0–9%	378	304	253	205	132	79	50	34	24	16	8	3
Steatosis ≥10%	159	117	96	74	52	35	20	17	9	5	3	0

(e)



Number of patients at risk

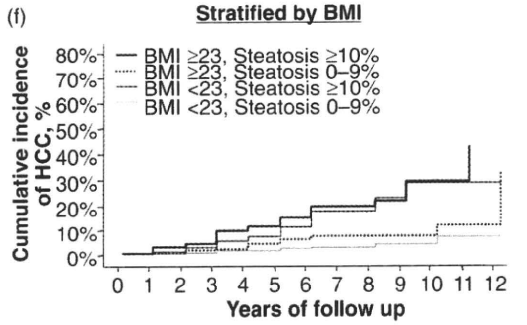
Male

Steatosis 0–9%	470	389	319	265	169	126	90	65	46	30	17	7
Steatosis ≥10%	173	134	98	73	54	40	21	21	15	8	6	1

Female

Steatosis 0–9%	457	372	301	238	151	101	71	52	31	19	10	3
Steatosis ≥10%	179	137	109	84	59	43	33	27	17	9	3	0

(f)



Number of patients at risk

BMI ≥23

Steatosis 0–9%	417	346	269	213	129	94	66	49	31	19	8	4
Steatosis ≥10%	226	176	137	101	71	55	34	33	20	10	5	0

BMI <23

Steatosis 0–9%	510	415	351	290	191	133	95	68	46	30	19	6
Steatosis ≥10%	126	95	70	56	42	28	20	15	12	7	4	1

Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC) among patients with steatosis (solid line) and without steatosis (dotted line), stratified by other risk factors. The cumulative incidence of HCC was (a) significantly higher in patients with a steatosis grade of 10% or greater ($P < 0.0001$ by the log-rank test), even after (b) stratification by the response to interferon therapy ($P < 0.0001$ for sustained virological response [SVR] and non-SVR by the log-rank test), (c) stratification by the stage of fibrosis ($P < 0.0001$ for F0–2 and $P = 0.0036$ for F3–4 by the log-rank test), (d) stratification by age ($P = 0.0001$ for age ≥60 and $P < 0.0001$ for age <60 by the log-rank test), (e) stratification by sex ($P < 0.0001$ for men and women by the log-rank test), and (f) stratification by body mass index (BMI) ($P < 0.0001$ for BMI ≥23 kg/m^2 and <23 kg/m^2 by the log-rank test). The number of patients at risk is shown below each graph.

age, sex, HCV genotype and stage of fibrosis.[19] In this study, only ALT and albumin were identified as predictors of HCC and steatosis was not. The authors acknowledged the small size of the cohort as a limitation and emphasized the need for larger cohort studies. The third study analyzed explanted liver from cirrhotic patients who underwent liver transplantation and included 32 patients with HCC and 62 patients without HCC.[18] The authors found that older age, higher α-fetoprotein levels and steatosis were significantly associated with HCC. The major advantage of this study was the standardization of fibrosis stage to cirrhosis. On the other hand, a limitation was the retrospective nature of the study; steatosis was evaluated after the diagnosis of HCC, when cirrhosis already was present (fibrosis stage F4). Because steatosis has been reported to decrease once cirrhosis has developed, this study may have underestimated the grade of steatosis present prior to the development of HCC. Thus, we cannot simply apply their findings to a clinical setting where biopsies are usually obtained before the development of cirrhosis and years before the development of HCC. Based on that background, the principal aim of this study was to analyze the association between hepatic steatosis and the development of HCC in chronic hepatitis C patients, adjusting for known risk factors. We found that steatosis was an independent risk factor by the multivariate Cox proportional hazards regression analysis and by the Kaplan–Meier method and log-rank test after stratification by other risk factors. To our surprise, the adjusted risk ratio of hepatic steatosis was higher than that of older age, male sex, non-SVR to IFN and higher BMI.

How steatosis contributes to the development of HCC remains unclear. Several studies including ours,[10] indicated that hepatic steatosis promotes the progression of hepatic fibrosis,[11–15] which potentiates the risk of HCC indirectly. On the other hand, the ob/ob mouse model of NAFLD showed that hepatic neoplasia developed in the absence of advanced fibrosis, supporting the concept that metabolic abnormalities related to obesity initiate the neoplastic process.[8] Leptin, an adipocytokine related to steatosis in chronic hepatitis C,[21] was shown recently to be mitogenic in human liver[22] and thus may be a link between steatosis and HCC development. Otherwise, steatosis may be responsible for increased lipid peroxidation and reactive oxygen species which induce genetic damage.[23–25] Another study showed that mice transgenic for the HCV core gene developed hepatic steatosis early in life and thereafter HCC which indicates that the HCV core protein has a chief role in the development of both steatosis and HCC development.[26] The precise mechanism of the association between steatosis and carcinogenesis needs further investigation.

