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Hepatitis B Virus X protein elevates Parkin-mediated mitophagy through Lon Peptidase in starvation

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ABSTRACT

Hepatocellular Carcinoma (HCC) is the fifth most prevalent cancer worldwide. Specially, Hepatitis B viurs X protein (HBx) is a leading factor in the progression of Hepatitis B viurs-related HCC. Nutrient-deprived tumor microenvironment also contributes to tumor development. However, the role of HBx in nutrient-deprived HCC has received little investigation. Here, we show that HBx elevates PINK1-Parkin mediating mitophagy in starvation. HBx not only increases the PINK1/Parkin gene expression but also accelerates Parkin recruitment to partial mitochondria. Further analysis indicates that, HBx either promotes mitochondrial unfolded protein response, with remarkable mitochondrial LONP1 increases, or reduces LONP1 expression in cytosol inducing LONP1-Parkin pathway, both consequently enhancing mitophagy. Moreover, the enhanced mitophagy lowers mitochondrial apoptosis in starved hepatoma cells, and Bax is implied in the machinery. In addition, we define differential centrifuge, 3000 *g* or 12,000 *g* to pellet mitochondria, as an effective method to obtain distinct mitochondria. In collect, HBx regulates diverse aspects of LONP1 and Parkin, enhancing mitophagy in starvation. This study may shed new insights into the machinery development of hepatocellular carcinoma.

1. Introduction

Hepatocellular Carcinoma (HCC) is the fifth most common cancer in the word and the cases are rising [1]. More over 50% of HCC is related to Hepatitis B virus (HBV) infection [2]. Specially, HBV-encoded oncoprotein Hepatitis B Virus X protein (HBx) is involved in HCC with various mechanisms including proteins interaction, modulating signal transductions, affecting cell cycles and apoptosis [3–5]. HBx is located either within nucleus acting as a trans-activator, or within mitochondria, affecting mitochondrial functions [6,7].

Nutrient deprivation is a common phenomenon during tumor development [8]. And, it is reported that ^{18}F-FDG PET, which takes advantage of the glucose analog, has a high average false-negative rate of 40–50% in the detection HCC due to the starvation in liver tumors [9]. The certain tumor microenvironment of nutrient deprivation contributes to tumor development [10]. The role of HBx in nutrient-deprived tumor microenvironment is not well known. HBx reduces starvation-induced cell death by inhibiting mitochondrial apoptosis pathway [11,12]. And elevated autophagy by HBx is implied during the process [13]. Notably, mitophagy, which is to selectively degrade mitochondria by autophagy, also decreases mitochondrial apoptosis. Given that HBx is distributed in mitochondria, it's reasonably to hypothesize that HBx may have a role in mitophagy during starvation. However, it receives little investigation.

Of note, HBx elevates PTEN induced Putative Kinase 1 (PINK1)-Parkin mediating mitophagy through the lowered mitochondrial potential, in nutritional sufficiency environment [14]. Accordingly, there are two stimulators for mitophagy, one is the loss of mitochondrial potential [15], the other is the induction of mitochondrial unfolded-protein response (UPR^{mt}), a transcriptional response during mitochondrial dysfunction or the accumulation of unfolded proteins within mitochondria to promote cell survival along with the repair and recovery of defective mitochondria [16].

The increased Lon Peptidase 1 (LONP1), an enzyme located in the mitochondria, mediating the degradation of misfolded polypeptides in mitochondrial matrix, is a marker of UPR^{mt} [17]. On the other hand,

Abbreviations: HCC, Hepatocellular Carcinoma; HBV, Hepatitis B virus; HBx, Hepatitis B Virus X protein; PINK1, PTEN Induced Putative Kinase 1; UPR^{mt}, Mitochondrial Unfolded-Protein Response; LONP1, Lon Peptidase 1; EBSS, Earle's balance salt solution; LC3, microtubule-associated protein 1 light chain 3B; 3-MA, 3-Methyladenine

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the lowered LONP1 resulting in the accumulation of unfolded proteins in mitochondrial matrix, stimulates the UPR^{mt}. And it's reported that the downregulation of LONP1 contributes to the induction of PINK1/Parkin-mediated mitophagy [18]. So, the relationship between LONP1 and tumor is intricate, either upregulated or reduced in variant tumors [18]. Additionally, the status of LONP1 in HCC remains unknown.

