BMS-788329 combination therapy thereafter. HCV RNA levels decreased only slightly in two mice when treated with telaprevir alone, indicating that the introduced NS3 V36A mutation conferred resistance against telaprevir. HCV RNA levels declined to undetectable levels in one of the mice (**Figure 2a**) and hovered near the limit of detection in the other mouse (**Figure 2b**).

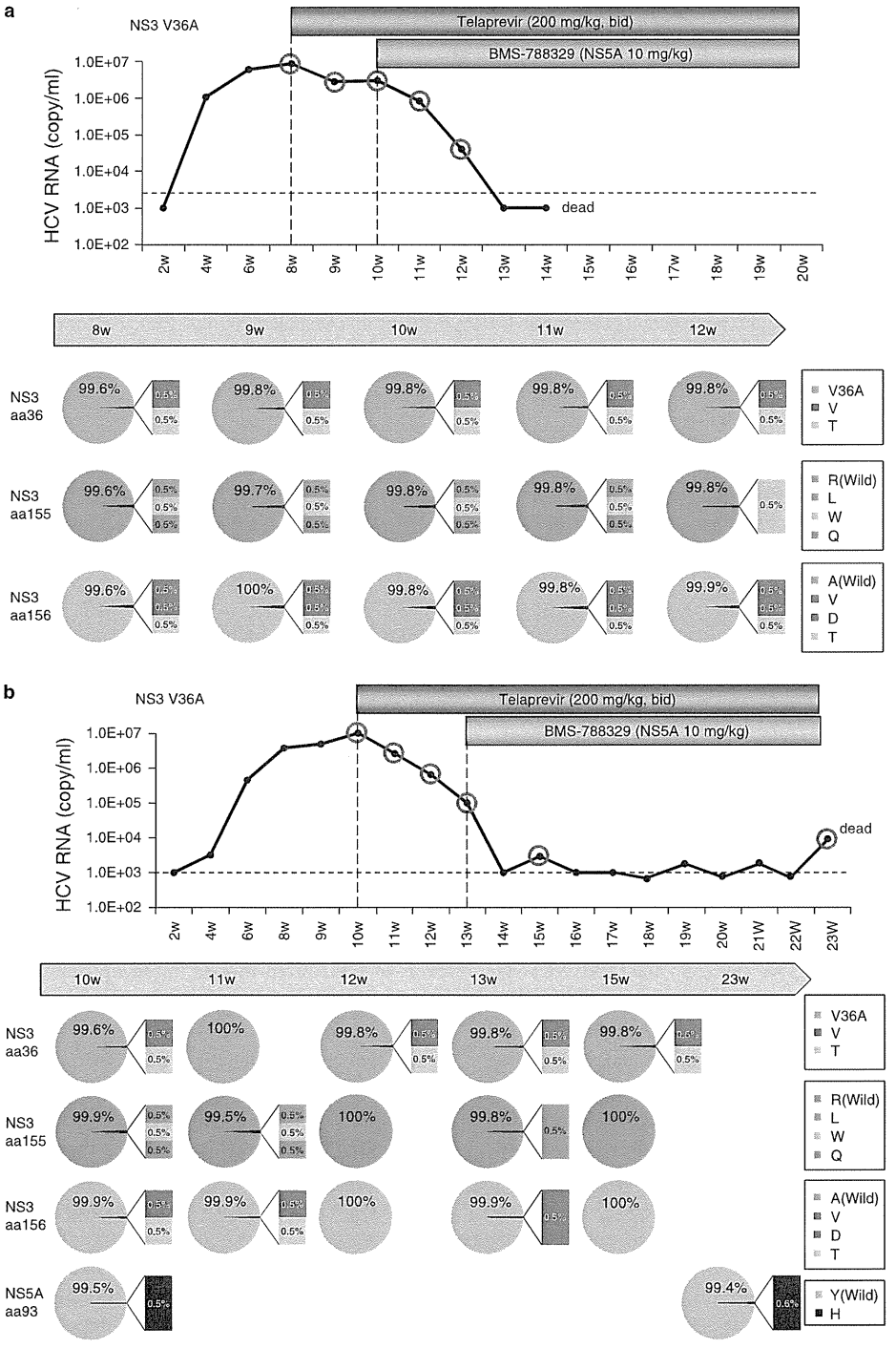

Figure 2. Combination treatment with telaprevir and BMS-788329 in human hepatocyte chimeric mice infected with an HCV clone containing NS3 V36A telaprevir resistance mutation. We infected mice with an infectious clone harboring a telaprevir-resistant NS3 V36A mutation. (**a**, **b**) Mice received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). HCV, hepatitis C virus; w, weeks.

Ultra-deep sequencing data showed that the introduced V36A mutation in the NS3 region of KT-9 was conserved in >99.5% of the viral sequences examined. In addition, amino acids 155 and 156 in NS3, which are also associated with telaprevir resistance, also remained unchanged (**Figure 2a,b** and **Supplementary Tables 1** and **2** online). Although mutant sequences were detected at very low frequency (<0.5%), these sequences may be due to sequencing errors or artifacts introduced during the amplification step because similar low-frequency variants were detected when sequencing a plasmid to establish the error threshold for detection of rare variants (data not shown). However, we detected a small amount of a new resistance mutation, Y93H in the NS5A region, in mice treated with telaprevir and BMS-788329 combination therapy for 10 weeks (**Figure 2b** and **Supplementary Table 2** online). These data indicate that sequential administration of telaprevir and NS5A inhibitor may result in emergence of a doubly resistant strain.

Effect of telaprevir and BMS-788329 combination therapy in human hepatocyte chimeric mice infected with an HCV clone containing NS5A L31V resistance mutation

We established clonal infection with an HCV KT-9 NS5A L31V mutant clone, which we expected to be resistant against NS5A inhibitor. Mice were treated with BMS-788329 alone for the first 2 weeks to confirm resistance and then treated with telaprevir plus BMS-788329 combination therapy thereafter. In both mice, HCV RNA levels declined rapidly in the first week of BMS-788329 monotherapy but then rose again sharply during the second week (**Figure 3a,b**). A second mutation, NS5A Y93C, emerged and replaced the wild-type strain during the initial 2 weeks of BMS-788329 monotherapy in both mice (**Figure 3a,b** and **Supplementary Tables 3** and **4** online). HCV RNA levels declined to undetectable levels in one of the two mice (**Figure 3a**). In the other mouse, viral breakthrough occurred during combination therapy with the two drugs (**Figure 3b**). The frequency of the NS3-resistant V36A strain increased to 97.8% during the course of combination therapy (**Figure 3b**, **Supplementary Table 4** online). These results indicate that the NS5A L31V strain may rapidly accumulate an additional V36A mutation. Such strains may easily develop resistance against telaprevir, as well.

Effect of BMS-788329 in combination with telaprevir or NS5B inhibitor in mice infected with clones with multiple drug-resistant mutations

We also established a HCV genotype 1b KT-9 clone with both L31V and Y93H mutations in the NS5A region in a chimeric mouse. The mouse was treated with BMS-788329 alone for the first 2 weeks to confirm resistance and then treated with telaprevir plus BMS-788329 combination therapy for 14 weeks. At that point (week 24), telaprevir was replaced with NS5B inhibitor in combination with BMS-788329 for a further 5 weeks (**Figure 4a**). HCV RNA levels in this mouse showed poor response to BMS-788329 alone. Furthermore, HCV RNA rebounded during combination treatment with BMS-788329 and telaprevir. A drug-resistant NS3-V36A strain predominated at weeks 12 and 14, and by weeks 16 and 17 an NS3 T54A strain had emerged (**Figure 4a** and **Supplementary Table 5** online). When we withdrew telaprevir and treated the mice with a combination of BMS-788329 and NS5B inhibitor, HCV RNA rapidly declined and became undetectable. However, the virus rebounded almost immediately and rapidly increased to almost 10^6 copies/ml (**Figure 4a**). Sequencing of the virus detected a resistant NS5B P495S strain (**Figure 4a** and **Supplementary Table 5** online). At week 29, direct sequencing indicated a mixture of wild-type and mutant strains at NS3 aa36 and 54 (data not shown).

Finally, we established an infection in a chimeric mouse with a HCV genotype 1b KT-9 clone with triple resistance mutations (NS3 V36A, NS5A L31V, and NS5A Y93H). The mouse was treated with BMS-788329 plus telaprevir combination therapy for 2 weeks, followed by combination therapy with BMS-788329 and NS5B inhibitor (**Figure 4b**). As expected, HCV RNA did not decrease during the initial BMS-788329 and telaprevir combination therapy. In contrast, HCV RNA levels declined rapidly during BMS-788329 and NS5B inhibitor combination therapy. HCV RNA remained negative until 11 weeks after cessation of the therapy, after which it increased gradually to nearly pre-treatment levels. Sequence analysis of the virus revealed four resistance mutations: NS3 V36A, NS5A L31V, NS5A Y93H, and NS5B P495S (**Figure 4b,c**). This indicates that mutant strains resistant against all recently developed DAAs might emerge following inappropriate use of drugs and that sequential use of these DAA should be avoided.

DISCUSSION

Although the approval of telaprevir and boceprevir has improved the eradication rate of HCV in patients treated with triple therapy (2–9), the therapy is approved only for genotype 1. Furthermore, severe side effects such as anemia, neutropenia, thrombocytopenia, and appetite loss limit patient eligibility to young and relatively healthy individuals without advanced liver diseases. Unexpected development of severe skin disease results in premature termination of the therapy. Therapies without interferon and ribavirin such as DAA combination therapies (20–22) may provide more tolerable therapy for older patients as well as those with cirrhosis.

