Carlsbad, CA). Based on the recent studies [17, 19–21] as well as our analysis using an oligonucleotide DNA chip, Genopal (Mitsubishi Rayon CO., LTD. Tokyo, Japan) which can detect 208 genes related to innate immune responses (data not shown), we selected the following ISGs and IFN-λs: *ISG15, A20, zc3h12a*, ring finger protein 125 (*RNF125*), myxovirus resistance protein A (*MxA*), *IL1β, IL10*, interferon regulatory transcription factor 1 (*IRF1*), *SOCS1, SOCS2, SOCS3*, 2'-5'-oligoadenylate synthetase 1 (*OAS1*), double stranded RNA-dependent protein kinase (*PKR*), *IL28A, IL28B*, and *IL29*. We then quantified their mRNA levels by real-time detection polymerase chain reaction (PCR). The primers and probes for *IL28A* and *IL28B* were designed according to the previous report [22], and those of other genes were obtained from Applied Biosystems (Carlsbad, CA) as TaqMan Gene Expression Assays (Table A in S1 File). Amplification and detection were carried out using an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Levels of mRNAs for ISGs were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control, and those for IFN-λs were measured using the calibration curves for each cDNA clone.

Statistical Analysis

Categorical variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorical variables by the Mann-Whitney U test. Correlations between continuous variables were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was considered significant in all tests.

Results

Patient characteristics and distribution of *IL28B* genetic variants

The baseline clinical characteristics of the study population are described in Table 1. The unfavorable *IL28B* genotype, TG or GG (TG/GG) at rs8099917 was possessed by 38% (19/50) of the

Table 1. Baseline clinical characteristics of the 50 chronic hepatitis C patients treated with PEG-IFN, RBV and protease inhibitor.

Characteristic	(n = 50)
Male gender	30 (60%)
Age, years	55 (29–70)
Hemoglobin, g/dL	14.8 (12.0–17.1)
Platelet count, $\times 10^4$ /μL	16.2 (9.8–27.9)
ALT, IU/L	34 (13–212)
γ-GTP, IU/L	28 (12–258)
HCV RNA, log IU/ml	6.7 (4.8–7.5)
rs8099917, TT / TG / GG	31 / 16 / 3
Fibrosis stage, F0 / 1 / 2 / 3 / 4 / N.D.	5 / 20 / 6 / 3 / 1 / 15
Prior treatment	
naïve / IFN mono / IFN +RBV / PEG-IFN+RBV	14 / 2 / 2 / 32
Treatment efficacy of PEG-IFN+RBV, TVR / NVR	19 / 13

Abbreviations: ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; N.D., not determined; IFN, interferon; RBV, ribavirin; PEG-IFN, pegylated interferon; TVR, transient virological response; NVR, non-virological response.

rs8099917: TT is favorable for treatment efficacy.

Data are expressed as numbers for categorical data or the median (range) for continuous data.

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patients. Fourteen patients were treatment-naive. Of the 32 patients previously treated with PEG-IFN/RBV, 19 and 13 had TVR and NVR, respectively. Of the 13 NVR patients, 4 were null responders, defined as having an HCV RNA decrease of < 2 log IU/mL at week 12 after the start of therapy, relative to baseline. In addition, the clinical characteristics of the subsets of patients receiving telaprevir or faldaprevir are described in Table B in S1 File. The proportions of patients with an unfavorable *IL28B* genotype and NVR on prior PEG-IFN/RBV therapy were higher in patients who received faldaprevir.

All 31 patients with a favorable *IL28B* genotype and 13 of 19 with an unfavorable genotype achieved SVR on PEG-IFN/RBV/PI treatment. Hence, the total SVR rate was 88% (44/50). The detailed information of the six non-SVR cases are described as follows: one patient did not response to PEG-IFN/RBV/telaprevir up to week 12 (quantity of HCV RNA at week 12 was 3.7 log IU/mL) and the therapy was discontinued; three had virological breakthrough at week 17, 38, 40 during PEG-IFN/RBV/faldaprevir and the therapies were discontinued; two were relapsed after the completion of PEG-IFN/RBV/faldaprevir. Thus these six patients resulted in non-SVR, though they were given enough doses of drugs. In four SVR cases, the therapies were discontinued at week 9, 11, 18, 20 during PEG-IFN/RBV/telaprevir due to adverse events. Other clinical characteristics of the patients according to *IL28B* genotype and treatment efficacy are described in Table C in S1 File.

Gene expression of ISGs and IFN-λs induced by PEG-IFN/RBV/PI in patients stratified according to *IL28B* genotype

Eight hours after the initial administration of PEG-IFN/RBV/PI, levels of mRNAs for *A20, SOCS1*, and *SOCS3* known to be genes suppressing antiviral activity via the IFN signaling pathway, as well as *IRF1* were found to be significantly higher in patients with TG/GG at rs8099917, an unfavorable *IL28B* genotype ($P = 0.007$, 0.026, 0.0004, and 0.0006, respectively). In contrast, the levels of mRNAs for *IL28A, IL28B*, and *IL29* were not different regardless of the *IL28B* genotype, although the expression of *IL28B* itself tended to be higher in patients with a favorable *IL28B* type (Fig. 1). There were also no significant differences in the levels of other mRNAs for *ISG15, IL1β, RNF125* (Fig. 1), *zc3h12a, MxA, IL10, SOCS2, OAS1* or *PKR* at 8 h (data not shown). We analyzed changes in expression of the genes for *A20, SOCS1, SOCS3* and *IRF1* between baseline and 8 h and found that the fold-changes of *SOCS3* and *IRF1* were significantly higher in patients with an unfavorable *IL28B* genotype ($P = 0.005$ and 0.030, respectively) (Fig. 2).

Correlations of gene expression in PEG-IFN/RBV/PI treatment

We evaluated the correlations of the levels of mRNAs for genes implicated in suppressing the antiviral state each other and with those promoting it, (*ISG15* and *IL28B*), in all 50 cases. The expression levels of most of the mRNAs for suppressive genes such as *A20, SOCS1* and *SOCS3*, as well as *IRF1* were significantly correlated with each other at 8 h (Fig. 3) as well as at baseline (Figure A in S1 File) and 24h (Figure B in S1 File). However, they did not correlate with those of *ISG15* and *IL28B* at 8 h (Figure C in S1 File) as well as at baseline and 24h (data not shown).

Associations between ISGs including suppressive genes against the antiviral state and prediction of treatment efficacy

To examine the association between the expression of genes suppressing antiviral activity and treatment efficacy, we divided the patients into three groups according to *IL28B* genotype and treatment outcome, as follows: TT: SVR (n = 31); TG/GG: SVR (n = 13); TG/GG: non-SVR

