

*Featured Article*

# Coexpression of Matrix Metalloproteinase-7 (MMP-7) and Epidermal Growth Factor (EGF) Receptor in Colorectal Cancer: An EGF Receptor Tyrosine Kinase Inhibitor Is Effective against MMP-7–Expressing Cancer Cells

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**ABSTRACT**

**Purpose:** Matrix metalloproteinase-7 (MMP-7) plays an important role in carcinoma invasion and metastasis of cancer. Recent studies focus on diverse roles of MMP-7, other than as a protease, during cancer progression. MMP-7 activates the epidermal growth factor (EGF) receptor by releasing an EGF ligand, tumor growth factor (TGF)- $\alpha$ .

**Experimental Design:** We examined expression of MMP-7 and EGF receptor in an immunohistochemical study of 40 colorectal cancer (CRC) cases. To determine the relationship between the EGF receptor and MMP-7, with a potential curative application, we compared the antitumor activity of the EGF receptor tyrosine kinase inhibitor (gefitinib) between MMP-7 transfectant, KYSE150 and HT29, and control cells.

**Results:** We found a statistically significant correlation ( $P = 0.04$ ) between MMP-7 and activated (phosphorylated) EGF receptor expression, both being positive in six (15%) cases. Gefitinib reduced the cell number ratio more for MMP-7 transfectant than mock cells, and the proportion of

apoptotic cells was 1.5 times higher in MMP-7 transfectant than mock cells by annexin/propidium iodide staining. This was mediated by activation of a TGF- $\beta$  signal as confirmed by the abundant expression of TGF- $\beta$  protein, the cytoplasmic to nuclear translocation of Smad4 protein by the administration of gefitinib, and the quantitative assay of the plasminogen activator inhibitor-1 promoter/luciferase construction.

**Conclusions:** We propose that there are some cancers with up-regulated MMP-7 expression that leads to the activation of apoptotic activity of TGF- $\beta$ , which is susceptible to treatment with EGF receptor tyrosine kinase inhibitor.

**INTRODUCTION**

Matrix metalloproteinase-7 (MMP-7) is a member of the MMP family and has a wide spectrum of varied substrates. It is reported to play an important role in carcinoma invasion and metastasis through extracellular matrix degradation. We have reported overexpression of MMP-7 in esophageal cancer, gastric cancer, and colorectal cancer (CRC) and disclosed that the magnitude of MMP-7 expression is a determining factor in the malignant potential of those cancers (1–4). Besides the role of MMP-7 as a protease that degrades extracellular matrix during cancer cell invasion or metastasis, we identified an intriguing finding that MMP-7 is involved in tumor growth itself or in promotion of the malignant potential via an autocrine or paracrine mechanism. Compared with the control untransfected esophageal cancer cell line KYSE150, MMP-7–transfected KYSE150 had augmented tumor growth and increased in immunostaining for factor VIII, cell cycle promotion, apoptotic inhibitory activity, and genomic instability.<sup>4</sup>

Pai *et al.* (5) recently reported that activation of the epidermal growth factor (EGF) receptor is initiated by release of the EGF ligand transforming growth factor (TGF)- $\alpha$  caused by MMP activity. Therefore, we considered that the above diverse roles of MMP-7 other than as a protease might be involved in the tyrosine-phosphorylated EGF receptor. The present study initially disclosed expression of MMP-7 and phosphorylated EGF receptor in clinical human CRC cases by an immunohistochemical study.

In the present study, we also disclose a synergistic activation of TGF- $\beta$ , which can induce apoptosis in MMP-7–overexpressing cells. With respect to the relationship between TGF- $\beta$

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**Note:** K. Mimori and K. Yamashita contributed equally to this work.

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and the EGF receptor, Goldkorn *et al.* (6) reported that TGF- $\beta$  increased the phosphorylation and kinase activity of the EGF receptor. Therefore, we attempt to clarify the relationship between MMP-7, the EGF receptor, and TGF- $\beta$ .

The EGF receptor tyrosine kinase inhibitor (gefitinib) recently has been used against cancers, such as non-small-cell lung cancer (NSCLC) or CRC, either alone or in combination with other therapies (7–12). It is important to identify susceptible cases or potential genes that may be treated with gefitinib; however, there have been few such studies to date. In the present study, we performed basic research to clarify the relationship between MMP-7, the EGF receptor, and TGF- $\beta$  expression to try to establish new therapeutic models for EGF receptor tyrosine kinase inhibitor use against cancers with high malignant potential and overexpression of MMP-7.

## MATERIALS AND METHODS

**Cancer Cases and Cell Lines.** Forty Japanese cases of CRC were examined. Cancer patients underwent surgery at the Medical Institute of Bioregulation, Kyushu University, Beppu, Japan and Oita Prefectural Hospital, Oita, Japan. There were 15 male and 25 female patients of an average age of 66 years (range, 26 to 88 years). There was no familial history of CRC. We obtained adequate informed consent from all of the patients before starting the experiments. Among the 40 cases, 8, 15, 13, and 4 were classified as tumor-node-metastasis stages I, II, III, and IV, respectively.

