Extracellular signal-regulated kinase and Akt activation play a critical role in the process of hepatocyte growth factor-induced epithelial-mesenchymal transition

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Abstract. Epithelial-mesenchymal transition (EMT) has recently been studied to elucidate mechanisms of the liver metastatic process. We investigated EMT in the process of liver metastasis and the effects of chemotherapy on EMT cells as therapeutic strategy for colorectal liver metastasis. We used the CT26 murine colorectal carcinoma cell line to create an *in vivo* mouse liver metastasis model. Liver tumors were stained immunohistochemically. Expression of proteins associated with TGF-β/Smad and hepatocyte growth factor (HGF)/c-Met pathways were investigated by western blotting. Cells with c-Met mRNA knockdown by siRNA techniques showed clearly reduced liver metastases compared with regular cells at 21 days. TGF-β and HGF induced EMT expression, but signal transduction was quite different. TGF-β induced ERK, but not Akt phosphorylation. HGF mediated both ERK and Akt phosphorylation. Akt inhibitor blocked Akt phosphorylation but did not affect TGF-β-induced activation of ERK, Snail and Slug. U-0126 did not reduce Snail activity by TGF-β at a concentration to block ERK phosphorylation. However, Akt inhibitor and U-0126 completely inhibited HGF-induced Slug activation. 5-FU mediated cell death in the EMT process induced by TGF-β more effectively than HGF. ERK/Akt signaling, but not the Smad pathway, may be one of the main processes in HGF-induced EMT, despite the Smad pathway, but not ERK/Akt, being critical for TGF-β-induced EMT. The MAPK/Akt pathway is indispensable in HGF/c-Met signaling. The ERK/Akt pathway particularly may be critical in the HGF-induced EMT process. However, long-term use of chemotherapeutic agents may induce drug resistance and distant metastases through EMT-related signaling pathway activation.

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Introduction

Colorectal cancer (CRC) is the third most common epithelial malignancy worldwide: approximately 1,000,000 new cases and 500,000 deaths occur each year (1). Liver metastases develop in 40-50% of patients with CRC and represent one of the most common causes of death. Surgical resection remains an expected procedure to ensure long-term survival or cure (2). After hepatectomy to treat metastatic liver tumor, activation of the signaling pathway from c-Met-related hepatocyte growth factor (HGF) becomes important in the progress of liver regeneration. Because the HGF/c-Met pathway also plays a critical role in the carcinogenesis of CRC (3), an increased level of serum HGF following hepatectomy has been feared to prompt cancer growth. We previously reported that c-Met overexpression was closely associated with liver metastasis, but c-Met expression is reduced in liver metastatic lesions compared with that seen in primary lesions (4). Therefore, surgical resection might unfavorably affect cancer cell progression, but the mechanism and outcome of c-Met expression remain unclear.

During key biological processes such as embryonic development, tissue remodeling, restitution, or wound repair, epithelial cells must escape from their rigid structural constraints through a well-known process termed epithelial-mesenchymal transition (EMT) (5). As an inducer of EMT in normal mammary epithelial cells, transforming growth factor-β (TGF-β) was first described and evaluated as a key factor (6). The TGF-β family of polypeptides is associated with a wide variety of biological functions, and its effect is elicited through activation of Smad2/3/4 for translocation from receptor to the nucleus. In CRC cells, loss of TGF-β sensitivity is frequently due to loss of or mutation in the signaling pathway, notably to its receptor and to the Smad process (7,8). TGF-β induces non-Smad pathways including those of mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) (9). Substantial activation of the HGF/c-Met pathway also leads to scattering and invasion of cancer cells through activation of the cell signaling pathway, and it may regulate EMT (10).

The purpose of the present study was to further develop our previous study on c-Met expression in CRC (4), to investigate EMT in the process of liver metastases, and to evaluate the

effects of chemotherapy on EMT cells as a therapeutic strategy for colorectal liver metastasis.

Materials and methods

Cell lines and culture conditions. Cells from the CT26 murine colorectal carcinoma cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM HEPES buffer, 1 mM sodium pyruvate solution, and 1% penicillin-streptomycin-amphotericin solution (all from Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere of 5% CO_2/95% air at 37˚C. Cells were passaged twice a week.

Animals housing and in vivo experiments. Male 5-week-old BALB/c mice were purchased from SRL (Hamamatsu, Japan) and housed in the animal facilities of the Division of Animal Experiment, Life Science Research Center, Gifu University with free access to water and food. A liver metastatic model of CRC was created by injection of $1.0x10^6$ CT26 cells into the spleen of BALB/c mice as described previously (4). At 21 days after injection, murine spleen and liver were removed and evaluated by western blot analysis, in an immunohistochemical study. Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Gifu University Graduate School of Medicine, and the UCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia.

Cell proliferation assay. Cell growth was assessed by a standard 3-(4,5-dimethyl-thiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) assay (11,12), which detects the dehydrogenase activity in viable cells. A total of $3x10^3$ CT26 cells were seeded into each of the 96-well culture plates or the same density of cells was seeded in 6-cm dish plates overnight and kept in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. The medium was exchanged for serum-free RPMI-1640 medium, and after 48-h incubation, growth stimulation by growth factors was started by adding 5 ng/ml of TGF-β and 20 or 40 ng/ml of HGF to each well in the same condition. Recombinant TGF-β1 and recombinant HGF were purchased from R&D Systems (Minneapolis, MN). After 72 h, the culture medium was removed, and 100 µl of a 0.5-mg/ml solution of MTT (Sigma-Aldrich) was added to each well. The plates were then incubated for 4 h at 37˚C. The culture medium was replaced with 100 µl of dimethyl sulfoxide (Wako) per well, and the absorbance at the 540-nm wavelength was measured using a 2104 EnVision Multilabel Reader (Perkin-Elmer, Waltham, MA).

