

Hepatitis B virus X protein activates E3 ubiquitin ligase Siah-1 to control virus propagation via a negative feedback loop

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Abstract

The seven in absentia homologue 1 (Siah-1) protein is an E3 ubiquitin ligase that induces ubiquitin-dependent proteasomal degradation of HBx, the principal regulatory protein of hepatitis B virus (HBV); however, its role in HBV propagation remains unknown. Here, we found that HBx upregulates Siah-1 levels in HepG2 but not in Hep3B cells, in which p53 is absent. For this effect, HBx sequentially activated ataxia telangiectasia mutated kinase and checkpoint kinase 2 via phosphorylation at the Ser-1981 and Thr-68 residues, respectively, which led to the activation of p53 via phosphorylation at the Ser-15 and Ser-20 residues. As a result, HBx was heavily ubiquitinated by Siah-1 and degraded by the ubiquitin–proteasome system in HepG2 cells, whereas this effect was marginal or undetectable in Hep3B cells. Knock-down of p53 in HepG2 cells downregulated Siah-1 levels and subsequently upregulated HBx levels, whereas ectopic p53 expression in Hep3B cells upregulated Siah-1 levels and subsequently downregulated HBx levels. In addition, Siah-1 knock-down impaired the ubiquitination and proteasomal degradation of HBx in HepG2 cells, whereas ectopic Siah-1 expression induced ubiquitin-dependent proteasomal degradation of HBx in Hep3B cells. The effects of HBx on p53 and Siah-1 were exactly reproduced in a 1.2-mer HBV replicon system, mimicking the natural course of HBV infection. In particular, Siah-1 knock-down upregulated the levels of HBx derived from the HBV replicon, resulting in an increase in HBV production. In conclusion, HBx modulates its own protein level via a negative feedback loop involving p53 and Siah-1 to control HBV propagation.

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) is a leading cause of human hepatic diseases, including hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) [1]. As a member of the *Hepadnaviridae* family, HBV replicates via reverse transcription of a pregenomic RNA intermediate and encapsidates a partially double-stranded circular DNA genome with about 3200 base pairs [2]. Of the four open reading frames in the HBV genome, the shortest one encodes a multifunctional regulatory protein, HBx. Several reports have speculated on the possible roles of this protein in HBV propagation and viral pathogenesis [3, 4]. In particular, the positive regulation of HBV propagation by HBx has been demonstrated by several experimental systems, including chimeric mice with humanized livers [5], HBV transgenic mice [6], tail vein-injected mice [7], anti-HBx short hairpin RNA [8] and cell-culture models [9, 10]. For this role, HBx appears to deregulate cellular signal transduction pathways, such as the cytosolic calcium signalling [11]

and phosphatidylinositol 3-kinase/AKT pathways [12]. In addition, the transcriptional transactivation function of HBx seems to be essential for proper viral replication, as it augments pre-genomic RNA synthesis from covalently closed circular DNA [6, 13]. However, the precise role of HBx and its action mechanism in the HBV life cycle remain unclear.

Seven in absentia homologue 1 (Siah-1) is a mammalian homologue of the *Drosophila* seven in absentia protein [14]. As an E3 ubiquitin (Ub) ligase of the Ub-proteasome system, Siah-1 is involved in the ubiquitination and proteasomal degradation of target proteins and thereby modulates diverse biological processes, including apoptosis [15, 16], mitosis [17], estrogen signalling [18], Ras-mediated transformation [19, 20], hypoxia response [21] and DNA damage-induced cell death [22]. In addition, Siah-1 mediates the potential of p53 to induce cell-cycle arrest, tumour suppression and apoptosis in mammalian cells [15, 23, 24]. Therefore, Siah-1 acts as a tumour suppressor and its inactivation

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Abbreviations: ATM, ataxia telangiectasia mutated; CHK2, checkpoint kinase 2; CHX, cycloheximide; DAPI, 4', 6-diamidino-2-phenylindole; DNMT, DNA methyltransferase; HA, heamagglutinin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IP, immunoprecipitation; MSP, methylation-specific PCR; MDM2, mouse double minute 2; Siah-1, seven in absentia homologue 1; shRNA, small hairpin RNA; siRNA, small interfering RNA; Ub, ubiquitin; UPS, ubiquitin-proteasome system; 5-Aza-2'dC, 5-Aza-2'deoxyctidine.

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correlates closely with tumour progression in human cancer, including HCC [25].

In general, p53 is activated in response to cellular stress, such as DNA damage and oncogene activation. Activation of p53 in response to DNA damage involves the successive phosphorylation-mediated activation of DNA damage sensors, such as ataxia telangiectasia mutated kinase (ATM) and checkpoint kinase 2 (CHK2), which induces a series of post-translational modifications of p53 and mouse double-minute 2 (MDM2), ultimately facilitating dissociation of the MDM2–p53 complex and stabilization of p53 [26, 27]. In addition, p53 is activated via another route involving p14 and MDM2 [28]. Expression of *p14* is usually induced in response to aberrant proliferation signalling from Myc and Ras through the E2F transcription factor [29, 30]. The activated p14 then binds directly to MDM2 and prevents it from targeting p53 for degradation by inhibiting the E3 ligase activity of MDM2 [31, 32], promoting rapid degradation of MDM2 [33] and preventing nuclear export of MDM2 and p53 [28], and ultimately activating p53.

Several previous reports have suggested close correlations among HBx, p53 and Siah-1. First, HBx upregulates p53 levels to enhance transcriptional activity [34–38]. Second, p53 transcriptionally activates Siah-1 expression through p53 response elements located within its promoter [15, 23, 24]. Third, either p53 activation [39, 40] or ectopic Siah-1 overexpression [41] induces Ub-dependent proteasomal degradation of HBx. Therefore, it is very challenging to investigate whether HBx controls its own protein level via a negative feedback loop involving p53 and Siah-1. In the present study, we first examined whether HBx transcriptionally activates Siah-1 expression via upregulation of p53 levels in both ectopic expression and HBV infection systems. Secondly, we investigated the mechanism by which HBx upregulates p53 levels in human hepatocytes. Thirdly, we investigated whether Siah-1 differentially induces ubiquitination and proteasomal degradation of HBx depending on the status of p53. Lastly, we attempted to elucidate the possible role of this regulatory circuit in HBV propagation.

