Combining Onartuzumab with Erlotinib Inhibits Growth of Non–Small Cell Lung Cancer with Activating EGFR Mutations and HGF Overexpression

Yuji Sano, Eri Hashimoto, Noriaiki Nakatani, Masaichi Abe, Yasuko Satoh, Kiyoya Sakata, Toshihiko Fujii, Kaori Fujimoto-Ouchi, Masamichi Sugimoto, Shigehisa Nagahashi, Masahiro Aoki, Hiroshi Motegi, Eiichi Sasaki, and Yasushi Yatabe

Abstract

Erlotinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR-TKI), benefits survival of patients with non–small cell lung cancer (NSCLC) who harbor activating EGFR mutations. However, elevated expression of hepatocyte growth factor (HGF), a ligand of the MET receptor tyrosine kinase, causes erlotinib resistance. Because onartuzumab, a monoclonal antibody to MET, blocks HGF-induced MET activation, the addition of onartuzumab to erlotinib may improve therapeutic efficacy. We engineered the human NSCLC cell line PC-9 (MET-positive cells harboring an exon 19 deletion of EGFR) to overexpress hHGF and evaluated the effects of an onartuzumab and erlotinib combination in vitro and in vivo in xenograft models. A stable clone of PC-9/hHGF was less sensitive to erlotinib than the parental PC-9, and the addition of onartuzumab to erlotinib suppressed the proliferation of these cells in vitro. In PC-9/hHGF xenograft tumors, onartuzumab or erlotinib alone minimally inhibited tumor growth; however, combining onartuzumab and erlotinib markedly suppressed tumor growth. The total MET protein level was decreased in PC-9/hHGF cells, because MET is constitutively phosphorylated by autocrine HGF, leading to its ubiquitination and degradation. Onartuzumab reduced phospho-MET levels, inhibited MET ubiquitination, and consequently restored MET protein levels. Moreover, in NSCLC clinical specimens harboring activating EGFR mutations, more than 30% of patients expressed high levels of HGF. Our findings raised the possibility that patients with NSCLC with EGFR mutations who express high levels of HGF may benefit from onartuzumab and erlotinib combination therapy, and that HGF can be a novel biomarker for selecting such patients.

Introduction

Lung cancer, one of the leading causes of cancer-related mortality, is classified into two histologic subgroups: non–small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC; ref. 1). The treatment of NSCLC, which accounts for approximately 85% of lung cancer, has improved with the development of molecular targeted therapies (2). Tyrosine kinase inhibitors of epidermal growth factor receptor (EGFR-TKI), such as erlotinib and gefitinib, are efficacious in patients with NSCLC with activating EGFR mutations (3–6). However, continuous administration of the drug decreases efficacy in once-responsive patients; the acquired EGFR T790M mutation, which causes resistance to EGFR-TKI, has been seen in half of these treatment-resistant patients (7–9). Some studies show that other than EGFR T790M, MET amplification and high expression of hepatocyte growth factor (HGF) also cause treatment resistance (10–13). High HGF expression levels in tumors, even in patients with activating EGFR mutations, are associated with a poor response to EGFR-TKI (14, 15) because the HGF–MET pathway, an alternative signaling route to EGFR, is also activated (16).

MET belongs to the receptor tyrosine kinase family. It was originally found as a fusion protein, TPR-MET, which was produced by translocation in a sarcoma cell line treated with a carcinogen (17). MET and its ligand, HGF, are known to regulate numerous biologic activities, including cell proliferation, migration, invasion, angiogenesis, survival, and metastasis (18–21). MET is highly expressed in various cancers (20), including NSCLC, and elevated MET and HGF levels in patients correlate with a higher pathologic tumor stage (22).

Unlike other anti-MET antibodies that may activate MET signaling (23, 24), onartuzumab was designed to be monovalent, to avoid agonistic activity (25), and it blocks the binding between MET and HGF (26, 27). Onartuzumab is produced in Escherichia coli and does not have antibody-dependent cellular cytotoxicity (ADCC; ref. 28). Demonstrating the ligand blocking activity of onartuzumab in xenograft models is problematic because mouse HGF does not activate human MET and thus has no effect on the proliferation of grafted human tumors (29, 30).

Erlotinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR-TKI), benefits survival of patients with non–small cell lung cancer (NSCLC) who harbor activating EGFR mutations. However, elevated expression of hepatocyte growth factor (HGF), a ligand of the MET receptor tyrosine kinase, causes erlotinib resistance. Because onartuzumab, a monoclonal antibody to MET, blocks HGF-induced MET activation, the addition of onartuzumab to erlotinib may improve therapeutic efficacy. We engineered the human NSCLC cell line PC-9 (MET-positive cells harboring an exon 19 deletion of EGFR) to overexpress hHGF and evaluated the effects of an onartuzumab and erlotinib combination in vitro and in vivo in xenograft models. A stable clone of PC-9/hHGF was less sensitive to erlotinib than the parental PC-9, and the addition of onartuzumab to erlotinib suppressed the proliferation of these cells in vitro. In PC-9/hHGF xenograft tumors, onartuzumab or erlotinib alone minimally inhibited tumor growth; however, combining onartuzumab and erlotinib markedly suppressed tumor growth. The total MET protein level was decreased in PC-9/hHGF cells, because MET is constitutively phosphorylated by autocrine HGF, leading to its ubiquitination and degradation. Onartuzumab reduced phospho-MET levels, inhibited MET ubiquitination, and consequently restored MET protein levels. Moreover, in NSCLC clinical specimens harboring activating EGFR mutations, more than 30% of patients expressed high levels of HGF. Our findings raised the possibility that patients with NSCLC with EGFR mutations who express high levels of HGF may benefit from onartuzumab and erlotinib combination therapy, and that HGF can be a novel biomarker for selecting such patients.
only paracrine models available to date are those that supply human HGF via implanted osmotic pumps (26), by human HGF transgenic mice (31), or by the coinoculation of cancer cells with HGF-producing human fibroblast (32). However, onartuzumab has shown high efficacy in autocrine models that use the U-87MG and KP4 cell lines, which produce human HGF (26, 27).

In a phase II study of patients with second-line or third-line NSCLC, where most of the patients possess wild-type EGFR, erlotinib combined with onartuzumab improved progression-free survival (PFS) and overall survival (OS) in MET-positive patients, as determined by immunohistochemistry (IHC; refs. 33, 34). A phase III (MetLung) study was conducted with the same study design as the phase II study, where high HGF levels were not an inclusion criterion for patient selection. Interim analysis of the phase III data could not confirm the efficacy observed in the phase II study (35).

Here, we modified EGFR-mutated cell lines to overexpress human HGF, and observed the additive effect of onartuzumab and erlotinib on tumor growth inhibition. Our results suggested the possibility that the combination of onartuzumab and erlotinib may be more efficacious in patients with activating EGFR mutations who are erlotinib-resistant due to an elevated HGF expression.

Materials and Methods

Cell lines

PC-9, a human lung adenocarcinoma cell line harboring an activating EGFR mutation (deletion of E746–A750) was purchased from and characterized by Immuno-Biological Laboratories in 2009. HCC827, a human lung adenocarcinoma cell line with an activating EGFR mutation (deletion of E746–A750), and U-87MG, a glioblastoma cell line, were purchased from and characterized by the American Type Culture Collection in 2006 and 2005, respectively. KP4, a pancreatic cancer cell line, was purchased from and characterized by RIKEN Bio Resource Center in 2001. All cell lines were cultured according to suppliers' instructions. No further cell line authentication was conducted for all these cell lines.

Reagents

Erlotinib was obtained from Hoffman-La Roche. Onartuzumab was obtained from Genentech, Inc. Gefitinib was purchased from Kemprotex. Crizotinib, a MET and ALK dual inhibitor, was prepared in-house.

Cell growth inhibition assay

Cells were plated in 96-well plates. After incubation for 1 day, various concentrations of erlotinib were added. For the combination assay, 30 μg/mL of onartuzumab was combined with various concentrations of erlotinib. To examine the effect of HGF on the sensitivity of PC-9 to erlotinib, 25 or 50 ng/mL of recombinant human HGF (R&D Systems) was used together with various concentrations of erlotinib. The cells were incubated for a further 3 days, and viable cells were counted by WST-8 assay (Dojindo Laboratories).

