Clinical Microbiology and Infection 25 (2019) 906.e1–906.e7

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

# The novel HBx mutation F30V correlates with hepatocellular carcinoma *in vivo*, reduces hepatitis B virus replicative efficiency and enhances anti-apoptotic activity of HBx N terminus *in vitro*

R. Salpini [1], M. Surdo [1], M.F. Cortese [1, 2], G.A. Palumbo [3], L. Carioti [1], G. Cappiello [4], A. Spanò [4], P. Trimoulet [5], H. Fleury [5], J. Vecchiet [6], C. Pasquazzi [7], C. Mirabelli [1, 8], R. Scutari [1], A. Sacco [1], M. Alkhatib [1], G. Missale [9], S. Francioso [10], L. Sarmati [11], M. Andreoni [11], M. Angelico [10], F. Ceccherini-Silberstein [1], M. Levrero [3, 12], C.F. Perno [1, 13], L. Belloni [3, 14], V. Svicher [1, *]

[1] *Department of Experimental Medicine and Surgery, University of Rome 'Tor Vergata' Rome, Italy*
[2] *Liver Pathology Unit, Departments of Biochemistry and Microbiology, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain*
[3] *Department of Internal Medicine-DMISM, Sapienza University, Rome, Italy*
[4] *'S. Pertini Hospital', Rome, Italy*
[5] *Hôpital Pellegrin Tripode, Bordeaux, France*
[6] *'SS Annunziata' Hospital, Chieti, Italy*
[7] *'S Andrea' Hospital, Rome, Italy*
[8] *University of Michigan Medical School, Ann Arbor, MI, USA*
[9] *Hospital of Parma, Parma, Italy*
[10] *Hepatology Unit, Tor Vergata University Hospital, Rome, Italy*
[11] *Infectious Diseases Unit, Tor Vergata University Hospital, Rome, Italy*
[12] *INSERM U1052 - Cancer Research Centre of Lyon, 69008 Lyon, France*
[13] *Haematology and Oncohaematology, University of Milan, Italy*
[14] *Centre for Life NanoSciences, IIT-Sapienza, Rome, Italy*

ARTICLE INFO

*Article history:*
Received 26 July 2018
Received in revised form
6 November 2018
Accepted 9 November 2018
Available online 23 November 2018

Editor: L. Kaiser

*Keywords:*
Apoptosis
HBx
Hepatitis B
Hepatitis B virus replication
Hepatocellular carcinoma

ABSTRACT

*Objective:* We aimed to investigate HBx genetic elements correlated with hepatitis B virus (HBV) -related hepatocellular carcinoma (HCC) and their impact on (a) HBV replicative efficiency, (b) HBx binding to circular covalently closed DNA (cccDNA), (c) apoptosis and cell-cycle progression, and (d) HBx structural stability.

*Methods:* This study included 123 individuals chronically infected with HBV: 27 with HCC (77.9% (21/27) genotype D; 22.1% (6/27) genotype A) and 96 without HCC (75% (72/96) genotype D; 25.0% (24/96) genotype A). HepG2 cells were transfected by wild-type or mutated linear HBV genome to assess pre-genomic RNA (pgRNA) and core-associated HBV-DNA levels, HBx-binding onto cccDNA by chromatin immunoprecipitation-based quantitative assay, and rate of apoptosis and cell-cycle progression by cytofluorimetry.

*Results:* F30V was the only HBx mutation correlated with HCC (18.5% (5/27) in HCC patients versus 1.0% (1/96) in non-HCC patients, p 0.002); a result confirmed by multivariate analysis. *In vitro*, F30V determined a 40% and 60% reduction in pgRNA and core-associated HBV-DNA compared with wild-type ($p < 0.05$), in parallel with a significant decrease of HBx binding to cccDNA and decreased HBx stability. F30V also decreased the percentage of apoptotic cells compared with wild-type ($14.8 \pm 6.8\%$ versus $19.1 \pm 10.1\%$, $p < 0.01$, without affecting cell-cycle progression) and increased the probability of HBx-Ser-31 being phosphorylated by PI3K-Akt kinase (known to promote anti-apoptotic activity).

*Conclusions:* F30V was closely correlated with HBV-induced HCC *in vivo*, reduced HBV replicative efficiency by affecting HBx-binding to cccDNA and increased anti-apoptotic HBx activity *in vitro*. This suggests that F30V (although hampering HBV's replicative capacity) may promote hepatocyte survival, so potentially allowing persistent production of viral progeny and initiating HBV-driven

* Corresponding author. V. Svicher, Department of Experimental Medicine and Surgery, University of Rome 'Tor Vergata' Rome, Italy.
*E-mail address:* valentina.svicher@uniroma2.it (V. Svicher).

https://doi.org/10.1016/j.cmi.2018.11.017

hepatocarcinogenesis. Investigation of viral genetic markers associated with HCC is crucial to identify those patients at higher risk of HCC, who hence deserve intensive liver monitoring and/or early anti-HBV therapy. **R. Salpini, Clin Microbiol Infect 2019;25:906.e1–906.e7**


## Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide [1]. The lifetime risk of developing HCC is 10- to 100-fold greater for chronic hepatitis B virus (HBV) carriers than non-infected individuals. In contrast with hepatitis C virus, a substantial number of HBV-infected individuals develop HCC without signs of liver damage [2], highlighting the existence of intrinsic HBV-mediated pro-oncogenic mechanisms. Among HBV proteins, HBx represents one of the central players of liver oncogenesis and could be an attractive therapeutic target for HCC suppression [1,3].