RESULTS

HBx upregulates p53 levels but downregulates MDM2 levels in human hepatocytes

Previous reports have demonstrated that HBx upregulates p53 levels in human hepatocytes [34–38]. Consistently, both transient and stable expression of HBx upregulated p53 levels in HepG2 cells (Fig. 1a). Treatment with a universal proteasomal inhibitor, MG132, dramatically upregulated p53 levels in HepG2-vector cells, whereas the effect was weaker in HepG2-HBx cells (Fig. 1b). Accordingly, the potential of HBx to upregulate p53 levels was abolished by MG132 (Fig. 1b), suggesting that HBx upregulates p53 levels by preventing proteasomal degradation.

To prove that HBx actually increases the half-life of p53, we treated HepG2-vector and HepG2-HBx cells with

cycloheximide (CHX) to block further protein synthesis while chasing MDM2 and p53 levels in these cells. As shown in Fig. 1(c), the half-life of MDM2 in HepG2-HBx cells was much shorter (approximately 27 min) compared to that in HepG2-vector cells (over 120 min). In contrast, the half-life of p53 was shorter in control cells (approximately 30 min) compared to that in HepG2-HBx cells (over 120 min). Accordingly, MDM2 levels were higher in control cells, whereas p53 levels were higher in HepG2-HBx cells (Fig. 1c). These results indicate that HBx upregulates p53 levels, whilst it downregulates MDM2 levels by oppositely affecting their protein stability.

To confirm that HBx differentially affects the degradation of MDM2 and p53 via the Ub–proteasome system (UPS), we introduced heamagglutinin (HA)-tagged Ub into HepG2-vector and HepG2-HBx cells and immunoprecipitated the Ub-complexed products. As a result, more polyubiquitinated forms of MDM2 were detected in HepG2-HBx cells (Fig. 1d, upper panel), whereas the ubiquitination of p53 was severely impaired in these cells (Fig. 1d, lower panel). Taken together, we concluded that HBx elevates p53 levels but lowers MDM2 levels by differentially inducing their degradation via the UPS.

HBx upregulates p53 levels via activation of the ATM–CHK2 pathway

Next, we investigated the mechanism by which HBx upregulates p53 levels. The p53 protein is usually accumulated in response to stress signals, such as DNA damage and hyperproliferation via the activation of ATM and p14, respectively [27, 30]. Therefore, we first examined whether HBx activates the p14–MDM2 pathway in human hepatocytes. Previous reports have demonstrated that HBx inhibited expression of tumour suppressor genes, including E-cadherin and insulin-like growth factor binding protein-3 via DNA methylation [42, 43]. Consistently, HBx downregulated the p14 levels in HepG2 cells and this effect was abolished by treatment with a universal DNA methyltransferase inhibitor, 5-Aza-2'-deoxycytidine (5-Aza-2'dC), in a dose-dependent manner (Fig. 2a), suggesting that HBx represses p14 expression via DNA methylation. Indeed, HBx induced promoter hypermethylation of p14 and downregulated its RNA and protein levels (Fig. 2b). These effects were almost completely abolished by treatment with 5-Aza-2'dC (Fig. 2b). In spite of p14 downregulation, higher p53 and lower MDM2 levels were detected in HepG2-HBx cells, (Fig. 2b). In addition, treatment with 5-Aza-2'dC, which almost completely abolished the potential of HBx to downregulate p14 levels, weakly affected the potential of HBx to modulate MDM2 and p53 levels (Fig. 2b). These results suggest that the p14–MDM2 pathway is not responsible for the activation of p53 by HBx.

We next investigated whether HBx upregulates p53 levels via activation of the ATM–CHK2 pathway. Consistent with previous reports [44, 45], the potential of HBx to upregulate the p53 levels was abolished by treatment with an ATM inhibitor, KU-55933, in a dose-dependent manner (Fig. 2c). In addition, HBx activated ATM via phosphorylation at the