Establishment of stable PC-9/hHGF and HCC827/hHGF clones

We transfected human HGF expression plasmids into PC-9 or HCC827 cells by electroporation (Bio-Rad). Transfected cells were selected in medium containing 300 μg/mL of zeocin (Life Technologies), and resistant colonies were expanded. We detected human HGF in the culture medium of clones by enzyme-linked immunosorbent assay (ELISA) and selected one clone for PC-9, and two clones for HCC827 secreting a high level of HGF for further analysis.

Flow cytometric analysis

Cells were suspended in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA). The anti-MET monoclonal antibody prepared in-house was added, and cells were then incubated on ice for 30 minutes. After washing with PBS containing 0.5% BSA, FITC-conjugated anti-mouse secondary antibody (Dako) was added. After washing with PBS containing 0.5% BSA, the samples were analyzed with Epics XL flow cytometer (Beckman–Coulter), or BD FACsVerse (Becton Dickinson) using the CellQuest Pro software (Becton Dickinson), or BD FACSVerse software (Becton Dickinson). The antibody-binding capacity of the cell surface MET was calculated using a QIFI kit (Dako).

Western blotting

Cells were lysed with the cell lysis buffer [100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 10% glycerol, 1% Triton X-100]. Tumor samples were homogenized with a multi-beads shoker cell disruptor (Yasui Kikai), or a Biomasher (Funakoshi). Protein concentration in the lysates was determined using the Bio-Rad Protein Assay (Bio-Rad), and 50 μg of total protein was resolved by SDS-PAGE. After transfer of proteins onto nitrocellulose membranes, the membranes were probed with specific antibodies against MET (Life Technologies), HGF (LifeSpan Bioscience), EGFR (1005), beta-actin (C-2; Santa Cruz Biotechnology), phospho-EGFR (Y1068), phospho-MET (Y1234/1235), ERK, phospho-ERK (T202/Y204), AKT and phospho-AKT (S473), all from Cell Signaling Technology. After washing with Tris-buffered saline containing 0.05% Tween-20 (TBS-T), membranes were probed with species-specific IR-dye 680–or 800CW-conjugated secondary antibodies (LI-COR Bioscience). After washing with TBS-T, membranes were scanned and analyzed with an Odyssey infrared imaging system (LI-COR Bioscience).

Ubiquitination assay

Cells were treated with 50 μmol/L of MG132, a proteasome inhibitor (Sigma–Aldrich) and/or 30 μg/mL of onartuzumab for 12 hours. Cell lysate protein (800 μg) was used for immunoprecipitation with 1.2 μg of in-house–prepared anti-MET chimeric antibody (in which the Fc region was replaced with human Fc), immunoprecipitation proceeded on ice for 12 hours. After protein A sepharose was added to lysates, samples were rotated gently at 4°C for 2 hours. After extensive washing, immunoprecipitates were resolved by SDS-PAGE and proteins transferred onto nitrocellulose membranes. Ubiquitinated MET was detected by Western blotting using anti-ubiquitin Ab (P4D1; Santa Cruz Biotechnology).

Xenograft models

All animal studies were performed in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) of Chugai Pharmaceutical Co., Ltd. PC-9, PC-9/hHGF, HCC827, or HCC827/hHGF cells were inoculated subcutaneously into female nude mice. After tumors reached approximately...
200 mm³ in volume, onartuzumab in PBS (30 mg/kg body weight, intraperitoneally, once) was administered alone or in combination with erlotinib in capsitol (Cydex: 50 mg/kg body weight, orally, daily). Control animals received PBS and capsitol as vehicle. Each group comprised 5 mice. Tumor volume was measured twice per week.

