Research Article
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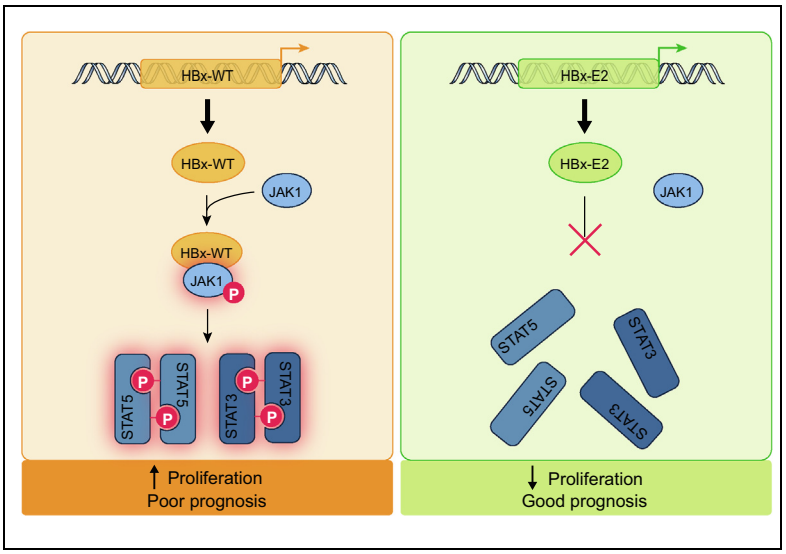
A novel HBx genotype serves as a preoperative predictor and fails to activate the JAK1/STATs pathway in hepatocellular carcinoma

Graphical abstract



Highlights

- Three HBx genotypes were identified in tumor and non-tumor tissues from patients with HBV-related HCC.
- HBx-E2 indicated better recurrence-free survival and overall survival for patients with HBV-related HCC.
- HBx-E2 preoperatively predicted the prognosis of patients with BCLC stage B HCC after resection.
- HBx-E2 and HBx-E2-N lost the ability to promote the proliferation of HCC cells and normal hepatocytes.
- HBx-E2 and HBx-E2-N failed to interact with JAK1 and activate the JAK1/STAT3/STAT5 signaling pathway.

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Lay summary

We classified a novel genotype of the full-length hepatitis B virus X gene (HBx), HBx-E2. This genotype was identified in tumor and nontumor tissues from patients with hepatitis B virus-related hepatocellular carcinoma. HBx-E2 could preoperatively predict the prognosis of patients with intermediate stage hepatocellular carcinoma, after resection.

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A novel HBx genotype serves as a preoperative predictor and fails to activate the JAK1/STATs pathway in hepatocellular carcinoma

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Background & Aims: Genetic variability in the hepatitis B virus X gene (HBx) is frequently observed and is associated with hepatocellular carcinoma (HCC) progression. However, a genotype classification based on the full-length HBx sequence and the impact of genotypes on hepatitis B virus (HBV)-related HCC prognosis remain unclear. We therefore aimed to perform this genotype classification and assess its clinical impact.

Methods: We classified the genotypes of the full-length HBx gene through sequencing and a cluster analysis of HBx DNA from a cohort of patients with HBV-related HCC, which served as the primary cohort (n = 284). Two independent HBV-related HCC cohorts, a validation cohort (n = 171) and a serum cohort (n = 168), were used to verify the results. Protein microarray assay analysis was performed to explore the underlying mechanism.

Results: In the primary cohort, the HBx DNA was classified into 3 genotypes: HBx-EHBH1, HBx-EHBH2, and HBx-EHBH3. HBx-EHBH2 (HBx-E2) indicated better recurrence-free survival and overall survival for patients with HCC. HBx-E2 was significantly correlated with the absence of liver cirrhosis, a small tumor size, a solitary tumor, complete encapsulation and Barcelona Clinic Liver Cancer (BCLC) stage A-0 tumors. Additionally, HBx-E2 served as a significant prognostic factor for patients with BCLC stage B HCC after hepatectomy. Mechanistically, HBx-E2 is unable to promote proliferation in HCC cells and normal hepatocytes. It also fails to activate the Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3)/STAT5 pathway.

Conclusion: Our study identifies a novel HBx genotype that is unable to promote the proliferation of HCC cells and suggests a potential marker to preoperatively predict the prognosis of patients with BCLC stage B, HBV-associated, HCC.

Lay summary: We classified a novel genotype of the full-length hepatitis B virus X gene (HBx), HBx-E2. This genotype was identified in tumor and nontumor tissues from patients with hepatitis B virus-related hepatocellular carcinoma. HBx-E2 could preoperatively predict the prognosis of patients with intermediate stage hepatocellular carcinoma, after resection.

Introduction

Chronic hepatitis B virus (HBV) infection is the dominant risk factor for the development of hepatocellular carcinoma (HCC) in the Asia-Pacific region due to inflammation, cirrhosis and direct viral oncogenic factors.[1] The hepatitis B virus X gene (HBx) gene, 1 of 4 open reading frames of HBV, encodes a 17 kDa protein and has been shown to be strongly linked to the development of HCC.[2] As a transactivator, the HBx protein activates various viral and cellular promoters and enhancers via protein–protein interactions and affects various signal-transduction pathways, such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Wnt/β-catenin, nuclear factor-κB (NF-κB) and protein kinase B/Akt pathways.[3,4] Furthermore, HBx has been shown to modulate a wide range of cellular functions, including proliferation, the cell cycle, apoptosis, autophagy, metastasis, and metabolism, which lead to the development of HCC.[5]

HBx shows high genetic variability, which is due to an inaccurate reverse transcriptase and a lack of proofreading activity, in HBV.[6] According to the results of genetic analyses of the HBx DNA, 2 main types of HBx genetic variations have been detected in patients with HBV-related liver diseases. First, some point mutations in the HBx gene, including nucleotides 1630, 1721, 1762, and 1764, and particularly double substitutions (A1762T and G1764A), are more frequent in patients with advanced liver diseases and HCC.[7,8] Second, distal C-terminally truncated HBx mutants are selected in tumor tissues and play a role in

Keywords: HBx; Genotype; BCLC staging; Prognosis; Cell proliferation.
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hepatocarcinogenesis.[9,10] HBx variants have been shown to play an important role in HCC progression. The A1762T/G1764A mutation increases the risk of HCC and is independently predictive of postoperative survival in patients with HCC.[11–12] C-terminally truncated HBx promotes the development and progression of cancer by increasing cell proliferation, invasiveness and metastasis.[13,14] The overall activity of HBx mutants appears to increase the expression of hypoxia-inducible factor-1α (HIF-1α), which is associated with poor outcomes for patients with HCC.[15] To study the variability of the HBx gene, it is helpful to further clarify the role of HBx in the progression of HCC and to identify the key locus that affects HBx functions.

Previous studies focusing on HBx gene variants generally concentrated on 2 mutation types. These studies failed to recognize the full-length HBx DNA sequence when classifying the genotype. They also failed to explore differences among genotypes and the biological effects resulting from multiple site variants. The primary amino acid sequence of the HBx protein is organized into a negative regulatory domain and a transactivation domain. Furthermore, 6 (A–F) regions in HBx exhibit different modular organizations and functions.[16,17] Interactions between the transactivation domain of HBx with different target molecules have been mapped to different regions.[18] Thus, the functional alterations in HBx resulting from variations at multiple sites in different regions between various genotypes are quite complicated and remain unclear.

In the present study, we focused on classifying HBx genotypes through a cluster analysis of variants in full-length HBx DNA sequences and refined the effect of different genotypes on the prognoses of patients with HCC. Interestingly, we identified a novel genotype of HBx in both the liver tissues and serum of patients with HBV-related HCC that was correlated with a better prognosis and disrupted function.

Patients and methods

Patients

This study used 3 independent cohorts of patients with HBV-related HCC. The primary cohort enrolled 284 patients from the Eastern Hepatobiliary Surgery Hospital (EHBH) in Shanghai, the validation cohort included 80 patients from the Mengchao Hepatobiliary Hospital of Fujian Medical University and 91 patients from the Affiliated Tumor Hospital of Guangxi Medical University in Nanning, and the serum cohort included 168 patients from the EHBH. Sera were collected from patients with HBV-induced hepatitis, cirrhosis and HCC at EHBH. The clinical data of 3 cohorts are presented in Table S1 and the clinical features of the primary cohort, validation cohort and serum cohort are listed in Tables S2, S3 and S4, respectively. The study protocol was approved by the local institutional review board and all patients signed an informed consent form.

Synthesis of 5 HBx DNA sequences

Five HBx DNA sequences were synthesized by the Generay Biotechnology Company (Shanghai, China) to further study the biological functions of HBx. These 5 genotypes are listed below. i) The HBx-positive (HBx-P) sequence, which has been confirmed to have specific biological functions, was used as a positive control.[19] ii) All HBx DNA sequences obtained from the primary cohort were compared, and the nucleotide present at the highest frequency at each site in the HBx region was considered a wild-type nucleotide. Thus, the wild-type HBx sequence was obtained and named HBx-WT. iii) The HBx-EHBH2 (HBx-E2) sequence was obtained by substituting corresponding sites in the HBx-WT sequence with variants present in the HBx-E2 genotype. iv) The corresponding sites of the HBx-WT sequence were substituted for the nonsynonymous loci of variants of the HBx-E2 genotype to acquire the HBx-E2-nonsynonymous (HBx-E2-N) sequence. v) The HBx-E2-synonymous (HBx-E2-S) sequence was obtained by substituting the corresponding bases of the HBx-WT sequence with synonymous loci of variant sites of HBx-E2. The DNA sequences are presented in the Supplementary Materials.

