

Activation of β -Catenin by Hypoxia in Hepatocellular Carcinoma Contributes to Enhanced Metastatic Potential and Poor Prognosis

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Abstract

Purpose: Aberrant activation of β -catenin contributes to the malignant phenotype in hepatocellular carcinoma (HCC). Hypoxia is also known to promote HCC invasion and metastasis. However, the association between β -catenin and the proinvasive role of hypoxia remains unclear. We investigated the role of β -catenin in the proinvasive consequences of hypoxia in HCC.

Experimental Design: We established *in vitro* and *in vivo* hypoxic models to investigate the expression of β -catenin in hypoxic HCC cells and its role in hypoxia-induced aggressiveness. The clinical significance of β -catenin and/or hypoxia-induced factor-1 α (HIF-1 α) was evaluated using HCC tissue microarrays.

Results: Hypoxia induced β -catenin overexpression and/or intracellular accumulation in four HCC cell lines through downregulating the endogenous degradation machinery, and promoted *in vitro* invasion and *in vivo* metastasis of MHCC97 and Hep3B cells. Besides morphologic changes, hypoxic MHCC97 and Hep3B cells exhibited molecular alterations consistent with epithelial-mesenchymal transition, characterized by the loss of epithelial cell markers (E-cadherin and plakoglobin) and upregulation of mesenchymal markers (vimentin and N-cadherin), as well as the increase of matrix metalloproteinase 2. However, silencing β -catenin in these hypoxic cells reversed epithelial-mesenchymal transition and repressed metastatic potential. Positive expression of β -catenin in HCC tissue microarray was associated with the expression of HIF-1 α ($P = 0.034$), and coexpression of β -catenin and HIF-1 α in HCC was correlated with shorter overall survival and time to recurrence.

Conclusion: β -Catenin in HCC is activated by hypoxia and contributes to hypoxia-induced metastatic potential. *Clin Cancer Res*; 16(10); 2740–50. ©2010 AACR.

The expansion of tumors and the inadequacy of their local vasculature results in hypoxia (1). This is a common feature of hepatocellular carcinoma (HCC) and is associated with poor tumor outcome (2). Recent studies have shown that hypoxia promotes tumor invasion and metastasis through the activation of several signaling pathways, such as Ras-extracellular signal-regulated kinase, which modulate hypoxia-related biological effects (3). These

include increased glycolysis, induction of angiogenesis, regulation of pH, and, notably, arrest of cell proliferation (4).

The Wnt/ β -catenin pathway is an important signaling pathway in HCC (5). Although it is involved in multiple biological effects in HCC, proliferation is regarded as one of the most promalignant mechanisms (5, 6). β -Catenin is the chief downstream effector of this pathway. It has been reported that one third of HCCs are associated with the aberrant expression of β -catenin (5, 7).

In this study, we examined alterations in β -catenin expression in hypoxic HCC cells and evaluated its significance in hypoxia-induced metastatic phenotypes. These experiments were based on several recent clinical and preclinical observations. First, given the critical role of β -catenin in the stimulation of HCC cell proliferation and the opposing effect of hypoxia on cell growth, the significance of β -catenin on the promalignant properties of hypoxia required investigation. Second, recent reports in colon, non-small cell lung, and kidney cancers indicated that hypoxia induced the inactivation of β -catenin signaling through the combination of hypoxia-induced factor-1 α (HIF-1 α) with human arrest defective 1 (8), although this suppressive effect has not been confirmed in HCC.

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Translational Relevance

Induction of severe hypoxia starves hepatocellular carcinomas (HCC) of oxygen and is considered to be one of the most important therapeutic mechanisms behind hepatic artery ligation, transarterial chemoembolization, and antiangiogenic agents. However, the hypoxia-induced proinvasive consequences cannot be neglected. In this study, we showed for the first time that β -catenin signaling in HCC cells was activated under hypoxic conditions and contributed to their hypoxia-induced metastatic potential. The combination of β -catenin expression and hypoxia in HCC correlates with metastasis and poor prognosis, and may represent a potential biomarker to aid treatment planning. The fact that the deletion of β -catenin abrogated the enhanced aggressiveness of HCC caused by hypoxia supports the use of combined therapeutic hypoxia and blocking of β -catenin activity for the treatment of HCC.

Third, recent findings from *C. elegans* (9) and colon cancer (9, 10) revealed a functional switch for β -catenin under hypoxic conditions, suggesting that β -catenin plays a completely new role in cancer progression, other than modulating proliferation. However, this has not been confirmed in HCC and this potentially new or enhanced function required detailed investigation.

Materials and Methods

Cell lines and animals. PLC/PRF/5, Hep3B, and HepG2 cells (nonmetastatic or low metastatic potential human HCC cell lines, American Type Culture Collection) and MHCC97 cells (human HCC cell lines with high metastatic potential, established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China; ref. 11) were used in this study. The cells were deprived of O₂ and cultured under hypoxic conditions (1% O₂, 5% CO₂, and 94% N₂) for at least 3 days. Cells maintained under normoxic conditions (20% O₂, 5% CO₂, and 75% N₂) were used as controls. The stable red fluorescent protein-expressing MHCC97-R and Hep3B-R cell lines, derived from MHCC97 and Hep3B cells, respectively, were kindly provided by W.Z. Wu, PhD. (the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China; ref. 12) and were used for *in vivo* experiments.

Male BALB/c *nu/nu* mice at ages 4 to 6 weeks and weighing ~20 g were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science, and were maintained under specific pathogen-free conditions. The experimental protocol was approved by the Shanghai Medical Experimental Animal Care Commission.

Cell transfection and clone selection. The functional role of β -catenin in hypoxia-induced malignant behavior was

examined using the short interfering RNA (siRNA)-mediated stable silencing method in HCC cell lines with (MHCC97 or MHCC97-R; ref. 13) or without (Hep3B or Hep3B-R) β -catenin mutations (14). Sequences targeting the β -catenin short hairpin RNAs (shRNA) were obtained from the Web site of the RNAi consortium at the Broad Institute and cloned into the pLKO.1 TRC cloning vector. The effect of HIF-1 α on β -catenin expression was examined by transient HIF-1 α silencing. This was achieved by transfecting cells with a pRNAT-U6.1/Neo vector (GenScript Corp.) expressing a siRNA targeting the coding region of the HIF-1 α gene. Detailed cloning information and nucleotide sequences were shown in Supplementary Materials and Methods.

Drug treatment. Cells were treated with the proteasome inhibitor MG132 (Sigma-Aldrich) at a final concentration of 20 μ mol/L for 6 hours before analysis for β -catenin levels in cell lysates. In addition, they were treated by the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (40 μ mol/L; Sigma) for 72 hours to examine the expression of glycogen synthetase kinase-3 β (GSK-3 β) expression.

