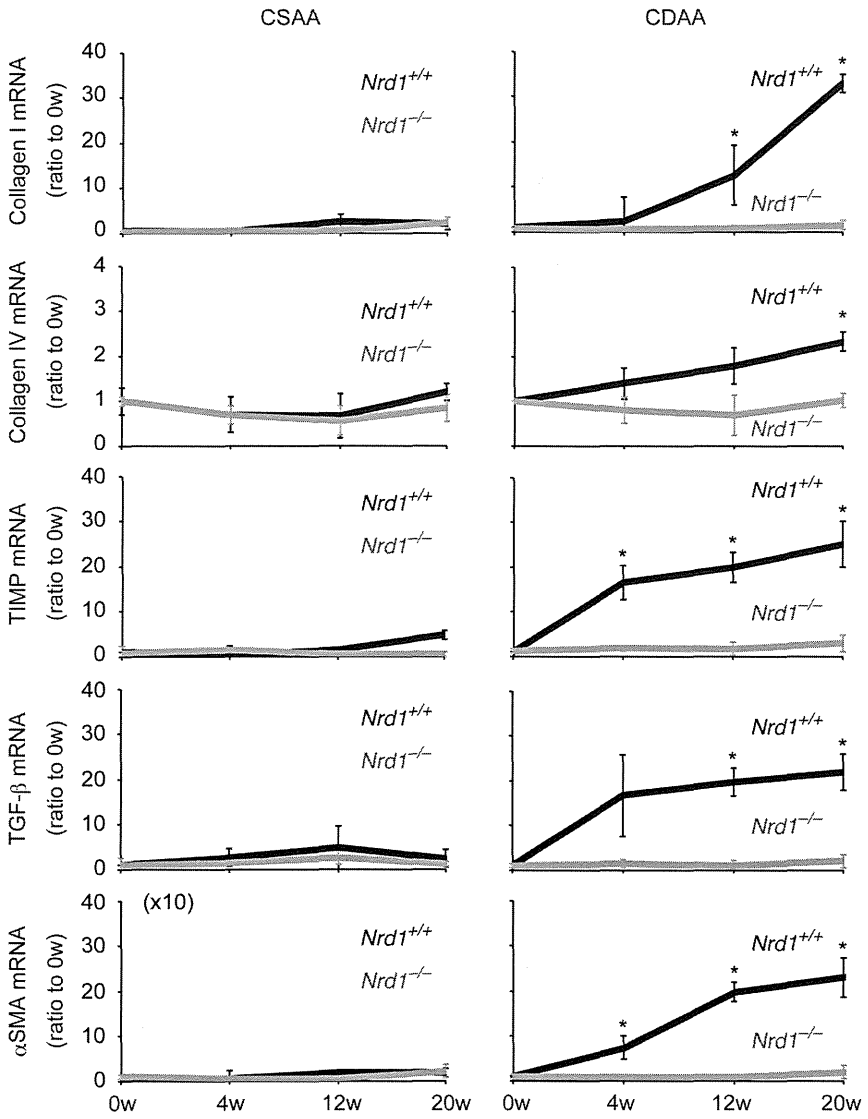

Figure 6. Fibrogenic factors were not elevated in *Nrd1*$^{-/-}$ mice fed the CDAA diet. During CDAA diet administration, mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA were significantly increased in the livers of *Nrd1*$^{+/+}$ mice but not in those of *Nrd1*$^{-/-}$ mice. Those factors were not altered by administration of the CSAA diet in *Nrd1*$^{+/+}$ or *Nrd1*$^{-/-}$ mice. *$P<0.05$.
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in *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice fed the CSAA diet (Figure 3B). qRT-PCR showed that mRNA expression of CCR2, a recruited macrophage marker, was significantly increased in *Nrd1*$^{+/+}$ mice, but not in *Nrd1*$^{-/-}$ mice (Figure 3C). This suggested that macrophages are not sufficiently recruited in *Nrd1*$^{-/-}$ mice. At 20 weeks of a CDAA feeding, production of TNF-α protein was significantly upregulated in both *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mouse livers (Figure 4A, produced TNF-α), but the increase in TNF-α protein secretion from liver specimens into the conditioned medium was decreased significantly (0.46-fold) by *Nrd1* knockout (Figure 4A, secreted TNF-α). In contrast, production of IL6 and IL1-β proteins were not increased in *Nrd1*$^{-/-}$ mice fed a CDAA diet (Figure 4B). These data suggested that nardilysin was required for the shedding of TNF-α in mice fed the CDAA diet and possibly the induction of inflammation. To further investigate that possibility, we examined whether blocking TNF-α suppresses the production of IL6 and IL1-β. We used *Nrd1*$^{+/+}$ mouse peritoneal macrophages as substitutes for Kupffer cells and recruited macrophages in the liver, and examined the effect of pre-incubation with anti-TNF-α neutralizing antibodies on the production of IL6 and IL1-β. Following LPS stimulation mRNAs and secreted proteins of both IL6 and IL1-β from macrophages were significantly increased, and administration of anti-TNF-α neutralizing antibodies significantly suppressed the production of IL6 and IL1-β (Figure 4C). This also suggested that TNF-α secretion played an important role to induce IL6 and IL1-β production in mice.

Nrd1$^{-/-}$ mice were resistant to CDAA diet-induced liver fibrotic changes

Persistent steatohepatitis results in hepatic fibrosis [1–4]. Using Sirius red staining we investigated whether secretion/production of inflammatory cytokines enhanced by nardilysin was associated with the development of liver fibrotic changes. Four weeks after CDAA feeding, fibrotic changes were not prominent in both *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice (Figure 5A and B). Twelve weeks after CDAA feeding, fibrotic changes were observed in *Nrd1*$^{+/+}$ mice,

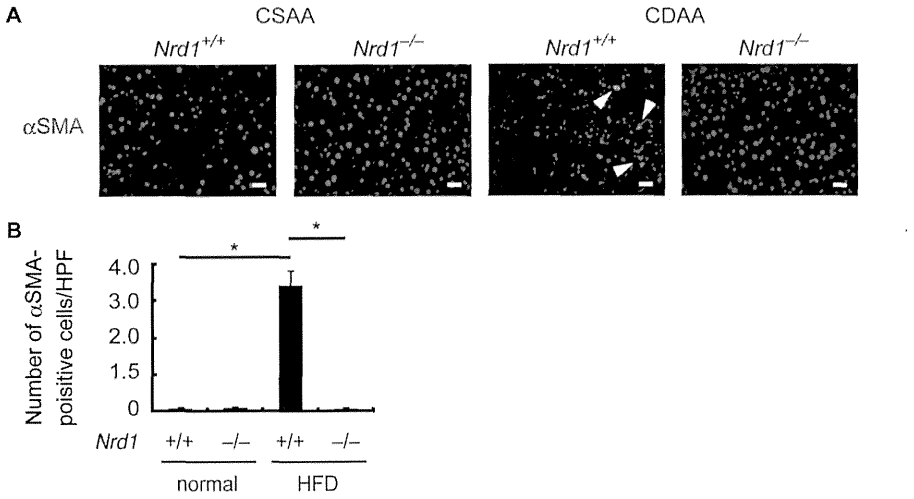

Figure 7. Activated myofibroblasts were not observed in *Nrd1*$^{-/-}$ mice fed the CDAA diet. A. Immunostainings for αSMA demonstrated that activated myofibroblasts were detected in *Nrd1*$^{+/+}$ mice fed a CDAA diet for 20 weeks, but not in *Nrd1*$^{+/+}$ mice. Activated myofibroblasts were hardly detected by administration of the CSAA diet in *Nrd1*$^{+/+}$ or *Nrd1*$^{-/-}$ mice. Bars indicate 100 μm. B. The number of αSMA-positive cells/×400 high-power field (HPF) in livers increased only in *Nrd1*$^{+/+}$ mice fed the CDAA diet for 20 weeks. *$P<0.05$.
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whereas such changes were not prominent in *Nrd1*$^{-/-}$ mice (Figure 5A and B). At 20 weeks of CDAA diet administration, fibrotic changes in *Nrd1*$^{+/+}$ mice became more prominent, while they were not observed in *Nrd1*$^{-/-}$ mice (Figure 5A and B). Fibrotic changes were not observed throughout the experiments in both *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice fed a CSAA diet (Figure 5A and B). Consistently, the increased mRNA expression of fibrogenic markers such as collagen I, collagen IV, TIMP1, TGF-β, and αSMA in *Nrd1*$^{+/+}$ mouse livers were not observed in *Nrd1*$^{-/-}$ mice fed the CDAA diet (Figure 6). Immunostainings for αSMA demonstrated that activated myofibroblasts were detectable only in *Nrd1*$^{+/+}$ mice fed a CDAA diet (Figure 7A and B). Thus, nardilysin played a pivotal role in the development of liver fibrosis caused by the CDAA diet.

Nrd1$^{-/-}$ mice were resistant to high fat diet-induced liver fibrogenesis

To further confirm the role of nardilysin in the development of steatohepatitis followed by liver fibrotic changes, *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice were also fed HFD. Similar to the CDAA diet, HFD administration for 20 weeks induces hepatic steatosis and liver fibrogenesis [16]. In the present study, steatosis was observed more prominently in *Nrd1*$^{+/+}$ mice compared to *Nrd1*$^{-/-}$ mice at 20 weeks of HFD administration, but not in mice fed a normal control diet (Figure 8A). Consistently, triglyceride in the liver were elevated in *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice (Figure 8B). However, serum ALT levels were significantly increased in *Nrd1*$^{+/+}$ mice upon 20-week administration of the HFD, whereas they were not increased in *Nrd1*$^{-/-}$ mice fed the HFD (Figure 8C). Furthermore, fibrotic changes were detected only in *Nrd1*$^{+/+}$ mice fed a HFD (Figure 8D and E). Consistent with this finding, qRT-PCR showed that the mRNA expression of IL1-β was significantly increased only in *Nrd1*$^{+/+}$ mice at 20 weeks of HFD feeding, but not in that of *Nrd1*$^{-/-}$ mice (Figure 9A). mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA were significantly increased in the livers of *Nrd1*$^{+/+}$ mice fed a HFD for 20 weeks, but not in those of *Nrd1*$^{-/-}$ mice (Figure 9B). Therefore, nardilysin also played an important role in the development of steatohepatitis and liver fibrogenesis induced by HFD in mice.