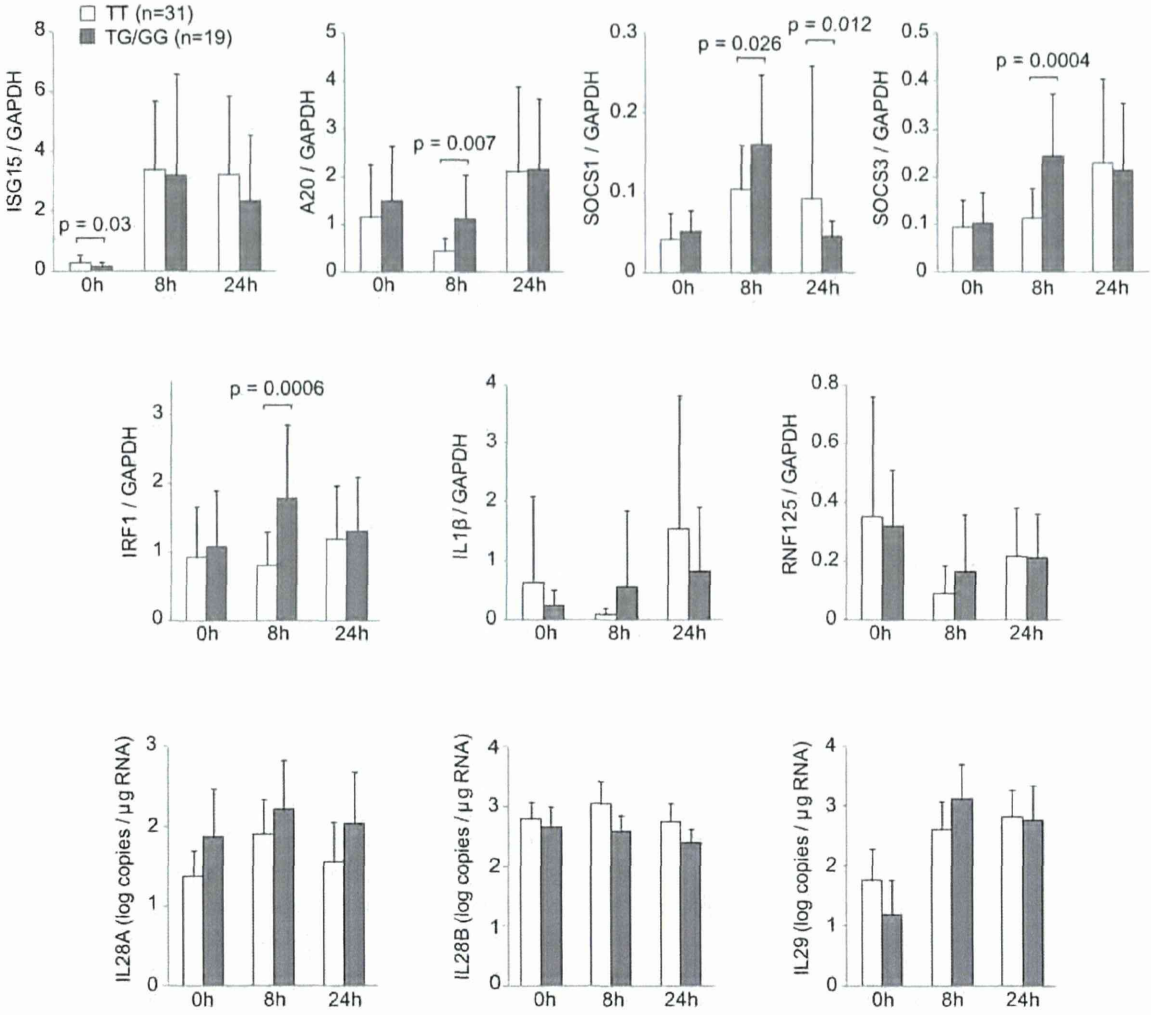

Fig 1. Expression of interferon-stimulated genes (ISGs) and interferon-lambdas (IFN-λs) in peripheral blood mononuclear cells at baseline, 8, and 24 hours after the initial administration of pegylated interferon, ribavirin, plus NS3/4A protease inhibitor, in patients stratified according to *IL28B* genotype. Levels of mRNAs for ISGs were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and those for IFN-λs were measured using the calibration curves for each cDNA clone. Bars and error bars represent means and standard deviations, respectively. TT and TG/GG at rs8099917 is a favorable and an unfavorable *IL28B* genotype for treatment responses, respectively.

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(n = 6) (Table C in S1 File). We then compared the levels of mRNAs for *A20, SOCS1, SOCS3, IRF1, ISG15*, and *IL28B* among the groups. We found that the levels of mRNAs for *A20, SOCS3* and *IRF1* at 8 h were significantly higher in TG/GG: non-SVR than in TT: SVR ($P = 0.002$, 0.001, and 0.002, respectively). Moreover, the levels of mRNAs for *SOCS3* and *IRF1* were also higher in TG/GG: SVR than in TT: SVR ($P = 0.012$ and 0.015, respectively) (Fig. 4A). Whereas the level of mRNA for *IL28B* tended to be higher in the order TT: SVR, TG/GG: SVR, TG/GG: non-SVR, there were no significant differences among the three groups. Although we also compared the expression levels of these genes at baseline and 24h among the same three groups, we could not find the definite tendency (data not shown). Next, we analyzed the changes in expression of *A20, SOCS1, SOCS3*, and *IRF1* from baseline to 8 h and found that the fold-change of *IRF1* was significantly higher in TG/GG: non-SVR than in TG/GG: SVR as well as in

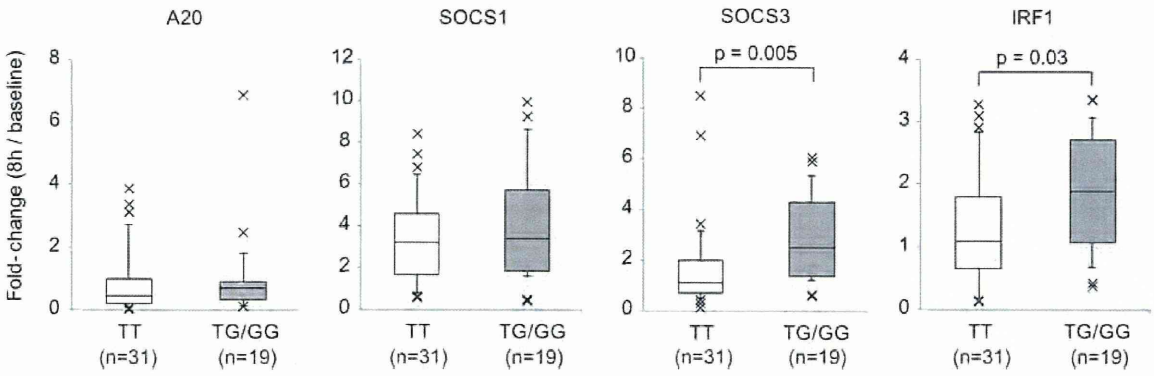

Fig 2. Fold-changes of mRNAs including suppressive genes in PBMCs at 8 hours relative to baseline in patients stratified according to *IL28B* genotype. TT and TG/GG at rs8099917 is a favorable and an unfavorable *IL28B* genotype for treatment responses, respectively. Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

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TT: SVR ($P = 0.035$ and 0.003, respectively). Similarly, the fold-change of *SOCS3* was higher in TG/GG: non-SVR and SVR than in TT: SVR ($P = 0.021$ and 0.032, respectively) (Fig. 4B). Collectively, one can conclude that levels of expression of mRNAs including these suppressive genes early after the initial administration of PEG-IFN/RBV/PI were different in patients with different *IL28B* genotypes and different treatment efficacies.

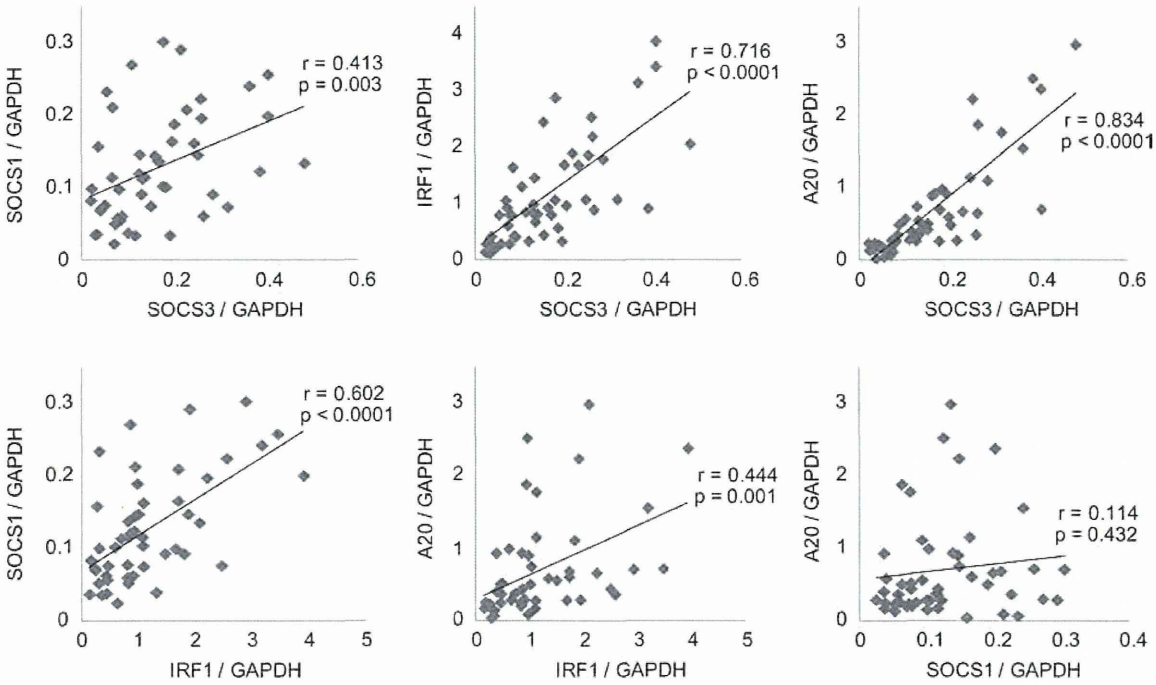

Fig 3. Relationships between levels of mRNAs including suppressive genes in PBMCs 8 hours after the initial administration of pegylated interferon, ribavirin, plus NS3/4A protease inhibitor in all patients. Levels of mRNAs including suppressive genes were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