The higher incidence of HCC in patients with significant steatosis has important clinical implications. The most important question is whether therapeutic interventions aimed at reducing steatosis could reduce the risk of HCC in chronic hepatitis C. Because the adjusted risk ratio of hepatic steatosis was higher than that of older age, male sex, non-SVR to IFN and higher BMI, we hypothesize that modification of lifestyle and the amelioration of hepatic steatosis may efficiently prevent hepatocarcinogenesis in patients having concomitant risk factors. Apparently, further prospective studies focusing on this point are necessary. Weight reduction may provide an important treatment strategy because one study indicated that weight reduction in chronic hepatitis C leads to a reduction in steatosis and an improvement in fibrosis despite the persistence of HCV infection.[27] Alternatively, insulin resistance may be another target of therapy because a study showed that the administration of pioglitazone led to metabolic and histological improvement in subjects with non-alcoholic steatohepatitis.[28] A limitation of the present study was that data for the plasma insulin concentration was not available and thus insulin resistance could not be assessed. Whether insulin resistance plays a role in hepatocarcinogenesis or its amelioration could improve steatosis and ultimately prevent development of HCC in chronic hepatitis C awaits future investigation.

Another important finding of the present study was that steatosis was a significant risk factor for the development of HCC in patients with SVR to IFN therapy. Thus, steatosis may play a role in carcinogenesis in patients who have cleared HCV. Several studies have shown that the incidence of HCC is reduced but not eliminated in those with SVR to IFN.[29–31] Because the predictors of HCC development in SVR patients have not been established to date, steatosis may be used to identify patients who need intensive surveillance and long-term follow up, even after the clearance of HCV. In conclusion, we showed that hepatic steatosis is significantly associated with the development of HCC in chronic hepatitis C independent of age, sex, BMI, degree of fibrosis and response to previous IFN therapy. Steatosis may be a useful marker for identifying patients at higher risk for HCC. Further studies are needed to evaluate the hypothesis that therapeutic interventions aimed at reducing steatosis may prevent hepatocarcinogenesis.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection

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Abstract
Background Pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy is currently the de-facto standard treatment for hepatitis C virus (HCV) infection. The aims of this study were to analyze the clinical and virological factors associated with a higher rate of response in patients with HCV genotype 1b infection treated with combination therapy.
Methods We analyzed, retrospectively, 239 patients with chronic hepatitis C-1b infection who received 48 weeks of combination therapy. We assessed clinical and laboratory parameters, including age, gender, pretreatment hemoglobin, platelet counts, HCV RNA titer, liver histology, the number of interferon sensitivity determining region (ISDR) mutations and substitutions of the core amino acids 70 and 91. Drug adherence was monitored in each patient. We carried out univariate and multivariate statistical analyses of these parameters and clinical responses.
Results On an intention-to-treat (ITT) analysis, 98 of the 239 patients (41%) had sustained virological responses (SVRs). Patients with more than two mutations in the ISDR had significantly higher SVR rates ($P < 0.01$). Univariate analyses showed that stage of fibrosis, hemoglobin, platelet counts, ISDR mutations, serum HCV RNA level, and adherence to PEG-IFN plus RBV were significantly correlated with SVR rates. Multivariate analysis in subjects with good drug adherence extracted the number of ISDR mutations (two or more: odds ratio [OR] 5.181).
Conclusions The number of mutations in the ISDR sequence of HCV-1b (≥ 2) is the most effective parameter predicting a favorable clinical outcome of 48-week PEG-IFN plus RBV therapy in patients with HCV genotype 1b infection.

Keywords Hepatitis C virus (HCV) · Chronic hepatitis C · PEG-IFN plus RBV therapy · Combination therapy · Interferon sensitivity determining region (ISDR)

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon
PEG	Polyethylene glycol
PEG-IFN	Pegylated-interferon-alpha 2b
RBV	Ribavirin
ISDR	Interferon sensitivity determining region
BMI	Body mass index

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ALT	Alanine transaminase
dM	Double mutant
ITT analysis	Intention-to-treat analysis
PP analysis	Per protocol analysis
SVR	Sustained virological response
ETR	End of treatment response
PKR	Double stranded RNA-dependent protein kinase
TLR	Toll-like receptor
MyD88	Myeloid differentiation primary response gene 88

