Article

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HBx Modulates Drug Resistance of Sorafenib-Resistant Hepatocellular Carcinoma Cells

Yaping Liu[1], Xu Liu[2], Miaosha Luo[1], Yarui Li[1], Hongxia Li[1,*]

[1]Department of Gastroenterology, The First Affiliated Hospital of Xi'an Jiaotong University, 710061 Xi'an, Shaanxi, China
[2]Department of Gastroenterology, Mianxian Hospital, 724200 Hanzhong, Shaanxi, China
*Correspondence: lhongxia2023@163.com (Hongxia Li)
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Background: Approximately 50% of hepatocellular carcinoma (HCC) arises due to the infection by hepatitis B virus X protein (HBx). Sorafenib, a unique targeted oral kinase inhibitor, is the therapeutic agent of choice for advanced HCC. The mechanism of HBx in drug resistance of sorafenib-resistant HCC cells was evaluated in this study.
Methods: Employing a stepwise increase of the sorafenib content, Hep3B and HepG2 cells were iteratively induced to establish drug-resistant cell lines (Hep3B/R and HepG2/R). The survival rate of Hep3B, Hep3B/R, HepG2, and HepG2/R cells was estimated using the cell counting kit-8 (CCK-8) assay. The IC_{50} values of sorafenib were calculated, exploring its effects under varying concentrations. The HBx content was quantified via quantitative reverse transcription PCR (RT-qPCR) and Western Blot. HBx overexpression and interfering virus vectors were constructed and transfected into Hep3B/R and HepG2/R cells. Cell viability and metastasis were assessed by colony formation, wound healing, and transwell assays. E-cadherin, N-cadherin, Vimentin, Slug, and Snail content was evaluated via Western Blot.
Results: HBx content was significantly elevated in Hep3B/R and HepG2/R subgroups compared to Hep3B and HepG2 subgroups. The proliferation, clonogenicity, invasiveness, and migratory abilities of Hep3B/R and HepG2/R cells in the HBx subgroup were markedly enhanced; E-cadherin content was significantly reduced, whereas the content of N-cadherin, Vimentin, Slug, and Snail was significantly elevated in the HBx subgroup. Conversely, in the sh-HBx subgroup, the proliferation, clonogenicity, invasion, and migration of Hep3B/R and HepG2/R cells were significantly reduced, E-cadherin content was markedly increased, and N-cadherin, Vimentin, Slug, and Snail content was significantly reduced, compared to the sh-negative control (NC) subgroup.
Conclusions: HBx knockout may affect the development of HCC by reducing the proliferation, invasion, and migration of Hep3B/R and HepG2/R cells through the inhibition of Epithelial-Mesenchymal Transition (EMT).

Keywords: hepatocellular carcinoma; hepatitis B virus X protein; sorafenib; drug resistance

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and the second-leading cause of cancer-related mortality [1]. Hepatocellular carcinomas have higher tendency to be associated with floridly reactive mesothelial cells [2]. According to the epidemiological data reported by Globocan 2020, more than 900,000 new cases of liver cancer are diagnosed each year, and about 830,000 cases result in fatality due to HCC [3]. Although interventions such as surgical resection, local ablation, and liver transplantation serve as beneficial modalities for early-stage liver cancer treatment [4], the prognostic outlook for HCC remains unfavorable, primarily attributed to its proclivity for late-stage diagnosis, expedited progression, and recurrent manifestation [5,6]. Therefore, it is crucial to understand the molecular mechanism of the occurrence and development of HCC.

Infection with the hepatitis B virus (HBV) can sequentially progress to hepatitis, cirrhosis, and eventually HCC. The hepatitis B virus X protein (HBx) is a multifunctional regulatory protein, pivotal in the activation of numerous oncogenes, thereby playing a key role in the oncogenesis and progression of HCC [7–9]. HBx can modulate an array of genes and epigenetic entities, resulting in disorders of various pathways and processes in HCC [10]. Notably, HBx induces a significant upregulation of ETS variant 4 (ETV4), with the elevated expression of ETV4 amplifying HCC cell invasion and metastasis through the upregulation of dishevelled-2 (DVL2) [11]. Recent insights by Dong *et al*. [12] underscore the HBx-mediated augmentation of DEAD-Box Helicase 17 (DDX17) as a contributory factor to HBV-related hepatocellular carcinoma tumorigenesis. Additionally, the implications of HBx in drug resistance have been extensively documented [13,14], with Liu *et al*. [15] positing the HBx protein as a potential etiological factor for multidrug resistance in HBV-related HCC, implicating the nuclear factor kappa-B (NF-κB) pathway in this alteration.

