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Nitric oxide inhibits autophagy and promotes apoptosis in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related mortality worldwide. The expression of nitric oxide synthase (NOS) and the inhibition of autophagy have been linked to cancer cell death. However, the involvement of serum nitric oxide (NO), the expression of NOS and autophagy have not been investigated in HCC. In the present study, we first established that the NO level was significantly higher in hepatitis B virus-related HCC than in the liver cirrhosis control (53.60 ± 19.74 vs 8.09 ± 4.17 μmol/L, $t = 15.13$, $P < 0.0001$). Using immunohistochemistry, we found that the source of NO was at least partially attributed to the expression of inducible NOS and endothelial NOS but not neuronal NOS in the liver tissue. Furthermore, in human liver cancer cells, NO-induced apoptosis and inhibited autophagy. Pharmacological inhibition of autophagy also induced apoptosis, whereas the induction of autophagy could ameliorate NO-induced apoptosis. We also found that NO regulates the switch between apoptosis and autophagy by disrupting the Beclin 1/Vps34 association and by increasing the Bcl-2/Beclin 1 interaction. Overall, the present findings suggest that increased NOS/NO promotes apoptosis through the inhibition of autophagy in liver cancer cells, which may provide a novel strategy for the treatment of HCC.

KEYWORDS
apoptosis, autophagy, hepatocellular carcinoma, nitric oxide, nitric oxide synthase

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and the second most common cause of cancer-related mortality worldwide. More than 600 000 people are newly diagnosed each year, and approximately the same number die annually. The incidence of HCC has increased in the USA and Western Europe over the past 25 years. HCC is difficult to treat because patients may be asymptomatic until the cancer has developed to an advanced stage. Although various treatment options are available, including surgical resection, radiation, ablation, chemotherapy, transplantation, sorafenib and combination therapy, the 5-year survival rate is less than 5%.[1] The precise molecular mechanisms responsible for the development of HCC have not yet been clarified. Therefore,

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TABLE 1 Demographic data and clinical characteristics of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) patients

Parameter	LC (n = 54)	HCC (n = 54)
Age (years)	48.3 ± 8.1	47.7 ± 7.7
Sex (M/F)	48/6	48/6
ALB (g/L)	33.6 ± 4.5	31.7 ± 3.8
TBIL (μmol/L)	21.3 ± 9.3	26.8 ± 10.7
γ-GT (U/L)	76.5 ± 34.2	177 ± 68.1
ALP (U/L)	67.3 ± 2.6	96.4 ± 38.6
ALT (U/L)	36 ± 30.23	52 ± 45.19
INR	1.3 ± 0.3	1.2 ± 0.3
PLT (10⁹/L)	58 ± 34	72 ± 41
AFP (μg/L)	5.3 ± 4.82	587 ± 1538.60
Child-Pugh score (A/B/C)	24/22/8	30/17/7

γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALB, albumin; ALP, alkaline phosphate; ALT, alanine aminotransferase; INR, international normalized ratio; PLT, platelet count; TBIL, total bilirubin.

searching for HCC-associated molecules may enable the development of effective strategies to improve the overall prognosis for patients with HCC.

Nitric oxide (NO) is a ubiquitous, short-lived physiological messenger that plays important roles in modulating tumor growth progression and carcinogenesis.[2] NO is synthesized by nitric oxide synthase (NOS) through a series of redox reactions involving L-arginine. Following its synthesis by NOS, the half-life of endogenous NO is extremely short, approximately 1 second. Thus, the endogenous production of NO is highly regulated by the activity of NOS. In mammals, three distinct genes encode the NOS isoenzyme, neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) genes. nNOS and eNOS are constitutively present in peripheral nerves and vascular endothelial cells, respectively, whereas iNOS is inducible, mainly in pathological conditions, by mesenchymal cells and parenchymal cells through the stimulation of endotoxins and cytokines. The expression of NOS has been detected in various cancers such as breast,[3] bladder,[4] stomach,[5] prostate,[6] lung,[7] colon,[8] pancreas[9] and renal cancers.[10] In liver cancer, the expression pattern of NOS remains unknown. One study investigated iNOS expression in cancer tissue and pair-matched non-tumor liver tissue and found lower iNOS expression in HCC.[11] However, in the aforementioned study, iNOS expression in HCC was not compared to that in cirrhosis patients without HCC, because most of the HCC cases developed in the background of cirrhosis. Moreover, eNOS and nNOS, which are also important for NO synthesis, were not tested in parallel. Therefore, the NOS expression and serum NO levels need to be further investigated in patients with HCC.

Nitric oxide modulates different cancer-related events including apoptosis, cell cycle progression, invasion and metastasis.[12] NO is being intensively investigated for therapeutic purposes; NO donors or NO inhibitors are used alone or in combination with other cytotoxic agents. In rat hepatoma cells[13] and cultured rat hepatocytes,[14]

NO increased cell death. However, there is considerable controversy and confusion regarding its role in HCC, at least in part due to a lack of data directly from cancer patients. In liver cancer, intratumoral injection of microencapsulated NOS-2 expressing cells reduced tumor growth in a xenograft mouse model. Administration of a NO donor or NOS-3 overexpression increased cell death receptor expression and reduced tumor cell growth after implantation of HepG2 cells in a xenograft mouse model.[15] Those observations in cell lines or animals markedly expanded our understanding of the importance of NO in HCC. However, whether NO is implicated in HCC and the underlying mechanism remains incompletely understood.