Cell lines

The Huh7 HCC cell line was obtained from the Chinese Academy of Sciences Cell Bank. The SMMC-7721 HCC cell line was obtained from the Second Military Medical University. The immortalized human hepatocyte LO2 cell line was obtained from the National Center for Liver Cancer (Shanghai, China). HepaRG cells showing susceptibility to HBV infection were obtained from the National Center for Liver Cancer (Shanghai, China).[20] The primary HCC cell lines and primary hepatocyte lines were acquired from tumor and nontumor tissues of patients with HCC (HBV genotype C) as previously described.[21] All the cell lines were identified by short tandem repeat typing and hepatic gene expression to exclude HeLa contamination.

Results

Identification of different HBx genotypes in tumor and nontumor tissue samples from the primary cohort

The complete HBx gene was sequenced in 284 tumor and adjacent nontumor tissue samples from patients with HCC in the HBV surface antigen-positive primary cohort (Fig. 1A). Of the 284 tissue samples, distal C-terminal truncations were found in 22.9% (65/284) of the tumor and 6.0% (17/284) of the nontumor samples. The full-length HBx DNA (except distal C-terminal truncations) was successfully sequenced in 219 (77.1%) tumor and 267 (94.0%) nontumor samples. Compared with the HBV/ayr subtype of the HBx sequence, 51 variant sites (with a frequency over 5%) were obtained. The variations were generally similar between the tumor and nontumor tissues (Fig. 1B). High-frequency variant sites were A1605C, C1655T, A1762T, G1764A and C1827A compared with the HBV/ayr subtype. In addition to these variant sites, internal deletions were found in 5.0% (14/284) of the tumor and 3.2% (9/284) of the nontumor samples. The breakpoint between 1762 and 1770 was the major form of deletion.

For the classification of HBx genotypes, each HBx DNA site was classified as mutated or nonmutated based on whether the loci at 51 variant sites underwent a variation, and the tumor and nontumor tissue samples were subjected to a cluster analysis. Three HBx genotypes were identified in both the tumor and nontumor tissues (Fig. 1C). We named these genotypes HBx-EHBH1 (HBx-E1), HBx-EHBH2 (HBx-E2), and HBx-EHBH3 (HBx-E3). The prevalence of these genotypes in the tumor and nontumor samples was the following: HBx-E1 (9.1% (20/219) *vs.* 6% (16/267)); HBx-E2 (20.5% (45/219) *vs.* 22.1% (59/267)); HBx-E3 (70.3% (154/219) *vs.* 71.9% (192/267)). Among the patients (n = 213) with full-length HBx sequences in both tumor and paired nontumor tissues, the percentages of identical HBx genotypes in tumor and paired nontumor tissues were 6.6% (for HBx-E1), 21.1% (for HBx-E2) and 66.2% (for HBx-E3).

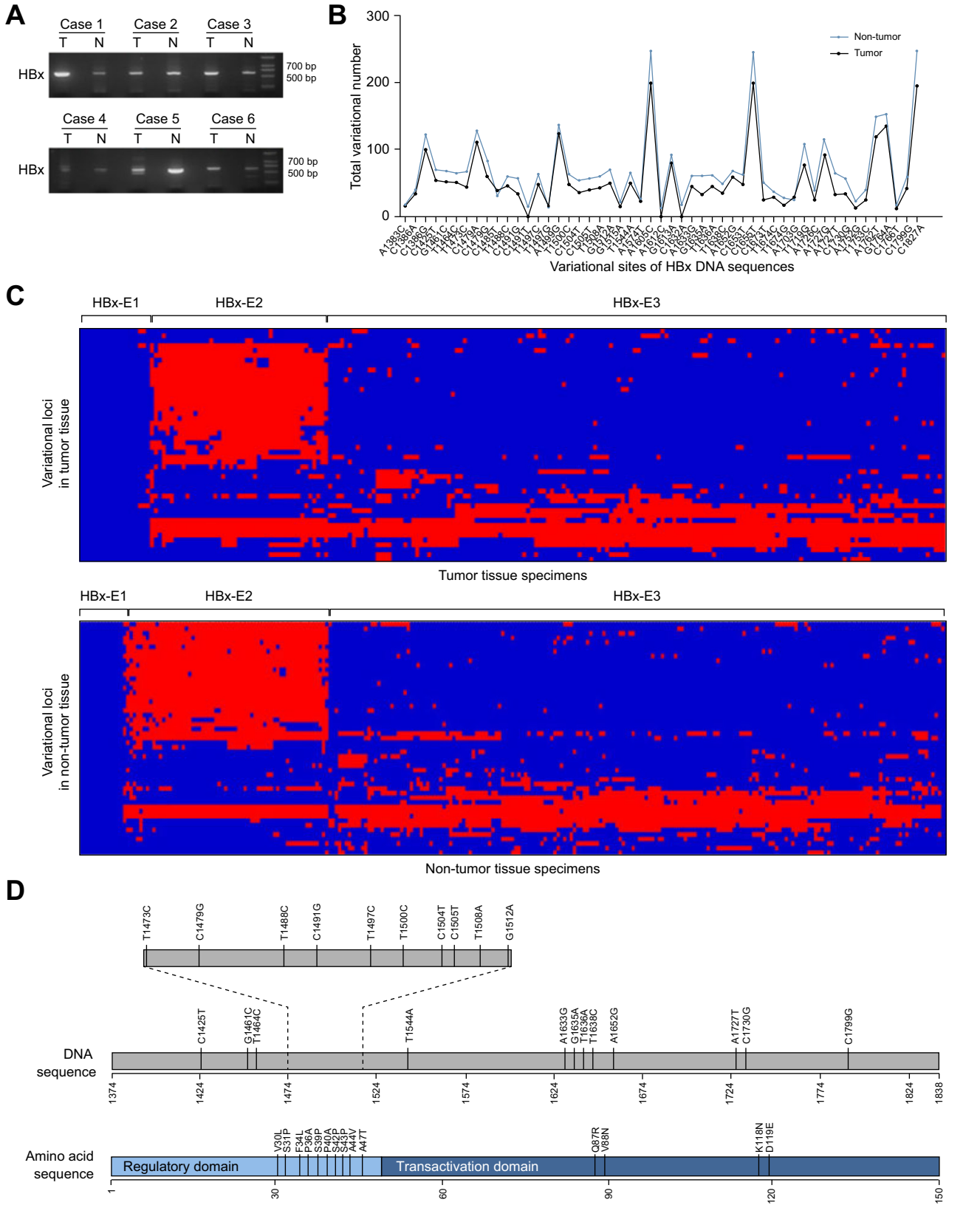


Fig. 1. Three HBx genotypes were identified by sequencing the HBx gene and a cluster analysis of variant sites in the tumor and adjacent nontumor tissues from the primary cohort. (A) Representative results indicating the presence of the full-length HBx DNA in HCC tumors (T) and their corresponding nontumor liver tissues (N). (B) Frequency of variant sites of HBx DNA sequences in the tumor (n = 219) and nontumor tissues (n = 267) of the primary cohort (HCC cohort from Shang Hai, n = 284) compared with the HBV/ayr sequence. (C) Three HBx genotypes were identified through a cluster analysis of variant loci in the HBx DNA in tumor (n = 219) and nontumor (n = 267) tissues samples from the primary cohort. Red indicates a mutation, and blue indicates no mutation. (D) Variant loci in the DNA and amino acid sequences of HBx-E2. The N-terminal part of HBx (light blue) is the negative regulatory domain of the X protein. The COOH-terminal part (blue) is the transactivation domain. HBx, hepatitis B virus X gene; HCC, hepatocellular carcinoma.

Remarkably, HBx-E2 contained a group of variant sites that included 15 nonsynonymous loci and 7 synonymous loci, which are shown in Fig. 1D. We identified 3 HBx genotypes through a cluster analysis of the variant sites. The clinical significance of the 3 genotypes was subsequently further analyzed.

