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Expression of carbonic anhydrase IX is associated with poor prognosis through regulation of the epithelial-mesenchymal transition in hepatocellular carcinoma

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Abstract. Carbonic anhydrase 9 (CA9) is a plasma membrane-associated isoenzyme that catalyzes pH regulation under hypoxic conditions. CA9 is transcriptionally regulated by hypoxia-inducible factor 1. Recent studies reported that hypoxia also promoted the epithelial-mesenchymal transition (EMT) in various cancers. In the present study, we evaluated the relationship between CA9 expression and EMT *in vitro* with two hepatoma cell lines. We also examined the clinical significance of CA9 expression in 117 consecutive patients that underwent hepatectomies for hepatocellular carcinoma (HCC). We evaluated CA9 expression and EMT induction under hypoxia with quantitative RT-PCR, western blot analysis and immunofluorescence staining, in HuH7 and HepG2 cells. We knocked down CA9 expression with small interfering RNA to evaluate the relationship between CA9 and EMT. We found that hypoxia induced CA9 expression in HCC cells and promoted EMT, evidenced by a loss of E-cadherin and an increase in N-cadherin. Twist, a transcriptional regulator of EMT, was also upregulated with hypoxia. The CA9 deficiency attenuated hypoxia-induced changes in E-cadherin and N-cadherin. Immunohistochemical evaluations of patient samples showed that CA9 was expressed in 50.4% of patients (59/117). However, patients with and without CA9 expression were not significantly different in clinicopathological factors. Nevertheless, a multivariate analysis showed that CA9 expression was an independent factor for both recurrence and prognosis among patients that underwent curative surgery for HCC. In conclusion, this study revealed that CA9 expression was a pivotal predictive factor for poor prognosis after radical surgery for HCC. Moreover, the CA9 regulation of the expression of EMT-related molecules represented a mechanism that enhanced malignant potential.

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Abbreviations: CA9, carbonic anhydrase 9; EMT, epithelial-mesenchymal transition; HIF1, hypoxia-inducible factor 1; RT-PCR, reverse transcription PCR

Key words: hepatocellular carcinoma, carbonic anhydrase 9, epithelial-mesenchymal transition, hypoxia, prognosis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies; it is the third leading cause of cancer-related deaths worldwide (1). HCC arises when the liver is damaged by various liver diseases, such as chronic viral hepatitis or alcoholic liver cirrhosis (2-4). The prognosis of patients with HCC is generally poor, because most patients are diagnosed in the advanced stages, with intra- or extra-hepatic metastasis (5,6), and without an indication of surgery. Consequently, postoperative recurrence rates are high, even when radical resections are performed (7). The postoperative recurrence rates at 3 and 5 years were 61.2 and 85.7%, respectively (8). Therefore, it is crucial to establish new molecular targeting therapies for HCC by searching for molecular markers that correlate with tumor progression and poor prognosis.

Members of the carbonic anhydrase (CA) family are zinc metalloenzymes that catalyze the hydration of carbon dioxide and produce bicarbonate and protons. In various living organisms, CAs function as a modulator of pH and ion transports in many biological processes. Carbonic anhydrase IX (CA9) is an integral plasma membrane isoenzyme with an extracellular catalytic domain (9,10). CA9 expression is elevated in various types of tumors compared to its expression in non-tumor tissues, and it is strongly induced under hypoxic conditions. In response to hypoxia, CA9 gene transcription is sensitively regulated by hypoxia-inducible factor 1 (HIF1). HIF1 plays a pivotal role in the hypoxic response and it mediates many phenomena (11).

Several studies have shown that CA9 expression in tumor cells was related to poor prognoses. The finding suggested that

CA9 might be a useful marker of aggressive malignant potential and progression in various cancers, including lung (12,13), breast (14,15), renal (16), bladder (17) and cervical (18) cancers. Although the detailed function of CA9 remains to be clarified, a few studies have suggested that upregulation of CA9 worsened prognoses by enhancing the malignant potential of tumors.

The epithelial-mesenchymal transition (EMT) is a process where epithelial cells lose cell polarity and cell adhesion capabilities and they gain migratory and invasive capacities. Therefore, EMT plays important roles in progression, because it allows tumor cells to invade to other tissues and migrate to distant organs (19). Recent reports have also shown that hypoxic conditions correlated with the induction of EMT in various tumor cells (20-22). Moreover, Svastová *et al* (23) reported that upregulation of CA9 expression promoted a loss of cell-to-cell adhesion via the downregulation of E-cadherin expression.

This study aimed to clarify the role of CA9 in HCC progression. To that end, we evaluated the correlation between CA9 and EMT in two hepatoma cell lines, and we also examined the clinical significance of CA9 expression in 117 consecutive patients that underwent curative resections for HCC.

Materials and methods

Cell culture. The human hepatoma cell lines, HuH7 and HepG2, were purchased from the Japan Cancer Research Resources Bank (Tokyo, Japan). Cells were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 500 μg/ml penicillin-streptomycin, at 37˚C in a humidified incubator with 5% CO_2 in air.

Drugs and reagents. Cobalt chloride hexahydrate ($CoCl_2$•$6H_2O$), which produces pseudo-hypoxia by inducing HIF1α expression, was purchased from Sigma-Aldrich (St. Louis, MO, USA). We used the following antibodies for immunohistochemistry, western blot analyses and immunofluorescence detection: monoclonal mouse anti-human CA9 antibody (Abcam, Cambridge, UK); monoclonal rabbit anti-human CA9 antibody (Cell Signaling Technology, Beverly, MA, USA); polyclonal rabbit anti-human E-cadherin antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); monoclonal mouse anti-human N-cadherin antibody (Santa Cruz Biotechnology); polyclonal rabbit anti-human Twist antibody (Santa Cruz Biotechnology); and monoclonal rabbit anti-human ZEB1 antibody (Cell Signaling Technology).

Hypoxic conditions. For treatments with hypoxia, cells were maintained in a humidified incubator with 1% O_2, 5% CO_2 and 94% N_2. For chemically-induced hypoxia, $CoCl_2$ was added to the medium at 200 μM. Cells cultured under normoxic conditions were used as the control.

Transfection of small interfering RNA (siRNA). Small interfering RNAs that targeted CA9 (siRNA-CA9) was purchased from Invitrogen (Waltham, MA, USA). The siRNA-CA9 sequences were: 5'-GGAAGAAAACAGUGCCUAUtt-3' and 5'-AUAGGCACUGUUUUCUUCCgg-3'. Cells were transfected with 40 μmol/l siRNA and RNAiMAX (Invitrogen). After an overnight incubation, cells were incubated in normoxia or hypoxia. Mock-transfected cells were used as a negative control.

Patients and tumor samples. From 2000 to 2010, 117 patients (92 men, 25 women, aged 36-84 years) with primary HCC underwent hepatectomies at the Osaka University Hospital. These patients had no history of transcatheter arterial chemoembolization (TACE), and they underwent radical surgery without macroscopic residual tumors. Surgical specimens were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin for histological evaluation.