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Sorafenib, a small molecular kinase inhibitor, often applied in the treatment of advanced HCC, can promote apoptosis, reduce angiogenesis, and inhibit tumor cell proliferation [16]. Despite its efficacy in addressing advanced HCC, the emergence of drug resistance to sorafenib is escalating. Only about 30% of patients benefit from sorafenib, typically developing drug resistance within a six-month timeframe [17]. Consequently, utilizing HCC drug-resistant cell lines (Hep3B/R and HepG2/R), this study engineered a series of stable HBx expression cell lines via lentivirus infection to elucidate the mechanistic role of HBx within sorafenib-resistant hepatocellular carcinoma cells.

Materials and Methods

Cell Culture

Hep3B and HepG2 were purchased from Wuhan Punosai Life Technology Co., Ltd. (CL-0102, Wuhan, China). Cells were cultured in DMEM medium (Gibco, C11965500B, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), and 5% CO_2 at 37 °C. Cells were authenticated using DNA-based methods and checked against the International Committee for Cell Line Accreditation (ICLAC) database for any misidentification, ensuring no cross-contamination. The genetic characteristics of cell lines were established by Short Tandem Repeat (STR) information, and no cross-contamination was detected. The cells tested negative for mycoplasma. Cells were split at 80% confluency using 0.5 mL trypsin (Solarbio, T1350, Beijing, China) until the cell was deformed and exfoliated. The complete medium was supplemented to terminate digestion and transferred to the new 25T cell culture flask according to a certain proportion to continue culture.

CCK-8 Assay

Cells were seeded into a 96-well plate and incubated for 48 h, after which 10 mL cell counting kit-8 (CCK-8) solution (Solarbio, CA1210, Beijing, China) was added to each well. The absorbance (OD) value at 450 nm was recorded, with each experiment performed in triplicate.

Construction of Drug-Resistant Cell Lines (Hep3B/R and HepG2/R)

Sorafenib was obtained from MCE (HY-10201, Princeton, NJ, USA). Drug-resistant cell lines (Hep3B/R and HepG2/R) were developed by increasing sorafenib concentrations (0, 4, 6, 8, 10, 12 µmol/L) in Hep3B and HepG2 cells. The survival rate of Hep3B, Hep3B/R, HepG2, and HepG2/R cells was evaluated using the CCK-8 assay. The IC_{50} values of sorafenib were calculated at different sorafenib concentrations.

Quantitative Reverse Transcription PCR (RT-qPCR)

The total RNA in the cell was extracted by the kit. CDNA synthesis kit (Thermo Fisher Scientific, R211-01, Waltham, MA, USA) was applied for reverse transcription, qPCR SYBR Green Master Mix reagent (Thermo Fisher Scientific, Q111-02, Waltham, MA, USA) was applied for RT-qPCR and the primers were synthesized by Beijing Huada Genome Company (Beijing, China). HBx-F: 5′-TACCGTCCCTTGCTTTCTCT-3′; HBx-R: 5′-CAGAGGTGAAGCGAAGTGC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F: 5′-AAGGATTCCTATGTGGGCGAC-3′; GAPDH-R: 5′-CGTACAGGGATAGCACAGCC-3′. 94 °C pre-denaturation for 30 s; 94 °C denaturation for 5 s, 60 °C annealing for 15 s, 72 °C extension for 10 s, amplification of 45 cycles. Results were analyzed quantitatively using the comparative threshold method. Data were calculated using the $2^{-\Delta\Delta CT}$ method.

