indeterminate genotype were found in 15 (50.0%), three (10.0%), 11 (36.7%) and zero (0%) of the 30 patients with acute liver diseases, and six (3.1%), 63 (32.8%), 101 (52.6%) and 21 (11.0%) of the 192 patients with chronic liver diseases, respectively. In contrast, one each of the patients (1.0%) with acute (case 1) and chronic (case 2) liver diseases had a HBV genotype other than A, B or C. The demographic and clinical features of the two patients were as follows.

A 27-year-old bisexual man (case 1) working in the adult entertainment industry was diagnosed as having acute hepatitis caused by HBV, and the genotype of the infecting HBV strain was identified as genotype H by the PCR-INVADER method. He received highly active antiretroviral therapy because of co-infection with HIV, and the serum HBV DNA titers decreased to less than the detectable level, with positivity for serum anti-HBs antibody developing 25 months later.

A 57-year-old man (case 2) was diagnosed as having chronic hepatitis caused by HBV, and the infecting HBV strain was classified as genotype F by the PCR-INVADER method, despite the genotype being classified as indeterminate by the EIA method. His deceased father had lived in Brazil in his youth and his elder brother had been diagnosed as being a HBV carrier at another hospital. He received oral entecavir at a daily dose of

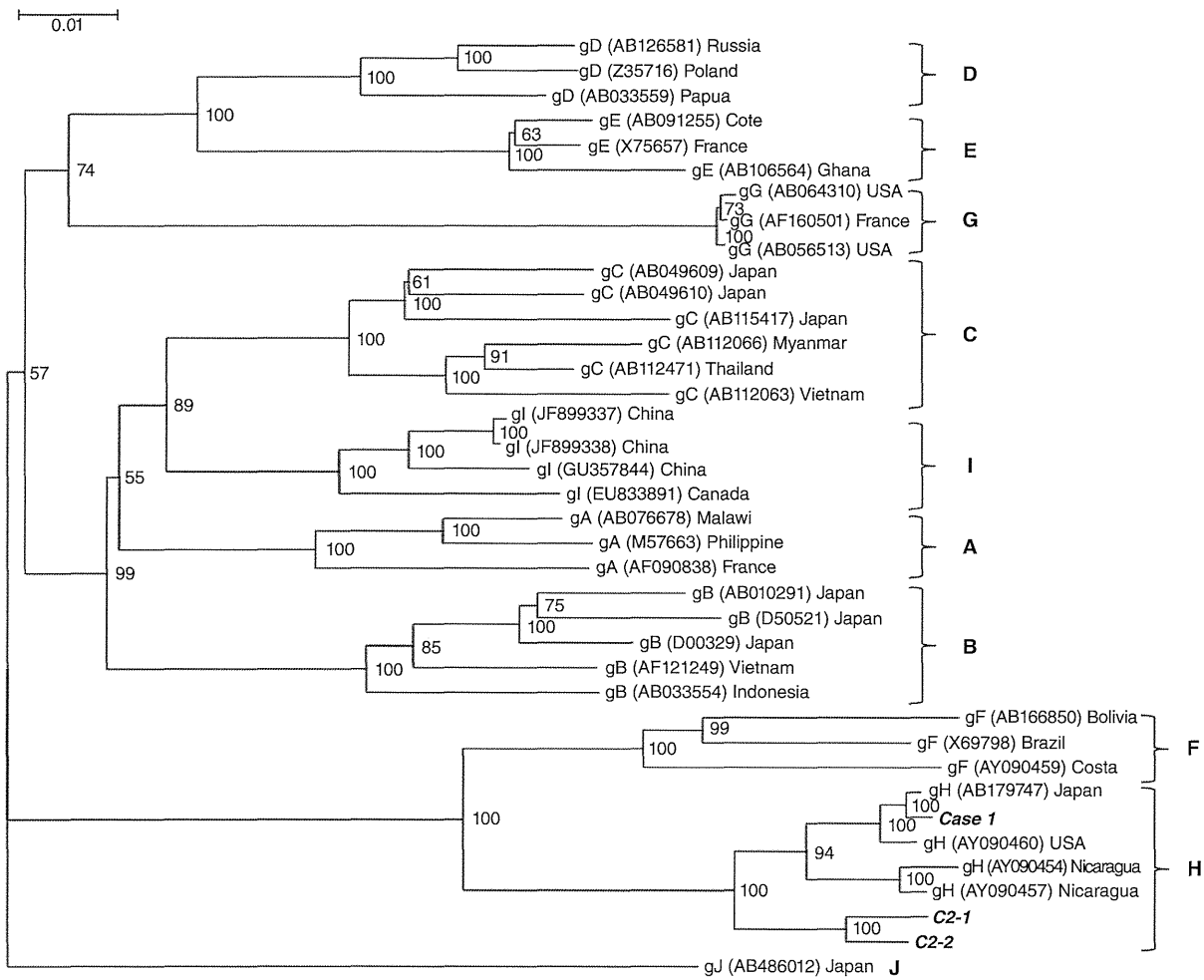


**Figure 1** A phylogenetic tree constructed based on the full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 and case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance.

0.5 mg, and the serum HBV titers decreased from 5.3 log copies/mL to a level less than 2.1 log copies/mL by 3 months of treatment.

## Full-length nucleotide sequences of the isolated HBV strains that were different from genotypes A, B and C

The nucleotide sequences of the HBV strains isolated from cases 1 and 2 were analyzed. A phylogenetic tree constructed based on the full-length sequence of HBV genome led to classification of the HBV strain isolated from case 1 as genotype H, showing an overall identity of 99.8% (3210/3215 bp) to the Thailand strain of genotype H (EU498228) (Figs 1,2). A similar analysis using a phylogenetic tree led to classification of the HBV strain isolated from case 2 as genotype H (Figs 1,3) despite it being classified as indeterminate and genotype F by EIA and PCR-INVADER assay, respectively. The full-length nucleotide sequence analysis showed an overall identity of 97.1% (3125/3218 bp) to genotype H strain isolated from a patient in Mexico (AB375164).

The nucleotide sequence of the HBV strains isolated from case 2 was further analyzed depending on the ORF, because the identity of the full-length nucleotide sequences to that of previously reported strains was less in case 2 than that in case 1. Consequently, the nucleotide sequence between DR2 (1590–1600 nt) and DR1 (1824–1834 nt) in the X region showed a similarity to that of the corresponding region of a genotype B strain isolated in Malaysia (JQ027316) and Indonesia (JQ429079), with identities of 98.4% (241/245 bp) and 98.0% (240/245 bp) (Fig. 4a). Moreover, analysis of the nucleotide sequence between 2023 and 2262 nt in the precore/core regions revealed that several different clones existed as quasispecies among HBV strains isolated from case 2, and two major clones, C2-1 and C2-2, were separated following cloning and sequencing of whole-genome nucleotides. Both C2-1 and C2-2 clones

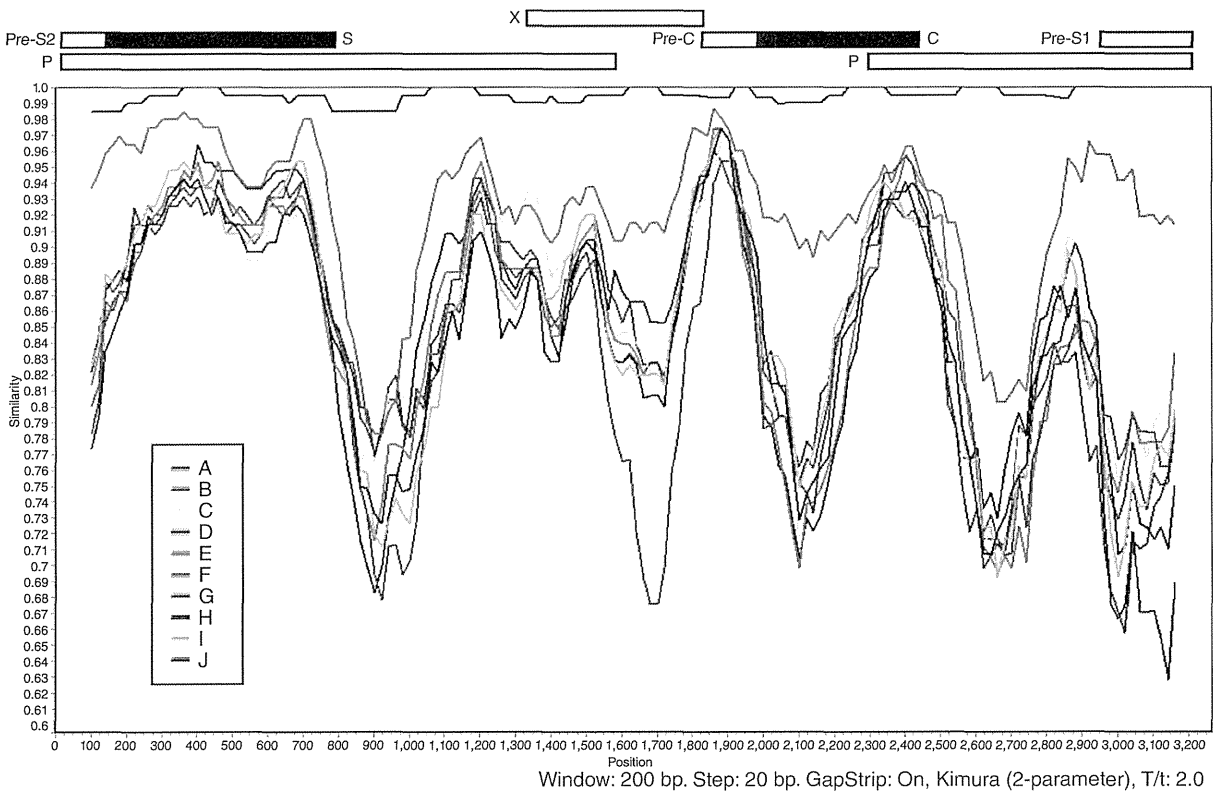


**Figure 2** Nucleotide similarity comparison of a full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size and gap-stripped alignments).