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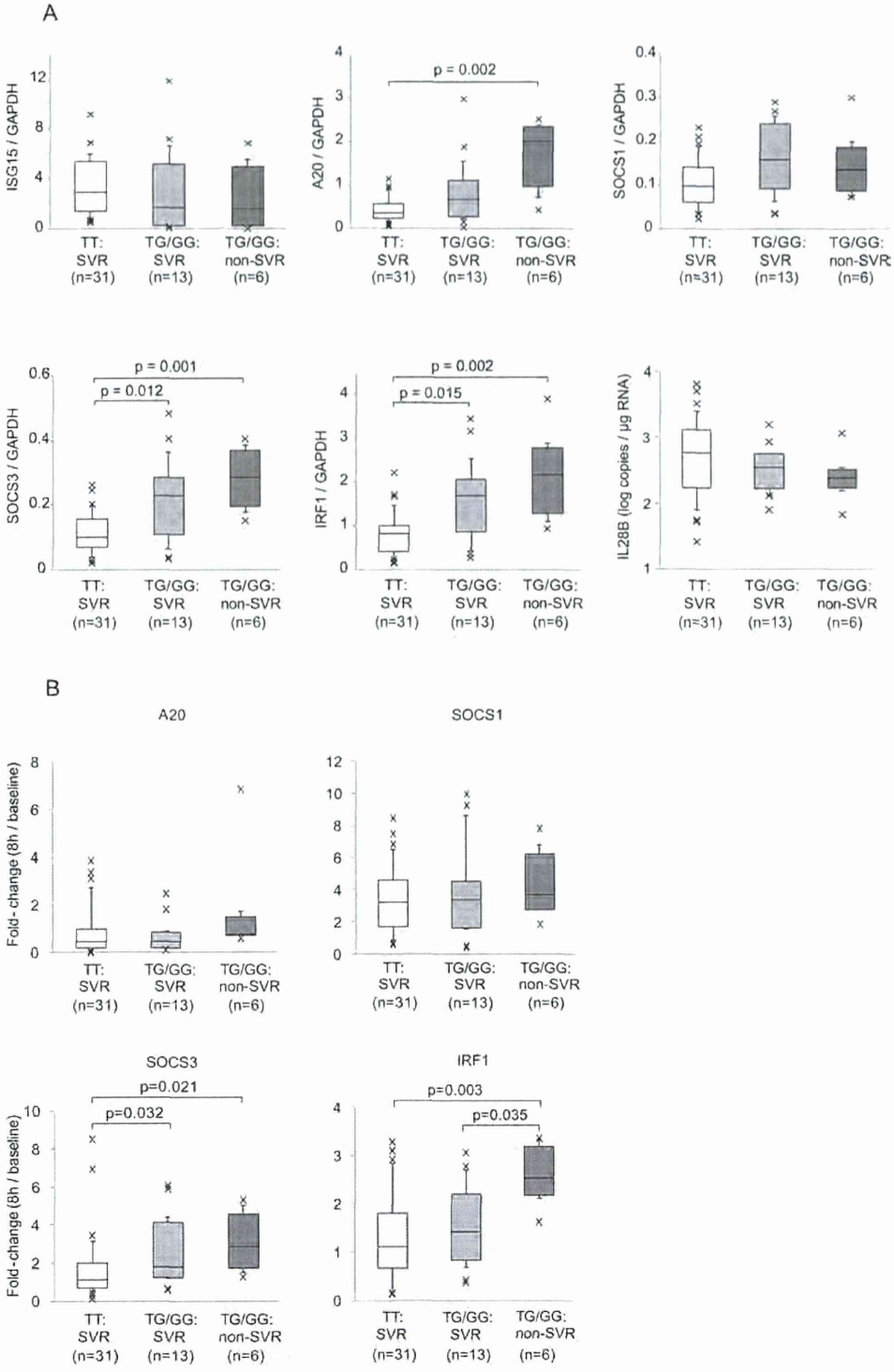

Fig 4. Associations between the expression of ISGs or IFN-λ3 and treatment efficacy. Patients were divided into three groups according to *IL28B* genotype at rs8099917 and treatment outcome: TT; SVR (n = 31), TG/GG; SVR (n = 13), and TG/GG; non-SVR (n = 6). (A) Expression of *ISG15, IL28B* and suppressive genes in PBMCs at 8 hours after the initial administration of pegylated interferon, ribavirin, plus NS3/4A protease inhibitor in each group. (B) Fold-changes of mRNAs including suppressive genes at 8 hours relative to baseline in each group. Levels of mRNAs including suppressive genes and

ISG15 were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and those for *IL28B* were measured using the calibration curves of cDNA clone. TT and TG/GG at rs8099917 is a favorable and an unfavorable *IL28B* genotype for treatment responses, respectively. Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

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Discussion

In the present study, we determined that mRNAs for *A20, SOCS1* and *SOCS3*, known to be genes suppressing antiviral activity via the IFN signaling pathway, as well as *IRF1* were highly expressed in PBMCs early after the initial administration of PEG-IFN/RBV/PI in patients with an unfavorable *IL28B* genotype, especially the non-SVR group. The correlations of mRNA expression levels of these genes, *ISG15*, and *IL28B* suggest that the expression levels of these suppressive genes show similar dynamics independently with the genes promoting the antiviral state in the interferon signaling pathway. Asahina *et al.* showed that the induction of several ISGs in PBMCs after the initial administration of PEG-IFN/RBV tended to be stronger in SVR than in NVR, but in their study the difference was not statistically significant [20]. The HCV NS3/4A protease cleaves and inactivates two important signaling molecules in the innate immune system, the mitochondrial antiviral signaling protein (MAVS), an essential component of the RIG-I pathway [23], and the Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), an adaptor in the TLR3 pathway [24]. Because PI inhibits the function of NS3/4A protease, it is expected to affect these pathways and the expression of ISGs. Indeed, Kalkeri *et al.* showed that PIs including telaprevir, boceprevir, and simeprevir can restore innate immunity by directly inhibiting NS3/4A protease-mediated cleavage of MAVS at clinically achievement concentrations *in vitro* using HCV replicon cells [25]. Therefore, in PEG-IFN/RBV/PI therapy, the expression of ISGs, IFN-λs, and molecules related to the innate immune system may be more markedly altered early after the start of this therapy than PEG-IFN/RBV therapy without PI. This may be the reason why we were able to determine the differences of expression of these suppressive gene mRNAs. We preliminarily compared the mRNA levels of the suppressive genes, *ISG15, MAVS* and *TRIF* in PBMCs between in patients of this study (data of two patients were unavailable) and in those with PEG-IFN/RBV therapy, whose characteristics are described in Table D in S1 File. There were no differences for these genes at 8h/baseline, however, the inductions of mRNA for several genes such as *A20, IRF1, SOCS3*, and *MAVS* at 24h/baseline were greater in PEG-IFN/RBV/PI (Figure D in S1 File). In general, previous studies have shown that HCV mainly could replicate in liver and lympho-trophic HCV would be minor, therefore it is not main event that HCV NS3/4A cleaves MAVS or TRIF in PBMCs. We speculate that inhibiting cleavages of MAVS and TRIF by PI in liver more strongly induces IRF3 activation and subsequent IFN-α/β and ISGs production, resulting in the activation of RIG-I, TLR3, and IFN signaling pathway in livers and PBMCs. For these reasons, we guess that the several genes related with these pathways were more strongly induced at 24 h in patients treated with PEG-IFN/RBV/PI. Further studies will be required to evaluate the effect of PI itself on the IFN signaling pathway in PBMCs or liver. In the present study, levels of mRNAs for IFN-λs as well as common ISGs promoting the antiviral state at baseline and during therapy were not found to be significantly associated with the *IL28B* genotype or treatment efficacy. Recently, Honda *et al.* showed that there was no difference of pretreatment mRNA expression of ISGs as well as *IL28A/B* in blood between *IL28B* genotypes or responses to PEG-IFN/RBV [26]. These results support our data at baseline. Interestingly, they also indicated that the expression of ISGs at baseline correlated significantly between liver and blood in patients with a favorable *IL28B* genotype, not in those with an unfavorable genotype [26].

