DNA fragments of IRF7 were inserted into the vector pcDNA4/TO/myc-His (Invitrogen). The expression plasmids for p50 and p65 were kindly provided by Dr. Rongtuan Lin (Lady Davis Institute for Medical Research, Baltimore, MD). pcDNA4/TO/myc-His vector (Invitrogen) was used as control for mock transfection.

BLC were transfected with *IFNλ4* plasmids or control plasmids by electroporation using Gene Pulser Xcell Electroporation System (BIO RAD, Hercules, CA). After 24 h, cells were treated with mock, recombinant IFNα-2b (100 IU/ml) (Schering-Plough) for 24h. *IFNλ4* plasmids and *IRF7* plasmids or control plasmids were co-transfected into HEK293T cells with Lipofectamine LTX reagent (Invitrogen) and Opti-MEM medium, according to the manufacturer's instructions. Total RNA was extracted and quantified by real-time qRT-PCR.

Luciferase assays. *IFNλ4* or control plasmids were transfected into HEK293/IL28B-luc cells and the cells were treated with IFNα for 24h next day. HEK293/IL28B-luc cells were co-transfected with IFNλ4 plasmids and IRF7, p50: p65 or control plasmids and incubated for 24h. MTS viability and single luciferase assays were conducted by 1420 Multilabel Counter (ARVO MX, PerkinElmer, Boston, MA) using a CellTiter 96 AQueous One Solution System (Promega, Madison, WI) and a Bright-Glo Luciferase Assay System (Promega), as previously described [25, 26].

Statistical analyses. The data were analyzed using the Welch's *t* test for continuous variables and the chi-square test for categorical data. p values < 0.05 were considered statistically significant.

Results

Genotype of *IL28B* SNP and expression of *IL29*, *IL28A*, and *IL28B* mRNA in PBMC.
Three SNPs near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were genotyped. The number of patients with each genotype is shown in Table 1. In agreement with a recent

20141225

report from the HapMap Project in Asia [21], the genotype of ss469415590 was completely correlated with that of rs12979860 in this study, while 3 of 50 patients have different genotype between ss469415590 and rs8099917. Baseline mRNA expression levels of *IL29*, *IL28A*, and *IL28B* were not influenced by the rs12979860 genotype (Fig. 1A). However, when PBMCs were stimulated with IFNα and poly(I:C), the induction of *IL28B* expression was significantly lower in patients with the *IL28B*-unfavorable genotype (rs12979860 CT/TT) than in those without (rs12979860 CC) (p = 0.049) (Fig. 1B).

Relationship of therapy response with *IL29*, *IL28A*, and *IL28B* mRNA levels in PBMC. We assessed the relationship between the expression level of the *IFNλ*s and the virological response to PEG-IFNα/RBV therapy. At baseline, there was no significant difference in *IFNλ*s expression between the SVR, relapser, and NR patients (data not shown). On the other hand, the induction of *IL28B* expression by IFNα and poly(I:C) decreased with the patients' response to therapy (Fig. 2A). The mRNA levels of NR patients were significantly lower than those for relapsers (p = 0.04) as well as VR (p=0.005). The induction of *IL29* expression of NR patients were lower than those for VR (p=0.048). In contrast, the induction of *IL28A* did not reveal any association between mRNA levels and treatment response.

When the *IL28B* induction levels of VR and NR patients were further stratified by genotype, it was significantly lower in NR than in VR patients in both rs12979860 CC and CT/TT subgroups (p = 0.01 and 0.02, respectively) (Fig. 2B).

Furthermore, 11 of 32 non-SVR patients were re-treated with NS3 protease inhibitor (TVR or SMV) plus PEG-IFNα/RBV triple therapy; 2 of them were IL28B-favorable and 9 were unfavorable. Even treated with NS3 protease inhibitor, it should be noted that IL28B inductions in non-SVR of the triple therapy were significantly lower than those in SVR of the PEG-IFNα/RBV therapy or triple therapy (p=0.017). IL28B inductions in non-SVR were also

20141225

lower than those in SVR of triple therapy (3.5 vs 12.1 fold induction). IL28A inductions in non-SVR of the triple therapy were also significantly lower than those in SVR (p=0.042) (Fig. 2C).

Impact of *IL28B* genotype and induction on *IFNλ4* mRNA expression. We measured the expression level of *IFNλ4* in PBMCs derived from CHC patients. Because we could not detect *IFNλ4* mRNA in PBMCs with RNA sequencing nor the previously reported TaqMan real-time quantitative RT-PCR system [21], we designed a new highly sensitive RT-PCR system using 4 sets of primers. The detection threshold was as low as 1–10 copies/assay (Supplementary Table 1; Supplementary Fig. 1A). This RT-PCR assay allowed us to confirm the full length mRNA sequence of *IFNλ4* in poly(I:C)-treated HepG2, HeLa, HEK293T cells, and BLC from ss469415590-ΔG/ Δ G patients by amplicon sequencing (Supplementary Figs. 1B,C).

Using this system, we tested PBMCs from 47 CHC patients for the presence of *IFNλ4* mRNA. Among the 23 patients with *IL28B*-unfavorable rs12979860 [T] and ss469415590 [ΔG]-allele, *IFNλ4* mRNA was detected in 12 patients (7 in non-stimulated PBMCs and 8 in IFN-poly(I:C)-stimulated PBMCs). In marked contrast, *IFNλ4* mRNA was not detected in any of the *IL28B*-favorable patients (Supplementary Fig. 2). There was no significant difference in baseline expression of *IFNλ*s between patients with or without detectable *IFNλ4* expression (Fig. 3A). However, the induction of *IL28B* expression by IFN-poly(I:C) was significantly lower in patients with *IFNλ4* mRNA than those without detectable *IFNλ4* (p = 0.008) (Fig. 3B). Even among *IL28B*-unfavorable patients (rs12979860 CT/TT), *IL28B* induction levels were significantly lower in *IFNλ4*-positive patients (p = 0.04) (Fig. 3B). Although induction of *IL28A* was lower in *IFNλ4*-positive patients than *IFNλ4*-negative patients (p = 0.04), there was no significant relation between *IFNλ4* expression and the induction of *IL28A* and *IL29* among *IL28B*-favorable patients.

