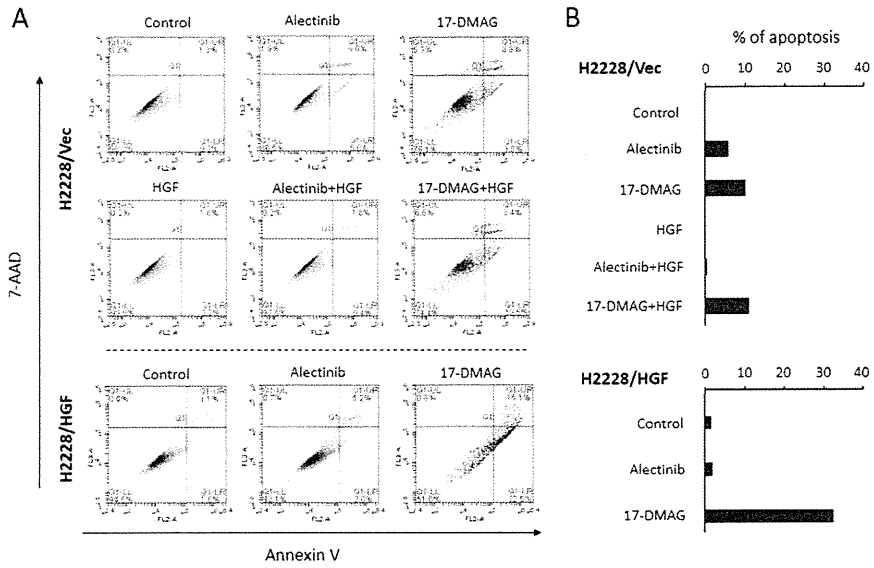

Figure 5: 17-DMAG induced apoptosis of EML4-ALK NSCLC cells, even in the presence of HGF. A. Apoptotic cells were evaluated by the 7-AAD cell viability assay . as described in the Materials and Methods. B. Quantification of apoptotic cells.

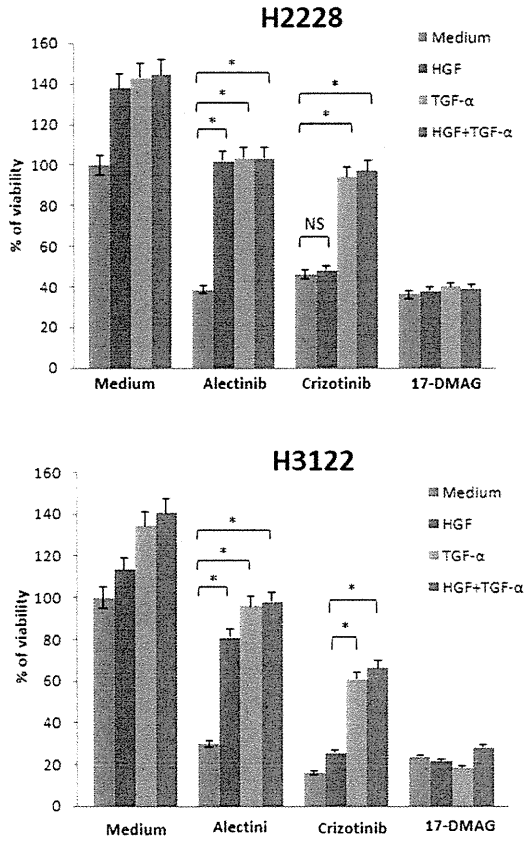

Figure 6: 17-DMAG reduced viability of EML4-ALK NSCLC cells, even in the presence of both HGF and TGF-α. H2228 and H3122 cells were incubated with or without alectinib (0.1 μmol/L), crizotinib (0.1 μmol/L), and/or HGF (50 ng/mL) and TGF-α (100 ng/mL), and cell viability was determined after 72 h by MTT assay. The percentage of cell viability is shown relative to controls without HGF or TGF-α treatment. * , $P < 0.001$ (one-way ANOVA). NS, not significant. Data shown are representative of at least 3 independent experiments. Error bars indicate SD of triplicate cultures.

EGFR (EGF, HB-EGF, and TGF-α) triggered resistance to alectinib in *ALK*-rearranged NSCLC cells, and that the Hsp90 inhibitor 17-DMAG overcame the resistance triggered by these receptor ligands. 17-DMAG inhibited protein levels of ALK, EGFR, and MET, even in the presence of ligand activation, and suppressed of AKT and ERK1/2 phosphorylation, thereby inducing apoptosis of *ALK*-rearranged NSCLC cells, irrespective of the presence of HGF or EGFR ligands. Since the Hsp90 inhibitor by itself could inhibit both driver (from rearranged *ALK*) and resistance signals (from activated receptors; MET and EGFR), it may be an ideal agent for overcoming ligand-triggered alectinib resistance in *ALK*-rearranged NSCLC.