Gelatin zymography, immunoblotting, and immunofluorescence analysis. Matrix metalloproteinase 2 or 9 (MMP-2 and MMP-9) activities in conditioned medium were visualized by electrophoresis on gelatin-containing polyacrylamide gels as described (15). Total cellular protein was isolated in immunoprecipitation buffer (16). Cytoplasmic and nuclear extracts from cultured cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Immunoblotting was done as previously described (17) with the following antibodies: HIF-1 α (1:1,000; Sigma), β -catenin (1:1,000; BD Biosciences), E-cadherin (1:500; Santa Cruz), plakoglobin (1:1,000; BD), vimentin (1:1,000; Sigma), N-cadherin (1:1,000; Santa Cruz), von Hippel-Lindau (1:1,000; Santa Cruz), GSK-3 β (1:500; BD), Akt (1:1,000; Cell Signaling), and phosphorylated (p)-Akt (1:1,000; Cell Signaling). Coimmunoprecipitation was carried out in above buffer, followed by immunoblotting to identify precipitates and coprecipitates. Cell lysates were precleared by incubation with protein A agarose beads for 1 hour and divided into two: one for immunoprecipitation with the anti-HIF-1 α antibody and the other with the anti- β -catenin antibody was used for immunoblotting. Immunofluorescence experiments were done according to previous methods (17).

Establishment of *in vivo* hypoxic model by hepatic artery ligation and metastasis assays. Hepatic artery ligation (HAL) was done in a spontaneous metastatic HCC model to induce intratumoral hypoxia (17). For the highly metastatic MHCC97-R cell line, 1×10^7 MHCC97-R, MHCC97-R-Mock (MHCC97-R-pLKO.1-scramble), and MHCC97-R-shRNA (MHCC97-R-pLKO.1- β -catenin) cells were orthotopically inoculated into the liver parenchyma of nude mice, according to the method previously described (17). After 14 days, HAL was performed by ligating the main branch of the hepatic artery under an operating microscope ($n_{\text{MHCC97-R}} = 12$, $n_{\text{MHCC97-R-Mock}} = 6$, and $n_{\text{MHCC97-R-shRNA}} = 6$). Sham-operated mice underwent

laparotomy with exposure of the liver and dissection of the vascular structures, but without interruption of the hepatic blood flow ($n_{\text{MHCC97-R}} = 12$, $n_{\text{MHCC97-R-Mock}} = 6$, and $n_{\text{MHCC97-R-shRNA}} = 6$). The animals were monitored once every 3 days for 35 days and were anesthetized then killed by cervical dislocation. Another six animals with MHCC97-R-shRNA xenografts in the sham-operated and HAL groups were maintained until 56 days after implantation, when the tumor size with MHCC97-R-shRNA was equal to that of matched MHCC97-R and MHCC97-R-Mock groups at 35 days. A parallel model using low metastatic potential Hep3B-R cell lines was also established and received similar treatment. Visceral organs, including the lungs and liver, were examined histopathologically by standard methods (17). Distant metastases were visualized using fluorescence stereomicroscopy (Leica Microsystems Imaging Solutions, Ltd.) and quantitatively analyzed by integrated absorbance measurements (12).

Patients and follow-up. HCC specimens used in tissue microarray (TMA) analysis were obtained with informed consent from patients who underwent radical resection between 2000 and 2002 at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China). The study was approved by the research ethics committee of Zhongshan Hospital. A total of 200 cases were used to

examine the relationship of HIF-1 α and/or β -catenin expression and poor prognosis in HCC. The patients' clinicopathologic features were described in Supplementary Table S1. They were followed up until March 2008 with a median observation time of 59.0 months. Additional 119 independent cases with a median follow-up of 39.0 months were used in the validation analysis. Clinicopathologic features of this cohort of patients were shown in Supplementary Table S2. The entrance criteria for all patients in this study were found in Supplementary Materials and Methods.

TMA and immunostaining. TMAs were constructed and immunostaining was done using a two-step protocol, as previously described (18). The assessment of HIF-1 α and β -catenin expression was described in Supplementary Materials and Methods.

Statistical analysis. Analysis was done using SPSS 13.0 for Windows (SPSS, Inc.). Pearson's χ^2 test or Fisher's exact test was used to compare qualitative variables. Quantitative variables were analyzed using the *t* test or Pearson's correlation test. Kaplan-Meier analysis was used to determine survival. The log-rank test was used to compare patients' survival between subgroups. The Cox regression model was used to perform multivariate analysis. $P < 0.05$ was considered statistically significant.

