

HO-1 is a favorable prognostic factor for HBV-HCC patients who underwent hepatectomy

Chun-Nan Yeh¹
Ren-Chin Wu²
Chi-Tung Cheng¹
Chun-Yi Tsai¹
Yau-Ren Chang¹
Ta-Sen Yeh¹
Tsung-Han Wu¹
Wei-Chen Lee¹
Kun-Chun Chiang³

¹Department of Surgery, Liver Research Center, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan;

²Department of Pathology, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan;

³General Surgery Department, Chang Gung Memorial Hospital, Chang Gung University, Keelung, Taiwan

Background: More than 500,000 people suffered from hepatocellular carcinoma (HCC) annually and the relative incidence to mortality rate indicates its unfavorable prognosis. Several studies have proved that heme-oxygenase-1 (HO-1) is indirectly engaged in the invasion and the metastasis of some types of malignancies, including breast cancer, prostate cancer, and lung cancer. The role of HO-1 in hepatitis B virus (HBV)-related HCC is still not clarified.

Materials and methods: The Western blot, doubling time, cell cycle analysis, migration assay, invasion assay, gene transfection, xenograft animal model, immunohistochemistry staining, and clinical validation study were applied in this study.

Results: HO-1 overexpression not only decreased the growth but also inhibited the migration and invasion in human HBV-HCC cells (Hep-3B vs PLC/PRF/5). The inhibitory effect on growth, migration, and invasion is further demonstrated by the overexpression of HO-1 in Hep-3B cell by transfection study. Furthermore, HO-1 decreasing the growth of HBV-HCC was confirmed in animal study. The clinical validation illustrated that higher HO1 expression was also associated with favorable disease-free survival of HBV-HCC patients who underwent hepatectomy.

Conclusions: We identified HO-1 as a favorable prognostic factor for HBV-HCC patients who underwent hepatectomy.

Keywords: HO-1, HBV, HCC, hepatectomy

Introduction

Hepatocellular carcinoma (HCC), comprising 80% of primary hepatic cancers, affects more than 500,000 people annually and ranked number four of tumor-related deaths globally.¹ The relative incidence to HCC's mortality rate indicates its unfavorable prognosis.¹ Chronic hepatitis B virus (HBV) infection is the most important etiologic agent of HCC accounting for nearly 55%–60% causes of HCC worldwide.²

Currently, partial hepatectomy still remains the treatment of choice for patients with resectable HCC in noncirrhotic liver. Growing evidences have suggested that liver transplantation and radiofrequency ablation (RFA) of tumor could provide comparable survival benefits when compared with partial hepatectomy, especially when the size of tumors are smaller than 3 cm.³ Unfortunately, most patients with HCC (70%–80%) are diagnosed at intermediate to advanced stage⁴ and there is no available and effective treatment currently.^{5,6} For all these advanced HCC patients, who are not fit for radical therapies and have poor response to traditional chemotherapy and radiotherapy, the general survival time is usually <6 months. Under these dismal conditions, it is always a priority to develop a new ammunition in battling HCC.

Correspondence: Chun-Nan Yeh
Department of Surgery, Liver Research Center, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan 5, Fu-Hsing Street, Kwei-Shan, Taoyuan, Taiwan
Tel +886 33 28 1200 ext 3219
Fax +886 33 28 5818
Email yehchunna@gmail.com

Kun-Chun Chiang
General Surgery Department, Chang Gung Memorial Hospital, Chang Gung University, Keelung 204, Taiwan
Tel +886 28 71 2121 ext 2508
Fax +886 28 75 7858
Email robertviolet6292@gmail.com

Heme-oxygenase (HO), the rate-limiting enzyme in the metabolism of heme, works as the catabolism to degrade the heme group into carbon monoxide (CO), free iron, and biliverdin.⁷ Several research studies also showed that HO-1 overexpression is involved in the pathogenesis and progression of some types of malignancies.⁸ In tumor-bearing animal model, overexpression of HO-1 induces increased proliferation, viability, and angiogenic potential of melanoma cells as well as augmented metastasis.⁹ Recent studies have indicated that HO-1 is indirectly implicated in invasion and metastasis of some types of malignancies, such as breast cancer,^{10,11} prostate cancer,^{12,13} and lung cancer.¹⁴

The role of HO-1 in HCC is still not well clarified, although HO-1 has been shown to repress invasive ability of Hep G2 cell and tumor growth in vivo.¹⁵ This study demonstrated the role of HO-1 in HCC in vitro, in vivo, and its clinical impact, especially for hepatitis B-related HCC patients.

Materials and method

Cell culture

Hep3B and PLC/PRF/5 cells (human HCC cells that express HBV) were purchased from Bioresource Collection and Research Center (60434 and 60223, Hsinchu, Taiwan) and maintained a previously described.¹⁶

The assay of cell proliferation by WST-1 kit

Hep3B, PLC/PRF/5 cells, HO-1 transfected Hep3B, and the mock-transfected Hep3B cells were placed at proximate 1,000 cells per cm² in a Costar® 48 Well Clear TC-Treated Multiple Well Plates (#3548; Corning Incorporated, Corning, NY, USA). The viable cells were evaluated by Cell Proliferation Reagent WST-1 (#11 644 807 001; Hoffman-La Roche Ltd., Basel, Switzerland).

Transwell filter migration assay

The migration assay was conducted as described previously.¹⁷ After 24 hours, the migrating cells were stained and counted under four random high-power microscopic fields (100×) per filter. The experiments were performed in triplicates.

Matrigel invasion assay

The matrigel invasion assay was performed as previously described.¹⁷ Briefly, 600 µL of RPMI 1640 medium with 10% FBS was added to the lower chamber of the 24 well-plate. Next, 100 µL of cells suspension at a density of 2×10⁵ cells/mL were cultured in serum-free RPMI 1640 medium in the upper well. The plates were incubated in 5% CO₂ atmosphere

at 37°C for 24 hours. The cells that migrated to the opposite side of the matrigel-coated membrane were fixed in 4% paraformaldehyde (0.4% NaOH, 4% paraformaldehyde, and 3.8% hydrated sodium borate, pH 9.5). The number of invading cells were digitally photographed and counted under the microscope (IX71; Olympus Corporation, Tokyo, Japan). Experiments were performed in triplicates and repeated at least three times.