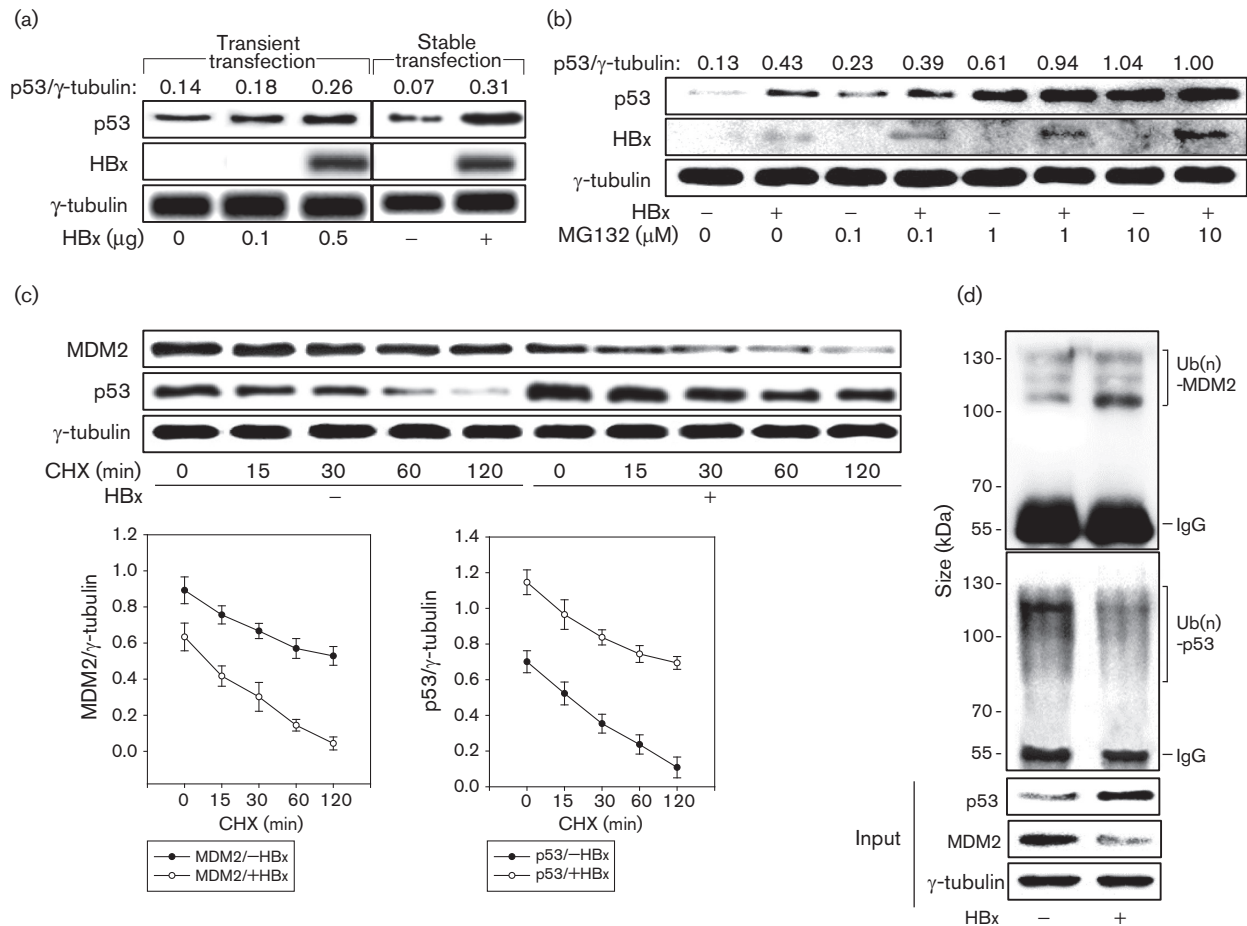


Fig. 1. HBx upregulates p53 levels by suppressing Ub-dependent proteasomal degradation. (a) The protein levels of p53, HBx and γ -tubulin in HepG2 cells transiently transfected with the indicated amount of HBx expression plasmid for 48 h and two stable cell lines, HepG2-vector and HepG2-HBx, were determined by Western blotting. Each band was quantified using ImageJ image-analysis software (NIH, USA); the values indicate the level of p53 relative to the loading control (γ -tubulin). (b) HepG2-vector and HepG2-HBx cells were either mock-treated or treated with an increasing concentration of MG132 for 4 h and then subjected to Western blotting. The values indicate the level of p53 relative to the loading control. (c) HepG2-vector and HepG2-HBx cells were treated with 50 μ g ml⁻¹ cycloheximide (CHX) for the indicated time, followed by Western blotting (upper panels). The MDM2, p53 and γ -tubulin levels were determined by densitometry; the strength of MDM2 and p53 was normalized to that of γ -tubulin at each time point. Data represent the mean \pm SD from three independent experiments (lower panels). (d) HepG2-vector and HepG2-HBx cells were transfected with the HA-Ub expression plasmid for 48 h. Total MDM2 and p53 proteins were immunoprecipitated with an anti-MDM2 antibody and an anti-p53 antibody, respectively, and then subjected to Western blotting using an anti-HA antibody to detect the Ub-complexed MDM2 and p53. The input shows the MDM2 and p53 levels in whole-cell lysates.

Ser-1981 residue and subsequently activated CHK2 via phosphorylation at the Thr-68 residue, without affecting their total protein levels (Fig. 2d). As a result, p53 was phosphorylated at the Ser-15 and Ser-20 residues by the activated ATM and CHK2, respectively, which led to p53 stabilization (Fig. 2d). The potential of HBx to induce phosphorylation of CHK2 and p53 was almost completely abolished by treatment with KU-55933, which downregulated the levels of p53 in HepG2-HBx cells to those in the control cells (Fig. 2d). The potential of HBx to downregulate MDM2 levels was also abolished by treatment with KU-55933. Based on these observations, we concluded that HBx upregulates p53 levels via activation of the ATM-CHK2 pathway.

HBx upregulates Siah-1 levels via p53 activation to downregulate its protein level

Having established that HBx upregulates p53 levels, we next investigated whether this effect leads to the activation of Siah-1 expression. According to the immunofluorescence assay, HBx was primarily detected as speckles in the cytoplasm of HepG2 and Hep3B cells (Fig. 3a), as previously demonstrated [46, 47]. HBx dramatically strengthened the Siah-1 signal in HepG2 cells, whereas this effect was not observed in Hep3B cells, suggesting that HBx upregulates Siah-1 levels in a p53-dependent manner. Indeed, transient expression of HBx dose-dependently elevated the p53 and Siah-1 levels in HepG2 cells (Fig. 3b), but not in Hep3B