HBx-E2 serves as a prognostic factor for patients with HCC

To identify the clinical significance of the 3 genotypes in the primary cohort, the relationships between the HBx genotypes and the prognosis of patients with resected HCC were first examined. A Kaplan-Meier analysis was used for subgroup comparisons between HBx-E1 (+) and HBx-E1 (−), HBx-E2 (+) and HBx-E2 (−), HBx-E3 (+) and HBx-E3 (−) in the tumor and nontumor samples from the primary cohort. Remarkably, patients with the HBx-E2 genotype in the tumor ($p = 0.001$, $p = 0.029$) and nontumor ($p = 0.001$, $p = 0.007$) tissues had better recurrence-free survival (RFS) and overall survival (OS) (Fig. 2A-B, Fig. S1A-B), whereas HBx-E1 and HBx-E3 were not related to the prognosis of patients with HCC. According to the Cox multivariate proportional hazards model, HBx-E2 was an independent prognosticator of RFS in tumor ($p = 0.006$) and nontumor ($p = 0.037$) samples (Table S5). We then determined whether the HBx-E2 genotype was correlated with clinicopathological characteristics. In tumor tissues, HBx-E2 was strongly associated with the absence of liver cirrhosis ($p = 0.044$), a small tumor size ($p = 0.004$), a solitary tumor ($p = 0.013$), complete encapsulation ($p = 0.004$), Barcelona Clinic Liver Cancer (BCLC) stage A-0 tumors ($p = 0.018$), and a 5-Gene

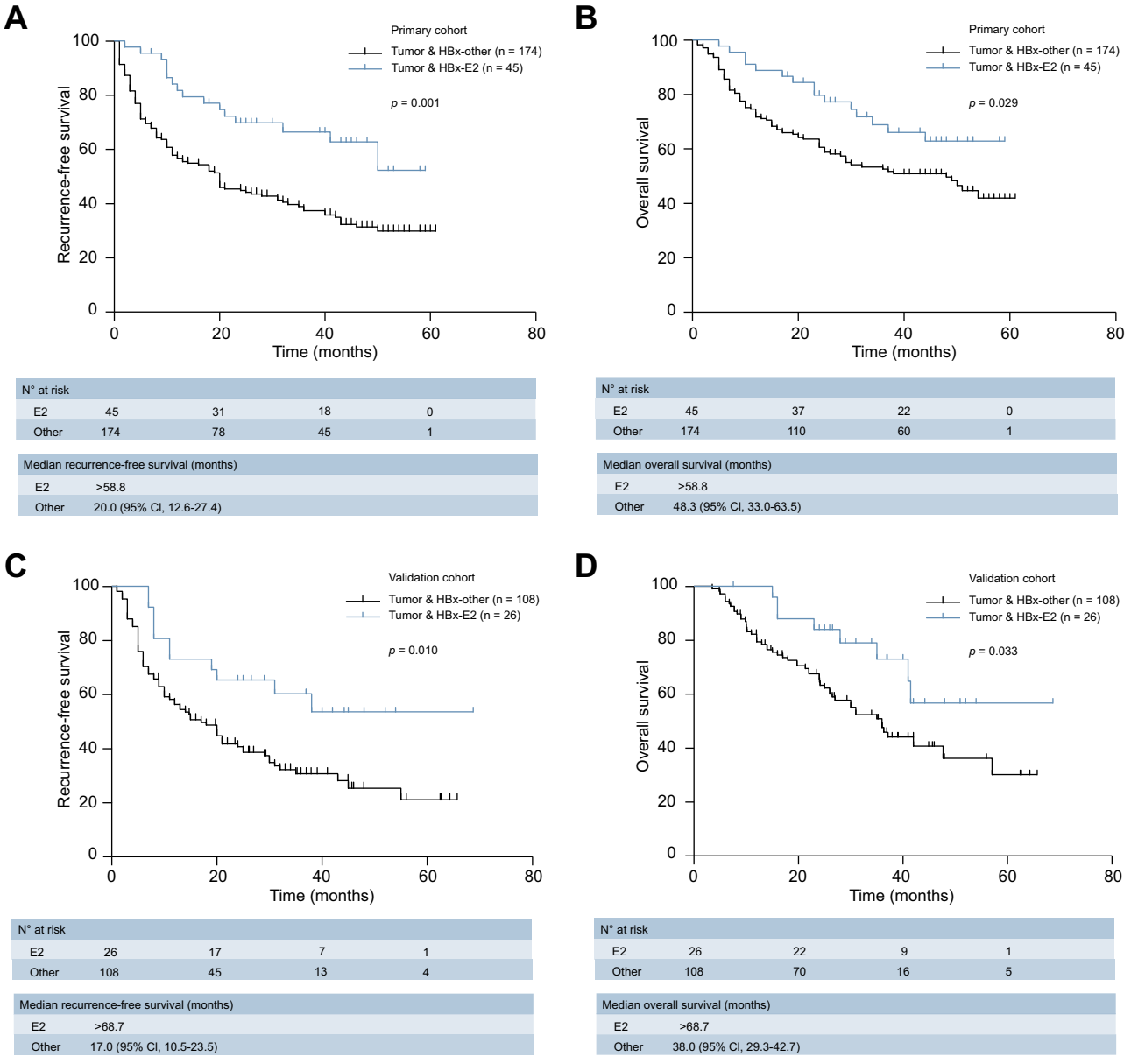


Fig. 2. HBx-E2 serves as a prognostic factor for patients with HCC in the primary cohort, which was validated in the validation cohort. (A,B) The HBx-E2 group had a better RFS (A) and OS (B) than the HBx-other group, as determined through an analysis of tumor (n = 219) tissue samples from patients with HCC in the primary cohort. (C,D) RFS (C) and OS (D) curves for the tumor tissue samples (n = 134) from the validation cohort (HCC cohort from Fu Jian and Guang Xi, n = 171). The Kaplan-Meier method and log-rank test were used. HBx, hepatitis B virus X gene; HCC, hepatocellular carcinoma; OS, overall survival; RFS, recurrence-free survival.

Score <0 ($p = 0.001$);[22] consistent data were obtained from nontumor tissues (Table S6).

Additionally, the results from the primary cohort were validated with samples from an independent HCC cohort from Fujian (n = 80) and Guangxi Province (n = 91) (validation cohort; n = 171). Similarly, 134 (78.4%) and 165 (96.5%) full-length HBx DNA sequences were obtained from tumor and adjacent nontumor samples, respectively. According to the results of the genotype analysis of the validation cohort, HBx-E2 was also detected in 19.4% (26/134) and 21.2% (35/165) of the tumor and nontumor samples, respectively. The presence of the HBx-E2 genotype in tumor and nontumor tissues indicated better RFS ($p = 0.010$; $p = 0.003$) and OS ($p = 0.033$; $p = 0.025$) for the validation cohort (Fig. 2C-D, Fig. S1C-D). Additionally, the correlation analysis of clinicopathological characteristics also showed that HBx-E2 was associated with the absence of liver cirrhosis and a small tumor size (Table S7).

In conclusion, the HBx-E2 genotype predicted the clinical progression of the disease, including recurrence and survival, in patients with HBV-related HCC in 2 independent cohorts from 3 different centers.

HBx-E2 predicts the prognosis of patients with BCLC stage B HCC after resection

The BCLC staging system is commonly accepted as a guideline for HCC treatment, although some of its treatment recommendations for patients with intermediate and advanced HCC remain controversial.[23,24] Based on the results from our previous study, partial hepatectomy is superior to transcatheter arterial chemoembolization (TACE) in patients with stage BCLC B[25]. Nevertheless, factors that affect the postoperative outcomes of patients with BCLC stage B, HBV-associated, HCC require further study.

Patients in the primary cohort were stratified according to the BCLC stage (0-A *vs.* B) and regrouped by different HBx genotype to further investigate the prognostic impact of HBx-E2. Kaplan-Meier analysis revealed that BCLC stage B patients with the HBx-E2 genotype exhibited better RFS and OS than BCLC stage B patients carrying other HBx genotypes in their tumor ($p = 0.008$ for RFS, $p = 0.009$ for OS) and nontumor ($p = 0.043$ for RFS, $p = 0.024$ for OS) samples (Fig. 3A-B, Fig. S2A-B). Furthermore, no significant differences in the RFS and OS were observed between BCLC stage B patients carrying HBx-E2 genotype and BCLC stage 0-A patients with and without the HBx-E2 genotype ($p >0.05$). Additionally, consistent results were observed in the validation cohort (Fig. 3C-D, Fig. S2C-D). Thus, HBx-E2 is a significant prognostic factor for patients with an intermediate stage of HBV-related HCC after hepatectomy.

Association of serum HBx-E2 with postoperative prognosis and clinicopathological characteristics

Serum samples were collected from 20 patients with HCC in the primary cohort to verify whether HBx-E2 was present in the blood of patients with HBV-related HCC. Nontumor tissues from 10 patients were positive for HBx-E2, and those from 10 other patients were negative for HBx-E2. The presence of the HBx-E2 genotype in the tumor, adjacent nontumor tissues and serum samples from the same patient was compared (Fig. 4A). The presence of HBx-E2 in the serum was consistent with its presence in the nontumor tissues, suggesting that serum HBx-E2 could serve as a prognostic biomarker. Additionally, we recollected tumor, nontumor and serum samples from different patients and cloned the purified PCR products of the HBx gene. For each sample, 30 clones were sequenced. The results showed that the detected HBx genotypes represent the vast majority of the viral population (Table S8).

