Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue

Takao Inoue,[1, 5] Hiroaki Kataoka,[2, 5] Kouichiro Goto,[3] Koki Nagaike,[2] Ko Igami,[4] Daiji Naka,[4] Naomi Kitamura[1] and Keiji Miyazawa[1, 3, 6]

[1]Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Yokohama 226-8501; [2]Second Department of Pathology, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-1692; [3]Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Hongo, Tokyo 113-0033; and [4]Yokohama Research Center, Mitsubishi Chemical Co., Kamoshida, Yokohama 227-8502

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c-Met is a high-affinity receptor for hepatocyte growth factor (HGF) and plays a crucial role in embryonic development, as well as in the process of tissue repair. Overexpression and amplification of c-Met are often observed in various cancer tissues, especially in gastric carcinoma. It has, however, been unclear whether the overexpression leads to activation of the c-Met receptor. To address this point, we prepared an antibody (anti-phospho-Met) which specifically recognizes c-Met that is phosphorylated at Y1235, a major phosphorylation site of c-Met. Normal as well as cancerous gastric tissue was positive for anti-total-Met staining, whereas only cancerous tissue was strongly positive for anti-phospho-Met staining; cells near the basal layer were moderately positive, and the proliferative zone in normal tissue was only weakly positive. Among cancerous tissues from seven patients examined in the present study, those from six patients were strongly positive for phospho-Met staining. These results indicate that c-Met is actually activated in gastric carcinoma tissue, and may trigger proliferation/anti-apoptotic signals. (Cancer Sci 2004; 95: 803–808)

C-Met was originally identified as a cellular counterpart of a chemically-induced oncogene *tpr-met* found in a human osteosarcoma cell line (HOS).[1)] It is composed of an extracellular α-subunit and a transmembrane β-subunit with tyrosine kinase activity.[2)] c-Met was found to be amplified, overexpressed and activated in a human gastric tumor cell line, GTL-16.[3)] Subsequently c-Met was reported to be a high-affinity receptor for hepatocyte growth factor (HGF)[4–6)] and shown to be essential for development of the liver, placenta and skeletal muscle.[7)] c-Met is also implicated in the process of carcinogenesis.[8)] Amplification and overexpression of *c-met* have been reported in human cancer tissue from liver, prostate, colon, breast, stomach, thyroid gland and skin. Missense mutations of c-Met have been identified in hereditary papillary renal carcinoma,[9)] hepatocellular carcinoma[10)] and gastric carcinoma.[11)] Although it is presumed that overexpression of c-Met leads to receptor activation, it remains unclear whether overexpressed c-Met is activated in carcinoma tissues or not. Thus, the involvement of c-Met in carcinogenesis remains to be demonstrated. In the present study, we prepared an antibody specific to c-Met that is phosphorylated at the major phosphorylation site (Y1235). Using the antibody, we obtained direct evidence that c-Met is activated in human gastric carcinoma tissue, where it is overexpressed.

Materials and Methods

Reagents. Recombinant human HGF was from the Research Center of Mitsubishi Chemical Corp. Commercially available antibodies used were as follows: anti-human Met (SC-161, Santa Cruz), anti-epidermal growth factor (EGF) receptor (MBL), anti-phosphotyrosine PY20 (Transduction Laboratories), anti-Ki67 MIB-1 (Sigma), anti-α-tubulin (T9026, Sigma). Anti-mouse c-Met was described previously.[6)]

Preparation of an anti-phospho-Met antibody. A peptide CYDKEYY(PO_3)SVHNK, which corresponds to the sequence surrounding the major phosphorylation site of c-Met (Y1235), was synthesized and conjugated to keyhole limpet hemocyanin (Calbiochem) using succinimidyl 4-(*p*-maleimidophenyl) butyrate (Pierce). The conjugate was injected into rabbit in Freund's complete adjuvant and anti-serum was collected. The anti-serum exhibited cross-reactivity to nonphosphorylated c-Met as well as other phosphorylated tyrosine kinase receptors, such as the EGF receptor. Thus, the anti-phospho-Met antibodies were purified using four steps of column chromatography. For affinity purification, keyhole limpet hemocyanin, the peptides CYDKEYY(PO_3)SVHNK and CYDKEYYSVHNK were coupled to Affigel-10 (Bio-Rad) at 1 mg protein (peptide)/ml gel. The antiserum was treated at 56°C for 30 min, and clarified by centrifugation. Then the serum was diluted 2-fold using Tris-buffered saline (pH 7.4) and passed through an anti-KLH column. The flow-through fraction was applied to the phosphopeptide column, and bound proteins were eluted with 100 m*M* glycine-HCl (pH 2.3) containing 10% ethyleneglycol. The eluate was subsequently passed through the dephosphopeptide column and an *o*-phospho-tyrosine agarose column (Sigma). The flow-through fraction was used as an affinity-purified anti-phospho-Met antibody.