Cell viability assay. Cell viability was analyzed with the methyl tetrazolium (MTT) assay. Briefly, cells treated with siRNA-CA9 or mock control were seeded at $5x10^3$ cells/well in 96-well plates. The MTT assay was performed at 24 and 48 h. For the assay, 10 μl of 5 mg/ml MTT (Invitrogen) was added to each well, and cells were incubated for 4 h; then, 100 μl dimethyl sulfoxide (DMSO) was added. After the MTT crystals were completely dissolved, the absorbance of each well was measured at 490 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription polymerase chain reaction analysis. Complementary DNA (cDNA) was generated from 1 μg RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were performed with the LightCycler and detection system (Roche Diagnostics GmbH, Mannheim, Germany) as previously described (24). Gene expression was measured in duplicate. The PCR conditions for CA9, E-cadherin, N-cadherin and vimentin amplifications were: one denaturing cycle at 95˚C for 10 min, followed by 45 cycles of: 95˚C for 15 sec, a suitable annealing temperature for 10 sec, 72˚C for 30 sec and a final extension at 72˚C for 10 min. The annealing temperatures for CA9, E-cadherin, N-cadherin and vimentin were 67, 64, 62 and 62˚C, respectively. The housekeeping gene, beta actin (β-actin), was quantitatively amplified concurrently to verify the integrity of the RNA. The primer sequences were as follows: CA9 forward primer, 5'-GATGAGAAGGCAGCAC AGAAGG-3' and CA9 reverse primer, 5'-CTCTGGCTGG CTTCTCACATTC-3'; E-cadherin forward primer, 5'-GAGA AACAGGATGGCTGAAGG-3' and E-cadherin reverse primer, 5'-TGAGGATGGTGTAAGCGATGG-3'; N-cadherin forward primer, 5'-TGTTGACTATGAAGGCAGTGG-3' and N-cadherin reverse primer, 5'-TCAGTCATCACCTCCAC CAT-3'; vimentin forward primer, 5'-AGCTAACCAACGAC AAAGCC-3' and vimentin reverse primer, 5'-TCCACTTTGC GTTCAAGGTC-3'; β-actin forward primer, 5'-GGCGGCAC CCCATGTACCCT-3' and β-actin reverse primer, 5'-AGGGG CCGGACTCGTCATACT-3'.

Western blot analysis. Western blotting was performed as previously described (25). Briefly, cell cultures were lysed with RIPA Buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA), according to the manufacturer's protocol. Aliquots (15 μg) of proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels containing 10% Tris-HCl

(Bio-Rad Laboratories). The separated proteins were transferred to polyvinylidene difluoride membranes and incubated with primary antibodies overnight at 4˚C.

Immunofluorescence staining. For immunofluorescence staining, cells were seeded on 12-well plates and stained according to procedures previously described (25). Briefly, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min. Then, the cells were incubated with anti-human CA9, anti-human E-cadherin, or anti-human N-cadherin antibodies overnight at 4˚C. After washing, the cells were further incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) or Alexa Fluor 546 goat anti-mouse IgG (Invitrogen) for 30 min at room temperature. Finally, the cells were washed and incubated with Hoechst staining solution for 3 min. Preparations were analyzed with fluorescence microscopy (Keyence Corp., Osaka, Japan).

Immunohistochemistry. Immunohistochemical staining was performed to determine CA9 expression in samples resected from patients with HCC. Briefly, formalin-fixed, paraffin-embedded, 4-μm thick sections were deparaffinized, then treated with an antigen retrieval procedure. Sections were incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. Sections were then incubated with a normal protein-blocking serum solution and a biotin-blocking solution (Vector Laboratories, Burlingame, CA, USA), as recommended by the manufacturer. Next, the sections were incubated overnight at 4˚C with a mouse monoclonal anti-human CA9 antibody (Abcam; 1:200). After washing in PBS, the sections were incubated with biotin-conjugated secondary antibody (horse anti-mouse IgG) and with peroxidase-conjugated streptavidin. The peroxidase reaction was then developed with 0.02% of 3,30-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution with 0.03% hydrogen peroxidase. Finally, the sections were counter-stained with Meyer's hematoxylin. Negative control sections were treated the same, except that the primary antibody was replaced with Tris-buffered saline.

Evaluation of immunohistochemistry. Immunohistochemically stained sections were evaluated independently by two investigators; both were unaware of the clinical data. The sections were first scanned with light microscopy at low magnification (x40); then, all fields were examined at a final magnification of x400. Results were expressed as the percentage of positively stained cells (a) and the staining intensity (b). The percentage of positively stained cells was graded as follows: 0 (no staining), 1 (≤10% of cells stained), 2 (11-50% of cells stained), 3 (51-75% of cells stained), or 4 (>75% of cells stained). The staining intensity was scored as follows: 0 (no staining), 1 (weaker than the positive control), or 2 (equal to the positive control; Fig. 7Aa-c). The immunoreactivity score (IRS) was calculated as follows: IRS = a x b, range 0-8. Tumors with IRS values of 0-1 were considered CA9-negative and tumors with IRS scores of 2-8 were considered CA9-positive.

Statistical analysis. Data from *in vitro* experiments and all clinicopathological indicators were compared with the Fisher's exact test. Continuous variables were compared with the Student's t-test. Survival curves were calculated with the Kaplan-Meier method, and differences between the survival curves were compared with the log-rank test. To evaluate the risk associated with prognostic variables, we applied the Cox model to determine the hazard ratio and the 95% confidence interval (95% CI). All statistical analyses were performed with the statistical software JMP, version 11 (SAS Institute, Inc., Cary, NC, USA). Two-sided $P<0.05$ were considered statistically significant.

Results

Expression of CA9 under hypoxic conditions in HCC cells. First, we evaluated whether CA9 was induced under hypoxic conditions in HCC cell lines. *CA9* gene expression in HuH7 cells was approximately four times higher than that in HepG2 cells under normoxia. *CA9* gene expression increased after 24 h of exposure to 1% oxygen in both HuH7 and HepG2 cells (Fig. 1A). CA9 protein levels also increased under hypoxic conditions after 24 h in both cell lines (Fig. 2). We used $CoCl_2$, a chemical inducer of HIF-1α, to mimic hypoxic conditions *in vitro*. In HuH7 cells, *CA9* gene expression increased within 48 h of exposure to $CoCl_2$. In HepG2 cells, *CA9* gene expression significantly increased within 24 h of exposure to $CoCl_2$ (Fig. 3A).

Morphological changes and E-cadherin and N-cadherin expression in hypoxia. Next, we examined cells for morphological changes and determined whether EMT marker expression was altered in hypoxic conditions. We found that both HuH7 and HepG2 cells became spindle-shaped after 72 h of hypoxia (Fig. 4B). Moreover, under hypoxic conditions, the relative mRNA levels indicated a decrease in the expression of E-cadherin, which plays a pivotal role in the behavior of cells on epithelium, and an increase in N-cadherin expression. We also evaluated the expression of vimentin under hypoxia. Vimentin gene expression increased under hypoxia in both HuH7 and HepG2 cells although the difference was not significant in HepG2 cells (Fig. 1B). Western blot analyses also revealed that hypoxia downregulated the expression of E-cadherin and upregulated the expression of Twist, a transcriptional regulator of the EMT (Fig. 2).