Quantitative reverse transcription polymerase chain reaction

For quantitative reverse transcription polymerase chain reaction (qRT-PCR), cultured cells and 124 NSCLC specimens, which were comprised of 123 adenocarcinomas and one squamous cell carcinoma, were examined. Total RNA was extracted from snap-frozen tissues using an RNeasy kit (Qiagen), and cDNAs were synthesized using a SuperScriptIII First-strand Synthesis System (Life Technologies). The synthesized cDNA was used for HGF, or MET qRT-PCR with SYBR Green (Life Technologies); levels were normalized to those of a housekeeping gene (GAPDH). Primer sequences were as follows: HGF forward: 5’-CTGCTCCCCCATGCCATCCCCTATG-3’, HGF reverse: 5’-TCTAGGTTAGCTTCTGGCTATTITGG-3’, MET forward: 5’-CAGTGAGAACGCTTAAACTGGAACCAAAAGATG-3’, MET reverse: 5’-GACCGGTCAGAGGTAATTTAATATGTTGCGC-3’, GAPDH forward: GTGAAGGCTCGGAGTCAACG-3’, GAPDH reverse: 5’-TACGCTTCAATGAAGGGGCTGCCATCCCCTATG-3’. Primer sequences were as follows:

Statistical analysis

All preclinical data were analyzed by the Student t test. For prevalence studies of MET-IHC and HGF qRT-PCR results, the χ² test or Fisher exact test was used (two-tailed). P values < 0.05 were considered as statistically significant.

Results

Erlotinib sensitivity of PC-9 cells

PC-9 is a representative NSCLC cell line harboring a deletion of exon 19 of EGFR, and is well known to be particularly sensitive to EGFR-TKI (37). We confirmed that PC-9 is more sensitive than other NSCLC cell lines to erlotinib (Supplementary Fig. S1A). We also confirmed MET expression on the surface of PC-9 cells by flow cytometric analysis (Supplementary Fig. S1B).

Effect of HGF on erlotinib sensitivity of NSCLC cell lines

Next, we examined the effect of HGF on the erlotinib sensitivity of PC-9 cells. As shown in Fig. 1A, HGF addition reversed the efficacy of erlotinib in PC-9 cells in a dose-dependent manner. To further assess the effect of HGF on the phosphorylation status of downstream molecules in the HGF–MET pathway, we analyzed lysates of cells treated with erlotinib and/or HGF by Western blotting. Treatment of PC-9 cells with erlotinib decreased phospho-EGFR levels and subsequently decreased phospho-ERK and phospho-AKT levels. Cotreatment of PC-9 cells with HGF and erlotinib reversed the effect of erlotinib, and restored phospho-ERK and phospho-AKT levels. Because the phospho-EGFR levels were still reduced and the phospho-MET levels were increased by this cotreatment, we assumed that activation of the HGF–MET pathway compensates for erlotinib-induced inhibition of the EGFR pathway (Fig. 1B).

We examined the effect of HGF on erlotinib-sensitivity of HCC2827 cells, another cell line harboring an activating EGFR mutation, to confirm that PC-9 is not the only cell line to show erlotinib resistance upon treatment with HGF. Similar results were obtained in HCC2827 cells in growth inhibition and Western blotting assays (Supplementary Fig. S1C and S1D). We also examined the effect of HGF on erlotinib sensitivity of EGFR wild-type cell lines such as NCI-H292, NCI-H322, and Calu-3, which show relatively high sensitivity to erlotinib (Supplementary Fig. S1A). As shown in Supplementary Fig. S1E, HGF had a moderate effect on erlotinib resistance in these EGFR wild-type cells.

Establishment of PC-9/hHGF cell line

To establish both an intrinsic and an acquired EGFR-TKI resistance model, a human HGF expression plasmid was transfected into PC-9 cells. After zebrafish selection, we performed HGF ELISA and detected increased HGF levels in the culture media of
transfectants; one such clone (PC-9/hHGF) was chosen for further analysis (Supplementary Fig. S2A).

We then verified that the mature form of HGF was expressed in these cells by Western blotting (Fig. 2A).

**EGFR-TKI sensitivity of PC9-/hHGF cells**

We treated PC-9/hHGF cells with various concentrations of gefitinib or erlotinib to examine whether PC-9/hHGF showed resistance to EGFR-TKI. As expected, the sensitivity of PC-9/hHGF to EGFR-TKI was decreased compared with that of the PC-9 parent cells (Supplementary Fig. S2B).

**Effect of onartuzumab and erlotinib on PC-9/hHGF cell proliferation**

To confirm whether HGF overexpression renders PC-9 dependent upon HGF–MET signaling in addition to EGFR signaling, we examined the effect of onartuzumab on cell growth. Onartuzumab alone had no growth-inhibitory effect on PC-9 cells, but had a minimal effect on PC-9/hHGF cells (Fig. 2B).