Discussion

In the present study, we demonstrated that steatosis was induced by the CDAA diet in both *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice, although fatty changes were less prominent in *Nrd1*$^{-/-}$ mice. Importantly, steatohepatitis followed by liver fibrotic changes was observed only in *Nrd1*$^{+/+}$ mice and not in *Nrd1*$^{-/-}$ mice. Secretion of TNF-α, and the production of inflammatory cytokines and fibrogenic factors were not upregulated in *Nrd1*$^{-/-}$ mice as compared with *Nrd1*$^{+/+}$ mice. In the HFD model, steatohepatitis and liver fibrogenesis were hardly observed in *Nrd1*$^{-/-}$ mice. These data suggested that nardilysin plays an important role in the development of steatohepatitis followed by liver fibrosis.

In mice fed with the CDAA diet, the levels of hepatic triglyceride content were lower in *Nrd1*$^{-/-}$ mice compared with those in *Nrd1*$^{+/+}$ mice, suggesting the possibility that nardilysin is involved in the regulation of hepatic lipid synthesis. A decreased steatosis in *Nrd1*$^{-/-}$ mice may partly affect hepatic inflammation. However, steatosis did occur in the liver of *Nrd1*$^{-/-}$ mice; on the other hand, hepatic inflammation was not observed despite the presence of steatosis in *Nrd1*$^{-/-}$ mice. This indicated that nardilysin has an important role in the initiation and/or promotion of inflammatory responses induced by the CDAA diet. Persistent inflammation distinguishes steatohepatitis from simple hepatic steatosis [1–3]. Among pro-inflammatory factors, TNF-α is one of the key molecules that initiate inflammatory cascades, and its role in the progression of NASH has been discussed [4–7]. For example, apoptotic change in the liver, which contributes to the progression of NASH, is inhibited by an anti-TNF receptor neutralizing antibody or pentoxifylline in a mouse model of NASH [20]. The absence of TNFR1, a receptor for TNF-α, reduces IL6 mRNA production in the liver fed with the HFD even in the presence of elevated serum TNF-α [21]. The absence of TNFR1 also reduces liver lipid accumulation and macrophage accumulation in livers of HFD-fed mice [21]. Thus, inhibition of TNF-α signaling appears to plays a pivotal role to suppress inflammatory reactions in NASH as well as other inflammatory disorders [22]. Although clinical application of anti-TNF-α therapy has not been established in the treatment of human NASH, anti-TNF-α neutralizing antibodies are effectively used to treat various human

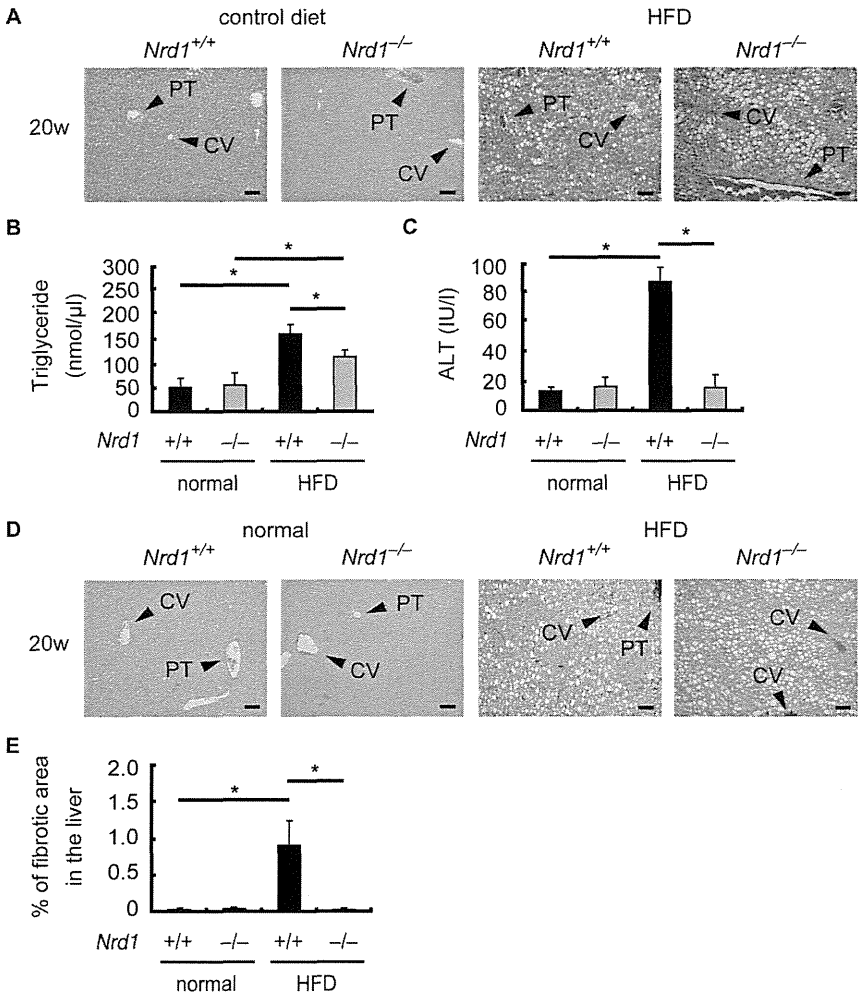

Figure 8. Liver fibrogenesis was not observed in *Nrd1*$^{-/-}$ mice fed the HFD. A. Steatosis was observed in both *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice after 20-week HFD administration (right), but not in those fed a normal control diet (left). Bars indicate 100 μm. B. Quantification of triglyceride in the liver. Triglyceride was elevated in the livers of both *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice after 20-week HFD administration, although it was significantly higher in *Nrd1*$^{+/+}$ mice. n = 4, each. *P<0.05. C. Serum ALT levels were significantly elevated in *Nrd1*$^{+/+}$ mice upon administration of the HFD, but were not elevated in other mouse groups. *P<0.05. D. Fibrotic area was less prominent in *Nrd1*$^{-/-}$ mice than in *Nrd1*$^{+/+}$ mice (right). Bars indicate 100 μm. E. Fibrotic area was observed only in the livers of *Nrd1*$^{+/+}$ mice fed the HFD (right). n = 5, each. *P<0.05.
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inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel diseases [6,7]. We previously reported that nardilysin is essential for the sufficient activation of TNF-α in cooperation with TACE [10–12]. By the knockdown of *Nrd1*, TNF-α secretion is decreased concomitantly with decreased TACE activity, and the production of inflammatory cytokines such as IL6 and IL1-β is significantly suppressed [10–12]. In the present study, it is worth noting that TNF-α secretion from liver specimens was decreased significantly in *Nrd1*$^{-/-}$ mice fed the CDAA diet, while TNF-α production was not different between *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice fed the CDAA diet. Consistently, the production of various inflammatory cytokines were not increased in the livers of *Nrd1*$^{-/-}$ mice. Although the precise mechanism of the decreased inflammatory responses in *Nrd1*$^{-/-}$ mice was not clear, it appeared likely that the impaired release of TNF-α in *Nrd1*$^{-/-}$ mouse livers was one of the reasons for the reduced inflammatory reactions in *Nrd1*$^{-/-}$ mice. As well, impaired recruitment of macrophages into the liver may also contribute to the reduced inflammatory reactions in *Nrd1*$^{-/-}$ mice. It would be also possible that different activation status of TNF-α and inflammatory responses conversely affect difference of fatty contents between *Nrd1*$^{+/+}$ and *Nrd1*$^{-/-}$ mice. Whatever the case, nardilysin seemed to play an important role in the development of steatohepatitis and liver fibrosis presumably through TNF-α activation.

Previous studies have shown that Kupffer cells and recruited macrophages interact with hepatic stellate cells, accelerate their activation, and promote the fibrogenic responses [4,17]. Activated myofibroblasts also promote the remodeling of the extracellular matrix and contribute to liver fibrosis [5]. Indeed, our immunohistochemical analyses showed that Kupffer cells and macrophages were major producers of TNF-α in the livers of mice fed the CDAA diet, and that αSMA-positive myofibroblasts were not prominent in *Nrd1*$^{-/-}$ mice. Decreased release of TNF-α from Kupffer cells and recruited macrophages could be one of the mechanisms for the suppression of diet-induced steatohepatitis in *Nrd1*$^{-/-}$ mice, and thus nardilysin in Kupffer cells and recruited macrophages may be required for the progression of NASH and liver fibrosis, concomitantly with the recruitment of myofibroblasts. However, we could not completely exclude the possible

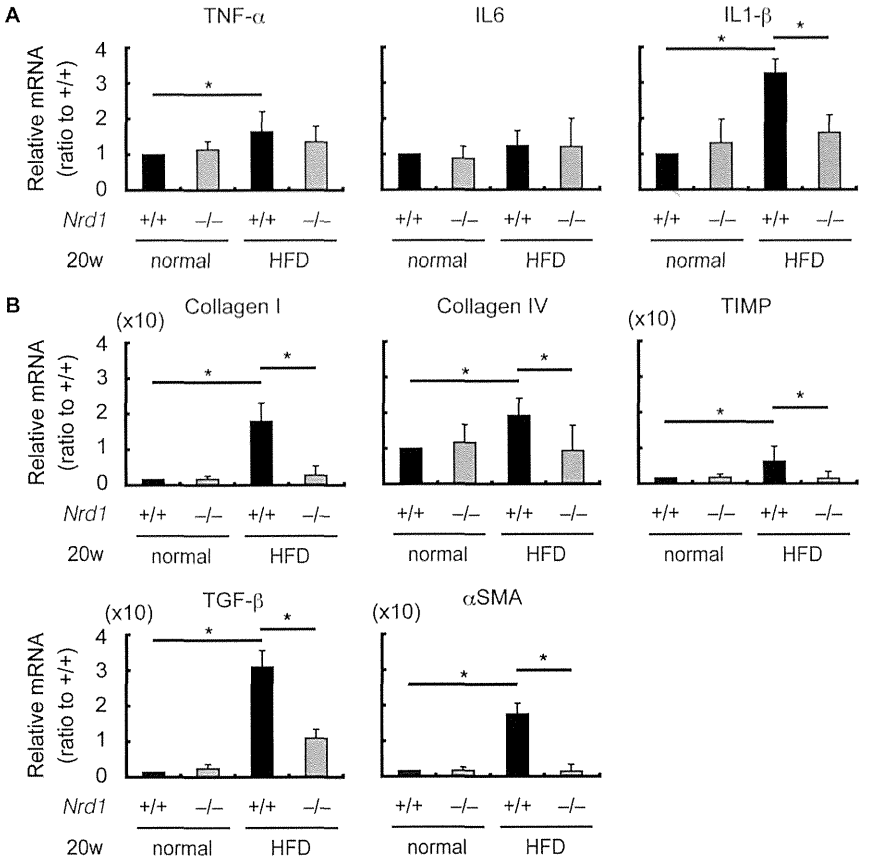

Figure 9. Inflammatory and fibrogenic factors were not increased in *Nrd1*$^{-/-}$ mice fed the HFD. A. mRNA of TNF-α was slightly increased in both *Nrd1*$^{+/+}$ (significantly) and *Nrd1*$^{-/-}$ mice. In contrast to *Nrd1*$^{+/+}$ mice, the mRNA expression level of IL1-β was not increased in *Nrd1*$^{-/-}$ mice. *$P<0.05$. B. mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA in the livers of *Nrd1*$^{+/+}$ mice fed a HFD for 20 weeks were significantly higher than the respective values of *Nrd1*$^{+/+}$ mice fed the control diet. However, they were not altered by HFD in *Nrd1*$^{-/-}$ mice. *$P<0.05$.
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contribution of nardilysin in other cells such as hepatocytes or endothelial cells for the development of NASH and liver fibrosis. Therefore, genetically-engineered mice lacking or strongly expressing nardilysin in Kupffer cells and macrophages may be required to confirm our hypothesis in future studies.

