Rac Activation Is Associated with Hepatocellular Carcinoma Metastasis by Up-regulation of Vascular Endothelial Growth Factor Expression

Terence K. Lee,1 Ronnie T.P. Poon,1 Anthony P. Yuen,1 Kwan Man,1 Zhen Fan Yang,1 Xin Yuan Guan,2 and Sheung Tat Fan1

Abstract

Purpose: Hepatocellular carcinoma (HCC) is associated with a propensity for vascular invasion and metastasis, which contribute to poor prognosis. Angiogenesis is a crucial process contributing to tumor growth and metastasis. Recently, Rac has been suggested to play a role in angiogenesis. However, the actual role of Rac in HCC angiogenesis remains unclear. Given that vascular endothelial growth factor (VEGF) is an important angiogenic factor in HCC, the purpose of this study was to evaluate the possible correlation between Rac activation and VEGF expression in HCC tumor samples, as well as the mechanism involved in Rac-induced HCC angiogenesis.

Experimental Design: We evaluated Rac and VEGF expression in the HCC tissue microarray of paired primary and metastatic HCC samples using immunohistochemical staining. The role of Rac-induced HCC angiogenesis was also evaluated in vitro in HCC cell lines.

Results: We first showed that activation of Rac was correlated with HCC metastasis (P < 0.001), and its expression was significantly correlated with VEGF expression by tissue microarray. Ectopic Rac-dominant active transfection in Hep3B cells increased VEGF secretion, which induced the morphologic change and proliferation of human umbilical vein endothelial cells, resulting in the promotion of angiogenesis. Rac induced the transcriptional activation of VEGF by direct interaction with hypoxia-inducible factor-1α (HIF-1α) expression. In hypoxic conditions, Rac promoted angiogenesis through an increase in HIF-1α stabilization.

Conclusion: This study shows that Rac is a novel angiogenic factor for HCC through the enhancement of HIF-1α protein stability, which provides a possible therapeutic target in the development of inhibitors of angiogenesis.

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world (1, 2). The long-term prognosis remains unsatisfactory even after surgical resection because of a high recurrence rate (3). The high recurrence rate is commonly associated with the propensity of HCC for vascular invasion and metastasis. Tumor growth and metastasis depend on the ability of the tumor to induce its own blood supply. This process is dependent on the induction of angiogenesis mediated by angiogenic factors secreted by the tumor cells. The involvement of a wide variety of angiogenic factors in the development of a microvasculature in the tumor has been reported. Among the identified angiogenic factors (3–6), vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors (7). Previous studies from our group have reported that VEGF is a potent angiogenic factor leading to HCC invasiveness and metastasis (8, 9). Hypoxia-inducible factor-1α (HIF-1α) is a major regulator of VEGF (10). In hypoxic conditions such as malignant tumors, HIF-1α is stabilized, which stimulates angiogenesis in order to increase the oxygen and nutrient supply by increasing VEGF production (11). Although the role of HIF-1α in angiogenesis has been previously suggested, the regulatory mechanism of HIF-1α stabilization is still not well understood.

The Rho family of small GTPases, including Cdc42, Rac, and RhoA, has been implicated in diverse cellular functions, including reorganization of the actin cytoskeleton, cell growth control, transcription regulation, and membrane trafficking (12). Recently, the Rho family has been found to play an important role in the modulation of distinct actin cytoskeleton changes required for cell adhesion, migration, and invasion leading to metastasis (13). However, the effects of Rac, the major member of the Rho family, on migration and invasion, seem to be cell type– and cell substrate– specific (14, 15). Apart from its role in cell motility, a few studies have also suggested that Rac might be involved in tumor angiogenesis (16–19). Given the central role of VEGF in HCC angiogenesis, it is of great interest to elucidate whether Rac activation promotes HCC angiogenesis by interacting with HIF-1α expression.
Recently, our group was the first to report that Rac activation induced HCC cell motility through the stress-activated protein kinase/c-Jun-NH₂ kinase pathway (20). However, the relationship between Rac activation and HCC angiogenesis has not been investigated. In this study, we first showed increased Rac activation in human metastatic HCC as compared with primary HCC by tissue microarray (TMA), and its activation was significantly correlated with VEGF expression. The direct interaction of Rac and VEGF was examined by ectopic transfection of Rac-dominant active (Rac-DA) and Rac-dominant negative (Rac-DN) cells into Hep3B and MHCC-97H cells, respectively. Rac-DA transfection in Hep3B increased VEGF expression through the direct activation of VEGF promoter, resulting in the promotion of angiogenesis and increased proliferation of human umbilical vein endothelial cells (HUVEC). Rac played a role in hypoxia-induced angiogenesis by increased HIF-1α stabilization. This line of evidence strongly provides an insight into the regulatory mechanism of HIF-1α by Rac activation. Our results show the novel angiogenic role of Rac in HCC metastasis, and most importantly, provides a therapeutic target for the inhibition of HCC angiogenesis and metastasis.

