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ABOUT COVER

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Basic Study

Reactive oxygen species-induced activation of Yes-associated protein-1 through the c-Myc pathway is a therapeutic target in hepatocellular carcinoma

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Abstract

BACKGROUND

The Hippo signaling pathway regulates organ size by regulating cell proliferation and apoptosis with terminal effectors including Yes-associated protein-1 (YAP-1). Dysregulation in Hippo pathway has been proposed as one of the therapeutic targets in hepatocarcinogenesis. The levels of reactive oxygen species (ROS) increase during the progression from early to advanced hepatocellular carcinoma (HCC).

AIM

To study the activation of YAP-1 by ROS-induced damage in HCC and the involved signaling pathway.

METHODS

The expression of YAP-1 in HCC cells (Huh-7, HepG2, and SNU-761) was quantified using real-time polymerase chain reaction and immunoblotting. Human HCC cells were treated with H₂O₂, which is a major component of ROS in living organisms, and with either YAP-1 small interfering RNA (siRNA) or control siRNA. To investigate the role of YAP-1 in HCC cells under oxidative stress, MTS assays were performed. Immunoblotting was performed to evaluate the signaling pathway responsible for the activation of YAP-1. Eighty-eight surgically resected frozen HCC tissue samples and 88 nontumor liver tissue samples were used for

Review Board (CHAMC 2018-02-037). All the human tissues were provided by the Bundang CHA Biobank of Bundang CHA Medical Center. For the gene expression analyses, 88 surgically resected frozen HCC tissue samples and 88 nontumor liver tissue samples were analyzed. Cases were prospectively and consecutively identified at Bundang CHA Medical Center between 2012 and 2018.

Institutional animal care and use committee statement: The *in vivo* study protocol was approved by the Institutional Animal Care and Use Committee (IACUC-180027) of CHA University. All the *in vivo* surgical procedures were performed under anesthesia with 2,2,2-tribromoethanol, and all efforts were made to minimize suffering.

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gene expression analyses.

RESULTS

H₂O₂ treatment increased the mRNA and protein expression of YAP-1 in HCC cells (Huh-7, HepG2, and SNU-761). Suppression of YAP-1 using siRNA transfection resulted in a significant decrease in tumor proliferation during H₂O₂ treatment both *in vitro* and *in vivo* (both $P < 0.05$). The oncogenic action of YAP-1 occurred *via* the activation of the c-Myc pathway, leading to the upregulation of components of the unfolded protein response (UPR), including 78-kDa glucose-regulated protein and activating transcription factor-6 (ATF-6). The YAP-1 mRNA levels in human HCC tissues were upregulated by 2.6-fold compared with those in nontumor tissues ($P < 0.05$) and were positively correlated with the ATF-6 Levels (Pearson's coefficient = 0.299; $P < 0.05$).

CONCLUSION

This study shows a novel connection between YAP-1 and the UPR through the c-Myc pathway during oxidative stress in HCC. The ROS-induced activation of YAP-1 *via* the c-Myc pathway, which leads to the activation of the UPR pathway, might be a therapeutic target in HCC.

Key Words: Hepatocellular carcinoma; Yes-associated protein-1; C-Myc; Reactive oxygen species; Unfolded protein response; Activating transcription factor-6

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Core Tip: We found a novel connection between Yes-associated protein-1 (YAP-1) and the unfolded protein response (UPR) through the c-Myc pathway during oxidative stress in hepatocellular carcinoma (HCC). As the Hippo pathway and c-Myc pathway share many important functions, including the regulation of growth, death and survival in cells and the regulation of stress resistance and life spans in organisms, we speculate that the interaction between YAP-1 and c-Myc is a point of convergence that allows HCC proliferation. The reactive oxygen species-induced activation of YAP-1 *via* the c-Myc pathway, which leads to the activation of the UPR pathway, might be a therapeutic target in HCC.

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INTRODUCTION

Reactive oxygen species (ROS), such as H₂O₂ superoxide radicals, and hydroxyl radicals, contribute to tumor progression by enhancing DNA damage and altering cell signaling pathways^[1,2]. It has been recently suggested that ROS are involved in tumor metastasis, which is a complex process that includes angiogenesis, epithelial-to-mesenchymal transition, invasion, and migration within the tumor micro-environment^[3]. ROS also control the expression of matrix metalloproteinases and mitogen-activated protein kinases (MAPKs), the activation of the Ras pathway, and the downregulation of E-cadherin expression^[4].

Hepatocellular carcinoma (HCC) is one of the common fatal malignancies which results in approximately one million worldwide deaths every year^[5]. Oxidative stress is known to be the most important factor of HCC development^[6,7]. The major etiologies of HCC, including chronic hepatitis B or C, alcohol-related liver disease, and nonalcoholic fatty liver disease, increase ROS levels^[8,9]. ROS levels are also positively correlated with HCC progression^[10,11].

The Hippo signaling pathway regulates organ size by regulating both cell proliferation and apoptosis with terminal effectors such as yes-associated protein

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(YAP)^[12,13]. The key components of the Hippo pathway include sterile 20-like kinases (Mst1 and Mst2; homologues of D. hippo), large tumor suppressors (Lats1 and Lats2; homologues of warts), YAP, its paralog protein transcriptional coactivator with PDZ-binding motif (TAZ), transcriptional coactivators, and homologues of yorkie. Inactivation of the Hippo pathway leads to uncontrolled cell proliferation in epithelial cells and stem cells^[14,15] and oncogenic transformation^[16], both of which are mediated by the upregulation of YAP. Dysregulation of the Hippo pathway has been proposed as one of the therapeutic targets in hepatocarcinogenesis^[17-19]. A previous study showed that YAP is an independent predictive marker for the overall survival and disease-free survival of HCC patients and that it is associated with tumor differentiation^[20]. The Hippo pathway, which regulates tumorigenesis, also has an important role in mediating oxidative stress^[21]. Shao *et al*^[13] suggested the involvement of YAP in causing cardiomyocyte survival during oxidative stress^[13].

Thus, the activation of YAP-1 by ROS-induced damage has been hypothesized to exacerbate the progression of HCC, but it remains unclear which signaling pathway is involved. Here, we investigated ROS-induced YAP-1 activation in HCC and the associated signaling pathway.

MATERIALS AND METHODS

Cell lines and coculture

Human HCC cell lines including Huh-7 and HepG2, which are well-differentiated HCC cell lines, and SNU-761, which is a poorly differentiated HCC cell line were used in this study. We used Dulbecco's modified Eagle medium (DMEM; Huh-7 and HepG2) or in RPMI 1640 (SNU-761) supplemented with 10% fetal bovine serum (FBS), 100000 U/L penicillin, and 100 mg/L streptomycin, with or without 100 nmol/L insulin for cell culture.

Cell proliferation analysis (MTS assay)

HCC cell proliferation was measured with the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, United States), on the basis of the cellular conversion of the colorimetric reagent 3, 4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) into soluble formazan by the dehydrogenase enzyme found in metabolically proliferating cells. Following each treatment, 20 μ L of the dye solution was added to each well of a 96-well plate and incubated for 2 h. Then, the absorbance was recorded at a wavelength of 490 nm using an enzyme-linked immunosorbent assay plate reader (Molecular Devices, Sunnyvale, CA, United States).