HO-1 expression vector and stable transfection

The full-length of the human HO-1cDNA clone (MGC: 45655) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The human HO-1 expression vector was constructed by cloning the HO-1 cDNA into the pcDNA3.1/Zeo expression vector (Thermo Fisher Scientific), with Hind III and Eco R1 sites. Proper ligation was confirmed by extensive restriction mapping and sequencing. Electroporation was conducted using an ECM 830 Square Wave Electroporation System (BTX, San Diego, CA, USA), set at 180 V, 70-ms pulse length, and one pulse setting. Transfected Hep3B cells were selected by 100 µg/mL of Zeocin (Thermo Fisher Scientific). For the construction of the mock-transfected HCC cells, the cells were transfected with a controlled pcDNA3.1/Zeo expression vector (Thermo Fisher Scientific) and were clonally selected in the same manner as described above.

Cell cycle analysis

Cells were first serum starved for 24 hours before analysis as previously described.¹⁷ Cell cycle analysis was performed using FACS-Calibur cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA, USA).

Western blot

The procedures for protein extraction, blocking, and immunodetection were described previously.¹⁸ The primary antibodies used in this study were monoclonal antibodies against HO-1 (1:1,000, GENETEX #GTX101147; Gene-Tex Inc., Irvine, CA, USA), CDK4 (#2906; Cell Signaling Technology, Irvine, CA, USA), CDK6 (#3136; Cell Signaling Technology), cyclin D3 (#2936; Cell Signaling Technology), E-cadherin (1:1,000, #3195; Cell Signaling Technology), N-cadherin (1:1,000, #13116; Cell Signaling Technology), Snail (1:1,000, #3879; Cell Signaling Technology), and Vimentin (1:400, #V6630; Sigma-Aldrich Co., St. Louis, MO USA). The secondary antibodies were anti-rabbit (111-035-003; Jackson ImmunoResearch

Laboratories, Inc., West Grove, PA, USA) or anti-mouse secondary antibodies (Zymed 81–6520; 1:5,000). The blots were detected using ECL reagents (WBKLS0500; EMD Millipore, Billerica, MA, USA). Membranes were detected by VersaDoc™ Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA) for analysis.

F-actin staining

The cells were seeded onto glass bottom culture dishes (MatTek, Ashland, MD, USA) precoated with 50 mL fibronectin and allowed to attach overnight. Then the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 1% BSA at room temperature. F-actin protein expression was detected by incubation with Texas Red X-Phalloidin, and immunofluorescence was examined using confocal microscopy (LSM510 Meta; Zeiss, Oberkochen, Germany) as previously described.¹⁹

Xenograft animal model

The animal use protocol had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Chang Gung Memorial Hospital (CGMH) (IACUC 2014032605), and the Committee recognized that the proposed animal experiment follows the Animal Protection Law by the Council of Agriculture. Executive Yuan, R.O.C. and the guidelines as shown in the Guide for the Care and Use of Laboratory Animals as promulgated by the Institute of Laboratory Animal Resources, National Research Council, USA. In all the procedures, every effort was made to minimize the suffering of the laboratory animals and the number of animals used as described previously. We obtained male nude mice (BALB/cAnN-Foxn1) from the National Laboratory Animal Center, Taiwan, at age 4 weeks. One week later, the mice were anesthetized by intraperitoneal ketamine (100 µg/mL)-xylazine (10 µg/mL) at a dose of 50 µL/Kg body weight. The cells were detached from cell flask by treatment with 0.5 µM EDTA in PBS solution and washed with RPMI1640 medium with 10% FCS. Then, the cells were resuspended in a PBS solution at the concentration indicated. About 1×10^7 cells/100 µL were injected subcutaneously on the right and left lateral back wall in close proximity to the shoulder of each mouse. We then measured the growth of the xenografts by Vernier caliper measurements, taken every 2–3 days. The tumor volume will be determined by the formula: $\text{volume} = \frac{\pi}{6} \times \text{larger diameter} \times (\text{smaller diameter})^2$.¹⁷ The pathological examination will be performed by two investigators at the Department of Pathology, CGMH.

Immunohistochemical staining of 70 human HCC tissues for HO-1

A total of 70 HBV-HCC specimens were obtained from patients admitted to the Chang Gung Memorial Hospital for curative hepatectomy. Curative hepatectomy is defined as patient with HCC undergoing hepatic resection with negative microscopic margin. The protocol was approved by the IRB of the Chang Gung Memorial Hospital (Approval: IRB 103-1827B; this study was conducted in accordance with the Declaration of Helsinki). All the 70 HBV-HCC patients provided written informed consent. HO-1 expression levels in the aforementioned 70 HCC patients were examined by immunohistochemical staining. Tissue sections (4 µm) prepared from the formalin-fixed, paraffin-embedded hepatectomy specimens were incubated with the primary antibody against HO-1 (#ab52947, 1:250 dilution; Abcam, Cambridge, UK) at 4°C overnight. After washing three times with TBST (5 minutes each), the signals were visualized with the Dako Labelled Streptavidin-Biotin2 (LSAB2) System-HRP (No. K0675; Dako Denmark A/S, Glostrup, Denmark). Control slides were incubated with the secondary antibody only. For the assessment of immunohistochemical staining, the percentage of stained target cells was evaluated in ten random microscopic fields per tissue section (400× magnification), and their averages were subsequently calculated. Staining intensities were scored as 1 (mild), 2 (moderate), or 3 (strong). H scores were calculated as the percentage of positive staining (0–100) × the corresponding staining intensity (0–3). Specimens with H-scores of <60 or ≥60 were classified as having low or high expression, respectively (range: 5–295; median 100).

Statistical analysis

All data were presented as percentage of patients or mean with SD. Numerical data were compared by independent two-sample *t* tests. Nominal data were compared by Pearson chi-squared test, Fisher's exact test, or multiple forward stepwise logistic regression test when appropriate. Survival was calculated and plots constructed according to the Kaplan–Meier method. Furthermore, the log-rank test was performed for a statistical univariate analysis of prognostic variables. All aforementioned factors were input for Cox proportional hazard model once statistical significance proved by univariate analysis. An “enter-selection” procedure to select the most relevant prognostic factors and only factors that remained significant ($P < 0.05$) were included in the final model. We performed all statistical analyses using IBM SPSS Statistics for Windows (ver. 20.0; IBM Corporation, Armonk, NY, USA). Furthermore, $P < 0.05$ was considered statistically significant.

Results

Increasing intracellular HO-1 protein levels decreased cell growth, invasion, and migration between Hep3B and PLC/PRF/5 cells

Significantly decreased intracellular HO-1 protein level was shown in Hep3B cells compared with PLC/PRF/5 cells (Figure 1A). As shown in Figure 1B, the double time of Hep3B cells or PLC/PFR/5 cells is 56.6 ± 3.3 or 66.2 ± 0.5 hours, respectively. To evaluate if HO-1 could affect cell metastatic ability of Hep3B or PLC/PFR/5 cells, migration and invasion assays were thus conducted. Figure 1C shows that PLC/PFR/5 cells had only $17\% \pm 8.4\%$ migration ability when compared with Hep3B cells (100%). HO-1 overexpression also reduced cell invasion ability to $22\% \pm 16.1\%$ in PLC/PFR/5 cells when compared with Hep3B cells (100%) (Figure 1D).