In preparing the protein samples, cells were treated with Akt inhibitor (BioVision, Inc., Mountain View, CA), and U-0126 (Cayman Chemical, Ann Arbor, MI) for 24 h before administration of growth factors. 5-FU (Kyowa-Kirin, Tokyo, Japan) was administered after 96 h of contact with growth factors.

Western blot analysis and antibodies. Treatment of the specimens was as described previously (13-15). Cell lysates were boiled in Sample Buffer Solution (Wako). Total cell protein extracts (20 µg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using SuperSep™ (Wako) and were electrophoretically transfected onto polyvinyl difluoride membranes. The membranes were blocked with PVDF blocking reagent (Toyobo, Osaka, Japan) for 1 h. The membranes were then incubated with primary antibodies against β-actin, E-cadherin, vimentin, Snail (Snail1), Slug (Snail2), Smad pathway proteins (p-Smad2, p-Smad3), p-ERK (extracellular signal-regulated kinase), ERK, p-Akt, Akt, p-JNK (c-jun N-terminal kinase), JNK, and caspase-3 (1:5,000; Cell Signaling Technology, Danvers, MA) overnight at 4˚C. The primary antibodies were diluted with Can Get Signal Solution 1 (Toyobo). The membranes were then washed with Dako Washing Buffer (Dako, Glostrup, Denmark) and incubated with the appropriate secondary antibodies (1:25,000; Millipore, Darmstadt, Germany), which were diluted with Can Get Signal Solution 2 (Toyobo). The immunoreactive proteins were visualized by chemiluminescence using ImmunoStar LD reagents (Wako), and images were captured by a LAS-4000 (Fuji film, Tokyo, Japan).

Immunohistochemistry. An LSAB kit (Dako) was used for immunohistochemical analysis (16,17). In brief, sections were pretreated by microwave treatment in citrate buffer for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2/methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Sections were incubated with the following primary antibodies: c-Met, 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) and E-cadherin, 1:100 dilution (Cell Signaling Technology). Sections were incubated with primary antibody for 1 h at 25˚C followed by incubations with biotinylated anti-rabbit/mouse IgG and peroxidase-labelled streptavidin for 10 min each. Staining was completed by incubation for 10 min with substrate-chromogen solution. Sections were counterstained with 0.1% hematoxylin. No specific staining was observed in the negative control slides prepared without primary antibody.

Transfection and small interfering RNA experiments for c-Met. CT26 cells were cultured in a medium without antibiotics for 24 h before transfection to 50-70% confluence. Cells were transfected with a small interfering RNA (siRNA) oligonucleotide using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in a final siRNA concentration of 40 nmol/l in serum-free Opti-MEM (Invitrogen) according to the manufacturer's instructions. At 6 h after transfection, the medium was replaced with RPMI-1640 medium supplemented with 10% FBS. The total proteins were extracted 48 h later, and expression levels of the c-Met protein were analyzed by western blotting. siRNA oligonucleotides for c-Met were purchased from Invitrogen.

Statistical analysis. The data were examined using the Student's t-test, χ^2 test, and ANOVA or Kruskal-Wallis test (with appropriate post hoc analysis for multiple comparisons) to determine statistical significance. p-value of <0.05 was regarded as statistically significant.

Results

Effect of c-Met on cancer progression. HGF induced c-Met phosphorylation in the CT26 colorectal cancer cell line after

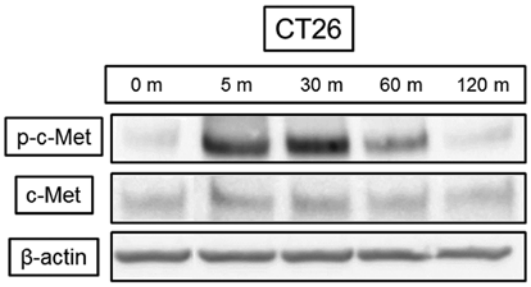


Figure 1. Effect of HGF on c-Met phosphorylation. HGF induced c-Met phosphorylation in the CT26 colorectal cancer cell line. Each band was obtained from western blotting as described in Materials and methods.

5 min with no change in the total amount of protein (Fig. 1). HGF at both the 20- and 40-ng/ml concentrations resulted in significant cell proliferation of 110% and 115%, respectively, ($p<0.05$) compared with control at 72 h (data not shown).

Expression of c-Met was decreased with the increase of cell density, 39% on day 11 and 13% on day 14, compared with day 7, and E-cadherin expression was increased, 104% on day 11 and 139% on day 14 (Fig. 2). Immunohistochemical study revealed the expression of both c-Met and E-cadherin to diminish in the metastatic liver tumors as shown in Fig. 3. Cells in which c-Met mRNA was knocked down by siRNA techniques (Fig. 4A) clearly showed reduced liver metastases compared with regular cells at day 21 in the *in vivo* BALB/c mouse model (Fig. 4B).

