しても，感染レセプター，感染系，cccDNA (covalently closed circular DNA)生合成とその維持機構，動態，病態との関連，HBVポリメラーゼのアッセイ系，詳細な複製メカニズムなど，多くの謎が残されたままである．

HBV感染レセプターの分離・同定は，初代培養アヒル肝細胞(primary duck hepatocyte = PDH)を用いたDHBVを中心に進められた．HBV吸着因子を含め，候補にあげられた因子は，重合アルブミンレセプター[5]から始まって，gp120[6]，gp180[7]などいくつかあるが，繰り返し述べたように感染系の構築には至っていない．これ程までに困難なHBV感染レセプターの分離・同定に向けてどのようなアプローチが必要なのか，遺伝子挿入HBVの作製とそれを用いたレセプターの同定戦略を考案した．

## 2 *In vitro* HBV感染系

前述のごとく，簡便な特に*in vitro*感染系が存在しないことが，HBV感染レセプターの分離・同定にとって決定的な障壁となっていることはいうまでもないが，*in vitro* HBV感染系(初代培養肝細胞を除く)構築の努力は続けられている．当然のことながら，HBVは正常肝実質細胞へ高親和性を示し，増殖能を発揮することから，肝実質細胞由来と考えられるHepG2，HuH7などのヒト肝癌培養細胞株が感染系として試されてきた．これらの細胞はHBVの複製サイクルと考慮して構築したHBV発現ベクターをトランスフェクションすることにより，感染性粒子を産生することがわかっている[8,9]．生体においてどのような形質をもつ肝実質細胞に感染するのか全く検討もつかないが，一般的に正常肝細胞は高分化状態にあると考えられるため，これらの細胞をステロイド，インスリン，dimethyl sulfoxide (DMSO)を用いて分化誘導し，感染実験を試みたという報告がある[10]．またこれらの細胞を用いて，肝臓という臓器状態をできるだけ再現する目的で高密度三次元培養によるHBV感染系の構築の試みもあるが，感染効率，コストパフォーマンスを考えるとHBV感染系としての価値は評価するレベルにないといわざるを得ない．

一般培養細胞系感染系としては，最近(といっても，すでに10年も経過する)樹立されたHCV感染肝癌から樹立されたHepaRGを用いたHBV感染系が唯一であると思われる[11]．この細胞を2～4% DMSOで数週間分化誘導すると20%程度のHBV感染効率が得られるとされる．しかしながら，一般的な培養肝癌細胞ながら商業用で直径約10 cmのディッシュで10万円強もする高価な細胞であり，とても日常的に使用できる細胞ではない．cccDNA形成など，本来の感染サイクルが進行していると思われるが，Dane様粒子の存在が示されている一方，感染性粒子を排出できないなどの問題点もある[12]．

HBV感染レセプターの分離・同定から，感染系の構築を目指す場合にこのHepaRGの有用性が議論されている[13]．最近の報告[14]では，小管構造の形成に関わる肝細胞の側底側細胞膜(basolateral membrane)側からHBVは感染する．初代培養ヒト肝細胞(primary human hepatocyte = PHH)では接着細胞の表面側一面に小管構造マーカーであるmultidrug resistance protein 2 (MRP2)の発現がみられ，8000 multiplicity of genome equivalent (mge)以上でほぼ100%のHBV感染性がみられるのに対し，DMSO分化誘導HepaRGでは，限られた集属する領域にMRP2の発現がみられ，同じmgeを用いた感染実験でその集団の周辺部にHBV感染が観

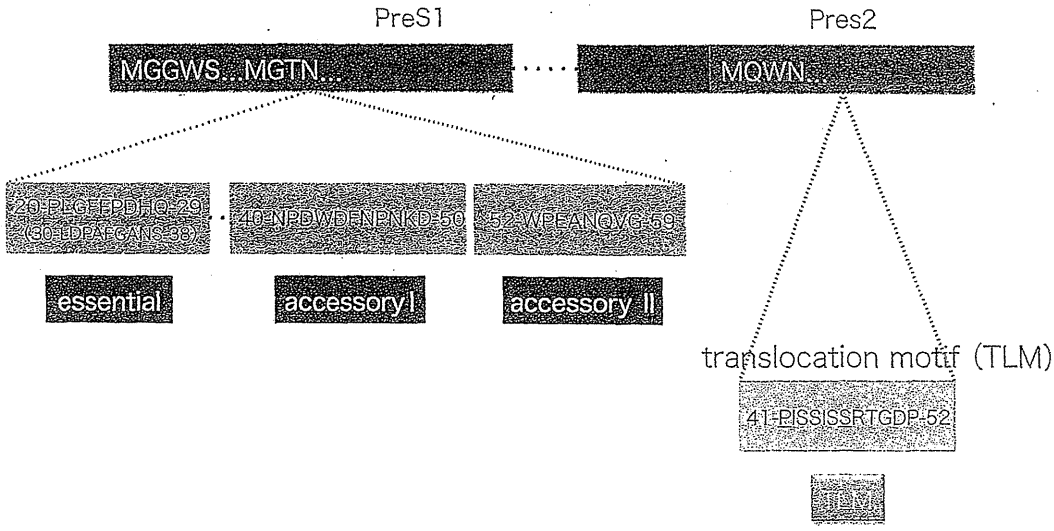


図2　preSの機能配列
preS1内の付着・侵入に必須な機能配列(20～29aa)，付随的機能配列(accessory IおよびII)とpreS2内の侵入過程で重要なTLMを示す．

察されることや，この現象がEGTAで促進される，集団の内部へも感染が確認できるようになる，ことから導き出された．本知見は生体における肝実質細胞の形質は，培養肝癌細胞株に比べ著しい高分化状態にあり，培養肝癌細胞では容易には達成し難い形質を有することを想像させる．この高分化状態がHBV感染レセプターの発現に関わるのか，活性に関わるのか定かではないにせよ，興味深い問題である．

## 3 付着・融合・侵入に関わるHBV因子

培養肝癌細胞のひとつ，HepG2細胞のHBV感染効率はないに等しいと考える研究者は多いと思われるが，当初はこの細胞を用いたHBV感染粒子の付着機構に関する研究は多々報告された[15,16]．最近ではD型肝炎ウイルス(HDV)なども利用してHepaRG細胞を用いた研究が多い[17]．HBVの感染性粒子の形成には所謂small S（SS若しくは単にHBs)膜蛋白とmiddle S（MS)膜蛋白に加え，large S（LS)膜蛋白が必須の因子となるが，感染性HBV粒子の細胞への付着に関して，特にpreS1領域の機能が重要視されている[18]．preS1領域は部分的にアミノ酸配列の相同性の低いところがあるが，おおむね高い相同性があると考えて良く，付着・膜融合に関わる必須な領域，2つの付随的な領域が存在すると考えられている．preS2領域も相同性は高く，細胞内侵入に関わると思われる領域(translocation motif＝TLM)が想定されている．またSS膜蛋白N末にも膜融合に関わる領域が予測されている．

以上のような情報をもとにして，preS1内の必須領域を介して，肝細胞膜表面因子に結合(付着)し，レセプター分子集合・構造変化，SS膜蛋白N末を加えたHBV粒子膜-細胞膜融合が起こり，TLMを介した細胞内侵入といったHBVの付着・膜融合・侵入機構が想像される．

## 6 HBV感染レセプターの分離・同定

さて，HBV感染レセプターの分離・同定は倫理的な制約も絡んで，PDH-DHBV系を中心に行われてきた．DHBVにはHBVのMSに相当する膜蛋白はなく，すなわちpreS2に