Recently, autophagy has been implicated in cancer initiation and development.[16] Autophagy is a highly conserved intracellular degradation process in which cellular organelles, lipids, proteins and invading microbes are sequestered in autophagosomes and are eventually fused with lysosomes for degradation. The autophagy pathways are controlled by more than 30 autophagy-related genes (ATG). The roles of autophagy in hepatocarcinogenesis remain controversial, with experimental evidence suggesting that autophagy both prevents and promotes tumor development. Genetic models have shown that the inhibition of autophagy favors the development of liver tumors. Mice heterozygous for Beclin 1, homologs of Atg6, showed a high frequency of spontaneous HCC. Deletion of Atg5 or Atg7 led to the development of multiple liver tumors. In contrast, tumor cells can also use autophagy as a cell survival mechanism against cellular stress or apoptosis. Sorafenib, a new drug that has been proven to have some survival benefits in patients with HCC, can induce apoptosis. Chloroquine (CQ), a lysosomotropic drug that raises the intralysosomal pH and impairs autophagic protein degradation, markedly increased sorafenib-induced tumor suppression in vitro and in vivo. Moreover, the inhibition of autophagy sensitizes histone deacetylase inhibitor-induced cell death in HCC cells. In HEK293 cells, NO inhibits autophagy,[17] whereas NO induces autophagy in melanoma cells.[18] However, whether and how NO regulates autophagy in liver cancer remains unknown.

In this study, we attempted to investigate the NO level and sources of NO and explore the regulatory effect of NO on autophagy and the potential underlying molecular mechanisms in liver cancer cells.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

Fifty-four patients with hepatitis B virus (HBV)-related HCC undergoing curative hepatectomy in the First Affiliated Hospital of Xi'an Jiaotong University between 2012 and 2015 were included in this study. This hospital currently has 2541 beds for inpatients and is the biggest general hospital directly under the administration of the Chinese Ministry of Health in northwestern China.

The diagnosis of HCC was based on α-fetoprotein and consistent findings from at least two separate imaging studies (including computed tomography, magnetic resonance imaging and hepatic angiography).

Each patient's liver function was classified according to the Child-Pugh class and the Model for End-Stage Liver Disease score.

The diagnoses were confirmed by histopathological study. None of the patients received preoperative chemo- or radiotherapy. Control subjects comprised 54 HBV-related liver cirrhosis (LC) patients with matched sex-/age-distribution and liver function. The clinical parameters of the controls and HCC patients are listed in Table 1. All patients provided written informed consent to participate in this research, which was approved by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University. Blood samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

2.2 | NO concentration detection

To determine NO levels in cirrhosis patients and HCC patients, a Griess assay (Qiagen, Hilden, Germany) was performed using plasma by strictly following the manufacturer's instructions.

2.3 | Immunohistochemical staining of iNOS, eNOS, nNOS and p62

Tumor samples free of necrosis or hemorrhage upon macroscopic inspection were selected, fixed in 4% paraformaldehyde and embedded in paraffin for histopathological diagnosis and immunohistochemical staining with specific antibodies: polyclonal rabbit anti-human iNOS (Abcam, Cambridge, MA, USA), eNOS (Abcam), nNOS (Abcam), and SQSTM1/p62 (Novus Biologicals, Littleton, CO, USA).

2.4 | Evaluation of iNOS, eNOS and nNOS expression

To score the staining of iNOS, eNOS and nNOS, only noticeable buffy-colored cytoplasmic staining was considered positive. The degree of staining was reviewed and scored independently by two observers, based on the area and intensity of staining. Staining intensity was graded on a score of 0-3, according to the following criteria: 0, negative staining; 1, weak staining; 2, moderate positive staining; and 3, strongly positive staining. The extent of positive staining was expressed as the percentage of whole cancer areas and were scored as follows: 0, negative; 1, positive staining in 1%-25%; 2, positive staining in 26%-50%; 3, positive staining in 51%-75%; and 4, positive staining in 76%-100%. The percentage scores for the positive tumor cell area were multiplied by the staining intensity scores to produce the weighted scores (degree × extent) for all tumor specimens. The values of the weighted scores ranged from 0 to a maximum of 12; 0-3 was defined as negative and 4 or higher was defined as positive.

2.5 | Quantitation of apoptosis

To quantitate apoptosis, 4′,6-diamidino-2-phenylindole (DAPI) staining and flow cytometry were used. First, 2.0×10^4 HepG2 cells were seeded in each well of 12-well plates. The following day, cells were washed with new media and exposed to various reagents as indicated. At the time of harvest, for DAPI staining, the cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 10 minutes at room temperature. DAPI was added for 5 minutes and nuclei were observed directly under a fluorescence microscope. For flow cytometry, the cells were washed twice with cold PBS and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Then, 5 μL of FITC Annexin V (BD Biosciences, San Jose, CA, USA) and 10 μL of propidium iodide were added to each 100 μL of cell (1×10^5 cells) suspension for 15 minutes at room temperature in the dark. In addition, 400 μL of 1× binding buffer was added and mixed gently. Flow cytometry analyses were performed with BD FACSJazz (BD Biosciences).

2.6 | Co-immunoprecipitation assay for protein binding

Cells lysates were prepared in lysis buffer and incubated with ProteinA/G-Sepharose beads at 4°C for 3 hours. Precleared lysates were incubated with appropriate antibody at 4°C for 12 hours with gentle rotation. Protein-A/G-Sepharose beads were added and incubated for 3 hours. Immunoprecipitates were collected by centrifugation, washed five times with lysis buffer, heated at 100°C for 5 minutes and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis.

