Surgical resection has been generally accepted to be the best treatment for hepatocellular carcinoma. However, recurrence and metastasis remain the major obstacles for long-term survival after surgery. Even after curative resection of small hepatocellular carcinoma, the recurrence rate is high (1). Therefore, studies focusing on tumor metastasis and recurrence are important to improve the prognosis of hepatocellular carcinoma patients. With a combination of pathologic and genetic approaches, recurrence of hepatocellular carcinoma has been classified into two types: multicentric occurrence of new tumors and intrahepatic metastasis of the original hepatocellular carcinoma (2–4). Several clinicopathologic studies have shown that the incidence of intrahepatic metastasis is higher in hepatocellular carcinoma with an infiltrative growth pattern than that with an expansive growth pattern (5). At the molecular level, a high incidence of loss of heterozygosity in chromosome 8 is associated with intrahepatic metastasis (6). Currently, there is no chemotherapeutic agent that is effective in preventing hepatocellular carcinoma metastasis.

Cell motility is a key determinant of tumor progression and metastasis. Recently, we have first shown that activation of Rac signaling pathway played a significant role in hepatocellular carcinoma cell motility (7). This novel finding implicated Rac signaling pathway as a potential therapeutic target for suppression of hepatocellular carcinoma metastasis. FTY720, an inhibitor of Rac activity, was found to effectively suppress hepatocellular carcinoma cell motility in vitro through down-regulation of Rac signaling pathway (7). The data led us to investigate the in vivo antimetastatic potential of FTY720. Unfortunately, among various human hepatocellular carcinoma cell lines, metastatic potential was rarely mentioned. Recently, two human hepatocellular carcinoma clones, MHCC-97H and MHCC-97L, with high and low metastatic potentials from the parental cell line MHCC97 have been established by Lee et al. (8). Two clones of the same genetic background but with different degrees of lung and intrahepatic metastases were established. They are
valuable models for the study of hepatocellular carcinoma metastasis and antimitotic potential of FTY720 in vivo.

In the present study, we aimed to examine the in vivo effect of FTY720 on tumor metastasis in a metastatic nude mice model of hepatocellular carcinoma. First, we showed in vitro that an elevated Rac-GTP level was found in MHCC-97H when compared with MHCC-97L, and a higher Rac-GTP level was correlated with higher metastatic potential. FTY720 effectively suppressed cell motility of MHCC-97H and MHCC-97L at the doses of 8 μM and 5 μM, respectively, accompanied with a corresponding decrease of the Rac-GTP level. FTY720 significantly decreased the primary tumor volume of MHCC-97H and MHCC-97L in a dose-dependent manner. Histologically, tumors without FTY720 treatment showed infiltrative growth with more aggressive features, whereas FTY720-treated tumors showed expansive growth patterns with fewer aggressive features. FTY720 significantly suppressed lung and intrahepatic metastases of MHCC-97H and MHCC-97L, accompanied with a decrease of Rac, vascular endothelial growth factor (VEGF) expression, and microvessel density (MVD), in a dose-dependent manner. These results suggest that FTY720 may be a novel therapeutic agent that is clinically applicable for suppression of hepatocellular carcinoma metastasis.

Materials and Methods

Cell lines and drugs and cell transfection. Human hepatocellular carcinoma cell lines MHCC-97L and MHCC-97H (from Liver Cancer Institute, Fudan University, Shanghai, China; ref. 8), Huh-7 (a gift from Dr. H. Nakabayashi, Hokkaido University School of Medicine, Sapporo, Japan; ref. 9), Hep3B (American Type Culture Collection, Manassas, VA), PLC (Japanese Cancer Research Bank, Tokyo, Japan), and PC3 (a gift from Prof. Y.C. Wong of Department of Anatomy of the University of Hong Kong, Hong Kong, China) were maintained in DMEM with high glucose (Life Technologies Bethesda Research Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies Bethesda Research Laboratories), 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Life Technologies Bethesda Research Laboratories) at 37°C in a humidified atmosphere containing 5% CO₂. FTY720 was kindly provided by Novartis Pharmaceuticals Ltd. (Basel, Switzerland). MHCC-97H was transfected transiently with Rac-DN (a gift from Dr. D.Y. Jin, Department of Biochemistry of the University of Hong Kong, Hong Kong, China) or empty vector pCDNA3.1(+)alone using FuGENE6 according to the manufacturer’s protocol (Boehringer Mannheim GmbH, Mannheim, Germany).