Introduction

Hepatitis C virus (HCV) is one of the major pathogens causing chronic hepatitis [1, 2] and eradication of the virus by the host occurs infrequently during the natural course of infection once it becomes chronic. Interferon (IFN) has been used widely as the most effective antiviral agent for chronic hepatitis C. Although ribavirin (RBV), a synthetic guanosine analog, alone does not decrease the serum HCV RNA level [3–5], it has been shown that combination therapy with IFN-alpha (given 3 times weekly) and daily RBV gives a higher sustained response rate than IFN monotherapy [6–8]. Pegylation is the process by which an inert molecule of polyethylene glycol (PEG) is covalently attached to a protein, and the addition of PEG to IFN produces a biologically active molecule with a longer half-life and more favorable pharmacokinetics than the natural molecule. These characteristics allow more convenient, once-weekly dosing [9]. Pegylated (PEG)-IFN plus RBV is significantly more effective than IFN plus RBV or PEG-IFN alone for the treatment of chronic hepatitis C, with sustained virological response rates of ~50% in patients infected with HCV genotype 1b [10].

We reported previously a close correlation between the number of mutations in the nonstructural 5A (NS5A) region of the HCV genome encoding amino acids (aa) at positions 2209–2248 [the IFN sensitivity determining region (ISDR)] and IFN efficacy in patients with HCV genotype 1b infection [11–13]. The aims of this study were to analyze clinical and virological factors associated with a higher rate of response by patients with HCV genotype 1b infection who were treated with combination therapy with pegylated-IFN-alpha 2b (PEG-IFN) plus RBV, and to clarify the relationship between ISDR mutations and virological response to the combination therapy.

Methods

Patients and methods

We analyzed, retrospectively, 239 patients with chronic HCV-1b infection who received combination therapy with PEG-IFN plus RBV between December 2004 and April 2008 at Tokyo Medical and Dental University Hospital (Tokyo, Japan) and associated hospitals participating in the Ochanomizu-Liver Conference Study Group. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by reverse transcription polymerase chain reaction (RT-PCR). Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age; gender; body mass index (BMI); previous IFN therapy; grade of inflammation and stage of fibrosis on liver biopsy; pretreatment biochemical parameters, such as hemoglobin, alanine transaminase (ALT) level, platelet count, low density lipoprotein (LDL) cholesterol, serum HCV RNA level (Log IU/ml); and the amino acid sequence of the IFN sensitivity determining region (aa 2209–2248, ISDR). Liver biopsy specimens were evaluated according to the grade of inflammation and the stage of fibrosis; this was done blindly by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of our hospital, and informed written consent was obtained from each patient.

Nucleotide sequencing of the *NS5A* gene

The serum samples were frozen at −80°C until use. Extraction of RNA from serum and RT-PCR were performed as described previously [14]. The PCR and sequencing primers were synthesized with a DNA synthesizer (model 391; Applied Biosystems Japan, Chiba, Japan).

To determine the nucleotide sequence of the *NS5A 2209–2248* region, we amplified nucleotides (nt) 7296–7320 of HCV complementary DNA by using the outer pair of primers [5′ outer primer, 5′-TGG ATG GAG TGC GGT TGC ACA GGT A-3′ (nt 6703–6727 of HC-J4); 3′ outer primer, 5′-TCT TTC TCC GTG GAG GTG GTA TTG C-3′ (nt 7296–7320)]. We transferred 1 μl of the first PCR product to the second PCR reaction along with the nested 5′ and 3′ primers [5′ inner primer, 5′-TGT AAA ACG ACG GCC AGT CAG GTA CGC TCC GGC GTG CA-3′ (nt 6722–6741), with the M13 forward primer sequence underlined; and 3′ inner primer, 5′-CAG GAA ACA GCT ATG ACC GGG GCC TTG GTA GGT GGC AA-3′ (nt 7275–7294), with the M13 reverse primer sequence underlined]. An M13 forward primer and an M13 reverse primer were attached to the 5′ terminal of the 5′ and 3′ inner primers, respectively, to facilitate direct sequencing with an automated DNA sequencer (model 373S; Applied Biosystems Japan).

Both strands of the PCR products were sequenced with the PRISM dye termination kit (Applied Biosystems Japan), according to the manufacturer's instructions. The sequencing primer was the M13 forward primer for the sense strand and the M13 reverse primer for the antisense strand. Deduced aa sequences of *NS5A* 2209–2248 were compared with the *NS5A* 2209–2248 sequences of HCV-J [15], which are prototypic sequences of HCV-1b. The results of the sequencing analysis were confirmed as consistent for each sample by repeating the experiment twice with different PCR products, to rule out the possibility of selection and amplification of minor NS5A quasi species variants in the low-titer specimens.

