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Functional interplay between hepatitis B virus X protein and human miR-125a in HBV infection

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ABSTRACT

The hepatitis B virus (HBV) is a widespread human pathogen and chronic HBV infection is a major risk factor for hepatocellular carcinoma (HCC). Some cellular microRNAs are emerging as important regulators of virus–host interaction, indirectly or directly modulating HBV replication and pathogenesis. miR-125a binds the viral transcript encoding the surface antigen and interferes with its expression, thus inhibiting viral replication. Intriguingly, liver miR-125a expression has been found increased in patients with high levels of hepatic HBV-DNA. The present study investigates the mechanism by which liver exposure to HBV induces the expression of miR-125a. The analyses were first performed on liver biopsies from HBV patients, showing that the expression of the viral transactivator X protein (HBx) paralleled the increase of miR-125a expression. Then, transfection of HCC cell lines with an HBx-expressing vector showed a substantial increase of miR-125a expression. Overall, the available data depict a self-inhibitory feedback loop in which HBV, through HBx, increases the expression of miR-125a, that in turn interferes with expression of HBV surface antigen, thus repressing viral replication.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at post-transcriptional level by affecting both the stability and translation of complementary mRNAs [1]. miRNAs play crucial role in development, cell differentiation and apoptosis [2,3]. Moreover, several studies indicate that miRNA genes act as oncogenes or tumor suppressors [4,5]. Finally, recent studies have shown that miRNAs are important regulators of virus–host interactions [6–8].

Several reports have been dedicated to the role of miRNAs in hepatitis B virus (HBV) replication and pathogenesis, since HBV affects over 350 million people worldwide and is one of the leading causes of liver disease, often progressing to cirrhosis and hepatocellular carcinoma (HCC) [9–11]. Some cellular miRNAs modulate HBV replication either by targeting cellular transcription factors required for HBV gene expression or by a direct binding to HBV transcripts. In particular, some miRNAs promote HBV replication by targeting negative regulators of HBV activity (miR-372/373, miR-501) [12,13] or by enhancing a positive regulator (miR-1) [14]. Other miRNAs suppress HBV replication by targeting positive regulators of HBV (miR-29c, miR-122 and miR-141) [15–18] or by directly targeting HBV transcripts (miR-210, mir-199a-3p and miR-125a-5p) [19,20]. With regard to the last regulatory mechanisms, several reports have been dedicated to the role of human miR-125a-5p (miR-125a) in HBV infection. We first demonstrated that miR-125a is able to target the expression of HBV surface antigen (HBsAg). The interaction of miR-125a with the viral sequence was first demonstrated with a validation test based on cultured hepatic cells and luciferase reporter genes. Then, miR-125a was shown to interfere with viral translation in PLC/PRF/5, a cell line that contains several fragments of HBV DNA integrated in its genome and secretes HBsAg in the culture medium. In this system, transfection of a miR-125a mimic induced a marked decrease of the expression of HbsAg, whereas the transfection of a miR-125a inhibitor enhanced the expression of HBsAg [20]. Moreover, an inspection of nucleotide sequences of HBV genomes deposited at NCBI revealed that the target site of miR-125a is well conserved in the viral population [8], supporting a significant role in HBV–host interaction. Later, an independent study has confirmed the ability of miR-125a to inhibit HBsAg translation: in a screening of

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HBV replication-related miRNAs, a pri-miR-125a expression vector could repress HBsAg synthesis in HepG2 cells [18]. Then, another study has confirmed the implication of miR-125a in HBV–host interaction: in the HepG2.2.15 cell model of hepatitis B virus replication, the expression analysis of a panel of 814 miRNAs revealed that iron treatment, which increases HBV replication, resulted in a decreased miR-125a expression, whereas TGF-β treatment, that decreases HBV replication, resulted in an increased miR-125a expression, with it being at the top of the up-regulated miRNAs. These data, along with the results of transfection experiments with a miR-125a mimic and an inhibitor that reproduced the effects of TGF-β and iron on viral replication, strongly support the ability of hepatic miR-125a to counteract viral replication [21]. We have recently extended these studies to HBV patients, showing that the expression of liver miR-125a correlates with the HBV load and severity of liver disease [22]. In particular, we found an approximately threefold increase in the expression of liver miR-125a in patients with high levels of hepatic HBV-DNA, thus suggesting that liver exposure to HBV induces the expression of miR-125a.