In summary, the present study indicates that nardilysin contributes to the development of diet-induced NASH and liver fibrotic changes by regulating chronic liver inflammation. Nardilysin could be an attractive target for anti-inflammatory therapy against NASH and liver fibrosis.

Author Contributions

Conceived and designed the experiments: SIH HS. Performed the experiments: SIH YM KI MT Yoshito Kimura YT Yuto Kimura YN KK. Analyzed the data: SIH HS KK. Contributed reagents/materials/analysis tools: EN HK. Wrote the paper: SIH HS TC.

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Original article

Daclatasvir combined with peginterferon alfa-2a and ribavirin in Japanese patients infected with hepatitis C genotype 1

Namiki Izumi[1], Osamu Yokosuka[2], Norifumi Kawada[3], Yukio Osaki[4], Kazuhide Yamamoto[5], Michio Sata[6], Hiroki Ishikawa[7], Tomoko Ueki[7], Wenhua Hu[8], Fiona McPhee[8], Eric A Hughes[9], Hiromitsu Kumada[10]*

[1]Musashino Red Cross Hospital, Tokyo, Japan
[2]Chiba University, Chiba, Japan
[3]Osaka City University, Osaka, Japan
[4]Osaka Red Cross Hospital, Osaka, Japan
[5]Okayama University, Okayama, Japan
[6]Kurume University, Kurume, Japan
[7]Bristol-Myers KK, Tokyo, Japan
[8]Bristol-Myers Squibb, Wallingford, CT, USA
[9]Bristol-Myers Squibb Research & Development, Princeton, NJ, USA
[10]Toranomon Hospital, Tokyo, Japan

*Corresponding author e-mail: nizumi@musashino.jrc.or.jp

Background: New direct-acting antiviral agents are currently being developed to treat chronic HCV. The efficacy and safety of daclatasvir combined with peginterferon alfa-2a (alfa-2a) and ribavirin were assessed in a randomized, double-blind Phase IIa study of Japanese patients with chronic HCV genotype-1 infection.

Methods: Japanese patients who were treatment-naive (n=25) or prior null (n=12) or partial (n=5) responders received once-daily daclatasvir 10 mg or 60 mg or placebo in combination with alfa-2a and ribavirin. Daclatasvir recipients with a protocol-defined response (HCV RNA<15 IU/ml at week 4 and undetectable at week 12) were treated for 24 weeks; placebo recipients and patients without a protocol-defined response were treated for 48 weeks.

Results: Sustained virological response at 24 weeks post-treatment (SVR_{24}) was achieved by 89% and 100% of treatment-naive patients receiving daclatasvir 10 mg and 60 mg, respectively, versus 75% in placebo recipients. Virological failure was more frequent in prior non-responder patients, with 50% and 78% achieving SVR_{24} in daclatasvir 10 mg and 60 mg groups, respectively. Adverse events occurred with similar frequency among treatment groups and were consistent with the adverse event profile of alfa-2a/ribavirin alone. The most commonly reported adverse events included pyrexia, alopecia, anaemia, lymphopenia, neutropenia, pruritus and diarrhoea. Three patients discontinued treatment due to anaemia.

Conclusions: Daclatasvir combined with alfa-2a/ribavirin in treatment-naive patients showed greater efficacy than alfa-2a/ribavirin alone and was generally well tolerated. The 60-mg dose of daclatasvir achieved the highest rates of SVR_{24} in both treatment-naive and non-responder populations and will be evaluated in a Phase III clinical trial.

Introduction

Chronic HCV infection is estimated to affect approximately 160 million people worldwide, and 3 to 4 million people become infected with HCV every year [1,2]. In Japan, approximately 1.5 to 2 million people are infected with chronic HCV, primarily HCV genotype 1b [3]. While HCV genotype 1 is the most common, it is also the most challenging to treat.

Treatment for chronic HCV infection has consisted primarily of a combination of interferon and ribavirin. Among treatment-naive patients with HCV genotype 1 infection treated with peginterferon-alfa (alfa) plus ribavirin, the rates of sustained virological response (SVR) ranged from 34% to 46% [4–6]. Recent clinical trials that combined the direct-acting anti-HCV agents

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boceprevir or telaprevir with alfa plus ribavirin achieved higher rates of SVR (68 to 75%) in treatment-naive patients with HCV genotype 1 infection compared with alfa/ribavirin alone [7–12]. However, the high pill burden and associated side effects of these new treatment regimens underscore the need for direct-acting agents with different mechanisms of action, simplified dosing regimens and more desirable tolerability profiles.

Daclatasvir (BMS-790052) is a highly selective, first-in-class HCV non-structural protein 5A (NS5A) replication complex inhibitor with broad genotypic coverage (HCV genotypes 1 to 6) *in vitro* [13,14]. Daclatasvir inhibits HCV RNA replication through interactions with the NS5A protein, a critical component for HCV viral replication [13,15]. Early clinical studies of daclatasvir demonstrated potent antiviral activity and a pharmacokinetic profile that supports once-daily dosing [16]. A previous Phase IIa study demonstrated that a combination of daclatasvir 10 mg or 60 mg once daily plus alfa-2a and ribavirin for 48 weeks resulted in high rates of SVR (83%) in treatment-naive patients with primarily HCV genotype 1a [17]. The tolerability and side effect profile of this triple therapy regimen was similar to that reported with alfa/ribavirin alone.

The efficacy and safety of daclatasvir combined with alfa-2a (Pegasys®) and ribavirin were assessed in Japanese patients with chronic HCV who were naive to treatment or non-responsive to prior treatment with alfa/ribavirin. Response-guided therapy was utilized to determine whether a shorter treatment duration (24 weeks) with daclatasvir would achieve SVRs.

Methods

Study design

This double-blind, randomized, Phase IIa study (Clinicaltrials.gov identifier NCT01017575) assessed the antiviral activity and safety of daclatasvir in combination with alfa-2a/ribavirin in Japanese patients with HCV genotype 1 infection, who were either treatment-naive or prior non-responders (null or partial) to treatment with alfa-2a/ribavirin or alfa-2b/ribavirin. Written informed consent was obtained from all patients. The study was approved by institutional review boards/independent ethics committees at each site and was conducted in compliance with Good Clinical Practice Guidelines and local regulatory requirements, and in accordance with the ethical principles that have their origin in the Declaration of Helsinki.

Patients were enrolled at six sites in Japan from 3 December 2009 through 30 April 2010. Randomization was conducted by the sponsor at a central randomization centre, which provided the patient identification number upon receipt of the investigator's patient registration form. Investigators received treatment kit number assignments by fax from the randomization centre for eligible screened patients.

The main objective of the study was to identify a safe and efficacious dose of daclatasvir in combination with alfa-2a/ribavirin that will allow further evaluation in larger studies.

Patient eligibility criteria

Study participants included Japanese men and women 20 to 70 years of age chronically infected with HCV genotype 1 (HCV RNA$\geq 10^5$ IU/ml) who were treatment-naive (defined as those who had never been exposed to any HCV therapy with interferon-containing regimens, including alfa-2a/ribavirin, or those containing direct-acting agents against HCV), or who were non-responders to previous therapy (defined as patients who failed to achieve ≥ 2 $\log_{10}$ reduction of HCV RNA at week 12 [null responder] or had achieved a ≥ 2 $\log_{10}$ reduction but never attained undetectable HCV RNA levels after at least 12 weeks [partial responder] of the current standard of care, alfa-2a/ribavirin or alfa-2b/ribavirin). Women of childbearing potential were required to use effective methods of contraception, due particularly to the use of ribavirin.

Patients with a history of hepatocellular carcinoma, coinfection with HBV or HIV, other chronic liver disease or evidence of hepatic decompensation were not eligible to participate. Other reasons for exclusion included liver cirrhosis confirmed by laparoscopy, imaging studies or liver biopsy within 24 months prior to screening, alanine aminotransferase (ALT)$\geq 5\times$ upper limit of normal, total bilirubin ≥ 2 mg/dl, international normalized ratio ≥ 1.7, albumin ≤ 3.5 g/dl, haemoglobin <12 g/dl, white blood cells $<3\times 10^9$/l, absolute neutrophil count $<1.5\times 10^9$/l, platelets $<90\times 10^9$/l, creatinine clearance <50 ml/min, inability to tolerate oral medication or gastrointestinal disease, or surgical procedure that may impact absorption of study drug. Patients exposed to any investigational drug (including direct-acting agents) or placebo within 4 weeks prior to dosing of study therapy, or previous exposure to new or investigational HCV therapeutic agents could not participate. Prohibited medications included proton pump inhibitors, moderate/strong inducers or inhibitors of CYP3A4, erythropoiesis-stimulating agents to achieve inclusion criteria and long-term treatment with immunosuppressive agents or agents associated with a high risk of hepatotoxicity or nephrotoxicity.

Treatments

Patients were randomly assigned to receive once-daily oral daclatasvir 10 mg or 60 mg or placebo (treatment-naive only) in combination with weight-based,

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twice-daily ribavirin (600, 800 or 1,000 mg/day for patients weighing ≤60 kg, >60 kg to ≤80 kg or >80 kg, respectively) and once weekly subcutaneous alfa-2a (180 μg; Figure 1). Patients receiving daclatasvir plus alfa-2a/ribavirin who achieved a protocol-defined response (PDR) were treated for 24 weeks. PDR was defined as HCV RNA less than the lower limit of quantification (LLOQ; 15 IU/ml) at week 4 and undetectable at week 12. Patients not achieving PDR received daclatasvir plus alfa-2a/ribavirin for 48 weeks. Patients treated with placebo (treatment-naive only) received alfa-2a/ribavirin for 48 weeks.