HBx is a 154-amino-acid protein (17 kDa) [4,5] characterized by two functional domains: the N-terminal domain (amino acids 1–50) endowed by anti-apoptotic effect, and the C-terminal domain with pro-apoptotic activity and involved in transactivation mechanisms [6,7]. Considering these dual properties, it has been postulated that the capability of HBx to induce HCC may depend on the equilibrium between the anti-apoptotic N-terminal and the pro-apoptotic C-terminal domains [7–9].

Deletions of the HBx C terminus are the most frequently reported HBx modifications associated with HCC. A higher rate of truncated HBx has been described in hepatocarcinoma cells rather than in untransformed hepatocytes, highlighting the potential role of these deletions and, in turn, of the N-terminal domain, in tumour development [8,10]. Furthermore, some point mutations (particularly K130M/V131I) in the HBx C terminus have been implicated in affecting the functions of the HBx C terminus and HCC onset [11,12]. Conversely, there is a paucity of information available on the impact of mutations within the N-terminal domain in oncogenic transformation of hepatocytes.

In this light, our study is aimed at evaluating the correlation of HBx mutations, particularly in the N-terminal domain, with HBV-induced HCC *in vivo*, and their impact on (a) HBV replicative and transcriptional efficiency, (b) HBx-binding to circular covalently closed DNA (cccDNA), (c) apoptosis and cell-cycle progression and, (d) HBx structural stability.

## Materials and methods

### *Study population*

This study included 123 chronically HBV-infected patients: 27 patients with HBV-related HCC and 96 consecutive patients with no clinical evidence of cirrhosis and HCC (as reference group), followed in different Italian and French clinical centres. The distribution of HBV genotype D and genotype A in HCC and non-HCC patients was comparable (77.9% (21/27) and 22.1% (6/27) in individuals with HCC versus 75% (72/96) and 25% (24/96) in non-HCC patients, respectively), minimizing the possible influence of HBV genotypes in the analysis of HBx mutations.

The HBV-related HCC cases were included in the study according to a radiological or histological diagnosis of hypervascular liver tumour mass (one or more liver cancer nodules) in patients with chronic HBV infection. No patients (0/123) had a co-infection with human immunodeficiency virus, hepatitis delta virus or hepatitis C virus.

Approval by the Ethics Committee was not necessary because the study was based on anonymized viral DNA samples obtained from clinical routines (legislative decrees 211/2003 (art.6,9) and 196/2003).

### *Population-based sequencing of the HBx region*

The population-based sequencing of the HBx region was performed on the 123 plasma samples, following a home-made protocol reported in the Supplementary material (Appendix S1).

### *Association of mutations with HCC*

HBx sequences were used to assess the association of specific HBx mutation(s) with HBV-related HCC. Mutations were defined according to the reference sequence of each specific HBV genotype (reference sequences: X65259.1 for genotype D and X02763.1 for genotype A). The prevalence of each HBx mutation was calculated in both HCC and non-HCC patients. Statistically significant differences in the prevalence of HBx mutations between the two groups of patients were assessed by Fisher's exact test.

The prevalence of HBx mutations associated with HCC was also calculated in two independent data sets of HBx sequences: the former obtained by GenBank and consisting of 40 HBx sequences from chronically HBV-infected patients with HCC (only genotypes D and A) [13,14], the latter obtained from The Hepatitis B virus Database (HBVdb) [15] and comprising 2428 HBx sequences (only genotypes D and A).

A logistic regression analysis was also performed using the statistical software IBM SPSS (v.19), considering the following variables: gender, age, nationality, HBV-DNA, hepatitis B “e” antigen (HBeAg) status, alanine aminotransferase (ALT), HBV genotype and HBx mutation(s) associated with HCC. After stepwise elimination for optimized Akaike information criterion, only variables showing a p-value <0.200 in univariate analysis were included in multivariate analysis.

### *Cell cultures*

HepG2 cells were cultured in a T25 flask (Costar; Corning, NY, USA) at a density of $5 \times 10^5$ cells/mL in Dulbecco's modified Eagle's medium (high glucose; Sigma, St Louis, MO, USA) supplemented with 100 U/mL penicillin + 100 mg/L streptomycin, 2 mM GlutaMAX and heat inactivated 10% fetal bovine serum. Cells were grown in a 37°C humidified atmosphere containing 5% $CO_2$.

### *Transfection experiments and quantification of HBV replicative parameters*

The HBx mutation F30V associated with HCC was introduced into a 1.0× genome-length HBV genotype D, HBeAg-negative clone by site-directed mutagenesis. Wild-type (WT) and mutated clones were transfected into HepG2 cells using the Mirrus Bio-trans-IT-LT1 (Mir2300A). All transfections included 0.1 μg green fluorescence protein (GFP) expression vector to assess transfection efficiency. Both cell lysates and supernatants were harvested at 48 and

72 hours post-transfection. Three experiments, each in triplicate, were performed to test the effects of F30V (with respect to WT-HBx) on intracellular core-associated HBV DNA and HBV pre-genomic RNA (pgRNA) quantified by real-time PCR using protocols described in the Supplementary material (Appendix S2).

### *cccDNA-specific chromatin immunoprecipitation-based quantitative assay*

Chromatin immunoprecipitation (ChIP)-based quantitative assay was used to evaluate the amount of F30V-HBx (with respect to WT-HBx) bound onto cccDNA according to the protocol reported in the Supplementary material (Appendix S3). Two independent experiments were performed.