Then, we investigated whether a combination of onartuzumab and erlotinib increased the growth-inhibitory effect of erlotinib. PC-9/hHGF cells were cotreated with various concentrations of erlotinib and 30 μg/mL of onartuzumab. When combined with onartuzumab, erlotinib showed increased growth inhibition of PC-9/hHGF cells, compared with the effects of onartuzumab alone (25%–30% growth inhibition; Fig. 2C). Next, we cotreated PC-9/hHGF cells with various concentrations of onartuzumab and 1 μmol/L of erlotinib and also found increased growth inhibition compared with the effect of erlotinib alone (20% growth inhibition; Supplementary Fig. S2C). Similar results were obtained when replacing onartuzumab with crizotinib, a MET/ALK-TKI that interferes with the MET–HGF pathway (Fig. 2D, Supplementary Fig. S2D).

We also established HGF-overexpressing clones of HCC827 cells and confirmed the high HGF expression level by qRT-PCR (Supplementary Fig. S2E). We examined

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**Figure 2.** Establishment of a stable PC-9 cell line expressing human HGF. A, PC-9 cells were transfected with a human HGF-expression vector. These PC-9/hHGF cells overexpressed mature human HGF, as detected by Western blotting. B, onartuzumab inhibited growth of PC-9/hHGF, but not PC-9 cells. Cells were treated with 30 μg/mL of onartuzumab for 3 days. Viable cells were counted with WST-8 assay, and growth inhibition activity was calculated (n = 6 wells; error bars, SD). C, increased growth-inhibitory effect of erlotinib in combination with onartuzumab in PC-9/hHGF cells. A constant concentration of onartuzumab (30 μg/mL) was combined with various concentrations of erlotinib and used for a growth inhibition assay in PC-9 and PC-9/hHGF (n = 3 wells; error bars, SD). D, MET-TKI in combination with erlotinib showed a synergistic effect on growth inhibition of PC-9/hHGF cells. Cells were treated with 1 μmol/L of crizotinib, a MET/ALK dual inhibitor (as a MET-TKI), and/or 1 μmol/L of erlotinib for 3 days. Viable cells were measured by WST-8, and growth inhibition was calculated (n = 4 wells; error bars, SD). E, combination of onartuzumab and erlotinib decreased phospho-AKT (pAKT) and phospho-ERK (pERK) levels in PC-9/hHGF cells. Cells were treated with 30 μg/mL of onartuzumab and/or 100 nmol/L of erlotinib for 6 hours. Cell lysates were prepared, and the phospho-AKT and phospho-ERK levels were detected by Western blotting.
whether the combination of onartuzumab and erlotinib increased
growth inhibition of HCC827/hHGF cells and found the same
results as for PC-9/hHGF cells (Supplementary Fig. S2F). These
results indicated that autocrine HGF overexpression contributes
to EGFR-TKI resistance in cells harboring activating EGFR
mutations (Fig. 2B–2D and Supplementary Fig. S2B–S2D and S2F).

Effect of onartuzumab and erlotinib on phosphorylation of
AKT and ERK
To confirm this additive effect of onartuzumab and erlotinib, we analyzed the phosphorylation status of signaling molecules in
cells treated with onartuzumab and/or erlotinib by Western
blotting. In PC-9 cells, treatment with erlotinib alone caused a
significant reduction of both phospho-AKT and phospho-ERK
levels, but onartuzumab alone had no impact on the phosphor-
ylation status of these molecules, and combining onartuzumab
with erlotinib caused no further reduction. On the other hand, in
PC-9/hHGF cells, erlotinib treatment could not reduce phospho-
AKT or phospho-ERK levels as much as it could in PC-9 cells,
whereas a combination of the two drugs resulted in an additive or
synergistic effect on phospho-AKT and phospho-ERK reduction
(Fig. 2E).

