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Human Cytokine Genetic Variants Associated With HBsAg Reverse Seroconversion in Rituximab-Treated Non-Hodgkin Lymphoma Patients

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Abstract: Hepatitis B virus (HBV) reactivation has been noted in HBV surface antigen (HBsAg)-seronegative patients with CD20$^+$ B-cell non-Hodgkin lymphoma (NHL) undergoing rituximab treatment. Clinically, hepatitis flares are usually associated with the reappearance of HBsAg (reverse seroconversion of HBsAg, HBV-RS). It is unclear whether human genetic factors are related to rituximab-associated HBV reactivation.

Unvaccinated HBsAg-seronegative adults (n = 104) with CD20$^+$ NHL who had received rituximab-containing therapy without anti-HBV prophylaxis were enrolled. Eighty-nine candidate single nucleotide polymorphisms (SNPs) of 49 human cytokine genes were chosen and were analyzed using the iPLEX technique. Competing risk regression was used to identify the factors associated with HBV-RS.

Participants had a median age of 66.1 years and 56.7% were male (n = 59). The anti-HBs and anti-HBc positivity rates were 82.4% and 94.1%, respectively, among patients for whom data were available (approximately 81%). A mean of 7.14 cycles of rituximab therapy were administered, and a total of 14 (13.4%) patients developed HBV-RS. Nine SNPs showed significant differences in frequency between patients with or without HBV-RS: *CD40* rs1883832, *IL4* rs2243248 and rs2243263, *IL13* rs1295686, *IL18* rs243908, *IL20* rs1518108, and *TNFSF13B* rs12428930 and rs12583006. Multivariate analysis showed that ≥6 cycles of rituximab therapy, *IL18 rs243908*, and the *IL4* haplotype rs2243248∼rs2243263 were independently associated with HBV-RS. The *IL4* haplotype rs2243248∼rs2243263 was significantly associated with HBV-RS regardless of anti-HBs status.

Polymorphisms in human cytokine genes impact the risk of rituximab-associated HBV-RS.

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Abbreviations: anti-HBc = antibody to hepatitis B virus core antigen, anti-HBs = antibody to hepatitis B virus surface antigen, CD20$^+$ NHL = CD20-positive B-cell non-Hodgkin lymphoma, CI = confidence interval, FL = follicular lymphoma, HBsAg = hepatitis B virus surface antigen, HBV = hepatitis B virus, HBV-RS = reverse seroconversion of hepatitis B virus surface antigen, HCV = hepatitis C virus, IL = interleukin, SHR = subhazard ratio, SNP = single nucleotide polymorphisms.

INTRODUCTION

Rituximab, an anti-CD20 monoclonal antibody, is used to treat patients with CD20-positive B-cell non-Hodgkin lymphoma (CD20$^+$ NHL)[1] or rheumatoid arthritis.[2] Hepatitis B virus (HBV) reactivation has been noted in hepatitis B virus surface antigen (HBsAg)-seronegative patients with CD20$^+$ NHL, and at 10% risk of reverse seroconversion of hepatitis B virus surface antigen (HBV-RS).[3–7] Clinically, hepatitis flares are frequently associated with the reappearance of HBsAg (i.e., HBV-RS).[5] Among the risk factors for HBV-RS in HBsAg-seronegative patients with CD20$^+$ NHL, HBV serological status prior to rituximab therapy, including antibody to hepatitis B virus core antigen (anti-HBc) seropositivity and antibody to hepatitis B virus surface antigen (anti-HBs) seronegativity, has been shown to be associated with a significantly increased risk in some reports.[5,8] A higher number of cycles of rituximab therapy has also been highlighted as a risk factor.[6,7] However, the anti-HBc and anti-HBs seropositivity rate is relatively high in unvaccinated HBsAg-seronegative adults in HBV-hyperendemic areas,[3,4,6–9] for example, at least 70% in Taiwan,[4–7,9] which was the 1st country to initiate universal HBV vaccination, in 1984.[10] Therefore, it is necessary to investigate whether human genetic factors are related to rituximab-associated HBV reactivation.

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Few studies investigated the association between genetic background and the adverse effects of rituximab therapy. Rossi et al[11] evaluated 19 single nucleotide polymorphisms (SNPs) in 106 patients with diffuse large B cell lymphoma who underwent treatment with rituximab combination with cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) and found that a variant of NAD(P)H oxidase subunits, *NCF4* rs1883112, was an independent predictor against hematologic, infectious, and cardiac toxicities. However, the candidate genes were chosen mainly based on their metabolic involvement with the chemotherapeutic agents, rather than with rituximab. It remains unclear whether human genetic factors are related to rituximab-associated HBV reactivation. However, because the pathogenesis of HBV infection is mainly immune-mediated,[12] HBV reactivation and HBV-RS might be associated with human genetic factors responsible for immune responses.

As few previous studies have examined this issue, this pilot study aimed to analyze SNPs of candidate genes. The genes involved in T-cell immune responses are known to be involved in both HBV reactivation and rituximab therapy, and they were chosen for analysis, for several reasons. First, the degree of T-cell immune response and the interaction of several cytokines are known to influence seroconversion, severity, and chronicity in HBV infection.[12,13] Several studies have investigated polymorphisms of the genes encoding these cytokines, including tumor necrosis factor (TNF) and interferon gamma (INF γ).[14,15] Second, evidence from a mouse model[16] and from patients with various underlying diseases indicated that rituximab-induced B-cell depletion may also influence T-cell immune responses. Hilchey et al[17] found that rituximab killed follicular lymphoma (FL) cells via the elicitation of an FL-specific T-cell response. Rituximab caused reversion of the T-cell immune response in patients with immune thrombocytopenic purpura,[18] and patients with systemic lupus erythematosus and rheumatoid arthritis.[19] However, rituximab-induced B-cell depletion might exacerbate T-cell-dependent immune-mediated diseases[20] and is known to impair the vaccine response.[21,22] These findings implied that in $CD20^+$ NHL patients, rituximab therapy would have damaged the balance between T-cell immune responses and occult HBV infection harbored within hepatocytes, finally resulting in HBV reactivation, HBV-RS, and hepatitis flare.