The present study investigates the mechanism of induction of liver miR-125a by HBV, focusing on HBV X protein (HBx), a transcriptional transactivator whose activity has already been correlated to changes in miRNA expression [23,24].

2. Methods

2.1. Patients

Twenty-seven consecutive HBsAg/anti-HBe/HBV-DNA-positive and HbeAg-negative Caucasian patients who were naive to nucleos(t)ide analogs and interferon therapy and who had no marker of HCV, HDV or HIV infection, no history of alcohol intake and no clinical, biochemical or US signs of liver cirrhosis were included in this investigation. These 27 patients underwent a diagnostic liver biopsy from April 2007 to March 2011 and fragments of 1.5 mm in length were stored at −80 °C in RNAlater solution (Qiagen) for subsequent molecular analyses. Necroinflammatory lesions (histological activity index, HAI) and fibrosis were assessed according to the Ishak scoring system [39]. Plasma samples of each patient were collected on the same day of liver biopsy and liver function tests were performed by routine methods as part of routine care by the attending physicians. All patients provided written, informed consent for the collection and storage of biological samples and for the anonymous use of their data in clinical research. The study was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria of the Second University of Naples.

2.2. Cell cultures and transfections

Human hepatocarcinoma cell lines, HepG2 and HuH-7, were cultured in RPMI 1640 and DMEM respectively, containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 100 μg/ml streptomycin. The day before transfection, cells were trypsinized and seeded in medium without antibiotics in 12-well plates. Plasmid transfections were performed with cells at 80–90% of confluence by using Lipofectamine2000 (Invitrogen), as described by the manufacturer. After 6 h, transfection mix was replaced with complete medium. The analyses were performed 72 h after transfection.

2.3. RNA purification and real-time PCR analyses

Total RNA was extracted by mirVanaTM miRNA isolation kit (Ambion) from liver tissues homogenized by TissueLyser (Qiagen GmbH, Hilden, Germany) for 30 s at 30 Hz. Total RNA purification from cell cultures was performed by miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The RNA concentration was determined spectrophotometrically (NanoDrop 2000c, ThermoScientific).

HBx expression in liver was quantified by RT-qPCR: 200 ng of RNA were retrotranscribed by Transcriptor High Fidelity cDNA Synthesis Sample kit (Roche) using random examer primer; 2 μl aliquots of RT reaction were then used to amplify HBx transcript (primers: 5′-GCGCACCTCTCTTTACGCGG-3′ and 5′-CGTTCACGGTG GTCTCCATGC-3′) with GAPDH used as a reference gene (primers: 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGA TTT-3′). miR-125a was detected and quantified along with RNU6B (reference gene) by RT-qPCR with TaqMan® miRNA assays from Applied Biosystems according to the manufacturer's protocol. The expression levels of HBx and miR-125a were normalized to their respective reference genes by using the $2^{-\Delta Ct}$ method and reported as arbitrary units (AU).

All liver samples were tested for HBV DNA, as previously described [22]. Hepatitis B virus genotypes were determined by phylogenetic analysis of sequences of 400 nt of the S region, as previously described [40].

Comparison of the data sets was performed by Student's *t*-test and a value of $p < 0.05$ was considered significant.

2.4. Cloning of HBx DNA sequence

HBx coding sequence was isolated by PCR from HBV-infected liver cDNA obtained as described above. PCR amplification was carried out with the following primers: FpcX, 5′-CGGAATTC*AC-C**ATG***GCTGCTAGGCTGTGCT-3′, containing *Eco*RI restriction site (underlined) and the Kozak sequence (italic) with the ATG start codon (bold); RpcX, 5′-GCTCTAGA**TTA**GGCAGAGGTGAAAAAGTT G-3′, containing *Xho*I restriction site (underlined) and the stop codon (bold). The PCR product was cloned in the *Eco*RI/*Xho*I sites of pcDNA3.1 vector and fully sequenced.

