Epirubicin directly upregulated HBV replication in vitro in a concentration-dependent manner. Exposure to epirubicin for 24 h induced >11- and 6-fold increases in the levels of intracellular and secreted HBV DNA, respectively. In concordance with the elevated levels of HBV DNA, the expression levels of HBV pregenomic RNA, intracellular HBV surface and HBV core antigens, and secreted HBV e antigen were significantly increased by treatment with 0.5 µM epirubicin. Notably, epirubicin promoted cellular excretion of HBV nucleocapsids, which are closely associated with the pathological effects of HBV, including acute liver failure. In conclusion, epirubicin exhibited a direct stimulatory effect on HBV replication and this may be a novel mechanism of HBV reactivation following cytotoxic anticancer chemotherapy.
Collection, Rockefeller, MD, USA) were transfected with pneo-CH9/HBV1.1 (Center for Molecular Biology, Heidelberg University, Heidelberg, Germany; recipient, Dr Lanlin, Southwest Hospital affiliated to Third Military Medical University, Chongqing, China) (7), which contains the cytomegalovirus promoter and 1.1 copies of the HBV genome, and then selected with G418 antibiotic (1,200 µg/ml; Merck and Co., Inc., Rahway, NJ, USA). Isolated cell colonies with the HBV genome integrated were selected subsequent to confirmation by Southern blotting and western blot analysis.

Cell culture and treatment. HepG2, HepG2-HBV1.1 and HepG2.2.15 (Xiangya School of Medicine, Central South University, Changsha, China) cells were cultured in minimum essential medium (MEM; Gibco-BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Hyclone, Shanghai, China) and maintained in a humidified incubator with 5% CO₂ at 37°C. For cytotoxic chemotherapy treatment, the cells were incubated with epirubicin (0-1 µM, Hisun Chemical Co., Ltd., Zhejiang, China) for 24 h. The medium was then replaced with fresh serum-free MEM medium for a further 48 h incubation and the cells were then harvested.

Effects of epirubicin on the viability of HepG2.2.15 and HepG2-HBV1.1 cells by trypsin blue exclusion assay. HepG2.2.15 (4x10⁵ cells/well) and HepG2-HBV1.1 (6x10⁵ cells/well) cells were seeded in six-well plates. Following adherence of the cells, epirubicin was added to the medium at various concentrations (0-1 µM) for 24 h, followed by 48 h incubation in a drug-free culture medium. The cells in each well were washed with phosphate-buffered saline twice following the addition of 200 µl trypsin, and then placed in a 37°C 5% CO₂ incubator for 1-2 min. Subsequently 800 µl 10% FBS MEM was added to each well to terminate the reaction, and the cell suspension by pipetting several times to disperse the cells evenly and count the cell number. The viability of cells = live cells / total cells.

MTT assay. HepG2.2.15 (1x10⁵ cells/well) and HepG2-HBV1.1 (1x10⁵ cells/well) cells were inoculated in 96-well plates. Following adherence of the cells, epirubicin was added to the medium at various concentrations (0-1 µM) for 24 h, and then 20 µl 0.5% MTT solution was added to each well for 4 h. Subsequently, the medium in each well was discarded and 150 µl DMSO was added. The plate was agitated for 10 min in order to dissolve the crystals and the absorbance of each well was measured at an optical density of 490 nm.

Quantification of HBV DNA copies by fluorescent quantitative polymerase chain reaction (qPCR). HBV replicative intermediates in the cells were obtained as follows. Cells from one 35-mm diameter dish were lysed with 0.5 ml lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% NP-40 and 2% sucrose at 37°C for 10 min. The cell debris and nuclei were removed by centrifugation at 13,000 x g and the supernatant was mixed with 200 µl 35% polyethylene glycol (PEG) 8000 (Beyotime Institute of Biotechnology, Shanghai, China) containing 1.5 M NaCl. Subsequent to incubation on ice for 2 h, the viral nucleocapsids were pelleted by centrifugation at 12,000 xg for 10 min at 4°C, followed by 24 h digestion at 37°C in 400 µl digestion buffer containing 0.5 mg/ml pronase (Takara Bio, Inc., Shiga, Japan), 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl (pH 8.0) and 10 mM EDTA. The digestion mixture was extracted twice with phenol, and the DNA was precipitated with ethanol and dissolved in Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer. In order to collect viral particles instead of free viral DNA in the culture medium, the supernatant was subjected to 35% PEG 8000 precipitation overnight according to the methods of a previous study (8) and then the precipitates were digested as previously described.