Small interfering RNA transfection

Cells were seeded in a 6-well culture plate (2×10^5 cells per well) in 2 mL antibiotic-free medium supplemented with 10% FBS. Once the cells reached 60%-80% confluence, they were transfected with small interfering RNA (siRNA) using the siRNA Transfection Reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) according to the manufacturer's instructions. The cells were treated with siRNA for 6 h at 37 °C, and then, growth medium containing 20% FBS and antibiotics was added. After 18 h, the medium was replaced with fresh medium containing 10% FBS and antibiotics. Twenty-four hours after transfection, the cells were used in further experiments.

In vivo subcutaneous xenograft model

Briefly, H₂O₂ (100 μ mol/L)-treated MH134 cells (5×10^7 cells per mouse) were subcutaneously transplanted into the flanks of C3H mice in the control group ($n = 10$). The tumor volume was measured using a Vernier caliper and calculated as $[\text{length} \times (\text{width})^2]/2$. YAP-1 siRNA transfected MH134 cells were subcutaneously implanted on the flank of mice in YAP siRNA group, and control siRNA transfected MH134 cells were implanted in control siRNA group. The maximal diameter of each nodule was measured every day for 13 d.

Immunoblot analysis

The cells were lysed for 20 min on ice with lysis buffer and centrifuged at 14000 g for 10 min at 4 °C. The samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blotted with the

appropriate primary antibodies at a dilution of 1:1000, and treated with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, United States). The bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, United States) and exposed to Kodak X-OMAT film (Kodak, New Haven, CT, United States). The primary antibodies, including rabbit anti-phospho-p42/44 MAPK, anti-phosphorylated-Akt, and rabbit anti-c-Myc, were purchased from Cell Signaling Technology (Danvers, MA, United States). The goat anti- β -actin antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States). The densitometric analyses were performed with Image J software (National Institutes of Health, Bethesda, MD, United States).

Real time-polymerase chain reaction analysis

The total ribonucleic acids (RNAs) were extracted from Huh-7, HepG2, and SNU-761 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, United States). The complementary deoxyribonucleic acid (cDNA) templates were prepared using oligo (dT) random primers and Moloney Murine Leukemia Virus (MoMLV) reverse transcriptase. After the reverse transcription reaction, the cDNA template was amplified by polymerase chain reaction (PCR) using Taq polymerase (Invitrogen). YAP-1 mRNA expression was quantified by real-time PCR (Light Cycler; Roche Molecular Biochemicals, Mannheim, Germany) using SYBR green as the fluorophore (Molecular Probes, Eugene, OR, United States). The primers for YAP-1 were as follows: Forward: 5'-TGAACAAACGTCCAGCAAGATAC-3'; and reverse: 5'-CAGCCCCCAAATGAACAGTAG-3'. The primers for c-Myc were as follows: Forward: 5'-CCCGCTTCTCTGAAAGGCTCTC-3'; and reverse: 5'-CTCTGCTGCTGCTGCTGCTGGTAG-3'. For the unfolded protein response (UPR) markers, the following primers were used: Glucose-regulated protein 78 (GRP78), forward: 5'-GACGGGCAAAGATGTCAGGAA-3' and reverse: 5'-TCATAGTAGACCGGAACAGATCCA-3'; XBP1, forward: 5'-TTGTCACCCCTCCAGAACATC-3' and reverse: 5'-TCCAGAATGCCCAACAGGAT-3'; activating transcription factor-6 (ATF-6), forward: 5'-TTGGCATTATTAATACTGAACATATGGA-3' and reverse: 5'-TTTGATTGTCAGGGCTCAC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as a control. The level of YAP-1 mRNA expression was calculated as the relative intensity of the PCR product band compared with that of the GAPDH gene using the $2^{-\Delta\Delta C_t}$ method. All the PCR experiments were performed in triplicate.

Statistical analysis

The statistical analyses were performed using PASW version 21.0 (SPSS Inc., Chicago, IL, United States). All the experimental results were obtained from three independent experiments using cells from three separate isolations and are presented as the mean \pm standard deviation (SD). For comparisons between groups, the data were analyzed by the Mann-Whitney *U* test or one-way ANOVA. For all the tests, $P < 0.05$ was regarded as statistically significant.

Ethics statement

Ethical approval was obtained from the ethics committee at CHA University. We carried out this study in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The *in vivo* study protocol was approved by the Institutional Animal Care and Use Committee (IACUC-180027) of CHA University. All the *in vivo* surgical procedures were performed under anesthesia with 2, 2, 2-tribromoethanol, and all efforts were made to minimize suffering.

All the experiments using human tissues were approved by the Bundang CHA Medical Center Institutional Review Board (CHAMC 2018-02-037). All the human tissues were provided by the Bundang CHA Biobank of Bundang CHA Medical Center. For the gene expression analyses, 88 surgically resected frozen HCC tissue samples and 88 nontumor liver tissue samples were analyzed. Cases were prospectively and consecutively identified at Bundang CHA Medical Center between 2012 and 2018.

RESULTS

ROS enhanced the mRNA and protein expression of YAP-1 in HCC cells

To analyze the potential ROS-induced changes in YAP-1 expression in HCC cells, we treated human HCC cells (Huh-7, HepG2, and SNU-761 cells) with 150 $\mu\text{mol/L}$ H_2O_2 . Real-time PCR and immunoblot analyses indicated that H_2O_2 treatment increased the mRNA (Figure 1A) and protein (Figure 1B) expression of YAP-1 in the HCC cells. These effects were inhibited following treatment of the cells with the antioxidant N-acetylcysteine (NAC) (Figure 1C). The antioxidant treatment significantly suppressed the protein expressions of YAP-1 in HCC cells.

Modulation of YAP-1 expression in ROS-exposed HCC cells showed antitumor effects in vitro

Next, to investigate whether exposure to H_2O_2 impacts HCC cell survival, HCC cells were treated with H_2O_2 (0–350 $\mu\text{mol/L}$), and the ROS levels were increased by intervals of 50 $\mu\text{mol/L}$. As shown in Figure 2A, exposure to H_2O_2 (0–350 $\mu\text{mol/L}$) did not reduce HCC cell survival. Then, we examined the efficacy YAP-1 siRNA transfection with real-time PCR. YAP-1 siRNA transfection significantly suppressed YAP-1 mRNA expression compared to control siRNA transfection in HCC cells (Figure 2B; $P < 0.05$). Next, we performed an MTS assay to evaluate whether YAP-1 modulates HCC cell proliferation. Suppression of YAP-1 using siRNA transfection or verteporfin treatment (YAP-1 inhibitor) resulted in a significant decrease in tumor proliferation during exposure 150 $\mu\text{mol/L}$ H_2O_2 *in vitro* (Figure 2C and D; both $P < 0.05$).