**Table 1**
Characteristics of study population ($n = 123$)

| | Patients ($n = 27$) with HCC (%) | Patients ($n = 96$) without HCC (%) | p |
|---|---|---|---|
| Patient characteristics | | | |
| Male, *n* (%) | 24 (88.9) | 74 (77.1) | 0.28 |
| Italian nationality, *n* (%) | 19 (70.4) | 64 (66.7) | 0.81 |
| Age (years), median (IQR) | 67 (54–70) | 50 (38–63) | <0.001 |
| HBeAg negative, *n* (%) | 21 (77.8) | 68 (70.8) | 0.62 |
| HBV-DNA (log IU/mL), median (IQR) | 4.1 (2.6–5.9) | 5.0 (3.9–6.7) | 0.04 |
| ALT (IU/L), median (IQR) | 59 (28–107) | 45 (31–90) | 0.57 |
| HBV genotype, *n* (%) | | | |
| D | 21 (77.9) | 72 (75) | 1 |
| A | 6 (22.1) | 24 (25) | |
| HCC characteristics, *n* (%) | | | |
| Single nodule HCC | 15 (55.6) | | |
| Multifocal-HCC | 6 (22.2) | | |
| Unknown | 6 (22.2) | | |
| Tumour size | | | |
| <2 cm | 3 (11.1) | | |
| 2–5 cm | 4 (14.8) | | |
| >5 cm | 8 (28.6) | | |
| Unknown | 12 (44.4) | | |
| α-fetoprotein at HCC (ng/mL), median (IQR) | 520 (9–11 216) | | |
| HCC treatment | | | |
| Chemoembolization | 7 (25.9) | | |
| Surgery | 5 (18.5) | | |
| Thermoablation | 2 (7.4) | | |
| None | 3 (11.1) | | |
| Unknown | 10 (37.0) | | |
| Liver status | | | |
| Diagnosis of cirrhosis | 14 (51.9) | | |
| Child–Pugh Classification | | | |
| A | 11 (40.7) | | |
| B | 6 (22.2) | | |
| C | 3 (11.1) | | |
| Unknown | 7 (25.9) | | |

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IQR, interquartile range.

### *Cell-cycle and apoptosis analysis*

At 72 hours post-transfection, $10^6$ cells were harvested and permeabilized using 2 mL of 70% ethanol overnight at 4°C. The next day, cells were washed with PBS and incubated for 1 hour at 37°C in the presence of propidium iodide (50 mg/L) and of RNAase (250 mg/L) (Life Technologies). Propidium iodide was used to concomitantly differentiate phases of cell cycle and apoptotic cells based on cellular DNA content by flow cytometry (BD FACSCalibur; BD, Franklin Lakes, NJ, USA) [16]. Apoptotic cells were recognized as a distinct hypoploid cell population (sub-G1 peak) as a result of cell shrinkage, nuclear condensation and internucleosomal fragmentation. Debris, residuals of necrotic cells (with minimal DNA fluorescence and reduced diameter compared with apoptotic cells) and doublets were excluded from the analysis. The percentage of cells in sub-G1 peak, G0/G1, S and G2/M phases was assessed by ModFIT and confirmed by FlowJo softwares.

The results are reported as the mean of three independent experiments. The statistical relevance of the change in both cell cycle and apoptosis was evaluated using a test of proportions based on Pearson test.

### *In silico prediction of three-dimensional structure of HBx*

The three-dimensional structure of HBx was predicted *in silico* using a reference-validated HBx model [17] as a custom-added modelling constraint elaborated by I-TASSER [18].

The fold-stability change (ΔΔG) between WT and F30V mutants was calculated by STRUM, with ΔΔG(WT−mutated) <0 indicating a reduced stability in the presence of the mutation [19]. Furthermore, the probability of phosphorylation at HBx position 31 by phosphatidylinositol-4,5-bisphosphate 3-kinase protein kinase B (PI3K-Akt) (a process known to be implicated in HBV anti-apoptotic activity, and hence to be associated with neoplastic transformation) [20–22], was calculated for WT-HBx and F30V-HBx by two prediction algorithms: Scansite [23] and NetPhos [24], both aimed at identifying amino acid motifs phosphorylated by Ser/Thr-kinases (details reported in the Supplementary material, Appendix S4).

## Results

### *Patients' characteristics*

This study included 27 individuals with a diagnosis of HBV-related HCC. Most were male (88.9%, 24/27), with a median age of

**Table 2**
Multivariate analysis: factors associated with hepatocellular carcinoma

| Variables[a] | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | Crude OR (95% CI) | p | Adjusted OR (95% CI) | p |
| Gender (female versus male[b]) | 0.42 (0.12–1.53) | 0.714 | 0.45 (0.10–1.9) | 0.285 |
| Age (for 1 year increase) | 1.06 (1.02–1.11) | 0.003 | 1.05 (1.02–1.08) | 0.003 |
| Nationality (foreign versus Italian[b]) | 0.84 (0.33–2.13) | 0.720 | 1.7 (0.50–5.72) | 0.402 |
| HBeAg | 1.44 (0.53–3.95) | 0.478 | 0.51 (0.12–2.11) | 0.354 |
| HBV-DNA, $\log_{10}$ IU/ml | 0.77 (0.61–0.98) | 0.03 | 0.78 (0.60–1.01) | 0.07 |
| ALT, U/L | 1.00 (0.99–1.01) | 0.688 | 1.00 (0.99–1.01) | 0.74 |
| HBV Genotype (A versus D[b]) | 0.86 (0.31–2.372) | 0.767 | 1.21 (0.30–4.89) | 0.79 |
| HBx-F30V | 21.59 (2.4–194.519) | 0.006 | 20.02 (2.05–195.00) | 0.001 |

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IQR, interquartile range.

[a] The logistic regression analysis was performed on 123 chronically HBV-infected patients with or without HCC. The following variables were considered: gender, age, year of sample collection, nationality, HBeAg status, HBV-DNA log10, ALT, HBV genotype, HBx mutation associated with HCC (F30V).

