KEY MESSAGES
1. miR-21 overexpression is critical to promote early carcinogenesis of hepatocytes upon hepatitis B virus infection.
2. HBx-induced IL-6-STAT3 pathway upregulates miR-21 expression.
3. The high dependency of miR-21 expression on HBx is a unique viral oncogenic pathway aberrantly affecting gene expression network.
4. Inhibition of miR-21 attenuates the anchorage-independent colony formation and subcutaneous tumour growth of non-tumour hepatocytes.

Introduction
Hepatocellular carcinoma (HCC) associated with hepatitis B virus (HBV) infection exhibits a higher degree of aggressiveness than non-infected tumours. HBV X protein (HBx) is encoded from the HBV genome and is involved in the pathogenic mechanism of HBV-associated HCC. It has multiple molecular functions in human hepatocytes via interactions with various transcription factors and modulates numerous cellular signalling pathways of the host. The role of HBx protein in HCC biology has been studied, but the gene expression network affected by the viral protein is not fully understood.

HBx protein induces differential expression of microRNAs (miRNAs). Aberrant expression of miRNAs has been implicated in numerous cancer types including HCC. Nonetheless, HBV-associated miRNAs that drive the transformation of normal hepatocytes and HCC carcinogenesis are poorly studied. In addition, the mechanism by which HBx alters the expression of miRNAs is largely unexplored. In this project, we demonstrated that induction of miR-21 was dependent on HBx-activation of the IL-6-STAT3 pathway. Expression of miR-21 is essential in transforming non-tumour hepatocytes to gain the ability to form anchorage-independent colonies and in vivo tumours, and implies a critical role during early HCC development.

Methods
This study was conducted from December 2011 to December 2013.

Cell culture and drug treatment
A human hepatoma cell line, Hep3B, immortalised non-tumourigenic hepatocyte cells, MIHA, and 293T, were maintained under standard procedures. Cells were treated with STAT3 inhibitor, cucurbitacin (Tocris, Bristol, UK), at a dose of 0.5 µM for 72 hours, or with recombinant human IL-6 (Invitrogen) at designated doses and durations.

Cancer-associated miRNA profiling in HBx-expressing stable cell line
MIHA cells were transfected with pcDNA3.1/myc containing COOH-terminal truncated HBx cDNA (HBx-∆35). After transfection, the cells were incubated with G418 (Invitrogen) for ~2 weeks. Differential expression of cancer-associated miRNA in HBx protein expressing MIHA cells was measured by Cancer MicroRNA qPCR Array with QuantiMir (System Biosciences). As an internal control, U6 primers were used for RNA template normalisation.

Lentivirus packaging and transduction
Myc-tagged full-length human HBx (HBx-FL) and HBx-∆35 were cloned into a lentiviral vector coexpressing EGFP (pLIG). Lentiviral vector carrying transgene of small hairpin RNA targeting STAT3 (shSTAT3) (BLOCK-it RNAi system) (Invitrogen) or miRZip-21 (MZIP21-PA-1) (System Biosciences) was purchased. Lentiviruses containing either myc-tagged HBx, HBx-∆35, shSTAT3 or miRZip-21 were packaged and transduced into MIHA cells with the presence of 8 µg/mL polybrene (Sigma). Cells were
sorted for GFP expression by the BD FACSArria III cell sorter (BD Biosciences, New Jersey, USA) to obtain pure populations of MIHA-HBx and MIHA-Δ35.

**Soft agar assay**

Cells (5x10^4) were re-suspended in 1.5 ml 0.35% agar in DMEM, and were layered onto six wells containing solidified 0.5% agar. Colonies were allowed to grow for 4-6 weeks before being stained with crystal violet.

**Subcutaneous xenograft tumour models**

All animal experiments were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. MIHA cells, MIHA-LIG, MIHA-HBx and MIHA-HBx-Δ35 (2x10^6) were injected subcutaneously into nude mice (n=5). Tumour volume was calculated by the equation: volume=(length x width^2)/2.