Effects of onartuzumab and erlotinib in xenograft models
Next, we examined the in vivo efficacy of the combination of
onartuzumab and erlotinib. We confirmed that HGF levels in the
sera of PC-9/hHGF-engrafted mice whose tumor size was around
200 mm³ were comparable with those of HGF-high patients in the
onartuzumab phase I study (Supplementary Fig. S3A; ref. 38).
Consistent with the in vitro data, erlotinib, but not onartuzumab,
showed strong growth inhibition of xenograft tumors derived
from the parent PC-9 line (Fig. 3A). On the other hand, erlotinib
was less efficacious in PC-9/hHGF xenograft models than in PC-9
models, and onartuzumab demonstrated minimal effects. How-
ever, a combination of the two drugs significantly increased the
efficacy of tumor growth inhibition (Fig. 3B). Similar results were
obtained in the HCC827 and HCC827/hHGF clone 9 xenograft
model (Fig. 3C and D). Surprisingly, the efficacy of the drug
combination in the PC-9/hHGF xenograft model lasted for more
than 100 days, showing that the onartuzumab and erlotinib
combination has a marked and durable effect (Supplementary
Fig. S3B).
We also examined the phosphorylation status of downstream
molecules in PC-9/hHGF tumors and found that the combination
of onartuzumab and erlotinib decreased phospho-ERK and

Figure 3.
In vivo efficacy of onartuzumab and erlotinib combination in xenograft models. A, adding onartuzumab to erlotinib did not increase therapeutic efficacy in a PC-9 xenograft model. Onartuzumab was administered at 30 mg/kg body weight intraperitoneally by injection once. Erlotinib was administered daily at 50 mg/kg orally (n = 5 mice in each group; error bars, SD). B, additive efficacy of combination therapy in the PC-9/hHGF xenograft model. Dosing and administration schedule were the same as described for the PC-9 xenograft model (n = 5; error bars, SD). C, absence of an additive efficacy of combination therapy in the HCC827 xenograft model. Dosing and administration schedule were as described for the PC-9 xenograft model (n = 5; error bars, SD). D, additive efficacy of combination therapy in the HCC827/hHGF clone 9 xenograft model. Dosing and administration schedule were as described for the PC-9 xenograft model (n = 5; error bars, SD).
phospho-AKT levels more than the administration of the single drugs did (Supplementary Fig. S3C). We also performed Ki-67 immunostaining in formalin-fixed paraffin-embedded (FFPE) xenograft tumor tissue sections and confirmed the combination effect (Supplementary Fig. S3D). Thus, we concluded that both onartuzumab and erlotinib are required to inhibit the proliferation of PC-9/hHGF and HCC827/hHGF tumors (Supplementary Fig. S3E).

MET expression in Japanese lung cancer patients with activating EGFR mutations

To assess the ratio of MET-positive Japanese NSCLC patients with activating EGFR mutations, we performed MET-IHC on their FFPE lung cancer tissue sections, using the same method and scoring system as in the onartuzumab clinical trials (Table 1, Supplementary Fig. S4). According to an onartuzumab phase II clinical trial that was conducted mainly in the United States, MET-IHC diagnostic positivity, which is defined as a score of 2+ and 3+, was estimated to be around 50% in patients with NSCLC (34).

Table 1. MET-diagnostic positive ratio in Japanese lung cancer patients

<table>
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<tr>
<th>MET diagnosis</th>
<th>EGFR status</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>Positive ratio (%)</th>
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</thead>
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<tr>
<td>Positive</td>
<td>Mutation</td>
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<td>53</td>
<td>82</td>
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<tr>
<td></td>
<td>Wild-type</td>
<td>20</td>
<td>109</td>
<td>129</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>49</td>
<td>162</td>
<td>211</td>
<td>23.2</td>
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</tbody>
</table>

NOTE: Statistical significance between mutation and wild-type, \( P = 0.00135 \). No tumor was detected in 8 patients. MET-IHC was performed with FFPE sections of lung cancer specimens.
However, in our study, 23.2% of Japanese patients were found to be MET-positive (Table 1), a discrepancy that may be associated with ethnic differences. Interestingly, 35.4% of patients with lung cancer with EGFR mutations were found to be MET-positive, as compared with 15.5% of patients without EGFR mutations. This result implied that the activation of the EGF pathway might be involved in inducing MET transcription and/or stabilizing the MET protein.