We then investigated whether $CoCl_2$ treatment altered the expression of E-cadherin and N-cadherin. After 48 h of exposure to conditions that mimicked hypoxia, both HuH7 and HepG2 cells showed relative reductions in E-cadherin mRNA levels and relative increases in N-cadherin mRNA levels (Fig. 3B). Immunofluorescence staining showed that E-cadherin was expressed on the membranes of cells, and that CA9 was not expressed in normoxic conditions. After exposing the cells to conditions that mimicked hypoxia, CA9 expression increased on cell membranes and in the cytoplasm, E-cadherin expression was lost and N-cadherin expression was gained (Fig. 4A). Thus, we confirmed that both hypoxia and exposure to $CoCl_2$ caused CA9 induction and E-cadherin repression.

siRNA-mediated knockdown of CA9: influence on cell growth. To clarify the effects of CA9 on cell growth, we performed a

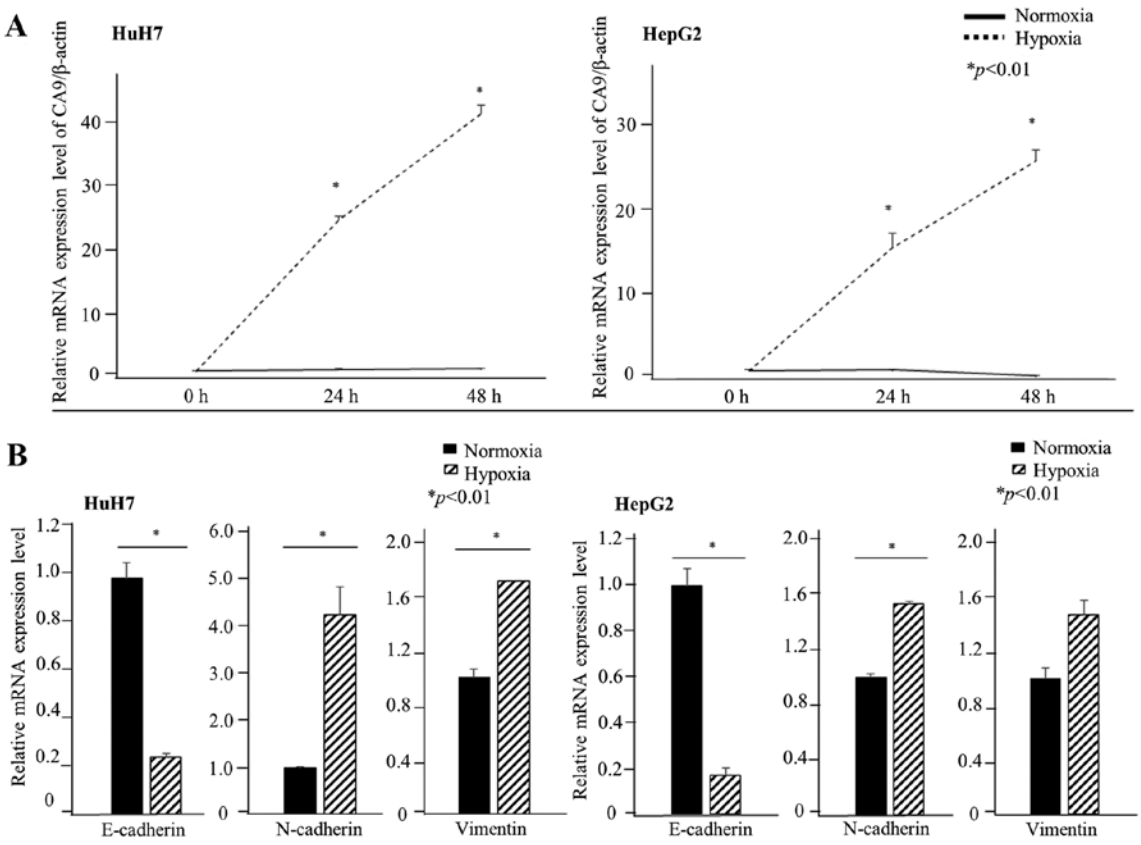

Figure 1. Hypoxia alters CA9, E-cadherin and N-cadherin gene expression. (A) Hypoxia significantly induced gene CA9 expression at 24 and 48 h in both HuH7 and HepG2 cell lines. (B) Hypoxia significantly decreased E-cadherin expression and significantly increased N-cadherin expression in HuH7 and HepG2 cells. Vimentin gene expression also significantly increased under hypoxia in HuH7 cells although there was no significant difference in HepG2 cells. *P<0.01 vs. normoxia.

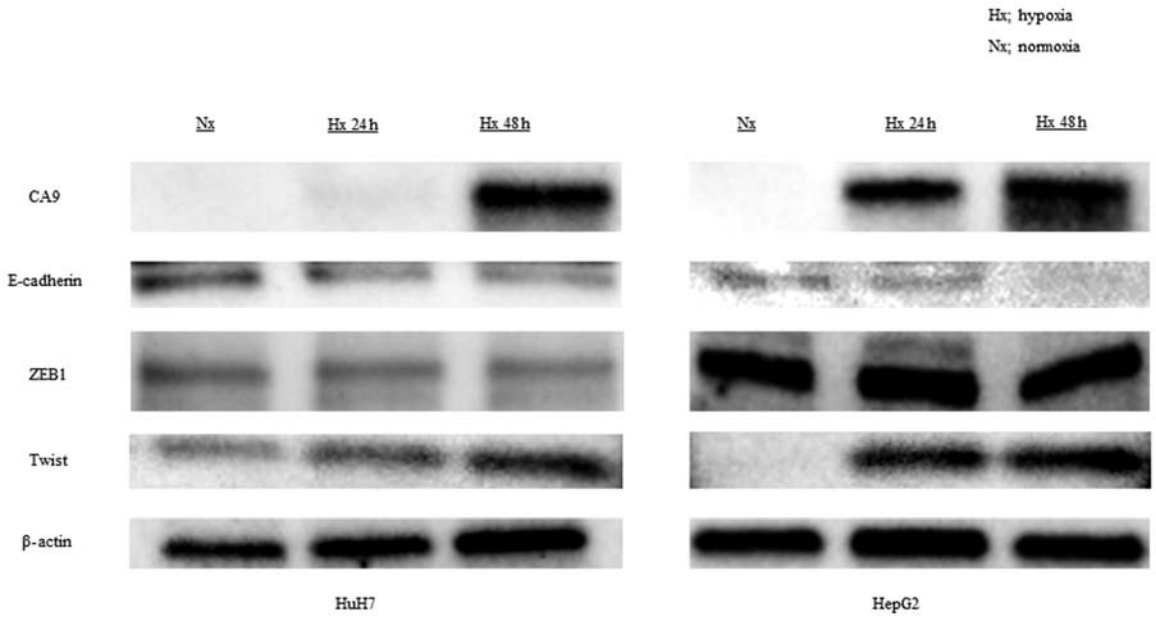

Figure 2. Hypoxia effects on CA9, E-cadherin, ZEB1 and Twist protein expression in HCC cells. Western blot analyses of HuH7 and HepG2 cells show that CA9 expression increased with hypoxia (Hx) compared to normoxia (Nx). Twist expression also increased with hypoxia (Hx), however, E-cadherin expression decreased with hypoxia, compared to normoxia (Nx).

knockdown of CA9 with a CA9-specific siRNA. Transfection of CA9 siRNA into HuH7 and HepG2 cells repressed the CA9 mRNA level to <20% of the control (mock-transfected). Under normoxic conditions, HuH7 cells with the CA9 knockdown showed no significant difference in relative cell viability compared to controls. However, in HepG2 cells, the CA9