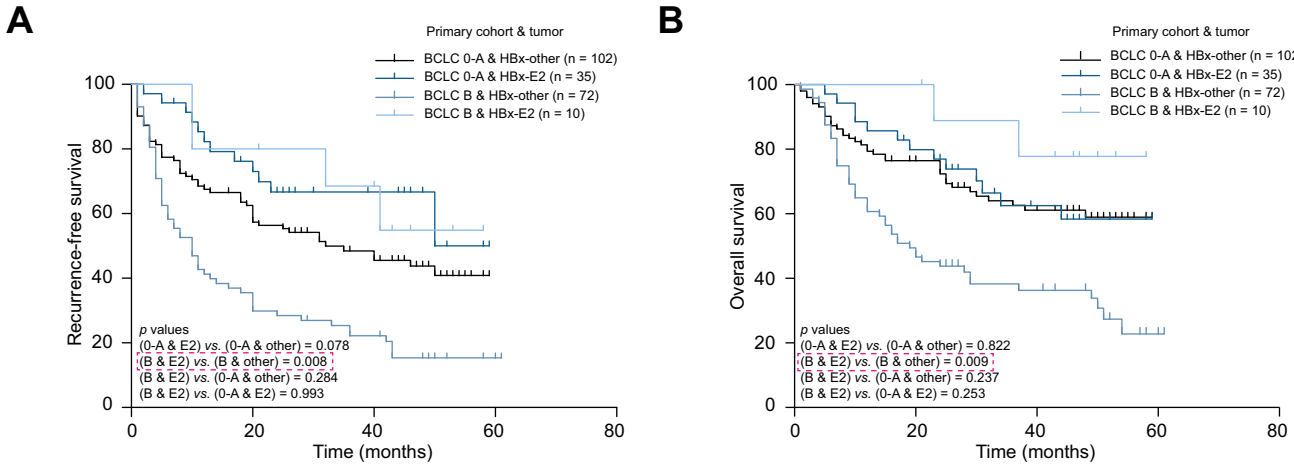
We then collected tissues and serum from patients with HBV-induced hepatitis (n = 60), cirrhosis (n = 60) and HCC (n = 60) and then measured the presence of HBx-E2 in the samples. The presence of HBx-E2 decreased from hepatitis to cirrhosis and HCC (Fig. 4B), implying a decreasing trend in the presence of HBx-E2 during malignant transformation. Subsequently, 168 serum samples were collected from patients with HBV-related HCC (serum cohort) to further clarify the clinical significance of serum HBx-E2. We obtained 160 full-length HBx DNA sequences (95.2%) by extracting the viral DNA and sequencing. The genotype analysis revealed the presence of the HBx-E2 genotype in 31.3% (50/160) of the samples. A survival analysis was then performed to explore whether serum HBx-E2 was correlated with patient prognosis (Fig. 4C-D). The RFS and OS of the patients in the HBx-E2 group were significantly better than those of the patients in the HBx-other group ($p = 0.013$ and $p = 0.045$, respectively). In addition, the comparison analysis for HBx-E2 with the clinicopathological characteristics revealed that serum HBx-E2 was strongly associated with the absence of liver cirrhosis ($p <0.0001$), a small tumor size ($p = 0.001$) and a solitary tumor ($p = 0.028$) (Table S9).

Subsequently, patients were further compared based on BCLC stage and HBx genotype. The RFS and OS of the BCLC stage B patients carrying the HBx-E2 genotype were significantly better than those of the BCLC stage B patients carrying other HBx-other genotypes ($p = 0.009$ and $p = 0.020$, respectively) but not statistically different from those of BCLC stage 0-A patients with or without the HBx-E2 genotype ($p >0.05$; Fig. 4E-F). In summary, these data support the hypothesis that serum HBx-E2 could be used to preoperatively predict the prognosis of patients with HCC, particularly intermediate HCC.

HBx-E2 and HBx-E2-N lose the ability to promote the proliferation of HCC cells and normal hepatocytes *in vitro* and *in vivo*

We assessed the serum HBV genotype, serum HBV-DNA levels, intrahepatic HBV DNA, intrahepatic HBV covalently closed circular (ccc) DNA, intrahepatic HBV pregenomic (pg) RNA, the interaction between HBx protein and cccDNA, expression of the HBc protein and expression of the HBx protein in the tissues to explore why HBx-E2 indicated a better prognosis. No significant differences in these measurements were observed between the HBx-E2 group and the HBx-other group (Tables S10–13 and Fig. S3A-B). Five HBx DNA sequences, specifically the HBx-positive (HBx-P), HBx-wild-type (HBx-WT), HBx-E2, HBx-E2-nonsynonymous (HBx-E2-N) and HBx-E2-synonymous (HBx-E2-S) sequences, were then synthesized to further investigate the biological functions of HBx-E2 in cells (Fig. S3C). The HCC cell lines (SMMC-7721 and Huh7) and an immortalized hepatocyte cell line (LO2) stably expressing HBx genotypes were established by lentivirus delivery (Fig. S3D).

HBx-P, HBx-WT and HBx-E2-S promoted cell growth and survival, and HBx-E2 and HBx-E2-N did not impact cell proliferation, as assessed by Cell Counting Kit 8 (CCK8) assays and clone formation assays (Fig. 5A-B, Fig. S4A-D). Subsequently, the flow cytometry analysis showed a reduction in the percentage of cells in the G1 phase and an increase in the percentages of cells in the S and G2 phase among the HBx-P, HBx-WT and HBx-E2-S

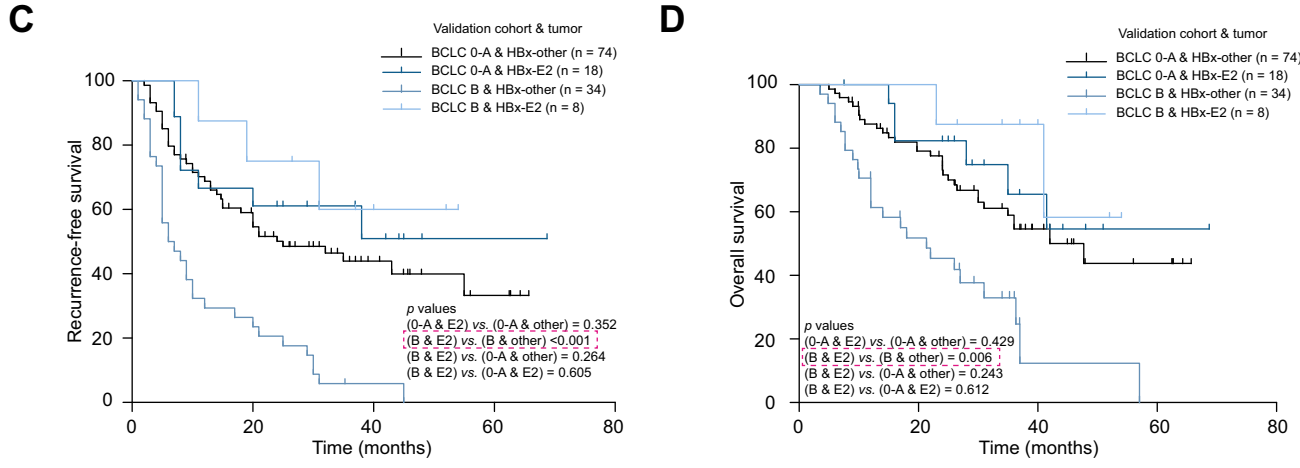


N° at risk				
0-A & other	102	56	30	0
0-A & E2	35	23	13	0
B & other	72	21	14	1
B & E2	10	8	5	0

Median recurrence-free survival (months)	
0-A & other	32.0 (95% CI, 13.5-50.5)
0-A & E2	>59.0
B & other	10.0 (95% CI, 6.4-13.6)
B & E2	>58.0

N° at risk				
0-A & other	102	75	41	0
0-A & E2	35	27	15	0
B & other	72	33	18	1
B & E2	10	10	7	0

Median recurrence-free survival (months)	
0-A & other	>59.0
0-A & E2	>59.0
B & other	19.0 (95% CI, 10.8-27.2)
B & E2	>58.0



N° at risk				
0-A & other	74	37	12	4
0-A & E2	18	11	5	1
B & other	34	8	1	0
B & E2	8	6	2	0