Western Blot

The protein content was assessed after the total cell protein extraction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Beyotime, P0690, Shanghai, China) electrophoresis was performed using a 10% gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, 24937-79-9, Waltham, MA, USA). The membrane was blocked with the purchased sealing solution for 0.5 h at room temperature. Subsequently, diluted primary antibodies for HBx (Abcam, ab157480, Cambridge, MA, USA), E-cadherin (Bioswamp, PAB46093, Wuhan, China), N-cadherin (1:1000, Bioswamp, PAB47911, Wuhan, China), Vimentin (1:1000, Bioswamp, PAB36390-P, Wuhan, China), Slug (1:1000, Bioswamp, PAB30534, Wuhan, China) and Snail (1:1000, Bioswamp, PAB33921, Wuhan, China), were added and incubated overnight at 4 °C. Then horseradish peroxidase (HRP) labeled sheep anti-rabbit second antibody (1:3000, Bioswamp, SAB48169, Wuhan, China) was supplemented and incubated for 2 h. Chemiluminescence system was applied to scan the images, and GAPDH (1:2000, Bioswamp, MAB45855, Wuhan, China) was applied as the internal reference for quantitative analysis. The blot densities were quantified by ImageJ software (1.8.0, NIH, Bethesda, MD, USA), and the results were expressed as normalized ratios to the densitometry units of GAPDH.

Construction and Transfection of HBx Vector

HBx overexpression and interference vectors were constructed by targeting specific genes. Among them, HBx overexpression plasmid and HBx control virus vector were applied to infect the corresponding cells. Finally, the stable hepatoma strains with stable expression of HBx were obtained, that was, the high expression subgroup of HBx and the negative control (NC) subgroup. The cells with high expression of HBx were infected with interfering virus sh-HBx (pLKD-CMV-R&PR-U6-shRNA-HBx) and interfering control virus sh-NC (pLKD-CMV-RFP-2A-Puro-U6-shRNA-NC), respectively. At 72 h, puromycin