Signal pathway to EMT by TGF-β and HGF. We compared the expression of E-cadherin and vimentin by HGF or TGF-β (Fig. 5). The 20-ng/ml concentration of HGF reduced the level of E-cadherin as well as the 5-ng/ml concentration of TGF-β in a time-dependent manner (TGF-β decreased to 47.3% and HGF decreased to 60.8% at 96 h), but knockdown of c-Met diminished the decrease of E-cadherin by HGF. HGF increased the expression of vimentin similarly to TGF-β in a time-dependent manner (increased to 308% by TGF-β and 221% by HGF at 96 h). Next, we observed activation of EMT transcription factors (Fig. 6). Expression of Snail was detected with the addition of TGF-β starting at 24 h; it peaked at 48 h and remained peaked continuously to 96 h. However, expression of Snail was not detected at any time after the addition of HGF. Slug expression was increased at 24 h by the addition of both TGF-β and HGF.

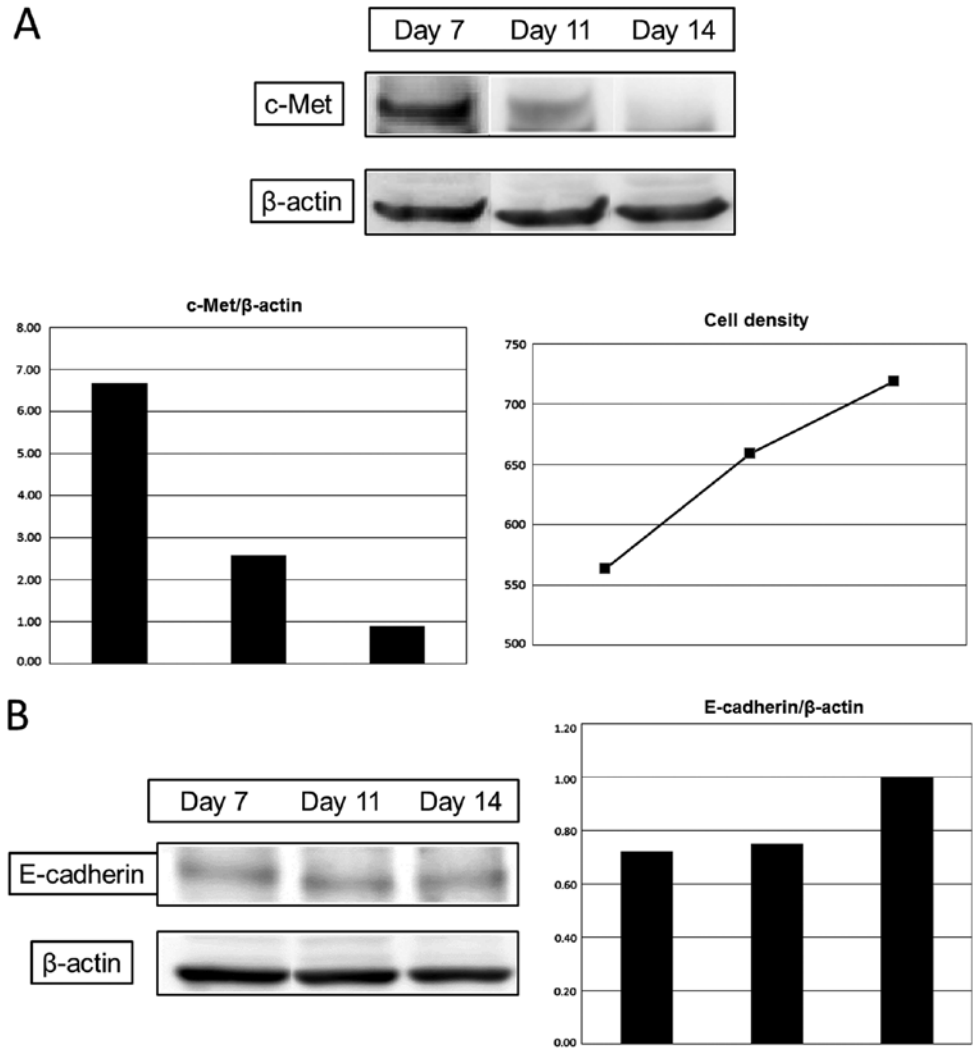


Figure 2. Expression of cellular proteins during cell proliferation. Expression of c-Met proteins decreased depending on time and increase in cell density (A). In contrast, expression of E-cadherin was increased (B). Protein bands were obtained from western blotting.

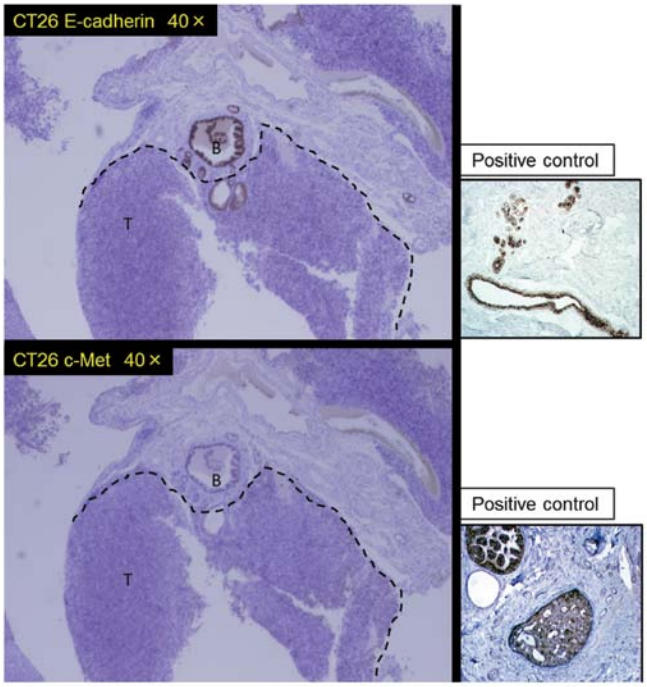


Figure 3. Immunohistochemical staining. Metastatic liver tumor was not stained by the c-Met antibody and E-cadherin antibody in the CT26 liver metastasis model. 'T' indicates metastatic liver tumor and 'B' indicates bile duct. Each positive control was obtained from human breast cancer.