Materials and Methods

Patient samples. TMA specimens were obtained from 60 patients who underwent hepatectomy for HCC between 1995 and 1999 at the Eastern Hepatobiliary Surgery Hospital, Shanghai, China, and who subsequently developed intrahepatic or extrahepatic metastases. Matched pairs of primary and metastatic HCC samples were obtained for the TMA. Thirty-nine out of 60 patients with HCC (65%) had a cirrhotic background.

Construction of TMA. The HCC TMA was constructed as previously described (21). Briefly, all tissue samples embedded in paraffin for array studies were freshly sectioned and stained with H&E. The representative regions of the lesion were reviewed carefully and defined by two pathologists. Based on the clinicopathologic information, the specimens were grouped in tissue cylinders and a diameter of 0.6 mm was taken from the selected regions of the donor block and then punched precisely into a recipient paraffin block using a tissue array instrument (Beecher Instruments, Silver Spring, MD). Consecutive 5 μm sections of the microarray blocks were made with a microtome. Finally, a TMA section with 60 pairs of primary and matched metastatic HCC samples (including 31 intrahepatic and 29 extrahepatic metastases) were constructed.

Immunohistochemistry. Formalin-fixed and paraffin-embedded sections with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. After pretreatment in a microwave oven [12 minutes in sodium citrate buffer (pH 6)], the endogenous peroxidase was inhibited by 0.3% H₂O₂ for 30 minutes, and the sections were incubated with 10% normal goat serum for 30 minutes. Primary antibodies rabbit polyclonal anti-Rac (1:500; Cytoskeleton, Denver, CO) and mouse monoclonal anti-VEGF (1:100; R&D Systems, Minneapolis, MN) were applied overnight in a moist chamber at 4°C, respectively. A standard avidin-biotin peroxidase technique (DAKO, Carpinteria, CA) was applied. The reaction was finally developed by Dako Liquid DAB+ substrate chromogen solution (DAKO).

Cell lines. Two metastatic human HCC cell lines, MHCC-97L and MHCC-97-H (low and high metastatic potential, respectively, from the Liver Cancer Institute, Fudan University, China; ref. 22), and three nonmetastatic HCC cell lines, Huh-7 (a gift from Dr. H. Nakabayashi, Hokkaido University School of Medicine, Japan; ref. 23), Hep3B (American Type Culture Collection, Manassas, VA), and PLC (Japanese Cancer Research Bank, Tokyo, Japan), were maintained in DMEM with high glucose (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 mg/mL of penicillin G, and 50 μg/mL of streptomycin (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO₂.

Plasmids and reagents. Rac-DA and Rac-DN was a gift from Dr. D.Y. Jin, Department of Biochemistry, the University of Hong Kong (20). Recombinant human VEGF (rhVEGF) was purchased from R&D Systems. VEGF neutralizing antibody and Flk-1 kinase inhibitor (40 nM/mL) were purchased from R&D Systems and Calbiochem (San Diego, CA), respectively.

Luciferase promoter assay. Hep3B (5 × 10⁵ cells per well) was plated into 24-well culture plates and allowed to grow for 24 hours. pGL3-V109, pGL3-V411, and pGL3-V2274 (these three VEGF promoters were kindly provided by Dr. K. Xie from the University of Texas, Houston, TX), and pRL-CMV-Luc were cotransfected with either Rac-DA or pcDNA into the cells using Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN). The cells were lysed 48 hours after transfection and were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was measured 48 hours after transfection and the reading was then normalized with the Renilla luciferase activity, which served as an internal control for transfection efficiency. Each experiment was done at least thrice in duplicate wells and each data point represents the mean and SD. The percentage of increase in luciferase activity of the Rac-DA-transfected cells with various VEGF promoters was calculated relative to that of the vector controls. The mean percentage increase (or decrease) in luciferase activity was presented as the final result, and the SD of the means was used as error bars.

Secretory VEGF protein quantitation by ELISA. An equal number of Hep3B-pcDNA and Hep3B-Rac-DA transfectants (2 × 10⁵ cells per well) were plated in six-well plates in DMEM containing 10% FBS. The cells were allowed to grow for 24 hours until they reached ~60% to 70% confluency. The growth medium was then removed and replaced with fresh DMEM containing 1% FBS. The cells were incubated for another 24 hours until they reached ~80% confluency. The medium was then harvested and filtered for the measurement of secretory VEGF. The remaining cells were collected and the viable cells were counted. VEGF present in the growth medium was measured using a Quantikine Human VEGF ELISA kit according to the manufacturer’s instructions (R&D Systems). The concentration of VEGF was measured as picograms per milliliter (pg/mL) in the growth medium and the results were then calculated as picograms of secreted VEGF per cell. Each experiment was done at least thrice and the mean concentration of VEGF secretion was presented as the final result. The SD of the means was used as error bars.