We have transfected MMP-7-expressing plasmid into eight esophageal cancer cell lines (TE1, TE3, TE5, KYSE30, KYSE50, KYSE70, KYSE110, and KYSE150) and four colorectal cancer cell lines (COLO201, COLO205, WiDr, and HT-29). The TE series was from Nishihira *et al.* of the Second Department of Surgery, Tohoku University School of Medicine (13), and the KYSE series was from Shimada *et al.* of the First Department of Surgery, Faculty of Medicine, Kyoto University, Japan (14). Only KYSE150 and HT29 transfectants showed intriguing data, such as activated EGF receptors and TGF- $\beta$  activation by EGF receptor inhibitors. Therefore, we disclosed these data from both cell lines in the present study.

**MMP-7 and Phosphorylated EGF Receptor Immunohistochemistry.** Resected surgical specimens from the cancer and corresponding normal tissues were stored at  $-20^{\circ}\text{C}$  until used. Frozen sections (4  $\mu\text{m}$ ) on silicon-coated slide glasses were fixed by periodate-lysine-paraformaldehyde (PLP) solution, which was prepared just before use as follows: stock solution B [8% paraformaldehyde (PFA)] was mixed with stock solution A [0.1 mol/L lysine and 0.05 mol/L phosphate buffer (pH 7.4)] in a ratio of 3B to 1A, and 0.1 volume of 0.1 mol/L  $\text{NaIO}_4$  then was added. The PLP-fixed slides were washed with PBS three times (10 minutes each wash) and air-dried.

We used the tyrosine phosphorylated (activated) EGF receptor (Mouse IgG; BD Biosciences, San Jose, CA) antibody, which is specific for the activated EGF receptor (15–17). Anti-hMMP-7 (clone no. 141-7B2, purified IgG monoclonal antibody; Fuji Chemical Co. Takaoka, Japan) is specific for human MMP-7 (18, 19).

The Envision kit/horseradish peroxidase (DAB, code no. K1390; Dako, Glostrup, Denmark) was used for staining with

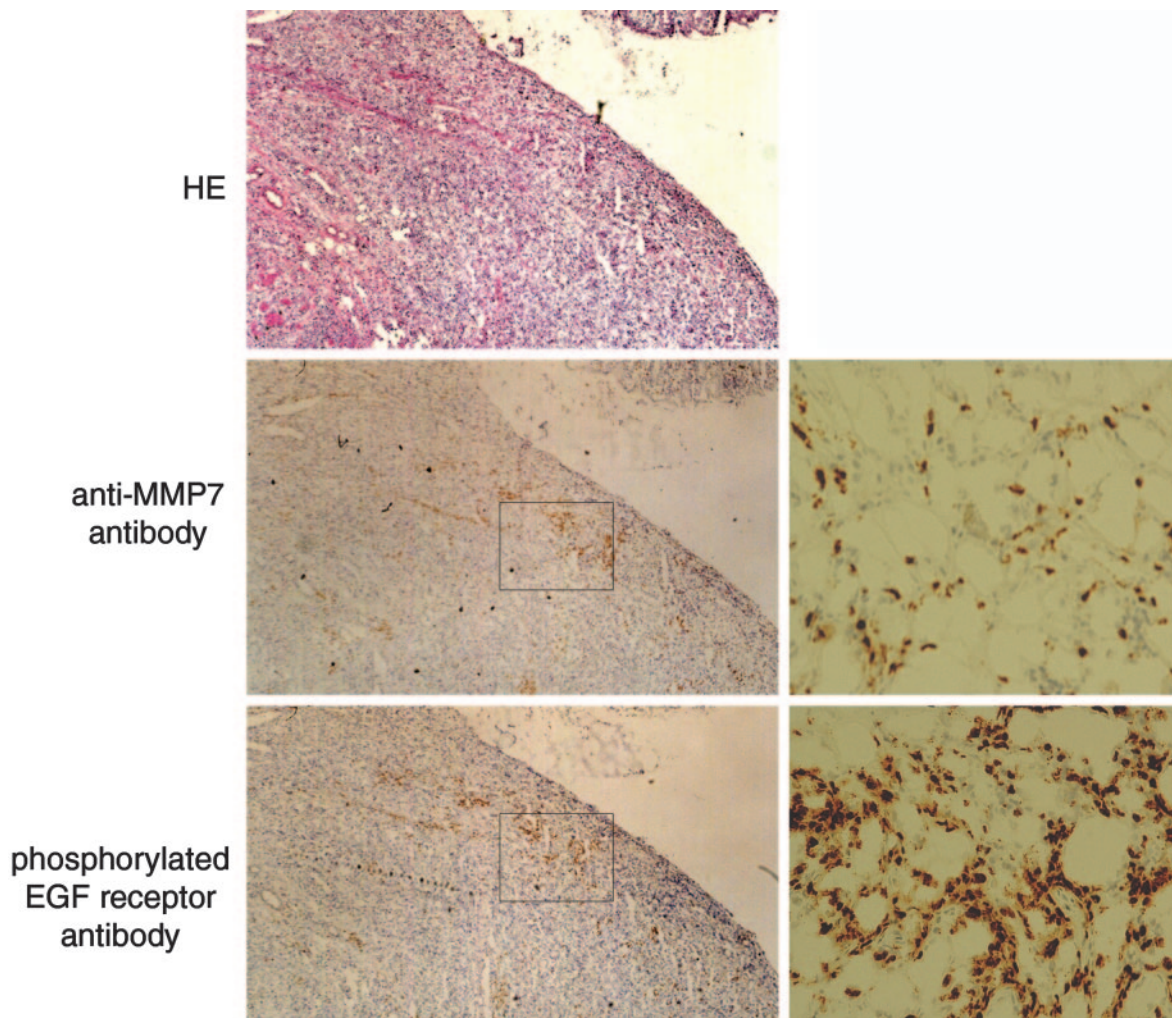
both antibodies. In brief, 0.3%  $\text{H}_2\text{O}_2$  blocking was performed for 10 minutes at room temperature. Washing was with distilled water (DW) for 5 minutes  $2\times$  at room temperature. Primary MMP-7 antibody was diluted 200 times and that for activated EGF receptor 100 times and incubated with the slides for 60 minutes at room temperature. After washing in Tris-buffered saline for 5 minutes  $2\times$  at room temperature, incubation with the polymer chemical reagent (bottle 3) was done for 60 minutes at room temperature. Subsequent washing with Tris-buffered saline for 5 minutes  $2\times$  at room temperature was followed by DAB color development for 10 minutes at room temperature. Counterstaining was performed with hematoxylin (code no. S2020; Dako) for 1 minute, and the slides then were washed with water and dehydrated for mounting.

