Hepatitis B Surface Antigen Genetic Elements Critical for Immune Escape Correlate With Hepatitis B Virus Reactivation Upon Immunosuppression

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Hepatitis B virus (HBV) reactivation during immunosuppression can lead to severe acute hepatitis, fulminant liver failure, and death. Here, we investigated hepatitis B surface antigen (HBsAg) genetic features underlying this phenomenon by analyzing 93 patients: 29 developing HBV reactivation and 64 consecutive patients with chronic HBV infection (as control). HBsAg genetic diversity was analyzed by population-based and ultradeep sequencing (UDS). Before HBV reactivation, 51.7% of patients were isolated hepatitis B core antibody (anti-HBc) positive, 31.0% inactive carriers, 6.9% anti-HBc/anti-HBs (hepatitis B surface antibody) positive, 6.9% isolated anti-HBs positive, and 3.4% had an overt HBV infection. Of HBV-reactivated patients, 51.7% were treated with rituximab, 34.5% with different chemotherapeutics, and 13.8% with corticosteroids only for inflammatory diseases. In total, 75.9% of HBV-reactivated patients (vs. 3.1% of control patients; $P < 0.001$) carried HBsAg mutations localized in immune-active HBsAg regions. Of the 13 HBsAg mutations found in these patients, 8 of 13 (M103I-L109I-T118K-P120A-Y134H-S143L-D144E-S171F) reside in a major hydrophilic loop (target of neutralizing antibodies [Abs]); some of them are already known to hamper HBsAg recognition by humoral response. The remaining five (C48G-V96A-L175S-G185E-V190A) are localized in class I/II–restricted T-cell epitopes, suggesting a role in HBV escape from T-cell-mediated responses. By UDS, these mutations occurred in HBV-reactivated patients with a median intrapatient prevalence of 73.3% (range, 27.6%-100%) supporting their fixation in the viral population as a predominant species. In control patients carrying such mutations, their median intrapatient prevalence was 4.6% (range, 2.5%-11.3%; $P < 0.001$). Finally, additional N-linked glycosylation (NLG) sites within the major hydrophilic loop were found in 24.1% of HBV-reactivated patients (vs. 0% of chronic patients; $P < 0.001$); 5 of 7 patients carrying these sites remained HBsAg negative despite HBV reactivation. NLG can mask immunogenic epitopes, abrogating HBsAg recognition by Abs. *Conclusion*: HBV reactivation occurs in a wide variety of clinical settings requiring immune-suppressive therapy, and correlates with HBsAg mutations endowed with enhanced capability to evade immune response. This highlights the need for careful patient monitoring in all immuno-suppressive settings at reactivation risk and of establishing a prompt therapy to prevent HBV-related clinical complications. (HEPATOLOGY 2015;61:823-833)

Hepatitis B virus (HBV) reactivation is defined as the abrupt reappearance of HBV (serum HBV DNA >100 IU/mL) in the serum of a person with previously resolved infection or a marked increase of HBV replication (>2 log increase of serum HBV DNA from baseline level) in an immunosuppressed patient with previously stable chronic infection (American Association for the Study of Liver Diseases (AASLD) Emerging Trends Conference, Arlington, Virginia, March 21-22, 2013). The potential HBV reactivation in patients with resolved HBV infection is related to the persistence in the nuclei of hepatocytes (after the primary infection) of a stable HBV-DNA minichromosome named covalently closed circular DNA (cccDNA). The cccDNA serves as a template to generate all RNAs necessary for viral replication, and its activity is controlled by the immune system. The balance between viral replication and immune control can explain why immunosuppression can increase HBV replication in chronically infected patients and reactivate "dormant" HBV in individuals considered as recovered.[1,2] The acute rise in serum HBV DNA can, in turn, lead to severe acute hepatitis, synthetic dysfunction, fulminant liver failure, and death.[1-3] HBV reactivation is most commonly reported in patients receiving cancer chemotherapy for hematological malignancies and those receiving bone marrow or stem cell transplantation.[4] Nevertheless, this event can also occur in a wide variety of clinical settings, including patients receiving chemotherapy for solid tumors, recipients of solid organ transplants, and patients receiving immune-suppressive therapy for treatment of tumors, as well as gastrointestinal, rheumatologic, or dermatological inflammatory diseases.[1,2]

The incidence of HBV reactivation in the immunocompromised host is not accurately defined owing to the lack of large-cohort prospective studies; however, high percentages of HBV reactivation, ranging from 24% to 88%, have been reported in different clinical settings.[1,2] A lamivudine (LMV)-based prophylaxis has been thus far proposed to prevent HBV reactivation.[5-7] Nevertheless, despite the use of LMV, a reliable risk of HBV reactivation still persists, reaching nearly the 20% of patients under immune-suppression status.[6] Additionally, several issues remain, including the optimal timing and treatment length as well as the follow-up range of preventive LMV treatment.[8]

Anecdotic clinical case descriptions have highlighted a high degree of genetic variability in the S gene in patients developing HBV reactivation.[9-14] The S gene is composed of the pre-S1, pre-S2, and S region, coding for the three forms of HBV surface glycoproteins: the small-sized HBV surface antigen (S-HBsAg) as well as the medium- (pre-S2+S) and large-sized surface antigen (pre-S1+pre-S2+S). An in-depth characterization of the extent of genetic variability throughout the entire S gene in patients undergoing immunosuppression-related HBV reactivation is still missing. Understanding this point can help in the understanding of biological processes underlying HBV persistence and viral reactivation. This may also provide the basis for the design of more-effective prophylaxis in terms of potency and duration of treatment.

In light of this, our study aims at investigating, by both population and ultradeep pyrosequencing (UDPS),

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the extent of genetic diversity and heterogeneity throughout the entire S gene in a well-characterized cohort of patients experiencing immunosuppression-related HBV reactivation, as well as to investigate the existence of viral genetic signatures underlying this phenomenon.

Patients and Methods

Study Population. This study included 93 patients: 29 developing immunosuppression-related HBV reactivation and 64 consecutive chronically HBV-infected patients used as a reference group, followed in different clinical centers in Italy ("Tor Vergata" University Hospital of Rome, "S.M. Goretti Hospital" of Latina, "S.S. Filippo e Nicola" Hospital" of Avezzano, and Second University of Naples). Ethical approval was deemed unnecessary because, under Italian law, biomedical research is subjected to previous approval by ethics committees only in the hypothesis of clinical trials on medicinal products for clinical use (art. 6 and art. 9, leg. Decr. 211/2003). The research was conducted on viral DNA samples (used for clinical routine), and data previously anonymized, according to the requirements set by Italian Data Protection Code (leg. decree 196/2003).

