

# Gender disparity of hepatocellular carcinoma: role of hepatitis B virus X protein and androgen receptor

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## KEY MESSAGES

1. The natural COOH-terminal truncated HBx was more frequently detected in hepatocellular carcinoma (HCC) in male than female patients.
2. There was no significant clinicopathological correlation with the HBx status or remarkable changes in androgen receptor (AR) expression in the presence of different truncated HBx mutants compared with full-length HBx.
3. Lower expression of AR was associated with

more aggressive tumour phenotype and a higher metastatic potential of HCC.

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## Introduction

Hepatocellular carcinoma (HCC) is especially prevalent in Hong Kong due to prevalent hepatitis B virus (HBV) infection. HBV-associated HCC has a male predominance, with a male-to-female ratio of about 4-5:1. This is in contrast to HCV-associated HCC that has a male-to-female ratio of 1.5-2:1. HBV is a partially double-stranded DNA virus containing four overlapping open reading frames. Among them, the X gene is most frequently integrated into the host genome naturally. Naturally integrated X gene is frequently found to be deleted at the 3'-end, leading to COOH-terminal truncated HBx protein.

In our previous study, we showed that in HBV-positive patients with HCC, full-length HBx (HBxFL) was detected in all non-tumorous livers ( $n=51$ ). However, HBxFL (HBx 1-154 amino acids) was found in only 53% of the tumours, whereas natural COOH-truncated HBx (HBx 1-130 amino acids) was found in the remaining 47%. The presence of natural COOH-truncated HBx significantly correlated with the presence of venous invasion ( $P<0.01$ ), a hallmark of metastasis, suggesting COOH-truncation of HBx plays a significant role in enhancing cell invasiveness and metastasis in HCC.<sup>1</sup> In addition, HBx mutants with different COOH-truncated length cause HCC by altering intracellular distribution and consecutive modulation of the important proliferative STAT/SOCS signalling, indicating a role of various shorter HBx mutants in tumourigenesis.<sup>2</sup> The mislocalisation of HBx truncated mutants can be explained in part by the finding that HBx protein contains a functional nuclear export sequence from amino acids 89 to 100.<sup>3</sup> Therefore, the COOH-terminal truncated form of HBx has been demonstrated to play a tumourigenic role in HCC and its oncogenicity may vary according to the truncated length.

Among male HBV carriers, the risk of HCC development is significantly increased in those with higher androgen receptor (AR) activity. HBV infection may, therefore, particularly cooperate with male-specific AR signalling to accelerate hepatocarcinogenesis. Moreover, HBx has been shown to enhance AR transcriptional activity through GSK-3 $\beta$  kinase pathways.<sup>4</sup> However, no report has shown a correlation between AR signalling and COOH-terminal truncated HBx protein.

In this study, we hypothesised that HBx cooperated with male-specific AR signalling to enhance hepatocarcinogenesis, and natural HBx truncated mutants had different effects on this AR signalling. The prevalence of natural COOH-terminal truncated HBx mutants (HBx ΔC1 and HBx <ΔC1) was slightly higher in male than female with HCC, but no significant clinicopathological association was observed with the expression of COOH-terminal truncated HBx mutants in HCCs. The COOH-terminal truncated HBx mutants were not associated with the mRNA expression of AR in the same HCC specimens, but the mRNA expression of AR showed a significant correlation with the metastatic potential, cellular differentiation and staging of HCC.

## Methods

This study was conducted from April 2013 to April 2014.

### Patient samples

A total of 99 pairs of human HCCs from patients who underwent liver resection for HCC between 1992 and 2001 at Queen Mary Hospital, Hong Kong, were selected. All these 99 patients (77 men and 22 women) aged 19 to 74 years were positive for serum hepatitis B surface antigen (HBsAg). All specimens

were snap-frozen in liquid nitrogen and kept at -80°C. Frozen sections were cut from HCC blocks separately and stained for histological examination to ensure a homogenous cell population of tissues.

### RNA extraction and reverse-transcriptase polymerase chain reaction

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad [CA], USA) following the manufacturer's instructions. cDNA was synthesised by using Gold RNA PCR Core Kit (Applied Biosystems, Foster City [CA], USA) following the manufacturer's instructions.