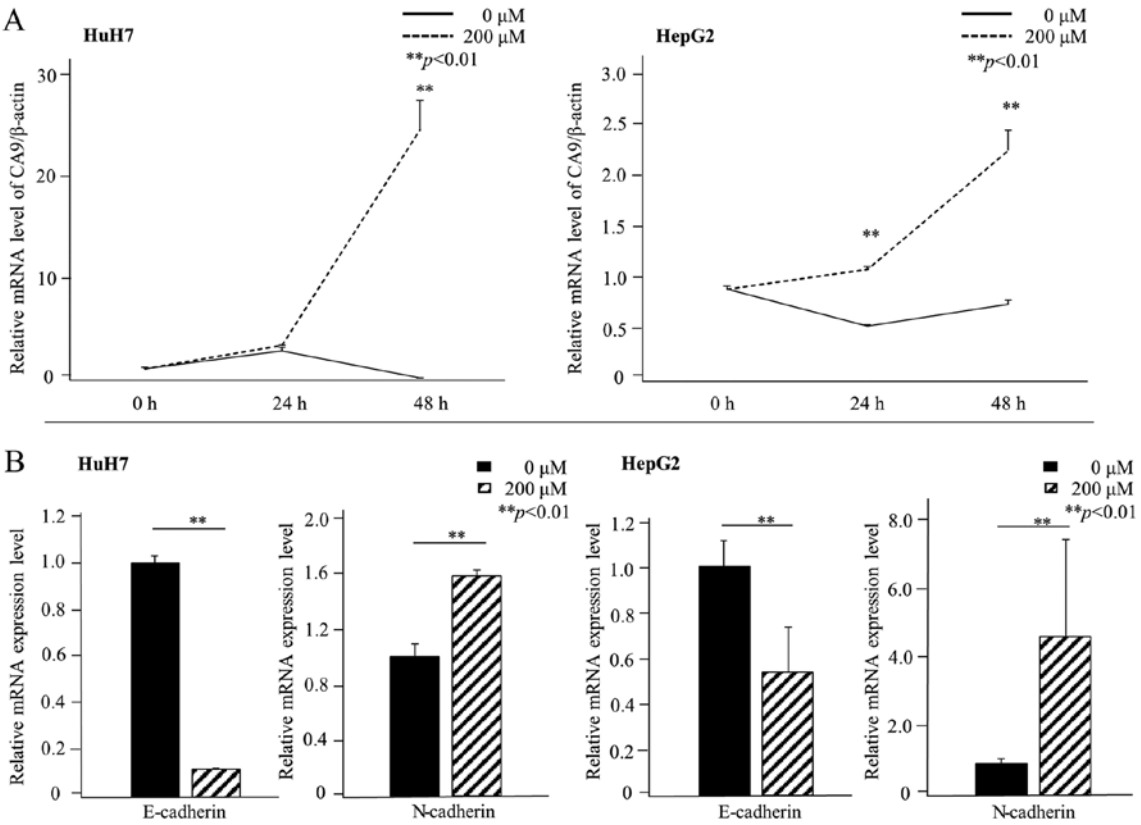

Figure 3. Hypoxia mimicked with $CoCl_2$ altered the expression of CA9, E-cadherin and N-cadherin genes. $CoCl_2$ was used to mimic hypoxic conditions. (A) With $CoCl_2$, CA9 expression significantly increased at 48 h in both HuH7 and HepG2 cells. (B) With $CoCl_2$, E-cadherin expression significantly decreased and N-cadherin expression significantly increased in both HuH7 and HepG2 cells. *P<0.01 vs. no $CoCl_2$.

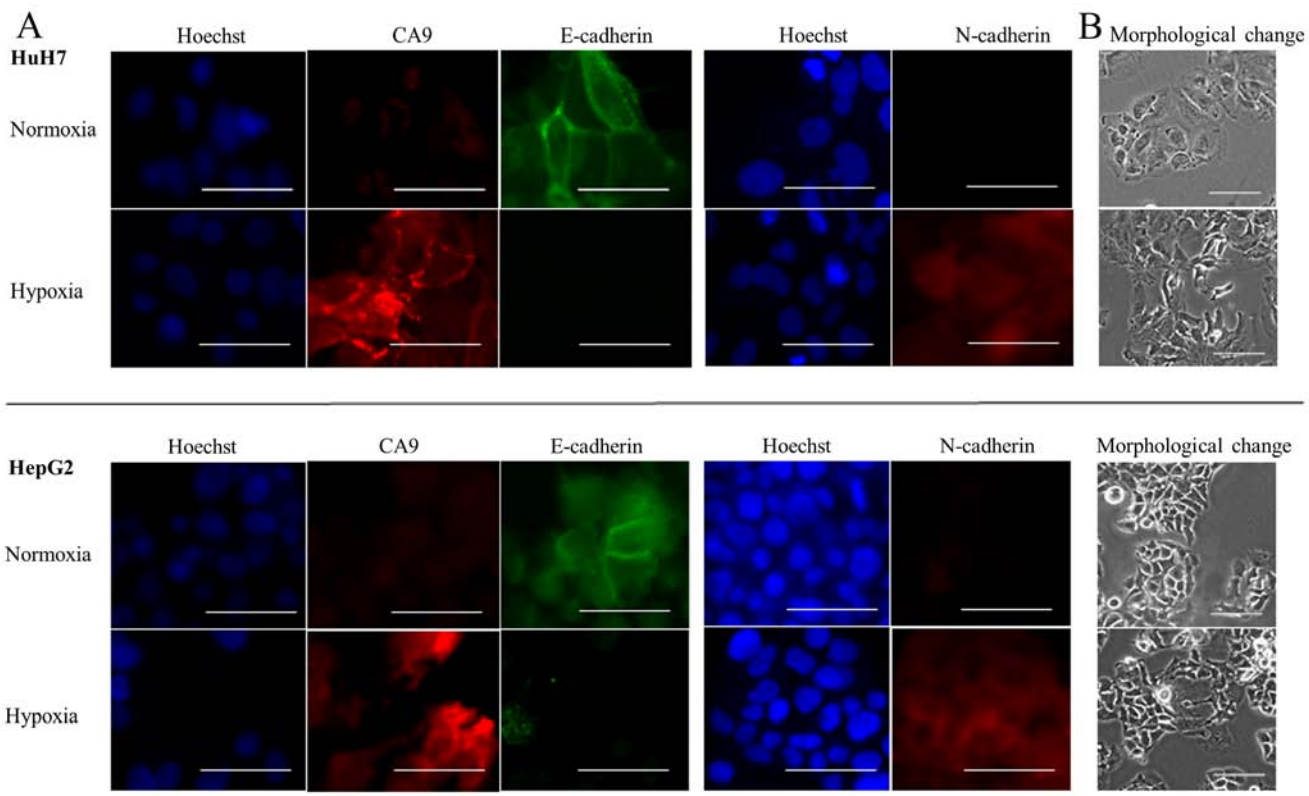

Figure 4. Hypoxia altered protein expression and morphological changes in HCC cells. (A) Immunofluorescence staining shows protein expression of CA9 (red), E-cadherin (green) and N-cadherin (red) in HuH7 and HepG2 cells. Nuclear staining (blue) was performed with Hoechst. Under hypoxia, CA9 and N-cadherin were upregulated and E-cadherin was downregulated. The scale bars, 50 μM. (B) Representative cell morphological changes under hypoxia. Hypoxia induced both HuH7 and HepG2 cells to become spindle-shaped. The scale bars, 100 μM.