As previously reported, *SOCS1* suppresses the Jak/STAT pathway, specifically STAT1 [27]. *SOCS3* inhibits expression of ISGs such as *OAS1* and *PKR* through inactivation of the Jak-STAT pathway [28]. *A20* is a suppressive factor of the nuclear factor-kappa B pathway [29] and a candidate negative regulator of the signaling cascade leading to *IRF3* activation in the innate antiviral response [30]. *IRF1* is well known as a transcription factor that activates the expression of *IFN-β*, leading to enhancement of IFN signaling [31, 32]. However, Moore *et al.* showed that *IRF1* enhances the expression of *SOCS1* using rat pancreatic β-cells, and suggested that *IRF1* provides a negative feedback on STAT1 and downstream signaling via STAT1 dephosphorylation by SOCS1 up-regulation [33]. Furthermore, in our preliminary *silico* analysis, *IRF1* is expected to bind the promoter region of *A20* (data not shown), and thus may influence the functional expression of *A20* through transactivation of *A20* promoter, resulting in negative regulation of IFN signaling cascade. Collectively, these suppressive factors may negatively affect the IFN signaling pathway and the production of ISGs or IFN in HCV infection. Abe *et al.* showed that pretreatment intrahepatic levels of two ISGs suppressing the antiviral state, *A20* and *Zc3h12a*, were significantly higher in patients with a favorable *IL28B* genotype, and that a high level of *SOCS1* was a predictive factor for NVR. In contrast, they found that levels of most of the ISGs promoting the antiviral state via the IFN signaling pathway and *IL28* were significantly lower in patients with a favorable *IL28B* genotype [34]. Thus, the expression of these suppressive genes in the liver might influence treatment efficacy. Taking this previous report together with our results using PBMCs presented here, we may conclude that the levels of mRNAs for suppressive genes in liver and PBMCs are associated with *IL28B* polymorphisms.

The mechanism of interaction between IFN-λ and ISG expression in liver or PBMC resulting in the elimination of HCV has not yet been elucidated. Using primary hepatocytes from humans and chimpanzees, Thomas *et al.* found that type III but not type I IFNs are primarily induced after HCV infection, and that their degree of induction is closely correlated with the levels of ISGs [35]. These results strongly suggest that hepatic IFN-λ production may have important roles and could be a principal driver of ISG induction in response to HCV infection. On the other hand, in a chronically HCV-infected chimeric mouse model, larger amounts of IFN-λs were produced by HCV-infected human hepatocytes with a favorable *IL28B* genotype on treatment with IFN-α [36]. Recently, it has been shown in *ex vivo* experiments that a certain subset of dendritic cells (DCs) within human PBMCs recognized HCV and produced large amounts of IFN-λs [37, 38], and that the capacity for producing IFN-λ3 was superior in subjects with a favorable *IL28B* genotype [38]. Furthermore, IFN-α directly affected DC function and significantly increased IFN-λ production [37]. These findings suggest that in addition to HCV-infected hepatocytes, DCs within PBMCs may play a crucial role in the response to IFN treatment via production of IFN-λs and ISGs. We speculate that the levels of several suppressive ISGs in liver and DCs might be different according to the *IL28B* genotype, implying a difference of response to treatment. In addition, it has not been fully elucidated how IFN-λs or ISGs influence effector cells such as natural killer (NK) cells or cytotoxic T lymphocytes in HCV infection. Although we also investigated several cytokines such as IL-2, 4, 5, 6, 10, 12, IFN-γ, and tumor necrosis factor (TNF)-α in patients′ serum during PEG-IFN/RBV/PI, we did not find any differences attributable to *IL28B* genotype or any associating with treatment efficacy (data not shown). Intriguingly, recent study showed that infiltration of various immune cells including DCs, NK cells, and T cells, and expression of various chemokines in liver were repressed in patients with an unfavorable *IL28B* genotype, and their up-regulation of intrahepatic ISGs was mediated by multiple factors, including *IL28A/B*, IFN-λ4, and wingless-related MMTV integration site 5A [26]. Further studies will be required to identify the role of ISGs suppressing the antiviral state in hepatocytes or DCs, and how IFN and ISGs effect the elimination of HCV.

This study has several limitations. The treatment regimens were different for different patients, including the type of PI, its dose, and duration of therapy, especially in the patients receiving faldaprevir, even though faldaprevir dose and treatment duration reportedly had little influence on SVR rates in some clinical trials [39]. Furthermore, there was bias in that the proportion of intractable cases was higher in the patients receiving faldaprevir. Second, the number of analyzed cases was small, especially the non-SVR cases. Third, we analyzed the expression of the selected genes in PBMCs at baseline and the only early periods after the initial administration of PEG-IFN/RBV/PI. Further comprehensive gene expression analysis including more prolonged kinetics of genes are necessary in a large number of patients treated with the same regimen to verify the results of the present study.

The findings in this study contribute to our understanding of immune response to HCV during PEG-IFN/RBV/PI therapy. IFN-free therapy is expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. Future study should evaluate immune responses under IFN-free therapy as well as IFN-based therapy to clarify the mechanism of HCV elimination.

In conclusion, the expression of several genes, which suppress antiviral activity by interfering IFN signaling pathway, in PBMCs during PEG-IFN/RBV/PI was found to be different according to the patient's *IL28B* genotype and treatment response.

Supporting Information

S1 File. Table A, Primers and probes for quantitative real-time PCR of ISGs and IFN-λs. Table B, Clinical characteristics of chronic hepatitis C patients treated with PEG-IFN/RBV plus telaprevir or faldaprevir. **Table C,** Clinical characteristics of chronic hepatitis C patients according to *IL28B* genotype and treatment efficacy. **Table D,** Clinical characteristics of chronic hepatitis C patients treated with PEG-IFN/RBV. **Figure A,** Correlations between levels of mRNAs including suppressive genes at baseline. **Figure B,** Correlations between levels of mRNAs including suppressive genes at 24 hours after the initial administration PEG-IFN, RBV, plus NS3/4A protease inhibitor. **Figure C,** Correlation between levels of mRNA including suppressive genes and those for *IL28B* or *ISG15* at 8 hours after the initial administration PEG-IFN, RBV, plus NS3/4A protease inhibitor. **Figure D,** Fold-changes of mRNAs for ISGs, *TRIF* and *MAVS* in PBMCs at 8, 24 hours relative to baseline in PEG-IFN/RBV and PEG-IFN/RBV/PI therapy.
(PDF)

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Author Contributions

Conceived and designed the experiments: SI KM YT. Performed the experiments: SI KO TF KI. Analyzed the data: SI KM. Contributed reagents/materials/analysis tools: KM EI TM KF NS AK ME SN TJ YT. Wrote the paper: SI KM TW YT.

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Original Article

Multicenter cooperative case survey of hepatitis B virus reactivation by chemotherapeutic agents

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Aim: The purpose of this multicenter cooperative study was to elucidate the clinical features of hepatitis B virus (HBV) reactivation by chemotherapeutic agents and the patient outcomes after HBV reactivation by a retrospective review of accumulated patients' medical records.

Methods: Records of a total of 27 patients (hematological malignancy, 14 patients; solid tumor, 13 patients) from 11 institutions who were diagnosed between June 2005 and October 2010 as having HBV reactivation following chemotherapy were reviewed.

