Hypoxia-induced des-γ-carboxy prothrombin production in hepatocellular carcinoma

KAZUMOTO MURATA[1], HIDETO SUZUKI[1], HIROSHI OKANO[3], TAKASHI OYAMADA[1], YOSHIKAZU YASUDA[2] and ATSUSHI SAKAMOTO[1]

[1]Center for Community Medicine, [2]Surgery Jichi Medical University, Tochigi 329-0498; and [3]Internal Medicine, Suzuka Chuo General Hospital, Suzuka, Mie, Japan

Received July 16, 2009; Accepted September 8, 2009

DOI: 10.3892/ijo_00000487

Abstract. Des-γ-carboxy prothrombin (DCP) is an established HCC tumor marker, but the precise mechanism of its production is still unclear. Recently, we demonstrated that cytoskeletal changes during epithelial-to-fibroblastoid conversion (EFC) or epithelial mesenchymal transition (EMT) induced by chemicals plays a critical mechanistic role in DCP production via impairment in vitamin K uptake. Our proposed mechanism of DCP production is consistent with substantial clinical evidence. Supplementary vitamin K2 analogues reduced serum DCP levels in hepatocellular carcinoma (HCC) patients. HCC patients with high serum DCP are associated with vascular invasion, metastasis and tumor recurrence. On the other hand, hypoxia has been reported to induce EMT or cytoskeletal changes. Therefore, we examined whether hypoxia induced DCP production during EFC or EMT in HCC cells. Indeed, hypoxic stimulation induced hepatoma cell lines (HepG2 or PLC/PRF/5 cells) to undergo EFC or EMT and these cells produced DCP. Immunofluorescence study demonstrated that hypoxic stimulation impaired labeled low-density lipoprotein uptake, which was a surrogate for vitamin K uptake. In addition, fine filamentous actin network, which has crucial role for clathrin-mediated endocytosis of vitamin K, was disrupted in DCP producing cells by hypoxic stimulation. Thus, hypoxic stimulation induced HCC cells to produce DCP in the same mechanism as chemicals. Furthermore, immunohistochemical study using surgically resected HCC samples showed that a positive staining of nuclear hypoxia inducible factor (HIF)-1α was more frequently observed in HCC cells with stronger staining intensity of DCP. Importantly, clinical observations that DCP as an HCC tumor marker was more useful in larger tumors, which is likely to be exposed with hypoxia during tumor development, support our results.

Correspondence to: Dr Kazumoto Murata, Center for Community Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
E-mail: atarum@jichi.ac.jp

Key words: hypoxia, des-γ-carboxy prothrombin, epithelial-to-fibroblastoid conversion, E-cadherin, hepatocellular carcinoma, epithelial mesenchymal transition, clathrin-mediated endocytosis, cytoskeletal rearrangement

Introduction

Prothrombin is a vitamin K-dependent blood coagulation protein that is synthesized only in the liver, and biological active form of prothrombin is produced when vitamin K functions as a cofactor in the posttranslational carboxylation of specific glutamyl residues. In the absence of vitamin K or when its action is antagonized by vitamin K antagonists (warfarin), abnormal prothrombin (des-γ-carboxyl prothrombin; DCP), also known as 'protein induced by vitamin K absence or antagonist II' (PIVKA-II), is released into the blood (1,2).

Recently, we demonstrated the mechanism of DCP production in HCC, which demonstrated that impairment of vitamin K uptake through cytoskeletal changes during chemical induction of epithelial-to-fibroblastoid conversion (EFC) has a crucial role for DCP production (3,4). Importantly, our proposed mechanism is supported by several clinical observations. Supplementary administration of vitamin K2 analogues reduced serum DCP levels in HCC patients (5), and vitamin K2 inhibited DCP production *in vitro* as well (6). This would be explained that supplementary vitamin K2 recover total uptake of vitamin K into HCC cells through a functionally impaired-filamentous (F)-actin-dependent or an F-actin-independent pathway (4) since DCP is not actively produced by HCC unlikely to α-fetoprotein (AFP), but shortage of vitamin K uptake into HCC cells resulted in biological inactive form of prothrombin (DCP) production. This would be a similar mechanism, that high serum DCP is observed when utilization of vitamin K is impaired by warfarin administration.

Cancer cells gain migratory and/or invasive properties when they were exposed to noxious stimuli such as chemicals (12) or hypoxia (13,14) in an attempt to escape from an unfavorable microenvironment. Various cancer treatments may stimulate epithelial mesenchymal transition (EMT) and thus enhance invasion and metastasis (15). Indeed, chronic chemotherapy induces EMT in colorectal cancer cells *in vitro*

(16) and chemotherapy-resistance is associated with EMT in several cancer cell lines (17-19). We also demonstrated that E-cadherin repression, which is involved in the early recurrence of HCC after surgical treatment (10) or metastasis (11), is observed in DCP-producing HCC cells during EFC (3,4). Clinical evidence that high serum DCP in HCC patients is associated with a high risk of vascular invasion (7,8), intrahepatic metastasis (8) and tumor recurrence (9) may support our results because E-cadherin is a central component of cell-cell adhesion junctions. Hypoxia or overexpression of hypoxia inducible factor (HIF)-1α also promotes EMT and metastatic phenotypes in human carcinoma cells (20). The presence of hypoxia within the tumor is generally an independent marker of invasion, metastasis (21), and of poor prognosis (13). In addition, disruption of the cytoskeletal network by hypoxia has been reported in diverse tissues such as vessel walls, brain, and kidney (22) and rat sinusoidal endothelial cells (23).

From the above accumulating evidence, we examined if hypoxia, which is a common feature during cancer development, could induce EFC or EMT, resulting in DCP production in HCC cells through the same mechanism as chemicals (3,4).

Materials and methods

Cell culture. HepG2 and PLC/PRF/5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells were incubated in normoxic (O_2=21%, 5% CO_2) or hypoxic (O_2=1%, 5% CO_2) condition. Cells were also incubated with indicated concentration of $CoCl_2$ (Sigma, St. Louis, MO) in normoxic condition (O_2=21%). For the immunofluorescence study, cells were cultured in chamber slides (Nagle Nunc International, Rochester, NY).

Enzyme-linked immunosorbent assay (ELISA) for DCP. Cells were seeded at a density of $2x10^5$ cells into 24-well plates. The next day, the medium was replaced with medium containing various concentrations of $CoCl_2$, and the cells were cultured for another 48 h. In other experiments, cells were cultured in hypoxic condition (1% O_2, 5% CO_2) for 48 h after replacement with new 10% DMEM. The supernatant of the culture medium was subjected to ELISA for DCP. The DCP concentration was determined using a PIVKA-II ELISA kit (Eisai, Tokyo, Japan) according to the manufacturer's instructions.

