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Original Research

Hepatitis B virus X protein promotes hepatocellular carcinoma transformation through interleukin-6 activation of microRNA-21 expression

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Abstract Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and chronic hepatitis B virus (HBV) infection is the major risk factor of HCC. The virus encodes HBV X (HBx) protein that plays a critical role in the development of HCC. Studies have revealed numerous HBx-altered genes and signalling pathways that heavily contribute to tumourigenesis of non-tumour hepatocytes. However, the role of HBx in regulating other critical gene regulators such as microRNAs is poorly understood, which impedes the exploration of a complete HBx-associated carcinogenic network. Besides, critical microRNAs that drive the transformation of non-tumour hepatocytes are yet to be identified. Here, we overexpressed C-terminal truncated HBx protein in a non-tumour hepatocyte cell line MIHA, and measured a panel of cancer-associated miRNAs. We observed that oncogenic miR-21 was upregulated upon ectopic expression of this viral protein variant. HBx-miR-21 pathway was prevalent in HCC cells as inhibition of HBx in Hep3B and PLC/PRF/5 cells significantly suppressed miR-21 expression. Subsequently, we showed that the upregulation of miR-21 was mediated by HBx-induced interleukin-6 pathway followed by activation of STAT3 transcriptional factor. The high dependency of miR-21 expression to HBx protein suggested a unique viral oncogenic pathway that could aberrantly affect a network of gene expression. Importantly, miR-21 was essential in the HBx-induced transformation of non-tumour hepatocytes. Inhibition of miR-21

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effectively attenuated anchorage-independent colony formation and subcutaneous tumour growth of MIHA cells. Our study suggested that overexpression of miR-21 was critical to promote early carcinogenesis of hepatocytes upon HBV infection.

1. Introduction

Chronic hepatitis B virus (HBV) infection is a major risk factor of hepatocellular carcinoma (HCC) [1]. Recent studies showed that HCCs with HBV infection exhibit a higher degree of aggressiveness than non-infected tumours. HBV X protein (HBx) is encoded from the HBV genome [2,3] that is involved in the pathogenic mechanism of HBV-associated HCC [4]. It has multiple molecular functions in human hepatocytes via interacting with various transcription factors and modulating numerous cellular signalling pathways of the host [4–12]. Though studies were rigorously conducted to reveal the role of HBx protein in HCC biology, the gene expression network affected by the viral protein is not fully understood.

Recently, studies showed that HBx protein induced differential expression of microRNAs (miRNAs). MiRNAs have sizes ranged from 20 to 23 nucleotides, and they participate in many biological processes including embryonic development, cell differentiation, proliferation and apoptosis [13–15]. Aberrant expression of miRNAs has been implicated in numerous cancer types including HCC. However, HBV-associated miRNAs that drive the transformation of normal hepatocytes and HCC carcinogenesis are poorly studied. Besides, the mechanism for HBx to alter the expression of miRNAs is still largely unexplored. Here, we demonstrated that induction of miR-21 was dependent on HBx-activation of interleukin-6 (IL-6)-STAT3 pathway. Expression of miR-21 was essential in transforming non-tumour hepatocytes to gain the ability to form anchorage independent colonies and *in vivo* tumour, which implied a critical role during early HCC development.

2. Materials and methods

2.1. Cell culture and drug treatment

Human hepatoma cell lines (Hep3B and PLC/PRF/5), immortalised non-tumourigenic hepatocyte cells (MIHA [14] and L02) and HEK293T were maintained as previously described [16]. Cells were treated with STAT3 inhibitor, cucurbitacin (Tocris, Bristol, United Kingdom (UK)), at a dose of 0.5 μM [17] for 72 h. Cells were treated with recombinant human IL-6 (Invitrogen) at indicated concentrations.

2.2. 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-Δ35). 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). Signals were normalised by U6 level.

2.3. Lentivirus packaging and transduction

Lentiviruses were packaged according to our protocol [18]. The list of lentiviral vectors used, transduction and cell sorting methods were described in supplementary materials and methods.

2.4. Subcutaneous xenograft tumour models

Subcutaneous injection of MIHA cells was conducted as previously described [19]. Details of the assay were described in supplementary materials and methods.

3. Results

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

Stable MIHA cells expressing HBx-Δ35 were established to emulate HBV-induced transformation. Overexpression of HBx-Δ35 was confirmed by Western blotting (Fig. 1A). Subsequently, we profiled cancer-associated miRNA expression in MIHA cells by Cancer MicroRNA qPCR Array with QuantiMir™. Differential expression of miRNAs was observed upon the HBx-Δ35 overexpression. MiRNAs with fold change larger than 1.5 were considered as biologically significant (Fig. 1B and C). In the subsequent validation, we attempted to identify miRNAs critical in the transformation of non-tumour hepatocytes. Among upregulated miRNAs, those with a cancer promoting role such as miR-373 and miR-21 were selected (Fig. 1B). For the downregulated miRNAs, miR-137 and miR-126 were selected for validation as they were shown to have tumour suppressive functions (Fig. 1C). Although other miRNAs from the profiling might have greater alteration in level, their

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