Results: Of the 27 patients with reactivation, 16 patients were hepatitis B surface antigen (HBsAg) positive and 11 were HBsAg negative prior to the commencement of chemotherapy. Of the 11 patients who were HBsAg negative prior to the chemotherapy, 10 had hematological malignancies and one had a solid tumor. Of the 14 patients with hematological malignancies with HBV reactivation enrolled in the study, the reactivation occurred more than 12 months after the completion of chemotherapy in five patients (36%); on the other hand, none of the patients (0%) with solid tumors developed HBV reactivation more than 12 months after the completion of chemotherapy. Of the 24 patients who had acute liver dysfunction at the diagnosis of HBV reactivation, nine (38%) had severe hepatitis and seven (29%) died of liver failure.

Conclusion: Most of the patients with HBV reactivation who were HBsAg negative prior to the chemotherapy had underlying hematological malignancies. Furthermore, patients with hematological malignancies often developed late-onset HBV reactivation. The prognosis of patients who develop acute liver dysfunction as a complication of HBV reactivation is extremely dismal.

Key words: case survey, chemotherapy, hepatitis B virus, hepatitis B virus DNA, reactivation

INTRODUCTION

A VARIETY OF anticancer drugs and their metabolites are known to cause liver dysfunction. In addition, chemotherapy can trigger rapid multiplication of the virus in patients harboring hepatitis B virus (HBV), that can result in fatal liver dysfunction. Such rapid increase in the hepatitis virus load is referred to as viral hepatitis reactivation.[1–4] The frequency and risk of HBV reactivation have been reported to depend on the degree of immunosuppression and the HBV infection status prior to the start of the treatment causing immunosuppression. Immunosuppression of varying degrees is known to occur with

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Table 1 Patient characteristics

Patient no.	Before chemotherapy						Underlying malignancy	Chemotherapy	
	Age	Sex	HBsAg	HBs Antibody	HBc Antibody	HBV DNA (log copies/mL)		Regimen	Combined use of glucocorticoid
1	50	Female	+		+	NA	Malignant lymphoma	R + cyclophosphamide + vincristine	
2	53	Female	+		+	NA	Malignant lymphoma	R CHOP + methotrexate intrathecal	+
3	84	Male	+	NA	NA	NA	Malignant lymphoma	R THP COP	+
4	57	Male	+		+	5 3	AML	Idarubicin + Ara C, HD Ara C	+
5	62	Male	+	NA	NA	NA	Brain tumor	Temozolomide + RT	
6	49	Female	+	NA	NA	NA	Breast cancer	Doxorubicin + CPA	+
7	53	Female	+	NA	NA	NA	Colorectal cancer	FOLFOX	+
8	51	Female	+	NA	+	NA	Gastric cancer	Cisplatin + S 1	+
9	58	Female	+	+	+	NA	HCC	Cisplatin (intra arterial infusion)	
10	71	Male	+	NA	+	6 9	HCC	TACE with epirubicin	
11	68	Male	+		+	NA	HCC	UFT + mitoxantrone	
12	53	Male	+	+	+	4.4	ICC	Gemcitabine + RT	+
13	62	Male	+		+	NA	ICC	Gemcitabine + S 1	+
14	60	Male	+	NA	NA	NA	Lung cancer	Cisplatin + irinotecan	+
15	78	Male	+	NA	NA	NA	Pancreatic cancer	Gemcitabine	+
16	64	Male	+		+	<2.1	Rectal carcinoid	Experimental drug*	
17	39	Male		+	+	UDL	Malignant lymphoma	HD CPA, whole body RT, AlloUCBT	
18	65	Female		NA	NA	NA	Malignant lymphoma	R CHOP	+
19	76	Male		NA	NA	NA	Malignant lymphoma	R CHOP	+
20	84	Female		NA	NA	NA	Malignant lymphoma	R THP COP	+
21	84	Female		NA	NA	NA	Malignant lymphoma	THP COP	+
22	70	Male		+	+	UDL	Multiple myeloma	Melphalan +cisplatin +thalidomide	+
23	87	Female		+	+	<1.8	Multiple myeloma	Melphalan +prednisolone	+
24	60	Female		+		NA	Multiple myeloma	MP, MCP, AutoPBSCT	+
25	61	Female		+	+	<2.6	Multiple myeloma	VAD, HD CPA, HD Melphalan, AutoPBSCT	+
26	48	Male			+	NA	ALL	HD CPA, whole body RT, AlloUCBT	
27	67	Male		NA	NA	NA	HCC	TACE followed by TSU 68	

*the name is not opened because it is under development.
Clinical diagnosis: Elevation of the serum aspartate aminotransferase and/or alanine aminotransferase levels with the detection of HBV DNA positivity and improvement observed in response to antiviral therapy
Complete recovery: complete recovery of AST/ALT and HBV DNA, Incomplete recovery: incomplete recovery of AST/ALT and HBV DNA
ALL, acute lymphoblastic leukemia; ALT, alanine aminotransferase; AlloBMT, allogenic bone marrow transplantation; AlloUCBT, allogenic umbilical cord blood transplantation; AML, acute myeloblastic leukemia; AST, aspartate aminotransferase; Ara-C; xxx; AutoPBSCT, autologous peripheral blood stem cell transplantation; CHOP, cyclophosphamide + doxorubicin + vincristine + prednisolone; CPA, cyclophosphamide; CVP, cyclophosphamide + vincristine + prednisolone; FOLFOX, 5-fluorouracil + leucovorin + oxaliplatin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HD, high dose; ICC, intrahepatic cholangiocarcinoma; MCP, ranimustine + cyclophosphamide + prednisolone; MP, melphalan + prednisolone; NA, not assessed; R, rituximab; RT, radiation therapy; S-1, tegafur + gimeracil + oteracil; TACE, transarterial chemoembolization; THP-COP, pirarubicin + cyclophosphamide + vincristine + prednisolone; TSU-68, xxx; UDL, under detected limit; UFD, xxx; UFT, xxx; VAD, vincristine + doxorubicin + dexamethasone.

Interval from initiation of chemotherapy to HBV reacti vation (days)	Interval from completion of chemotherapy to HBV reacti vation (days)	At occurrence of reactivation				Outcome after reactivation
		Diagnosis of reactivation	HBV DNA (log copies/mL)	Severity of liver dysfunction	Antiviral drug	Complete recovery
637	441	Clinical diagnosis	6.9	Acute hepatitis	Entecavir	Incomplete recovery
760	539	Clinical diagnosis	5.3	Acute hepatitis	Lamivudine	Liver failure and death
1317	1210	HBV DNA titer elevation	8.8	Severe hepatitis	Entecavir	Complete recovery
147	55	Clinical diagnosis	7.6	Acute hepatitis	Lamivudine → entecavir	Complete recovery
448	319	Clinical diagnosis	5.8	Acute hepatitis	Entecavir	Complete recovery
42	23	Clinical diagnosis	5.7	Severe hepatitis	Lamivudine	Liver failure and death
209	34	Clinical diagnosis	8.6	Fulminant hepatitis	Lamivudine	Complete recovery
87	25	Clinical diagnosis	9.0	Acute hepatitis	Entecavir	Incomplete recovery
143	40	Clinical diagnosis	7.1	Acute hepatitis	Lamivudine	Incomplete recovery
309	309	Clinical diagnosis	6.9	Acute hepatitis	Entecavir	Incomplete recovery
93	37	Clinical diagnosis	5.9	Acute hepatitis	Lamivudine	Liver failure and death
130	16	HBV DNA titer elevation	8.0	Fulminant hepatitis	Entecavir	Incomplete recovery
103	17	Clinical diagnosis	5.7	Acute hepatitis	Entecavir	Complete recovery
103	18	Clinical diagnosis	5.5	Acute hepatitis	Entecavir	Incomplete recovery
28	14	Clinical diagnosis	2.8	Acute hepatitis	Entecavir	Complete recovery
51	9	Clinical diagnosis	2.6	Acute hepatitis	None	Complete recovery
340	339	HBV DNA( ) → (+)	6.0	Without hepatitis	Lamivudine → entecavir	Liver failure and death
309	182	HBsAg( ) → (+)	7.4	Severe hepatitis	Lamivudine	Liver failure and death
407	202	HBsAg( ) → (+)	9.7	Fulminant hepatitis	Entecavir	Liver failure and death
528	79	HBsAg( ) → (+)	6.5	Fulminant hepatitis	Entecavir	Complete recovery
721	69	HBsAg( ) → (+)	7.7	Acute hepatitis	Entecavir	Incomplete recovery
937	155	HBV DNA( ) → (+)	<2.1 (+)	Without hepatitis	Entecavir	Liver failure and death
700	553	HBsAg( ) → (+)	8.5	Severe hepatitis	Entecavir	Complete recovery
355	84	HBsAg( ) → (+)	6.2	Acute hepatitis	Entecavir	Complete recovery
354	233	HBV DNA( ) → (+)	2.4	Without hepatitis	Entecavir	Incomplete recovery
416	415	HBsAg( ) → (+)	8.6	Severe hepatitis	Entecavir	Complete recovery
132	14	HBsAg( ) → (+)	6.9	Acute hepatitis	Entecavir	