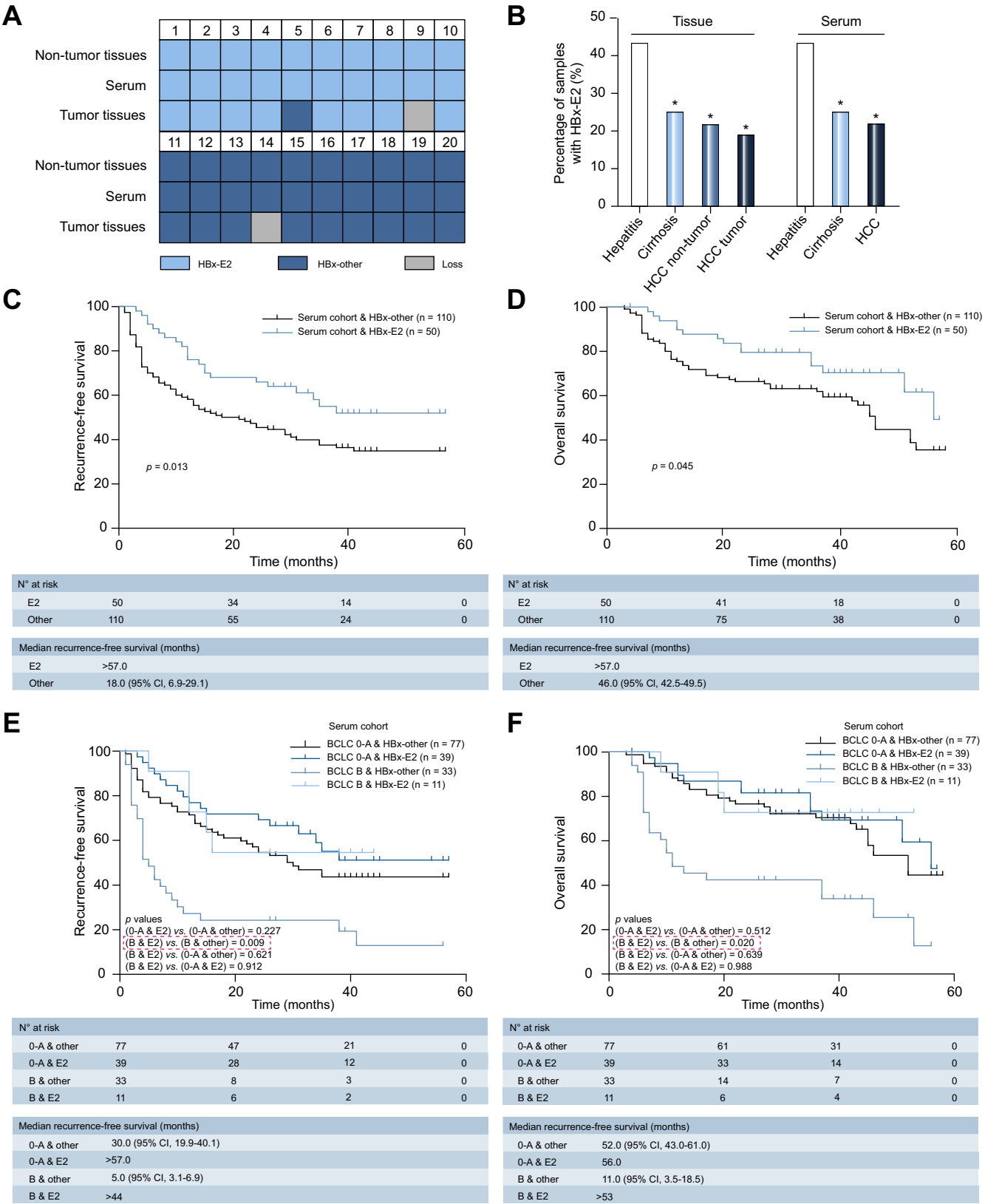
Median recurrence-free survival (months)	
0-A & other	25.0 (95% CI, 11.8-38.2)
0-A & E2	>68.7
B & other	6.0 (95% CI, 1.7-10.3)
B & E2	>54.0

N° at risk				
0-A & other	74	54	15	5
0-A & E2	18	14	6	1
B & other	34	16	1	0
B & E2	8	8	3	0

Median recurrence-free survival (months)	
0-A & other	47.7 (95% CI, 33.4-62)
0-A & E2	>68.7
B & other	21.3 (95% CI, 9.8-33.0)
B & E2	>54.0

Fig. 3. HBx-E2 is significantly correlated with a good postoperative prognosis for patients with BCLC stage B HCC in the primary and validation cohorts. The (A,C) RFS and (B,D) OS curves of tumor samples from the primary cohort and validation cohort were stratified by BCLC staging (0-A *vs.* B) and HBx genotypes (HBx-E2 *vs.* HBx-other). The Kaplan-Meier method and log-rank test were used. BCLC, Barcelona Clinic Liver Cancer; HBx, hepatitis B virus X gene; HCC, hepatocellular carcinoma; OS, overall survival; RFS, recurrence-free survival.

groups. In the HBx-E2 and HBx-E2-N groups, the ratios of cells in the G1, S and G2 phase were not statistically significantly different from the ratios of the control group (Fig. 5C, Fig. S4E–F). Additionally, HBx-P, HBx-WT and HBx-E2-S obviously inhibited cells apoptosis, whereas HBx-E2 and HBx-E2-N had no effect on apoptosis, as assessed by annexin V/PI double staining, flow cytometry analysis and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays (Fig. 5D, Fig. S4G–I). Furthermore, we acquired 20 primary HCC cell lines (n = 10 for HBx-E2; n = 10 for HBx-other) and 20 primary hepatocyte lines (n = 10 for HBx-E2; n = 10 for HBx-other) from tumor and nontumor tissues of patients with



N° at risk				
E2	50	34	14	0
Other	110	55	24	0

Median recurrence-free survival (months)	
E2	>57.0
Other	18.0 (95% CI, 6.9-29.1)

N° at risk				
E2	50	41	18	0
Other	110	75	38	0

Median recurrence-free survival (months)	
E2	>57.0
Other	46.0 (95% CI, 42.5-49.5)

N° at risk				
0-A & other	77	47	21	0
0-A & E2	39	28	12	0
B & other	33	8	3	0
B & E2	11	6	2	0

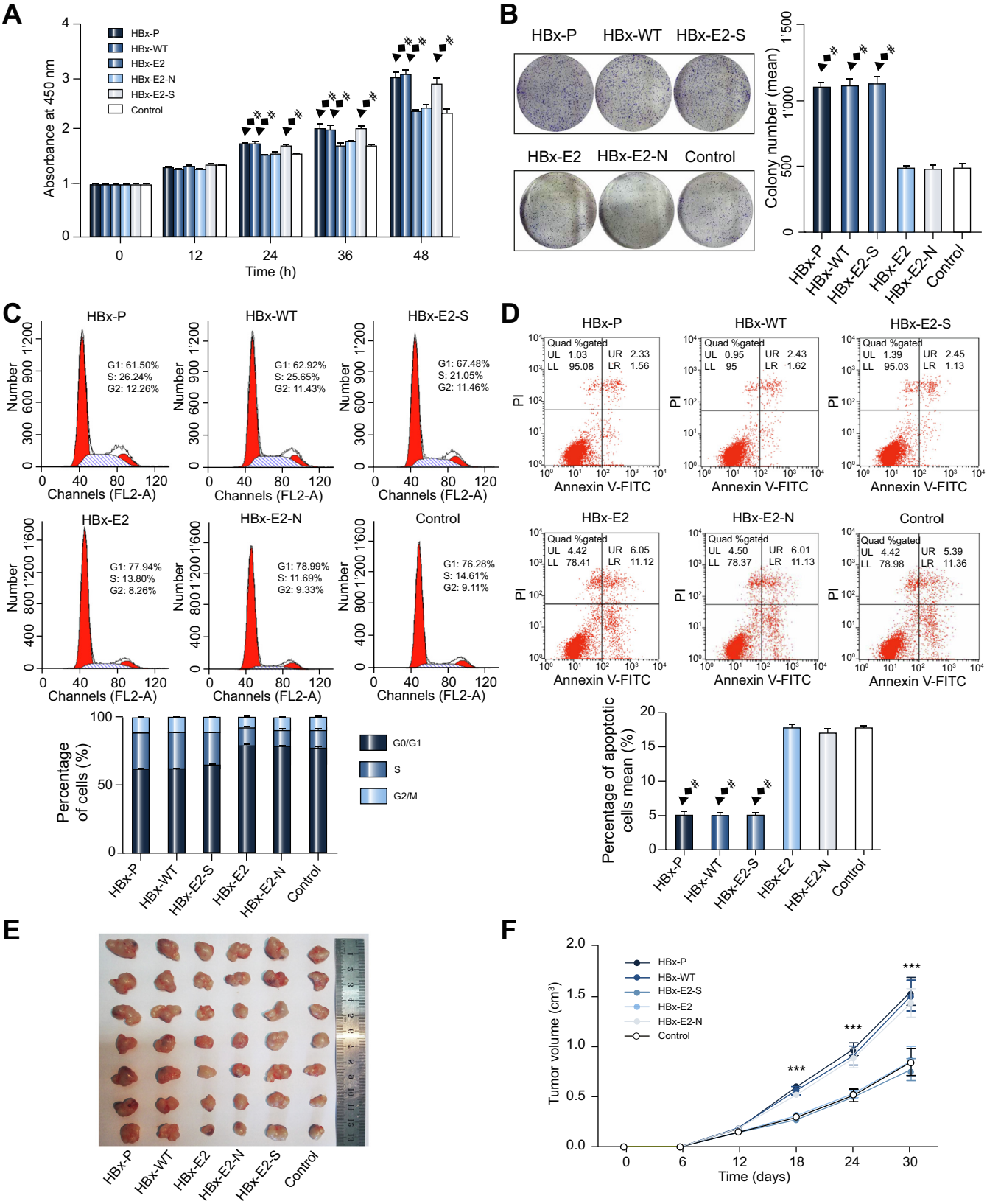
Median recurrence-free survival (months)	
0-A & other	30.0 (95% CI, 19.9-40.1)
0-A & E2	>57.0
B & other	5.0 (95% CI, 3.1-6.9)
B & E2	>44

N° at risk				
0-A & other	77	61	31	0
0-A & E2	39	33	14	0
B & other	33	14	7	0
B & E2	11	6	4	0

Median recurrence-free survival (months)	
0-A & other	52.0 (95% CI, 43.0-61.0)
0-A & E2	56.0
B & other	11.0 (95% CI, 3.5-18.5)
B & E2	>53

Fig. 4. The presence of HBx-E2 in the serum decreased during HCC development, and serum HBx-E2 was used to predict prognosis. (A) The detection of HBx-E2 in the tumor tissues, adjacent nontumor tissues and the serum of 20 patients with HCC revealed a consistent HBx-E2 presence in the serum and nontumor tissues. (B) Percentage of HBx-E2-positive tissue and serum samples from patients with chronic hepatitis (n = 60), cirrhosis (n = 60) and HCC (n = 60). *p <0.05 compared with the chronic hepatitis group. Chi-square test was used. (C,D) The survival analysis of the HBx-E2 group and the HBx-other group in the serum cohort (the independent HCC cohort from Shang Hai, n = 168) showed that the RFS (C) and OS (D) of the HBx-E2 group was significantly superior to those of the HBx-other group. The Kaplan-Meier method and log-rank test were used. (E,F) The RFS (E) and OS (F) curves for the serum cohort were stratified by BCLC staging (0-A *vs.* B) and HBx genotypes (HBx-E2 *vs.* HBx-other). The Kaplan-Meier method and log-rank test were used. HBx, hepatitis B virus X gene; HCC, hepatocellular carcinoma; OS, overall survival; RFS, recurrence-free survival.