Patients with immunosuppression-related HBV reactivation met the criteria defined by the "Emerging Trends Conference: Reactivation of Hepatitis B" held in Arlington, Virginia, on 21-22 March 2013, promoted by the AASLD (http://www.aasld.org/LIVERLEARNING%C2%AE/Pages/etc.aspx). Patients experienced a reappearance of serum HBV DNA ($>$100 IU/mL) or a marked rise of serum HBV DNA ($>$2 log IU/mL from baseline level), during or after the administration of immunosuppressive therapy. Inactive carrier patients were defined as patients that, before HBV reactivation, had a persistently serum HBV DNA $<$2,000 IU/mL, persistently normal transaminases, and were hepatitis B envelope antigen (HBeAg) negative and hepatitis B envelope antibody (anti-HBe) positive.

Plasma samples were collected at the time of the first diagnosis of HBV reactivation. Chronically HBV-infected patients were naïve to anti-HBV drugs and had no clinical evidence of cirrhosis and hepatocellular carcinoma. No patients had a co-infection with human immunodeficiency virus (HIV), hepatitis delta virus (HDV), and hepatitis C virus (HCV).

Population-Based Sequencing of the Full-Length S Gene (Pre-S1, Pre-S2, and S). The population-based sequencing of the full-length S gene was performed on plasma samples, following a home-made protocol, as previously described, and optimized in order to cover the pre-S1 and pre-S2 region.[15] Details are reported in text I of the Supporting Information.

UDPS. The extent of genetic heterogeneity in the S gene coding for the S-HBsAg was also investigated by UDPS (Roche 454 Junior; Roche Diagnostics Corporation, Basel, Switzerland). UDPS was restricted to 19 reactivation cases and 22 chronically HBV-infected patients (randomly selected). The UDPS protocol is reported on in text II of the Supporting Information.

Evolutionary Divergence. The evolutionary divergence of the full-length S sequences (pre-S1, pre-S2, and S region), obtained by population-based sequencing, was estimated as the extent of nucleotide substitutions per site determined by the Tajima-Nei model of MEGAv5, applying a gamma distribution with shape parameter = 1.0. The estimate variance was assessed from the bootstrap method with shape parameter = 1,000, and the test was conducted for nucleotide alignment. Evolutionary divergence was calculated for the pre-S1, pre-S2, and S region and in the functional domains of the S-HBsAg: N-terminal region (amino acids [aa] 1-98); major hydrophilic region (aa 99-169), and membrane-embedded C-terminus (aa 170-226).

Analysis of the N-Linked Glycosylation Sites. N-linked glycosylation (NLG) analysis was performed on the full-length S-HBsAg aa sequences included in the study using the program N-Glycosite (available at: http://www.hiv.lanl.gov/). An NLG site is identified in the aa sequence by the motif NXS/T. Thus, the NLG sequon has to begin with an asparagine (N) followed by any aa except Proline. The next aa residue has to be either a threonine (T) or a serine (S).

Mutation Prevalence. The full-length S sequences obtained by population-based sequencing were used to assess the association of specific mutations with HBV reactivation. Only mutations present in $>$3 HBV-reactivated patients were considered for the analysis. In particular, the prevalence of each mutation was calculated in the group of HBV-reactivated patients and in the group of chronically HBV-infected patients. Fisher's exact test was performed to verify whether the differences in frequency among the two groups of patients were statistically significant. In order to support their correlation with HBV reactivation, a multivariable logistic regression was performed. The following variables were considered: patients' age; serum HBV DNA; alanine aminotransferase (ALT) and aspartate aminotransferase (AST); and presence of at least one mutation associated with HBV reactivation.

Sequences obtained by UDPS were used to define the intrapatient prevalence of mutations associated with HBV reactivation and of mutations that are known to affect S-HBsAg recognition by antibodies (Abs) whose list is available at the following website: http://hbv.bioinf.mpi-inf.mpg.de/index.php (updated October 2013).

Statistical Analysis. Data were analyzed using the statistical software package SPSS (v17.0; SPSS Inc., Chicago, IL).

Impact of NLG Sites on HBsAg Quantification. In order to investigate the ability of the NLG sites identified in this study on HBsAg recognition and quantification, a plasmid encoding the HBsAg linked to a streptavidin tag (strep-tag) was used to transfect HepG2 cells. The amount of strep-tagged HBsAg released in culture supernatants was then quantified using different enzyme-linked immunosorbent assays (ELISAs): a specifically designed ELISA capable of recognizing the Strep-tag linked to the HBsAg (defined as Strep-tag-based ELISA) and two ELISAs directed against the HBsAg protein (Architect [Abbott, Abbott Park, IL] and Mono-lisa [Bio-Rad, Hercules, CA]). Two independent experiments were carried out, each in duplicate. Details are reported in text III of the Supporting Information.

Results

Patients' Characteristics. This study included 29 patients developing immunosuppression-related HBV reactivation (Table 1). Most patients were male (65.5%) with a median (interquartile range; IQR) age of 63 (61-72) years. At the diagnosis of HBV reactivation, median (IQR) serum HBV DNA was 6.1 (4.1-8.0) log IU/mL and median (IQR) of ALT and AST were 315 (35-552) and 91 (29-267) IU/L, respectively.

Median duration of immunosuppression was 15 (9-45) months. Regarding the serological status of HBV infection before viral reactivation, 51.7% (15 of 29) of patients were isolated hepatitis B core antibody (anti-HBc) positive, 31.0% (9 of 29) were inactive carriers, 6.9% (2 of 29) were anti-HBc and hepatitis B surface antibody (anti-HBs) positive, 6.9% (2 of 29) were isolated anti-HBs positive, and 3.4% (1 of 29) had an overt HBV infection. Before immunosuppression, patients negative for HBsAg and positive for anti-HBc had serum HBV DNA <20 IU/mL, and inactive carriers had serum HBV DNA ranging from 20 to 441 IU/mL. The patient with overt HBV infection had serum HBV DNA of 51,896 IU/mL. A total of 51.7% of HBV-reactivated patients were treated with rituximab for hematologic malignancies and 34.5% were treated with other immunosuppressive drugs (including fludarabine, everolimus+mycophenolate, methotrex-