Nucleotide sequencing of the core gene

Substitutions of amino acids 70 and 91 in HCV-core region were determined according to core sequences obtained as described previously [16, 17]. The pattern of glutamine/histidine (mutant) at aa 70 and methionine (mutant) at aa 91 was evaluated as the double-mutant (dM) type, while the other patterns were non-double-mutant (non dM) type. Two patterns of mutants and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type (dW), while the other patterns were considered non-double-wild-type (non dW).

Study design and treatment regimens

Patients were treated with combination therapy with PEG-IFN (Peg-Intron; Schering-Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 μg/kg subcutaneously and RBV (Rebetol; Schering-Plough Nordic Biotech) (body weight [b.w.] < 60 kg, 600 mg po daily; b.w. 60–80 kg, 800 mg po daily; b.w. > 80 kg, 1000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 48 weeks. Treatment reduction was permitted, to escape side effects, but extended treatment of 72 weeks is not included in this analysis. Achieved rates of PEG-IFN and RBV administration were calculated as the percentage of the actual total dose administered of a standard total dose of 48 weeks according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, and 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy. Biochemical and hematological testing was done by a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4-weekly intervals, and after therapy at 4-weekly intervals for 24 weeks, by a quantitative PCR assay with a sensitivity of 100 copies/ml (National Genetics Institute, Los Angeles, CA, USA).

Outcomes

The primary end point was a sustained biochemical and virological response. Sustained virological response (SVR) was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were end-of-treatment virological responses (HCV RNA undetectable in serum). In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response were explored.

Statistical analysis

SPSS software package (SPSS 12J for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis, which was carried out using the χ^2 or Fisher's exact probability test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P* values were two-tailed and those lessthan 0.05 were considered statistically significant.

Results

Clinical characteristics and response to therapy

The clinical characteristics of the 239 patients are summarized in Table 1. On an intention-to-treat (ITT) analysis, serum HCV RNA levels were undetectable by the end of treatment in 172 of the 239 patients (72%) who were treated with PEG-IFN plus RBV, and among them, 98 of the 239 patients (41%) had an SVR (Table 2). The SVR rate decreased with drug discontinuation and dose

Table 1 Baseline characteristics of participating patients infected with HCV genotype 1b

Total number	239
Age (years)[a]	57 (21–78)
Gender (male/female)	142/97
Body mass index (kg/m^2)[a]	23.3 (15.3–31.0)
Previous interferon therapy (no/yes)	167/72
Histology at biopsy	
Grade of inflammation	
A0/1/2/3	3/65/102/10
Stage of fibrosis	
F0/1/2/3/4	4/73/57/37/9
Hemoglobin (g/dl)[b]	14.3 ± 1.3
ALT (IU/L)[b]	86 ± 67
Platelet count ($\times 10^3/\mu l$)[b]	160 ± 58
LDL cholesterol (mg/dl)[b]	74 ± 19
Serum HCV-RNA level (Log(IU/ml))[b, c]	6.1 ± 0.6
Type of mutations in the core (dM/non dM)	30/166
Type of mutations in the core (dW/non dW)	65/131
Type of ISDR sequence (0/1/2/3/4 or more)	126/45/11/5/18

HCV hepatitis C virus, *LDL* low density lipoprotein, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 2 Sustained response rates to treatment according to drug adherence

Characteristic	Number/total number (%)
Overall	
End of treatment	172/239 (72)
End of follow up	98/239 (41)
PEG-interferon-α2b adherence	
End of treatment	
>80%	131/154 (85)
60–80%	19/27 (70)
<60%	22/58 (38)
End of follow up	
>80%	86/154 (56)
60–80%	7/27 (26)
<60%	5/58 (9)
Ribavirin adherence	
End of treatment	
>80%	113/134 (84)
60–80%	37/46 (80)
<60%	22/59 (37)
End of follow up	
>80%	74/133 (56)
60–80%	18/47 (38)
<60%	6/59 (10)

PEG pegylated

reduction. The SVR rates of patients who received a total cumulative treatment dose of PEG-IFN of more than 80% were almost twice as high as the rates of patients who received less than 80% (56%, 26%, and 9% with >80%, 60%–80% and <60% of the PEG-IFN dose, $P < 0.001$). The SVR rates did not decrease with RBV reduction, as long as the cumulative treatment dose of RBV was more than 60%, but when the RBV reduction fell below 60%, the SVR rates were significantly lower (56%, 38%, and 10% with >80%, 60%–80%, and <60% of the RBV dose, $P < 0.001$).