Raising levels of intracellular HO-1 protein by transfecting cells with the HO-1 expression vector significantly decreased cell growth and induces cell cycle arrest at G1 phase in Hep3B cells

To evaluate the effect of HO-1 overexpression on Hep3B cell growth, HO-1 was overexpressed in Hep3B cells

represented by Hep3B HO-1 cells (Hep3B cell with HO-1 overexpression) and mock overexpression of HO-1 in Hep3B cells represented by Hep3B DNA cells (Hep3B cell with mock overexpression of HO-1) (Figure 2A). As shown in Figure 2B, the double time of Hep3B DNA cells or Hep3B HO-1 cells is 17 ± 0.8 or 36 ± 2 hours, respectively. To further investigate how HO-1 influenced Hep3B cell growth, the distribution of cell cycle of Hep3B DNA cells and Hep3B HO-1 cells was then monitored by flow cytometry. Figure 2C demonstrates that Hep3B HO-1 cells had higher percentage of G1 phase cells than Hep3B DNA cells ($60\% \pm 2\%$ vs $48\% \pm 1.2\%$). The result of Western blot showed that HO-1 overexpression repressed CDK4, CDK6, and Cyclin D3 expression in Hep3B cells (Figure 2D).

HO-1 overexpression decreased the metastatic potential of Hep3B cells

To evaluate if HO-1 could affect cell metastatic ability of Hep3B cells, migration and invasion assays were thus conducted. Figure 3A shows that Hep3B HO-1 cells had only $22\% \pm 3\%$ migration ability when compared with Hep3B DNA cells (100%). HO-1 overexpression also reduced cell invasion ability to $17\% \pm 3\%$ in Hep3B

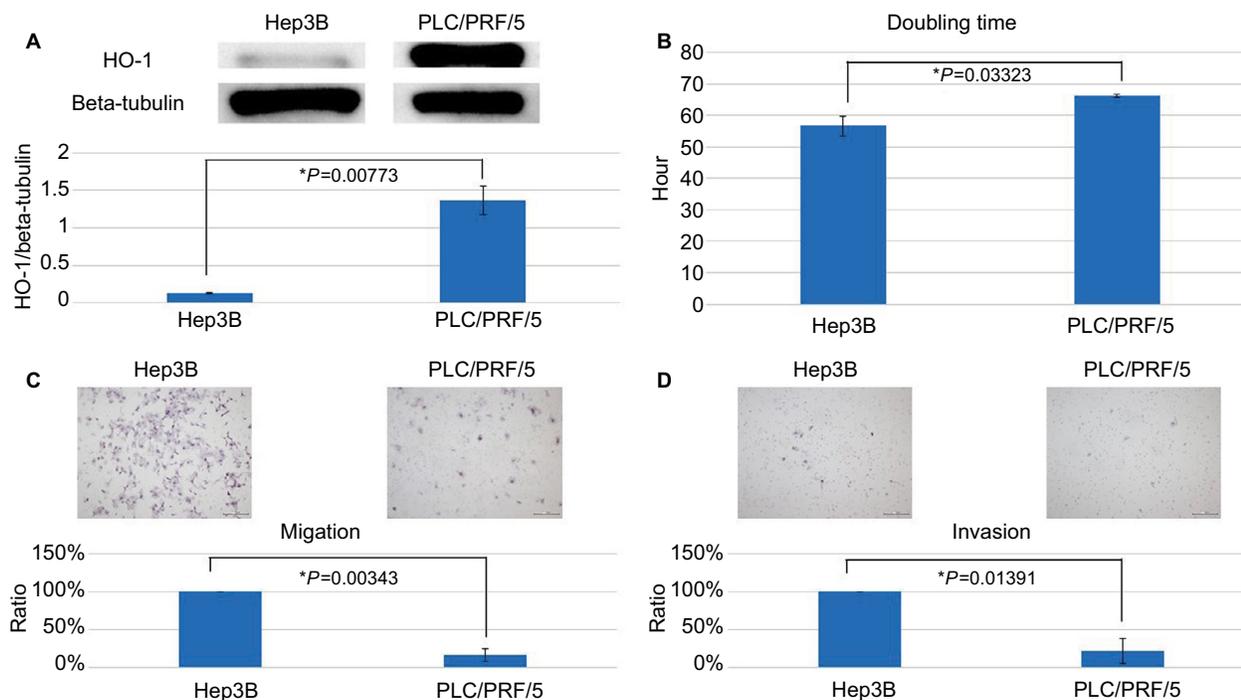


Figure 1 Influence of intracellular HO-1 protein levels on cell growth, invasion, and migration between Hep3B and PLC/PRF/5 cells. **Notes:** (A) Significantly decreased intracellular HO-1 protein level was shown in Hep3B cells compared with PLC/PRF/5 cells. (B) The double time of Hep3B cells or PLC/PFR/5 cells is 56.6 ± 3.3 or 66.2 ± 0.5 hours, respectively. (C) To evaluate if HO-1 could affect cell metastatic ability of Hep3B or PLC/PFR/5 cells, migration and invasion assays were thus conducted. PLC/PFR/5 cells had only $17\% \pm 8.4\%$ migration ability when compared with Hep3B cells (100%). (D) HO-1 overexpression also reduced cell invasion ability to $22\% \pm 16.1\%$ in PLC/PFR/5 cells when compared with Hep3B cells (100%).

Abbreviation: HO-1, heme-oxygenase-1.