Capillary tube formation assay. Hep3B, Hep3B-pcDNA and Hep3B-Rac-DA transfectants were plated onto six-well plates (2 × 10⁵ cells per well) in DMEM containing 10% FCS. After 48 hours, the medium was removed and fresh DMEM was added. The cells were allowed to grow for 24 hours until they were ~80% confluent. The growth medium was collected and filtered for the treatment of HUVECs. HUVECs at passage 4 (104 cells per well) were seeded onto three-dimensional collagen gel (Chemicon International, Temecula, CA) in 9-well plates in the growth medium of Hep3B, Hep3B-pcDNA, and Hep3B-Rac-DA transfectants. DMEM (1% FBS) containing rhVEGF (20 ng/mL, R&D Systems) and DMEM only were also used to culture HUVECs as positive and negative controls, respectively. Tube formation in each treatment was inspected under an inverted light microscope after incubation for 10 hours at 37°C and images were captured at a single level beneath the monolayer.

Bromodeoxyuridine staining. HUVECs (5,000 cells per well) were grown on chamber slides in EGM2 medium for 24 hours. The culture medium was removed and replaced with growth medium of Hep3B, Hep3B-pcDNA, and its Rac-DA transfectants (20 ng/mL rhVEGF in DMEM with 1% FCS, EGM2, and DMEM containing 1% FCS, respectively) for 24 hours of incubation. The treatment medium was then removed and EGM2 was added for another 24 hours of
incubation. The cells were then stained with bromodeoxyuridine (10 μmol/L) for 2 hours and stained with FITC antibody against bromodeoxyuridine. At least 1,000 cells were counted in each experiment and results are presented as the percentage of bromodeoxyuridine-positive cells.

**Reverse transcription-PCR.** Total RNA was isolated using Trizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). cDNA was synthesized using the SuperScript First Strand Synthesis System (Invitrogen) and was then amplified by PCR with HIF-1α and VEGF primer: HIF-1α-f, 5′-GAGTGGAGGCTGCAGACACAT-3′ (24), VEGF-f, 5′-GATGGAGGTGGGTTGACG-3′ and HIF-1α-r, 5′-GAGTGGAGGCTGCAGACACAT-3′ and VEGF-r, 5′-GAGAATGGAGAATCAGTCTGG-3′ (25). The PCR cycling protocol was as follows: 30 cycles for 10 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 57°C, 1 minute at 72°C, and 10 minutes at 72°C. 18S was amplified as an internal control. The PCR products were electrophoresed on 2% agarose gel and were analyzed using a gel documentation system.

**Western blotting.** The cells were lysed and protein extraction was done. The samples were separated in 10% sodium dodecyl-sulfate acrylamide gel and electrophoretically transferred to polyvinylidene difluoride membrane (Amersham, Buckinghamshire, United Kingdom). The membrane was blocked with 10% nonfat milk, washed and then probed with Rac (Cytoskeleton), actin, HIF-1α and VHL (Santa Cruz Biotechnology, Santa Cruz, CA), and VEGF (R&D Systems). After washing, the membrane was incubated with hors eradish peroxidase–conjugated rabbit anti-mouse, goat anti-rabbit, and rat anti-goat antibodies (Amersham), and then visualized by enhanced chemiluminescence plus according to the manufacturer’s protocol.

**Rac activation assay.** The cells were seeded onto a 14-cm diameter tissue culture plate to 70% confluence. The cells were serum-starved for 24 hours and stimulated with 100 ng/mL of platelet-derived growth factor-β for 30 minutes and then lysed with 0.5 mL of ice-cold lysis buffer. The protein lysate was incubated with PAK-PBD beads for 1 hour at 4°C and washed thrice with 1 × wash buffer [25 mmol/L Tris-HCl (pH 7.5), 1 mmol/L DTT, 30 mmol/L MgCl2, 40 mmol/L NaCl, 1% Nonident P-40] and twice with the same buffer without Nonident P-40. The bead pellet was finally suspended in 20 μL of Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with Rac antibody (Cytoskeleton).

**Immunofluorescence of filamentous actin.** To visualize the effect of distributions of filamentous actin proteins, Hep3B was fixed with 4% formaldehyde dissolved in PBS for 10 minutes at room temperature and permeabilized for 15 minutes with 0.1% Triton X-100. Cells were incubated in 1% bovine serum albumin in PBS for 30 minutes to block nonspecific antibody binding, and then incubated with 1 μg/mL FITC-phalloidin (Sigma Chemical Co., St. Louis, MO) overnight at 37°C. The slides were analyzed using an image analysis system (Eclipse E600; Nikon, Tokyo, Japan).