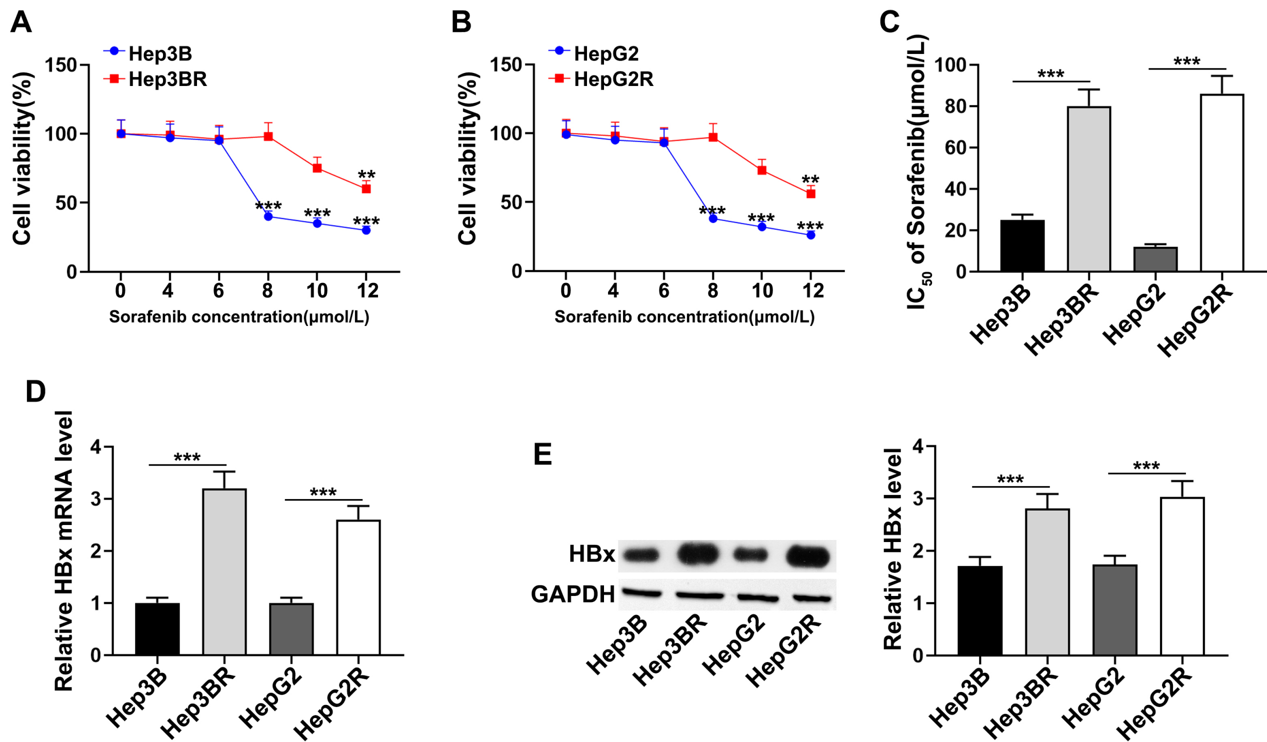

Fig. 1. Assessment of hepatitis B virus X protein (HBx) content and cellular survival in Hep3B/R and HepG2/R cells. (A,B) The survival rate of Hep3B, Hep3B/R, HepG2, and HepG2/R cells was estimated using the cell counting kit-8 (CCK-8) assay. ** $p < 0.01$, *** $p < 0.001$, compared to 0 µmol/L subgroup. (C) IC_{50} values of Hep3B/R and HepG2/R cells. (D) HBx content in Hep3B, Hep3B/R, HepG2, and HepG2/R cells (quantitative reverse transcription PCR (RT-qPCR)). (E) HBx protein content in Hep3B, Hep3B/R, HepG2, and HepG2/R cells was assessed by Western blot. ** $p < 0.01$, *** $p < 0.001$, compared to Hep3B/HepG2 subgroup. N = 3.

(Yeasen, 60210ES, Shanghai, China) was supplemented, the concentration of 2 mg/mL was maintained, and the stable strains of HBx interference subgroup and interference control subgroup were obtained. Sh-HBx (5′-GCACCAUCAACUAUGAUGATT-3′) and HBx overexpression plasmid were obtained from GenePhama (Shanghai, China).

Clone Formation Assay

The cells of each subgroup were collected, the concentration of cell suspension was adjusted, and the cells were gently rotated in a 6-well plate (2000 cells/well, 2 mL per well) so that the cells were evenly dispersed and cultured in a cell incubator with 5% CO_2 at 37 °C. The culture was terminated when a clone visible to the naked eye appeared on the culture plate. 2 mL of 4% paraformaldehyde was supplemented to fix cells for 15 minutes at 4 °C. The fixed solution was removed, and 1 mL Wright-Giemsa composite dye (Solarbio, G1020, Beijing, China) was supplemented for 20 minutes; then, the dye was washed slowly with running water, dried, and photographed.

Wound Healing Assay

The marker pen was applied to draw 5 straight lines uniformly on the back of the 6-hole plate with a ruler. 1×10^6 cells were supplemented to each well. The next day, scratches were made perpendicular to the straight lines on the back. Post-scratching, cells were washed with PBS, treated according to different subgroups with serum-free medium, and further incubated in a 5% CO_2 incubator at 37 °C. Images were captured at 0 h and 48 h to assess wound healing.

Transwell Assay

Cells were dispersed using 0.25% trypsin and seeded in 24-well plates with Matrigel Chambers used for invasion assays. 5×10^4 transfected cells were placed in the upper chamber, Matrigel matrix gel was added, and medium with 10% FBS was placed in the lower chamber. After incubating the cells for 24 h, cells that failed to migrate were removed from the upper chamber. The transwell membrane was fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet. After rinsing well in tap water, the cells were counted under an inverted microscope. All experiments were performed in triplicate and repeated three times.