Activation of bypass signals is a common resistance mechanism for targeted drugs. For example, EGFR-TKI resistance could be caused by *MET* amplification [27], HGF-triggered MET activation [23], Gas6-triggered AXL activation [28], and *HER2* amplification [29] in *EGFR* mutant lung cancer. BRAF inhibitor resistance could be caused by HGF-triggered MET activation [30], and IGF-1 triggered its receptor activation [31] in *BRAF* mutant melanoma. Crizotinib resistance could be caused by EGFR ligand-triggered EGFR activation [11], and stem cell factor (SCF)-triggered amplified cKIT activation [8] in EML4-ALK NSCLC. Therefore, HGF and EGFR ligands may be common resistance triggers that activate bypass survival signal via their receptor activation. The results in the present study are consistent with previous research indicating that alectinib resistance was induced by HGF and EGFR ligands.

Previous studies reported that several signaling pathways, including PI3K/AKT, MEK/ERK, and STAT3, are essential for survival and/or resistance to ALK inhibitors in *ALK*-rearranged NSCLC cells [12, 32]. Accordingly, we found that alectinib inhibited STAT3 and ALK phosphorylation. In the presence of alectinib, HGF or EGFR ligands restored AKT and ERK1/2, but not STAT3, phosphorylation and thereby made EML4-ALK cells insensitive to alectinib. These observations indicate that, when activated by their ligands, AKT and ERK signals from MET or EGFR play pivotal roles in alectinib resistance of EML4-ALK NSCLC cells.

It is of interest in the present study that HGF and EGFR ligands induced not only ALK-TKI resistance but also increased cell growth of EML4-ALK NSCLC cells. HGF and EGFR ligands also induced morphological change of H2228 cells (Supplementary Fig. 3). Therefore, these receptor ligands may modulate various cancer phenotypes of EML4-ALK NSCLC cells. HGF-MET and EGFR-ligands-EGFR axises play pivotal roles in progression of various types of tumors (33, 34). We are planning further studies to explore the molecular mechanisms of this morphological change and co-relation between the expression of receptor ligands in patient specimens and clinical characteristics in *ALK*-rearranged NSCLC.

Inter- and/or intra-tumor heterogeneity is a critical obstacle in cancer therapy with targeted drugs [35]. This is also the case in ALK-TKI resistance. Intra-tumor heterogeneity caused by crizotinib resistance results from the L1196M gatekeeper *ALK* mutation, and other *ALK* secondary C1156Y mutations co-existed in malignant pleural effusion of a patient who acquired crizotinib resistance [6]. Moreover, activation of different two receptors, EGFR and amplified *KIT* (both of which could induce crizotinib resistance), also co-existed in one crizotinib-resistant tumor [8].

Hsp90 inhibitors have been reported to overcome crizotinib resistance caused by several mechanisms, including *ALK* amplification, L1196M gatekeeper *ALK* mutation, other secondary *ALK* mutations (including F1174L), and epithelial to mesenchymal transition. Furthermore, we demonstrated that the Hsp90 inhibitor may overcome alectinib resistance, even when ligands of MET and EGFR co-exist. A new generation of Hsp90 inhibitors, including ganetespib, has recently been developed, and remarkable efficacy has been reported in a co-clinical model and early phase clinical studies [36]. Therefore, Hsp90 inhibition using new generation inhibitors may be a promising strategy to treat *ALK*-rearranged NSCLCs that acquire resistance to alectinib.

MATERIALS AND METHODS

Cell culture

The H2228 human lung adenocarcinoma cell line, with EML4-ALK fusion protein variant3 (E6;A20), was purchased from the American Type Culture Collection (Manassas, VA). The H3122 human lung adenocarcinoma cell line, with EML4-ALK fusion protein variant1 (E13;A20), was kindly provided by Dr. Jeffrey A. Engelman of the Massachusetts General Hospital Cancer Center (Boston, MA) [37]. H2228 and H3122 cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 μg/mL) in a humidified CO_2 incubator at 37°C. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for *Mycoplasma* using a MycoAlert *Mycoplasma* Detection Kit (Lonza , Basel, Swiss).

Reagents

Alectinib, crizotinib, and 17-DMAG were purchased from Seleck Chemicals (Houston, TX). Recombinant EGF, TGF-α, and HB-EGF were purchased from R&D Systems (Minneapolis, MN). Recombinant HGF was prepared as described in a previous study [38].

Cell growth assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl terazolium bromide (MTT) dye reduction method [39]. Tumor cells were harvested at 80% confluence, seeded at 2 × 10^3 cells per well in 96-well plates, and incubated in appropriate medium for 24 h. Several concentrations of alectinib, crizotinib, 17-DMAG, and/or EGF, TGF-α, HB-EGF, and HGF were added to each well, and incubation continued for another 72 h. Fifty μL MTT (2 mg/mL; Sigma, St.Louis, MO) was added to each well, followed by incubation for 2 h at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL of dimethyl sulfoxide (DMSO). Absorbance was measured with an MTP-120 Microplate reader (Corona Electric , Hitachinaka, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth was calculated relative to untreated controls. Each assay was carried out at least in triplicate, with results based on 3 independent experiments.