We assessed the effect of combination of BMS-788329 plus telaprevir or BMS-821095 using human hepatocyte chimeric mice. We chose these combinations because daclatasvir, which is a close analog of BMS-788329, shows potent antiviral effects with few side effects (30). Furthermore, when we performed a clinical trial of the combination of daclatasvir and asunaprevir, some patients had elevated transaminases and hyperbilirubinemia, probably due to the side effects of asunaprevir (22). We thus attempted to find out a better dual combination of DAAs. Although the combination of telaprevir and BMS-788329 effectively reduced serum virus levels in mice infected with genotype 1b serum, we observed minimal change in HCV RNA levels in mice infected with genotype 2 (32). These results are consistent

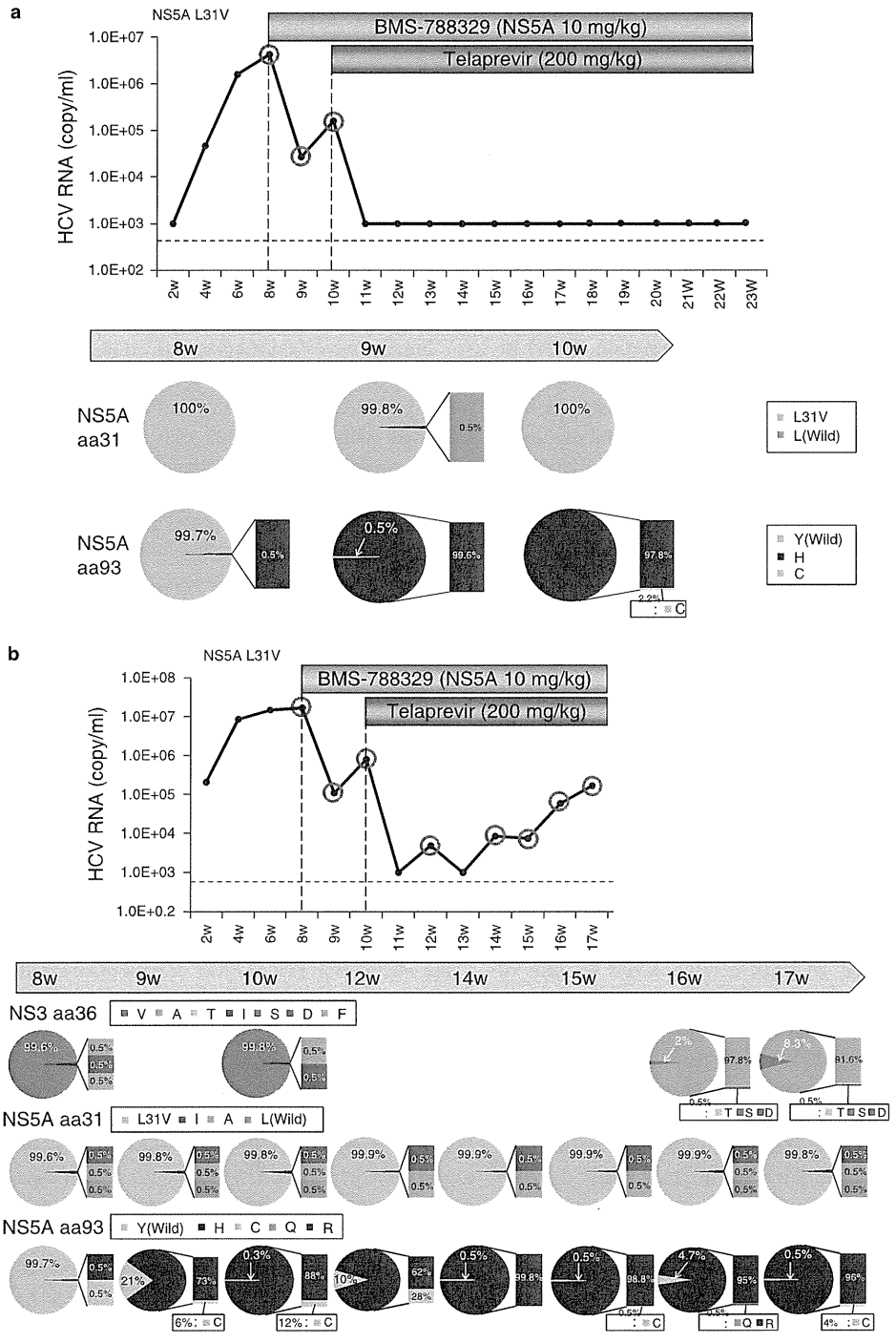

Figure 3. Effect of telaprevir and BMS-788329 combination therapy in human hepatocyte chimeric mice infected with an HCV clone containing NS5A L31V resistance mutation. We infected mice with an infectious clone harboring an NS5A inhibitor-resistant NS5A L31V mutation. (**a**, **b**) Mice received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). HCV, hepatitis C virus; w, weeks.

with *in vitro* experiments showing higher ED50 levels of these drugs against genotype 2 (30,31). The combination of BMS-788329 and telaprevir thus might be a good candidate for clinical trial in patients with genotype 1b infection. Higher dosage or a next-generation NS3 protease inhibitor should be considered for treatment of patients infected with genotype 2.

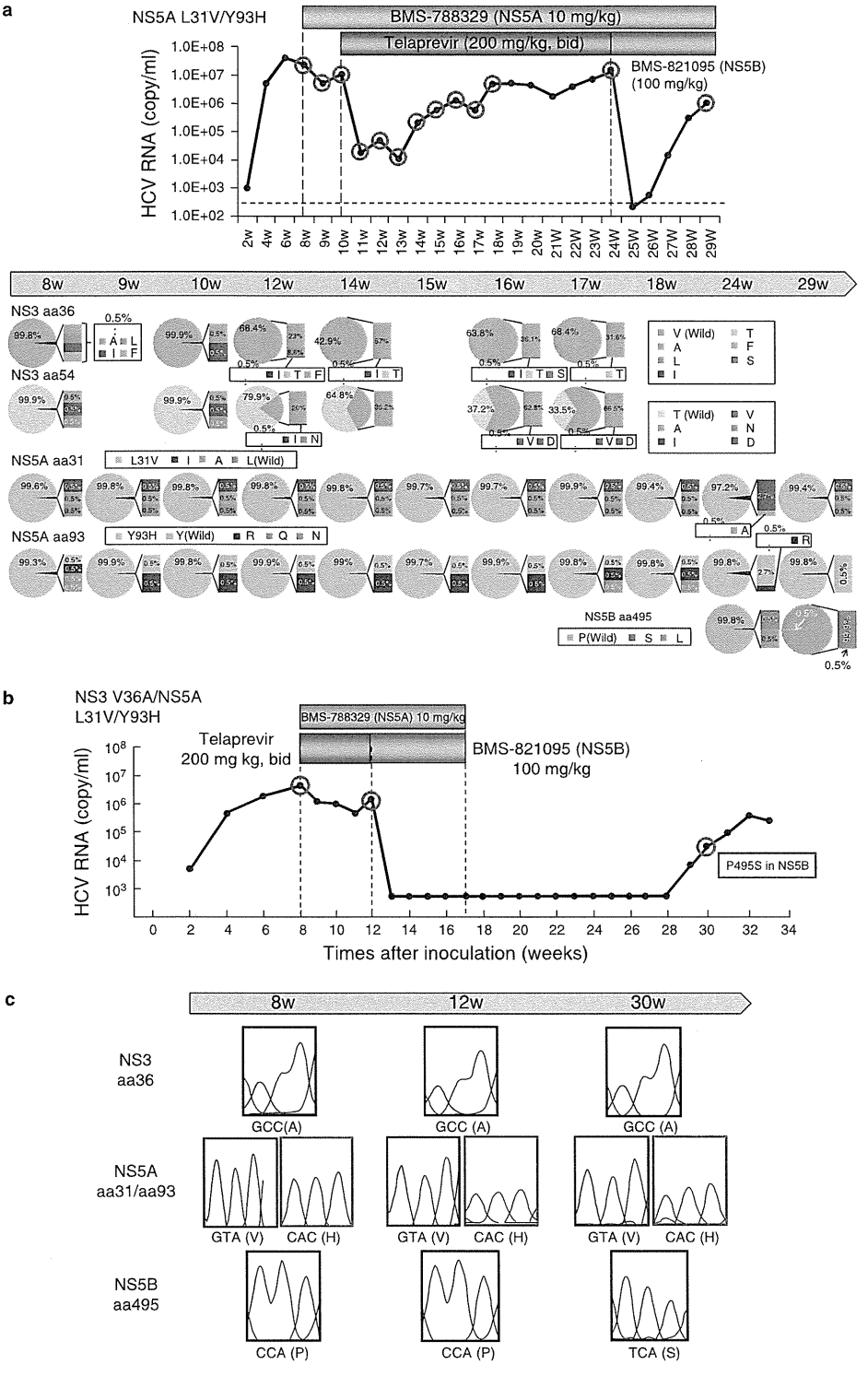

Figure 4. Effect of BMS-788329 in combination with telaprevir or NS5B inhibitor in mice infected with clones with multiple drug-resistant mutations. (**a**) We infected a mouse with an infectious clone harboring NS5A inhibitor-resistant NS5A L31V and Y93H mutations. The mouse received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). After 14 weeks of telaprevir plus BMS-788329 combination therapy, we replaced telaprevir with 100 mg/kg (mouse body weight) of NS5B inhibitor and continued combination therapy with BMS-788329 for an additional 5 weeks. (**b**, **c**) We infected a mouse with an infectious clone harboring resistance mutations against both telaprevir (NS3 V36A) and NS5A inhibitor (NS5A L31V and Y93H). The mouse received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor) for 2 weeks, followed by combination therapy with BMS-788329 and 100 mg/kg (mouse body weight) of NS5B inhibitor. W, weeks.

As one of the mice treated with the combination of BMS-788329 and telaprevir showed poor response followed by relapse, we decided to analyze the effect of pre-existing resistance mutations on response to therapy. We infected mice with HCV clones having introduced mutations known to be associated with resistance to specific DAAs. Combination therapy with BMS-788329 and telaprevir effectively suppressed replication of HCV BMS-788329-resistant NS3 V36A HCV (**Figure 2**). This combination might be useful for patients who have a naturally occurring drug resistance profile. In contrast, two mice with a BMS-788329-resistant NS5A L31V mutation easily acquired an additional Y93C mutation, which has been reported to confer very strong resistance against the drug (**Figure 3**) (28). These factors should be considered when we establish future DAA combination therapies.

When we treated a mouse infected with a NS5A L31V and Y93H double mutation with BMS-788329 and telaprevir, the mice rapidly developed resistance against telaprevir. Furthermore, we observed the rapid emergence of an NS5B P495S mutant during combination therapy with BMS-788329 and BMS-821095 (NS5B inhibitor) (**Figure 4**). Such mutant strains with triple resistance features were also observed when the virus reappeared after cessation of a similar treatment (**Figure 4**). These results imply that mutant strains resistant to all three drugs can emerge after sequential use of these DAAs.

DAA combination therapy without interferon and ribavirin is expected to become a primary treatment option in the near future. As we showed in this study, however, multidrug-resistant strains may appear after incomplete, sequential use of DAAs (**Figure 4**). Although simultaneous use of three drugs is the strongest therapy against HCV, side effects related to drug interactions may occur. Therefore, we should further examine possible combinations of DAAs to establish the best combination therapy to eradicate HCV from all treated patients without incurring serious side effect.

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CONFLICT OF INTEREST

Guarantor of the article: Kazuaki Chayama, MD, PhD.

Specific author contributions: H. Abe, N. Hiraga, and M. Imamura designed and performed the experiments. C. N. Hayes analyzed the data. H. Abe and C. N. Hayes wrote the manuscript. M. Tsuge, D. Miki, S Takahashi, and H. Ochi participated in data analysis and discussion. K. Chayama initiated and directed the entire study, designed experiments and wrote the manuscript.

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Potential competing interests: K.C. is a speaker for BMS, MSD and Roche.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ DAAs against HCV have recently been developed.
- ✓ DAAs have been recommended to be used as interferon-based regimen.
- ✓ DAAs without interferon must be used in combination because of development of resistant strain.
- ✓ Development of multidrug-resistant strains remains to be characterized.

WHAT IS NEW HERE

- ✓ Resistant strains easily develop from cloned virus strains after sequential DAAs combination therapy.
- ✓ Sequential use of DAAs must be avoided to prevent a development of resistant strains.

