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HBx induces chemoresistance in diffuse large B cell lymphoma by inhibiting intrinsic apoptosis via the NF-κB/XIAP pathway

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Diffuse large B cell lymphoma (DLBCL) is the predominant subtype of malignant lymphoma in adults with high heterogeneity. Hepatitis B virus (HBV) has been shown to infect B lymphocytes and has been associated with a higher risk of developing DLBCL, most clearly in countries where HBV is endemic. Accumulating evidence suggests that the standard chemotherapy regimens for DLBCL patients with HBV infection exhibit limited efficacy and unfavorable outcomes. The HBx antigen, encoded by the X gene in the four open reading frames of HBV, may be a key molecule promoting the heightened malignant biological characteristics of DLBCL, but whether it affects the chemotherapy response and the mechanism in DLBCL remains unclear. Through the implementation of *in vitro* and *in vivo* experiments, our study demonstrates that the HBx antigen triggers excessive activation of the NF-κB pathway, resulting in increased expression of the X-linked inhibitor of apoptosis protein (XIAP). This upregulation inhibits caspase-3-mediated intrinsic apoptosis and enhances resistance to first-line chemotherapeutic agents like epirubicin and vincristine in DLBCL. These findings offer insights into the development of innovative combination therapies for DLBCL patients with HBV infection.

INTRODUCTION

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, possesses a genome of approximately 3.2 kb in length, characterized by partially double-stranded circular DNA. The HBV gene comprises four overlapping open reading frames (pre-S/S, pre-C/C, X, P), and encodes various proteins that include Pre-S1, Pre-S2, HBsAg, HBeAg, HBcAg, HBx, and DNA polymerase.[1] According to the World Health Organization, an estimated 2 billion individuals globally have experienced a past or present HBV infection, with approximately 257 million people (3.5% of the world's population) identified as chronic carriers.[2]

HBV exhibits a hepatophilic nature and also demonstrates an affinity for lymphocytes. The incidence of HBV infection is significantly higher in patients with non-Hodgkin lymphoma (NHL) than in the general population and patients with non-hepatocellular carcinoma solid tumors.[3,4] Among the various pathologic subtypes of NHL, people with diffuse large B-cell lymphoma (DLBCL) have the highest prevalence of HBV infection at 10.58%.[5,6] Our previous clinical investigation demonstrated that DLBCL patients with HBV infection exhibit lower response rates to first-line immunochemotherapy with R-CHOP (rituximab, cyclophosphamide, epirubicin [EPI], vincristine [VCR], and prednisone). Specifically, the overall response rates for patients with and without HBV were 77.4% and 90.1%, respectively (p = 0.001), and the complete response rates were 51.2% and 64.6%, respectively (p = 0.017).[7] This suboptimal treatment efficacy is associated with disease refractory, relapse, and unfavorable prognosis. Nevertheless, there is a lack of comprehensive research on the mechanisms driving disease progression and unfavorable outcomes in HBV-associated DLBCL.

In the R-CHOP regimen, rituximab, a human-mouse chimeric anti-CD20 monoclonal antibody, exerts its therapeutic effects primarily through targeting the CD20 antigen expressed on the surface of B cells, and the presence of HBV infection does not reduce the expression of CD20 on the lymphoma cell surface.[8] Several clinical studies have also demonstrated a higher therapeutic response rate to the R-CHOP regimen compared to the CHOP regimen in DLBCL patients with HBV infection.[9,10] First-line chemotherapeutic agents EPI and VCR may be associated with a low response rate to first-line therapy in DLBCL patients with HBV infection. The modulation of apoptosis by inhibitors of apoptosis proteins (IAPs), particularly the X-linked inhibitor of apoptosis protein (XIAP) belonging to the nuclear factor (NF)-κB-dependent IAP family,[11,12] significantly impacts apoptosis induced by chemotherapeutic agents. XIAP serves

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as a regulator of the caspase cascade by inhibiting both initiator and effector caspase activities. Elevated XIAP expression has been observed in various solid tumors, including ovarian cancer, renal cancer, prostate cancer, colorectal cancer, liver cancer, and thyroid cancer,[13,14] and is linked to heightened malignancy, treatment resistance, and unfavorable prognosis. Nevertheless, the relationship between XIAP expression and chemotherapy sensitivity in HBV-associated DLBCL has yet to be investigated.

The clinical outcome of HBV infection depends on the complex interplay between HBV replication and host immune response. The human body initially carries the virus for a period of time and subsequently expresses the relevant antigens, including HBsAg and HBeAg, thereby eliciting immune response by stimulating and activating the BCR signaling pathway. B cells produce antibodies HBsAb, HBeAb, and HBcAb to block further infection and support effective viral clearance.[15] Previous studies have not identified the presence of HBV-encoded HBsAg, HBcAg, and HBeAg antigens in the pathological tissues of DLBCL.[16,17] The X region, the smallest open reading frame within the HBV genome, encodes the HBx antigen, which has pleiotropic properties that support virus gene expression and replication, increase the resistance of infected hepatocytes to immune-mediated lysis, and promote resistance to chemotherapy and targeted therapies.[18,19] Studies conducted by Wang et al.[16] and Huang et al.[17] have both demonstrated a high prevalence of positive expression of the HBx antigen in the pathological tissues of DLBCL patients with HBV infection. The role of the HBx antigen in the initial therapeutic response of DLBCL and the underlying mechanism remains unclear.

HBx protein has been found to enhance the expression of multidrug resistance genes, including MDR1, MRP1, LRP1, and ABCG2,[20,21] while also influencing the expression of specific anti-apoptotic genes through activation of the NF-κB signaling pathway in hepatocellular carcinoma.[22,23] This dual effect ultimately results in resistance to chemotherapy agents such as fluorouracil and doxorubicin. HBx-positive hepatocellular carcinoma pathological tissues exhibit elevated NF-κB activity and increased tumor malignancy.[24] Bernal-Mizrachi et al. found that NF-κB pathways are important in the resistance to apoptosis in lymphoma models, which is mediated by the overexpression of different downstream anti-apoptotic targets, such as Bcl-xL, and members of the IAP family (XIAP and cellular IAPs [cIAPs]). The overexpression of Bcl-xL and XIAP impedes the intrinsic apoptosis pathway, thereby inhibiting the effect of different activators of apoptosis, such as doxorubicin.[25] These results suggest that HBx could enhance the resistance to chemotherapy-induced apoptosis through the hyperactivation of the NF-κB pathway in DLBCL. Based on the above premise, we aim to establish a theoretical basis for advancing mechanistic research and clinical strategy development through the implementation of *in vitro* and *in vivo* experiments.

RESULTS

HBx induces resistance to EPI and VCR in DLBCL *in vitro*

In vitro, Lenti-HBX-GFP-Puro (lentiviral vector carrying the X gene, GFP marker, and puromycin resistance), and Lenti-GFP-Puro (lentiviral vector carrying only the GFP marker and puromycin resistance) were transfected with SUDHL-4 (germinal center B-cell-like subtype) and U2932 (non-germinal center B-cell-like subtype) cells, respectively. The transfection efficiency was assessed through quantitative reverse-transcription PCR (RT-qPCR) and western blotting (Figures 1A and 1B). Notably, immunofluorescence staining revealed the presence of HBx protein in both the cytoplasm and nucleus of HBx-SUDHL-4 and HBx-U2932 cells, while it was absent in GFP-SUDHL-4 and GFP-U2932 cells (Figures 1C and S1A).