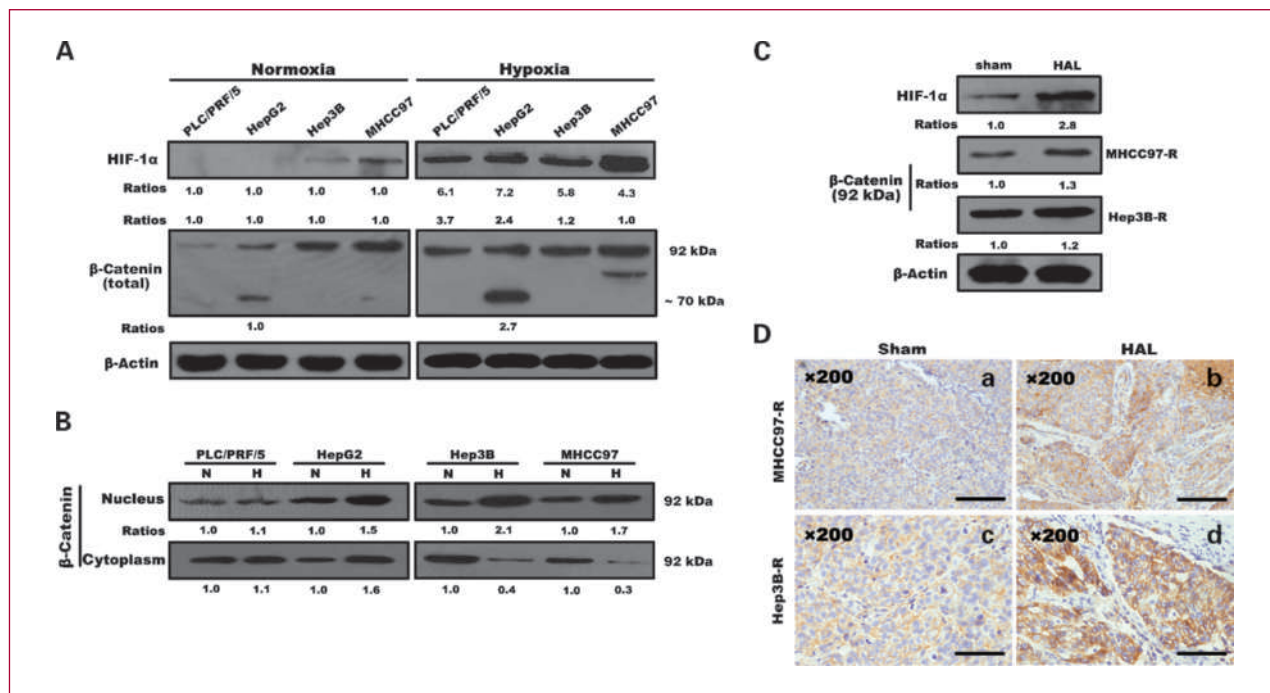


Fig. 1. Expression of HIF-1 α and β -catenin in hypoxic HCC cell lines and HAL-treated xenografts. Immunoblotting showed that hypoxia increased β -catenin protein levels in PLC/PRF/5 and HepG2 cells (A), and induced its intracellular translocation in Hep3B and MHCC97 cells (B). The induction of HIF-1 α expression by hypoxia was detected in all cells with respect to their normoxic controls. Blots were normalized to β -actin protein in each lane. The ratios of each protein relative to matched normoxic cells are listed below each blot. C, immunoblotting of tumor tissues showed that HAL markedly increased HIF-1 α expression in MHCC97-R and Hep3B-R xenografts compared with those in sham-operated groups but slightly upregulated β -catenin protein level. Ratios relative to corresponding sham-operated control were normalized to β -actin. D, immunostaining revealed that most β -catenin-positive cells in above tumor tissues showed a cytoplasmic/nuclear accumulation.

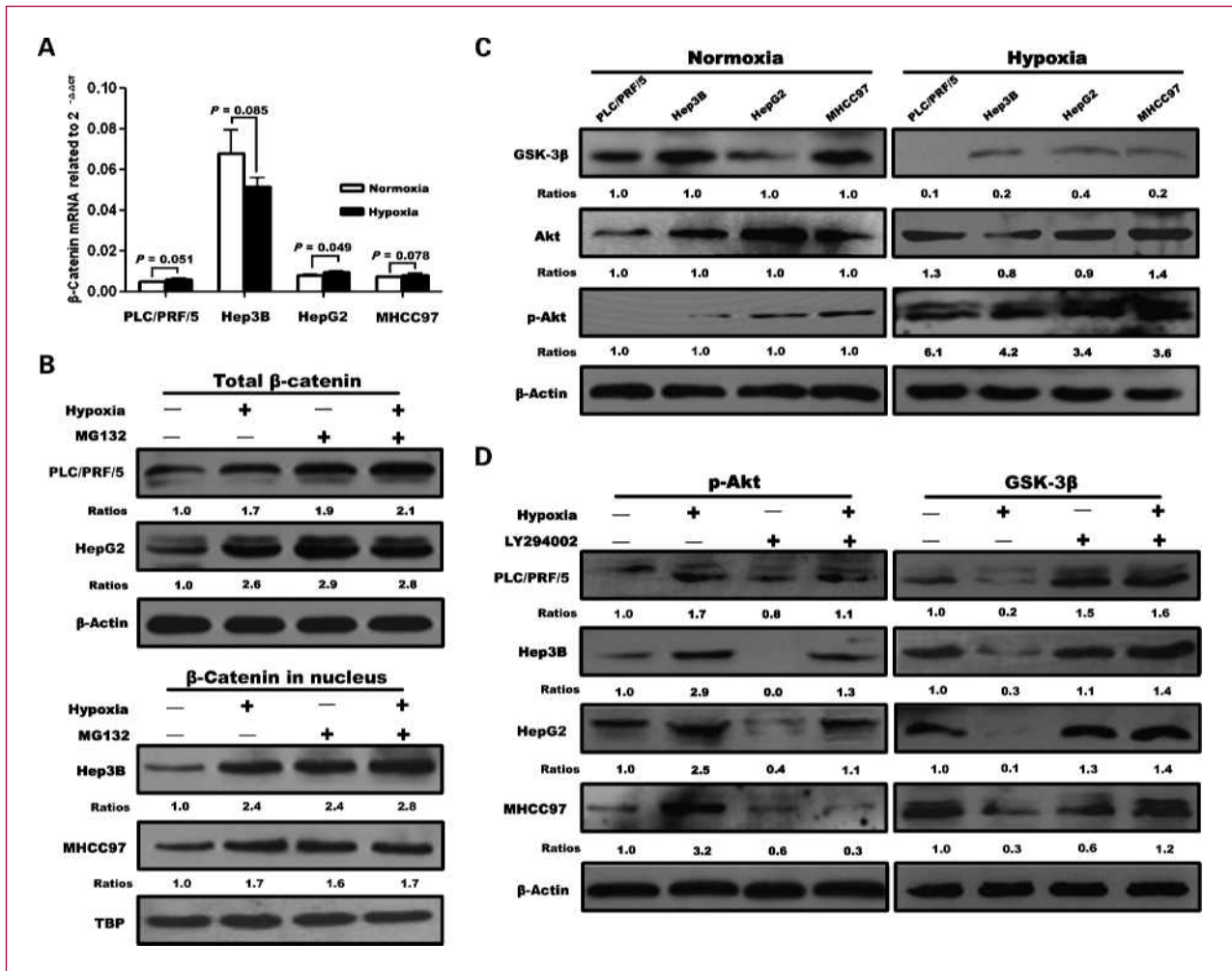


Fig. 2. Effect of hypoxia on the transcription and stabilization of β -catenin in HCC cells. **A**, qRT-PCR showed that hypoxia did not significantly increase β -catenin mRNA levels in HCC cells, except in HepG2 cells. **B**, immunoblotting revealed that the effects of hypoxia on elevated levels of β -catenin in PLC/PRF/5 and HepG2 cells, and on nuclear accumulation of β -catenin in Hep3B and MHCC97 cells, were similar to the effects of the proteasome inhibitor MG132 on β -catenin in these cells. The ratios of each protein relative to nontreated cells were normalized to β -actin. **C**, alterations of β -catenin in hypoxic HCC cells coincided with p-Akt expression, assayed by immunoblotting, which both were inversely related to changes in GSK-3 β levels. The ratios of each protein relative to normoxic cells were normalized to β -actin. **D**, immunoblotting showed that LY294002 remarkably repressed hypoxia-induced GSK-3 β degradation and augmentation of p-Akt. The ratios of each protein relative to normoxic cells were normalized to β -actin.

Results

Hypoxia induced overexpression or internalization of β -catenin in HCC. Under normoxic conditions, little or no expression of HIF-1 α and β -catenin were detected in HCC cells (Fig. 1A). Three days after induction of hypoxia, all HCC cells used here exhibited a notable upregulation of HIF-1 α (Fig. 1A). In addition, a pronounced increase in total β -catenin levels was observed in PLC/PRF/5 and HepG2 cells (>2.4-fold), but not in Hep3B and MHCC97 cells (<1.2-fold). The latter showed a marked intracellular translocation of β -catenin (>1.7-fold) as evidenced by subcellular fractionation assays (Fig. 1B). Immunofluorescence staining further confirmed these changes in β -catenin (Supplementary Fig. S1). In the *in vivo* MHCC97-R and

Hep3B-R xenograft models, immunoblotting showed that the increase in β -catenin expression due to HAL was slight (<1.3-fold), compared with their sham-operated controls (Fig. 1C). However, immunostaining revealed significant intracellular accumulation in both tumor tissues (Fig. 1D).