Table 1. Patients' Characteristics

Patients' Characteristics	HBV-Reactivated Patients* (N = 29)	Chronically Infected Patients*[†] (N = 64)
Male, N (%)	19 (65.5)	43 (68.3)
Italian nationality, N (%)	29 (100)	48 (80)
Median age, Yyears (IQR)	63 (61-72)	51 (39-65)
Median HBV DNA, log IU/mL (IQR)	6.1 (4.1-8.0)[‡]	4.7 (3.2-6.2)
Median ALT, IU/L (IQR)	315 (35-568)[‡]	49 (31-102)
Median AST, IU/L (IQR)	91 (29-267)[‡]	40 (25-71)
HBV genotype D, N (%)	29 (90.6)	64 (100)
Prereactivation HBV status, N (%)		
Isolated anti-HBc positive	15 (51.7)	–
Anti-HBc positive/anti-HBs positive	2 (6.9)	–
Inactive carrier[§]	9 (31.0)	–
Isolated anti-HBs positive	2 (6.9)	–
Active carrier	1 (3.4)	–
Median duration of immunosuppression (IQR)[‖]	15 (9-45)	
Immunosoppressive therapy, N (%)		
Rituximab-containing chemotherapy	15 (51.7)	–
Corticosteroids alone	4 (13.8)	–
Other chemotherapies[¶]	10 (34.5)	–
Immune-suppressive therapy at HBV reactivation, N (%)		
Ongoing	13 (44.8)	–
Concluded	14 (48.3)	–
Unknown	2 (6.9)	–
Pathology requiring immune-suppressive therapy, N (%)		
Non-Hodgkin lymphoma/ other lymphomas	13 (44.8)	–
Chronic lymphoid leukemia/acute myeloid leukemia	6 (20.7)	–
Chronic inflammatory diseases[#]	4 (13.8)	–
Multiple myeloma	2 (6.9)	–
Kidney transplantation	3 (10,3)	–
Other cancers	1 (3.4)	–
LMV prophylaxis, N (%)	9 (31.0)	–
Outcome		
Acute hepatitis B phase resolution	16 (55.2)	–
Chronicization of HBV infection	1 (3.4)	–
Death	7 (24.1)	–
Unknown	1 (3.4)	–

*No patients were coinfected with HIV, HCV, and HDV.

[†]Chronically HBV-infected patients were consecutively collected. They were naïve to anti-HBV drugs without evidence of end-stage liver disease.

[‡]At the time of diagnosis of HBV reactivation.

[§]Inactive carriers were defined as patients that, before HBV reactivation, had a serum HBV DNA <2,000 IU/ml, persistently normal transaminases, and were HBeAg negative and anti-HBe positive.

[‖]Datum available for 22 of 29 patients.

[¶]Other chemotherapies included the following treatments: fludarabine; evero-limus+mycophenolate; methotrexate; and vincristine+dexamethasone.

[#]Chronic inflammatory diseases include: chronic obstructive pulmonary disease; nodose panarteritis; and psoriasis-related arthritis.

ate, and vincristine+dexamethasone) for different neoplasias; of interest, the remaining patients (13.8%) underwent a therapy only based on corticosteroids for autoimmune or inflammatory diseases, such as chronic

obstructive pulmonary disease, panarteritis nodosa, and psoriatic arthritis.

Notably, in 48.3% of patients, HBV reactivation occurred after discontinuation of immunosuppressive therapy (range, 1-14 months). Patients developing HBV reactivation after discontinuation of immunosuppression were significantly older than patients developing HBV reactivation during immunosuppression (67 [63-75] vs. 60 [52-66] years; $P = 0.034$; Supporting Table 1). Conversely, a negative correlation was observed between the use of corticosteroids for chronic inflammatory diseases and HBV reactivation after completing immunosuppression (30.8% vs. 0%; $P = 0.025$; Supporting Table 1). No other correlations were found.

Overall, 24.1% of patients (7 of 29) died within 6 months after HBV reactivation: 4 for liver failure and 3 for progression of the underlying pathology requiring immunosuppressive therapy.

Drug Resistance in Patients With HBV Reactivation Despite LMV Prophylaxis. In our study, 9 patients developed HBV reactivation despite LMV prophylaxis (median [IQR] duration: 12 [11-29] months). The primary LMV resistance mutations, rtM204V or rtM204I, were detected in 3 patients at reactivation. rtM204V was detected in 2 patients along with other mutations (rtL180M, rtV173L, and rtA181S), whereas rtM204I was present alone in a single patient. As a result of the overlapping between the RT and S-HBsAg gene, rtM204V corresponded to sI195M in S-HBsAg in both patients, rtM204I to sI196L, rtV173L to sE164D, and rtA181S to sW172C.

In a single patient, rtQ215S, potentially affecting LMV efficacy, was found. Mutations associated with alkyl-phosphonate resistance (rtA181T/V, rtN236T, and rtA194T) were not detected.

Genetic Determinants in the Full-Length S Gene Underlying Immunosuppression-Related HBV Reactivation In Vivo. The extent of genetic diversity in pre-S1, pre-S2, and S-HBsAg underlying immunosuppression-related HBV reactivation was investigated. A group of 64 consecutive, nonselected, chronically HBV D-genotype-infected patients was used as a reference group. These patients had a median (IQR) serum HBV DNA of 4.7 (3.2-6.2) log IU/mL and median ALT and AST of 49 (31-102) and 40 IU/ mL (25-71), respectively (Table 1). All of them were naïve to any anti-HBV drug treatment.

The mean evolutionary divergence of S-HBsAg sequences was significantly higher in patients with HBV reactivation than in chronically infected patients (mean evolutionary divergence: 0.017 [+ 0.005] vs. 0.009 [+ 0.004]; $P < 0.001$). The highest mean evolu-

tionary divergence was observed in the major hydrophilic region, target of neutralizing Abs (mean evolutionary divergence: 0.032 [± 0.012] vs. 0.018 [± 0.009], $P \leq 0.001$). The mean S-HBsAg evolutionary divergence did not correlate with the duration of immunosuppression (rho = −0.14; $P = 0.54$).

Differently from HBsAg, no differences in mean evolutionary divergence were observed in the pre-S1 and pre-S2 region (0.025 [± 0.009] vs. 0.032 [± 0.010], $P = 0.4$ for pre-S1; 0.032 [± 0.015] vs. 0.032 [± 0.015], $P = 0.4$ for pre-S2).