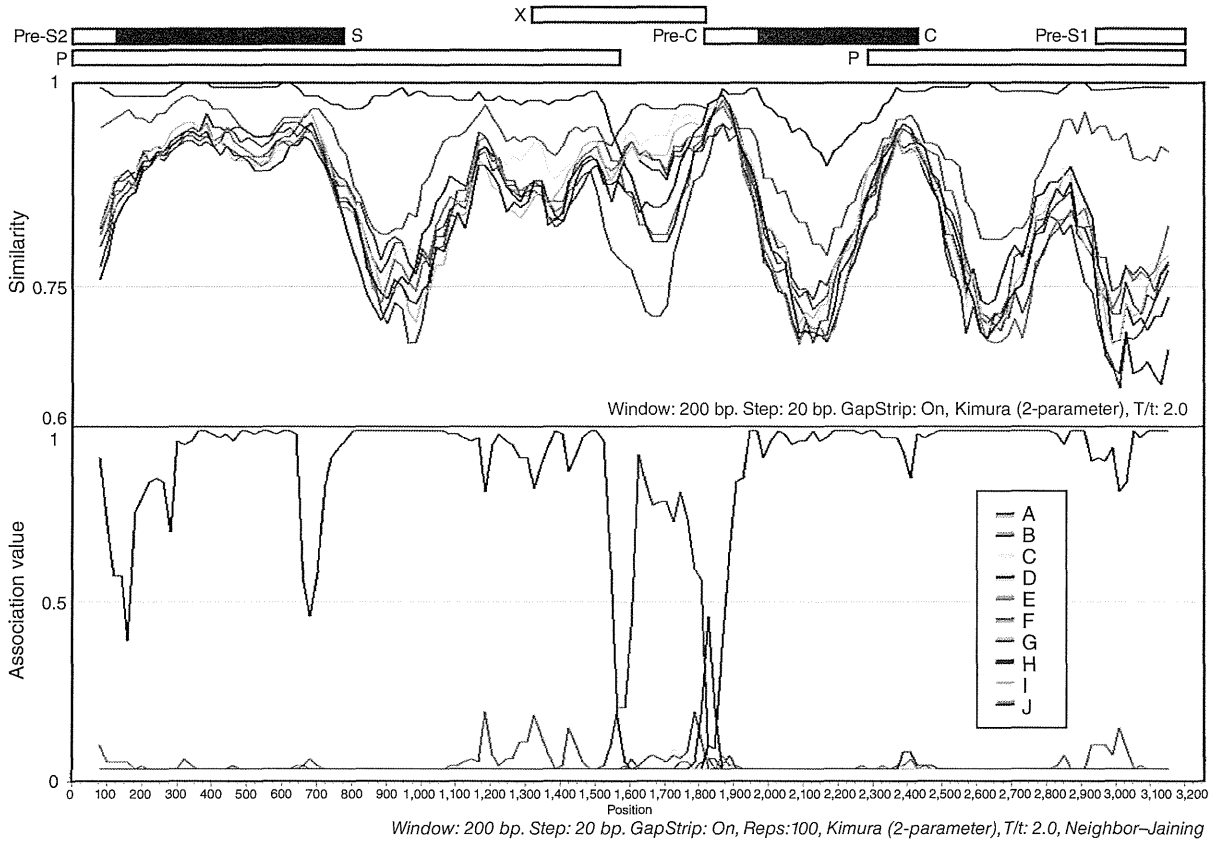


**Figure 3** Nucleotide similarity comparison of the full-length sequence of the hepatitis B virus (HBV) strain isolated from case 2 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size, 100 bootstrap replicates, gap-stripped alignments and neighbor-joining algorithm).

were classified as genotype H according to full-length nucleotide sequence analysis, with an identity of 96.4% to 95.8% to each other, and as genotype B based on analysis of the nucleotide sequence between DR2 and DR1, with an identity of 96.9% to 95.8%, respectively. However, the nucleotide sequence between 2023 and 2262 nt in the precore/core regions showed no similarity to that of any previously reported HBV strains. In these regions, the C2-1 and C2-2 clones showed nucleotide sequences with an identity of 98.6% to each other, and the nucleotide divergences in comparison to strains of genotypes A–J ranged 9.6–30.0% in the C2-1 clone and 8.1–28.5% in the C2-2 clone (Table 1). A phylogenetic tree constructed based on these regions revealed that both strains may be classified into the novel cluster of HBV (Fig. 4b). Also, the amino acid sequence divergences from previously reported HBV strains ranged from 18.1% to 27.9% in the C2-1 clone and 17.1% to 26.9% in the C2-2 clone.

The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession number AB818694 for case 1, AB819065 for the C2-1 and AB819066 for the C2-2 strain.

## DISCUSSION

IN THE PRESENT paper, the genotypes of the HBV strains isolated from 222 patients with acute and chronic hepatitis B were evaluated by EIA and/or PCR-INVADER assay, and HBV genotype A strains, commonly isolated in Africa, Europe and India, were found in 9.4% of the patients; genotype A strains were isolated from 50.0% of patients with acute liver diseases and 3.1% of patients with chronic liver diseases. These values were almost in line with those reported from other institutions in Japan.[11–13] HBV genotype A strains are known to be frequently isolated from patients with

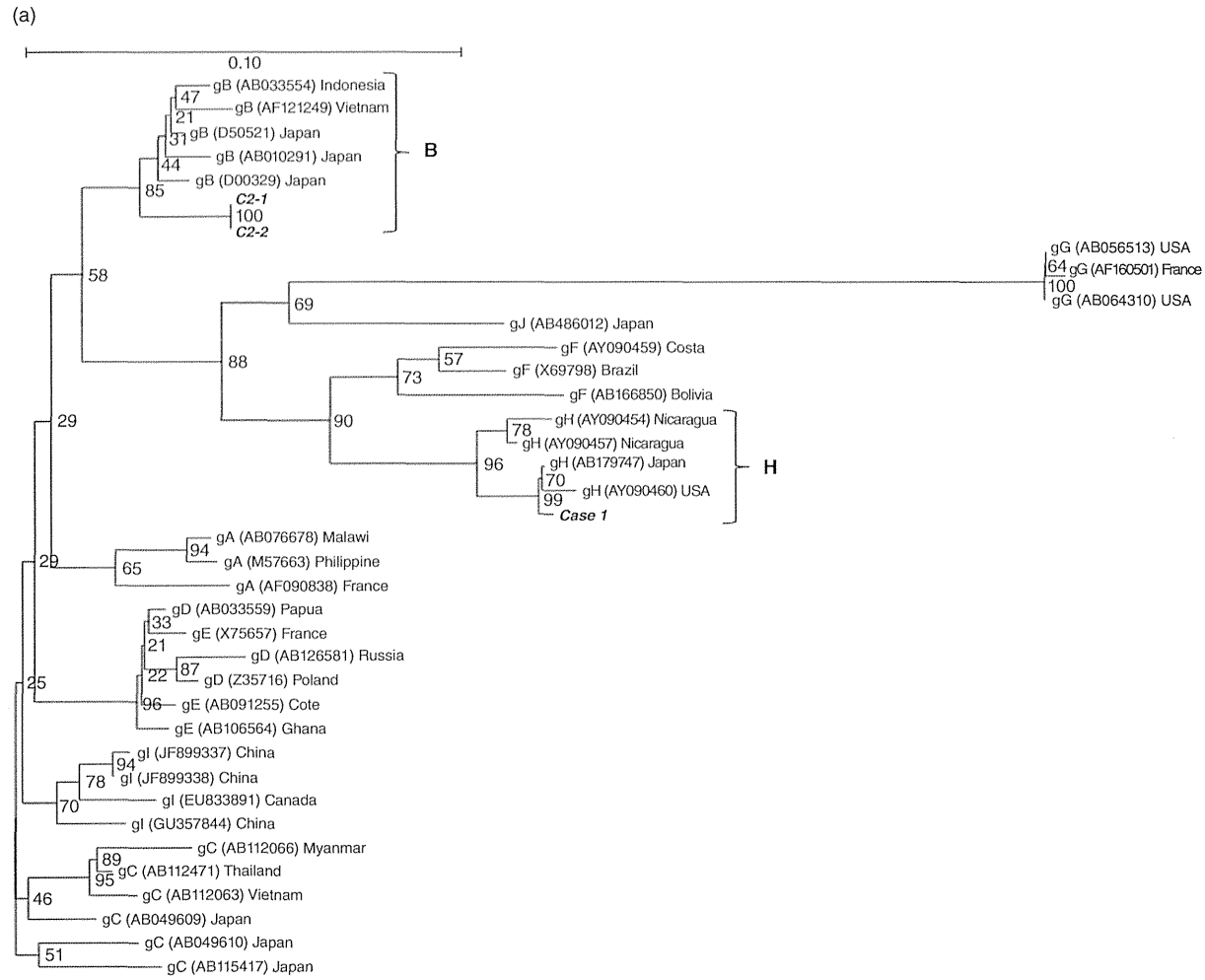


**Figure 4** A phylogenetic tree constructed based on the sequence of the hepatitis B virus (HBV) strain isolated from case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance. The regions included in the analysis were: (a) nucleotide sequence between DR2 (1590 nt) and DR1 (1834 nt) in the X region, (b) between 2023 and 2262 nt in the precore/core region.

acute liver diseases caused by HBV, especially in urban areas as compared to the countryside,[29] suggesting that globalization and diversification of the sex industry may change the distribution pattern of the HBV genotypes in Japan, including in Saitama Prefecture, the area around our institution.