Immunoblotting. Immunoblotting analysis was conducted after separation of the proteins on 6% SDS-PAGE. For analysis of phospho-Met specificity, c-Met was immunoprecipitated from a lysate of HepG2 cells as described previously.[13)]. To prepare dephospho-Met, the immunoprecipitates were treated with calf intestine alkaline phosphatase (TaKaRa) at 37°C for 60 min. To prepare phospho-Met, the immunoprecipitates were incubated with 1 m*M* ATP and 20 m*M* $MgCl_2$ in 40 m*M* HEPES (pH 7.5) at 37°C for 60 min. EGF receptor was immunoprecipitated with anti-EGF receptor, followed by the *in vitro* phosphorylation procedure. For analysis of phospho-Y1235 specificity, chimeric receptor constructs EMR and EMR(Y1233F) were used.[12)] EMR consists of the extracellular domain of human EGF receptor and the transmembrane and cytoplasmic domains of mouse c-Met. Y1233F in mouse c-Met corresponds to Y1235 in human c-Met, and the peptide sequences surrounding the tyrosine residue are conserved in mouse and human c-Met. COS7 cells were transiently transfected using FuGene6 (Roche) and cultured for 24 h before analysis. Cell lysates were prepared, and EMRs were immunoprecipitated with anti-mouse c-Met antibody,[12)] followed by the *in vitro* phosphorylation procedure. For analysis of phosphorylation of endogenous c-Met in HGF-treated HepG2 cells, cells lysates were prepared after

[5]These two authors equally contributed to the work.
[6]To whom all correspondence should be addressed at Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo.
E-mail: keiji-miyazawa@umin.ac.jp

stimulation with HGF (100 ng/ml) at 37°C for 10 min, and 30 μg of protein was used for immunoblotting analysis.

Antibody concentrations used were 1 μg/ml for anti-human Met, 1:200 dilution for anti-mouse Met, 30 ng/ml for anti-phosphotyrosine PY20, 2 μg/ml for anti-phospho-Met, and 0.1 μg/ml for anti-α-tubulin.

Immunocytochemical staining. HepG2 cells were plated on an 8-well Culture Slide (BD Falcon) coated with type IV collagen, and stimulated with HGF (100 ng/ml) for 10 min at 37°C. Cells were then fixed in cold acetone/methanol (1:1), and treated with the primary antibodies (anti-human Met and anti-phospho-Met, 1 μg/ml) and the secondary anti-rabbit IgG (biotinylated, 1:1000 dilution, Vector). Intracellular localization was determined after incubation with FITC-labeled avidin DCS (1:1000 dilution, Vector) by confocal laser scanning microscopy. Cell nuclei were stained with propidium iodide (PI).

Immunohistochemical staining. All human tissue was obtained from surgical specimens. Formalin-fixed, paraffin-embedded tissue specimens were prepared according to routine procedures. Sections were dewaxed in xylene and rehydrated in decreasing ethanol solutions and water. After antigen retrieval (5 min of autoclaving in 10 m*M* citrate buffer, pH 6.0), the sections were treated with 3% H_2O_2 in PBS for 10 min and washed in PBS twice, followed by blocking in 3% bovine serum albumin in PBS for 1 h at room temperature. Then the sections were incubated with the primary antibody (2 μg/ml in PBS) for 16 h at 4°C. For the adsorption test, the antibody was pretreated with a 20-fold excess of the antigen peptide at 4°C overnight. The sections were then washed with PBS containing 0.05% Tween 20, followed by incubation with Envision-labeled polymer reagent (DAKO) for 45 min at room temperature. The reaction was detected with nickel, cobalt-3,3′-diaminobenzidine (ImmunoPure Metal Enhanced DAB Substrate Kit, Pierce), and counterstained with Mayer's hematoxylin.