In light of the importance of HIF-1 α stabilization in the cellular response to hypoxia, we investigated if HIF-1 α played a direct role in the alterations in β -catenin expression and translocation. The interaction between HIF-1 α and β -catenin in hypoxia was confirmed by coimmunoprecipitation assays using MHCC97 and Hep3B cells (Supplementary Fig. S2A). Despite HIF-1 α knockdown using siRNA, β -catenin still exhibited a pronounced intracellular accumulation in response to hypoxia, suggesting that this

effect was independent of HIF-1 α (Supplementary Fig. S2B). Conversely, β -catenin silencing by shRNA inhibited the increase in HIF-1 α protein levels due to hypoxia in both cell lines. In addition, the expression of von Hippel-Lindau (a specific suppressor of HIF-1 α) in β -catenin-null cells remained unaffected by hypoxia but was markedly down-regulated in hypoxic parental cells (Supplementary Fig. S2C). These results suggest that the hypoxia-induced alterations in β -catenin have a substantial effect on HIF-1 α expression in HCC cells.

Hypoxia activated β -catenin in HCC cells through downregulating endogenous degradation machinery. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) revealed no marked upregulation of β -catenin expression in HCC cells in response to hypoxia (Fig. 2A). Although hypoxic HepG2 cells exhibited a higher

β -catenin mRNA levels compared with controls, the difference was barely significant ($P = 0.049$). We thus examined the posttranscriptional stability of β -catenin. The four HCC cell lines were treated with or without the proteasome inhibitor, MG132, and the cell lysates were assayed for β -catenin by immunoblotting. In the absence of MG132, total levels of β -catenin in hypoxic PLC/PRF/5 and HepG2 cells were 1.7- and 2.6-fold higher, respectively, compared with corresponding normoxic cells (Fig. 2B). Nuclear β -catenin protein levels in hypoxic Hep3B and MHCC97 cells were also increased 2.4- and 1.7-fold, respectively, compared with matched controls (Fig. 2B). When MG132 was added, β -catenin accumulated in control cells but there was little further accumulation in the hypoxic cells ($P > 0.05$; Fig. 2B). Subsequent analysis by immunoblotting showed a reduction of GSK-3 β protein levels in hypoxic HCC cells

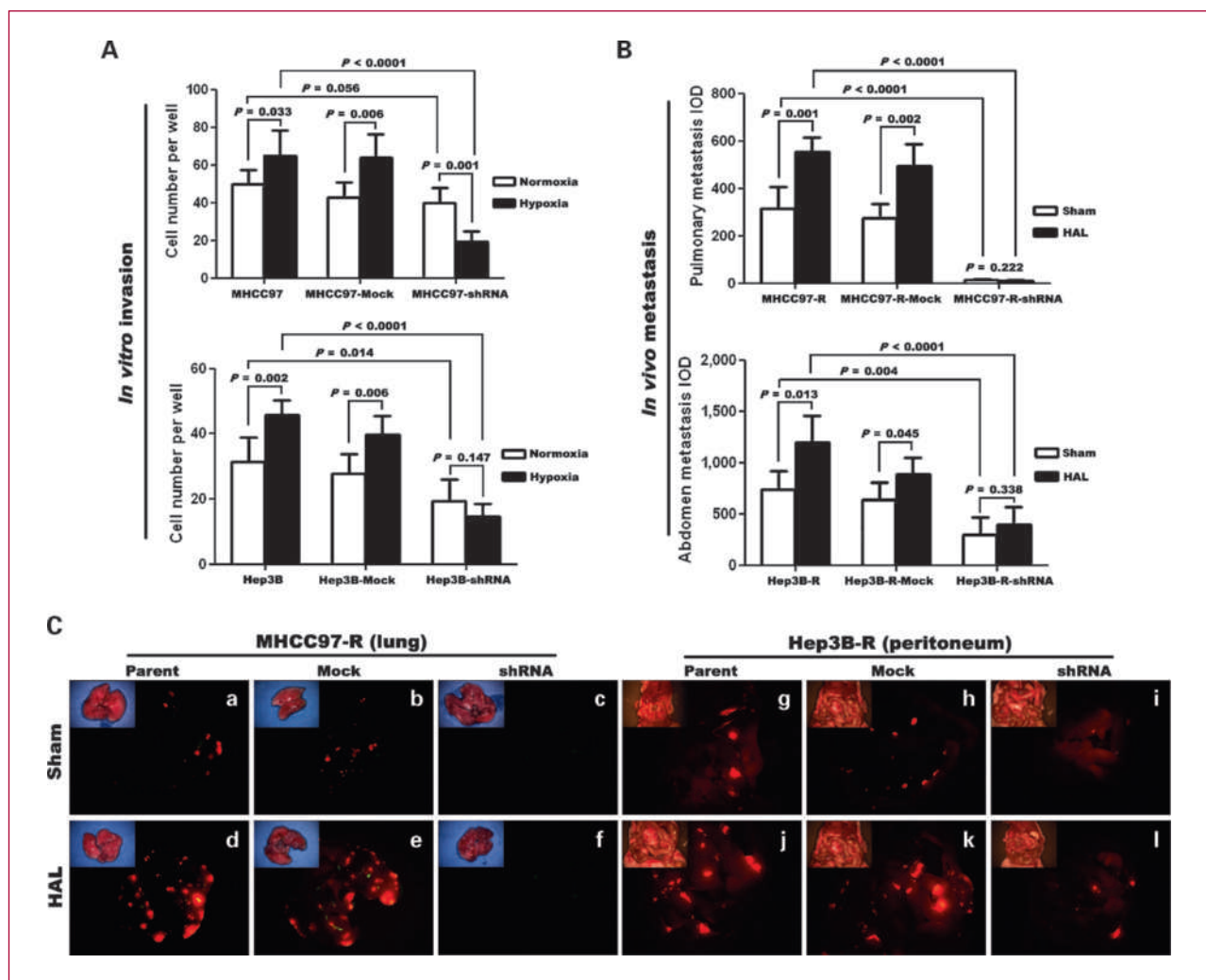


Fig. 3. Effect of silencing β -catenin on the invasion and metastasis of MHCC97 and Hep3B cells under normoxic and hypoxic conditions. **A**, *in vitro* Transwell analysis revealed that depletion of β -catenin completely inhibited hypoxia-enhanced invasiveness of MHCC97 and Hep3B cells, and partially impaired their original invasiveness in normoxia. **B**, quantification of bioluminescence showed that HAL accelerated pulmonary metastasis in MHCC97-R xenografts and increased peritoneal seeding in Hep3B-R xenografts, compared with matched controls. However, the enhanced metastatic potential was abrogated significantly by silencing β -catenin. **C**, representative bioluminescence images visualized tumor metastatic loci in lung and peritoneum.