Subsequently, we explored the impact of HBx on the sensitivity of DLBCL cells to EPI and VCR. CCK-8 results indicated that as the concentrations of EPI and VCR increased, the cytotoxicity of the HBx-overexpression group (HBx-SUDHL-4 and HBx-U2932) and the control group (GFP-SUDHL-4 and GFP-U2932) both increased. However, within the same dose range of EPI or VCR, the cytotoxicity of the HBx-overexpression group was found to be lower than that of the control group, as illustrated in Figure 1D. The findings suggest that HBx attenuates the toxicity induced by EPI and VCR on DLBCL cells.

EPI and VCR mainly induce apoptosis in lymphoma cells and are classified as non-specific cell-cycle-blocking agents. The half-maximal inhibitory concentration (IC_{50}) values of EPI or VCR were administered to each group of DLBCL cells for 48 h, the apoptosis rates in the HBx-overexpression group were found to be significantly lower compared to those in the control group, with statistical significance ($p < 0.05$) (Figures 2A and 2B). To eliminate the effect of baseline cell apoptosis, we defined the ratio of cell apoptosis rates before and after drug treatment as the drug sensitivity index. The HBx-SUDHL-4 and HBx-U2932 cells exhibited a lower drug sensitivity index for EPI and VCR, with statistically significant differences ($p < 0.05$) (Figure 2C). These findings suggest that HBx weakens the pro-apoptotic effects of EPI and VCR on DLBCL cells.

Caspase-3 and poly (ADP-ribose) polymerase-1 (PARP-1) also serve as crucial indicators of endogenous apoptosis.[26] After activation of the apoptotic pathway by chemotherapeutic agents, caspase-3 and PARP-1 undergo cleavage and inactivation, leading to the formation of cleaved caspase-3 and cleaved PARP-1. Our findings showed that after treatment with EPI or VCR, the levels of cleaved caspase-3 and cleaved PARP-1 proteins were lower in the HBx-overexpression group compared with the control group (Figure 2D). Moreover, EPI or VCR had little effect on HBx protein expression (Figure 2E).

XIAP-mediated apoptosis is associated with resistance to EPI and VCR in HBx-positive DLBCL cells

We examined the relative mRNA expression levels of multidrug resistance molecules (MDR1, MRP1, LRP1, and ABCG2) (Figure 3A) and IAPs family members (cIAP1, cIAP2, XIAP, Survivin, and Livin) in the control and HBx-overexpression groups of DLBCL cells. There were no significant differences in the expression of multidrug resistance molecule expression between the two groups. The IAP family members play a crucial role in apoptosis regulation and the

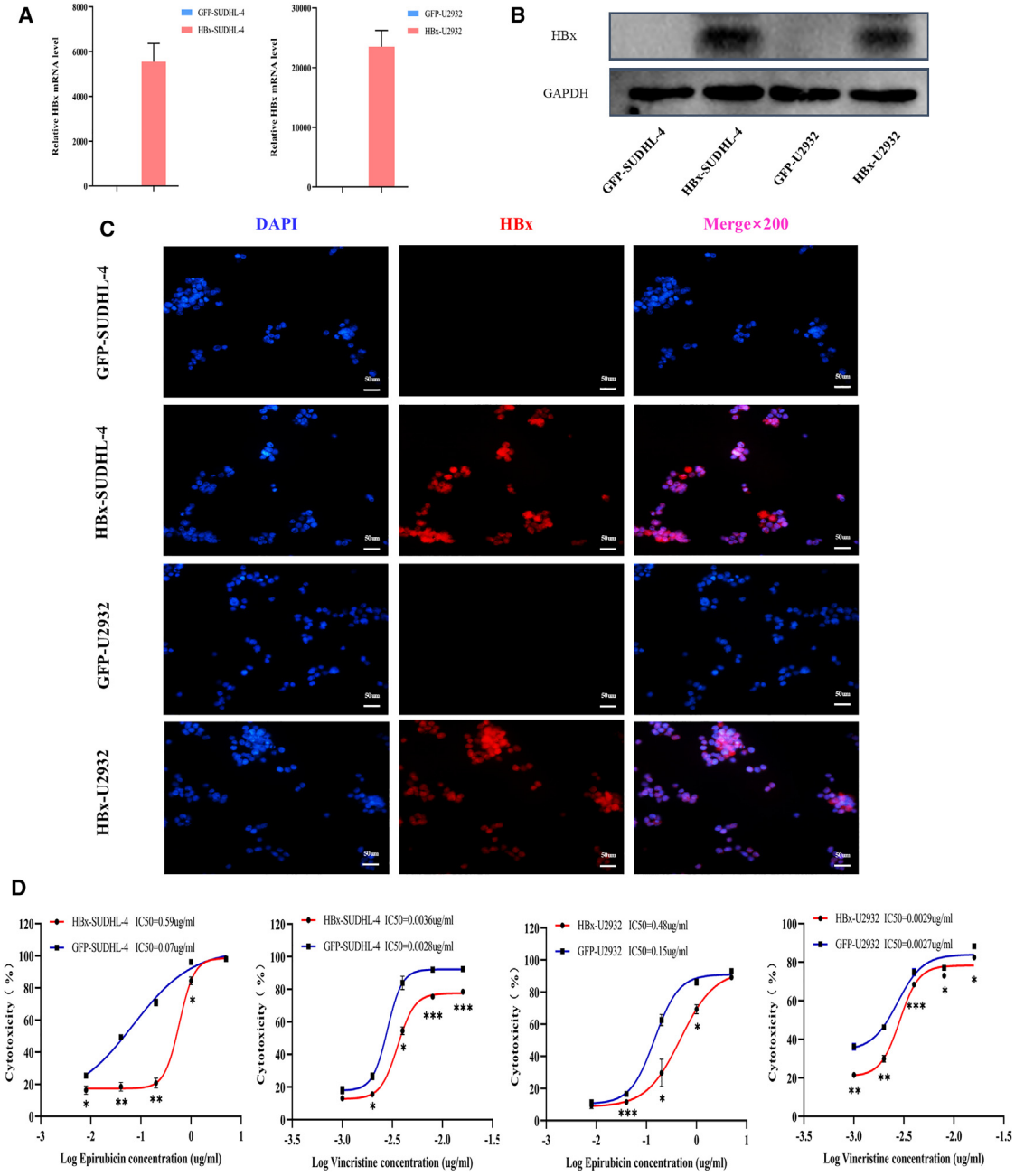


Figure 1. HBx attenuates the toxicity induced by EPI and VCR on DLBCL cells

(A and B) RT-qPCR (A) and western blotting (B) were performed to detect the HBx overexpression in SUDHL-4 and U2932 cells. (C) Immunofluorescence staining was performed to observe the expression and position of HBx proteins (200×). (D) The toxicity of different concentrations of EPI and VCR on DLBCL cells by CCK-8 assay. The results were determined in triplicate, and the error bars represent the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

modulation of chemotherapy drug cytotoxicity.[27] Notably, both XIAP mRNA and protein were highly expressed in HBx-SUDHL-4 and HBx-U2932 cells (Figures 3B and 3C). To further validate the correlation between XIAP-mediated apoptosis and resistance to EPI and VCR in DLBCL cells, we transfected with either negative control small interfering RNA (siRNA) or XIAP-specific siRNA individually in GFP-SUDHL-4 and HBx-SUDHL-4, as well as in GFP-U2932 and HBx-U2932 cells, and then exposed to EPI and VCR. Results showed that XIAP-specific siRNA significantly augmented apoptosis induced by EPI and VCR in GFP-SUDHL-4, HBx-SUDHL-4, GFP-U2932,