Results

Preparation of an antibody that recognizes activated form of c-Met. The intrinsic activity of receptor-type tyrosine kinases is usually suppressed, but can be activated by autophosphorylation induced by ligand stimulation.[14] The major phosphorylation site to activate the c-Met kinase was identified as

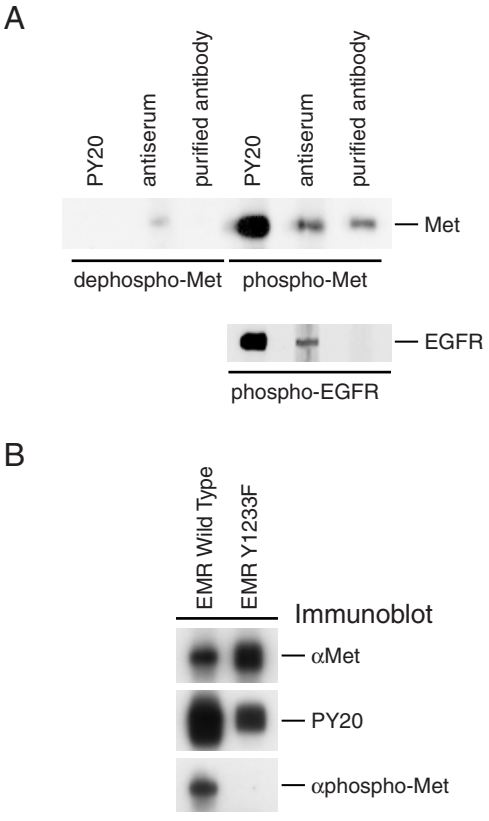

Fig. 1. Confirmation of the specificity of the anti-phospho-Met antibody. (A) Reactivity of the affinity-purified anti-phospho-Met antibody to dephosphorylated c-Met, as well as phosphorylated c-Met and EGF receptor, was examined using Screener Blotter (Sanplatec Corp.). Anti-phosphotyrosine PY20 was used to confirm the phosphorylation state of c-Met and EGF receptor. (B) Specificity of the affinity-purified anti-phospho-Met antibody for the major phosphorylation site was examined using EMR and EMR(Y1233F) that contain the mouse c-Met intracellular domain. Y1233F mutation in mouse c-Met corresponds to Y1235 mutation in human c-Met. Reactivity of each protein with the anti-phospho-Met antibody was examined after *in vitro* phosphorylation.