### Semi-quantitative PCR

For semi-quantitative PCR, the reaction was set up using AmpliTaqGOLD enzyme (Applied Biosystems, Carlsbad [CA], USA) and carried out with Takara PCR Thermal Cycler. PCR products were detected by 1.5% ethidium bromide-agarose gel electrophoresis at 100V for 15 minutes and visualised on a gel documentation system. The primers for PCR were as follows: HBx ATG: 5'-CCC AAG CTT ATG GCT GCT AGG CTG TGC T-3'; HBx full length (HBxFL): 5'-CGA ATT CTT AGG CAG AGG TGA AAA AGT TG-'; HBx ΔC1: 5'-CGA ATT CTT ACT TTA ATC TAA TCT CCT CCC C-3'; HBx <ΔC1: 5'- CTT ATG TAA GAC CTT GGG CAA CAT-3'

### Quantitative real-time PCR

The reaction was set up using SYBR Green Real-Time PCR Master Mixes (Applied Biosystems) and was carried out with Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol. All reactions were performed in triplicate and the expression of targeted gene relative to hypoxanthine guanine phosphoribosyl transferase was determined using  $2^{-\Delta CT}$  method. The primer sequences for real-time PCR were as follows: AR F: 5'-CTC ACC AAG CTC CTG GAC TC-3'; AR R: 5'-GAA AGG ATC TTG GGC ACT TG-3'; HPRT F: 5'-CTTGCTGACCTGCTGGATT-3'; HPRT R: 5'-CTGCATTGTTGCCAGTGT -3'.

## Results

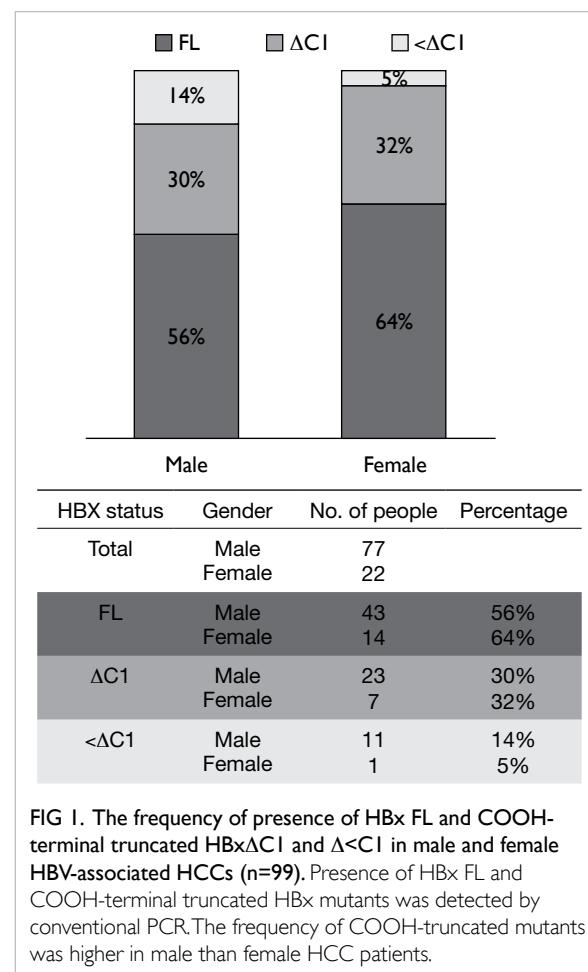
### The prevalence of natural COOH-terminal truncated HBx mutants (HBx ΔC1 and HBx <ΔC1) was slightly higher in HCCs from male than female patients

The HCCs were screened for the presence of COOH-terminal truncated HBx mutants, ΔC1 (HBx 1-130 amino acids) and <ΔC1 (HBx 1-95 amino acids). The expression pattern of the truncated HBx mutants between male and female patients was compared. Among the 99 HCC samples, the natural COOH-terminal truncated HBx mutants (HBx ΔC1

and HBx <ΔC1) were more frequently detected in HCCs in males than females (44% vs 37%, Fig 2). The frequency of HBx ΔC1 in HCCs in males was similar to that in females (30% vs 32%), but a more remarkable difference was observed for HBx <ΔC1 mutants (14% vs 5%, Fig 1). These indicated that the COOH-terminal truncated HBx mutants that are shorter than ΔC1 may be found more frequently in male HCCs and may be more associated than HBx ΔC1 with the male predominance in HCC.