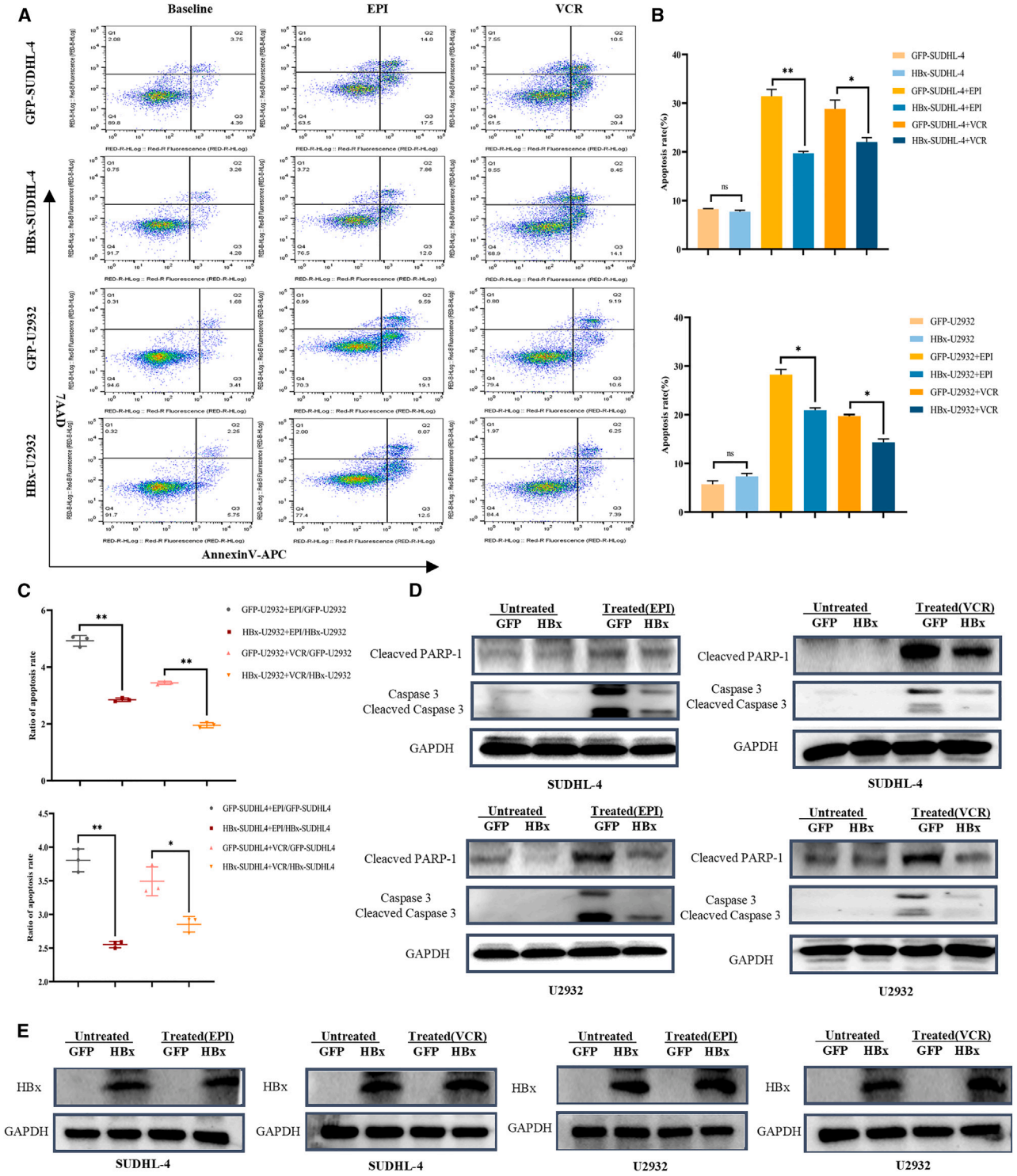
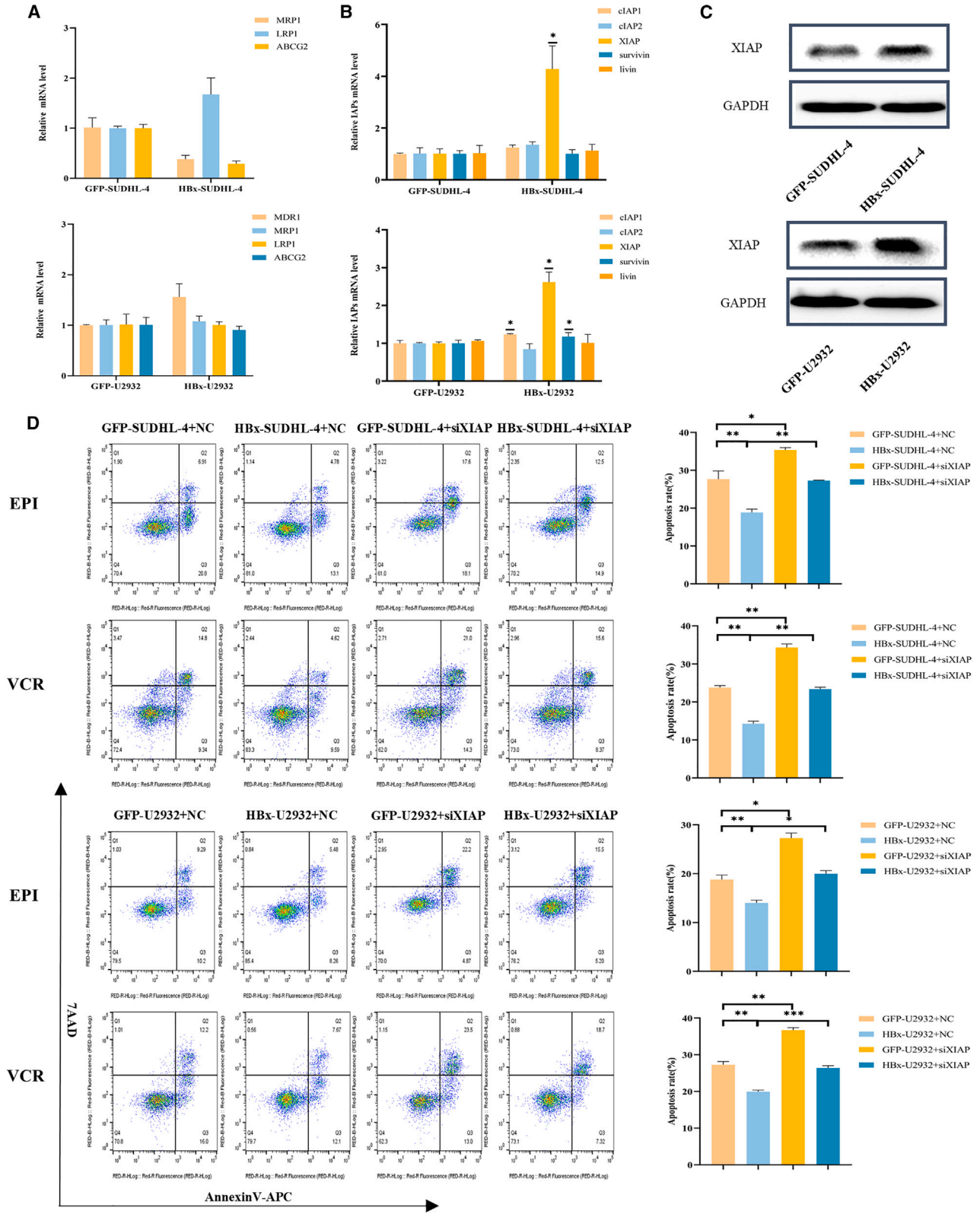