Our study aimed to study the role of HBx in mitophagy within malignant liver cells in starvation. We found that HBx elevated mitophagy in starvation via upregulating PINK1 and Parkin expression and promoting the translocation of Parkin to partial mitochondria. Furthermore, by immunofluorescence and mitochondrial isolation, we demonstrated that HBx modulated LONP1 in two ways to promote mitophagy. Firstly, HBx elevated mitochondrial LONP1 to enhance UPR^{mt}. Secondly, HBx decreased the cytosolic LONP1 leading to the upregulation of PINK1 and Parkin amounts. Finally, we verified HBx attenuated apoptosis in hungered malignant liver cells through activating mitophagy. Additionally, as for the methods, we demonstrated that using two kinds of differential centrifuge, 3000 *g* or 12,000 *g*, to pellet mitochondria, was able to obtain various mitochondria. And with the method, we illustrated that HBx induced Parkin only to partial mitochondria, indicating that not all mitochondria were involved in mitophagy.

2. Material and methods

2.1. Cell culture and plasmids

HepG2 cells and HepG2.2.15 cells, both kindly given by Professor Xu Lin (Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Fujian Medical University, China), SMMC-7721 cells, provided by Cell Bank (Chinese Academy of Sciences), and 293 A cells, a gift from Professor Yan-ling Chen (Fujian Institute of Hepatobiliary Surgery, Fujian Medical University, China), were respectively maintained in high-glucose DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), especially HepG2.2.15 were cultured with 0.38 μg/ml G418. For nutrient deprivation, cells were removed cultured media and washed with PBS for 3 times, then incubated with EBSS for 3 h. To inhibit mitophagy, HepG2 cells and HepG2.2.15 were treated with 10 mM 3-MA (Sigma, #M9281) for 8 h, and SMMC-7721 were treated with 2 mM 3-MA for 24 h.

HBx-expressing plasmids pcDNA3.1-flag-HBx (adw subtype) and pcDNA3.1-vector, as well as HBx-expressing adenovirus HBx-Ad (adw subtype) and vector-adenovirus, were all gifts from professor Xu Lin (Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Fujian Medical University, China). The shRNA plasmids targeted to LONP1 were bought from Genechem (China). Plasmids were extracted using EndoFree Plasmid Maxi Kit (QIAGEN, # 12362). Lipofectamin 3000 (Invitrogen, # L3000-008) was used for plasmids transfection. Adenoviruses were amplified with 293A cells. Considering the HepG2 cells had a low MOI with virus and low transfect efficiency with plasmids, meanwhile, the HBx- and vector-adenovirus emit green fluorescence with the expression of GFP, the HBx-expressing adenoviruses and plasmids were applied as follows: For whole cell lysate or subcellular cell lysate, HepG2 cells were infected with expression adenovirus, SMMC-7721 cells were transfected with expression plasmids; For immunofluorescence or apoptosis analysis with flow cytometry, both HepG2 cells and SMMC-7721cells were applied with expression plasmids.

2.2. Quantitative RT-PCR

The RNA was obtained with Trizol (Invitrogen, #15596026), followed by reverse PCR using cDNA synthesis kit (Thermo scientific, #K1622) according to the manufacturer's protocol. Then the quantitative PCR was conducted with SYBR Green (Roche, #17747200). The primers used were as follows, PINK1 forward 5'-ATCTGGTTCAACAGGGCATC-3', PINK1 reverse 5'-ACGTACCAGCTGCTGAAGG-3', Parkin forward 5'-CGCAACAAATAGTCGGAACA-3', Parkin reverse 5'-AAGGCAGGGAGTAGCCAAGT-3', LONP1 forward 5'-GAAGTTGGTTGAGCTGCTGAG-3', LONP1 reverse 5'-ATCTCATGGATCTGGGCAAA-3', ACTB forward 5'- CTCCATCCTGGCCTCGCTGT-3', β-actin reverse 5'-GCTGTCACCTTCACCGTTCC-3'. The relative gene expression levels were calculated with the method of $2^{-\Delta\Delta Ct}$ values.