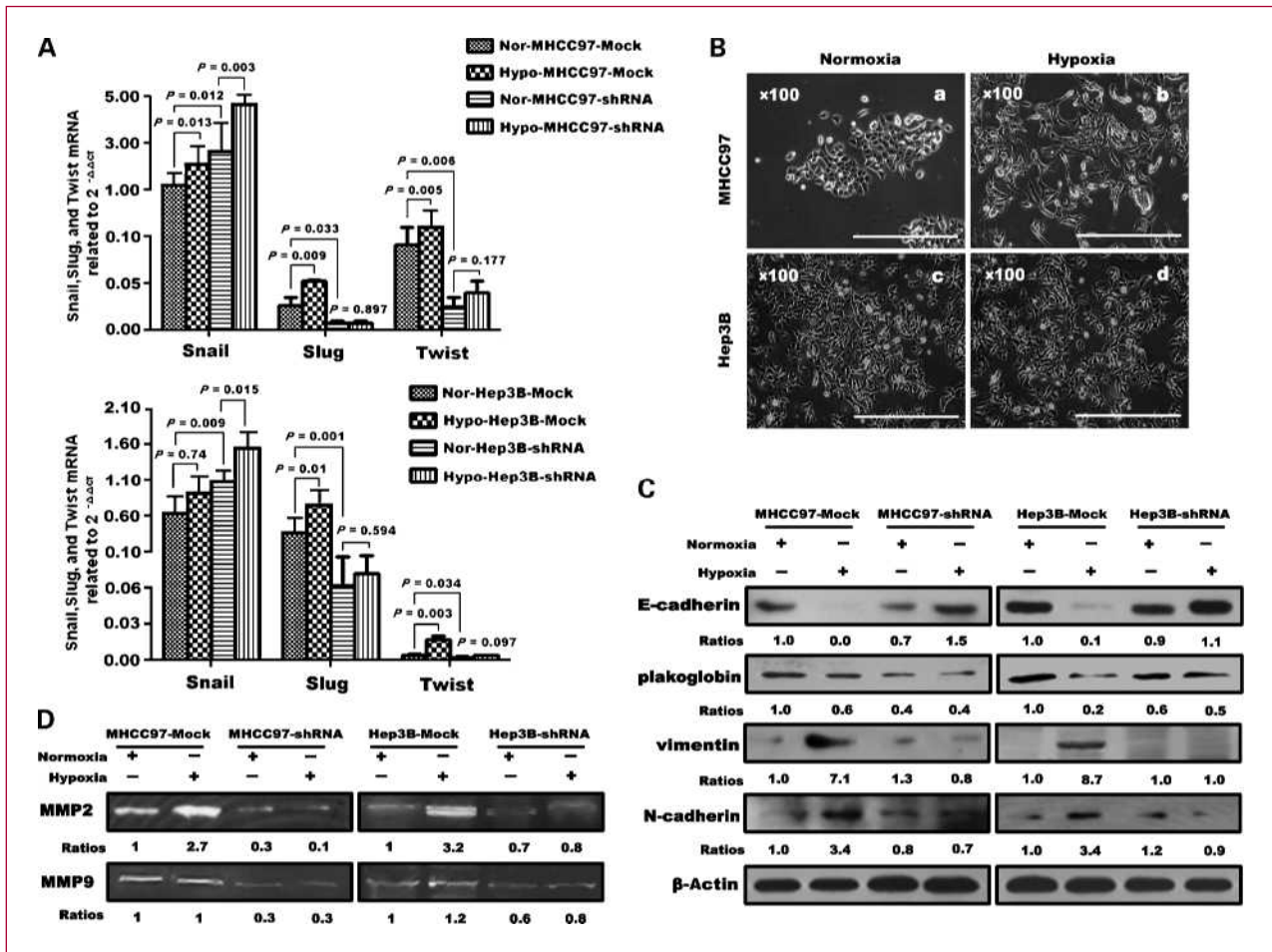


Fig. 4. Effect of β -catenin knockdown on hypoxia-induced molecular changes consistent with EMT in HCC cells. **A**, qRT-PCR revealed an augmentation of transcription factors Snail, Slug, and Twist expression in hypoxic MHCC97 and Hep3B cells with respect to normoxic controls. When β -catenin in these cells was knocked down by shRNA, the upregulation of Slug or Twist due to hypoxia were inhibited, whereas the level of Snail mRNA was higher than that in parental cells. **B**, morphologic changes consistent with EMT (spindle-shaped cells with loss of polarity and increased intercellular separation) were observed in MHCC97 and Hep3B cells in response to hypoxia. **C**, immunoblotting showed that hypoxia-elicited EMT in MHCC97-Mock and Hep3B-Mock cells, characterized by the loss of E-cadherin and plakoglobin as well as the upregulation of vimentin and N-cadherin, was repressed by silencing β -catenin. The ratios of each protein relative to corresponding vector-transfected cells in normoxia were normalized to β -actin. **D**, gelatin zymography showed that hypoxia increased the expression of MMP-2 in HCC cells, which was reversed by silencing β -catenin. The activity of MMP-9 was not affected. Ratios relative to vector-transfected cells in normoxia were normalized to β -actin.

accompanied by the upregulation of β -catenin (Fig. 2C), suggesting that hypoxia activated β -catenin through inhibiting GSK-3 β expression. In addition, the loss of GSK-3 β coincided with the upregulation of p-Akt, a hallmark of PI3K/Akt activation (Fig. 2C). The PI3K inhibitor LY294002 not only reduced p-Akt expression but also suppressed hypoxia-induced GSK-3 β degradation (Fig. 2D).

β -Catenin deletion abrogated the increased invasiveness of HCC cells due to hypoxia. The *in vitro* invasion assay showed that the numbers of invasive MHCC97 and MHCC97-Mock cells were 49.6 ± 7.5 and 42.7 ± 8.1 under normoxic conditions, which were slightly higher than that of MHCC97-shRNA cells (39.8 ± 8.1 ; $P > 0.05$). After the induction of hypoxia, the numbers of invasive MHCC97 and MHCC97-Mock cells were significantly increased (65 ± 13.2 and 63.8 ± 12.4 ; $P < 0.05$) compared with

those of the respective normoxic cells, but the number of invasive MHCC97-shRNA cells declined in response to hypoxia (19.2 ± 5.9), compared with hypoxic MHCC97 and MHCC97-Mock cells, as well as normoxic-MHCC97-shRNA cells ($P < 0.05$; Fig. 3A).

Similar results were also achieved in Hep3B cells as shown in Fig. 3A. However, no statistically significant difference in the number of invasive Hep3B-shRNA cells was found between in normoxia and in hypoxia ($P = 0.147$).