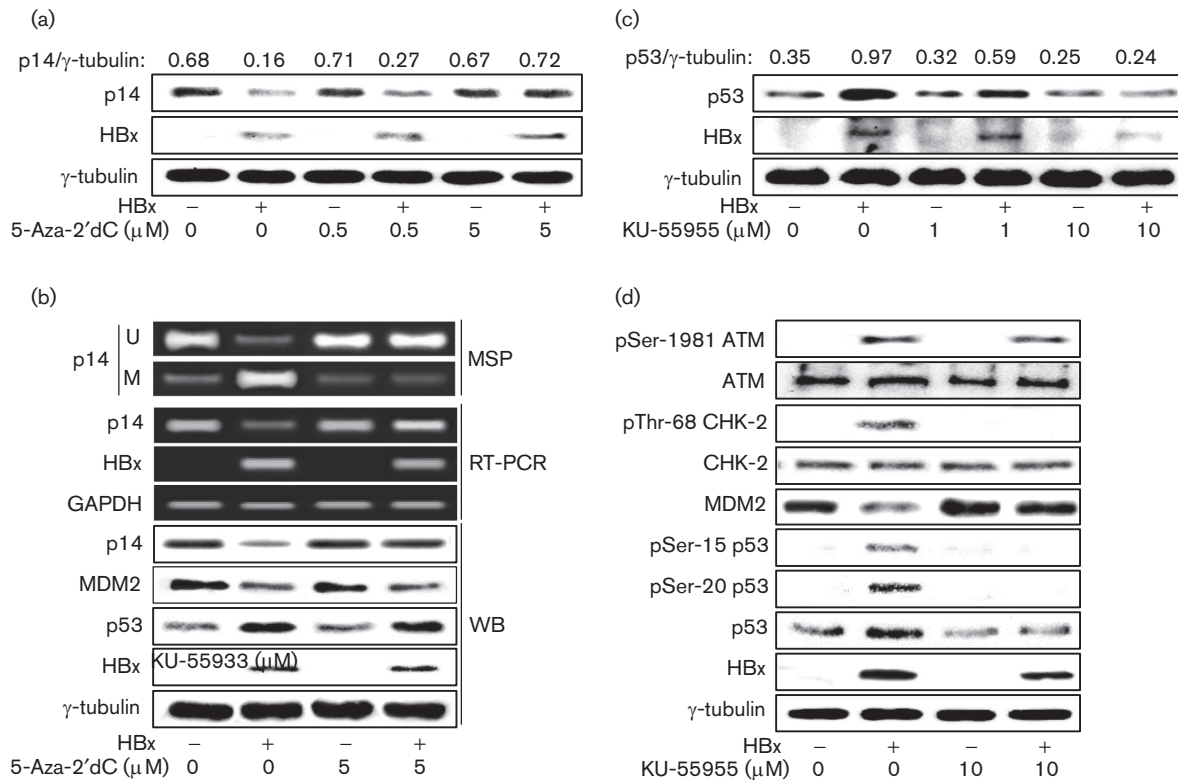


Fig. 2. HBx upregulates p53 levels by activating the ATM–CHK2 pathway. (a) HepG2-vector and HepG2-HBx cells were treated with an increasing concentration of 5-Aza-2'dC for 24 h, followed by Western blotting. The values indicate the level of p14 relative to the loading control. (b) HepG2-vector and HepG2-HBx cells were either mock-treated or treated with 5 μ M 5-Aza-2'dC for 24 h. Methylation-specific PCR (MSP) was performed to determine whether the CpG sites within the p14 promoter were unmethylated (U) or methylated (M) (upper panels). The RNA levels of p14, HBx and GAPDH were determined by conventional RT-PCR (middle panels). The levels of the indicated proteins were determined by Western blotting (lower panels). (c) HepG2-vector and HepG2-HBx cells were treated with an increasing concentration of KU-55933 for 1 h and then subjected to Western blotting. The values indicate the level of p53 relative to the loading control. (d) HepG2-vector and HepG2-HBx cells were either mock-treated or treated with 10 μ M KU-55933 for 1 h before being harvested and then subjected to Western blotting.

cells, in which p53 was absent (Fig. 3c), suggesting that p53 is required for the HBx-mediated activation of Siah-1 expression.

To prove that HBx upregulates Siah-1 levels via the activation of p53, we attempted to knock-down p53 in HBx-expressing HepG2 cells using a specific small hairpin RNA (shRNA). Indeed, p53 knock-down almost completely abolished the potential of HBx to upregulate Siah-1 levels in HepG2 cells (Fig. 1d). In addition, ectopic p53 expression in Hep3B cells dose-dependently upregulated Siah-1 levels in the presence of HBx (Fig. 1e). Taken together, we concluded that HBx upregulates Siah-1 levels via the activation of p53 in human hepatocytes.

According to a previous report, Siah-1 interacts with HBx and facilitates its polyubiquitination and proteasomal degradation [41]. The immunofluorescence assay clearly and consistently showed colocalization of HBx and Siah-1 in the cytoplasm of HepG2 cells (Fig. 3a). Therefore, we investigated whether the HBx-mediated upregulation of Siah-1

affects the HBx levels. Indeed, downregulation of Siah-1 levels as a result of p53 knock-down led to upregulation of HBx levels in HepG2 cells (Fig. 3d), whereas upregulation of Siah-1 levels as a consequence of ectopic p53 expression led to downregulation of HBx levels in Hep3B cells (Fig. 3e). Moreover, Siah-1 knock-down using a specific small interfering RNA (siRNA) upregulated HBx in HepG2 cells, resulting in further upregulation of p53 levels (Fig. 3f), whereas ectopic Siah-1 expression downregulated HBx levels in Hep3B cells in the absence of p53 (Fig. 3g). Therefore, we concluded that HBx downregulates its own protein level via the activation of Siah-1 expression.

Siah-1 downregulates HBx levels via Ub-dependent proteasomal degradation

We next investigated whether Siah-1 induces Ub-dependent proteasomal degradation of HBx. For this purpose, we introduced HA-tagged Ub into HBx-expressing cells and immunoprecipitated the Ub–HBx complexes. Consistent with a previous report [48], the ubiquitinated forms of HBx