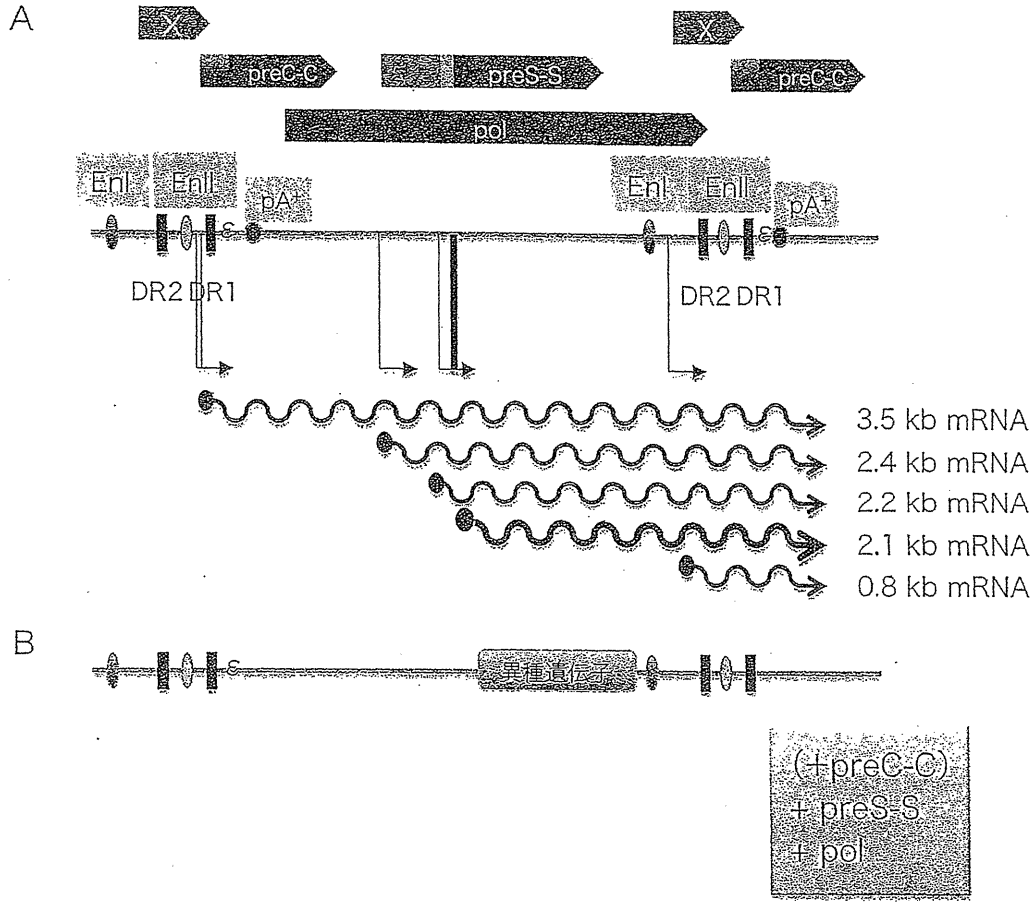


図3　HBVベクター型遺伝子挿入HBVデザイン

A：HBVゲノムを直鎖状に描いている．上段のボックス矢印はORFを示す．pgRNAを発現するための必須基本ユニットを示した．En：エンハンサー．pA：ポリA付加シグナル．鍵矢印は各転写産物の開始部位，波線は転写産物を示す．

B：SSの位置に異種遺伝子を挿入したデザインを示した．この場合，挿入により，preC-C遺伝子，X遺伝子を除くそのほかのHBV関連遺伝子はすべて破壊されるため，トランスに供給する必要がある．挿入部位によってはpreC-Cの供給も必要となる

該当する領域は存在しない．LS内のpreS領域に付着・膜融合に関わる必須な領域，2つのTLMに相当する領域が同定されている．

DHBVで同定された最も有力なDHBV付着因子はgp180（carboxypeptidase D）であった[19]．本因子は膜表面をビオチン化したPDHをDHBVと付着させた後，抗DHBV膜蛋白抗体を用いて免疫沈降することによって分離・同定されたものである．本因子の同定は，HBVの付着・膜融合・侵入に関わるHBV感染レセプターの本質にも迫る因子として期待されたが，LMH細胞（chicken由来の培養肝癌細胞株でDHBV感染を許容しない）をDHBV感染許容細胞へ変化させることはできなかったし，またgp180に対する抗体もアヒル初代培養肝細胞へのDHBV感染を阻止するに至らなかった．

付着因子の存在が報告されているHepG2細胞からもHBV感染が許容される分化誘導したHepaRG細胞からも，依然としてHBV感染レセプターの本体に迫る因子の分離・同定はされていない．現時点における生化学，分子生物学などの技術を駆使しても，確固としたHBV膜蛋白結合因子すらこれらの細胞

から同定されないのはなぜであろうか？ーますますHBV感染レセプターの謎は深まり，逆に興味深くもあるが，HBV感染レセプターの分離・同定には相当の工夫と努力とを余儀なくされると思われる．

## 7 遺伝子挿入HBVの作製

これまでの経緯から，HBV感染レセプターの分離・同定へ向けた新たな局面を拓くために遺伝子挿入HBV-いわば，組換え型HBV或はpseudotype HBV作製を試みている．この2つの遺伝子挿入HBVは，前者のごとくHBVの複製機構を理解したHBVベクター型か，膜粒子のみをHBV型に変えたpseudotype HBV型に分けられる．本アプローチはこれまでに試みられなかったHBV感染性粒子を基盤にした感染性を指標にするアッセイ系の構築を可能にする．

### 1. HBVベクター型

本ベクターの構築には，HBVの複製過程を正確に理解する必要がある．HBVは，部分二重鎖DNAという独特のゲノム構造をもつことは勿論，さらに蛋白プライミングによって逆転写複製を行うという極めて特徴的な複製サイクルをもつ[20]．

HBVは感染成立後，部分二重鎖DNAを修復し，いわゆるcccDNAというエピゲノムを形成する．本ゲノムは肝実質細胞核内にあって，転写許容構造体として逆転写の鋳型となる3.5 kbプレゲノムRNAをはじめ，いくつかのHBV関連転写産物産生の鋳型として機能する．3.5 kbプレゲノムRNAはpreC翻訳領域内でDR1（direct repeat 1）を含む形で転写され，3'側にDR2，さらに5'のDR1を含んだやや下流でpolyA付加シグナルが認識されて，すなわち5'と3'が重複される形で集結する．

本稿ではHBV複製サイクルの詳細については省略するが，このベクター構築上最低限必要なエレメントとして，プレゲノムRNA転写開始部位を含めた転写開始装置，5' DR1，ε，DR2，3' DR1配列の基本情報を残しておく必要があり，またどの転写unitを使うか考慮する必要がある（図3A, B）．ウイルス蛋白因子として，実際の逆転写過程から部分二重鎖DNA合成に関わるHBVポリメラーゼ（HBVpol），コア粒子を供給するHBVコア蛋白（HBV-C），膜粒子を構成するpreS-S蛋白（HBV preS-S）を適宜トランスに供給する必要がある（図3B）．またゲノムサイズも厳密に影響すると思われるし，プレゲノムRNAの転写効率を上げるために適宜異種遺伝子エンハンサーを考慮する必要があるかもしれない．

このタイプの遺伝子挿入型HBVはHBVの生活環を踏襲するので，理論上は感染後速やかにcccDNAが形成され遺伝子発現が可能となると予測される．蛍光蛋白遺伝子などで感染をモニターすることにより，HBV感染レセプターの遺伝子の分離・同定に役立てることが可能である．ただし，HBV cccDNAにはγヘルペスウイルスにみられる潜伏感染複製オリジン（ori-P）は存在せず，細胞分裂の際に複製・分配・維持されることなくしだいに希釈されていくと想定される．

私どもは肝実質細胞を標的とした遺伝子治療を達成する目論見で，約20年前に本タイプの遺伝子挿入HBVベクターの開発に挑んだ経緯がある．その後Schallarらを中心として本タイプの遺伝子挿入HBVベクターが作製可能であることが示されている[21]．