### The expression of COOH-terminal truncated HBx mutants in human HCCs showed no significant clinicopathological or survival correlation

To examine whether the presence of natural COOH-terminal truncated HBx mutants was associated with a particular tumour biological behaviour and consequent prognosis, the expression of full-length HBx and truncated HBx mutants was correlated with different clinicopathological features and survival rates in the 99 HCC patients. The presence of COOH-terminal truncated HBx mutants (ie the group with either HBx ΔC1 or HBx <ΔC1) did not show any significant correlation with the clinicopathological features (Fig 2a). In addition, there was no significant



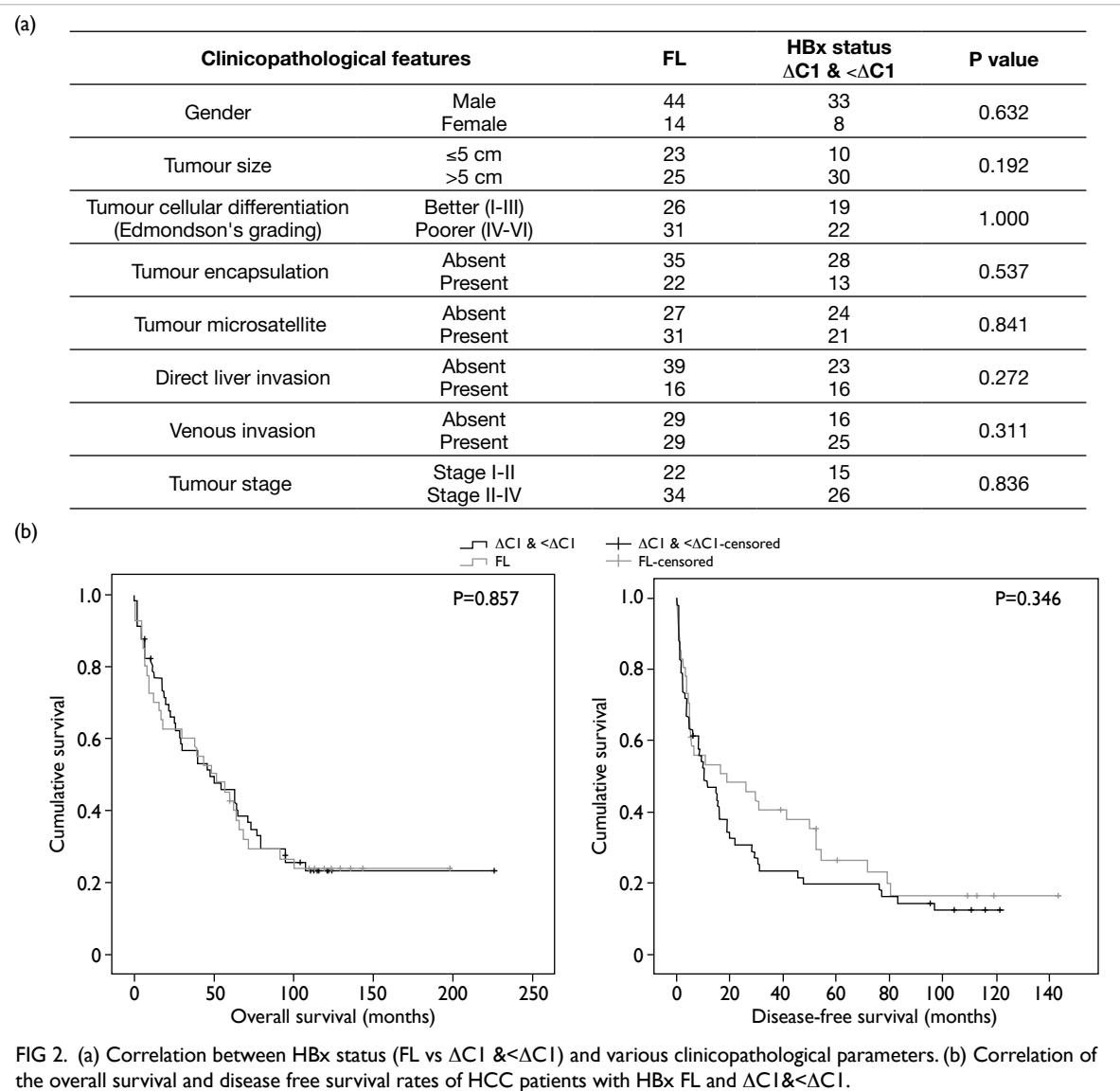


FIG 2. (a) Correlation between HBx status (FL vs ΔC1 & <ΔC1) and various clinicopathological parameters. (b) Correlation of the overall survival and disease free survival rates of HCC patients with HBx FL and ΔC1 & <ΔC1.

difference between the presence of HBx mutants and the disease free or overall survival (Fig 2b). This demonstrated that the presence of COOH-terminal truncated HBx mutants might not be associated with tumour biological behaviour.