Uptake of low-density lipoprotein (LDL). Cells were cultured in serum-free DMEM containing 1% bovine serum albumin with or without 100 μM $CoCl_2$, or cultured in 1% O_2 for 24 h. Cells were incubated with 1 μg/ml of 3,3'-dioctadecylindocarbocyanin (Dil)-LDL (Biomedical Technologies, Stoughton, MA) and 10 μg/ml of human LDL for 20 min at the same conditions, then fixed with 4% paraformaldehyde. Anti-mouse PIVKA-II (anti-DCP) (MU-3, 1:100, a kind gift from Eisai Co.) was applied overnight at room temperature after fixation. Then, monolayers were incubated with FITC anti-mouse IgG.

Immunofluorescent studies. Cells were incubated in each indicated condition for 24 h. After cells were fixed with 4% paraformaldehyde, primary antibodies were applied to monolayers of the cells overnight at room temperature. Anti-rabbit E-cadherin (1:50, IBL Co., Fujioka, Japan) or anti-mouse-PIVKA-II were used as primary antibodies. After washing, cells were incubated with FITC-conjugated anti-rabbit IgG (Sigma) or Alexa Fluor 568-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA). The cell nuclei were labeled with mounting medium containing DAPI (Vector, Burlingame, CA). FITC-conjugated phalloidin (Sigma) was applied for 40 min at room temperature to reveal the F-actin. Images were obtained using the 'All-In-One Immunofluorescence Microscopy' system (Keyence Inc., Tokyo).

Western blot analysis. Cells were resolved using SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with anti-vimentin (1:1000, Chemicon, Billerica, MA), anti-HIF-1α (1:1000, BD Transduction Laboratories, San Jose, CA), anti-Snail (1:1000, BD Transduction Laboratories) or anti-E-cadherin (1:2500, BD Transduction Laboratories). Antibodies against ß-actin (1:5000, Abcam, Tokyo) were used for an internal control. The proteins were detected using electro-chemiluminescence techniques (Pierce Chemicals, IL). Densitometric quantification was performed using Image Quant 5.2 (GE Healthcare, Piscataway, NJ).

Human HCC samples. Surgically resected specimens were collected from 40 patients between 2005-2007 at Jichi Medical University Hospital and Koseiren Suzuka Chuo Hospital, Japan. The ethics committees of both hospitals approved the protocol to use liver samples. Both serum DCP and AFP were measured in all patients. The liver specimens were fixed in 10% formalin immediately after resection. Formalin-fixed samples were embedded into paraffin and prepared into 4-μm sections. These specimens were routinely stained with hematoxylin and eosin.

Immunohistochemistry. The specimens were immunohistochemically examined with the avidin-biotin complex (ABC) method. Anti-E-cadherin (1:400, BD Transduction Laboratories), anti-PIVKA-II (1:100) and anti-HIF-1α (H1-α67, 1:500, Sigma) were used as primary antibodies. ABC kit was obtained from Vector Laboratories (Burlingame, CA). After deparaffinization, the sections were retrieved by autoclave. After blocking endogenous peroxidase by immersion into 0.3% H_2O_2, each primary antibody was applied and reacted overnight at room temperature. For color development, 0.05% wt/vol 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used. The section was counterstained with hematoxylin. The immunohistochemical staining as well as the histologic grade of HCC were independently evaluated by three authors (K.M., H. S., and A. S.) without knowledge of any clinical information. When the evaluation varied among the three, they re-evaluated and discussed to reach agreement. Staining intensity of DCP was evaluated into 4 grades: negative, weekly positive, moderately positive and strongly positive. Positive staining of nuclear HIF-1α was counted per 1000 hepatocytes in the area where the strongest DCP staining was observed in the section.