MET levels in PC-9/hHGF
We were surprised to observe that MET protein levels in the HGF-transfected cells were less than in the parent cells for both PC-9 and HCC827 cell lines (Fig. 4A). Moreover, the decreased MET levels recovered upon treatment of PC-9/hHGF with onartuzumab or MET-TKI, even though phospho-MET levels were reduced (Fig. 2E, Supplementary Fig. S5A). Taken together, this indicated that HGF overexpression stimulates MET downregulation as well as cell proliferation.

We also examined the change in MET levels at the cell surface by flow cytometric analysis. In PC-9 cells, onartuzumab treatment did not shift the MET peak, but in the PC-9/hHGF cells, onartuzumab treatment resulted in a shift of the MET peak (Fig. 4B). Similar results were obtained with MET flow cytometric analysis when PC-9 parent cells were treated with recombinant HGF in the presence of onartuzumab (Supplementary Fig. S5B). We confirmed that onartuzumab administration resulted in increased MET protein levels as quantified by densitometric analysis of Western blots of proteins obtained from a PC-9/hHGF xenograft model (Fig. 4C). On the basis of these results, we speculated that when onartuzumab reduces the phospho-MET level, MET is no longer recognized by Cbl, a ubiquitin ligase of MET, and escapes from ubiquitin-dependent degradation.

To prove this hypothesis, we examined the effect of onartuzumab on MET ubiquitination. We treated cells with MG-132, a proteasome inhibitor, with or without onartuzumab. Cell lysates were immunoprecipitated using an anti-MET antibody, and ubiquitinated MET was then detected with an anti-ubiquitin antibody on Western blots. As shown in Fig. 4D, MET was ubiquitinated in the presence of MG132 in PC-9/hHGF cells; however, its level was decreased by onartuzumab treatment, suggesting that inhibition of MET-ubiquitination by onartuzumab treatment causes recovery of the total MET level.

MET levels in other HGF-autocrine cell lines
Next, we also investigated whether this event was observed in native cancer cell lines. We selected U-87MG and KP4 cells, both of which are well known for overexpressing HGF; onartuzumab has also been shown to have strong growth-inhibition activity in xenograft tumors derived from these cell lines (26, 27). Similar to our findings in PC-9/hHGF cells, MET protein levels were increased by onartuzumab treatment in U87-MG and KP4, although phospho-MET levels were decreased (Fig. 4E). The difference in MET protein levels did not derive from the transcriptional level, as no significant changes in mRNA levels were observed between the control and the onartuzumab-treated samples (Fig. 4F). We also obtained similar results when we used a MET-TKI or HGF siRNA instead of onartuzumab to inhibit MET–HGF activation (Supplementary Fig. SSC and SSD). We also confirmed that HGF siRNA significantly inhibited proliferation of U-87MG and KP4 cells (Supplementary Fig. SSE).

By screening lung cancer cell lines with HGF qRT-PCR, we found that HCC2108 expressed high levels of endogenous HGF (Supplementary Fig. S6A), and the proliferation of HCC2108 was inhibited by HGF siRNA, suggesting that the proliferation depends on HGF (Supplementary Fig. S6B). Next, we treated HCC2108 cells with onartuzumab or crizotinib to observe the changes in MET protein levels. As expected, treatment with onartuzumab or crizotinib resulted in increased MET protein levels (Supplementary Fig. S6C) without significant changes in the mRNA levels (Supplementary Fig. S6D). We therefore concluded that the MET degradation pathway is activated, in addition to activation of a proliferation signal, in most HGF-autocrine cells (Fig. 4G).

HGF mRNA levels in NSCLC specimens
We then performed HGF qRT-PCR using NSCLC clinical samples. Of NSCLC patients with activating EGFR mutations, 34.4% (21 of 61) expressed HGF levels higher than that seen in U-87MG cells, in which tumor growth was repressed by treatment with more than 7.5 mg/kg onartuzumab (28), suggesting that HGF levels in U-87MG cells are sufficient for onartuzumab to be highly effective. These 34.4% patients with NSCLC with high levels of HGF and activating EGFR mutations may not respond well to erlotinib monotherapy, but a combination of onartuzumab and erlotinib may be more efficacious in these patients.