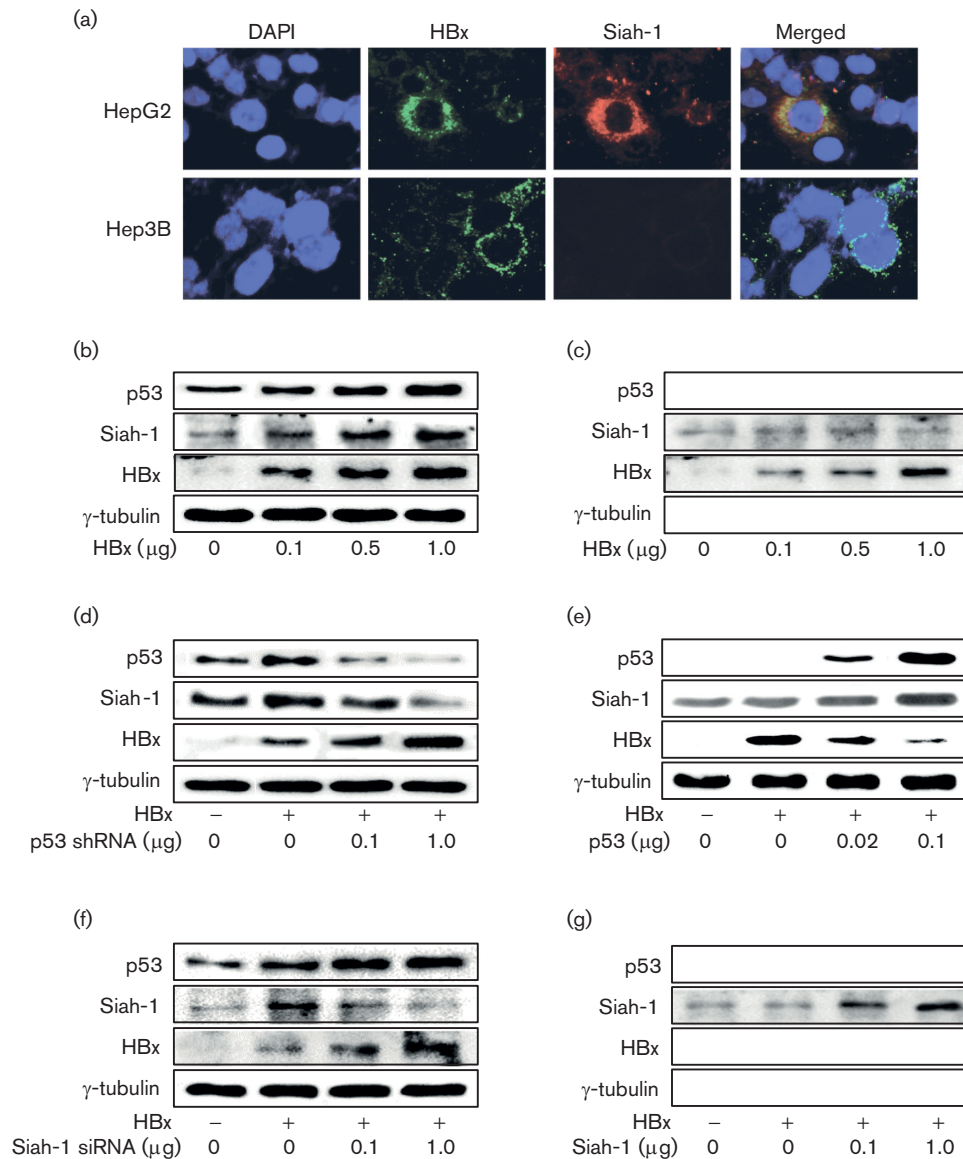


Fig. 3. HBx upregulates Siah-1 levels via p53 activation to downregulate its protein level. (a) HepG2 and Hep3B cells grown on coverslips were transfected with HBx expression plasmid for 48 h, fixed and processed for double-label indirect immunofluorescence. Cells were incubated with anti-HA and anti-Siah-1 antibodies, and then reacted with anti-mouse IgG-FITC and anti-rabbit IgG-rhodamine antibodies to visualize HBx (green) and Siah-1 (red), respectively. Nuclei (blue) were stained with 4', 6-diamidino-2-phenylindole (DAPI). (b) HepG2 cells were transiently transfected with an increasing amount of HBx expression plasmid for 48 h and then subjected to Western blotting. (c) Hep3B cells were transiently transfected with an increasing amount of HBx expression plasmid for 48 h and then subjected to Western blotting. (d) HepG2-vector and HepG2-HBx cells were transiently transfected with the indicated amount of p53 shRNA plasmid for 48 h and then subjected to Western blotting. (e) Hep3B-vector and Hep3B-HBx cells were transiently transfected with the indicated amount of p53 expression plasmid for 48 h and then subjected to Western blotting. (f) HepG2-vector and HepG2-HBx cells were transiently transfected with the indicated amount of Siah-1 siRNA for 48 h and then subjected to Western blotting. The total amount of siRNA in the transfection mixtures was equalized by adding non-silencing control siRNA. (g) Hep3B-vector and Hep3B-HBx cells were transiently transfected with the indicated amount of Siah-1 expression plasmid for 48 h and then subjected to Western blotting.

were detected as a ladder or smear on SDS-PAGE, but formed a main band of approximately 120 kDa (Fig. 4a, b), indicating that around 10 Ub moieties tend to be attached to the 20 kDa HA-HBx protein. Consistent with a previous

report [41], Siah-1 knock-down decreased the ubiquitinated forms of HBx in HepG2 cells (Fig. 4a), whereas ectopic Siah-1 expression increased the ubiquitinated forms of HBx in Hep3B cells (Fig. 4b). In addition, treatment with MG132

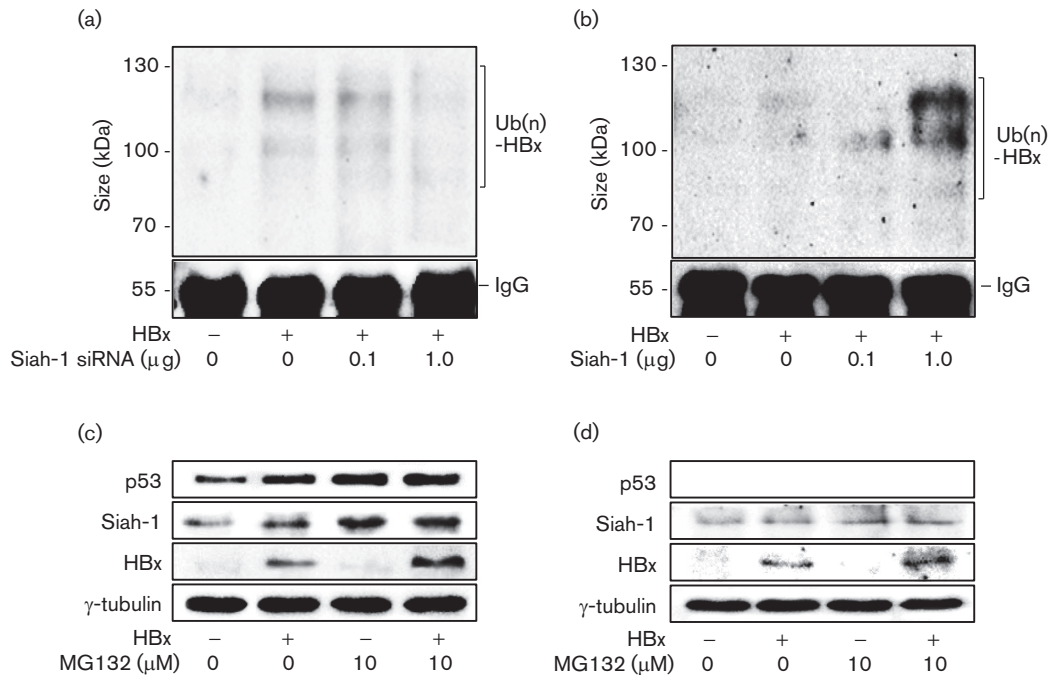


Fig. 4. Siah-1 downregulates HBx levels via Ub-dependent proteasomal degradation. (a) HepG2-vector and HepG2-HBx cells were transfected with the indicated amount of Siah-1 siRNA, along with the HA-Ub expression plasmid, for 48 h. Total HBx proteins were immunoprecipitated with an anti-HBx antibody and then subjected to Western blotting using an anti-Ub antibody to detect the Ub-complexed HBx. (b) Hep3B-vector and Hep3B-HBx cells were transfected with the indicated amount of Siah-1 expression plasmid, along with the HA-Ub expression plasmid, for 48 h. An IP-coupled Western blot assay was performed as described in (a). (c) HepG2-vector and HepG2-HBx cells were either mock-treated or treated with 10 μ M MG132 for 4 h and then subjected to Western blotting. (d) Hep3B-vector and Hep3B-HBx cells were either mock-treated or treated with 10 μ M MG132 for 4 h and then subjected to Western blotting.