**Invasion assay.** Invasion assays were done with 24-well BioCoat Matrigel Invasions Chambers (Becton Dickinson, Franklin Lakes, NJ) using 5 × 104 cells in serum-free DME and plated onto either control or Matrigel-coated filters. Conditioned medium from MHCC-97H or MHCC-97H-Rac-DN was placed in the lower chambers as chemo-attractants. After 22 hours in culture, cells were removed from the upper surface of the filter by scraping with a cotton swab. Cells that invaded through the Matrigel and were adherent to the bottom of the membrane were stained with crystal violet solution. The cell-associated dye was eluted with 10% acetic acid and its absorbance was determined at 595 nm. Each experiment was done in triplicate and the mean values ± SE are presented.

**Statistical analysis.** Continuous data were expressed as the median and range and compared between groups using the Mann-Whitney U test. Categorical variables were compared using the χ2 test (or Fisher’s exact test where appropriate). All statistical analyses were done using a statistical software (SPSS 9.0 for Windows; SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

---

**Results**

**Rac activation correlated significantly with HCC metastasis and VEGF expression.** The role of Rac in tumor metastasis is controversial and seems to be cell type– and cell substrate– specific (14, 15). We evaluated Rac expression from 60 samples of primary HCC and their matched metastatic HCC by immunostaining. Rac expression was found in both the cytoplasm and nucleus of the tumor cells (Fig. 1A). Many in vitro studies have indicated that translocation of Rac to the nucleus occurred when Rac was in an active state (26, 27). Therefore, we regarded immunostaining of nuclear Rac protein as positive staining. Rac expression in primary HCC and matched metastatic HCC is summarized in Table 1. Rac expression was significantly associated with HCC metastasis (P < 0.001). To assess the possible correlation of Rac expression with angiogenesis, we evaluated the protein expression of VEGF in another set of corresponding TMA specimens. Rac correlated positively with VEGF expression (P < 0.001; r = 0.58; Fig. 1A). The VEGF expression in TMA is summarized in Table 1. To confirm this relationship in vitro, we employed five HCC cell lines with
various metastatic potentials. The Rac-GTP level was elevated in metastatic HCC cell lines, MHCC-97H and MHCC-97L, compared with noninvasive Huh-7, PLC, and Hep3B cell lines by Rac-GTP pull-down assay, although there was no significant difference in total Rac level (Fig. 1B). Accompanied by the elevated Rac-GTP level, increases of VEGF mRNA and protein expressions were also observed (Fig. 1B). Both Rac activation and increased VEGF expression were found in metastatic HCC cell lines.

**Significance of Rac-mediated VEGF expression in HCC cell lines.** Given the significant correlation between Rac and VEGF in metastatic HCC samples, we then investigated whether Rac increased metastatic potential by up-regulation of VEGF in HCC cells. In this study, we transiently transfected Rac-DA into Hep3B and examined the effect on VEGF at mRNA and protein levels. After Rac-DA transfection, up-regulation of VEGF protein levels was observed when compared with empty vector controls (Fig. 2A). Ectopic Rac-DA transfection increased both VEGF protein expression and VEGF mRNA level (Fig. 2A). Using ELISA, there was an ~111% increase in VEGF protein secretion in Rac-DA transfectants, when compared with the empty vector control (Fig. 2B). To examine the effect of Rac on HCC invasiveness, we transiently transfected Rac-DN into MHCC97H. Using Matrigel invasion assay, an ~55% decrease in cell invasion was observed in Rac-DN-transfected MHCC-97H cells when compared with empty vector controls (Fig. 2C). The addition of exogenous rhVEGF (20 ng/mL) increased cell invasion in Rac-DN-transfected MHCC-97H cells (Fig. 2C).

### Table 1. Rac and VEGF expression in HCC TMA

|               | Rac | VEGF                      |
|---------------|-----|---------------------------|
|               | Negative (-ve) | Nuclear staining (+) | Negative (-ve) | Weak (+) | Moderate-strong (+++) |
| Primary HCC   | 40/57 (70%)     | 17/57 (30%)             | 21/59 (35.6%) | 18/59 (30.5%) | 20/59 (33.9%) |
| Metastatic HCC| 19/57 (33%)     | 38/57 (67%)             | 4/59 (6.8%)   | 10/59 (17.0%) | 45/59 (76.2%) |