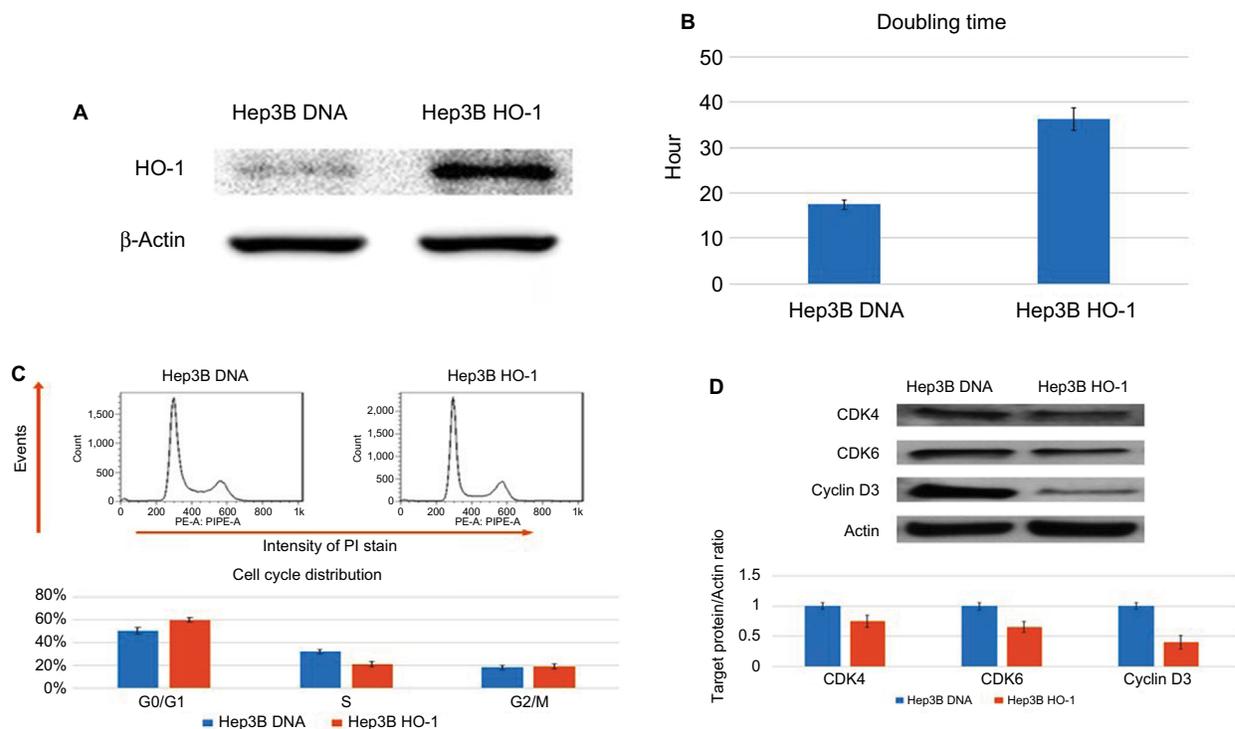


Figure 2 Increasing intracellular HO-1 protein levels by transfecting cells with the HO-1 expression vector significantly decreased cell growth and induced cell cycle arrest at G1 phase in Hep3B cells.

Notes: (A) HO-1 was overexpressed in Hep3B cells represented by Hep3B HO-1 cells (Hep3B cell with HO-1 overexpression) and mock overexpression of HO-1 in Hep3B cells represented by Hep3B DNA cells (Hep3B cell with mock overexpression of HO-1). (B) The double time of Hep3B DNA cells or Hep3B HO-1 cells is 17 ± 0.8 or 36 ± 2 hours, respectively. (C) The cell cycle distribution of Hep3B DNA cells and Hep3B HO-1 cells were then analyzed by flow cytometry. Hep3B HO-1 cells had higher percentage of G1 phase cells than Hep3B DNA cells ($60 \pm 2\%$ vs $48 \pm 1.2\%$). (D) The Western blot showed that HO-1 overexpression repressed CDK4, CDK6, and Cyclin D3 expression in Hep3B cells.

Abbreviations: HO-1, heme-oxygenase-1.

HO-1 when compared with Hep3B DNA cells (100%) (Figure 3B).

Evaluation of the effects of HO-1 on E-cadherin, N-cadherin, Snail, Vimentin, and F-actin expressions in Hep3B cells

Since E- and N-cadherin play vital roles during cell migration and invasion, we investigated whether HO-1 would influence E- and N-cadherin expression in Hep3B cells. Figure 3C shows that N-cadherin expression was inhibited to 0.6 ± 0.08 fold by HO-1 overexpression while E-cadherin expression was increased to 6.2 ± 0.18 fold by HO-1 overexpression in Hep3B cells. Epithelial–mesenchymal transition (EMT) is a process which could make cells more invasive. As shown in Figure 3C, EMT-inducing transcriptional factor, Snail, was downregulated by HO-1 in Hep3B cells with the mesenchymal cell marker, Vimentin, also being inhibited. The HO-1 effect on F-actin synthesis in Hep3B cells was further studied by immunofluorescent stain. Figure 3D shows that Hep3B HO-1 cells had lower F-actin expression than Hep3B DNA cells.

Evaluation of HO-1 effect on tumor growth and tumorigenesis of Hep3B cells in vivo

As we conducted xenograft to Hep3B DNA cells and Hep3B HO-1 cells into nude mice, 4 weeks later, the tumor volume increased to $176 \pm 12\%$ in Hep3B DNA cell group (Figure 4). As for Hep3B HO-1 cells, all the xenografted tumors disappeared after 3 weeks of implantation.

Correlations between the expression of HO-1 and clinicopathological characteristics in patients with HBV-HCC undergoing hepatectomy

Table 1 summarizes the clinicopathological characteristics of 70 HBV-HCC patients, and we continued to analyze the correlations between HO-1 expression efficacy and clinicopathological characteristics in 70 HBV-HCC patients. Among specimens from 70 HBV-HCC patients undergoing hepatectomy, 37 (52.9%) revealed strong HO-1 staining intensity (H-scores ≥ 50). The interesting finding is that HO-1