Figure 2. HBx weakens the pro-apoptotic effects of EPI and VCR on DLBCL cells

(A) Flow assay for apoptosis in each group of DLBCL cells (GFP-SUDHL-4, HBx-SUDHL-4, GFP-U2932, HBx-U2932) treated with EPI and VCR at IC_{50} for 48 h. (B and C) Quantification of apoptosis rate (B) and drug sensitivity index (C). (D and E) Western blotting to detect the expression of apoptotic proteins cleaved caspase-3 and cleaved PARP-1, as well as HBx. The results were determined in triplicate, and the error bars represent the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.



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and HBx-U2932 cells compared to negative control siRNA ($p < 0.05$) (Figure 3D). XIAP-specific siRNA interference in HBx-SUDHL-4 and HBx-U2932 cells resulted in a restoration of apoptosis rates comparable to those observed in control cells following treatment with EPI and VCR.

HBx confers resistance to EPI and VCR in DLBCL *in vivo*

To further confirm the potential role of HBx in conferring resistance to EPI and VCR in DLBCL, an *in vivo* study was conducted using DLBCL tumor-bearing mice. Control DLBCL cells (transfected with an empty vector) and HBx-overexpression DLBCL cells in logarithmic growth phase were grafted into the nonobese diabetic-severe combined immunodeficiency disease mice. Tumor formation was observed subcutaneously in mice 12 days after injection of tumor cells. When tumor volume exceeded 400 mm^3, three treatment regimens were administered: single-agent EPI, single-agent VCR, and a combination of EPI with VCR. Following drug treatment, the body weights of DLBCL tumor-bearing mice initially decreased and gradually rebounded after 2 weeks (Figure 4A), and tumor volumes were measured daily (Figure 4B). The analysis of tumor inhibition rates indicated a greater reduction in tumor burden in the control group compared to the HBx-overexpression group, regardless of treatment with single-agent EPI, single-agent VCR, or a combination of EPI and VCR, with statistical significance ($p < 0.05$) (Figure 4C). Based on gross pictures and ultrasound images of the tumor, we similarly observed that HBx-overexpression DLBCL tumor-bearing mice had reduced sensitivity to EPI and VCR compared to the control group (Figures 4D, and 4E).

Serum alanine aminotransferase levels in DLBCL tumor-bearing mice were evaluated using ELISA, and liver function in all groups remained within normal parameters (Figure S1C). Histological examination of mouse liver tissue through H&E staining did not reveal any significant abnormalities (Figure S1D), indicating that HBx antigen and chemotherapy did not impair the liver function of mice.

Both the control group and HBx-overexpression group demonstrated high expression of B cell lymphoma markers CD20 and PAX5, consistent with the pathological features of DLBCL. Prior to chemotherapy, the tumor tissue of the HBx-overexpression group exhibited high levels of HBx and XIAP expression (Figure S2A), consistent with findings from *in vitro* studies. As shown in Figure 5, the results of H&E staining indicated lower tumor tissue density in the control group following chemotherapy in comparison to the HBx-overexpression group. We also performed semiquantitative analysis and statistical tests on the immunohistochemistry (IHC) staining results. In all treatment regimens, the HBx-overexpression group consistently showed elevated XIAP expression and reduced expression of apoptotic markers cleaved caspase-3 and cleaved PARP-1 (Figure 4F).

HBx-mediated NF-κB hyperactivation upregulates XIAP *in vitro*

Immunofluorescence results indicated that HBx is localized in both the nucleus and cytoplasm of DLBCL cells. HBx can directly interact with transcription factors within the nucleus to modulate downstream target gene expression. Upon further investigation into the specific mechanism by which HBx regulates XIAP, we conducted a screening of five transcription factors (NF-κB p65, cyclic AMP response element binding protein 1 [CREB1], p53, Sp1, and C/EBPα) that potentially interact with HBx and have binding sites in the XIAP promoter region. This screening was informed by a review of the literature and software prediction (LASAGNA-Search 2.0).[28–31] RT-qPCR results revealed that the mRNA expression levels of NF-κB p65 and CREB1 mRNA were significantly higher in HBx-SUDHL-4 and HBx-U2932 cells compared to the control group ($p < 0.05$) (Figure 6A). Transient transfection experiments were conducted using plasmids containing NF-κB p65 or CREB1 genes in various groups of DLBCL cells. The results showed that the overexpression of NF-κB p65 led to a significant increase in XIAP mRNA expression ($p < 0.05$) (Figure 6B). Furthermore, luciferase reporter assays indicated that transcription factor NF-κB p65 markedly enhanced XIAP promoter activity compared to CREB1 (Figure 6C). These findings highlight the crucial role of the NF-κB pathway in the transcriptional activation of XIAP.

Additionally, we analyzed the impact of HBx on NF-κB protein expression, phosphorylation modification, and nuclear translocation. Both total NF-κB protein and phosphorylated p65 (536) levels were increased in HBx-SUDHL-4 and HBx-U2932 cells compared to the control group (Figure 6D). A distinct pattern of cytoplasmic and nuclear NF-κB p65 protein expression is observed, with GFP-SUDHL-4 and GFP-U2932 cells showing higher cytoplasmic NF-κB p65 levels, while HBx-SUDHL-4 and HBx-U2932 cells exhibit elevated nuclear NF-κB p65 expression. The nuclear-to-cytoplasmic ratio of NF-κB p65 expression was significantly higher in the HBx-overexpression group compared to the control group ($p < 0.05$) (Figures 6E and 6F). Immunofluorescence staining showed enhanced nuclear NF-κB p65 fluorescence in the HBx-overexpression DLBCL cells (Figure 7A). Collectively, these results suggested that the HBx-overexpression DLBCL cells exhibit hyperactivation of the NF-κB pathway. We chose the NF-κB p65 inhibitor BAY 11–7082 (blocking p65 entry into the nucleus) at varying concentrations (1, 2.5, and 5 μM) to treat the HBx-positive DLBCL cells, respectively. With increasing concentrations of BAY 11–7082, the levels of XIAP mRNA and protein gradually decreased (Figures 7B and 7C), underscoring the close

Figure 3. XIAP-mediated apoptosis is associated with resistance to EPI and VCR in HBx-positive DLBCL cells