**Fig. 2.** Rac increased VEGF secretion leading to increased HCC invasiveness. **A,** Rac expression in Hep3B-pCDNA3.1 and Rac-DA transfectant. The transfection efficiency was ~60% after quantitation by the transfection of green fluorescent protein into Hep3B. Accompanied by increased Rac protein levels, VEGF mRNA and protein levels were up-regulated. **B,** secretory VEGF was significantly increased in Rac-DA transfectant. VEGF protein concentration was measured by ELISA and results are presented as picogram per cell. To examine the effect of Rac on HCC invasiveness, we transiently transfected Rac-DN into MHCC97H. **C,** using Matrigel invasion assay, MHCC-97H-Rac-DN cells showed decreased invasion when compared with MHCC-97H-pcDNA and parental MHCC-97H cell lines. Cell invasion in MHCC-97H-Rac-DN cells could be elevated by the addition of exogenous rhVEGF (20 ng/mL), whereas little increase was found in MHCC-97H cells.
This showed that Rac-induced VEGF up-regulation might increase HCC cell invasiveness.

**Rac activated VEGF promoter activity in the HCC cell line.** Next, we determined whether Rac activated VEGF at the transcriptional level by cotransfection of the Rac-DA together with a luciferase reporter harboring three different lengths of VEGF promoter (pG3-V109, pG3-V411, and pG3-V2274) and generated transient transfectants. We found that VEGF promoter activity was increased significantly by up to ~10.2-fold in pG3-V2274 (VEGF promoter that contained HIF-1α binding site) when compared with pG3-V109 and pG3-V411 in Rac-DA transfectants (Fig. 3A). To examine whether Rac activated VEGF using HIF-1α, Western blotting was done to examine HIF-1α expression in Rac-DA transfectants. HIF-1α protein levels, but not mRNA levels, were increased in Rac-DA transfectants when compared with empty vector controls (Fig. 3B). Taken together, Rac induced VEGF expression by up-regulation of the HIF-1α protein.

**Angiogenic effect of secretory VEGF on HUVECs.** The effect of secretory VEGF protein by Rac-DA transfectant on HUVECs was studied in terms of angiogenic and proliferative abilities. After culturing the cells in Rac-DA transfectant medium for 10 hours in a capillary tube formation assay, HUVECs showed an elongated morphology with a network structure, in contrast to the scattered and round-shaped morphology in DMEM or in Hep3B parental cell or Hep3B-pcDNA3.1 media (Fig. 4A). The addition of rhVEGF (20 ng/mL) to HUVECs also led to an elongated network structure (Fig. 4A). Furthermore, the proliferation rate of HUVECs in Rac-DA transfectant medium was ~4.2-fold higher than HUVECs cultured in the media of Hep3B parental or Hep3B-pcDNA cells. Increased proliferation was observed in medium supplemented with 20 ng/mL rhVEGF (Fig. 4B). The angiogenic role of increased VEGF secretion by Rac-DA transfection on HUVECs was examined by treatment with VEGF neutralizing antibody and an inhibitor of VEGF receptor Flk-1, SU11498. Disappearance of the elongated morphology and decreased proliferation rate of HUVECs were observed after the addition of SU11498 (40 μmol/L) and VEGF neutralizing antibody (0.1 μg/mL) in the culture medium of rhVEGF and Rac-DA transfectant (Fig. 4C and D).

**Rac plays a role in hypoxia-induced angiogenesis by an increase in HIF-1α stabilization.** Hypoxia is a common feature of metastatic tumor, especially in the central regions (28). HIF-1α is a crucial factor in hypoxia-induced angiogenesis. Because we found that Rac induced VEGF expression by up-regulation of HIF-1α, we hypothesized that Rac played a role in hypoxia-induced angiogenesis in HCC. To confirm this, we first examined the expression level of Rac-GTP, VEGF, and HIF-1α both in normoxic and hypoxic conditions. From phalloidin staining, membrane ruffling and increased lamellipodia formation were observed in Hep3B in hypoxic conditions, showing hypoxia-induced Rac activation (Fig. 5A). Using Western blotting, Rac-GTP, VEGF, and HIF-1α protein levels were increased in hypoxic conditions when compared with normoxia (Fig. 5B). To further confirm this hypothesis, we transiently transfected Rac-DN into Hep3B cells and examined the expression of Rac, VEGF, and HIF-1α protein in hypoxic conditions. Using Western blotting, we found inhibition of Rac activation in hypoxic conditions (Fig. 5C). Upon inhibition of Rac activation, no apparent increase in both VEGF and HIF-1α protein levels were observed in hypoxic conditions (Fig. 5C). To examine whether Rac played a role in hypoxia-induced angiogenesis by an increase in HIF-1α protein stabilization, we monitored the levels of Rac-induced HIF-1α protein synthesis with the addition of cycloheximide to block HIF-1α protein synthesis. To this end, we investigated
the decay of hypoxia-stabilized HIF-1α after transfer of vector control cells from hypoxia to normoxia. Rapid decay of HIF-1α was observed after 20 minutes and was undetectable after 60 minutes in the normoxic condition (Fig. 5D). In contrast, Rac-induced HIF-1α protein levels in normoxia remained constant even 60 minutes after the addition of cycloheximide (Fig. 5D). VHL and p53 protein are two of the most important molecules in the ubiquitination and proteasomal degradation of HIF-1α (29, 30). The Western blot in Fig. 5E shows a significant increase in VHL protein level when normoxic conditions are restored in empty vector controls, suggesting HIF-1α protein degradation by VHL protein. However, no significant change in the VHL protein levels were seen in Rac transfectants (Fig. 5E). These results suggested that Rac increased the stability of HIF-1α protein by the inhibition of VHL protein. For p53 protein, we did not detect any expression in either empty vector or Rac-transfected Hep3B cell lines due to p53 deletion.