Discussion
Several studies have confirmed the efficacy of erlotinib and gefitinib in patients with NSCLC with activating EGFR mutations (39–41); therefore, these drugs have been approved for use as first-line treatment. However, not all patients with activating EGFR mutations respond to erlotinib, indicating that activating EGFR mutation is not the sole factor affecting erlotinib sensitivity.

Yano and colleagues observed that about 29% (13 of 45) of patients who did not respond to EGFR-TKI despite having EGFR mutations showed high HGF expression in their tumors (15). Several reports have also shown that high HGF expression is associated with acquired and intrinsic resistance to EGFR-TKI (13, 15, 16). We here confirmed that activation of MET signaling by HGF overexpression causes EGFR-TKI resistance in PC-9/hHGF cells. Because both MET–HGF and EGFR pathways are simultaneously stimulated in PC-9/hHGF cells, neither a MET inhibitor nor an EGFR inhibitor alone was sufficient to completely inhibit
levels. In addition, onartuzumab showed high efficacy in the PC-9/hHGF model, the combined use of onartuzumab and erlotinib decreased not only phospho-EGFR, but also phospho-MET in PC-9 and PC-9/hHGF cells (Fig. 2E), suggesting that mutated EGFR trans-activates MET signaling. These results imply a rationale for MET and EGFR combination therapy in patients with NSCLC.

In the ongoing MetDriver study, which is a phase III clinical trial using a combination of erlotinib and onartuzumab, or erlotinib alone, as first-line treatment in patients with NSCLC with activating EGFR mutations, MET expression status was tested by IHC, and only MET-diagnostic positive patients (MET-IHC scores of 2+ and 3+) were included; however, HGF-high patients were not prospectively selected (ClinicalTrial.gov, NCT01887886). In our PC-9/hHGF model, the combined use of onartuzumab and erlotinib showed high efficacy, despite the low MET expression levels. In addition, onartuzumab showed high efficacy in U-87MG and KP4 cells, in which elevated HGF levels resulted in decreased MET protein levels. We also observed that MET protein levels in HGF-autocrine cells, including PC-9/hHGF, U-87MG, and KP4 cells, were less than in A549 or HOP-92 cells, representative cell lines with MET-IHC scores of +2 (Supplementary Fig. S7). We demonstrated that autocrine HGF activates not only proliferation but also MET downregulation via the ubiquitin-proteasome pathway, in which Cbl triggers ubiquitination and degradation of MET by binding to phospho-MET (42, 43). These data suggested that measuring HGF expression levels in addition to MET-IHC may be important for selecting patients who are likely to benefit from combination therapy. Although the small sample number confounded the correlation between blood HGF levels and survival rate in the phase II trial of onartuzumab and erlotinib (38), it is likely that high HGF levels, not in the blood, but in the tumor region, can be a novel biomarker for onartuzumab use in future.

In conclusion, we showed that the combined use of onartuzumab and erlotinib has an additive effect in a model that expresses high levels of human HGF in cells with an activating EGFR mutation. This suggests that the use of a combination of onartuzumab and erlotinib may achieve higher efficacy in the first-line treatment of patients with NSCLC with activating EGFR mutations and high levels of HGF expression.

Disclosure of Potential Conflicts of Interest
Y. Yatabe has speakers' bureau honoraria from Chugai Pharmaceuticals Co., Ltd. and Ventana Roche and is a consultant/advisory board member for Ventana Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: Y. Sano, E. Hashimoto, N. Nakatani, M. Abe, Y. Yatabe Development of methodology: Y. Sano, N. Nakatani, Y. Satoh, Y. Yatabe Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sano, E. Hashimoto, N. Nakatani, Y. Yatabe Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sano, E. Hashimoto, N. Nakatani, K. Sakata, T. Fujii, M. Sugimoto, H. Moteigi, E. Sasaki, Y. Yatabe Writing, review, and/or revision of the manuscript: Y. Sano, E. Hashimoto, N. Nakatani, K. Fujimoto-Ouchi, M. Sugimoto, H. Moteigi, Y. Yatabe Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Sano, E. Hashimoto, Y. Yatabe Study supervision: Y. Sano, N. Nakatani, K. Fujimoto-Ouchi, M. Sugimoto, S. Nagahashi, M. Aoki, Y. Yatabe

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Yuji Sano, Eri Hashimoto, Noriaki Nakatani, et al.

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