chemotherapy, such as that following hematopoietic stem cell transplantation and organ transplantation, rituximab based chemotherapy and chemotherapy for solid tumors. The HBV infection status prior to chemotherapy is deter mined by the serum profile of HBV associated markers (hepatitis B surface antigen [HBsAg], hepatitis B e antigen [HBeAg], hepatitis B core antibody [HBcAb], hepatitis B surface antibody [HBsAb]) and the viral load of HBV DNA.[1–4] However, there have been few comprehensive reports on HBV reactivation, and the clinical background factors involved in HBV reactivation, including the circum stances of the chemotherapy AND the characteristics of the

reactivation, and the clinical outcomes following HBV reactivation have not yet been clearly elucidated. We there fore conducted a retrospective clinical review of the medi cal records of patients who developed HBV reactivation following treatment with chemotherapeutic agents. The purpose of this multicenter cooperative study was to eluci date the clinical features of HBV reactivation and the pa tient outcomes after HBV reactivation.

METHODS

Patients

WE CONDUCTED A retrospective clinical review of the medical records of patients with HBV reactiva tion induced by anticancer drugs accumulated at each in stitution. This clinical study was conducted with the approval of the ethics committee of the National Cancer Center, and in accordance with epidemiological research guidelines.

We defined HBV reactivation as follows: (i) increase of the HBV DNA titer by more than 10 fold or conversion to a HBeAg positive from HBeAg negative status in patients determined to be HBsAg positive after the commencement of chemotherapy; (ii) conversion from a HBsAg negative to HBsAg positive status after the commencement of che motherapy; and (iii) increase of the HBV DNA titer to above the detection limit in patients with HBV DNA titers below the detection limit of the assay after the commence ment of chemotherapy.[1,2] In addition, elevation of the se rum aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) levels along with HBV DNA posi tivity and improvement in response to antiviral therapy was also defined as HBV reactivation in this study.

Variables examined

The variables examined in the patients with HBV reactiva tion are listed below. Patient background factors were age, sex, the underlying malignancy, presence/absence of liver metastasis, presence/absence of concomitant liver disease and history of alcohol consumption.

Factors related to the chemotherapy inducing the HBV reactivation were chemotherapeutic regimen used, the day of commencement of chemotherapy, the day of dis continuation of chemotherapy and concomitant use of glucocorticoid.

Status at the occurrence of reactivation included date of diagnosis of HBV reactivation, symptoms associated with the HBV reactivation, the antiviral drugs used for treating the HBV reactivation, date of start of antiviral drug admin istration, concomitant treatments for HBV reactivation, severity of the liver dysfunction caused by the reactivation and outcome after the reactivation.

Laboratory tests before and after the HBV reactivation consisted of hemogram (leukocytes, neutrophils, lympho cytes, hemoglobin, platelets), serum biochemistry (total bilirubin, AST, ALT, alkaline phosphatase), coagulation parameters (prothrombin time) and hepatitis B virus marker profile (HBsAg, HBsAb, HBeAg, hepatitis B e anti body, HBcAb, HBV DNA load).

RESULTS

Patient characteristics before the commencement of chemotherapy

THE RECORDS OF a total of 27 patients with HBV reactivation diagnosed between June 2005 and October 2010 were accumulated from 11 institutions (Table 1). The patient characteristics before the com mencement of chemotherapy are shown in Table 2. The patients consisted of 15 men and 12 women, with a me dian age of 62 years (range, 39 87). Among the patients with HBV reactivation, 16 were HBsAg positive and 11 patients were HBsAg negative prior to the commence ment of chemotherapy. The underlying malignancies were hematological malignancies in 14 patients and solid tumors in 13 patients; among the hematological malig nancies, malignant lymphoma was the most common, while among the solid tumors, hepatocellular carcinoma was the most common. Among the 11 patients who were HBsAg negative prior to the chemotherapy, 10 had un derlying hematological malignancies and only one had a solid tumor. The chemotherapy inducing the HBV reac tivation was the chemotherapeutic regimen administrated with hematopoietic stem cell transplantation in four patients, a rituximab based regimen in five patients, platinum combination regimen in four patients and gemcitabine alone or combination regimen in three patients. A glucocorticoid was used concomitantly in 18 patients.

Findings at the time of HBV reactivation

At the time of reactivation, 12 patients presented with symptoms, including fatigue, anorexia, nausea/vomiting, jaundice, pyrexia and drowsiness (Table 3). Of the 27 pa tients, in 24, the HBV reactivation was diagnosed by checking for elevation of the HBV DNA titers after detec tion of increase of the serum AST and/or ALT level, while in the remaining three patients, reactivation was diagnosed by observing conversion from HBsAg negative to HBsAg positive or an increase of the HBV DNA load in the absence of elevation of the serum AST and/or ALT levels (patients

Table 2 Patient characteristics before chemotherapy

Variables		*n*	(%)
All patients		27	
Age (years)	Median [range]	62	39 87
Sex	Male	15	(56)
	Female	12	(44)
Serological marker of hepatitis B viral infection	HBsAg (+)	16	(59)
	HBsAg ( )	11	(41)
	HBsAg ( ), and anti HBs or anti HBc (+)	6	(22)
	HBsAg ( ), no data on anti HBs and anti HBc	5	(19)
Tumor type			
Hematological tumor	All	14	(52)
	Malignant lymphoma	8	(30)
	Multiple myeloma	4	(15)
	Leukemia	2	(7)
Solid tumor	All	13	(48)
	Hepatocellular carcinoma	4	(15)
	Bile duct cancer	2	(7)
	Others	7	(26)
Chemotherapeutic regimen	Hematopoietic stem cells transplant	4	(15)
	R CHOP	5	(19)
	Platinum combination	4	(15)
	Gemcitabine alone or combination	3	(11)
	Others	11	(40)
Concomitant use of a glucocorticoid	Present	18	(67)
Liver metastases	Present	3	(11)
Complication of liver disease	Chronic hepatitis type C	1	(4)
Alcohol abuse	Habitual drinker	6	(22)
	Social drinker	10	(37)

Anti-HBs, hepatitis B surface antibody; anti-HBc antibody, hepatitis B core antibody; HBsAg, hepatitis B surface antigen, R-CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone combined with rituximab

17, 22 and 25). All of the three latter patients with under lying hematological malignancies were HBsAg negative and HBcAb positive prior to the commencement of che motherapy, and HBV reactivation was detected by monthly measurements of the HBsAg or HBV DNA. The median interval from completion of chemotherapy to HBV reactivation and median interval from initiation of chemotherapy to HBV reactivation were 79 days (range, 9 1210) and 309 days (range, 28 1317), respectively. In none of the 13 patients (0%) with solid tumors did HBV develop more than 12 months after the completion of che motherapy, while in five of the 14 patients (36%) with un derlying hematological malignancy, it developed more than 12 months after the completion of chemotherapy.

Outcome after HBV reactivation

Of the 27 patients, 26 were treated with antiviral drugs such as entecavir or lamivudine at the time of HBV reacti vation, while one patient improved spontaneously (pa tient 16) (Table 3). Acute liver dysfunction developed at the time of the reactivation in 24 patients, while the re maining three patients showed no evidence of liver dysfunction (patients 17, 22 and 25). Of the 27 patients, five (28%) and four (15%) had severe hepatitis and fulmi nant hepatitis, respectively, and seven patients (26%) died of liver failure.