To our surprise, HBV genotype H strains, which are mainly prevalent in Central America, were isolated from two patients, one each with chronic and acute liver diseases. The HBV strain isolated from the patient with acute liver disease (case 1) showed a nucleotide sequence with 99.8% identity to the Thailand strain (EU498228), which has recently been reported to be isolated from Japan as well as Central America.[30] Considering that case 1 was a bisexual male with HIV co-infection contracted as a result of sexual activities with a number of unspecified Japanese partners, the HBV strain isolated from this patient may be resident in Japanese persons engaging in unusual sexual activities. On the other hand, HBV genotype A strains, especially the genotype A2/Ae strain, have been isolated increasingly frequently from patients with HBV and HIV co-infection.[31] These observations prompted us to postulate that HBV genotype H strains as well as genotype A

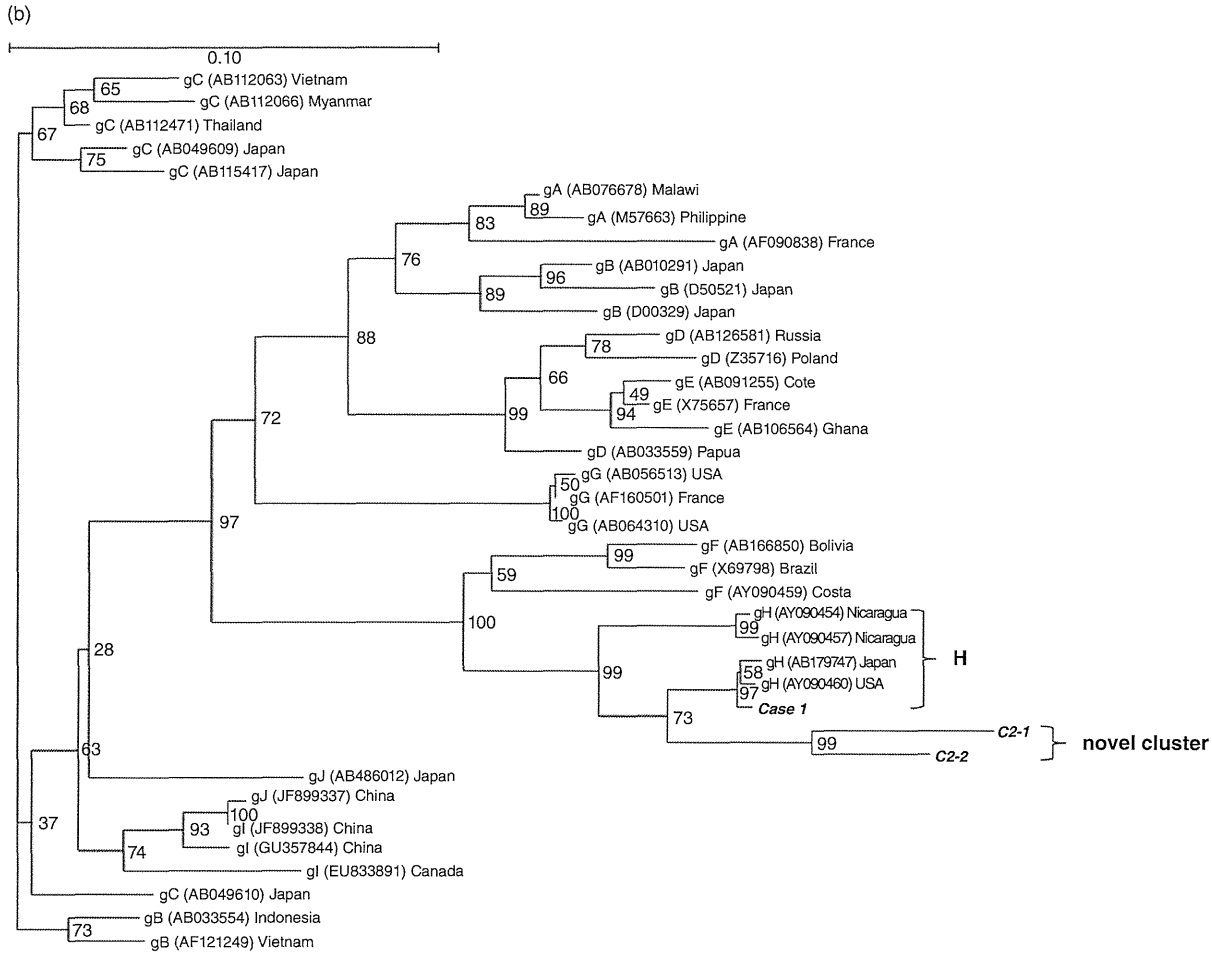


Figure 4 *Continued*

strains seem to spread among Japanese persons with unusual sexual habits. Previously, Tanaka *et al.* reported a HIV-infected patient in whom co-infection of both HBV genotype H and G strains was observed.[32] In case 1, however, co-infection of HBV genotype G strain was not detected.

It is noteworthy that HBV genotype H strains were isolated even from a Japanese patient with chronic liver disease (case 2), which showed recombination with a genotype B strain. The recombination breakpoint was estimated at positions 1590 and 1834 nt, located between DR2 and DR1 in the X region (Fig. 5): the nucleotide sequence in the X region of this strain showed an identity of 97.2% to that of genotype B strains in Malaysia (JQ027316) and Indonesia (JQ429079) despite the full-length nucleotide sequence showing 97.1% identity to a genotype H strain isolated from Mexico (AB375164). In the present study, nucleotide sequences were analyzed using two fragments (WA2 and gN2), suggesting that the possible recombination points exist in the overlapping regions of both fragments. However, the possibility that both genotypes B and H HBV strains existed as quasispecies in case 2 was neglected, because the sequences of the overlapping regions (1702–1780 and 1908–2081 nt) showed 100% identity between WA2 and gN2 fragments. It is well known that a HBV genotype B2/Ba strain, widely prevalent in Asian countries, shows nucleotide sequences identical to genotype C strains in the precore/core region due to the inter-genotype recombination

**Table 1** Percentages of differences in the nucleotide and amino acid sequences of hepatitis B virus (HBV) strains isolated from case 2 (C2-1 and C2-2) and representative strains of genotypes A–J HBV

| | | Percentages of differences to representative HBV strains of genotypes | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A (3) | B (5) | C (6) | D (3) | E (3) | F (3) | G (3) | H (4) | I (4) | J (1) |
| C1-1 | Nucleotide | 25.9–30.0 | 25.6–28.6 | 24.4–26.9 | 26.9–29.6 | 28.5–29.8 | 17.6–17.9 | 26.2–26.7 | 9.6–13.0 | 24.8–26.5 | 26.1 |
| | Amino Acid | 18.6–25.7 | 21.3–25.1 | 23.8–27.9 | 22.8–25.5 | 24.2–25.7 | 18.1–18.2 | 22.8–24.2 | 18.1–19.4 | 22.7–27.3 | 24.6 |
| C2-2 | Nucleotide | 24.4–28.5 | 24.1–27.1 | 22.9–25.4 | 25.4–28.1 | 27.0–28.3 | 16.1–16.4 | 24.7–25.2 | 8.1–11.5 | 23.3–25.0 | 24.6 |
| | Amino Acid | 17.6–24.7 | 20.3–24.1 | 22.8–26.9 | 21.8–24.5 | 23.3–24.7 | 17.1–17.2 | 21.8–23.3 | 17.1–18.4 | 21.7–26.3 | 23.6 |

Values in parenthesis indicate the number of HBV strains.

between B and C strains.[33] Also, HBV strains developing as a consequence of the inter-genotype recombination between A and D, A and E, A and C, C and D, and C and G have been reported from Africa, Vietnam, Tibet and Thailand.[34–37] Moreover, recombination among HBV strains of the same genotype, the so-called intra-genotype recombination, has been proposed to occur especially in HBV genotype A, D, F and H strains.[38] However, HBV genotype H strains showing recombination with other genotype strains have not ever been reported. Considering the fact that the father of case 2 had lived in Brazil in his youth, the sequences of genotype H in case 2 strains might have originated in Brazilian strains. In Brazil, genotypes A and D HBV strains are predominantly distributed with frequencies of 49.5% and 24.3%, respectively, while genotype B HBV strains are only 2.9%.[39] Thus, the recombination event with the genotype B HBV strain might have developed following the emigration of his father to Japan. To clarify the area and era in which the recombination developed, the full-length nucleotide sequence of the HBV strain isolated from the elder brother of case 2 needs to be evaluated, but, unfortunately, the brother, receiving medical examination at another institution, rejected further viral genome analysis.

Although the mechanisms involved in the development of inter-genotype and intra-genotype recombination of the HBV genomes remains unclear, several observations reported in previous publications prompted us to postulate the "non-random pathway"; DR1 (1830 nt) in the X gene, a possible origin of viral replication, is considered to be a hot spot that may be responsible for recombination of HBV genomes among different strains.[40,41] Hino *et al.* reported, based on *in vitro* recombination assay, that HBV DNA fragments containing the region spanning DR1 increased the recombination events reproducibly in the presence of extracts from actively dividing HCC cells.[40] Also, Pineau *et al.* revealed that the integration sites of covalently closed circular HBV DNA were usually located in the nucleotide sequence between 1600 and 2000 nt, when the HBV genomes chromosomally integrated in the host genomes were evaluated in human HCC tissues.[41] These *in vitro* and *in vivo* observations were consistent with the results obtained from the analysis of the HBV strains isolated from case 2, showing that the genome of the HBV genotype B strains were integrated in that of the HBV genotype H strain between DR2 and DR1.