Rac activation assay. Cells were seeded onto a 14-cm-diameter tissue culture plate to 70% confluence. For Rac activation assay, the cells were serum starved for 24 hours, stimulated by 100 ng/mL platelet-derived growth factor BB 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 an 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% NP40] and twice with the same buffer without NP40. The bead pellet was finally suspended in 20 μL of Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transferred into nitrocellulose membrane, and blotted with Rac antibody (Cytoskeleton, Denver, CO).

Wound-healing assay and three-dimensional growth of cells in extracellular matrix. Cell migration was assessed by measuring the movement of cells into an area scraped free with a 200-μL pipette tube (time 0) and three-dimensional growth of cells in the extracellular matrix. Cell concentration was first adjusted to 4 to 6 × 10⁶/mL, and 100 μL of cell suspension was then transferred to an Eppendorf tube to which 100 μL of collagen type I solution (Sigma Chemicals, St. Louis, MO) was added. These were mixed gently and dropped on a 60-mm Petri dish for 30 minutes until solidification. Aliquots of 3 to 5 mL of RPMI (Life Technologies Bethesda Research Laboratories) were added slowly to the gel. The development of organoids in this three-dimensional culture model was dependent on migration of single cells into cell aggregates. Organoids development was monitored on day 7 using inverted light microscope (Zeiss Axioscope 25) and photographed with a Polaroid camera at ×100.

Evaluation of metastatic potential of MHCC-97H and MHCC-97L. Approximately 1 × 10⁶ cells in 0.2 mL of cell medium were injected s.c. into the right flank of the mice, which were then observed daily for signs of tumor development. Once the s.c. tumor reached 1 to 1.5 cm in diameter, it was removed and cut into about 1 to 2-mm cubes, which were implanted into the left liver lobe of the nude mice, using the method described previously (10, 11). A week later, the nude mice were randomized into a group of 12 and were treated either with 0.9% sodium chloride, 5 or 10 mg/kg FTY720 for 35 days, or without FTY720 treatment. After 35 days of FTY720 treatment, the animals were sacrificed and autopsied. The liver, lung, kidney, and spleen suspected of tumor metastasis were sampled for tissue sectioning. To examine the intrahepatic, lung, kidney, and spleen metastases, 100 sequential sections (4 μm) were cut and stained with H&E as described previously (8).

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-Rac1 and anti-VEGF and rat anti-mouse CD34) were applied overnight in a moist chamber at 4°C. A standard avidin-biotin peroxidase technique (DAKO, Carpinteria, CA) was applied. Briefly, biotinylated goat anti-rabbit immunoglobulin and goat anti-rat immunoglobulin and avidin-biotin peroxidase complex were applied for 30 minutes each, with 15-minute washes in PBS. The reaction was finally developed by DAKO Liquid DAB⁰ Substrate-Chromogen System (DAKO).

Western blot. Western blot was performed as described previously (12). Protein suspension from the whole-cell lysates or nuclear fraction (20 μg) was mixed with 6× protein sample buffer [0.35 mol/L Tris-HCl (pH 6.8), 30% glycerol, 21.4% β-mercaptoethanol, 10% SDS] and then boiled for 5 minutes. The boiled protein was then loaded onto a 12% SDS-polyacrylamide gel for electrophoresis using constant current (20 mA per gel) and then transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ) using constant voltage of 100 V. The membrane was then blocked with 10% nonfat milk at room temperature for 1 hour, washed with large volume of TBS-T (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20), and probed with rabbit polyclonal anti-Rac1 and anti-VEGF for 1 hour at room temperature. After washing with TBS-T, the membrane was incubated with secondary antibody against rabbit IgG for 1 hour at room temperature and then washed again with TBS-T. A chemiluminescence signal was developed by adding the premixed Enhanced Chemiluminescence Plus Western blotting reagent (Amersham) to the membrane surface, and the signal was then visualized with the X-ray film.

Evaluation of microvessel density. Details of MVD determination have been described in a previous study (13). Briefly, tissue sections were immunostained with rat anti-mouse CD34 monoclonal antibody (Biogenex, San Ramon, CA). At low power field (×40), the tissue sections were screened, and five areas with the most intense neoangiogenesis (hotspots) were selected. Microvessel counts of these areas were done at high power fields (×200). To reduce observer-related variation, counting of the microvessels was done with a computer image analyzer (MetaMorph Imaging System version 3.0; Universal Imaging, West Chester, PA), which is an integrated system of a Windows-based software especially designed for immunohistochemical analysis. Any positively stained endothelial
cell or endothelial cell cluster that was clearly separated from adjacent microvessels and tumor cells and connective tissues was counted as one microvessel, irrespective of the presence of a vessel lumen. An automated microvessel count/field was computed in each hotspot, and the mean microvessel count of the five most vascular areas was taken as the MVD, which was expressed as the absolute number of microvessels/high power fields. Evaluation of the microvessel count was assessed by two independent observers.