Modulation of YAP-1 expression in ROS-exposed HCC cells showed antitumor effects in an in vivo xenograft tumor mouse model

The antitumor effects of YAP-1 siRNA were examined using an *in vivo* xenograft model. First, we evaluated whether exposure to ROS changes the expression of YAP-1 in the murine HCC cell line MH134. H_2O_2 treatment significantly increased the proliferation of the MH134 cells (Figure 3A; $P < 0.05$). We also confirmed that suppression of YAP-1 using siRNA transfection resulted in significantly decreased mRNA expression of YAP-1 in the MH134 cells treated with 150 $\mu\text{mol/L}$ H_2O_2 (Figure 3B). In the xenograft tumor model, the YAP-1 siRNA group showed significantly suppressed tumor growth compared to the control siRNA group at days 11, 12, and 13 after tumor budding (Figure 3C; all $P < 0.05$).

The oncogenic action of YAP-1 was reciprocally activated by the c-Myc pathway in ROS-exposed HCC cells

The immunoblot assay results showed that the downregulation of YAP-1 caused by siRNA transfection or verteporfin treatment decreased the protein expression of c-Myc in the ROS-exposed HCC cell lines (Figure 4A and B). When the ROS-exposed HCC cells were treated with a c-Myc inhibitor (10058-F4, 60 $\mu\text{mol/L}$), the protein expression of YAP-1 was significantly decreased compared with that in the control-treated cells (Figure 5A). Moreover, treatment with the antioxidant NAC downregulated the expression of c-Myc in the ROS-exposed HCC cell lines (Figure 5B). We also performed real-time PCR and immunoblot analyses to evaluate whether up-regulation of the c-Myc pathway was dependent on YAP-1 expressions. YAP-1 siRNA transfection significantly suppressed c-Myc mRNA expression compared to control siRNA transfection in ROS-exposed HCC cells (Figure 5C; all $P < 0.05$). Immunoblot analyses of c-Myc also revealed that ROS-exposed HCC cells transfected with YAP-1 siRNA showed suppressed protein expression of c-Myc as compared to those transfected with control siRNA (Figure 5D).

The ROS-induced oncogenic action of YAP-1 in HCC cells led to an enhanced UPR

To determine whether the oncogenic action of YAP-1, which occurs *via* the activation of the c-Myc pathway, leads to the upregulation of components of the UPR, we performed real-time PCR on cells treated with or without H_2O_2 for 78-kDa GRP78/BiP, ATF-6, and XBP1 (Figure 6A). ROS exposure significantly enhanced the mRNA expression of GRP78, ATF-6, and XBP1 in the HCC cell lines. The downregulation of YAP-1 by siRNA transfection also significantly suppressed the expression of the UPR markers compared to control siRNA transfection. We also performed immunoblot analysis to evaluate the endoplasmic reticulum (ER) stress marker phosphorylated eIF-2 α (Figure 6B); the results revealed that the transfection of YAP-1 siRNA attenuated the protein expression of phosphorylated eIF-2 α compared to control siRNA

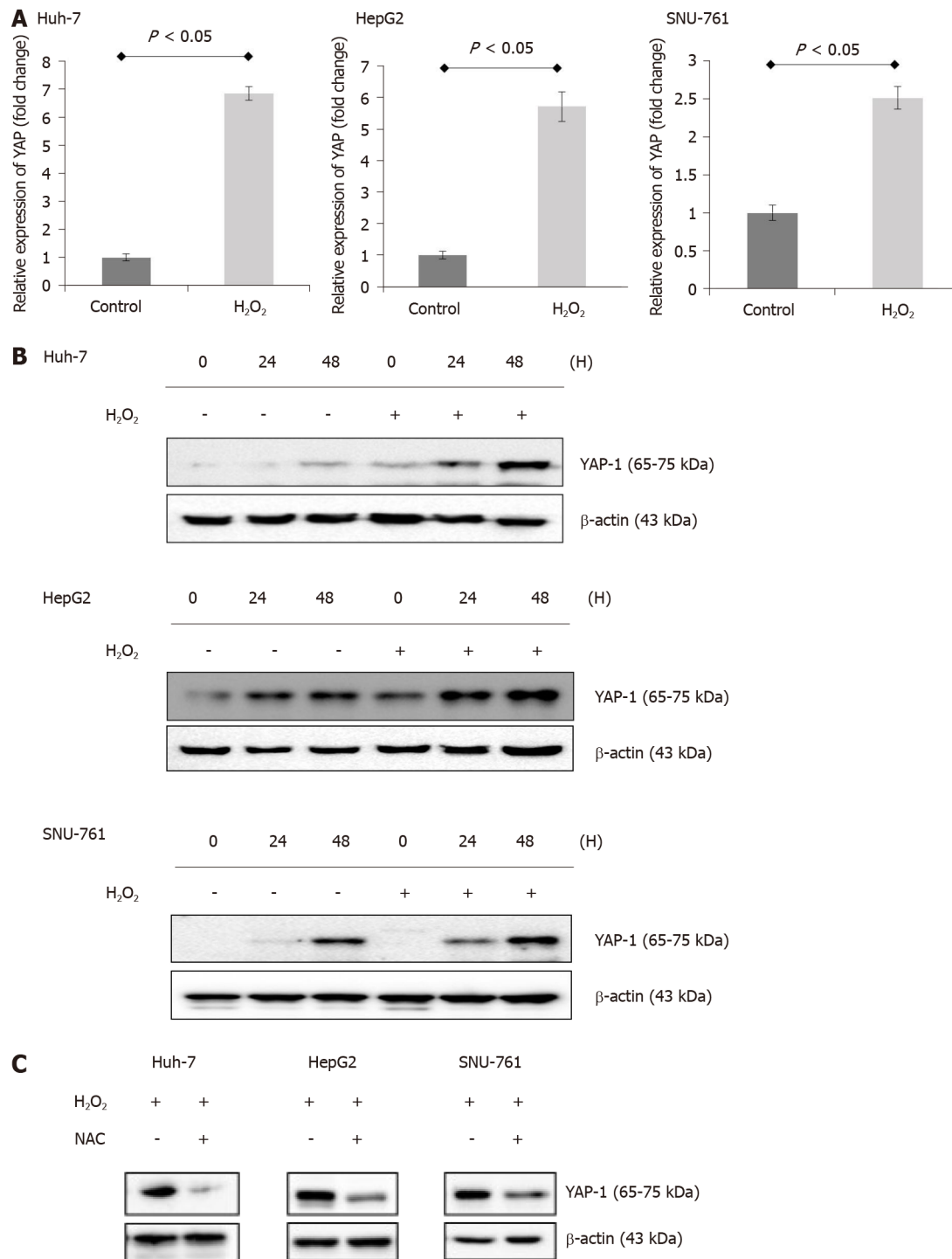


Figure 1 Reactive oxygen species enhanced the mRNA and protein expression of yes-associated protein-1 in hepatocellular carcinoma cells. **A:** Yes-associated protein-1 (YAP-1) mRNA was significantly enhanced in hepatocellular carcinoma (HCC) cells treated with 150 $\mu\text{mol/L}$ H_2O_2 . YAP-1 mRNA expression was quantified using quantitative polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression. The experiment was repeated three times. The data are expressed as the mean \pm SD. The error bars represent the SD; **B:** The protein expression of YAP-1 in HCC cells was significantly enhanced when the HCC cells were exposed to 150 $\mu\text{mol/L}$ H_2O_2 , especially at 48 h. The experiment was repeated three times; **C:** Treatment with the antioxidant N-acetylcysteine inhibited the protein expression of YAP-1 in HCC cells. The experiment was repeated three times. YAP-1: Yes-associated protein-1; HCC: Hepatocellular carcinoma; SD: Standard deviation; NAC: N-acetylcysteine.

transfection.