[b] Reference group.

67 years (interquartile range (IQR) 54–70 years) (Table 1). At HCC diagnosis, median HBV-DNA and ALT were 4.1 (IQR 2.6–5.9) logIU/mL and 59 (IQR 30–110) U/L, respectively (Table 1). Notably, 40.7% (11/27) of patients received an HCC diagnosis in a condition of low-grade liver disease (Child–Pugh class A) (Table 1). As expected, at HCC diagnosis, most patients showed elevated α-fetoprotein (AFP) levels with median serum AFP 519 (IQR 9–11 216) ng/mL (Table 1). In most patients, liver imaging showed the presence of a single-nodule HCC with a median size of 5.0 (IQR 2.5–7.6) cm for each tumour (Table 1). Among 17 patients undergoing HCC treatment, trans-catheter arterial chemoembolization was the most used strategy for HCC treatment (in 25.9% (7/27) of patients) whereas in 5/17 the progression of HCC required a surgical resection (Table 1).

*Association of HBx mutation F30V with HBV-related HCC*

Analysing all HBx positions occurring in the HCC and non-HCC populations, one specific HBx mutation was significantly correlated with HCC: F30V, localized in the HBx N terminus (see Supplementary material, Fig. S1). In particular, 18.5% (5/27) of HCC patients versus 1.0% (1/96) of non-HCC patients harboured a virus with F30V (p 0.002). Notably, AFP levels at HCC diagnosis were higher in patients with F30V than in those without this mutation (median AFP 2600 (IQR 756–10 950) versus 56 (IQR 5–6508) ng/mL).

Multivariate analysis confirmed the independent association of F30V with a higher probability of developing HCC (adjusted OR 20.0, 95% CI 2.1–195.0; p 0.001), after correction for patients' demographics, HBeAg status, HBV genotype, serum HBV-DNA and ALT (Table 2).

In an independent data set of 2428 HBx sequences (only genotypes D and A) obtained from a publicly available HBVdb [15], the prevalence of F30V was 4.5% (109/2428), thus excluding the polymorphic nature of this mutation. Conversely, in an independent data set of 40 HBx sequences specifically isolated from patients with HCC and retrieved from GenBank, the prevalence of F30V was comparable to that observed in our cohort of HCC patients (12.5% (5/40) versus 18.5% (5/27), p 0.51), supporting its association with HCC.

The presence of K130M/V131I (already known to increase HBV oncogenic potential) was also investigated in HCC and non-HCC groups, as well as their co-occurrence with F30V. K130M/V131I were found in 53% (14/27) of patients with HCC and in 37.5% (36/96) of non-HCC patients (p 0.3). The co-occurrence of F30V and K130M/V131I was never found, suggesting divergent genetic pathways contributing to HCC development.

*HBx-F30V inhibits HBV transcription and replication activity in vitro*

HBx is known to up-regulate cccDNA transcription [4,25]. Hence, the impact of HBx-F30V on HBV replicative and transcriptional parameters was evaluated. After transfecting HepG2 cells with a WT or mutated linear HBV genome, the amount of pgRNA and intracellular core-associated HBV-DNA (Fig. 1a,b) was quantified at 48 hours post-transfection. HBx-F30V determined a 40% reduction in pgRNA levels ($p < 0.05$) and a 60% reduction in core-associated HBV-DNA ($p < 0.01$) compared with WT, suggesting a detrimental impact of F30V on HBV transcriptional and replicative capacity.

*HBx-F30V hampers HBx capability to bind cccDNA*

HBx can potentiate the expression of viral genes by its direct recruitment onto cccDNA [4,25], so we investigated whether F30V can affect HBx recruitment onto cccDNA. Using cccDNA ChIP assay, we showed a significant decrease of HBx-binding to cccDNA in the presence of F30V (Fig. 1c), that parallels with a reduced HBV transcription and replication.