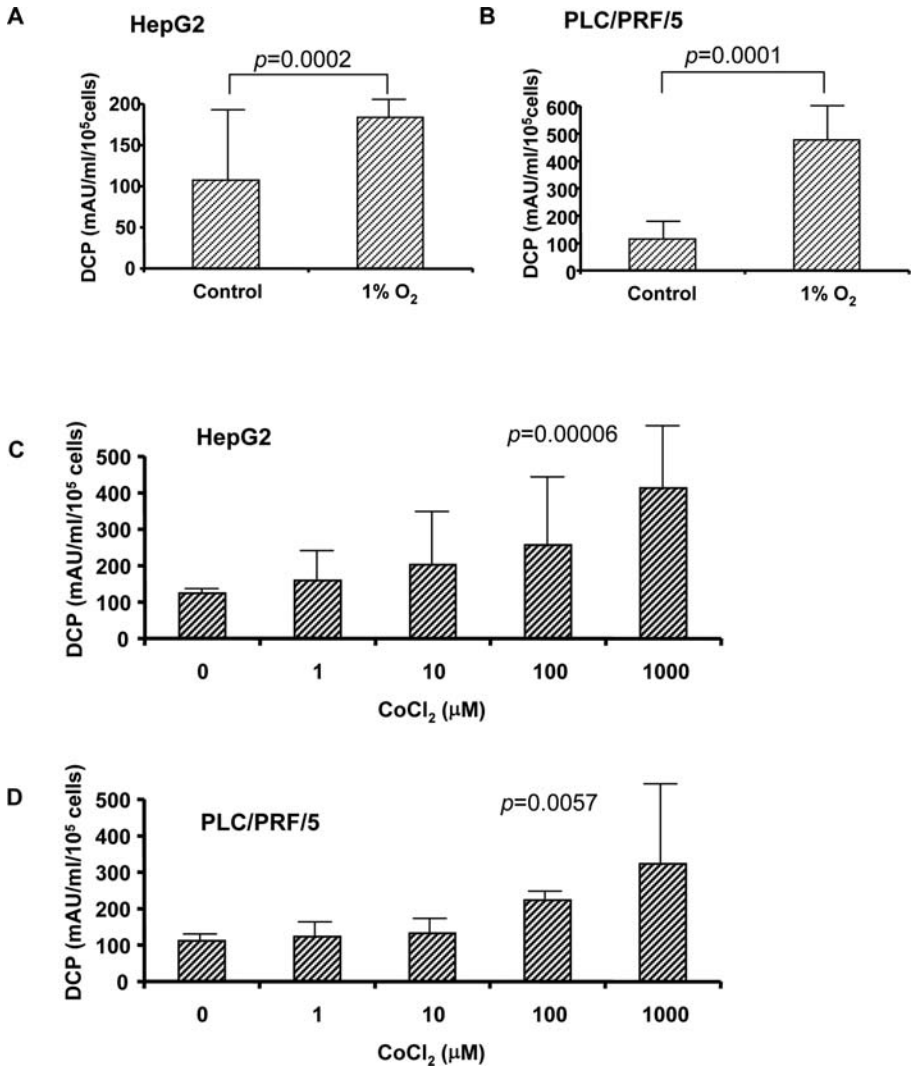

Figure 1. Hypoxia-induced DCP production (ELISA). HepG2 (A) or PLC/PRF/5 (B) cells incubated in hypoxic condition (1% O_2) for 48 h significantly induced DCP production (p=0.0002, p=0.0001, respectively; paired t-test). Hypoxia mimetic $CoCl_2$ induced HepG2 (C) or PLC/PRF/5 (D) cells to produce DCP in a dose-dependent manner (p=0.00006, p=0.0057, respectively; ANOVA). Values are mean ± SEM.

Statistical analysis. All data are expressed as mean ± SD. Multiple comparisons were performed using one-way ANOVA. Intergroup comparisons were performed using paired t-tests and Bonferroni's correction for multiple comparisons. Values of $p<0.05$ were considered to be statistically significant.

Results

Hepatoma cells produced DCP in hypoxic condition. ELISA demonstrated that HepG2 (Fig. 1A) and PLC/PRF/5 (Fig. 1B) cells significantly produced DCP when cultured in hypoxic condition (O_2=1%) for 48 h. Hypoxia-mimetic $CoCl_2$ also significantly induced both HepG2 (Fig. 1C) and PLC/PRF/5 (Fig. 1D) cells to produce DCP in a dose-dependent manner.

E-cadherin expression in DCP-producing cells. To confirm whether hypoxia induced hepatoma cells to undergo EMT and whether these cells produced DCP during EMT, immunofluorescent studies and Western blotting were performed. Immunofluorescent studies demonstrated that hypoxia reduced E-cadherin expression in both HepG2 (Fig. 2A) and PLC/PRF/5 (Fig. 2B) cells, whereas linear E-cadherin expression around the cell boundary was observed clearly in both control cells. $CoCl_2$-treated cells or cells cultured in 1% O_2 became scattered and adopted a fibroblast-like appearance, whereas control cells showed round-shaped and clustered forms, although it was not prominent as chemicals (3,4). In addition, hypoxia-stimulated cells with reduced E-cadherin expression produced DCP. Western blotting (Fig. 2C) demonstrated that hypoxic stimulation (both $CoCl_2$ and 1% O_2 culture) up-regulated both HIF-1α and Snail in both cell types. Hypoxic stimulation down-regulated E-cadherin expression in both cell types, which was consistent with immunofluorescent studies. By contrast, hypoxic stimulation up-regulated vimentin expression in PLC/PRF/5 cells whereas no vimentin expression was observed before and after hypoxic stimulation in HepG2 cells.

Impairment of LDL uptake in DCP-producing cells. We previously reported that vitamin K uptake was impaired in