DISCUSSION

IN 2001, DERVITE *et al.* reported, for the first time, HBV reactivation in a HBsAg negative patient who had re ceived rituximab based chemotherapy.[5] It became clear then that reactivation could occur not only in HBsAg pos itive patients, but also in HBsAg negative and HBcAb/HBsAb positive patients. Since then, HBV reactiva tion has begun to attract much interest in clinical practice. However, the factors associated with, and the outcomes of, reactivation have not yet been sufficiently characterized. Therefore, we conducted a clinical survey of the data of pa tients with HBV reactivation, and case reports of 27 pa tients with HBV reactivation occurring following chemotherapy were collected from 11 institutions. This study focused on the clinical courses of the patients who developed HBV reactivation, and both patients who

Table 3 Condition at occurrence and outcomes in patients with reactivation of hepatitis B viral infection

Variables		*n*	(%)
Symptom	Present	12	(44)
	Malaise	7	(26)
	Anorexia	7	(26)
	Nausea/vomiting	2	(7)
	Jaundice	1	(4)
	Fever	1	(4)
	Somnolence	1	(4)
Criteria for diagnosis of HBV reactivation	Clinical Diagnosis*	14	(52)
	Positive conversion of HBsAg	8	(30)
	Increase of the HBV DNA titer to above the detection limit	3	(11)
	Increase of the HBV DNA titer by more than 10 fold	2	(7)
Interval from completion of chemotherapy to HBV reactivation	Median [range], days	79	[9 1210]
	Solid tumor, median [range], days	23	[9 319]
	Hematological malignancy, median [range], days	218	[55 1210]
Treatment for HBV reactivation	Antiviral drug	26	(96)
	Entecavir	20	(74)
	Lamivudine	8	(30)
	Glycyrrhizin	12	(44)
	Ursodeoxycholic acid	4	(15)
	Interferon	4	(15)
	Steroids	2	(17)
	Plasma exchange	1	(4)
Type of liver dysfunction	Acute hepatitis	15	(55)
	Severe hepatitis	5	(19)
	Fulminant hepatitis	4	(15)
	None	3	(11)
Outcome after reactivation	Complete improvement of the serum AST/ALT and HBV DNA titer to normal range	12	(44)
	Incomplete improvement of the serum AST/ALT and/or HBV DNA titer	8	(30)
	Liver failure and death	7	(26)

*Clinical diagnosis: Elevation of the serum aspartate aminotransferase and/or alanine aminotransferase levels with the detection of HBV DNA positivity and improvement observed in response to antiviral therapy

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus infection.

underwent adequate screening and follow up for HBV re activation and those who did not undergo adequate screening and follow up were included in this study. In ad dition, patients with various malignant diseases, receiving various treatment regimens, and any HBsAg status were in cluded in this study. Furthermore, not only patients in whom the HBV reactivation was diagnosed on the basis of increased HBV DNA titers and conversion of the HBeAg or HBsAg status, but also those in whom the diagnosis was made based on elevation of the serum AST and/or ALT levels along with HBV DNA positivity and improvement in response to antiviral therapy were included. Therefore, we obtained comprehensive data on patients developing HBV reactivation in actual clinical practice. Thus, even though the number of patients with HBV reactivation was limited in this study, accumulation of such patients with HBV reactivation may be expected to contribute to a further understanding of HBV reactivation and also lead to the development of some novel countermeasures against HBV reactivation.

In this study, while reactivation in patients with a HBsAg positive status prior to chemotherapy was observed in both patients with underlying hematological malignancies and solid tumors, reactivation in patients with a HBsAg negative status prior to chemotherapy occurred predomi nantly in patients with underlying hematological malig nancies. Previous reports of HBV reactivation in HBsAg negative patients have rarely been reported in the patients with solid tumors, including breast cancer,[6] hepatocellular carcinoma,[7,8] brain tumors,[9] rectal cancer,[10] pharynx and esophageal cancer,[11] and lung cancer,[11] and in patients re ceiving drug regimens including cyclophosphamide, doxo rubicin plus 5 fluorouracil, temozolomide, and mitomycin plus hydroxycamptothecin.[7] Our present re port serves to emphasize that caution against reactivation must be exercised even in HBsAg negative patients with

solid tumors. Glucocorticoids were used in combination with the chemotherapy to increase the therapeutic efficacy and/or prevent emetic reaction in 18 of the 27 patients in our study. Glucocorticoids have been mentioned as risk factors for HBV reactivation,[12] and it appears indeed that glucocorticoid use may influence the risk of HBV reactiva tion. It is necessary to pay attention not only to the anti cancer drugs used, but also to whether glucocorticoids were also used in combination with the drugs as antiemetics.

In regard to the interval from completion of chemother apy to HBV reactivation, HBV reactivation developed within 12 months after the completion of chemotherapy in all 13 patients (100%) with solid tumors. However, in five of the 14 (36%) patients with hematological malig nancies, HBV reactivation occurred more than 12 months after the completion of chemotherapy. The maximum in terval from completion of chemotherapy to HBV reactiva tion in this series was 3.3 years in a patient with malignant lymphoma treated with THP COP therapy (pirarubicin, cyclophosphamide, vincristine plus prednisolone). This late onset was thought to be related to a delayed immune recovery because of prolonged suppressive effects of the in tensive chemotherapy for hematological malignancy and glucocorticoid treatment, although some patients might have been due to discontinue prophylactic antiviral drug treatment. On the other hand, the immunosuppressive ef fects of chemotherapy for solid tumors may not be so prolonged,[1–3,13] although almost all patients with solid tu mors may die before the late onset of HBV reactivation be cause of the generally dismal prognosis. Thus, follow up for HBV reactivation is obviously necessary for a long pe riod of time after completion of chemotherapy in patients with hematological malignancies, although the follow up for HBV reactivation is recommended for limited periods, such as 12 months, at least 12 months and 2 6 months, af ter the completion of chemotherapy by some guidelines and consensus statement.[14–16]

Among the 24 patients who developed acute liver dys function at the time of the reactivation, nine patients (38%) had severe or fulminant hepatitis and seven pa tients (29%) died of liver failure. As previously re ported,[17,18] the prognosis of patients who develop liver dysfunction as a complication of HBV reactivation remains poor. This finding suggests that periodic monitoring of liver function is insufficient to prevent liver function related deaths associated with HBV reactivation, and coun termeasures to prevent liver dysfunction due to HBV reac tivation, such as prophylactic administration of antiviral drug(s) before the commencement of chemotherapy and periodic monitoring of the HBV DNA levels, is important in patients receiving chemotherapy.

Consensus statements regarding HBV reactivation were published by the Asian Pacific Association for the Study of the Liver (APASL) in 2005,[19] the Practice Guidelines by the American Association for the Study of Liver Diseases (AASLD) in 2007,[20] the Consensus Development Confer ence Management of Hepatitis B by the National Institutes of Health (NIH) in 2008,[16] and the Clinical Practice Guideline by the European Association for the Study of the Liver (EASL) in 2009,[12] and, in Japan, the Guidelines for Countermeasures against the Onset of Hepatitis B due to Immunosuppression and Chemotherapy were pub lished in 2009.[13] In all of these guidelines, preventive treat ments with antiviral drugs for HBsAg positive patients receiving chemotherapy are recommended. Furthermore, all guidelines, except the AASLD guideline, recommend periodic monitoring for HBV DNA and deferred preemp tive administration of antiviral drug(s) after positive con version of HBV DNA in HBsAg negative HBcAb/HBsAb positive patients. However, evidence is yet to be established to support these recommendations, and these recommendations were based on clinical experiences and ideal aspects. Therefore, some clinical studies to clarify their usefulness have been conducted both in Japan and abroad.[1] In the future, even firmer evidence of counter measures for HBV reactivation is expected to be demonstrated.

This study had some limitations. HBcAb and HBsAb were measured in only 59% and 52% of patients, respec tively. Therefore, the diagnostic basis for HBV reactivation may be inadequate, because patients with HBV reactiva tion diagnosed clinically, based on elevation of the serum AST and/or ALT followed by detection of HBV DNA posi tivity and improvement observed in response to antiviral therapy, were also included in this study. In addition, there were some missing data in this study, inevitable on ac count of the retrospective nature of the study. Finally, we could not clarify the frequency of HBV reactivation in pa tients under chemotherapy who were HBsAg positive or HBsAg negative and HBcAb/HBsAb positive, because the number of such patients during the study period could not be determined in all of the institutions. However, the frequency of HBV reactivation according to the HBsAg status could be clarified from the results of some prospec tive studies on the risk of HBV reactivation in patients with solid tumors or hematological malignancies receiving chemotherapy conducted by our colleagues (UMIN no. 000005369 and 000001299). However, despite these limitations, the analyses were meaningful, because

information about HBV reactivation following chemother apy available to date is rather limited.