**Western Blot and Northern Blot Analyses in MMP-7 Transfectants.** Expression of EGF receptor (Fig. 1; Novacastra Inc., Newcastle, United Kingdom) and antiphosphotyrosine antibody (pY) (NeoMarkers Inc., Fremont, CA) in cell extracts from MMP-7-transfected KYSE150 (1) and mock cells was examined by Western blot analysis as performed previously (20). The expression of TGF- $\beta$  mRNA in mock cells or MMP-7-transfected KYSE150 cells, and that of EGF receptor neutralizing antibody (Upstate Biotechnology Inc., Lake Placid, NY) were examined by Northern blot analysis as reported previously (21).

**MTT Assay to Evaluate the Antitumor Effect of Gefitinib.** We used the established and reliable MMP-7-transfected cell line KYSE150 (an esophageal cancer cell line) and their control cell line as described previously (Fig. 2; ref. 1). In brief, 100- $\mu\text{L}$  suspensions of  $1.5 \times 10^3$  cells were grown in microtiter plate wells (96 flat-bottomed wells) in a final volume of 100  $\mu\text{L}$  of culture medium per well in a humidified atmosphere ( $37^{\circ}\text{C}$  and 6.5%  $\text{CO}_2$ ). We prepared gefitinib from the tablet of Iressa (250 mg; AstraZeneca, Wilmington, DE), which was ground down and dissolved into DMSO to a final concentration of 0.01, 0.1, 1.0, 10, 30, 60, and 100  $\mu\text{mol/L}$  (22, 23), and added these concentrations into the 96 wells and then incubated for 72 hours. After the incubation, we added 10  $\mu\text{L}$  of MTT labeling reagent (Roche Molecular Biochemicals, Tokyo Japan; final concentration, 0.5  $\mu\text{g/mL}$ ) to each well. The microtiter plate was incubated for 4 hours in a humidified atmosphere. The solubilization solution (100  $\mu\text{L}$ ) was added to each well, and plates were allowed to stand overnight in the incubator in a humidified atmosphere. After checking for complete solubilization of the purple formazan crystals, the spectrophotometric absorbance of the samples was measured at 570 to 655 nm using a microtiter plate (ELISA) reader.

The growth inhibition rate by gefitinib was calculated as follows: inhibition rate =  $(a - x)/(a - b)$  with a, nontreated tumor cells; x, treated tumor cells; and b, tumor background (Roswell Park Memorial Institute 1640).