2.7 | Immunoblot

Protein extracts were prepared by lysing cells in radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 1 mmol/L ethylenediaminetetraacetic acid [EDTA] and 50 mmol/L TrisHCl, pH 8.0) supplemented with complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO, USA). Cleared lysates were separated on 14% Tris-glycine gels or 8%-13% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) followed by being transferred to nitrocellulose. After blocking with 5% skim milk, blots were probed using the following primary antibodies: rabbit anti-LC3 (Cell Signaling, Boston, MA, USA) or SQSTM1/p62 (Novus Biologicals). Bands were detected using horseradish peroxidase-labeled secondary antibodies. Blots were developed using an enhanced chemiluminescence detection system.

2.8 | Statistical analysis

The results are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS software 13.0. Student's t-test or a one-way ANOVA followed by Tukey's or Kruskal-Wallis post-hoc tests, depending on the data distribution, were used for comparing the differences in variables between the

HCC and control groups. The reported *P*-values are two-sided, and *P* < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Serum NO levels increased in HCC

As a multipotent mediator, the NO level in serum is elevated in breast,[19] colon[20] and bladder[21] cancer. Here, we investigated serum NO levels in HCC. Because most of the HCC cases developed on the basis of cirrhosis and our preliminary data and published work showed increased NO in cirrhosis, matched patients with cirrhosis were selected as a control. We found a significant increase in the NO concentrations in patients with HCC when compared with the concentrations in the cirrhotic patients (53.60 ± 19.74 vs 8.09 ± 4.17 μmol/L, *t* = 15.13, *P* < 0.0001) (Figure 1).

3.2 | Source of NO

Endogenous NO production is tightly regulated by the activity of NOS. Thus, we tested the in vivo expression of NOS in tissue. Negative immunostaining (Figure S1) was observed in liver tissues of patients with cirrhosis and patients with HCC when we immunostained with a specific antibody against nNOS. The negative staining was not attributed to the incompetency of the antibody, because our parallel staining of brain tissue displayed a positive reaction (data not shown). Next, we investigated the expression of iNOS and eNOS in liver tissues from patients with cirrhosis or with HCC (Figure 2). Granular staining of iNOS and eNOS were found in the cytoplasm of the tumor cells and hepatocytes adjacent to the cancerous tissue. In contrast, iNOS and eNOS were barely detectable in patients with cirrhosis.
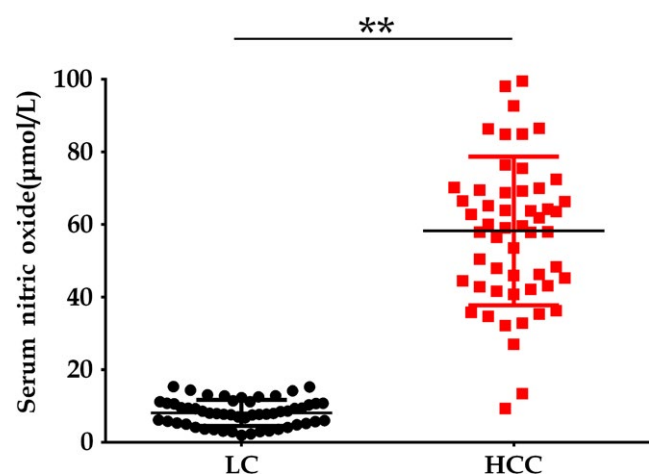

FIGURE 1 Serum nitric oxide (NO) levels increased in hepatocellular carcinoma (HCC). Plasma from hepatitis B virus-related HCC patients and cirrhosis patients were tested for nitrite (end product of NO) using the Griess reaction. The NO level in the HCC patients was significantly higher than that in the cirrhosis patients. ****P < 0.01. LC, liver cirrhosis

3.3 | NO induces apoptosis in HepG2 cells

Among all the NO donors, sodium nitroprusside (SNP) is the only compound approved for human use and thus was selected for subsequent experiments. By using DAPI staining, the effect of NO on apoptosis in human HCC cells was assessed. As shown in Figure S2, NO induces apoptosis in HCC cells in a dose- and time-dependent manner. We selected 100 μmol/L and 24 hours for the subsequent treatment for more potent effects.

Next, we tested whether NO induces HCC cell apoptosis through autophagy. CQ is a lysosomotropic drug that raises the intralysosomal pH and impairs autophagic protein degradation. CQ treatment also leads to apoptotic changes in HepG2 cells (Figure 3). Rapamycin, a drug that induces autophagy by inhibiting mammalian target of rapamycin (mTOR), protected HepG2 cells from NO-induced apoptosis (Figure 3).

3.4 | NO inhibits autophagy

Nitric oxide is a multipotent mediator and its effect on autophagy largely depends on the cell type and cellular environment. In HEK293 cells, NO inhibits autophagy,[17] whereas NO induces autophagy in melanoma cells.[18] To date, no data have reported the effect of NO on autophagy in liver cancer cells. Incubation with the NO donor SNP decreased LC3-II expression and increased p62 expression, which was observed in a concentration- (Figure S3A,D) and time-dependent manner (Figure S3B,E). The effects on autophagy are best assessed by analyzing LC3-II degradation resulting from the lysosomal proton pump inhibitor bafilomycin A1 (BA), a well-established method for monitoring autophagy flux. HepG2 cells were treated with SNP in the presence or absence of BA. Because BA blocks the degradation of the autophagosome cargo and the fusion of the autophagosome with the lysosome, an increased level of LC3-II was seen in cells treated with BA alone. However, SNP treatment results in a decreased LC3-II level, even in the presence of BA (Figure S3C,F), suggesting that the initiation step of autophagy was inhibited.