Upregulated mRNA expression of YAP-1 was correlated with the expression of ATF-6 in human HCC tissues

For the gene expression analyses, 88 surgically resected frozen HCC tumor tissue samples and 88 paired nontumor liver tissue samples were evaluated. The majority of the patients ($n = 71$, 80.7%) had stage I HCC according to the American Joint

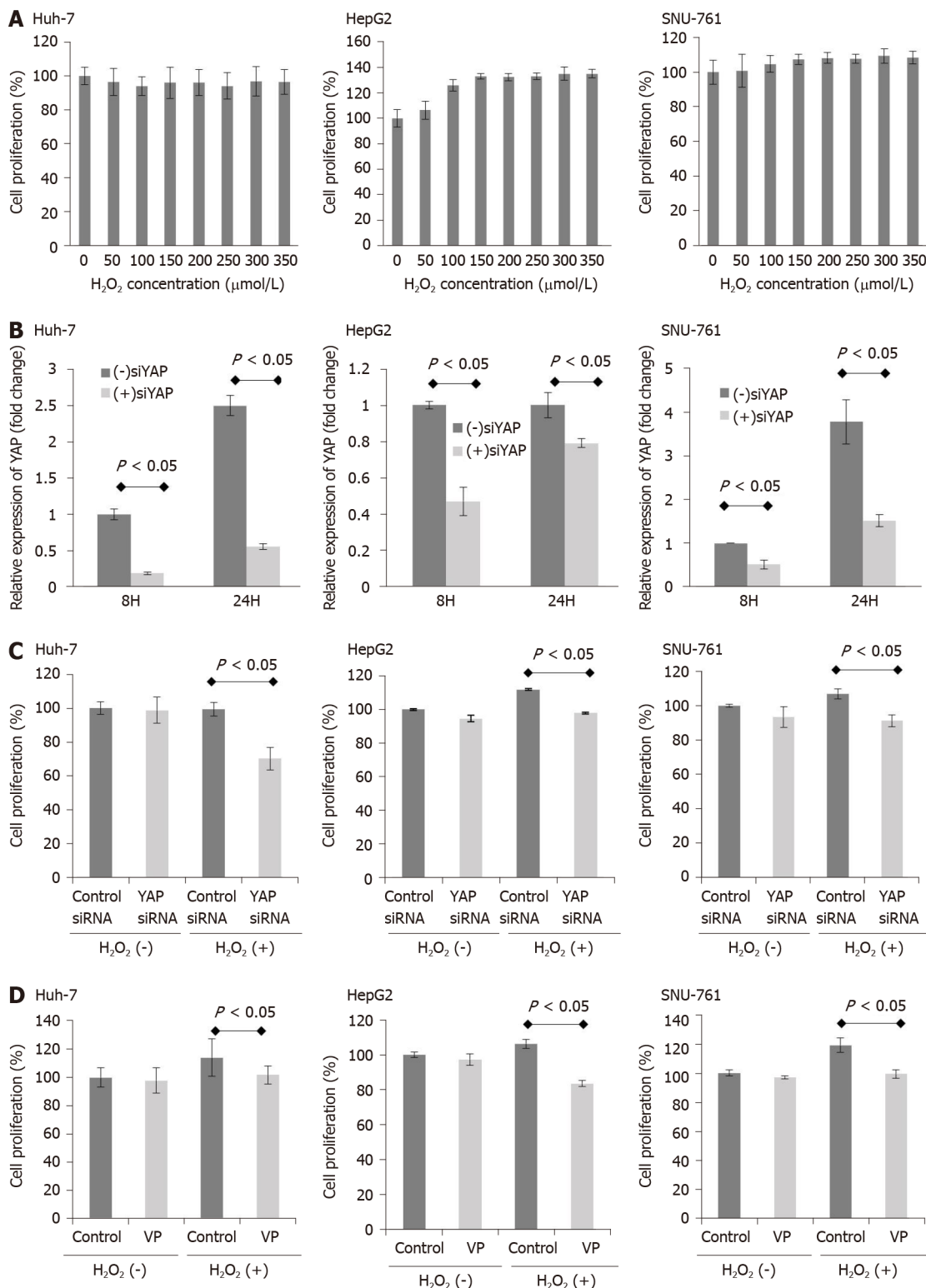


Figure 2 The effects of Yes-associated protein-1 on the proliferation of reactive oxygen species (ROS)-exposed hepatocellular carcinoma (HCC) cells. A: An MTS assay was performed on hepatocellular carcinoma (HCC) cells that were treated with H₂O₂ (0-350 μmol/L), and the reactive oxygen species levels were increased by intervals of 50 μmol/L. The data are expressed as the mean ± SD of percent changes of optical densities. The experiment was repeated three times; B: Yes-associated protein-1 (YAP-1) small interfering RNA (siRNA) transfection significantly suppressed YAP-1 mRNA expression compared to control siRNA transfection in HCC cells ($P < 0.05$). The data are expressed as the mean ± SD. The experiment was repeated three times; C: When HCC cells were transfected with YAP-1 siRNA, the proliferation of HCC cells was significantly decreased compared with control siRNA transfection based on the MTS assay results ($P < 0.05$). The data are expressed as the mean ± SD of percent changes of optical densities. The experiment was repeated three times; D: When HCC cells were treated with verteporfin (1000 nmol/L), the proliferation of HCC cells was significantly decreased compared with the control treatment based on the MTS assay results ($P < 0.05$). The data are expressed as the mean ± SD of percent changes of optical densities. The experiment was repeated three times. YAP: Yes-associated protein; siRNA: Small interfering RNA; VP: Verteporfin.

Commission on Cancer 8th edition HCC staging system. 11 patients (12.5%) and 6 patients (6.8%) had stage II and stage III HCC, respectively. No patient had major vascular invasion or lymph node/distant metastasis. The expression of YAP-1 was further determined in the resected HCC tissues and adjacent nontumor tissues using real-time PCR. The mean mRNA expression of YAP-1 was upregulated by 2.6-fold in the HCC tissues compared with the nontumor tissues (Figure 7A; $P < 0.05$). Among the 88 HCC tumor tissues, YAP-1 RNA expression was upregulated in 42 samples (47.7%) compared to the nontumor tissues, and YAP-1 expression was positively correlated with ATF-6 expression (Figure 7B; Pearson's coefficient = 0.299; $P < 0.05$). For one patient whose YAP-1 expression in HCC tissue was 15.5-fold higher than that in nontumor tissue, we performed immunohistochemical staining for YAP-1 with HCC tissue, which is shown in Figure 7C.