**Evaluation of Apoptosis by Annexin and Propidium Iodide Staining.** Cell cycle analysis was performed by DNA-propidium iodide (PI) binding and analysis with a fluorescence-activated cell-sorting (FACS) flow cytometer. MMP-7-transfected KYSE150 and mock cells were starved in serum-free medium for 48 hours and then stimulated with 10 ng/mL of EGF (15). After EGF treatment, we added 20  $\mu\text{mol/L}$  of EGF receptor protein kinase inhibitor and incubated for 24 hours and 48



*Fig. 1* Immunohistochemistry with anti-MMP-7 antibody and antiphosphorylated EGF receptor antibody. Simultaneous staining for MMP-7 and the phosphorylated EGF receptor was observed mainly in the cancer cells in a representative colorectal cancer case. HE, hematoxylin and eosin staining. Left shows low magnification ( $\times 40$ ), and the right shows high magnification of the squared area ( $\times 200$ ).

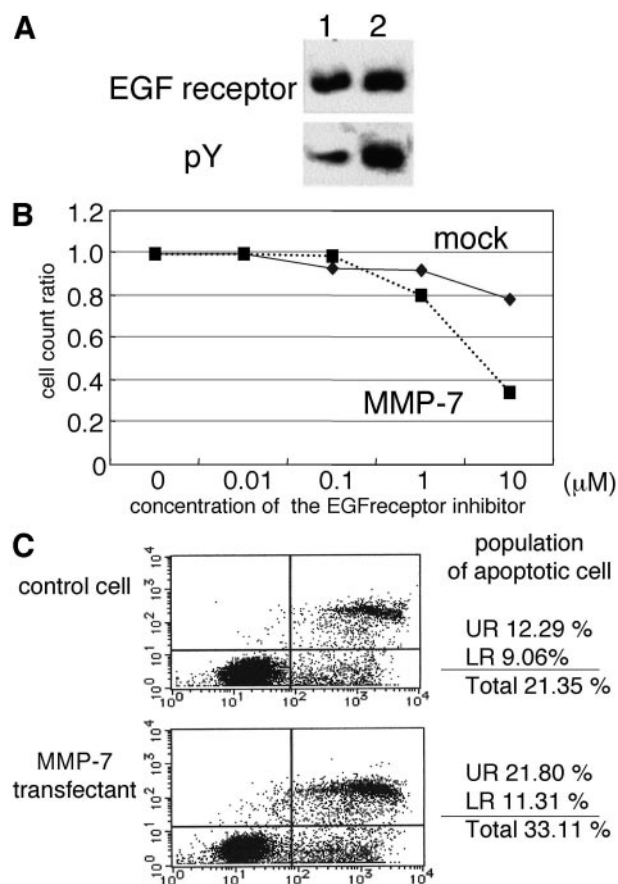
hours. Cells were detached with 0.05% trypsin/0.02% EDTA and harvested by centrifugation, counted, and prepared for cell cycle analysis. Cells ( $1 \times 10^6$ ) were fixed in 70% EtOH, stained with PI (50  $\mu\text{g}/\text{mL}$ ) and analyzed with an EPICS XL (Beckman Coulter Corp., Fullerton, CA). For apoptosis analysis, cells also were stained with annexin V conjugated with FITC (Becton Dickinson, Franklin Lakes, NJ). Analysis was performed with a FACScan (Becton Dickinson).

#### Immunofluorescence Staining of Cells for Smad4.

MMP-7-transfected KYSE150 and mock cells ( $1 \times 10^5/\text{mL}$ ) in 2 mL culture medium were plated into Dab-Tek II chamber slides (NUNC, Tokyo, Japan). After 24 hours of incubation, they were put on ice and washed three times with cold PBS. Two milliliters of cold PFA 2% were added, and slides were incubated for 30 minutes at  $4^\circ\text{C}$  and then washed three times with cold PBS. To permeabilize the cell membrane, cells were incubated for 10 minutes in 2 mL PBS/0.5% Triton X at  $4^\circ\text{C}$ . Primary antibody solution (100  $\mu\text{L}$ ) was layered on the cells

(Smad4 protein usually containing 1  $\mu\text{g}$  of Mab in 0.5% Triton X) and incubated for 1 hour at  $4^\circ\text{C}$ . Slides were washed  $3 \times$  for 5 minutes with cold PBS/3% BSA/0.05% saponin and then  $1 \times$  for 5 minutes with PBS/3% BSA/0.05% Triton X. Secondary antibody (FITC labeled, 100  $\mu\text{L}$ ) was layered at an empirically determined concentration in PBS/3% BSA/0.05% Triton X. Finally, quenching solution (1 drop phenylenediamine dissolved in 50% glycerol/PBS) was added per slide and then sealed with nail enamel (Fig. 3).