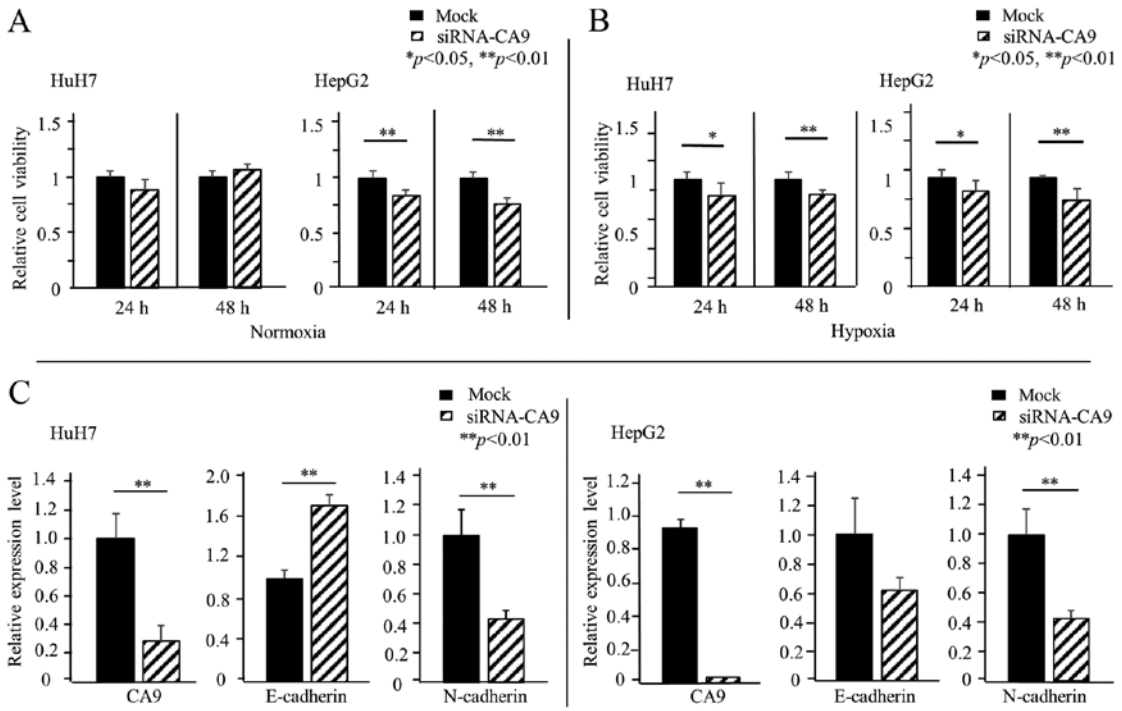

Figure 5. CA9 knockdown affects cell viability and EMT-related molecule expression under hypoxic conditions. MTT assay results show (A) the relative cell viabilities after knocking down CA9 in HuH7 (left panel) and HepG2 (right panel) cells, under normoxic conditions. Relative cell viability was not significantly different between transfected HuH7 cells (striped bars) and controls (black bars). However, the CA9 knockdown repressed relative cell viability in HepG2 cells. *P<0.05 and **P<0.01 vs. mock-transfected cells. (B) Under hypoxic conditions, the CA9 knockdown repressed relative cell viabilities in both HuH7 and HepG2 cells. *P<0.05 and **P<0.01 vs. mock-transfected cells. (C) qRT-PCR results show gene expression in hypoxic conditions. (C, left panel) In HuH7 cells, the CA9 knockdown attenuated the hypoxia-induced suppression of E-cadherin and stimulation of N-cadherin gene expression. (C, right panel) In HepG2 cells, the CA9 knockdown did not significantly alter the E-cadherin gene expression, but it attenuated the hypoxia-induced stimulation of N-cadherin gene expression. *P<0.01 vs. mock-transfected cells.

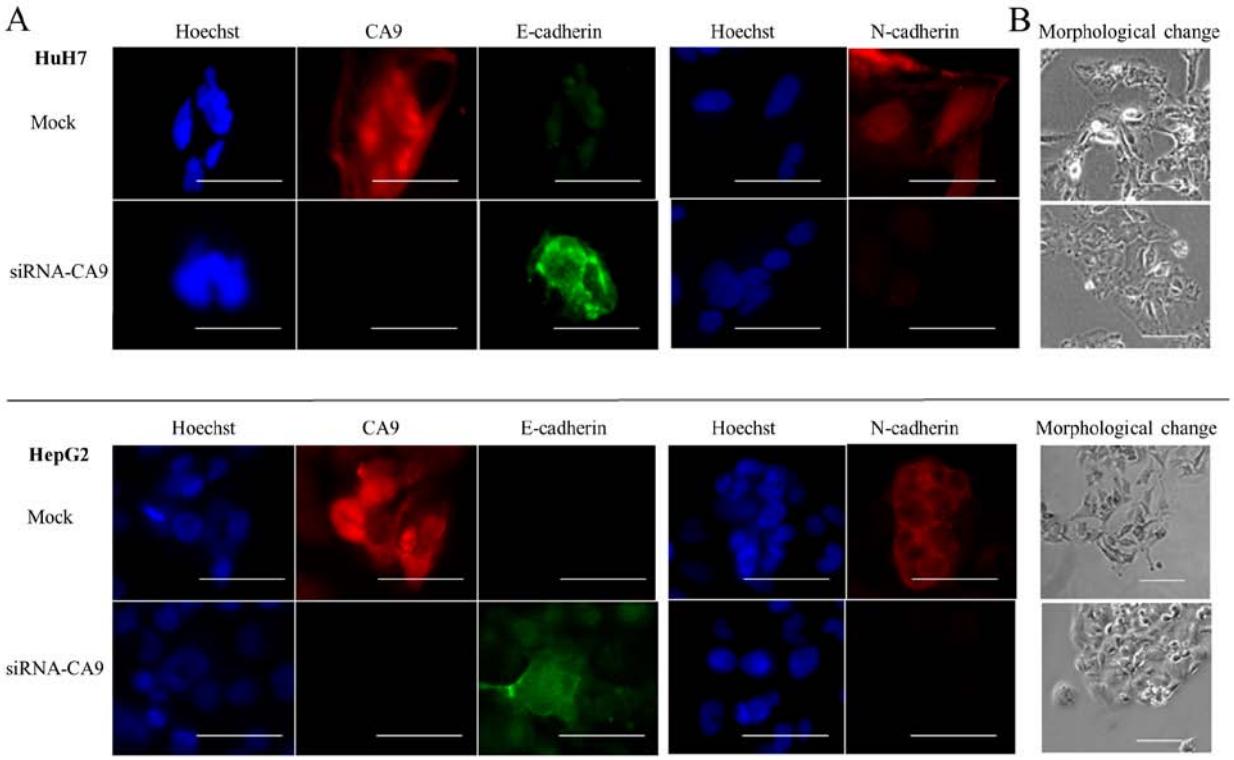

Figure 6. Hypoxia-induced changes in morphology and protein expression were eliminated after knocking down CA9 in HCC cells. (A) Immunofluorescence staining shows expression of CA9 (red), E-cadherin (green) and N-cadherin (red) in cell cultures. Nuclear staining (blue) was performed with Hoechst. (Top rows) Hypoxia induced a downregulation of E-cadherin and an upregulation of N-cadherin, in HuH7 and HepG2 cells. (Bottom rows) The siRNA-CA9 knockdown counteracted the effects of hypoxia. Scale bars, 50 μm. (B) Representative morphological (spindle-shapes) changes in cells under hypoxia (top panels) were attenuated in both HuH7 and HepG2 CA9-knockdown cells (bottom panels). Scale bars, 100 μm.