### 2. HBV pseudotype particles（HBVpp）型

このタイプの遺伝子挿入型HBVは，VSV-Gを使ったpantropic retrovirus，あるいはlenti-

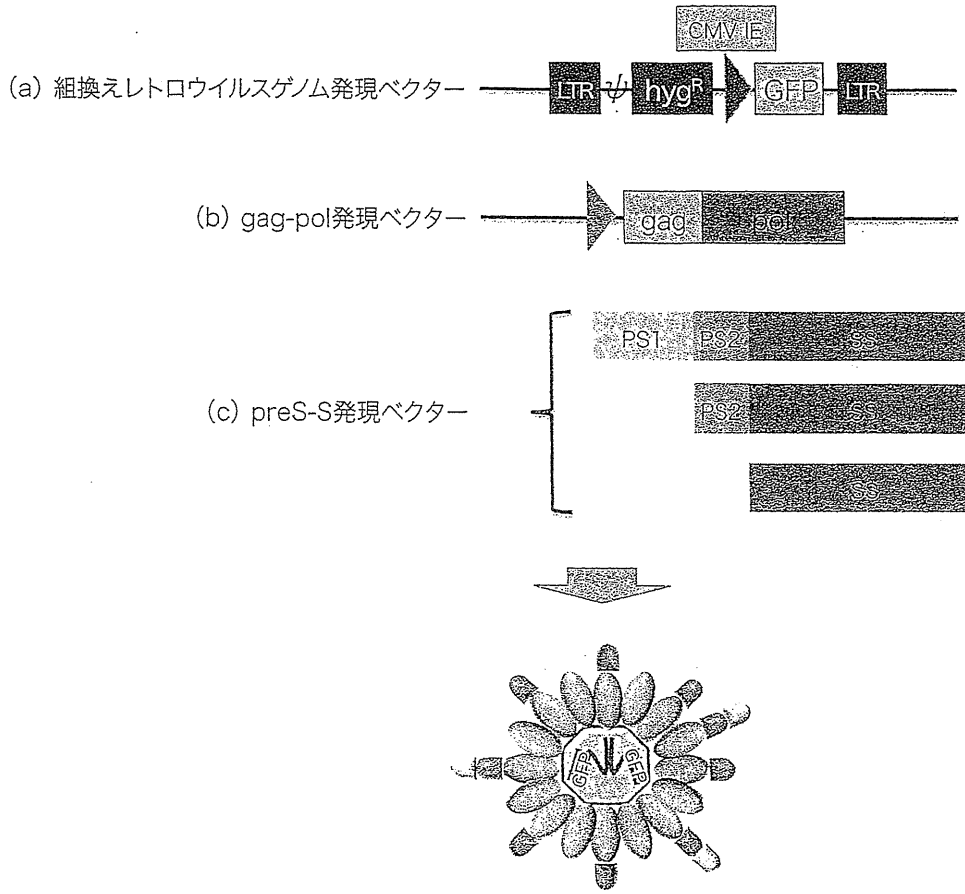


図4　HBVpp型のデザイン
このタイプではレトロウイルスのRNAゲノムを供給する発現ベクター（a），コア粒子と逆転写過程からプロウイルス合成に必要なpolを供給するgag-pol発現ベクター，HBV膜粒子を供給するHBVの3つの膜蛋白を供給する発現ベクターが必要となる．(b)にHIV型を用い，(a)にHIV（レンチウイルス）型のゲノムを供するrev responsive element（RRE）などの必要なエレメントを携えておけば，レンチウイルス型のレトロウイルスベクターとなる．(a)あるいは(b)をあらかじめ安定型培養細胞株として樹立してパッケージング細胞として使用することも可能である．LTR: long terminal repeat. $hyg^R$: hygromycine resistance gene. CMV IE: cytomegalovirus immediate early enhancer-promoter. GFP: green fluorescent protein. ψ：レトロウイルスパッケージングシグナル．▷：エンハンサー-プロモーター．

virusにおいてVSV-GをHBV膜蛋白に変えたpseudo-HBVである．HCVでもpseudotype particles（HCVpp）として試みられている[22]．HBVppは付着・侵入はHBVの過程を辿り，それ以降の過程はレトロウイルスの生活環に従う．最終的には逆転写過程を経て，プロウイルスとして安定的に感染宿主細胞ゲノムに組み込まれるので，培養細胞など，継代が基本的に可能な細胞を用いた場合，ウイルス遺伝子として蛍光蛋白遺伝子や薬剤耐性遺伝子を挿入しておけば，感染細胞はクローン化できることになる．

問題となるのは，通常のレトロウイルスベクターを用いた場合，感染後，プロウイルスを形成し，安定的な挿入遺伝子の発現まで数日から1週間程度の時間を要する点である．この意味でlentivirus型のレトロウイルスをベースにしたHBVppが望ましいかもしれない．

私どもの研究室では，肝実質細胞のcDNA

ライブラリーを培養肝癌細胞へ導入後，HBVppの感染性を指標にしたHBV感染レセプターの分離・同定を試みる戦略で通レトロウイルス型HBVppの構築を独自に試みた．まずレトロウイルスのgag-pol遺伝子を発現する細胞にEGFP（EGFP）とハイグロマイシン耐性遺伝子（HygR）を挿入したレトロウイルスベクターゲノムを組込んだパッケージング細胞を作製し，この細胞に3つのHBV膜蛋白を発現させることで培養上清中にHBV膜蛋白を被ったウイルス粒子が産生されるかどうかを検討した．抗HBs抗体による免疫沈降，ウイルスゲノム抽出，RT-PCRで免疫沈降したサンプルに挿入したEGFP遺伝子が確認された．セシウム密度勾配超遠心法でもHBV粒子密度に近い1.22 g/m*l*近傍に粒子が集積することや，電顕によっても粒子形成されていることが確認された[23]．なお，本HBVppの感染能についていくつかの知見を得ているが本稿では差し控えたい．

## 8 おわりに

以上，遺伝子挿入HBVベクターについて概説した．HBVベクタータイプもHBVppタイプも作製可能であり，それぞれの利点を活かしたHBV感染レセプターの分離・同定における利用価値は高いと思われる．またこれら2つの遺伝子挿入HBVベクターは感染性を指標にしたアッセイ系を組み立てることにより，HBV感染レセプター分離・同定後の*in vitro*，および*in vivo*におけるHBV生活環や病態解析解明，治療法の開発とその評価系の構築に有用性は極めて高いものと思われる．

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# Comparison of the Efficacy of Ribavirin Plus Peginterferon Alfa-2b for Chronic Hepatitis C Infection in Patients With and Without Coagulation Disorders

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Many patients with coagulation disorders are infected with hepatitis C virus (HCV) that advances to end stage liver disease, resulting in an increased number of deaths. The efficacy of ribavirin and peginterferon combination therapy for chronic HCV infection in patients with coagulation disorders has not been clarified fully. The aim of this study was to evaluate the efficacy and tolerability of combination therapy in this patient population compared with patients who are infected with HCV and do not have coagulation disorders. A total of 226 consecutive chronic hepatitis C patients were treated with combination therapy and divided into two groups: patients with (n = 23) and without coagulation disorders (n = 203). Clinical characteristics, sustained virological response rates obtained by an intention-to-treat analysis, and combination therapy discontinuation rates were compared between the two groups. The sustained virological response rates did not differ significantly between patients with and without coagulation disorders (65.2% vs. 47.8% by intention-to-treat analysis). According to a multivariate analysis, age, alanine aminotransferase, gamma-glutamyltransferase, and HCV genotype were associated significantly with a sustained virological response, whereas whether a patient had a coagulation disorder did not affect the sustained virological response. In conclusion, combination therapy for chronic hepatitis C was comparably effective between patients with and without coagulation disorders and did not result in adverse bleeding. ***J. Med. Virol. 85:228–234, 2013.*** © 2012 Wiley Periodicals, Inc.



**KEY WORDS:** chronic hepatitis C; interferon; ribavirin; coagulation disorders; hemophilia

## INTRODUCTION

Hepatitis C virus (HCV) infection is a widespread viral infection that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Until the 1980s, most patients with coagulation disorders became infected with HCV because of the extensive use of untreated factor concentrate. Some of these patients were infected with both hepatitis C and human immunodeficiency virus (HIV) [Brettler et al., 1990; Troisi et al., 1993; Yee et al., 2000; Franchini et al., 2001]. These patients with liver diseases and persistent abnormal transaminase progress to end stage liver disease, resulting in an increased number of liver disease-related deaths. In cases of co-infection with the HIV, the progression of liver disease is more rapid [Sanchez-Quijano et al., 1995; Soto et al., 1997; Benhamou et al., 1999; Ragni and Belle, 2001; De Luca et al., 2002] with a higher mortality rate than

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during HCV monoinfection [Darby et al., 1997; Yee et al., 2000]. The need for treating infection with HCV in patients with coagulation disorders is increasing worldwide.

Sustained virological responders who are negative for serum HCV RNA 6 months after the end of treatment with interferon (IFN) are likely to remain in virological and biochemical remission with histologic improvement [Marcellin et al., 1997; Shiratori et al., 2000]. In addition, IFN therapy reduces the risk of hepatocellular carcinoma among virological or biochemical responders [Imai et al., 1998; Ikeda et al., 1999; Yoshida et al., 1999]. Ribavirin is now used generally in combination with IFN or pegIFN to treat chronic hepatitis C and combination therapy is more effective than IFN monotherapy [Lai et al., 1996; McHutchison et al., 1998; Poynard et al., 1998; Manns et al., 2001].