HCC (HBV genotype C) and different HBx genotypes, and found that the proliferation capacity of primary HCC cells and primary hepatocytes with HBx-E2 was weaker than that of the HBx-other groups (Fig. S5A,B). Moreover, the growth of HepaRG cells infected with HBV-HBx-E2 particles from the serum of patients with HCC (n = 10) was slower than that of the cells infected with



HBV-HBx-other particles (n = 10), in which HBV DNA was quantified by qPCR (Fig. S5C, Table S14).

We next investigated the roles of HBx genotypes in HCC growth *in vivo*. SMMC-7721 cells expressing different HBx genotypes or controls were subcutaneously injected into nude mice. The tumors in the HBx-P, HBx-WT and HBx-E2-S groups grew faster and had a larger volume, whereas the tumor volume and growth rate of the HBx-E2 and HBx-E2-N groups were not statistically significantly different from the values found for the control group (Fig. 5E-F). In addition, the marker staining of xenografts further verified the roles of HBx-E2 and HBx-E2-N in cell proliferation (Ki67 and proliferating cell nuclear antigen (PCNA)), the cell cycle (p21) and apoptosis (TUNEL) (Fig. S5D–H).

These data demonstrated that HBx-E2 variants led to a disruption of HBx-mediated regulation of cell proliferation, cell cycling and apoptosis in HCC cells and normal hepatocytes, consistent with a role for HBx-E2 in the prognosis of HCC. Moreover, the alterative effects of HBx-E2 on cell function depend on the variants at nonsynonymous sites in HBx-E2.

HBx-E2 and HBx-E2-N fail to interact with JAK1

The biological functions of HBx primarily depend on pleiotropic protein–protein interactions.[26] To determine whether the HBx-E2 variant had lost the function to promote proliferation through variation of the binding proteins, we isolated recombinant HBx-WT and HBx-E2 proteins (Fig. S6A-B) and used a protein microarray assay to globally screen the discrepancies between HBx-E2 interactors and HBx-WT interactors *in vitro*.[27]

The top 50 proteins ranked by fluorescence intensity that interact with HBx-E2 or HBx-WT are listed in Table S15. Among them, 42 proteins could interact with both HBx-E2 and HBx-WT. There were 16 proteins that interact with only one HBx genotype. Three (JAK1, RBBP4, CAP2) of the 16 binding proteins are associated with the development of HCC. JAK1, which transduces cytokine signals from membrane receptors to STATs,[28] was among the proteins that was found to participate in stronger interactions with HBx-WT and not with HBx-E2 (Fig. 6A). Moreover, JAK1 has previously been shown to interact with HBx and plays a key role in cell proliferation.[29–31] Hence, we further validated the physical interaction between HBx proteins and JAK1 by performing coimmunoprecipitation (CoIP) experiments in both cells and patient tissues with different HBx genotypes. Based on the results from the reciprocal CoIP assay, HBx-P, HBx-WT and HBx-E2-S interact with JAK1. Conversely, an interaction between HBx-E2 and JAK1, HBx-E2-N and JAK1 was not observed in Huh7, SMMC-7721 and LO2 cells, and tumor and nontumor tissues from patients with HCC (Fig. 6B-C, Fig. S6C). Consistently, evidence of intracellular colocalization or non-colocalization was obtained through immunofluorescence staining and confocal microscopy in cells and patient tissues (Fig. 6D-E, Fig. S6D-E). Based on these results, the nonsynonymous HBx-E2 variants cause the HBx protein to fail to interact with JAK1.

HBx-E2 and HBx-E2-N do not activate the JAK1/STAT3/STAT5 signaling pathway

We then tested the activation of the JAK1/STAT signaling pathway in cells expressing various HBx genotypes. As shown in Fig. 7A–C, an obvious increase in the phosphorylation of JAK1, STAT3 and STAT5 was induced by HBx-P, HBx-WT and HBx-E2-S, but not HBx-E2 or HBx-E2-N, in Huh7, SMMC-7721 and LO2 cells. In addition, tumor and nontumor tissues from 40 patients with HCC who carry the HBx-E2 genotype and 40 patients who did not carry HBx-E2 genotype from the primary cohort were selected for immunohistochemical staining to further ascertain the activation of JAK1/STAT signaling in clinical samples. Lower levels of phosphorylated JAK1, STAT3 and STAT5 were observed in both the tumor and nontumor tissues from the HBx-E2 group than in tissues from the HBx-other group (Fig. 7D). These data demonstrated that HBx-E2 and HBx-E2-N lost the function to activate the JAK1/STAT3/STAT5 signaling pathway.

To further confirm the involvement of JAK1 in the loss of the proliferation-promoting function of HBx-E2, we stably overexpressed JAK1 and activated p-JAK1 in cells with HBx-E2 and HBx-E2-N using a JAK1-expressing recombinant lentivirus (Fig. S7A). The activation of p-JAK1 obviously increased the proliferation-promoting effects of HBx-E2 and HBx-E2-N in Huh7 and LO2 cells *in vitro* (Fig. S7B–E). Additionally, we inoculated different clones of Huh7 subcutaneously into nude mice. The activation of p-JAK1 obviously promoted tumor growth in the HBx-E2 group *in vivo* (Fig. 7E & F). Moreover, we treated SMMC-7721 cells expressing HBx-P, HBx-WT and HBx-E2-S with 50 nM upadacitinib (ABT-494), a JAK1-specific inhibitor, and found that the proliferation-promoting effects were significantly reduced using CCK8 and clone formation assays (Fig. S7F–H). These results suggest that the loss of JAK1 activation may lead to a loss of the proliferation-promotion function of HBx-E2.

Discussion

Previous reports of HBx genetic variations have mainly concentrated on point mutations and truncations. Few studies have focused on multilocus variants between the full-length HBx genomic sequences from the perspective of a large HCC cohort. Thus, we classified the HBx full-length sequence into various genotypes to clarify the clinical significance and biological functions of multilocus variants between genotypes.

Fig. 5. HBx-E2 and HBx-E2-N lost their abilities to affect proliferation, the cell cycle and apoptosis in HCC cells *in vitro* and *in vivo*. (A & B) HBx-P, HBx-WT, and HBx-E2-S promoted SMMC-7721 cell proliferation, whereas HBx-E2 and HBx-E2-N did not alter cell proliferation, as measured by the Cell Counting Kit 8 (CCK8) assay (n = 4, A) and the clone formation experiment (n = 4, B). (C) Flow cytometry results showing that HBx-P, HBx-WT and HBx-E2-S reduced the proportion of cells in the G1 phase and increased the proportions of cells in the S and G2 phases, whereas HBx-E2 and HBx-E2-N had no significant effects on the cell cycle of SMMC-7721 cells (n = 4). (D) Annexin V/PI double staining showed that HBx-P, HBx-WT, and HBx-E2-S inhibited the apoptosis of SMMC-7721 cells, whereas HBx-E2 and HBx-E2-N did not affect cell apoptosis (n = 4). ▲p <0.01 compared with the HBx-E2 group. ◆p <0.01 compared with the HBx-E2-A group. #p <0.01 compared with the control group. Student's t test was used. (E & F) The subcutaneous xenograft model was established by subcutaneously injecting 1×10^6 cells into the right axilla of each nude mouse (n = 7 for each group). The tumor volume was measured every week. (E) Photographs of the tumors removed from each group. (F) Tumor growth rate curve. ***p <0.001 compared with the control group. The Mann-Whitney U test was used. All data are presented as the means ± SD. HBx, hepatitis B virus X gene; HCC, hepatocellular carcinoma; WT, wild-type.