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PLOS ONE

Genetic Analyses Reveal a Role for Vitamin D Insufficiency in HCV-Associated Hepatocellular Carcinoma Development

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Abstract

Background: Vitamin D insufficiency has been associated with the occurrence of various types of cancer, but causal relationships remain elusive. We therefore aimed to determine the relationship between genetic determinants of vitamin D serum levels and the risk of developing hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

Methodology/Principal Findings: Associations between *CYP2R1*, *GC*, and *DHCR7* genotypes that are determinants of reduced 25-hydroxyvitamin D (25[OH]D_3) serum levels and the risk of HCV-related HCC development were investigated for 1279 chronic hepatitis C patients with HCC and 4325 without HCC, respectively. The well-known associations between *CYP2R1* (rs10993116, rs10741657), *GC* (rs2282679), and *DHCR7* (rs7944926, rs12785878) genotypes and 25(OH)D_3 serum levels were also apparent in patients with chronic hepatitis C. The same genotypes of these single nucleotide polymorphisms (SNPs) that are associated with reduced 25(OH)D_3 serum levels were found to be associated with HCV-related HCC ($P=0.07$ [OR = 1.13, 95% CI = 0.99–1.28] for *CYP2R1*, $P=0.007$ [OR = 1.56, 95% CI = 1.12–2.15] for *GC*, $P=0.003$ [OR = 1.42, 95% CI = 1.13–1.78] for *DHCR7*; ORs for risk genotypes). In contrast, no association between these genetic variations and liver fibrosis progression rate ($P>0.2$ for each SNP) or outcome of standard therapy with pegylated interferon-α and ribavirin ($P>0.2$ for each SNP) was observed, suggesting a specific influence of the genetic determinants of 25(OH)D_3 serum levels on hepatocarcinogenesis.

Conclusions/Significance: Our data suggest a relatively weak but functionally relevant role for vitamin D in the prevention of HCV-related hepatocarcinogenesis.

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Competing Interests: The authors have declared that no competing interests exist.

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‡ The members of the Swiss Hepatitis C Cohort Study Group are provided in the Acknowledgments

Introduction

Chronic hepatitis C is associated with important morbidity, resulting in liver cirrhosis and its complications in a significant proportion of infected individuals [1]. Due to the rising age of the hepatitis C virus (HCV)-infected population in the Western world, a dramatic increase of cases with advanced liver cirrhosis and hepatocellular carcinoma (HCC) has been predicted for the next decade [1]. Due to the insufficient treatment options for advanced HCC as well as limited number of liver allograft donors for patients with curable disease, improved strategies to screen for early HCC or to prevent the development of HCC are warranted [2]. Recently, two genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in the region of *DEPDC5* and *MICA* as susceptibility loci for HCC development in patients with chronic hepatitis C [3,4]. Although these genetic variations were strongly associated with HCV-induced HCC, with potentially important implications for the identification of patients at high risk of developing HCC, it might be challenging to translate these findings into novel therapeutic strategies for HCC.

Vitamin D insufficiency is common in different populations worldwide and has been associated with the presence of various types of cancer, including HCC [5–7]. These observations attracted considerable interest because vitamin D can be easily supplemented, with only infrequent side-effects and little costs. Yet, it has remained mostly unclear whether there is a causal association between vitamin D insufficiency and cancer development, or whether reduced vitamin D serum levels are simply surrogates for other circumstances in cancer patients (e.g. malnutrition, limited exposure to sunlight) [5–7]. Recently, two large GWAS have identified SNPs at three loci (*GC*, encoding the vitamin D binding protein; *DHCR7*, encoding 7-dehydrocholesterol reductase; and *CYP2R1*, encoding a liver 25-hydroxylase) as genetic determinants of reduced 25(OH)D_3 serum levels [8,9]. We hypothesized, that associations between these genetic variations and the occurrence of malignant or other diseases may provide a stronger argument for a causal relationship between vitamin D and the development of such diseases than the investigation of (punctual) vitamin D serum levels. In view of the high prevalence of severe vitamin D deficiency in patients with chronic hepatitis C [10], we therefore sought to investigate the association between genetic variations in *CYP2R1*, *GC*, and *DHCR7* and HCV-induced HCC.

Methods

Objectives

Genetic variations in three independent genes, *CYP2R1*, *GC*, and *DHCR7*, are associated with life-long reduced 25(OH)D_3 serum levels [9]. Vitamin D insufficiency has been associated with the occurrence of various types of cancer, but causal relationships remain elusive. Therefore, we hypothesized that genetic determinants of 25(OH)D_3 serum levels may be associated with HCV-related HCC if there is a causal relationship between vitamin D metabolism and HCC development in chronic hepatitis C patients. Hence, we assessed associations between the presence of HCC in HCV infected patients and genetic variants in *CYP2R1*, *GC*, and *DHCR7* in the primary analysis of the present study.

Participants

For the primary analysis of the present study, the association between HCV-induced HCC and genetic variants in *CYP2R1*, *GC*, and *DHCR7*, four independent cohorts of HCV-infected individuals were investigated, two cohorts including Caucasians and two cohorts including Japanese individuals. All cohorts include consecutive patients from various outpatient clinics; hence the prevalence of HCC in our cohorts is not representative of the prevalence of HCC in HCV-infected patients in general.

The first Caucasian cohort was selected from patients enrolled in the Swiss Hepatitis C Cohort Study (SCCS). The SCCS is a multicenter study pursued at 8 major Swiss hospitals and their local affiliated centers, including a total of 3,648 patients with chronic or resolved HCV infection [11]. For the present analysis, SCCS patients were included in our study if they had chronic hepatitis C, had provided written informed consent for genetic testing, had genomic DNA available for testing, if they were Caucasian, and if the duration of infection with HCV was known. Patients with hepatitis B virus infection were excluded from the discovery cohort, as well as from the replication cohorts. A second, independent cohort of Caucasian patients with chronic HCV infection with or without HCC was identified (designated as Berlin/Bonn cohort in the following); these patients were recruited at the University Hospital Departments of Gastroenterology and Hepatology in Bonn, Berlin and Leipzig in Germany, as described in Nischalke *et al* [*12*]. In addition, two independent cohorts of Japanese patients with chronic hepatitis C with or without HCC were included (a detailed description of these cohorts is provided in Miki *et al.* [*4*]; the cohorts are designated as Japanese GWAS and Japanese Replication cohort, as in Miki *et al.*). Importantly, the duration of infection with HCV was known in patients of the SCCS but not in the three additional cohorts.

In addition to the primary analysis of this study, a number of sub-analyses were performed in order to validate our research strategy. These sub-analyses investigated possible associations between genetic variations in *CYP2R1*, *GC* and *DHCR7* and i) liver fibrosis progression rate (FPR), ii) response to treatment with pegylated interferon-α (PEG-IFN-α) and ribavirin, and iii) 25(OH)D_3 serum levels. These sub-analyses were performed in SCCS patients only because of the thorough documentation of these end-points together with the known duration of infection in the SCCS. FPR was defined as a dichotomized phenotype ($<$ *vs.* $\geq$ sex-adjusted median FPR), which was calculated on the basis of the ratio of the METAVIR fibrosis score to the estimated duration of infection in years until liver biopsy (METAVIR units per year), as described previously [13]. Hence, all SCCS patients with at least one available liver biopsy with fibrosis staging prior to antiviral treatment and with known date of infection were included in the analyses of FPR. The treatment response analyses was restricted to SCCS patients who were treated under clinical practice conditions with either PEG-IFN-α2a or PEG-IFN-α2b in combination with weight-based ribavirin, with standard treatment durations (48 weeks for HCV genotype 1 and 4, 24 weeks for HCV genotype 2 and 3), and if they had received ≥80% of the recommended dose of both agents during the first 12 weeks of therapy. SVR was defined as HCV RNA below the limit of detection in a sensitive assay ≥24 weeks after treatment completion, and all patients who failed to achieve SVR were classified as nonresponders. Serum concentrations of 25(OH)D_3 were determined in all SCCS patients with chronic hepatitis C in whom a plasma sample at baseline of antiviral therapy or at the time of a liver biopsy was available. Demographic and clinical characteristics were extracted from clinical databases. High alcohol intake was defined as consumption >40 g per day over a period of ≥5 years. Liver biopsies were evaluated by experienced local pathologists. Liver fibrosis was classified according to the METAVIR score. Necroinflammatory activity was stratified into two groups, absent to mild activity *vs.* moderate to high activity. Steatosis was classified as absent or present. HCC was diagnosed

either by biopsy or, in selected cases, by typical presentation in two independent imaging modalities. The study was approved by local ethical committees.

Description of Investigations Undertaken

In a recent GWAS, Wang *et al.* have identified three loci (*CYP2R1*, *GC*, and *DHCR7*) to be associated with reduced $25(OH)D_3$ serum levels [9]. Importantly, a second independent GWAS by Ahn *et al.* yielded similar results and confirmed the association between the three loci and $25(OH)D_3$ serum levels [8]. From these studies, we selected the most significant SNPs for each locus (rs1993116 and rs10741657 in *CYP2R1*; rs2282679 in *GC*; rs7944926 and rs12785878 in *DHCR7*). rs10741657 and rs1993116 in *CYP2R1* as well as rs7944926 and rs12785878 in *DHCR7* are in high LD (Table S1). Hence, these SNPs can be substituted by each other for an investigation of the association between the indicated loci and HCV-related HCC, and either rs7944926 or rs12785878 in *DHCR7* and either rs10741657 or rs1993116 in *CYP2R1* were genotyped in each of the four cohorts. Specific SNPs were selected according to availability in our databases, as genotyping of these SNPs was either performed in the context of previous GWA studies of our cohorts [4,14], or by using a fluorescent-based competitive allele-specific PCR genotyping system (KBioscience, UK) using the primers listed in Table S2. SNPs in *CYP27B1*, which we have previously shown to be associated with SVR [10,15], were not included in the present study as they are believed to be predominantly important during the rapid regulation of calcitriol tissue levels in inflammatory responses, and because they have no impact on $25(OH)D_3$ serum levels.

Measurement of $25(OH)D_3$ was performed as described previously [10].

Ethics

The study was approved by local ethical committees of each affiliation (Switzerland: Universitätsspital Basel, Basel; Inselspital, Bern; University Hospital Geneva, Geneva; CHUV, Lausanne; Ospedale Moncucco, Lugano; Hôpital Neuchâtelois, Neuchatel; Kantonsspital St. Gallen, St. Gallen; Universitätsspital Zürich, Zürich; Germany: University of Berlin, Berlin; University of Bonn, Bonn; Japan: University of Hiroshima, Hiroshima), and written informed consent was received from all participants.

