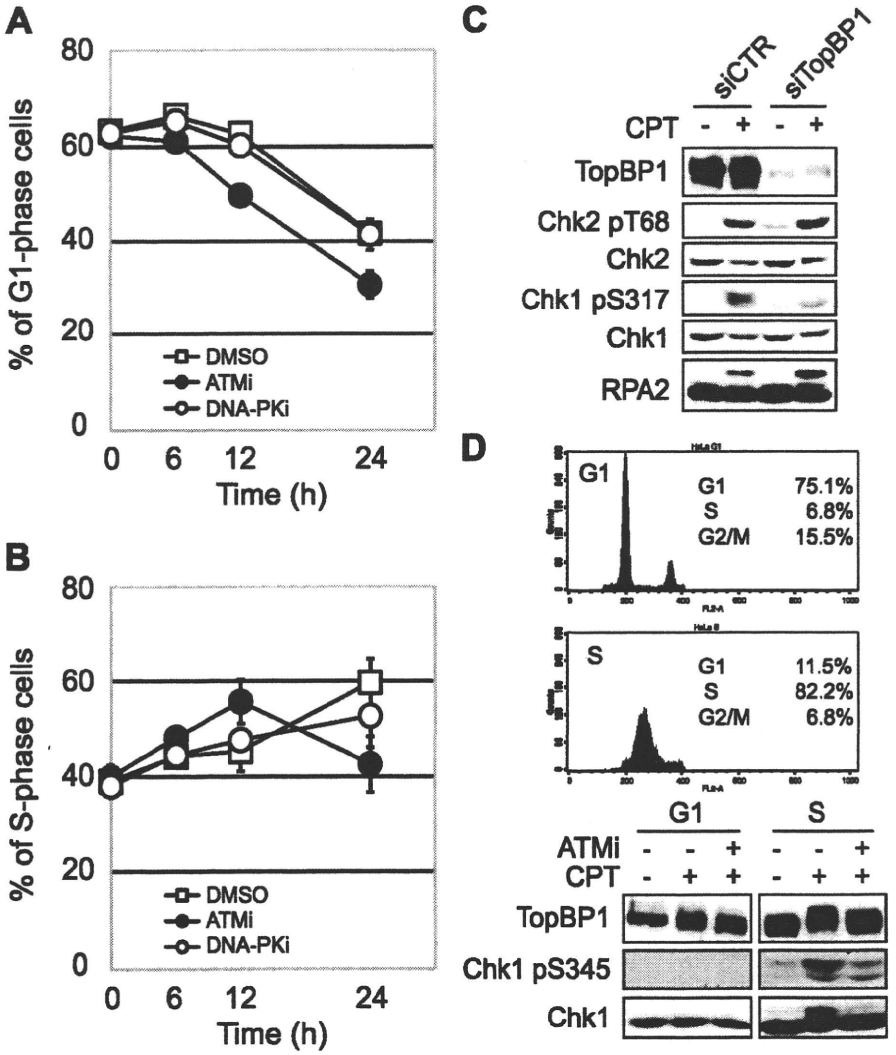

FIGURE 4. **CPT induces ATM-dependent G_1/S and S phase checkpoint arrest.** *A* and *B*, U2OS cells were treated with 1 μM CPT following treatment with ATM (KU-55933; 10 μM, 1 h) or DNA-PK inhibitor (NU7026; 10 μM, 1 h). After incubation for the indicated time, cells were fixed with ethanol for propidium iodide staining. Cell cycle distribution was analyzed by flow cytometry, and the percentage of cells in G_1 phase and S phase was plotted in *A* and *B*, respectively. *Error bars* show S.E. calculated from three independent experiments. *C*, CPT-induced Chk1 phosphorylation is TopBP1-dependent. HeLa cells transfected with control (*siCTR*) or TopBP1 siRNA (*siTopBP1*) were treated with CPT (2 μM, 1 h), and then Chk2, Chk1, and RPA2 phosphorylation were analyzed by Western blotting with the indicated antibodies. *D*, CPT-induced TopBP1 phosphorylation is restricted to S phase. To synchronize cell cycle in the G_1 and S phase, HeLa cells were released from nocodazole block and thymidine block, respectively. Both cell populations were treated with CPT (2 μM, 1 h) with or without ATM inhibitor (KU-55933; 10 μM, 1 h). TopBP1, Chk1, and Chk1 phosphorylation was detected by Western blotting using the indicated antibodies.

DSEs in cells treated with ATM inhibitor or vehicle. As expected, CPT treatment caused an increase in comet tail moment, reflecting the generation of DNA strand breaks (Fig. 5*C*). Cells co-treated with CPT and the ATM inhibitor demonstrated an ~2.5-fold increase in comet tail moment compared with cells treated with CPT alone, supporting the contention that accumulation of DSEs is responsible for DNA-PK hyperactivation in the absence of functional ATM. Finally, as predicted based on the DNA-PK autophosphorylation results, the accumulation of DNA strand breaks in CPT and ATM inhibitor-treated cells was reduced by DRB treatment (Fig. 5*C*). These results are consistent with a model whereby transcription-mediated strand breaks are carried over from G_1 phase to S phase in ATM-inhibited cells, where they are converted into DSEs upon collision with DNA replication forks.

Substantiation of the above model required us to test whether CPT-induced transcription-mediated strand breaks arising in the G_1 phase are in fact responsible for DNA-PK hyperactivation. To test this possibility, U2OS cells were synchronized in the G_1 phase as schematically indicated in Fig. 5*D*. G_1-synchronized cells were incubated with KU-55933 for 1 h prior to CPT exposure. CPT treatment was performed only for 1 h during G_1 phase to avoid the possibility of direct DSE generation in S phase. At 0 h, 1 h, and 5 h after CPT addition, DNA-PKcs autophosphorylation was analyzed by Western blotting. There was no significant difference in S phase percentages between untreated and ATM inhibitor-treated cells in the absence of CPT, indicating that ATM inhibition does not affect S phase entry in this system (Fig. 5*D*). We found that CPT-induced DNA-PKcs autophosphorylation was enhanced in cells treated with the ATM inhibitor in the G_1 phase *versus* cells treated with CPT alone (Fig. 5*D*). Overall, the findings demonstrate that ATM activates a G_1/S checkpoint in response to CPT-mediated transcriptional collapse. Failure of this checkpoint leads to the carryover of transcription-mediated strand breaks into the S phase, resulting in DSE generation and hyperactivation of DNA-PK.

DNA-PK Promotes CPT-induced Cell Death in the Absence of ATM—Given that inhibition of ATM led to DNA-PK hyperactivation in CPT-treated cells, we reasoned that dual inhibition of ATM and DNA-PK would lead to synergistic cell killing by CPT. To address this possibility, the effect of ATM or/and DNA-PK inhibition on CPT-sensitivity was analyzed in HCT116 colon carcinoma cells. In HCT116 cells, we found that ATM inhibition caused moderate CPT hypersensitivity, whereas DNA-PK inhibition actually led to a small, yet reproducible, increase in HCT116 cell survival following CPT treatment (Fig. 6*A*). This result is consistent with previous findings showing that DNA ligase IV- or Ku70-deficient chicken DT40 cells display partial CPT resistance (24, 25). Interestingly, we found that DNA-PK inhibition rescued the CPT-hypersensitive phenotype of ATM-

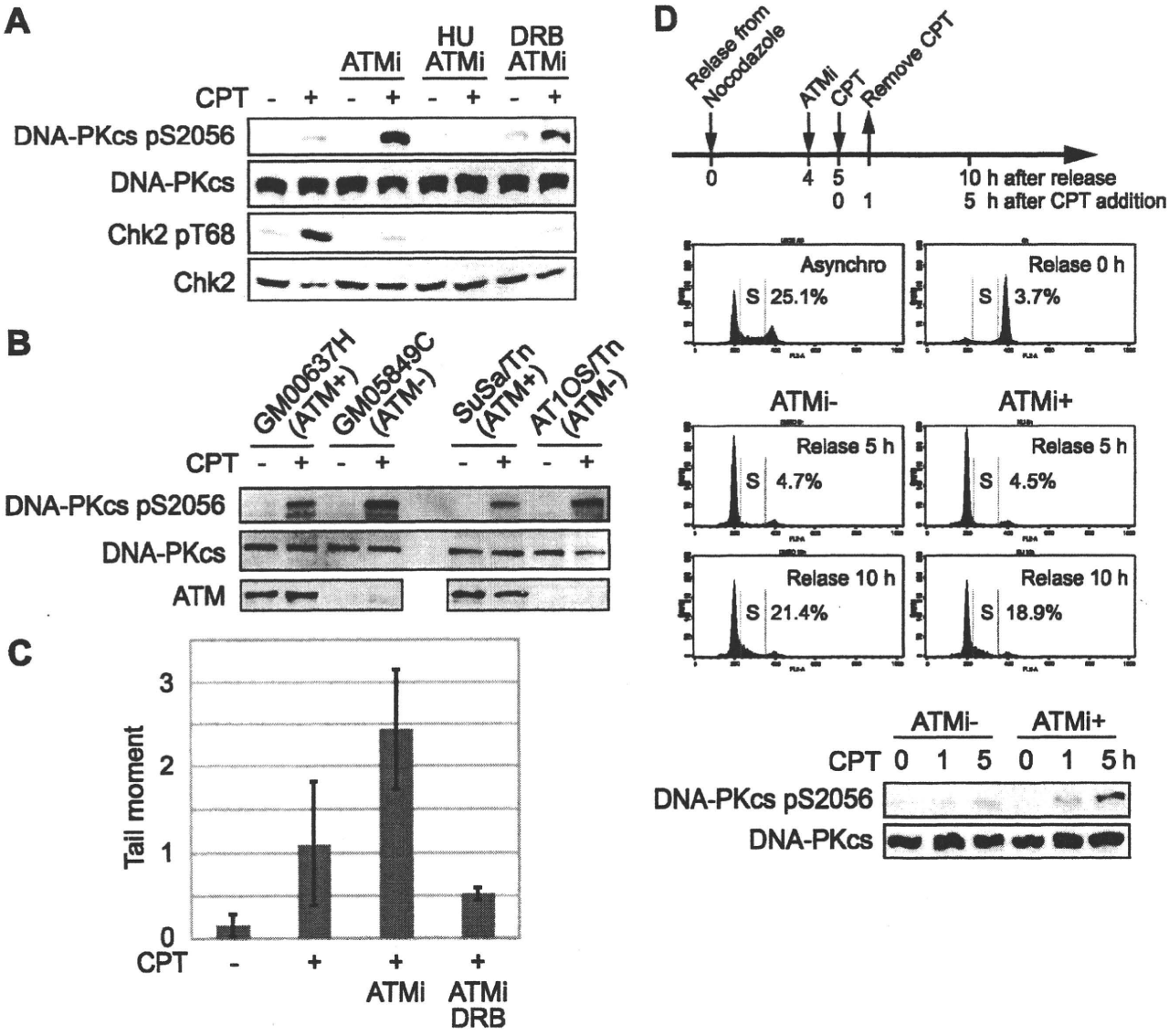

FIGURE 5. **ATM suppresses DNA-PK activation in response to CPT.** *A*, effects of ATM, replication, and transcription inhibitors on CPT-induced DNA-PKcs autophosphorylation. HeLa cells were treated with CPT (2 μM, 1 h) following ATM inhibitor (KU-55933; 10 μM, 1 h) with HU (2 mM, 10 min) or DRB (100 μM, 2 h) treatment, and DNA-PKcs autophosphorylation and Chk2 phosphorylation were analyzed by Western blotting. *B*, DNA-PK activation in ATM-deficient cells. Control cells (GM00637H or SuSa/Tn) and ATM-deficient cells (GM05849C or AT1OS/Tn) were treated with CPT (2 μM, 1 h), and DNA-PKcs autophosphorylation were analyzed by Western blotting. *C*, comet assay for detection of CPT-induced DNA strand breaks. HeLa cells were treated with CPT (0.25 μM) for 1 h following ATM inhibitor (10 μM, 1 h) treatment in the absence or presence of DRB (100 μM, 2 h), and induced-DNA strand breaks were detected by neutral comet assay. The comet tail moments were averaged in triplicate experiments, where the median among 100 cells was calculated in each experiment. *Error bars* represent S.E. calculated from three independent experiments. *D*, inhibition of ATM leads to DNA damage carry over from G_1 to S phase. U2OS cells were synchronized in M phase with nocodazole and released into G_1 and S phase upon incubation with fresh medium. Flow cytometry histograms show cell cycle profiles after release from the nocodazole block in the absence or presence of KU-55933 (10 μM, 1 h). Where indicated, cells were treated with CPT (5 μM, 1 h). The status of DNA-PK activation in the different experimental samples was determined by Western blotting with anti-phospho-DNA-PK antibodies.

inhibited cells (Fig. 6*A*). At a low dose (5 nM) of CPT, HCT116 cells exposed to both DNA-PK and ATM inhibitors showed a 10-fold increase in colony formation *versus* HCT116 cells cultured only in the presence of the ATM inhibitor. This finding suggested that cytotoxicity of CPT is associated with DNA-PK activation.