With TGF-β, the peak was detected at 24 h and continued to 48 h, and with HGF, the peak was detected at 24 h and slightly decreased at 48 h. The increase in Slug expression was diminished at 96 h for both TGF-β and HGF. TGF-β induced the activation of Smad2 at 30 min, which continued to 90 min, and Smad3, which peaked at 30 min, despite no differences in the total amounts of Smad2/3, Smad4, and Smad7 present (data not shown). In contrast, HGF exerted no action on these factors, and c-Met knockdown had no effect on the TGF-β-induced cell signal pathway (data not shown).

We then examined the cellular signaling pathway induced by TGF-β or HGF (Fig. 7A) and found that TGF-β induced phosphorylation of ERK from 30 min with weak phosphorylation continuing to 90 min, but no phosphorylation of Akt and JNK. HGF mediated phosphorylation of both ERK and Akt from 30 min, which continued over 90 min, but not JNK. Akt inhibitor blocked phosphorylation of Akt but had no effect on TGF-β-induced activation of ERK, Snail, and Slug. U-0126, which is a MAPK kinase inhibitor, did not reduce Snail activity either by TGF-β at a concentration that blocked ERK phosphorylation (Fig. 7B). In contrast, HGF-induced Slug activation was completely inhibited by both Akt inhibitor and U-0126 (Fig. 7C), and changes in E-cadherin and vimentin phosphorylation by HGF were also blocked by these inhibitors (data not shown).

Effect of chemotherapeutic agent on the EMT process. 5-Fluorouracil (5-FU), one of the most common and basic of chemotherapeutic agents, mediated cell death of the present CT26 cell line (IC_{50}: 4.87±0.61 μM) for 72 h. We evaluated the effects of 5-FU on EMT transcription factors (Fig. 8) and found that 1 μM 5-FU increased expression of Snail, which peaked at 24 h and gradually decreased at 96 h; Slug, which began at 24 h and clearly peaked at 48 h; and vimentin, which increased at