20141225

Association between *IFNλ4* expression and clinical response to antiviral therapy. The rate of virological non-response was significantly higher in patients with *IFNλ4* mRNA than in all those without detectable *IFNλ4* (p = 0.003; Fig. 3C). Among the *IL28B*-unfavorable patients (rs12979860 CT/TT), the virological non-response rate also tended to be higher in patients expressing *IFNλ4* (p = 0.08).

Suppression of *IL28B* induction by *IFNλ4* in vitro. The mechanism behind the lower induction of *IL28B* mRNA in CHC patients expressing *IFNλ4* was investigated by testing whether the expression of *IL28B* is influenced by overexpression of *IFNλ4* in vitro. When IFNλ4 was overexpressed, baseline expression of IL28B was significantly increased in HEK293, BLC (Supplementary Fig. 3). However, as shown in Fig. 4A, IL28B expression was increased by IFNα (1.8 fold induction, p=0.012) but that induction was suppressed in the presence of IFNλ4 (1.2 fold induction, p=0.28) in BLC. As *IL28B* promoter is known to be activated by the transcription factors such as IRF7 and NFκB [11, 31], we next evaluated IL28B induction by IRF7. *IL28B* mRNA was induced by IRF7 in dose dependent manner and the induction levels were suppressed by *IFNλ4* overexpression significantly (Fig. 4B). *IL28B* promoter activities induced by IFNα, IRF7 and p50:p65 were also inhibited by *IFNλ4* overexpression (Fig. 4C-E).

Discussion

The present study shows that the inducibility of *IL28B* expression is associated with virological responsiveness to IFNα in CHC patients, and it is also related to the *IL28B* genotype. Furthermore, we detected *IFNλ4* mRNA in PBMCs using an original sensitive RT-PCR system. *IFNλ4* suppressed *IL28B* induction and associated with virological non-responses to IFNα-based antiviral therapy.

20141225

Earlier studies reported the lower production of *IL28B* in blood cells of *IL28B*-unfavorable CHC patients [5, 27]. However, the relationship between *IL28B* genotype and expression level remained controversial, probably due to the very low expression level of *IL28B*. In the present study, there was no significant difference in baseline expression level between the *IL28B* genotypes. However, stimulation to PBMCs with IFNα and poly(I:C) raised *IL28B* expression, and this induction was significantly lower in *IL28B*-unfavorable CHC patients. More importantly, the degree of *IL28B* induction was positively correlated to the responsiveness to PEG-IFNα/RBV therapy.

Our findings are consistent with a previous study showing ex vivo induction of *IL28B* by TLR7 agonists [28], and we further confirmed *IL28B* inducibility using IFNα and poly(I:C), which mimic exogenous IFNα administration in HCV patients. Because IFNλ is an essential element of innate anti-HCV responses [16, 29, 30], our data suggest that inadequate induction of IL28B is primarily responsible for virological non-response to IFNα-based therapy.

To elucidate the mechanisms responsible for the genotype-specific inducibility of *IL28B*, we focused on *IFNλ4*. We report, for the first time, the presence of *IFNλ4* mRNA in PBMCs derived from CHC patients with the *IL28B*-unfavorable allele. We could not detect *IFNλ4* mRNA with the previously reported TaqMan real-time RT-PCR system [21]. *IFNλ4* expression was confirmed with a highly sensitive RT-PCR system we designed for this study, which could detect even a single copy of *IFNλ4* mRNA per assay. Although *IFNλ4* mRNA was not detected in 16 of the 23 unstimulated PBMC samples of CHC patients with the *IL28B*-unfavorable genotype, we cannot exclude the presence of *IFNλ4* mRNA under the detection limit of this RT-PCR system in these patients. However, it is important to mention that detectable level of *IFNλ4* expression was associated with NR and more severe impairment of *IL28B* induction. These data suggest that the baseline expression of *IFNλ4* in

PBMCs is responsible for the non-response to IFNα treatment through suppression of *IL28B* induction.

Our in vitro experiments in cell lines demonstrated that *IL28B* induction by IFNα, IRF7 or NFκB was suppressed by IFNλ4 overexpression. These data are consistent with the relationship between *IFNλ4* and *ISG* induction [20-22]. Our finding of base line *IL28B* induction by *IFNλ4* is also reasonable because *IFNλ* promoters contain IFN-stimulated response element (ISRE) sites [11, 31] that could be activated by IFNλ4 through STAT1 and STAT2 phosphorylation [21]. IFNλ4 may pre-activate IL28B promoter through ISRE activation, and moreover, it may influence NFκB-induced promoter activity by unknown mechanism. Our in vitro data support our observation in the clinical samples, and suggest that the expression of *IFNλ4* in immune cells of *IL28B*-unfavorable CHC patients may weakly induce basal *IL28B* expression, which may be insufficient for HCV eradication [32]. But it may prevent additional induction of *IL28B* by exogenous IFNα treatment through impairment of *IL28B* promoter activity. The molecular mechanism by which *IFNλ4* suppresses *IL28B* mRNA induction and promoter activation should be further investigated, although *IFNλ4* may also have important functions affecting IFN regulation [20, 33, 34].