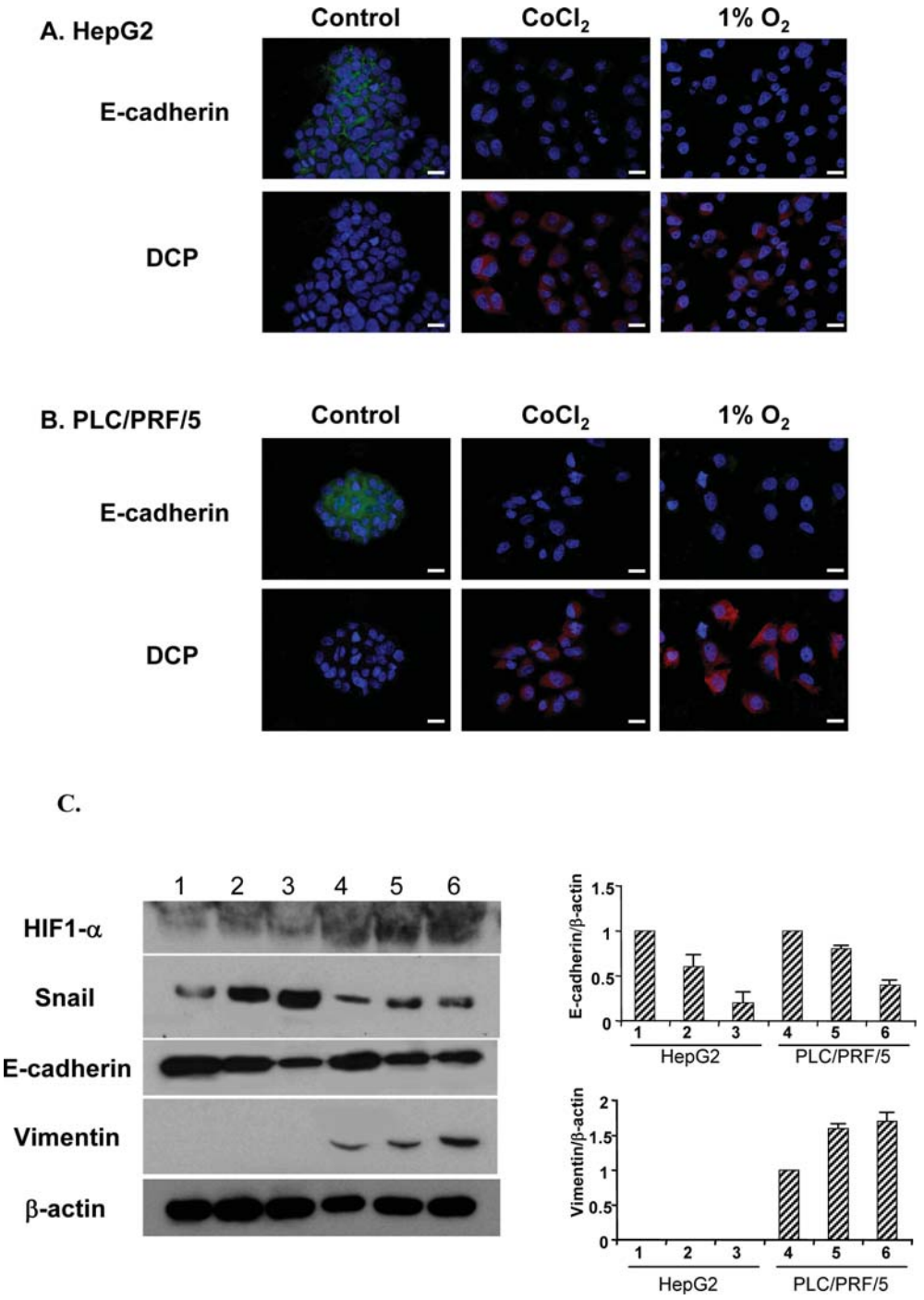

Figure 2. DCP production in cells with reduced E-cadherin expression. In an immunofluorescent study, DCP production (red) was observed clearly in $CoCl_2$-treated or 1% O_2-incubated HepG2 (A) and PLC/PRF/5 (B) cells along with reduced E-cadherin expression. In both control cells, linear E-cadherin expression (green) was observed clearly around the cell boundary. Cell nuclei were stained with DAPI (blue). Bars represent 20 μm. (C) Western blotting. $CoCl_2$ treatment or 1% O_2 incubation up-regulated HIF-1α and Snail expression in both cells. Both $CoCl_2$ treatment or hypoxic incubation down-regulated E-cadherin expression in both HepG2 and PLC/PRF/5 cells in comparison with control cells. Both hypoxic stimulations up-regulated vimentin expression only in PLC/PRF/5 cells. Lanes 1 and 4, control; lanes 2 and 5, $CoCl_2$; lanes 3 and 6, 1% O_2 culture. Values are mean ± SEM.

DCP-producing HCC cells during chemical induction of EFC (3,4). Therefore, we examined whether hypoxia might impair vitamin K uptake in hepatoma cells as well. We used labeled-LDL (3,3'-dioctadecylindocarbocyanin LDL: Dil-LDL) uptake as a surrogate for vitamin K uptake as previously reported (3,4). Fluorescent studies demonstrated prominent accumulation of Dil-LDL in the cytoplasm of control HepG2 (Fig. 3A) and PLC/PRF/5 (Fig. 3B) cells, but these accumulations were impaired by $CoCl_2$ treatment or 1% O_2 culture. Interestingly, cells with an impairment in Dil-LDL uptake produced DCP, while cells with marked accumulation of Dil-LDL did not produce DCP.

F-actin rearrangement in DCP-producing cells. In our previous study (3,4), disruption of F-actin during EFC or EMT was a crucial mechanism of DCP production in hepa-

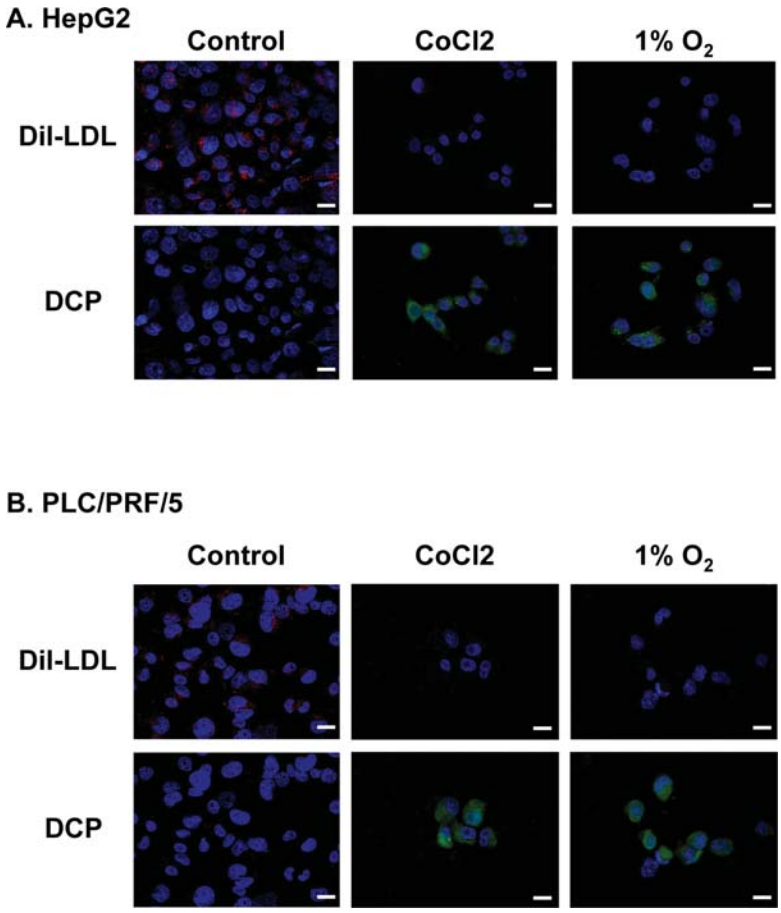

Figure 3. Immunofluorescent studies of labeled-LDL (Dil-LDL) uptake and DCP production. Dil-LDLs (red) were clearly accumulated in the control HepG2 (A) or PLC/PRF/5 (B) cells, but impaired in $CoCl_2$-treated cells or cells incubated in 1% O_2. DCP production (green) was observed in cells with impairment of Dil-LDL uptake. Cell nuclei were stained with DAPI (blue). The white bars represent 20 μm.