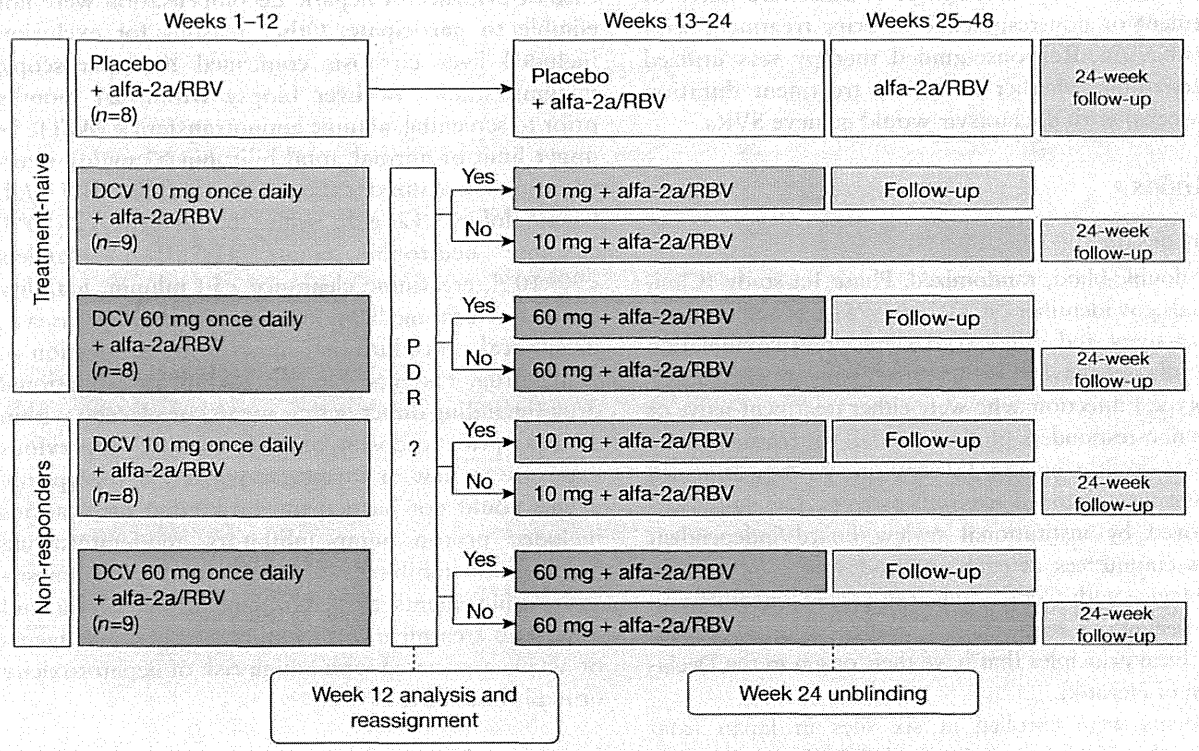
Randomized treatment assignment was double-blind and placebo-controlled for daclatasvir during the first 24 weeks of treatment. The study was unblinded at week 24 and conducted subsequently as open label for patients who did not achieve PDR or were receiving placebo.

Efficacy and safety assessments

The primary efficacy assessment was the proportion of patients with extended rapid virological response (eRVR), defined as undetectable HCV RNA at both week 4 and week 12. Secondary efficacy assessments included the proportion of patients with rapid virological response (RVR), defined as undetectable HCV RNA at week 4; the proportion of patients with complete early virological response (cEVR), defined as undetectable HCV RNA at week 12; the proportion of patients with SVR (defined as undetectable HCV RNA) at week 12 (SVR_{12}) and week 24 (SVR_{24}) post-treatment.

The possible presence of daclatasvir-resistant variants was analysed using stored plasma specimens. Total RNA was isolated, the NS5A region amplified by reverse transcription-PCR, and the resultant amplicon assessed by population sequencing [15]. Resistance testing was performed centrally on all samples at baseline, and on samples indicative of virological failure when HCV RNA was ≥1,000 IU/ml. Virological failure was defined as virological breakthrough (confirmed >1 $\log_{10}$ increase in HCV RNA over nadir or confirmed HCV RNA>LLOQ after confirmed undetectable HCV RNA while on treatment), <1 $\log_{10}$ decrease in HCV RNA from baseline at week 4 of treatment, failure to achieve EVR (defined as <2 $\log_{10}$ decrease in HCV RNA from

Figure 1. Study design



DCV, daclatasvir; PDR, protocol-defined response: HCV RNA< lower limit of quantification (15 IU/ml) at week 4 and undetectable at week 12; alfa-2a, peginterferon alfa-2a; RBV, ribavirin.

baseline at week 12 of treatment), detectable HCV RNA at week 12 and HCV RNA>LOQ at week 24 of treatment, detectable HCV RNA at end of treatment (EOT, including early discontinuation) and relapse (defined as detectable HCV RNA during follow-up after undetectable HCV RNA at EOT).

Assessments including HCV RNA, physical examination, adverse events, laboratory tests, pregnancy test and concomitant medications, were conducted at screening, study day 1 (baseline), weeks 1, 2, 4, 6, 8 and 12, then every 4 weeks until the end of therapy and post-treatment weeks 4, 12 and 24. Twelve-lead electrocardiograms were recorded at screening and on-treatment at weeks 4, 12, 24 and 48. Serum HCV RNA levels were determined at a central laboratory (SRL Inc., Tokyo, Japan) using Roche COBAS® TaqMan® HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan), with LLOQ of 15 IU/ml. HCV genotype and subtype were determined at the central laboratory by PCR amplification and sequencing. *IL28B* genotype was determined by PCR amplification and sequencing of the rs12979860 single nucleotide polymorphism.

Statistical analysis

Using a target sample size of 8 patients per treatment group, a safety event with an incident rate of 19% could be detected with 80% probability. Categorical variables were summarized with counts and percentages, and continuous variables were summarized with univariate statistics. CIs were two-sided with 80% confidence levels; CIs for binary end points were exact binomial and CIs for continuous end points were based on the normal distribution. All statistical analyses were conducted using SAS/STAT® version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Patient disposition and demographic characteristics

A total of 55 patients were enrolled in the study; 2 patients withdrew consent and 10 patients were excluded due to: HCV RNA viral load $<10^5$ IU/ml at screening (2 patients), chronic infection with HCV other than genotype 1 (2 patients), albumin levels ≤3.5 g/dl (3 patients), gastrointestinal disease/surgical procedure potentially impacting study drug absorption (2 patients), abnormal thyroid function or haemoglobin levels <12 g/dl (1 patient had both low albumin and low haemoglobin levels). In total, 42 patients met study criteria and were randomized and treated; 1 patient was randomized but did not receive treatment due to an enlarged lymph node.

Twenty-five patients were naive to treatment. The non-responder group included 12 null responders (<2 $\log_{10}$ decrease in HCV RNA after ≥12 weeks of alfa-2a/ribavirin or alfa-2b/ribavirin) and 5 partial responders (≥2 $\log_{10}$ decrease but never attained undetectable HCV RNA after ≥12 weeks of alfa-2a or alfa-2b/ribavirin).

The majority of Japanese patients were infected with HCV genotype 1b (Table 1), reflecting the high proportion of this subtype in Japan; two treatment-naive patients in the 10 mg daclatasvir dose group were HCV genotype 1a. Other than an imbalance in gender distribution, baseline characteristics were similar across treatment groups (Table 1). Mean HCV RNA levels ranged from 6.5 to 6.9 $\log_{10}$ IU/ml and most patients had a high baseline viral load (>800,000 IU/ml). Treatment-naive patients (19/25) were primarily *IL28B* genotype CC (rs12979860), consistent with the overall distribution of *IL28B* genotypes in Japan (Table 1). Prior non-responders (16/17) were primarily *IL28B* genotypes CT or TT (Table 1).

Table 1. Baseline demographic and disease characteristics

	Treatment-naive patients			Non-responder patients	
Baseline parameter	Placebo (*n*=8)	DCV 10 mg (*n*=9)	DCV 60 mg (*n*=8)	DCV 10 mg (*n*=8)	DCV 60 mg (*n*=9)
Median age, years (range)	54 (41–65)	56 (28–66)	57 (31–67)	53 (26–68)	55 (36–67)
Male gender, *n* (%)	3 (38)	4 (44)	2 (25)	6 (75)	5 (56)
HCV genotype 1b, *n* (%)	8 (100)	7 (78)	8 (100)	8 (100)	9 (100)
Mean HCV RNA, $\log_{10}$ IU/ml (SD)	6.5 (0.65)	6.9 (0.28)	6.5 (0.77)	6.7 (0.43)	6.7 (0.36)
Response to prior alfa/RBV					
Null response, *n* (%)	N/A	N/A	N/A	5 (63)	7 (78)
Partial response, *n* (%)	N/A	N/A	N/A	3 (38)	2 (22)
IL28B genotype (rs12979860)					
CC, *n* (%)	6 (75)	8 (89)	5 (63)	0	0
CT, *n* (%)	1 (13)	1 (11)	3 (38)	6 (75)	8 (89)
TT, *n* (%)	0	0	0	2 (25)	0
Not reported, *n* (%)	1 (13)	0	0	0	1 (11)

alfa, peginterferon alfa; DCV, daclatasvir; N/A not applicable; RBV, ribavirin.

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A total of 39 of 42 patients completed the 24-week double-blind phase of the study. Three patients discontinued due to anaemia; 1 treatment-naive patient receiving 10 mg daclatasvir discontinued at week 12, and 2 prior non-responder patients receiving 60 mg daclatasvir discontinued at week 14 and week 17 of treatment. Twelve patients continued treatment in the open-label phase of the study, 3 of whom discontinued treatment due to virological breakthrough (1 treatment-naive patient receiving 10 mg daclatasvir, 1 non-responder patient receiving 10 mg daclatasvir and 1 non-responder patient receiving 60 mg daclatasvir). One patient in the placebo group requested to discontinue treatment.

A similar number of patients in each treatment group required dose reductions of alfa-2a and ribavirin. In total, 17 patients required alfa-2a reductions (4 in the placebo group, and 2 to 4 in each of the daclatasvir treatment groups), and 35 patients required ribavirin reductions (7 in the placebo group and 6 to 8 in each of the daclatasvir treatment groups).