To further confirm the inhibition of autophagy by NO, tandem monomeric green fluorescent protein (mGFP)-monomeric red fluorescent protein (mRFP)-LC3 was transfected into HepG2 cells. The GFP signal is sensitive to the acidic and/or proteolytic conditions of the lysosomal lumen, whereas mRFP is more stable. Therefore, colocalization of both GFP and RFP fluorescence (yellow) indicates a compartment that has not fused with a lysosome. In contrast, an mRFP signal without GFP corresponds to a fusion of the autophagosome with the lysosome. As shown in Figure 4, at the basal level, the HepG2 cells displayed slight autophagy flux, which was consistent with the low autophagy activity observed in the HCC tissue. Incubation with rapamycin, which induces autophagy through mTOR inhibition, resulted in increased mRFP and colocalization of GFP and RFP fluorescence (Figure 4), indicating the induction of autophagy. However, after incubation with SNP at 100 nmol/L for 24 hours, a decrease in both GFP puncta and colocalization of GFP and RFP
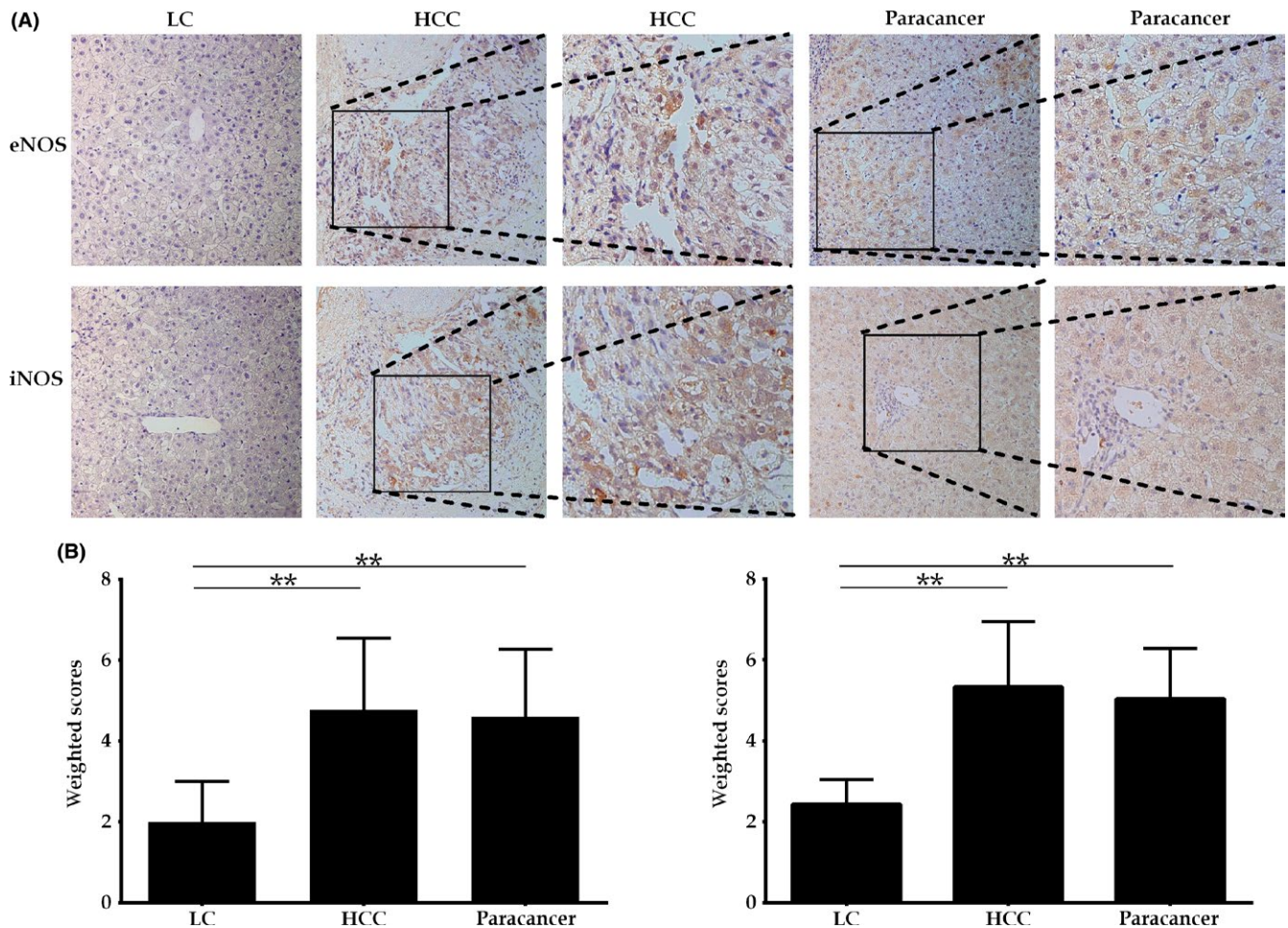
(A)

(B)

FIGURE 2 Endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression in liver tissues. Immunohistochemical eNOS and iNOS staining were conducted using human cirrhosis liver tissue, hepatocellular carcinoma (HCC) tissue and HCC paracancerous tissue. Weighted scores were produced by multiplying the percentage scores of the positive tumor cell area by the staining intensity scores (degree × extent) (original magnifications ×100 and ×200). LC, liver cirrhosis

fluorescence were observed, suggesting an inhibition of autophagy. Together, these data support the idea that NO inhibits autophagy flux in liver cancer cells.

3.5 | NO increases Beclin 1-Bcl2 interaction and reduces Beclin 1-hVps34 interaction

Nitric oxide induces apoptosis and autophagy. Beclin 1, the mammalian ortholog of yeast Atg6, interacts with several cofactors and regulates the switch between apoptosis and autophagy. Beclin 1 is a molecular switch that induces autophagy when it binds to Vps34, whereas it prompts apoptosis and inhibits autophagy when it binds to Bcl-2 and disrupts the Beclin 1/Vps34 complex. We tested whether NO regulates the Beclin 1/Bcl2 and Beclin 1/Vps34 interactions.