Table I. Clinicopathological factors of 117 patients with resected hepatocellular carcinoma.

Variables	CA9(+) (N=59)	CA9(-) (N=58)	P-value
Baseline characteristics			
Age (years)[a]	68.7±1.3	66.7±1.3	0.15
Sex (male/female)	50/9	42/16	0.12
HBs-Ag (+/-)	13/49	14/44	0.83
Anti-HCV Ab (+/-)	30/29	32/26	0.71
Child-Pugh classification (A/B)	50/9	51/6	0.58
AFP (ng/ml)[a]	7553±12913	18952±13024	0.73
PIVKA-II (mAU/ml)[a]	4912±6047	9267±6099	0.69
Pathologic characteristics			
Tumor size (cm)[a]	3.5±0.3	3.8±0.3	0.73
Number of tumors (St/Mt)	43/16	44/14	0.83
Macroscopic Vp (+/-)	8/51	5/53	0.56
Histological type (well, mod/por)	30/29	26/32	0.58
Liver cirrhosis (NL, CH/LC)	37/22	32/26	0.84
Microscopic vp (+/-)	17/42	15/43	0.83

CA9, carbonic anhydrase-9; (+), (-), positive or negative staining; HBs-Ag, hepatitis B surface antigen; Anti-HCV Ab, anti-hepatitis-C virus antibodies; AFP, α-fetoprotein; PIVKA-II, prothrombin induced by vitamin K absence-II; St, Mt, single tumor or multiple tumor; well, mod, por: well differentiated or moderately differentiated; NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; Vp, portal vein tumor thrombus at clinical diagnosis; vp, portal vein tumor thrombus at postoperative diagnosis. [a]Data were shown as mean ± standard deviation.

knockdown significantly decreased the relative cell viability in normoxic conditions (Fig. 5A). Under hypoxic conditions, the CA9 knockdown decreased relative cell viabilities in both HuH7 and HepG2 cells (Fig. 5B).

Effect of the CA9 knockdown on hypoxia-induced EMT. In HuH7 and HepG2 cells transfected with siRNA-CA9, we evaluated the expression of E-cadherin and N-cadherin in hypoxic conditions. Both qRT-PCR and immunofluorescence staining results showed that the CA9-knockdown abrogated hypoxia-mediated E-cadherin repression in HuH7, but not HepG2, cells. The CA9 knockdown also attenuated hypoxia-induced N-cadherin expression in both HuH7 and HepG2 cells (Figs. 5B and 6A). Moreover, cells with the CA9-knockdown exhibited less spindle-shaped morphology than cells with unaltered CA9 expression (Fig. 6B). These results suggested that knocking down CA9 counteracted hypoxia-induced EMT. Thus, CA9 might regulate hypoxia-promoted EMT.

Correlations between tumor CA9 expression and clinicopathological findings. To elucidate the clinical significance of CA9 expression in HCC, we performed immunohistochemical evaluations of HCC tissue samples acquired from 117 patients that underwent radical surgery. We detected little or no CA9 reactivity in hepatocytes from liver tissue adjacent to the tumor, but we detected moderate to strong CA9 reactivity in the interlobular bile ducts. The expression of CA9 in HCC was heterogeneous and there were no specific areas in which CA9 was overexpressed, such as central lesions or marginal lesions. The IRS evaluations indicated that, of the 117 tumors, 59 (50.4%) showed positive CA9 staining and 58 (49.6%) showed negative CA9 staining.

Next, we evaluated correlations between CA9 expression and clinicopathological factors. Table I shows the relationship between immunohistochemical detection of CA9 expression and clinicopathological characteristics of 117 patients with HCC. We found no significant difference in clinicopathological factors between the CA9-positive and CA9-negative groups.

Univariate and multivariate analyses of associations between patient survival and CA9 expression. Fig. 7 shows the disease-free survival (DFS) and overall survival (OS) after surgery for patients with and without CA9 expression. The CA9-positive group showed significantly shorter DFS and OS, compared to the CA9-negative group. The 1-, 3- and 5-year DFS rates were 62.5, 41.7 and 29.9%, respectively, for patients with positive CA9 expression, and 79.1, 56.3, 43.7%, respectively, for those with negative CA9 expression (P=0.045). The 1-, 3- and 5-year OS rates were 96.6, 78.2 and 57.6% for patients with positive CA9 expression, and 98.3, 94.7 and 94.7%, respectively, for those with negative CA9 expression (P=0.0002).

We also evaluated the prognostic factors for DFS and OS in univariate and multivariate analyses. The univariate analysis of DFS data revealed several factors that were significantly associated with postoperative recurrence, including serum AFP (P=0.0048), number of tumors (P=0.0088), macroscopic vascular invasion (P=0.026), liver cirrhosis (P=0.026), microscopic vascular invasion (P=0.0011) and CA9 expression (P=0.046). The multivariate analysis of DFS data revealed four significant independent prognostic factors, including serum AFP (P=0.029), liver cirrhosis (P=0.009), microscopic

Table II. Factors related to disease-free survival (DFS), based on univariate and multivariate analyses.

	Univariate			Multivariate		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥70 vs. <70 years)	2.00	6.30-0.50	0.21			
Sex (male vs. female)	1.75	0.97-3.43	0.06			
HBs-Ag (yes vs. no)	0.60	0.31-1.08	0.09			
HCV-Ab (yes vs. no)	1.54	0.96-2.54	0.07			
Child-Pugh classification (A vs. B)	0.86	0.45-1.85	0.67			
AFP (≥20 vs. <20 ng/ml)	1.97	1.23-3.17	**<0.01**	1.99	1.19-3.39	**<0.01**
PIVKA-II (≥100 vs. <100 U/ml)	1.59	0.99-2.60	0.06			
Tumor size (≥20 vs. <20 mm)	1.10	0.95-12.7	0.06			
Number of tumors (St vs. Mt)	0.49	0.30-0.83	**<0.01**	0.92	0.52-1.65	0.76
Macroscopic Vp (yes vs. no)	2.44	1.12-4.73	**0.03**			
Histological type (well, mod/por)	0.68	0.42-1.09	0.11			
Liver cirrhosis (NL+CH vs. LC)	0.58	0.36-0.94	**0.03**	0.51	0.30-0.85	**<0.01**
Microscopic vp (yes vs. no)	2.53	1.47-4.23	**<0.01**	2.19	1.25-3.77	**<0.01**
CA9 (positive vs. negative)	1.63	1.01-2.64	**0.05**	1.18	1.07-2.96	**0.03**

HR, hazard ratio; CI, confidence interval; HBs-Ag, hepatitis B surface antigen; Anti-HCV Ab, anti-hepatitis-C virus antibodies; AFP, α-fetoprotein; PIVKA-II, prothrombin induced by vitamin K absence-II; St, Mt, single tumor or multiple tumor; well, mod, por, well differentiated or moderately differentiated, poorly differentiated; NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; Vp, portal vein tumor thrombus at clinical diagnosis; vp, portal vein tumor thrombus at postoperative diagnosis; CA9, carbonic anhydrase-9; positive or negative, immunohistochemical staining result. Bold, statistically significant.