Previous studies have investigated the efficacy of IFN monotherapy in patients with coagulation disorders and chronic hepatitis C [Makris et al., 1991], and the efficacy of combination therapy with ribavirin and PegIFN in patients with coagulation disorders [Fried et al., 2002a; Mancuso et al., 2006; Posthouwer et al., 2007]. However, there are no reported comparisons of this combination therapy between patients infected with HCV with and without coagulation disorders. In this study, the efficacy and tolerability of ribavirin plus pegIFN were evaluated retrospectively in patients with coagulation disorders and chronic hepatitis C and the results were compared with the responses of patients infected with HCV but without coagulation disorders.

## MATERIALS AND METHODS

### Patients and Methods

A total of 226 consecutive patients with chronic hepatitis C and a high viral load (serum HCV RNA levels greater than 100 kilo-international units [KIU]) were treated with a combination of pegIFN and ribavirin between December 2004 and March 2007 at Nagoya University Hospital and Ogaki Municipal Hospital. These patients included 23 patients with coagulation disorders (17 with hemophilia A, 4 with hemophilia B, and 2 with von Willebrand disease). All patients were under 75 years old, were anti-HCV antibody-positive, and had serum HCV RNA levels greater than 100 KIU/ml by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0; Roche Molecular Systems, Pleasanton, CA) within 12 weeks preceding the therapeutic period. Patients were excluded if they had pretreatment hemoglobin (Hb) levels <10 g/dl, tested positive for serum hepatitis B surface antigen, a history of drug addiction, alcohol abuse, autoimmune hepatitis, primary biliary cirrhosis, a serious psychiatric or medical illness, or were pregnant. To exclude patient bias, only complete cohorts from each hospital were enrolled. HCV genotypes were determined by PCR using genotype-specific primers [Okamoto et al., 1994; Simmonds et al., 1994].

All patients were treated with 1.5 μg/kg of pegIFN α-2b (Peg-Intron®, MSD, Tokyo, Japan) once weekly for 24 weeks in patients infected with HCV genotype 2 or 3 and for 48 weeks in patients infected with HCV genotype 1 or 4. For the 17 patients infected with HCV genotype 1, the treatment duration was extended to 72 weeks because of higher efficacy compared to that obtained after 48 weeks of treatment, but only in cases in which HCV RNA was positive at 12 weeks and negative at 24 weeks from the start of therapy. Treatment was discontinued when a patient's Hb concentration fell below 8.5 g/dl because of drug-induced hemolytic anemia or when a patient's white blood cell count fell below 1,000/mm$^3$, neutrophil count fell below 500/mm$^3$, or platelet count fell below 50,000/mm$^3$. Some patients discontinued treatment because the virus could not be eradicated after 24 weeks, as determined by the physician. The pegIFN alfa-2b dose was reduced to 50% of the assigned dose when the white blood cell count was below 1,500/mm$^3$, the neutrophil count below 750/mm$^3$ or the platelet count below 8,000/mm$^3$. Oral ribavirin (Rebetol®, MSD, Tokyo, Japan) was administered for the same duration as pegIFN at 600 mg/day for patients who weighed 60 kg or less, 800 mg/day for those who weighed more than 60 kg but less than 80 kg, and 1,000 mg/day for those who weighed more than 80 kg during the treatment period. The ribavirin dose was reduced by 200 mg/day when the patient's Hb concentration fell below 10 g/dl because of drug-associated hemolytic anemia. Ribavirin was discontinued when pegIFN therapy was discontinued. Informed consent was obtained from each patient and the study was performed in accordance with the 1975 Declaration of Helsinki.

### Liver Histology

Pretreatment liver biopsy specimens were classified based on a fibrosis scale of F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) and in terms of necroinflammatory activity on a scale of A0 to A3 (A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity) [Bedossa and Poynard, 1996; Fried et al., 2002b]. In patients with coagulation disorders, a liver biopsy was performed using factor concentrate, provided the patients gave informed consent.

### Assessment of Efficacy

The virological response was assessed by a qualitative HCV RNA assay with a lower sensitivity limit of 100 copies/ml (Amplicor HCV version 2.0; Roche Molecular Systems). According to the qualitative HCV RNA results, responses were defined as a sustained virological response if no HCV RNA was detected at the end of the 24-week follow-up period after the treatment was completed. A patient was considered to have an end of treatment virological response if no HCV RNA was detected at the end of treatment.

### Comparison of Characteristics and Treatment Efficacy Between Patients With and Without Coagulation Disorders

Sex ratio, age, body weight, body mass index (BMI), baseline serum alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT), pretreatment Hb level, platelet counts, HCV genotype and viral load, histologic activity, and fibrosis were compared between patients with and without coagulation disorders. The sustained virological response rates obtained by an intention-to-treat analysis and per-protocol analysis, ribavirin and pegIFN dose reduction rates, and combination therapy discontinuation rates were compared between the two groups. The end of treatment virological response rate was obtained by intention-to-treat and per-protocol analyses and then compared between the two groups. Next, the variable accession method in a multivariate analysis was used to examine factors associated with a sustained virological response after combination therapy, including the following factors: sex, age, BMI, baseline serum ALT, GGT, platelet counts, genotype, HCV RNA concentration, and presence of a coagulation disorder.

Because efficacy differed by the HCV genotype and the patient age, and since all coagulation disorder patients were male, the analysis focused on male, age-matched patients infected with HCV genotype 1. The characteristics and efficacy of treatment were compared in males, and age-matched patients with and without coagulation disorders who were infected with HCV-genotype 1.

### Statistical Analysis

Values are expressed as the means ± SDs. Between-group differences in mean quantitative values were analyzed by Student's *t*-test, and differences in nonparametric data were analyzed by the Mann–Whitney *U*-test. Differences in proportions were examined by the Chi-squared test. Multiple logistic regression analysis was used to identify factors related to a sustained virological response. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC). All $P$ values were two-tailed, and $P < 0.05$ was considered statistically significant.

## RESULTS

### Patient Characteristics

The patients included 127 men and 99 women aged 22–74 years (mean ± SD, 54.7 ± 11.6). The mean age of patients without coagulation disorders was 56.3 ± 10.9 years and most patients were in their 50s and 60s. In contrast, the mean age of patients with coagulation disorders was 41.5 ± 9.8 years with an age distribution ranging from 20 to 50 years. The clinical characteristics of the two study groups are shown in Table I. All patients with coagulation disorders in this study were male because of inherited, sex-linked hemophilia, and two patients in this study had male von Willebrand disease. Patients with coagulation disorders were significantly younger than patients without coagulation disorders ($P < 0.0001$). Although body weight was not different between the two groups, patients with coagulation disorders had a significantly lower BMI than patients without coagulation disorders. Patients without coagulation disorders were infected with HCV genotypes that are not unique to Japan, such as genotypes 1a, 3a, and 4a. Four patients with coagulation disorders were infected with human immunodeficiency virus and one of these patients had achieved a sustained virological response.

### Response to Therapy

The ribavirin dose reduction rate tended to be higher in patients without coagulation disorders than in patients with coagulation disorders ($P = 0.0643$). The treatment discontinuation rate did not differ significantly between the two groups. As a result, the sustained virological response rate by an intention-to-treat analysis did not differ significantly between the

TABLE I. Clinical Characteristics of Patients Treated With Combination Therapy

| | Total patients (n = 226) | Patients without coagulation disorders (n = 203) | Patients with coagulation disorders (n = 23) | *P* value |
|---|---|---|---|---|
| Sex ratio (male/female) | 127/99 | 104/99 | 23/0 | <0.0001 |
| Age (years) | 54.7 ± 11.6 | 56.3 ± 10.9 | 41.5 ± 9.8 | <0.0001 |
| Body weight (kg) | 60.2 ± 11.1 | 60.5 ± 11.5 | 60.5 ± 8.1 | 0.9972 |
| Body mass index | 22.9 ± 3.1 | 23.1 ± 3.1 | 21.5 ± 2.5 | 0.0226 |
| Baseline serum ALT (IU/L) | 63.3 ± 56.8 | 60.9 ± 54.9 | 84.4 ± 69.1 | 0.0598 |
| GGT (IU/L) | 54.2 ± 63.9 | 51.4 ± 62.2 | 78.6 ± 74.4 | 0.0526 |
| Hemoglobin (g/dl) | 14.1 ± 1.3 | 14.1 ± 1.3 | 14.4 ± 1.3 | 0.2714 |
| Platelets ($\times 10^4/\mu l$) | 17.8 ± 5.2 | 17.7 ± 5.2 | 19.0 ± 5.6 | 0.2597 |
| Genotype (1a/1b/2a/2b/3a/4a) | 7/160/40/15/3/1 | 0/150/39/14/0/0 | 7/10/1/1/3/1 | <0.0001 |
| HCV RNA (KIU/ml) | 1,898.0 ± 1,448.3 | 1,923.1 ± 1,464.5 | 1,676.6 ± 1,305.1 | 0.4404 |
| Activity (A0/A1/A2/A3) | 2/108/71/11 | 2/101/64/11 | 0/7/7/0 | 0.3442 |
| Fibrosis (F0/F1/F2/F3) | 17/104/49/22 | 16/97/45/20 | 1/7/4/2 | 0.5351 |

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCV RNA, hepatitis C virus RNA; KIU, kilo-international units.