After treatment with the NO donor SNP, the binding of Bcl-2 to Beclin 1 was increased in HepG2 cells expressing Flag-tagged Beclin 1 (Flag-Beclin 1) and Myc-tagged Bcl-2 (Myc-Bcl-2), suggesting that the apoptosis-inducing effects of NO were due to increased association of the Bcl-2 with Beclin 1 (Figure 5A). Furthermore, endogenous Bcl-2 and Beclin 1 formed a complex in HepG2 cells and the SNP

treatment significantly increased their interaction, as demonstrated by a co-immunoprecipitation assay (Figure 5B). Beclin 1 regulates the initiation of autophagosome formation as a part of the hVps34/PI3K complex. Consistent with the NO-mediated increase in Bcl-2-Beclin 1 association, the NO donor SNP reduced Beclin 1-hVps34 interaction in HepG2 cells expressing Flag-hVps34 and Myc-Bcl-2 (Figure 5C), suggesting that the inhibitory effects of NO on autophagy were due to a decreased association of hVps34 with Beclin 1 that disrupted the initiation of autophagosome formation.

3.6 | Autophagy was inhibited in liver cancer

Our in vivo data exhibited that NO is implicated in HCC, and our in vitro data showed that NO inhibits autophagy and promotes apoptosis in hepatocytes, raising the question of whether in vivo autophagy in hepatocytes is inhibited. Thus, we investigated the autophagy activity in liver tissue from patients with HCC. As shown in Figure 6, both in cancerous tissue and tissue adjacent to liver cancer, hepatocytes displayed low autophagic activity, as manifested by increased p62.
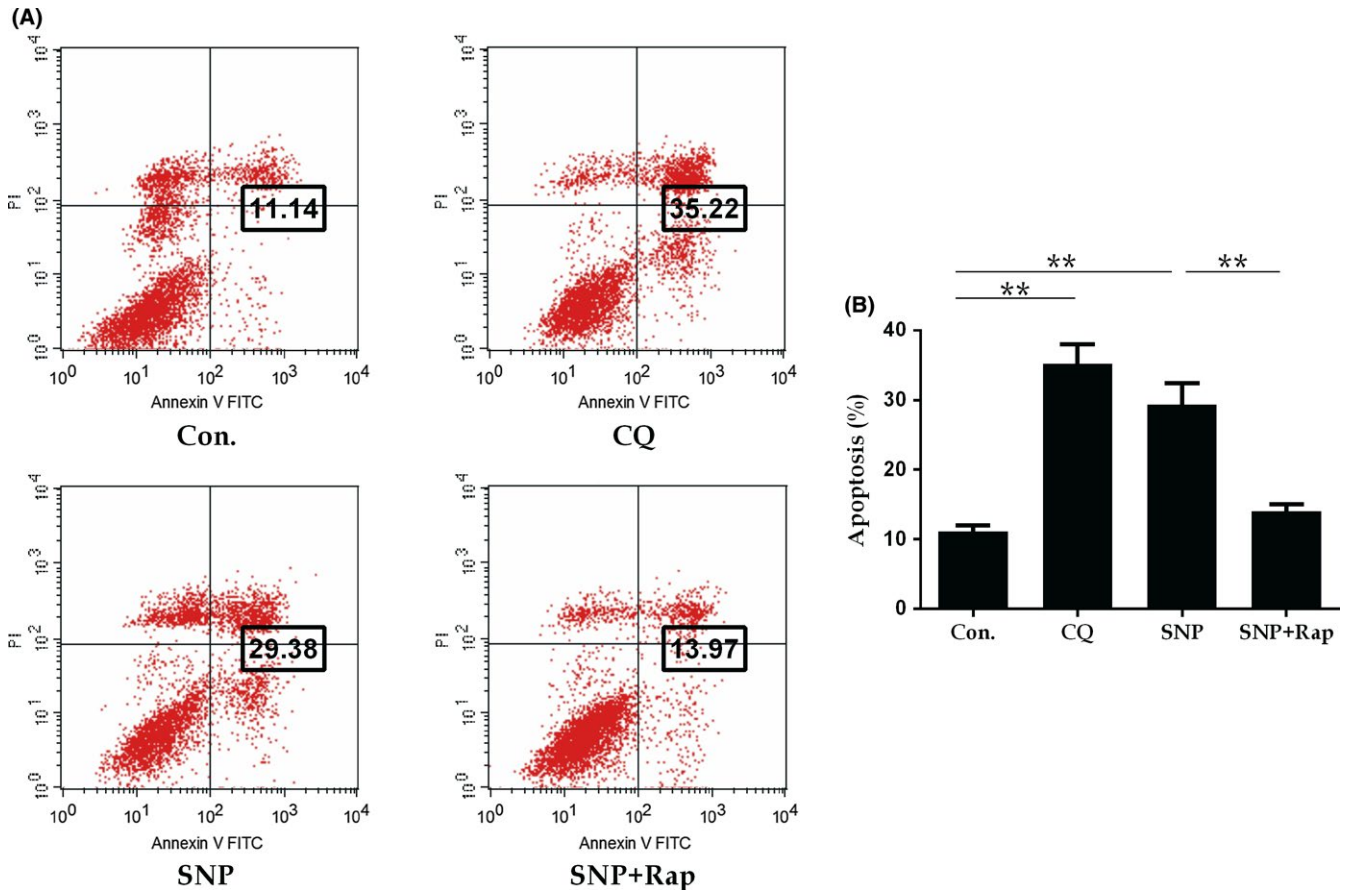
FIGURE 3 Autophagy rescues nitric oxide-induced apoptosis in HepG2 cells. A, HepG2 cells, in a logarithmic growth phase, were treated with 50 µmol/L chloroquine (CQ) and 100 µmol/L sodium nitroprusside with or without 50 nmol/L rapamycin (Rap) for 24 hours. Then, apoptosis was evaluated by flow cytometry. B, The percentage of apoptotic cells in three independent experiments are presented as mean ± standard error (error bar). *P < 0.05, **P < 0.01. Con, control; SNP, sodium nitroprusside