Statistical Analyses

Testing for Hardy-Weinberg equilibrium and linkage disequilibrium (LD) was performed with the genhw and pwld packages in Stata (version 9.1, StataCorp, College Station, TX). Associations of SNPs with risk of HCV-induced HCC (dichotomic variable HCC *vs.* no HCC) were assessed with χ^2 contingency tables, and – in the SCCS in patients with known duration of infection – in uni- and multivariate Cox regression models. Associations between SNPs and treatment outcome (SVR *vs.* no SVR) and with FPR (below *vs.* above sex-adjusted median FPR) were assessed in logistic regression models and Cox regression models, respectively. In regression models, SNPs were analyzed using an additive model (none, one or two copies of the minor allele were coded 0, 1 and 2, respectively, assuming greater effect with increased copy number of the minor allele), unless otherwise specified.

Results

Patient Characteristics

Out of a total of 3,648 patients enrolled in the SCCS, 1661 patients with known duration of infection were included in the primary analyses of the present study based on the selection criteria defined above. Out of these patients, 50 individuals developed HCC. The three additional cohorts included a total number of 1229 patients with chronic hepatitis C and HCC, as well as 2714 patients with chronic hepatitis C without HCC at the time of the analysis. Thus, the primary analysis of the present study included 1279 chronic hepatitis C patients with HCC and 4325 without HCC. Baseline characteristics of these patients are summarized in Table 1.

In addition, 963 and 750 SCCS patients were eligible to assess the impact of genetic variations in *CYP2R1*, *GC*, and *DHCR7* on FPR and on the outcome of standard treatment with PEG-IFN-α and ribavirin, respectively. Serum levels of $25(OH)D_3$ were only available in a minority for 496 SCCS patients.

Table 1. Baseline characteristics of included patients.

	SCCS		Japanese GWAS		Japanese Repl.		Bonn/Berlin	
	HCC	Control	HCC	Control	HCC	Control	HCC	Control
N	50	1611	310	1252	803	1253	116	209
Age (SD)[1]	28* (15)	21 (10)	–	–	–	–	61* (10.6)	48 (12.4)
Male sex, n (%)[1]	31 (62)	991(62)	230 (74)*	725 (58)	515 (64)*	570 (45)	73 (58)	114 (55)
Alcohol (≥40 g/d ≥5 years), n (%)[2]	3 (8)	220 (17)	–	–	–	–	–	–
Diabetes, n (%)	9 (18)*	91 (6)	–	–	254 (32)*	219 (18)	–	–
HCV Genotype, n (%)[3]								
1, 4	27 (60)	1014 (64)	307 (99)	1241 (99)	540 (72)*	837 (67)	–	–
2, 3	17 (40)	570 (36)	2 (1)	10 (1)	213 (28)*	416 (33)	–	–

*These comparisons between HCC cases and controls are statistically significant ($P<0.05$). Repl, replication. The cohort labeling "Japanese GWAS" and "Japanese Replication" is based on the initial description of the cohorts in Miki *et al.*, for the present study both cohorts served as replication cohorts.

[1]Age and sex data was missing in 5 patients in the Bonn/Berlin cohort. For the SCCS, age of infection is shown. Age of infection was unknown for the Bonn/Berlin cohort, for this cohort age at diagnosis is shown.

[2]Alcohol consumption data was missing in 12 HCC and 290 non-HCC patients from the SCCS.

[3]HCV genotype was missing in 5 HCC and 23 non-HCC patients from the SCCS, in 1 HCC and 2 non-HCC patients from the Japanese GWAS, and 50 HCC patients from the Japanese replication cohorts.

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Association between Genetic Determinants of 25(OH)D_3 Serum Levels and HCV-induced HCC

Median 25(OH)D_3 serum levels in patients with chronic hepatitis C with or without HCC in the SCCS were 12.7 and 14.3 ng/mL, (range 4.9–40.9 and 3.9–76.9) respectively ($P = 0.19$, values available in 496 patients). However, 25(OH)D_3 serum levels fluctuate strongly during seasons and as a consequence of numerous circumstances such as exposure to sunlight, nutrition, accompanying diseases, vitamin D supplementation and others. We therefore believe that genetic variants in *CYP2R1*, *GC*, and *DHCR7*, with their proven impact on 25(OH)D_3 serum levels, should be used as surrogates for long-term 25(OH)D_3 serum levels. Hence, we genotyped the most relevant tagging SNPs for these loci (rs1993116/rs10741657 for *CYP2R1*, rs2282679 for *GC*, rs7944926/rs12785878 for *DHCR7*; data on LD and Hardy Weinberg equilibrium for these SNPs are shown in Table S1), and assessed their association with HCV-related HCC.

Table 2 summarizes results of the primary analysis for associations between HCV-related HCC and genetic variations in *CYP2R1*, *GC*, and *DHCR7* in the four independent patient cohorts. In the combined analyses of these cohorts, the strongest association with HCV-induced HCC was found for *GC* ($P = 0.007$, OR = 1.56, 95% CI = 1.12–2.15) and *DHCR7* ($P = 0.003$, OR = 1.42, 95% CI = 1.13–1.78), whereas *CYP2R1* was almost significantly associated with HCV-induced HCC ($P = 0.07$, OR = 1.13, 95% CI = 0.99–1.28). Remarkably, in each independent cohort, frequencies and ORs for the risk alleles at each locus showed an association in the similar direction, even though statistical significance was not reached for all subgroups.

Of note, genotyping of rs1287578 in *DHCR7* revealed huge differences of T allele frequencies between Caucasian and Japanese cohorts. These differences are in line with allele frequencies reported in HapMap. Since previous GWAS on vitamin D serum levels did not include relevant numbers of Asian

Table 2. Summary of associations between SNPs in *CYP2R1*, *GC*, and *DHCR7*, and HCV-related hepatocellular carcinoma development.

CYP2R1

			Cases			Controls			Risk allele frequencies			
SNP	**Study**	**Allele 1/2**	**11**	**12**	**22**	**11**	**12**	**22**	**Case**	**Control**	***P***	**OR (95% CI)**
rs1993116	SCCS	A/G	6	16	28	199	774	634	0.72	0.64	0.02	1.95 (1.18–3.41)
rs1993116	Japanese GWAS	A/G	41	136	133	163	621	468	0.65	0.62	0.07	1.26 (0.98–1.61)
rs10741657[1]	Japanese Replication	A/G	106	377	320	174	597	482	0.63	0.62	0.5	1.06 (0.88–1.27)
rs1993116	Bonn-Berlin	A/G	17	48	47	25	98	81	0.63	0.64	0.7	1.10 (0.67–1.76)
	Combined	A/G	***170***	***577***	***528***	***561***	***2090***	***1665***	***0.64***	***0.63***	***0.07***	***1.13 (0.99–1.28)***

GC

SNP	**Study**	**Allele 1/2**	**11**	**12**	**22**	**11**	**12**	**22**	**Case**	**Control**	**P**	**OR (95% CI)**
rs2282679	SCCS	T/G	26	20	4	855	641	104	0.28	0.27	0.6	1.25 (0.44–3.54)
rs2282679	Japanese GWAS*	T/G	153	125	31	679	475	97	0.30	0.27	0.19	1.33 (0.87–2.03)
rs2282679	Bonn-Berlin	T/G	55	46	15	117	77	14	0.33	0.25	0.06	2.01 (0.97–4.38)
	Combined	***T/G***	***234***	***191***	***50***	***1651***	***1193***	***215***	***0.31***	***0.27***	***0.007***	***1.56 (1.12–2.15)***

DHCR7

SNP	**Study**	**Allele 1/2**	**11**	**12**	**22**	**11**	**12**	**22**	**Case**	**Control**	**P**	**OR (95% CI)**
rs7944926	SCCS	T/C	17	10	4	462	310	55	0.29	0.25	0.9	1.04 (0.51–2.14)
rs7944926	Japanese GWAS	T/C	127	156	27	599	534	119	0.34	0.31	0.03	1.32 (1.03–1.70)
rs12785878[2]	Japanese Replication	T/G	84	336	383	153	543	557	0.69	0.66	0.15	1.13 (0.95–1.36)
rs12785878[2]	Bonn-Berlin	T/G	63	44	9	113	77	18	0.27	0.27	1.00	1.00 (0.63–1.58)
	Combined[#]	***T/C***	***144***	***166***	***31***	***1061***	***844***	***174***	***0.33***	***0.29***	***0.003***	***1.42 (1.13–1.78)***

Allele 2 indicates the risk allele, according to Wang et al. [9]. *P*-values and ORs were calculated for risk genotypes using favorable genotypes as a reference, i.e. for *CYP2R1* by comparing GG *vs.* GA/AA genotypes, for *GC* by comparing TT/TG vs. GG genotypes, and for *DHCR7* by comparing TT vs. TC/CC genotypes.

[#]Only patients from the SCCS and Japanese GWAS were included in the combined analysis for this locus, because of the different allele frequencies for rs12785878 in Japanese patients compared to Caucasian patients. Data remain significant after inclusion of the Bonn-Berlin cohort (P = 0.018, OR = 1.27 [95% CI = 1.04–1.56]).

*Genotyping of this SNP failed in the Japanese Replication cohort due to limited amounts of DNA. Please note that the total number of patients with available genotypes is not equal between different loci due to limited amount of DNA or genotyping failure in some cases.

[1]rs1993116 and rs10741657 are in complete LD in the Caucasian and Japanese population ($R^2 = 0.95$ and = 1.00, respectively), the major alleles of both SNPs have a similar impact on 25(OH)D_3 serum levels, indicating that both SNPs can be used equivalently [9].

[2]rs7944926 and rs12785878 are in complete LD in the Caucasian and Japanese population ($R^2 = 1.00$ and = 1.00, respectively), the major alleles of both SNPs have a similar impact on 25(OH)D_3 serum levels, indicating that both SNPs can be used equivalently [9].

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individuals, a functional interpretation of rs1287578 genotyping data in Japanese appears to be difficult, as the risk allele for this SNP in Asians has not yet been clearly identified. Therefore, data for rs1287578 genotype in the Japanese replication cohort were not included in the combined analysis shown in Table 2.

The known duration of infection allowed an additional cox regression analyses of the risk of HCC in patients from the SCCS. Figure 1 shows the cumulative incidence of HCC in patients with the *CYP2R1* risk genotype rs1993116 GG compared to those with the favorable genotypes GA and AA ($P=0.037$, hazard ratio (HR) = 1.81 (95% confidence interval (CI) = 1.03–3.13).

To exclude a possible selection bias within the SCCS, a subanalysis was performed in which the inclusion criterion "known duration of infection", which was specific for the SCCS, was omitted. As shown in Table S3, results of this case-control study are largely comparable to the primary analysis of our study.