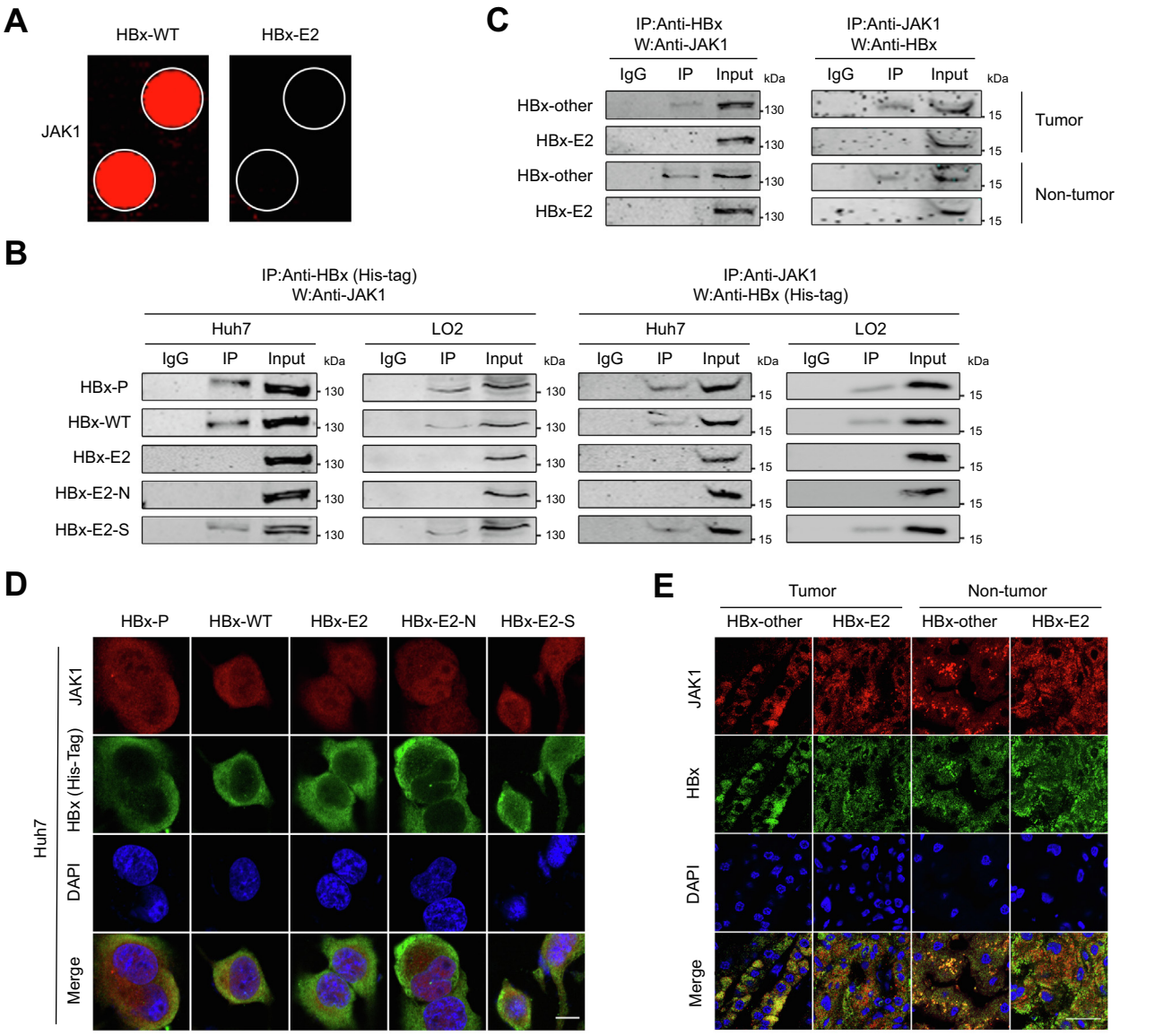


Fig. 6. HBx-E2 and HBx-E2-N lose the function to interact with JAK1. (A) A protein microarray assay was performed using the HBx-WT protein and HBx-E2 protein. The left panel represents the specific binding between the HBx-WT protein and JAK1. The right panel shows the lack of interaction between the HBx-E2 protein and JAK1. (B) Lysates were prepared from Huh7 and LO2 cells expressing various HBx genotypes and immunoprecipitated with either anti-IgG, anti-JAK1 or anti-His (for His-tagged HBx) antibodies. Immunoprecipitates were analyzed by Western blotting. (C) Immunoprecipitation of whole tissue lysates from patient tumor and nontumor samples carrying HBx-E2 or HBx-other was performed using either anti-IgG, anti-JAK1 or anti-HBx antibodies. Immunoprecipitates were analyzed by Western blotting. (D) JAK1 (red) and HBx (green) were detected in Huh7 cells expressing different HBx genotypes through immunofluorescence staining. The colocalization of JAK1 and HBx-P, HBx-WT or HBx-E2-S and the non-colocalization of JAK1 and HBx-E2 or HBx-E2-N were evaluated by confocal laser scanning microscopy as indicated (scale bar, 10 μm). (E) Immunofluorescence staining of patient tumor and nontumor samples with HBx-E2 or HBx-other using an anti-JAK1 (red) antibody and an anti-HBx (green) antibody. Nuclei were stained by DAPI (blue). The colocalization and the noncolocalization were evaluated by confocal laser scanning microscopy. (scale bar, 20 μm). HBx, hepatitis B virus X gene; WT, wild-type.

Through sequencing of HBx DNA in the large samples, 3 types of HBx gene variants, including distal C-terminal truncations, single site variations and internal deletions, were detected in tumor and nontumor tissues. The distal C-terminal truncations were significantly more frequent in tumors than in nontumor tissues, consistent with previous reports.[13,32,33] This resulted in a significant decrease in the amount of the full-length HBx sequence obtained in tumor samples compared with that in the adjacent nontumor samples and serum samples. The dual mutations A1762T/G1764A are related to a poor prognosis for patients with HCC, which was verified in our study (data not shown). The internal deletion and distal C-terminal truncation in the tumor samples from the primary cohort were not significantly related to prognosis.

In this manuscript, we report a novel HBx genotype (HBx-E2) that was identified through sequencing and cluster analyses of HBx DNA in HCC cohorts with large numbers of samples. The presence of HBx-E2 in tumor tissues and adjacent nontumor tissues was approximately the same, but HBx-E2 was only observed in nontumor tissues and not the corresponding tumor tissues from the 3.2% (7/213) of patients from whom full-length HBx sequences were obtained in both tumor and nontumor tissues. This difference might be because HBV genomes with favorable fitness outgrow genomes with less fitness and HBx