2.5. Western blot analysis

Total proteins from cultured cells were prepared in lysis buffer (50 mM Tris–Cl, pH 8.0, 137 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). 40 μg proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (GE Healthcare) following standard protocols. Antibody against HBx (Abcam) and β-actin (Sigma–Aldrich) were used for immunodetection according to the manufacturer's instructions. Protein bands were visualized by autoradiogram using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

3. Results and discussion

3.1. HBx and miR-125a expression in HBV patients

HBx is a 154-amino acid transcriptional transactivator of viral and cellular promoters and enhancers [25]. It is not endowed with a DNA binding domain but is able to modulate gene expression through the binding to transcription factors or other components of the basal transcription machinery [26,27]; it is also able to induce epigenetic modifications [28] and modify various signal transduction pathways [29,30]. We measured the expression of HBx in liver biopsies from 27 patients with chronic hepatitis B. Their demographic, biochemical, virological, histological and biomolecular data are shown in Table 1. In a previous study on the same cohort of patients, we showed that miR-125a expression in individuals with high viral load (HBV DNA > 0.14 IU/cell) was

about threefold higher that observed in those with low viral load (HBV DNA ⩽ 0.14) [22]. The analysis of the same two groups of patients for HBx revealed that its expression level is also very different. In the patients with high viral load the expression of HBx was 3.6-fold higher than in those with a low viral load, thus following the same increase that was observed for miR-125a (Fig. 1). A direct correlation between HBx expression and liver HBV DNA is also shown in Fig. 2.

HBx expression was also analyzed according to the patients' AST and ALT serum values, HAI and fibrosis scores. Significantly higher HBx expression was observed in patients with abnormal AST serum levels (mean ± SD: 2.70 ± 2.17 vs. 0.63 ± 0.16, $p = 0.0003$), in those with abnormal ALT serum levels (mean ± SD: 3.88 ± 3.17 vs. 0.61 ± 0.20, $p = 0.0002$), in those with HAI >5 and in those with fibrosis >1 (Table 2). Even though liver disease was mild in most cases (abnormal ALT serum values in 37.1% and higher HAI and fibrosis scores in 25.9%), a clear correlation between high HBx levels and the severity of the liver disease was observed. These findings are consistent with a recognized role of HBx in the hepatocarcinogenesis [31–33], with the HBx gene often found selectively over-expressed in HCC tissues [34,35].

Overall, these data suggest that (1) HBx expression is correlated with the severity of liver disease, and (2) HBx expression is correlated with liver HBV load and may have a role in inducing miR-125a expression in HBV-infected hepatocytes.

3.2. HBx increases miR-125a expression in HCC cell lines

To investigate the possible effect of HBx on miR-125a expression, HCC cell lines were transfected with an HBx-expressing vector. The HBx coding sequence was isolated by RT-PCR from one of the liver biopses and cloned in the expression vector pcDNA3.1 to obtain the expression plasmid pcHBX. Sequencing analysis indicated that HBx coding sequence belonged to HBV subtype ayw (GeneBank entry X65257.1), as expected. Two different hepatoma cell lines, HepG2 and HuH-7, were then transfected with pcHBX and the expression of the viral protein was verified by Western blot. As shown in Fig. 3A, both cell lines expressed significant amounts of the protein, visible as a band of ~17 kDa. Measurement of miR-125a in the same samples then revealed a substantial increase of the miRNA in the HBx-expressing cells. In particular,

Table 1
Demographic, biochemical, virological and histological characteristics of the enrolled patients.