Factors associated with sustained virological response

Seven parameters that influenced the SVR rate were identified by univariate analysis, including stage of fibrosis at liver biopsy, hemoglobin, platelet count, serum HCV RNA level, the type of ISDR sequence, and adherence to PEG-IFN plus RBV (Table 3). On the other hand, the SVR rate was not related to gender ($P = 0.07$), age or BMI. The amino acid substitution pattern was not significant in the overall analysis, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve SVR. As shown in Table 4, gender differences existed in the mutations in ISDR and core regions based on therapeutic responses. Because there were rather fewer female than male patients, the type of ISDR sequence did not significantly influence the SVR in females. We also analyzed types of mutations in the core, and the amino acid substitution pattern was not significant in the male patients, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve an SVR, as mentioned above. We also compared results between treatment-naïve patients and those who had failed previous IFN therapy (Table 5). As there were some differences in stage of fibrosis, platelet count, grade of inflammation, and gender in univariate analysis, treatment was comparably effective in both groups.

Finally we performed multivariate analysis in subjects with good drug adherence (Table 6), which identified only one parameter that influenced the SVR rate independently by variable selection: the number of mutations in the ISDR sequence (two or more: odds ratio [OR] = 5.181, $P < 0.05$). This regression model was always obtained regardless of the variable selection method used, including conditional parameter estimation, Wald statistic, and

Table 3 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on therapeutic response

	SVR (n = 98)	Non-SVR (n = 141)	P value
Age (years)[a]	56 (27–69)	58 (23–72)	NS
Gender (male/female)	65/33	77/64	0.070
Previous interferon therapy (no/yes)	68/30	99/42	NS
Grade of inflammation (A0–1/2–3)	31/50	37/62	NS
Stage of fibrosis (F0–2/3–4)	68/13	67/33	*0.009*
Body mass index (kg/m^2)[a]	23.3 (15.5-28.1)	23.3 (15.3-31.0)	NS
Pretreatment Hemoglobin (g/dl)[b]	14.6 ± 1.1	14.0 ± 1.4	*<0.001*
Pretreatment ALT (IU/ml)[b]	87 ± 68	86 ± 67	NS
Pretreatment platelet count ($\times 10^3$/μl)[b]	178 ± 63	148 ± 51	*<0.001*
Pretreatment LDL cholesterol (mg/dl)[b]	78 ± 21	72 ± 18	NS
Pretreatment serum HCV-RNA level (Log(IU/ml))[b, c]	5.9 ± 0.7	6.2 ± 0.4	*<0.001*
No. of mutations in the ISDR (0–1/2 or more)	66/23	105/11	*0.002*
Type of mutations in the core (dM/non dM)	9/76	21/90	NS
Type of mutations in the core (dW/non dW)	31/54	34/77	NS
PEG-interferon adherence (>80/60–80/<60%)	85/7/6	68/20/53	*<0.001*
Ribavirin adherence (>80/60–80/<60%)	72/19/7	60/28/53	*<0.001*

IFN interferon, *RBV* ribavirin, *SVR* sustained virological response, *NS* not significant, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$, *core* substitution of amino acids 70 and 91, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 4 Mutations in the ISDR and core regions analyzed separately for gender based on therapeutic response

	SVR (n = 98)	Non-SVR (n = 141)	P value
No. of mutations in the ISDR (0–1/2 or more)			
Male	36/21	56/8	*0.002*
Female	30/2	49/3	NS
Type of mutations in the core (dM/non dM)			
Male	8/46	11/48	NS
Female	1/30	10/42	*0.026*
Type of mutations in the core (dW/non dW)			
Male	18/36	16/43	NS
Female	13/18	18/34	NS

likelihood ratio statistic in combination with forward or backward variable selection methods.

Comparison of SVR rates according to the number of mutations in the ISDR sequence

We analyzed first the percentage of patients with more than two mutations in the ISDR among 762 patients who received IFN therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. The percentage of patients with more than two mutations in the ISDR was between about 20% and 30% for all ages (Fig. 1a).

Secondly, we analyzed responses to PEG-IFN plus RBV treatment and serum levels of HCV RNA in relation to the number of mutations in the ISDR. In Fig. 1b, patients with SVR are indicated by open circles and those with non-SVR, by closed circles. Although the rate of SVR tended to be higher in patients with increasing numbers of mutations in the ISDR, 5 patients with more than two mutations in the ISDR who experienced drug discontinuation and dose reduction resulted in non-SVR.

We confirmed changes over time in VR rates in patients treated with PEG-IFN plus RBV (Fig. 1c). Patients with more than two mutations in the ISDR are indicated in the figure by open circles and those with none or one mutation in the ISDR, by closed circles. The VR rates tended to be high early in the treatment in patients with more than two mutations in the ISDR.