TABLE II. Efficacy of Combination Therapy

| | Total patients (n = 226) | Patients without coagulation disorders (n = 203) | Patients with coagulation disorders (n = 23) | *P* value |
|---|---|---|---|---|
| SVR rate (intention-to-treat) | 49.6 (112/226) | 47.8 (97/203) | 65.2 (15/23) | 0.1130 |
| SVR rate (per-protocol) | 54.4 (111/204) | 52.7 (97/184) | 70.0 (14/20) | 0.1405 |
| ETR rate (intention-to-treat) | 84.1 (190/226) | 84.7 (172/203) | 78.3(18/23) | 0.4218 |
| ETR rate (per-protocol) | 89.1 (179/201) | 89.6 (163/182) | 84.2 (16/19) | 0.4772 |
| Ribavirin dose reduction rate | 44.2 (100/226) | 46.3 (94/203) | 26.1 (6/23) | 0.0643 |
| PegIFN dose reduction rate | 34.1 (77/226) | 33.5 (68/203) | 39.1 (9/23) | 0.5891 |
| Combination therapy discontinuation rate | 9.8 (22/226) | 9.4 (19/203) | 13.0 (3/23) | 0.5722 |

SVR, sustained virological response; ETR, end of treatment virological response; PegIFN, peginterferon.

two groups. The sustained virological response rate of patients with coagulation disorders by a per-protocol analysis was higher than that of patients without coagulation disorders, but there was no significant difference. In addition, based on both intention-to-treat and per-protocol analyses, the end of treatment virological response rate did not differ significantly between the two groups (Table II).

Factors associated with a sustained virological response in combination therapy were determined by a multivariate analysis. HCV genotype 1 and 4 versus 2 and 3 ($P = 0.001$, odds ratio 4.353 [95% CI, 1.810–10.469]), baseline serum GGT ($P = 0.003$, odds ratio 1.018 [1.006–1.030]), age ($P = 0.006$, odds ratio 1.053 [1.015–1.093]), and baseline serum ALT ($P = 0.014$, odds ratio 0.991 [0.983–0.998]) were associated significantly with a sustained virological response, but whether or not a patient had a coagulation disorder was not associated significantly with a sustained virological response.

### Characteristics and Response of Male, Age-Matched Patients Infected With HCV Genotype 1

The clinical characteristics of the two study groups in the male, age-matched patients infected with HCV genotype 1 are shown in Table III. Body weight, BMI, and Hb levels were significantly lower in patients with coagulation disorders than patients without coagulation disorders ($P = 0.0003$, 0.0027, and 0.0103, respectively).

The treatment discontinuation rate of patients with coagulation disorders did not differ between the two groups. The sustained virological response rate by intention-to-treat and per-protocol analyses did not differ significantly between the two groups (Table IV). Factors associated with a sustained virological response in the male, age-matched, genotype 1 patients treated with combination therapy were determined by a multivariate analysis. BMI ($P = 0.036$, odds ratio 1.810 [1.041–3.145]) and baseline serum GGT ($P = 0.037$, odds ratio 0.981 [0.963–0.999]) were associated significantly with a sustained virological response, but whether or not a patient had a coagulation disorder was not associated significantly with a sustained virological response.

### Adverse Events

The reasons for discontinuing combination therapy and the times at which the therapy was discontinued are shown in Table V. Once treatment was discontinued, therapy was not restarted even after the initial symptoms or illness disappeared. There were no bleeding episodes in the patients with coagulation disorders, including patients who received a liver biopsy.

TABLE III. Clinical Characteristics of Male, Age-Matched Patients With Genotype 1 Treated With Combination Therapy

| | Total patients (n = 36) | Patients without coagulation disorders (n = 18) | Patients with coagulation disorders (n = 18) | *P* value |
|---|---|---|---|---|
| Age (years) | 42.8 ± 8.0 | 44.9 ± 5.9 | 40.7 ± 9.3 | 0.1136 |
| Body weight (kg) | 66.1 ± 11.0 | 73.4 ± 9.3 | 60.4 ± 8.7 | 0.0003 |
| Body mass index | 22.7 ± 2.8 | 24.3 ± 2.3 | 21.4 ± 2.5 | 0.0027 |
| Baseline serum ALT (IU/L) | 69.8 ± 54.3 | 63.5 ± 31.7 | 76.2 ± 70.5 | 0.4919 |
| GGT (IU/L) | 72.7 ± 64.2 | 74.3 ± 71.1 | 71.2 ± 58.5 | 0.8869 |
| Hemoglobin (g/dl) | 14.9 ± 1.2 | 15.4 ± 1.0 | 14.4 ± 1.2 | 0.0103 |
| Platelets ($\times 10^4/\mu l$) | 19.3 ± 5.4 | 18.8 ± 4.5 | 19.8 ± 5.6 | 0.5773 |
| HCV RNA (KIU/ml) | 2,050.8 ± 1,273.4 | 2,322.8 ± 1,249.1 | 1,778.8 ± 1,273.5 | 0.2044 |
| Activity (A0/A1/A2/A3) | 0/12/11/0 | 0/6/5/0 | 0/6/6/0 | 0.6723 |
| Fibrosis (F0/F1/F2/F3) | 2/11/8/2 | 1/5/4/1 | 1/6/4/1 | 0.9392 |

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCV RNA, hepatitis C virus RNA; KIU, kilo-international unit.

TABLE IV. Efficacy of Combination Therapy in Male, Age-Matched Patients With Genotype 1

| | Total patients (n = 36) | Patients without coagulation disorders (n = 18) | Patients with coagulation disorders (n = 18) | *P* value |
|---|---|---|---|---|
| SVR rate (intention-to-treat) | 58.3 (21/36) | 61.1 (11/18) | 55.6 (10/18) | 0.7353 |
| SVR rate (per-protocol) | 69.0 (20/29) | 64.7 (11/17) | 75.0 (9/12) | 0.5551 |
| ETR rate (intention-to-treat) | 77.8 (28/36) | 83.3 (15/18) | 72.2 (13/18) | 0.4227 |
| ETR rate (per-protocol) | 93.1 (27/29) | 88.2 (15/17) | 100.0 (12/12) | 0.2182 |
| Ribavirin dose reduction rate | 22.2 (28/36) | 16.7 (3/18) | 27.8 (5/18) | 0.7175 |
| PegIFN dose reduction rate | 36.1 (13/36) | 27.8 (5/18) | 44.4 (8/18) | 0.2979 |
| Combination therapy discontinuation rate | 5.6 (2/36) | 0 (0/18) | 16.7 (3/18) | 0.0704 |

SVR, sustained virological response; ETR, end of treatment virological response; PegIFN, peginterferon.

## DISCUSSION

A previous randomized trial in patients infected with HCV with inherited bleeding disorders showed that the sustained virological response rate improved significantly for patients who were treated with IFN and ribavirin compared to those treated with IFN alone [Fried et al., 2002a]. In addition, both chronic hepatitis C patients with and without coagulation disorders responded similarly to pegIFN and ribavirin combination therapy [Franchini et al., 2006; Posthouwer et al., 2006]. However, the efficacy and tolerability of this combination therapy differed based on the HCV genotype as well as the age, gender, and race of the patients; therefore it is difficult to compare patients with and without coagulation disorders under the same conditions. No report has examined that patients infected chronic hepatitis C with and without coagulation disorders at the same institution and during the same observation period. In addition, there are no reports on the efficacy of combination therapy in patients with chronic hepatitis C with and without coagulation disorders in age-matched patients infected with HCV genotype 1. Therefore, a retrospective study was conducted to evaluate the efficacy and tolerability of ribavirin plus pegIFN in chronic hepatitis C patients with and without coagulation disorders. In the per-protocol analysis, there were no significant differences, but the sustained virological response rate was higher in patients with coagulation disorders than in patients without coagulation disorders. Mancuso et al. [2006] reported that combination therapy with pegIFN alfa-2b plus ribavirin is highly efficacious in hemophiliacs with chronic hepatitis C. In an overall analysis, patients with coagulation disorders had a lower mean age than patients without coagulation disorders. In addition, the BMI of the patients with coagulation disorders was lower than that of patients without coagulation disorders. A multivariate analysis showed that the HCV genotype, baseline serum GGT, age, and baseline ALT were factors associated significantly with a sustained virological response and whether patients had coagulation disorders was not associated with a sustained virological response. Age, especially younger than 40 years old, was a good predictive factor for a sustained virological response, as was reported previously [Poynard et al., 2000; Fried et al., 2002b].