No of patients	27
Age, years, median (range)	41 (22–61)
Males no (%)	17 (63%)
Risks factors, no (%):	
Sex	3 (11.1)
Familiarity	4 (14.8)
Surgery	16 (59.3)
Transfusion	1 (3.7)
Not determined	3 (11.1)
AST × n.v., mean ± SD	1.24 ± 1.49
ALT × n.v., mean ± SD	1.82 ± 2.47
PLT, cell/ml, mean ± SD	234,630 ± 63,253
PT, %, median (range)	93 (72–110)
γGT, IU/ml, mean ± SD	27 ± 18
ALP, IU/ml, mean ± SD	129 ± 98
HBV-DNA, IU/ml, mean ± SD	1.89E6 ± 6.71E6
Patients with HBV genotype D, no (%)	27 (100)
HAI, mean ± SD	4.74 ± 2.45
Fibrosis score, mean ± SD	1.33 ± 1.17
Patients with steatosis, no (%)	8 (29.6)

AST: aspartate-aminotransferase; n.v.: normal value; SD: standard deviation; ALT: alanine-aminotrasferase; PLT: platelet blood test; PT: prothrombin time; γGT: gammaglutamil-transferases; ALP: alkaline phosphatase; HAI: histological activity index.

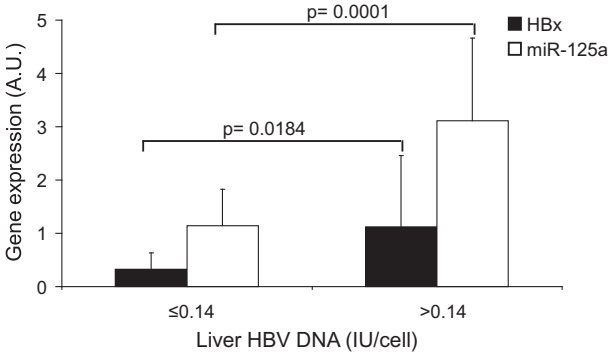

Fig. 1. HBx and miR-125a expression in liver biopsies from HBV patients. Expression was evaluated by real-time PCR in 19 patients with liver HBV DNA ⩽ 0.14 IU/cell and 8 patients with liver HBV DNA > 0.14 IU/cell. Data are the mean ± SD.

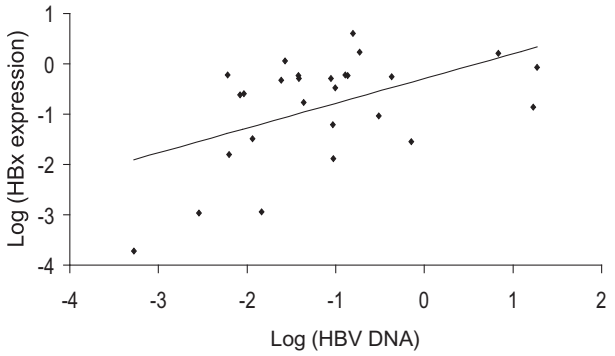

Fig. 2. HBx expression is correlated with HBV DNA levels in liver biopsies from HBV patients. Data were collected by real-time PCR and $\log_{10}$ transformed. Correlation coefficient $r = 0.50$.

Table 2
HBx expression according to liver disease.

Parameters	Number of patients	HBx expression, A.U., mean ± SD	*p*-Value
AST			
⩽1 × n.v.	19	0.27 ± 0.31	
>1 × n.v.	8	1.25 ± 1.24	0.003
ALT			
⩽1 × n.v.	17	0.27 ± 0.31	
>1 × n.v.	10	1.05 ± 1.18	0.016
HAI score			
⩽5	16	0.26 ± 0.31	
>5	11	1 ± 1.13	0.019
Fibrosis score			
⩽1 (0–1)	17	0.29 ± 0.32	
>1 (2–3)	10	1.03 ± 1.19	0.022

the expression was increased by 2-fold in HepG-2 cells and 3.5-fold in HuH-7 (Fig. 3B).