DISCUSSION

This study revealed that the ROS-induced activation of YAP-1 *via* the c-Myc pathway, which leads to the activation of the UPR, might be a therapeutic target in HCC. We have elucidated the molecular mechanism by which YAP-1 mediates the survival of HCC cells under oxidative stress.

Carcinogenesis leads to the accumulation of misfolded proteins in the ER^[22]. Then, the UPR is activated to restore normal cellular function by degrading the misfolded proteins and activating the production of chaperones, such as GRP78. However, under pathological conditions, prolonged UPR activation can promote apoptosis, leading to cell death. Overall, if ER stress is too severe, the UPR leads to translational arrest and induces specific factors for cell survival or cell death. In several cancers, the expression of UPR components is enhanced, indicating the dependency of these cancers on the UPR^[23]. Thus, there is a possibility that modification of the UPR might have anticancer effects.

Hypoxia is one of the major mediators of UPR-inducing pathways. Human fibrosarcoma and lung carcinoma cells upregulated GRP78 expression and XBP1 splicing under hypoxic conditions *in vitro*^[24]. Tumor formation with aberrant microcirculation might lead to hypoxic conditions, which induce the UPR. Gradually, the UPR increases cell survival and tumor proliferation, which thereby increases hypoxia in the core of the tumor. After the sequestration of GRP78 by misfolded proteins, ATF-6, inositol requiring protein 1, and protein kinase RNA-like endoplasmic reticulum kinase (PERK) act as transducers to transmit the ER stress signal to the cytosol and nucleus. Activated ATF-6 translocates to the Golgi, where proteases cleave it and release its fragments into the cytosol^[25]. Indeed, enhanced nuclear translocation of the ATF-6 fragment is observed in various cancers, including HCC. In this study, we identified the potential of ATF-6 to act as an effector of HCC under oxidative stress.

The c-Myc pathway undergoes chromosomal translocation and gene amplification in many cancers, including HCC. Activated c-Myc pathway upregulates oncogenes which are involved in ribosome biogenesis. Previous studies reported that elevated protein synthesis due to increased c-Myc expression in cancer cells lead to UPR activation^[26,27]. Activation of UPR signaling promotes autophagy in tumor cells under conditions of hypoxia, oxidative stress, and nutrient limitation. Our findings suggest a key link between YAP-1-mediated oncogenic transformation and HCC cell survival *via* the c-Myc-mediated UPR under oxidative stress.

There are increasing lines of evidence suggesting that the loss-of-function mutations in components of the Hippo pathway and hyperactivation of YAP-1 have been observed in many cancers. Thus, we speculate that the regulating the YAP-1-c-Myc pathway might be a crucial mechanism through which the Hippo pathway regulates hepatocarcinogenesis.

Several multikinase inhibitors that have been approved for advanced HCC, including sorafenib, regorafenib, and lenvatinib, have shown modest survival advantages^[28,29]. Recent evidence suggests that long-term treatment of HCC leads to hypoxia-mediated sorafenib resistance in patients with HCC because tumor-driving pathways, including YAP-1, become activated^[30-32]. However, the molecular mechanism of sorafenib resistance is unclear. Here, we found that ROS are the primary triggers of YAP-1-c-Myc-UPR signaling hyperactivation during oxidative stress, and this phenomenon is also observed in human HCC tissues.

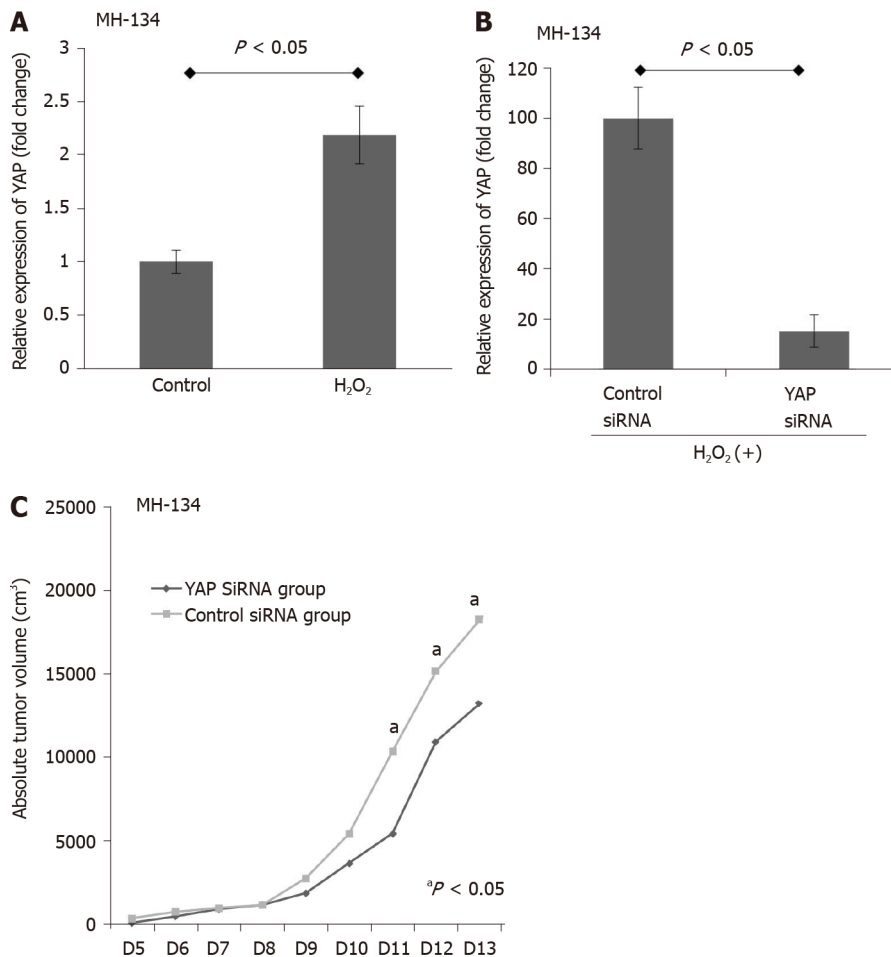


Figure 3 Modulation of yes-associated protein-1 in reactive oxygen species-exposed hepatocellular carcinoma cells showed antitumor effects in an *in vivo* xenograft tumor mouse model. A: H_2O_2 treatment significantly increased the proliferation of MH134 cells based on the MTS assay results ($P < 0.05$). The data are expressed as the mean \pm SD. The experiment was repeated three times; B: Yes-associated protein-1 (YAP-1) small interfering RNA (siRNA) transfection significantly suppressed YAP-1 mRNA expression compared to control siRNA transfection in reactive oxygen species-exposed MH134 cells ($P < 0.05$). The data are expressed as the mean \pm SD. The experiment was repeated three times; C: In the xenograft tumor model, the YAP-1 siRNA group showed significantly suppressed tumor growth compared to the control siRNA group at days 11, 12, and 13 after tumor budding (all $^aP < 0.05$). The data are expressed as the mean \pm SD. YAP: Yes-associated protein; siRNA: Small interfering RNA.