(A and B) Relative mRNA expression of multidrug resistance molecules (A) and IAP family members (cIAP1, cIAP2, XIAP, Survivin, and Livin) (B) in DLBCL cells of each group. (C) Western blotting for XIAP protein expression. (D) Flow assay and quantification plots of the effects of EPI and VCR on apoptosis of GFP-SUDHL-4, HBx-SUDHL-4, GFP-U2932, and HBx-U2932 cells after knockdown XIAP. Data are presented as the mean ± SEM. The results were determined in triplicate, and the error bars represent the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

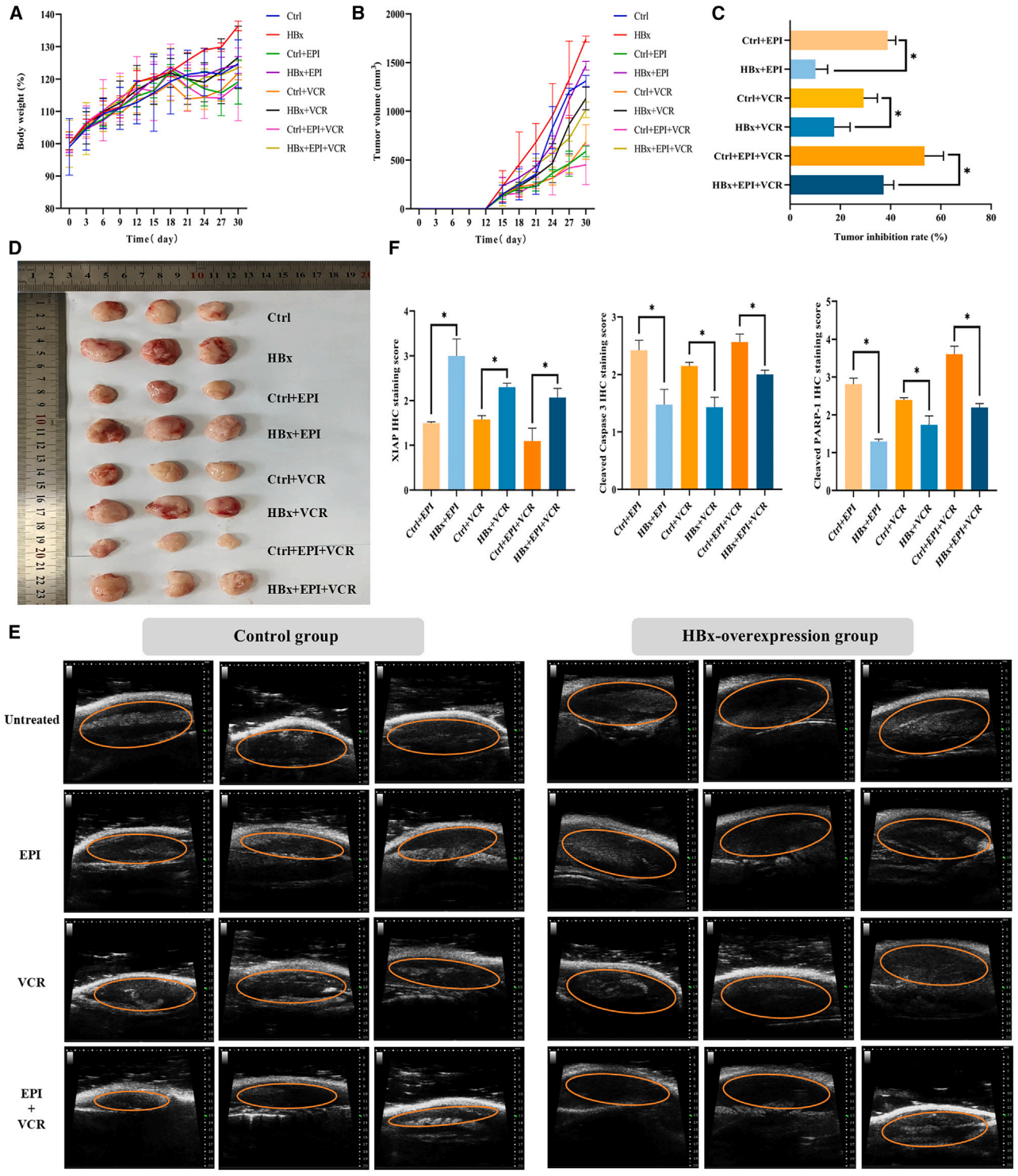


Figure 4. HBx confers resistance to EPI and VCR in DLBCL *in vivo*

(A and B) Changes in body weight (A) and tumor volume (B) in tumor-bearing mice. (C) Statistical plots of tumor inhibition rate. (D and E) Gross pictures (D) and ultrasonographic images (E) of tumor tissues. (F) Semiquantitative analysis of the IHC staining. The results were determined in triplicate, and the error bars represent the mean ± SD. *$p < 0.05$.

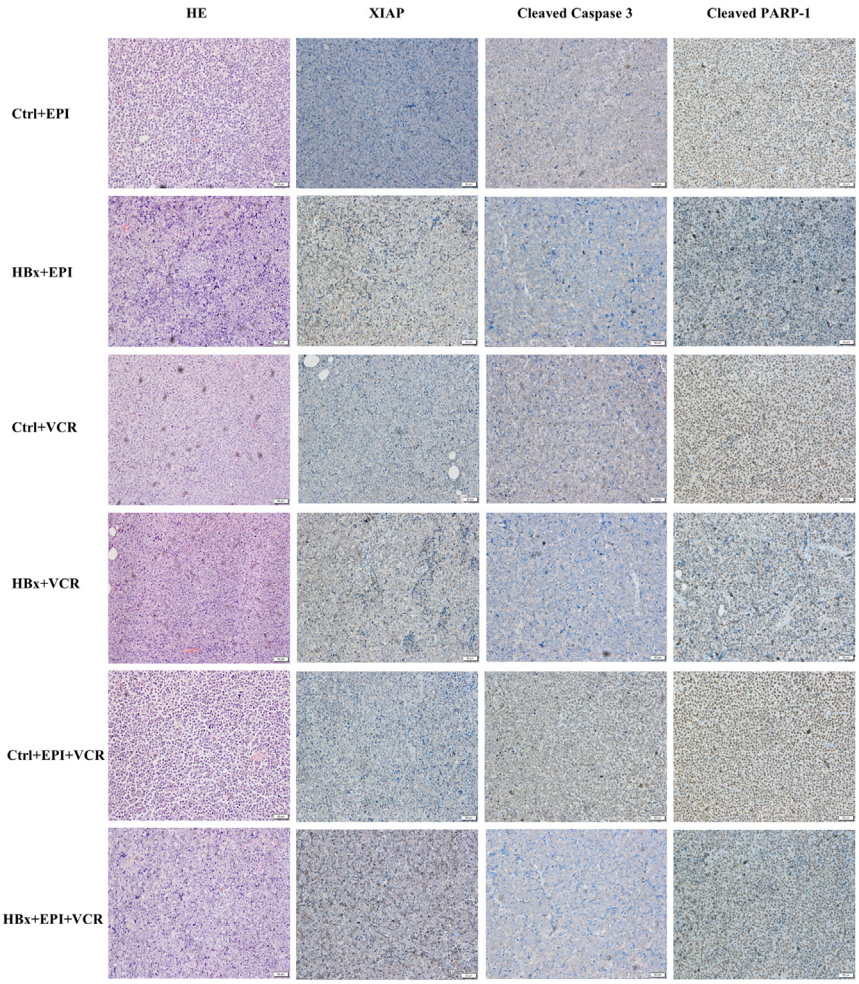


Figure 5. H&E staining and immunohistochemical staining of tumor tissues in the chemotherapy group detecting the expression of XIAP, cleaved caspase-3, and cleaved PARP-1

association between HBx-induced XIAP upregulation and NF-κB p65 nuclear translocation.