The quantification of HBV copies was performed by SYBR-Green assays using FastStart Universal SYBR-Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany). Primers for amplification of the HBV DNA fragments were designed specifically for the conserved region of the HBV gene by Huada Gene Sci-Tech Company (Shenzhen, China) and the sequences were as follows: Forward primer (F2150), 5'-CTTATTGTGATGTTATGTCAAC-3'; and reverse primer (R2300), 5'-TCTATAAGCTGGAGGAGTGCCAGGA-3'. The plasmid pneo-CH9/HBV1.1 at different concentrations (5x10⁵, 5x10⁶, 5x10⁷, 5x10⁸, 5x10⁹ copies/µl) served as a template to make the standard curve.

Quantification of HBV pregenomic (pg) RNA by fluorescent qPCR. Total RNA was extracted by a DNA-free RNA Mini Extraction kit (Watson, Shanghai, China) and 1 µg total RNA was used for cDNA synthesis, which was conducted by reverse transcription using the PrimeScript RT reagent kit (Perfect Real Time; Takara Bio, Inc.). Relative quantification of the target genes (HBV 3.5 kb mRNA) was performed using SYBR Green assays, with β-actin mRNA as an endogenous control. The expression values of the target genes were calculated using the 2⁻ΔΔCt method.

Southern blot analysis. HBV replicative intermediates were extracted from the cells or the supernatant of the culture medium according to the methods previously described and then separated on 0.8% agarose gels. The DNA samples were transferred to nylon membranes (Roche Diagnostics GmbH). Subsequent to ultraviolet crosslinking and prehybridization, the membranes were hybridized with a digoxigenin-labeled HBV-specific probe using a Random-Primed DNA Labeling kit (Roche Diagnostics GmbH). The signal was detected by exposure on an X-ray film and scanning using the Versa Doc Imaging system (Bio-Rad, Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). The levels of HBsAg and HBV e antigen (HBeAg) in the culture medium and cell extracts were assessed by ELISA using Antibody to Hepatitis B Surface Antigen and Antibody to Hepatitis B Virus E Antigen ELISA kits, according to the manufacturer's instructions (Kehua Biotech Co, Ltd, Shanghai, China). The levels of HBV core antigen (HBeAg) in the culture medium were assessed using an ELISA kit (Disease Diagnosis Reagent and Vaccine Engineering Technology Research Center of China Infectious State, Xiamen University, Xiamen, China). This kit contained two types of 96-well plate. One was coated with HBsAb, to capture only the Dane particle. The other was
coated with HBcAb, therefore it detected the total HBcAg from the Dane particles and the HBV nucleocapsids. Each experiment was performed in triplicate and independently repeated three times.

**Western blot analysis of HBcAg expression.** Cellular proteins were extracted using radio immunoprecipitation buffer supplemented with phenylmethylsulfonyl fluoride. Protein concentrations were determined using a bicinchoninic acid assay protein concentration determination kit (Beyotime Institute of Biotechnology, Shanghai, China). Equal quantities of sample were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with monoclonal mouse anti-HBcAg (6D1D10E4; Huazhong University of Science and Technology, Wuhan, China; diluted 1:150) or monoclonal rabbit anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; diluted 1:2,500). Following incubation, the membranes were washed three times, and goat anti-rabbit or goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.; diluted 1:2,500) was added for 1 h. The membranes were washed three times with Tris-buffered saline and Tween 20 buffer, and signals were detected using the Enhanced Chemiluminescence Detection system (Pierce Biotechnology, Inc., Rockford, IL, USA).