In conclusion, HBV reactivation has been observed in patients with a variety of malignancies, but almost all of the patients who developed HBV reactivation from a HBsAg negative status had underlying hematological malignancies. Because late onset of HBV reactivation was often observed in patients with hematological malignan cies, follow up for HBV reactivation is obviously necessary for a long period of time after completion of chemother apy in patients with hematological malignancies. As the prognosis of patients who develop liver dysfunction as a complication of HBV reactivation remains poor, counter measures to prevent liver dysfunction due to HBV reac tivation is important in patients receiving chemotherapy. To establish firm evidence of HBV reactivation, further well designed clinical trials are warranted.

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ORIGINAL ARTICLE LIVER, PANCREAS, AND BILIARY TRACT

Clinicopathological characteristics and diagnostic performance of *Wisteria floribunda* agglutinin positive Mac-2-binding protein as a preoperative serum marker of liver fibrosis in hepatocellular carcinoma

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Abstract

Background *Wisteria floribunda* agglutinin positive Mac-2-binding protein (WFA$^+$-M2BP) is a novel serum marker of liver fibrosis identified in glycoproteomic biomarker screening studies, and its clinicopathological characteristics have yet to be elucidated sufficiently for clinical utilization.

Methods We retrospectively analyzed the clinicopathology data and serum WFA$^+$-M2BP levels in 376 hepatocellular carcinoma patients undergoing liver surgery. WFA$^+$-M2BP was quantified in frozen serum samples collected at the time of surgery using the FastLec-Hepa method.

Results Significant independent determinants of serum WFA$^+$-M2BP levels included pathological diagnosis of cirrhosis, female gender, hepatitis C virus (HCV) infection, and liver dysfunction characteristics, such as abnormal indocyanine green retention rate at 15 min, platelet counts, albumin levels, alanine aminotransferase levels, and total bilirubin levels. Serum WFA$^+$-M2BP levels increased with the pathological fibrosis stage and liver dysfunction severity. HCV infection significantly affected serum WFA$^+$-M2BP levels throughout the pathological and functional progression of liver fibrosis, and the effect of gender was significant only in F4 stage patients with severe liver dysfunction. The diagnostic thresholds for cutoff index values for cirrhosis were 1.435 and 4.615 in HCV-negative and HCV-positive patients, respectively. Serum WFA$^+$-M2BP levels at the time of operation were a significant predictor of hepatocellular carcinoma recurrence and overall survival in both HCV-negative and HCV-positive patients.

Conclusions Serum WFA$^+$-M2BP levels reflected both the pathological and functional progression of liver fibrosis comprehensively and continuously. Elevated WFA$^+$-M2BP levels were a significant risk factor for tumor recurrence and decreased overall survival after liver surgery independent of HCV infection.

Keywords Biomarker · *Wisteria floribunda* agglutinin positive Mac-2-binding protein · Hepatocellular carcinoma · Liver fibrosis · Cirrhosis

Abbreviations

AFP	Alpha fetoprotein
AFP L3	*Lens culinaris* agglutinin reactive fraction of alpha fetoprotein
ALT	Alanine aminotransferase

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COI	Cutoff index
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
ICG-R15	Indocyanine green retention rate at 15 min
M2BP	Mac-2-binding protein
PIVKA II	Protein induced by vitamin K absence II
WFA	*Wisteria floribunda* agglutinin
WFA^+-M2BP	*Wisteria floribunda* agglutinin positive Mac-2-binding protein

Introduction

In liver surgery for hepatocellular carcinoma (HCC), optimal patient selection is critically important to decrease surgical morbidity and mortality [1, 2]. Liver cirrhosis underlies HCC in most patients and diminishes the postoperative prognosis as a risk factor for operative complications, postoperative liver dysfunction, and tumor recurrence [3 5]. Therefore, preoperative assessment of liver fibrosis is an important step before liver surgery for HCC. The gold-standard method for the diagnosis of liver fibrosis is liver biopsy; however, needle biopsy poses potential risks of life-threatening complications and sampling errors. Therefore, a number of alternative noninvasive methods for diagnosis of liver fibrosis, including serological examinations, scoring systems, and ultrasound elastography, have been developed [6 8].

Wisteria floribunda agglutinin positive Mac-2-binding protein (WFA^+-M2BP) is a novel serum marker of liver fibrosis developed in recent glycoproteomic biomarker-screening studies [9, 10]. WFA^+-M2BP consists of Mac-2-binding protein (M2BP) glycoforms with strong and specific affinity for *Wisteria floribunda* agglutinin (WFA). M2BP expression is elevated by hepatitis viral infection and progression of liver fibrosis, and M2BP was identified as a serum marker of liver fibrosis on the basis of a proteomic analysis in HCV-infected patients [11, 12]. Furthermore, our previous antibody-overlay lectin microarray analysis demonstrated that WFA distinguished fibrosis-associated M2BP glycoforms from total M2BP with high accuracy and a high signal-to-noise ratio [9]. M2BP is a secretory N-glycoprotein that contains seven highly glycosylated N-linked glycosylation sites, and ten to 16 M2BP monomers form a large ringlike oligomer under physiological conditions [13 15]. Because of such structural features, the binding affinity of WFA^+-M2BP for WFA is so strong that WFA^+-M2BP can be quantified without sample preparation. Therefore, serum WFA^+-M2BP levels can be clinically measured by an automated glycan-based immunoassay within 20 min [9].

Serum WFA^+-M2BP levels showed significant increases with the increasing severity of liver fibrosis in several clinical studies. The areas under the receiver operating characteristic curves for WFA^+-M2BP levels for the diagnosis of F1 F4, F2 F4, F3 F4, and F4 stage liver fibrosis (see "Patients, samples, and clinicopathology data" for an explanation of the stages) were 0.698 0.778, 0.790 0.838, 0.812 0.876, and 0.795 0.960, respectively [9, 16 18]. The diagnostic accuracy of WFA^+-M2BP levels was almost comparable with that of ultrasound elastography and was superior to that of magnetic resonance imaging, the aspartate transaminase to platelet ratio index, hyaluronic acid levels, and type IV collagen levels [17].

WFA^+-M2BP is a promising surrogate marker of liver fibrosis. However, the characteristics of WFA^+-M2BP have not been elucidated sufficiently to establish diagnostic criteria for its clinical utilization. In the current study, we analyzed the clinicopathological characteristics and behaviors of serum WFA^+-M2BP levels in HCC patients with the aim of determining appropriate conditions for clinical application, and assessed the clinical significance of serum WFA^+-M2BP levels in liver surgery for HCC.

Methods

Patients, samples, and clinicopathology data

We retrospectively analyzed the clinicopathology data, including serum WFA^+-M2BP levels, of 376 HCC patients who underwent primary hepatectomy at Hokkaido University Hospital between May 2001 and February 2012. Frozen serum samples collected at the time of surgery were available in all cases, and patients with a preoperative diagnosis of distal metastasis were excluded from this study. Serum WFA^+-M2BP levels were quantified using the newly developed glycan-based immunoassay named FastLec-Hepa at the Research Center for Medical Glycoscience of the National Institute of Advanced Industrial Science and Technology (Ibaraki, Japan). The clinicopathology data analyzed in this study included gender, age, hepatitis B virus (HBV) infection, HCV infection, indocyanine green retention rate at 15 min (ICG-R15), prothrombin time, platelet counts, albumin levels, alanine aminotransferase (ALT) levels, total bilirubin levels, alpha fetoprotein (AFP) levels, *Lens culinaris* agglutinin reactive fraction of AFP (AFP L3) levels, protein induced by vitamin K absence II (PIVKA II) levels, the number of tumors, the diameter of the largest tumor, lymph node metastasis, vascular invasion, tumor differentiation, the extent of liver resection (operation), the FIB4 index, and the liver fibrosis stage. Pathological stages of liver fibrosis were diagnosed in surgical specimens according to the new