**Results**

**miR-21 was upregulated by ectopic HBx expression in MIHA cells**

We profiled cancer-associated miRNAs expression in HBx-Δ35 overexpressing MIHA cells by Cancer MicroRNA qPCR Array with QuantiMir. MiRNAs with fold changes larger than 1.5 were considered biologically significant, and oncomiRs including miR-21 and miR-373 were upregulated while tumour suppressor miRNAs miR-126 and miR-137 were downregulated after HBx-Δ35 overexpression (Fig 1a). To investigate the association between HBx and the miRNAs, we generated lentivirus carrying full length HBx (HBx-FL) or HBx-Δ35 transgene to transduce MIHA cells. We showed that both HBx-FL and HBx-Δ35 significantly promoted MIHA cell proliferation (Fig 1b). We hypothesised that HBx proteins could induce differential miRNAs expression, contributing to the altered phenotypes of non-tumour hepatocytes.

Subsequently, we validated the miRNA profiling in both HBx-FL- and HBx-Δ35-expressing MIHA cells by qRT-PCR. MiR-21 expression was significantly increased upon ectopic HBx expression (Fig 1c), whereas miR-126, miR-137 and miR-373 showed no significant change (data not shown). To prove the presence of HBx-miR-21 pathway in HCC cells, we inhibited HBx by siRNAs in Hep3B cells that were positive for HBx expression. MiR-21 expression was significantly inhibited upon knockdown of HBx in Hep3B cells (Fig 1d). The level of other miRNAs was also measured, but knockdown of HBx failed to alter their expression (data not shown). Inhibition of miR-21 activity by miRZip-21 in HBx-FL expressing MIHA cells could upregulate a putative miR-21 target programmed cell death 4 (PDCD4) [Fig 1e]. Taken together, we illustrated that miR-21 was an important downstream regulator of HBx proteins during HBV-mediated hepatocarcinogenesis.

**Upregulation of IL-6 by HBx protein induced miR-21 expression**

We studied the association between the HBx-IL-6 pathway and miR-21 induction during early hepatocyte transformation. Overexpression of HBx-FL and HBx-Δ35 significantly increased the expression of IL-6 in MIHA cells (Fig 1f). HBx-Δ35 induced a remarkably higher IL-6 expression in cells compared with HBx-FL, suggesting that such variant could activate the IL-6 pathway more robustly. We further treated MIHA cells with recombinant human IL-6, and the level of miR-21 in cells was increased both time-dependently (Fig 1g) and dose-dependently (Fig 1h). Recombinant IL-6 treatment also increased both pri-miR-21 (Fig 1i) and pre-miR-21 levels (Fig 1j) time-dependently. The concurrent upregulation of primary, precursor and mature miR-21 suggested that IL-6 transcriptionally activated the transcription of the miR-21 gene in non-tumour hepatocytes, and subsequently elevated mature miR-21 level. Furthermore, knockdown of IL-6 by siRNAs significantly reduced the expression of miR-21 in Hep3B cells (Fig 1k), suggesting that IL-6 signalling was pivotal to HBx-mediated induction of miR-21 expression in HCC cells.