Virological response and outcomes

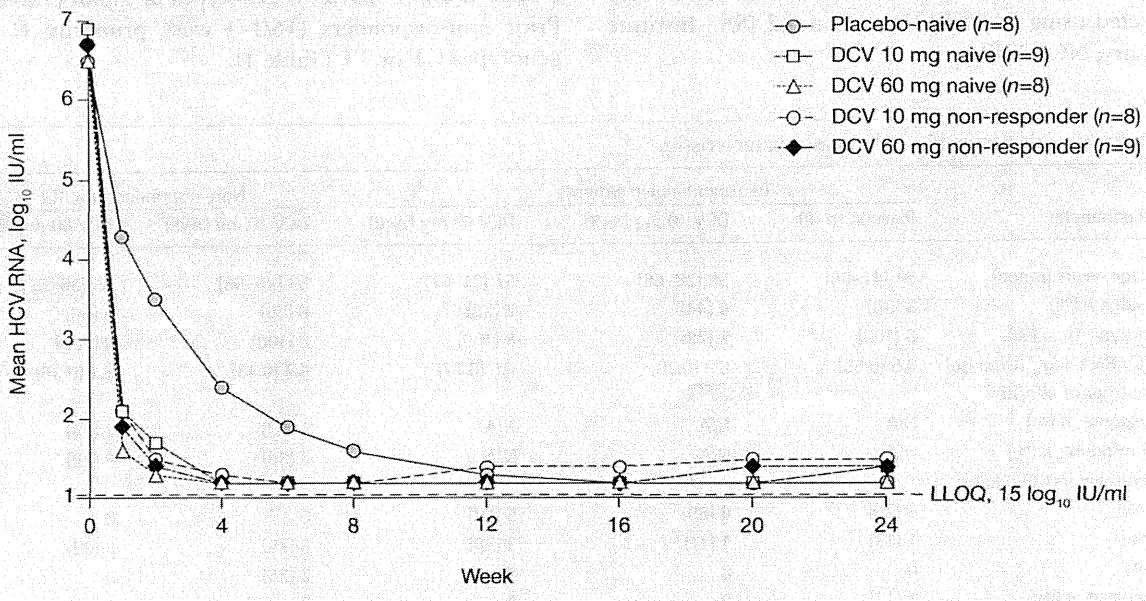
HCV RNA suppression was more rapid in patients treated with daclatasvir compared with patients receiving placebo (Figure 2). By week 2, the mean reduction from baseline in HCV RNA was 5.1 to 5.3 $\log_{10}$ IU/ml in the daclatasvir treatment groups compared with 3.1 $\log_{10}$ IU/ml in the placebo group. The mean improvement in HCV RNA was maintained through week 48 for all daclatasvir dose groups.

Table 2 shows the virological outcomes (RVR, eRVR, cEVR, end of treatment response [EOTR] and SVR_{24}) in all patients and in daclatasvir recipients who achieved PDR. The primary efficacy end point, eRVR, was achieved by 63% to 78% of patients in the daclatasvir treatment groups compared with 13% in the placebo group (Table 2). The rate of eRVR was highest in the non-responder daclatasvir 60 mg dose group. A total of 29 patients (15/25 treatment-naive and 14/17 non-responders) in the daclatasvir treatment groups achieved PDR and completed treatment at week 24 with 24 weeks of follow-up.

Treatment-naive patients

Overall, SVR_{24} was achieved by 89% and 100% of treatment-naive patients receiving daclatasvir 10 mg and 60 mg, respectively, versus 75% of those receiving placebo. PDR was achieved by 78% (7/9) and 100% (8/8) of treatment-naive patients receiving daclatasvir 10 mg and 60 mg, respectively, compared with 13% (1/8) of patients receiving placebo (Table 2). All treatment-naive patients who achieved PDR subsequently achieved EOTR and SVR_{24} after 24 weeks of therapy. One treatment-naive patient in the daclatasvir 10 mg dose group, who was infected with HCV genotype 1b, did not achieve PDR and also failed to achieve SVR_{24}. In treatment-naive patients with *IL28B* genotype CC

Figure 2. HCV RNA reductions through week 24



Virological outcomes in treatment-naive and non-responder patients. DCV, daclatasvir; LLOQ, lower limit of quantification.

Table 2. Virological outcomes in treatment-naive and non-responder patients

	Treatment-naive			Prior non-responders	
	Placebo (*n*=8)	DCV 10 mg (*n*=9)	DCV 60 mg (*n*=8)	DCV 10 mg (*n*=8)	DCV 60 mg (*n*=9)
All patients					
HCV RNA undetectable, week 4 (RVR)	1/8 (12.5; 1.3, 40.6)	7/9 (77.8; 51.0, 93.9)	5/8 (62.5; 34.5, 85.3)	5/8 (62.5; 34.5, 85.3)	8/9 (88.9; 63.2, 98.8)
HCV RNA undetectable, week 12 (cEVR)	5/8 (62.5; 34.5, 85.3)	8/9 (88.9; 63.2, 98.8)	8/8 (100; 75.0, 100.0)	7/8 (87.5; 59.4, 98.7)	8/9 (88.9; 63.2, 98.8)
HCV RNA undetectable, weeks 4 and 12 (eRVR)	1/8 (12.5; 1.3, 40.6)	6/9 (66.7; 40.1, 87.1)	5/8 (62.5; 34.5, 85.3)	5/8 (62.5; 34.5, 85.3)	7/9 (77.8; 51.0, 93.9)
HCV RNA undetectable, EOTR	8/8 (100; 75.0, 100.0)	8/9 (88.9; 63.2, 98.8)	8/8 (100; 75.0, 100.0)	7/8 (87.5; 59.4, 98.7)	8/9 (88.9; 63.2, 98.8)
SVR_{24}	6/8 (75.0; 46.2, 93.1)	8/9 (88.9; 63.2, 98.8)	8/8 (100; 75.0, 100.0)	4/8 (50.0; 24.0, 76.0)	7/9 (77.8; 51.0, 93.9)
Virological breakthrough[a]	0/8 (0)	1/9 (11.1)[b]	0/8 (0)	1/8 (12.5)[b]	1/9 (11.1)[b]
Post-treatment relapse[a]	1/8 (12.5)[c]	0/9 (0)	0/8 (0)	3/8 (37.5)[d]	1/9 (11.1)[b]
Patients with PDR					
HCV RNA<15 IU/ml at week 4, undetectable at week 12 (PDR)	1/8 (12.5; 1.3, 40.6)	7/9 (77.8; 51.0, 93.9)	8/8 (100; 75.0, 100.0)	7/8 (87.5; 59.4, 98.7)	7/9 (77.8; 51.0, 93.9)
HCV RNA undetectable, EOTR	1/1 (100; 10.0, 100.0)	7/7 (100; 72.0, 100.0)	8/8 (100; 75.0, 100.0)	7/7 (100; 72.0, 100.0)	7/7 (100; 72.0, 100.0)
SVR_{24}	1/1 (100; 10.0, 100.0)	7/7 (100; 72.0, 100.0)	8/8 (100; 75.0, 100.0)	4/7 (57.1; 27.9, 83.0)	6/7 (85.7; 54.7, 98.5)

Data are end point (*n*/total *n* [%; 80% CI]) unless otherwise indicated. [a]Data are end point (*n*/total *n* [%]). [b]Genotype *IL28B* CT. [c]Genotype *IL28B* CC. [d]Two patients had *IL28B* CT, one patient had *IL28B* TT. cEVR, complete early virological response; DCV, daclatasvir; EOTR, end of treatment response; eRVR, extended rapid virological response; PDR, protocol-defined response: HCV RNA< lower limit of quantification week 4 and undetectable week 12; RVR, rapid virological response; SVR_{24}, sustained virological response at 24 weeks.

(rs12979860), HCV RNA was undetectable at week 12 (cEVR) in 92% of daclatasvir recipients (7/8 daclatasvir 10 mg and 5/5 daclatasvir 60 mg recipients, respectively) and in 67% (4/6) of patients receiving placebo; by post-treatment week 24 (SVR_{24}), HCV RNA was undetectable in all daclatasvir recipients with *IL28B* genotype CC. Among the small number of treatment-naive patients with *IL28B* genotype CT, 0/1 receiving daclatasvir 10 mg, 3/3 receiving daclatasvir 60 mg and 1/1 receiving placebo achieved SVR_{24}.

Non-responder patients

In the non-responder population, all of whom were non-CC *IL28B* genotype, SVR_{24} was achieved by 50% (4/8) receiving daclatasvir 10 mg and 78% (7/9) receiving daclatasvir 60 mg. PDR was achieved by 88% (7/8) and 78% (7/9) of non-responder patients receiving daclatasvir 10 mg and daclatasvir 60 mg, respectively (Table 2). All non-responder patients who achieved PDR maintained response through the end of therapy. Among patients with PDR, SVR_{24} was achieved by 57% (4/7) of non-responders receiving daclatasvir 10 mg and 86% (6/7) of non-responders receiving daclatasvir 60 mg. In the non-responder group of patients who did not achieve SVR_{24}, five were *IL28B* genotype CT (three daclatasvir 10 mg, two daclatasvir 60 mg) and one patient (daclatasvir 10 mg) was *IL28B* genotype TT.

Virological failure

As expected, virological failure was less frequent in treatment-naive patients than in non-responder patients (Table 2). Treatment-naive recipients of daclatasvir 60 mg had no virological failures, no virological breakthrough and no post-treatment relapse. One treatment-naive patient receiving daclatasvir 10 mg had viral breakthrough, with emergence of the NS5A variants L31V–Y93H. Emergence of the NS5A variants L28M–Y93H, L31M/V–Y93H or R30H–L31V was detected in six prior non-responder patients who failed treatment (four receiving daclatasvir 10 mg, two receiving daclatasvir 60 mg). The predominant resistance pathway to virological failure in patients infected with HCV genotype 1b in this study was via the emergence of substitutions at L31–Y93. Most patients with virological failure had *IL28B* non-CC genotypes (rs12979860), including all six non-responder patients and one treatment-naive patient receiving daclatasvir 10 mg. Y93H was detected at baseline in one treatment-naive patient and in one prior non-responder; both of these patients subsequently achieved SVR. No patient had detectable L31 polymorphisms at baseline.