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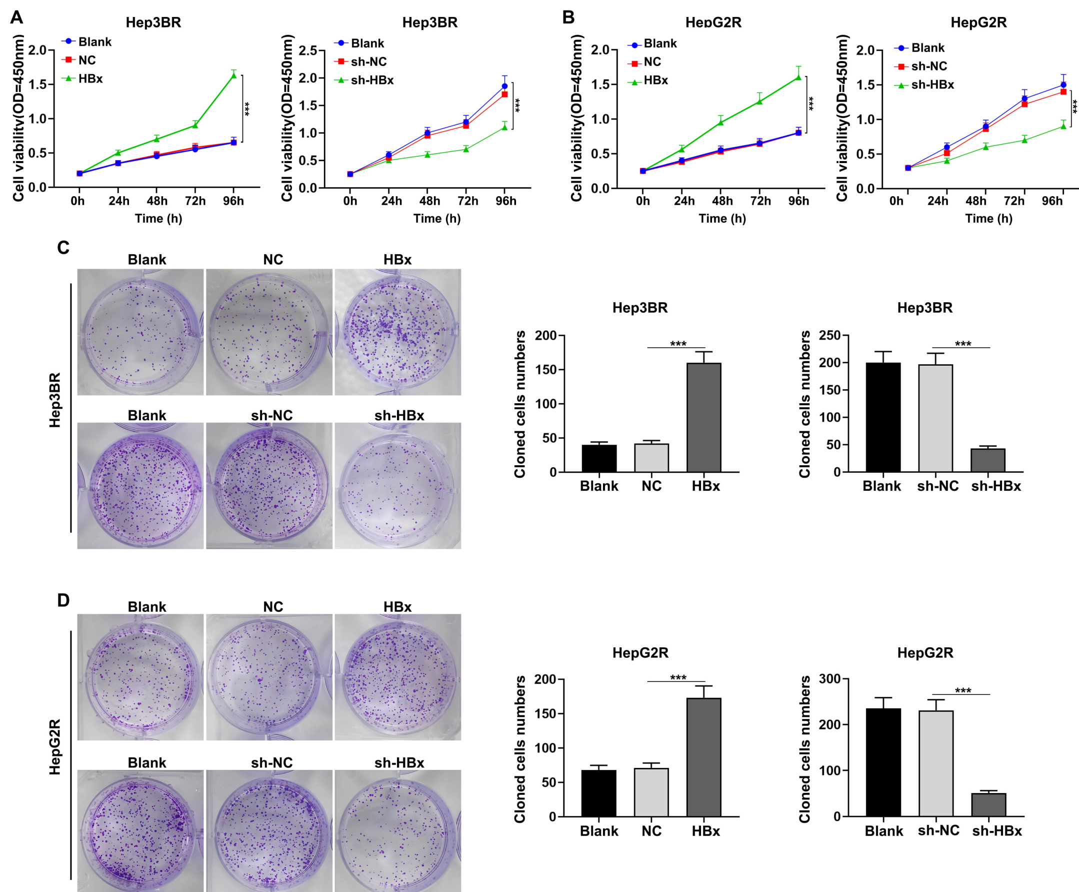

Fig. 2. HBx amplifies the activity of Hep3B/R and HepG2/R cells. Hep3BR and HepG2R were transfected with HBx or sh-HBx. (A,B) Cell viability was appraised by CCK-8 assay in Hep3BR and HepG2R cells. (C,D) Cell viability was appraised by colony formation assay in Hep3BR and HepG2R cells. ***$p < 0.001$, compared to NC (negative control)/sh-NC subgroup. N = 3.

Statistical Analysis

All values were presented as mean $\pm$ SD of at least triplicate determinations. Comparisons of variables between the 2 groups were conducted by 2-tailed independent sample *t*-test. One-way analysis of variance (ANOVA) was performed for multiple groups, followed by the Bonferroni post hoc test (SPSS 13.0; SPSS Inc., Chicago, IL, USA). In all cases, $p < 0.05$ was considered statistically significant.

Results

HBx Content in HCC-Resistant Cells

We discovered significant increases in proliferative ability and IC_{50} in the Hep3B/R and HepG2/R subgroups compared to the Hep3B and HepG2 subgroups respectively (Fig. 1A–C). The elevated HBx content in Hep3B/R subgroup compared to Hep3B subgroup (Fig. 1D,E), provides a plausible interconnection and rationale for further investigation.

HBx Augments Proliferation, Migration, and Invasion of HCC-Resistant Cells

Building upon our initial findings of increased HBx content in resistant cells, we sought to understand the consequential impact of increased HBx levels on various cellular activities. Our observations demonstrated that the proliferative ability of Hep3B/R and HepG2/R cells in the HBx subgroup was notably elevated compared to the NC subgroup, as illustrated in Fig. 2A,B. Furthermore, an exploration into clonal expansion showed a noticeable increase in the number of clones in the HBx subgroup compared to the NC subgroup, a noticeable decrease in the number of clones in Hep3B/R and HepG2/R cells in sh-HBx subgroup compared to sh-NC subgroup (Fig. 2C,D). A closer investigation of cellular migration highlighted that the HBx subgroup demonstrated a remarkable surge in migration ability in contrast to the NC subgroup, a remarkable decrease in migration ability in sh-HBx subgroup compared to sh-NC subgroup, as depicted in Fig. 3A–D.