Discussion

HCC metastasis is commonly associated with poor prognosis because of the lack of effective systemic chemotherapy. Recently, family members of the Rho-like GTPases, including Cdc42, Rac1, and RhoA, have been found to play important roles in cell adhesion, migration, and invasion leading to metastasis (13). However, their effects on migration and invasion are controversial. Rac promotes tumor cell migration and invasion in renal and breast carcinomas (14). On the contrary, activation of Rac inhibits migration and invasion of Madin-Darby canine kidney cells (15). In this study, we first reported that Rac activation in tumor tissues correlated with HCC metastasis by TMA immunostaining. This result confirmed the finding of our previous in vitro study which showed the activation of Rac signaling pathways in metastatic HCC cell lines (20). Recent studies have shown that Rac might be involved in tumor angiogenesis. Angiogenesis is fundamental in tumor metastasis (31). Among various factors, VEGF seems to play a central role in tumor angiogenesis and is a reliable marker of HCC angiogenesis (8, 9). By immunostaining of TMA, we found a significant correlation between Rac activation and VEGF (P < 0.001, r = 0.58). Consistent with this positive correlation, we found up-regulated VEGF expression in metastatic cell lines showing elevated Rac-GTP when compared with primary nonmetastatic cell lines. Ectopic Rac-DA introduction not only increased VEGF mRNA and protein expression, but also increased secretory VEGF in the culture medium of Rac-DA transfectants when compared with empty vector controls. Rho family member RhoA and cdc42 have been previously linked to VEGF regulation of endothelial cells (32, 33). To examine whether RhoA and cdc42 also increased the VEGF expression level, we transfected Rho-DA and cdc42 into Hep3B. Interestingly, we did not find any increase in VEGF at both mRNA and protein levels for RhoA transfectant, and found only a slight increase in cdc42 transfectants (data not shown). This result showed that Rac-induced VEGF
expression in HCC tumor cells might be cell type–specific. Secretory VEGF produced by Rac-DA transfectants in the culture medium were able to induce the cell proliferation of HUVECs, indicating that VEGF produced by Rac-DA transfectants were functionally active, able to promote endothelial proliferation, and form new blood vessels. This result was further confirmed by the finding that the addition of SU1498 and VEGF neutralizing antibody into the culture medium of Rac-DA transfectants resulted in the disappearance of the elongated structure and decreased proliferation rate of HUVECs. We further showed that suppression of Rac by Rac-DN transfection also decreased HCC cell invasiveness, as shown in our matrix gel invasion assay. Interestingly, we observed that HCC cell invasiveness was regulated by VEGF. This mechanism was confirmed by increased cell invasion after the addition of exogenous rhVEGF to Rac-DN transfected MHCC-97H cells. In our unpublished data, MHCC-97H was found to be VEGFR-1- and VEGFR-2-positive. Western blot showed an increase in VEGF-B expression upon Rac activation (data not shown). Recent studies showed that VEGF-1 on tumor cells might promote migration and invasion (34, 35). Therefore, it suggests that VEGF produced by Rac-DA transfectants might increase HCC invasion by activation of the VEGF-1 receptor.