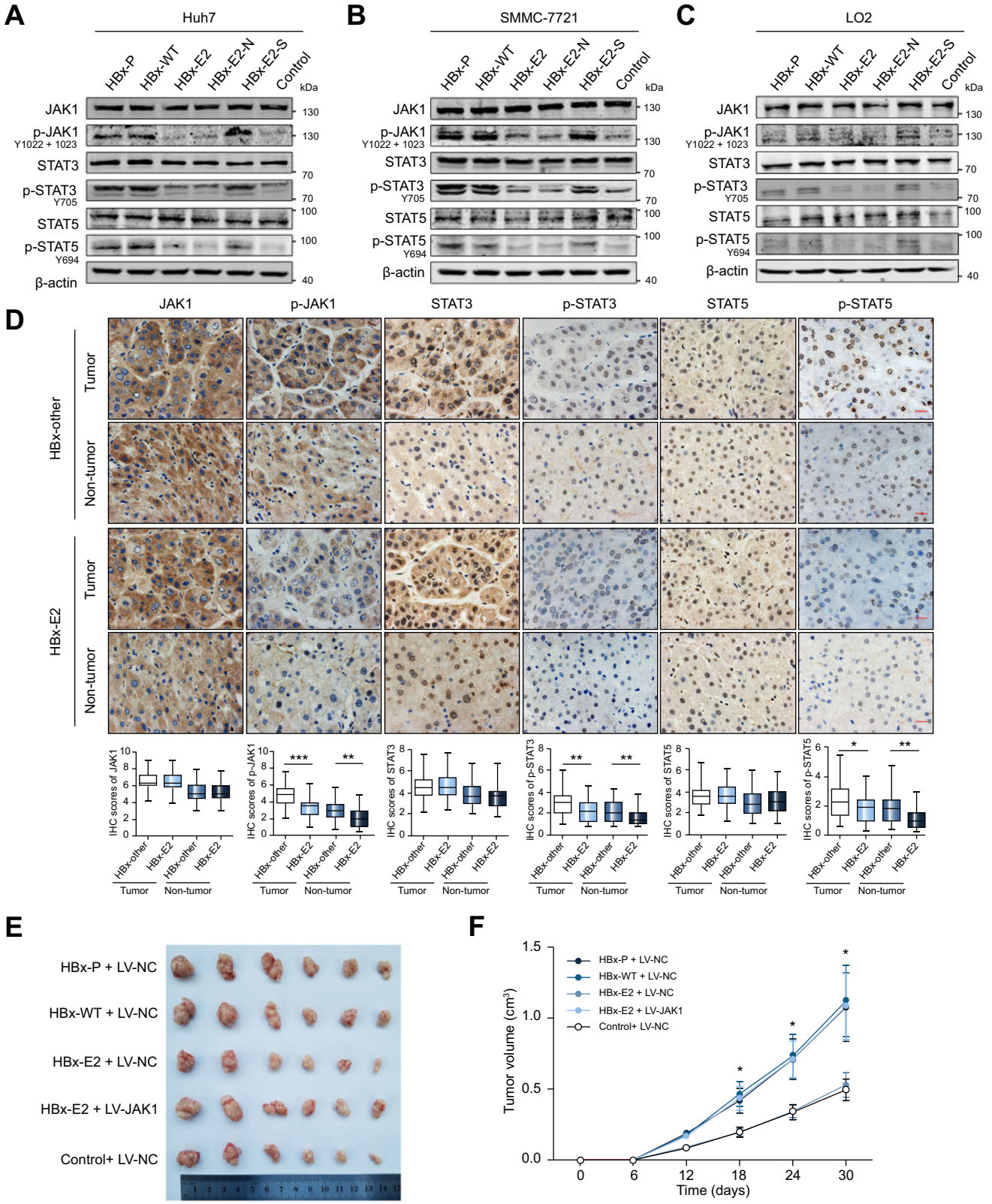


Fig. 7. HBx-E2 and HBx-E2-N fail to activate the JAK1/STAT3/STAT5 signaling pathway. (A–C) The total JAK1, phosphorylated JAK1 (p-JAK1), total STAT3, phosphorylated STAT3 (p-STAT3), total STAT5 and phosphorylated STAT5 (p-STAT5) levels were determined through a Western blot analysis of the indicated cell lysates. β-actin was used as a loading control. (D) Immunohistochemical staining showing the expression levels of the JAK1, p-JAK1, STAT3, p-STAT3, STAT5 and p-STAT5 proteins in tumor and nontumor tissues with HBx-E2 (n = 40) or HBx-other (n = 40) from the primary cohort. *p <0.05. **p <0.01. ***p <0.001. The Mann-Whitney U test was used. Scale bar, 20 μm. (E & F) Subcutaneous tumor model of JAK1 overexpressed HBx-Huh7 cells (n = 6 for each group). (E) Photographs of the tumors removed from each group. (F) Tumor growth rate curve. *p <0.05 compared with the control group. The Mann-Whitney U test was used. All data are presented as the means ± SD. HBx, hepatitis B virus X gene.

genotypes are selected during the development of HCC, resulting in the different HBx genotypes observed in tumor and adjacent nontumor tissues. Seven patients with the distal C-terminal truncations in tumor tissue carried the HBx-E2 in their corresponding nontumor tissue. Patients carrying the distal C-terminal truncations in nontumor tissues did not carry HBX-E2 in their corresponding tumor tissues. Patients carrying the internal deletions in tumor or nontumor tissues did not carry HBx-E2 in their corresponding nontumor or tumor tissues. Based on the results of the correlation analysis, HBx-E2 is strongly associated with multiple clinicopathological characteristics, consistent with the inability of HBx-E2 to increase the proliferation of cancer cells. Additionally, according to the results of the prognosis analysis, patients with the HBx-E2 genotype have better prognoses than patients with other HBx genotypes. Furthermore, HBx-E2 lost the ability to promote malignant transformation. This study provides the first demonstration that the HBx genotype is related to a better prognosis among patients with HCC and fails to show malignant biological functions in HCC progression. Remarkably, HBx-E2 was detected in the serum and thus might be used to preoperatively predict the prognosis of patients with HCC. According to previous studies of HBx and HBx variants, HBx promotes liver diseases (HCC and liver cirrhosis), and mutations at some loci further intensify the carcinogenic abilities of HBx. The reported sites include A1383C, R1479C/T, C1485T, C1631T, A1762T, G1764A, and T1800C.[34] The HBx-E2 loci were not included. Thus, our study does not contradict the results from previous studies.

BCLC staging is the most frequently used system to stage patients with HCC and to assign patients to the best treatment option for their respective clinical situation.[35] However, some disputes exist, particularly regarding the treatment strategy for patients with BCLC stage B disease, for whom only TACE is recommended.[24–25,36] In this study, we proved that HBx-E2 could predict the prognosis of patients with BCLC stage B HCC after resection. Notably, the surgical outcomes of patients with BCLC stage B who carried the HBx-E2 genotype were not inferior to the outcomes of patients with BCLC stage 0-A, implying that hepatectomy may be an optional treatment for BCLC stage B patients with HBx-E2 genotype. The detection of HBx genotypes may also have implications for strategies in disease monitoring and adjunctive therapies. The additional benefit of HBx-E2 cannot be found in BCLC 0-A patients, which may be attributed to the more radical operation that BCLC 0-A patients undergo. Nault *et al.* reported that the molecular 5-gene score is associated with outcomes in patients with HCC treated by resection.[22] We assessed the correlation of HBx-E2 presence and its association with clinical outcomes with the 5-gene score. The presence of HBx-E2 is associated with the 5-gene score in tumor and nontumor tissues (Table S6). The survival analysis showed that a molecular 5-gene score <0 was associated with better RFS and OS in overall patients and BCLC 0-A patients with full-length HBx sequences in both tumor and nontumor tissues from the primary cohort (data not shown). However, the 5-gene score was not related to the prognosis of the patients at the BCLC B stage. Thus, HBx-E2 was more effective than the 5-gene score in predicting the prognosis of patients at the BCLC B stage in the primary cohort. This may be because all the patients included in this study were HBV-infected patients.

In the process of exploring the reasons for better prognosis indicated by HBx-E2, we first eliminated the factors of HBV genotype, HBV-DNA levels, cccDNA, pgRNA and HBx protein levels. The insignificant difference in pgRNA levels between the HBx-E2 group and the HBx-other group indicated that HBx-E2 does not impact cccDNA transcription. Additionally, the HBx gene overlaps with both the ribonuclease H (RNaseH) domain of the viral polymerase and the N-terminus of the preC/C open reading frame. Fourteen loci of HBx-E2 are located within the RNaseH domain, which is involved in viral genome replication.[37] The levels of HBV DNA, HBV cccDNA and HBV pgRNA were not significantly different between the HBx-E2 group and the HBx-other group, implying that the HBx-E2 variant may not affect RNaseH function. Hence, we focused on the biological functions of the HBx-E2 protein. Despite extensive studies, the actual roles of HBx in cell growth, cell cycle progression and apoptosis remain controversial, partly because HBx exerts different effects on different cell lines and partly due to the distinct genotypes employed in the studies.[38–41] In this study, we employed HCC cell lines and a normal hepatocyte cell line expressing 5 different HBx genotypes and showed that different HBx genotypes exert different effects on cell functions. The changeable HBx genotypes may explain the complicated HBx functions. The recurrence of HCC after hepatic resection is attributed to either metastasis from the primary tumor or the development of *de novo* tumors. The impaired cell growth function of HBx-E2 in both HCC cells and normal hepatocytes accounts for better outcomes in patients with HCC carrying the HBx-E2 genotype after hepatectomy.

The JAK/STAT signaling pathway is closely related to the proliferation, cell cycle, apoptosis, and differentiation of cells.[42–44] HBx has been shown to interact with JAK1, leading to the activation of the Jak1-STAT pathway.[29,30] In this study, HBx-E2 variants in nonsynonymous sites were found to cause the HBx protein to lose the ability to interact with JAK1 and activate JAK1/STAT signaling. Furthermore, the inhibition of JAK1 induced an obvious reduction in the proliferation-promoting effects of HBx-P, HBx-WT and HBx-E2-S, indicating that the impaired function of HBx-E2 and HBx-E2-N might be mainly mediated by a lack of activation of JAK1/STAT signaling. Moreover, the activation of the JAK/STAT pathway is involved in hepatic fibrogenesis,[45] which may at least partly explain why HBx-E2 is associated with the absence of liver cirrhosis. Additionally, the difference in liver function associated with HBx genotypes is more evident in BCLC B patients (Tables S16 & S17), which may be another reason why HBx-E2 more significantly influences the prognosis of patients with intermediate HCC.

HBx-E2 was obtained from the full-length HBx sequence and was presented in the serum. Furthermore, unlike the distinct patterns of viral insertion between tumor and adjacent nontumor tissues,[46] the presence of HBx-E2 in tumor tissues and adjacent nontumor tissues was approximately the same. Therefore, the HBx-E2 variant should present in the cccDNA sequence and not be derived from host genome integrated sequences. HBx is a nonstructural protein that can fold to form a secondary structure under specific conditions.[47] As a result, its interactions with target proteins are altered. This characteristic provides the theoretical basis that explains the differences in the biological functions of HBx-E2 compared with other genotypes. Fifteen nonsynonymous loci contained in HBx-E2 could change the structure of the HBx protein and alter its regulatory effects on downstream target molecules, such as JAK1, which may ultimately lead to different biological functions. The nonsynonymous sites of HBx-E2 are all located within the central region

(residues 26–142), which has been proven to be responsible for STAT activation,[29] implying that these loci may be crucial for changing the structure of the central area. The integrated effects of the variations in HBx-E2 on the functional domains and structure of the HBx protein remain to be addressed.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

WZ, FY, HL and QX designed the study, analyzed results, supervised experiments, and wrote the manuscript. QX, SY, QT, JY, JC, KL, JM, DD performed most of the experiments and analyzed results. YY, HL, FG, ZW, LZ, XG, ZH collected clinical samples and supervised clinicopathological data. YF, SS provided reagents and laboratory apparatus. YZ revised the text of the article.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.01.007.

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Author names in bold designate shared co-first authorship

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