The lower induction of *IL28B* might be caused by the decrease of the frequency of IFNλs producing cells. However, in the present study, because we measured the expression of *IFNλ*s in all PBMCs, we could not specify the subset of IFNλ4 producer cells. A recent study demonstrated that blood dendritic cell antigen 3 (BDCA3)[+] dendritic cells (DCs) produce IFNλ3 and expression levels of *IL28B* from BDCA3+ DCs were significantly higher in subjects with *IL28B* major than those with minor type in response to HCV infection [35]. In their experiment, large volumes of blood samples (i.e., 400 ml) were required to sort very small populations of BDCA3+DC (0.054% of all PBMCs), but obtaining such a large amount

20141225

of blood per patient was ethically impossible in our study. We also considered that *IFNλ4* mRNA levels might be higher when analyzed in those specific IFNλ producer cells.

In conclusion, the induction of *IL28B* mRNA expression by ex vivo stimulation with IFNα and poly(I:C) in PBMCs was significantly associated with virological responsiveness in CHC patients treated with IFNα-based therapy. The impaired induction of *IL28B* was associated with the expression of *IFNλ4,* generated by unfavorable dinucleotide polymorphisms near the *IL28B* gene. These data improve our understanding of IFN resistance and may lead to the development of new antiviral therapies targeting the IFNλ induction system.

20141225

Reference

1 Alter MJ. Epidemiology of hepatitis C. Hepatology 1997;26:62S-65S.

2 Ghany MG, Strader DB, Thomas DL, et al. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 2009;49:1335-1374.

3 Ghany MG, Nelson DR, Strader DB, et al. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. Hepatology 2011;54:1433-1444.

4 Forestier N, Zeuzem S. Triple therapy with telaprevir: results in hepatitis C virus-genotype 1 infected relapsers and non-responders. Liver Int 2012;32 Suppl 1:44-50.

5 Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.

6 Ge D, Fellay J, Thompson A, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.

7 Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.

8 Rauch A, Kutalik Z, Descombes P, et al. Genetic Variation in IL28B Is Associated With Chronic Hepatitis C and Treatment Failure: A Genome-Wide Association Study. Gastroenterology 2010;138:1338-U1173.

9 Kotenko S. The family of IL-10-related cytokines and their receptors: related, but to what extent? Cytokine Growth Factor Rev 2002;13:223-240.

10 Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63-68.

20141225

11 Osterlund P, Pietilae T, Veckman V, et al. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J Immunol 2007;179:3434-3442.

12 Ank N, Iversen M, Bartholdy C, et al. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol 2008;180:2474-2485.

13 Ank N, West H, Bartholdy C, et al. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 2006;80:4501-4509.

14 Marcello T, Grakoui A, Barba-Spaeth G, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology 2006;131:1887-1898.

15 Dolganiuc A, Kodys K, Marshall C, et al. Type III Interferons, IL-28 and IL-29, Are Increased in Chronic HCV Infection and Induce Myeloid Dendritic Cell-Mediated FoxP3+Regulatory T Cells. PLoS One 2012;7.

16 Li M, Liu X, Zhou Y, et al. Interferon-lambda s: the modulators of antivirus, antitumor, and immune responses. J Leukoc Biol 2009;86:23-32.

17 Asahina Y, Nakagawa M, Kakinuma S, et al. Polymorphism Near the Interleukin-28B Gene and Anti-Hepatitis C Viral Response. J Clin Transl Hepatol 2013;1:39-44.

18 Asahina Y, Izumi N, Hirayama I, et al. Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response. Gastroenterology 2008;134:1396-1405.

19 Asahina Y, Tsuchiya K, Muraoka M, et al. Association of gene expression involving innate immunity and genetic variation in interleukin 28B with antiviral response. Hepatology 2012;55:20-29.

20141225

20 Sarasin-Filipowicz M, Oakeley EJ, Duong FH, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A. 2008;105:7034-7039.

21 Prokunina-Olsson L, Muchmore B, Tang W, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 2013;45:164-171.

22 Amanzada A, Kopp W, Spengler U, et al. Interferon-λ4 (IFNL4) Transcript Expression in Human Liver Tissue Samples. PLoS One 2013;8:e84026.

23 Sakamoto N, Nakagawa M, Tanaka Y, et al. Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b. J Med Virol 2011;83:871-878.

24 Aly H, Watashi K, Hijikata M, et al. Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes. J Hepatol 2007;46:26-36.

25 Tasaka M, Sakamoto N, Nakagawa M, et al. Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response. J Gen Virol 2007;88:3323-3333.

26 Nakagawa M, Sakamoto N, Tanabe Y, et al. Suppression of hepatitis C virus replication by cyclosporin A is mediated by blockade of cyclophilins. Gastroenterology 2005;129:1031-1041.

27 Langhans B, Kupfer B, Braunschweiger I, et al. Interferon-lambda serum levels in hepatitis C. J Hepatol 2011;54:859-865.

28 Murata K, Sugiyama M, Kimura T, et al. Ex vivo induction of IFN-λ3 by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients. J Gastroenterol 2013.

29 Stone AE, Giugliano S, Schnell G, et al. Hepatitis C virus pathogen associated molecular pattern (PAMP) triggers production of lambda-interferons by human plasmacytoid dendritic cells. PLoS Pathog 2013;9:e1003316.

30 Thomas E, Gonzalez VD, Li Q, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology 2012;142:978-988.

31 Onoguchi K, Yoneyama M, Takemura A, et al. Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 2007;282:7576-7581.

32 Makowska Z, Heim M. Interferon signaling in the liver during hepatitis C virus infection. Cytokine 2012;59:460-466.

33 Booth D, George J. Loss of function of the new interferon IFN-lambda 4 may confer protection from hepatitis C. Nat Genet 2013;45:119-120.