4 | DISCUSSION

It has been known for many years that NO plays an important pro-survival/pro-apoptosis role in various tumors.[22] However, whether and how NO is implicated in HCC remains controversial. Our data show that the NO level was significantly higher in HBV-related HCC. Because most of the patients developed HCC on the basis of cirrhosis, we selected patients with cirrhosis as a control group, supporting the idea that an elevated NO level was specifically attributed to HCC but not to cirrhosis. Similar with our results, Moussa et al[23] also demonstrated an increased plasma nitrite/nitrate level in patients with HCV-related HCC compared with the plasma levels of patients with cirrhosis. However, recently, Zhou et al[11] compared NO levels between cancer tissue and non-malignant liver tissue in patients with HCC and found that the NO levels in the cancerous tissue were significantly lower than those in the non-cancerous tissue. The discrepancy in NO levels between serum and cancerous tissues may arise from sampling error. Because the half-life of endogenous NO is extremely short, approximately 1 second, the NO level in the liver tissue is largely dependent on NOS expression. As shown in Figure 2, the expression of NOS is highly inhomogeneous. In HCC, the tumor may outgrow its blood supply, and the center of the tumor, the area with the poorest blood supply, may undergo necrosis and thus contain the lowest level of NO. However, the blood supply of the surrounding areas will be much higher and will thus have a relatively high NO level.

One of the interesting issues in this study is the identification of the source of NO in HCC. In mammals, three distinct genes encode the NOS isoenzymes, neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) genes.[24] nNOS is exclusively present in peripheral nerves and, consistently, nNOS was completely absent from patients with cirrhosis and patients with HCC. eNOS is constitutively present in vascular endothelial cells. During the pathophysiological process of HCC, an increase in vascularity is very common. Consistent with an increased serum NO level, our data showed that there was a marked increase in eNOS immunoreactivity. iNOS, produced mainly in pathological conditions by mesenchymal cells and parenchymal cells through the stimulation of endotoxin and cytokines, is expressed under transcriptional and post-transcriptional control. Our study also showed that iNOS was highly expressed in cancer tissue. Therefore, the systemic increase in NO in HCC patients could be due, at least partially, to the overexpression of eNOS and iNOS but not nNOS.
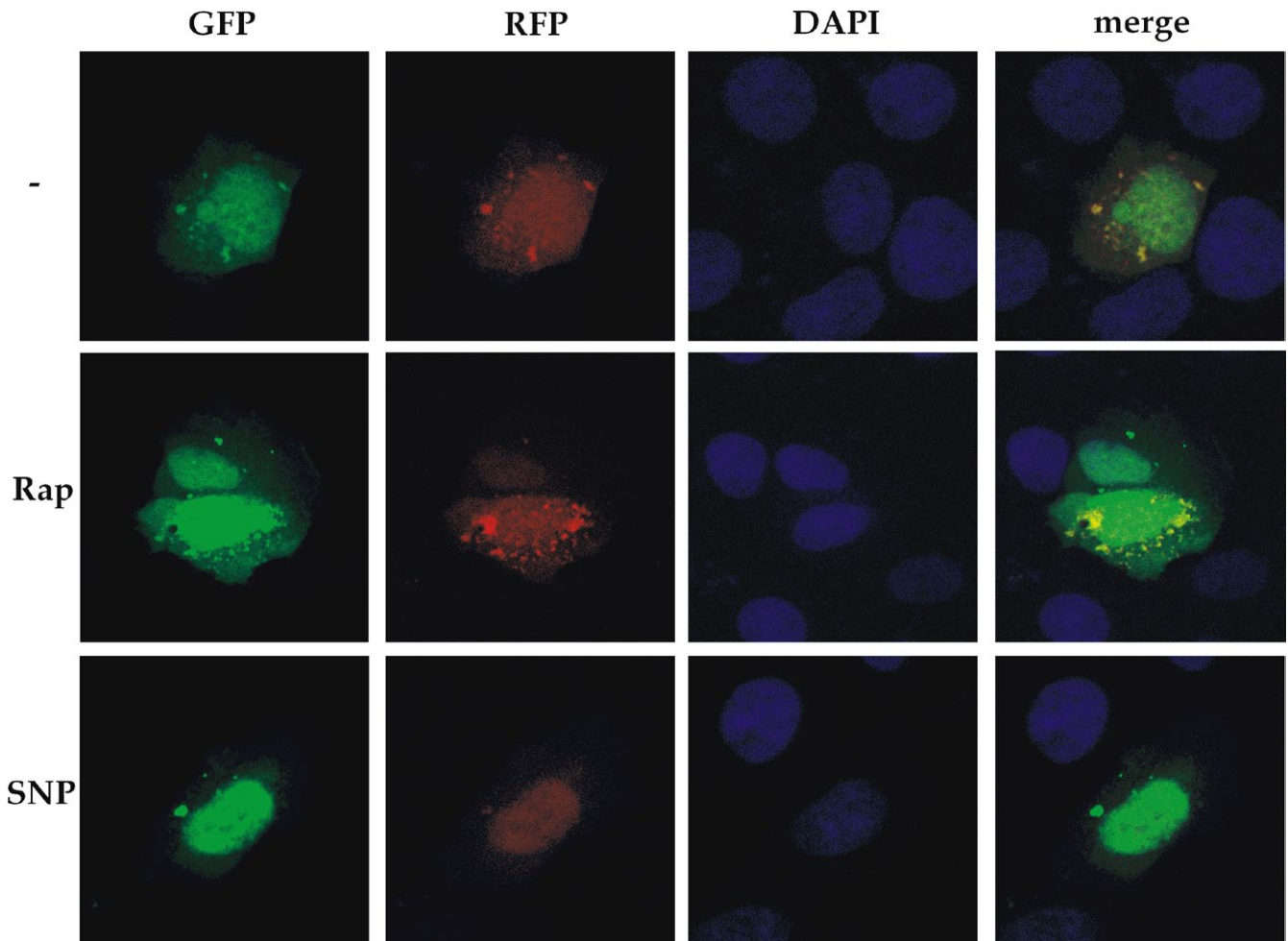
FIGURE 4 Nitric oxide inhibits autophagy in HepG2 cells. monomeric green fluorescent protein (GFP)-red fluorescent protein (RFP)-LC3 was transfected into the HepG2 cell line, followed by treatment with rapamycin (50 nmol/L) or sodium nitroprusside (100 µmol/L) for 24 hours. Colocalization of GFP and RFP was imaged. DAPI, 4′,6-diamidino-2-phenylindole; SNP, sodium nitroprusside
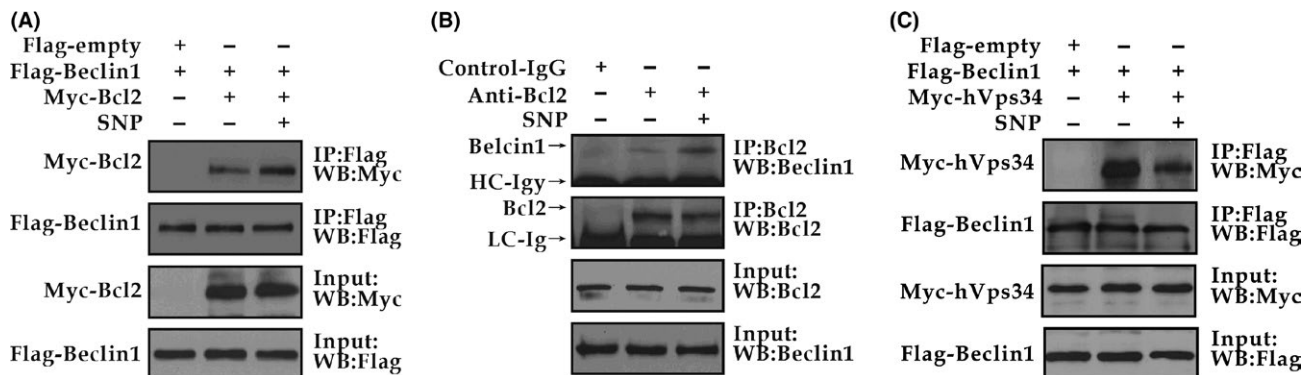