**Statistical analysis.** Continuous data were expressed as 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**

Elevated Rac-GTP level was correlated with higher metastatic potential, and FTY720 suppressed the cell motility of MHCC-97H cell line with elevated Rac-GTP level at a higher dose in vitro. Recently, two human hepatocellular carcinoma clones (MHCC-97H and MHCC-97L) with different metastatic potentials from the parent cell line MHCC97 have been established (8). These two cell lines provide a very useful tool for the study of the mechanism of hepatocellular carcinoma metastasis. A significant increase of Rac-GTP level was found in both MHCC-97H and MHCC-97L compared with noninvasive Huh-7, Hep3B, and PLC cell line by Rac-GTP pull-down assay (Fig. 1A). MHCC-97H cell line, which has a higher metastatic ability, was found to have a higher level of Rac-GTP than MHCC-97L (Fig. 1A). To determine the effect of FTY720 on the Rac-GTP level in MHCC-97H and MHCC-97L, Rac activity was evaluated by Rac-GTP pull-down assay at the dosages of 2, 5, and 8 μmol/L (IC₅₀ for MHCC-97H were 11 and 18 μmol/L; IC₅₀ for MHCC-97L were and 9 and 16 μmol/L, respectively). FTY720 remarkably decreased the Rac activity at 8 and 5 μmol/L, respectively (Fig. 1B). Upon decreased Rac activity by FTY720, significantly decreased cell motility was observed by wound-healing assay and three-dimensional growth of cells in matrix gel (Fig. 1C and D).
The proliferation of these two cell lines upon administration of FTY720 at the above doses did not affect the result significantly as evidenced by Ki67 staining (data not shown).

FTY720 significantly decreased primary liver tumor volume in vivo. The therapeutic effect of FTY720 was examined using the metastatic liver tumor nude mice model. Continuous FTY720 treatment at the doses of 5 or 10 mg/kg was started after 7 days of orthotopic implantation of liver tumors originated from MHCC-97H or MHCC-97L cells. During the experiment, FTY720-treated mice showed no sign of toxicity (infection, diarrhea, or loss of body weight). There was no significant change of body weight in the control group (25 ± 3.4 kg), 5 mg/kg FTY720–treated group (24.1 ± 4.2 kg), and 10 mg/kg FTY720–treated group (23.9 ± 5.2 kg). FTY720 significantly decreased primary liver tumor volume from 1,421 ± 383 to 329 ± 19 and 53.5 ± 10.2 mm³ at the doses of 5 and 10 mg/kg, respectively, for MHCC-97H (Fig. 2A). The primary tumor volume was decreased from 1,313 ± 277 to 58 ± 8.2 and 19.1 ± 2.1 mm³ at the doses of 5 and 10 mg/kg, respectively, for MHCC-97L (Fig. 2B).

FTY720 significantly suppressed lung and intrahepatic metastases of hepatocellular carcinoma in vivo. Lung metastases were observed 35 days after orthotopic transplantation of MHCC-97H (Fig. 3A). After FTY720 treatment, lung metastasis was dramatically suppressed (Fig. 3B). The number of mice with lung and intrahepatic metastases in the treatment group and control group are shown in Table 1. All the control group showed no kidney and spleen metastasis. To gain a better insight on the mechanism of suppression of hepatocellular carcinoma metastasis, tissue sections of the tumor were examined histologically. For both MHCC-97H and MHCC-97L, venous infiltrative invasion was frequently observed in the control group (Fig. 3C and D). Morphologically, both MHCC-97H and MHCC-97L showed a trabecular pattern in the inner portion of the tumor with an infiltrative growth pattern, which was consistent with typical human hepatocellular carcinoma. On the contrary, FTY720-treated groups showed expansive growth patterns without venous invasion (Fig. 3E-H).

FTY720 significantly decreased Rac expression of tumor cells. From our in vitro study, we found that the elevated Rac-GTP level was correlated with higher metastatic potential. FTY720 inhibited cell motility by down-regulation of the Rac-GTP level. To further confirm this result, we have done the immunohistochemistry to compare Rac1 expression of the tumor cells between the control and treatment group. By immunostaining, Rac1 expression was found in nucleus (active form) and abundantly in tumor cells with lung metastasis, intrahepatic metastasis, and venous invasion in the control group (Fig. 4A-C). After FTY720 treatment, less nuclear staining of Rac protein was observed in the treatment group for MHCC-97H (Fig. 4E and F). To quantitate the levels of Rac protein after FTY720 treatment, nuclear fraction of the protein was isolated. We found that FTY720 down-regulated the nuclear protein expression of Rac in a dose-dependent manner (Fig. 4G).