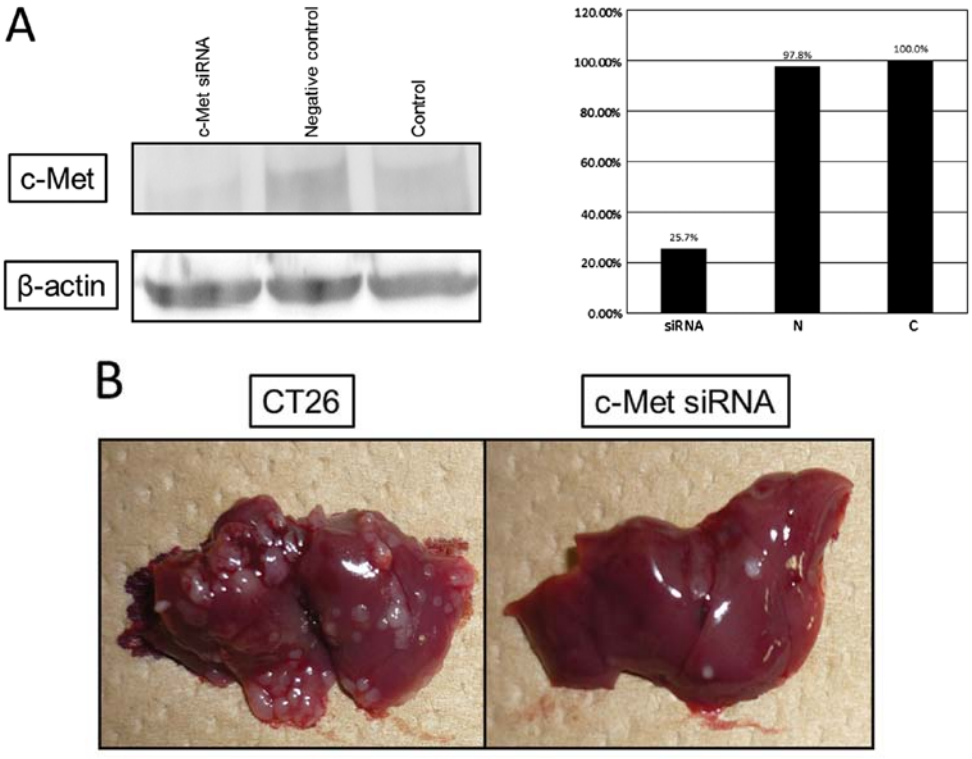
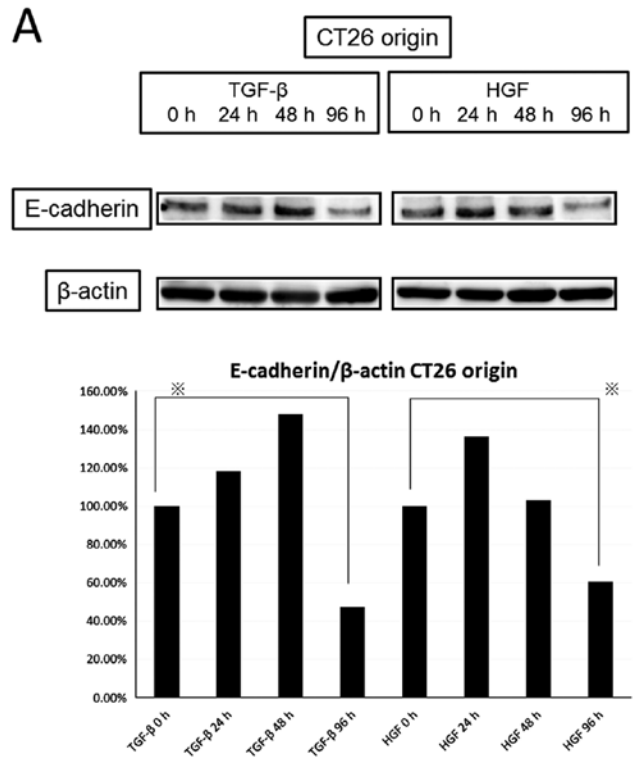
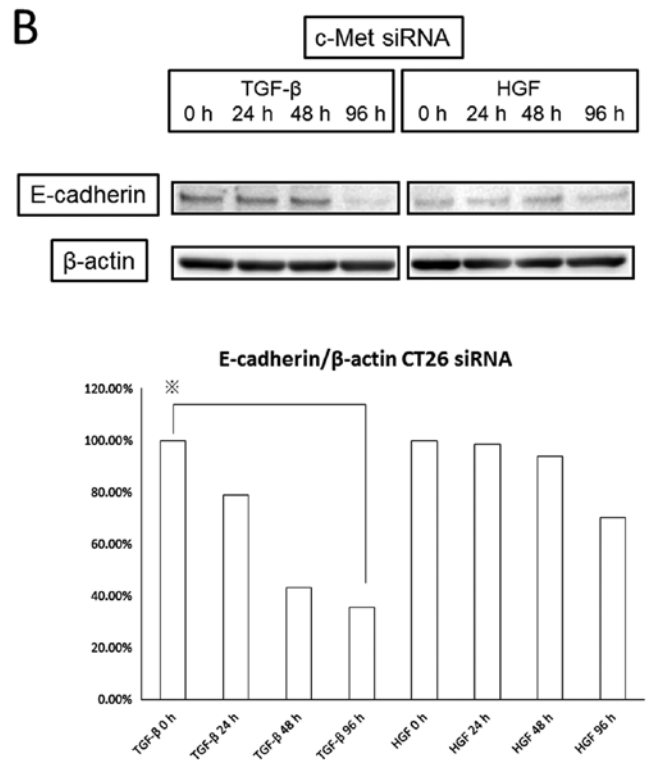


Figure 4. Effect of c-Met on liver metastasis. The protein of c-Met was knocked down to about 30% of the control value by siRNA (A). On the 21st day after injection of cancer cells in the spleen, numerous metastatic tumors were detected in the liver, but cancer cells in which c-Met expression was knocked down clearly resulted in fewer tumors (B).





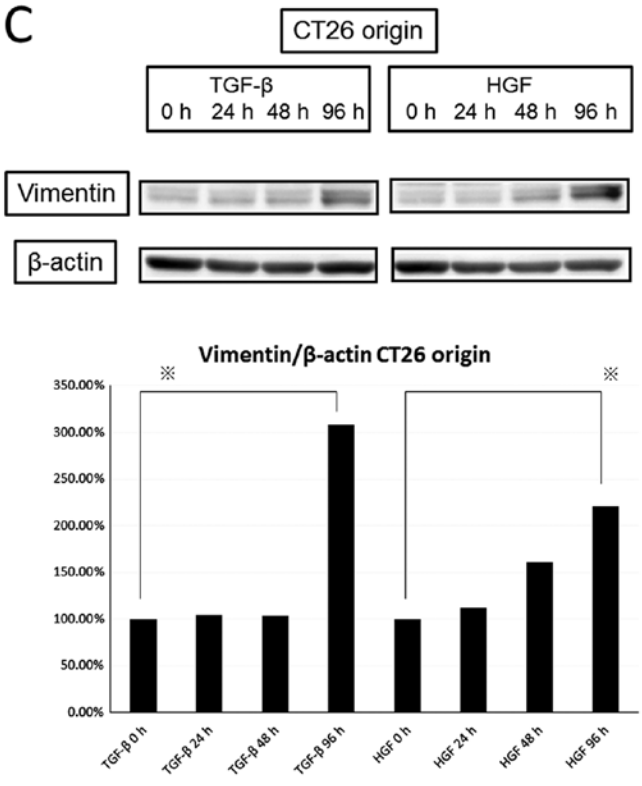


Figure 5. Expression of EMT-related proteins. (A), HGF 20 ng/ml reduced the level of E-cadherin as well as did TGF-β 5 ng/ml in a time-dependent manner. (B), However, knockdown of c-Met diminished the HGF-induced decrease of E-cadherin expression. (C), HGF increased the expression of vimentin as well as TGF-β in a time-dependent manner. *p<0.05.