Given that CPT-induced DSEs are repaired predominantly through HR-dependent pathways in the S phase (2), we reasoned that hypersensitivity of ATM-inhibited cells might be linked to defective HR repair. To test this idea, we measured the formation of Rad51 foci, a surrogate marker for HR, in HeLa cells exposed to CPT in the presence of ATM and DNA-PK inhibitors. CPT treatment for 6 h increased the percentage of cells displaying Rad51 foci from 4% to 11% (Fig. 6*B*). Co-exposure of cells to KU-55933 attenuated CPT-induced Rad51 foci formation, suggesting that ATM contributes to HR repair of these lesions. Surprisingly, the DNA-PK inhibitor treatment also suppressed the percentage of cells with Rad51 foci, and the combination of ATM and DNA-PK inhibition further reduced the percentage of Rad51 foci-positive cells. These findings imply that DNA-PK and ATM cooperatively contribute to the HR pathway in response to CPT and that phe-

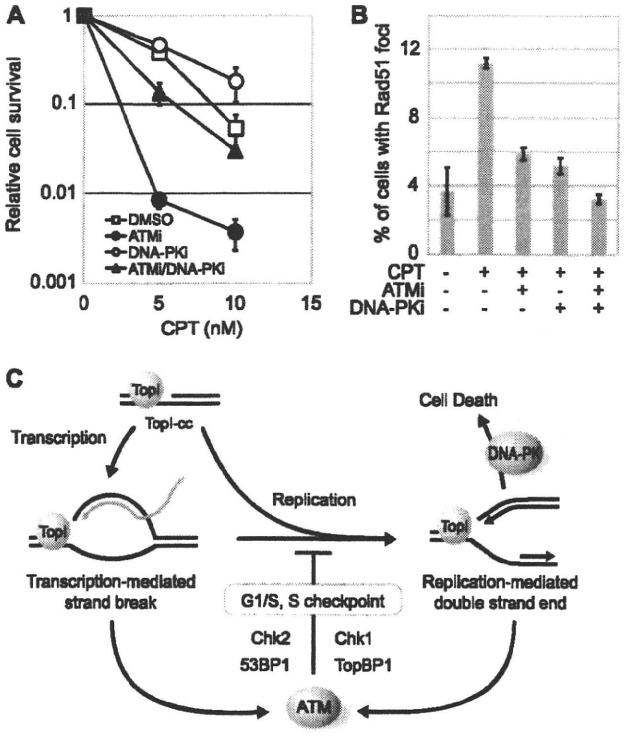

FIGURE 6. **DNA-PK activation promotes cells death in response to CPT.** *A*, clonogenic survival assays of HCT-116 cells treated with CPT in the absence or presence of ATM and/or DNA-PK inhibitors. Cells pretreated with ATM inhibitor (KU-55933; 10 μM, 1 h) and/or DNA-PK inhibitor (NU7026; 10 μM, 1 h) were cultured in the presence of CPT for 2 days at the indicated concentration. *Error bars* show S.E. calculated from three independent experiments. *B*, effects of ATM and/or DNA-PK inhibition on Rad51 foci formation. HeLa cells were treated with CPT (2 μM, 6 h) following pretreatment with ATM (10 μM, 1 h) and/or DNA-PK (10 μM, 1 h) inhibitors, and then cells were stained with anti-Rad51 antibody. The cells with over 10 foci were considered Rad51 foci-positive, and the percentage of Rad51 foci-positive cells is depicted graphically. *Error bars* show S.E. calculated from three independent experiments. *C*, schematic model summarizing the CPT-induced cellular responses. Transcription-mediated strand breaks caused by CPT in G_1 phase are converted to double strand end by DNA replication. ATM is activated against both DNA damages and induces G_1/S and S phase checkpoints to prevent DNA-PK hyperactivation leading to cell death.

notypic rescue of CPT hypersensitivity in cells co-treated with ATM and DNA-PK inhibitors is unlikely due to restoration of HR repair.

DISCUSSION

In this study we have explored the complex mechanisms of signal transduction following exposure of cancer cell lines to a TopI poison, CPT. The most salient findings in the study include: (i) CPT induces transcription-dependent and -independent activation of ATM leading to 53BP1 foci formation and checkpoint activation; (ii) the absence of ATM-dependent G_1/S checkpoint leads to severe DNA damage and DNA-PK hyperactivation in the S phase; (iii) hyperactivation of DNA-PK by CPT in the absence of ATM causes cell death. Taken together, the present findings illuminate important details of the cellular response to a clinically relevant class of chemotherapeutics.

The mechanism of DNA strand breakage induced by CPT and clinically useful derivatives, irinotecan and topotecan, has been the subject of intensive study, and it is well established that TopI-cc are converted to frank DSBs during S phase (1). Transcription-mediated strand breaks are thought to be structurally distinct from DSBs caused by other DNA-damaging agents, such as IR or bleomycin. Because transcription bubbles comprised unwound DNA and nascently synthesized RNA, it is possible that transcription-mediated strand breaks contain a DNA·RNA hybrid end. Consistent with this hypothesis, CPT-induced DNA-PK activation is markedly dependent on DNA replication and was very weak in cells synchronized in G_1 phase. In 1986, Mimori and Hardin (26) reported reduced affinity of the Ku heterodimer for DNA·RNA hybrid probes compared with DNA·DNA double-strand probes. The strong preference of Ku/DNA-PK complexes for double strand DNA *versus* DNA·RNA hybrids plausibly explains why DNA-PK is not activated by CPT in G_1 phase cells. On the other hand, the transcription-dependent activation of ATM, which has been reported here and recently reported by Sordet *et al.* (7), implies that ATM can be activated by DNA·RNA hybrids, although the precise mechanism is not known.

Our results suggested that one end result of transcription-dependent ATM activation is the induction of Chk2 phosphorylation and G_1/S checkpoint arrest. ATM also clearly participates in S phase checkpoint signaling in response to CPT. In S phase cells, ATM promoted TopBP1 phosphorylation and TopBP1-dependent Chk1 activation. The finding implies that ATM and ATR function in a linear pathway, as has been proposed for IR-induced responses (27). Interestingly, CPT-induced RPA2 phosphorylation was not TopBP1-dependent in our hands. Given that ATR is involved in RPA2 phosphorylation (22, 28), this finding implies that TopBP1 is required for only a subset of ATR-dependent phosphorylation events. To summarize these findings, we found that ATM is critical for both G_1 and intra-S phase arrest in response to CPT, which activates two independent ATM activation pathways: a transcription-dependent pathway that is active in the G_1 phase and signals through Chk2, and a DNA replication-dependent pathway that is active in the S phase and signals through TopBP1 and Chk1 (Fig. 6*C*).

In the absence of an ATM-dependent G_1/S checkpoint, transcription-mediated strand breaks caused by CPT are carried over into the S phase, where they are converted into frank DSBs, presumably as a consequence of collisions between stalled transcription complexes and DNA replication forks. As a result, DNA-PK is hyperactivated. In HCT116 cells, the hyperactivation of DNA-PK in the absence of functional ATM leads to a dramatic loss of colony-forming activity. In addition, treatment with only the DNA-PK inhibitor imparted moderate CPT resistance to HCT116 cells, indicating that DNA-PK influences cell survival independent of ATM status. The rescue of CPT sensitivity in ATM-inhibited cells by DNA-PK inhibition cannot be explained by enhanced HR repair because Rad51 foci formation was not promoted by DNA-PK inhibition. Adachi *et al.* (24) have reported similar results showing that DNA ligase IV deletion rescued CPT sensitivity of Rad54-deficient chicken DT40 cells. Although the mechanistic basis for DNA-PK-dependent loss of viability in CPT-treated cells is not clear, we envision two nonexclusive models: first, activation of DNA-PK

could lead to deleterious nonhomologous end joining, which antagonizes survival by promoting deleterious DNA end-joining reactions that preclude HR (24); second, DNA-PK harbors an intrinsic proapoptotic function that is triggered by replication-mediated DSEs. The participation of DNA-PK in apoptosis is complex, with the reports showing prosurvival and proapoptosis functions (29–32). Thus, in the absence of ATM, it is possible that CPT activates a latent, proapoptotic function of DNA-PK. Viewing from a different perspective, our findings raise a question as to how ATM antagonizes DNA-PK-dependent apoptosis in CPT-treated cells. The simplest explanation is that ATM prevents the accumulation of catastrophic S phase damage required to activate DNA-PK. However, it is possible that a more direct antagonism between ATM and DNA-PK exists, at the level of undefined substrate phosphorylation.

In summary, we have shown that ATM responds to CPT-caused transcription collapse and activates cell cycle checkpoints with subsequent prevention of DSE generation and DNA-PK-mediated cell death. These findings indicate that DNA-PK functional status may be an important predictor of CPT treatment efficacy and that pharmacologic abrogation of the G_1/S checkpoint should enhance CPT sensitivity of tumors. These findings provide rationale for further development and preclinical testing of ATM inhibitors and other G_1/S checkpoint modifiers as adjuvants to TopI poison-based therapies.

REFERENCES

1. Pommier, Y. (2006) *Nat. Rev. Cancer* **6,** 789–802
2. Arnaudeau, C., Lundin, C., and Helleday, T. (2001) *J. Mol. Biol.* **307,** 1235–1245
3. Klein, H. L., and Kreuzer, K. N. (2002) *Mol. Cell* **9,** 471–480
4. Cliby, W. A., Lewis, K. A., Lilly, K. K., and Kaufmann, S. H. (2002) *J. Biol. Chem.* **277,** 1599–1606
5. Wang, J. L., Wang, X., Wang, H., Iliakis, G., and Wang, Y. (2002) *Cell Cycle* **1,** 267–272
6. Wu, J., and Liu, L. F. (1997) *Nucleic Acids Res.* **25,** 4181–4186
7. Sordet, O., Redon, C. E., Guirouilh-Barbat, J., Smith, S., Solier, S., Douarre, C., Conti, C., Nakamura, A. J., Das, B. B., Nicolas, E., Kohn, K. W., Bonner, W. M., and Pommier, Y. (2009) *EMBO Rep.* **10,** 887–893
8. Nakamura, H., Fukami, H., Hayashi, Y., Kiyono, T., Nakatsugawa, S., Hamaguchi, M., and Ishizaki, K. (2002) *J. Radiat. Res.* **43,** 167–174
9. Sakasai, R., and Tibbetts, R. (2008) *J. Biol. Chem.* **283,** 13549–13555
10. Dodson, G. E., and Tibbetts, R. S. (2006) *J. Biol. Chem.* **281,** 1692–1697
11. Wang, B., Matsuoka, S., Carpenter, P. B., and Elledge, S. J. (2002) *Science* **298,** 1435–1438
12. Rappold, I., Iwabuchi, K., Date, T., and Chen, J. (2001) *J. Cell Biol.* **153,** 613–620
13. Dimitrova, D. S., and Gilbert, D. M. (2000) *Exp. Cell Res.* **254,** 321–327
14. Mailand, N., Bekker-Jensen, S., Faustrup, H., Melander, F., Bartek, J., Lukas, C., and Lukas, J. (2007) *Cell* **131,** 887–900
15. Kolas, N. K., Chapman, J. R., Nakada, S., Ylanko, J., Chahwan, R., Sweeney, F. D., Panier, S., Mendez, M., Wildenhain, J., Thomson, T. M., Pelletier, L., Jackson, S. P., and Durocher, D. (2007) *Science* **318,** 1637–1640
16. Huen, M. S., Grant, R., Manke, I., Minn, K., Yu, X., Yaffe, M. B., and Chen, J. (2007) *Cell* **131,** 901–914
17. Sehgal, P. B., Derman, E., Molloy, G. R., Tamm, I., and Darnell, J. E. (1976) *Science* **194,** 431–433
18. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998) *Genes Dev.* **12,** 755–762
19. Dubois, M. F., Bellier, S., Seo, S. J., and Bensaude, O. (1994) *J. Cell. Physiol.* **158,** 417–426
20. Lin, C. P., Ban, Y., Lyu, Y. L., Desai, S. D., and Liu, L. F. (2008) *J. Biol. Chem.* **283,** 21074–21083
21. Liu, S., Bekker-Jensen, S., Mailand, N., Lukas, C., Bartek, J., and Lukas, J. (2006) *Mol. Cell. Biol.* **26,** 6056–6064
22. Sakasai, R., Shinohe, K., Ichijima, Y., Okita, N., Shibata, A., Asahina, K., and Teraoka, H. (2006) *Genes Cells* **11,** 237–246
23. Sakasai, R., Teraoka, H., and Tibbetts, R. S. (2010) *DNA Repair* **9,** 76–82
24. Adachi, N., So, S., and Koyama, H. (2004) *J. Biol. Chem.* **279,** 37343–37348
25. Hochegger, H., Dejsuphong, D., Fukushima, T., Morrison, C., Sonoda, E., Schreiber, V., Zhao, G. Y., Saberi, A., Masutani, M., Adachi, N., Koyama, H., de Murcia, G., and Takeda, S. (2006) *EMBO J.* **25,** 1305–1314
26. Mimori, T., and Hardin, J. A. (1986) *J. Biol. Chem.* **261,** 10375–10379
27. Yoo, H. Y., Kumagai, A., Shevchenko, A., Shevchenko, A., and Dunphy, W. G. (2007) *J. Biol. Chem.* **282,** 17501–17506
28. Anantha, R. W., Vassin, V. M., and Borowiec, J. A. (2007) *J. Biol. Chem.* **282,** 35910–35923
29. Bozulic, L., Surucu, B., Hynx, D., and Hemmings, B. A. (2008) *Mol. Cell* **30,** 203–213
30. Gurley, K. E., Moser, R., Gu, Y., Hasty, P., and Kemp, C. J. (2009) *EMBO Rep.* **10,** 87–93
31. Cobb, L. J., Liu, B., Lee, K. W., and Cohen, P. (2006) *Cancer Res.* **66,** 10878–10884
32. Callén, E., Jankovic, M., Wong, N., Zha, S., Chen, H. T., Difilippantonio, S., Di Virgilio, M., Heidkamp, G., Alt, F. W., Nussenzweig, A., and Nussenzweig, M. (2009) *Mol. Cell* **34,** 285–297