FIGURE 5 Nitric oxide (NO) increased Bcl-2-Beclin 1 and decreased Beclin 1-hVps34 interactions. A, Immunoprecipitation with anti-Flag M2 affinity agarose gel and immunoblotting with anti-Myc antibody showed that the NO donor sodium nitroprusside (SNP) increased the interaction of Flag-Beclin 1 with Myc-Bcl-2 in HepG2 cells transfected with Myc-Bcl-2 along with either empty Flag or Flag-Beclin 1. Asterisk denotes immunoglobulin G band. B, Endogenous Bcl-2 and Beclin 1 interact with each other in HepG2 cells. Co-immunoprecipitation was performed by incubating whole cell lysates (400 µg) with an anti-Bcl-2 antibody, and the precipitates were blotted with anti-Beclin 1 antibody. C, Immunoprecipitation with anti-Flag M2 affinity agarose gel and immunoblotting with anti-Vps34 antibody shows that the NO donor SNP decreased the Flag-Beclin1-hVps34 interaction in HepG2 cells transfected with hVps34 along with either empty Flag or Flag-Beclin 1
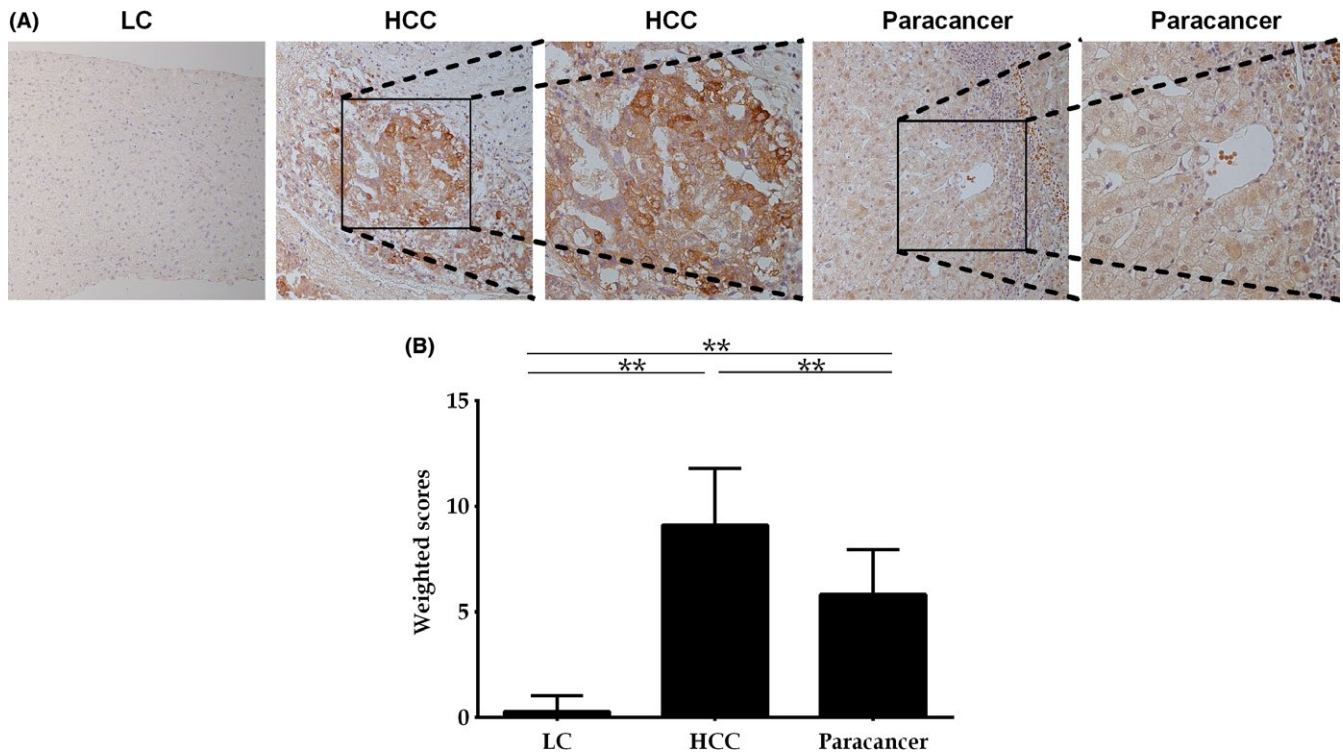
FIGURE 6 Autophagy was inhibited in the liver tissues of hepatocellular carcinoma (HCC) patients. Immunohistochemical SQSTM1/p62 staining was conducted using human cirrhosis liver tissue, HCC tissue and HCC paracancerous tissue. The weighted scores were produced by multiplying the percentage scores for the positive tumor cell area by the staining intensity scores (degree × extent). Original magnification ×100 and ×200. LC, liver cirrhosis