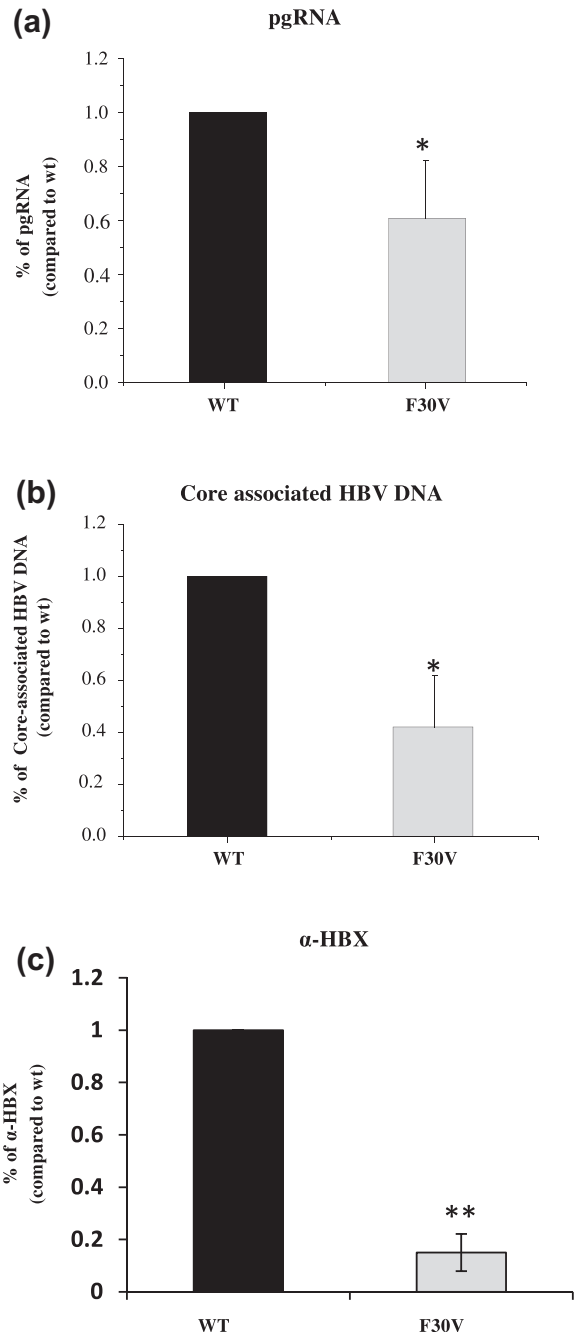


**Fig. 1.** Impact of F30V-HBx mutant on hepatitis B virus (HBV) replication. Production of intracellular pre-genomic RNA (pgRNA) (a) and core-associated HBV DNA (b) measured at 48 hours following transfection of full-length linear monomer of HBV genotype D wild-type (WT) or F30V-mutated into HepG2. pgRNA and core-associated HBV DNA were measured by real-time PCR. (c) Cross-linked chromatin was immunoprecipitated with a relevant control IgG or specific anti-HBx antibodies and analysed by real-time PCR with HBV circular covalently closed DNA (cccDNA) selective primers. The amount of pgRNA, core-associated HBV-DNA and HBX-bound cccDNA was expressed as percentage considering the amount of the wild-type as 100%. Histograms show mean values from three experiments, each led in triplicate in (a) and (b), and from two experiments in (c); bars indicate Standard Deviation. p values were determined using Student's *t* test. *p <0.05, **p <0.01.

*Cell cycle and apoptosis*

In order to support the association of F30V with HBV-induced hepatocarcinogenesis, we investigated the impact of this mutation on apoptosis and on cell-cycle progression by flow cytometry.

F30V decreased the percentage of apoptotic cells compared with WT-HBx (14.8 ± 6.8% versus 19.1 ± 10.1%, p <0.01) (Fig. 2a; see Supplementary material, Fig. S2). Conversely, by analysing cell-cycle progression, the percentage of cells in the different phases of the cell cycle was comparable between F30V and WT (Fig. 2b). These results suggest that F30V, by increasing anti-apoptotic HBx activity, can enhance hepatocyte survival, providing the basis for oncogenic transformation of hepatocytes.

*Impact of HBx-F30V three-dimensional structure: implications for anti-apoptotic effect*

By *in silico* prediction of three-dimensional HBx structure, amino acid position 30 lies in a coil region of the HBx N terminus, known to act as a negative regulatory domain (Fig. 3). In line with the replicative defect, the introduction of F30V determined a reduction in HBx stability (ΔΔG(WT−F30V) = −1.2 Kcal/mol).

Moreover, recent studies demonstrated that the phosphorylation of HBx-Ser-31 by PI3K-Akt is implicated in increasing HBx anti-apoptotic activity [20–22]. Interestingly, F30V increased the *in silico* probability of Ser-31 phosphorylation by PI3K-Akt with respect to WT (F30V 0.54 versus WT 0.48 and F30V 0.76 versus WT 0.72 according to Scansite and NetPhos, respectively). This increased phosphorylation of HBx-Ser-31 might explain the decreased rate of apoptosis observed *in vitro* in the presence of F30V, supporting its role in promoting hepatocyte survival.

## Discussion

This study has identified and characterized a specific HBx genetic element (F30V) significantly correlated with HBV-induced