β -Catenin deletion repressed the enhanced metastatic potential of HCC xenografts after HAL. Both MHCC97-R-shRNA and Hep3B-R-shRNA cells possess stable downregulation of β -catenin at least for 3 months compared with their matched controls (Supplementary Fig. S3). In addition red fluorescent protein expression in these cells were maintained throughout this time period (12). After

orthotopic injection of MHCC97-R cells and their derived cells (including MHCC97-R-Mock, MHCC97-R-shRNA cells) into nude mice, all the groups successfully formed liver tumors. Three weeks after HAL treatment, the tumor sizes of MHCC97-R and MHCC97-R-Mock xenografts were $3.1 \pm 1.0 \text{ cm}^3$ and $2.9 \pm 0.8 \text{ cm}^3$, respectively, both of which were significantly smaller than those of corresponding sham-operated controls ($4.2 \pm 0.5 \text{ cm}^3$ in MHCC97-R and $3.9 \pm 0.7 \text{ cm}^3$ in MHCC97-R-Mock; $P < 0.05$). After silencing β -catenin, the tumor size in the HAL group ($2.3 \pm 0.4 \text{ cm}^3$) and sham-operated group ($2.5 \pm 0.5 \text{ cm}^3$) were both reduced, compared with their respective controls. No significant difference in tumor size of MHCC97-R-shRNA was observed in between HAL-treated and sham-operated xenografts ($P = 0.378$). In the HAL group, the pulmonary metastasis rate and metastatic tumor cluster per mouse were 100% (12 of 12) and 77 ± 20 , respectively, in the MHCC97-R xenografts, and 100% (6 of 6) and 68 ± 22 , respectively, in the MHCC97-R-Mock xenografts, which were both markedly higher than those in the sham-operated groups [58% (7 of 12) and 31 ± 12 in MHCC97-R xenografts and 33% (2 of 6) and 26 ± 12 in MHCC97-R-Mock xenografts]. However, the pulmonary metastasis rate and metastatic tumor cluster per mouse were 0% (0 of 6) and 0, respectively, in MHCC97-R-shRNA mice receiving either sham-operation

or HAL (compared with that of MHCC97-R, $P < 0.05$; Supplementary Fig. S4). Similar results were also obtained by bioluminescence (Fig. 3B and C). To rule out the possibility that the reduced pulmonary metastasis in the MHCC97-R-shRNA group was due to the decreased xenograft size, rather than to silencing of β -catenin, we performed a tumor size-matched comparison of the incidence of pulmonary metastases in MHCC97-R-shRNA animals versus controls (MHCC97-R and MHCC97-R-Mock). All MHCC97-R-shRNA cells in the sham-operated and HAL groups failed to transfer to the lung and showed statistically significant differences from their respective tumor size-matched controls ($P < 0.05$).

The findings that HAL retarded tumor growth but simultaneously promoted metastasis were also achieved in Hep3B-R xenografts. Bioluminescence revealed that peritoneal seeding of Hep3B-R or Hep3B-R-Mock xenografts was dramatically increased by HAL and was repressed by silencing β -catenin (Fig. 3B and C). However, histologic analysis after H&E staining failed to detect any visible pulmonary metastatic loci in these mice, regardless of the presence or absence of HAL.

β -Catenin is critical for hypoxia-induced epithelial-mesenchymal transition. qRT-PCR showed the pronounced increase of β -catenin putative targets Slug or Twist mRNA in hypoxic MHCC97 and Hep3B cells with respect to

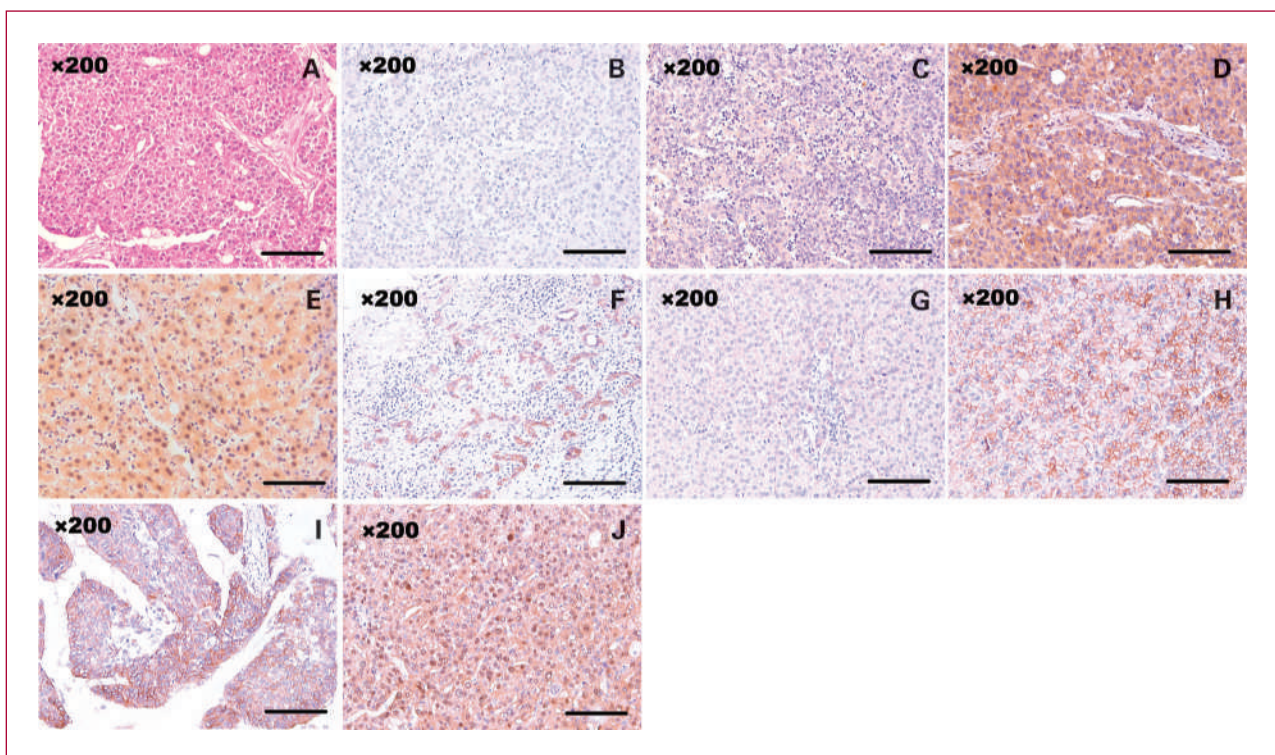


Fig. 5. Expression patterns of HIF-1 α and β -catenin immunostaining in HCC TMAs. Representative examples of H&E staining (A), negative control staining (B), weak cytoplasmic staining (C), moderate cytoplasmic staining (D), and strong nuclear staining (E) for HIF-1 α ; constitutive expression of β -catenin in normal bile ducts as positive control (F); weak membranous staining (G), strong membranous staining (H), cytoplasmic staining (I), and nuclear staining (J) for β -catenin in cancer tissues.