Safety

There were no consistent differences in adverse events between groups receiving either dose of daclatasvir

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Table 3. Adverse events and selected haematological and laboratory abnormalities

	Treatment-naive patients			Non-responder patients	
	Placebo (*n*=8)	DCV 10 mg (*n*=9)	DCV 60 mg (*n*=8)	DCV 10 mg (*n*=8)	DCV 60 mg (*n*=9)
Event					
Grade 3/4 adverse events, *n*	7	6	6	6	6
Discontinuations due to adverse events, *n*	0	1	0	0	2
Serious adverse events, *n*	0	2	0	0	0
Adverse events (grade 1–4) occurring in >25% of patients in any treatment group					
Pyrexia, *n*	5	6	5	6	8
Anaemia, *n*	5	6	5	4	5
Decreased appetite, *n*	5	2	5	4	4
Alopecia, *n*	6	3	5	2	3
Lymphopenia, *n*	5	2	4	5	3
Malaise, *n*	5	1	4	2	5
Neutropenia, *n*	4	4	3	3	2
Fatigue, *n*	4	4	2	3	2
Headache, *n*	4	3	0	4	4
Insomnia, *n*	2	4	3	2	3
Arthralgia, *n*	2	2	2	2	3
Rash, *n*	3	5	2	0	1
Leukopenia, *n*	3	1	2	2	2
Cough, *n*	1	2	2	1	5
Pruritis, *n*	3	3	3	3	1
Diarrhoea, *n*	3	1	1	1	3
Back pain, *n*	2	2	1	0	4
Nasopharyngitis, *n*	3	1	3	1	1
Cheilitis, *n*	3	2	0	1	0
Injection site reaction, *n*	1	4	0	1	0
Chills, *n*	4	0	0	0	0
Vomiting, *n*	3	0	0	1	0
Thrombocytopenia, *n*	0	0	0	3	0
Grade 3/4 events					
Anaemia, *n*	0	2	1	0	2
Neutropenia, *n*	4	4	3	3	2
Thrombocytopenia, *n*	0	0	0	2	0
Elevated ALT, *n*	0	0	0	0	0
Elevated AST, *n*	0	0	0	1	0
Elevated bilirubin, *n*	0	0	0	0	0

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCV, daclatasvir.

or placebo, nor were there apparent differences in the adverse events profile between treatment-naive versus non-responder patients in the daclatasvir dose groups. No unique adverse events were identified that were attributable to daclatasvir. The most commonly reported adverse events in all dose groups were pyrexia, alopecia, anaemia, lymphopenia, neutropenia, pruritus and diarrhoea (Table 3). The frequency of grade 3/4 adverse events was comparable across treatment groups (Table 3). Severe anaemia was the cause of three patients discontinuing treatment. The events resolved after treatment in one treatment-naive patient in the daclatasvir 10 mg dose group, and without treatment in two non-responder patients receiving daclatasvir 60 mg. There were two serious adverse events (acute pancreatitis and back pain), both occurring in treatment-naive patients receiving daclatasvir 10 mg. No deaths occurred during the study. Overall, the adverse events observed in both the placebo and daclatasvir-containing treatment groups were consistent with the adverse event profile of alfa-2a/ribavirin alone.

Table 3 shows the haematological and laboratory abnormalities by treatment group and between treatment-naive and non-responder patients. The most common abnormalities were haematological related, similar to those frequently observed with alfa-2a/ribavirin. There were no consistent differences in haematological or laboratory abnormalities among groups receiving placebo or daclatasvir (Table 3).

Discussion

The combination of direct-acting antiviral agents, boceprevir or telaprevir, plus alfa/ribavirin has been shown to improve the rates of SVR over that achieved with alfa/ribavirin alone. These newer agents, however, have a high pill burden, are complicated by inconvenient dosing schedules and are associated with frequent adverse events that can be severe, such as rash and anaemia, which may impact tolerability and adherence. Thus, there is a need for more tolerable direct-acting antivirals that provide better efficacy than the standard of care [7,8,11,12].

The results from this Phase IIa study demonstrated rapid antiviral activity when daclatasvir was combined with alfa-2a/ribavirin. The primary antiviral activity end point for this study was the proportion of patients with eRVR (defined as undetectable HCV RNA at both weeks 4 and 12). Both doses of daclatasvir (10 mg and 60 mg) in combination with alfa-2a/ribavirin exhibited greater efficacy than placebo (alfa-2a/ribavirin alone) in inducing eRVR, (63% to 78% versus 13%, respectively). The rate of eRVR was highest for non-responder patients receiving daclatasvir 60 mg compared with the non-responder daclatasvir 10 mg dose group and the treatment-naive daclatasvir dose groups.

A high proportion of treatment-naive patients receiving daclatasvir plus alfa-2a/ribavirin achieved PDR and were treated for only 24 weeks, and all of these patients achieved SVR_{24}. Compared with treatment-naive patients, a similar proportion of patients with prior non-response to alfa/ribavirin achieved PDR, although post-treatment relapse was more frequent in these patients. Rates of post-treatment relapse were comparable in treatment-naive patients and prior non-responder patients who received daclatasvir 60 mg, and more frequent in non-responder patients in the daclatasvir 10 mg dose group. No virological failures were reported in treatment-naive patients receiving daclatasvir 60 mg. One patient receiving daclatasvir 10 mg and one placebo recipient had virological failure (virological breakthrough and post-treatment relapse, respectively). As anticipated, virological failure was more frequent in the non-responder patients (4/8 receiving daclatasvir 10 mg, 2/9 receiving daclatasvir 60 mg). Collectively, these data suggest the higher dose of daclatasvir (60 mg) in combination with alfa-2a/ribavirin can achieve better virological outcomes than the 10 mg daclatasvir dose in both treatment-naive and non-responder patient populations.

In a Phase III study of treatment-naive patients with HCV genotype 1 who were treated with the direct-acting agent telaprevir combined with alfa-2a/ribavirin, the rate of SVR_{24} was 79% among patients with HCV genotype 1b [8]. Kumada *et al.* [18] reported similar results among treatment-naive Japanese patients chronically infected with HCV genotype 1b. The SVR_{24} rates were 73% in patients who received telaprevir combined with alfa-2b and ribavirin compared with 49% in those receiving alfa-2b/ribavirin, although skin disorders, rash and anaemia were more frequent in patients receiving telaprevir [18]. In the present study, the overall rates of SVR_{24} in the treatment-naive population receiving both doses of daclatasvir compared favourably (100% daclatasvir 60 mg, 89% daclatasvir 10 mg) with the studies of telaprevir-treated patients. The SVR rates in this study were also comparable to those reported in a previous dose-finding Phase II study (83%) in which treatment-naive patients infected with HCV genotype 1 (primarily genotype 1a) received 48 weeks of daclatasvir (10 mg or 60 mg) combined with alfa-2a/ribavirin [17].

The efficacy results of this Phase IIa trial clearly demonstrate that the addition of daclatasvir to alfa-2a/ribavirin increases the antiviral activity of the regimen. As might be anticipated, however, response to the addition of a single direct-acting agent such as daclatasvir to alfa-2a/ribavirin appears to remain somewhat dependent on patients' response to alfa-2a/ribavirin, as suggested by the different antiviral activity observed in the treatment-naive and non-responder populations. Of note, the addition of a second direct-acting antiviral may improve the interferon non-responsiveness in this population. A recent study demonstrated SVR rates exceeding 90% in non-responder patients receiving daclatasvir (60 mg) combined with the NS3 protease inhibitor asunaprevir and alfa/ribavirin [19]. This last study suggests that the dependence of a regimen with a single direct-acting antiviral to the patient's response to alfa-2a/ribavirin can be overcome by the addition of a second direct-acting antiviral.

IL28B genotype has been shown to be a strong predictor of alfa-2a/ribavirin-responsiveness, with CC genotype showing the strongest response [20,21]. Data in the present study are limited but generally consistent with a similar effect with this regimen. All treatment-naive patients with *IL28B* CC genotype achieved SVR_{24}, as did three of the four treatment-naive patients with *IL28B* CT genotype. All patients in the non-responder group had *IL28B* non-CC genotypes, which may have contributed to their previous lack of response to alfa/ribavirin as well as the higher rate of virological failure in the present study. Only two patients had signature daclatasvir resistance polymorphisms (Y93H) at baseline, and both achieved SVR. Future studies in larger populations are needed to determine the influence of *IL28B* genotype and baseline NS5A polymorphisms on virological responses with this regimen.

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This study showed that both doses of daclatasvir in combination with alfa-2a/ribavirin were generally well tolerated. Adverse events in patients receiving daclatasvir plus alfa-2a/ribavirin were similar to those observed with alfa-2a/ribavirin alone. The observed safety profile of daclatasvir appears consistent with previous reports of daclatasvir administered alone [16] or in combination with alfa/ribavirin [17,22,23] or other direct-acting antivirals [19,24,25]. In contrast to the recently approved direct-acting antivirals, telaprevir and boceprevir, the addition of daclatasvir to alfa-2a/ribavirin did not appear to impact haematological safety. Patients receiving telaprevir combined with alfa/ribavirin had a higher incidence of adverse events, including anaemia, rash and gastrointestinal disorders, compared with patients receiving alfa/ribavirin alone [8,12,18]. Serious to potentially life-threatening skin reactions have been reported when telaprevir was used in combination with alfa/ribavirin [10]. Anaemia was also reported more frequently in patients receiving boceprevir plus alfa/ribavirin compared with alfa/ribavirin alone [7,11].

The limitations of this Phase IIa study include the relatively small sample size, which precludes definitive conclusions regarding efficacy outcomes as well as safety and tolerability of daclatasvir in combination with alfa-2a/ribavirin. The study population consisted of a Japanese cohort, and thus the data may not be extrapolated to other ethnic populations. Additionally, while most of the patients in this study were HCV genotype 1b, two patients were HCV genotype 1a.

This study demonstrates that addition of daclatasvir to alfa/ribavirin confers a markedly more rapid early virological response in patients with primarily genotype 1b infection. These data, coupled with similarly positive results from a companion study with alfa-2b [26], suggest that further evaluation of daclatasvir in combination with both types of alfa is warranted. Phase III clinical trials of daclatasvir (60 mg once daily) in combination with alfa/ribavirin and/or asunaprevir are currently ongoing.