**Statistical analysis.** The data are presented as the mean ± standard deviation of at least three independent experiments. Statistical significance was determined by Student's t-test. The differences between groups were assessed by one-way analysis of variance using the Bonferroni post hoc test and P≤0.05 was considered to indicate a statistically significant difference. All analyses were performed using the Statistical Package for the Social Sciences statistical software for Windows, version 10.1.4 (SPSS, Inc., Chicago, IL, USA).

**Results**

**Effect of epirubicin on the viability of stable HBV-expressing cell lines.** Epirubicin, as a cytotoxic chemotherapy agent, induces cell apoptosis or necrosis at high concentrations (8,9), which may be associated with HBV replication. The aim of the present study was to investigate whether epirubicin promotes HBV replication at lower concentrations. The cytotoxicity of epirubicin on HepG2.2.15 and HepG2-HBV1.1 cells was examined using trypan blue exclusion and MTT assays. From the results of the two assays (Fig. 1), 0.5 µM epirubicin was revealed to exhibit no significant cytotoxicity to either HepG2.2.15 or HepG2-HBV1.1 cells. Therefore, in subsequent experiments, 0.5 µM served as the working concentration of epirubicin.

**HBV DNA and pgRNA levels increase concentration-dependently in stable HBV-expressing cell lines following epirubicin treatment.** HepG2.2.15 (10) and HepG2-HBV1.1 (11) cells were employed as stable HBV-expressing cell lines. HepG2.2.15 (4x10^5 cells/well) and HepG2-HBV1.1 (6x10^5 cells/well) cells were treated with epirubicin at various concentrations (0-0.5 µM) for 24 h, followed by 48 h incubation in drug-free culture medium. Intracellular HBV nucleocapsids were extracted at 72 h after drug treatment, and viral replication intermediates were subsequently measured by fluorescent qPCR and Southern blot analysis. The number of HBV DNA copies was concentration-dependently upregulated upon epirubicin stimulation (Fig. 2A). The number of intracellular HBV DNA copies increased 6.35±0.49- and 11.26±1.31-fold in the HepG2.2.15 and HepG2-HBV1.1 cells, respectively, with 0.5 µM epirubicin treatment. Similarly, the concentration-dependent effect of epirubicin on HBV replication in the cells was confirmed by Southern blot analysis (Fig. 2B). The HBV DNA was extracted from the supernatant of the culture medium following precipitation by PEG 8000 and analyzed using qPCR (Fig. 2C) and Southern blotting (Fig. 2D). Epirubicin upregulated the levels of HBV DNA in the supernatant in a concentration-dependent manner. Additionally, 0.5 µM epirubicin increased the secretion of HBV DNA copies by 6.30±0.57- and 6.31±1.17-fold in the HepG2.2.15 and HepG2-HBV1.1 cells, respectively. To further determine the effects of epirubicin on the levels of HBV RNA, the levels of HBV pgRNA were measured by qPCR using primers targeting the 3.5 kb mRNA of the viral genome. The levels of HBV pgRNA increased markedly with epirubicin treatment (Fig. 3).

![Figure 1. Effects of epirubicin on the viability of stable HBV-expressing cell lines.](image-url)
Epirubicin promotes HBV protein expression. To elucidate the effects of epirubicin on the HBV proteins expressed, the levels of HBeAg, HBcAg and HBsAg were detected by ELISA and western blot analysis. With 0.5 µM epirubicin treatment, the levels of HBeAg in the supernatant were upregulated, as detected by ELISA (Fig. 4A), and the levels of HBcAg expression in the cytoplasm increased markedly, as detected by western blot analysis (Fig. 4B). Notably, epirubicin increased the levels of HBsAg expression in the cytoplasm (Fig. 4C), while the levels of HBsAg secretion were not markedly increased (Fig. 4D).