HGF-gene transfection

One day before transfection, aliquots of 1 × 10^5 H2228 cells in 1 mL of antibiotic-free medium were plated on 6-well plates. The full-length *HGF* cDNA cloned into the BCMGSneo expression vector [40] was transfected using Lipofectamine 2000 according to the manufacturer's instructions. After incubation for 24 h, the cells were washed with phosphate buffered saline (PBS) and incubated for an additional 72 h in antibiotic-containing medium. Then, the cells were selected in G418 sulfate (Calbiochem, Jolla, CA). After limiting dilution, the HGF-producing cells, H2228/HGF, were established. HGF production by H2228/HGF was confirmed by enzyme linked immunosolvent assay (ELISA).

HGF production

Cells (2 × 10^5) were cultured in RPMI-1640 medium with 10% FBS for 24 h. The cells were washed with PBS and incubated for 48 h in 2 mL of RPMI-1640 medium with 10% FBS. Then, culture medium was harvested and centrifuged, and the supernatant was stored at −70°C until analysis. HGF concentrations were determined by IMMUNIS HGF EIA (Institute of Immunology, Tokyo) according to the manufacturer's protocols. All samples were run in duplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves, and the HGF detection limit was 100 pg/mL.

Apoptosis assay

Cell apoptosis induced by alectinib and 17-DMAG was measured by the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) which detects and quantifies apoptotic cells with phycoerythrin (PE) Annexin V and 7-amino-actinomycin (7-AAD) staining. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Western blotting

Sodium dodesyl sulfate (SDS) polyacrylamide gels (Bio-Rad , Hercules, CA) were loaded with 40 μg total protein per lane; following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), which were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature, followed by overnight incubation at 4°C with anti-ALK (C26G7), anti-phospho-ALK (Tyr1604), anti-phospho-EGFR (Tyr1068), anti-STAT3 (79D7), anti-phospho-STAT3 (Y705), anti-AKT, anti-phospho-AKT (Ser473), anti-ErbB4 (111B2), anti-phospho-ErbB4 (Tyr1284), anti-MET (25H2), anti-phospho-MET (Y1234/Y1235) (3D7), or anti-β-actin (13E5) antibodies (1:1,000 dilution each; Cell Signaling Technology , Danvers, MA), or with anti-human EGFR (1 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (Erk)1/Erk2 (0.2 μg/mL), or anti-phospho-Erk1/Erk2 (T202/Y204) (0.1 μg/mL) antibodies (R&D Systems). After washing 3 times, the membranes were incubated for 1 h at room temperature with secondary antibodies (horseradish peroxidase-conjugated species-specific antibodies).

Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce, Osaka, Japan). Each experiment was independently carried out at least 3 times.

Statistical analysis

Differences were analyzed by one-way ANOVA. All statistical analyses were carried out using GraphPad StatMate 4 (GraphPad Software, Inc ., San Diego, CA). $P < 0.05$ was considered significant.

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OPEN

Triple Inhibition of EGFR, Met, and VEGF Suppresses Regrowth of HGF-Triggered, Erlotinib-Resistant Lung Cancer Harboring an *EGFR* Mutation

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Introduction: Met activation by gene amplification and its ligand, hepatocyte growth factor (HGF), imparts resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in *EGFR*-mutant lung cancer. We recently reported that Met activation by HGF stimulates the production of vascular endothelial growth factor (VEGF) and facilitates angiogenesis, which indicates that HGF induces EGFR-TKI resistance and angiogenesis. This study aimed to determine the effect of triple inhibition of EGFR, Met, and angiogenesis on HGF-triggered EGFR-TKI resistance in *EGFR*-mutant lung cancer.

Methods: Three clinically approved drugs, erlotinib (an EGFR inhibitor), crizotinib (an inhibitor of anaplastic lymphoma kinase and Met), and bevacizumab (anti-VEGF antibody), and TAS-115, a novel dual TKI for Met and VEGF receptor 2, were used in this study. *EGFR*-mutant lung cancer cell lines PC-9, HCC827, and *HGF*-gene–transfected PC-9 (PC-9/HGF) cells were examined.

Results: Crizotinib and TAS-115 inhibited Met phosphorylation and reversed erlotinib resistance and VEGF production triggered by HGF in PC-9 and HCC827 cells in vitro. Bevacizumab and TAS-115 inhibited angiogenesis in PC-9/HGF tumors in vivo. Moreover, the triplet erlotinib, crizotinib, and bevacizumab, or the doublet erlotinib and TAS-115 successfully inhibited PC-9/HGF tumor growth and delayed tumor regrowth associated with sustained tumor vasculature inhibition even after cessation of the treatment.