In this study, we evaluated human genetic variants that might be responsible for anti-HBV immune responses and the adverse effects of rituximab through mass screening of 89 SNPs among 49 candidate genes.

PATIENTS AND METHODS

Patients and Data Collection

For consecutive adult patients ($\geq$18 years of age) with the diagnosis of $CD20^+$ NHL between January 2000 and December 2010 at Taipei Veterans General Hospital,[7] those who were seronegative for HBsAg at diagnosis, received rituximab alone or combined with chemotherapy, and had genomic DNA archived were eligible for the study. Patients were excluded if any of the following criteria were met: seropositive for hepatitis C virus (HCV) or human immunodeficiency virus at diagnosis, HBV vaccination, prophylactic use of anti-HBV agents, and organ or hematopoietic stem cell transplantation administered before the event of HBV reactivation or at last follow-up.

The collected data included demographics, NHL subtypes and treatments, liver function tests, HBV serology tests (HBsAg, anti-HBs, and anti-HBc) and viral load, and outcome. The HBV-RS event was identified, and additional data on liver function, viral serology, and virological tests were collected. This study was approved by the Institutional Review Board of the Taipei Veterans General Hospital.

Rituximab-Containing Therapy and Monitoring of HBV Reactivation

The induction therapy for patients with aggressive $CD20^+$ NHL included 6 to 8 cycles of rituximab (375 mg/m^2 per cycle) in combination with CHOP or a CHOP-like regimen every 3 weeks.[1] At diagnosis, serological tests for HIV, HBsAg, and anti-HCV were routinely performed, and testing for anti-HBs and anti-HBc were performed more frequently subsequent to the late 2000s. Liver function tests were performed before every cycle of rituximab-containing therapy, and during the follow-up period, including alanine aminotransferase (normal ranges $<$40 IU/L), aspartate aminotransferase, alkaline phosphate, and total bilirubin.[4,7] For clinical suspicion of HBV reactivation during and after rituximab-containing therapy, HBsAg and anti-HCV were tested again, and, in addition, quantitative measurement of HBV viral load in the serum was performed, using the COBAS Taqman HBV test (Roche Diagnostics, Switzerland), with a detection limit of 12 IU/mL.[4,7] HBeAg (and anti-HBe) testing was usually performed when the results of the HBsAg test were positive.[4,23]

In this study, HBV-RS was used to represent HBV reactivation and was defined as reappearance of HBsAg in the serum, and an HBV viral load $>$2000 IU/mL if applicable.[4,7] Hepatitis flare preceded or accompanied by HBV-RS was defined if serum alanine aminotransferase level was above 100 IU/L.[4,7] The cycle number of rituximab-containing therapy was recorded until HBV-RS was noted, or the final follow-up if HBV-RS did not develop. The overall survival (OS) was defined as the time between the beginning of rituximab-containing therapy and the final follow-up.[7]

Selection and Genotyping of Candidate Genes and SNPs

This pilot study was part of the Genetic Polymorphisms Influencing the Impact of HBV Infection on $CD20^+$ NHL Patients study, in terms of those relevant to toxicities and susceptibility. Candidate genes and SNPs driving immune responses were chosen and analyzed via 3 steps. First, except for SNPs of *NCF4*,[11] candidate genes and their SNPs were chosen based on a comprehensive literature review of articles relevant to immune response. The articles were collected via a PubMed search on Sep. 30, 2012 using different combinations of several keywords, including the combination of ''lymphoma, susceptibility, and polymorphism'' and ''(cytokine, or chemokine, or innate immunity);'' the combination of ''hepatitis B virus and polymorphism'' and ''(cytokine, or chemokine, or innate immunity).'' Those SNPs with sufficient information, including statistical significance, were chosen. Second, SNPs with a known allele frequency $<$5% in Asian or Chinese populations were excluded, based on data in the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp/). Finally, only those SNPs for which primers were available for the iPLEX genotyping technique (Sequenom, Inc., MI) were analyzed. A total of 89 SNPs of 49 genes were analyzed in 3 multiplex reactions (supplemental Table 1, http://links.lww.com/MD/A767).

Genomic DNA was used for genotyping and was mainly collected from the peripheral blood or bone marrow of $CD20^+$ NHL patients at diagnosis. Genotyping of SNPs was performed

using iPLEX SNP Genotyping (Sequenom, Inc., MI) at the National Center for Genomic Medicine, Taiwan. The iPLEX assay can detect sequence differences at the single nucleotide level based on the primer extension process, in which the primer is extended dependent upon the template sequence, resulting in allele-specific differences in mass between extension products.[24]

Statistical Analysis

Patients with and without HBV-RS were categorized as the case and control subgroups, respectively. Clinical variables were compared between the case and control subgroups. The Chi-squared test (or Fisher exact test when applicable), Student's *t*-test (or one-way analysis of variance when applicable), and the Mann–Whitney *U*-test were applied for categorical, parametrically, and nonparametrically continuous variables between subgroups, respectively.