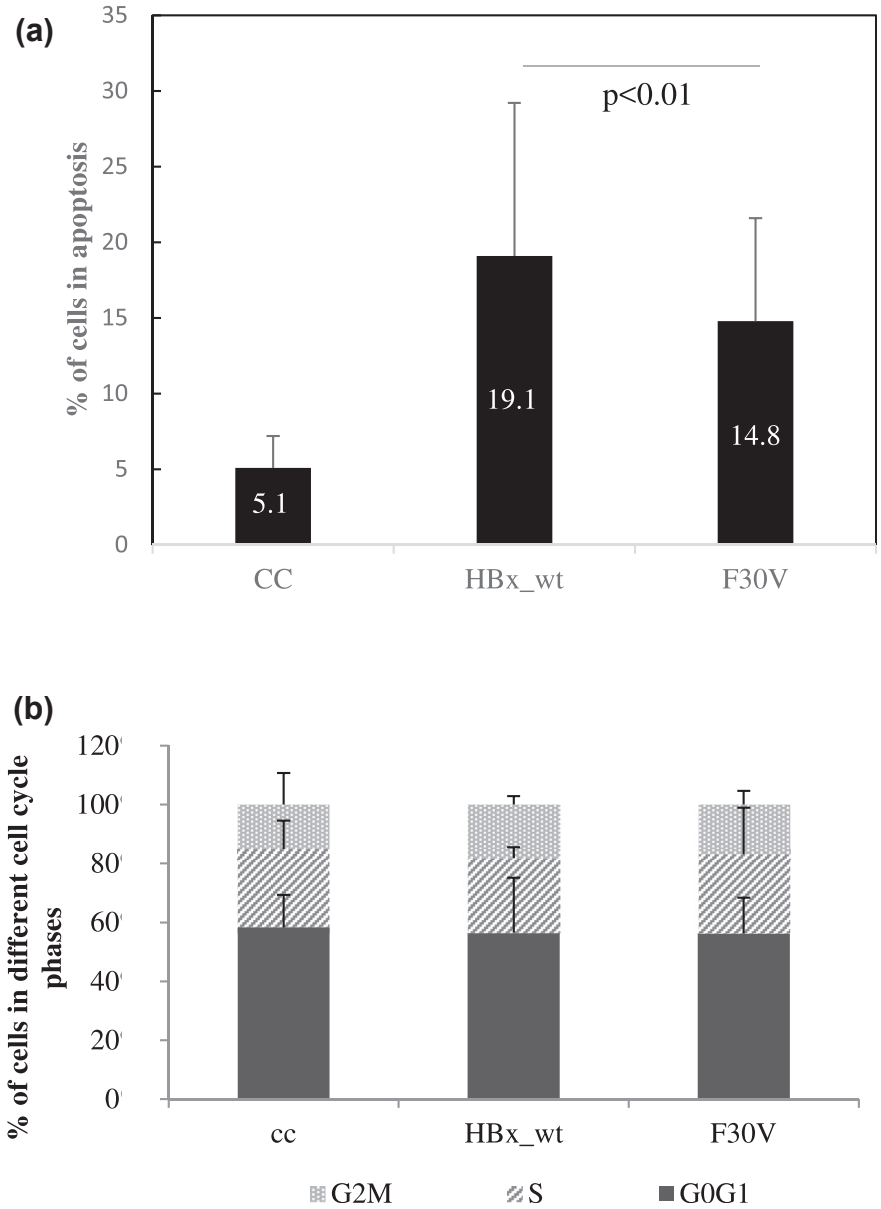


**Fig. 2.** Percentage of cells in apoptosis (a) and in different phases of cell cycle (b). HepG2 cells were transfected with hepatitis B virus (HBV) genotype D full-length linear monomers including wild-type (WT) and mutated HBx. $10^6$ cells were harvested at 72 hours post-transfection and permeabilized using 2 mL 70% ethanol overnight at 4°C. The next day cells were washed with PBS and incubated for 1 hour at 37°C in the presence of propidium iodide (PI) (50 mg/L) and of RNAase (250 mg/L) (Life Technologies). The percentage of cells in apoptosis and in G0/G1, S and G2/M cell-cycle phases was assessed by flow-cytometry (BD FACSCalibur) and analysed by ModFIT and FlowJo softwares. The results are reported as mean of three independent experiments. The statistical relevance of the change in both cellular cycle and apoptosis was evaluated using a test of Proportions based on Pearson Test. Abbreviations: WT, wild-type; CC, cell controls not transfected.

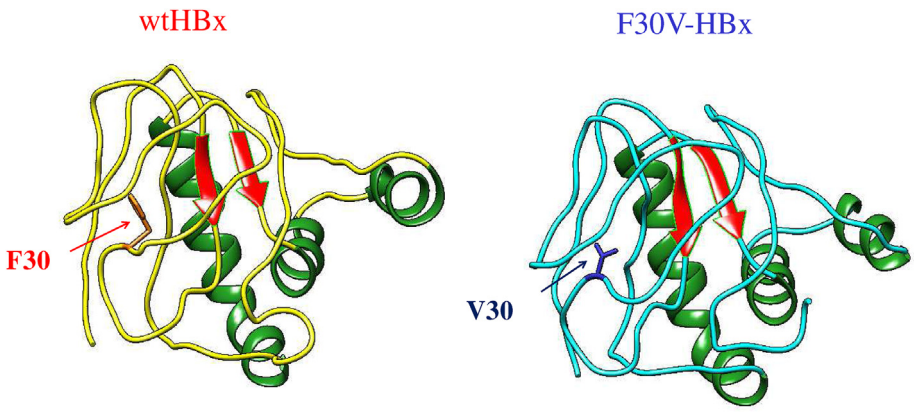


**Fig. 3.** Three-dimensional structure of wild type HBx and in presence of F30V mutation. Wild-type (WT) and mutated three-dimensional HBx were predicted by using a homology-modelling approach, based on Van Hemert et al., 2012 [17] and constructed by I-TASSER.

HCC *in vivo*. To date, the investigation of HBx mutations associated with HCC onset has been mainly conducted in Asian studies [12,26,27]. To our knowledge, this is the first European study addressing this issue.

F30V was detected in 18.5% of HCC patients and only in 1% of non-HCC patients. The low prevalence of F30V in non-HCC patients supports the fact that this mutation cannot be considered a polymorphism. In line with this concept, the analysis of 2428 HBx sequences obtained from a publicly available database HBVdb [15] showed an overall F30V prevalence of 4.5%. The association of F30V with HCC is further corroborated from an independent data set of 40 HBx sequences specifically isolated from patients with HCC and retrieved from GenBank, showing a prevalence comparable to that observed in our cohort of HCC patients.

The association of mutations at HBx position 30 with neoplastic transformation of hepatocytes has been previously reported in an Asian study, analysing 60 HCC tumour tissues and paratumour tissues from China, further reinforcing the involvement of this HBx residue in mechanisms mediating the oncogenic potential of HBV [28].

F30V resides in the highly conserved N-terminal domain known to be indispensable for transforming activity and negative regulation of pro-apoptotic activity and known to overcome oncogene-induced senescence [6,8,9]. From *in vitro* experiments, F30V can reduce the rate of apoptotic cells suggesting its role in promoting anti-apoptotic activity with respect to WT-HBx. Prevention of apoptosis has been proposed as a mechanism contributing to the multi-step hepatocarcinogenetic process as it can avoid the elimination of damaged hepatocytes [29].

Furthermore, F30V is adjacent to the serine at HBx position 31. A previous study has shown that the phosphorylation of HBx position 31 by PI3K-Akt is a mechanism mediating HBx anti-apoptotic properties and, in turn, cell survival [21,22]. In line with this finding, a recent study has reported that HBx isoforms, more prone to be phosphorylated at Ser-31 by PI3K-Akt, are associated with a higher risk of HCC development [20]. By *in silico* analysis, we found that HBx-F30V increases the likelihood of Ser-31 being phosphorylated by PI3K-Akt. It is conceivable that F30V emergence may favour Ser-31 phosphorylation, so enhancing HBx anti-apoptotic activity.