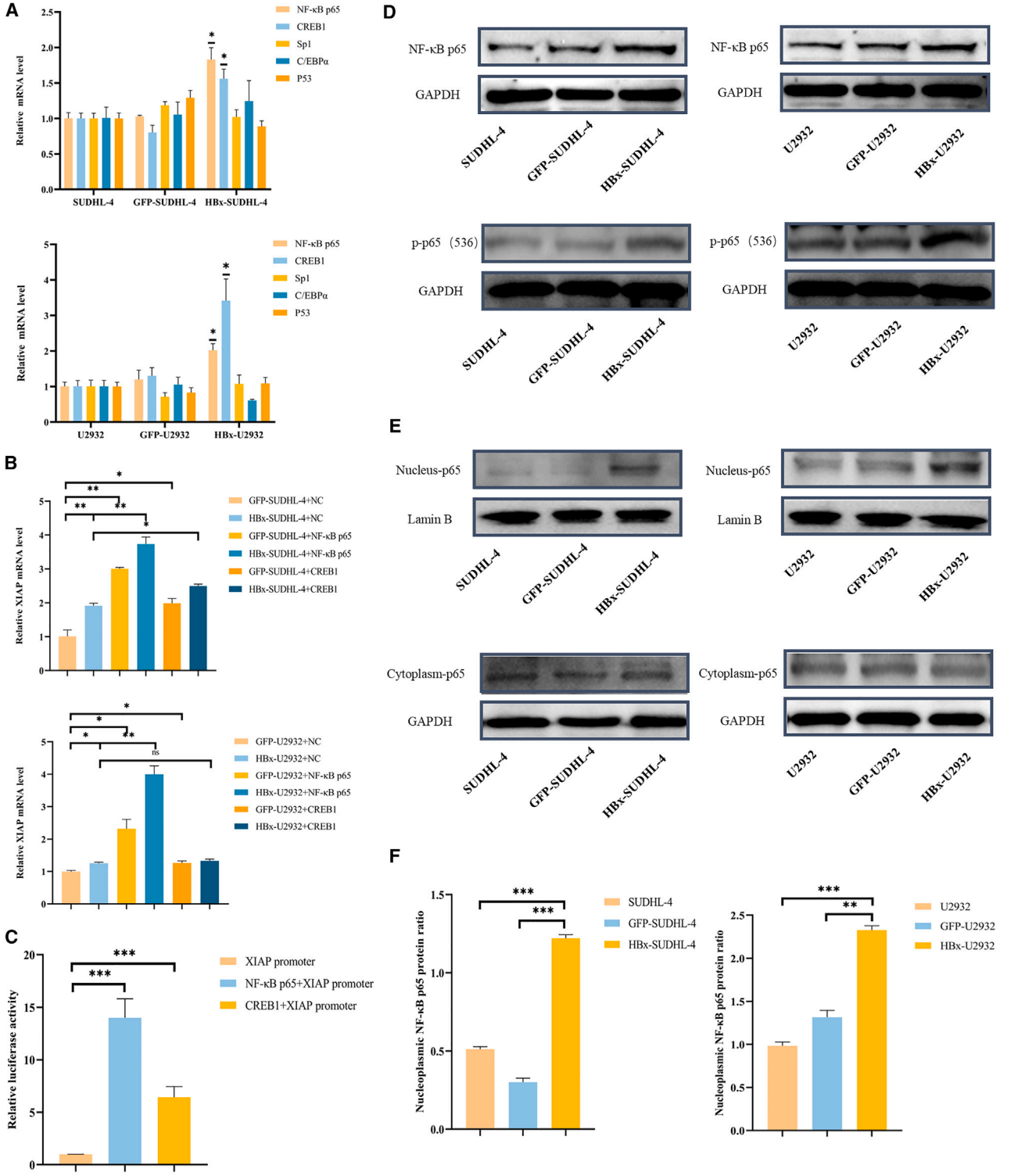
DISCUSSION

Several epidemiologic studies have established a strong association between HBV infection and aggressive B cell lymphomas, particularly DLBCL.[6,32,33] DLBCL patients with HBV infection exhibit suboptimal responses to initial standard therapy and unfavorable prognosis.[34,35] Whether in the era of conventional chemotherapy or immunotherapy, HBV infection remains an independent risk factor for prognosis in DLBCL patients.[7,36,37] Despite extensive investigation into the interaction between HBV infection and DLBCL, the precise mechanisms by which HBV infection induces resistance to first-line chemotherapy and poor prognosis are still not fully elucidated.

Previous experimental studies have shown that HBx, a functional protein encoded by the X gene of HBV, can contribute to reducing the sensitivity of GCB subtype DLBCL to second-line chemotherapeutic agents (MTX, Ara-C). Both MTX- and Ara-C-induced DNA damage significantly activate the CHK2 (a critical DNA damage-response protein) pathway, leading to cell-cycle arrest in the S phase and growth inhibition. HBx specifically inhibits CHK2 phosphorylation, thereby inducing resistance in DLBCL cells to S phase arrest drugs such as MTX and Ara-C.[38] Subsequent investigations conducted by the same research team uncovered that HBx directly upregulates the expression of lncNBAT1, which interacts with signal transducer and activator of transcription 1 and prevents it from enriching in the promoter region of the functional target gene apolipoprotein B mRNA editing enzyme catalytic subunit 3A (APOBEC3A), inhibiting APOBEC3A expression and inducing DLBCL to produce resistance to MTX and Ara-C.[39]

In hepatocellular carcinoma cells with persistent HBV infection, HBx protein is expressed in varying degrees, and it facilitates the transformation of normal liver cells into cancer cells,[40] the proliferation of hepatocellular carcinoma cells,[41] and confers resistance to chemotherapy and targeted therapies.[18,20] Our previous research identified high expression of HBx protein in lymphoma tissues from DLBCL patients with concurrent HBV infection.[17] We hypothesize that there is a potential association between HBx and resistance to first-line chemotherapy in DLBCL. To investigate this, we constructed DLBCL cell lines (GCB and ABC subtypes) stably expressing HBx *in vitro* and found that the upregulation of HBx promotes resistance in DLBCL cells to EPI and VCR. *In vivo* studies further support these results, demonstrating a significant reduction in tumor burden in HBx-negative mice compared to HBx-positive mice when treated with single-agent EPI, single-agent VCR, or a combination of both. These experimental outcomes are consistent with clinical observations.