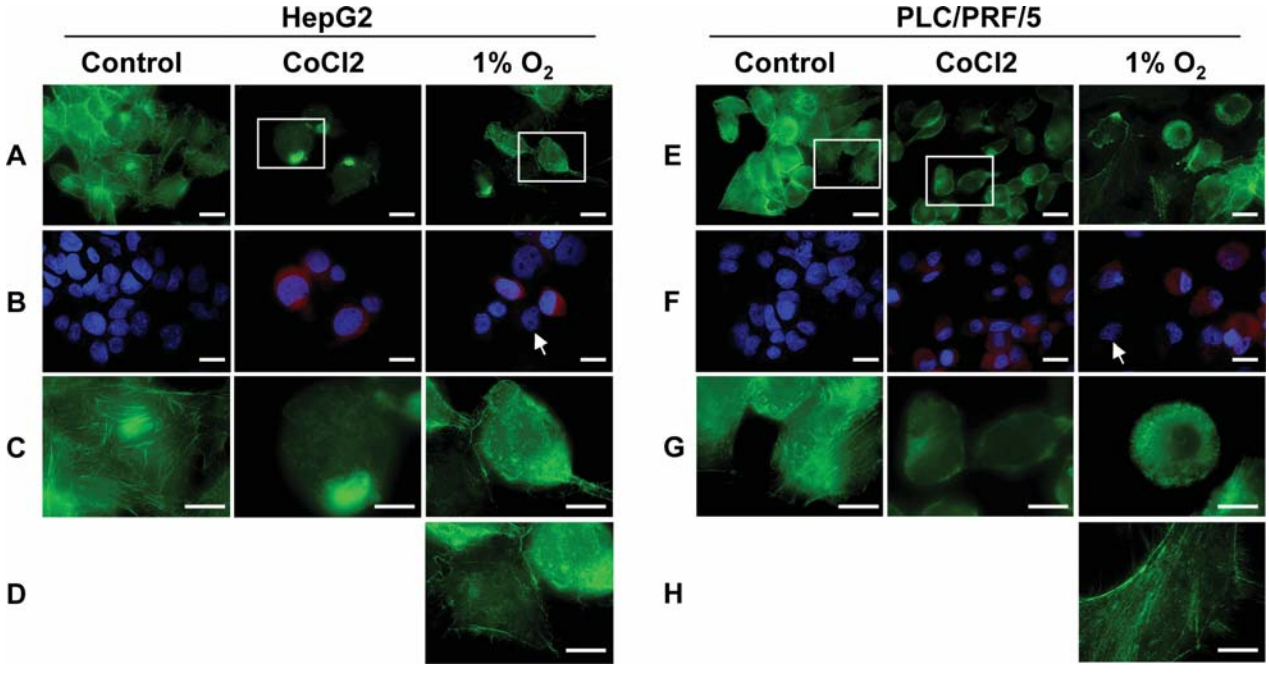

Figure 4. Disruption of F-actin and DCP production. (A and E) FITC-conjugated phalloidin staining showed fine linear F-actin in control cells, whereas F-actin was disrupted in both $CoCl_2$-treated cells and cells cultured in 1% O_2. The white bars represent 20 μm. (B and F) Double staining of phalloidin and DCP showed that cells with disruption of F-actin produced DCP (red) after hypoxic stimulations, whereas control cells with fine F-actin network did not produce DCP. The bars represent 20 μm. (C and G) Magnified images of the inlet of Fig. 4A and E. The bars represent 10 μm. (D and H) Cells with fine F-actin networks did not produce DCP (white arrow in Fig. 4B and F). The bars represent 10 μm.

Table I. Patient characteristics.

No.	Age	Cause	DCP	AFP	Histological grade	Vascular invasion
1.	70	C	24	12	W	-
2.	72	C	9,595	55,980	M	-
3.	60	C	3,564	20	P	+
4.	40	B	508	2,131	M	+
5.	55	B	2,692	45	W	-
6.	64	NBNC	42	15	W	-
7.	75	NBNC	217	4	W	-
8.	65	C	20	5	W	-
9.	78	B	11,266	2	M	+
10.	77	C	139	396	P-M	+
11.	71	C	18,192	15	M	++
12.	76	C	113	24	W	-
13.	74	NBNC	5,900	3	M	+
14.	63	NBNC	696	8	M	-
15.	65	C	1,735	210	M-P	-
16.	78	C	18	2,272	W	-
17.	69	C	205	52	W	-
18.	70	C	321	24	M	-
19.	44	B	274	50,000	W	-
20.	62	NBNC	1,880	1,060	M	+
21.	40	B	16,500	50,000	M-P	+
22.	74	C	2,340	31	M	-
23.	79	NBNC	6,520	4	M	-
24.	78	C	1,872	60	M	-
25.	74	C	83	5	W	-
26.	69	NBNC	1	2,772	M	+
27.	70	C	250	52	M	+
28.	68	C	21	514	W	-
29.	55	NBNC	108	64	M-P	-
30.	73	C	159	20,109	W	-

W, well-; M, moderate-; P, poorly-differentiated HCC.

toma cells. Dynamic polymerization of endogenous F-actin plays an important role in clathrin-mediated endocytosis, including endocytosis of fat-soluble vitamins (i.e., vitamin K). In addition, disruption of the cytoskeletal network by hypoxia has also been reported (22,23). Therefore, the contribution of cytoskeletal rearrangement during hypoxia to DCP production in hepatoma cells was investigated. Indeed, an immunofluorescent study using FITC-conjugated phalloidin revealed a subcortical typical network of F-actin in control HepG2 (Fig. 4A and C) and PLC/PRF/5 (Fig. 4E and G) cells, whereas these structures were disrupted and phalloidin-positive dots were observed after $CoCl_2$ treatment or 1% O_2 culture. In addition, DCP production was clearly observed in cells that lost linear F-actin structures, whereas DCP production was not observed in the control cells that showed a fine network of F-actin (Fig. 4B and F). DCP-producing cells and non-producing cells were simultaneously found in the same culture. Cells with linear F-actin structures (arrow) did not produce DCP even in hypoxic condition (Fig. 4D and H), emphasizing that disruption of F-actin plays a major role in DCP production as well as chemical induction of EFC or EMT (3,4).

An immunohistochemical study of HIF-1α and DCP in human HCC samples. A total of 40 patients with surgically resected HCC were collected. Out of 40 patients, we excluded 10 patients who were undergone transarterial embolization (TAE) before surgical resection because ischemic response