Hepatitis B virus strains isolated from case 2 were classified as quasispecies in accordance with the nucleotide sequence between 2023 and 2262 nt in the precore/

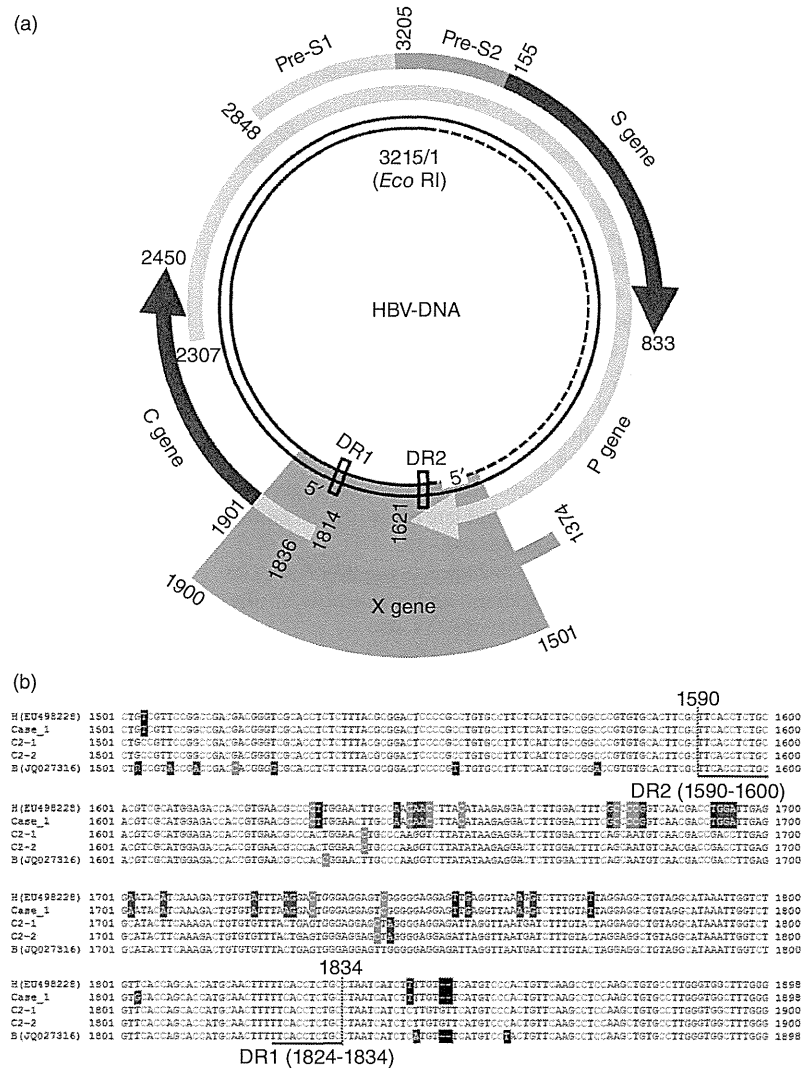


**Figure 5** Hepatitis B virus (HBV) genome and the open reading frame. (a) The sequence region (shaded in red) includes the recombination breakpoint at position 1590 and 1834 nt, located between DR2 and DR1 in X region. (b) Nucleotide alignments over the sequences spanning 1501–1900 nt in case 1, C2-1, C2-2 and reference strains of HBV genotype H (accession no. EU498228) and B (JQ027316). Dashed lines at 1590 and 1834 nt represent the recombination breakpoint.

core regions. Thus, the nucleotide sequences were analyzed following cloning of the HBV genome, and two major clones, C2-1 and C2-2, were isolated. Neither clone showed any similarity to any of the previously reported strains in the precore/core regions, and a phylogenetic tree constructed based on these regions revealed that these strains may be classified into the novel cluster of HBV; sequence divergences of nucleotides in the range of 8.1–30.0% and of amino acid in the range of 17.1–27.9% as compared to previously reported genotype A–J strains. The possibility that intergenotype recombination of the HBV genome between H and B strains may provoke mutation of the nucleotide sequence in the precore/core regions leading to development of a possible novel genotype HBV strain needs to be evaluated in the future.

In conclusion, HBV genotype H strains, which are prevalent in Central American countries, were isolated from Japanese patients with chronic as well as acute liver diseases. HBV strains isolated from the chronic liver disease patient showed recombination of the genome between genotype H and B strains, and no similarity was found in the nucleotide sequences of the precore/core regions in comparison with those of the previously reported HBV strains. Thus, globalization may promote development of a possible novel genotype of HBV through recombination between Central American and East Asian strains.

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## SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

**Table S1** Hepatitis B virus DNA-specific oligonucleotide primers used in the study.

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REVIEW ARTICLE

# A proposal for management of rheumatic disease patients with hepatitis B virus infection receiving immunosuppressive therapy

**Masayoshi Harigai · Satoshi Mochida · Toshihide Mimura · Takao Koike · Nobuyuki Miyasaka**

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**Abstract** Reactivation of hepatitis B virus (HBV) and de novo HBV hepatitis in patients with rheumatic diseases given intensive and long-term immunosuppressive therapy with or without biological disease-modifying antirheumatic drugs is of great concern, especially in regions where the virus is endemic, including Japan. To ascertain a better benefit–risk balance for immunosuppressive therapy for patients with rheumatic diseases, the Japan College of Rheumatology developed this proposal. All patients with rheumatic diseases commencing immunosuppressive therapy should be screened for hepatitis B surface antigen (HBsAg); those who are negative for HBsAg should be screened for hepatitis B core antibody (HBcAb) and hepatitis B surface antibody (HBsAb) as well. HBV carriers and serum HBV DNA positive patients with resolved infection should receive nucleoside analog as soon as possible, prior to commencing immunosuppressive therapy. For serum HBV DNA negative patients with resolved infection, careful monthly monitoring using serum levels of aspartate and alanine aminotransferases and HBV DNA is recommended during and at least 12 months after withdrawal of immunosuppressive therapy. If serum HBV DNA becomes positive, patients should receive nucleoside analog treatment as soon as possible, while ongoing immunosuppressive therapy should be continued to avoid severe or fulminant hepatitis development. To facilitate proper management of patients with HBV infection, collaboration between rheumatologists and hepatologists is strongly encouraged.

**Keywords** Hepatitis B virus · Reactivation · Rheumatic diseases · Immunosuppressive therapy · Glucocorticoid

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## Introduction

Epidemiological data have indicated that about 350 million people worldwide (6 % of the world population) are infected with hepatitis B virus (HBV) and that 200 million of those live in Asian countries [1, 2]. Previous studies estimated that the number of HBV carriers who are positive for hepatitis B surface antigen (HBsAg) in Japan is 1.0–1.5 million and that 23.2 % of the total Japanese population has been previously infected with HBV [3, 4]. Once hepatocytes are infected with HBV, replication-competent covalently closed circular DNA (cccDNA) is formed in the nuclei of the infected hepatocytes during the viral

replication process. The cccDNA serves as the main template for transcription of viral pregenome RNA, as well as messenger RNA (mRNA), and persists permanently in the cells [5]. Hence, HBV carriers and patients with resolved HBV infection [HBsAg negative and hepatitis B core antibody (HBcAb) and/or hepatitis B surface antibody (HBsAb) positive] are equivalent in terms of the presence of replication-competent HBV genome in their hepatocytes. Chemotherapy- or immunosuppressive therapy-associated immunosuppressed status may increase the risk for reactivation of HBV, both in patients who are HBV carriers and in patients with resolved HBV infection. Hepatitis following viral reactivation in patients with resolved HBV infection is called "de novo HBV hepatitis" and often leads a fatal and fulminant course, especially in patients with malignant lymphoma given chemotherapy containing rituximab, i.e., anti-CD20 chimeric antibody, and corticosteroids [6, 7].

Recent advances in treatment for rheumatoid arthritis (RA) have improved outcomes for patients. In Japan, six biological disease-modifying antirheumatic drugs (DMARDs) have been approved for RA since 2003, and the maximum approved dosage of methotrexate for RA was increased to 16 mg/week in February 2011. Together with these changes in medications, goal-oriented early aggressive therapy has been introduced in clinical practice, aiming at remission and maintenance of remission of the disease [8]. Similar therapeutic strategies have also been introduced for other rheumatic diseases, such as systemic lupus erythematosus and systemic vasculitides [9, 10]. As a result, patients with rheumatic diseases receive intensive remission-induction treatment with long-term maintenance therapy using corticosteroids, immunosuppressants, and/or biological DMARDs, which have potential risk for reactivation of HBV and de novo HBV hepatitis. During the past few years, several investigators reported reactivation of HBV in patients with rheumatic diseases given biological DMARDs, especially tumor necrosis factor inhibitors [11–16]. Development of HBV reactivation and fatal fulminant hepatitis was also reported in patients with rheumatoid arthritis given low-dose methotrexate [17–19]. These data strongly suggest that appropriate screening for HBV infection and monitoring for reactivation in HBV-infected patients are mandatory in rheumatology clinical practice (Fig. 1).

In this proposal we summarize epidemiological data on reactivation of HBV in Japan and in patients with rheumatic diseases. Based on the latest evidence and expert opinions, we indicate methods of proper management for HBV-infected patients with rheumatic diseases who will receive immunosuppressive therapy. The diagnosis of HBV infection and prophylaxis of reactivation are in accordance with the "Guidelines for prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B virus infection" that were jointly developed by the Intractable Hepatobiliary Disease Study Group of Japan and the Study Group for the Standard Antiviral Therapy for Viral Hepatitis in the Health and Labour Sciences Research [20, 21]. This proposal is subject to changes as advances occur in research in this and related medical fields. This proposal was originally published in Japanese on the website of the Japan College of Rheumatology on September 6, 2011, and revised on October 17, 2011 and September 5, 2012.