2.3. Mitochondria isolation

Mitochondria isolation were performed according to the introductions of mitochondria isolation kit for cultured cells (Thermo scientific, #89874). Briefly, cells were firstly harvested with 0.25% EDTA (Ethylene Diamine Tetraacetic Acid) trypsin, then homogenized with Dounce Tissue Grinder. Following, the postnuclear fractions were obtained by centrifuging at 700 × *g* for 10 min. Notably, two kinds of centrifugation were applied to the postnuclear fractions to pellet mitochondria: for method one, postnuclear fractions were centrifuged at 12,000 × *g* for 15 min at 4 °C for method two, to obtain a more purified fraction of mitochondria, with > 50% reduction of lysosomal and peroxisomal contaminants according to the instructions, the postnuclear fractions were centrifuged at 3000 × *g* for 15 min. At last, the mitochondrial pellet was subjected to mitochondria lysis buffer (Beyotime, CAS#C3601-4, China), then processed with western blot analysis. Additionally, the postmitochondrial fractions were obtained by centrifuging the supernatant of mitochondrial pellet at 12,000 × *g* for 20 min.

2.4. Western blot

Whole-cell lysates were obtained with RIPA lysis buffer (Beyotime, # P0013B, China). Then, the protein was denatured with 5*SDS-PAGE loading buffer, and boiled at 95–100 °C for 5 min, followed by western blot. Briefly, the electrophoresis was performed with SDS-PAGE gels, then the transfer process was conducted with NC (nitrocellulose) membrane in wet condition at a constant 300 mA. After blocking with 5% non-fat milk solution and incubating with primary antibodies overnight at 4 °C, then incubated with secondary antibodies for 1 h at room temperature, the NC membrane was detected the signals detected with ECL and exposed to X-ray films. The antibodies used were as follows: Anti-flag antibody produced in mouse (sigma, # F1804), Anti-Hepatitis B Virus Antibody, core Antigen (Millipore, # MAB16988), Anti-LC3B (Sigma, #L7543), Anti-LONP1 antibody produced in rabbit (sigma, #HPA002192), Anti-PINK1 antibody (Abcam, #ab137361), Anti-Parkin antibody (Abcam, # ab15954), Anti-caspase-3 antibody (Abcam, #ab184787), Anti-Bax antibody (Cell signaling technology, #5023), Anti-Bcl-2 antibody (Beyotime, #AB026, China), Anti-β-actin antibody (ZSGB-BIO, # TA-09, China), Anti-Tom20 antibody (Cell signaling technology, #13929). The gray value of bands was evaluated by Image J software.

2.5. Immunofluorescence

Cells were grown on poly-L-lysine coated cover-lips inside a Petri dish. When reached to 50–60% confluency, cells were stained with 300 nM MitoTracker® Red CMXRos (Invitrogen, #M7512) for 30 min at 37 °C according to the instructions, then fixed with 4% parafomaldehyde for 15 min, permeabilized with 0.3% Triton X-100 for 15 min, and blocked with 10% goat or donkey serum in PBST for 1 h. Following, cells were immunostained with primary antibodies overnight at 4 °C, then subjected to secondary antibodies for 1 h at room temperature. The secondary antibodies used were as follows: Goat anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 647 conjugate (Invitrogen, #A-21245), Donkey anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor 488 conjugate (Invitrogen, A-21206). To the end, cells were observed with fluorescence confocal microscopy (Zeiss FM780). And