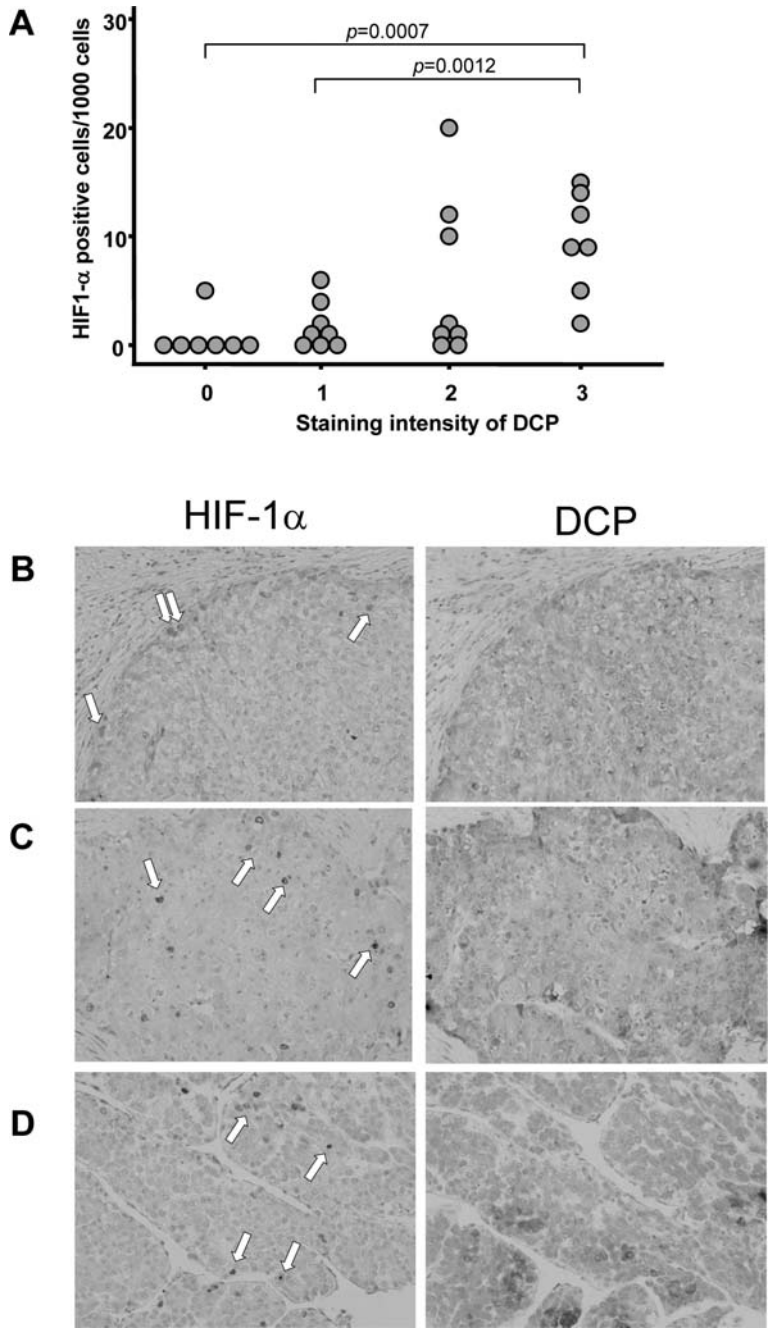

Figure 5. Immunohistochemical studies with HIF-1α and DCP staining. (A) The number of nuclear HIF1-α was positively correlated with staining intensity of DCP in the same area. Typical staining patterns of DCP and HIF-1α in case 5 (B), 11 (C) and 17 (D) are shown. The arrows indicated some cells with positive nuclear HIF-1α. Bars represent 100 μm.

caused by TAE may affect HIF-1α expression. Characteristic features of enrolled patients are summarized in Table I. The median age was 67±11 years (male: female = 23:7) at the time of operation. The causes of chronic liver diseases were hepatitis B infection (n=5), hepatitis C infection (n=17) and cryptogenic cirrhosis (n=8). Immunohistochemistry using serial sections demonstrated that the staining intensity of DCP positively correlated with the number of positive staining of nuclear HIF-1α in the same lesions (γ=-0.635, p<0.0001) (Fig. 5A). Typical staining patterns are shown in Fig. 5B (Case 5), 5C (case 11) and 5D (case 17). In these cases, a positive staining of nuclear HIF-1α was observed more frequently in HCC cells with stronger intensity of DCP. These findings are consistent with our *in vitro* observations that hypoxia induced DCP production.

Discussion

DCP is a well-established tumor marker for HCC (24), and the majority of the case-controlled studies have validated the utility of DCP for the diagnosis of HCC (25-28). However, serum levels of DCP are not always parallel to serum levels of AFP in patients with HCC. Sensitivity of DCP is lower than AFP for the diagnosis of small HCC although DCP has an extremely high specificity for HCC (29). On the contrary, in large HCCs, the utility of DCP for the diagnosis of HCC

was higher than that of AFP (30,31). In addition, serum DCP levels are reported to have a correlation with large tumor sizes (32). Cancer cell growth is limited by the extent of vascularization. Without compensatory vascular adaptation, hypoxia generally occurs according to tumor development because diffusion limit of oxygen is within 100-200 μm from blood vessels (33). Therefore, large HCC cells are likely to be exposed in hypoxic conditions. Hypoxia promotes EMT or metastatic phenotypes *in vitro* (20) and *in vivo* (13,21). In addition, the incidence of intrahepatic or extrahepatic tumor spread of HCC was significantly higher in larger HCCs as well (34,35). Taken together this evidence and our proposed mechanism (3,4), hypoxic stimulation could induce phenotypic changes to EMT, resulting in DCP production. Indeed, our ELISA demonstrated that two hepatoma cell lines (HepG2 and PLC/PRF/5 cells) were induced to produce DCP when they were cultured in 1% O_2. In addition, hypoxic mimetic $CoCl_2$ induced DCP production in a dose-dependent manner.

During EMT, E-cadherin expression is down-regulated, and vimentin, an intermediate filament normally expressed in cells of mesenchymal origin, is up-regulated in cells involved in pathological or physiological processes which require epithelial cell migration, especially during wound healing (11) or tumor invasion (36,37). Both the immunofluorescent study and Western blotting showed that hypoxic stimulation induced both cells to down-regulate E-cadherin expression and PLC/PRF/5 cells to up-regulate vimentin. Therefore, hypoxia induced HepG2 cells to EFC, and induced PLC/PRF/5 cells to EMT. E-cadherin is reported to repress through up-regulation of transcriptional genes such as Snail, Slug or TWIST1 (38), and an inverse correlation between Snail and E-cadherin was observed in our experiments as well. Therefore, it is likely that hypoxia-induced E-cadherin repression is mediated by up-regulation of Snail in HCC cells although some other pathways were not determined in this study. On the other hand, it is not clarified yet how vimentin is up-regulated during EMT. These issues should be addressed in the future.

Next, we examined Dil-LDL uptake because its uptake was impaired during chemical induction of EFC, which resulted in DCP production (3,4). In this study, accumulation of Dil-LDL was impaired in cells cultured in hypoxic conditions whereas prominent accumulation of Dil-LDL in cells cultured in normoxic condition. In addition, cells with an impairment in Dil-LDL uptake produced DCP, while cells with marked accumulation of Dil-LDL did not produce DCP, as seen in hepatoma cells with chemical induction of EFC (3,4). Actin-based cytoskeletal reorganization affects several processes required to reshape the plasma membrane, including different forms of endocytic internalization and the protrusion of lamellipodia and filopodia during cell migration (39-41). In addition, the cytoskeleton is dramatically reorganized during EMT (42,43). Therefore, we next examined if hypoxia could contribute to cytoskelatal rearrangement. Actually, fine network of F-actin was absent in the $CoCl_2$-treated cells or cells cultured in 1% O_2. In addition, DCP production was clearly observed in cells that lost linear F-actin structures, whereas DCP production was not observed in the control cells that showed a fine network of actin filaments. Thus, hypoxia induced HCC cells to produce DCP in the same mechanism as chemicals (3,4). In other words, HCC cells change their phenotypes in an unfavorable microenvironment, which induce HCC cells to produce DCP by impairment of vitamin K through cytoskeletal rearrangement. In these situations, it is possible that E-cadherin repression affects cytoskeleton since the cytoplasmic domain of E-cadherin interacts with catenin molecules that mediate binding to the actin filaments (44).