Research Article

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Evolution of hepatitis B genotype C viral quasi-species during hepatitis B e antigen seroconversion

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Background & Aims: Although the evolution of viral quasi-species may be related to the pathological status of disease, little is known about this phenomenon in hepatitis B, particularly with respect to hepatitis B e antigen (HBeAg) seroconversion.

Methods: Nucleotide sequences of the hepatitis B virus (HBV) *X/precore/core* region was analyzed at five time-points in four groups of chronic hepatitis B patients, interferon-induced seroconverters (IS, $N = 9$), interferon non-responders (IN, $N = 9$), spontaneous seroconverters (SS, $N = 9$), and non-seroconverters (SN, $N = 9$) followed during 60 months on an average. Only patients with genotype C were studied.

Results: Analysis of 1800 nucleotide sequences showed that there was no statistical difference between the nucleotide genetic distances of seroconverters (IS and SS; 6.9×10^{-3} substitutions (st)/site and 6.7×10^{-3} st/site, respectively) and those of non-seroconverters (IN and SN; 5.3×10^{-3} st/site and 3.8×10^{-3} st/site, respectively) before seroconversion. Compared to non-seroconverters (IN and SN; 5.1×10^{-3} st/site and 5.9×10^{-3} st/site, respectively), the sequence diversity of seroconverters (IS and SS; 10.9×10^{-3} st/site and 9.9×10^{-3} st/site, respectively) was significantly higher after seroconversion ($p < 0.05$), and was higher in seroconverters after seroconversion than before seroconversion ($p < 0.05$), while this changed very little in non-seroconverters during the observation period. Phylogenetic trees showed greater complexity in secoconverters than non-seroconverters. Parsimony-based estimation of the direction of sequence change between descendants and ancestors before HBeAg seroconversion, revealed higher frequencies of transversional A to T substitution in seroconverters (0.06 *vs.* 0.02, $p = 0.0036$) that coincided with the dynamics of quasi-species possessing A1762T mutation.

Conclusions: The distinctly greater viral diversity in HBeAg seroconverters after seroconversion could be related to escape mutants resulting from stronger selection pressure.

Keywords: Chronic hepatitis B; Quasi-species; Hepatitis B e antigen seroconversion.

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Abbreviations: SC, seroconversion; ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IS, interferon-induced HBeAg seroconverters; IN, IFN non-responders; SS, spontaneous seroconverters; SN, non-seroconverters.

Introduction

Hepatitis B virus (HBV) is a major human pathogen which can cause severe hepatic disease, including chronic hepatitis, cirrhosis (LC), and hepatocellular carcinoma (HCC). Quasi-species comprises a complex and dynamic distribution of non-identical but related genomes [1]. The evolution of viral quasi-species has been reported as important in the pathogenesis of RNA viruses such as hepatitis C virus [2–6] and human immunodeficiency virus [7–10], but little is known about HBV. HBV is a hepatotropic, non-cytopathic DNA virus replicated by an error-prone polymerase through an RNA intermediate. Because of this feature, the replication of HBV lacks fidelity. This results in a complex distributions of genomes with naturally-acquired mutations or mutations selected by either antiviral therapy or the immune response of the host. HBV quasi-species have not been subjected to detailed investigation, especially in the context of hepatitis B e antigen (HBeAg) seroconversion (SC), an immunologically mediated event. Whether there is a causal relationship between HBV seroconversion and HBV quasi-species remains unclear. HBV-related disease is known to be mediated both virologically and immunologically. Several studies have depicted the dynamic evolution of HBV quasi-species during lamivudine resistance or multiple drug resistance. This highlights the importance of HBV molecular evolution in revealing the mechanism of drug resistance [11,12]. HBV-specific cytotoxic T-cells play a significant role in the control of replication of HBV, which has been well documented in the literature [13–16].

Precore/core protein is the target of immunologically mediated HBeAg seroconversion. When the *precore/core* gene in HBV DNA is transcribed and translated, HBeAg is produced and secreted into the circulation [17,18]. But the synthesis and secretion of HBeAg are aborted by the emergence of a point mutation from G to A at nucleotide (nt)1896 (G1896A). Convincing lines of evidence have indicated a close association between HBeAg/anti-HBe seroconversion and the emergence of *precore* and *core* promoter mutations [19,20].

Research Article

The purpose of this study was to elucidate the evolution of HBV quasi-species during HBeAg seroconversion. The results might help us to better understand the pathogenic mechanisms of HBV. We selected patients with well-characterized clinical phenotypes and compared their viral diversity based on the nucleotide sequences of the *X*/precore/core region. *Precore* and *core* promoter mutations were also investigated in detail before and after HBeAg seroconversion.

Materials and methods

Patients

Sera from 36 chronic hepatitis B patients with well-characterized clinical follow-up for >5 years were selected from a chronic hepatitis B database (77 seroconverters and 67 non-seroconverters) at Chiba University Hospital. Only patients with genotype C (subtype C2) were studied to ensure that differences found in viral evolution were not due to genotypic variation. Nine patients in each group were selected randomly if they fulfilled the following criteria and had sufficiently long follow-up. The index group comprised patients with documented HBeAg seroconversion (spontaneous seroconverters, SS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−25.2 ± 6.2/months), time-point II (−11.6 ± 2.7/months), time-point III (1 ± 2.3/months), time-point IV (12.5 ± 3.3/months), and time-point V (25 ± 3.6 months). Untreated control patients included those who were followed for a similar period of time and were persistently HBeAg positive (non-seroconverters, SN), and they were matched for average age of seroconversion and time-point intervals of the SS group. A second index group of patients with interferon (IFN)-induced HBeAg seroconversion (IFN seroconverters, IS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−24.3 ± 3.1/months), time-point II (−11.2 ± 1.9/months), time-point III (1 ± 1.2/months), time-point IV (12.7 ± 1.7/months), and time-point V (25.4 ± 2.2/months). Control patients were persistently HBeAg-positive despite IFN therapy (IFN non-responders, IN). Controls were matched for the average age of seroconversion, sex and time-point intervals of the IS group.

HBeAg seroconversion was defined as the loss of HBeAg and the development of anti-HBe. The serial serum samples in this study were taken at five time-points for each patient, as described above. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological examination

HBsAg, HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL). HBV genotype was determined from the patients' sera by ELISA (HBV genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan), based on the method described by Usuda et al. [21]. Serum HBV DNA levels were monitored using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 $\log_{10}$ copies/ml, at each time-point.

Cloning and sequencing

Total DNA was extracted from 200 μl of each serum sample using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 200 μl distilled water. Because HBeAg seroconversion is associated with a decrease in HBV DNA levels, nested PCR was performed for all the samples. The primers for the first round of PCR were 5′-TCG CAT GGA GAC CAC CGT GA-3′ (sense, nt1604–1623) and 5′-ATA GCT TGC CTG AGT GC-3′ (antisense, nt 2076–2060). The primers for the second round of PCR were 5′-CAT AAG AGG ACT CTT GGA CT-3′ (sense, nt 1653–1672) and 5′-GGA AAG AAG TCA GAA GGC-3′ (antisense, nt 1974–1957).

Amplification was performed with 2 μl of DNA template (extracted DNA from serum samples for the first round PCR and the first round PCR products for the second round PCR) in 50 μl reaction under the following conditions: an initial 2 min of denaturation at 94 °C and 36 cycles of 94 °C denaturation for 1 min, annealing at either 54 °C or 52 °C for 1 min, in the first and second round respectively, and 72 °C extension for 1 min. The last cycle was followed by a final extension at 72 °C for 10 min. A 473-base pairs fragment (nt 1604–2076) containing the *X/precore/core* region was amplified.

PCR reactions were followed by cloning using TOPO® TA cloning kits (Invitrogen, Carlsbad, CA). All PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), then cloned into the TOPO vector, and transformed into *Escherichia coli*. At least 15 clones per one cloning for samples from PCR reactions proceeded subsequent to the electrophoretic size separation on 1.2% agarose gel. Ten positive clones per cloning for samples from each PCR reaction were sequenced using BigDye® Terminator and a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The cloning PCR and sequencing primers were M13-forward, 5′-GTA AAA CGA CGG CCA GT-3′, and M13-reverse, 5′-GGA AAC AGC TAT GAC CAT G-3′.

Sequence analysis

The DNAPARS program from PHYLIP v3.65 package, implemented in Simmonic Sequence Editor version 1.5 [22], was used for sequence analysis. To evaluate quasi-species-based evolution of HBV strains in chronic patients, sequences of clones ($N = 10$) isolated at each time-point ($N = 5$) from individual patients ($N = 36$) were subjected to alignment and used to generate one parsimonious ancestral sequence. Maximum nucleotide composition distances were evaluated pair-wise between the ancestral sequence and the sequences of each of the 10 clones with a mean value estimated for each patient at a given time-point (*MEGA* version 4 [23]). All patients were categorized into four groups with respect to seroconversion status and the mean distance value for each group was calculated for each time-point.

The differences in genetic distance among clinical groups and time-points, and diversity at each time-point, were analyzed using ANOVA (analysis of variance). Student's *t*-test was also performed to determine the average of genetic diversities in non-seroconverters. All graphical data are presented as means ± standard deviation (SD). Results were considered statistically significant at $p < 0.05$. The statistical analysis was performed with SPSS (2004; SPSS Inc., Tokyo, Japan).

Construction of phylogenetic trees

To examine the evolution of the viral sequence and whether this evolution was elicited by quasi-species or mutagenesis, phylogenetic trees were constructed using the Neighbor-Joining (NJ) model with the Simmonic Sequence Editor version 1.5, based on the genomic sequences of HBV. Moreover, to investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 were further analyzed phylogenetically. Neighbor-Joining trees were constructed at time-points 1 and 2 (Fig. S1 and S2, respectively) using all groups of sequences.

Results

Baseline clinical characteristics of the patients and sequential levels of serum ALT and HBV DNA

The clinical and laboratory characteristics of all patients are listed in Table 1. The levels of alanine aminotransferase (ALT) and HBV DNA over time are illustrated in Fig. 1A and B, respectively. Serum ALT levels, a marker of hepatocyte damage, normalized after seroconversion and, for all groups except the interferon non-responders, were <40 IU/L at the end-point of observation. HBV DNA loads decreased markedly in seroconverters (<3 $\log_{10}$ copies/ml, $p < 0.0001$) but changed very little in non-seroconverters. It is noteworthy that, at the second year after seroconversion, serum HBV DNA loads increased in interferon-induced seroconverters compared to spontaneous seroconverters, without statistical significance ($p^H = 0.1087$) (Fig. 1B).