CONCLUSION

In conclusion, our study shows a novel connection between YAP-1 and the UPR through the c-Myc pathway during oxidative stress in HCC. As the Hippo pathway and c-Myc pathway share many important functions, including the regulation of growth, death and survival in cells and the regulation of stress resistance and life spans in organisms, we speculate that the interaction between YAP-1 and c-Myc is a point of convergence that allows HCC proliferation. The ROS-induced activation of YAP-1 *via* the c-Myc pathway, which leads to the activation of the UPR pathway, might be a therapeutic target in HCC.

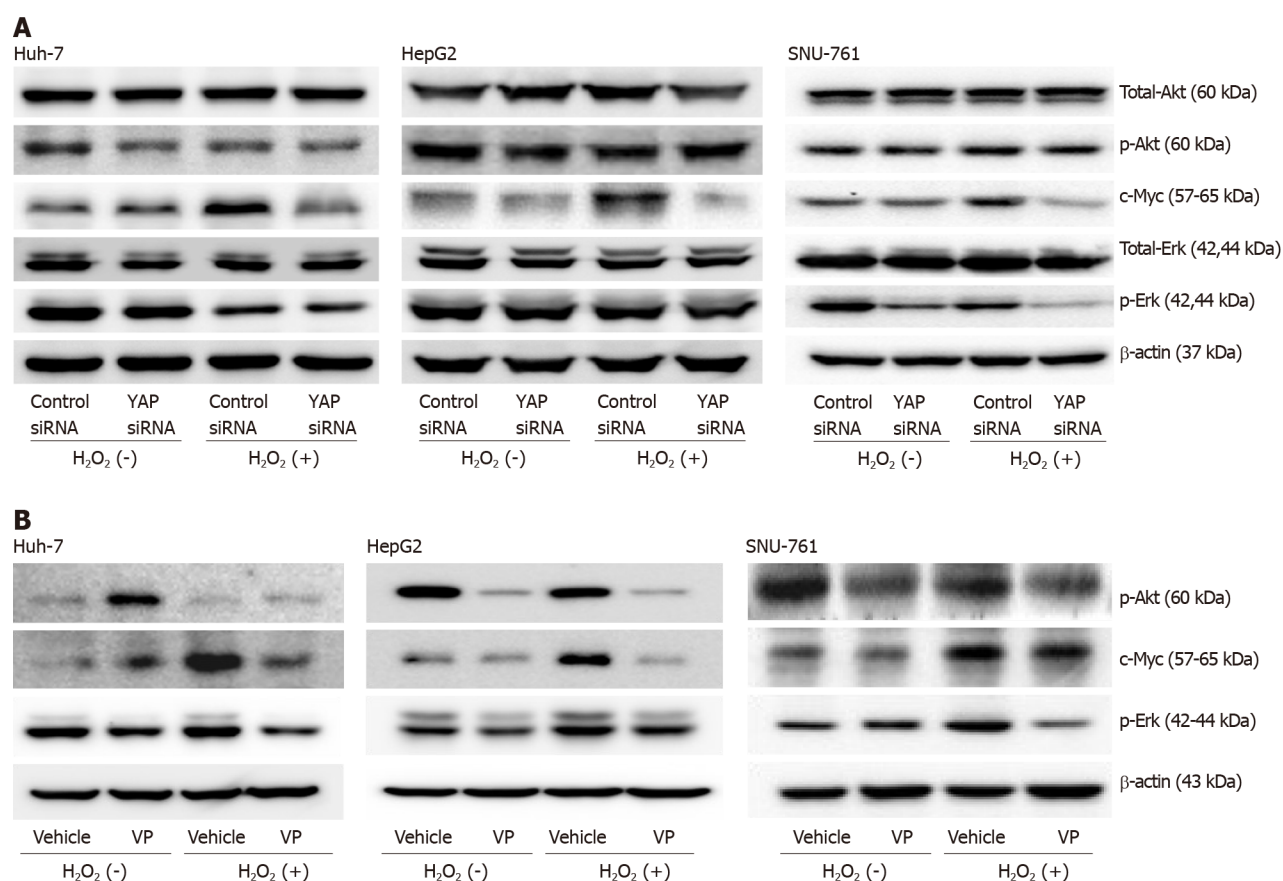


Figure 4 The oncogenic action of yes-associated protein-1 was activated by the c-Myc pathway in reactive oxygen species-exposed hepatocellular carcinoma cells. A: Immunoblot analyses of phosphorylated-Akt, total-Akt, c-Myc, phosphorylated-p42/44 (Erk), total-p42/44 (Erk), and yes-associated protein-1 (YAP-1) were performed in reactive oxygen species (ROS)-exposed hepatocellular carcinoma (HCC) cells transfected with YAP-1 small interfering RNA (siRNA) or control siRNA. The experiment was repeated three times; B: Immunoblot analyses of phosphorylated-Akt, total-Akt, c-Myc, phosphorylated-p42/44 (p-Erk), total-p42/44 (Erk), and YAP-1 were performed in ROS-exposed HCC cells treated with verteporfin or control. The experiment was repeated three times. YAP: Yes-associated protein; siRNA: Small interfering RNA; VP: Verteporfin.