TABLE V. Reasons for Discontinuing Combination Therapy

| Reason | Number | Weeks after starting treatment |
|---|---|---|
| Patients with coagulation disorders | | |
| Peritonitis due to appendicitis | 1 | 16 |
| Pneumoniae | 1 | 18 |
| No HCV eradication | 3 | 24, 28, 29 |
| IDDM | 1 | 44 |
| Patients without coagulation disorders | | |
| Fatigue | 5 | 1, 2, 4.9, 19 |
| Bleeding from duodenal varies | 1 | 8 |
| Dizziness | 1 | 12 |
| Palpitation | 1 | 13 |
| Cholecystitis | 1 | 16 |
| Symptom of Parkinson's disease | 1 | 16 |
| Fundal hemorrhage | 1 | 17 |
| Hepatocellular carcinoma | 2 | 19, 21 |
| Suspicion of Interstitial pneumonia | 1 | 20 |
| Gastric cancer | 2 | 21, 36 |
| Self-discontinuation | 1 | 24 |
| Neutropenia | 1 | 25 |
| Eruption | 1 | 25 |
| No HCV eradication | 7 | 24, 25, 25, 27, 28, 29, 29 |

These results suggest that male patients who are infected with HCV genotype 1 and have coagulation disorders will have a higher sustained virological response than patients without coagulation disorders, if the coagulation disorder patients do not discontinue treatment. However, these results do not account for the differences in age. Therefore, male, age-matched patients infected with HCV genotype 1 were evaluated. The characteristics that differed between patients with and without coagulation disorders were body weight, BMI and baseline Hb levels.

In male, age-matched patients infected with HCV genotype 1, the sustained virological response rate based on both intention-to-treat and per-protocol analyses was not different between patients with and without coagulation disorders.

Using a multivariate analysis, whether patients had coagulation disorders was not associated significantly with a sustained virological response. Only BMI and GGT were identified as factors associated with a sustained virological response to combination therapy in male, age-matched patients infected with HCV genotype 1. A previous report showed that GGT levels may represent a surrogate marker of tumor necrosis factor-alpha expression in the liver and explain the importance of serum analyses to in predict the treatment outcome [Taliani et al., 2002]. Several studies revealed that GGT is one predictor of a sustained virological response [Taliani et al., 2002, 2006; Villela-Nogueira et al., 2005]. In western countries, obesity and a high BMI are associated with the absence of a sustained virological response to combination therapy of pegIFN or IFN with ribavirin [Bressler et al., 2003; Camma et al., 2004]. However, in Japan, most of the patients who are treated with combination therapy are not obese and have lower BMIs than patients in western countries. In this population, the mean BMI was 22.7 $\pm$ 2.8. In this low BMI population, a higher BMI would be associated with a sustained virological response. However, the reason why a low BMI is associated with the absence of a sustained virological response has not elucidated.

Adverse effects are thought to increase in patients with coagulation disorders; however, there was not a significant difference in adverse effects necessitating discontinuation of pegIFN and ribavirin between patients with and without coagulation disorders (13.0% vs. 9.4%). In addition, severe adverse effects and bleeding adverse effects were not associated with coagulation disorders. A previous report showed that IFN and ribavirin combination therapy may reduce the use of clotting factors in hemophilia patients with chronic hepatitis C [Honda et al., 2005; Yamamoto et al., 2006]. Ribavirin may reduce the side effect of bleeding during combination therapy. In this study, patients with coagulation disorders did not experience an adverse effect of bleeding.

In conclusion, treatment of chronic hepatitis C with combination therapy was effective comparably between patients with and without coagulation disorders and there were no adverse effects of bleeding.

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# Prevalence of Hepatitis C Virus Genotype 1a in Japan and Correlation of Mutations in the NS5A Region and Single-Nucleotide Polymorphism of Interleukin-28B With the Response to Combination Therapy With Pegylated-Interferon-Alpha 2b and Ribavirin

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Hepatitis C virus (HCV) genotype 1a is rare in Japanese patients and the clinical characteristics of this genotype remain unclear. The interferon (IFN) sensitivity-determining region (ISDR) and single-nucleotide polymorphisms (SNPs) of interleukin-28B (IL28B) among patients with HCV genotype 1b are associated with IFN response, but associations among patients with genotype 1a are largely unknown. This study investigated the clinical characteristics of genotype 1a and examined whether genomic heterogeneity of the ISDR and SNPs of IL28B among patients with HCV genotype 1a affects response to combination therapy with pegylated-IFN-α2b and ribavirin. Subjects comprised 977 patients infected with HCV genotype 1, including 574 men and 412 women (mean age, 55.2 ± 10.6 years). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or core regions and confirmed by direct sequencing of the NS5A region. HCV genotypes 1a (n = 32) and 1b (n = 945) were detected. Twenty-three (71.9%) of the 32 patients with genotype 1a were patients with hemophilia who had received imported clotting factors. Prevalence of genotype 1a after excluding patients with hemophilia was thus 0.9%. Of the 23 patients with genotype 1a who completed IFN therapy, 11 (47.8%) were defined as achieving sustained virological response. Factors related to sustained virological response by univariate analysis were IL28B and ISDR. In conclusion, HCV genotype 1a is rare in Japan. The presence of IL28B genotype TT, and more than two mutations, in the ISDR are associated with a good response to IFN therapy in patients with HCV genotype 1a. ***J. Med. Virol. 84:438–444, 2012.*** © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** hepatitis C virus; genotype 1a; NS5A; IL 28B; interferon

## INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma [Seeff, 2002]. HCV infection is a significant global health problem, affecting 170 million individuals worldwide. HCV can be divided into six genotypes and several subtypes according to genomic heterogeneity [Simmonds et al., 2005]. Each genotype shows a unique distribution and clinical characteristics such

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as interferon (IFN) responsiveness [Ghany et al., 2009]. HCV genotypes 1b, 2a, and 2b are the major types encountered in Japan [Enomoto et al., 1990; Hayashi et al., 2003]. Genotype 1a is common worldwide, but is rare in Japan except among individuals with hemophilia who have received imported clotting factors [Fujimura et al., 1996; Otagiri et al., 2002; Hayashi et al., 2003]. The prevalence and clinical characteristics, including IFN responsiveness, of Japanese patients with HCV genotype 1a are unclear. HCV NS5A protein reportedly includes a domain associated with IFN response. This domain, located in the NS5A region of HCV genotype 1b, is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR) [Enomoto et al., 1996]. IFN acts to inhibit viral replication by inducing double-stranded RNA-dependent protein kinase (PKR). The ISDR is located at the 5′ end of the PKR-binding domain and is inhibited by PKR in vitro [Gale et al., 1998]. ISDR heterogeneity of genotype 1b is thus an important factor that may affect response to IFN [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. Several studies have reported a relationship between ISDR and IFN responsiveness among patients with HCV genotype 1a [Hofgärtner et al., 1997; Zeuzem et al., 1997; Kumthip et al., 2011; Yahoo et al., 2011]. However, this remains controversial for genotype 1a, and the utility of ISDR sequences for predicting IFN responsiveness has not been investigated for HCV genotype 1a in Japan due to the rarity of this genotype. Both genetic heterogeneity of the HCV genome and host genetics contribute to IFN responsiveness. Several genome-wide association studies have thus been performed to clarify host factors associated with IFN responsiveness, revealing that interleukin-28B (IL28B) polymorphisms are strongly associated with response to IFN therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009]. Combined use of the single-nucleotide polymorphisms (SNPs) of IL28B and amino acid substitutions in the core region and ISDR could thus improve the prediction of response to IFN in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. However, the effects of a combined evaluation of the SNPs of IL28B and amino acid substitutions in the ISDR in patients with HCV genotype 1a on IFN response are unclear. The aim of the present study was to determine whether genomic heterogeneity of the ISDR and SNPs of IL28B among patients with HCV genotype 1a affect response to combination therapy with pegylated-IFN-α2b and ribavirin.