34 Lupberger J, Felmlee DJ, Baumert TF. Interferon-lambda polymorphisms and hepatitis C virus clearance revisited. Hepatology 2013;58:439-441.

35 Yoshio S, Kanto T, Kuroda S, et al. Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-λ in response to hepatitis C virus. Hepatology 2013;57:1705-1715.

20141225

Figure legends

Fig. 1. Comparison of *IFNλs* expression levels between chronic hepatitis C patients with rs12979860 CC or CT/TT. (a) Baseline mRNA levels of *IL29*, *IL28A,* and *IL28B* in PBMCs expressed relative to the internal control (/int.cont.). (b) Fold changes in *IL29*, *IL28A,* and *IL28B* expression in PBMCs stimulated for 8 h with poly(I:C) (10 μg/ml) after a 12-h pretreatment with IFNα-2b (100 IU/ml). Columns represent means ± SEM.

Fig. 2. Impact of *IFNλs* expression levels on therapy response in chronic hepatitis C patients. Fold changes in *IL29*, *IL28A*, and *IL28B* expression in PBMCs stimulated with IFNα-2b and poly(I:C). IFNλ induction levels were compared between (a) SVR (sustained virological responders), relapsers, and NR (non-virological responders) for peg-IFNα/ RBV (P/R) therapy. (b) VR (virological responders) and NR in patients with distinct IL28B genotypes (rs12979860 CC or CT/TT). (c) SVR for P/R, SVR for protease inhibitor (PI) plus P/ R triple therapy, and non-SVR for the triple therapy. Columns represent means ± SEM.

Fig. 3. Impact of *IFNλ4* on *IFNλs* expression and therapy response. Relationship of *IFNλ4* expression with (a) baseline expression of *IFNλs*, (b) *IFNλs* induction and (c) therapy response were compared in chronic hepatitis C patients with distinct *IL28B* genotypes (rs12979860 CC or CT/TT). The *IL28B*-unfavorable (CT/TT) group were subdivided into undetectable (−) or detectable (+) *IFNλ4* mRNA patients. (a) Baseline expressions of *IL29*, *IL28A*, and *IL28B* in PBMC. (b) Fold changes in *IL29*, *IL28A*, and *IL28B* expression in PBMCs stimulated f with IFNα-2b and poly(I:C). (c) Virological non-response rates for PEG-IFNα/ RBV therapy. Columns represent means ± SEM.

20141225

Fig. 4. Manipulating *IFNλ4* expression regulates *IL28B* induction and promoter activity.

(a) Fold inductions of *IL28B* mRNA in BLCs transfected with *IFNλ4* and treated with IFNα

(100U/ml). (b) Fold inductions of *IL28B* mRNA in HEK293T cells co-transfected with *IFNλ4*

and IRF7 (control, 100ng, 500ng, 1000ng). Induction rates were expressed as fold change

relative to control-transfected cells. (c) Fold inductions of *IL28B* promoter activity in

HEK293/IL28B-Luc cells transfected with IFNλ4 and treated with IFNα (0, 10, 100, 1000

IU/ml). (d, e) Fold inductions of *IL28B* promoter activity in HEK293/IL28B-Luc cells co-

transfected with IFNλ4 and (d) IRF7 (control, 200ng, 500ng) or (e) p50:p65 (control, 200ng).

Luciferase activities and cell viabilities were expressed as fold change relative to untreated or

control-transfected cells. The error bars indicate standard deviation. *P<0.05.

20141225

Table 1. Characteristics of patients analyzed for IFNλ expression levels.

| Characteristic	(n = 50)
Age median (range), year	64 (29-79)
Sex, n (%) male/female	19 (38) / 31 (62)
ALT median (range), IU/L	22 (5-157)
γGTP median (range), IU/L	23 (10-343)
LDL-C median (range), mg/dL	100 (38-169)
Hemoglobin median (range), g/dL	13.4 (9.3-16.8)
Platelet count median (range), ×10^4/μL	15.5 (5.2-23.6)
Fibrosis stage, n (%)	
F1,2 / F3,4	28 (70) / 12 (30)
Viral load median (range), log IU/mL*	6.8 (4.8-7.6)
HCV core 70 a.a. n(%)[†]	
wild / mutant / ND	15 (30) / 21 (42) / 14 (28)
HCV core 91 a.a. n (%)	
wild / mutant / ND	18 (36) / 18 (36) / 14 (28)
ISDR substitutions, n (%)[‡]	
0,1 / 2≦ / ND	26 (52) / 6 (12) / 18 (36)
IL28B SNP (rs8099917), n (%)	
TT / TG, GG	27 (54) / 23 (46)
IL28B SNP (rs12979860), n (%)	
CC / CT, TT	24 (48) / 26 (52)
IL28B SNP (ss469415590), (%)	
TT / ΔG	24 (48) / 26 (52)
Effect of previous therapy, n (%)	
SVR / Relapse / NR	18 (36) / 14 (28) / 18 (36)