The above-mentioned results prompted us to investigate the existence of specific genetic elements in S-HBsAg specifying immunosuppression-related HBV reactivation.

Overall, 13 S-HBsAg-mutations were significantly correlated with immunosuppression-related HBV reactivation (Fig. 1A; P values from <0.001 to 0.03). Twenty-two of twenty-four (75.9%) patients with HBV reactivation (vs. only 2 of 64 chronically infected patients) carried more than one of these mutations.

Conversely, in our cohort of 64 chronically infected patients, such mutations were completely absent (0 of 64 for sC48G, sV96A, sM103I, sL109I, sT118K, sT120A, sY134H, sS143L, sD144E, sS171F, sL175S, sG185E, and sV190A) or nearly absent (only 1 of 64 for sT118K and sL175S). Their prevalence ranged from 10.3% to 20.7% in patients with HBV reactivation, compared to 0%-1.6% in the 64 chronically infected patients. By multivariable analysis, the presence of at least one of these S-HBsAg mutations was independently correlated with immunosuppression-related HBV reactivation therapy after correction for patient age, serum HBV DNA, and ALT and AST (91.04 [14.10-586.30]; $P < 0.001$; Table 2).

Beyond these specific mutations, five additional S-HBsAg positions (100, 114, 115, 145, and 154) were found mutated more frequently in patients with HBV reactivation than in chronically infected patients (Fig. 1B). Prevalence of these mutated positions ranged from 10.3% to 20.7% in patients with HBV reactivation, compared to 0%-3.1% in the 64 chronically infected patients ($P < 0.05$).

No mutations in the pre-S1 and pre-S2 region were correlated with HBV reactivation status.

Localization of S-HBsAg Residues Correlated With Immunosuppression-Related HBV Reactivation. Of the 13 S-HBsAg mutations associated with HBV reactivation, 7 of 13 (sM103I, sL109I, sT118K, sP120A, sY134H, sS143L, and sD144E) reside in a major hydrophilic loop (aa: 99-169), the main B-cell
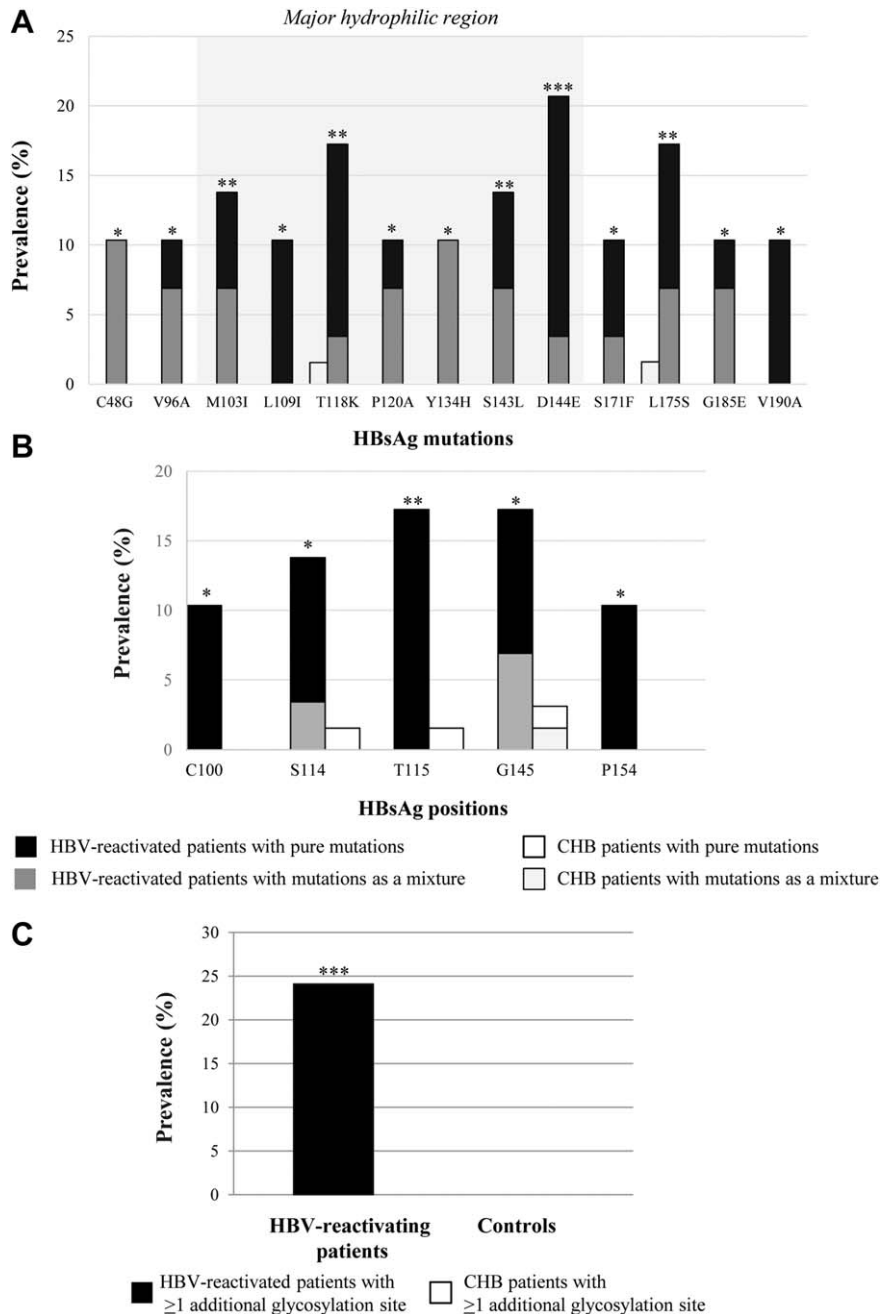
Fig. 1. Histogram reports the 13 S-HBsAg mutations whose prevalence was significantly higher in HBV-reactivated patients than in chronically infected patients (CHB patients; used as a reference group) (A), the five S-HBsAg positions found mutated more significantly in HBV-reactivated patients than in CHB patients (B), and the percentage of patients with at least one additional glycosylation site in the major hydrophilic region (C). Statistically significant differences were assessed by Fisher's exact test. This analysis was led in 29 HBV-reactivated patients and 64 chronically infected patients all infected with HBV genotype D. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. In (A) and (B), black and dark gray bars indicate the percentage of HBV-reactivated patients in which the S-HBsAg mutations were detected as pure mutation and as a mixture, respectively. White and light gray bars indicate the percentage of CHB patients in which the S-HBsAg mutations were detected as pure mutation and as a mixture, respectively. In (C), black and white bars indicate the percentage of HBV-reactivated patients with at least one additional NLG site in HBsAg. No additional NLG sites were detected in patients with chronic HBV infection. Abbreviation: CHB, chronic hepatitis B.