Allele and genotype frequencies of SNPs were calculated, and those with a call rate of <90% that deviated from the Hardy–Weinberg equilibrium (with a cut-off value of $P < 0.05$) in the control subgroup, or that were monomorphic were excluded from subsequent association analysis. Using HBV-RS as a binary response variable, an association analysis of single SNPs with HBV-RS was performed using logistic regression, and results are stated using odds ratios (ORs) with a relative 95% confidence interval (CI). The best inheritance model (codominant, dominant, recessive, over-dominant, or log-additive) was selected, according to values for *P*, Akaike Information Criterion (AIC), and Bayesian Information Criterion (BIC). Furthermore, those SNPs with a *P*-value near 0.05 in the single SNP association analysis were further analyzed in terms of the presence of linkage disequilibrium (LD) and haplotype. For LD, matrices with selected statistics (D, D′, Pearson *r*, and associated *P*-values) are shown. The association of haplotype with HBV-RS was shown as the global score test.[25]

Finally, clinical variables, genotypes, and haplotypes that differed significantly between the case and control subgroups were considered to be potential factors influencing the development of rituximab-associated HBV-RS, and were further evaluated using regression analysis. With ''death in the absence of HBV-RS'' considered as a competing event, the cumulative incidence of HBV-RS was calculated, and the Fine and Gray model was used to assess the impact of different variables on the cumulative incidence of HBV-RS,[7] and reported using subhazard ratios (SHRs) with a relative 95% CI. Finally, the multivariate regression analysis was used to identify factors that were independently influencing the development of rituximab-associated HBV-RS, after adjusting for the factors that had $P < 0.1$ in univariate analyses. A *P*-value <0.05 was thought statistically significant. The testing for Hardy–Weinberg equilibrium, linkage disequilibrium, association, and descriptive analysis of SNPs were performed using the SNPstata web site (http://bioinfo.iconcologia.net/SNPstats).[26] Other analyses were done using the Statistical Package for the Social Sciences (SPSS version 17.0), Stata version 11.0 (Stata Corp., College Station, TX), and XLSTAT (Addinsoft SARL, Paris, France).

RESULTS

Patient Characteristics According to the Presence of HBV-RS

As shown in Table 1, 104 unvaccinated HBsAg-seronegative patients with $CD20^+$ NHL were enrolled. The median age was 66.1 years and more patients were male than female (n = 59 males, 56.7%). The major histological subtypes were diffuse large B cell lymphoma and FL, which accounted for 66.7% (n = 59) and 23.1% (n = 24) of patients, respectively. Information on anti-HBs and anti-HBc status was available for 81.7% and 82.7% of patients, respectively, with respective positive rates of 82.4% and 94.1%.

At time of last follow-up or HBV-RS, a mean of 7.14 cycles of rituximab therapy had been administered, and approximately 70% of patients had received >6 cycles. A total of 14 (13.4%) patients developed HBV-RS, and all of those for whom data were available were seropositive for anti-HBc (10/10). Eleven (79%) patients experienced hepatitis flare. Compared with their counterparts, patients with HBV-RS had a lower rate of anti-HBs-positivity (58.3% vs 86.3%, $P = 0.033$), and more cycles and longer durations of rituximab therapy. No significant differences in OS were observed between patients with and without HBV-RS.

Candidate SNPs Associated With HBV-RS

The allele and genotype frequencies of the 89 SNPs were analyzed by iPLEX assay and are listed in supplemental Table 2, http://links.lww.com/MD/A767. For 84 of the SNPs, genotyping had a call rate of 90.38% to 100%; only 5 SNPs (*CXCL8* rs4073, *CXCR4* rs2228014, *IL1B* rs1143643, *IL12RB1* rs12564159, and *IL18* rs187238) had a low call rate. Three SNPs (*CARD15/NOD2* rs2066844, *NLRC5* rs9936269, and *TLR2* rs5743708) were monomorphic. In addition, the genotype frequencies of 7 SNPs deviated from Hardy–Weinberg equilibrium, including *CD40LG* rs3092933, *FCGR2A* rs10800309, *IFNL3* rs8099917 and rs12980275, *IFNL4(IL28B)* rs12979860, *IL12A* rs485497, and *TNFSF13B* rs1224141. The remaining 74 SNPs were included in the HBV-RS association analysis (supplemental Table 3, http://links.lww.com/MD/A767). Finally, without correction of multiple comparisons, 9 candidate SNPs showed a possible association with HBV-RS, with the cut-off value of uncorrected $P < 0.06$, including *CCL5* rs2107538, *CD40* rs1883832, *IL4* rs2243248 and rs2243263, *IL13* rs1295686, *IL18* rs243908, *IL20* rs1518108, *TNFSF13B* rs12583006, and rs12428930 (Table 2). Of these, the *IL4* rs2243248 T/G and rs2243263 C/G genotypes were present in 20% (n = 18) and 23.3% (n = 21) of the total patients, respectively, none of these developed HBV-RS (Table 2).

Haplotypes Associated with HBV-RS

The LD analysis of these 9 candidate SNPs showed LD between *IL4* rs2243248 and *IL4* rs2243263 ($r^2 = 0.9178$) and between *TNFSF13B* rs12583006 and *TNFSF13B* rs12428930 ($r^2 = 0.9813$) (supplemental Tables 4 and 5, http://links.lww.com/MD/A767). The 18 patients (17.3% of 104) who had *IL4* rs2243248 T/G and did not develop HBV-RS also had *IL4* rs2243263 C/G (supplemental Tables 3 and 5, http://links.lww.com/MD/A767). Of note, the *IL4* rs2243248∼rs2243263 haplotype was significantly associated with the development of rituximab-associated HBV-RS (global $P = 0.033$) (Table 3).