Moreover, we investigated the expression of multidrug resistance and apoptosis inhibitor molecules in different groups of DLBCL cells. Our findings revealed a significant upregulation of XIAP in HBx-positive DLBCL cells and murine tumor tissues. When XIAP was downregulated using siRNA in HBx-positive cells, the sensitivity to EPI and VCR in DLBCL cells was restored, and the apoptotic rate could be restored to the level observed in the control group, suggesting a potential correlation between the HBx-induced upregulation of XIAP and chemotherapy resistance in DLBCL (Figure 8). XIAP, a crucial member of the IAP family, uniquely binds to caspases-3, -7, and -9, effectively inhibiting apoptosis and acting as a pivotal factor in



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impeding the cellular apoptosis process.[42,43] Other members of the IAP family, such as Survivin, also rely on XIAP to exert their effects. The Survivin-XIAP complex enhances the stability of XIAP, preventing its degradation through the ubiquitination and proteasome pathway, ultimately inhibiting caspase activation and contributing to anti-apoptotic mechanisms.[44] Elevated levels of XIAP can hinder intrinsic apoptosis mediated by caspase-3 and enhance the threshold of chemotherapy-induced apoptosis activation. By suppressing XIAP expression, the apoptotic threshold can be reduced, allowing chemotherapy agents to effectively induce cell apoptosis and partially overcome lymphoma chemoresistance.[14] Cillessen et al. found that small-molecule XIAP antagonists were equally effective in both chemotherapy-resistant and chemotherapy-sensitive samples of DLBCL.[45] Sensitivity to XIAP antagonists is characterized by high expression of XIAP and low expression of Bcl-2.[45] These findings indicate that incorporating XIAP expression into the current prognostic stratification system in clinical practice may enhance the accuracy of predicting and identifying chemotherapy-resistant patients who could benefit from XIAP antagonists. For this subgroup of DLBCL, XIAP antagonists in combination with chemotherapy could be a promising therapeutic strategy. However, research on drugs targeting XIAP and other IAP family members is predominantly in the early stages of preclinical and clinical development.

HBx may not possess the structural domains required for DNA binding, but it is capable of binding to specific transcription factors that regulate the expression of relevant target genes.[46] Common cellular transcription factors such as CREB and NF-κB are activated and translocate to the nucleus to exert *trans*-activating effects. We validate that NF-κB p65 promotes XIAP transcriptional expression through the dual luciferase reporter gene assay. Additionally, we analyzed the impact of HBx on NF-κB protein expression, phosphorylation modification, and nuclear localization. The results showed that HBx upregulates NF-κB p65 expression in DLBCL, induces it to undergo post-protein modification (phosphorylation), and promotes translocation of NF-κB p65 from the cytoplasm to the nucleus. Chen et al. demonstrated that HBx facilitates the translocation of NF-κB p65 from the cytoplasm to the nucleus and directly binds to the promoter of NF-κB p65 in hepatocytes, leading to its transcriptional activation.[28] Another study observed that HBx promoted phosphorylation of NF-κB p65 in HK-2 cells in a time- and concentration-dependent manner.[47] It has also been suggested that tumor necrosis factor receptor 1 (TNFR1) is an essential pathway for HBx-mediated NF-κB activation.[48] The precise mechanism by which HBx induces NF-κB p65 phosphorylation and nuclear translocation remains to be fully elucidated. We chose the NF-κB p65 inhibitor BAY 11–7082 at varying concentrations to treat the HBx-positive DLBCL cells, the levels of XIAP mRNA and protein decreased. Several previous studies have shown the antitumor effect of BAY 11–7082 in xenograft models without significant toxic effects, including prostate cancer, gastric cancer, lung cancer, esophageal cancer, and colorectal cancer.[49] It is also worth noting that many studies focus on the synergistic effects of BAY 11–7082 combined with other therapy approaches. Furthermore, the limited availability of clinical samples in this study precluded a thorough investigation of the relationship between XIAP expression levels and the prognosis of DLBCL patients with HBV infection. Future research endeavors will involve collaborative efforts with multiple medical centers to address this knowledge gap.

MATERIALS AND METHODS

Cell culture and chemotherapeutic drugs

Human DLBCL cell line SUDHL-4 (GCB subtype) and U2932 cell line (ABC subtype) were purchased from Nanjing Kobai Biological Company. Cells were cultured in RPMI-1640 medium (BioChannel Biological Technology) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO_2 in a cell culture incubator. The following chemotherapeutic drugs were used: EPI (Pfizer) and VCR (HETERO).

Lentiviral transfection and plasmid transfection

DLBCL cells were infected with lentivirally packaged Lenti-HBX-GFP-Puro (Bard) and subsequently selected with puromycin at a concentration of 2 μg/mL. CREB1 and RELA (NF-κB p65) vector plasmids were constructed by Keeneco Biologicals, with plasmid details provided in Figure S2B. Plasmid transfection was conducted according to the protocol supplied with DNA-Hieff Trans liposomal nucleic acid transfection reagent.

siRNA

The siRNA sequences in this study were as follows: negative control siRNA (anti-sense: ACGUGACACGUUCGGAGAATT, sense: UUCUCCGAACGUGUCACGUTT), XIAP-specific siRNA (anti-sense: UUACUUUAUCACCUUCACCUA, sense: GGUGAAGGUGAUAAAGUAAAG).

CCK-8

The cells were inoculated in 96-well plates at a density of 5×10^5/mL (SUDHL-4 cell) or 4×10^5/mL (U2932 cell) and treated with different concentrations of EPI or VCR for 48 h. The cells were then treated with CCK-8 reagent (Biosharp), with 10 μL per well for 2 h. The optical density at 450 nm of the medium was measured with a microplate reader. The IC_{50} values were calculated using GraphPad Prism 8.0 software.

Figure 6. HBx-mediated NF-κB hyperactivation upregulates XIAP *in vitro*

(A) Relative expression of transcription factors NF-κB p65, CREB1, Sp1, C/EBPα, and P53 mRNAs in each group of DLBCL cells. (B) Effect of transfection of NF-κB p65 and CREB1 plasmids on the expression of XIAP. (C) Dual-luciferase reporter gene assay for the impact of transcription factors NF-κB p65 and CREB1 on XIAP promoter activity. (D and E) Western blotting to detect total NF-κB p65, phosphorylated NF-κB p65 (D), and the nuclear and cytoplasmic NF-κB p65 protein (E). (F) Semiquantitative analysis of the nuclear-to-cytoplasmic ratio of NF-κB p65 protein. The results were determined in triplicate, and the error bars represent the mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$.