upregulated HBx levels in HepG2 cells (Fig. 4c), confirming that HBx is subjected to the Siah-1-mediated proteasomal degradation. The effect of MG132 on HBx was weak but still detectable in Hep3B cells (Fig. 4d), suggesting that one or more additional pathways are also involved in the proteasomal degradation of HBx. Treatment with MG132 also upregulated Siah-1 levels in HepG2 (Fig. 4c) but not in Hep3B cells (Fig. 4d), probably due to the elevated p53 levels in HepG2 cells. Taken together, we concluded that Siah-1 induces ubiquitination and proteasomal degradation of HBx.

HBx downregulates its own protein level via a negative feedback loop involving p53 and Siah-1 to control HBV propagation

Finally, we investigated whether the Siah-1-mediated proteasomal degradation of HBx affects HBV propagation. For this purpose, we employed a 1.2 mer HBV replicon system, which mimics the natural course of HBV infection [9]. Transfection with an HBV replicon, 1.2-mer-WT, but not its HBx-null counterpart, 1.2-mer HBx-null, upregulated both p53 and Siah-1 levels in HepG2 cells (Fig. 5a). In addition, p53 knock-down in HepG2 cells transfected with 1.2-mer-WT HBV replicon downregulated Siah-1 levels, which

led to upregulation of HBx levels (Fig. 5b). Moreover, Siah-1 knock-down in these cells upregulated HBx levels (Fig. 5c), which led to a significant increase in HBV propagation (Fig. 5d). Siah-1 knock-down also upregulated p53 levels (Fig. 5c), probably due to the increased action of HBx. These results were consistent with data from HBx overexpression (Fig. 3). Taken together, we concluded that HBx induces its own proteasomal degradation via upregulation of Siah-1 expression to negatively regulate HBV propagation in p53-positive hepatocytes.

DISCUSSION

Despite a number of studies, the effect of HBx on p53 remains unclear. Early studies implicated HBx in the progression of HCC through its ability to inhibit the potential of p53 to induce apoptosis [49, 50]. For this effect, HBx binds to and partially sequesters p53 in the cytoplasm, inhibiting several p53 activities, such as DNA sequence-specific binding and transcriptional transactivation [49, 51, 52]. However, later studies proposed a completely opposite role for HBx in the regulation of p53, wherein HBx upregulates p53 levels to stimulate the expression of its target genes, such as *p21*, *Siah-1*, and several proapoptotic genes, including *Bax*, *Fas* and *Nox*, which ultimately leads to

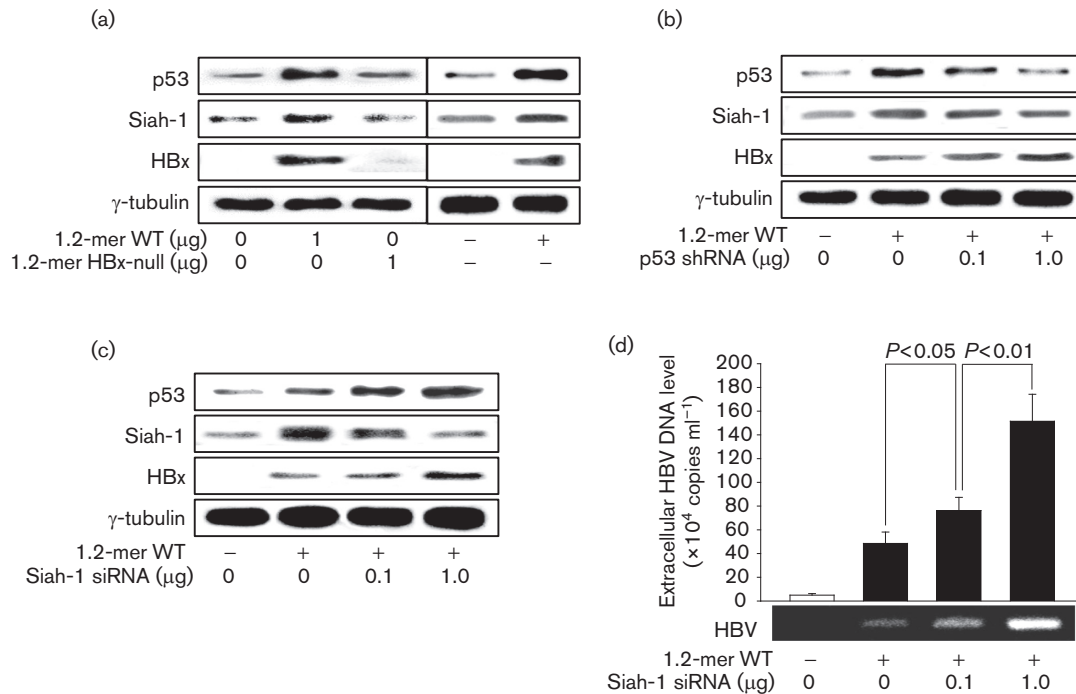


Fig. 5. HBx downregulates its own protein level via a negative feedback loop to restrain HBV propagation. (a) HepG2 cells transiently transfected with either a 1.2-mer WT HBV replicon or its HBx-null derivative for 48 h, or stably transfected with either an empty vector or a 1.2-mer WT HBV replicon, were subjected to Western blotting. (b) HepG2 cells stably transfected with either an empty vector or a 1.2-mer WT HBV replicon were transiently transfected with the indicated amount of p53 shRNA plasmid and then subjected to Western blotting. (c) HepG2 cells stably transfected with either an empty vector or a 1.2-mer WT HBV replicon were transiently transfected with the indicated amount of Siah-1 siRNA and then subjected to Western blotting. (d) Levels of HBV particles released from the cells prepared in (c) were determined by conventional PCR and IP-coupled real-time PCR as described in the Methods section. Data represent the mean \pm SD from four independent experiments.

growth inhibition and apoptotic death of the hepatocytes [34–38]. The present study consistently showed that HBx upregulates p53 levels to transcriptionally activate Siah-1 expression in human hepatocytes. It is not easy to reconcile the apparently opposite effects of HBx on p53. Several factors, including cell context, HBx variation, expression level and other experimental conditions, may affect the interactions between HBx and p53 [36, 53]. However, it is important to note that the effect of HBx was also demonstrated in the present study using an HBV replicon system, which mimics the natural course of HBV infection.