Table III. Factors related to overall survival (OS), based on univariate and multivariate analyses.

	Univariate			Multivariate		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥70 vs. <70 years)	0.97	0.39-2.19	0.94			
Sex (male vs. female)	1.83	0.69-6.28	0.24			
HBs-Ag (yes vs. no)	0.44	0.10-1.28	0.14			
HCV-Ab (yes vs. no)	2.57	1.08-7.05	**0.03**	2.48	1.02-6.92	**0.05**
Child-Pugh classification (A vs. B)	0.61	0.48-4.35	0.39			
AFP (≥20 vs. <20 ng/ml)	2.63	1.17-6.44	**0.02**	1.59	0.62-4.35	0.34
PIVKA-II (≥100 vs. <100 U/ml)	2.35	1.02-6.05	**0.04**	1.42	0.53-4.10	0.50
Tumor size (≥20 vs. <20 mm)	1.61	0.56-6.81	0.41			
Number of tumor (St vs. Mt)	0.58	0.26-1.41	0.22			
Macroscopic Vp (yes vs. no)	4.28	1.55-10.2	**<0.01**			
Histological type (well, mod/por)	0.49	0.21-1.08	0.08			
Liver cirrhosis (NL+CH vs. LC)	0.54	0.24-1.19	0.13			
Microscopic vp (yes vs. no)	3.29	1.44-7.31	**<0.01**	3.29	1.30-8.44	**0.01**
CA9 (positive vs. negative)	4.79	2.34-12.6	**<0.01**	5.84	2.36-16.2	**<0.01**

HR, hazard ratio; CI, confidence interval; HBs-Ag, hepatitis B surface antigen; Anti-HCV Ab, anti-hepatitis-C virus antibodies; AFP, α-fetoprotein; PIVKA-II, prothrombin induced by vitamin K absence-II; St, Mt, single tumor or multiple tumor; well, mod, por, well differentiated or moderately differentiated, poorly differentiated; NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; Vp, portal vein tumor thrombus at clinical diagnosis; vp, portal vein tumor thrombus at postoperative diagnosis; CA9, carbonic anhydrase-9; positive or negative, immunohistochemical staining result. Bold, statistically significant.

vascular invasion (P=0.018), and CA9 expression (P=0.02; Table II).

Univariate analyses of OS data showed several factors that were significantly associated with postoperative survival,

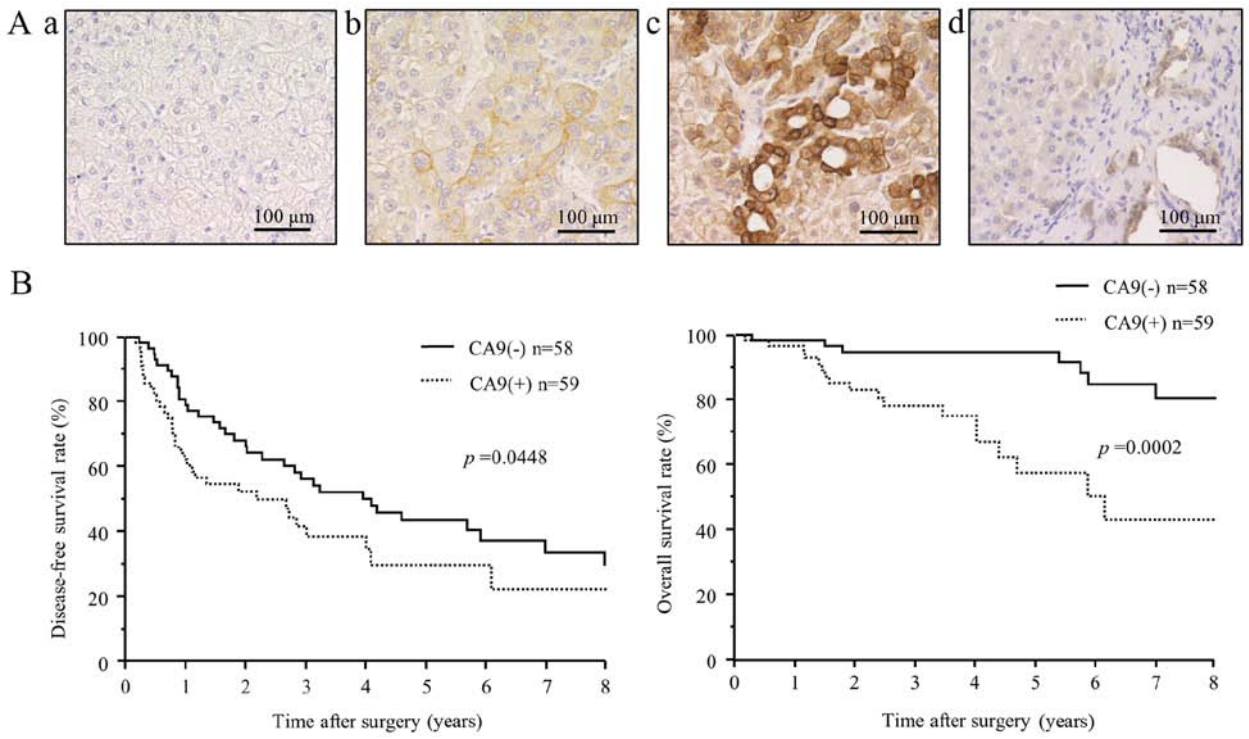

Figure 7. Immunohistochemical expression and clinical significance of CA9 in hepatocellular carcinoma. (A) Immunohistochemical staining of CA9 in cancer cells and adjacent non-cancerous liver tissue. Representative images show different CA9 tissue expression levels, with intensity scores of: (top left, a) 0, no staining, (top right, b) 1, weak staining, and (bottom left, c) 2, strong staining. (Bottom right, d) CA9 was rarely expressed in normal liver parenchyma, but strong expression was detected in intrahepatic bile ducts. Scale bars, 100 μm. (B) Kaplan-Meier disease-free survival curve (top) and overall survival curve (bottom) of patients with CA9-positive tumors (dotted lines) or CA9-negative tumors (solid lines). The CA9-positive group showed significantly shorter disease-free survival (P=0.0448) and overall survival (P=0.0002) than the CA9-negative group.

including HCV infection (P=0.037), serum AFP (P=0.019), serum PIVKA-II (P=0.044), macroscopic vascular invasion (P=0.007), microscopic vascular invasion (P=0.005) and CA9 expression (P=0.0003). The multivariate analysis of OS data revealed three significant independent prognostic factors, including HCV infection (P=0.05), microscopic vascular invasion (P=0.011) and CA9 expression (P<0.001; Table III).

Discussion

Under hypoxic conditions, a number of chemical substances are induced intracellularly in cancer cells, which facilitate survival in harsh environments. These substances play crucial roles in the invasiveness, metastasis, and growth of cells. Generally, hypoxic stimuli induce the expression of HIF1α, a key molecule in O_2 homeostasis (26). HIF1α stimulates the production of hypoxia-related molecules, such as GLUT1, VEGF and CA9 (27-29). Among these molecules, CA9 is a hypoxia-associated endogenous enzyme that mainly regulates intracellular and extracellular pH levels.

In the present study, we first evaluated the expression of CA9 under hypoxic conditions in two HCC cell lines, HuH7 and HepG2. We showed that CA9 mRNA and protein levels gradually increased after exposure to hypoxia, in both HuH7 and HepG2 cells. We also confirmed upregulation of CA9 under pseudo-hypoxic conditions, with $CoCl_2$, which induces conditions that mimic hypoxia by stabilizing HIF1α (30). These results were consistent with the results previously reported (31).