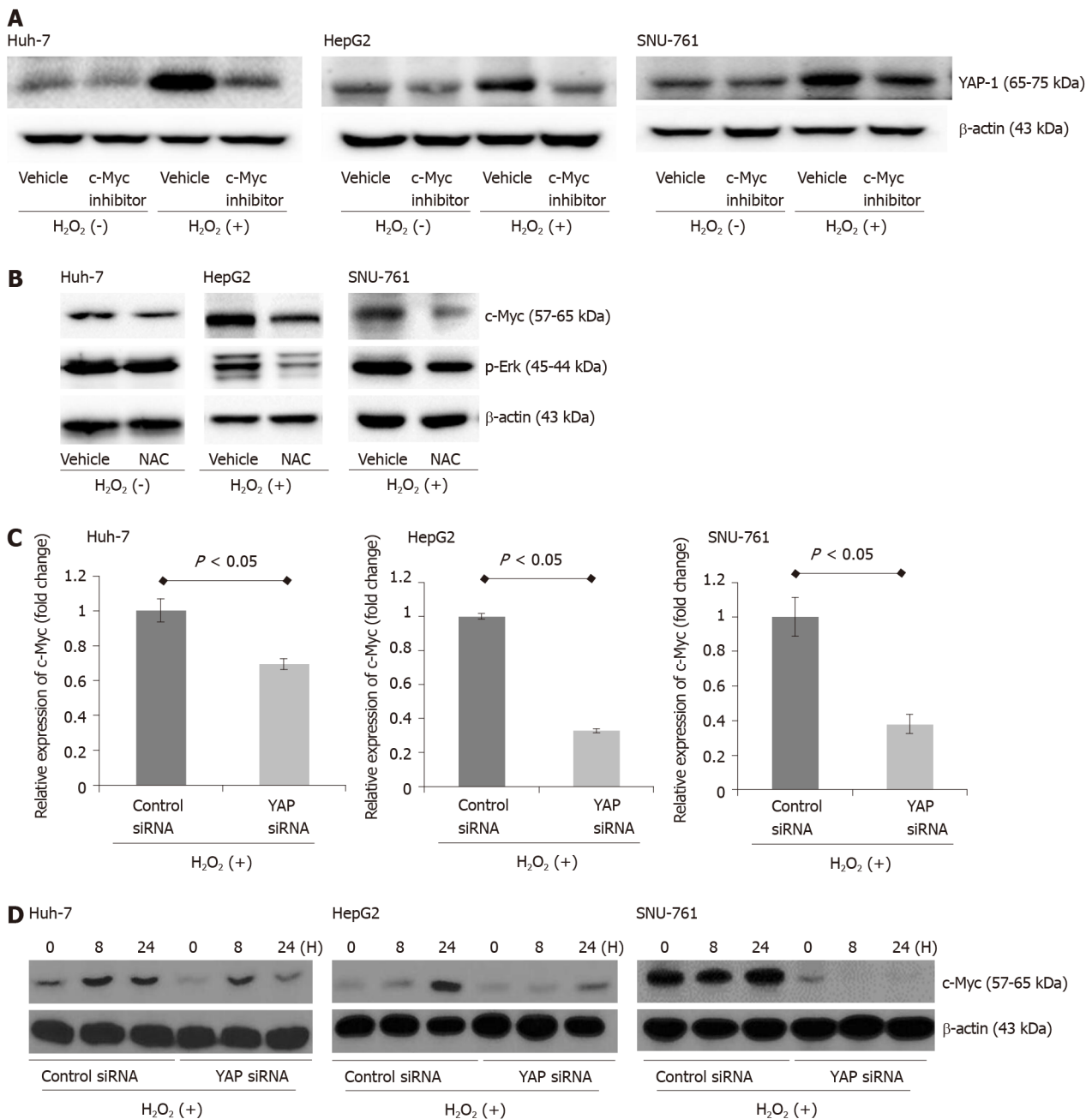


Figure 5 Up-regulation of the c-Myc pathway was dependent on yes-associated protein-1 expressions in reactive oxygen species-exposed hepatocellular carcinoma cells. A: A c-Myc inhibitor (10058-F4, 60 μmol/L) significantly decreased the protein expression of yes-associated protein-1 (YAP-1) in reactive oxygen species (ROS)-exposed hepatocellular carcinoma (HCC) cells. The experiment was repeated three times; B: N-acetylcysteine treatment downregulated c-Myc protein expression in ROS-exposed HCC cell lines. The experiment was repeated three times; C: YAP-1 small interfering RNA (siRNA) transfection significantly suppressed c-Myc mRNA expression compared to control siRNA transfection in ROS-exposed HCC cells (all $P < 0.05$). The c-Myc mRNA expression was quantified using quantitative PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression. The data are expressed as the mean \pm SD. The experiment was repeated three times; D: Immunoblot analyses of c-Myc were performed in ROS-exposed HCC cells transfected with YAP-1 siRNA or control siRNA. The experiment was repeated three times. YAP: Yes-associated protein; siRNA: Small interfering RNA; NAC: N-acetylcysteine.