It has been found that HBx alters the mitochondrial membrane potential to increase cellular reactive oxygen species production [54, 55]. In addition, HBx induces the accumulation of γ -H2AX, a marker of DNA double-strand breaks, and increases the levels of phosphorylated ATM [45] and phosphorylated CHK-2 [44]. Consistent with these reports, the present study showed that HBx successively activates ATM and CHK2 via their phosphorylation at the Ser-1981 and Thr-68 residues, respectively, which leads to the activation of p53 via its phosphorylation at the Ser-15 and Ser-20 residues. Based on this and other studies, it was possible to propose a model for the regulation of p53 by HBx (Fig. 6).

For the negative regulation of p53, HBx downregulates the p14 levels via DNA methylation and upregulates the MDM2 levels, which leads to the Ub-dependent proteasomal degradation of p53. Direct protein–protein interactions between HBx and p53 may also contribute to the HBx inactivation of p53, as previously demonstrated [49, 51, 52]. For the positive regulation of p53, HBx sequentially activates ATM and CHK2, leading to the phosphorylation and stabilization of p53. This regulatory network can be more complicated if other pathways are considered, such as the transcriptional activation of MDM2 by p53 and the Siah-1-mediated feedback inhibition of HBx. The overall p53 protein level, which is apparently affected by several experimental conditions, might be determined by the balance between the dual effects of HBx on p53. Based on this model, it is possible to conclude that the positive regulation is dominant and thus overrides the negative regulation of p53 by HBx under our experimental conditions.

Several reports have demonstrated that p53 decreases the protein stability of HBx. For example, p53 overexpression downregulates the HBx levels, whereas p53 knock-down by siRNA upregulates its levels in HCC cell lines [39]. However, the underlying mechanism is still unclear. The present

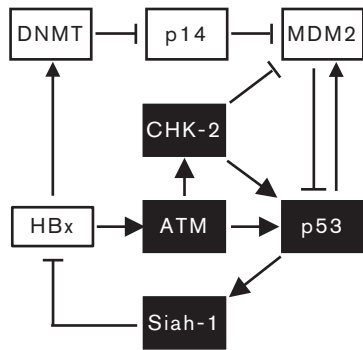


Fig. 6. A proposed model for the regulation of HBx via a negative feedback loop involving p53 and Siah-1. HBx upregulates the protein levels and enzyme activities of DNA methyltransferases (DNMTs) to repress p14 expression via DNA methylation, resulting in upregulation of the MDM2 levels and subsequent downregulation of the p53 levels. In addition, p53 levels are also negatively regulated by MDM2 itself, which is the product of a p53-inducible gene. Thus, the two molecules are linked to each other through an autoregulatory negative feedback loop aimed at maintaining low p53 levels, especially in the absence of HBx. For the positive regulation of p53, HBx sequentially activates ATM and CHK2, which leads to MDM2 inactivation and p53 activation. The latter pathway, indicated by the thicker arrows, appears to be dominant in our experimental conditions, as evidenced by the accumulation of p53 in HBx-expressing cells. The p53 protein then transcriptionally activates Siah-1 expression, which in turn induces Ub-dependent proteasomal degradation of HBx and subsequent inhibition of HBV propagation.

study provides several lines of evidence that suggest that p53 destabilizes HBx in a Siah-1- and Ub-dependent mechanism. First, treatment with MG132 dramatically increased the HBx levels in HepG2 cells, whereas the effect was mild or negligible in Hep3B cells, indicating that p53 is implicated in the proteasomal degradation of HBx. Second, HBx upregulated the p53 and Siah-1 levels in HepG2 cells but not Hep3B cells, suggesting that HBx activates Siah-1 expression via p53 activation. Third, Siah-1 knock-down decreased the ubiquitination of HBx and upregulated its protein levels in HepG2 cells, as previously demonstrated [41]. Fourth, Siah-1 overexpression increased the ubiquitination of HBx and downregulated its protein levels in Hep3B cells, indicating that Siah-1 activation alone is sufficient for the downregulation of HBx.

A number of studies have clearly demonstrated the positive regulatory role of HBx in HBV propagation using diverse experimental systems, including cell-culture models [9, 10, 56]. The present study, however, demonstrated that HBx can negatively regulate HBV propagation by downregulating its own protein level via a negative feedback loop involving p53 and Siah-1. The biological significance of this regulatory system is unknown. The UPS is known to negatively regulate virus propagation, serving as an effective host protection system against virus infection [57]. For example, E6AP induces polyubiquitination and degradation of the

hepatitis C virus (HCV) core protein to inhibit virus propagation [58]. Therefore, the Siah-1-mediated ubiquitination and proteasomal degradation of HBx may simply represent a host protection system against HBV infection. However, many viruses, including HBV, are also known to manipulate the UPS to favour their propagation by redirecting cellular E3 ligases or encoding their own enzymes to enable propagation, egress and immune evasion [59, 60]. It is thus possible to speculate that HBV uses this system to evade host immune responses by minimizing virus particle production, especially during the so-called immunotolerant phase, wherein levels of HB viremia are surprisingly constant. Reevaluation of the present study in animal and human clinical models may clarify the biological significance of the HBx regulatory circuit and its effect on HBV propagation.