Next, we examined whether hypoxic conditions induced the EMT phenotype in HCC cell lines. We found that hypoxia and $CoCl_2$-mediated pseudo-hypoxia promoted EMT and regulated EMT, by promoting the transcription factor, Twist, in HCC cell lines. Several previous reports have shown that hypoxia could induce the EMT phenotype via upregulation of HIF1 expression (32,33). In HCC, Zhang *et al* (34) showed that hypoxia upregulated HIF1, which activated Snail; subsequently, this pathway resulted in E-cadherin depletion and N-cadherin augmentation. Several reports have also revealed that hypoxia upregulated transcription factors that promoted EMT, such as Twist, ZEB1 and Slug, in various tumor cells (34-36).

Many studies have shown that upregulation of CA9 under hypoxic conditions was correlated with cell survival and growth. However, the mechanism underlying the upregulation of CA9 and its contribution to survival and growth of tumor cells had not been fully clarified. CA9 catalyzes the reversible hydration of carbon dioxide into bicarbonate and protons; this conversion allows cells to thrive under hypoxic conditions (10,37). On the other hand, Yu *et al* (38) reported that CA9 expression inhibited hexokinase II inhibitor-induced apoptosis, and Lock *et al* (39) showed that CA9 was critical for hypoxia-mediated cancer stem cell expansion in breast cancer cells.

In this study, we conducted MTT assays to evaluate cell survival after the CA9 knockdown. In HuH7 cells, the CA9 knockdown did not affect the relative rate of cell survival in normoxia; however, the CA9 knockdown significantly reduced the relative cell survival rate under hypoxic conditions. In

HepG2 cells, the CA9 knockdown significantly reduced the relative cell survival rate compared to controls, under both normoxic and hypoxic conditions. Based on these results, we speculated that HuH7 cell survival might not depend on CA9 expression under normoxic conditions, because HuH7 expressed little or no CA9 in normoxia. On the other hand, HepG2 cell survival may depend on CA9 expression under both hypoxic and normoxic conditions.

Our results showed that a CA9 knockdown with siRNA attenuated the hypoxia-induced loss of E-cadherin and augmentation of N-cadherin. This counter-effect was confirmed with both genetic and protein analyses in HuH7 cells, and with the protein analysis in HepG2 cells. Furthermore, morphological evaluations showed that the CA9 knockdown also attenuated the tendency of both HuH7 and HepG2 cells to become spindle-shaped in hypoxia. Recent studies have proposed that CA9 participated in tumor microenvironment acidification and the loss of tumor cell-to-cell adhesion. Generally, E-cadherin instability drives the EMT, because E-cadherin plays a central role in cell-to-cell adhesion junctions (40). Previous studies have proposed a few mechanisms that might contribute to the loss of cell-to-cell adhesion with CA9 upregulation. One hypothesis held that CA9 expression inhibited or competed against adapter molecules, such as β-catenin and α-catenin, at adherent junctions. This interaction could disturb connections between adapter molecules and the intracellular domain of E-cadherin, which could lead to E-cadherin instability (23).

Lock *et al* (39) showed that hypoxia-induced CA9 could regulate Snail, a transcription factor that promotes EMT, in the stem cell compartment of breast cancer cells. They also hypothesized that CA9 expression might regulate EMT through a potential mechanism involving mTORC1 signaling. Other transcription factors that promote EMT include ZEB1, ZEB2, Slug and Twist (41-44). In this study, we found that Twist displayed upregulated expression under hypoxic conditions. Further investigation is necessary to reveal the correlation between CA9 and mTORC1 signaling in HCC. We thought these two pathways were main mechanisms in the regulation of EMT by CA9.

In HuH7 cells, CA9 regulated E-cadherin transcription. On the other hand, in HepG2 cells, a CA9 knockdown did not affect E-cadherin transcription. These results suggested that the two cell lines might employ different mechanisms for regulating EMT via CA9. For example, CA9 may drive E-cadherin instability at the post-transcriptional level in HepG2 cells.

We also investigated the clinical and pathological significance of CA9 expression in 117 patients with HCC. The rate of CA9-positive staining was 50.4% (59/117) but there was no significant difference between the CA9(+) and CA9(-) groups in clinicopathological factors. A previous study showed a 15% rate of CA9-positive staining in HCC samples. However, that study included only 17 immunohistochemically-stained HCC samples. Another study showed a 21.5% rate of CA9-positive staining. That study included patients that were preoperatively treated with TACE and radiofrequency ablation (RFA). Therefore, those studies and this study had different study designs (45,46). Huang *et al* reported a 48.5% rate of CA9 positive expression. They selected patients with HCC that had not received any anticancer therapies (e.g., TACE) before a curative liver resection. Their study design and cohort were similar to those of the present study, and accordingly, the rate of CA9 positive samples was also similar (47).

Expression of CA9 generally predicts a poor prognosis in various cancers (12-17,46,47). The present study showed that CA9 expression was associated with a poor prognosis and early recurrence in HCC. Two previous reports evaluated correlations between CA9 expression and prognosis with immunohistochemical analyses of HCC samples. Kang *et al* (46) performed immunohistochemical anti-CA9 staining on microarrays of HCC tissues and non-neoplastic liver tissues. They employed a training cohort of 838 patients and a validation cohort of 225 patients. They showed that the CA9(+) group had a worse prognosis than the CA9(-) group, for both the DFS and OS. They also showed that CA9 expression was an independent prognostic factor for DFS and OS in a multivariate analysis. However, the rate of CA9-positive samples was quite low, and some patients in the training cohort had undergone liver transplantation or had received previous treatment for HCC. Furthermore, they could not show a correlation between CA9 expression and DFS in the validation cohort. In another study, Huang *et al* (47) showed that CA9 expression predicted poor DFS and OS in univariate analyses, but in a multivariate analysis, CA9 expression was only an independent prognostic factor for DFS. That study included 227 patients that had received a hepatectomy for HCC from 1988 to 1996. One limitation of that study was the older age of the cohort. In this study, CA9 expression was an independent prognostic factor for both DFS and OS in multivariate analyses among patients with HCC that spanned a large age range (36-84 years) and had no previous history of treatment.

In the present study, total recurrence rates of CA9 between the positive group and negative group for 10 years after primary curative surgery was similar 62.1% (36 cases) and 61.0% (36 cases), respectively. However, overall survival rate of CA9-positive group was higher than that of CA9-negative group. To address this difference, we evaluated the recurrence pattern of our cohort. In the CA9-positive group, 36 cases experienced recurrence and 16 cases (44.4%) among them have recurrence exceeding the Milan criteria. In the CA9-negative group, 36 cases experienced recurrence and only 6 cases (16.7%) among them have recurrence exceeding the Milan criteria. This aggresive recurrent pattern could contribute to poor prognosis in the CA9-positive group.

In conclusion, the present study showed that CA9 expression was a pivotal predictive factor for HCC recurrence and prognosis after radical surgery. Our results suggested that one mechanism for enhancing malignant potential was CA9 regulation of the expression of EMT-related molecules. Therefore, CA9 represents a potential therapeutic target for future HCC treatments. Future studies are necessary to confirm the finding that CA9 expression can enhance malignant potential in HCC.

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