HIF-1α is known to be translocated into nucleus under hypoxia, following gene transcription (45). The expression and localization of HIF-1α was reported to be well correlated with tissue hypoxia (46). In our immunohistchemistry using surgically resected HCC samples, the intensity of DCP was positively correlated with the number of nuclear HIF-1α positive cells in the same area. These observations may confirm our *in vitro* experiments that hypoxic stimulation induced EFC or EMT, resulting in DCP production in HCC cells. However, all DCP producing cells did not show positive staining of nuclear HIF-1α. Adaptation to hypoxia through HIF-1α are particularly relevant to cancer progression, and include angiogenic factors such as VEGF, cell proliferation and survival factors such as insulin-like growth factors, and glycolytic enzymes, invasion or metastatic factors such as matrix metalloproteinases (47). Thus, sequential adaptation may stabilize HIF-1α, resulting in switch-off of HIF-1α signaling. In addition, cytoskeletal changes might still remain after switch-off of its signaling. These could be one of the reasons why staining of HIF1-α and DCP was not always matched in HCC tissues.

According to our results, transarterial chemoembolization (TACE), which induce hypoxia within HCC cells, have a potential risk to induce phenotypic changes to EMT in survival HCC cells after treatment and, therefore, these cells could become aggressive phenotypes to cause invasion or metastasis. Indeed, sarcomatous appearance was most frequent in cases with repeated TAE (48). In addition, TACE combined with radiofrequency ablation (RFA) therapy was superior to TACE alone in improving survival for patients with HCC (49). RFA combined with TACE in patients with early-stage HCC provides overall and disease-free survival rates similar to those achieved by hepatectomy (50). It is possible that RFA might eradicate surviving HCC cells that would become aggressive phenotypes after TACE, although some other factors such as increase of ablative zone after TAE may contribute to it.

In conclusion, we demonstrated here that hypoxia induced HCC cells to produce DCP with the same mechanism as chemicals. In addition, our serial studies may suggest the ecology of HCC cells, with special reference to the mechanism of DCP production; HCC cells can become an aggressive phenotype in an unfavorable microenvironment such as chemicals or hypoxia. During these changes, HCC cells produce DCP by impairment of vitamin K uptake through cytoskeletal rearrangement.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society. We thank Dr T.

Murata, pathology section, Suzuka Chuo Hospital for providing us their paraffin sections of resected HCC.