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NI has received lecture fees from Chugai Co., MSD Co. and Bristol-Myers Squibb in 2012. KY has received research funding from Chugai Co., MSD Co. and Bristol-Myers Squibb in 2012. OY has received lecture fees from Bristol-Myers Squibb, MSD Co. and Chugai Co. in 2012. WH, EAH, HI, FM and TU are employees of Bristol-Myers Squibb. NK, HK, YO and MS have no conflicts to disclose.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Survey of survival among patients with hepatitis C virus-related hepatocellular carcinoma treated with peretinoin, an acyclic retinoid, after the completion of a randomized, placebo-controlled trial

Kiwamu Okita · Namiki Izumi · Kenji Ikeda · Yukio Osaki · Kazushi Numata · Masafumi Ikeda · Norihiro Kokudo · Kazuho Imanaka · Shuhei Nishiguchi · Shunsuke Kondo · Yoichi Nishigaki · Susumu Shiomi · Kazuomi Ueshima · Norio Isoda · Yoshiyasu Karino · Masatoshi Kudo · Katsuaki Tanaka · Shuichi Kaneko · Hisataka Moriwaki · Masatoshi Makuuchi · Takuji Okusaka · Norio Hayashi · Yasuo Ohashi · Hiromitsu Kumada · The Peretinoin Study Group

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Abstract
Background This study examined the effects of peretinoin, an acyclic retinoid, on the survival of patients with hepatitis C virus-related hepatocellular carcinoma (HCC) who had completed curative therapy and participated in a randomized, placebo-controlled trial.
Methods This study was an investigator-initiated retrospective cohort study. Subjects were all patients who were administered the investigational drug (peretinoin 600 mg/day, peretinoin 300 mg/day, or placebo) in the randomized trial. Survivals between the groups were compared using the log-rank test, and hazard ratios were estimated by Cox regression.
Results Survey data were collected from all patients ($n = 392$) who participated in the randomized trial, all of whom were then divided into the peretinoin 600 mg/day ($n = 132$), peretinoin 300 mg/day ($n = 131$), and placebo ($n = 129$) groups. At the median follow-up of 4.9 years, 5-year cumulative survival rates for patients in the 600 mg/day, 300 mg/day, and placebo groups were 73.9, 56.8, and 64.3 %, respectively. Comparison of overall survival among patients classified as Child-Pugh A revealed that

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K. Okita (✉)
Shimonoseki Kohsei Hospital, Kamishinchi-Machi 3-3-8, Shimonoseki, Yamaguchi 750-0061, Japan
e-mail: icb68895@nifty.com

N. Izumi
Musashino Red Cross Hospital, Sakai-minami-cho 1-26-1, Musashino, Tokyo 180-8610, Japan

K. Ikeda · H. Kumada
Toranomon Hospital, Toranomon 2-2-2, Minato-ku, Tokyo 105-8470, Japan

Y. Osaki
Osaka Red Cross Hospital, Fudegasaki-cho 5-30, Tennoji-ku, Osaka 543-8555, Japan

K. Numata · K. Tanaka
Yokohama City University Medical Center, Urafune-cho 4-57, Minami-ku, Yokohama 232-0024, Japan

M. Ikeda
National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277-8577, Japan

N. Kokudo
The University of Tokyo Hospital, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan

K. Imanaka
Osaka Medical Center for Cancer and Cardiovascular Diseases, Nakamichi 1-3-3 Higashinari-ku, Osaka 537-8511, Japan

S. Nishiguchi
Hyogo College of Medicine Hospital, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan

S. Kondo · T. Okusaka
National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan

Y. Nishigaki
Gifu Municipal Hospital, 7-1 Kashima-cho, Gifu 500-8513, Japan

S. Shiomi
Osaka City University Hospital, Asahi-machi 1-5-7, Abeno-ku, Osaka 545-8585, Japan

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survival of the 600 mg/day group ($n = 105$) was significantly longer than that of the placebo group ($n = 108$) (hazard ratio 0.575, 95 % CI 0.341–0.967; $P = 0.0347$).
Conclusions Administration of 600 mg/day peretinoin to patients with hepatitis C virus-related HCC who have completed curative therapy may improve survival for those classified as Child-Pugh A, for whom liver function is relatively stable.

Keywords Hepatocellular carcinoma · Hepatitis C virus · Multicentric carcinogenesis · Overall survival · Peretinoin

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, affecting 740,000 people annually [1]. The incidence of HCC has been rising due to an increase in hepatitis C virus (HCV) infections [2–4]. HCC comprises 94 % of all primary liver cancers in Japan; one prominent cause is infection by HCV [4]. Hepatitis C virus-related HCC (HCV-HCC) comprises 75 % of all HCC cases, while HCC due to hepatitis B virus infection (HBV-HCC) comprises 10–15 % [5].

Early diagnosis and curative treatment for HCC, such as hepatectomy or radiofrequency ablation (RFA, a localized therapy), has become more prevalent in recent years. However, high recurrence rates and unfavorable prognoses remain even after curative treatment for HCV-HCC. Cumulative recurrence rates of HCV-HCC are high, with reported rates of 24, 76, and 92 % at 1, 3, and 5 years, respectively, after treatment for the initial onset of liver cancer [6]. Characteristics of HCC recurrence include metastases within the liver, and many patients experience multicentric cancer onset due to underlying liver conditions (e.g., cirrhosis) related to viral liver disease. As underlying liver disease can provide optimal sites for oncogenesis, suppression of multicentric cancer onset is particularly important for improving HCC prognosis, even after curative treatment. Hepatitis virus-related HCC is often treated effectively by antiviral therapies such as interferon [7–9]. However, strategies to control recurrence following cured HCC have not yet been established [10–12].

One method used to control cured HCC recurrence employs a retinoid-based chemoprevention strategy [13]. Peretinoin [(2*E*,4*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,4,6,10,14-pentaenoic acid] is a retinoid with a vitamin A-like structure discovered by Muto et al. (1980) [14], and currently is one of the potential drugs anticipated to suppress HCC recurrence [15–17].

According to a randomized trial [18], administration of 600 mg/day peretinoin suppressed HCV-HCC recurrence following curative therapy. In Japan, 75 % of patients with HCC test positive for HCV, and compared to other causes of HCC, HCV has the highest risk of recurrence. Thus, the randomized trial aimed to examine the suppressive effects of peretinoin on recurrence among a population with a homogeneously high risk for recurrence, and thus focused on patients who tested positive for HCV following curative therapy. The trial was a multicenter, parallel-group, double-blind, randomized, placebo-controlled trial. Patients who tested positive for HCV (negative for HBV) and underwent liver resection or RFA to treat initial onset or initial recurrence, and who were classified as Child-Pugh A or B, were assigned to one of the following three groups: peretinoin 600 or 300 mg/day, or placebo. The investigational drug was administered orally once a day, for no longer than 2 years. Of the 401 patients registered in the randomized trial between March 2005 and July 2007, 268 were administered the investigational drugs (peretinoin 600 mg/day, $n = 134$; peretinoin 300 mg/day, $n = 134$; placebo, $n = 133$). Our analysis revealed no superiority of the treatment groups (peretinoin 600 and 300 mg/day) over the placebo group with respect to the primary endpoint of recurrence-free survival ($P = 0.434$). However, the highest 3-year cumulative recurrence-free survival rate (43.7 %) was found in the group administered 600 mg/day peretinoin. The peretinoin 600 mg/day group also showed a significantly lower recurrence rate after 2 years relative to that of the placebo group (hazard ratio 0.27; 95 % CI

K. Ueshima · M. Kudo
Kinki University School of Medicine, 377-2, Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan

N. Isoda
Jichi Medical University, Yakushiji 3311-1, Shimotsuke-shi, Tochigi 329-0498, Japan

Y. Karino
Sapporo Kosei General Hospital, Kita 3-jo Higashi 8-choume-5, Chuo-ku, Sapporo, Hokkaido 060-0033, Japan

S. Kaneko
Kanazawa University Hospital, Takara-machi 13-1, Kanazawa, Ishikawa 920-8641, Japan

H. Moriwaki
Gifu University Hospital, Yanagido 1-1, Gifu 501-1194, Japan

M. Makuuchi
Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo 150-8935, Japan

N. Hayashi
Kansai Rosai Hospital, Inabaso 3-1-69, Amagasaki, Hyogo 660-8511, Japan

Y. Ohashi
Chuo University, Kasuga 1-13-27, Bunkyo-ku, Tokyo 112-8551, Japan

0.07–0.96). Sub-group analysis revealed that within the peretinoin 600 mg/day group, the recurrence risk also decreased significantly among those classified as Child-Pugh A (hazard ratio to the placebo group, 0.60; 95 % CI 0.41–0.89) and those with tumor size under 2 cm (hazard ratio to the placebo group, 0.41; 95 % CI 0.23–0.73). Notably, the follow-up duration for the randomized trial was short (median, 2.5 years), and we did not evaluate overall survival among groups.

This study was conducted as an investigator-initiated research, independently of the randomized trial to evaluate the effects of peretinoin on the overall survival up to 6 years of follow-up.

Methods

Study design

This study was a retrospective cohort study which assessed survival outcomes of all participating patients to the randomized trial from the time the trial ended through December 31, 2011. The randomized trial was conducted in accordance with Good Clinical Practice guidelines.

Setting and participants

The principal investigator obtained permission to conduct the study from the ethics committee of Shimonoseki Kohsei Hospital. Approval for data provision was obtained in accordance with the respective institution's regulations. The study was conducted in compliance with the Declaration of Helsinki, as well as the "Ethical Guidelines for Epidemiological Research" set in Japan by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labour, and Welfare.

Of the 41 medical institutions where the randomized trial was conducted, we targeted all the institutions that provided written consent to participate in this cohort study and used a data collection form to collect data in the medical records. The collected data included the dates of any deaths up through December 31, 2011 and the last recorded date of survival (in cases where observation was not possible). Data collection forms were collected between January 1, 2012 and December 31, 2012. Data collection forms were mailed from the data center to the medical institutions providing the data. At the medical institutions providing the data, either the physician in charge of the study or the person delegated by this physician provided answers to the questions according to existing resources (medical records, etc.). Anonymized data collection forms were then sent back to the data center.

The primary endpoint was overall survival, defined by the time period beginning on the date of patient registration for the randomized trial until death from all causes or the final date when survival was confirmed. Patients for whom survival was last confirmed before December 2011 were treated as "lost to follow-up."

At the medical institutions providing the data, if a delegate of the physician in charge of the study completed the data collection form, the physician validated the data such that data reliability was guaranteed. The data center conducted an independent and careful review of the collected data according to the data management plan, and then created and maintained the database.