**Role of STAT3 in HBx-induced miR-21 expression**

We further delineated the molecular pathway of IL-6-mediated miR-21 upregulation. IL-6 and STAT3 pathway is prevalent in human HCC. Nonetheless, the role of HBx proteins in regulation of the IL-6-STAT3 pathway was not clear. We showed that phosphorylated STAT3 was significantly elevated after overexpression of HBx-FL and HBx-Δ35 (Fig 2a). Similar to the upregulation of IL-6 mRNA expression, HBx-Δ35 induced a higher level of phosphorylated STAT3 compared with HBx-FL (Fig 2a). HBx was highly associated with activation of the IL-6-STAT3 pathway in HCC cells, as we further showed that phosphorylation of STAT3 was effectively inhibited when IL-6 was depleted by siRNAs in Hep3B cells (Fig 2b). To reveal the importance of STAT3 in HBx-induced miR-21 activation, lentiviral vector containing shSTAT3 was transduced in MIHA cells with or without HBx-FL and HBx-Δ35 expression. Western blotting showed that the level of total STAT3 was drastically prohibited in all MIHA cell lines after lentiviral infection (Fig 2c). In turn, we measured the change in miR-21 level in the STAT3-depleted cells. As expected, upregulation of mature miR-21 was significantly attenuated in both HBx-FL and HBx-Δ35 overexpressed MIHA cells (Fig 2d). In contrast, depletion of STAT3 in parental and control lentivirus infected MIHA cells did not alter miR-21 expression.
FIG 1. Ectopic HBx expression induced miR-21 expression in non-tumour hepatocytes via IL-6 signalling. Differential expression of cancer-associated miRNAs was measured in MIHA cells upon overexpression of HBx protein, and (a) upregulated miRNAs including miR-21 and miR-373, and downregulated miRNAs including miR-126 and miR-137 were identified. (b) Cell proliferation assay showed that ectopic expression of HBx-FL or HBx-Δ35 significantly increased the cell proliferation rate of MIHA cells compared with parental and lentivirus control cells. (c) Lentiviral overexpression of HBx-FL and HBx-Δ35 upregulated the level of miR-21 in MIHA cells. (d) Knockdown of HBx inhibited the expression of miR-21 in Hep3B cells. (e) Inhibition of miR-21 activity in HBx-expressed MIHA cells through second infection of lentivirus expressing miRZip-21 significantly increased the transcript level of PDCD4. (f) Both HBx-FL and HBx-Δ35 protein induced the expression of IL-6 in MIHA cells, while HBx-Δ35 induced a significantly higher IL-6 level compared with HBx-FL. Recombinant human IL-6 upregulated the expression of mature miR-21 in a (g) time-dependent and (h) dose-dependent manner in parental MIHA cells. (i) Precursor miR-21 and (j) primary miR-21 were upregulated with IL-6 treatment time-dependently. (k) Knockdown of IL-6 by siRNAs significantly inhibited the level of endogenous miR-21 in Hep3B cells. (Reproduced with permission from Elsevier)
expression (Fig 2d). This implied that the regulation of miR-21 by STAT3 in non-malignant hepatocytes was dependent on HBx, which proved that aberrant miR-21 expression was driven by HBx-IL-6-STAT3 signalling.

Phosphorylation of STAT3 enhanced miR-21 promoter occupancy

Chromatin immunoprecipitation (ChIP) analysis revealed a significant increase in STAT3 occupancy in the miR-21 promoter upon ectopic expression of HBx-FL or HBx-∆35 (Fig 2e). Western blotting analysis revealed an increase in STAT3 phosphorylation after treatment with recombinant IL-6 in MIHA cells (Fig 2f), and ChIP analysis showed that exposure of IL-6 to MIHA cells significantly enriched STAT3 proteins at the promoter of miR-21 gene (Fig 2g).

To further demonstrate the importance of STAT3 activity in the HBx-miR-21 pathway, a selective JAK/STAT3 signalling pathway inhibitor,
FIG 3. Role of miR-21 in HBx-induced hepatocarcinogenesis: (a) left panel: soft agar assay demonstrated that MIHA cells expressing HBx-FL or HBx-Δ35 were capable of forming anchorage-independent colonies. (a) right panel: MIHA cells expressing HBx-Δ35 exhibited a higher tendency to form anchorage-independent colonies compared with HBx-FL. (b) Co-expression of miRZip-21 with HBx proteins effectively abrogated the colony forming ability of HBx-expressed MIHA cells. (c) Inhibition of miR-21 significantly reduced the number of colonies formed in both HBx-FL and HBx-Δ35 expressing MIHA cells. (d) Tumour xenografts were extracted from nude mice injected with MIHA cells with or without the expression of HBx or miRZip-21. There were three and four out of five nude mice respectively injected with HBx-FL and HBx-Δ35 expressing MIHA cells. Coexpression of miRZip-21 was able to inhibit the subcutaneous tumour formation ability of HBx expressing cells. (e) Inhibition of miR-21 by miRZIP-21 significantly reduced the tumour weight compared with HBx-FL and HBx-Δ35. (f) Schematic diagram illustrating the HBx-triggered miR-21 hepatocarcinogenesis pathway. (Reproduced with permission from Elsevier)
Role of miR-21 in HBx-induced hepatocarcinogenesis