METHODS

Plasmids

The HBx expression plasmid, pCMV-3 × HA1-HBX3, encoding HBx downstream of three copies of the influenza virus HA epitope was described previously [36]. The 1.2-mer-WT HBV replicon containing 1.2 units of the HBV genome (genotype D) and its HBx-null counterpart were described previously [9]. The plasmid pSiah-1-Myc WT, which expresses Myc-tagged Siah-1, were described previously [61]. The p53 shRNA plasmid was obtained from Santa Cruz Biotechnology. The plasmids pHA-Ub encoding HA-tagged Ub and pCMV p53-WT were gifts from Y. Xiong (University of North Carolina at Chapel Hill, USA) and C. W. Lee (Sungkyunkwan University, Korea), respectively.

Cell culture and transfection

HepG2 (KCLB no. 58065) and Hep3B (KCLB no. 88064) were obtained from the Korean Cell Line Bank. For transient expression, 2×10^5 cells per 60 mm dish were transfected with 1 µg of appropriate plasmid(s) using the TurboFect transfection reagent (Thermo Scientific) following the manufacturer's instructions. The HepG2 and Hep3B cells transfected with pCMV-3 × HA1-HBX3 were selected in the presence of $500 \mu\text{g ml}^{-1}$ G418 (Gibco) to establish the HBx-expressing stable cell lines, HepG2-HBx and Hep3B-HBx.

HBV replicon system

The HBV-producing cell lines were established by stable transfection with the 1.2-mer-WT HBV replicon, followed by selection with $500 \mu\text{g ml}^{-1}$ G418 (Gibco). Immunoprecipitation (IP)-coupled polymerase chain reaction (PCR) was performed to measure the levels of extracellular HBV particles. Briefly, HBV particles were immunoprecipitated from culture supernatant using an anti-HBs antibody (Santa Cruz Biotechnology) according to the protocol described in the IP section. HBV genomic DNA was purified from the precipitated HBV particle-antibody complexes using the QIAamp DNA mini kit (Qiagen). For conventional PCR analysis of HBV DNA, the genomic DNA was amplified

using the Solg 2 × Taq PCR Smart mix 1 (Solgent) and a primer pair, HBV 1399F (5'-TGG TAC CTC CGC GGG ACG TCC TT-3') and HBV 1632R (5'-AGC TAG CGT TCA CGG TGT CTC C-3'). Quantitative real-time PCR assay (qPCR) of HBV was carried out, as described previously [62]. Briefly, HBV DNA was amplified using the SYBR premix Ex Taq II (Takara Bio) and a primer pair, HBV 379F (5'-GTG TCT GCG GCG TTT TAT CA-3') and HBV 476R (5'-GAC AAA CGG GCA ACA TAC CTT-3') in a Rotor Gene Q PCR machine (Qiagen).

RNA interference

The Siah-1 siRNA duplexes were synthesized as previously described [35]. Based on the target sequence of siRNA for Siah-1 (5'-AAC TCC TGC CTC CTT ATG TAT TT-3') and the non-silencing siRNA (control siRNA) sequence (5'-AAG AGC CGT CAG ACT GCT ACA-3'), siRNA duplexes were synthesized and purified by Qiagen. For knock-down of Siah-1 expression, 2 × 10⁵ cells per 60 mm dish were transfected with the indicated amount of Siah-1 siRNA using TurboFect transfection reagent (Thermo Scientific). The control siRNA was included to equalize the amount of siRNA in the transfection mixtures. For knock-down of p53, cells were transiently transfected with p53 shRNA plasmid as described in the cell culture and transfection section.

Western blot analysis

Cells were lysed in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 % SDS, 1 % NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Membranes were then incubated with antibodies to p14, pSer-1981 ATM, pSer-15 p53, pSer-20 p53 and pThr-68 CHK2 (Cell Signaling); p53, ATM, CHK2, MDM2, Ub and Siah-1 (Santa Cruz Biotechnology); HBx (Millipore); and γ -tubulin (Sigma). After this, they were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse, anti-goat, anti-human, or anti-rabbit IgG (H+L)-HRP (Bio-Rad). The ECL kit (Amersham) was used to visualize the protein bands via the ChemiDoc XRS imaging system (Bio-Rad).

Immunoprecipitation (IP)

Exactly 2 × 10⁶ cells per 100 mm diameter plate were transfected with pHA-Ub for 48 h. An IP assay was performed using a Classic Magnetic IP/Co-IP assay kit (Pierce), according to the manufacturer's specifications. Briefly, the whole-cell lysates were incubated overnight at 4 °C with an appropriate antibody. Pierce protein A/G magnetic beads were then added and incubated for an additional 1 h. The beads were then collected using a magnetic stand (Pierce) and the antigen/antibody complexes eluted from them were subjected to Western blotting.

Immunofluorescence analysis

Cells grown on coverslips were fixed in 4 % formaldehyde at room temperature for 15 min and then permeabilized in methanol at -20 °C for 10 min. The HBx and Siah-1

proteins in cells were reacted with anti-HA (Roche) and anti-Siah-1 (Santa Cruz Biotechnology) antibodies overnight at 4 °C and then with anti-mouse IgG-FITC and anti-goat IgG-rhodamine antibodies (Santa Cruz Biotechnology), respectively, at room temperature for 1 h. Slides prepared with UltraCruz mounting medium (Santa Cruz Biotechnology) were visualized with an Eclipse fluorescence microscope (Nikon).

Methylation-specific PCR (MSP)

Genomic DNA (1 μ g) denatured in 50 μ l of 0.2 N NaOH was modified by treatment with 30 μ l of 10 mM hydroquinone (Sigma) and 520 μ l of 3 M sodium bisulfite (pH 5.0; Sigma) at 50 °C for 16 h. For MSP, the modified DNA (100 ng) was amplified with Taq polymerase using both methylated and unmethylated primer pairs of p14, as previously described [63].

Reverse-transcription PCR (RT-PCR)

The total RNA (3 μ g) extracted from cells using the RNeasy mini kit (Qiagen) was reverse-transcribed with the corresponding reverse primer. One-quarter of the reverse-transcribed RNA was amplified with Taq polymerase to detect the p14, HBx and GAPDH RNA levels, as previously described [42, 64].

Statistical analysis

The values indicate means \pm standard deviations (SD) from at least three independent experiments. The two-tailed Student's *t*-test was used for all statistical analyses. A *P* value <0.05 was considered statistically significant.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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