References

1. Liebman HA, Furie BC and Furie B: Hepatic vitamin K-dependent carboxylation of blood-clotting proteins. Hepatology 2: 488-494, 1982.
2. Blanchard RA, Furie BC, Jorgensen M, Kruger SF and Furie B: Acquired vitamin K-dependent carboxylation deficiency in liver disease. N Engl J Med 305: 242-281, 1981.
3. Murata K and Sakamoto A: Impairment of clathrin-mediated endocytosis via cytoskeletal change by epithelial to fibroblastoid conversion in HepG2 cells: a possible mechanism of des-γ-carboxy prothrombin production in hepatocellular carcinoma. Int J Oncol 33: 1149-1155, 2008.
4. Murata K, Suzuki H, Okano H, Oyamada T, Yasuda Y and Sakamoto A: Cytoskeletal changes during epithelial-to-fibroblastoid conversion as a crucial mechanism of des-γ-carboxy prothrombin production in hepatocellular carcinoma. Int J Oncol 35: 1005-1014, 2009.
5. Mizuta T, Ozaki I, Eguchi Y, *et al*: The effect of menatetrenone, a vitamin K2 analog, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment. Cancer 106: 867-872, 2006.
6. Wang Z, Wang M, Finn F and Carr BI: The growth inhibitory effects of vitamin K and their actions on gene expression. Hepatology 22: 876-882, 1995.
7. Koike Y, Shiratori Y, Sato S, *et al*: Des-γ-carboxy prothrombin as a usuful predisposing factor for the development of portal venous invasion in patients with hepatocellular carcinoma. Cancer 91: 561-569, 2001.
8. Suehiro T, Matsumata T, Itasaka H, Taketomi A, Yamamoto K and Sugimachi K: Des-gamma-carboxy prothrombin and proliferative activity of hepatocellular carcinoma. Surgery 117: 682-691, 1995.
9. Shimada M, Yonemura Y, Ijichi H, *et al*: Living donor liver transplantation for hepatocellular carcinoma: a special reference to a preoperative des-gamma-carboxy prothrombin value. Transplant Proc 37: 1177-1179, 2005.
10. Inayoshi J, Ichida T, Sugitani S, *et al*. Gross appearance of hepatocellular carcinoma reflects E-cadherin expression and risk of early recurrence after surgical treatment. J Gastroenterol Hepatol 18: 673-677, 2003.
11. Lee TK, Poon RTP, Yuen AP, *et al*. Twist overexpression correlates with hepatocellualr carcinoma metastasis through induction of epithelial-mesenchymal transition. Clin Cancer Res 12: 5369-5376, 2006.
12. Yang AD, Fan F, Camp ER, van Buren G, *et al*: Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. Clin Cancer Res 12: 4147-4153, 2006.
13. Sullivan R and Graham CH: Hypoxia-driven selection of the metastatic phenotype. Cancer Metastasis Rev 26: 319-331, 2007.
14. Cannito S, Novo E, Compagnone A, *et al*: Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. Carcinogenesis 29: 2267-2278, 2008.
15. Wever O, Pauwels P, Craene B, *et al*: Molecular and pathological signatures of epithelial-mesenchymal transition at the cancer invasion front. Histochem Cell Biol 130: 481-494, 2008.
16. Yang AD, Fan F, Camp ER, *et al*: Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. Clin Cancer Res 10: 295-305, 2008.
17. Shah AN, Summy JM, Zhang J, Park SI, Parikh NU and Gallick GE: Development and characterization of gemcitabine-resistant pancreatic tumor cells. Ann Surg Oncol 14: 3629-3637, 2007.
18. Thompson S, Buck E, Petti F, *et al*: Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. Cancer Res 65: 9455-9462, 2005.
19. Hiscox S, Jiang WG, Obermeier K, *et al*: Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of ß-cathenine phosphorylation. Int J Cancer 118: 290-301, 2006.
20. Yang MH, Wu MZ, Chiou SH, *et al*: Direct regulation of TWIST by HIF-1α promotes metastasis. Nat Cell Biol 10: 295-305, 2008.
21. Zhong H, De Marzo AM, Laughner E, *et al*: Simons JW. Over expression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res 59: 5830-5835, 1999.
22. Bouvry D, Planes C, Malbert-Colas L, Escabasse V and Clerici C: Hypoxia-induced cytoskeleton disruption in alveolar epithelial cells. Am J Respir Cell Mol Biol 35: 519-527, 2006.
23. Nishimura Y, Romer LH and Lemasters J: Mitochondrial dysfunction and cytoskeletal disruption during chemical hypoxia to cultured rat hepatic sinusoidal endothelial cells: The pH paradox and cytoprotection by glucose, acidotic pH, and glycine. Hepatology 27: 1039-1049, 1998.
24. Liebman H, Furie BC, Tong MJ, *et al*: Des-γ-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma. N Engl J Med 310: 1427-1431, 1984.
25. Takikawa Y, Suzuki K, Yamazaki K, *et al*: Plasma abnormal prothrombin (PIVKA-II): a new and reliable marker for the detection of hepatocellular carcinoma. J Gastroenterol Hepatol 7: 1-6, 1992.
26. Grazi GL, Mazziotti A, Legnani C, *et al*: The role of tumor markers in the diagnosis of hepatocellular carcinoma, with special reference to the des-gamma-carboxy prothrombin. Liver Transpl Surg 1: 249-255, 1995.
27. Marrero JA, Su GL, Wei W, *et al*: Des-gamma-carboxyprothrombin can differenciate hepatocellular carcinoma from non-malignant chronic liver disease in American patients. Hepatology 37: 1114-1121, 2003.
28. Durazo FA, Blatt LM, Corey WG, *et al*: Des-γ-carboxyprothrombin, alpha-fetoprotein and AFP-L3 in patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma. J Gastroenterol Hepatol 23: 1541-1648, 2008.
29. Okuda H, Nakanishi T, Takatsu K, *et al*: Comparison of clinicopathological features of patients with hepatocellular carcinoma seropositive for α-fetoprotein alone and those seropositive for des-γ-carboxy prothrombin alone. J Gastroenterol Hepatol 16: 1290-1296, 2001.
30. Nakamura S, Nouso K, Sakaguchi K, *et al*: Sensitivity and specificity of des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinoma varies according to tumor size. Am J Gastroenterol 101: 2038-2043, 2006.
31. Tsai SL, Huang GT, Yang PM, Sheu JC and Chen DS: Plasma des-gamma-carboxyprothrombin in the early stage of hepatocellular carcinoma. Hepatology 11: 481-488, 1990.
32. Kasahara A, Hayashi N, Fusamoto H, *et al*: Clinical evaluation of plasma des-gamma-carboxy prothrombin as a marker protein of hepatocellular carcinoma in patients with tumors of various sizes. Dig Dis Sci 38: 2170-2176, 1993.
33. O'Reilly MS, Holmgren L, Chen C and Folkman J: Angiostatin induces and sustains dormancy of human primary tumors in mice. Nat Med 2: 689-692, 1996.
34. Hockel M and Vaupel P: Tumor hypoxia: Definitions and current clinical, biological, and molecular aspects. J Natl Cancer Inst 93: 266-276, 2001.
35. Yuki K, Hirohashi S, Sakamoto M, Kanai T and Shimosato Y: Growth and spread of hepatocellular carcinoma. A review of 240 consecutive autopsy cases. Cancer 66: 2174-2179, 1990.
36. Gilles C, Polette M, Piette J, *et al*: Vimentin expression in cervical carcinomas: association with the invasive and the migratory phenotype of tumor cells. J Pathol 180: 175-80, 1996.
37. Ramekers FC, Haag D, Kant A, *et al*: Coexpression of keratin- and vimentin-intermediate filaments in human metastatic carcinoma cells. Proc Nat Acad Sci USA 80: 2618-2622, 1983.
38. Lester RD, Jo M, Montel V, Takimoto S and Gonias SL: uPAR induces epithelial-mesenchymal transition in hypoxic breast cancer cells. J Cell Biol 178: 425-436, 2007.
39. Thiery JP and Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7: 131-142, 2006.
40. Kaksonen M, Toret CP and Durbin DG: Harnessing actin dynamics for clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 7: 404-414, 2006.
41. Engqvist-Goldstein AE and Drubin DG: Actin assembly and endocytosis: from yeast to mammalians. Annu Rev Cell Dev Biol 19: 287-332, 2003.
42. Savagner P: Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. BioEssays 23: 912-923, 2001.
43. Gotzmann J, Huber H, Thallinger C, *et al*: Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-ß1 and Ha-Ras: steps towards invasiveness. J Cell Sci 115: 1189-1202, 2002.

44. Noren NK, Liu BP, Burridge K and Kreft B: p120 catenin regulates the actin cytoskeleton via Rho GTPases. J Cell Biol 150: 567-579, 2000.
45. Semenza GL: Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15: 551-578, 1999.
46. Vukovic V, Haugland HK, Nicklee T, Morrison AJ and Hedley DW: Hypoxia-inducible factor-1α is an intrinsic marker for hypoxia in cervical cancer xenografts. Cancer Res 61: 7394-7398, 2001.
47. Nasimuzzaman M, Waris G, Mikolon D, Stupack DG and Siddiqui A: Hepatitis C virus stabilizes hypoxia-inducible factor 1α and stimulates the synthesis of vascular endothelial growth factor. J Virol 81: 10249-10257, 2007.
48. Kojiro M, Sugihara S, Kakizoe S, Nakashima O and Kiyomatsu K: Hepatocellular carcinoma with sarcomatous change: a special reference to the relationship with anticancer therapy. Cancer Chemother Pharmacol 23: S4-S8, 1989.
49. Cheng BQ, Jia CQ, Liu CT, *et al*: Chemoembolization combined with radiofrequency ablation for patients with hepatocellular carcinoma larger than 3 cm. A randomized controlled trial. JAMA 299: 1669-1677, 2008.
50. Yamakado K, Nakatsuka A, Takaki H, *et al*: Early-stage hepatocellular carcinoma: radiofrequency ablation combined with chemoembolization versus hepatectomy. Radiology 247: 260-266, 2008.