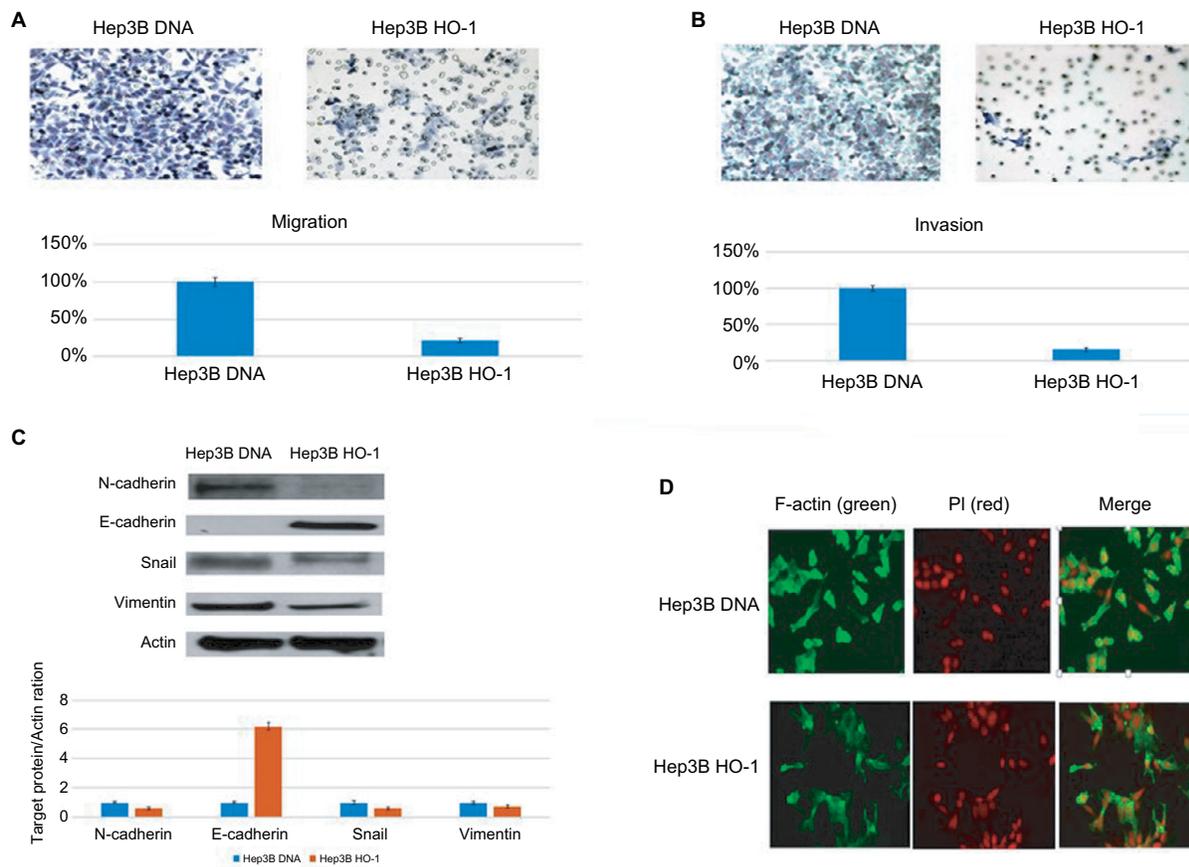


Figure 3 HO-1 overexpression decreased metastatic potential of Hep3B cells.

Notes: (A) Hep3B HO-1 cells had only 22%±3% migration ability when compared with Hep3B DNA cells (100%). (B) HO-1 overexpression also reduced cell invasion ability to 17%±3% in Hep3B HO-1 when compared with Hep3B DNA cells (100%) (Figure 3B). (C) N-cadherin expression was inhibited to 0.6±0.08 fold as HO-1 overexpression, whereas E-cadherin expression was increased to 6.2±0.18 fold by HO-1 overexpression in Hep3B cells. EMT-inducing transcriptional factor, Snail, was downregulated by HO-1 in Hep3B cells with the mesenchymal cell marker, Vimentin, also being inhibited. (D) Hep3B HO-1 cells had lower F-actin expression than Hep3B DNA cells.

Abbreviations: EMT, epithelial–mesenchymal transition; HO-1, heme-oxygenase-1.

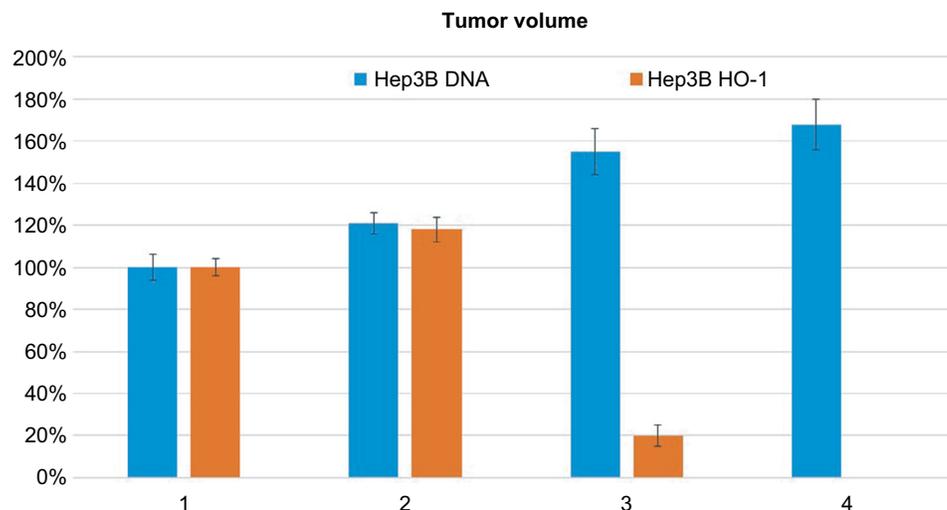


Figure 4 Evaluation of HO-1 effect on tumor growth and tumorigenesis of Hep3B cells in vivo.

Notes: As we xenografted Hep3B DNA cells and Hep3B HO-1 cells into the nude mice, 4 weeks later, the tumor volume increased to 176%±12% in Hep3B DNA cells group. As for Hep3B HO-1 cells, all the xenografted tumors disappeared after 3 weeks of implantation.

Abbreviation: HO-1, heme-oxygenase-1.

upregulation significantly correlated with smaller tumor size, less vascular invasion, and less tumor recurrence; however, no parameter was independently associated with HO-1 overexpression (Table 2).

HO-1 overexpression has potential to be a favorable prognostic indicator for HBV-HCC patients undergoing hepatectomy

Univariate log-rank analysis was then conducted to identify the factors that adversely influenced the disease-free survival (DFS) rate in the HCC patients mentioned above. We found that both the presence of symptoms of the disease (vascular

invasion and satellite lesion) and the weak staining intensity of HO-1 correlated with inferior DFS (Table 3). However, vascular invasion and the weak staining intensity of HO-1 were identified as individualized predictors for an inferior DFS rate in the patients after multivariate Cox proportional hazard analysis (Table 3; Figure 5).

Discussion

Zou et al ever used in vitro and in vivo studies to show that HO-1 inhibits the migration and tumor growth of human HCC cells by suppressing IL-6 expression, irrespective of hepatitis infection.¹⁵ However, the function of HO-1 in human HBV-HCC has not been well determined.

HO, the rate-limiting enzyme in the metabolism of heme, works as the catabolism to degrade the heme group into CO, free iron, and biliverdin.⁷ Isoforms of three mammalian HO have been identified, including HO-1, HO-2, and HO-3, with patterns of distinct tissue-specific expression. For example, HO-1 is inducible, highly expressed in the spleen and liver,^{8,20} and HO-2 expression is constitutively seen in most tissues except testes and the brain.²¹ The HO-1 upregulation has been regarded as cyto-protection and reactive response to oxidative stress and other stimuli,^{22,23} including reactive oxygen species (ROS).²⁴ Chronic virus infection such as HBV may enhance ROS production and cause oxidative stress in host cells.²⁵ Yuan et al ever illustrated that the production of HBV-induced mitochondrial ROS represses the SOCS3 expression through the silencing of Snail-mediated epigenetic, leading to the sustained activation of IL-6/STAT3 pathway and extremely affecting hepato-carcinogenesis.²⁶ This may explain HO-1 expression in most HBV-HCC patients (almost 60% in our clinical cohort).