**Plasminogen Activator Inhibitor-1 Promoter/Luciferase Construct.** We considered that gefitinib increased TGF- $\beta$  activity in MMP-7-transfected KYSE150 cells and HT29 cells. We used the quantitative bioassay for TGF- $\beta$  based on its ability to induce plasminogen activator inhibitor-1 (PAI-1) expression (24). D. B. Rifkin (Department of Cell Biology, New York University School of Medicine, New York, NY) originally provided the PAI-1 plasmid. First, to determine the appropriate concentration of gefitinib, we eval-



**Fig. 2** Comparison of an antitumor effect of EGF receptor tyrosine kinase inhibitor. **A**, expressions of EGF receptor and phosphorylated EGF receptor by Western blot analysis. Comparing mock cells (*Lane 1*), MMP-7-transfected KYSE150 cells (*Lane 2*) showed higher level of phosphorylation; however, expression of EGF receptor in both cells is almost identical. **B**, At concentrations of 0.01  $\mu\text{mol/L}$ , 0.1  $\mu\text{mol/L}$ , 1.0  $\mu\text{mol/L}$ , and 10.0  $\mu\text{mol/L}$  of gefitinib, we calculated the cell count ratio compared with the number of cells with no drug. The reduction in cell number ratio was more for MMP-7-transfected KYSE150 cells than for mock cells and was most notable at the 10.0  $\mu\text{mol/L}$  concentration. **C**, Annexin PI staining was performed for MMP-7 transfectant cells and mock cells at a 10.0  $\mu\text{mol/L}$  concentration of gefitinib. The proportion of apoptotic cells was 33.1% and 21.5% in MMP-7-transfected KYSE150 and mock cells, respectively.

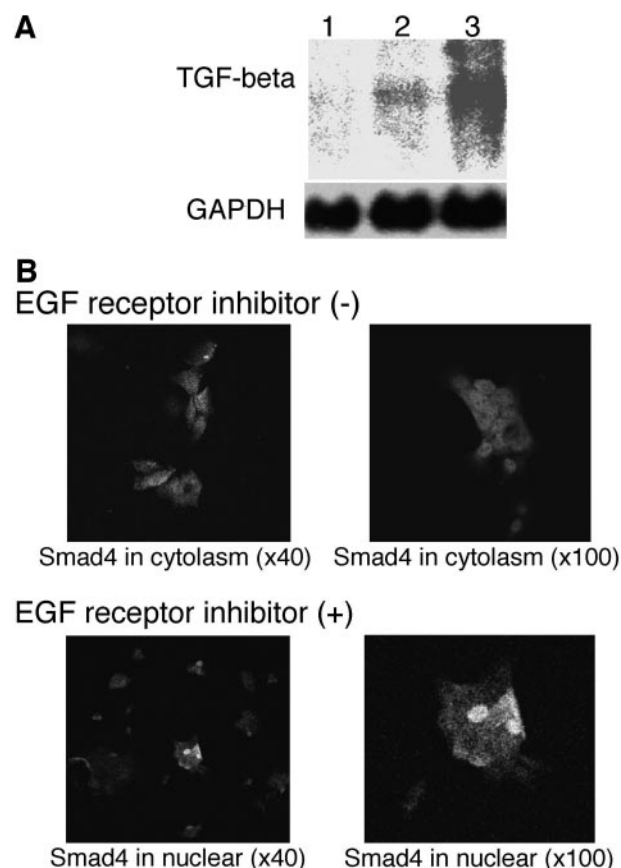
uated TGF- $\beta$  activity in MMP-7 transfectants KYSE150 and HT29 with gefitinib (0  $\mu\text{mol/L}$ , 30  $\mu\text{mol/L}$ , and 60  $\mu\text{mol/L}$ ) and recombinant TGF- $\beta$  (Genzyme, Cambridge, MA) using the Dual-Luciferase Reporter Assay System kit (Promega Corporation, Madison, WI) and performed as Abe *et al.* (24) reported previously (Fig. 4A). We then used TGF- $\beta$ -neutralizing antibody (100 ng/mL) (Genzyme) to inhibit TGF- $\beta$  activity; in Fig. 4B, the left panel is MMP-7 transfected into KYSE150, and the right panel is MMP-7 transfected into HT-29 [*Lane 1*, gefitinib (30  $\mu\text{mol/L}$ ) only; *Lane 2*, gefitinib + TGF- $\beta$  antibody (100 ng/mL); *Lane 3*, control (no treatment); *Lane 4*, TGF- $\beta$  antibody only; *Lane 5*, rTGF- $\beta$  only; and *Lane 6*, rTGF- $\beta$  + TGF- $\beta$  antibody].

Conversely, in Fig. 4C, we examined the direct evidence of

TGF- $\beta$  for apoptosis in MMP-7 transfectants KYSE150 and HT29 with gefitinib (30  $\mu\text{mol/L}$ ). We used TGF- $\beta$ -neutralizing antibody (100 ng/mL) and analyzed the results with a FACS flow cytometer as described previously.