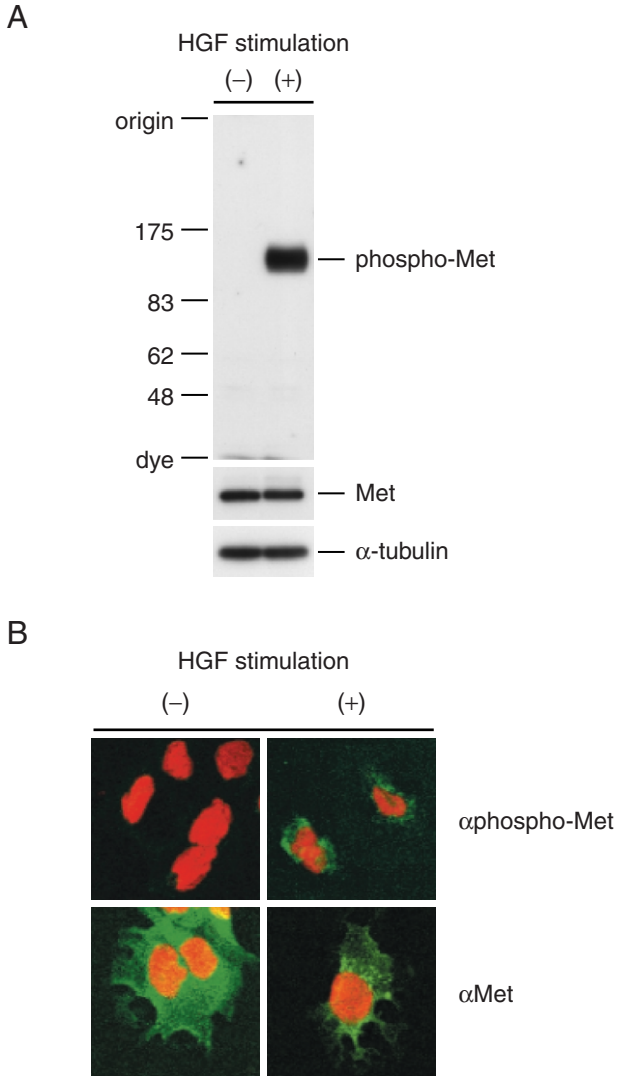

Fig. 2. Phosphorylation of c-Met in HepG2 cells treated with HGF. HepG2 cells were stimulated with HGF (100 ng/ml) for 10 min at 37°C, and phosphorylation of c-Met was analyzed by immunoblotting (A) and immunocytochemical staining (B). Antibodies used were anti-phospho-Met (A, B), anti-human c-Met (A, B) and anti-α-tubulin (A). Molecular mass markers are indicated in kilodaltons on the left (A). Subcellular localization of phospho-Met (upper panels) and total Met (lower panels) is shown in green. Nuclear staining is shown in red (B).

Y1235.[15, 16] It is thus appropriate to use an anti-phospho-Met antibody that recognizes this crucial tyrosine residue to detect the activated form of c-Met.[17] Anti-phospho-Met antibody was prepared by immunizing a rabbit with chemically synthesized peptide CYDKEYY(PO_3)SVHNK that corresponds to the sequence surrounding the major phosphorylation site of c-Met (Y1235), followed by an affinity purification procedure to make it specific for phospho-Met. The final preparation did not cross-react with nonphosphorylated c-Met or phosphorylated epidermal growth factor receptor (Fig. 1A). Specificity for c-Met phosphorylated at Y1235 was further confirmed using EMR and EMR(Y1233F) which are chimeric receptor constructs with the mouse c-Met intracellular domain (for details of these constructs, see "Materials and Methods"). Y1233 in mouse c-Met corresponds to Y1235 in human c-Met, and the peptide sequence surrounding the tyrosine is conserved in both species. EMR and EMR(Y1233F) were overexpressed in COS7 cells, followed by immunoprecipitation and *in vitro* phosphorylation. Tyrosine phosphorylation of EMR and EMR(Y1233F) was first determined by immunoblotting using anti-phosphotyrosine antibody PY20 (Fig. 1B). EMR was strongly tyrosine-phosphorylated, while EMR(Y1233F) was rather weakly phosphorylated probably because the major phosphorylation site was mutated. The anti-phospho-Met antibody recognized wild-type EMR, but not EMR(Y1233F). These results suggest that the anti-phospho-Met antibody specifically recognizes c-Met phosphorylated at the major phosphorylation site.

We next tried to detect phosphorylation of endogenous c-Met in response to HGF stimulation using the anti-phospho-Met antibody. HepG2 cells were stimulated with HGF, and the phosphorylation of c-Met Y1235 was determined by immunoblotting, as well as by immunocytochemical staining (Fig. 2). On immunoblotting analysis, we observed a strong positive band of phospho-Met after HGF treatment, while the amount of total c-Met protein was not significantly altered (Fig. 2A). When we stained HepG2 cells before and after HGF-stimulation, we observed HGF-dependent punctate signals in the cytosol (Fig. 2B, upper panels), which were probably attributable to internalized receptors. Staining with anti-total Met antibody also revealed the punctate localization of c-Met after HGF-stimulation (Fig. 2B, lower panels).

Immunohistochemical staining of human gastric mucosa using the anti-phospho-Met antibody. We then used the antibody to examine the phosphorylation state of c-Met in human gastric mucosa. A representative result is shown in Fig. 3. In these serial sections, normal gastric mucosa (right half) and well-differenti-