Epirubicin promotes the excretion of HBV nucleocapsids in stable HBV-expressing cells. To investigate why the levels of HBsAg secretion did not increase with epirubicin treatment, the HBcAg levels in the supernatant were detected by ELISA. As the HBV virus cannot secrete free HBcAg into the culture medium, HBcAg in the supernatant can only be from HBV Dane particles or HBV nucleocapsids. Therefore, the HBcAg in the supernatant represents the levels of HBV Dane particles or nucleocapsids. However, no significant change in the levels of Dane particles in the supernatant was identified following treatment with epirubicin, although the overall levels of HBcAg from Dane particles and nucleocapsids in the supernatant markedly increased following 0.5 µM epirubicin.
treatment (Fig. 5). This result suggests that epirubicin induced HepG2-HBV1.1 cells to excrete HBV nucleocapsids instead of HBV Dane particles.

**Discussion**

HBV reactivation is an emerging clinical challenge in HBV carriers receiving anticancer chemotherapy. All types of anticancer drugs used in chemotherapy have been associated with HBV reactivation, including the classic cytostatics, monoclonal antibodies and steroids (12). The rate of HBV reactivation is highest in patients with breast cancer (41-56%; 2,13) and lymphoma (24-67%; 14,15). This high incidence may be explained in part by the intensive chemotherapy required to treat the diseases.

Epirubicin is one of the most commonly used drugs to treat breast cancer and lymphoma, particularly in patients who have undergone surgery to remove the tumor. Similar to other cytotoxic drugs, epirubicin exhibits immunosuppressive activity, which is considered to be the main mechanism of HBV reactivation. However, there may be other mechanisms responsible for the HBV reactivation caused by cytotoxic anticancer drugs in addition to immunosuppression.

In the present study, the effects of epirubicin on HBV replication in HepG2.2.15 and HepG2-HBV1.1 cells were observed. The number of HBV DNA copies was concentration-dependently increased by epirubicin in the cytoplasm and the supernatant of the culture medium. Furthermore, the levels of intracellular HBsAg and HBcAg, and secreted HBeAg also increased following epirubicin treatment. However, epirubicin did not markedly affect the levels of HBsAg secretion. This
result signified that the abundant secretion of HBV DNA copies stimulated by epirubicin was not extracted from HBV Dane particles, as HBsAg is the main component of the outer layer of the particles. By detection of the levels of HBcAg in the supernatant of the cell culture, it was demonstrated that epirubicin promoted the cells to excrete HBV nucleocapsids instead of Dane particles. This corresponds with the lack of significant change in the levels of HBsAg secretion following epirubicin treatment. These results indicate that epirubicin increased the levels of HBV DNA and RNA production, and protein expression but did not affect the levels of secreted virus particles. Similar to this phenomenon, corticosteroids have been demonstrated to increase the total intracellular levels of HBV DNA, RNA and HBsAg without affecting the levels of secreted HBsAg in previous studies (16,17).

HBV nucleocapsids are closely associated with HBV pathogenesis. The outer layer of HBV nucleocapsids consists of HBcAg, which is the main factor responsible for HBV-associated acute liver failure (ALF). Studies have suggested that HBcAg is able to directly activate B cells to produce specific antibodies (IgG1 and IgM anti-HBc), without the aid of T lymphocytes. This implicates the importance of B cell immunity in the pathogenesis of HBV-associated ALF (18,19). In the present study, epirubicin stimulated stable HBV-expressing cells to excrete HBV nucleocapsids, which may be the principle cause of the severe liver damage induced by cytotoxic anticancer drugs.

It has been confirmed that the recovering immune system following withdrawal of cytotoxic drugs plays a role in liver damage. However, the mechanisms for the marked increase in the levels of HBV replication in the early stages of anticancer treatment remain unclear. In the current study, epirubicin increased the levels of HBV DNA, RNA and protein expression, and directly promoted HBV nucleocapsid secretion under cytotoxic stress. This may be a novel mechanism of HBV reactivation in HBV carriers receiving anticancer chemotherapy. One possible link between cytotoxic stresses and activation of HBV replication is that cell cycle arrest may be induced by cytotoxic drugs (20-22). The present study also demonstrated that the cell cycle was inhibited at the G2/M-phase by epirubicin (data not shown). Further studies are required to confirm these hypotheses.

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