Table 1 Demographic data of 70 HBV-HCC patients

Age (years)	52.48±14.39
Gender (M:F)	54:16
Tumor size (cm)	6.88±4.86
AFP (ng/mL)	2440.48±3911.91
Cirrhosis	36 (51.4%)
Pathological features	
Grading (Edmonson and Stainer)	
Grade 1	3 (4.3%)
Grade 2	32 (45.7%)
Grade 3	21 (30.0%)
Grade 4	5 (7.1%)
Capsule formation	43 (69.4%)
Tumor rupture	7 (5.6%)
Vascular invasion	27 (38.6%)
Satellite lesions	49 (71.0%)
HO-1 expression	
Low expression	33 (47.1%)
High expression	37 (52.9%)

Abbreviations: AFP, α -fetoprotein; F, female; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HO-1, heme-oxygenase-1; M, male.

Table 2 Association analysis of HO-1 expression among clinicopathological features of 70 HCC patients with hepatitis B infection

	HO-1 low expression	HO-1 high expression	P-value
Age (years)	50.79±14.25	53.98±14.53	0.358
Gender (M:F)	23:10	31:6	0.161
Tumor size (cm)	9.91±5.56	4.85±2.95	<0.001
AFP (ng/mL)	2,985.08±4,210.63	1,952.76±3,613.44	0.274
Cirrhosis	15 (45.5%)	21 (56.8%)	0.345
Grading (Edmonson and Stainer)			
Low grade (I+II)	14 (53.8%)	21 (60%)	0.794
High grade (III+IV)	12 (46.2%)	14 (40%)	
Capsule formation	18 (66.7%)	25 (71.4%)	0.687
Vascular invasion	17 (51.5%)	10 (27.0%)	0.036
Satellite lesions	25 (78.1%)	24 (64.9%)	0.226
Recurrence	31 (93.9%)	25 (67.6%)	0.006

Abbreviations: AFP, α -fetoprotein; F, female; HCC, hepatocellular carcinoma; HO-1, heme-oxygenase-1; M, male.

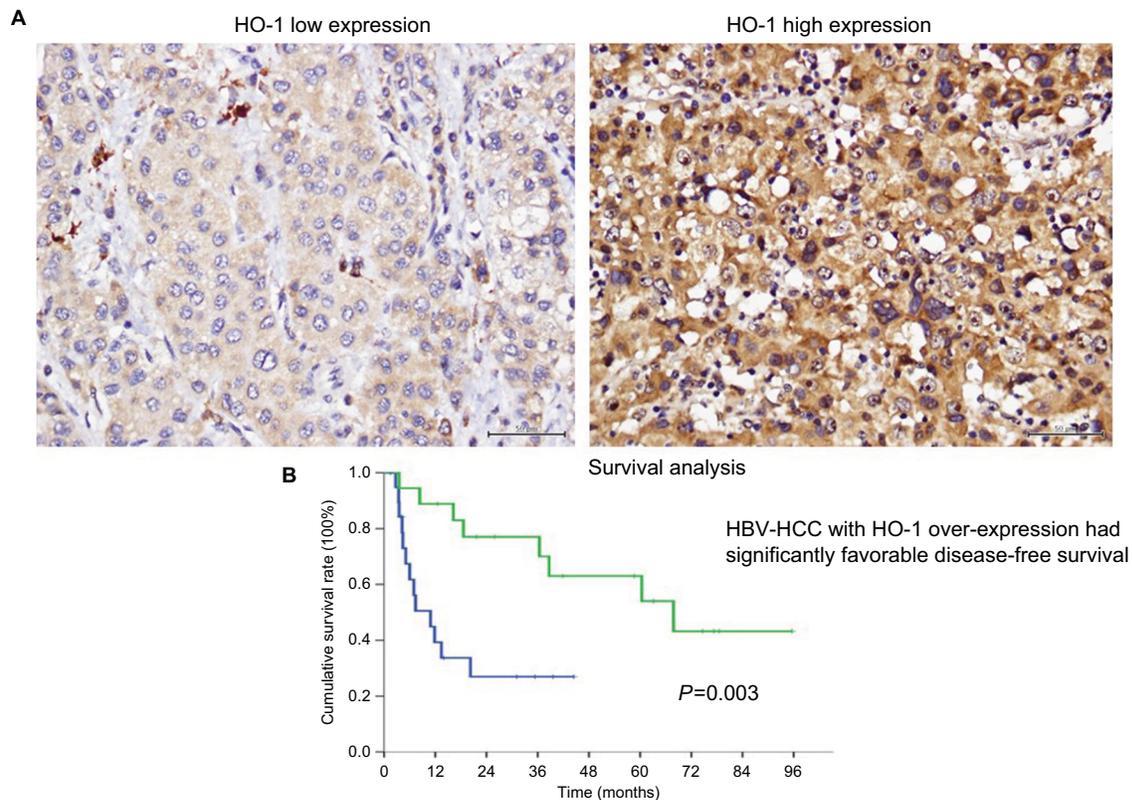


Figure 5 Correlations between HO-1 expression and clinicopathological features in patients with HBV-HCC who underwent hepatectomy.

Notes: (A) Immunohistochemical staining of human HBV-HCC specimen with different intensity scores for HO-1 expression (low and high, respectively; $\times 400$, scale bar = 50 μm). (B) Kaplan–Meier plot of disease-free survival in HBV-HCC patients based on HO-1 expression levels. The high HO-1 expression group had favorable disease-free survival ($P=0.003$). HO-1 high expression is indicated by the green line. HO-1 low expression is indicated by the blue line.

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HO-1, heme-oxygenase-1.

Our current results strongly suggest that HO-1 may be a suppressor gene in human HBV-HCC. As shown in Figure 1A and B, human HCC cell (PLC/PRF/5) expressed higher levels of HO1 and has shorter cell doubling time than Hep3B cell. This result together with our finding that higher HO-1 expression attenuated the migration and invasion growth of Hep3B (Figure 1C and D).

The above observation is further supported by our finding that Hep3B HO-1 cell has longer cell doubling times (Figure 2A and B). During the progression of cell cycle, the regulative interaction of cyclins and cyclin-dependent kinases plays a vital role during cell cycle phase transition. Cyclins and cyclin-dependent kinase complexes are suppressed by CKIs, resulting in cell cycle arrest. In Figure 2C, two main CDKs are shown to be responsible for the G1/S transition; CDK4 and CDK6 were inhibited by HO-1, resulting in decreasing cell cycle progression into S phase with increasing G1 phase cell population (Figure 2C and D). D-type cyclins (D3) bind CDK4 and CDK6, and the activity of cyclin D/CDK complexes promote the entry into cell cycle.^{27,28} Cyclin D/CDK complexes are shown to promote cell cycle progression.