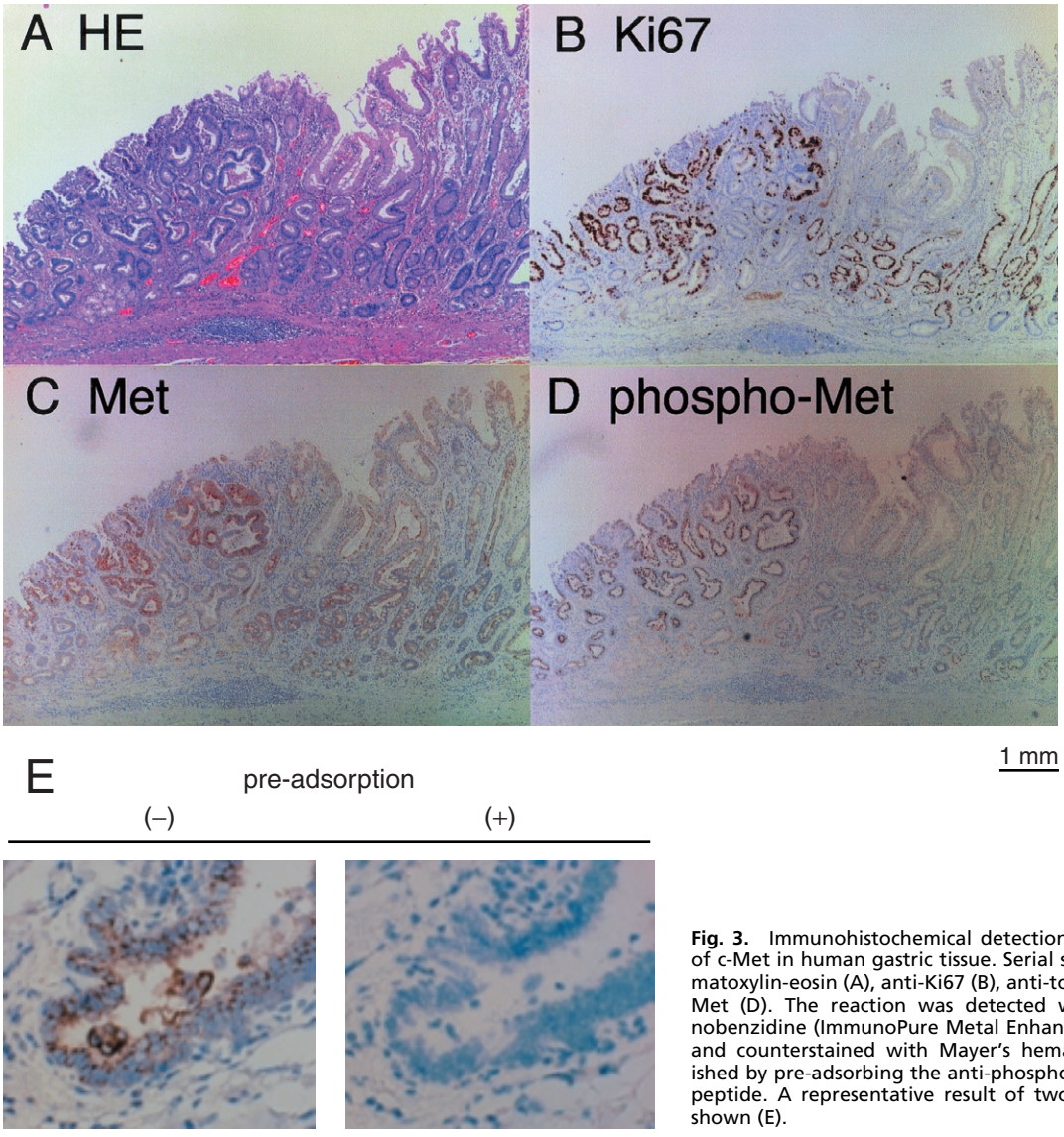

Fig. 3. Immunohistochemical detection of the phosphorylated form of c-Met in human gastric tissue. Serial sections were stained with hematoxylin-eosin (A), anti-Ki67 (B), anti-total-Met (C) and anti-phospho-Met (D). The reaction was detected with nickel, cobalt-3,3′-diaminobenzidine (ImmunoPure Metal Enhanced DAB Substrate Kit, Pierce) and counterstained with Mayer's hematoxylin. Staining was diminished by pre-adsorbing the anti-phospho-Met antibody with antigenic peptide. A representative result of two independent experiments is shown (E).