To investigate whether Rac regulates VEGF transcriptionally by interacting with the VEGF promoter, we did a promoter assay with different VEGF promoter lengths. We found that HIF-1α was an important site for Rac-induced VEGF activation because promoter activity was maximal in VEGF promoters harboring the HIF-1α-binding site. From the Western blot shown in Fig. 4B, we observed the up-regulation of HIF-1α in Rac-DA transfectants. However, the mRNA level of HIF-1α remained unchanged. These data suggested that Rac induced VEGF up-regulation through HIF-1α protein. HIF-1α is a heterodimeric transcription factor composed of two basic helix-loop-helix subunits (36). It is a major regulator of VEGF contributing to angiogenesis (10). In hypoxic conditions, HIF-1α is an important transcription factor regulating hypoxia-induced angiogenic factors such as VEGF (10). From the Western blot shown in Fig. 3B, the role of Rac in hypoxia-induced angiogenesis was suggested. A previous study has shown that Rac promoted angiogenesis through up-regulation of the HIF-1α protein (37). As shown in Fig. 5A and B, we found a significant elevation of Rac-GTP showing Rac activation in hypoxic conditions. In addition, hypoxia-induced Rac activation was confirmed by increased membrane ruffling and lamellipodia formation. Accompanied by Rac activation, HIF-1α and VEGF were also up-regulated. In Fig. 3B, we showed that Rac activated the VEGF promoter through HIF-1α regulation. Many activators of HIF-1α, such as hypoxia, have been shown to regulate HIF-1α protein expression by inhibiting ubiquitination and degradation (11). Other than hypoxia, several external stimuli, such as growth factors, hormones, nitric oxide, transition metals, and iron chelators were found to induce VEGF expression through an increase in HIF-1α protein stability (38–40). Therefore, HIF-1α protein stability played a crucial role in hypoxia-induced angiogenesis. To investigate whether Rac-induced HIF-1α protein accumulated through a similar mechanism of Hep3B cell transfection, we examined the decay of Rac-induced HIF-1α proteins in the absence of protein synthesis. HIF-1α protein stability in cycloheximide-treated cells showed that the degradation of HIF-1α protein was decreased in Rac-DA-transfected cells when compared with controls under hypoxic conditions, supporting the finding that

![Fig. 5.](image-url)
induction of HIF-1α protein levels by Rac activation was due to enhanced HIF-1α protein stability. VHL and p53 are two important molecules in the ubiquitination and proteasomal degradation of HIF-1α protein (29, 30). The importance of VHL in HIF-1α protein decay (Fig. 5E) showed increased VHL expression after returning to normoxic conditions. In contrast, there was no significant change in VHL protein after Rac transfection. Rac increased HIF-1α protein stability by suppression of VHL protein level. In clinical samples of HCC, >90% of them were hepatitis B virus–positive. Previous reports showed that ectopic transfection of HBx increased Rac and VEGF expression, respectively (41–43). Given the direct Rac-mediated VEGF expression in our study, it is postulated that HBx exhibits a more aggressive growth pattern which may be due to Rac-mediated VEGF secretion. Further investigations are needed to confirm this hypothesis.

In conclusion, we first showed that Rac is a novel angiogenic factor in HCC. Rac promoted hypoxia-induced HCC angiogenesis by the enhancement of HIF-1α protein stability. Our results not only provide a molecular basis for the role of Rac-mediated HCC angiogenesis, but also suggest a novel therapeutic target in the development of inhibitors of angiogenesis.