Based on these collective results, we concluded that HO-1 decreases the proliferation of Hep3B cell partially through the downregulation of cyclin D/CDK complexes.

The major cause of HCC-related death resulted from intra- or extra-hepatic metastasis of HCC. HCC cell migration and invasion are indispensable before the metastasis. Our studies illustrated that overexpression of HO-1 in Hep3B cells decreases tumor migration and invasion (Figure 3A and B). E-cadherin, which is an adhesion protein that regulates cell-cell adhesion, is inversely associated with cancer prognosis.²⁹ Its loss may result in cytoskeleton degradation, which increases the motility and invasion of tumor cell. On the contrary, aberrant expression of N-cadherin contributes to tumor migration and invasion.³⁰ In Figure 3C, ectopic expression of HO-1 is shown to increase E-cadherin expression and decrease N-cadherin expression, causing less invasiveness of Hep3B HO-1 cells. EMT, which is also a key factor during tumor metastasis, potentiates cancer cell mobility to metastasis, strengthens resistance to immune system and chemotherapy, and stimulates other stem cell-like characteristics of the cancer.^{31–33} EMT is also strongly associated with HCC metastasis.³⁴

Table 3 Univariate and multivariate analyses of factors influencing disease-free survival of 70 HCC patients with hepatitis B infection who underwent hepatectomy

Factors	Survival time (months)				P-value
	Median	95% CI of median	3-year (%)	5-year (%)	
Age (years)					0.276
≤60 (n=44)	10.13	6.96–13.30	29.5	24.8	
>60 (n=26)	34.39	0.00–71.41	49.4	29.9	
Gender					0.343
Male (n=54)	12.89	7.06–18.71	39.1	28.6	
Female (n=16)	9.47	1.63–17.30	27.8	20.8	
Tumor size (cm)					0.106
≤5 cm (n=34)	19.82	0.00–41.40	41.6	28.2	
>5 cm (n=35)	9.83	6.32–13.34	29.4	22.9	
AFP (ng/mL)					0.296
≤400 ng/mL (n=43)	14.53	6.95–22.11	37.4	29.1	
>400 ng/mL (n=27)	9.47	2.14–16.80	34.7	23.1	
Cirrhosis					0.514
No (n=34)	10.03	6.57–13.48	32.4	26.1	
Yes (n=36)	14.10	6.20–22.00	40.5	27.2	
Grading (Edmonson and Stainer)					0.818
Low grade (I+II) (n=35)	14.10	5.24–22.97	39.1	29.5	
High grade (III+IV) (n=26)	34.39	2.89–65.89	46.4	32.8	
Capsule formation					0.158
Absent (n=19)	10.09	9.16–11.03	26.3	21.1	
Present (n=43)	19.82	0.00–49.54	48.5	34.7	
Vascular invasion					0.004*
Absent (n=43)	19.82	0.00–47.25	47.1	36.5	
Present (n=27)	7.27	3.70–10.83	19.3	11.6	
Satellite lesions					0.036
Absent (n=20)	60.37	19.18–101.55	62.7	51.3	
Present (n=49)	10.13	6.84–13.41	27.3	18.9	
HO-1 expression					<0.001*
Low expression (n=33)	6.97	2.78–11.16	0	0	
High expression (n=37)	57.80	36.03–79.56	67.0	49.2	

Notes: *Statistically significant in Cox's proportional hazards analysis; relative risks (95% CI; P-value) for vascular invasion and HO-1 expression are 2.14 (1.20–3.81; 0.01) and 14.29 (6.25–33.3; <0.001), respectively.

Abbreviation: AFP, α -fetoprotein; HO-1, heme-oxygenase-1.

So far, at least three families of transcription factors are known to promote EMT, including the Snail/Slug, ZEB1/2, and Twist families.³⁵ As shown in Figure 3C, Hep3B HO-1 cells has higher expression of Snail than Hep3B DNA cells. Since the synthesis and polarization of F-actin play a vital role in cell migration,³⁶ we investigated the expression and distribution of F-actin in Hep3B HO-1 and Hep3B DNA cells by applying immunofluorescent staining. In Figure 3D, HO-1 overexpression suppressed the synthesis and polarization of F-actin in Hep3B cells, causing decreased tumor migration. Generally, our results suggested that HO-1 decreased the potential of Hep3B metastasis through E-cadherin upregulation, N-cadherin downregulation, suppression of EMT, and the synthesis and polarization of F-actin. HO-1 suppressing EMT via

TGF β 1-dependent ROS production³⁷ or via inhibiting Numb, a phosphotyrosine-binding domain protein, has been reported in various experimental scenarios.³⁸ However, the real mechanism responsible for the above associations regarding EMT between HBV-HCC and HO-1 needs further investigation.

To further verify the HO-1 functioning in vivo, we conducted xenograft of Hep3B-DNA cells and Hep 3B-HO-1 cells to the nude mice. In Figure 4, Hep 3B-HO-1 cells induced less tumorigenesis (as for Hep3B HO-1 cells, all the xenografted tumors even disappeared after 3 weeks of implantation) and cancer progression than Hep-3B-DNA cells in vivo. Combining the in vitro outcomes, these data strongly indicate that HO-1 plays as a suppressor gene for HBV-HCC.

All the preclinical results are further validated by clinical cohort, HO-1 upregulation strongly correlated with smaller tumor size, less vascular invasion, and less tumor recurrence, although no parameter was independently associated with HO-1 overexpression. Furthermore and more importantly, vascular invasion and weak HO-1 staining intensity independently associated with unfavorable DFS for HBV-HCC patients who underwent hepatectomy.

Conclusion

Based on our data, HO-1 may play an important role in HBV-HCC progression and tumor metastasis. The in vitro, in-vivo, and clinical correlation demonstrated that HO-1 overexpression decreased proliferation, migration, and invasion in HBV-HCC cells and predicted favorable prognosis for HBV patients who underwent hepatectomy.

Acknowledgment

This work was supported by the Chang Gung Memorial Hospital (CMRPG3B0532, CMRPG3E0702, and CMRPG3E0703 to CN Yeh).

Disclosure

The authors report no conflicts of interest in this work.