Finally we compared the PEG-IFN plus RBV treatment efficacy in two groups, divided based on ISDR mutations. Patients with more than two mutations in the ISDR had a significantly higher tendency to achieve SVR in both ITT and per-protocol (PP) analyses ($P < 0.01$) (Fig. 1d), and

Table 5 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on previous interferon therapy

Previous interferon therapy	No (n = 167)	Yes (n = 72)	P value
Sustained response rates	68/167 (41)	30/72 (42)	NS
Age (<65/≥65)	127/40	57/15	NS
Gender (male/female)	93/74	49/23	0.074
Grade of inflammation (A0–1/2–3)	55/72	13/40	*0.018*
Stage of fibrosis (F0–2/3–4)	103/24	32/21	*0.003*
Pretreatment hemoglobin (<14.5/≥14.5)	93/74	41/31	NS
Pretreatment platelet count (<160/≥160 $\times 10^3$)	84/83	50/22	*0.006*
Pretreatment Serum HCV RNA level[a] (<6/≥6)	54/112	25/46	NS
No. of mutations in the ISDR (0–1/2 or more)	116/22	55/12	NS
PEG-interferon adherence (>80/60–80/<60%)	110/18/39	43/9/20	NS
Ribavirin adherence (>80/60–80/<60%)	97/30/40	35/17/20	NS

[a] Data are shown as Log(IU/ml)

Table 6 Multivariate analysis for the clinical and virological factors related to sustained response to PEG-IFN plus RBV therapy in 104 patients who were not intolerant to PEG-IFN plus RBV therapy

Factor	Category	Odds ratio (95% CI)	P value
(a) Five-factor model			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	4.486 (0.922–21.74)	0.063
Pretreatment Hemoglobin (g/dl)		1.250 (0.853–1.833)	NS
Pretreatment Serum HCV RNA level[a]		0.510 (0.224–1.159)	NS
Stage of fibrosis	F 0/1/2	1	
	F 3/4	0.460 (0.153–1.382)	NS
Pretreatment Platelet count ($\times 10^3/\mu l$)		1.022 (0.949–1.101)	
(b) Step-wise variable selection			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	5.181 (1.129–23.81)	*0.034*

CI confidence interval, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209–2248}$

[a] Data are shown as Log(IU/ml)

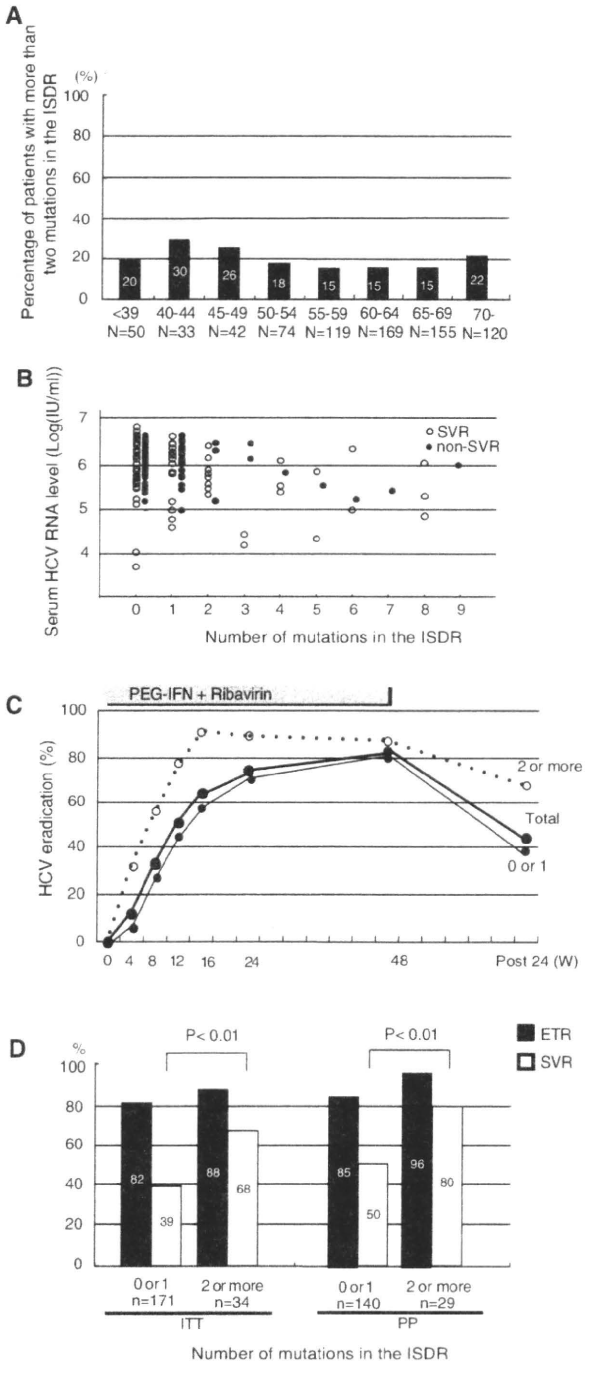
the SVR rates of the patients with good drug adherence was 80%.