## Fulminant hepatitis and late-onset hepatic failure in Japan

Since 1998, the Intractable Hepatobiliary Diseases Study Group of Japan has conducted an ongoing nationwide annual survey for fulminant hepatitis and late-onset hepatic failure (LOHF). By 2009, 1,186 patients with these hepatic disorders [19–21], 39 % of which were HBV related, had been accumulated. In Japan, HBV-related acute liver failure is classified into transient infection, acute exacerbation in HBV carrier, and indeterminate infection patterns; de novo HBV hepatitis due to viral reactivation in patients with resolved HBV infection is classified as one of the subgroups of acute exacerbation in HBV carriers [22]. According to this classification, the causes of HBV-related fulminant hepatitis and LOHF in Japan are transient infection (55 %), acute exacerbation in HBV carrier including reactivation in patients with resolved infection (35 %), and indeterminate infection pattern (10 %) [19–21]. The percentage of HBV carriers who developed fulminant hepatitis or LOHF gradually decreased from 1998 to 2004, but increased again in and after 2005 due to the increased number of patients with viral reactivation in resolved HBV infection [23–25]. Of 488 patients who developed fulminant hepatitis or LOHF during 2004 and 2009, 194 (40 %) were HBV related; causes of these infections were transient infection in 91 (47 %), acute exacerbation in HBV carrier including reactivation in patients with resolved infection in 72 (37 %), and indeterminate infection pattern in 31 (16 %). Among the 72 patients classified into acute exacerbation in HBV carrier, the investigators identified 17 patients with reactivation of HBV in patients with resolved infection; these patients had been initially classified as HBV carriers showing acute hepatitis exacerbation. Thirteen of these 17 patients were treated with rituximab-containing regimens, but some received other chemotherapy or immunosuppressive therapy. All of these patients died, pointing to an extremely unfavorable prognosis [21]. Although the "Guidelines for prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B virus infection" were first published in 2009 [26], more recent data from the Intractable Hepatobiliary Diseases Study Group of Japan

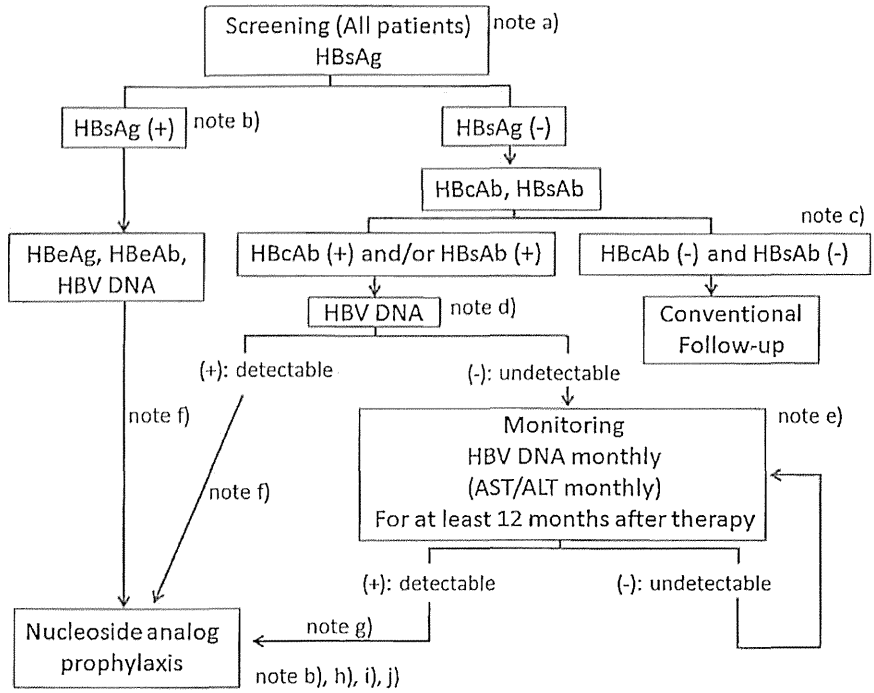


**Fig. 1** Algorithm for screening and management of hepatitis B virus infection in patients with rheumatic diseases. All patients with rheumatic diseases who start immunosuppressive therapy should be screened for hepatitis B virus (HBV) infection using this algorithm. HBV carriers or patients with resolved HBV infection should be managed accordingly. Notes: *a* All patients with rheumatic diseases commencing immunosuppressive therapy should be screened for HBsAg. Those who are negative for HBsAg should be screened for HBcAb and HBsAb as well to identify patients with resolved infection. Chemiluminescent immunoassay/chemiluminescent enzyme immunoassay (CLIA/CLEIA) is highly recommended to measure HBsAg, HBcAb, and HBsAb. *b* HBsAg positive patients are subject to consultation with a hepatologist. Consultation with a hepatologist is desirable in all patients subject to administration of nucleoside analog. *c* Detection of serum HBV DNA is desirable in those patients who have previously received immunosuppressive therapy and have no results of HBcAb and HBsAb before the start of the therapy. *d* Detection by real-time polymerase chain reaction (PCR) method (Taq-Man™ PCR method) is recommended. *e* Patients receiving rituximab plus corticosteroid combination therapy for malignant lymphoma or patients receiving hematopoietic stem cell transplantation are at particular risk for HBV reactivation and deserve careful attention. *f* Prophylactic nucleoside analogs should be started as soon as possible before starting immunosuppressive therapy. *g* Nucleoside analogs should be administered immediately when HBV DNA becomes positive during and after immunosuppressive therapy. *h* Entecavir is recommended as the nucleoside analog. HBV DNA is monitored monthly during administration of nucleoside analogs. *i* Criteria for discontinuation of nucleoside analog treatment are described in the text. *j* Patients should be closely observed for 12 months after treatment with nucleoside analogs as described in the text. Nucleoside analog should be readministered immediately when HBV DNA becomes positive during observation. *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *HBcAb* hepatitis B core antibody, *HBsAg* hepatitis B surface antigen, *HBsAb* hepatitis B surface antibody, *HBeAg* hepatitis B envelope antigen, *HBeAb* hepatitis B envelope antibody. Adapted and modified from Oketani et al. [21]

shows that an even larger number of patients developed reactivation from a resolved HBV infection status [24], indicating a necessity for broadened publicity of the guidelines among physicians of related specialties.

## Reactivation of HBV in patients with rheumatic diseases

The Health and Labour Sciences Research Group for "Clarification of current status for reactivation of hepatitis B virus associated with immunosuppressants and antineoplastics and establishment of the preventive measures" started a registry in 2009 for HBV-infected patients with solid cancers, hematopoietic malignancies, renal diseases, and rheumatic diseases [27, 28]. Rheumatic disease patients eligible for this study are those who are (1) positive for HBsAg, HBcAb or HBsAb, and (2) treated with corticosteroids (prednisolone equivalent dose $\geq$0.5 mg/kg body weight/day), immunosuppressive drugs or biological DMARDs approved in Japan, including infliximab, etanercept, adalimumab, tocilizumab, abatacept, and golimumab. As of March 2012, 127 patients from 19 medical institutions were enrolled in this study and were followed up according to the study protocol. An interim analysis of this prospective observation study found 11 of the 127 patients were HBV carriers; the remaining patients had resolved HBV infection. By the end of March 2012, nine patients with resolved

infection became positive for serum HBV DNA, two patients before and seven patients after commencing immunosuppressive therapy. Overall, 7.8 % of the 116 patients with resolved HBV infection had viral reactivation. All of these patients were successfully treated according to the guidelines developed by the Intractable Hepatobiliary Diseases Study Group of Japan [26], and none of them developed hepatitis.

## Screening for HBV infection

### Patients who should be screened for HBV infection

According to this proposal, all patients with rheumatic diseases who commence immunosuppressive therapy in clinical practice should be screened for HBV infection. At present, immunosuppressive therapy in this proposal includes moderate or high doses of corticosteroids, biological DMARDs, synthetic DMARDs with immunosuppressive potential, (e.g., methotrexate, tacrolimus, leflunomide, and mizoribine), and immunosuppressants (e.g., azathioprine, cyclophosphamide, cyclosporine A, and mycophenolate mofetil). Other immunosuppressants will be added to this list following their approval by the Japanese Ministry of Health, Labour, and Welfare.

### Recommended methods for screening

All rheumatic disease patients commencing immunosuppressive therapy should be screened for HBsAg. Those negative for HBsAg should be screened for HBcAb and HBsAb as well. Among various methods currently available for measurement of these HBV-associated antigens and antibodies, chemiluminescent immunoassay/chemiluminescent enzyme immunoassay (CLIA/CLEIA) is highly recommended because of its sensitivity and specificity. An assay system for HBsAg with even higher sensitivity is under development; application of such an assay system for clinical practice should be considered in the future. Rheumatologists are encouraged to consult hepatologists regarding HBV carriers and patients with resolved HBV infection with rheumatic diseases prior to commencing immunosuppressive therapy. Patients positive for HBsAb alone due to previous HBV vaccination are not subject to the following management.

## Management of high-risk patients with rheumatic diseases

### Management of HBV carriers

Hepatitis B envelope antigen (HBeAg), anti-HBe antibody, and serum HBV DNA should be measured for HBV carriers. The real-time polymerase chain reaction (PCR) method (Taq-Man™ PCR method) is highly recommended for quantification of HBV DNA in sera because of its high sensitivity and specificity. Analyses for genotype of HBV and precore and core promoter gene mutation may also be indicated.

HBV carriers should receive nucleoside analog as soon as possible prior to commencing immunosuppressive therapy and should be concurrently followed up by both rheumatologists and hepatologists. Entecavir hydrate, lamivudine, and adefovir pivoxil are currently approved nucleoside analogs in Japan. We recommend 0.5 mg of entecavir hydrate, once a day at fasting as a first choice because emergence of entecavir hydrate-resistant HBV variants has been reported at a very low rate [29–33]. Nucleoside analog treatment should be continued during and at least 12 months after withdrawal of immunosuppressive therapy with careful monitoring of patients using alanine aminotransferase, HBeAg, HBeAb, and serum HBV DNA [21, 34, 35]. If copy numbers of serum HBV DNA do not significantly decrease with nucleoside analog treatment, resistance to the drug is suspected and consultation with hepatologists is needed.

Discontinuation of nucleoside analog treatment is based on the status of serum viral markers: negative for HBeAg and positive for HBeAb, and low levels of HBV DNA, HBV core-related antigen, and HBsAg [36]. Consultation with hepatologists is recommended before discontinuing nucleoside analog treatment and for monitoring patients afterwards. Patients who discontinue nucleoside analog treatment should be strictly followed up for at least 12 months and restarted on the drug if serum HBV DNA levels increase.