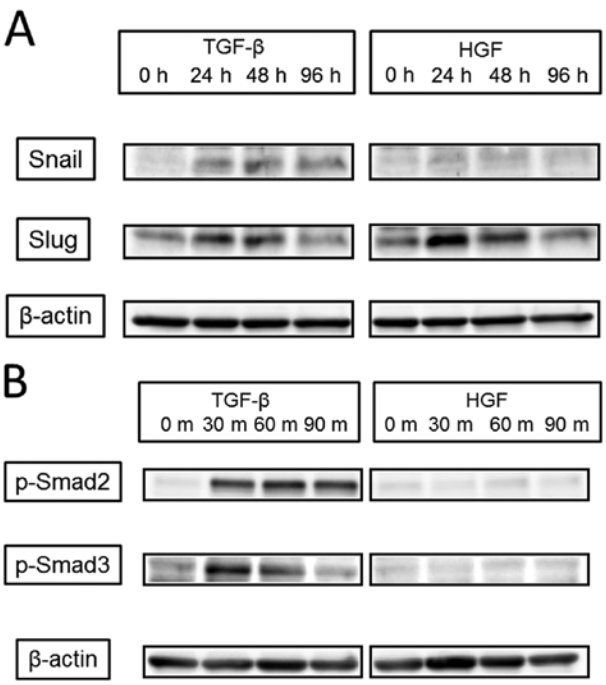


Figure 6. Differences in the EMT signaling pathway between TGF-β and HGF. (A, upper panel), Snail was detected by addition of TGF-β starting from 24 h, peaked at 48 h, and continued to 96 h, but was not detected at any time by addition of HGF. (A, lower panel), Slug was increased by both TGF-β and HGF. With TGF-β, the peak was detected at 24 h and continued to 48 h; with HGF, the peak was detected at 24 h with a slight decrease at 48 h. (B), TGF-β induced the activation of Smad2 for 30 min, which continued to 90 min, and Smad3, which peaked at 30 min; however, no activation was induced by HGF.

24 h and continued to 96 h. 5-FU phosphorylated ERK and Akt after 30 min but had no effect on Smad2/3. 5-FU also induced caspase-3 activation at 24 h that peaked at 48 h.

Finally, we studied the chemotherapeutic effect of 5-FU on cell death and signal transduction in the EMT process (Fig. 9). Pretreatment of CT26 cells with 5 ng/ml TGF-β for 96 h enhanced 5-FU-induced cell death by 10%, compared with the control, and 20 ng/ml HGF also augmented the chemothera-

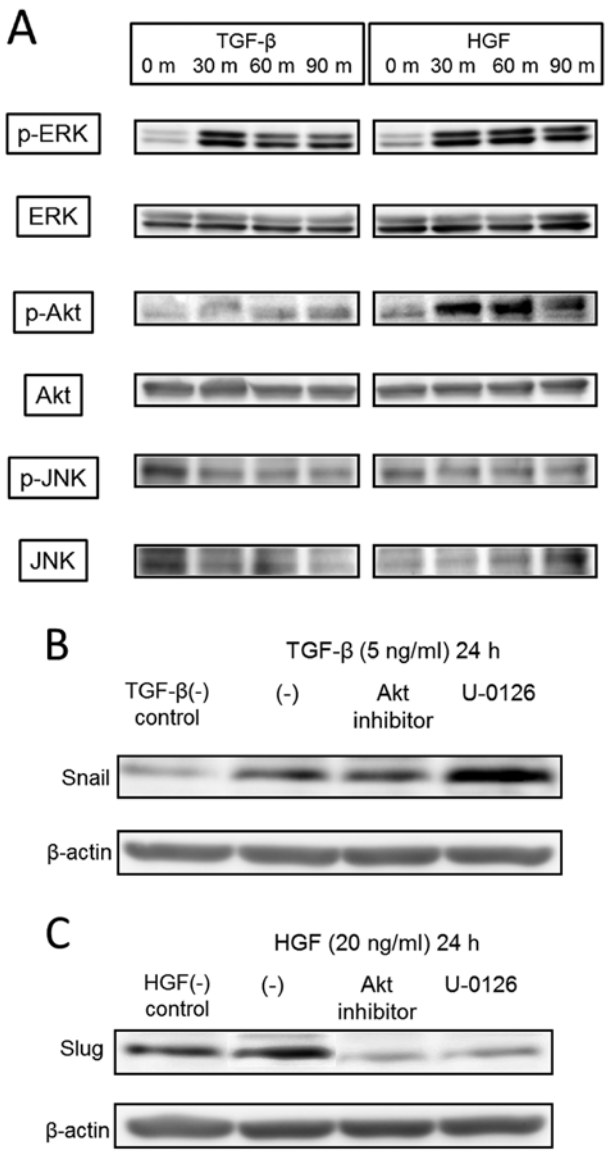


Figure 7. Activation of cellular signaling pathway and its effect. (A), TGF-β induced phosphorylation of ERK but not of Akt and JNK, and HGF mediated both ERK and Akt from 30 min, which continued over 90 min. (B), Snail activation by TGF-β was not decreased by inhibitors of Akt or ERK, which blocked phosphorylation of Akt or ERK. (C), In contrast, Snail activity was completely blocked by both inhibitors.