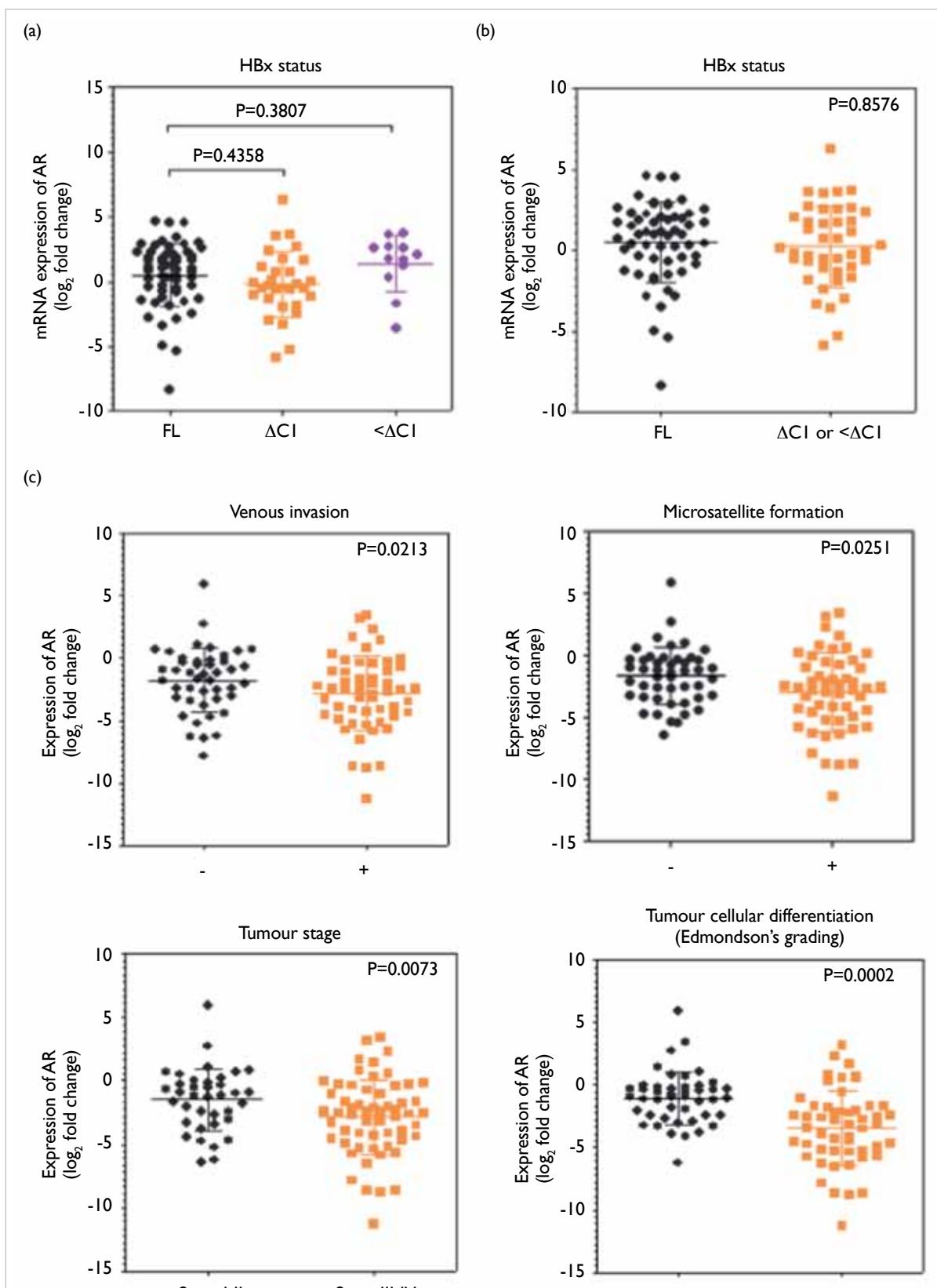
#### The COOH-terminal truncated HBx mutants had no significant correlation with the mRNA expression of AR in the same HCC specimens

To investigate the effect of different COOH-terminal truncated HBx mutants on the mRNA expression level of AR, the expression level of AR was examined and correlated with the presence of COOH-terminal truncated HBx mutants in the 99 HCCs. There was no significant difference between the mRNA levels of AR and the presence of COOH-terminal truncated HBx mutants or the full-length HBx (Fig 3a). Although there was a trend of association between the presence of HBxΔC1 and downregulation of AR ( $P=0.4358$ ), the mRNA levels of AR were not

significantly different upon further stratification of the HBx mutants into HBxΔC1 and HBx<ΔC1 (Fig 3b). These indicated that the COOH-terminal truncated HBx mutants, either HBx ΔC1 or HBx<ΔC1, may not regulate mRNA expression of AR.