Association between Genetic Determinants of 25(OH)D_3 Serum Levels and Liver Fibrosis Progression Rate (FPR) and Treatment Outcome

Thus far, we cannot completely exclude that the above described associations between genetic determinants of reduced 25(OH)D_3 serum levels and HCV-induced HCC are primarily mediated by an effect of the indicated SNPs on FPR or treatment outcome. We therefore performed sub-analyses to test whether FPR or treatment outcome are associated with variations in *CYP2R1*, *GC* and *DCHR7*. In 963 SCCS patients in whom FPR could be calculated, none of the SNPs was significantly associated with slow *vs.* fast FPR ($P=0.2$ for rs1993116 in *CYP2R1*; $P=0.5$ for rs2282679 in *GC*, and $P=0.3$ for rs7944926 in *DHCR7*; Table 3). In addition, in 750 SCCS patients who had received standard therapy with PEG-IFN-α and ribavirin, no significant associations were found between SNPs in *CYP2R1*, *GC* and *DHCR7* and treatment outcome (SVR *vs.* no SVR; $P=0.9$, 0.4, 0.2, respectively; Table 4), suggesting that the observed associations between these loci and HCC are specific for (HCV-induced) hepatocarcinogenesis. Finally, we calculated 25(OH)D_3 serum levels according to *CYP2R1*, *GC*, and *DHCR7* genotypes. 25(OH)D_3 serum levels were 14.9, 13.4 and 12.4 ng/mL in patients with *CYP2R1* rs1993116 genotype AA, AG, and GG ($P=0.41$), respectively; 14.4, 14.2, 11.7 in patients with *GC* rs2282679 genotype TT, TG, GG ($P=0.037$); and 14.3, 14.4, and 13.1 in patients with *DHCR7* rs7944926 genotype TT, TC, and CC ($P=0.44$).

Discussion

The present study shows that genetic variations in *CYP2R1*, *GC*, and *DHCR7* are associated with progression to HCC in patients with chronic hepatitis C. The SNPs investigated in *CYP2R1*, *GC*, and *DHCR7* have been identified by two independent GWAS as relevant genetic determinants of reduced 25(OH)D_3 serum levels [8,9]. Although genetic association studies cannot prove causal relationships between genetic variations and specific phenotypes, the association here identified between three genetically independent, but functionally related susceptibility loci of vitamin D deficiency and HCV-induced HCC suggest that an impaired vitamin D metabolism (functionally) contributes to hepatocarcinogenesis in HCV-infected patients. Importantly, the lack of association of these SNPs in *CYP2R1*, *GC*, and *DHCR7* with FPR and response to treatment with PEG-IFN-α and ribavirin suggests a specific effect of these genetic variations on HCV-induced hepatocarcinogenesis.

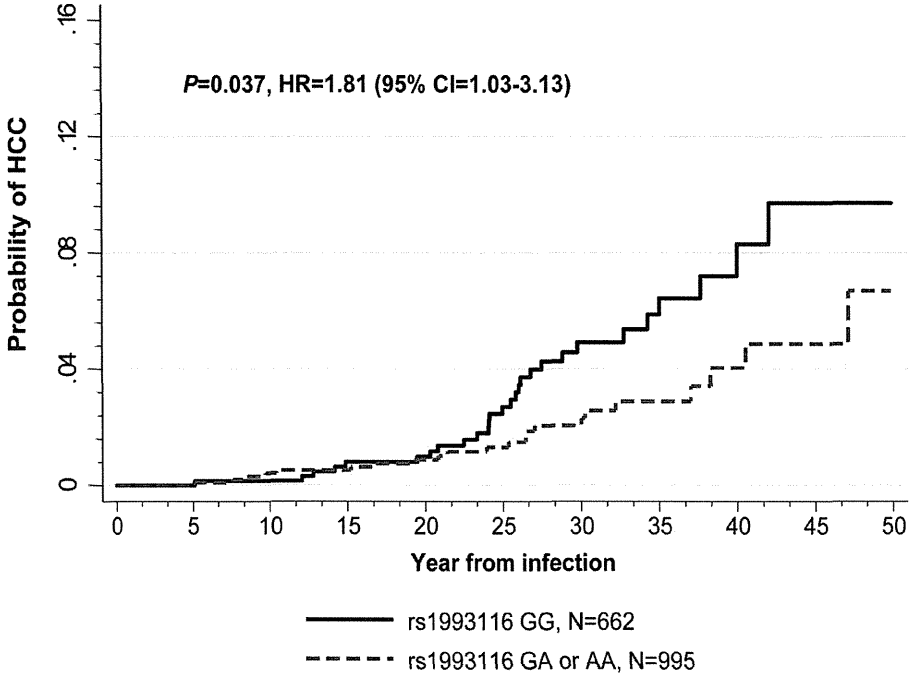

Figure 1. Risk of hepatocellular carcinoma (HCC) development in SCCS patients with chronic hepatitis C and known duration of infection, according to *CYP2R1* rs1993116 genotypes. The probability to develop HCC from the time of hepatitis C virus infection by *CYP2R1* rs1993116 genotypes (GG *vs.* GA/AA) was assessed by using cumulative incidence curves, with censoring of data at the date of last follow-up or death. Statistics are shown for univariate Cox regression analysis. CI, confidence interval; HR, hazard ratio.
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Table 3. Association of SNPs in *CYP2R1*, *GC*, and *DHCR7* with liver fibrosis progression rate (FPR) in patients with chronic hepatitis C.

			Patients, n (%)			
Gene	**SNP**	**Gt**	**Slow FPR***	**Fast FPR***	**P**	**OR (95% CI)**
CYP2R1	rs1993116	GG	183 (41)	192 (37)		
		GA	213 (48)	263 (51)	0.2	1.20 (0.93–1.56)
		AA	47 (11)	65 (13)		
GC	rs2282679	TT	236 (53)	266 (51)		
		TG	175 (40)	217 (42)	0.5	1.08 (0.84–1.39)
		GG	31 (7)	34 (7)		
DHCR7	rs7944926	TT	160 (54)	221 (58)		
		TC	115 (39)	139 (36)	0.3	0.85 (0.63–1.16)
		CC	22 (7)	22 (6)		

*FPR, liver fibrosis progression rate. Slow *vs.* fast FPR was defined as FPR<vs. ≥ sex-adjusted median FPR in METAVIR-units per year. Statistics are shown for logistic regression analyses based on the additive model of inheritance. The usage of other models of inheritance (recessive, dominant) did not result in significant associations as well (not shown).

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Table 4. Association of SNPs in *CYP2R1*, *GC*, and *DHCR7* with response to treatment of pegylated interferon-α and ribavirin in patients with chronic hepatitis C.

			Patients, n (%)			
Gene	**SNP**	**Gt**	**without SVR**	**with SVR**	**P**	**OR (95% CI)**
CYP2R1	rs1993116	GG	109 (38)	171 (37)		
		GA	138 (48)	238 (52)	0.9	1.03 (0.76–1.39)
		AA	42 (15)	52 (11)		
GC	rs2282679	TT	166 (57)	248 (54)		
		TG	105 (36)	186 (40)	0.42	1.14 (0.85–1.54)
		GG	19 (7)	26 (6)		
DHCR7	rs7944926	TT	127 (53)	230 (58)		
		TC	95 (40)	143 (36)	0.2	0.81 (0.59–1.12)
		CC	17 (7)	22 (6)		

Statistics are shown for logistic regression analyses based on the additive model of inheritance. The usage of other models of inheritance (recessive, dominant) did not result in significant associations as well (not shown).

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Vitamin D insufficiency has been previously linked to the development of HCC [16]. However, causal relationships remained mostly unclear because these studies were small or concentrated on the assessment of $25(OH)D_3$ serum levels at the date of HCC, which may result in false-positive associations due the influence of impaired liver function on circulating $25(OH)D_3$ [5,16–18]. In addition, randomized controlled clinical trials evaluating the effect of vitamin D or its analogues on HCC development are lacking. Thus, the results of the present, large-scale genetic association study add a significant argument to evaluate the possible chemo-preventive effect of vitamin D supplementation in HCV-infected patients. However, although our data suggest a causal role of vitamin D metabolism in HCV-induced HCC, there is currently no published evidence that vitamin D supplementation would translate into a benefit in HCV-infected patients with advanced liver disease. In this regard, caution is advisable since some placebo-controlled studies evaluating the supplementation of other vitamins have reported a reduced overall survival rate in some verum groups [19]. Yet, in view of our present data, and in view of the high prevalence of severe vitamin D deficiency in patients with chronic hepatitis C [10,20,21], randomized controlled clinical trials in HCV-infected patients with advanced liver disease appear to be justified.

Limitations

Our study has potential limitations. First, $25(OH)D_3$ serum levels were only available in a relatively small subgroup of patients. Nevertheless, we observe the same trends for $25(OH)D_3$ serum levels according to *CYP2R1, GC,* and *DHCR7* genotypes as described previously, and HCV-infected patients with HCC had slightly lower $25(OH)D_3$ serum levels compared to those without HCC. More important, we believe that analyses of associations between punctual $25(OH)D_3$ serum levels and endpoints such as HCC can be misleading, since $25(OH)D_3$ serum levels strongly fluctuate during seasons, with age, and as a consequence of numerous other conditions (liver fibrosis, diabetes, obesity, supplementation, etc.) [6,7]. A second limitation of our study is the relatively weak level of statistical significance in the analyses of the individual cohorts, with the consequence that associations for the three loci with HCC became only fully significant in the combined analyses of all included patients. Most likely, this can be explained by the relatively low frequency of the risk genotypes (especially of *GC* and *DHCR7*), as well as by the numerous variables with influence $25(OH)D_3$ serum levels in a given individual [9]. In this regard, it is important to note that ORs for the risk genotype of all genes (*CYP2R1, GC,* and *DHCR7*) were similarly directed in the discovery cohort as well as in all replication cohorts, confirming a true association between these loci and HCV-induced HCC. In addition, the strengths of the associations between these loci and HCV-induced HCC were largely comparable to the strength of the associations between these loci and $25(OH)D_3$ serum levels (strong effect of *GC* and *DHCR7*, moderate effect of *CYP2R1*, according to Wang *et al.*) [9]. Nevertheless, the observed subtle differences between the different cohorts included in our study may not only be explained by the relatively small sample size of individual cohorts, but also by specific cohort features such as different recruitment strategies (e.g. cohort study *versus* case-control studies). In this regard, our study also cannot fully rule out whether all investigated genes (*CYP2R1, GC, DHCR7*) have the same impact on progression to HCV-related HCC at different stages of liver disease or in populations of different ancestries. Furthermore, our study cannot clearly characterize whether the observed findings apply for patients who did or did not respond to antiviral therapy. Though we show in the SCCS (in a minority of the whole study population), that SNPs in *CYP2R1, GC,* and *DHCR7* were not associated with treatment outcome, it remains unclear whether these genetic variations are associated with HCC in both individuals with or without treatment-induced eradication of HCV.

The heterogeneity of the four independent cohorts included in our analyses might be perceived as another limitation. This applies for important cohort features such as race, recording of treatment modalities, HCC frequency, or different allele frequencies for some SNPs (especially rs1278578).

Conclusions

In conclusion, we provide evidence for a functionally relevant contribution of reduced $25(OH)D_3$ serum levels to HCV-induced

HCC. Controlled clinical trials to evaluate the impact of vitamin D supplementation on HCC risk and overall survival in patients with chronic hepatitis C appear to be justified.