Conclusion: These results suggest that triple inhibition of EGFR, HGF/Met, and VEGF/VEGF receptor 2, by either a triplet of clinical drugs or TAS-115 combined with erlotinib, may be useful for controlling progression of *EGFR*-mutant lung cancer by reversing EGFR-TKI resistance and for inhibiting angiogenesis.

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Lung cancer is the leading cause of cancer-related deaths worldwide. Recent advances in molecular biology have identified driver oncogenes such as epidermal growth factor receptor (EGFR) mutations or the echinoderm microtubule–associated protein-like 4/anaplastic lymphoma kinase (ALK) fusion gene in non–small-cell lung cancer (NSCLC). In the treatment of NSCLCs harboring these driver oncogenes, the use of EGFR tyrosine kinase inhibitors (TKIs; such as gefitinib and erlotinib) and an ALK inhibitor (such as crizotinib) to block driver oncogene survival signals resulted in marked tumor regression.[1–4] Despite these clinical successes, tumors acquire resistance to those agents in almost all cases during the course of therapy.[5]

Recently, several mechanisms of EGFR-TKI resistance have been identified and classified as follows: (1) alteration of the target *EGFR* gene (e.g., T790M gatekeeper mutation)[6,7]; (2) activation of bypass resistance signals (e.g., *Met* gene amplification,[8] hepatocyte growth factor [HGF] overexpression,[9] and activation of the nuclear factor-kappa B (NFkB) pathway[10] and Gas6-AXL axis)[11]; and (3) other mechanisms such as transformation to small-cell lung cancer,[12–14] epithelial-to-mesenchymal transition,[15–17] alteration of microRNA,[18] and down-regulation of MED12.[19] Previously, we demonstrated that HGF activates, through the Met/PI3K/Akt pathway, bypass signals that trigger resistance; overexpression of HGF was observed more frequently than T790M and *Met* amplification in tumors from patients with NSCLC who acquired EGFR-TKI resistance in a Japanese cohort.[20] These findings indicate that HGF is a clinically relevant target for overcoming EGFR-TKI resistance in *EGFR*-mutant lung cancer.

Angiogenesis is essential for the progression of various types of solid tumors, including NSCLC. Vascular endothelial growth factor (VEGF) is the most prominent proangiogenic molecule and is considered to be a therapeutic target in NSCLC. We previously reported that overexpressed HGF

stimulates VEGF production by means of phosphorylation of Met/Gab1 and promotes tumor growth by stimulating angiogenesis in *EGFR*-mutant lung cancer models,[21] which indicates that HGF is a critical inducer of not only EGFR-TKI resistance but also angiogenesis in *EGFR*-mutant lung cancer. Therefore, we hypothesized that triple inhibition of the driver signal (EGFR), bypass resistance signal (Met), and angiogenesis (VEGF) may be beneficial for controlling the progression of *EGFR*-mutant lung cancer with HGF-triggered EGFR-TKI resistance.

EGFR-TKIs, erlotinib, gefitinib, ALK-TKI, crizotinib, and the anti-VEGF antibody bevacizumab have been clinically approved as molecularly targeted drugs in many countries. Crizotinib is known to have activity against Met in addition to ALK and c-ros oncogene 1, receptor tyrosine kinase (ROS1).[22,23] In the present study, we investigated the therapeutic effect of triple inhibition against HGF-triggered, EGFR-TKI–resistant lung cancer harboring an *EGFR* mutation by using clinically available targeted drugs, namely, erlotinib, crizotinib, and bevacizumab. We further assessed the therapeutic potential of erlotinib and TAS-115 (Supplementary Figure 1, Supplementary Digital Content 1, http://links.lww.com/JTO/A570), a novel VEGF receptor 2 (VEGFR-2) inhibitor, which can be orally administered and has Met inhibitory activity, and we compared this doublet treatment with the clinically available triplet. In this study, we demonstrate that the doublet inhibited the progression of HGF-overexpressing *EGFR*-mutant lung cancer more efficiently than the clinically available triplet treatment. Moreover, TAS-115 combined with erlotinib also controlled tumor growth well and, remarkably, delayed regrowth even after cessation of the treatment.