ated tubular adenocarcinoma (left half) can be simultaneously observed (Fig. 3A, hematoxylin-eosin staining). Staining of serial sections with anti-Ki67 (MIB-1) (Fig. 3B), which detects proliferating cells, indicated that the adenocarcinoma (left) and the proliferative zone in normal glandular structure (right bottom) were strongly positive. Staining with anti-Met antibody indicated a widely distributed expression of c-Met in epithelial tissues and higher expression in adenocarcinoma (Fig. 3C); c-Met is thus overexpressed in the cancerous tissue of this specimen. A distinct staining pattern was observed when we used anti-phospho-Met (Fig. 3D). The adenocarcinoma region was strongly positive, indicating that c-Met is not only overexpressed, but also activated. In contrast, the proliferative zone in normal tissue was only weakly positive; cells near the basal layer were moderately positive. Cell growth of the proliferative zone appears to be independent of c-Met signaling. The specificity of staining with the anti-phospho-Met antibody was confirmed by pre-adsorbing the antibody with antigenic peptide (Fig. 3E).

Detailed observations of the staining pattern suggest that the extent of expression of c-Met and that of c-Met phosphorylation are not always correlated (Fig. 4, A–C). Fig. 4, A–C reveals representative pictures from Fig. 3 at higher magnification. In the adenocarcinoma region, c-Met was overexpressed and well phosphorylated (Fig. 4A). In normal tissue, c-Met was moderately expressed but not phosphorylated in most parts (Fig. 4B), while it was phosphorylated in a limited number of parts near the basal layer (Fig. 4C) as described above.

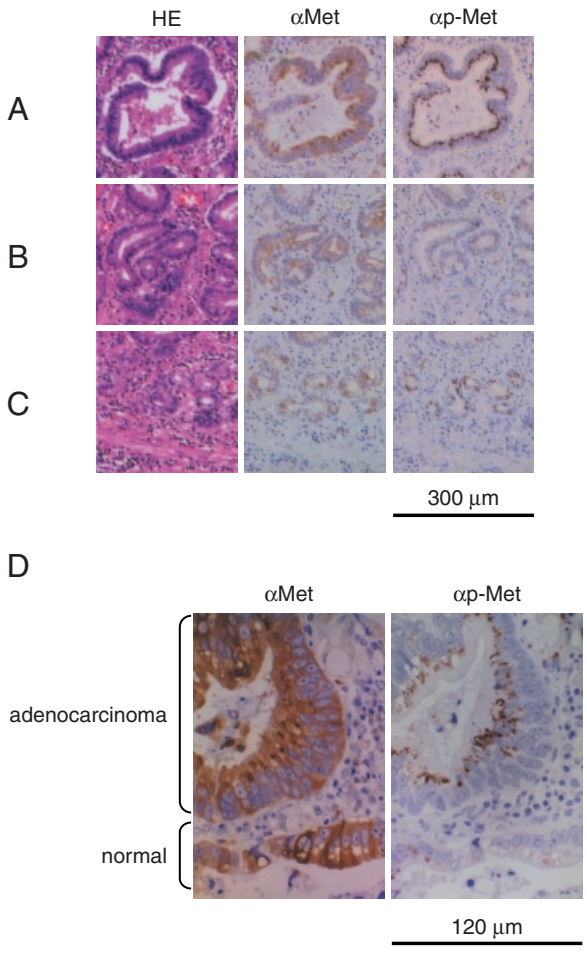

Fig. 4. Detailed comparison of staining in serial sections by anti-Met (αMet) and anti-phospho-Met (αp-Met). Higher magnification images from Fig. 3 are presented in A–C. A specimen from another patient was also stained (D).

Fig. 4D shows sections taken from another patient. In these sections, we can observe adenocarcinoma tissue characterized by multiple layers of cells (upper part), in close vicinity to normal tissue (bottom part). c-Met is distributed ubiquitously in cells, whereas phospho-Met was localized only to the lumen side of the glandular duct. Luminal staining of phospho-Met was also observed in other tissue specimens examined in the present study. These results may suggest that c-Met was stimulated from the lumen side of the cells in these cases. We also noticed that phospho-Met staining did not localize at the cell surface but rather localized at the inner part of the cells (Fig. 4D). It appears likely that phospho-Met is internalized to endosomes, consistent with the result obtained with cultured HepG2 cells (Fig. 2B).