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, Hepatitis C virus; ISDR, IFN sensitivity determining region; SVR, sustained virological responder; VR, virological responder; NR, non-responder; ND, not determined.
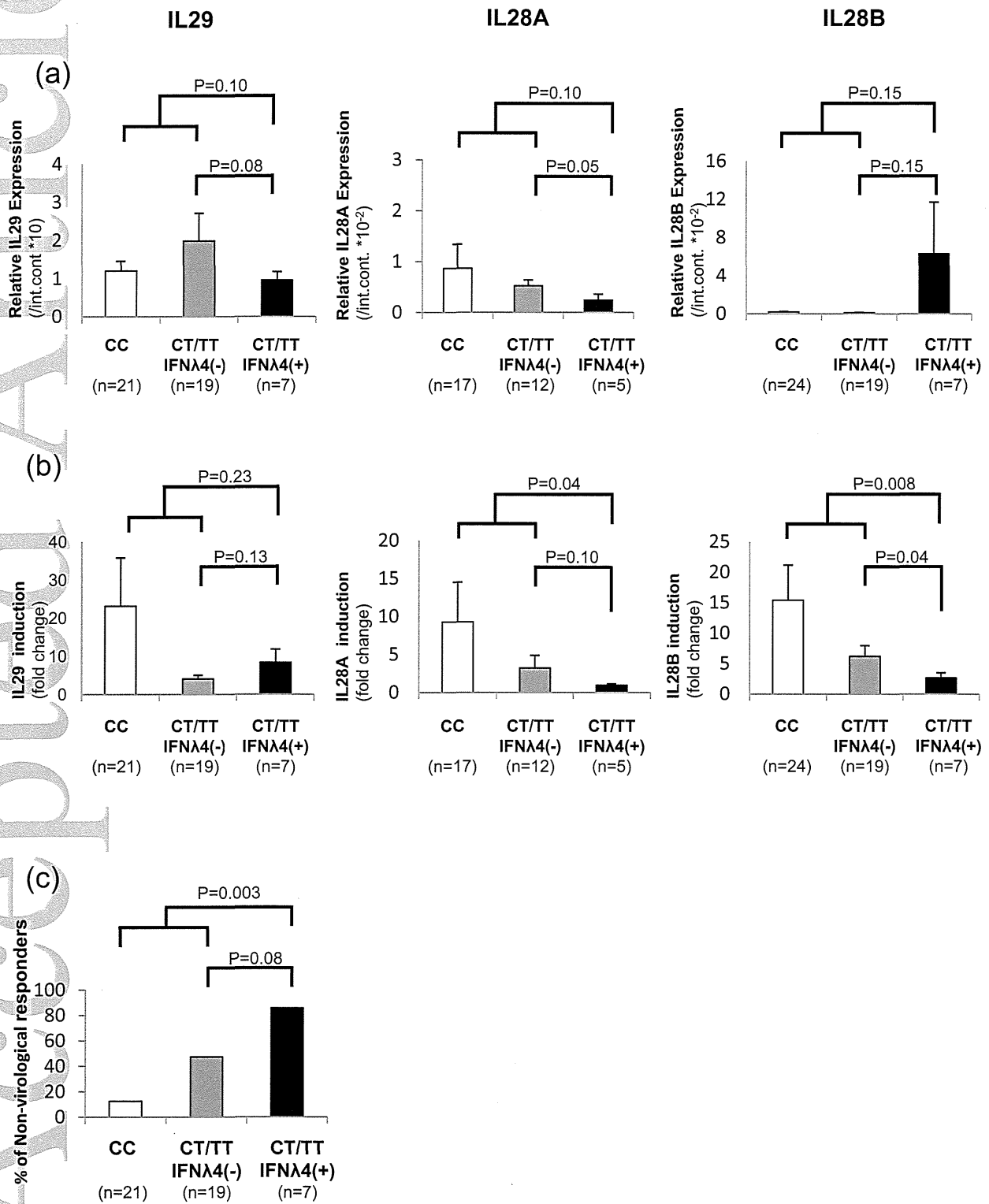
*HCV viral load was analyzed among Relapsers and Non-responders.