Viral nucleotide sequence diversity

Viral sequence diversity, phylogenetic trees, and mutation pattern based on 1800 HBV nucleotide sequences from clones of the *X/precore/core* region, were analyzed among selected patients.

Table 1. Baseline clinical features of patients.

	IFN Seroconverters (IS)	IFN Non-seroconverters (IN)	Spontaneous Seroconverters (SS)	Spontaneous Non-seroconverters (SN)
Age (y)	40 ± 8	40 ± 11	29 ± 10	34 ± 6
Male : Female	6:3	8:1	5:4	7:2
HBV DNA (log_{10} copies/ml)	6.8 ± 0.9	6.8 ± 1.0	6.8 ± 1.2	7.1 ± 0.8
ALT (IU/L)	88.3 ± 48.6	94.3 ± 144.4	89.8 ± 71.4	67.6 ± 48.7

Note: The IFN-induced group (seroconverters and non-responders) was older than the spontaneous group (seroconverters and non-responders). Males were the majority in all groups. Baseline serum HBV DNA and ALT levels are similar among the four groups. Data are shown as mean ± SD.

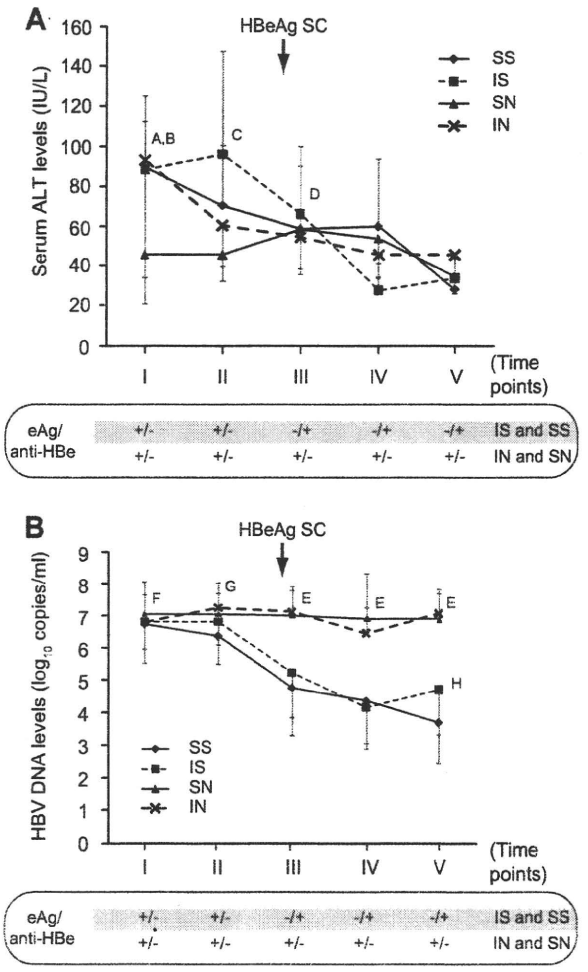

Fig. 1. Serum ALT and DNA levels in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk, and non-seroconverters controls (SN) is a solid line triangle. (A) $p^A = 0.0234$ comparing time-point I with time-point IV, $p^B = 0.0028$ comparing time-point I with time-point V, $p^C = 0.007$ comparing time-point II with time-point V, $p^D = 0.0068$ comparing time-point III with time-point V. (B) $p^E < 0.0001$ comparing seroconverters with non-seroconverters, $p^F < 0.0001$ comparing time-point I with III, IV, V, $p^G < 0.0001$ comparing time-point II with the other time-points, $p^H = 0.1087$ at time-point V in seroconverters.

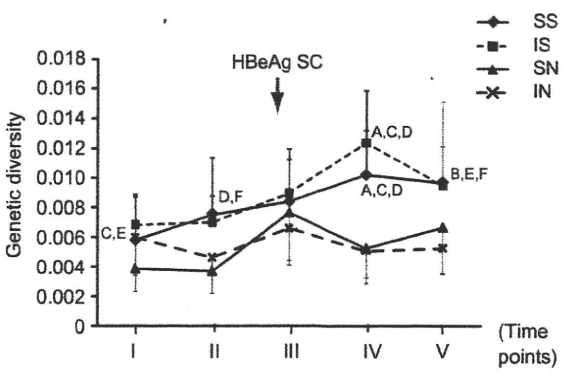

Fig. 2. Viral genetic diversity in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk and non-seroconverters controls (SN) is a solid line triangle. $p^A < 0.0001$ comparing seroconverters with non-seroconverters at time-point IV, $p^B = 0.0301$ comparing seroconverters with non-seroconverters at time-point V, $p^C = 0.0013$ and $p^D = 0.0025$ comparing I and II with time-point IV in seroconverters. $p^F = 0.0121$ and $p^F = 0.021$ comparing time-points I and II with V in seroconverters.

Striking differences in nucleotide sequence diversity were revealed between seroconverters and non-seroconverters before and after seroconversion (Fig. 2). The nucleotide sequence diversity of seroconverters was similar to that of non-seroconverters before seroconversion. Analysis of genetic distance showed that the viral sequence diversity of seroconverters was significantly greater than that of non-seroconverters after seroconversion (Fig. 2, $p^A < 0.0001$ at time-point IV, $p^B = 0.0301$ at time-point V) and was greater in seroconverters after seroconversion than before (Fig. 2, $p^C = 0.0013$ and $p^D = 0.0025$), while almost no changes were observed in non-seroconverters during the observation period.

It is noteworthy that, in interferon-induced seroconverters at the last time-point of observation, the nucleotide sequence diversity was less, although this increased clearly at the first year after seroconversion. This tendency of reversed change at the last two time-points was also seen in HBV DNA loads (Fig. 1B), namely, increase or decrease of the genetic diversity accompanied by decrease or increase of the viral load in interferon-induced seroconverters. On the other hand, the nucleotide sequence diversity increased continuously in spontaneous seroconverters, accompanied by a concurrent decrease of viral loads (Fig. 1B) during the follow-up period. Amino acid sequence diversity had an almost

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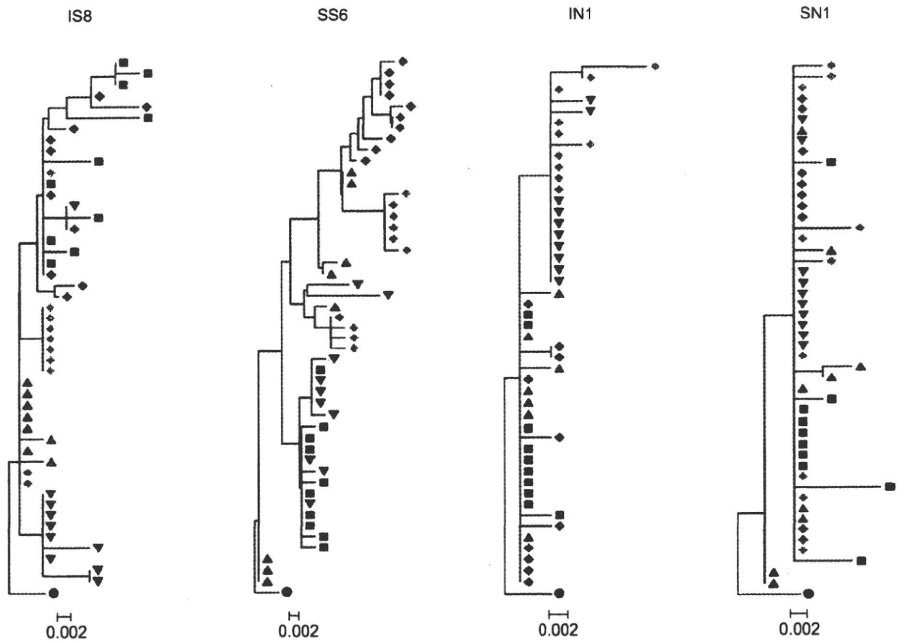

Fig. 3. Representative Neighbor-Joining phylogenetic trees of HBV sequences for each clinical group showing complex trees in seroconverters. HBV *X/precore/core* sequences from time-points I (purple filled triangle), II (blue filled inverted triangle), III (green filled square), IV (red filled diamond) and V (sky blue filled diamond) serum samples are analyzed phylogenetically and their positions are displayed on the trees. A sequence retrieved from the time-point I (red dot) of each group as outgroup in the trees, respectively. Scale bar represents 0.002% genetic variation. Seroconversion patients (IS, SS) show relatively complex branching patterns, forming clusters over time. With the pressure of seroconversion, the genetic diversity increased. In contrast, patients without seroconversion (IN, SN) were simply branching patterns and the genetic diversity in these patients changed very little over time.

identical pattern to that of DNA nucleotide sequence diversity (data not shown).

Construction of phylogenetic trees

Phylogenetic trees were complex for secoconverters and comparatively simple for non-seroconverters. In seroconverters (IS and SS), the arrangement and branch lengths of the trees were consistently more complex and longer than those for non-seroconverters. The genetic diversity was great after seroconversion in seroconverters (IS and SS) and less in non-seroconverters (IN and SN) (Fig. 3).

To investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 (before seroconversion) were further analyzed phylogenetically. Trees were reconstructed using Neighbor-Joining, ML (data not shown), and PAML methods (data not shown). In general, no clusters were seen to be supported by robust bootstrap values for any group or particular patient quasi-species. This indicates that the region of the HBV genome studied does not contain patterns of variability sufficient for robust phylogenetic relation reconstruction. However, variability of branch lengths in the tree indicated that seroconversion patient groups exhibit greater diversity of the quasi-species compared to patients without seroconversion. This is in agreement with the genetic distance plot (Fig. 2), showing greater deviation from the mean values observed in patients with seroconversion. The IN group exhibited least deviation on the distance plot (Fig. 2) and shortest branch lengths on the trees (Fig. 3).

Interclonal differences of the quasi-species

To investigate whether a particular mutation pattern of evolution of the quasi-species is associated with seroconversion, we further analyzed the sequence changes in all patients at time-points 1 and 2, corresponding to the time before seroconversion. Parsimony-based ancestral sequences were generated using the Simmonic Sequence Editor. Aligned sequences of time-points 1 and 2 from a single patient were used as the input. Frequencies of changes in 12 types of mutations, including 4 transitions (CT, TC, AG, and GA) and 8 transversions (AT, TA, AC, CA, CG, GC, GT, and TG) were evaluated between generated descendants and ancestral sequences for each clone of the patient. Statistical *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups is summarized in Table 2 and Supplementary Table 1.

Analysis of sequence changes indicated a higher frequency of transversional A to T in spontaneous seroconverters (SS *vs.* SN = 0.06 *vs.* 0.02, $p = 0.04$) and IFN-induced seroconverters (IS *vs.* IN = 0.05 *vs.* 0.01, $p = 0.05$) and A to C changes in IFN-induced seroconverters (IS *vs.* IN = 0.025 *vs.* 0.006, $p = 0.04$) before seroconversion. Comparison of seroconversion groups (SS and IS) indicated a higher frequency of transversional A to T mutation pattern ($p = 0.003$, Table 2) and the trend of G to A mutation is higher in seroconversion groups (SS and IS) (Table 2). Subsequently, alignments of the clones were generated. Visual inspection of the alignments indicated variation in the ratio of A1762T mutation in clones isolated from each patient at time-points 1 and 2 (Fig. 4). In contrast to non-seroconverters, seroconverters

Table 2. *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups.

	Seroconversion (n = 18)	Non-seroconversion (n = 18)	*p*
CT	0.117033	0.103750	0.637023
TC	0.156706	0.201328	0.155252
AG	0.125483	0.148372	0.498916
GA	0.196722	0.124511	0.073433
AT	0.061194	0.022128	0.003665
TA	0.049372	0.045417	0.778612
AC	0.027944	0.012550	0.145158
CA	0.017128	0.011094	0.523868
CG	0.009439	0.007744	0.835337
GC	0.018167	0.014894	0.748267
GT	0.009839	0.019217	0.272185
TG	0.041783	0.035528	0.731324

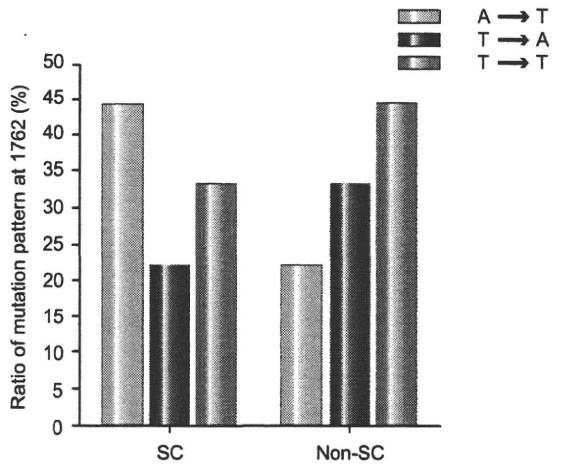

Fig. 4. The evolution of the *core* promoter mutation (A1762T) between seroconversion and control groups from time-point I to II. SC indicates seroconversion and non-SC, non-seroconversion. Alignment of the clones was carried out and the frequency of A1762T mutation in clones isolated from each patient at time-points 1 and 2 was determined. Subsequently, the evolutionary ratio of mutation from time-point I to II was calculated.

showed a higher frequency of A to T mutation pattern in the core promoter region from time-point I to II.