## RESULTS

**Simultaneous Expression of MMP-7 and Phosphorylated EGF Receptor.** Expression of MMP-7 was observed in 14 (35%) of 40 CRC cases, and strong staining was localized mainly in cancer cells. There was no significant association between the incidence of MMP-7-positive staining and any clinicopathologic factors. Furthermore, nine cases (23%) showed positive staining for activated EGF receptor, and similarly there was no clinicopathologic characteristic associated



**Fig. 3** EGF receptor tyrosine kinase inhibitor released a TGF- $\beta$  signal in MMP-7-overexpressing cells. **A**, Expression of TGF- $\beta$  mRNA in mock cells (*Lane 1*), MMP-7-transfected KYSE150 cells (*Lane 2*), and MMP-7 transfectants with EGF receptor-neutralizing antibody (*Lane 3*) was examined by Northern blot analysis. There was more abundant expression of TGF- $\beta$  in MMP-7-transfected KYSE150 cells than in mock cells. Moreover, the addition of EGF receptor inhibitor enhanced expression of TGF- $\beta$  remarkably higher than MMP-7-expressing cells. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control. **B**, Nuclear accumulation of Smad4 protein was recognized in MMP-7-transfected KYSE150 cells after gefitinib treatment, whereas mock cells (not shown) showed only cytoplasmic accumulation. Therefore, this figure indicates activated TGF- $\beta$  in MMP-7-transfected KYSE150 after treatment with gefitinib.

**Table 1** Association between matrix metalloproteinase-7 expression and the phosphorylated epidermal growth factor receptor

	Phosphorylated EGF receptor	
	Positive (9)	Negative (31)
MMP-7 expression		
Positive (14)	6	8
Negative (26)	3	23

Abbreviations: EGF, epidermal growth factor; MMP-7, matrix metalloproteinase-7.

Fisher's exact test ( $P = 0.04$ )

with phosphorylated EGF receptor expression. However, there was a significant correlation between the incidence of positive MMP-7 expression and an activated EGF receptor ( $P = 0.04$ ) by Fisher's exact test (Table 1). Moreover, we found an intriguing result that portions of the cancer tissue specimens stained with anti-MMP-7 antibody were almost identical to those stained by the activated EGF receptor antibody. A representative case is shown in Fig. 1. This distinct finding indicated that the phosphorylation of the EGF receptor occurred in CRC cells that had simultaneously accumulated MMP-7. However, we have to note that only 6 (15%) of 40 CRC cases showed simultaneous expression of both molecules.

#### Increased Phosphorylation of EGF Receptor in MMP-7.

MMP-7-transfected KYSE150 cells show much more phosphorylation (activation) of EGF receptor than mock cells evaluated by anti-EGF receptor antibody and pY antibody (Fig. 2).

#### Antitumor Effect of Gefitinib on MMP-7 Transfectant

**Cells.** Comparing the inhibitory activity of gefitinib against MMP-7-transfected KYSE150 cells and mock cells by the MTT assay, a much higher antitumor effect was observed in the former than the latter, depending on the inhibitor concentration. At a 10- $\mu$ mol/L concentration of gefitinib, the mock cell/MMP-7-transfected KYSE150 cell count ratio was 2.3 (Fig. 2), whereas at a 100- $\mu$ mol/L concentration, there was no difference in the remarkable inhibition of cell growth of both cell lines.

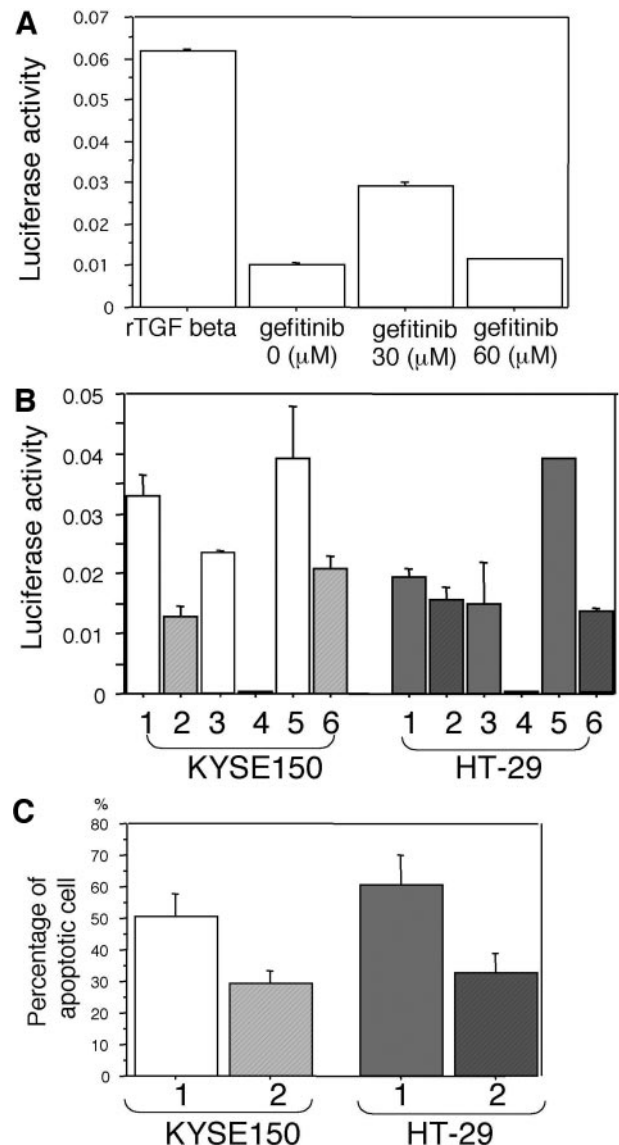
After addition of fetal bovine serum with EGF to quiescent cells to restart the cell cycle, the fraction of apoptotic cells in the MMP-7-transfected KYSE150 (33.1%) line was higher than that of the mock line (21.4%; Fig. 2). As per our previous statements, we considered that these findings might indicate that MMP-7-transfected KYSE150 undergoes apoptosis through the TGF- $\beta$  pathway more rapidly or frequently than mock cells after the addition of gefitinib (Fig. 3 and Fig. 4).