MATERIALS AND METHODS

Cell Cultures and Reagents

The *EGFR*-mutant human lung adenocarcinoma cell lines PC-9 (del E746_A750) and HCC827, with deletions in *EGFR* exon 19, were purchased from Immuno-Biological Laboratories Co. (Gunma, Japan) and from American Type Culture Collection (Manassas, VA) respectively.[21] Human *HGF*-gene transfectant (PC-9/HGF) and vector control (PC-9/Vec) cells were established as previously described.[24] These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. The human embryonic lung fibroblast cell line MRC-5 was purchased from the Health Science Research Resources Bank (Osaka, Japan). MRC-5 (P30–35) cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human dermal microvascular endothelial cells (HMVECs) were incubated in RPMI-1640 medium with 10% FBS (control), RPMI-1640 medium with 10% FBS plus VEGF, or HuMedia-MvG with different concentrations of TAS-115 for 72 hours. Thereafter, cell viability was determined by thiazolyl blue tetrazolium bromide (MTT) assay. Cells were regularly screened for mycoplasma by using MycoAlert Mycoplasma Detection Kits (Lonza, Rockland, ME). The cell lines were authenticated at the laboratory of the National Institute of Biomedical Innovation (Osaka, Japan) by short tandem repeat analysis. TAS-115 was synthesized by Taiho Co., Ltd (Tokyo, Japan). Erlotinib and crizotinib were obtained from Selleck Chemicals (Houston, TX). Bevacizumab was obtained from Chugai Pharma (Tokyo, Japan). Human recombinant HGF was prepared as previously described.[24]

Production of HGF and VEGF in Cell Culture Supernatants

Cells (2×10^5) were cultured in a 2 ml of culture medium with 10% FBS for 24 hours, washed with phosphate-buffered saline (PBS), and incubated for 48 hours in the medium supplemented with 10% FBS. In some experiments, HGF was added to the medium. The culture media was harvested and centrifuged, and the supernatants were stored at −80°C until analysis. The concentrations of HGF and VEGF were determined by IMMUNIS HGF EIA (Institute of Immunology, Tokyo, Japan) or Quantikine VEGF enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), respectively, according to the respective manufacturer's protocol. All samples were run in duplicate. Color intensity was measured at 450 nm by using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The detection limits for HGF and VEGF were 100 and 31 pg/ml, respectively.

Cell Viability Assay

Cell growth was measured using the MTT dye reduction method.[24] Tumor cells were plated into 96-well plates at a density of 2×10^3 cells/100 ml RPMI-1640 medium with 10% FBS per well. After 24-hour incubation, various reagents were added to each well, and the cells incubated for a further 72 hours, followed by the addition of 50 μl of MTT solution (2 mg/ml; Sigma, St. Louis, MO) to each well and incubation for 2 hours. The media containing MTT solution was removed, and the dark blue crystals were dissolved by adding 100 ml of dimethyl sulfoxide. The absorbance of each well was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth is shown relative to untreated controls. Each reagent concentration was tested at least in triplicate during each experiment, and each experiment was conducted at least three times.

Antibodies and Western Blotting

Protein aliquots of 25 μg each were resolved by sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, CA) electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). After washing four times, the membranes were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature and overnight at 4°C with primary antibodies to β-actin (13E5), Met (25H2), phospho-Met (Y1234/Y1235;3D7), phospho-EGFR (Y1068), Akt, phospho-Akt (Ser473; 736E11), VEGFR-2 (55B11), phospho-VEGFR-2 (Tyr951;15D2), human EGFR (1 μg/ml), human/mouse/rat Erk1/Erk2 (0.2 μg/ml), and p-Erk1/Erk2 (T202/Y204; 0.1 μg/ml; R&D Systems). After three washes,

the membranes were incubated for 1 hour at room temperature with species-specific, horseradish peroxidase–conjugated secondary antibodies. Immunoreactive bands were visualized with Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA) and an enhanced chemiluminescence substrate (Pierce Biotechnology, Rockford, IL). Each experiment was conducted at least three times independently.

Coculture of Lung Cancer Cells with Fibroblasts or Endothelial Cells

Cells were cocultured in Transwell collagen–coated chambers separated by an 8-mm (BD Biosciences, San Jose, CA) or 3-mm (Corning, Tewksbury, MA) pore size filter. Tumor cells (8×10^3 cells/800 ml) with or without TAS-115 (1.0 μmol/liter) or erlotinib (0.3 μmol/liter) in the lower chamber were cocultured with MRC-5 (1×10^4 cells/300 μl) cells in the upper chamber for 72 hours. The upper chamber was then removed, 200 μl of MTT solution was added to each well, and the cells were incubated for 2 hours at 37°C. The media was removed, and the dark blue crystals in each well were dissolved in 400 μl of dimethyl sulfoxide. Absorbance was measured with an MTP-120 Microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth was measured relative to untreated controls. All samples were assayed at least in triplicate, with each experiment conducted three times independently.