Supporting Information

Table S1 Linkage disequilibrium of SNPs in *CYP2R1*, *GC*, and *DHCR7* investigated in the present study.
(DOC)

Table S2 Primers for SNP genotyping assays.
(DOC)

Table S3 Summary of associations between SNPs in *CYP2R1*, *GC*, and *DHCR7*, and HCV-related hepatocellular carcinoma development, considering the SCCS as case-control study.
(DOC)

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Conceived and designed the experiments: CML ZK D. Moradpour PYB. Performed the experiments: CML D. Miki HO HDN JB SB AC JFD MG MHH RM BM FN DS ZK US TM TB KC D. Moradpour PYB. Analyzed the data: CML KM HO KM JG ZK KC D. Moradpour PYB. Contributed reagents/materials/analysis tools: CML D. Miki HO HDN JB SB AC JFD MG MHH RM BM FN DS ZK US TM TB KC D. Moradpour PYB. Wrote the paper: CML D. Miki HO HDN JB SB KM JG AC JFD MG MHH RM BM FN DS ZK TM US TB KC D. Moradpour PYB.

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Circulating MicroRNA-22 Correlates with MicroRNA-122 and Represents Viral Replication and Liver Injury in Patients with Chronic Hepatitis B

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Hepatitis B virus (HBV) infection is associated with increased expression of microRNA-122. Serum microRNA-122 and microRNA-22 levels were analyzed in 198 patients with chronic HBV who underwent liver biopsy and were compared with quantitative measurements of HBsAg, HBeAg, HBV DNA, and other clinical and histological findings. Levels of serum microRNA-122 and microRNA-22 were determined by reverse transcription-TaqMan PCR. Serum levels of microRNA-122 and microRNA-22 were correlated ($R^2 = 0.576$; $P < 0.001$), and both were elevated in chronic HBV patients. Significant linear correlations were found between microRNA-122 or microRNA-22 and HBsAg levels ($R^2 = 0.824$, $P < 0.001$ and $R^2 = 0.394$, $P < 0.001$, respectively) and ALT levels ($R^2 = 0.498$, $P < 0.001$ and $R^2 = 0.528$, $P < 0.001$, respectively). MicroRNA-122 levels were also correlated with HBV DNA titers ($R^2 = 0.694$, $P < 0.001$ and $R^2 = 0.421$, $P < 0.001$). Levels of these microRNAs were significantly higher in HBeAg-positive patients compared to HBeAg-negative patients ($P < 0.001$ and $P < 0.001$). MicroRNA-122 levels were also lower in patients with advanced liver fibrosis ($P < 0.001$) and lower inflammatory activity ($P < 0.025$). These results suggest that serum micro-RNA levels are significantly associated with multiple aspects of HBV infection. The biological meaning of the correlation between microRNA-122 and HBsAg and should be investigated further.
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KEY WORDS: HBsAg; histological activity; inflammation; microRNA

Abbreviations: ALT, alanine transaminase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-122, microRNA-122; miR-22, microRNA-22; PCR, polymerase chain reaction.

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INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped virus with a partially double-stranded 3.2 kb DNA genome belonging to the Hepadnaviridae family [Fields et al., 2007]. Chronic HBV infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [Beasley et al., 1981]. More than 350 million people are persistent carriers of HBV and many may progress to chronic liver disease [Lavanchy, 2004; McMahon, 2009].

MicroRNAs are a class of naturally occurring short non-coding RNAs that regulate the expression of a wide range of genes and play an important role in various biological functions including cell differentiation, development, immune responses, metabolism, and carcinogenesis. Circulating microRNAs are bound to Ago2 and remain in the serum for an extended period of time [Blumberg et al., 1965; Bala et al., 2009]. Liver damage ultimately results in alteration of hepatic and serum microRNA expression profiles [Bala et al., 2009]. Hepatocellular carcinoma-associated expression profiles have been reported by a number of laboratories [Murakami et al., 2006; Ji et al., 2009; Ura et al., 2009; Gao et al., 2011; Hou et al., 2011; Mizuguchi et al., 2011], but microRNA expression profiles may differ based on etiology, including differences among patients infected with HBV compared with patients infected with hepatitis C virus (HCV). HBV infection disrupts pathways involved in signal transduction, DNA damage, and cell death, whereas HCV infection tends to disrupt pathways involved in lipid metabolism, cell cycle regulation, and immune response [Ura et al., 2009].

Many of these cellular changes are mediated by changes in microRNA expression, suggesting that analysis of microRNA expression may improve understanding of HBV pathogenesis and uncover new avenues for risk assessment and therapy. A number of microRNAs associated with HBV infection have been reported [Bala et al., 2009], but in most cases little is known about the biological roles of the identified microRNAs. In this study, two microRNAs, microRNA-122 (miR-122) and microRNA-22 (miR-22), were examined as possible biomarkers for association with chronic HBV infection. miR-122 was selected due to its strong expression in the liver and central role in liver function, and because it directly suppresses HBV replication by binding to viral RNA [Qiu et al., 2010; Chen et al., 2011]. Serum miR-122 has been reported as a biomarker for various liver injuries and is correlated with levels of ALT, HBV DNA, and HBsAg [Zhang et al., 2010; Waidmann et al., 2012]. Circulating miR-122 is elevated in patients with chronic hepatitis B, especially in patients positive for HBeAg [Xu et al., 2010; Ji et al., 2011; Qi et al., 2011; Zhou et al., 2011; Waidmann et al., 2012]. miR-22 was selected for this study because it is also highly expressed in the liver and has been implicated in HCC and liver failure in patients infected with HBV [Ji et al., 2011; Jiang et al., 2011; Xu et al., 2011]. miR-22 is described in the literature both as a tumor-suppressor [Xu et al., 2011] and as a micro-oncogene [Liu et al., 2010] due to its central role in targeting multiple genes involved in determining cell fate, including PTEN [Liu et al., 2010], p21 [Tsuchiya et al., 2011], Mat1a and Mthfr [Koturbash et al., 2011], and senescence-associated transcripts CDK6, SIRT1, and Sp1 [Xu et al., 2011]. miR-22 also targets estrogen receptor alpha [Pandey and Picard, 2009], which compromises the protective effects of estrogen and leads to up-regulation of IL-1α in hepatocytes under conditions of oxidative stress, such as that caused resulting from activity of the HBx protein [Jiang et al., 2011]. HBV also evades senescence through hypermethylation of p16 and transcriptional interference in components of the stress-induced senescence pathway [Kim et al., 2010]. Changes in miR-22 expression may, therefore, reflect cellular changes leading to suppression of senescence and indicate an increased risk of dysplasia.

Because of their prominent roles in the liver and association with HBV infection, serum microRNA levels of miR-122 and miR-22 were compared between healthy individuals and patients with chronic HBV infection, and correlation with clinical and histological parameters were examined.

MATERIALS AND METHODS

Study Patients

One hundred and ninety-eight patients with chronic hepatitis B who visited Hiroshima University Hospital between January 2000 to December 2009 who underwent liver biopsy for diagnosis of chronic hepatitis and agreed to provide blood samples for a viral hepatitis study were examined. Histological diagnosis was evaluated as described previously [Desmet et al., 1994]. Anti-HBs and anti-HBc antibodies were also examined in 22 healthy controls, all of whom tested negative for HBsAg and anti-HBc and anti-HCV antibodies. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent for the study using a form approved by the ethical committee of Hiroshima University.

Viral Markers

Serum samples obtained at biopsy were kept frozen at −80°C prior to analysis. Serum HBsAg and HBeAg levels were measured quantitatively using the Abbott Chemiluminescence Immunoassay Kit (Abbott Japan, Tokyo, Japan). HBV DNA levels were determined by the Cobas TaqMan HBV standardized real-time polymerase chain reaction (PCR) assay (Roche Molecular Systems, Pleasanton, CA). Results are expressed in log10 international units per milliliter.

MicroRNA Analysis

Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion,

TABLE I. Clinical Characteristics of Hepatitis B Virus Patients and Healthy Controls

	HBV patients (n = 198)		Healthy controls (n = 22)	
Characteristic	N	Value	N	Value
Age (years)[a]	198	42 (13–71)	22	31.5 (25–39)
Sex (male/female)	198	140/58	22	10/12
Fibrosis (1/2/3/4)	198	58/75/43/22		
Activity (0/1/2/3)	198	2/53/109/34		
miR-122/cel-miR-238	198	0.144 (0.002–1.737)	22	0.02 (0.01–0.04)
miR-22/cel-miR-238	198	0.266 (0.019–1.652)	22	0.02 (0.11–0.49)
HBV DNA (LGE/ml)[a]	181	6.5 (2.6–8.8)		
AST (IU/l)[a]	197	51 (18–982)		
ALT (IU/l)[a]	197	73 (10–1,867)	20	16 (10–23)
γ-GT (IU/l)[a]	189	46 (9–536)		
ALB (g/dl)[a]	196	4.3 (2.6–5.2)		
PLT ($\times 10^4$/mm^3)[a]	197	17.1 (1.0–36.2)		
PT[a]	180	92 (19–146)		
AFP (ng/ml)[a]	186	6.5 (<5.0–8,928.0)		
HBsAg (IU/ml)	176	2,765 (<0.05–1,55,000)		
Anti-HBeAg (±/NA)	176	104/82/12		
HBeAb (±/NA)	176	85/96/17		

NA, not available.
[a]Median (range).

Austin, TX) according to the manufacturer's instructions. RNA was eluted in 80 μl of nuclease free water and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Japan Ltd, Tokyo, Japan). *Caenorhabditis elegans* miR-238 (cel-miR-238) was spiked to each sample as a control for extraction and amplification steps. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10× reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5× RT primer, 0.25 μl of RNase inhibitor, and 7.22 μl of nuclease free water in a total volume of 20 μl. The reaction was performed at 16°C for 30 min followed by 42°C for 30 min. The reaction was terminated by heating the solution at 85°C for 5 min. miR-122 and miR-22 were amplified using primers and probes provided by Applied Biosystems (Foster City, CA) using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2× Universal PCR Master Mix, 1.25 μl of 20× TaqMan Assay solution, 1 μl of reverse