#### Overexpression and Activation of TGF- $\beta$ in MMP-7 Transfectants by Gefitinib.

Fig. 3 shows the abundant expression of TGF- $\beta$  in MMP-7 transfectant cells than in mock cells. It is noteworthy that the expression of TGF- $\beta$  mRNA was obviously increased by the addition of EGF receptor-neutralizing antibody. We also confirmed that the inhibition of EGF receptor can release the TGF- $\beta$  induction of nuclear translocation of the Smad4 protein in MMP-7-transfected KYSE150 cells. In contrast, mock cells did not show any nuclear translocation, only a cytoplasmic location (not shown).

Moreover, it is distinctive that the increased activity of TGF- $\beta$  and TGF- $\beta$ -related apoptosis were associated with gefitinib in Fig. 4B and C. In Fig. 4B, in a comparison of Lane 3

with Lane 1, we found that the addition of gefitinib in MMP-7 transfectant showed a higher activity of TGF- $\beta$  than MMP-7 transfectant without gefitinib. Whereas, intriguingly, in a comparison of Lane 2 and Lane 4, TGF- $\beta$  activity in gefitinib-added cell was partially inhibited by TGF neutralization antibody (Lane



**Fig. 4** Evaluation of the TGF- $\beta$  activity by PAI-1 promoter/Luciferase (PAI/L) construct. **A.** To determine the appropriate concentration of gefitinib to use additional experiments, a concentration of 30  $\mu$ mol/L gefitinib was selected because it induces the highest TGF- $\beta$  activity than the other tested conditions, such as 0 or 60  $\mu$ mol/L. **B.** The left is MMP-7-transfected KYSE150, and the right is MMP-7-transfected HT-29. Lane 1, gefitinib (30  $\mu$ mol/L) only; Lane 2, gefitinib + TGF- $\beta$  antibody (100 ng/mL); Lane 3, control (no treatment); Lane 4, TGF- $\beta$  antibody only; Lane 5, recombinant TGF- $\beta$ ; and Lane 6, rTGF- $\beta$  + TGF- $\beta$  antibody. Lanes 5 and 6 are set up as a control for this assay. According to Lanes 1 and 3, both cell lines showed higher activity of TGF- $\beta$  than MMP-7 expression without gefitinib. In contrast, comparison between Lane 2 and Lane 4, TGF- $\beta$  activity in gefitinib-added cells was partially inhibited by TGF neutralization antibody (Lane 2),

2), TGF- $\beta$  activity in MMP-7-expressing cell without gefitinib was completely inhibited (*Lane 4*). This discrepancy has occurred because the EGF receptor, which was activated by MMP-7, may inhibit TGF- $\beta$  activity. Therefore, we supposed that TGF neutralization antibody works completely. Furthermore, we confirmed that the induction of apoptosis in MMP-7 transfectant cells by gefitinib was inhibited by TGF- $\beta$  neutralization antibody. Additional study is needed to determine the precise interaction among EGF receptor, MMP-7, and TGF- $\beta$ .