References

1. Kupfer H, Ye W, Broome U, et al. The risk of liver and biliary duct cancer in patients with chronic viral hepatitis, alcoholism, or cirrhosis. Hepatology 2001;34:714–8.
2. Harris CC. Hepatocellular carcinoma: recent advances and speculations. Cancer Cells 1990;5:146–8.
3. Poon RT, Fan ST, Wong J. Risk factors, prevention and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg 2000;232:211–7.
4. Kandel J, Bossy-Wetzel E, Radvanyi F, et al. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 1996;86:1095–104.
5. Leibovich SJ, Polverini PJ, Shepard HM, et al. Macrophage-induced angiogenesis is mediated by tumour necrosis factor α. Nature 1987;329:630–2.
6. Mitchell RA, Bucala R. Tumor growth-promoting properties of macrophage migration inhibitory factor (MIF). Semin Cancer Biol 2000;10:39–66.
7. Toi M, Matsumoto T, Bando H. Vascular endothelial growth factor: its prognostic, predictive, and therapeutic implications. Lancet Oncol 2001;2:667–73.
8. Ng IQ, Poon RT, Lee JM, et al. Microvesicle density, vascular endothelial growth factor and its receptors FRT-1 and FK-1/KDR in hepatocellular carcinoma. Am J Clin Pathol 2001;116:838–45.
9. Poon RT, Ng IQ, Lau C, et al. Serum vascular endothelial growth factor predicts venous invasion in hepatocellular carcinoma: a prospective study. Ann Surg 2002;233:227–35.
10. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604–13.
11. Bae MK, Jeong JW, Kim SH, et al. Tid-1 interacts with the von Hippel-Lindau protein and modulates angiogenesis by destabilization of HIF-1α. Cancer Res 2005;65:2520–5.
12. Van Aelst L, D’Souza-Schorey C. Rho GTPases and signaling networks. Genes Dev 1997;11:2299–322.
13. Hall A. Rho GTPases and the actin cytoskeleton. Science 1998;279:509–14.
14. Engers R, Springer E, Michels F, et al. Rac affects invasion of human renal cell carcinoma cells by up-regulating tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 expression. J Biol Chem 2001;276:41889–97.
15. Kleij PJ, Westwick JK, Whitehead IP, et al. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. Nature 1997;390:632–6.
16. Soga N, Connolly JO, Chehaila M, et al. Rac regulates vascular endothelial growth factor stimulated motility. Cell Commun Adhes 2001;8:1–13.
17. Cascone L, Giraudo E, Cacavian F, et al. Temporal and spatial modulation of Rho GTPases during in vitro formation of capillary vascular network. Adherenser junctions and myosin light chain targets as Rac1 and RhoA. J Biol Chem 2004;279:37208–9.
18. Hirota K, Fukuda R, Takahashi S, et al. Induction of hypoxia-inducible factor 1 activity by muscarinic acetylcholine receptor signaling. J Biol Chem 2004;279:14521–8.
19. Turcotte S, Desrosiers RR, Blevieu R. HIF-1α mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. J Cell Sci 2003;116:2247–60.
20. Lee TK, Man K, Ho JW, et al. Significance of Rac signaling pathway in HCC cell motility: implications for a new therapeutic target. Carcinogenesis 2005;26:831–7.
21. Hu L, Lau SH, Tzang CH, et al. Association of vimentin overexpression and hepatocellular carcinoma metastasis. Oncogene 2004;23:298–302.
22. Li Y, Tong ZY, Ye SL, et al. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97L. World J Gastroenterol 2001;7:630–6.
23. Nakabayashi H, Henkata K, Miyano K, et al. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–63.
24. Fredre S, Freitaj P, Otto T, et al. The proinflammatory cytokine interleukin 1α and hypoxia cooperatively induce the expression of adrenomedullin in ovarian carcinoma cells through hypoxia inducible factor 1 activation. Cancer Res 2005;65:4890–7.
25. Li Z, Wang D, Na X, et al. The VHL protein recruits a novel KRAB-A domain protein to repress HIF-1α trancriptional activity. EMBO J 2003;22:1857–67.
26. Buchanag FN, Elliot CM, Gibbs M, et al. Translocation of the Rac guanine nucleotide exchange factor Trar1 induced by platelet-derived growth factor and lysophosphatidic acid. J Biol Chem 2000;275:9742–7.
27. Hirsch E, Barberis L, Brancaccio M, et al. Defective Rac-mediated proliferation and survival after targeted mutation of the β1 integrin cytodomain. J Cell Biol 2002;157:481–92.
28. Ryan HE, Lo J, Johnson RS. HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005–15.
29. Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271–5.
30. Minet E, Mottet D, Michel G, et al. Hypoxia-induced activation of HIF-1: role of HIF-1α-Hsp90 interaction. FEBS Lett 1999;460:251–6.
31. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86:353–64.
32. Van Nieuwenhoven GP, Koolwijk P, Versteelen A, et al. Involvement of RhoA/Rho kinase signaling in VEGF-induced endothelial cell migration and angiogenesis in vitro. Arterioscler Thromb Vasc Biol 2003;23:211–7.
33. Soga N, Namba N, Mcalister S, et al. Rho family GTPases regulate VEGF-stimulated endothelial cell motility. Exp Cell Res 2001;269:73–87.
34. Yang AD, Capo ER, Fan F, et al. Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. Cancer Res 2006;66:46–51.
35. Wang FQ, So J, Reiersand S, et al. Vascular endothelial growth factor-regulated ovarian cancer invasion and migration involves expression and activation of matrix metalloproteinases. Int J Cancer 2006;111:879–88.
36. Sadhi A, Montaner S, Patel V, et al. The Kaposi’s sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1α. Cancer Res 2000;60:4873–80.
37. Xue Y, Bi F, Zhang X, et al. Inhibition of endothelial cell proliferation by targeting Rac1 GTPase with small interference RNA in tumor cells. Biochem Biophys Res Commun 2004;320:1309–15.
38. Zelzer E, Levy Y, Kahana C, et al. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1α/ARNT. EMBO J 1998;19:5085–94.
39. Kimura H, Weisz A, Kurashima Y, et al. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood 2000;95:189–97.
40. Laughner E, Taghavi P, Chiles K, et al. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1α-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001;21:3995–4004.
41. Lara-Pezzi E, Serrador JM, Montoya MC, et al. The Hepatitis B virus HBx protein induces adherens junction disruption in a src-dependent manner. Hepatology 2001;33:1270–81.
42. Chung TW, Lee YC, Kin CH. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3/KAT pathways: involvement of invasive potential. FASEB J 2004;18:1123–5.
43. Too YG, Oh SH, Park ES, et al. Hepatitis B virus X protein induces expression of Fas ligand gene through enhancing transcriptional activity of early growth response factor. J Biol Chem 2004;279:36242–9.