Subcutaneous Xenograft Models

Nude mice (male, 5–6 weeks old) were obtained from Clea (Tokyo, Japan). Cultured tumor cells (PC-9/Vec or PC-9/HGF) were implanted subcutaneously into the flanks of each mouse at 3×10^6 cells/0.1 ml. When tumor volumes reached 100 to 200 mm^3, the mice (n = 5 per group) were randomized to the following groups: (1) no treatment (control group), (2) only 50 mg/kg of erlotinib orally, (3) only 25 mg/kg of crizotinib orally, (4) only 100 μg/mouse of bevacizumab intraperitoneally, (5) only 75 mg/kg of TAS-115 orally, (6) erlotinib and crizotinib, (7) crizotinib and bevacizumab, (8) erlotinib and bevacizumab, (9) erlotinib, crizotinib, and bevacizumab, and (10) erlotinib and TAS-115. Each tumor was measured in two dimensions three times a week, and the volume was calculated using the following formula: tumor volume (mm^3) = 1/2 (length (mm) × (width (mm))^2). All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Approval No. AP-122505).

Histological Analyses

For detection of endothelial cells (CD31), 5-μm-thick frozen sections of xenograft tumors were fixed with cold acetone and washed with PBS. Then, endogenous peroxidase activity was blocked by incubation in 3% aqueous H_2O_2 for 10 minutes. After treatment with 5% normal horse serum, the sections were incubated with primary antibodies to mouse CD31 (MEC13.3; BD Biosciences). After probing with species-specific, biotinylated secondary antibodies, the sections were incubated for 30 minutes with avidin–biotinylated peroxidase complex by using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The 3,3′-diaminobenzidine tetrahydrochloride Liquid System (DAKO, Glostrup, Denmark) was used to detect immunostaining. Omission of the primary antibody served as a negative control. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate-biotin nick end-labeling staining was performed using the Apoptosis Detection System (Promega Corporation, Madison, WI). In brief, 5-μm-thick frozen sections of xenograft tumors were fixed with PBS containing 4% formalin. The slides were washed with PBS and permeabilized with 0.2% Triton X-100. The samples were then equilibrated, and DNA strand breaks were labeled with fluorescein-12-2-deoxy-uridine-5-triphosphate (fluorescein-12-dUTP) by adding the nucleotide mixture and the terminal deoxynucleotidyl transferase enzyme. The reaction was stopped with saline sodium citrate, and the localized green fluorescence of apoptotic cells was detected by fluorescence microscopy (×200). The five areas containing the highest numbers of stained cells within a section were selected for histologic quantitation by light or fluorescent microscopy at a ×400 magnification. All results were independently evaluated by three investigators (JN, TN, and ST).

Statistical Analysis

Differences were analyzed by one-way analysis of variance. All statistical analyses were carried out using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., La Jolla, CA). A p value of less than 0.01 was considered statistically significant.

RESULTS

Effect of Crizotinib and TAS-115 on Bypass Resistance Signals Triggered by Exogenous HGF In Vitro

In the first set of experiments, we examined the effect of crizotinib and TAS-115 on exogenously added HGF-triggered EGFR-TKI resistance in vitro. PC-9 and HCC827 cells are highly sensitive to erlotinib, whereas exogenously added HGF induces resistance to erlotinib in both cell lines. Crizotinib on its own discernibly inhibits the growth of PC-9 cell at high concentrations, consistent with its multikinase activities, and it remarkably sensitizes the cell to erlotinib even in the presence of HGF. TAS-115 does not affect the growth of PC-9 or HCC827 cells at concentrations less than 10 μmol/liter; however, the combined use of TAS-115 with erlotinib reverses HGF-induced resistance in the cell lines in a concentration-dependent manner (Figs. 1*A*, *B* and 2*A*, *B*, and Supplementary Figure 2, Supplementary Digital Content 2, http://links.lww.com/JTO/A571). We previously reported that stromal fibroblasts are a source of exogenous HGF for EGFR-TKI–naive NSCLC and that fibroblast-derived HGF induces resistance to gefitinib and erlotinib in PC-9 and HCC827 cells.[25] Crizotinib and TAS-115 reverse the erlotinib resistance of PC-9 cells induced by coculturing with MRC-5 cells (Supplementary Figure 3A, B, Supplementary Digital Content 3, http://links.lww.com/JTO/A572). These results indicate that both crizotinib and TAS-115 can reverse the EGFR-TKI resistance induced by exogenous HGF in vitro.