Core promoter (A1762T/G1764A) and precore (G1896A) mutations

Given that the *core* promoter/*precore* mutations influenced virus replication and HBeAg seroconversion, we analyzed the sequential change of *core* promoter (A1762T/G1764A)/*precore* (G1896A) mutations over time (Table 3). After seroconversion, patients with more than 50% *precore* mutant clone had higher HBV DNA loads than those with less than 50% of *precore* mutant clone (*precore* wild type) virus at time-point V [5.4 ± 1.3 (n = 5) *vs.* 3.8 ± 1.1 (n = 13), p = 0.0185] and 8 patients with a HBV DNA load less than 4.0 $\log_{10}$ copies/ml had all *precore* wild-type virus at time-point V (Table 3). Clinical progress of these patients was investigated over 10 years as median (range 1–20 years) after HBeAg seroconversion. HCC developed in 3 of 5 patients with *precore* mutant virus, compared to 1 of 13 patients with *precore* wild-type virus at time-point V (p = 0.017). On the other hand, 3 patients with ASC had all *precore* wild-type virus at time-point V (Table 3).

Discussion

In this study, analysis of 1800 nucleotide sequences from 36 HBV carriers showed that the viral diversity of seroconverters (IS and SS) after seroconversion was significantly greater than that of non-seroconverters (IN and SN) (Fig. 2, p <0.05) and was higher after seroconversion than before, in the seroconverters (Fig. 2, p <0.05). Phylogenetic analysis also generated complex trees for seroconverters and relatively simple trees for non-seroconverters. Analysis on interclonal differences in the quasi-specie showed a higher frequency of transversional A to T mutation pattern in seroconverters that coincided with the A1762T core promoter mutation. These findings suggested that HBeAg seroconversion involves dynamic shifts of the serum HBV quasi-species.

Osiowy et al. [24] examined viral quasi-species in eight HBeAg-negative patients at two time-points 25 years apart and obtained the evolutionary rate. Their results suggested that HBV diversity may be generated more rapidly than those estimated previously [25–29]. The higher evolutionary rate may be related to the seroconversion event driving quasi-specie complexity and diversification [24]. Our phylogenetic study showed that viral quasi-species populations appear to be replaced by new populations arising from a different clade after seroconversion.

Increased immune responses are accompanied by the reduction of viral loads and stronger immune pressure induces the selection of escape mutations, which leads to greater viral diversity [30]. According to this scenario, in our study, non-seroconverters have a high viral load and low quasi-species diversity and they obviously have a weak immune response.

Lim et al. [31] reported that viral genetic diversity in genotype B CHB patients was 2.4-fold greater in HBeAg seroconverters (spontaneous or IFN-induced) than in non-seroconverters before seroconversion. In this study of genotype C CHB patients, the nucleotide genetic distance was 1.49-fold greater in seroconverters (IS and SS) than in non-seroconverters before seroconversion but there was no statistical difference. This discrepancy might be due to the smaller region for analysis of genetic distance in our study than that of Lim et al. Another interpretation is that the host's immune response to the selection of mutant virus might differ between genotype B and genotype C. The natural course of CHB and the response to treatment could be affected by HBV genotype and there are some lines of evidence that indicate that the prevalence rates of *precore* and *core* promoter mutations vary among patients infected with HBV strains of different genotypes [32–34].

T-test comparison of mean values of nucleotide changes (Table 2) and linear logistic regression univariate analysis of mutations associated with seroconversion between seroconverters and non-seroconverters (data not shown) indicated a variation in the AT mutation pattern in the former (p = 0.003 and p = 0.006, respectively). This coincided with differences in the

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Table 3. *Core* promoter and *precore* mutations in seroconverters (IS and SS).

Patients	CP (ntA1762T/G1764A) (percent)			PC (ntG1896A) (percent)			DNA Loads (log_{10} copies/ml)			Histological diagnosis
	I	III	V	I	III	V	I	III	V	
IS1	100	100	100	0	0	70	5.7	3.8	4.8	CHB
IS2	100	100	100	90	100	90	7.6	7.2	7.6	HCC
IS3	70	100	100	10	0	10	6.5	5.2	5.5	CHB
IS4	90	100	10	0	10	0	7.6	6.2	3.3	CHB
IS5	100	40	20	0	0	0	7.6	3.6	4.1	CHB
IS6	70	90	90	20	10	90	5.7	4.1	4.5	HCC
IS7	100	100	90	0	10	10	7.2	3.1	3.4	LC
IS8	80	100	60	0	0	60	7.6	4.0	4.5	CHB
IS9	100	100	10	0	0	0	6.0	4.5	4.8	HCC
SS1	0	60	0	80	0	80	7.6	4.2	5.4	HCC
SS2	80	100	90	10	90	10	6.6	7.6	5.9	ASC
SS3	100	90	60	10	0	0	6.5	4.3	2.8	ASC
SS6	30	100	10	0	0	10	3.9	4.4	4.1	CHB
SS7	80	100	100	0	0	0	7.6	2.8	2.6	ASC
SS8	0	100	90	0	20	0	7.6	5.4	3.6	CHB
SS9	0	80	20	0	10	0	7.6	4.0	2.6	CHB
SS10	50	20	40	0	0	40	7.3	3.9	2.6	CHB
SS11	100	100	100	0	0	0	6.1	6.3	3.8	CHB

IS: interferon induced seroconverter; SS: spontaneous seroconverter; ASC: asymptomatic carriers; CHB: chronic hepatitis B; LC: cirrhosis; HCC: hepatocellular carcinoma.

ratio of T1762A quasi-species between seroconverters and non-seroconverters, indicating that it might be a marker preceding seroconversion in HBV/genotype C-infected patients as reported previously [35–37].

HBeAg seroconversion is an incomplete marker of immune control, although most patients experience some clinical benefit from it [38,39]. Previous studies have shown that the average rate of spontaneous HBeAg seroconversion in patients with chronic hepatitis B is about 10% per year [40,41]. HBeAg seroconversion associated with incomplete viral suppression may result in the emergence of the *precore* mutant and attendant chronic sequelae. Mutations in the *precore* and *core* promoter regions of the HBV genome have been reported in many HBeAg-negative CHB patients. Longitudinal studies found that the A1896 mutation emerges or is selected around the time of HBeAg seroconversion, and high *precore* mutant ratios have been associated with persistent hepatitis after anti-HBe seroconversion [42]. Patients who continued to have high HBV DNA titres after HBe seroconversion had a lower genetic heterogeneity but more often had the *precore* mutant.

The limitations of this study were, the small size of study group, only 10 clones per sample, and a small region for analysis of genetic distance. In addition, the *X/precore/core* region is a highly conserved region, investigation of another region of the HBV genome, such as the polymerase, might help us to better understand the evolution of quasi-species of HBV.

In conclusion, the distinctly greater viral diversity after seroconversion in HBeAg seroconverters could be related to increased HBV-specific T-cell responses and escape mutants which arise from selective pressure caused by host immune activity. Long-term follow-up is required to determine whether hepatitis B viral diversity decreases or remains at a high level. Further study will be needed to elucidate the relationship between seroconversion and viral quasi-species in relation to antiviral therapy.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References

[1] Domingo E, Holland JJ. RNA virus mutations and fitness for survival. Annu Rev Microbiol 1997;51:151–178.

[2] Arataki K, Kumada H, Toyota K, Ohishi W, Takahashi S, Tazuma S, et al. Evolution of hepatitis C virus quasispecies during ribavirin and interferon-alpha-2b combination therapy and interferon-alpha-2b monotherapy. Intervirology 2006;49:352–361.

[3] Laskus T, Wilkinson J, Gallegos-Orozco JF, Radkowski M, Adair DM, Nowicki M, et al. Analysis of hepatitis C virus quasispecies transmission and evolution in patients infected through blood transfusion. Gastroenterology 2004;127:764–776.

[4] Pawlotsky JM. Hepatitis C virus genetic variability: pathogenic and clinical implications. Clin Liver Dis 2003;7:45–66.

[5] Pellerin M, Lopez-Aguirre Y, Penin F, Dhumeaux D, Pawlotsky JM. Hepatitis C virus quasispecies variability modulates nonstructural protein 5A transcriptional activation, pointing to cellular compartmentalization of virus–host interactions. J Virol 2004;78:4617–4627.

[6] Fan X, Mao Q, Zhou D, Lu Y, Xing J, Xu Y, et al. High diversity of hepatitis C viral quasispecies is associated with early virological response in patients undergoing antiviral therapy. Hepatology 2009;50:1765–1772.

[7] da Silva J. The evolutionary adaptation of HIV-1 to specific immunity. Curr HIV Res 2003;1:363–371.

[8] Edwards CT, Holmes EC, Pybus OG, Wilson DJ, Viscidi RP, Abrams EJ, et al. Evolution of the human immunodeficiency virus envelope gene is dominated by purifying selection. Genetics 2006;174:1441–1453.

[9] Lipsitch M, O'Hagan JJ. Patterns of antigenic diversity and the mechanisms that maintain them. J R Soc Interface 2007;4:787–802.
[10] Ross HA, Rodrigo AG. Immune-mediated positive selection drives human immunodeficiency virus type 1 molecular variation and predicts disease duration. J Virol 2002;76:11715–11720.
[11] Villet S, Pichoud C, Villeneuve JP, Trepo C, Zoulim F. Selection of a multiple drug-resistant hepatitis B virus strain in a liver-transplanted patient. Gastroenterology 2006;131:1253–1261.
[12] Yim HJ, Hussain M, Liu Y, Wong SN, Fung SK, Lok AS. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 2006;44:703–712.
[13] Bertoletti A, Maini M, Williams R. Role of hepatitis B virus specific cytotoxic T cells in liver damage and viral control. Antiviral Res 2003;60:61–66.
[14] Huang CF, Lin SS, Ho YC, Chen FL, Yang CC. The immune response induced by hepatitis B virus principal antigens. Cell Mol Immunol 2006;3:97–106.
[15] Panther E, Spangenberg HC, Neumann-Haefelin C, Rosler K, Blum HE, von Weizsacker F, et al. The role of the virus specific T-cell response in acute and chronic HBV and HCV infection. Z Gastroenterol 2004;42:39–46.
[16] Tan AT, Koh S, Goh V, Bertoletti A. Understanding the immunopathogenesis of chronic hepatitis B virus: an Asian prospective. J Gastroenterol Hepatol 2008;23:833–843.
[17] Bruss V, Gerlich WH. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology 1988;163:268–275.
[18] Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol 1988;106:1093–1104.
[19] Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 1989;2:588–591.
[20] Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 1994;68:8102–8110.
[21] Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Meth 1999;80:97–112.
[22] Simmonds P. Recombination and selection in the evolution of picornaviruses and other Mammalian positive-stranded RNA viruses. J Virol 2006;80: 11124–11140.
[23] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24: 1596–1599.
[24] Osiowy C, Giles E, Tanaka Y, Mizokami M, Minuk GY. Molecular evolution of hepatitis B virus over 25 years. J Virol 2006;80:10307–10314.
[25] Bozkaya H, Ayola B, Lok AS. High rate of mutations in the hepatitis B core gene during the immune clearance phase of chronic hepatitis B virus infection. Hepatology 1996;24:32–37.
[26] Hannoun C, Horal P, Lindh M. Long-term mutation rates in the hepatitis B virus genome. J Gen Virol 2000;81:75–83.
[27] Simmonds P. The origin and evolution of hepatitis viruses in humans. J Gen Virol 2001;82:693–712.
[28] Fares MA, Holmes EC. A revised evolutionary history of hepatitis B virus (HBV). J Mol Evol 2002;54:807–814.
[29] Locarnini S. Molecular virology and the development of resistant mutants: implications for therapy. Semin Liver Dis 2005;25 (Suppl. 1):9–19.
[30] Stumpf MP, Pybus OG. Genetic diversity and models of viral evolution for the hepatitis C virus. FEMS Microbiol Lett 2002;214:143–152.
[31] Lim SG, Cheng Y, Guindon S, Seet BL, Lee LY, Hu P, et al. Viral quasi-species evolution during hepatitis Be antigen seroconversion. Gastroenterology 2007;133:951–958.
[32] Chan HL, Hussain M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 1999;29:976–984.
[33] Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J Infect Dis 1999;179:775–782.
[34] Blackberg J, Kidd-Ljunggren K. Genotypic differences in the hepatitis B virus core promoter and precore sequences during seroconversion from HBeAg to anti-HBe. J Med Virol 2000;60:107–112.
[35] Lindh M, Gustavson C, Mardberg K, Norkrans G, Dhillon AP, Horal P. Mutation of nucleotide 1, 762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. J Med Virol 1998;55:185–190.
[36] Kim YS, Kim SI, Hwang SG, Kim JO, Cho JY, Lee JS, et al. Diversity of core promoter mutations in immune clearance phase of chronic HBV infection. Eur J Gastroenterol Hepatol 1999;11:821–825.
[37] Liu CJ, Chen PJ, Lai MY, Kao JH, Chen DS. Evolution of precore/core promoter mutations in hepatitis B carriers with hepatitis B e antigen seroreversion. J Med Virol 2004;74:237–245.
[38] Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, et al. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 2002;35:1522–1527.
[39] Hui CK, Leung N, Shek TW, Yao H, Lee WK, Lai JY, et al. Sustained disease remission after spontaneous HBeAg seroconversion is associated with reduction in fibrosis progression in chronic hepatitis B Chinese patients. Hepatology 2007;46:690–698.
[40] Liaw YF, Chu CM, Su IJ, Huang MJ, Lin DY, Chang-Chien CS. Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis. Gastroenterology 1983;84:216–219.
[41] Lok AS, Lai CL, Wu PC, Leung EK, Lam TS. Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. Gastroenterology 1987;92:1839–1843.
[42] Chu CM, Yeh CT, Lee CS, Sheen IS, Liaw YF. Precore stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. J Clin Microbiol 2002;40:16–21.