### Management of patients with resolved HBV infection

Serum HBV DNA should be measured using the Taq-Man™ PCR method for patients with resolved HBV infection. If serum HBV DNA of a patient is positive (i.e., detectable with agarose gel electrophoresis or equal to or more than 2.1 log copies/ml), the patient should receive nucleoside analog treatment as soon as possible before commencing immunosuppressive therapy, as described for HBV carriers. Duration, monitoring, and discontinuation of nucleoside analog treatment for these patients are the same as those for HBV carriers. It should be mentioned that reactivation of HBV cannot be predicted by HBsAb titers at baseline or changes over time [21].

If serum HBV DNA levels in a patient are <2.1 log copies/ml and undetectable with agarose gel electrophoresis, careful monthly monitoring of patients using serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and HBV DNA is recommended during and for at least 12 months after withdrawal of immunosuppressive therapy. The median lag period

between elevation of serum HBV DNA and alanine aminotransferase levels was 18.5 weeks (range 12–28 weeks) [37]; starting nucleoside analog after the onset of hepatitis could not prevent progression to fatal hepatitis [21]. Therefore, if serum HBV DNA of a patient becomes positive, the patient should receive nucleoside analog treatment as soon as possible, as described above. Duration, monitoring, and discontinuation of nucleoside analog treatment for these patients are the same as those for HBV carriers. Ongoing immunosuppressive therapy should be continued to avoid restoration of host immunity against HBV, which may result in an immunological attack on infected hepatocytes and cause hepatitis.

## Differential diagnosis for patients with abnormal hepatic function test

If a patient with a rheumatic disease shows abnormal results of hepatic function tests during or after immunosuppressive treatment, major differential diagnoses include, in addition to reactivation of HBV, drug-induced liver disease, hepatic involvement of rheumatic diseases, alcoholic or nonalcoholic fatty liver disease, autoimmune liver diseases (e.g., autoimmune hepatitis and primary biliary cirrhosis), diseases of the bile duct and pancreas, acute hepatitis due to hepatitis A, B, C or E virus, acute hepatitis due to other viruses (e.g., *Epstein–Barr virus, cytomegalovirus, herpes virus, adenovirus, coxsackie virus, rubeola virus, rubella virus, human immunodeficiency virus*, and *parvovirus*), abnormal thyroid function, and other hepatic diseases, including malignancy.

## Points to consider for patients with rheumatic disease developing HBV reactivation or de novo hepatitis

### Reactivation of HBV or de novo HBV hepatitis in rheumatic disease patients without previous screening and monitoring for HBV

If reactivation of HBV or de novo HBV hepatitis develops in a patient with rheumatic disease who had not been screened or appropriately monitored for HBV infection, the patient should receive nucleoside analog as soon as possible and hepatologists should be consulted.

### Discontinuation and reintroduction of immunosuppressive therapy after reactivation of HBV or de novo HBV hepatitis

Discontinuation of immunosuppressive therapy in rheumatic disease patients with HBV reactivation or de novo HBV hepatitis should be carefully discussed with hepatologists because abrupt withdrawal of the therapy may induce severe or fulminant hepatitis. Based on currently available evidence and expert opinions, we recommend continuation of immunosuppressive therapy together with nucleoside analog treatment. Prospective observational studies are being implemented to address this issue in Japan [28]. For a patient who has successfully discontinued immunosuppressive therapy, benefit–risk balance should be carefully assessed before restarting immunosuppressive therapy for rheumatic diseases.

## Collaboration with board-certified hepatologists

In-house and regional collaborations between rheumatologists and hepatologists are encouraged and required to facilitate prompt and proper management of HBV carriers and rheumatic disease patients with resolved HBV infection. Lists of board-certified rheumatologists and board-certified hepatologists are available on the websites of the Japan College of Rheumatology (http://pro.ryumachi-net.com/index.php?option=com_content&view=article&id=49&Itemid=57) and the Japan Society of Hepatology (http://www.jsh.or.jp/specialist/list.html).

## Summary

Reactivation of HBV and subsequent de novo HBV hepatitis are preventable serious adverse events associated with immunosuppressive therapy for patients with rheumatic diseases. Before starting immunosuppressive therapy, it is highly recommended that all patients be thoroughly screened for current and resolved HBV infection according to the procedures described in this proposal. HBV carriers and patients with resolved HBV infection who are positive for serum HBV DNA should be treated with nucleoside analog prior to commencing immunosuppressive therapy. Close monitoring for reactivation of HBV is necessary for prompt intervention with nucleoside analog to mitigate subsequent hepatitis. Collaboration with hepatologists is encouraged and required to facilitate these management processes for patients with rheumatic diseases infected with HBV.

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ORIGINAL ARTICLE

# Cellular senescence checkpoint function determines differential Notch1-dependent oncogenic and tumor-suppressor activities

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Notch activity regulates tumor biology in a context-dependent and complex manner. Notch may act as an oncogene or a tumor-suppressor gene even within the same tumor type. Recently, Notch signaling has been implicated in cellular senescence. Yet, it remains unclear as to how cellular senescence checkpoint functions may interact with Notch-mediated oncogenic and tumor-suppressor activities. Herein, we used genetically engineered human esophageal keratinocytes and esophageal squamous cell carcinoma cells to delineate the functional consequences of Notch activation and inhibition along with pharmacological intervention and RNA interference experiments. When expressed in a tetracycline-inducible manner, the ectopically expressed activated form of Notch1 (ICN1) displayed oncogene-like characteristics inducing cellular senescence corroborated by the induction of G0/G1 cell-cycle arrest, Rb dephosphorylation, flat and enlarged cell morphology and senescence-associated β-galactosidase activity. Notch-induced senescence involves canonical CSL/RBPJ-dependent transcriptional activity and the $p16^{INK4A}$-Rb pathway. Loss of $p16^{INK4A}$ or the presence of human papilloma virus (HPV) E6/E7 oncogene products not only prevented ICN1 from inducing senescence but permitted ICN1 to facilitate anchorage-independent colony formation and xenograft tumor growth with increased cell proliferation and reduced squamous-cell differentiation. Moreover, Notch1 appears to mediate replicative senescence as well as transforming growth factor-β-induced cellular senescence in non-transformed cells and that HPV E6/E7 targets Notch1 for inactivation to prevent senescence, revealing a tumor-suppressor attribute of endogenous Notch1. In aggregate, cellular senescence checkpoint functions may influence dichotomous Notch activities in the neoplastic context.

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## INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is among the deadliest cancers known[1] and is a paradigm for the investigation of all types of squamous cell carcinomas (SCCs). Common genetic lesions associated with ESCC include p53 mutations, $p16^{INK4A}$ loss, cyclin D1 overexpression, epidermal growth factor receptor (EGFR) overexpression and telomerase activation.[2] Ectopically expressed telomerase (hTERT) or human papilloma virus (HPV) E6/E7 gene products immortalize human esophageal epithelial cells (keratinocytes) overcoming replicative senescence.[3,4] Oncogenes induce senescence in immortalized esophageal keratinocytes.[5–7] Senescence serves as a fail-safe mechanism to prevent oncogene-induced aberrant proliferation. In fact, malignant transformation of esophageal keratinocytes requires concurrent inactivation of the senescence checkpoint functions regulated by the p53 and Rb pathways to negate oncogene-induced senescence.[5,7–9]

The Notch pathway regulates cell fate and differentiation through cell–cell communication. The mammalian Notch family comprises four transmembrane receptor proteins (Notch1 to Notch4). Ligands (JAG1/2, DLL1, 3 and 4) bind Notch receptors through cell–cell contact to trigger γ-secretase-mediated proteolytic cleavage of Notch receptor proteins, resulting in nuclear translocation of the intracellular domain of Notch (ICN), the activated form of Notch. ICN of all Notch receptor paralogs forms a transcriptional activation complex containing a common transcription factor CSL (a.k.a. RBPJκ) and the coactivator Mastermind-like (MAML).[10] Notch1 target genes include the HES/HEY family of transcription factors, Notch3 and IVL, a marker of squamous-cell differentiation. Squamous-cell differentiation is impaired by *Notch1* loss, *CSL* loss or ectopic expression of dominant-negative MAML1 (DNMAML1) in the skin and esophagus in mice.[11–13]

The highly context-dependent nature of Notch functions adds complexity to its roles in cancers. Although Notch acts as an oncogene in T-cell acute lymphoblastic leukemia, both oncogenic and tumor-suppressor roles have been found in solid tumors even within identical tumor types.[14] Notch1 may be activated in SCCs.[15,16] The active form of Notch1 (that is, ICN1) transforms

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keratinocytes in concert with HPV E6/E7,[17,18] although Notch1 may be downregulated to sustain E6/E7 expression at the late steps of malignant transformation.[19] Multiple lines of evidence indicate a tumor-suppressor role of Notch in SCCs. They include loss-of-function mutations identified in primary SCCs, including ESCC[20–23] and tumor-prone phenotypes in genetically engineered mouse models targeting the Notch pathway.[24–30] By maintaining epidermal integrity and barrier functions, Notch may prevent the tumor-promoting inflammatory microenvironment in the skin.[30] It is unclear in what specific context Notch may act as an oncogene or a tumor suppressor in SCCs.

Notch1 is activated in vascular endothelial cells undergoing replicative senescence.[31,32] Although Notch1 has been implicated in cell-cycle arrest associated with squamous-cell differentiation,[12,33] it is unclear whether Notch1 induces or mediates senescence in cells of epithelial origin and how senescence may be linked to either the oncogenic or tumor-suppressor attributes of Notch1. Herein we investigated the functional consequences of Notch1 activation and inhibition in esophageal keratinocytes and ESCC cells, revealing unique interactions between Notch1 and cellular senescence checkpoint functions via transforming growth factor (TGF)-β signaling, which may influence dichotomous Notch1 functions in SCCs and other cancers.