We next stained sections from five other patients with gastric adenocarcinoma (Fig. 5). In four cases, including one with poorly differentiated adenocarcinoma, the phospho-Met antibody strongly stained adenocarcinoma, while the normal region was only weakly stained by the antibody (Fig. 5, A–C, E and F). There was, however, one case in which well-differentiated tubular adenocarcinoma was negative for phospho-Met staining (Fig. 5D).

Discussion

The mode of activation of c-Met in cancerous tissues remains unclear. The overexpression of c-Met alone does not appear to be sufficient to cause activation. One possible mechanism is that an autocrine or paracrine loop is formed in cancerous tissue. HGF is produced and secreted as a single-chain inactive form[18] and needs to be proteolytically processed to exert its biological activity.[19] Recently Kataoka *et al*. reported that HGF is proteolytically activated in colorectal carcinoma tissues, which probably leads to ligand-dependent receptor activation.[20] The second possible mechanism is that c-Met is somehow activated in an HGF-independent fashion. Wang *et al*. reported that cell attachment triggers the activation of c-Met when it is overexpressed and that cell-attachment-induced activation of c-Met is more tumorigenic than ligand-induced activation is.[21] Giordano *et al*. reported that Semaphorin 4D indirectly activates c-Met via interaction with its receptor Plexin B1.[22] There are several other possibilities, including missense mutations and chromatin rearrangements, and a lack of proper processing also cannot be excluded. The mechanism of c-Met activation in cancerous tissues may differ, depending on the microenvironment.

In the present study, we examined the activation of c-Met in human gastric cancer tissue and found that c-Met was dominantly activated on the lumen side of the glandular duct, while c-Met was ubiquitously distributed in the cells. Activation of c-Met on the lumen side may not be dependent on HGF-stimulation, because expression of HGF has been reported in cells of the stromal compartment.[23, 24] Recently Churin *et al*. reported that *Helicobacter pylori* infection causes activation of c-Met,[25] and could be one of the candidates for triggering activation of c-Met from the lumen side.

In the case of poorly differentiated adenocarcinoma (Fig. 5E), phospho-Met staining was not localized to the lumen side of tubular structures. At the invasion front too (Fig. 5, E right and F), phospho-Met staining exhibited a scattered pattern in cells. Activation of c-Met in these case could be induced by ligand stimulation from the stromal compartment.

Whatever the mechanism is, we have directly demonstrated that c-Met in human gastric carcinoma tissue is actually activated, and that this activation is likely to cause progression of gastric carcinoma. Recently successful examples of therapy based on inhibition of receptor tyrosine kinases have