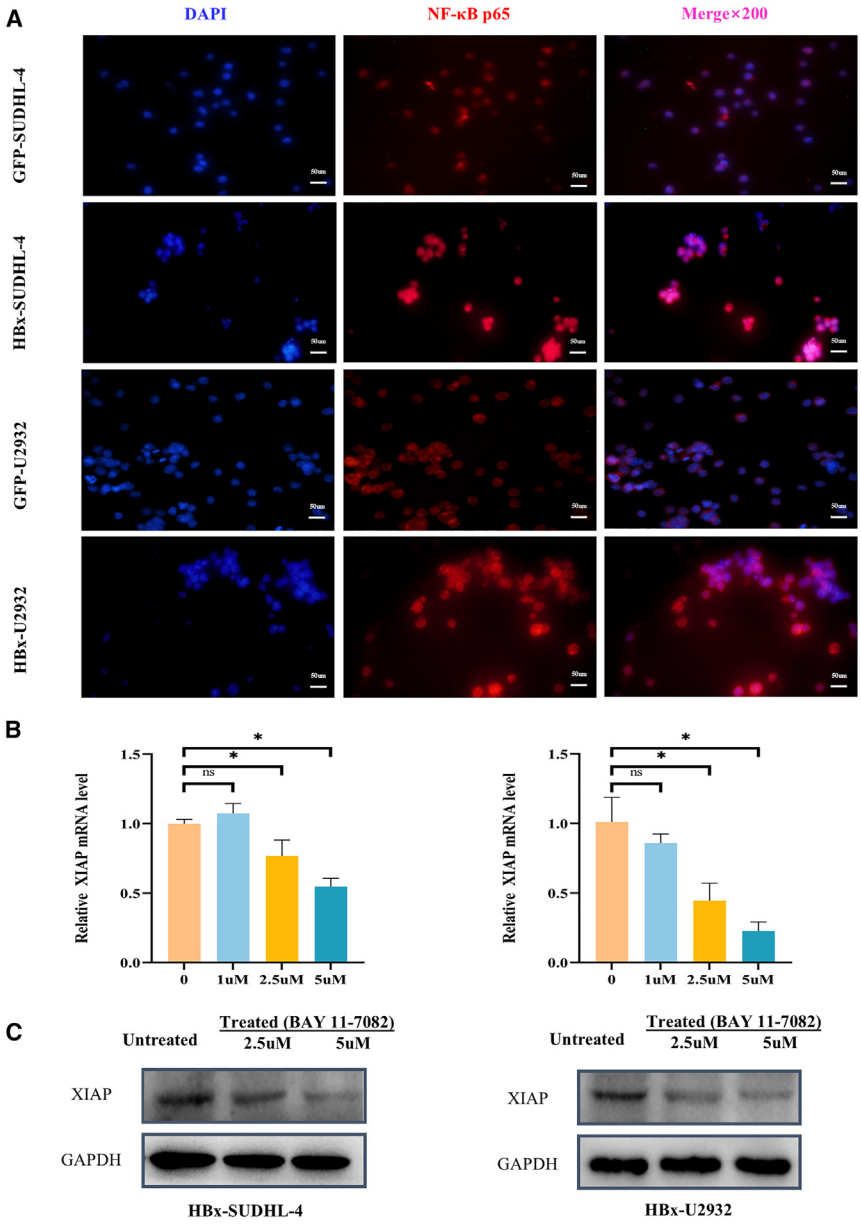

Figure 7. HBx-induced XIAP upregulation is closely related to NF-κB p65 nuclear translocation

(A) Immunofluorescence staining to observe the expression and location of NF-κB p65 proteins in each group of DLBCL cells (200×). (B and C) Effect of different concentrations of BAY 11–7082 on XIAP mRNA and protein in HBx-SUDHL-4 and HBx-U2932 cells, respectively.

TOLOBIO) with an ABI 7300 instrument. The primer sequences used in this study are shown in Table S2.

Western blotting and IHC

Western blotting and IHC were performed according to standard procedures.[50] The following antibodies were used: anti-HBX (bs-2147R, Bioss), XIAP (bs-1281R, Bioss), cleaved caspase-3 (341034, Zenbio), cleaved PARP-1 (380374, Zenbio), glyceraldehyde 3-phosphate dehydrogenase (bs-2188R, Bioss), NF-κB p65 (FNabo06089, FineTest), p65(Ser536) (HY-P80839, MCE), CD20 (no. 48750, CST), and PAX5 (no. 12709, CST).

Tumor xenografts

Five-week-old male SCID Beige mice were purchased from Beijing Vital River Laboratory Animal Technology. Control and HBx-overexpression DLBCL cells (1×10^7 each) in the logarithmic growth phase, suspended in 160 μL of mixed liquid (cell suspension and matrix gel mixed at a ratio of 1:1), were planted subcutaneously on the right dorsal side of mice. Daily observations included monitoring mouse weight and tumor progression. The mice were monitored to assess the tumor volume using the formula 1/2 (length × width2). Single-agent EPI (5.5 mg/kg) or single-agent VCR (0.15 mg/kg) or a combination of the two drugs was injected intraperitoneally when the tumor volume exceeded 400 mm^3. The mice were euthanized on day 30 post-injection. The tumor tissues were removed and weighed to calculate the tumor growth inhibition rate using the formula (1-average tumor weight of the treatment group/average tumor weight of the control group) × 100%. All experiments were approved by the Institutional Animal Care and Use Committee, Jilin University.

Apoptosis

The cells were inoculated in 24-well plates at a density of 5×10^5/mL (SUDHL-4 cell) or 4×10^5/mL (U2932 cell). Each well was treated with EPI or VCR at a final concentration of IC_{50} for 48 h. The apoptosis of DLBCL cells was detected by annexin V-APC/7-AAD method, and FlowJo software was applied for data analysis.

RT-qPCR

Total RNA was extracted from cells using RNAeasyTM (R0026, Beyotime). First-strand cDNA synthesis was performed using the ToloScript ALL-in-one RT kit (no. 22107, TOLOBIO). The qPCR analysis was performed using 2×Q3 SYBR Master Mix (no. 22204,

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.0 software, with data presented as means ± SDs. The independent-samples t test was utilized to assess group differences. Statistical significance was set as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

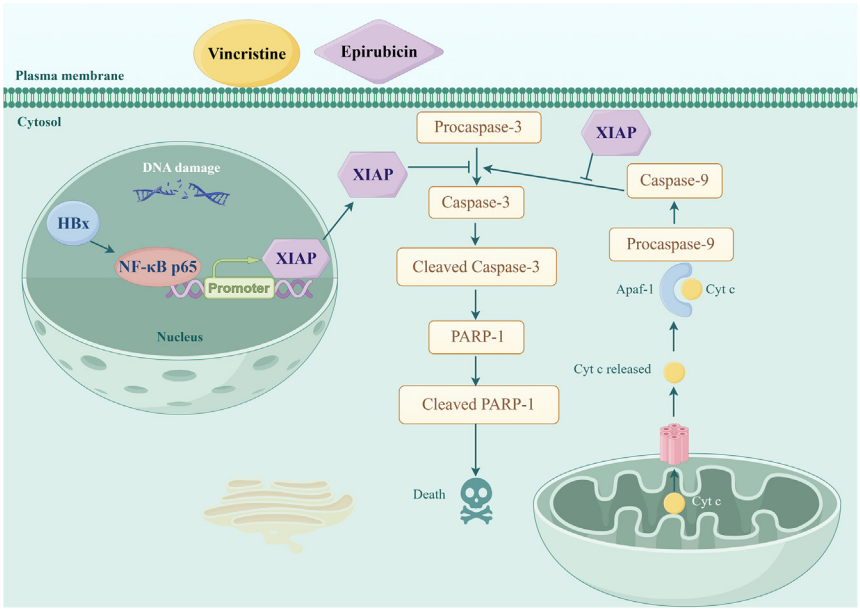

Figure 8. HBx induces chemoresistance in DLBCL by inhibiting intrinsic apoptosis via the NF-κB/XIAP pathway

DATA AND CODE AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the article and its supplemental information files or from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Z.Z., W.Y., and O.B. conceived the study. Z.Z. performed the experiments. Z.Z. and W.Y. wrote and revised the manuscript. W.G., X.W., J.L., Y.Z., B.W., and X.L. provided experimental support. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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