Competing-Risks Regression Analysis of the Association of Clinical Factors, SNPs, and Haplotypes With HBV-RS

When ''death in the absence of HBV-RS'' was considered as the competing risk, the cumulative incidence of HBV-RS was estimated to be 2%, 5%, and 13.3% at 6 months, 12 months, and 24 months after rituximab therapy, respectively (Figure 1).

TABLE 1. Characteristics of 104 Unvaccinated HBsAg-Seronegative Patients With CD20$^+$ NHL According to the Development of HBsAg Reverse Seroconversion

		HBsAg Reverse Seroconversion		
	Total, %	Yes, %	No, %	*P*
No. of patients	104 (100)	14 (100)	90 (100)	
Age (years) at diagnosis, median (range)	66.1 (30.9–85.4)	69.0 (47.6–81.7)	69.7 (30.9–85.4)	0.984
Gender: male/female	59/45 (56.7/43.3)	5/9 (35.7/64.3)	54/36 (60/40)	0.145*
Histological subtypes				
DLBCL	59 (66.7)	8 (57.1)	51 (66.7)	1*
FL	24 (23.1)	2 (14.3)	22 (24.4)	0.513*
Viral serology				
anti-HBs – available	85 (81.7)†	12 (86.7)	73 (81.1)	1*
anti-HBs – seropositive	70/85 (82.4)†	7/12 (58.3)	63/73 (86.3)	0.033*
anti-HBc – available	86 (82.7)†	10 (71.4)	76 (84.3)	0.258*
anti-HBc – seropositive	79/86 (94.1)†	10/10 (100)	69/76 (90.8)	1*
Rituximab therapy				
Cycle number, mean (SD)	7.14 (3.986)	9.29 (3.872)	6.81 (3.920)	0.030
≥6 cycles	73 (70.2)	14 (100)	59 (65.6)	0.009*
Durations (weeks), median (range)	21.5 (0–299.3)	29.2 (15.7–159.4)	21.3 (0–299.3)	0.088‡
Overall survival (weeks), mean (95% CI)	313.3 (271.0–355.6)	338.9 (237.3–440.6)	300.7 (256.4–344.9)	0.526

anti-HBc = antibody to hepatitis B virus core antigen, anti-HBs = antibody to hepatitis B virus surface antigen, CI = confidence interval, DLBCL = diffuse large B cell lymphoma, FL = follicular lymphoma, HBsAg = hepatitis B virus surface antigen, N = number of patients, NHL = non-Hodgkin lymphoma, SD = standard deviation.

* Fisher exact test.

† For 79 patients with both anti-HBs and anti-HBc status, the patient number of anti-HBs/anti-HBc: +/+, −/+, +/−, and −/− was 68 (86.1%), 5 (6.3%), 2 (2.5%), and 4 (5.1%), respectively.

‡ Mann–Whitney *U*-test.

TABLE 2. Candidate Single Nucleotide Polymorphisms Associated With HBsAg Reverse Seroconversion After Rituximab Therapy

Gene symbol*	SNP ID†	Genotype	HBV-RS, No	HBV-RS, Yes	OR‡	(95% CI)‡	*P* Value‡	AIC	BIC
CCL5	rs2107538	C/C-C/T	78 (86.7%)	14 (100%)	1.00		0.054	82.5	87.8
		T/T	12 (13.3%)	0 (0%)	0.00	(0.00-NA)			
CD40	rs1883832	C/C	26 (29.2%)	1 (7.1%)	1.00		0.052	82.1	87.4
		C/T-T/T	63 (70.1%)	13 (92.9%)	5.37	(0.69–43.15)			
IL4	rs2243248	T/T	72 (80%)	14 (100%)	1.00		0.016	80.4	85.7
		T/G	18 (20%)	0 (0%)	0.00	(0.00-NA)			
IL4	rs2243263	G/G	69 (76.7%)	14 (100%)	1.00		0.0089	79.3	84.6
		C/G	21 (23.3%)	0 (0%)	0.00	(0.00-NA)			
IL13	rs1295686	G/G-A/G	83 (92.2%)	10 (71.4%)	1.00		0.039	81.9	87.2
		A/A	7 (7.8%)	4 (28.6%)	4.74	(1.18–19.09)			
IL18	rs243908	T/T	67 (76.1%)	7 (50%)	1.00		0.052	81.8	87.1
		C/T-C/C	21 (23.9%)	7 (50%)	3.19	(1.00–10.14)			
IL20	rs1518108	C/C	58 (64.4%)	5 (35.7%)	1.00		0.043	82.1	87.4
		C/T-T/T	32 (35.6%)	9 (64.3%)	3.26	(1.01–10.57)			
TNFSF13B	rs12428930	A/A	32 (35.6%)	1 (7.1%)	1.00		0.018	80.6	85.9
		A/C-C/C	32 (35.6%)	13 (92.9%)	7.17	(0.90–57.37)			
TNFSF13B	rs12583006	T/T	31 (34.8%)	1 (7.1%)	1.00		0.02	80.5	85.8
		A/T-A/A	58 (65.2%)	13 (92.9%)	6.95	(0.87–55.63)			

AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, CI = confidence interval, HBsAg = hepatitis B virus surface antigen, HBV-RS = reverse seroconversion of hepatitis B virus surface antigen, OR = odds ratio, SNP = single nucleotide polymorphism.

* Used in the gene resource (http://www.ncbi.nlm.nih.gov/gene/).

† Used in the dbSNP resource (http://www.ncbi.nlm.nih.gov/snp/).

‡ Logistic regression, with no correction of multiple comparison.