## RESULTS

### Notch1 is activated in human esophageal keratinocytes undergoing replicative senescence

The role of Notch1 in senescing epithelial cells remains unknown. We examined Notch1 in well-characterized primary human esophageal keratinocytes EPC2, which undergo replicative senescence by 40–44 population doublings[3] with an increased doubling time (Figures 1a and b). The activated form of Notch1 (ICN1$^{Val1744}$) was upregulated at 43 population doubling in cells with senescent characteristics corroborated by Rb dephosphorylation, upregulation of p53, p16$^{INK4A}$ and p21 (CDKN1A), flat and enlarged cell morphology and the increased senescence-associated β-galactosidase (SABG) activity (Figures 1c–e). Pharmacological Notch inhibition by a γ-secretase inhibitor (GSI) suppressed ICN1$^{Val1744}$ and antagonized the above changes (Figure 1), suggesting that Notch1 may regulate replicative senescence in keratinocytes.

### ICN1 induces senescence via canonical CSL-dependent transcription

To delineate the functional consequences of Notch1 activation, we used the tetracycline-inducible system to express ICN1 ectopically. Doxycycline (DOX) induced ICN1 within 24 h to activate its downstream molecules, including *HES5* and *Notch3*, in a dose-dependent manner in EPC2-hTERT, a telomerase-immortalized EPC2 derivative (Figures 2a and b; Supplementary Figure S1a). ICN1 induced p16$^{INK4A}$, p21 and Rb dephosphorylation as a function of time to inhibit cell proliferation, leading to G0/G1 cell-cycle arrest (Figures 1b–d). Senescence was suggested by flat and enlarged cell morphology and DOX dose-dependent SABG induction (Figures 1e and f). ICN1 also induced its target genes and senescence in human esophageal keratinocytes EPC1 and its derivative EPC1-hTERT; however, p16$^{INK4A}$ was not detectable in the latter (Supplementary Figures S1b and S2), suggesting p16$^{INK4A}$ loss in EPC1 during telomerase-induced immortalization.[34] Of note, DOX alone did not induce senescence in parental cell lines nor in those carrying a control vector (data not shown), indicating that DOX *per se* did not induce senescence.

We next conducted RNA interference (RNAi) experiments to determine the role of canonical CSL. CSL knockdown prevented ICN1 from activating CSL-dependent transcription, allowing continued cell proliferation with antagonized Rb dephosphorylation and decreased SABG activation in EPC2-hTERT and EPC1-hTERT cells (Figure 3 and Supplementary Figure S3), suggesting that CSL-dependent transcription may mediate ICN1-induced senescence.

### ICN1-induced senescence may be impaired in transformed human esophageal cells

Malignant transformation may involve inactivation of the cellular senescence checkpoint functions, serving as a fail-safe mechanism against oncogene activation. We asked whether ectopically expressed ICN1 induces senescence in transformed human esophageal cells EPC2-T, EN60 and TE11. EPC2-T is a derivative of EPC2-hTERT carrying *EGFR*, *cyclin D1* and *p53$^{R175H}$* transgenes and that has been further modified to express either DNMAML1, a genetic pan-Notch inhibitor, or green fluorescent protein (GFP) as a control.[35] The p14$^{ARF}$-p53 and p16$^{INK4A}$-Rb pathways are compromised in EN60 cells carrying HPV *E6* and *E7*,[4] which target p53 and Rb for degradation or sequestration, respectively. TE11 cells show biallelic *p53* inactivation[36] and *INK4A* deletion.[37]

ICN1 activated CSL-dependent transcription in EPC2-T that is inhibited by DNMAML1 (Supplementary Figures S1c and S4b). Likewise, CSL knockdown prevented ICN1 from activating CSL-dependent transcription and SABG induction in EPC2-T cells (data not shown). In the absence of DNMAML1, ICN1 induced p16$^{INK4A}$ and Rb dephosphorylation to inhibit cell proliferation (Supplementary Figures S4b–e). Interestingly, the extent of ICN1-mediated SABG induction was limited in EPC2-T cells (40–50%) without DNMAML1 (Supplementary Figures S4d and e) as compared with parental EPC2-hTERT cells (60–80%) (Figure 2).

When tested in EN60 and TE11, ICN1 activated CSL-dependent transcription and induced Notch target genes; however, ICN1 affected little, if any, Rb phosphorylation, cell proliferation or SABG activity (Supplementary Figure S5), suggesting that oncogenic genetic alterations that limit cellular senescence checkpoint functions may suppress ICN1-induced senescence without affecting CSL-dependent transcriptional activity.

### The p16$^{INK4A}$-Rb pathway may have a regulatory role in ICN1-induced senescence

We next asked how the p14$^{ARF}$-p53 and p16$^{INK4A}$-Rb pathways may influence ICN1-induced senescence. Notch can either activate or inhibit p53 in a context-dependent manner.[38] p53 and p14$^{ARF}$ proteins, the latter a p53 stabilizing tumor suppressor, were unaffected or rather downregulated in EPC2-hTERT, EPC1 and EPC1-hTERT with ectopically expressed ICN1 (Figure 2b; Supplementary Figure S2a). We also examined *p21* and *BAX*, two genes induced by oncogenic Ras$^{G12V}$ in EPC2-hTERT in a p53-dependent manner.[5] ICN1 induced *p21*, but not *BAX*, mRNA in EPC1-hTERT and EPC2-hTERT cells (Supplementary Figures S6a and b). As ICN1 induces *p21* in a CSL-dependent manner,[12] the failure of *BAX* induction may suggest the lack of p53 activation in response to ICN1. Moreover, ICN1 induced neither *p21* nor *BAX* mRNA in EPC2-T cells expressing p53$^{R175H}$ (Supplementary Figure S6c). Finally, p53$^{R175H}$ did not prevent ICN1-induced senescence in EPC2-hTERT and EPC2-T (Supplementary Figures S7 and S4). Thus, p53 inactivation may be insufficient to negate ICN1-induced senescence; however, the above findings do not exclude the requirement of p14$^{ARF}$ at the onset of ICN1-induced senescence. p14$^{ARF}$ may also inhibit cell proliferation in a p53-independent manner,[39] prompting us to perform RNAi experiments to explore cell-cycle regulators, including p14$^{ARF}$.

Among the cyclin-dependent kinase inhibitors (CDKIs), ICN1 induced *p15$^{INK4B}$* and *p16$^{INK4A}$* CSL-dependently in EPC2-T as antagonized by DNMAML1 (Supplementary Figures S4a and S6c). We screened their involvement in ICN1-induced senescence with small interfering RNA (siRNA) sequences directed against *p15$^{INK4B}$* and the exon 3 of *INK4A* (Figure 4a), the latter shared by both *p14$^{ARF}$* and *p16$^{INK4A}$*. RNAi directed against both *p14$^{ARF}$* and