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Review Article

Management of hepatitis B: Consensus of the Japan Society of Hepatology 2009

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Recently, much progress has been made in the field of hepatitis B, such as natural history of the disease in relation to the amount of hepatitis B virus (HBV) DNA, genotypes of HBV influencing the natural course and treatment effects, mutations of HBV influencing the severity of the disease and development of hepatocellular carcinoma, and antiviral treatment such as nucleos(t)ide analogues and pegylated interferon. To make the consensus for the diagnosis, management and treatment of hepatitis B, a meeting was held during 45th annual meeting of Japan Society of Hepatology (JSH) in June 2009. In the meeting, recommendations and informative statements were discussed on the following subjects: (i) natural history of HBV infection; (ii) clinical implication of HBV genotypes; (iii) HBV mutations and their potential impact on pathogenesis of HBV infection; (iv) indications for antiviral treatment of chronic hepatitis B; (v) nucleos(t)ide analogues for chronic hepatitis B; and (vi) interferon therapy for chronic hepatitis B. The presenters reviewed the data on these subjects and proposed the consensus statements and recommendations. These statements were discussed among the organizers and presenters, and were approved by the participants of the meeting. In the current report, the relevant data were reviewed and the 12 consensus statements and nine recommendations on chronic hepatitis B were described.

Key words: genotype, hepatitis B virus, interferon, mutation, natural history, nucleotide analogue

Hepatitis B virus (HBV) is one of the most distributed viruses which infect humankind. More than 3 billion people, one half of the world's population, have been exposed to HBV during their life.[1] Acute infection in adults is self-limited in general whereas infection during early childhood will develop into persistent chronic infection in most individuals.[2] More than 400 million people worldwide are chronically infected with HBV and are at risk of developing life-threatening complications including liver cirrhosis and hepatocellular carcinoma (HCC).[1] HBV is a major public health problem worldwide especially in East Asia and Africa. In Japan, approximately 1.5 million people are infected with HBV and it is one of the major causes of HCC and chronic hepatic failure. Other complications of HBV infection include fulminant hepatitis and acute liver failure.

The consensus meeting for diagnosis, management and treatment for hepatitis B was held during the 45th annual meeting of the Japan Society of Hepatology (JSH) in June 2009 (Congress President: M Kudo), where the recommendations and informative statements were discussed. Although the JSH consensus meeting of hepatitis B had been held four times so far, recommendations were hitherto published only in Japanese and this is the first report in English. Established

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information for pathogenesis and contributing factors for disease progression which was agreed by the organizers and presenters are shown as "consensus statements", and clinically useful consensus are shown as "recommendations". The quality of recommendations or informative statements are required to show a "level" (assessing strength or certainty) of evidence and "grading" of recommendations or assessment according to a standard reporting system of clinical guidelines.[3]

NATURAL HISTORY OF HBV INFECTION

AN EVALUATION OF studies on the natural history of HBV infection was done using the scoring system proposed by MacMahon *et al.*[4] in the present analysis because scoring systems for treatment studies cannot always be applied directly to those using natural history. The proposed scoring system consists of levels 1 (1a, 1b), 2 (2a, 2b, 2c), and 3. Level 1a is defined as a population-based longitudinal cohort study with a hepatitis B surface antigen (HBsAg) negative comparison group. Level 1b is identical to level 1a, but with no comparison group. Level 2a is defined as a clinic-based longitudinal cohort study, level 2b is a population-based or clinic-based cohort nested case–control study, and level 2c is a cross-sectional clinic-based study. Level 3 is defined as an observation study case series.

The natural history of chronic HBV infection can be classified into several phases based on levels of alanine aminotransferase (ALT), hepatitis B e-antigen (HBeAg) status, amounts of HBV DNA, and estimated immunological states.[4–9] A representative classification of these phases is shown in Table 1. In the immune tolerance phase, HBeAg is positive, serum levels of ALT are normal, histological activities of hepatitis are absent or minimal, and levels of HBV DNA are elevated. The immune tolerance phase is thought to occur most frequently in individuals who are infected through perinatal transmission, and this phase usually lasts until adolescence or young adulthood.[10–12]

The chronic hepatitis B phase is characterized by elevated ALT and HBV DNA levels. In this phase, the host's immune system recognizes HBV as being foreign and initiates an immune response that results in hepatitis. In cases who are HBeAg positive, active hepatitis can be prolonged and may result in cirrhosis. However, chronic hepatitis B eventually transitions into an inactive phase with a loss of HBeAg positivity in the majority of patients. Seroconversion to anti-HBe and the fall of serum HBV DNA to low levels result in the disappearance of disease activity, despite persisting HBsAg and low levels of HBV DNA.[13–16] Seroconversion rates range 7–16% per year according to reports with higher evidence levels (levels 1b, 2a).[16–19] Factors associated with seroconversion are age (level 1b),[20] ALT levels (level 1b), occurrence of acute exacerbation of hepatitis (level 1b),[19,21] and genotype (level 2c).[22,23]

The seroconversion of HBeAg results in the transition from hepatitis phase to inactive carrier phase, which is generally thought to be a benign course for HBV carrier, but sometimes hepatitis can be reactivated spontaneously.[24] Patients experiencing reactivation undergo another transition, with increases in HBV DNA and ALT levels and disease activity without reappearance of HBeAg.[24] This phase is referred to as HBeAg negative chronic hepatitis B. Occasional severe hepatitis B flare-ups with middle range HBV DNA levels (3–8 log copies/mL) occur in this phase.[8,25] HBeAg negative chronic hepatitis B is caused by mutant strains of HBV unable to produce HBeAg,[25,26] and tends to develop into cirrhosis and complicate HCC more than HBeAg positive chronic hepatitis B.[27–30]

Table 1 Phases in the natural history of HBV carriers (modified from [4])

Phase	Hepatitis	Blood			Liver
		DNA	HBeAg	HBsAg	cccDNA
Immune tolerance	–	8–11	+	+	+
HBeAg positive Chronic hepatitis	Usually Persistent	6–10	+	+	+
HBeAg negative Chronic hepatitis	Often Fluctuating	3–8	–	+	+
Inactive carrier	–	<4	–	+	+
Recovery	–	–	–	–	+

HBV DNA: log copies/mL. cccDNA, covalently close circular DNA; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

Many factors that are associated with the development of HCC have been reported so far. Higher age (level 1a), male sex (level 1a), presence of cirrhosis (level 2a) and familial cluster of carriers (level 2c) are reported as host factors.[31,32] Viral factors include high viral load (level 1b),[33–36] existence of pre-core and core promoter mutations (level 2a), genotype C and high ALT levels (level 1b). High viral load should be considered as a factor in patients over 35–40 years of age. Co-infection with hepatitis C virus, hepatitis D virus or HIV (level 2a), drinking habit (level 2c) and exposure to aflatoxin (level 2c) are reported as social and environmental factors.[37–39] Other lifestyle-related factors, such as smoking habit, obesity and complications from diabetes mellitus, have been documented as well.

Consensus 1
In patients with chronic hepatitis B, seroconversion of HBeAg usually results in the transition from hepatitis phase to inactive carrier phase, which generally has low HBV replication and normal ALT levels. However, reactivation of chronic hepatitis can spontaneously occur without the reappearance of HBeAg. At this point, active hepatitis continues and the risk of complicating cirrhosis and HCC is high in patients with HBeAg negative chronic hepatitis B. (Level 1b.)

In the inactive carrier phase, HBV replication is continuously suppressed as a result of predominantly host immunological pressure against HBV. Patients in the inactive carrier phase generally have a benign course because active hepatitis subsides and the risk of HCC decreases.[19,20,24,40] However, regular follow up is required because reactivation of HBV sometimes occurs spontaneously or as a result of immunosuppressive therapy.[19,24]

Hepatitis B surface antigen is known to fall to undetectable levels in some inactive carriers. This HBsAg negative phase, referred to as the recovery phase, has no hepatitis and a low risk of HCC. Still, caregivers must be aware that patients who are old or cirrhotic have a relatively higher risk of HCC.[41,42] Disappearance of HBsAg in the recovery phase does not indicate complete eradication of HBV because the HBV genome remains as covalently close circular DNA (cccDNA) in the nucleus of hepatocytes.

Consensus 2
2-1 HBV can not be completely eradicated using any currently existing treatment measures. (Level 2a.)
2-2 Patients in the inactive carrier or recovery phase have a benign clinical course. However, regular follow up of such patients is required because reactivation of hepatitis B and ensuing HCC can occur. (Level 1b, 2a.)

Clinicians have to consider two types of hepatitis B reactivation: one during the inactive carrier phase and the other in the recovery phase.[4] Both types of reactivation have been attributed with increasing incidence to strong immunosuppressive therapies. De novo hepatitis B, a reactivation of hepatitis B in the recovery phase, tends to develop into fulminant hepatitis, which has a very high mortality rate.[43–46] Thus, establishment of effective measures to prevent reactivation of hepatitis B is necessary.

Consensus 3
3-1 Reactivation of hepatitis B can occur during the inactive carrier or recovery phases and stems mainly from strong immunosuppressive treatment courses. (Level 2a.)
3-2 Recent advances in medical care have increased the use of immunosuppressive agents and thus the incidence of hepatitis B reactivation. (Level 2a.)
3-3 Reactivation of hepatitis B tends to develop into fulminant hepatitis. (Level 2a.)

Recommendation 1
In addition to the loss or seroconversion of HBeAg, a substantial decrease in HBV viral load and subsequent disappearance of hepatitis are the primary targets in the treatment of patients with chronic hepatitis B. (Level 1b.)

Recommendation 2
The main goals of HBV carrier treatment are patients in the inactive carrier and recovery phases. However, caregivers should be aware that reactivation of hepatitis B and complication of HCC can occur even in these benign phases. (Level 1b.)

Recommendation 3
Reactivation of hepatitis B due to immunosuppressive therapy tends to develop into severe hepatitis, thus requiring the establishment of effective preventative measures. (Level 2a.)