TABLE 3. Haplotype Frequencies and Association Analysis of *IL4* and *TNSSF13B* According to the Development of HBsAg Reverse Seroconversion

Gene	SNP		Total	HBV-RS, No	HBV-RS, Yes	Cumulative Frequency	OR (95% CI)*	*P* Value*
IL4†	rs2243248	rs2243263						
Hap1	T	G	0.899	0.8833	1	0.899	1.00	–
Hap2	G	C	0.0865	0.1	NA	0.9856	0.00 (−Inf–Inf)	1
Hap3	T	C	0.0144	0.0167	NA	1	0.00 (−Inf–Inf)	1
TNSSF13B‡	rs12428930	rs12583006						
Hap1	A	T	0.5432	0.5554	0.4643	0.5432	1.00	–
Hap2	C	A	0.4771	0.4333	0.5357	0.9903	1.46 (0.66–3.20)	0.35
Hap3	A	A	0.0097	0.0112	NA	1	0.00 (−Inf–Inf)	1

CI = confidence interval, HBsAg = hepatitis B virus surface antigen, HBV-RS = reverse seroconversion of hepatitis B virus surface antigen, Inf = infinity, OR = odds ratio, SNP = single nucleotide polymorphism.

* Logistic regression.

† $P = 0.033$, by the global score test.

‡ $P = 0.48$, by the global score test.

In the univariate regression analysis (Table 4), the factors found to be independently associated with the development of HBV-RS were anti-HBs status, cycles of rituximab therapy (≥6 cycles), the SNPs *IL4* rs2243248 (Figure 2A) and rs2243263 (Figure 2B), *IL13* rs1295686 (Figure 2C), *IL18* rs243908 (Figure 2D), *IL20* rs1518108 (Figure 2E), and the haplotype *IL4* rs2243248∼rs2243263 (Figure 3A). In addition, the SNPs *TNFSF13B* rs12428930 and rs12583006, and the haplotype *TNFSF13B* rs12428930∼rs12583006 (Figure 3B) showed a trend of association with HBV-RS.

In the multivariate analysis adjusted for gender and histological subtype of all patients, the remaining independent factors associated with HBV-RS were cycles of rituximab therapy (≥6 cycles), the SNP *IL18 rs243908*, and the haplotype *IL4* rs2243248∼rs2243263 (Table 4). The SNP *IL20* rs1518108 showed a trend of association ($P = 0.076$).

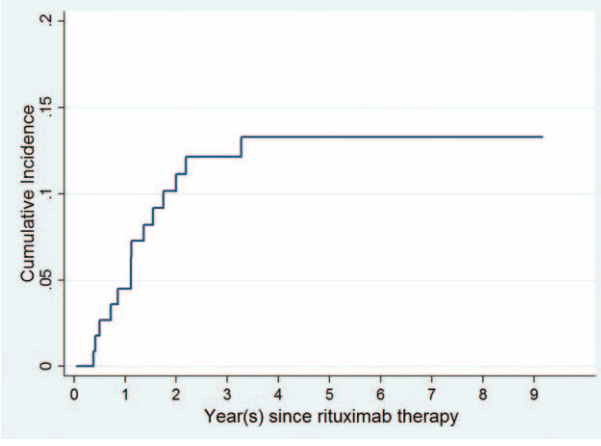

FIGURE 1. Cumulative incidence of rituximab-associated reverse seroconversion of HBsAg in 104 unvaccinated patients with $CD20^+$ NHL, with ''death in the absence of HBsAg reverse seroconversion'' as the competing risk. The *P* value was calculated by competing risk analysis based on the Fine and Gray model. $CD20^+$ NHL = CD20-positive B-cell non-Hodgkin lymphoma, HBsAg = hepatitis B virus surface antigen.

IL4 Haplotype Was Not Related to Anti-HBs Status in the Risk of HBV-RS

As 18.3% of patients were missing data for anti-HBs, anti-HBs was not included in the multivariate analysis. Subgroup analysis was performed to determine whether the *IL18* SNP and the *IL4* haplotype were associated with the risk of HBV-RS, according to anti-HBs status at diagnosis. In this analysis, *IL18* rs243908 was no longer significantly associated with HBV-RS in the anti-HBs seropositive and negative subgroups (see Figure 4A [SHR = 2.512; 95% CI, 0.574–11.001; $P = 0.222$] and 4B [SHR = 1.088; 95% CI, 0.206–5.749; $P = 0.921$] for the seropositive and seronegative subgroups, respectively). It should be noted that the association of *IL4* haplotype rs2243248∼rs2243263 with HBV-RS remained significant in both the anti-HBs seropositive and seronegative subgroups (Figure 4C and D for the seropositive [SHR = 1.51e-19; $P \approx 0$] and seronegative groups [SHR = 1.76e-19; $P \approx 0$], respectively).

DISCUSSION

To our knowledge, this pilot study was the 1st to explore the potential association of SNPs and haplotypes of human cytokine genes with rituximab-associated HBV-RS. The findings are particularly noteworthy because the regression analysis employed clinical factors, and because the association with a higher number of cycles of rituximab (≥6) noted here is similar to results published in our recent report.[4,7] First, both SNPs of *IL4*, rs2243248 and rs2243263, as well as the *IL4* haplotype rs2243248∼rs2243263 were found to be significantly associated with the development of rituximab-associated HBV-RS. IL4 is produced by type 2 helper T (Th2) cells and can costimulate B-cell proliferation.[27] For possible mechanisms by which IL4 influences rituximab-associated HBV-RS, IL4 could suppresses the expression and the replication of HBV in the hepatocellular carcinoma cell line Hep3B,[28] but rituximab was shown to cause a 12-fold peak-decrease of *IL4* mRNA in whole blood.[29] A lower IL4 level was associated with a lower anti-HBs level after HBV vaccination in HIV patients.[30] Total 6 SNPs of *IL4* were genotyped in this study (supplemental Table 1, http://links.lww.com/MD/A767), including rs2243248 located in the promoter region, rs2070874 in the 5′ untranslated