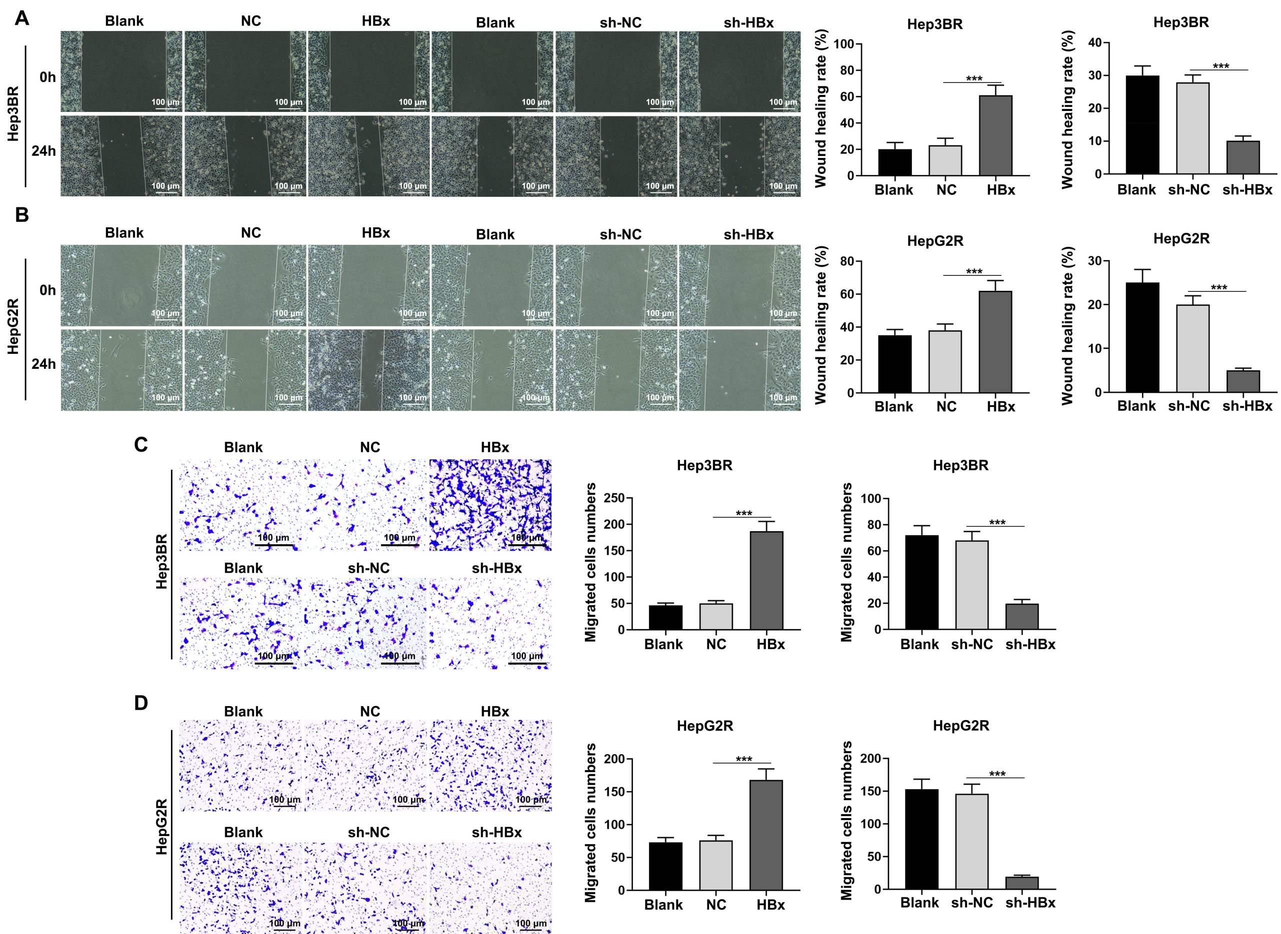

Fig. 3. Dissecting the impact of HBx on cellular migration in Hep3B/R and HepG2/R cells. Hep3BR and HepG2R were transfected with HBx or sh-HBx. (A,B) Cell migration was appraised by wound healing assay in Hep3BR and HepG2R cells. (C,D) Cell migration was appraised by transwell assay in Hep3BR and HepG2R cells. ***$p < 0.001$, compared to NC/sh-NC subgroup. N = 3.

HBx on HCC-Resistant Cells Invasion and EMT

Our investigations revealed a pronounced alteration in the invasion capabilities of Hep3B/R and HepG2/R cells in the HBx subgroup, which notably elevated compared to the NC subgroup, in addition, the invasion number of cells in the sh-HBx subgroup was significantly reduced compared to the sh-NC subgroup (Fig. 4A,B). This contrast in invasion capabilities highlighted the importance of the HBx content in modulating the invasive properties of HCC-resistant cells.