## PATIENTS AND METHODS

A total of 977 patients (569 men, 408 women) with chronic hepatitis C genotype 1 and high viral load (<100 KIU/ml) who were treated at Nagoya University Hospital and affiliated hospitals were enrolled in this study. Mean age of patients was 55.1 ± 12.2 years (range: 18–75 years). None of the patients had a history of chronic alcohol abuse, autoimmune disease, or metabolic disease. Patients with active intravenous drug use and immigrants were excluded from this study. The core region (aa 30–110) and ISDR (aa 2,209–2,248) of HCV were examined by direct sequencing. SNPs of IL28B (rs8099917) were identified using a real-time polymerase chain reaction (PCR) system. Patients received subcutaneous injections of pegylated-IFN-α2b (1.5 μg/kg) once each week along with oral ribavirin (600 mg/day for patients <60 kg, 800 mg/day for 60–80 kg, 1,000 mg/day for >80 kg) for 48 weeks. Patients who became negative for HCV-RNA between 16 and 36 weeks after initiating IFN treatment had the IFN treatment extended to 72 weeks, in accordance with Japanese guidelines [Kumada et al., 2010]. HCV-RNA levels in serum samples were examined at 12 weeks, at the end of IFN therapy, and at 6 months after the end of treatment. Serum was stored at −80°C for virological examination at pretreatment. Early virological response was defined as HCV-negative status at 12 weeks. Patients who were persistently negative for serum HCV-RNA at 24 weeks after withdrawal of IFN treatment were considered to show sustained virological response. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### Virological Analysis

HCV-RNA quantitative viremia load was determined by PCR. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or core regions as described previously and confirmed by direct sequencing of the NS5A region [Otagiri et al., 2002; Dal Pero et al., 2007; Hayashi et al., 2011a]. Genotypes were classified according to the nomenclature proposed by Simmonds et al. [2005]. Direct sequencing of the core and NS5A-ISDR regions was performed as reported previously [Dal Pero et al., 2007; Hayashi et al., 2011a]. In brief, RNA was extracted from 140 μl of serum using a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligos and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). The HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μl PCR reaction mixture contained 100 nM of each primer, 1 ng of template cDNA, 5 μl of GeneAmp 10× PCR buffer, 2 μl of dNTPs, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Primers for the core region were: sense, 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense, 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′. Primers for the NS5A-ISDR were: sense, 5′-GCCTGGAGCCCTTGTAGTC-3′ and

TABLE I. Clinical Characteristic of Patients With HCV Genotype 1a

| | N = 32 |
|---|---|
| Age (y.o.) | 36.4 ± 2.2 |
| Sex: male/female | 28/4 |
| AST (IU/L) | 48.8 ± 33.6 |
| ALT (IU/L) | 64.6 ± 57.8 |
| Platelet ($10^4$/μl) | 18.8 ± 6.0 |
| HCV RNA level (KIU/ml) | 2607.4 ± 3072.2 |
| Source (clotting factor/BTF/unknown) | 23/2/7 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

antisense, 5′-CTGCGTGAAGTGGTGGAATAC-3′. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed using the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-TGTTTCCCCCACGCACTAC-3′ and antisense 5′-TGATGGGCAGTTTTTGTTCTTC-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems).

### Genotyping Analysis

Detection of SNPs for IL28B (rs8099917) was conducted using a real-time PCR system. In brief, genomic DNA was extracted from 150 μl of whole blood with a commercial kit (QIAamp DNA Blood mini Kit; Qiagen) and dissolved in 50 μl of diethylpyrocarbonate-treated water. DNA (10 ng) was used for PCR and genotyping of IL28B SNP (rs8099917) was performed by TaqMan allelic discrimination (ABI-Prism 7300 SDS software; Applied Biosystems) with TaqMan SNP Genotyping Assays provided by Applied Biosystems (C__11710096_10).

### Statistical Analysis

Data are expressed as mean ± standard deviation (SD). The paired *t*-test was used to analyze differences in variables. A value of $P < 0.05$ was considered statistically significant. Statview 5.0 software (SAS Institute, Cary, NC) was used for all analyses.

## RESULTS

Thirty-two of the 977 patients (3.3%) were infected by genotype 1a. Clinical characteristics of patients with genotype 1a are summarized in Table I. Twenty-three cases involved patients with hemophilia who had received imported clotting factors. The prevalence of genotype 1a after excluding patients with hemophilia was 0.9%. A comparison of clinical characteristics according to hemophilia status is shown in Table II. No significant differences were apparent among the two groups. Differences in clinical characteristics between genotypes 1a and 1b are shown in Table III. Males were more frequent among patients with genotype 1a (87.5%) than among those with genotype 1b (57.2%), as the majority of patients with genotype 1a were young male patients with hemophilia. Sequence alignments of the core region at codons 71 and 90 showed arginine and cysteine, respectively, in all patients. The HCV core region of genotype 1a was thus well-conserved, with no significant mutations at codons 71 or 90. This is not similar to previous findings for genotype 1b [Akuta et al., 2005, 2011; Hayashi et al., 2011a,b; Kurosaki et al., 2011]. Alignment of the amino acid sequence for NS5A-ISDR is shown in Figure 1. The sequence of the HCV-1 strain was defined as the consensus sequence of genotype 1a, and the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. Sequences of the HCV-1 strain and HCV-1 strain with only one amino acid substitution were defined as wild-type, while ISDR sequences with more than two amino acid substitutions were defined as mutant-type. Twenty-seven strains were defined as wild-type and 5 strains were defined as mutant-type. IL28B genotypes could be obtained for 25 patients, and IL28B alleles were TT (n = 14) and TG (n = 11). Twenty-three patients received pegylated-IFN-α2b plus ribavirin therapy. Twenty patients were treated for 48 weeks, and 1 patient was treated for 72 weeks. Two patients were withdrawn at 24 weeks due to a

TABLE II. Clinical Characteristic According to Hemophilia

| | Patients with hemophilia (N = 23) | Patients without hemophilia (N = 9) | *P*-value |
|---|---|---|---|
| Age (y.o.) | 37.1 ± 9.2 | 37.1 ± 16.3 | 0.9966 |
| Sex: male/female | 22/1 | 6/3 | 0.0572 |
| AST (IU/L) | 51.2 ± 34.8 | 41.9 ± 30.9 | 0.5072 |
| ALT (IU/L) | 68.2 ± 55.8 | 54.0 ± 66.1 | 0.5566 |
| Platelet ($10^4$/μl) | 18.4 ± 6.8 | 19.8 ± 3.0 | 0.5602 |
| HCV levels (KIU/ml) | 2599.6 ± 3108.0 | 2630.0 ± 3176.5 | 0.9812 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

TABLE III. Clinical Characteristic According to Genotypes

| | Genotype 1a (N = 32) | Genotype 1b (N = 945) | *P*-value |
|---|---|---|---|
| Age (y.o.) | 36.4 ± 2.2 | 55.9 ± 11.6 | 0.0001 |
| Sex: male/female | 28/4 | 546/408 | 0.0004 |
| Patients with hemophilia | 23 | 4 | 0.0001 |
| AST (IU/L) | 48.8 ± 33.6 | 59.9 ± 45.0 | 0.1745 |
| ALT (IU/L) | 64.6 ± 57.8 | 64.6 ± 57.8 | 0.9894 |
| Platelet ($10^4/\mu l$) | 18.8 ± 6.0 | 17.2 ± 6.0 | 0.0918 |
| HCV levels (KIU/ml) | 2607.4 ± 3072.2 | 2011.5 ± 1453.8 | 0.0642 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus.

lack of response to IFN therapy. Frequency of early virological response, characterized by undetectable HCV at 12 weeks, was 30.4% (7/23). Virological response rate at the end of treatment was 47.8% (11/23). Finally, 11 of 23 patients (47.8%) achieved sustained virological response. Clinical characteristics were compared between patients who achieved sustained virological response and patients who did not (Table IV), revealing significant differences in two factors on univariate analysis: IL28B and ISDR.