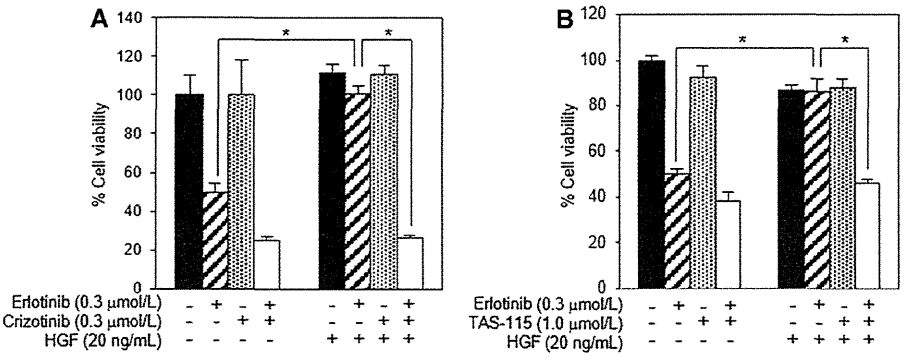

FIGURE 1. Combined use of crizotinib or TAS-115 with erlotinib reverses resistance to EGFR-TKI induced by exogenous HGF. *A* and *B*, PC-9 cells were incubated with or without erlotinib or crizotinib and TAS-115 in the presence or absence of HGF (20 ng/ml) for 72 hours. Cell viability was determined by MTT assay. Bars show SD. The data shown are representative of five independent experiments with similar results. EGFR, epidermal growth factor receptor; EGFR-TKI, EGFR-tyrosine kinase inhibitor; HGF, hepatocyte growth factor; MTT, Thiazolyl Blue Tetrazolium Bromide.

Effect of Crizotinib and TAS-115 on Bypass Resistance Signals Triggered by Endogenous HGF

Previously, we showed that HGF is predominantly present in tumor cells of patients with NSCLC with acquired resistance to EGFR-TKIs and that transient *HGF*-gene transfection into PC-9 cells results in resistance to EGFR-TKIs.[20] We, therefore, generated a stable *HGF*-gene transfectant in PC-9 cells (PC-9/HGF) and assessed the effects against continuously produced endogenous HGF. PC-9/HGF cells secrete high levels of HGF and become resistant to erlotinib, whereas PC-9 or the vector control PC-9/Vec cells do not. Although TAS-115 does not affect the growth of PC-9/HGF cells, crizotinib discernibly inhibits it at high concentrations. The combination of crizotinib or TAS-115 with erlotinib successfully reverses the resistance of PC-9/HGF cells (Fig. 2*A–G*). Using Western blotting, we examined the effects of crizotinib and TAS-115 on signal transduction in PC-9/Vec and PC-9/HGF cells (Fig. 2*H–I*). We found that erlotinib inhibits the phosphorylation of EGFR and ErbB3 in PC-9/Vec cells, thereby inhibiting the phosphorylation of Akt and extracellular signal-regulated kinase 1/2 (ERK1/2). Met phosphorylation is observed in PC-9/HGF cells but not in PC-9/Vec cells. However, erlotinib fails to inhibit phosphorylation of Akt or Erk1/2 in the presence of HGF. Both crizotinib and TAS-115 suppress the constitutive phosphorylation of Met but not EGFR, ErbB3, or downstream Akt and ERK1/2. HGF stimulates the phosphorylation of Met, but the combined use of crizotinib or TAS-115 with erlotinib inhibits the phosphorylation of Met, Akt, and Erk1/2. These results suggest that crizotinib and TAS-115, when combined with erlotinib, reverse HGF-triggered erlotinib resistance by inhibiting the Met/Gab1/PI3K/Akt pathway.

Effect of Crizotinib and TAS-115 on Angiogenesis In Vitro and In Vivo

As we reported previously,[21] exogenous and endogenous HGF stimulated VEGF production in the PC-9 cancer cell line. Both crizotinib and TAS-115 inhibit VEGF production, presumably because of inhibiting Met activation by HGF (Fig. 3*A*, *B*). We also assessed the effect of crizotinib, TAS-115, and bevacizumab on the growth of HMVECs. VEGF promoted HMVEC viability, whereas TAS-115 and bevacizumab, but not crizotinib, inhibit VEGF-stimulated viability of HMVECs in a dose-dependent manner (Fig. 3*C, D*). We also explored the potential of TAS-115 against VEGFR-2. Western blot analysis indicated that VEGFR-2 is phosphorylated by VEGF stimulation in HMVECs, and TAS-115 and bevacizumab show an inhibitory effect (Supplementary Figure 4, Supplementary Digital Content 4, http://links.lww.com/JTO/A573). We next examined the effect on in vivo angiogenesis by using short-term treatment models. Nude mice with established subcutaneous tumors (tumor volume approximately 100 mm^3) were treated with erlotinib with or without crizotinib, bevacizumab, and/or TAS-115, and tumor vascularization was determined on day 4 (Fig. 4*A*, *B*). In PC-9/Vec tumors, treatment with erlotinib alone, TAS-115 alone, or erlotinib with TAS-115 inhibited vascularization. PC-9/HGF tumors have more vascularization than PC-9/Vec tumors. In PC-9/HGF tumors, treatment with bevacizumab, but not erlotinib or crizotinib, inhibited vascularization. We found that TAS-115 inhibited vascularization more potently than bevacizumab. Under these experimental conditions, treatment with erlotinib plus crizotinib inhibited vascularization. Importantly, erlotinib plus TAS-115 more potently inhibited vascularization, compared with erlotinib plus crizotinib, with or without bevacizumab. These results indicate that TAS-115 has a high potential to inhibit angiogenesis in vivo in *EGFR*-mutant tumors that produce high levels of HGF. We also confirmed that treatment with crizotinib or TAS-115 inhibits the phosphorylation of EGFR and Met in vivo (Supplementary Figure 5, Supplementary Digital Content 5, http://links.lww.com/JTO/A574).