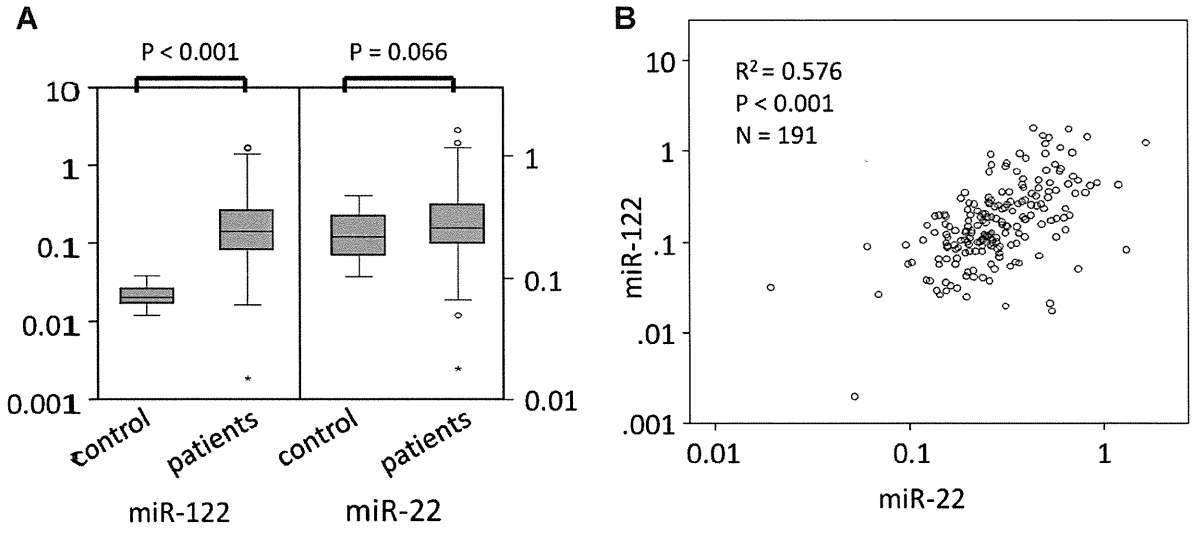

Fig. 1. Detection of miR-122 and miR-22 in patients infected with HBV and in healthy subjects and the relationship between miR-122 and miR-22. **A**: Serum levels of miR-122 and miR-22 in patients infected with HBV (171) and in healthy controls (22). Boxes represent 25–75 percentiles, and horizontal bars represent median values. Statistical analysis was performed using the Mann–Whitney U test. **B**: The relationship between miR-122 and miR-22 was analyzed using the Spearman rank correlation coefficient.

transcription product, and 10.25 μl of nuclease free water in a total volume of 25 μl. Amplification conditions were 95°C for 10 min followed by 50 denaturing cycles for 15 sec at 95°C and annealing and extension for 60 sec at 60°C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized microRNA was used to generate a standard curve that permitted absolute quantification of molecules. For miR-122 and miR-22, relative abundance was determined using standard curves generated with a dilution series of samples with high serum levels. miR-122 and miR-22 levels were calculated by normalizing based on cel-miR-238 measurement levels.

Statistical Analysis

Data were analyzed using the Mann–Whitney U test for continuous variables and the chi-squared or Fisher exact test for categorical variables using the R statistics package (http://www.r-project.org). Factors associated with high miR-122 and miR-22 levels were analyzed by multiple regression analysis using the rms library. Forward/backward stepwise selection of factors with a P-value < 0.05 in univariate analysis was used for model selection. The Spearman rank correlation coefficient was used to evaluate the strength of the association between continuous variables.

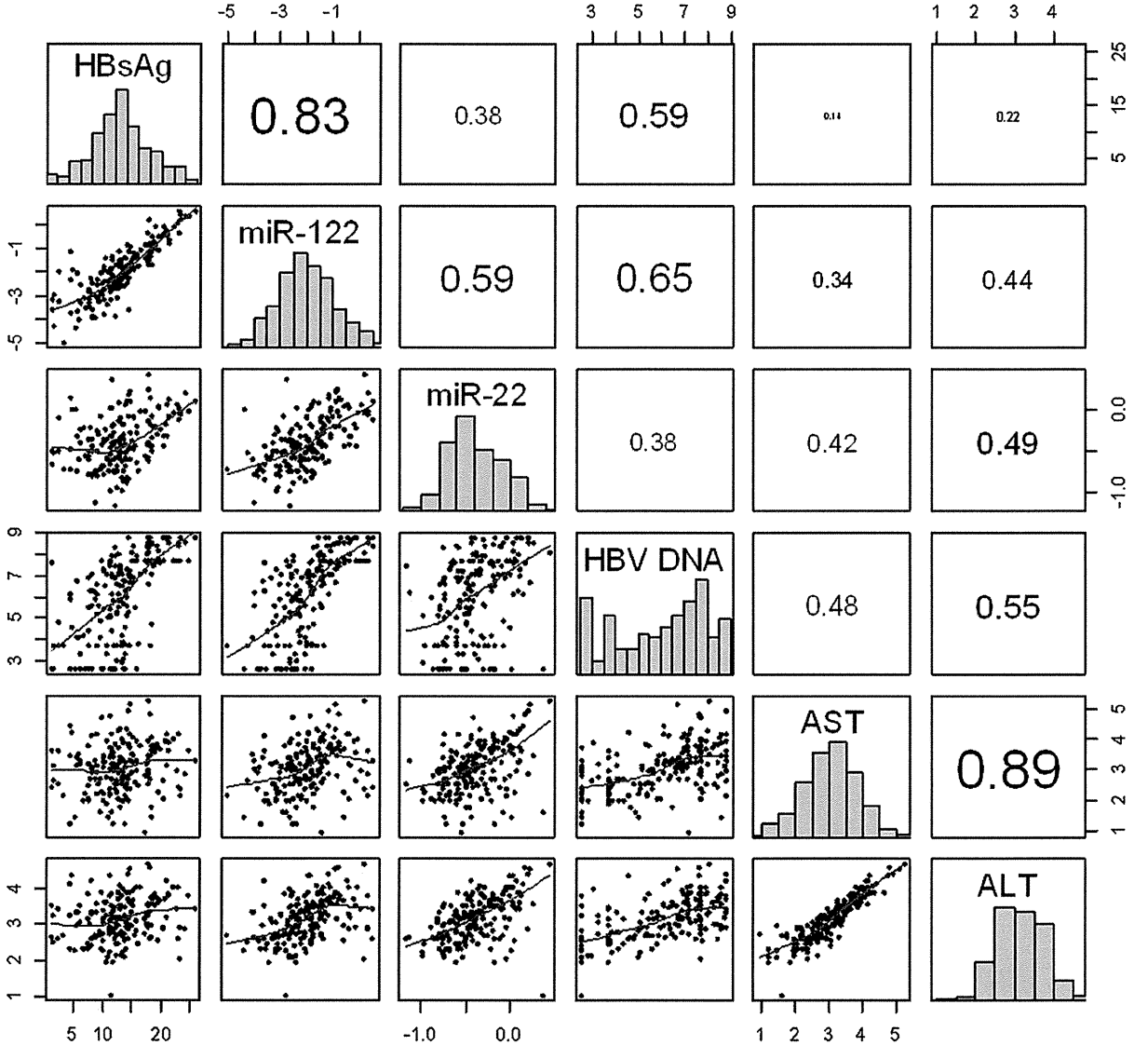

Fig. 2. Pairwise correlations of miR-122 and miR-22 with HBsAg, HBV DNA, ALT, and AST levels. Serum levels of miR-122 and miR-22 were compared with serum HBsAg and HBV DNA titers and with ALT and AST levels using the Spearman rank correlation coefficient.

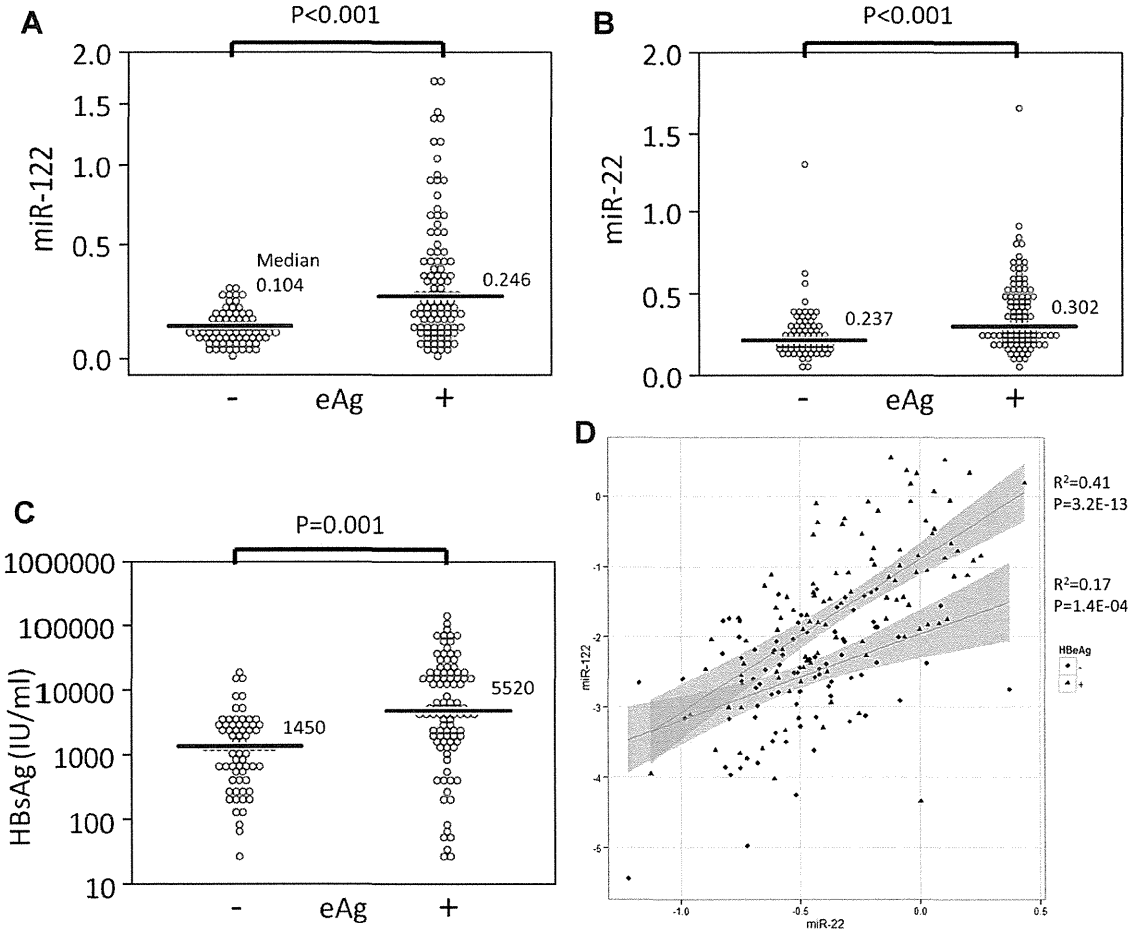

Fig. 3. Comparison of miR-122 and miR-22 with HBsAg levels between patients positive or negative for HBeAg. Serum levels of miR-122 (**A**), miR-22 (**B**), and HBsAg (**C**) were analyzed using the Mann–Whitney U test. Bars indicate median values.