[†]HCV core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants.
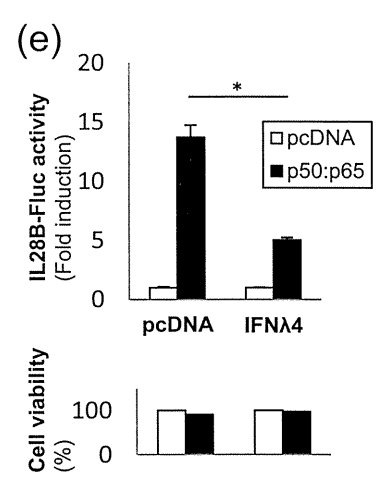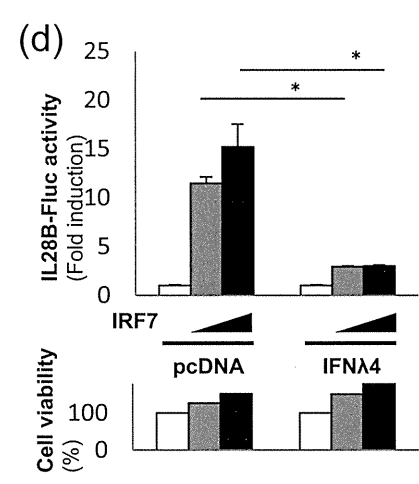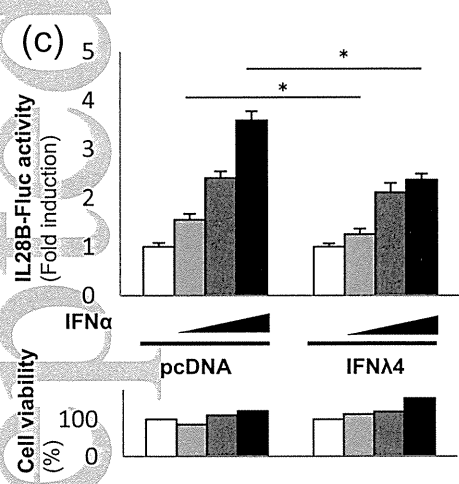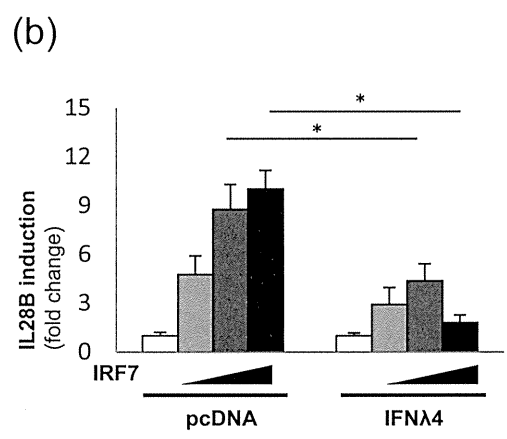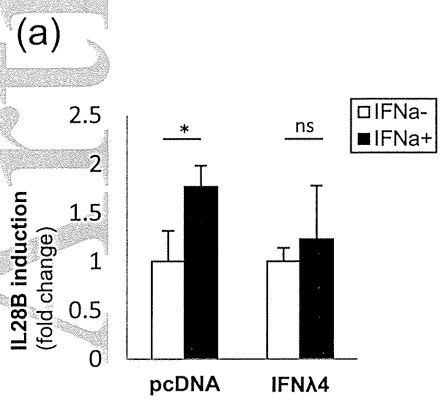
20141225
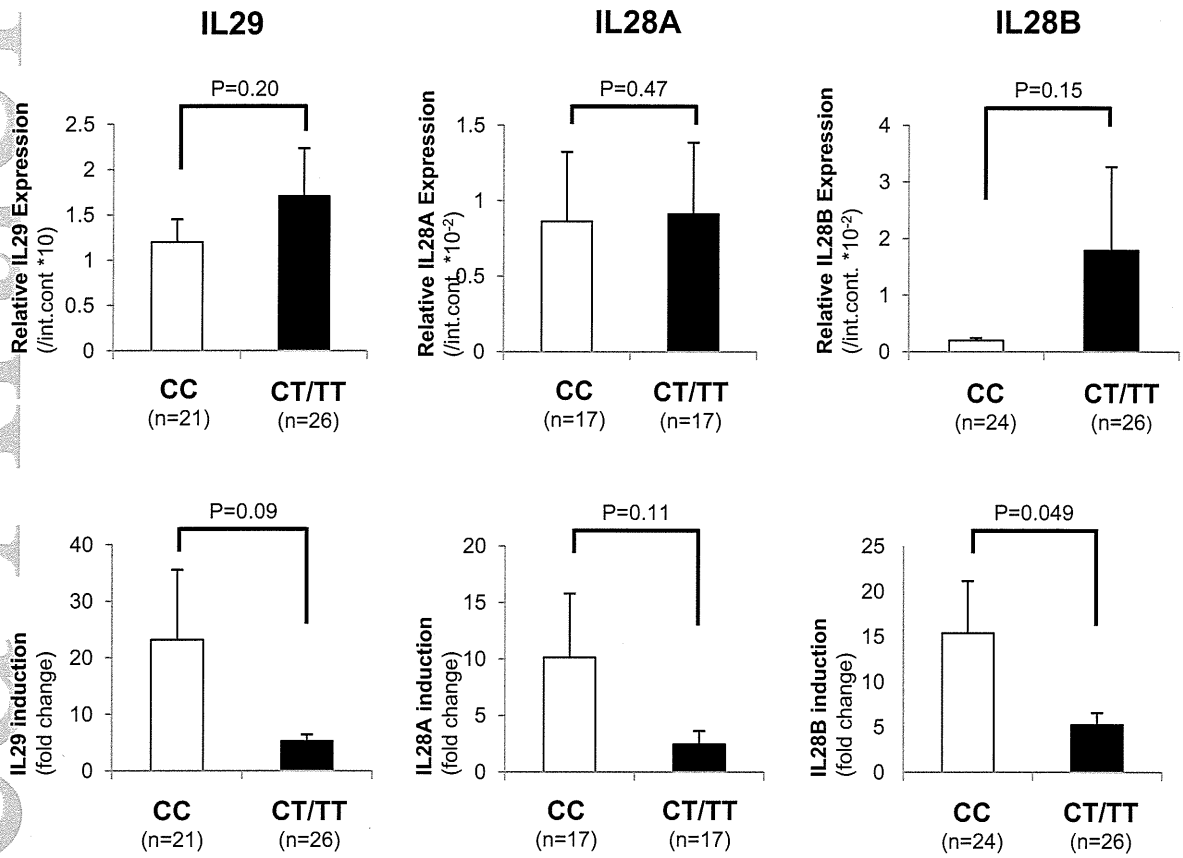
jgh_12902_f2.pdf