epitope of HBV. Among them, sT118K, sP120A, sY134H, sS143L, and sD144E are already known to hamper S-HBsAg recognition by Abs (http://hbv.bioinf.mpi-inf.mpg.de/index.php). The remaining five mutations (sC48G, sV96A, sS171F, sL175S, sG185E, and sV190A) are localized in T-cell epitopes belonging to class I (sC48G, sV96A, sL175S, and sG185E) or II (sS171F).[16] All the five positions (100, 114, 115, 145, and 154), found mutated more often in HBV-reactivated patients than in chronically infected patients, reside in the major hydrophilic region of the S-HBsAg.

Furthermore, among the 13 S-HBsAg mutations identified, seven correspond to mutations in reverse transcriptase (RT): sC48G (rtV56G); sM103I (rtV112I/L); sL109I (rtS117Y); sT118K (rtH126Q); sP120A (rtT128G/S); sY134H (rtV142A); and sV190A (rtL199V). Among them, only rtL199V localized close to the catalytically active YMDD motif encompassing the RT positions 203-206.

Data From UDPS: Intrapatient Distribution of Mutations Associated With HBV Reactivation.
UDPS analysis was led in 19 HBV-reactivated patients and 22 chronically infected patients. In

Table 2. Odds Ratio for the Association of S-HBsAg Mutations With Immunosuppression-Related HBV Reactivation From Fitting a Multivariable Logistic Regression Model

Variables	Univariable Analysis*		Multivariable Analysis*	
	Odds Ratio	P Value	Odds Ratio	P Value
Age (per 1-year increase)	**1.10 (1.04-1.14)**	**<0.001**	**1.08 (1.00-1.17)**	**0.048**
HBV DNA (per 1log IU/mL increase)	**1.42 (1.10-1.83)**	**0.006**	1.62 (0.94-2.79)	0.082
ALT (per 20-IU/mL increase)	**1.10 (1.04-1.17)**	**0.001**	**1.12 (1.01-1.23)**	**0.027**
AST (per 20-IU/ml increase)	1.06 (0.99-1.12)	0.091	–	–
At least 1 mutation associated with HBV reactivation[†]	**97.43 (18.81-504.79)**	**<0.001**	**91.04 (14.10-586.3)**	**<0.001**

*The analysis was performed on 93 patients infected by HBV of genotype D. For this analysis, the following variables were considered: age, ALT/AST values and the presence of at least one S-HBsAg mutation significantly correlated with immunosuppression-driven HBV reactivation. All variables with a $P < 0.01$ at univariable analysis were included in the multivariable analysis. Statistically significant P values in the multivariate analysis are in bold.

[†]The 13 S-HBsAg mutations associated with immunosuppression-driven HBV reactivation (Fig. 1A) were considered in this analysis.

HBV-reactivated patients, no statistically significant differences in demographic and clinical characteristics were found after stratification for UDPS availability (Supporting Table 2). Similarly, prevalence of the S-HBsAg-mutations associated with HBV reactivation was comparable between the group of HBV-reactivated patients with UDPS and that without UDPS.

In the reference group, all the demographic and clinical characteristics (with the exception of serum HBV DNA) were comparable (Supporting Table 2).

By UDPS, S-HBsAg mutations associated with HBV-reactivation occurred in 13 of 19 of HBV-reactivated patients with a median (IQR) intrapatient prevalence of 73.3% (27.6%-100%; Fig. 2A). Conversely, in chronically infected patients, the median (IQR) intrapatient prevalence of S-HBsAg mutations associated with HBV reactivation showed a remarkable decrease (4.6% [2.5%-11.3%]; $P = 0.007$). In 2 chronically infected patients, these mutations occurred with an intrapatient prevalence of 46.6% and 28.4%, respectively, and in 5 patients they occurred as minority species with an intrapatient prevalence from 0.7% to 11.3% (thus not detectable by the standard sequencing methodology; Fig. 2B).

A similar scenario was found analyzing mutations known to hamper HBsAg recognition by Abs (defined as immune-escape mutations; for their list, please cite http://hbv.bioinf.mpi-inf.mpg.de/index.php). Indeed, the median (IQR) intrapatient prevalence of these mutations was 55.8%(29.7%-97.9%) in HBV-reactivated patients and 6.8% (3.4%-79.7%) in chronically infected patients ($P = 0.03$).

An Enrichment of Genotypically Defined S-HBsAg NLG Sites Characterizes HBV Reactivation. By analyzing the S-HBsAg sequences, additional NLG sites in the major hydrophilic region were found in 24.1% (7 of 29) of patients with HBV reactivation, whereas they were completely absent in chronically infected patients ($P < 0.001$; Fig. 1C).

In 5 patients, a single additional NLG site was detected resulting from the point mutations, sT115N and sT123N, in 2 patients each, respectively, and from the insertion of an N between the S-HBsAg positions, 114 and 115, in the remaining patient (ins115N). Notably, 2 additional glycosylation sites were detected in 2 patients, resulting from the point mutations, sS113N+sT131N, in the former and from the mutation, sT117N, combined with the insertion of an N between the S-HBsAg positions, 113 and 114 (ins114N), in the latter.

Notwithstanding, among the 7 patients with additional glycosylation sites, 5 were isolated anti-HBc positive (before HBV reactivation) and remained HBsAg negative despite HBV reactivation.

N-Glycosylation Impact on HBsAg Quantification. The plasmid encoding the strep-tagged HBsAg, carrying the wild-type (WT) sequence or the above-mentioned NLG sites, were used to transfect HepG2 cells. The amount of strep-tagged HBsAg released in supernatants was then quantified using the ELISAs described in the *Materials and Methods* section. The additional NLG site resulting from the mutation, K160N, and known to affect HBsAg recognition was used as a control.