RESULTS

Detection of Circulating miR-122 and miR-22 and Their Correlation

Both miR-122 and miR-22 were detectable in all HBV patients, and median values were higher than in normal controls (Table I; Fig. 1A, $P < 0.001$ and $P = 0.066$, respectively). miR-122 and miR-22 expression levels were moderately correlated (Fig. 1B, $R^2 = 0.576$, $P < 0.001$).

miR-122 and miR-22 Levels and Viral Markers

Relationships between miR-122 and miR-22 levels and HBsAg, HBeAg, and ALT levels were examined (Fig. 2A). There was a strong linear correlation between HBsAg and miR-122 levels ($R^2 = 0.824$, $P < 0.001$). There was also a correlation between HBsAg and miR-22 levels ($R^2 = 0.394$, $P < 0.001$), although the correlation was not as strong as with miR-122. Both miR-122 and miR-22 were also correlated with HBV DNA titers ($R^2 = 0.694$, $P < 0.001$

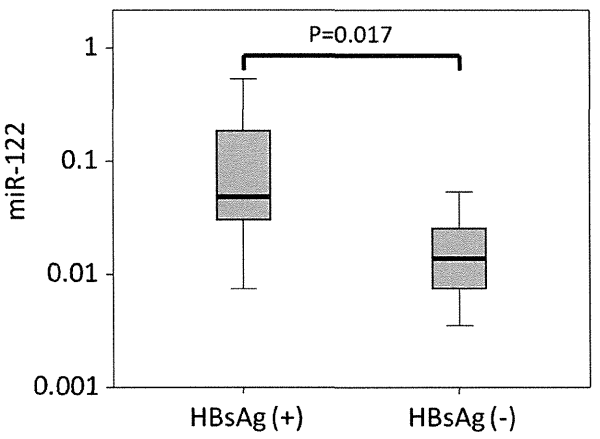

Fig. 4. miR-122 and HBsAg elimination. miR-122 levels before and after HBsAg elimination are shown for patients who became negative for HBsAg (n = 13). Bars represent median, minimum, and maximum levels, and boxes represent the 25th and 75th percentiles. Data were analyzed using the Mann–Whitney U test.

and $R^2 = 0.421$, $P < 0.001$, respectively) and ALT levels ($R^2 = 0.498$, $P < 0.001$ and $R^2 = 0.528$, $P < 0.001$, respectively). The correlation with ALT was slightly stronger with miR-22 ($R^2 = 0.528$) than with miR-122 ($R^2 = 0.498$). Patients who were positive for HBeAg had elevated levels of both miR-122 and miR-22 (Fig. 3A and B; $P < 0.001$ and $P < 0.001$) and had higher HBsAg titers (Fig. 3C; $P = 0.001$). The correlation between miR-122 and miR-22 expression was also stronger in HBeAg positive patients (Fig. 3D, $R^2 = 0.41$, $P = 3.2E{-}13$) compared to HBeAg negative patients ($R^2 = 0.17$, $P = 1.4E{-}04$).

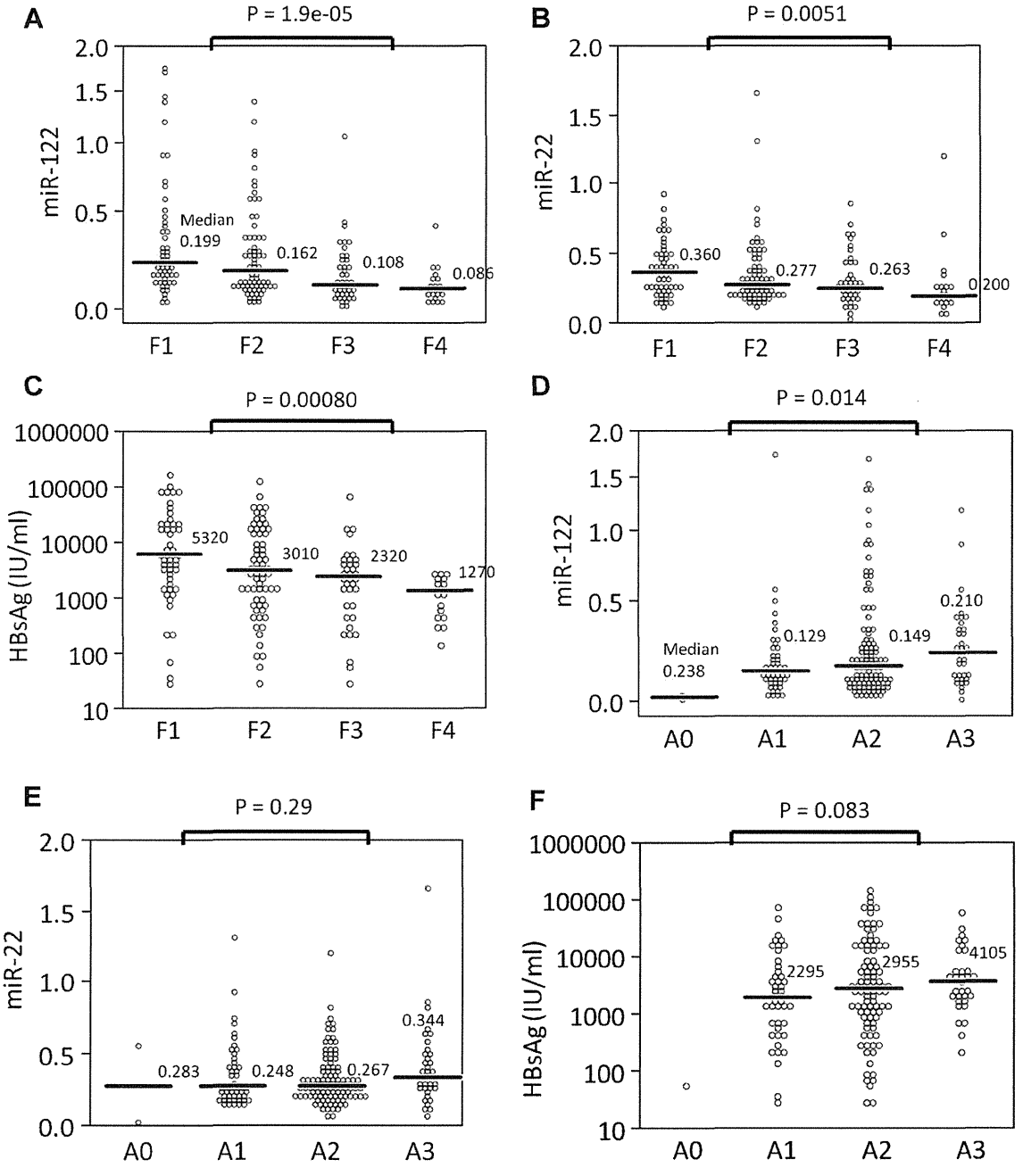

Fig. 5. Stage of fibrosis and histological inflammation activity by liver biopsy and miR-122, miR-22, and HBsAg levels. Serum levels of miR-122, miR-22, and HBsAg were plotted according to the stage of fibrosis (**A**, **B**, and **C**, respectively) and inflammation activity (**D**, **E**, and **F**). Median values are indicated as horizontal bars. Statistical analysis was performed using the Kruskal–Wallis non-parametric analysis of variance test.

miR-122 Levels in Patients Who Became Negative for HBsAg

To examine if the high miR-122 levels seen in chronic hepatitis B patients with high HBsAg levels result from active HBsAg production or represent individual characteristics that allow high-level HBsAg production, miR-122 levels were measured before and after elimination of HBsAg (observation period 4.5–16.5 years [median 9.0 years]). As shown in Figure 4, miR-122 levels in these patients declined significantly when they became negative for HBsAg ($P = 0.017$).

miR-122 and miR-22 Levels and Histological Findings

As shown in Figure 5A and B, both miR-122 and miR-22 were observed at progressively lower serum levels at more advanced stages of fibrosis ($P < 0.001$ and $P = 0.001$, respectively). HBsAg levels were also lower in patients with advanced fibrosis (Fig. 5C; $P = 0.001$). In contrast, serum levels of miR-122 and miR-22 were higher in patients with higher inflammatory activity (Fig. 5D and E; $P = 0.025$ and $P = 0.170$, respectively), although for miR-22 the difference was not significant. HBsAg levels were also marginally higher in patients with higher inflammatory activity (Fig. 5F; $P = 0.079$).

Factors Associated with Higher Serum miR-122 and miR-22 Levels

Clinical factors associated with elevated miR-122 and miR-22 levels were examined using multiple linear regression. As shown in Table II, HBsAg was most strongly associated with miR-122 ($P = 1.1E{-}67$), whereas serum AST levels were most strongly associated with miR-22 ($P = 4.7E{-}19$).

miR-122 and miR-22 Levels in Patients with Acute HBV Infection, Cirrhosis, and HCC

To examine miR-122 and miR-22 levels in patients with and without HBV infection, miR-122 and miR-22 levels were also measured in the following groups of patients: healthy controls (5), patients with acute (9) or chronic (9) HBV infection, liver cirrhosis (24),

TABLE II. Univariate and Multivariate Regression Analysis of Predictive Factors for MicroRNA-122 and MicroRNA-22 Expression Levels Relative to cel-miR-238

		Univariate			Multivariate	
MicroRNA	Variable	N	Coef.	*P*	Coef.	*P*
miR-122	Female	198	0.076	6.6E−01		
	Age	198	−0.030	2.9E−07***	0.007	1.7E−02*
	Fibrosis	198	−0.391	8.9E−07***	−0.143	3.8E−04***
	Activity	198	0.331	4.0E−03**		
	HBsAg	176	0.177	6.7E−46***	0.137	3.3E−32***
	HBeAg (±)	186	1.010	3.5E−11***		
	Anti-HBeAb (±)	181	−0.801	2.5E−07***		
	HBV DNA	181	0.357	2.1E−21***	0.064	1.4E−02*
	AST	197	0.472	6.1E−07***		
	ALT	197	0.816	2.0E−11***	0.281	4.1E−04***
	γ-GT	189	0.187	3.8E−01		
	Total bilirubin	196	−1.020	3.5E−02*	−0.596	9.8E−03**
	ALB	196	0.137	3.2E−02*		
	PT	180	0.020	1.3E−05***		
	AFP	186	0.000	1.5E−01		
	miR-22	198	2.010	1.9E−19***	0.739	4.2E−07***
miR-22	Female	198	−0.080	1.1E−01		
	Age	198	−0.009	3.8E−07***	−0.005	7.4E−04***
	Fibrosis	198	−0.085	2.9E−04***		
	Activity	198	0.053	1.1E−01		
	HBsAg	176	0.023	1.6E−07***	−0.016	2.1E−02*
	HBeAg (±)	186	0.192	1.8E−05***		
	Anti-HBeAb (±)	181	−0.143	1.5E−03**	−0.044	2.8E−01
	HBV DNA	181	0.058	4.6E−07***	−0.025	7.9E−02
	AST	197	0.161	1.5E−09***	0.116	2.6E−05***
	ALT	197	0.255	9.1E−14***		
	γ-GT	189	0.129	3.0E−02*		
	Total bilirubin	196	−0.170	2.2E−01		
	ALB	196	0.058	1.3E−03**		
	PT	180	0.006	1.6E−06***	0.004	2.3E−04***
	AFP	186	0.000	2.5E−01		
	miR-122	198	0.170	1.9E−19***	0.162	5.5E−06***

Forward/backward stepwise selection was used for model selection.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.00\,1$.