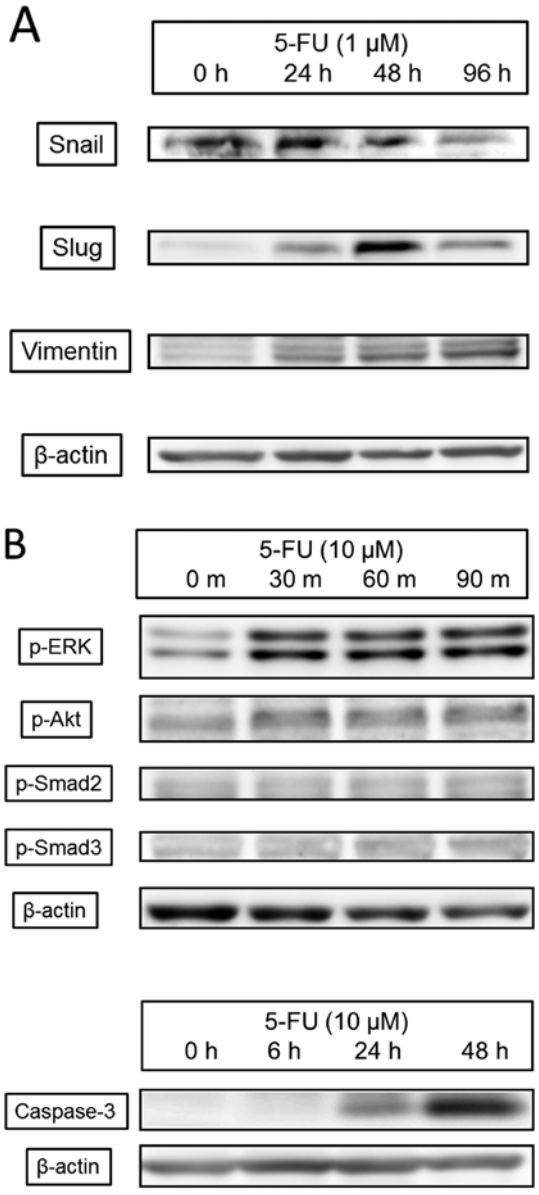


Figure 8. Effect of 5-FU on the cellular signaling pathway. (A), 5-FU 1 μM increased activations of both Snail and Slug and protein volume of vimentin. 5-FU also phosphorylated ERK and Akt after 30 min but had no effect on Smad2/3. 5-FU also induced caspase-3 activation for 24 h that peaked at 48 h (B).

peutic effect of 5-FU by 63%. During EMT induced by both TGF-β and HGF, 5-FU-induced stronger ERK phosphorylation than that in non-EMT-induced cells; however, no effect on caspase-3 was detected (data not shown).

Discussion

In recent years, EMT has been the focus of investigation into the mechanisms of the liver metastatic process (5,18,19). EMT changes the morphology of the cancer cell to that of a spindle shape with mediating migratory competence and invasive capacity overflow (20). Following metastasis of cancer cells promoted by EMT, the induction of MET (mesenchymal-epithelial transition) occurs, and cancer cells build up in a distant organ (18). This regulation is controlled by already known and newly discovered signaling pathways (18). Among factors such as EMT that stimulate scattering of epidermal cells, the HGF/c-Met pathway is also enhanced (10,21). The present study evaluated both HGF action on the process of liver metastasis and estimated which therapeutic procedure, hepatectomy and/or chemotherapy, is more beneficial for the treatment of liver metastases.

A hallmark of EMT represents the loss of E-cadherin, an important caretaker of the epithelial phenotype (18,22). E-cadherin is a cell-cell adhesion molecule, and its loss is consistently observed at sites of EMT during cancer metastases, indicating that its level of expression correlates with cancer progression (23). A recent study showed E-cadherin itself to interact with receptor tyrosine kinases such as c-Met through

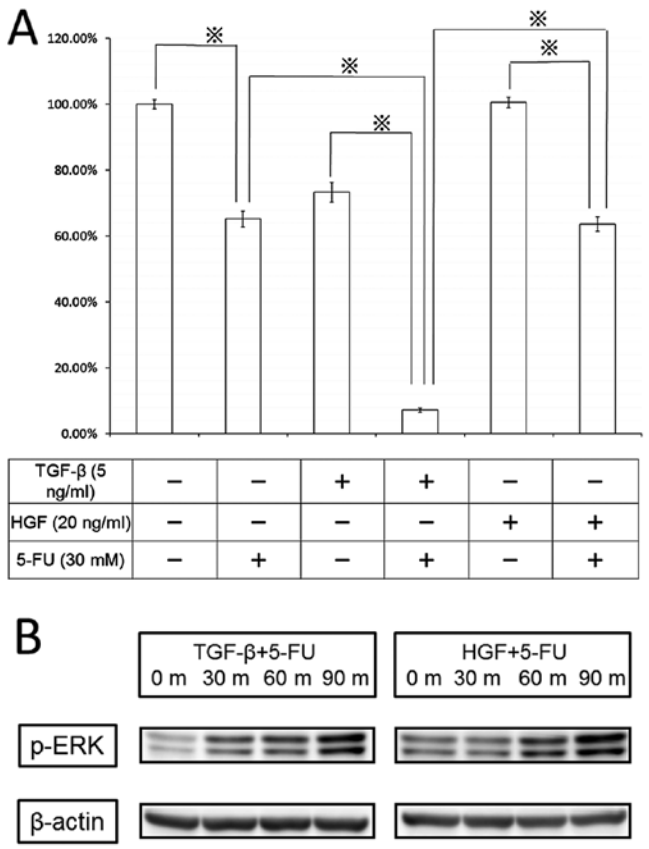


Figure 9. Chemotherapeutic effect of 5-FU on EMT-induced cells. (A), Pretreatment with 5 ng/ml TGF-β enhanced 5-FU-induced cell death by 10%, and 20 ng/ml HGF also augmented the chemotherapeutic effect of 5-FU by 63%. (B), During EMT induced by both TGF-β and HGF, 5-FU induced stronger ERK phosphorylation than that in non-EMT-induced cells ($^{*}p<0.05$).