Overall findings support the hypothesis that HBx genetic variability may favour the shift from pro-apoptotic to anti-apoptotic HBx activity, thus explaining the dual HBx function (either as pro-apoptotic or as anti-apoptotic protein) reported in the literature [7,30].

Conversely, F30V has no impact on capacity of HBx to modulate cell-cycle progression. Indeed, the percentages of cells in the different phases of the cell cycle were comparable in the presence or absence of F30V, suggesting that the HBx-induced transit through G1 to S phase does not seem to be a mechanism responsible for the association of F30V with HCC [31].

In our cohort of HCC patients, F30V never co-occurred with K130M/V131I (known to favour host cell proliferation by inducing cell-cycle progression) [11], suggesting two divergent evolutionary pathways contributing to HCC development: the former acting on apoptosis inhibition and the latter on cell-cycle progression [11,30,32].

Our *in vitro* experiments also showed that HBx-F30V is less effective in supporting HBV replication and in transactivating HBV genes with respect to HBx-WT. This has been supported by ChIP-based quantitative assay, showing an impaired HBx-binding to cccDNA and by structural analysis revealing decreased HBx stability in the presence of F30V.

Overall findings suggest that, although hampering HBV replicative capacity, F30V may enhance hepatocyte survival by increasing anti-apoptotic HBx activity, thus allowing persistence of an intrahepatic viral reservoir and long-term production of infectious progeny. Furthermore, by lowering the level of HBV replication, this mutation may also favour HBV evasion of the immune-response, further facilitating viral persistence. At the same time, this may pose the basis for clonal selection of hepatocytes harbouring this viral variant, favouring the development of liver cancer [11].

In conclusion, HBx mutation F30V is closely correlated with HBV-induced HCC *in vivo*, reduced HBV replicative efficiency by affecting HBx-binding to cccDNA and increased anti-apoptotic HBx activity *in vitro*. This suggests that F30V (although hampering the replicative capacity of HBV) may promote cell survival, supporting its involvement in initiating mechanisms underlying HBV-driven hepatocarcinogenesis. The investigation of such viral genetic elements provides the basis for the identification of viral biomarkers that can predict HCC onset. This is crucial to identify individuals at higher HCC risk who may deserve intensive HCC monitoring, and/or early anti-HBV therapy. This represents an unmet medical need, answering the issue ‘assess host genetic and viral markers to determine prognosis and optimize patients’ management’ raised by the EASL Guidelines (www.easl.eu).

## Acknowledgements

We thank Amalia Mastrofrancesco, Massimiliano Bruni and Andrea Biddittu for data management, and Patrizia Saccomandi for support with the *in vitro* experiments.

## Funding

This work was financially supported by FIRB project (RBAP11YS7K_001), by Bandiera Project, by the Italian Ministry of Instruction, University and Research, and by Aviralia Foundation.

## Transparency declaration

R. Salpini reports speaker fees and consulting fees from Bristol Myers Squibb, Gilead Sciences, Diasorin and FujiRebio, outside the submitted work. H. Fleury reports grants from Merck Sharp and Dohme, outside the submitted work. L. Sarmati reports grants from Gilead Sciences and from Merck Sharp & Dohme, outside the submitted work. M. Andreoni reports grants, personal fees and other from Bristol Myers Squibbs, grants, personal fees and other from Gilead Sciences, and grants, personal fees and other from Merck Sharp & Dohme, outside the submitted work. M. Angelico reports grants from Gilead Sciences, grants from Bristol Myers-Squibbs, and personal fees from Abbvie, outside the submitted work. F. Ceccherini-Silberstein reports grants, personal fees and other from Bristol Myers Squibbs, grants, personal fees and other from Gilead Sciences, grants, personal fees and other from Merck Sharp & Dohme, personal fees from Janssen-Cilag, grants and personal fees from Viiv Healthcare, and personal fees from Roche Diagnostics, outside the submitted work. M. Levrero reports personal fees from Bristol Myers Squibb, personal fees from Gilead Sciences, personal fees from Merck Sharp & Dohme, personal fees from Janssen-Cilag, and personal fees from Roche Diagnostics, outside the submitted work. C.F. Perno reports grants and personal fees from Bristol Myers Squibb, grants and personal fees from Gilead Sciences, personal fees from Merck Sharp & Dohme, personal fees from Abbott Diagnostics, and personal fees from Roche Diagnostics, outside the submitted work. V. Svicher reports grants and personal fees from Bristol Myers Squibbs, grants and personal fees from Gilead Sciences, grants and personal fees from Diasorin Diagnostics, and personal fees from Fujirebio, outside the submitted work. The other authors have no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2018.11.017.