CLINICAL IMPLICATION OF HBV GENOTYPES

DISTINCT CLINICAL AND/OR virological characteristics of the HBV infection have been reported in different geographical parts of the world and are increasingly associated with host factors, environmental factors and the genetic diversity of the infecting virus.[47] HBV is classified into at least eight genotypes (A–H) based on an intergroup divergence of 8% or more in the complete nucleotide sequence and a number of subgenotypes (Aa/A1, Ae/A2, Bj/B1, Ba/B2, Cs/C1, Ce/C2, D1, D2, and so forth) that are currently known to have distinctive association with ethnic and/or geographical distribution.[48]

Association between HBV genotype and clinical manifestation

Acute hepatitis

The universal vaccination program against HBV has significantly reduced the number of new infection cases in most countries with levels of endemicity estimated from intermediate to high.[49] However, efficiency of universal vaccination in countries with a low level of endemicity still remains controversial. Japan is one of the countries with a low level of endemicity and mainly vertical (mother to baby) transmission route.[50] In Japan, HBV vaccination in combination with HBV immunoglobulin treatment is the only recommended measure for infants born to HBsAg positive mothers. Studies in Japan indicated genotype C (subgenotype Ce/C2) to be the major type in the country and genotype B (subgenotype Bj/B1) is the second distributed. Surveillance studies have shown a recent trend toward increase in number of acute hepatitis B infection among young adults mainly through sexual contacts.[51,52] Although most cases are associated with genotype C infection, there is a continuous trend toward increase in prevalence of genotype A among acute hepatitis cases.[51,53–56] Patients infected with genotype C have been known to be rarely associated with development of chronic persistence after acute infection in immune competent adults in Japan (1%) in contrast to the higher rates of those infected with genotype A (6–23%).[53,54] A recent multicenter study in Japan indicated a trend among chronic hepatitis B patients toward increase in prevalence of genotype A (from 1.7% in 2002 to 3.5% in 2006), whereas other genotypes remained stable at their prevalence during the same period.[57] The shift in genotype prevalence with the increase of genotype A among chronically infected carriers can be explained by higher risk of genotype A to develop persistence. This is consistent with higher rates of chronic persistence after acute infection in adults in European countries where genotype A is prevalent (10%).[48,58] This is also consistent with results of *in vitro* and *in vivo* comparisons of different genotype strains showing different dynamics of replication: slow for genotype A and rapid by genotype C.[59,60] The surveillance study indicated that all patients treated with lamivudine (LVD) recovered from acute hepatitis, whereas none of the three patients who developed a chronic outcome had received antiviral treatment during their acute phase of infection, indicating that LVD might be able to prevent the chronic outcome.[54] Cumulatively, these data indicate the clinical importance of routine genotyping for acute hepatitis B patients.

Fulminant hepatitis

One of the most serious complications of acute HBV infection is fulminant hepatitis. In Japan, the annual number of fulminant hepatitis reported was approximately 400 cases, with approximately half of these caused by HBV infection. Despite its rather low incidence, fulminant hepatitis is a national problem because the mortality rate is extremely high.[61] It is important to understand factors predisposing for development of fulminant hepatitis. Viral factors associated with the development of fulminant hepatitis are mutations in the core promoter (T1762/A1764)[62] and the pre-core region (A1896).[54,63,64] However, these findings were not consistent with studies in Europe and the USA.[65–67] A large-scale cross-sectional study in Japan revealed association between genotype B (subgenotype Bj/B1) infection and development of fulminant hepatitis; on the other hand, no cases of fulminant hepatitis were registered among those infected with genotype A (subgenotype Ae/A2).[54] Differences in genotypes circulating in Asia and Europe/USA may indicate that distinct viral factors are playing roles in manifestation of infection by different genotype.

Chronic hepatitis

Chronic HBV infection is the most common cause of HCC in Asia.[68] Efficient surveillance and early diagnosis of development of this life complication requires risk stratification of chronic hepatitis B patients. Older age, male sex and liver cirrhosis are well recognized factors associated with increased risk of HCC.[69,70] In addition, recent large-scale population-based and clinical case–control studies carried out in Asia, have shown that infecting virus factors associated with a high risk of HCC, include HBV DNA levels,[71,72] HBV basal core promoter mutations,[35] genotype C (vs B),[22,36,73,74] and sub-

genotype Ce/C2.[71,75] There are data indicating that genotype C infection associated with a higher viral load than genotype B.[76] Association of genotype F with HCC was found to be higher than that of genotype C in Alaskan natives.[77,78] Unfortunately, there are few prospective studies examining other HBV genotypes for association with adverse outcomes. Genotype A (subgenotype Aa/A1) was found in association with development of HCC in young adults in South Africa.[79,80] However, very high rates of detection of subgenotype Aa/A1 among asymptomatic carriers suggest contribution of environmental factors (aflatoxin contained in food) for the development of HCC. In comparison with Aa/A1, HCC associated with Ae/A2 is found primarily in older individuals. In addition, the rate of complications, including HCC, for those infected with subgenotype Ae/A2 appears to be less than that found in those infected with genotype D, C or F1.[77,81] A prospective study in Spain showed that genotype A (presumably Ae/A2) infection was associated with a significantly higher cumulative rate of sustained biochemical remission, HBV DNA and HBsAg clearance in patients with chronic HBV infection than genotype D infection.[81]

Consensus 4

4-1 Recently, there is an increase of HBV genotype A proportion among acute hepatitis B infection cases in Japan. (Level 3.)

4-2 HBV genotype A acute infection has a tendency to evolve in chronic hepatitis compared to genotype B/C. (Level 3.)

4-3 Antiviral therapy of acute infection might be efficient in prevention of chronic carrier stage. (Level 3.)

4-4 Genotype C compared with genotype B is associated with higher risk of outcome in HCC in chronic carriers. (Level 2a, grade B.)

4-5 Genotype A compared with genotype D and F in chronic carriers is associated with better prognosis in terms of spontaneous ALT normalization and DNA clearance. (Level 2a, grade B.)

HBV MUTATIONS AND THEIR POTENTIAL IMPACT ON PATHOGENESIS OF HBV INFECTION

THE HBV GENOME consists of double-stranded DNA, 3200 bp in length. HBV replicates through reverse transcription of a RNA intermediate, the pregenome RNA, different from all known mammalian DNA viruses. HBV infection is characterized by high levels of virus production, however, the HBV reverse transcriptase is an error-prone enzyme lacking proof-reading capacity, resulting in a large number of nucleotide substitutions during replication. The misincorporation rate has been estimated to be of the order of 10^{10} incorrect nucleotide incorporations per day. As a result, HBV has a quasispecies distribution in infected patients.

Naturally occurring mutations identified in the HBV genome are more prevalent in patients with chronic hepatitis than in HBeAg positive asymptomatic carriers. Among them, several specific mutations have been shown to be associated with the pathogenesis of HBV infection.

HBeAg seroconversion

A HBV strain harboring stop codon mutation in the precore region was first reported in anti-HBe positive patients with chronic hepatitis.[25] The precore region located upstream of the core region is involved in the production and secretion of HBeAg protein. HBeAg is secreted into blood after removal of N-terminal 19 amino acids (a.a.) and C-terminal 34 a.a. from HBeAg precursor protein composed of precore and core regions. Nucleotide substitution of G to A at nt 1896 confers stop codon (TAG) mutation from tryptophan (TGG) at codon 28 in the precore region, resulting in a failure to produce HBeAg protein.[82–84] Although controversial, 10 genotypes have been identified tentatively so far[85] and genotypes affect the occurrence of stop codon mutation in the precore region. The stop codon mutation in the precore region (G1896A) is rarely encountered in HBV genomes of genotype A, some of genotype C and F, because they possess C at position 1858 that makes a pair with G at position 1896 in the stem-loop structure of the *cis*-encapsidation signal.[86]

The HBV core promoter regions located upstream of core region are involved in the transcription of precore mRNA and pregenomic RNA. Nucleotide substitution of A to T at nt 1762 combined with substitution of G to A at nt 1764 in the core promoter region give rise to a reduced transcription of precore mRNA and increased level of viral DNA, resulting in a decreased production of HBeAg protein and enhanced viral replication.[87–89]

Consensus 5

Nucleotide substitution G1896A confers stop codon mutation in the precore region. Nucleotide substitution A1762T combined with substitution G1764A in

the core promoter region give rise to a reduced transcription of precore mRNA. These nucleotide changes in combination with a reduction of HBeAg caused by suppressed replication of HBV are closely associated with HBeAg seroconversion. (Level 2b, grade B.)

Association between HBV mutations and clinical manifestation

Fulminant hepatitis

Precore and core promoter mutations are very frequent in patients with fulminant hepatitis from Asia[62,63,90] and the Middle East.[64] However, these mutations were not detected in those from Western countries.[65,67,91,92] This difference could be attributable to the difference of genotype prevalence, frequent genotype Ae and rare Bj in Western countries.[86] The patients infected with the former genotype rarely have precore mutant virus, while the latter frequently have the mutant virus. Stop codon mutation in the precore region is inhibited in genotype A because of C at position 1858 that makes a pair with G at position 1896 in the stem-loop structure of the *cis*-encapsidation signal.[93]

Ozasa *et al.* analyzed the difference of host and viral factors between 40 patients with fulminant hepatitis B and 256 with acute self-limited hepatitis B in a multicenter cross-sectional study,[54] and showed that precore stop codon mutation of G1896A and genotype Bj are associated with fulminant hepatitis in Japan. They also reported the marked enhancement of viral replication by introducing either G1896A or A1762T/G1764A mutation into the Bj clone in *in vitro* transfection study. Because this type of HBV mutant is found not only in patients with fulminant hepatitis but also in asymptomatic HBV carriers,[94] the interaction between the virus and the host's immune response might influence the outcome of HBV infection.

In addition to the mutants mentioned above, pre-S2 defective virus or HBV defective in secretion because of surface gene mutations are reported in patients with fulminant hepatitis. These mutant viruses showed a characteristic feature of virus retention in hepatocytes and misassembly with high replication capacity.[95–97]

HCC development

Evidence has been accumulating over the past decade that the risk of developing cirrhosis and HCC is influenced by the patient's viral status, such as genotype, viral load and genomic mutations. Naturally occurring mutations have been identified in the structural and non-structural genes as well as the regulatory elements of the virus, and these mutations are more prevalent in patients with chronic hepatitis than in HBeAg positive asymptomatic carriers.[98]

A double mutation, A1762T/G1764A in the basal core promoter region has been found in patients with advanced liver disease and HCC. Several case–control studies,[30,35,99–102] retrospective cohort studies[103,104] and one prospective cohort study[105] confirmed this finding, while some conflicting results were also reported in the case–control studies[106,107] and one prospective study.[108]

The role of deletions in the pre-S region of the HBV genome has been shown to be associated with the development of progressive liver diseases including HCC. Several case–control studies confirmed this finding.[27,107–110] A further mapping study of the pre-S region showed that all the deletion regions encompassed T- and B-cell epitopes and most of them lost one or more functional sites including the polymerized human serum albumin-binding site.[109] Deletion of these functional sites may cause intracellular retention of HBV envelope proteins and viral particles and contribute to more progressive liver damage and HCC development.

In addition to these common mutations, several other mutations, C1653T in the enhancer II region, T1753C/A/G in the core promoter region, and G1317A/T1341C/A/G in enhancer I region, have been reported to be associated with the development of HCC in some case–control studies.[30,107,111]

Consensus 6
There is some evidence that emergence of HBV genomic mutations arising during the course of chronic infection influence the outcome of chronic liver disease. Among them, core promoter mutations A1762T/G1764A might have a potential for developing progressive liver disease and HCC. (Level 2a, grade B.)

HBsAg escape mutant

The HBsAg mutant was first described in a child born to a HBsAg positive mother who developed acute hepatitis B in spite of vaccination and passive immunization against HBV.[112] This viral strain contained a substitution of glycine to arginine at position 145 (sG145R) and was able to escape the immune surveillance, resulting in an infection despite the presence of anti-HBs antibodies, vaccine escape mutant. Similar mutants have been detected all over the world.[113–115]

Patients after liver transplantation for HBV-related chronic liver disease who had received anti-HBs antibodies to prevent re-infection of the graft showed an "immune escape mutant".[116–118] Furthermore, "diagnosis escape mutants" have also been described because HBsAg detection assays are based on anti-HBs antibodies.[119] The emergence of these variants may contribute to occult HBsAg negative HBV infection.[120]

The HBV genome is organized in such a way that the envelope gene is overlapped by the polymerase gene; therefore, HBV with changes in the polymerase gene associated with resistance to the nucleos(t)ide analog which are described in detail in section 5 may have consequent changes in the envelope gene. A triple mutant causing LVD resistance (rtV173L + rtL180M + rtM204V), which have an enhanced replication capacity compared with rtL180M + rtM204V alone, causes two amino acid changes in the overlapping surface gene (sE164D + sI195M). This mutant reduces anti-HBs binding to levels seen only with the vaccine escape mutant sG145R.[121] Some patients treated with LVD showed seroclearance of HBsAg with detectable circulating HBV DNA. An sP120A mutation was associated with HBsAg seroconversion in these patients and this mutation produces a reduced anti-HBs binding which causes the failure to detect HBsAg.[122]

Consensus 7
Amino acid substitutions, deletions or insertions across the "a" determinant of HBsAg, such as a substitution sG145R, give rise to vaccine and immunoglobulin escape mutant. (Level 4, grade C.)