Nitric oxide induces apoptosis in hepatoma cells and rat hepatocytes. Consistent with this idea, our data revealed that the NO donor-induced HepG2 cell apoptosis in a time- and dose-dependent manner. In hepatic stellate cells, NO promotes caspase-independent apoptosis through autophagy inhibition.[25] The effect of NO on hepatocytes remains unknown. To further investigate the mechanism of apoptosis induced by NO, we explored the autophagy flux resulting from treatment with the NO donor. The autophagic effect of NO on liver cancer cells was evaluated with four standard methods, including LC3 lipidation, long-lived scaffolding protein p62 degradation, autophagic flux detection by analyzing LC3-II degradation with the lysosomal proton pump inhibitor bafilomycin A1 (BA) and fusion of lysosomes with autophagosomes. In our study, NO was identified as an autophagy inhibitor. Because NO is thought to have a wide range of biological functions, it is possible that NO has multiple entry points into the autophagic pathway depending on the cell type and cellular environment.

Beclin 1, a Bcl-2-homology (BH)-3 domain-only protein, plays an important role in autophagy through the formation of the Beclin/Vps34 complex.[26] The Beclin/Vps34 complex is negatively regulated by Bcl-2 family proteins, which bind to Beclin 1 and disrupt the interaction between Beclin 1 and Vps34, thereby inhibiting autophagosome formation.[27] DEA-NONOate and DETA-NONOate inhibit autophagy by inactivating JNK1 and thereby reduce Bcl-2 phosphorylation, which in turn leads to apoptosis by increasing the

Bcl-2-Beclin 1 interaction and to inhibition of autophagy by disrupting the Beclin 1/Vps34 association.[17] Our data clearly showed that SNP, a clinically approved reagent, increased the Bcl-2/Beclin 1 interaction and disrupted the Beclin 1/hVps34 association. Therefore, by disrupting the Beclin 1/hVps34 association and increasing the Bcl-2/Beclin 1 interaction, NO regulates autophagy and apoptotic cell death in HCC, which may be a clinical therapeutic target for HCC treatment.

Given that NOS/NO induces apoptosis in liver cancer cells, we explored the reason that eNOS and iNOS levels are increased in cancer cells. In the past, many studies have been conducted about the hallmarks of cancer, such as infinite proliferation, sustained angiogenesis, and activating invasion and metastasis, all of which are harmful to the host. A few studies have found that an anti-tumor response of the host existed. It has been reported that the acute phase protein response, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species (ROS) contributed to the host's defense against cancer. In a number of in vitro and in vivo studies, cancers have stimulated an acute phase protein response, such as that of serum amyloid A, fibrinogen and C-reaction protein, which have been shown to be cytotoxic to tumor cells either directly through cancer cell membrane destabilization and lysis or through the initiation of apoptosis in the cancer cell.[28] NADPH oxidase and related enzymes also participate in important cellular processes that are directly related to host defense, including signal

transduction, cell proliferation and apoptosis.[29] ROS, through their ability to induce cellular senescence and apoptosis, was harmful to tumor cells.[30] We hypothesize that NOS/NO may form another important anti-cancer host defense response, which may be a good approach for treating cancer.

We have shown that overexpression of eNOS and iNOS but not nNOS in cancer tissue contributes to increased NO levels in patients with HCC. We also found that NO inhibits autophagy by disrupting the Beclin 1/Vps34 association and promotes apoptosis by increasing the Bcl-2/Beclin 1 interaction in liver cancer cells. Our findings suggest that increased NO/NOS levels regulate autophagy and apoptotic cell death in HCC.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.