TABLE 4. Competing Risk Regression for Predictors of HBsAg Reverses Seroconversion After Rituximab Therapy*

	Univariate*			Multivariate*		
Factor	**SHR**	**(95% CI)**	***P***	**SHR**	**(95% CI)**	***P***
Gender						
Male	1	–	0.124	1	–	0.496
Female	2.363	0.791–7.058		1.690	0.373–7.659	
anti-HBc						
Seronegative	1	–	–†	–	–	–‡
Seropositive	3.80e+32	–†		–	–	
anti-HBs						
Seronegative	1	–	0.020	–	–	–‡
Seropositive	0.263	0.086–0.807		–	–	
Histological subtypes						
DLBCL						
No	1	–	0.801	1	–	0.248
Yes	1.142	0.407–3.204		1.940	0.631–5.970	
FL						
No	1		0.332	–	–	–
Yes	0.484	0.112–2.095		–	–	
Cycle number of rituximab therapy						
<6	1	–	–†	1	–	<0.001
≥6	4.84e+32	–†		1.60e+07	5.61e+06–4.55e+07	
SNP						
CCL5 rs2107538						
C/C-C/T	1	–	0.462	–	–	
T/T	0.779	0.401–1.516		–	–	
CD40 rs1883832						
C/C	1	–	0.120	–	–	–
C/T-T/T	5.322	0.645–43.910		–	–	
IL4 rs2243248						
T/T	1	–	–*	–	–	–§
T/G	1.94e-33	–*		–	–	
IL4 rs2243263						
G/G	1	–	–*	–	–	–§
C/G	1.48e-19	–*		–	–	
IL13 rs1295686						
G/G-A/G	1	–	0.026	1	–	0.834
A/A	3.753	1.173–12.014		1.208	0.205–7.111	
IL18 rs243908						
T/T	1	–	0.030	1	–	0.018
C/T-C/C	3.144	1.117–8.856		4.867	1.318–17.975	
IL20 rs1518108						
C/C	1	–	0.043	1	–	0.076
C/T-T/T	3.043	1.036–8.941		3.096	0.888–10.792	
TNFSF13B rs12428930						
A/A	1		0.056	–	–	–§
A/C-C/C	7.085	0.952–52.707		–	–	
TNFSF13B rs12583006						
T/T	1	–	0.060	–	–	–§
A/T-A/A	6.853	0.922–50.956		–	–	
Haplotype						
IL4 rs2243248∼rs2243263						
T/T∼G/G	1	–	–†	1	–	<0.001
T/G∼C/G	1.48e-19	–†		3.61e-08	1.26e-08–1.03e-07	
TNFSF13B rs12428930∼rs12583006						
A/A∼T/T	1	–	0.055	1	–	0.117
A/C∼A/A, C/C∼A/T	7.098	0.955–52.754		5.182	0.663–40.492	

anti-HBc = antibody to hepatitis B virus core antigen, anti-HBs = antibody to hepatitis B virus surface antigen, CI = confidence interval, DLBCL = diffuse large B cell lymphoma, FL = follicular lymphoma, HBsAg = hepatitis B virus surface antigen, SHR = subdistribution hazard ratio, SNP = single nucleotide polymorphism.

* Competing risk analysis was based on the Fine and Gray model, with ''death in the absence of HBV-RS'' considered as a competing event.

† Positive/negative infinity, with the value of P near 0.

‡ Not enrolled in the multivariate regression because of missing values in some patients.

§ Haplotype (rather than SNPs) enrolled in the multivariate regression because of a high LD.