FTY720 inhibited vascular endothelial growth factor–dependent tumor angiogenesis. VEGF is a potent angiogenic factor and plays a central role in tumor angiogenesis in hepatocellular carcinoma (14, 15). There is a close relationship between VEGF expression and MVD level (16), which is regarded as a quantitative means of angiogenesis. Recently, Rac has been reported to be an important component in mediation of VEGF-induced vascular permeability resulting in angiogenesis (17). In the present study, tumor VEGF expression, as detected by Western blot, was decreased upon administration of FTY720 at the doses of 10 mg/kg for MHCC-97H (Fig. 5A). Consistently, decreased cytoplasmic expression of VEGF in tumor cells was also observed by immunohistochemistry (Fig. 5B).
expression of Rac protein was significantly correlated with the decrease in VEGF protein level at the doses of 5 and 10 mg/kg FTY720 treatment \( (P < 0.05) \), suggesting that VEGF is at least in part down-regulated through Rac signaling pathway. The direct correlation between Rac inhibition and decreased VEGF protein level was examined by transient transfection of Rac-DN plasmid into MHCC-97H cell. Decreased VEGF protein expression was observed after transfection of Rac-DN, which suggested a direct link between Rac and VEGF protein (Fig. 5C). Accompanied with down-regulation of VEGF, FTY720 decreased MVD by 47% and 65%, respectively, at the doses of 5 and 10 mg/kg for MHCC-97H (Fig. 5D), whereas MVD was decreased by 69% and 81%, respectively, for MHCC-97L (Fig. 5D).

**Discussion**

Despite recent efforts made in diagnostic and therapeutic methods, the prognosis of hepatocellular carcinoma patients...
with metastasis is poor (18). The molecular mechanism of metastasis is not fully understood. Recently, we have found that Rac signaling pathway plays a crucial role in hepatocellular carcinoma cell motility, and FTY720 was found to effectively suppress hepatocellular carcinoma cell motility through the Rac/c-Jun NH2-terminal kinase pathway. In the present study, we showed the in vivo antimetastatic effect of FTY720 on a metastatic hepatocellular carcinoma nude mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>MHCC-97H</th>
<th>MHCC97-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume (mm³)</td>
<td>1421.4 ± 383</td>
<td>329.1 ± 19.7</td>
<td>53.5 ± 10.2</td>
</tr>
<tr>
<td>Intrahepatic metastasis (%)</td>
<td>18/24 (75%)*</td>
<td>1/12 (8.3%)*</td>
<td>0/12 (0%)*</td>
</tr>
<tr>
<td>Pulmonary metastasis (%)</td>
<td>22/24 (92%)*</td>
<td>3/12 (25%)*</td>
<td>0/12 (0%)*</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with the control group.

Table 1. The effect of FTY720 on liver growth and metastasis

**Fig. 4.** FTY720 decreased expression of Rac1 in a dose-dependent manner. Rac1 was abundantly expressed in tumors cells with (A) lung metastasis, (B) intrahepatic metastasis, and (C) venous invasion in MHCC-97H. H&E staining showed (D) tumor cells invading into the blood vessels in the consecutive section of (C) blood vessels (arrows, nuclear Rac staining). After FTY720 treatment, nuclear Rac1 expression was decreased remarkably at 5 mg/kg for MHCC-97H by immunostaining (E and F). By Western blot (G), nuclear Rac1 protein was decreased significantly after FTY720 treatment at 5 or 10 mg/kg for both MHCC-97H and MHCC-97L, respectively.

Rac1
Histone
From our in vitro study, we confirmed the role of Rac in hepatocellular carcinoma cell motility as evidenced by an elevated Rac-GTP level in MHCC-97H and MHCC-97L when compared with the noninvasive Huh-7, Hep3B, and PLC cell lines. A higher dose of FTY720 was needed to effectively suppress cell motility of MHCC-97H when compared with MHCC-97L. The suppression of cell motility was accompanied with a corresponding decrease in the Rac-GTP level. The above in vitro data have led us to investigate not only the effect but also the detailed mechanism of FTY720 in our metastatic hepatocellular carcinoma tumor model. FTY720 significantly decreased the tumor volume of both MHCC-97H and MHCC-97L in a dose-dependent manner. FTY720 was found to decrease tumor volume of MHCC-97L more significantly than MHCC-97H. This may be due to the difference in the proliferative rate (ref. 8; doubling time was 34.2 versus 60.0 hours for MHCC-97H and MHCC-97L, respectively).