By using the ELISA targeting the strep-tag linked to the HBsAg, the additional NLG sites did not affect the quantification of the strep-tagged HBsAg, which was comparable to that observed for the WT (Fig. 3). Conversely, all the NLG sites (except the pattern, S113N+T131N) determined a drastic reduction in the quantification of the strep-tagged HBsAg when the Architect and Bio-Rad assays (both targeting the HBsAg) were used (Fig. 3). This decrease was >90%
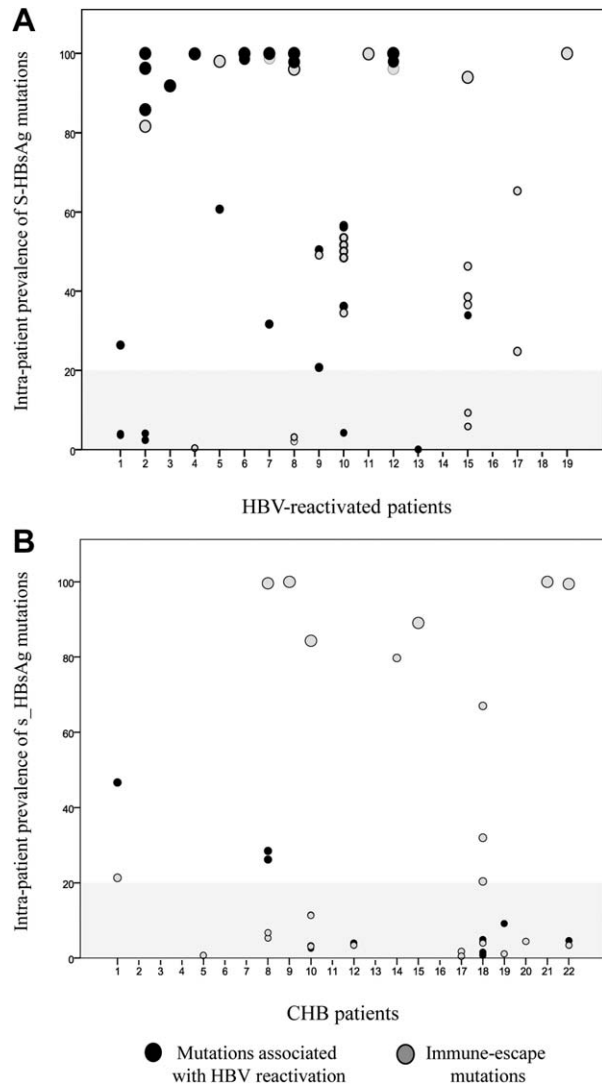
Fig. 2. Graph reports the intrapatient prevalence of S-HBsAg-mutations for each patient in the HBV-reactivated group (N = 19) (A) and in the group of chronically infected patients used as a reference (N = 22) (B). Intrapatient prevalence was expressed as percent of reads with the specific mutation. The relative dimension of black and gray dots represents the intrapatient prevalence of S-HBsAg mutations correlated with HBV reactivation and of S-HBsAg mutations known as immune-escape mutations, respectively. The list of S-HBsAg mutations associated with an altered S-HBsAg recognition by Abs is reported in http://hbv.bioinf.mpi-inf.mpg.de/index.php. The gray area includes mutations with an intrapatient prevalence <20% defined as minority species not detected by standard population-based sequencing. Abbreviation: CHB, chronic hepatitis B.

(compared to WT) for four of five glycosylation sites analyzed (98.5% for T115N, 94.1% for ins115N, 92% for T123N, and 90.2% for T117N+ins114N) by using the Bio-Rad assay and for three of five glycosylation sites (99.9% for T115N, 94.7% for ins115N, and 99.4% for T123N) by using the Architect assay (Fig. 3). These results show that the NLG sites identified in this study hamper HBsAg recognition and quantification without affecting HBsAg release.

Discussion

This study highlights a high degree of genetic complexity in the S-HBsAg sequences isolated from patients with immunosuppression-related HBV reactivation. It also provides a snapshot from real clinical practice on the clinical characteristics of HBV-reactivated patients. To our knowledge, this is one of the first European studies addressing this point in a relatively large cohort of patients.

We show that immunosuppression-related HBV reactivation can occur in a large variety of anti-HBV serological profiles. In particular, in our study, a large fraction (51.7%) of HBV-reactivated patients were isolated anti-HBc positive and 6.9% were anti-HBc and anti-HBs positive. The high prevalence of isolated anti-HBc-positive patients supports the notion that an undetectable anti-HBs level at the starting of immunosuppressive therapy correlates with an increased risk of HBV reactivation (Seto W, Chan T, Hwang Y, Choi O, Wong D, Fung J, et al. Interim analysis of hepatitis B reactivation in patients with prior HBV exposure undergoing hematopoietic stem cell transplant. The Liver Meeting for AASLD, 2013. Abstract #34). Moreover, 41.2% of anti-HBc-positive patients (+ anti-HBs) were receiving an immunosuppressive therapy not including rituximab or anti–tumor necrosis factor agents. This group of patients (thus far considered at low risk of HBV reactivation[1]) may thus require further attention and investigation, particularly in term of establishing *ab initio* an effective prophylaxis.

Thus far, little information is available on HBV reactivation in patients receiving corticosteroids alone. In our study, 13.8% (4 of 29) of patients developed HBV reactivation during treatment with corticosteroids for autoimmune or chronic inflammatory diseases. Among them, 2 were isolated anti-HBc positive and 2 were inactive carriers. Thus, patients undergoing therapy based only on corticosteroids is another category of patients requiring more attention in terms of screening and/or monitoring of HBV markers during immunosuppressive therapy.

Of note, in our study, HBV reactivation after discontinuation of immunosuppressive therapy was observed in 48.3% of patients and was positively correlated with patients' age. Such a correlation can be explained by the fact that older patients require more time to reconstitute the immune system. Thus, after discontinuation of immunosuppressive therapy, the status of immunosuppression can persist longer, predisposing the patients to HBV reactivation for a prolonged time.