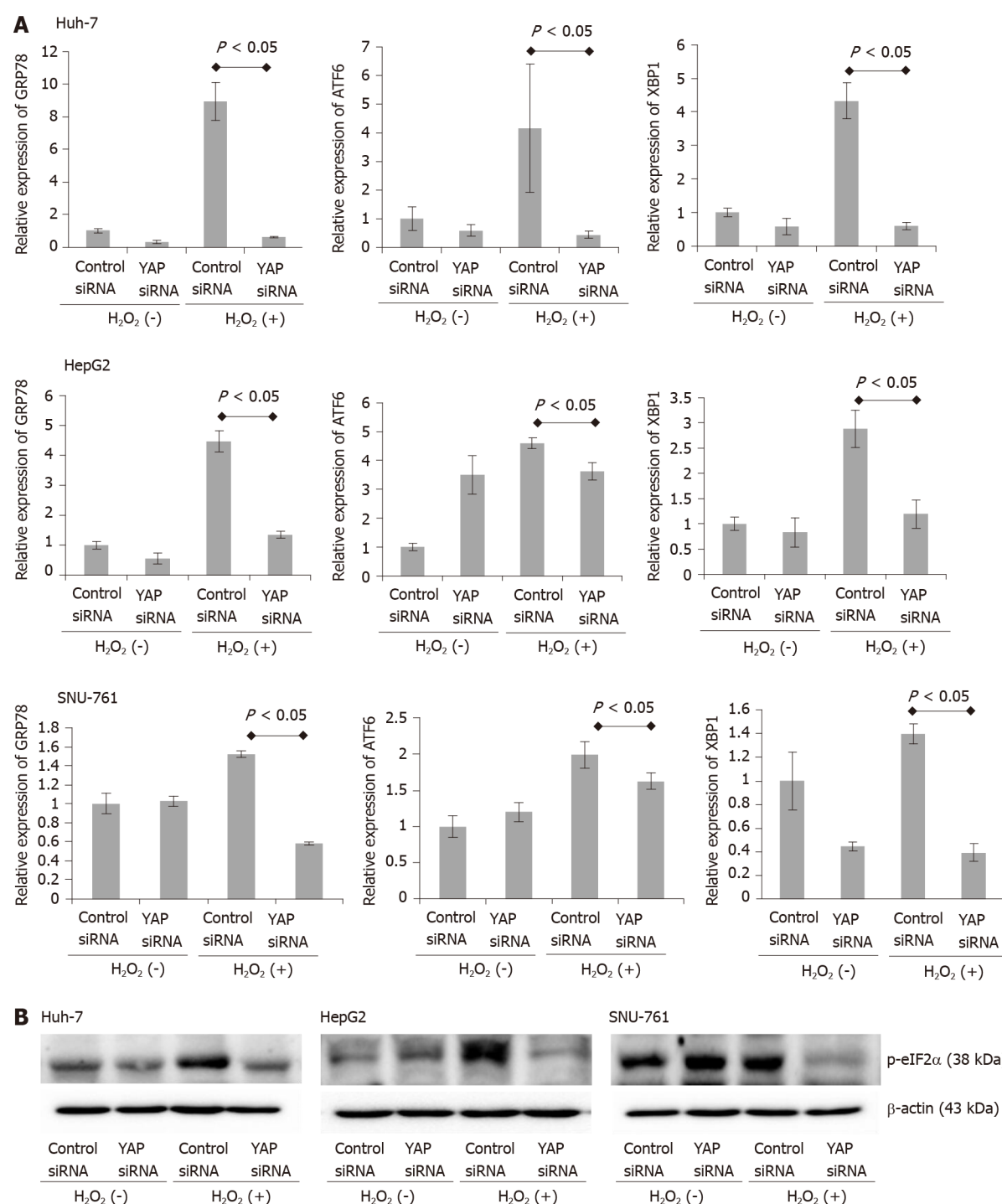


Figure 6 The reactive oxygen species-induced oncogenic action of yes-associated protein-1 in hepatocellular carcinoma cells led to an enhanced unfolded protein response. A: Yes-associated protein-1 (YAP-1) small interfering RNA (siRNA) significantly decreased the mRNA expression of unfolded protein response markers, including 78-kDa (glucose-regulated protein 78/BiP), activating transcription factor-6, and XBP1, in reactive oxygen species (ROS)-exposed hepatocellular carcinoma (HCC) cells ($P < 0.05$). The experiment was repeated three times. The data are expressed as the mean \pm SD; B: Immunoblot analyses of YAP-1 and phosphorylated-eIF-2 α were performed in ROS-exposed HCC cells transfected with YAP-1 siRNA or control siRNA. The experiment was repeated three times. GRP78: Glucose-regulated protein 78; ATF-6: Activating transcription factor-6; YAP: Yes-associated protein; siRNA: Small interfering RNA.

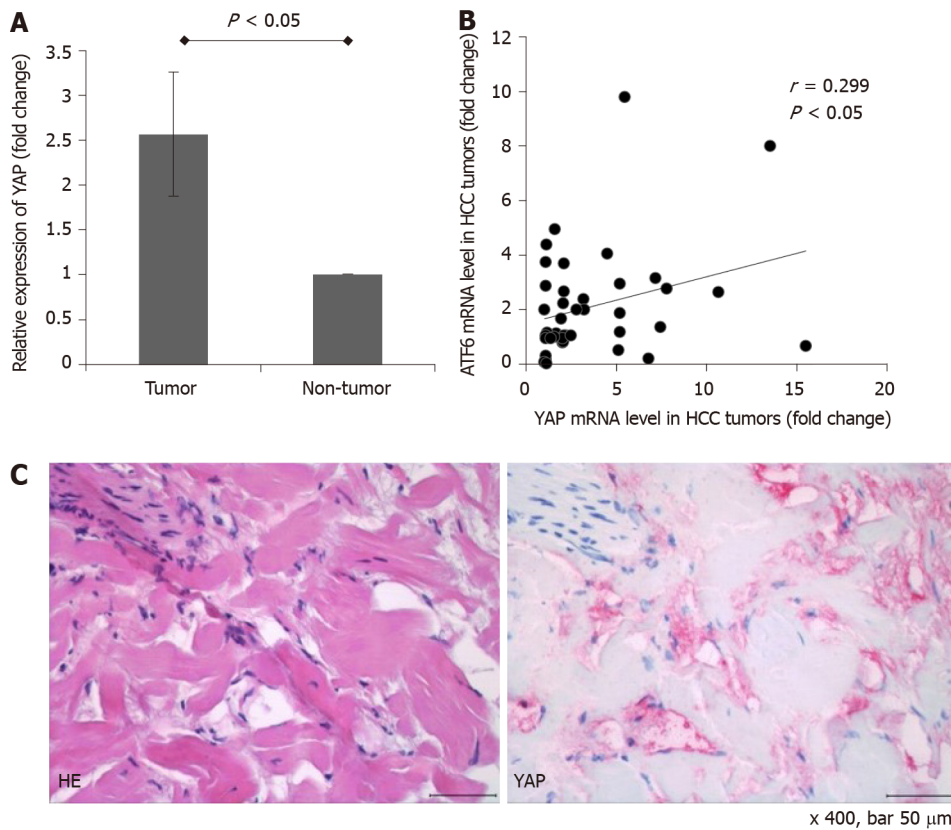


Figure 7 Upregulated mRNA expression of yes-associated protein-1 was correlated with the expression of activating transcription factor-6 in human hepatocellular carcinoma tissues. A: The mean mRNA expression of yes-associated protein-1 (YAP-1) was upregulated by 2.6-fold in hepatocellular carcinoma (HCC) tissues compared with nontumor tissues ($n = 88$). The data are expressed as the mean \pm SD; B: The mRNA expression of YAP-1 was positively correlated with the mRNA expression of ATF6 (Pearson's coefficient = 0.299; $P < 0.05$); C: The expression of YAP-1 in human HCC tissue was detected by immunohistochemistry (400 \times magnification). Scale bars, 50 μ m. YAP: Yes-associated protein; HCC: Hepatocellular carcinoma; SD: Standard deviation; ATF-6: Activating transcription factor-6; HE: Hematoxylin-eosin.

ARTICLE HIGHLIGHTS

Research background

Reactive oxygen species (ROS) contribute to tumor progression by promoting DNA damage and altering cell signaling pathways. It has been recently suggested that ROS are involved in tumor metastasis, which is a complex process that includes epithelial-to-mesenchymal transition, migration, invasion, and angiogenesis within the tumor microenvironment.

Research motivation

Oxidative stress is the most important causative factor of hepatocellular carcinoma (HCC). The major etiologies of HCC, including chronic hepatitis B or C, alcohol-related liver disease, and nonalcoholic fatty liver disease, increase ROS levels. Thus, the activation of yes-associated protein-1 (YAP-1) by ROS-induced damage has been hypothesized to exacerbate the progression of HCC.

Research objectives

We investigated the activation of YAP-1 by ROS-induced damage in HCC and the involved signaling pathway.

Research methods

The expression of YAP-1 was quantified using real-time PCR and immunoblotting. Human HCC cells were treated with H_2O_2 , and with either YAP-1 small interfering RNA (siRNA) or control siRNA. MTS assays were performed to evaluate HCC cell proliferation. To investigate the signaling pathway, immunoblotting was performed. Eighty-eight surgically resected frozen HCC tissues and 88 nontumor paired liver tissues were used for gene expression analyses.

Research results

H₂O₂ treatment increased the mRNA and protein expression of YAP-1 in HCC cells. Suppression of YAP-1 resulted in a significant decrease in tumor proliferation during H₂O₂ treatment both *in vitro* and *in vivo*. The oncogenic action of YAP-1 occurred *via* the activation of the c-Myc pathway, leading to the upregulation of components of the unfolded protein response, including 78-kDa glucose-regulated protein and activating transcription factor-6 (ATF-6). The YAP-1 mRNA levels in human HCC tissues were upregulated by 2.6-fold compared with those in nontumor tissues and were positively correlated with the ATF-6 Levels.

Research conclusions

This study shows a novel connection between YAP-1 and the unfolded protein response (UPR) through the c-Myc pathway during oxidative stress in HCC. We speculate that the interaction between YAP-1 and c-Myc is a point of convergence that allows HCC proliferation.

Research perspectives

The ROS-induced activation of YAP-1 *via* the c-Myc pathway, which leads to the activation of the UPR pathway, might be a therapeutic target in HCC.

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