Taken together, the data collected on the liver biopsies and the results obtained with the cell cultures strongly suggest that viral HBx protein induces the expression of human miR-125a. In this regard, it should be noted that it is not uncommon for a virus to modulate miRNA expression. For example, the Epstein–Barr virus latent membrane protein 1 was reported to activate miR-155 transcription [36]; the human T-lymphotropic virus Type 1 Tax protein up-regulates miR-146a [37]; HIV was reported to globally suppress host miRNA expression [38]. Despite not having inquired the molecular mechanism of miR-125a induction by HBx, the available

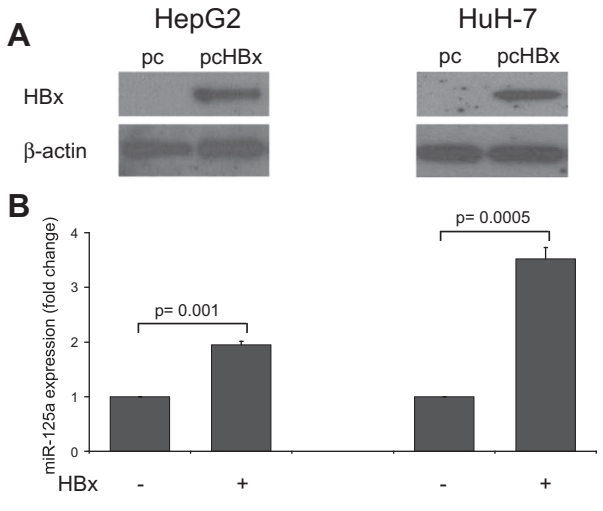

Fig. 3. Levels of miR-125a in HBx-expressing HCC cells. (A) Western blot analysis of HBx in HepG2 and HuH-7 cells transfected with parental pcDNA3.1 expression vector (pc) or its recombinant form containing the HBx coding sequence (pcHBX). Detection of β-actin was used as control. (B) Relative miR-125a expression in HepG2 and HuH-7 cells expressing (+) or not (−) HBx.

data depict a general model in which HBx protein is used to increase the expression of miR-125a, that in turn interferes with expression of HBV surface antigen, thus repressing viral replication (Fig. 4).

Co-evolution and adaptation between viruses and humans are often portrayed as a zero-sum biological arms race. Viruses enter host cells equipped with an array of mechanisms to evade the host defense responses and replicate and, host cells, in turn, build a defense mechanism to restrict the infection. Several studies have highlighted the role of cellular or viral miRNAs in the regulation of virus–host interaction: viruses encode miRNAs altering the expression of host genes to establish a cellular environment that is favorable to viral replication; on the other hand, host cells encode miRNAs counteracting viral replication.

In this study, the analyses performed on liver biopsies from HBV patients and two cultured cell lines provide a concrete evidence that HBV X protein plays a role in the up-regulation of liver miR-125a. In turn, this miRNA is known to interfere with the expression of HBV surface antigen, thus repressing viral replication. This self-inhibitory feedback loop (Fig. 4) may help HBV to fine-tune its replication, keeping it low to escape the immune system and establish a chronic infection. This mechanism would balance the virus–host interaction providing a condition that is beneficial for both the host survival and the viral spread in humans.

The regulatory circuit hypothesized in this study may have implications for the development of new strategies of anti-HBV intervention and prevention of hepatocarcinoma, since an outcome re-direction from a persistent infection, possibly progressing to HCC, to a complete recovery may be envisaged by unbalancing the molecules involved in the virus–host coexistence.

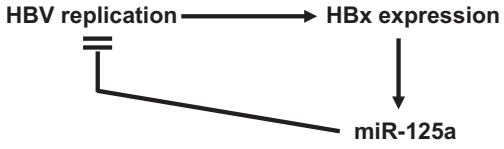

Fig. 4. A model depicting the HBV–host interaction based on miR-125a/HBx interplay. HBV infects hepatocytes and expresses HBx protein. As infection proceeds and viral load increases, HBx level raises leading to overexpression of miR-125a that, in turn, limits HBV replication.

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