## DISCUSSION

Wilson *et al.* (25) recently indicated that MMP-7 is regulated by  $\beta$ -catenin signaling in intestinal cancer. They reported that the occurrence of adenomas in *Min* mice can be strikingly blocked by MMP-7-targeted knockout for intestinal lineage cells, which suggested to us that tumorigenesis mediated by  $\beta$ -catenin signaling might be mediated through MMP-7. Conversely, Torrance *et al.* (26) reported that the EGF receptor inhibitor inhibited adenoma formation in *Min* mice, which indicated that the EGF receptor is supposedly regulated by  $\beta$ -catenin. Therefore, there has been focus on a direct relationship between MMP-7 and the EGF receptor, two important molecules in oncogenesis or malignant progression, and their regulated signals may be considered as targets for molecular therapy. In the present study, we identified coexpression of the activated EGF receptor and MMP-7 by immunohistochemistry; however, one cannot determine which is dominant by an immunohistochemistry study alone. Sundareshan *et al.* (27) have reported converse findings to ours in human prostate carcinoma cells, that regulation of matrilysin expression could not be regulated by the EGF receptor. Ueda *et al.* (28) also reported that matrilysin secreted by a cervical carcinoma cell was not affected by EGF. However, consistent with our results, Pai *et al.* reported that inhibition of MMPs blocked prostaglandin E2 (PGE2)-mediated EGF receptor transactivation and downstream signaling, indicating that PGE2-induced EGF receptor transactivation involves signaling transduced via TGF- $\alpha$  (an EGF ligand) released by c-Src-activated MMPs (5). Prenzel *et al.* (29) also reported that metalloproteinase activity for cleavage of pro-HB EGF recently has been described in EGF receptor. As for the activation of EGF receptor by MMPs, we have found that shedding of EGF ligands, such as amphiregulin, TGF- $\alpha$ , and HB-EGF, by autosomal expression of MMP-7 activates the EGF receptor signal, followed by promotion of transcriptional factors *in vitro*.<sup>4</sup> The present study showed that MMP-7 transfectants show more phosphorylation (activation) of EGF receptor, and gefitinib showed a remarkable antitumor effect with apoptosis on MMP-7-overexpressing cells compared with control cells, leading us to hypothesize that MMP-7 was dominant to EGF receptor activation in cancer progression.

Furthermore, we have disclosed that the inhibition of EGF receptor promotes apoptosis induced by the abundant expression of TGF- $\beta$  with the nuclear translocation of Smad4 protein, and this finding was observed more in MMP-7-overexpressing cells than in mock cells. We realize that communications among these and other signals may be complicated; however, we have speculated on one possible explanation how EGFR inhibition may up-regulate TGF- $\beta$  expression. First, we have obtained

direct evidence of TGF- $\beta$  activation evaluated by the PAI-1 promoter/luciferase assay as follows. In Fig. 4B, comparing *Lane 1* and *Lane 3*, TGF- $\beta$  is more activated with the addition of gefitinib. Moreover, comparing *Lane 2*, TGF- $\beta$  activity in MMP-7-expressing cells with EGF receptor activation was completely inhibited by TGF- $\beta$  antibody (*Lane 4*). We speculated that this discrepancy has occurred because activated EGF receptor by MMP-7 might inhibit TGF- $\beta$  activity; in other words, TGF- $\beta$  neutralization antibody works completely under the activated EGF receptor by MMP-7 in *Lane 4*. In Fig. 4C, we confirmed that the increased apoptosis of MMP-7 transfectant by gefitinib was regulated by TGF- $\beta$  activity.

Interestingly, there is supposed to be clinical evidence that pulmonary fibrosis is observed as a side effect in NSCLC patients treated with EGF receptor tyrosine kinase inhibitor. We speculate that it may be indicative of an activated TGF- $\beta$  signal by EGF receptor tyrosine kinase inhibitors. Further studies *in vitro* or *in vivo* will be required to investigate this.

In conclusion, we detected simultaneous expression of MMP-7 and phosphorylated EGF receptor in CRC cases. An EGF receptor inhibitor induced more apoptosis in MMP-7 transfectant cells than in control cells *in vitro*. EGF receptor tyrosine kinase inhibitor enhanced the expression and the activation of TGF- $\beta$ , which was confirmed by nuclear translocation of Smad4 and luciferase activity. The present data lead to the concept of a new molecular target for gene therapy, especially for colon cancers with severe malignant potential provoked by overexpression of MMP-7. We may be able to predict CRC cases responsive to treatment with EGF receptor tyrosine kinase inhibitor according to the status of MMP-7 expression. We found, however, that a limited number of cases, such as 15% of CRC cases and 2 (17%) of 12 cancer cell lines, exhibited the synergistic activation of EGF receptor and TGF- $\beta$  by MMP-7; therefore, we have to continue identifying other factors to mediate the efficiency of EGF receptor tyrosine kinase inhibitor against MMP-7-expressing tumors.

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## Coexpression of Matrix Metalloproteinase-7 (MMP-7) and Epidermal Growth Factor (EGF) Receptor in Colorectal Cancer: An EGF Receptor Tyrosine Kinase Inhibitor Is Effective against MMP-7–Expressing Cancer Cells

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