Moreover, Epithelial-Mesenchymal Transition (EMT) analysis disclosed significant changes in EMT markers, correlating with the altered HBx content. E-cadherin content in Hep3B/R and HepG2/R cells was notably reduced in the HBx subgroup compared to the NC subgroup. Conversely, N-cadherin, Vimentin, Slug, and Snail were elevated in the HBx subgroup compared to the NC subgroup (Fig. 4C,D). Additionally, a reversal in the content of these markers was observed in the sh-HBx subgroup, in stark contrast to the sh-NC subgroup, underscoring the influential role of HBx in dictating EMT transition dynamics.

Discussion

With the annual incidence rate of HCC manifesting a tripling in the past three decades [18], the role of sorafenib as a standard treatment for advanced HCC has become critical [19]. Despite its paramount importance, the intricacies of sorafenib resistance in HCC are not fully understood, rendering the resistance of HCC to sorafenib-targeted drugs a significant challenge. Thus, establishing a sorafenib resistance model is pivotal for investigating the underlying mechanisms and enhancing the sensitivity of HCC to sorafenib [20]. In this study, we engineered drug-resistant cell lines (Hep3B/R and HepG2/R), unveiling the potential of HBx knockout in impeding the proliferation, invasion, migration, and EMT of sorafenib-resistant hepatocellular carcinoma cells, subsequently restraining the progression of HCC.

Sorafenib is the first approved liver cancer drug. By inducing Hep3B and HepG2 cells with incrementing concentrations of sorafenib, we successfully modeled drug-resistant cell lines, demonstrating enhanced cellular ca-

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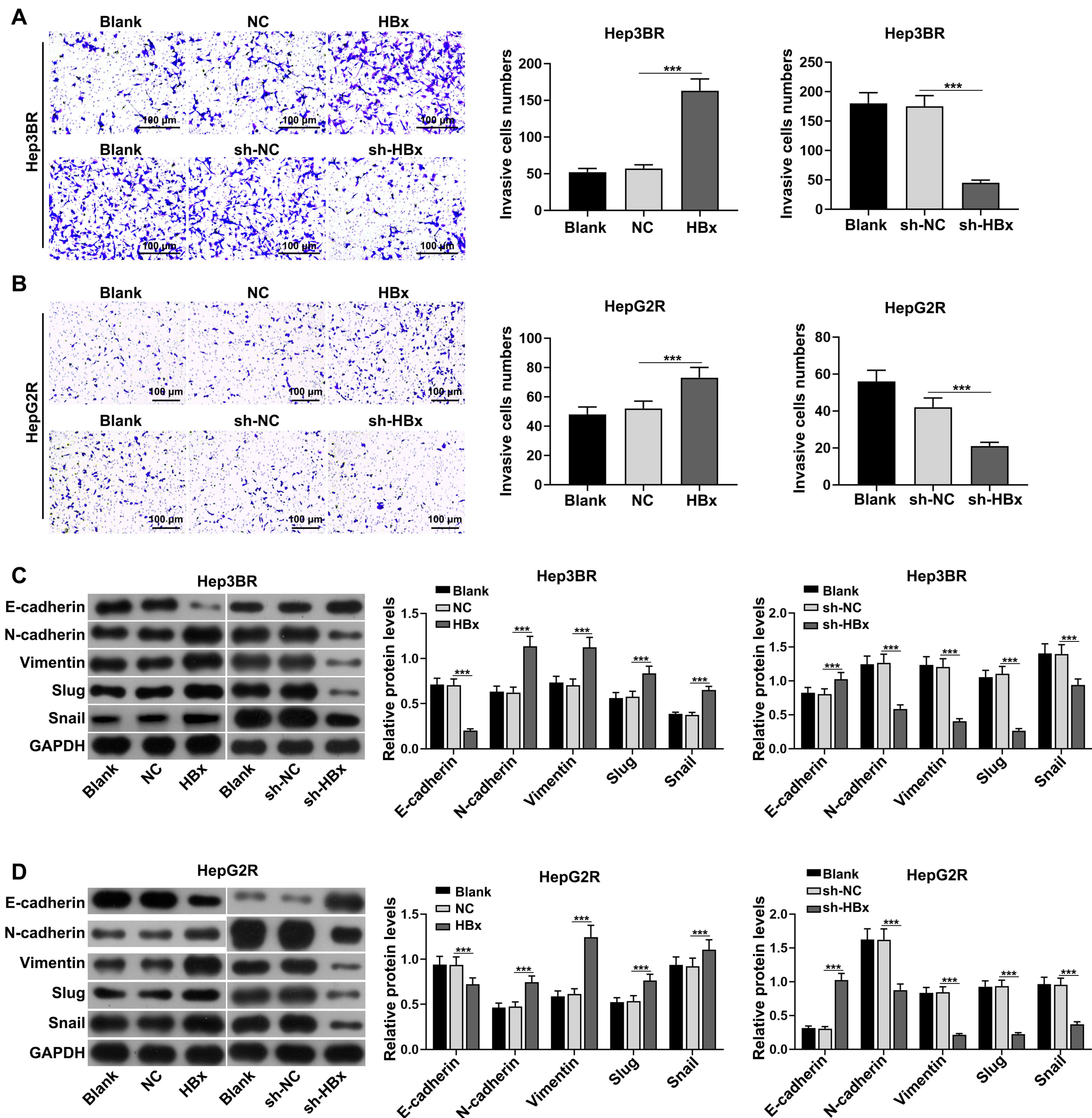