jgh_12902_f3.pdf

Original Article
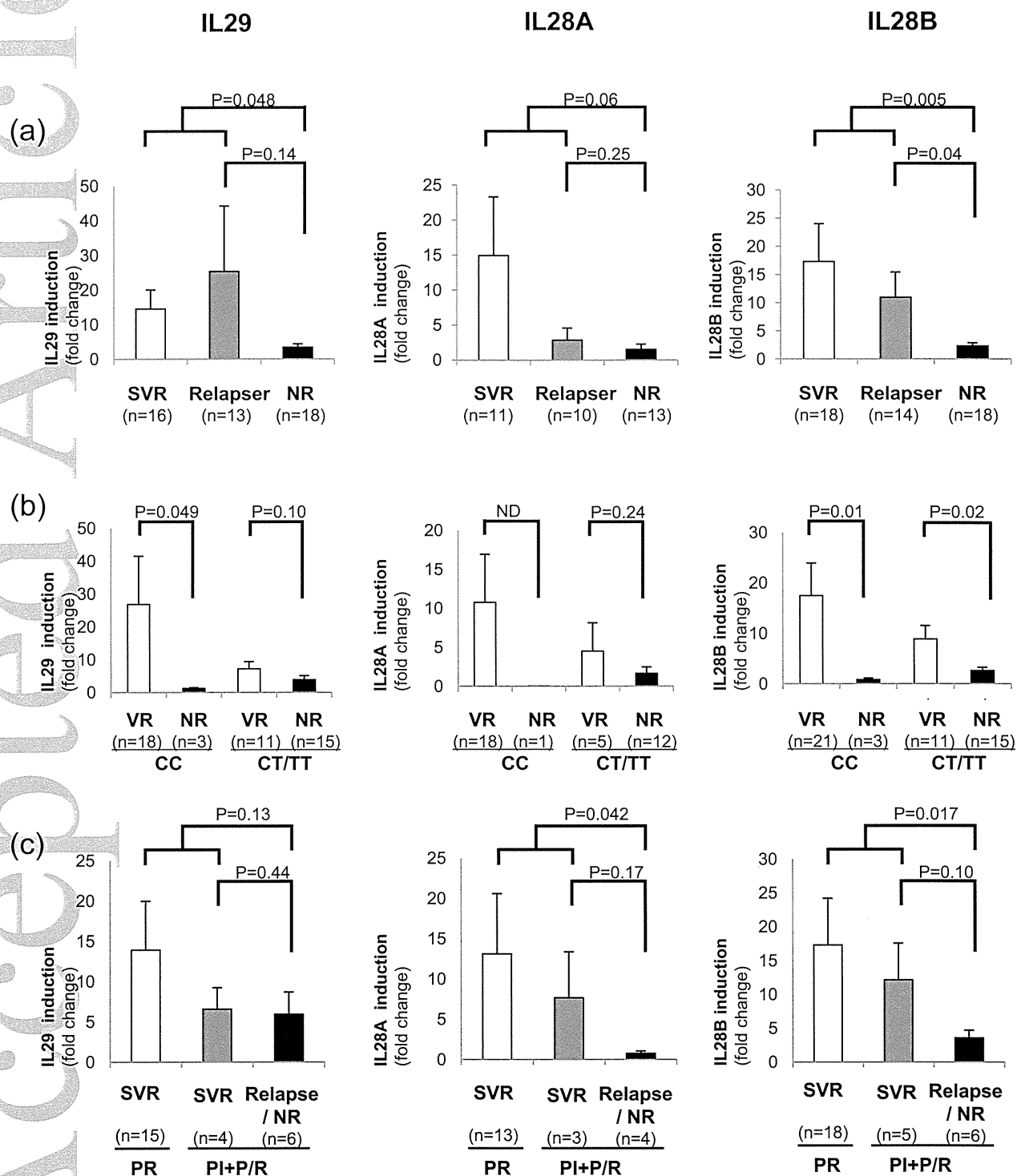
Changes in Plasma Vascular Endothelial Growth Factor at 8 Weeks After Sorafenib Administration as Predictors of Survival for Advanced Hepatocellular Carcinoma

Kaoru Tsuchiya, MD, PhD[1]; Yasuhiro Asahina, MD, PhD[2,3]; Shuya Matsuda, MD[1]; Masaru Muraoka, MD[1];
Toru Nakata, MD[1]; Yuichiro Suzuki, MD[1]; Nobuharu Tamaki, MD[1]; Yutaka Yasui, MD[1]; Shoko Suzuki, MD[1];
Takanori Hosokawa, MD[1]; Takashi Nishimura, MD, PhD[1]; Ken Ueda, MD[1]; Teiji Kuzuya, MD, PhD[1]; Hiroyuki Nakanishi, MD,
PhD[1]; Jun Itakura, MD, PhD[1]; Yuka Takahashi, MD, PhD[1]; Masayuki Kurosaki, MD, PhD[1]; Nobuyuki Enomoto, MD, PhD[4]; and
Namiki Izumi, MD, PhD[1]

BACKGROUND: A new predictive biomarker for determining prognosis in patients with hepatocellular carcinoma (HCC) who receive sorafenib is required, because achieving a reduction in tumor size with sorafenib is rare, even in patients who have a favorable prognosis. Vascular endothelial growth factor (VEGF) receptor is a sorafenib target. In the current study, the authors examined changes in plasma VEGF concentrations during sorafenib treatment and determined the clinical significance of VEGF as a prognostic indicator in patients with HCC. **METHODS:** Plasma VEGF concentrations were serially measured in 63 patients with advanced HCC before and during sorafenib treatment. A plasma VEGF concentration that decreased >5% from the pretreatment level at 8 weeks was defined as a "VEGF decrease." An objective tumor response was determined using modified Response Evaluation Criteria in Solid Tumors 1 month after the initiation of therapy and every 3 months thereafter. **RESULTS:** Patients who had a VEGF decrease at week 8 (n = 14) had a longer median survival than those who did not have a VEGF decrease (n = 49; 30.9 months vs 14.4 months; P = .038). All patients who had a VEGF decrease survived for >6 months, and the patients who had both a VEGF decrease and an α-fetoprotein response (n = 6) survived during the observation period (median, 19.7 months; range, 6.5-31.0 months). In univariate analyses, a VEGF decrease, radiologic findings classified as progressive disease, and major vascular invasion were associated significantly with 1-year survival; and, in multivariate analysis, a VEGF decrease was identified as an independent factor associated significantly with survival. **CONCLUSIONS:** A plasma VEGF concentration decrease at 8 weeks after starting sorafenib treatment may predict favorable overall survival in patients with advanced HCC. *Cancer* 2014;120:229-37. © 2013 The Authors. *Cancer* published by Wiley Periodicals, Inc. on behalf of American Cancer Society. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

KEYWORDS: antiangiogenic therapy, biomarker, hepatocellular carcinoma, prognosis, α-fetoprotein.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver (70%-85%) and a major cause of mortality. It is the fifth and seventh most frequent cancer and the second and sixth most frequent cause of cancer death in men and women, respectively.[1] At early stages or at Barcelona Clinic Liver Cancer stage A, a 5-year survival rate of 60% to 70% can be achieved in well selected patients with HCC who undergo surgical therapies (liver resection or transplantation) or locoregional procedures (ie, radiofrequency ablation).[2] However, treatment of advanced HCC that is not amenable to surgical or locoregional therapies remains a challenge in clinical practice.