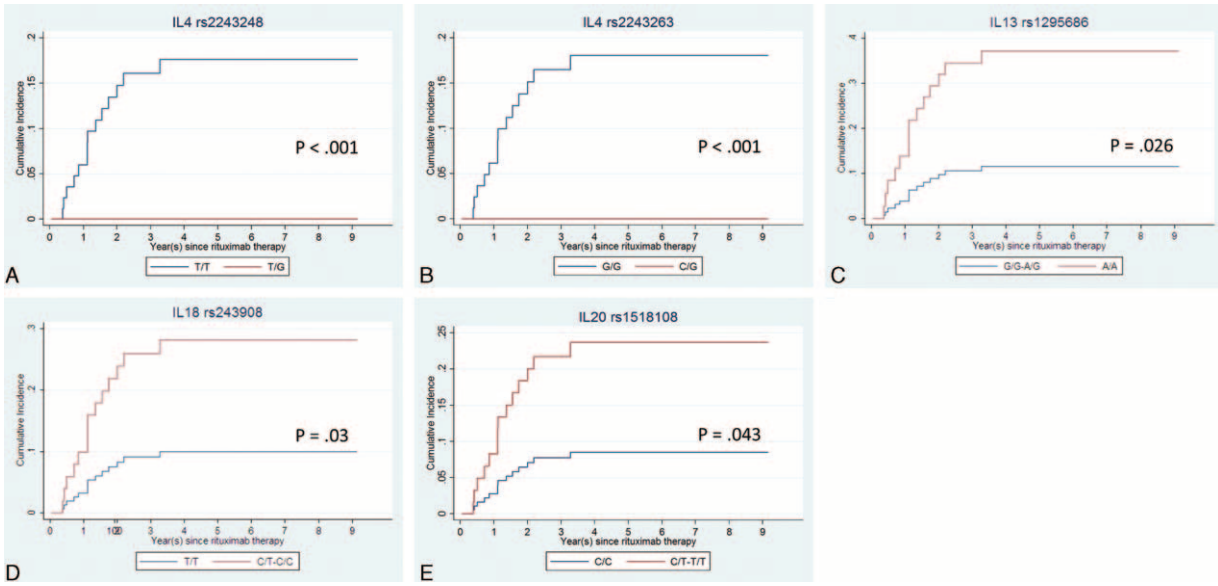

FIGURE 2. Cumulative incidence of rituximab-associated reverse seroconversion of HBsAg in 104 unvaccinated patients with $CD20^+$ NHL, with ''death in the absence of HBsAg reverse seroconversion'' as the competing risk, according to SNPs (A) *IL4* rs2243248, (B) *IL4* rs2243263, (C) *IL13* rs1295686, (D) *IL18* rs243908, and (E) *IL20* rs1518108. The *P* value was calculated by competing risk analysis based on the Fine and Gray model. $CD20^+$ NHL = CD20-positive B-cell non-Hodgkin lymphoma, HBsAg = hepatitis B virus surface antigen, SNP = single nucleotide polymorphism.

region, rs2227284, rs2243263, and rs2243268 all in the intron region, and rs2243291 in 3′ untranslated region. The allele frequencies of *IL4* rs2243248 and rs2243263 and the strong LD between them noted here are concordant with previous results.[31] *IL4* rs2243248 might influence the mRNA transcription of *IL4*, similar to the way of *IL4* rs2243250 (−590C>T),[32–34] which is also located in the promoter region, but was not included in the present analysis owing to technical limitations of iPLEX. On the other hand, *IL4* rs2243263 might participate in the regulation of *IL4* expression via binding with CTCF transcription factors.[35] Chen et al[36] reported that the *IL4* rs2243248 G allele was associated with nonresponse to HBV vaccine; however, it was found to be associated with a lower incidence of HBV-RS in our study (Table 4). In addition, *IL4* rs2243248 was associated with an increased the likelihood of high lytic antibody titers for human herpesvirus 8 infection.[37] Consequently, we speculated that $CD20^+$ NHL patients with different *IL4* rs2243248∼rs2243263 haplotypes will have different serum IL4 levels and differing T-cell responses during rituximab therapy, thus influencing the development of HBV-RS.

Second, *IL18* rs243908 was found to be associated with the development of HBV-RS in our patients (Table 4). As previously described,[38] IL18 is produced not only by immune cells but also by nonimmune cells. IL18 enhances the IL12-driven Th1 immune response, but it can also stimulate Th2 immune responses in the absence of IL12. IL18 could also influence HBV replication by activating resident intrahepatic natural killer cells and natural killer T cells to produce gamma interferon (IFN-γ), and by inducing IFN-α/β production in the liver.[39] Two *IL18* SNPs – rs243908 and rs187238 were genotyped in this study (supplemental Table 1, http://links.lww.com/MD/A767), based on the association of lymphoma[40] and HBV infection,[41] respectively. *IL18* rs187238 located in the promotor region of *IL18* was found to be associated with the

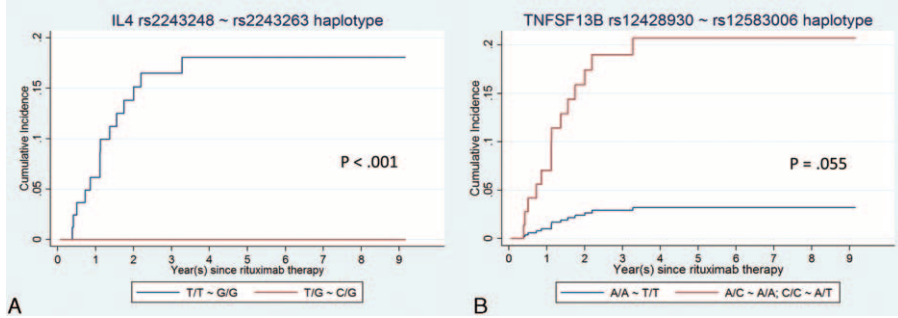

FIGURE 3. Cumulative incidence of rituximab-associated reverse seroconversion of HBsAg in 104 unvaccinated patients with $CD20^+$ NHL, with ''death in the absence of HBsAg reverse seroconversion'' as the competing risk, according to haplotypes (A) *IL4* rs2243248∼rs2243263 and (B) *TNFSF13B* rs12428930∼rs12583006. The *P* value was calculated by competing risk analysis based on the Fine and Gray model. $CD20^+$ NHL = CD20-positive B-cell non-Hodgkin lymphoma, HBsAg = hepatitis B virus surface antigen.