References

- Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer*. 2001;94(2):153–156.
- Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol*. 2008;48(2):335–352.
- Lau WY, Lai EC. The current role of radiofrequency ablation in the management of hepatocellular carcinoma: a systematic review. *Ann Surg*. 2009;249(1):20–25.
- Tsuzuki T, Sugioka A, Ueda M, et al. Hepatic resection for hepatocellular carcinoma. *Surgery*. 1990;107(5):511–520.
- Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet*. 2003;362(9399):1907–1917.
- Sangiovanni A, Del Ninno E, Fasani P, et al. Increased survival of cirrhotic patients with a hepatocellular carcinoma detected during surveillance. *Gastroenterology*. 2004;126(4):1005–1014.
- Maines MD. The heme oxygenase system: past, present, and future. *Antioxid Redox Signal*. 2004;6(5):797–801.
- Jozkowicz A, Was H, Dulak J. Heme oxygenase-1 in tumors: is it a false friend? *Antioxid Redox Signal*. 2007;9(12):2099–2118.
- Was H, Cichon T, Smolarczyk R, et al. Overexpression of heme oxygenase-1 in murine melanoma: increased proliferation and viability of tumor cells, decreased survival of mice. *Am J Pathol*. 2006;169(6):2181–2198.
- Lin CW, Shen SC, Hou WC, Yang LY, Chen YC. Heme oxygenase-1 inhibits breast cancer invasion via suppressing the expression of matrix metalloproteinase-9. *Mol Cancer Ther*. 2008;7(5):1195–1206.
- Kim DH, Kim JH, Kim EH, et al. 15-Deoxy-Delta12,14-prostaglandin J2 upregulates the expression of heme oxygenase-1 and subsequently matrix metalloproteinase-1 in human breast cancer cells: possible roles of iron and ROS. *Carcinogenesis*. 2009;30(4):645–654.
- Gueron G, De Siervi A, Ferrando M, et al. Critical role of endogenous heme oxygenase 1 as a tuner of the invasive potential of prostate cancer cells. *Mol Cancer Res*. 2009;7(11):1745–1755.
- Alaoui-Jamali MA, Bismar TA, Gupta A, et al. A novel experimental heme oxygenase-1-targeted therapy for hormone-refractory prostate cancer. *Cancer Res*. 2009;69(20):8017–8024.
- Liu PL, Tsai JR, Charles AL, et al. Resveratrol inhibits human lung adenocarcinoma cell metastasis by suppressing heme oxygenase 1-mediated nuclear factor-kappaB pathway and subsequently down-regulating expression of matrix metalloproteinases. *Mol Nutr Food Res*. 2010;54 (Suppl 2):S196–S204.
- Zou C, Zhang H, Li Q, et al. Heme oxygenase-1: a molecular brake on hepatocellular carcinoma cell migration. *Carcinogenesis*. 2011;32(12):1840–1848.
- Yeh CN, Pang ST, Chen TW, et al. Expression of ezrin is associated with invasion and dedifferentiation of hepatitis B related hepatocellular carcinoma. *BMC Cancer*. 2009;9(9):233.
- Chiang KC, Yeh CN, Chung LC, et al. WNT-1 inducible signaling pathway protein-1 enhances growth and tumorigenesis in human breast cancer. *Sci Rep*. 2015;5(5):8686.
- Chiang KC, Chen SC, Yeh CN, et al. MART-10, a less calcemic vitamin D analog, is more potent than 1 α ,25-dihydroxyvitamin D3 in inhibiting the metastatic potential of MCF-7 breast cancer cells in vitro. *J Steroid Biochem Mol Biol*. 2014;139:54–60.
- Juang HH, Chung LC, Sung HC, et al. Metallothionein 3: an androgen-upregulated gene enhances cell invasion and tumorigenesis of prostate carcinoma cells. *Prostate*. 2013;73(14):1495–1506.
- Sacca P, Caballero F, Batlle A, Vazquez E. Cell cycle arrest and modulation of HO-1 expression induced by acetyl salicylic acid in hepatocarcinogenesis. *Int J Biochem Cell Biol*. 2004;36(10):1945–1953.
- Liu N, Wang X, McCoubrey WK, Maines MD. Developmentally regulated expression of two transcripts for heme oxygenase-2 with a first exon unique to rat testis: control by corticosterone of the oxygenase protein expression. *Gene*. 2000;241(1):175–183.
- Takahashi T, Morita K, Akagi R, Sassa S. Heme oxygenase-1: a novel therapeutic target in oxidative tissue injuries. *Curr Med Chem*. 2004;11(12):1545–1561.
- Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol*. 2010;50:323–354.
- Finkel T. Oxidant signals and oxidative stress. *Curr Opin Cell Biol*. 2003;15(2):247–254.
- Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog*. 2006;5:14.
- Yuan K, Lei Y, Chen HN, et al. HBV-induced ROS accumulation promotes hepatocarcinogenesis through Snail-mediated epigenetic silencing of SOCS3. *Cell Death Differ*. 2016;23(4):616–627.
- Sherr CJ. D-type cyclins. *Trends Biochem Sci*. 1995;20(5):187–190.
- Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*. 2004;18(22):2699–2711.
- Birchmeier W, Hülsken J, Behrens J. Adherens junction proteins in tumour progression. *Cancer Surv*. 1995;24:129–140.
- Derycke LD, Bracke ME. N-cadherin in the spotlight of cell-cell adhesion, differentiation, embryogenesis, invasion and signalling. *Int J Dev Biol*. 2004;48(5–6):463–476.
- Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009;139(5):871–890.
- Scheel C, Eaton EN, Li SH, et al. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. *Cell*. 2011;145(6):926–940.
- Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;133(4):70432–71534.

34. Chen L, Guo P, He Y, et al. HCC-derived exosomes elicit HCC progression and recurrence by epithelial-mesenchymal transition through MAPK/ERK signalling pathway. *Cell Death Dis.* 2018;9(5):513.
35. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer.* 2007;7(6):415–428.
36. Stricker J, Falzone T, Gardel ML. Mechanics of the F-actin cytoskeleton. *J Biomech.* 2010;43(1):9–14.
37. Clark JS, Carter AJ, Dixit M, Arany I. Simvastatin inhibits epithelial-to-mesenchymal transition through induction of HO-1 in cultured renal proximal tubule cells. *In Vivo.* 2016;30(4):407–411.
38. Zhang Z, Qu J, Zheng C, et al. Nrf2 antioxidant pathway suppresses Numb-mediated epithelial-mesenchymal transition during pulmonary fibrosis. *Cell Death Dis.* 2018;9(2):83.

Cancer Management and Research

Publish your work in this journal

Cancer Management and Research is an international, peer-reviewed open access journal focusing on cancer research and the optimal use of preventative and integrated treatment interventions to achieve improved outcomes, enhanced survival and quality of life for the cancer patient. The manuscript management system is completely online and includes

Submit your manuscript here: <https://www.dovepress.com/cancer-management-and-research-journal>

a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Dovepress