## References

[1] Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet [Internet] 2012;379:1245–55.
[2] El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology 2012;142:1264–12673.e1.
[3] Neuveut C, Wei Y, Buendia MA. Mechanisms of HBV-related hepatocarcinogenesis. J Hepatol 2010;52:594–604.
[4] Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, et al. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. Proc Natl Acad Sci USA 2009;106:19975–9.
[5] Guerrieri F, Belloni L, D'Andrea D, Pediconi N, Le Pera L, Testoni B, et al. Genome-wide identification of direct HBx genomic targets. BMC Genomics 2017;18:184.
[6] Tang H, Oishi N, Kaneko S, Murakami S. Molecular functions and biological roles of hepatitis B virus x protein. Cancer Sci 2006;97:977–83.
[7] Slagle BL, Bouchard MJ. Role of HBx in hepatitis B virus persistence and its therapeutic implications. Curr Opin Virol 2018;30:32–8.
[8] Ma N-F, Lau SH, Hu L, Xie D, Wu J, Yang J, et al. COOH-terminal truncated HBV X protein plays key role in hepatocarcinogenesis. Clin Cancer Res 2008;14:5061–8.
[9] Chau DK-F, Chen GG, Zhang H, Leung BCS, Chun S, Lai PB-S. Differential functions of C- and N-terminal hepatitis B x protein in liver cells treated with doxorubicin in normoxic or hypoxic condition. PLoS One 2012;7:e50118.
[10] Liu X-H, Lin J, Zhang S-H, Zhang S-M, Feitelson M-A, Gao H-J, et al. COOH-terminal deletion of HBx gene is a frequent event in HBV-associated hepatocellular carcinoma. World J Gastroenterol 2008;14:1346–52.
[11] Siddiqui ZI, Farooqui SR, Azam SA, Afroz M, Wajid S, Parveen S, et al. A comparative study of hepatitis B virus X protein mutants K130M, V131I and KV130/131MI to investigate their roles in fibrosis, cirrhosis and hepatocellular carcinoma. J Viral Hepat 2017;24:1121–31.
[12] Al-Qahtani AA, Al-Anazi MR, Nazir N, Ghai R, Abdo AA, Sanai FM, et al. Hepatitis B virus (HBV) X gene mutations and their association with liver disease progression in HBV-infected patients. Oncotarget 2017;8:105115–25.
[13] Datta S, Ghosh A, Dasgupta D, Ghosh A, Roychoudhury S, Roy G, et al. Novel point and combo-mutations in the genome of hepatitis B virus-genotype D: characterization and impact on liver disease progression to hepatocellular carcinoma. PLoS One 2014;9:e110012.
[14] Elkady A, Tanaka Y, Kurbanov F, Oynsuren T, Mizokami M. Virological and clinical implication of core promoter C1752/V1753 and T1764/G1766 mutations in hepatitis B virus genotype D infection in Mongolia. J Gastroenterol Hepatol 2008;23:474–81.
[15] Hayer J, Jadeau F, Deleage G, Kay A, Zoulim F, Combet C. HBVdb: a knowledge database for hepatitis B virus. Nucleic Acids Res 2013;41:D566–70.
[16] Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 2006;1:1458–61.
[17] van Hemert FJ, van de Klundert MAA, Lukashov VV, Kootstra NA, Berkhout B, Zaaijer HL. Protein X of hepatitis B virus: origin and structure similarity with the central domain of DNA glycosylase. PLoS One 2011;6:e23392.
[18] Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinform 2008;9:40.
[19] Quan L, Lv Q, Zhang Y. STRUM: structure-based prediction of protein stability changes upon single-point mutation. Bioinformatics 2016;32:2936–46.
[20] Lee W-P, Lan K-H, Li C-P, Chao Y, Lin H-C, Lee S-D. Oncogenic circuit constituted by Ser31-HBx and Akt increases risks of chronic hepatitis and hepatocellular carcinoma. Biochim Biophys Acta 2016;1862:837–49.
[21] Rawat S, Bouchard MJ. The hepatitis B virus (HBV) HBx protein activates AKT to simultaneously regulate HBV replication and hepatocyte survival. J Virol 2015;89:999–1012.
[22] Lee W-P, Lan K-H, Li C-P, Chao Y, Lin H-C, Lee S-D. Pro-apoptotic or anti-apoptotic property of X protein of hepatitis B virus is determined by phosphorylation at Ser31 by Akt. Arch Biochem Biophys 2012;528:156–62.
[23] Obenauer JC, Cantley LC, Yaffe MB. Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res 2003;31:3635–41.
[24] Blom N, Sicheritz-Pontén T, Gupta R, Gammeltoft S, Brunak S. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 2004;4:1633–49.
[25] Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol 2011;55:996–1003.
[26] Ali A, Abdel-Hafiz H, Suhail M, Al-Mars A, Zakaria MK, Fatima K, et al. Hepatitis B virus, HBx mutants and their role in hepatocellular carcinoma. World J Gastroenterol 2014;20:10238–48.
[27] Li W, Goto K, Matsubara Y, Ito S, Muroyama R, Li Q, et al. The characteristic changes in hepatitis B virus X region for hepatocellular carcinoma: a comprehensive analysis based on global data. PLoS One 2015;10:e0125555.
[28] Wang Q, Zhang T, Ye L, Wang W, Zhang X. Analysis of hepatitis B virus X gene (HBx) mutants in tissues of patients suffered from hepatocellular carcinoma in China. Cancer Epidemiol 2012;36:369–74.
[29] Yeh C-T, Shen C-H, Tai D-I, Chu C-M, Liaw Y-F. Identification and characterization of a prevalent hepatitis B virus X protein mutant in Taiwanese patients with hepatocellular carcinoma. Oncogene 2000;19:5213–20.
[30] Lin S, Zhang Y-J. Interference of apoptosis by hepatitis B virus. Viruses 2017;9:230.
[31] Bouchard M, Giannakopoulos S, Wang EH, Tanese N, Schneider RJ. Hepatitis B virus HBx protein activation of cyclin A-cyclin-dependent kinase 2 complexes and G1 transit via a Src kinase pathway. J Virol 2001;75:4247–57.
[32] Mukherji A, Janbandhu VC, Kumar V. HBx-dependent cell cycle deregulation involves interaction with cyclin E/A–cdk2 complex and destabilization of p27 $^{Kip1}$. Biochem J 2007;401:247–56.