## DISCUSSION

The present study investigated 977 patients with genotype 1 using direct sequencing of core and NS5A regions, revealing that genotype 1a is rare (3.3%) in Japan. Of the 33 patients with genotype 1a, 23 (71.9%) were patients with hemophilia, confirming that the majority of cases with genotype 1a involve patients with hemophilia who have received imported clotting factors, as previously reported [Fujimura et al., 1996; Otagiri et al., 2002; Hayashi et al., 2003]. Analysis after excluding patients with hemophilia revealed the prevalence of genotype 1a in Japan was 0.9% (9/954). Recently, the distributions of HBV genotypes have been changing in Japan due to international exchange [Hayashi et al., 2007; Matsuura et al., 2009]. However, prevalences of HCV genotypes have remained stable because of the different modes of infection involved. The present study revealed that 11 (47.8%) of 23 patients achieved sustained virological response. The IFN responsiveness of HCV genotype 1a in Japanese patients was reported in 1999 from Okinawa, a far southern island in Japan [Sakugawa et al., 1997]. That study reported that the rate of sustained virological response tended to be higher in patients with genotype 1a than in those with genotype 1b, but no significant differences were identified because of the small number of patients with genotype 1a. Low virological response rates in both genotypes 1a and 1b were confirmed in the present Japanese patients, as in Caucasian patients [Manns et al., 2001; McHutchison et al., 2009]. No significant differences in sustained virological response rate were seen between genotypes 1a and 1b. Discriminating between genotypes 1a and 1b thus seems to have little clinical relevance in terms of IFN responsiveness. Viral factors associated with sustained virological response, including HCV genotype, have been studied most frequently studied and mutations in the core and NS5A regions of HCV genotype 1b have been associated with response to IFN therapy [Akuta et al., 2005, 2010, 2011; Okanoue et al., 2009; Nakagawa et al., 2010; Toyoda et al., 2010; Hayashi et al., 2011a; Hayes et al., 2011; Kumthip et al., 2011; Kurosaki et al., 2011]. These viral factors could improve prediction of sustained virological response for genotype 1a, as in 1b. Amino acid substitutions at positions 70 and 91 of the HCV core region in genotype 1b have been related to IFN responsiveness, liver steatosis, hepatic oxidative stress, insulin resistance, and carcinogenesis [Akuta et al., 2005, 2007, 2009; Tachi et al., 2010]. These substitutions may have substantial impacts on

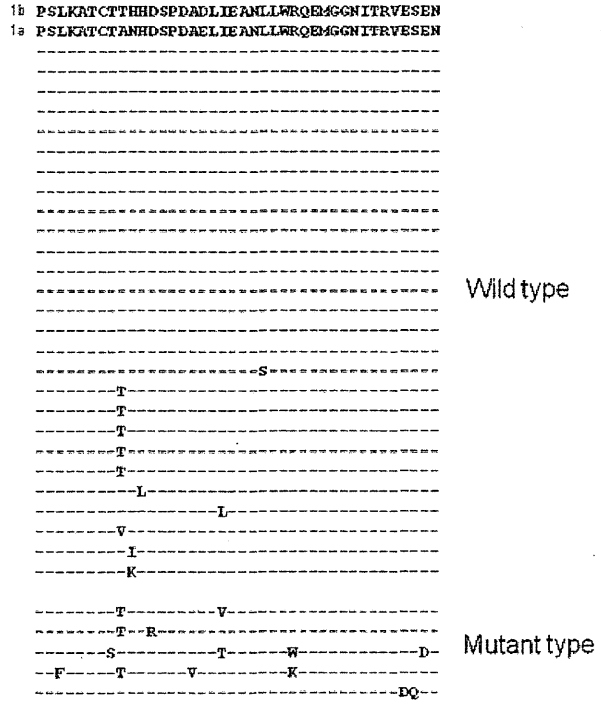


Fig. 1. Alignment of the amino acid sequence for the NS5A-ISDR. In the sequence alignment, dashes indicate amino acids identical to consensus sequence HCV1. Sequences of the HCV1 strain and HCV1 strains with one-nucleotide substitutions were defined as wild-type ISDR, and all other strains were defined as mutant-type ISDR. ISDR, interferon sensitivity-determining region.

TABLE IV. Univariate Analysis: Factors Predictive of Sustained Virologic Response

| Factors | Sustained virologic response (n = 11) | Non-sustained virologic response (n = 12) | *P*-value |
|---|---|---|---|
| Age (y.o.) | 37.9 ± 10.9 | 39.8 ± 11.3 | 0.6958 |
| Gender: male/female | 10/1 | 10/2 | 0.9999 |
| ALT (IU/L) | 78.2 ± 50.8 | 62.6 ± 68.1 | 0.5435 |
| AST (IU/L) | 51.4.4 ± 29.2 | 48.8 ± 40.4 | 0.8616 |
| PLT ($\times 10^4/mm^3$) | 19.0 ± 5.4 | 19.3 ± 5.7 | 0.8870 |
| HCV RNA level (KIU/ml) | 1323.1 ± 1077.3 | 2567.0 ± 2940.8 | 0.2481 |
| ISDR: wild/mutant | 7/4 | 12/0 | 0.0373 |
| IL28B:TT/TG | 9/1 | 4/8 | 0.0115 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; IL28B, interleukin 28B.

the pathogenesis of HCV genotype 1a infection. However, the HCV core region of genotype 1a is well-conserved and no significant mutations were seen in the core region, which is associated with IFN responsiveness. Several reports have also found that the HCV core region, including positions 70 and 91, of HCV genotype 1a is highly conserved [Alestig et al., 2011; Kumthip et al., 2011]. Mutations in the core region of genotype 1a would be rare, so this region might be unsuitable for routine clinical use, unlike in genotype 1b. However, the number of patients in this study was small, and large studies including from other countries are needed to clarify these issues. The ISDR in the NS5A region of HCV genotype 1b is closely associated with response to IFN therapy. ISDR mutations of genotype 1b are well known to be more important in predicting sustained virological response in Japanese patients than European patients [Hofgärtner et al., 1997; Zeuzem et al., 1997; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. European studies have failed to detect the specific amino acid substitutions in ISDR of genotype 1a associated with IFN responsiveness [Hofgärtner et al., 1997; Zeuzem et al., 1997]. In this study, sustained virological response was achieved in 36.8% of patients with wild-type ISDR and 100% of patients with mutant-type ($P = 0.0373$). The present analysis showed a close relationship between ISDR of genotype 1a and sustained virological response, as in genotype 1b. Recent investigations in Thailand and Iran have failed to identify the usefulness of ISDR for HCV genotype 1a in predicting sustained virological response [Kumthip et al., 2011; Yahoo et al., 2011]. The high virological response rate and low prevalence of patients with mutations in the ISDR do not favor the use of ISDR analysis in predicting IFN responsiveness [Herion and Hoofnagle, 1997; Yokozaki et al., 2011]. Rates of sustained virological response among these studies were much higher than those in the present study (68.4% and 75% vs. 47.8%). The mean number of mutations in patients who achieved sustained virological response in the studies by Kumthip et al. [2011] and Yahoo et al. [2011], and the present group were 1.4, 1.4, and 1.6, respectively. Differences in sustained virological response and the number of mutations to the ISDR might underpin this discrepancy in the evaluation of ISDR. Although the sample size in the present study was small, the results indicate that ISDR represents a strong indicator of progression to sustained virological response for patients with HCV genotype 1a. Amino acid substitutions in the ISDR of genotype 1a thus also play an important role in predicting sustained virological response in Japanese patients compared to patients from other countries. IL28B polymorphisms such as host genetics, as well as mutations in the HCV genome, contribute to IFN treatment outcomes. Rates of sustained virological response in patients in this study with TT and TG were 69.2% and 11.1%, respectively. The TG allele of the IL28B genotype was significantly associated with poor response to IFN therapy ($P = 0.0115$). SNPs of IL28B would regulate the expression of IFN-stimulated genes and affect IFN responsiveness. IL28B and ISDR thus exert independent effects on IFN responsiveness and both host and viral factors impacting IFN responsiveness would improve the prediction of sustained virological response. Several studies have thus reported that both the SNP of IL28B and mutations in the ISDR were associated with sustained virological response in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. In the present study of HCV genotype 1a, among the 9 patients who had simultaneously the TG allele for IL28B and wild-type ISDR, only 1 achieved sustained virological response (11.1%). The best-sustained virological response was achieved in patients with mutant-type ISDR and the T allele (100%). The combination of SNPs for IL28B and mutations in ISDR may thus predict response to IFN therapy in patients with HCV genotype 1a as well as genotype 1b. Given the small sample size in this investigation, larger cohorts are needed to confirm the present results. Furthermore, infection with genotype 1a in Japanese patients is rare, making large-scale studies difficult to perform.

In conclusion, the prevalence of HCV genotype 1a is rare in Japan and the majority of cases involve patients with hemophilia. The TG genotype of IL28B is associated with poor response, while mutant-type ISDR is associated with good response to combination therapy with pegylated-IFN-α2b and ribavirin in patients with HCV genotype 1a. Combined use of both IL28B and ISDR could improve the prediction of IFN response.

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