Side effects

Side effects leading to treatment discontinuation occurred in 53 patients (22%). Overall, 109 patients (46%) required reduction of the dose of one or both drugs during the treatment regimens (23% required PEG-IFN reduction and 35% required RBV reduction). The most common events leading to drug withdrawal were general fatigue and appetite loss (n = 15), hematologic abnormalities (n = 6), dermatological symptoms (n = 5), retinopathy (n = 5), neuro-psychiatric events (n = 4), and interstitial pneumonia, including severe cough (n = 4).

Discussion

Although the relationship between ISDR mutations and the clinical efficacy of IFN has been conflicting in Western countries [18–24], our results support previous studies reporting a close correlation between the number of mutations in the ISDR and IFN efficacy in patients with chronic HCV-1b infection [11–13]. Because most patients with 4 or more mutations in the ISDR (hereafter classified as the mutant type) experienced SVR with conventional IFN monotherapy, we reported previously that the number of amino acid substitutions in the ISDR was an independent predictor of the response to IFN therapy [12]. In the present study, we demonstrate that ISDR mutations are the most effective predictors of treatment outcome of 48-week



◀ **Fig. 1** **a** The percentages of patients with more than two mutations in the interferon sensitivity determining region in NS5A $_{2209-2248}$ (*ISDR*), according to age (*horizontal axis*) among 762 patients who received interferon (IFN) therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. **b** Responses to pegylated (PEG)-IFN plus ribavirin (RBV) treatment and serum levels of hepatitis C virus (HCV) RNA in relation to the number of mutations in the ISDR. Patients with sustained virological response (*SVR*) are indicated by *open circles* and those with non-SVR by *closed circles*. **c** Changes over time in VR rates in patients treated with PEG-IFN plus RBV. Patients with more than two mutations in the ISDR are indicated by *open circles* and those with no or one mutation in the ISDR by *closed circles*, *W* weeks. **d** PEG-IFN plus RBV treatment efficacy divided into two groups based on ISDR mutations. End-of-treatment response (*ETR*) and SVR are shown in both intention-to-treat (ITT) analysis (*left*) and per-protocol (PP) analysis (*right*)

PEG-IFN plus RBV therapy in patients with HCV genotype 1binfection.

In the present study, the SVR rate was not related to gender, age, or previous IFN therapy by univariate analysis. First of all, in regard to gender ($P = 0.07$), as male patients had a higher tendency to achieve SVR than female patients, further validation in larger-scale studies is required to clarify the significance of gender. Secondly, in regard to age, there was no relation to SVR in overall analysis with continuous variables, but younger patients, aged less than 65 years, had a higher rate of response than those aged more than 65 years ($P < 0.05$, data not shown). Actually there are some reports suggesting the relationship of age and SVR [25, 26]. Finally, in regard to previous IFN therapy, as shown in Table 5, treatment was comparably effective in both groups; previous IFN therapy did not affect the SVR rate. The reasons for equivalent response rates in subjects with prior IFN history, which was not expected, are unclear. In our study, the group with prior IFN history had more advanced liver fibrosis and a low platelet count, and stage of fibrosis was one of the factors extracted by univariate analysis as a useful pretreatment marker predicting SVR. We also analyzed the other three parameters extracted by univariate analysis. Although there was no difference in pretreatment hemoglobin, or number of ISDR mutations, the group with prior IFN history tended to have a low serum HCV-RNA level. Further, the group with prior IFN history had a high proportion of male patients. Although the SVR rate was not related to gender, male subjects had a higher tendency to achieve SVR than female subjects.

In our present study, the SVR rate was not related to core mutations. As described in previous reports [17, 27, 28], amino acid substitutions in the core region are regarded as predictors of response to PEG-IFN plus RBV therapy in Japanese patients infected with HCV genotype 1b. In the present study, the SVR rate was not related to the pattern of amino acid substitution in the overall analysis. The reasons for these discrepant results are unclear, but females with dual substitutions at amino acids 70 and 91 had a lower tendency to achieve SVR. Further studies are necessary to clarify the mechanism of action for amino acid substitutions in the core region of HCV.

Recent studies suggest that the mutations in the ISDR are associated with response to combination therapy with IFN and RBV [29–32]. Most recently, it has been reported

that amino acid substitutions in the core and mutations in the ISDR are predictive of virological response to the combination therapy in patients with HCV genotype 1b and a high viral load [28]. There are some reports suggesting that the mutations in the ISDR may not serve as a predictor for treatment outcome [33, 34], but as the numbers of subjects in these studies were around 30, a number which is not sufficient to evaluate the results, this factor may explain these discrepant results.