Conversely, HBV reactivation after discontinuing immunosuppression was negatively correlated with use
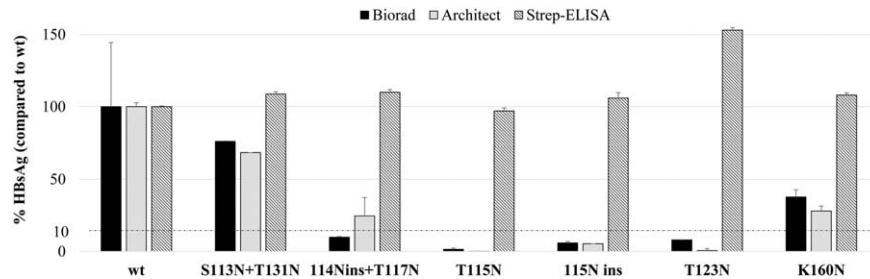
Fig. 3. Quantification of strep-tagged HBsAg released in supernatants of HepG2 cell cultures by different ELISAs are shown. For each mutant, the amount of strep-tagged HBsAg released in supernatants was expressed as a percentage, considering the amount of the WT strep-tagged HBsAg as 100%. The mutant, K160N, was used as a control. Results represent the mean values ($\pm$ standard deviation) of two experiments, each in duplicate. Dotted line indicates a 90% inhibition in HBsAg recongition and quantification.

of corticosteroids. This can be explained by the fact that the HBV genome contains a glucocorticoid-responsive element (GRE) whose stimulation enhances the rate of cccDNA transcription and, in turn, HBV replication.[17] In this light, it is conceivable that interruption of corticosteroids can shut down GRE stimulation, thus repressing cccDNA transcriptional activity.

No information is thus far available on mechanisms underlying immunosuppression-related HBV reactivation in patients with "resolved" or inactive HBV infection. In this study, by analyzing the full-length S gene (pre-S1, pre-S2, and S-HBsAg), we found an unexpectedly high degree of genetic diversity and complexity in S-HBsAg from patients with HBV reactivation. We also identified 13 S-HBsAg genetic signatures in immune-active S-HBsAg regions significantly correlated with the status of HBV reactivation. The majority of them reside in the major hydrophilic region; some of them are already known to act as immune-escape mutations. Among them, the mutations sY134H, sS143L, and sD144E reside in a particularly immunogenic segment encompassing the aa 139-149.[18] sD144E has been implicated in immune escape and/or diminished affinity for monoclonal Abs.[19] This mutation has been also described in a case of corticosteroid-induced reactivation of latent HBV infection in an HIV-positive patient.[12] Within the 139-149 segment, we also observed an enrichment of variability at the S-HBsAg position 145, known to abrogate the production of, as well as reduce the HBsAg affinity for, neutralizing Abs.[16,20] Mutations at position 145 as well as at positions 100, 109, 118, 134, 144, and 154 have been also detected during HBV reactivation in previous studies (based on clinical case description or lower number of patients).[9-14] The slight discrepancies in the patterns of S-HBsAg mutations reported in our and other studies can be mainly ascribed to the inclusion of different HBV genotypes. However, we cannot exclude that other factors, such as the type of immunosuppressive regimen, and serological status of HBV infection before reactivation, can influence the patterns of mutations detected during HBV reactivation.

To further corroborate the association of these mutations with HBV reactivation, their prevalence was also evaluated in two distinct groups of patients (all with HBV genotype D): the former composed by 44 acutely infected patients and the latter composed by 148 nucelos(t)ide analog (NUC)-treated chronically infected patients.[15] The association of the 13 HBsAg mutations with HBV reactivation was fully confirmed in these two data sets (P values ranging from 0.01 to 0.002 in acutely infected patients and from 0.01 to 0.004 in NUC-treated patients). In acutely infected patients, none of the 13 HBsAg-mutations associated with HBV reactivation were detected by population sequencing. Similarly, in NUC-treated patients, 8 of 13 HBsAg mutations were never detected and the remaining five occurred with a prevalence <1.5%. This range of prevalence is superimposable to that observed in drug-naïve patients (0%-1.6%).

By UDPS, the S-HBsAg-mutations associated with HBV reactivation status mainly occur as predominant species with a high intrapatient prevalence, suggesting their ability to confer a selective advantage. Conversely, in chronically infected patients, these mutations (if detected) mainly occur as minority species. We cannot establish whether these mutations are generated or have been selected from archived cccDNA during immunosuppressive therapy. However, the clustering of these mutations in S-HBsAg immune-active regions suggests that the suboptimal immune response during the initial weakening of the immune system can determine a remodulation of viral quasi-species structure leading to a shift toward highly mutated viral species endowed with enhanced potential to evade immune response. From an evolutionary point of view, the high degree of S-HBsAg genetic

variability observed in HBV-reactivated patients is in agreement with the theory known as "the survival of the flattest." This theory postulates that, under high mutation rates, the evolution does not necessarily favor the single viral variant with the faster replication capacity, but a more robust cloud of viral variants, interconnected by mutations, whose average replication rate is the highest.[21] Thus, the selection of multiple variants becomes the best way to counteract changes in the environment.

We also highlighted, in 24.1% of HBV-reactivated patients, an enrichment of additional NLG sites, in the major hydrophilic region of the S-HBsAg, not occurring in chronically infected patients. By using an ELISA capable of recognizing a strep-tag linked to the HBsAg, we found that, in the presence of NLG sites, the amount of strep-tagged HBsAg measured by the classical ELISA is drastically or totally abrogated, whereas the amount of strep-tagged HBsAg measured by the Strep-tag based ELISA is comparable to that observed for the WT strep-tagged HBsAg. These results support that the NLG sites identified in this study hamper HBsAg recognition and quantification without affecting HBsAg release. NLG sites play a critical role in viral evasion from humoral responses. Indeed, they can mask B-cell epitope and thus protein surface target of neutralizing Abs. The additional NLG sites observed in HBV-reactivated patients derived from complex mutational events, and the majority of them were HBsAg negative despite reactivation of viral replication. This corroborates the ability of these NLG sites to interfere with S-HBsAg recognition by Abs by masking B-cell epitope. This is in line with a recent study showing that S-HBsAg mutants with new N-glycosylation sites reacted weakly with anti-HBs. Native gel analysis of secreted virion in supernatants of transfected HuH7 cells indicated that mutants had better virion enveloping and secretion capacity than WT HBV.[22] This suggests that, beyond acting as immune-escape mutations, these additional NLG sites may also provide an advantage in terms of replicative capacity. *In vitro* experiments are necessary in order to verify the impact of these sites on the glycosylation process and HBV replication capacity

In our study, 9 patients developed HBV reactivation despite LMV prophylaxis. Most of them (6 of 9) did not develop rtM204I/V. It is known that pharmacological pressure cooperates with immunological pressure in suppressing HBV replication.[23] In the setting of immune suppression and abundant presence of S-HBsAg immune-escape mutations, the low-potent LMV may fail to prevent HBV recurrence. In this regard, a recent study showed a high efficacy of entecavir in preventing HBV reactivation in patients with patients with lymphoma and resolved HBV infection.[24]

In conclusion, HBV reactivation correlates with a complex quasi-species carrying HBsAg mutations in immune-active regions and with the presence additional NLG sites that can contribute to HBV escape by neutralizing and diagnostic Abs. This underlines the importance of a careful patient monitoring in all immunosuppressive settings at reactivation risk, a proper assessment of the best time of onset and duration of prophylaxis, and the prompt introduction of a potent therapy in order to prevent HBV-related clinical complications.