Statistical analysis

In accordance with the principle of the intention-to-treat analysis, this study analyzed data from all patients who had been administered the investigational drug in the randomized trial. Cumulative survival rates in each treatment group were calculated by the Kaplan–Meier method with the dates of each patient registration for the randomized trial as the starting point. The log-rank test was used to compare the 600 mg/day peretinoin and placebo groups, and the 300 mg/day peretinoin and placebo groups. We also calculated hazard ratios and 95 % confidence intervals (CIs) using the Cox proportional hazards model. Two-tailed statistical significance was set at $P < 0.05$, and P values were not adjusted for multiplicity. All data from December 31, 2011 onwards were treated as censored.

In sub-group analyses, the same survival time analysis method used to evaluate primary endpoints was employed on the classification factors which were set in advance. Classification factors included Child-Pugh classification (A, B), tumor size (<2 cm, ≥2 cm), curative treatment procedure (local ablation, resection), sex (male, female), and age when consent was provided (<65 years, 65–74 years, ≥75 years). All statistical analyses were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). This study is registered in the UMIN Clinical Trials Registry (UMIN000006728).

Results

Patients

Survey data were collected from all 392 patients (600 mg/day, $n = 132$; 300 mg/day, $n = 131$; placebo, $n = 129$) (Fig. 1). Patient demographic factors are summarized in Table 1. Sex, age, treatment approach, Child-Pugh classification, and primary tumor size were well balanced among the three treatment groups. Number of deaths in the

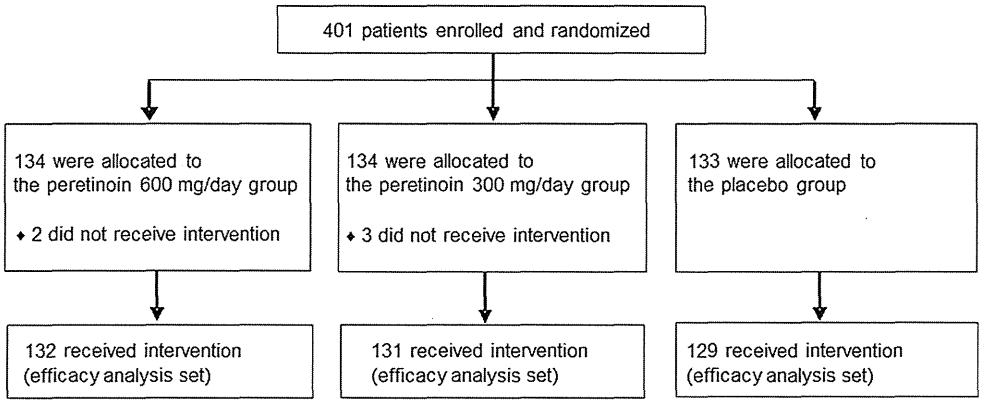

Fig. 1 Flow diagram of patient selection. For the randomized trial, a total of 401 individuals were registered and assigned randomized treatments (peretinoin 600 mg/day, $n = 134$; peretinoin 300 mg/day, $n = 134$; placebo, $n = 133$). Of these, those who were ultimately not administered the investigational drug (2 in the 600 mg/day group, 3 in the 300 mg/day group, and 2 in the placebo group) were excluded, and data from the remaining patients were analyzed (peretinoin 600 mg/day, $n = 132$; peretinoin 300 mg/day, $n = 131$; placebo, $n = 129$)

Table 1 Patient demographics

	Peretinoin		Placebo ($n = 129$)	P value (χ^2)
	600 mg/day ($n = 132$)	300 mg/day ($n = 131$)		
Sex				
Male	85 (64.4 %)	76 (58.0 %)	89 (69.0 %)	0.1806
Female	47 (35.6 %)	55 (42.0 %)	40 (31.0 %)	
Age (years)[a]				
<65	38 (28.8 %)	42 (32.1 %)	41 (31.8 %)	0.7331
65–74	69 (52.3 %)	62 (47.3 %)	57 (44.2 %)	
≥75	25 (18.9 %)	27 (20.6 %)	31 (24.0 %)	
Curative treatment				
Local ablation	85 (64.4 %)	84 (64.1 %)	85 (65.9 %)	0.9497
Resection	47 (35.6 %)	47 (35.9 %)	44 (34.1 %)	
Child-Pugh				
A	105 (79.5 %)	107 (81.7 %)	108 (83.7 %)	0.6842
B	27 (20.5 %)	24 (18.3 %)	21 (16.3 %)	
Tumor size				
<2 cm	58 (43.9 %)	58 (44.3 %)	59 (45.7 %)	0.9531
≥2 cm	74 (56.1 %)	73 (55.7 %)	70 (54.3 %)	

[a] Age at the time of registration in the randomized trial

600 mg/day group, the 300 mg/day group, and the placebo group were 36, 55, and 47, respectively. In the 600 mg/day group, the 300 mg/day group, and the placebo group, 19, 15, and 15 patients, respectively, were "lost to follow-up."

The median follow-up duration was 1,782 days (4.9 years), with a maximum of 2,364 days (6.5 years). Median and maximum follow-up durations for each of the groups were as follows: 1,815 days (5.0 years) and 2,364 days (6.5 years) for the 600 mg/day group, 1,681 days (4.6 years) and 2,320 days (6.4 years) for the 300 mg/day group, and 1,768 days (4.8 years) and 2,336 days (6.4 years) for the placebo group.

Overall survival

Table 2 shows cumulative survival rates determined using the Kaplan–Meier method. The 2-year survival rates in the 600 mg/day, 300 mg/day, and placebo groups were 93.1, 88.5, and 93.0 %, respectively. The 5-year survival rates in the 600 mg/day, 300 mg/day, and placebo groups were

Table 2 Patients with follow-up and survival rates

Group	*n*	Censored[a]	Event[c]	Survival rate	
				2 years	5 years
600 mg/day	132	96 (19[b])	36	93.1 %	73.9 %
300 mg/day	131	76 (15[b])	55	88.5 %	56.8 %
Placebo	129	82 (15[b])	47	93.0 %	64.3 %

Median follow-up time: 4.9 years

[a] Data from after 12/31/2011 were considered censored cases

[b] If the last date on which survival was confirmed was before December of 2011, then the patient was considered lost to follow-up

[c] Death due to any cause was considered an event

73.9, 56.8, and 64.3 %, respectively, with the maximum cumulative survival rate observed in the 600 mg/day group. Median survival time in the 300 mg/day and placebo groups were 2,102 days (5.8 years) and 2,165 days (5.9 years), respectively; this parameter could not be calculated for the 600 mg/day group due to good prognosis. Figure 2 shows the Kaplan–Meier curves for the 600 mg/day and placebo groups. With regard to overall survival, no significant difference was observed between the 600 mg/day and placebo groups (hazard ratio 0.726; 95 % CI 0.470–1.122; $P = 0.1475$ by the log-rank test). Similarly, no significant difference in overall survival was observed between the 300 mg/day and placebo groups (hazard ratio 1.253; 95 % CI 0.849–1.850; $P = 0.2547$ by the log-rank test).

Sub-group analysis (600 mg/day vs. placebo group)

Results from the sub-group analysis of survival time, which compared the 600 mg/day and placebo groups, are shown in Fig. 3. Factors from the sub-group analysis that were significant at the $P < 0.05$ level by the log-rank test included the Child-Pugh A classification (hazard ratio 0.575; 95 % CI 0.341–0.967; $P = 0.0347$ by the log-rank test) and primary tumor size under 2 cm (hazard ratio 0.447; 95 % CI 0.218–0.919; $P = 0.0245$ by the log-rank test). Kaplan–Meier curves by Child-Pugh A classification for the 600 mg/day and placebo groups (600 mg/day, $n = 105$; placebo, $n = 108$) are shown in Fig. 4.

Discussion

Early diagnosis and curative treatment for HCC has become widespread, but following completion of curative treatment for HCV-HCC, recurrence rates remain high and prognosis is poor. The 5-year cumulative survival rate in this cohort study was higher in the 600 mg/day peretinoin group than in the placebo group. Particularly for patients with the Child-Pugh A classification, the 600 mg group had significantly longer survival compared to the placebo group. We believe that this finding will contribute to future attempts for developing measures to prevent HCC recurrence.

The guidelines for clinical studies of HCC [19] recommend that only patients with the Child-Pugh A classification be incorporated into clinical studies, because death due to cirrhosis among patients classified as Child-Pugh B or Child-Pugh C could mask treatment effects. In this study, roughly 80 % of our patients were classified as Child-Pugh A, and thus our results relating to this group may be generalizable. In addition, the results of this study, expressed in patients with tumor size of under 2 cm, are consistent with the report of Nakashima et al. [20] that suggests the incidence of multicentric recurrence is high amongst

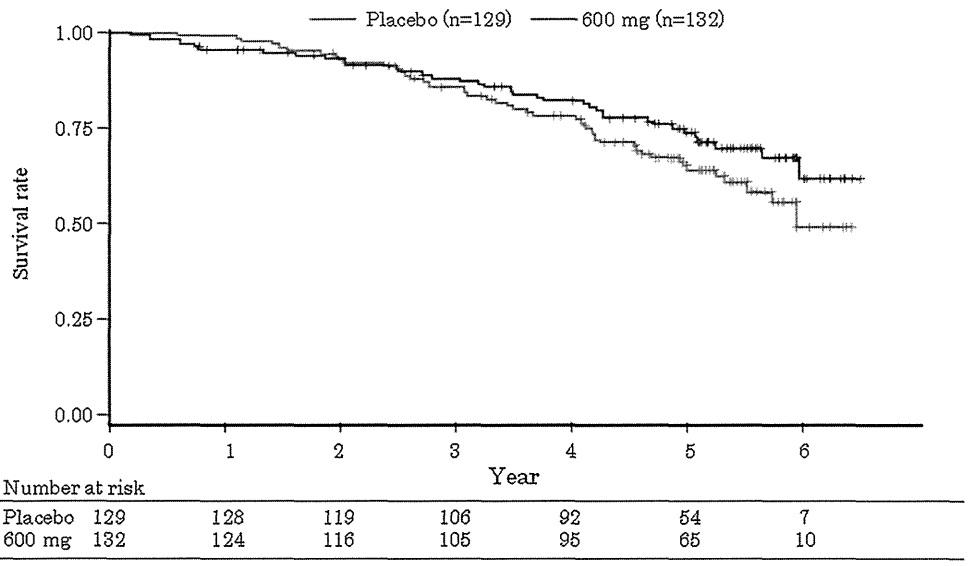

Fig. 2 Kaplan–Meier plot of overall survival: peretinoin 600 mg/day vs placebo. Hazard ratio 0.726; 95 % CI 0.470–1.122; $P = 0.1475$ by log-rank test