The mechanisms of IFN sensitivity in relation to the sequence of the HCV $NS5A_{2209-2248}$ region are not clear. However the "mutant-type" ISDR correlates with a low viral load, as reported previously [12, 35, 36]; most patients in the present study with two or more mutations in the ISDR had high levels of virus. Furthermore, stepwise multiple logistic regression analysis of the factors, including substitution of the ISDR and the viral load, revealed that both of them were independent predictive variables of SVR, and the odds ratio of the number of mutations in the ISDR was the highest in the pretreatment factors associated with SVR by multivariate analysis. The precise mechanism involved must be elucidated in further in vitro studies.

There have been several reports that suggest biological roles of the ISDR in the response to IFN and in HCV infection. Double-stranded RNA-dependent protein kinase (PKR) is a critical component of the cellular antiviral responses induced by IFN. Gale et al. [37, 38] have reported that mutations within the PKR-binding region of NS5A, including ISDR, can disrupt the NS5A–PKR interaction, possibly rendering HCV sensitive to the antiviral effects of IFN. Toll-like receptor (TLR) has also been reported to play various roles in many viral infections, and it has been reported that NS5A bound MyD88, a major adaptor molecule of TLR-mediated signaling, and inhibited the TLR–MyD88 signaling pathway by a direct interaction with the death domain of MyD88 through the ISDR [39]. Furthermore, it has been reported that the lipid droplet is an important organelle for HCV production, and NS5A is a key protein that recruits replication complexes to lipid droplets for the production of infectious viral particles [40]. While the mechanism of action of the ISDR in the response to IFN or viral replication remains to be proven, these findings suggest new aspects of HCV infections.

In our previous report [12], patients with 4 or more mutations in the ISDR experienced SVR with conventional IFN monotherapy, but in more effective therapy with PEG-IFN plus RBV combination therapy, the number of mutations as a predictor of SVR decreased from 4 to 2. Watanabe et al. [41] have also reported that the number and position of mutations in the ISDR correlated with IFN efficacy in HCV-1b infection. Moreover, it has been reported that patients with viruses mutated at positions 2209, 2216, or 2227 more frequently experienced SVR than did those without these mutations. Another group has also reported regarding statistical analysis, using a database of 675 individual ISDR sequences in HCV-NS5A and the IFN response [42]. They have shown that IFN-sensitive viruses contain a larger and more diverse collection of substitutions than IFN-resistant viruses. While it remains unknown how the numbers of mutations are involved in the biological role of ISDR, or which sites of mutation and changes of amino acid are also important for the response to IFN-based treatment, it is thought that the functional importance of numbers or sites of mutations can be explained in terms of interaction between NS5A and some target molecules such as PKR, MyD88, and lipid droplets.

In vitro studies have shown that the introduction of NS5A mutations enables an HCV replicon to replicate efficiently [10, 43, 44]. In our previous report, site-specific mutation of the ISDR also modulated HCV replication [45]. The ISDR was identified originally as the site that determines the sensitivity of HCV to IFN [12]. This indicates that the ISDR mutations are not lethal in vivo. Furthermore, mutations in the ISDR are closely associated clinically with decreased serum HCV RNA levels [42], whereas ISDR mutations in the HCV replicon enhance replication. While the explanation for this paradox has not become clear, a big difference between the environment of cultured cells and that in the human liver is thought contribute to this phenomenon.

We found that the percentage of patients with more than two mutations in the ISDR was between 20% and 30% for all ages; thus, around one-fifth of patients are thought likely to experience SVR. Indeed, the SVR rate among patients with two or more mutations in the ISDR sequence was 68% (ITT) and 80% (PP) compared to 39% (ITT) and 50% (PP) among those patients with no or one mutation in the present study. Furthermore, predictive factors such as serum HCV RNA level, stage of fibrosis, and hemoglobin also aid in the assessments of treatment, and we can use these parameters to develop a treatment strategy.

Several prospective randomized trials have shown that 72-week extended therapy improves SVR by 7.5%–12% in late viral responders [46, 47]. One cohort study showed that 72-week treatment for late viral responders achieved an even higher SVR, of 67.1%, which was 21% higher than the SVR achieved with 48-week treatment [48]. These reports demonstrate that tailoring of treatment duration by on-treatment viral response can further improve the outcomes of antiviral therapy. In our 48-week based treatment, 90% of patients with more than 2 ISDR mutations cleared the virus within 12 weeks of treatment (early viral response; EVR) and consequently achieved 30% higher SVR than those with 1 or no ISDR mutation. These results