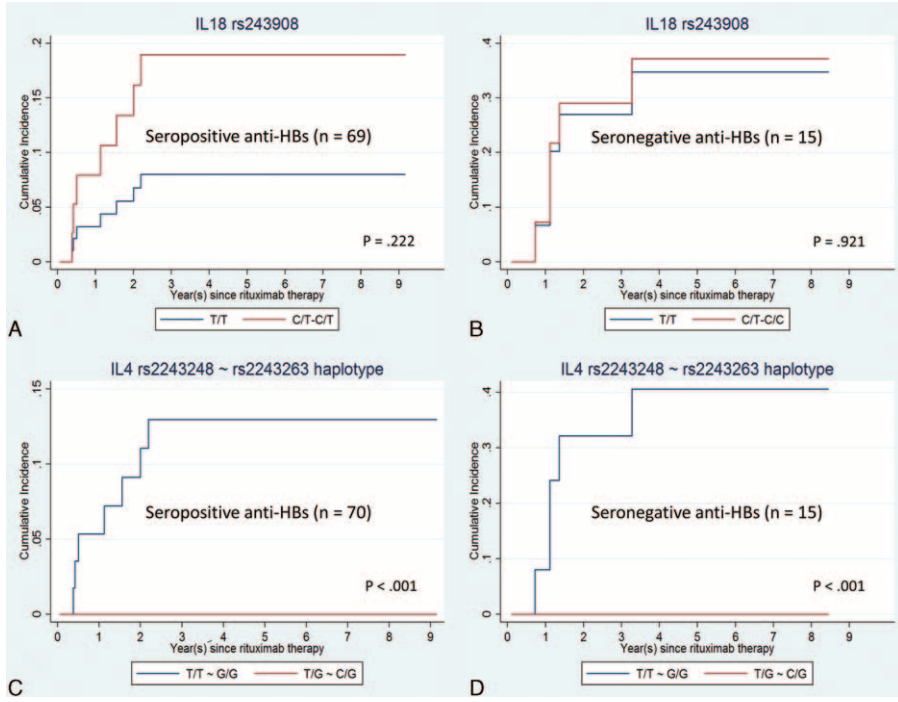

FIGURE 4. Cumulative incidence of rituximab-associated reverse seroconversion of HBsAg in 86 unvaccinated patients with CD20$^+$ NHL, with ''death in the absence of HBsAg reverse seroconversion'' as the competing risk, according to the anti-HBs serostatus at diagnosis and *IL18* SNP rs243908 (A, B), and *IL4* haplotype rs2243248~rs2243263 (C, D). The *P* value was calculated by competing risk analysis based on the Fine and Gray model. anti-HBs = antibody to hepatitis B virus surface antigen, CD20$^+$ NHL = CD20-positive B-cell non-Hodgkin lymphoma, HBsAg = hepatitis B virus surface antigen, SNP = single nucleotide polymorphism.

transcriptional activity of the *IL18* promoter,[42] and even with serum IL18 levels.[43] *IL18* rs187238 and several other *IL18* SNPs were reported to be associated with chronic HBV infection,[44] the development of hepatocellular hepatoma, and the clearance of HBV.[45] Unfortunately, we were unable to include rs187238 in our analysis because it showed a low call rate (supplemental Tables 1 and 2, http://links.lww.com/MD/A767). On the other hand, *IL18* rs243908 was also located in 5′ untranslated region, and the relationship with IL18 secretion was not understood. It may potentially be associated with HBV infection, as it showed modest LD with *IL18* rs187238 ($r^2 = 0.809$). The association of *IL18* rs243908 with HBV-RS was not found to be significant in the subgroup analysis according to anti-HBs serostatus (Figure 4A and B). The loss of significance was probably caused by the relatively low number of patients in each subgroup; its impact on HBV-RS might have been overshadowed by the effect of the anti-HBs serostatus group.

In addition, the multivariate analysis showed that *IL20* rs1518108 had a trend of association with the development of HBV-RS (Table 4). IL20, which belongs to the IL10 family, is a potent immunomodulatory cytokine, and the selection of *IL20* rs1518108 here was based on the potential association with HBV infection.[45] Similarly to the report published by Truelove et al,[7] which stated that the *IL20* rs1518108 C/T variant is associated with chronic HBV infection in African American, we demonstrated that patients with the variants C/T-T/T tended to have a higher risk of HBV-RS than those with the C/C variant (Table 4). Because all of the above cytokines are closely regulated and correlated with each other during infection in humans, it is possible that all of the *IL18* and *IL20* SNPs and the *IL4* haplotypes are participating in the process of HBV-RS. However, *IL4* haplotype may serve as a surrogate marker to represent host immune status and susceptibility to HBV-RS during rituximab treatment in CD20$^+$ NHL patients with resolved hepatitis B.

Our study has a few limitations. First, this pilot study was part of the Genetic Polymorphisms Influencing the Impact of HBV Infection on CD20$^+$ NHL Patients project, in terms of those relevant to toxicity and susceptibility, and candidate genes were selected from those relevant to lymphoma susceptibility and those relevant to HBV infection. Identification of the underlying mechanisms of the SNPs and haplotypes identified here is beyond the scope of this study, but should be investigated in future. Second, the retrospective design, the heterogeneity of patients, and the relatively small number of HBV-RS patients might obscure the potential significance of some SNPs. A larger number of patients would not only improve the degree of precision for these SNPs and haplotypes with a trend of association in this study, but also help us to further analyze the relationship between these SNPs and treatment outcome or drug toxicity. In addition, the association of these SNPs and haplotypes with HBV-RS should also be examined in CD20$^+$ NHL patients with other risk factors recently reported,[6,7] including anti-HCV seropositivity, histological subtype of post-transplant lymphoproliferative disorders, and succeeding hematopoietic stem cell transplantation. Third, we did not analyze the impact of baseline HBV DNA, because it was not routinely tested for our patients of this study. Our previous report showed that undetectable HBV viral load before chemotherapy did not

confer reactivation-free status of rituximab treated lymphoma patients.[4] Finally, the other factor – the intensity of combination chemotherapy (e.g., CHOP) was not analyzed here, because the risk of HBV-RS in $CD20^+$ NHL patients with resolved hepatitis B treated with CHOP alone was very low.[3]

In conclusion, this pilot study was the 1st to provide evidence that the development of rituximab-associated HBV-RS is likely to be influenced by host genetic background in lymphoma patients. Our findings provide a clinical indication to further stratify the risk of HBV-RS in $CD20^+$ NHL patients with resolved hepatitis B before administering rituximab treatment.

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