Fig. 4. The impact of HBx on Hep3B/R and HepG2/R invasion and Epithelial-Mesenchymal Transition (EMT). Hep3BR and HepG2R were transfected with HBx or sh-HBx. (A,B) Transwell assay was applied to evaluate cell invasion in Hep3BR and HepG2R cells. (C,D) E-cadherin, N-cadherin, Vimentin, Slug, and Snail content was appraised in Hep3BR and HepG2R cells. ***$p < 0.001$, compared to NC/sh-NC subgroup. N = 3.

pabilities, increased IC_{50}, and elevated HBx levels [13]. These insights highlight the potential issues associated with long-term sorafenib treatment, wherein patients are susceptible to developing drug resistance, thereby impacting the chemotherapy outcomes and contributing to the multidrug resistance seen in liver cancer. Our study and the role of HBx align with Song *et al.* [20] showing that HBx-mediated upregulation of Translation Regulatory Long Non-Coding RNA 1 (TRERNA1) facilitates sorafenib resistance and fosters cell proliferation in HCC by targeting Neuroblastoma RAS (NRAS) via *miR-22-3p* sequestration.

Moreover, we show that HBx not only promotes sorafenib resistance but also orchestrates the EMT process in HCC-resistant cells, corroborating the proven carcinogenicity of HBx in HCC and its prevalent involvement in the development of HBV-related HCC [21–23]. Our find-

ings highlight the extensive influence of HBx in boosting the occurrence of hepatocellular carcinoma by promoting cell metastasis, proliferation, and sorafenib resistance. Furthermore, our data suggest that overexpression of HBx significantly augments the activity, proliferation, invasion, and migration of cancer cells, while its knockdown can considerably curtail these cellular activities, indicative of its role in inducing hepatocyte carcinogenesis through autophagy mediated by arrestin beta-1 [8]. It is this broad spectrum of actions that positions HBx as a key factor in the malignancy of HCC and a potential prognostic indicator and therapeutic target for HBV-HCC [24]. In agreement with the overarching concept of EMT being a crucial step in tumor invasion and metastasis, our research reiterates that tumor cells achieve enhanced invasion and metastasis capabilities through the EMT process [25], with HBx mediating this process in HCC cells. This was illustrated by the notable elevation in E-cadherin content post-HBx knockdown, with a concurrent reduction in N-cadherin, Vimentin, Slug, and Snail content, indicating the potential of HBx knockout in modulating cellular multiplication by attenuating the EMT process of HCC cells.

Conclusions

By successfully contructing Hep3B/R and HepG2/R drug-resistant cell lines and implementing HBx overexpression and interfering virus vectors, our study offers a preliminary exploration into the impact of HBx on sorafenib-resistant HCC cells. Our results show that HBx knockout can alter the trajectory of HCC by inhibiting the proliferation, invasion, and migration of HCC-resistant cells and mitigating EMT. In subsequent studies, we aim to explore the detailed mechanisms by which HBx modulates sorafenib-resistant HCC cells, providing a more nuanced understanding and revealing new therapeutic possibilities.

Availability of Data and Materials

The dataset analyzed during the current study are available.

Author Contributions

YPL and HXL designed the research study. YPL and XL performed the research. XL and MSL provided help and advice on the western blot experiments. YRL analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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