cell-cell adhesion (24). The present study found that with the decreased expression of c-Met, both cell density and E-cadherin increased. Vimentin, another commonly used molecular marker for EMT, is well known (25), and its increase with the loss of expression of E-cadherin by HGF described in the present study may be involved in EMT for colon cancer cells. HGF is also a major driver of cancer progression (26) and regulates regular signaling pathways, such as those of Akt or ERK, to promote carcinogenesis (27). Because TGF-β, one of the most essential inducers of EMT, is important in the progression of carcinoma to an invasive state (28), we evaluated differences in the signal pathway.

Among the molecular factors related to EMT induction, such as Snail, Slug, Twist, EF1/ZEB1, and SIP1/ZEB2 (21), Snail is a zinc finger transcription factor that induces EMT by directly repressing E-cadherin expression and confers epithelial cells with migratory and invasive properties as an important step for metastasis (6,29). Slug, another zinc finger protein, is closely related to the Snail pathway and regulation of E-cadherin gene transcription (30). Furthermore, other cross-talk pathways have also been focused on (31), and the synergy between Ras signaling and Smad signaling was found to be critical in the induction of EMT. In fact, the receptor of TGF-β is well known to activate MAPKs, such as ERK, JNK, and p38 MAP kinases, PI3 kinase, and small GTPases (31). The present study showed that ERK/Akt signaling, but not the Smad pathway, might be the main pathway in HGF-induced EMT, despite the fact that the Smad pathway, but not ERK/Akt, was critical for induction of EMT by TGF-β. The MAPK/Akt pathway is indispensable in HGF/c-Met signaling (32), and activated Akt was reported to induce loss of cell-cell adhesion, morphological changes, and induction of cell motility (33). Additionally, HGF-induced cell scattering or invasive action is abrogated due to down-regulation of phosphorylated Akt (10). In contrast, in the induction of EMT, TGF-β cooperates with other signaling pathways, such as Wnt (34), Hedgehog (35), and Notch (36), which are all pathways linked to the stem cell renewal pathway (37). TGF-β-induced EMT or its repressors, such as Twist or Snail, also confer stem cell-like properties to non-carcinogenic, immortalized human mammary epithelial cells, providing the first link between EMT and 'stemness'. Indeed, the EMT process was found to generate stem-like properties in breast cancer cells (38). As a concept of cancer stemness, Snail includes not only stem cell-like properties but also resistance to chemo/radiation therapy under the self-renewal process (39). Namely, there is a possibility for cancer therapy through inhibition of EMT not only to reduce metastasis but also to improve drug sensitivity (40). Therefore, we are planning additional study of chemotherapeutic agents to evaluate a novel concept for therapeutic strategy.

Chemotherapeutic agents such as paclitaxel (41) or oxaliplatin (42) make cancer cells susceptible to EMT. The present study showed 5-FU to phosphorylate ERK/Akt and activate Snail and Slug, but not Smad, in an EMT-like manner. Because the ERK pathway has dual actions related to both cell proliferation and growth inhibition (12,13,43), it is still unclear whether 5-FU-induced ERK activation itself is directly related with EMT. However, there is another possibility, that 5-FU produces reactive oxygen species (ROS) and that these ROS inhibit phosphatase action of cell signaling-related proteins to lead to phosphorylation of ERK accordingly (11,12). ROS itself was shown to lead to EMT though the expression of Snail and to cause genomic instability and oxidative damage to DNA (44). Taken together, EMT was evaluated in relation to drug resistance of anticancer agents (41). However, the early response, but not the long-term reaction, to these agents is still unclear and controversial. Some reports showed high expression of E-cadherin to relate to higher chemosensitivity (45,46), whereas its expression has also been related to lower chemosensitivity (47). Further, expression of E-cadherin does not correlate with the effect of chemotherapy or reflect patient prognosis (48). Commonly, loss or low expression of E-cadherin in liver metastasis occurs more frequently in CRC related to poor patient prognosis (49). In the present study, low expression of E-cadherin was detected in the EMT process and high chemosensitivity for 5-FU was shown. In the signalling pathway as well, ERK activation by 5-FU-increased more when EMT was present, indicating ROS expression to be higher. Although the connection between E-cadherin expression and drug-sensitivity in the present study was unclear, EMT and the signaling thereof were affected by different agents, TGF-β and HGF. In particular, the ERK/Akt pathway might be critical in the process of HGF-induced EMT.

After hepatectomy for liver metastasis from CRC, serum HGF elevates for liver regeneration (50), but this does not increase the risk of new metastasis and aggressive tumor formation. As demonstrated in the present study, the reason

was due to diminished c-Met expression at the metastatic site. Chemotherapeutic agents should be effective even if c-Met expression results in regeneration of cancer cells. However, long-term use of chemotherapeutic agents might induce drug-resistant and distant metastases through the activation of the EMT-related signaling pathway. Further investigation will be necessary to determine therapeutic strategy with the use of anticancer agents.

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