By soft agar assay, overexpression of both full length HBx and HBx-Δ35 was able to induce anchorage-independent colony formation of MIHA cells, whereas neither parental MIHA cells nor the lentiviral control MIHA-LIG cells could form colonies (Fig 3a). MIHA cells expressing HBx-Δ35 could generate twice as many colonies as those expressing full length (Fig 3a). HBx-Δ35 possessed a greater ability to transform non-tumour hepatocytes compared with its full length counterpart.

In order to characterise the role of miR-21 in HBx-induced cell proliferation, MIHA cells were co-infected with lentivirus carrying miRZip-21 transgene. Soft agar assay was conducted after infection of the panel of MIHA cells with lenti-CTRL or lenti-miRZip-21. The colony forming capability of HBx-FL and HBx-Δ35 expressing MIHA cells was inhibited by miRZip-21 (Figs 3b and 3c). Moreover, MIHA cells expressing HBx and HBx-Δ35 proteins were injected subcutaneously into nude mice (n=5). Cells expressing HBx-FL developed tumours in three out of five mice, whereas those expressing HBx-Δ35 developed tumours in four out of five mice (Fig 3d). More importantly, no tumours were found in mice injected with cells coexpressing HBx-FL and miRZip-21. Inhibition of miR-21 also effectively abrogated the effect of HBx-Δ35 to induce tumour formation, in which only two out of five mice developed tumours with obviously reduced tumour volume and weight (Figs 3d and 3e).

Discussion

We aimed to identify HBx-induced miRNAs that have an important role during early HCC development. Overexpression of miR-21 occurred as soon as the hepatocytes were infected with HBV. Among various upstream pathways that regulate miR-21, the IL-6-STAT3 signalling pathway is closely related to cellular transformation and oncogenesis in HCC. High levels of IL-6 are found in patients with HBV infection and HBV-associated HCC.\(^3\) HBx-induced miR-21 expression was highly dependent on activation of the IL-6-STAT3 pathway, which demonstrated the specificity of miR-21 induction by HBx. Apart from overexpressing HBx in non-tumour hepatocytes, silencing IL-6 in HBx protein expressing HCC cell line Hep3B could result in a reduction in miR-21 level, which consolidated the interactions between HBx, IL-6-STAT3 and miR-21.

Our results from soft agar assay and xenograft assay suggest that HBx-induced miR-21 is pivotal in the transformation of non-tumour hepatocytes. More importantly, as inhibiting miR-21 with miRZIP-21 inhibited tumour xenograft growth, there is high therapeutic value to targeting miR-21 in HBV-associated HCC. Inhibition of miR-21 effectively prohibited MIHA cells from formation of both anchorage-independent colony and in vivo subcutaneous tumour, which suggests abrogation of the transformation of non-tumour hepatocytes.

Taken together, miR-21 was a critical downstream effector of HBx that drove the tumourigenesis of non-tumour hepatocytes. Ectopic HBx expression triggered the IL-6 pathway, promoted the phosphorylation and activation of transcriptional factor STAT3, and induced the expression of miR-21. Our in vitro and in vivo studies supported that miR-21 exhibited phenotypical change of the non-tumour cells during HBx-mediated hepatocarcinogenesis. Inhibiting miR-21 or attenuating its activation pathways (ie IL6-STAT3) could be promising when developing novel therapies for HBV-associated HCC. Given the important association between HBx and miR-21 in early HCC development, targeting miR-21 may also provide a novel means to prevent the development of HCC in HBV carriers.

Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#11100452).


References