References

1. Hoofnagle JH. Reactivation of hepatitis B. HEPATOLOGY 2009;49(5 Suppl):S156-S165.
2. **Hwang JP, Lok AS**. Management of patients with hepatitis B who require immunosuppressive therapy. Nat Rev Gastroenterol Hepatol 2014;11:209-219.
3. Hsu C, Tsou HH, Lin SJ, Wang MC, Yao M, Hwang WL, et al.; Taiwan Cooperative Oncology Group. Chemotherapy-induced hepatitis B reactivation in lymphoma patients with resolved HBV-infection: a prospective study. HEPATOLOGY 2014;59:2092-2100.
4. Keam B, Lee JH, Im SA, Yoon JH. Why, when, and how to prevent hepatitis B virus reactivation in cancer patients undergoing chemotherapy. J Natl Compr Canc Netw 2011;9:465-477.
5. Loomba R, Rowley A, Wesley R, Liang TJ, Hoofnagle JH, Pucino F, et al. Systematic review: the effect of preventive lamivudine on hepatitis B reactivation during chemotherapy. Ann Intern Med 2008;148:519-528.
6. Hsu C, Hsiung CA, Su IJ, Hwang WS, Wang MC, Lin SF, et al. A revisit of prophylactic lamivudine for chemotherapy-associated hepatitis B reactivation in non-Hodgkin's lymphoma: a randomized trial. HEPATOLOGY 2008;47:844-853.
7. Katz LH, Fraser A, Gafter-Gvili A, Leibovici L, Tur-Kaspa R. Lamivudine prevents reactivation of hepatitis B and reduces mortality in immunosuppressed patients: systematic review and meta-analysis. J Viral Hepat 2008;15:89-102.
8. Tsutsumi Y, Yamamoto Y, Shimono J, Ohhigashi H, Teshima T. Hepatitis B virus reactivation with rituximab-containing regimen. World J Hepatol 2013;5:612-620.
9. Martel N, Cotte L, Trabaud MA, Trepo C, Zoulim F, Gomes SA, et al. Probable corticosteroid-induced reactivation of latent hepatitis B virus infection in an HIV-positive patient involving immune-escape. J Infect Dis 2012;205:1757-1761.
10. Ceccarelli L, Salpini R, Sarmati L, Svicher V, Bertoli A, Sordillo P, et al. Late hepatitis B virus reactivation after lamivudine prophylaxis interruption in an anti-HBs-positive and anti-HBc-negative patient treated with rituximab-containing therapy. J Infect 2012;65:180-183.
11. Milazzo L, Ebranati E, Cattaneo D, Gabanelli E, Lai A, Zehender G, Gervasoni C. Recurrence of another hepatitis B virus escape mutant comes back in a patient infected with HIV and low CD4+ count. J Med Virol 2014;86:97-101.

12. Coppola N, Loquercio G, Tonziello G, Azzaro R, Pisaturo M, Di Costanzo G, et al. HBV transmission from an occult carrier with five mutations in the major hydrophilic region of HBsAg to an immuno-suppressed plasma recipient. J Clin Virol 2013;58:315-317.

13. Borentain P, Colson P, Coso D, Bories E, Charbonnier A, Stoppa AM, et al. Clinical and virological factors associated with hepatitis B virus reactivation in HBsAg-negative and anti-HBc antibodies-positive patients undergoing chemotherapy and/or autologous stem cell trans-plantation for cancer. J Viral Hepat 2010;17:807-815.

14. Rago A, Lichtner M, Mecarocci S, Marocco R, Cenfra N, Belvisi V, et al. Antiviral treatment including entecavir plus tenofovir disoproxil fumarate for HBV-reactivation following a rituximab-based regimen. Antivir Ther 2010;15:929-932.

15. Salpini R, Alteri C, Cento V, Pollicita M, Micheli V, Gubertini G, et al. Snapshot on drug-resistance rate and profiles in patients with chronic hepatitis B receiving nucleos(t)ide analogues in clinical practice. J Med Virol 2013;85:996-1004.

16. Desmond CP, Bartholomeusz A, Gaudieri S, Revill PA, Lewin SR. A systematic review of T-cell epitopes in hepatitis B virus: identification, genotypic variation and relevance to antiviral therapeutics. Antivir Ther 2008;13:161-175.

17. Chou CK, Wang LH, Lin HM, Chi CW. Glucocorticoid stimulates hepatitis B viral gene expression in cultured human hepatoma cells. HEPATOLOGY 1992;16:13-18.

18. Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. J Viral Hepat 1997;4(Suppl 1):11-20.

19. Protzer-Knolle U, Naumann U, Bartenschlager R, Berg T, Hopf U, Meyer zum Büschenfelde KH, et al. Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. HEPATOLOGY 1998;27:254-263.

20. Wu C, Deng W, Deng L, Cao L, Qin B, Li S, et al. Amino acid sub-stitutions at positions 122 and 145 of hepatitis B virus surface antigen (HBsAg) determine the antigenicity and immunogenicity of HBsAg and influence in vivo HBsAg clearance. J Virol 2012;86:4658-4669.

21. Wilke CO, Wang JL, Ofria C, Lenski RE, Adami C. Evolution of digi-tal organisms at high mutation rates leads to survival of the flattest. Nature 2001;412:331-333.

22. **Yu DM**, **Li XH**, Mom V, Lu ZH, Liao XW, Han Y, et al. N-glycosyla-tion mutations within hepatitis B virus surface major hydrophilic region contribute mostly to immune-escape. J Hepatol 2014;60:515-522.

23. Boni C, Laccabue D, Lampertico P, Giuberti T, Viganò M, Schivazappa S, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterol-ogy 2012;143:963-973.

24. **Huang YH**, **Hsiao LT**, Hong YC, Chiou TJ, Yu YB, Gau JP, et al. Randomized controlled trial of entecavir prophylaxis for rituximab-associated hepatitis B virus reactivation in patients with lymphoma and resolved hepatitis B. J Clin Oncol 2013;31:2765-2772.

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