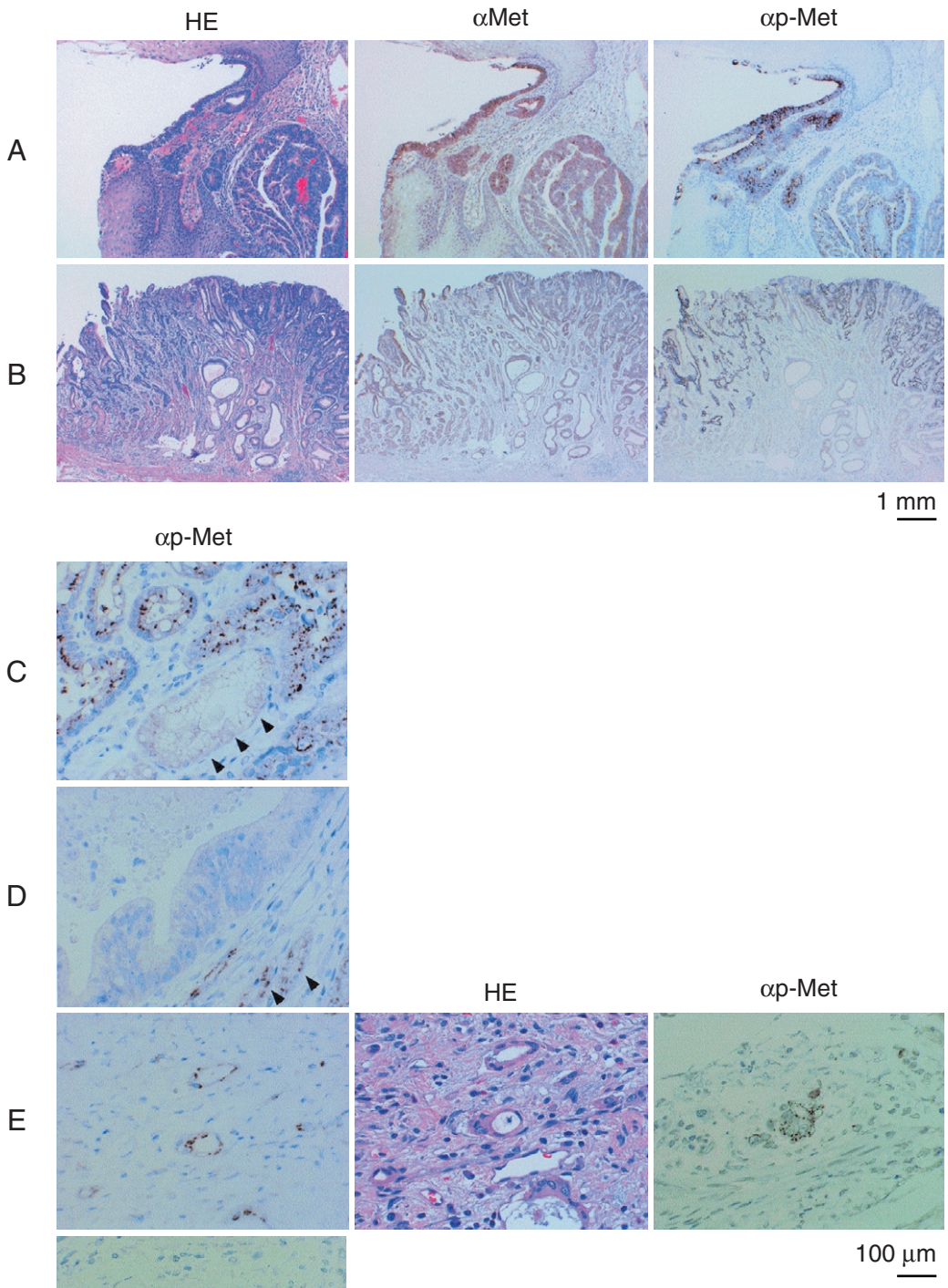

Fig. 5. Phospho-Met staining of gastric adenocarcinoma from other patients. (A)–(E) represent tissues from five independent patients. Phosphatase inhibitor cocktail (Sigma) was used during the staining procedure. (A) Moderately differentiated tubular adenocarcinoma. Esophageal stratified squamous epithelium was hardly stained with the anti-phospho-Met antibody. (B) Moderately differentiated tubular adenocarcinoma. (C) Well-differentiated or moderately differentiated tubular adenocarcinoma. Arrowheads indicate normal glandular structure that is negative for phospho-Met staining. (D) Well-differentiated tubular adenocarcinoma. The adenocarcinoma region in this case was negative for phospho-Met staining, while the normal glandular structure was weakly positive (arrowheads). (E) Poorly differentiated adenocarcinoma. Cells in tubular structures (left and middle panel), as well as in the invasion front (right panel), are positive for phospho-Met staining. (F) Less differentiated portion at the invasion front of moderately–differentiated tubular adenocarcinoma shown in (B).

emerged.[26] Christensen *et al*. reported a small molecule inhibitor, PHA-665752, which is selective for c-Met receptor kinase.[27] Interestingly, PHA-665752 exhibited a cytoreductive effects in a tumor xenograft model using GTL-16 human gastric carcinoma cells,[27] in which c-Met is overexpressed and activated.[3] Kinase inhibitors of c-Met would be useful in the

treatment of stomach cancers overexpressing c-Met. NK4, a competitive inhibitor of HGF-c-Met interaction, has also been reported to be therapeutically effective in tumor xenograft models.[28]

There have been many reports suggesting the presence of an autocrine or paracrine activation loop of c-Met in physiological as well as pathological situations. Co-localization of HGF and c-Met, however, does not always mean receptor activation of c-Met, because HGF has to be activated to exert its activity.[19] This antibody would thus be useful in examining the physiological as well as the pathological importance of c-Met tyrosine kinase.

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