INDICATIONS FOR ANTIVIRAL TREATMENT OF CHRONIC HEPATITIS B

ONCE THE LIVER is persistently infected with HBV, it is difficult to eradicate the virus. It is reported that the natural clearance rate of HBsAg in asymptomatic HBsAg carriers is approximately 1–2% per year.[123] Therefore, the first goal in treating chronic hepatitis B is to prevent patients from progression to cirrhosis and occurrence of HCC.

When the initiation of antiviral therapy for chronic hepatitis B is considered, it is very important to estimate the fibrosis stage of each patient. If possible, a liver biopsy should be performed in order to obtain sufficient information to determine the extent of hepatic fibrosis. When the fibrosis stage of patients with chronic hepatitis B is moderate to severe, or when the patients have cirrhotic liver, the administration of antiviral therapy should be considered. When inflammatory activity is high and the fibrosis seems to be progressive, the introduction of antiviral therapy should also be considered.

In order to prevent the occurrence of hepatic fibrosis and HCC, virological factors as well as biochemical factors are important. A long-term follow-up study of untreated HBsAg positive individuals in Taiwan in which the cumulative incidence of HCC and cirrhosis were studied for 13 years revealed that high baseline HBV DNA was associated with increased risk of HCC and cirrhosis. Incidence rate of HCC in patients whose viral load of HBV DNA was less than 300 copies/mL was 1.3%, whereas in patients whose viral load was more than 1 000 000 copies/mL the incidence rate was 14.9%.[33] Moreover, incidence of cirrhosis in patients whose viral load was less than 300 copies/mL was 4.5%, whereas it was 36.2% in patients whose viral load was more than 1 000 000 copies/mL.[124] Therefore, the introduction of antiviral therapy should be considered based on biochemical and virological findings.

As mentioned above, although high viral load of HBV DNA is one of the strong risk factors in predicting poor prognosis of HBV carriers, low HBV DNA level does not rule out risk in Asian patients. Among HBeAg positive patients, HBV DNA levels of less than 10^5 copies/mL predicted better histological outcome; however, 14.3% of patients still had established fibrosis.[125] The liver biopsy is also very useful for such cases.

Recommendation 4
- 4-1 Introduction of antiviral therapy should be considered on the biochemical and virological findings. (Level 2a, grade B.)
- 4-2 Antiviral therapy should be considered for patients with low virus load but progressed hepatic fibrosis. (Level 2a, grade B.)
- 4-3 Liver biopsy finding (if available) should be useful to determine the introduction of antiviral therapy. (Level 2a, grade B.)

On the other hand, when patients with HBV have obscure or mild fibrosis, a close observation without any medication could be considered for them. Once antiviral therapy with a nucleos(t)ide analogue is started, it is very difficult to stop. Therefore, for patients who are in an inactive carrier state and whose fibrosis stage is relatively mild, a coarse observation without any treatment could be a useful choice to treat the patients.

Young patients with chronic hepatitis B, especially those who are HBeAg positive, often face the flare-up of hepatitis. Because such patients are likely to achieve spontaneous HBe seroconversion and go into an inactive carrier state, unnecessary antiviral therapy should be avoided for them. A coarse observation without any medications should be considered for young patients or those with mild fibrosis.

Recommendation 5
Indication of antiviral therapy for chronic hepatitis B: Observation without therapy should be considered for young patients or those with mild fibrosis. (Level 3, grade B.)

NUCLEOS(T)IDE ANALOGUES FOR CHRONIC HEPATITIS B

AS STATED ABOVE, the goal of antiviral therapy in patients with chronic hepatitis B is to prevent cirrhosis and HCC. Maintaining persistent suppression of HBV replication reduces the development of cirrhosis and HCC. In the last decade, there has been a major advance in the treatment of chronic hepatitis B with nucleos(t)ide analogues such as LVD, adefovir (ADV), entecavir (ETV), telbivudine and tenofovir.[126–132] In treatment by nucleos(t)ide analogues for chronic hepatitis B in Japan, LVD, ADV and ETV are mainly used at present. Nucleos(t)ide analogues are potent inhibitors of the polymerase/reverse transcriptase and are easy to administrate p.o. to chronic hepatitis B patients because of low adverse effects and strong efficacy to suppress HBV replication. Thus, nucleotide analogue therapy could rescue liner decompensation, reduce fibrosis progression and prevent the development of HCC.[133–136] On the other hand, there are major disadvantages including requirement of prolonged or even indefinite therapy for most patients and the high incidence of antiviral resistance. Disadvantages of nucleos(t)ide analogues include the development of antiviral resistance.[137–140] Drug-resistant viruses emerge during the treatment and could be associated with flare-up of hepatitis. Due to no proof of reading activity of HBV polymerase, the spontaneous substitution rate of HBV genome is high in the natural course of the disease. Through the selection of pre-existing resistant variants and gradual accumulation of new a.a. substitutions, the mutations exhibiting the best replication capacity in the presence of the drug are selected under the circumstance of antiviral pressure. The level of intrinsic resistance and the replicative fitness determine the mutant spread and hence the annual incidence of drug resistance.

LVD

Lamivudine was the first nucleoside analogue licensed for the treatment of chronic HBV infection in Japan in 1999. LVD was given at a dose of 100 mg daily and has excellent safety and tolerability.[141–143]

Liaw *et al.* reported that continuous treatment with LVD delays the clinical progression of chronic hepatitis B with advanced fibrosis or cirrhosis by significantly reducing the incidence of hepatic decompensation and risk of HCC (level 1b).[134] Matsumoto *et al.* also showed that LVD therapy effectively reduces the incidence of HCC in Japanese patients with chronic hepatitis B.[144] Thus, it is generally considered that control of viral load using nucleos(t)ide analogues is effective to prevent complicating HCC in patients with active chronic hepatitis B.

Consensus 8
The control of viral load using nucleos(t)ide analogues reduces the risk of complicating HCC in patients with chronic hepatitis B. (Level 1b, grade B.)

Lamivudine resistance is characterized by the mutation of the highly conserved tyrosine, methionine, aspartate, aspartate (YMDD) nucleotide-binding motif in the catalytic domain of the enzyme. YMDD to YIDD (rtM204I) or YVDD (rtM204V) mutations are associated with LVD resistance.[142,145,146] These resistant mutants appear to replicate less efficiently than the wild-type virus *in vitro*, however, additional mutations such as rtV173L and rtL180M can restore partially the replication capacity *in vitro*.[147,148] LVD resistance occurred in approximately 20% of patients after 1 year, which increased to approximately 70% after 5 years (Fig. 1).

A meta-analysis, which included Asian patients and North American/European patients, indicated that HBV subtype ayw (genotype D) appears to respond significantly better to LVD treatment than does HBV subtype adw (genotype A). Insufficient suppression of the adw subtype during the early phase of treatment may lead to the high incidence of LVD resistance in HBV subtype adw.[149] In a study comparing the virological outcome among infections with HBV genotypes A, B and C, patients infected with genotype A had the lowest rate of HBV DNA clearance than those with genotype B or C, and had the highest incidence of resistant mutations.[150]

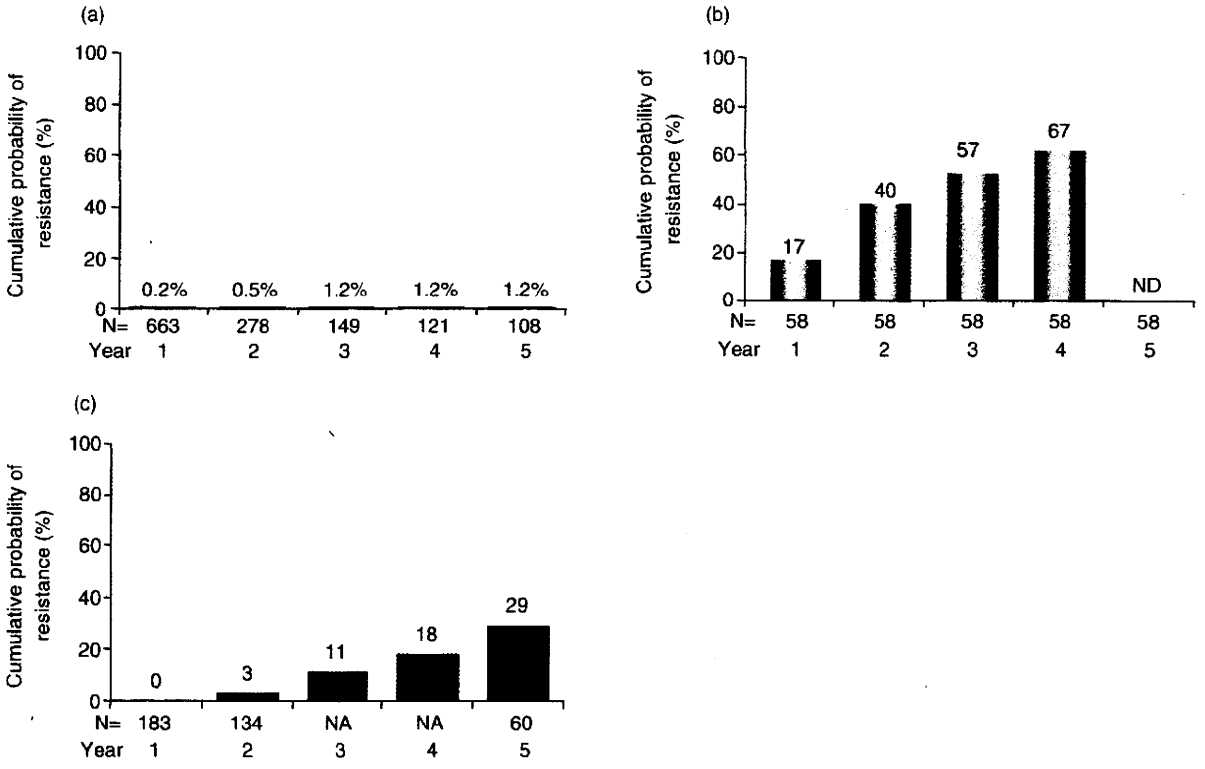

Figure 1 Cumulative probability of resistance after the initiation of entecavir (ETV), lamivudine (LVD) and adefovir (ADV) for patients with hepatitis B e-antigen. (a) Cumulative probability of resistance after the initiation of ETV.[159] (b) Cumulative probability of resistance after the initiation of LVD.[138] (c) Cumulative probability of resistance after the initiation of ADV.[153]

Lamivudine or hepatitis B immunoglobulin (HBIg) treatment induced vaccine/HBIg-escape mutations sP120T and sG145R in combination with LVD-resistance mutations. These mutations are associated with rtT128N and rtW153Q in the polymerase protein and have been found to partially restore the *in vitro* replicative capacity of LVD-resistant HBV.[121]

Another LVD resistant mutation, rtA181T, concomitantly generates a stop codon in the surface antigen (sW172stop), resulting in impaired secretion of HBsAg.[151] Neither the adefovir associated resistance mutation rtN236T nor the tenofovir associated resistance mutation rtA194T causes changes in the envelop protein.

ADV

Adefovir dipivoxil is a prodrug of ADV and has structural similarity to the natural substrate, dATP. Several studies have also been conducted using ADV.[128,152–154] In HBeAg positive patients, treatment with ADV for 1 year resulted in HBeAg seroconversion in 12%, serum HBV DNA in less than 10^3 copies/mL in 21% and normalization of ALT in approximately 48% of patients.[127] The rate of HBeAg seroconversion increased to 29% after 2 years and 43% after 3 years of treatment. In HBeAg negative patients, serum HBV DNA of less than 10^3 copies/mL and normalization of ALT were observed in 51% and 72%, respectively, after 1 year of ADV.[154] After 5 years of therapy, the serum HBV DNA were less than 10^3 copies/mL in 67% of patients, and ALT level normalized in 69%. The reported incidence of ADV resistance is 0% after 1 year, 3% after 2 years and 29% after 5 years of antiviral therapy (Fig. 1).[154] The primary mutations associated with ADV resistance are rtN236T and rtI233V in the D domain and rtA181V in the B domain of HBV polymerase. In comparison with more than 100-fold decrease in sensitivity to LVD associated with the two primary mutations, the rtN236T mutation confers only a 5–10-fold decrease in sensitivity to ADV *in vitro*,[155] which may explain the delayed emergence of this mutant.

In LVD-resistant patients treated with ADV monotherapy, the rate of antiviral resistance was 6–18% after