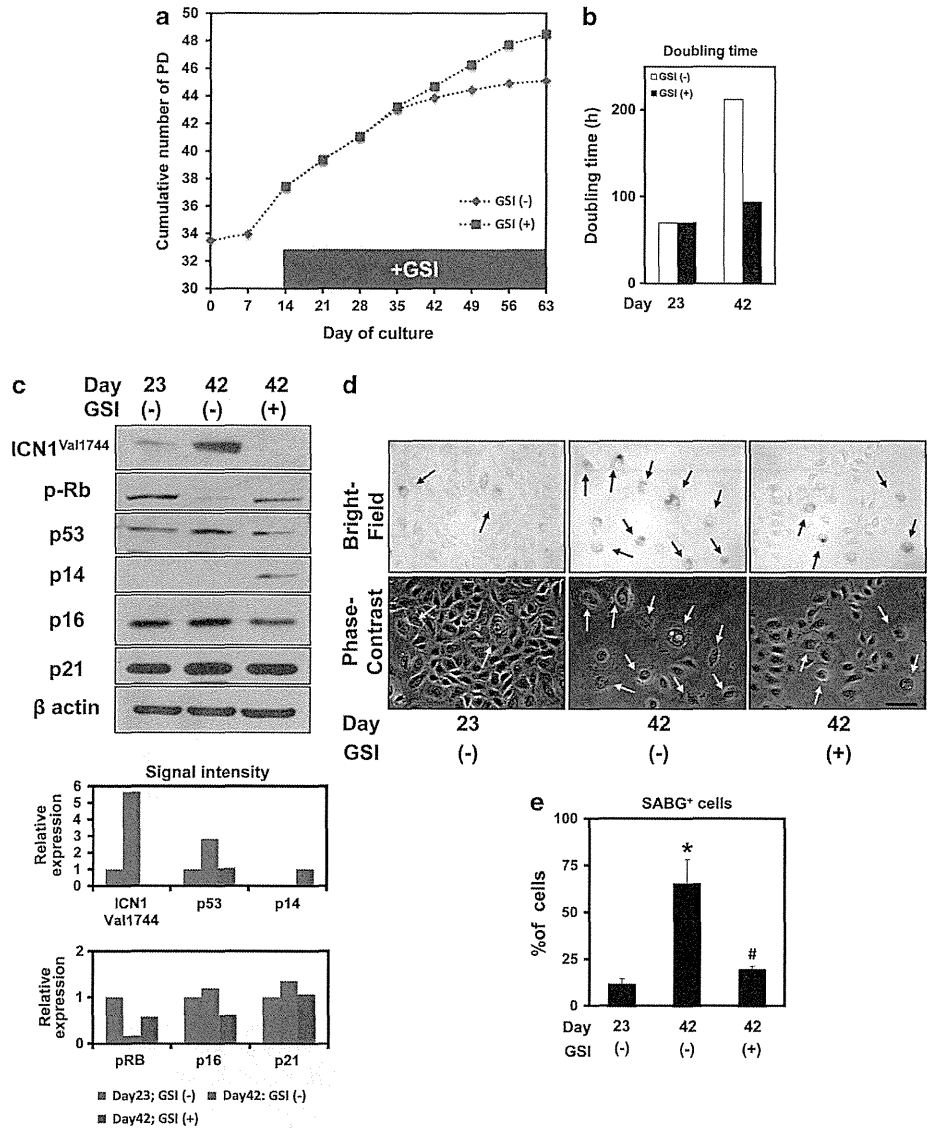


**Figure 1.** Notch1 is activated in EPC2 cells undergoing replicative senescence. A frozen vial of primary culture of EPC2 cells (27.5 population doubling) was thawed and grown in the presence or absence of GSI for a period indicated in panel (**a**). Cells were harvested at the indicated time points to determine population doubling (**a**) as well as doubling time (**b**) and subjected to western blotting (**c**) and SABG assays (**d**, **e**). In panel (**c**), β-actin served as a loading control; ICN1$^{Val1744}$, the activated form of Notch1; p-Rb, phospho-Rb$^{S780}$. In densitometry, the signal intensity for the molecule of interest was calibrated by that of β-actin at each time point. In panel (**d**), representative bright-field and phase-contrast images demonstrate SABG-positive cells and the corresponding cells with flat and enlarged cell morphology (arrows) as scored in panel (**e**); $^*P < 0.05$ vs Day 23 and GSI (−); $^\#P < 0.05$ vs Day 42 and GSI (−); $n = 6$. Note a reduced cell density at day 42 (43 population doubling) without GSI. Note that GSI suppressed ICN1$^{Val1744}$ (**c**), preventing the extension of doubling time (**b**) as well as the induction of SABG positive cells in panels (**d**) and (**e**).

*p16$^{INK4A}$*, but not *p15$^{INK4B}$*, significantly inhibited ICN1-mediated SABG activation in EPC2-T (Supplementary Figure S8), implicating the *INK4A* locus in ICN1-induced senescence.

To dissect the roles of *p14$^{ARF}$* and *p16$^{INK4A}$* in *ICN1*-induced senescence more specifically, we designed siRNA targeting non-overlapped sequences in *p16$^{INK4A}$* (exon 1α) and *p14$^{ARF}$* (exon 1β) (Figure 4a). When tested in EPC2-hTERT, EPC2-T and EPC1 cells that express both p14$^{ARF}$ and p16$^{INK4A}$, RNAi directed against p16$^{INK4A}$, but not p14$^{ARF}$, prevented ICN1 from inducing senescence as corroborated by decreased Rb dephosphorylation, continuous cell proliferation and reduced SABG activity in all cell lines (Figures 4b–e for EPC2-T; Supplementary Figures S9 and S10 for EPC2-hTERT and EPC1, respectively), suggesting that p16$^{INK4A}$ may have a predominant role in ICN1-induced senescence. Nevertheless, RNAi directed against *p14$^{ARF}$* or *p16$^{INK4A}$* revealed context-dependent functional interplays between *p14$^{ARF}$* and *p16$^{INK4A}$*, influencing basal cell proliferation and expression of other cell-cycle regulators as summarized in Supplementary Table S1.

As DNMAML1 prevented ICN1 from inducing *p16$^{INK4A}$* mRNA (Supplementary Figure S6c), we asked whether ICN1 may transcriptionally activate *p16$^{INK4A}$*. In transfection assays using a *pGL3-p16* reporter construct, ICN1 did not activate the 2.3-kb 5′-regulatory region of *p16$^{INK4A}$* in EPC2-hTERT and EPC2-T (data not shown). Of note, the ECR browser did not detect conserved CSL-binding *cis*-elements within this region *in silico*.[40] Thus ICN1

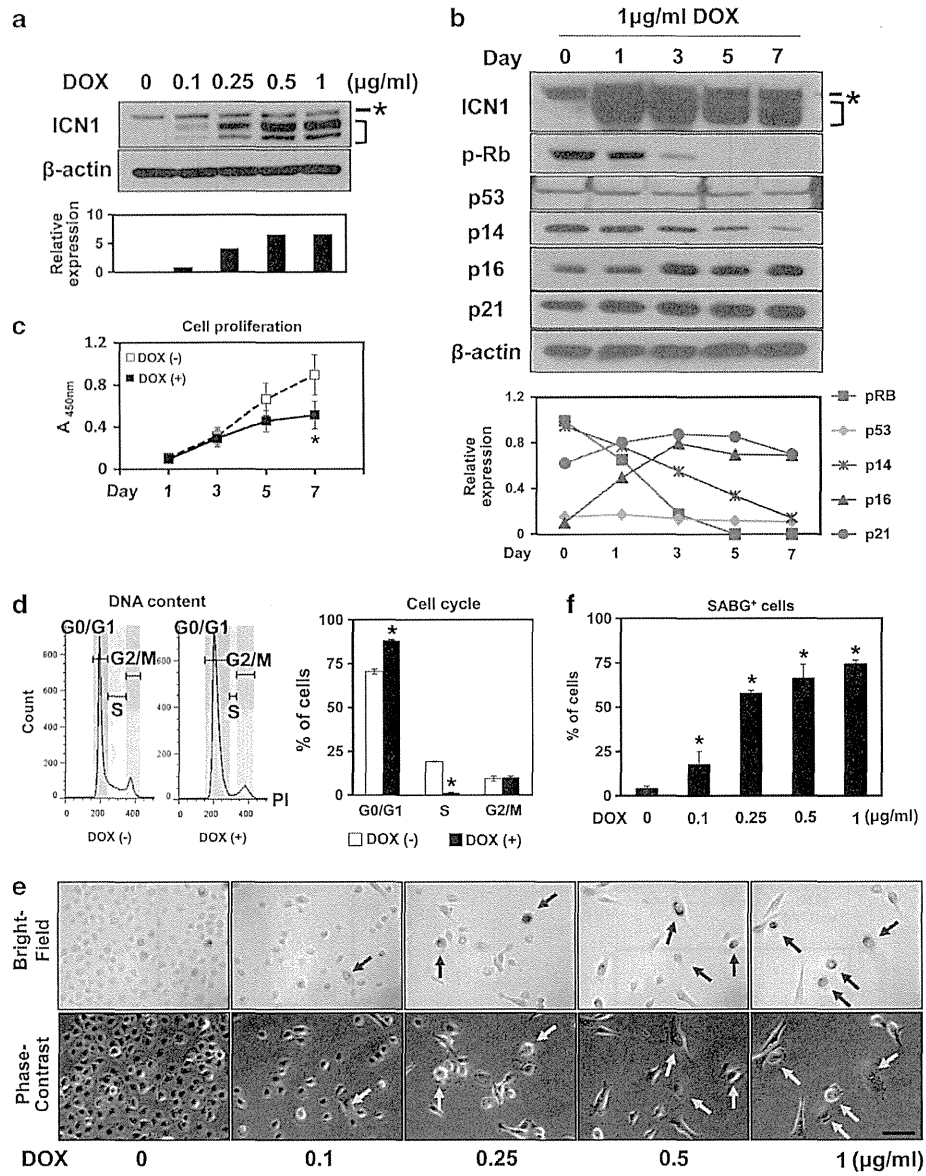


**Figure 2.** ICN1 induces Notch target genes and senescence in EPC2-hTERT cells. EPC2-hTERT carrying *ICN1*$^{Tet\text{-}On}$ was treated with the indicated concentrations of doxycycline (DOX); or 0 μg/ml [DOX (−)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1. In panels (**a**), (**d**), (**e**) and (**f**), cells were exposed to DOX for 7 days. In panels (**b**) and (**c**), cells were exposed to DOX for the indicated time period. Following DOX treatment, cells were analyzed by western blotting for ICN1, phospho-Rb$^{S780}$ (p-Rb), p53 and cell-cycle regulators at the indicated time points with densitometry in panels (**a**) and (**b**); WST1 assays for cell proliferation in panel (**c**); flow cytometry for cell cycle in panel (**d**); and SABG assays in panels (**e**) and (**f**). In panels (**a**) and (**b**), β-actin served as a loading control. *Denotes transmembrane/intracellular region of endogenous Notch1, which was suppressed by RNAi directed against Notch1 (data not shown). Bracket indicates lentivirally expressed ICN1 induced by DOX. A doublet appears consistently and may represent a posttranslational modification. Or note, anit-Notch1 (5B5) antibody was used to detect lentivirally expressed ICN1, which lacks the epitope recognized by anti-ICN1$^{Val1744}$ antibody. In panel (**c**), $^*P < 0.05$ vs DOX (−) at day 7 ($n = 6$). In panel (**d**), representative histogram plots are shown. Proportions of cells in G0/G1, S and G2/M cell-cycle phases were determined. $^*P < 0.05$ vs DOX (−) ($n = 3$). In panel (**f**), representative bright-field and phase-contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows) as scored in panel (**e**); $^*P < 0.05$ vs 0 μg/ml DOX ($n = 6$).

may not regulate *p16*$^{INK4A}$ transcriptionally through its proximal 5′-regulatory region.

### HPV E6/E7 may repress TGF-β signaling to prevent Notch1-mediated senescence in transformed human esophageal keratinocytes

The HPV E6 and E7 proteins inactivate p53 and Rb, respectively. To determine whether E6 and E7 may influence endogenous Notch activity, we performed RNAi experiments in EN60 cells carrying both *E6* and *E7* as a single fusion gene. siRNA sequences directed against either *E6* or *E7* suppressed both *E6* and *E7* transcripts (Figure 5a; and data not shown), resulting in induction of ICN1$^{Val1744}$ and cyclin-dependent kinase inhibitors with p53 stabilization, Rb dephosphorylation, reduced cell proliferation, G0/G1 cell-cycle arrest and SABG expression (Figures 5b–f; Supplementary Figure S11). Importantly, SABG activity was antagonized by concurrent knockdown of either Notch1 or