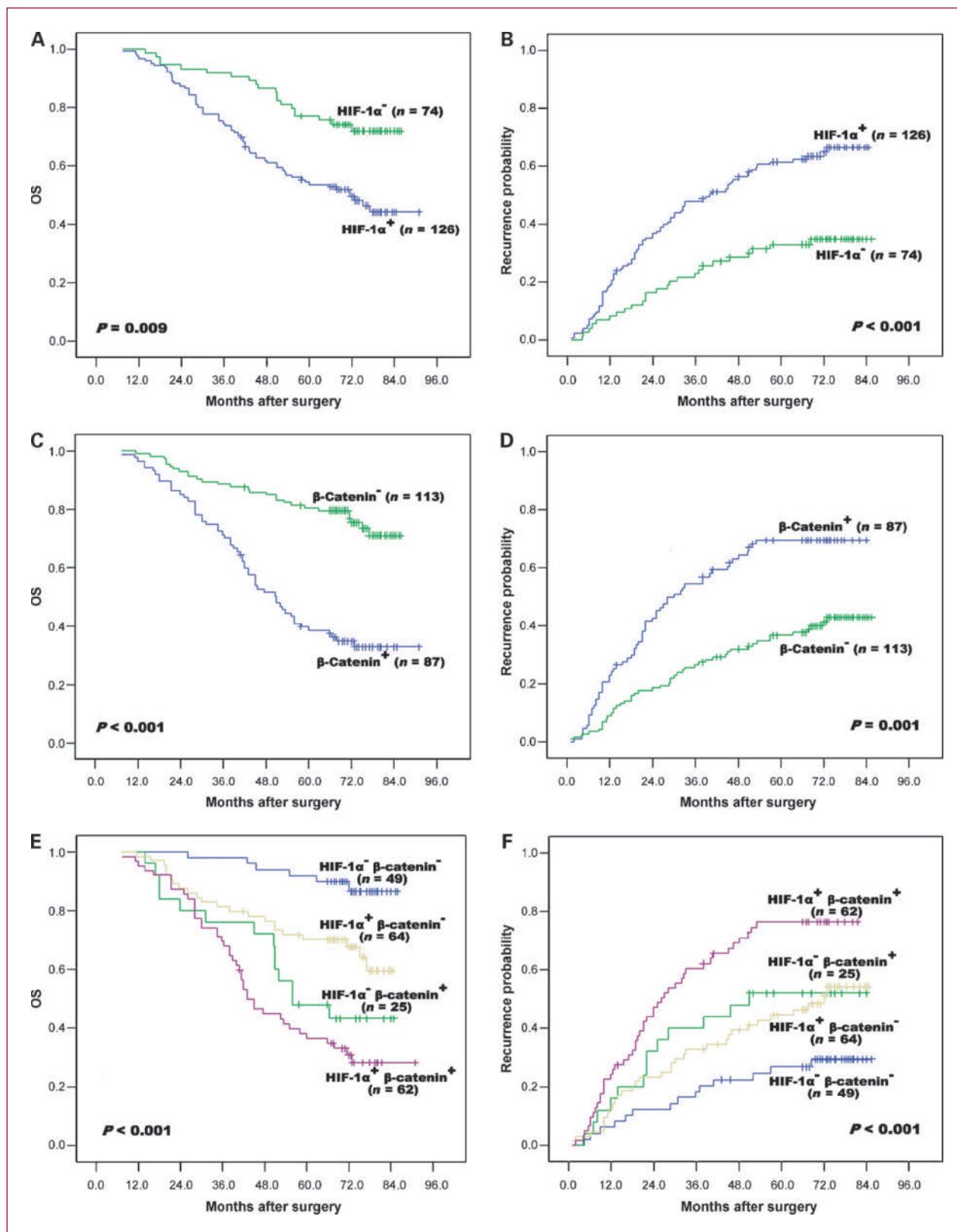


Fig. 6. Prognostic significance assessed by Kaplan-Meier survival estimates and log-rank tests. Comparisons of OS and TTR by HIF-1 α (A and B), β -catenin (C and D), and HIF-1 α / β -catenin (E and F).

controls, but these was abrogated by silencing β -catenin (Fig. 4A). Because Slug and Twist are epithelial-mesenchymal transition (EMT)-related transcription factors (19), we further identified EMT changes in hypoxic HCC cells. As shown in Fig. 4, besides morphologic changes such as loss of cell polarity, induction of EMT has been shown by immunoblotting in these cells, characterized by a shift in the expression of epithelial (E-cadherin and plakoglobin) to mesenchymal markers (vimentin and N-cadherin). Immunofluorescence staining of E-cadherin and vimentin further confirmed this EMT-associated shift in marker expression (Supplementary Fig. S5), but repression of β -catenin in hypoxic cells caused a complete or partial shift of EMT markers back to the pre-hypoxic state (Supplementary Fig. S5; Fig. 4). Additionally, gelatinase activity assays showed that MMP-2 expression was increased in hypoxic HCC cells, compared with control cells (>2.7-fold), whereas MMP-9 activity remained unaffected by hypoxia (<1.2-fold). Expression of both MMP-2 and MMP-9 in these cells was reduced after silencing of β -catenin and no significant differences in expression of these MMPs between normoxic and hypoxic conditions were observed (Fig. 4D).

Expression of β -catenin and combined expression of HIF-1 α and β -catenin were correlated with poor prognosis in HCC patients. Previous reports have suggested that HIF-1 α can be considered as a hallmark of tumor hypoxia (1). Here, TMA analysis showed HIF-1 α -positive staining in 63.0% (126 of 200) of cases, which was associated with the high incidence of intrahepatic metastasis ($P = 0.035$). β -Catenin-positive staining was detected in 43.5% (87 of 200) of tumors in the same cohort and was correlated with increased microvascular invasion ($P = 0.001$), poor tumor differentiation ($P = 0.041$), and high tumor-node-metastasis stage ($P = 0.018$; Supplementary Table S3). The representative immunostaining of two markers was shown in Fig. 5. Either HIF-1 α - or β -catenin-positive expression alone were associated with reduced overall survival (OS) and time to recurrence (TTR) in HCC (Fig. 6A-D), and both were independent risk factors for OS and TTR (Supplementary Table S4). These results support the prognostic roles for HIF-1 α and β -catenin in HCC. In addition, consistent with the relationship between hypoxia and β -catenin activation described above, there was a strong positive correlation between β -catenin and HIF-1 α expression levels in HCC tissues ($P = 0.034$), which was confirmed in the validation cohort (Pearson's χ^2 test, $P = 0.013$). Patients with concomitant HIF-1 α - and β -catenin-positive expression had a higher incidence of intrahepatic metastases and microvascular invasion compared with patients with expression of either HIF-1 α or β -catenin alone, or neither (30.6% versus 17.4%, $P = 0.035$ and 23.0% versus 16.7%, $P = 0.045$, respectively). The median OS and TTR in patients with coexpression (43 and 26 mo, respectively) were both significantly shorter than in other patients (Fig. 6E and F), for whom the median OS time and TTR were not reached during the follow-up period ($P < 0.001$ for both). The strong prognostic rele-

vance of combined HIF-1 α and β -catenin expression was also observed in the independent cohort of HCC patients (Supplementary Tables S5 and S6; Supplementary Fig. S6). These results provide clinical support for a pro-invasive role of β -catenin in hypoxia.