MHCC-97H and MHCC-97L have been reported to have lung and intrahepatic metastases after orthotopic implantation into the livers of nude mice (8). In our experiments, we found that both lung and intrahepatic metastases were significantly suppressed in a dose-dependent manner after FTY720 treatment. Throughout the experiment, there was no severe toxic effect to the nude mice, as all the nude mice remained healthy.

The infiltrative growth pattern was frequently observed in hepatocellular carcinoma clinical specimens with intrahepatic metastasis (5). From our MHCC97H and MHCC-97L implanted tumor tissues, those tumors with lung and...
intrahepatic metastases exhibited an infiltrative growth pattern. After FTY720 treatment, those without lung and intrahepatic metastases exhibited a change of growth pattern to an expansive one with a clear boundary between the tumor and mouse normal liver. Histologically, it confirmed the effect of FTY720 on suppression of lung and intrahepatic metastases in vivo. A recent study has shown that infiltrative growth at tumor boundary in vivo may reflect the motility of tumor cells in vivo (19). From our in vitro study, we found that FTY720 suppressed motility of both MHCC-97H and MHCC-97L effectively at different dosages without affecting the proliferation significantly (data not shown). Both our in vitro and in vivo observations suggested that FTY720 suppressed metastasis through inhibition of cell motility, which is a crucial factor for metastasis.

To further confirm the role of Rac in hepatocellular carcinoma cell motility, we did the immunostaining in vivo on MHCC-97H and MHCC-97L tumor tissues. We found both nuclear and cytoplasmic expression of Rac protein in tumor tissues. Many in vitro studies have indicated that translocation of Rac to nucleus occurred when Rac was in active state (20, 21). Therefore, we regarded immunostaining of nuclear Rac protein as positive signaling. First, nuclear Rac expression was frequently observed in both MHCC-97H and MHCC-97L implanted tissues. Second, nuclear Rac expression was abundantly expressed in tumor cells with lung and intrahepatic metastases and venous infiltration. This observation confirmed the role of Rac in hepatocellular carcinoma cell motility. By quantitation of nuclear Rac protein expression in Western blot in MHCC-97H and MHCC-97L, we found that the amount of nuclear expression of Rac protein in MHCC-97H was greater than that in MHCC-97L, which was consistent with the in vitro Rac-GTP pull-down assay results. After FTY720 treatment, Rac expression was dramatically decreased in a dose-dependent manner for both cell lines. This result confirmed the role of Rac in FTY720-suppressed cell motility.

Angiogenesis as assessed by MVD and tumor VEGF expression seems to play an important role in intrahepatic metastasis of hepatocellular carcinoma (16). In hepatocellular carcinoma clinical samples, there was a close correlation between VEGF expression and tumor MVD, suggesting that VEGF was an important factor for angiogenesis in hepatocellular carcinoma (16). FTY720 was previously shown to inhibit VEGF-induced vascular permeability, which is an important process for angiogenesis (22). To find out whether FTY720 has any antiangiogenic effect on our metastatic hepatocellular carcinoma model, we examined VEGF and MVD by CD34 expression levels. By immunostaining, cytoplasmic VEGF and MVD were decreased dramatically after FTY720 treatment in a dose-dependent manner. Rac was a key component in mediation of VEGF-induced vascular permeability (17). In our study, the result suggested that there might be a cross-talk between Rac and VEGF protein and CD34 expression. We found that there was a positive correlation between the decrease in Rac protein level and decrease in VEGF protein expression in tumor cells upon FTY720 treatment. To confirm the direct effect of Rac inhibition on VEGF protein expression, we transiently transfected Rac-DN into MHCC-97H. Accompanied with the Rac inactivation, VEGF protein expression was down-regulated. Therefore, FTY720 might at least, in part, inhibit Rac-mediated VEGF-induced vascular permeability resulting in suppression of metastasis. Despite the above data of the effect of FTY720 on inhibiting cell motility, by changing invasive tumor growth pattern and attenuating angiogenesis, the antimetastasis effect of FTY720 might contribute to substantial reduction in primary tumor burden due to significant decrease in tumor volume as shown in Fig. 2. It is consistent with our previous report that FTY720 significantly inhibited growth of liver tumor with induction of apoptosis (12). Therefore, FTY720 inhibition of hepatocellular carcinoma metastasis in our model might also due to its tumor-suppressive effect.

In conclusion, the present study showed that FTY720 exhibited a potent antimetastatic effect in vivo through inhibition of Rac-mediated cell motility and VEGF-mediated angiogenesis without notable side effects. FTY720 could be a novel pharmaceutical drug for suppression of hepatocellular carcinoma metastasis.

References


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