Sorafenib is an oral, small-molecule tyrosine kinase inhibitor that blocks the synthesis of several intracellular proteins considered to be important for tumor progression, including the platelet-derived growth factor receptor beta, raf kinase, and the vascular endothelial growth factor (VEGF) receptor. VEGF is a homodimeric glycoprotein with a molecular weight of 45 kDa. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and a structurally related molecule: placental growth factor. Three high-affinity VEGF tyrosine kinase receptors (VEGFRs) have been identified:

VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-2 is the principal receptor that promotes the proangiogenic action of VEGF-A and has been the principal target of antiangiogenic therapies, although additional studies have underlined the importance of signaling through VEGFR-1. In 2 phase 3, placebo-controlled, randomized trials, sorafenib treatment significantly improved the time to tumor progression (TTP) and overall survival (OS) of patients with advanced HCC.[3,4] In those trials, however, no statistically significant pretreatment factors that predicted responses after patients started receiving sorafenib were identified.[5] Therefore, in clinical practice, it is extremely important to identify a predictive post-treatment biomarker that is associated with the treatment efficacy of sorafenib and the prognosis of patients after they start receiving sorafenib.

In general, the efficacy of treating solid tumors with systemic chemotherapy agents is assessed by radiologic findings. In 2010, Lencioni and Llovet published a modification of the Response Evaluation Criteria in Solid Tumors (RECIST).[6] However, the modified RECIST can be used only for typical HCC. Advanced HCCs often have atypical vascular patterns; therefore, evaluating tumor response to sorafenib is difficult with radiologic findings alone. Alternatively, α-fetoprotein (AFP) is the most popular tumor marker for HCC, and it has been reported that early AFP responses are a useful surrogate marker for predicting treatment response and prognosis in patients with advanced HCC who receive cytotoxic and antiangiogenic agents.[7-9] However, approximately 30% of patients with advanced HCC in the Sorafenib HCC Assessment Randomized Protocol (SHARP) trial had normal AFP concentrations.[10] Therefore, the identification of a new biomarker that can complementarily predict the efficacy of sorafenib and the prognosis of patients is necessary.

In a mouse model, an increase in hepatic VEGF levels was observed at 24 hours, 72 hours, and 120 hours after the administration of sorafenib,[11] suggesting that a change in VEGF levels may also occur during sorafenib therapy in humans. Therefore, we evaluated plasma VEGF changes during sorafenib treatment in patients with advanced HCC to determine whether VEGF has potential as a new biomarker for the prediction of treatment efficacy and prognosis after sorafenib administration.

MATERIALS AND METHODS
Patient Selection
Between December 2009 and August 2012, 95 consecutive patients with advanced, inoperable HCC received treatment with sorafenib at Musashino Red Cross Hospital. The diagnosis of HCC was based on guidelines established by the Liver Cancer Study Group of Japan[12] and the American Association for the Study of Liver Diseases[13] or by pathologic examination. According to these guidelines, a diagnosis of HCC is confirmed by histology or by characteristic radiologic findings, such as typical arterial enhancement of the tumor followed by a washout pattern in the images in the portal venous phase or the equilibrium phase on dynamic spiral computed tomography (CT) imaging or contrast-enhanced magnetic resonance imaging. Inclusion criteria were predefined as follows: 1) patients were alive 8 weeks after beginning treatment; and 2) patients had plasma VEGF and serum AFP concentrations evaluated at baseline, at 4 weeks, and at 8 weeks. Of 95 patients, 23 were unavailable for a week-8 VEGF measurement for the following reasons: 7 patients stopped sorafenib therapy because of erythema multiforme (grade 2-3) and started other therapies (radiation therapy or cytotoxic chemotherapy) within 1 month after starting sorafenib, 4 patients moved to another location before week 8, 5 patients refused to undergo a plasma VEGF measurement at week 8, and 7 patients were not available for obtaining VEGF concentration results. These 23 patients and 9 other patients who died within 8 weeks were excluded from the study. Hence, in total, 63 patients fulfilled the inclusion criteria. At enrollment, all patients had metastatic or locally advanced HCC that was not amenable to surgery or locoregional therapies, including transcatheter arterial chemoembolization (TACE) and local ablation. Written informed consent was obtained from all patients, and the ethics committee at Musashino Red Cross Hospital approved the study in accordance with the Declaration of Helsinki.

Sorafenib Treatment
The initial daily dose of sorafenib was 800 mg in 28 patients, 400 mg in 28 patients, and 200 mg in 7 patients. A reduced initial dose was allowed for patients who had the following factors: advanced age (≥80 years), gastrointestinal varices with a risk of bleeding, low body weight (<50 kg), and a poor performance status (≥2). In total, 60 patients underwent multiphase-multidetector CT imaging before starting sorafenib, 1 month after starting sorafenib, and every 3 months thereafter. Radiologic responses to therapy were evaluated according to modified RECIST. In all patients, serial measurements of plasma VEGF and serum AFP concentrations were performed before and after the receipt sorafenib and every month thereafter, with an allowance of ± 1 week. The endpoint of the current study was OS. In the follow-up visit after sorafenib administration, the medication was discontinued if progressive disease