Discussion

The results of the current study showed the molecular and functional activation of β -catenin by hypoxia in HCC and showed its contribution to hypoxia-induced metastatic phenotypes. The induction of EMT was one of the proinvasive mechanisms augmented by β -catenin during hypoxia. The coexpression of β -catenin and HIF-1 α (a marker of hypoxia) in HCC was found to be correlated with metastases and poor prognosis in two independent cohorts of patients. These results confirm the importance of β -catenin in HCC under hypoxic conditions.

Hypoxia plays a critical role in tumor progression (1). Consistent with our previous report (17), it not only facilitated *in vitro* cell invasion in HCC but also resulted in peritoneal seeding and pulmonary metastasis in an *in vivo* HAL model. However, the growth of HCC cells and xenografts were suppressed by hypoxia. Further analysis revealed that this could be attributed to the arrest of cell proliferation rather than the induction of apoptosis (Supplementary Fig. S7). This phenomenon seems to contrast with the proinvasive characteristics of hypoxia.

Considering the central role of β -catenin in HCC biology (6) and the well-characterized association between β -catenin and proliferation (5, 6), it is essential to examine the role of β -catenin in hypoxia. This study revealed the effects of hypoxia on β -catenin in four HCC cell lines, characterized by elevated protein levels and/or intracellular accumulation. The downstream proteins Slug (20) and Twist (21) were also upregulated by hypoxia. These results suggest that β -catenin in HCC cells was activated by hypoxia. Using *in vitro* and *in vivo* hypoxic HCC models, we further showed that the aberrant activation of β -catenin was positively correlated with hypoxia-induced invasion and metastasis; repression of β -catenin by shRNA almost completely abrogated the enhanced aggressiveness of HCC cells in hypoxia. These observations indicate a contributory role for β -catenin in hypoxia-induced metastatic potential and agreed with clinical observations of a positive correlation between aberrant expression of β -catenin and hypoxia (reflected by HIF-1 α), with the fact that their combination predicted metastasis and short survival.

It has been well established that stabilization and/or accumulation of β -catenin is largely a result of transcriptional activation and proteasome inhibition (6, 22). However, the current study found no significant effect of hypoxia on β -catenin mRNA levels in HCC cells and the accumulation of β -catenin in these cells was attributed to the blockage of proteasome-mediated degradation. A more in-depth study showed a correlation between β -catenin accumulation and the downregulation of GSK-3 β in hypoxia. GSK-3 β

is a vital suppressor of β -catenin and is modulated by PI3K/Akt signaling (23). This is one of the most important signaling pathways associated with hypoxia-related malignant phenotypes (3) and its activation in hypoxic HCC cells, characterized by the upregulation of p-Akt, was confirmed in our previous (17) and present studies. Elevated p-Akt levels in these cells coincided with GSK-3 β degradation and were reversed by the PI3K/Akt inhibitor LY294002. These results suggest that activation of PI3K/Akt by hypoxia, through GSK-3 β degradation, leads to the activation of β -catenin in HCC cells.

The response to hypoxia showed in this study, as well as the results of recent studies, suggested an involvement of HIF-1 α in these effects. The aberrant expression of β -catenin due to hypoxia in HCC cells and clinical specimens coincided with HIF-1 α augmentation. An interaction between HIF-1 α and β -catenin in hypoxic HCC cells was also revealed by coimmunoprecipitation assays. However, knockdown of the *HIF-1 α* gene failed to show any effect of HIF-1 α on the expression of β -catenin, although silencing of β -catenin did prevent the upregulation of HIF-1 α by hypoxia. Giles et al. (24) found that this suppressive effect was due to the failure of β -catenin-induced activation of von Hippel-Lindau, a putative ubiquitin ligase involved in the degradation of HIF-1 α . These results indicate that the β -catenin modulation of HIF-1 α expression was more significant than the effect of HIF-1 α on β -catenin in hypoxic HCC cells.

Kaidi et al. (10) further clarified the significance of the effects of β -catenin on HIF-1 α function, confirming a dynamic role for β -catenin in hypoxia in switching from the promotion of cell proliferation to HIF-1 α transcriptional activity. As shown in our study, the expression of the β -catenin target genes *c-Myc* and *cyclinD1* (markers of tumor proliferation) were reduced by hypoxia (Supplementary Fig. S7D), whereas the expression of its other targets Slug and Twist, which are involved in cell invasion, were markedly increased. These results help explain the findings that hypoxia slows down cell proliferation but concomitantly enhances metastatic potential. Slug and Twist are also putative transcription factors involved in EMT (19), which has been shown to be elicited by hypoxia and amplified HCC aggressiveness (17, 25). EMT therefore seems to be the phenotypic consequence of changes in β -catenin expression in hypoxia. The activation of β -catenin, in addition to the upregulation of EMT-related transcription factors such as Slug, was also related to the morphologic and molecular changes consistent with EMT in hypoxic HCC cells. Knockdown of β -catenin by shRNA resulted in the partial or complete repression of these changes. These complementary results indicate a role for β -catenin in hypoxia-induced metastatic potential. Regarding the

significance of PI3K/Akt signaling in hypoxia-elicited EMT and β -catenin activation, we propose a molecular pathway from hypoxia to HCC progression that includes the activation of PI3K/Akt, degradation of GSK-3 β , and subsequent upregulation of β -catenin, which in turn increases Slug and Twist expression and induces EMT. Interestingly, hypoxic stimulation results in the upregulation of snail in cells both with and without β -catenin (Fig. 4A), suggesting that β -catenin is not upstream of snail and that snail expression is not sufficient to control EMT in the absence of β -catenin.

The potential role of β -catenin in hypoxia highlights its relevance in the tumor microenvironment in HCC development. In addition to the promalignant consequences of β -catenin mutations themselves (5–7), environmental stressors such as hypoxia have also been shown to activate β -catenin in HCC cells and to subsequently promote invasion and metastasis, extending the significance of β -catenin in HCC progression. The results of studies on β -catenin also emphasize its ability to alter its functions to meet the ever-changing needs of the tumor cells, allowing it to adapt to potentially wild fluctuations in its microenvironment. Accordingly, this relationship provides the potential for novel therapeutic strategies against HCC. Although it has been accepted that blocking blood vessel supply can maximize survival benefit in HCC patients (26, 27), it results in intratumoral hypoxia, with significant promalignant consequences (17, 28). Recognition of the hypoxia- β -catenin interaction suggests that hypoxia-related therapy, in combination with β -catenin inhibitors, could synergistically potentiate its effects in HCC. This potential is supported by the demonstration that primary tumor burden, as well as distant metastases, were reduced more by the combination of HAL and siRNA against β -catenin than by HAL alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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