#### The mRNA expression of AR showed a significant correlation with the metastatic potential, cellular differentiation and staging of HCC

To evaluate the association of the mRNA expression of AR with a particular tumour biological behaviour and prognostic significance in HCC, the mRNA levels of AR correlated with different clinicopathological features. Lower expression of AR in the HCC was associated with the presence of tumour venous invasion ( $P=0.0213$ ), tumour microsatellite formation ( $P=0.0251$ ), poorer tumour cellular differentiation ( $P=0.0002$ ) and higher tumour stage ( $P=0.0073$ ) [Fig 3c]. This correlation



**FIG 3.** (a, b) The AR mRNA levels were correlated with the presence of HBxFL and different COOH-terminal truncated HBx mutants in human HBV-associated HCC tissues (n=99). (c) The mRNA expression of AR was correlated with different tumour biological features including presence of tumour venous invasion, tumour encapsulation, direct liver invasion, tumour microsatellite formation, tumour cellular differentiation, tumour size, background liver disease, gender and tumour staging. Lower expression of AR was associated with the presence of venous invasion, microsatellite formation, poorer cellular differentiation and higher tumour stage in HCC.

study demonstrated that lower expression of AR was associated with a higher metastatic potential and more aggressive tumour phenotype in HCC.

## Discussion

The frequency of natural COOH-terminal truncated HBx (HBx ΔC1 and HBx<ΔC1) was higher in HCCs of male than female patients (44% vs 37%). More importantly, when the mutants were stratified into the group with HBx<ΔC1 only and the other one with HBx FL or HBx ΔC1, the frequency of HBx mutants in male and female HCCs (14% vs 5%) was even more obvious. With a higher frequency of HBx<ΔC1 in HCCs of males and the differential regulatory roles of shorter truncated HBx mutants in HCC due to nuclear mislocalisation,<sup>2</sup> these imply that HBx<C1 may play a role in hepatocarcinogenesis in male HCC patients and may contribute in part to the male predominance of HCC. Nonetheless, the underlying mechanisms require further investigation. In addition, the expression status of HBx in the HCCs showed no significant correlation with any clinicopathological features or patient survival. This might have been a result of technical limitations in examining HBx gene truncation, where the co-existence of HBx FL and COOH-terminal truncated mutants in the same specimen cannot be detected by conventional PCR. The HCC tumours with HBx FL detected might also harbour HBxΔC1 and/or HBx<ΔC1, and this may influence the comparison among groups of various parameters.

Full-length HBx protein has been shown to enhance AR transcriptional activity through modulation of c-Src and GSK-3β kinase pathways.<sup>5</sup> In addition, HBx protein enhances AR-responsive gene expression depending on androgen level.<sup>6</sup> Therefore, the regulatory roles of full-length HBx on AR activity are well demonstrated. In the present study, we examined the consequence of COOH-terminal truncated HBx on AR transactivation. The results showed no significant correlation between the presence of X truncation and mRNA expression of AR in HCCs when compared with the full-length HBx, suggesting that COOH-truncated HBx mutants may not exert a more potent activation on AR than the full-length HBx. Nevertheless, a lower expression of AR was associated with more aggressive tumour behaviour. This finding was consistent with that in other studies that report hepatic AR suppression of HCC metastasis through modulation of cell migration and anoikis,<sup>7</sup> enhanced cell adhesion and decreased cell migration via modulating β1-integrin-AKT signalling in HCC cells.<sup>8</sup> In contrast to our results, several reports found that the activation and overexpression of AR promoted cell invasion and migration in HCC and tumour staging.<sup>9</sup> Therefore, the potential roles of AR in hepatocarcinogenesis remain elusive and warrant further investigation.

## Conclusion

The HBx truncated mutants are more frequent in HCCs of males than females, and this may implicate a role of HBx truncated mutants in the male predominance of HCC. Nonetheless, the correlation study of the presence of HBxC1 or HBx<ΔC1 and the different clinicopathological features of HCC patients and the mRNA levels of AR implied that HBx truncated mutants alone may not exert a significant effect on the tumour biological behaviour or induce a more potent transactivation of AR than HBxFL. AR may play a role in hepatocarcinogenesis, in which lower expression of AR is associated with more aggressive tumour phenotype and a higher metastatic potential of HCC.

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