Effect of Combined Treatment on Growth of HGF-Overexpressing Tumors In Vivo

Nude mice bearing established subcutaneous tumors (tumor volume approximately 100 mm^3) were treated with erlotinib with or without crizotinib, bevacizumab, and/or TAS-115 for 39 days. The treatment was feasible, and no

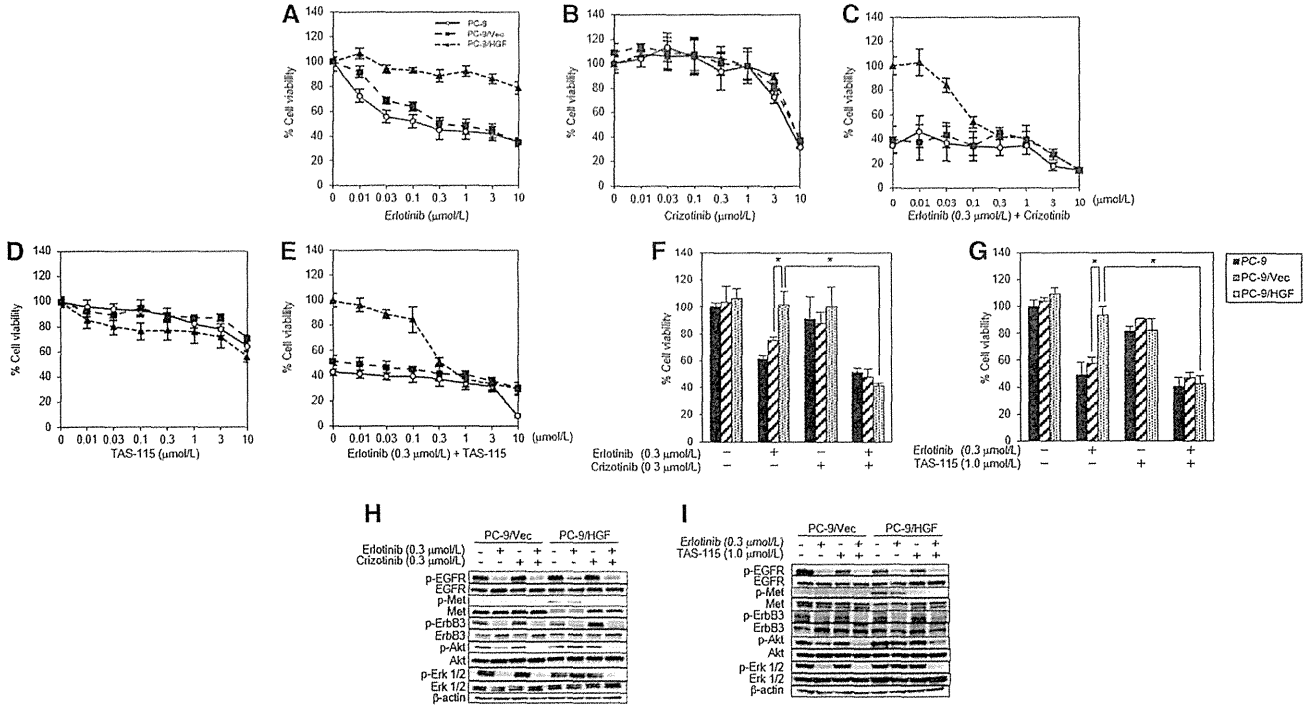

FIGURE 2. Combined use of crizotinib or TAS-115 with erlotinib reverses resistance to EGFR-TKI induced by endogenous HGF. *A*, PC-9/Vec and PC-9/HGF cells were incubated with or without erlotinib for 72 hours. Cell viability was determined by MTT assay. Bars show SD. *B* and *D*, PC-9/Vec and PC-9/HGF cells were treated with crizotinib or TAS-115 for 72 hours. *C–G*, PC-9/Vec and PC-9/HGF cells were incubated with or without erlotinib (0.3 μmol/liter) with or without crizotinib (0.3 μmol/liter) and TAS-115 (1.0 μmol/liter) for 72 hours. The data shown are from three independent experiments with similar results. *H* and *I*, PC-9/HGF cells were incubated with TAS-115 (1.0 μmol/liter) or crizotinib (0.3 μmol/liter) and/or erlotinib (0.3 μmol/liter) for 1 hour. Thereafter, cell lysates were harvested, and phosphorylation of the indicated proteins was determined by Western blot analysis. EGFR, epidermal growth factor receptor; EGFR-TKI, EGFR-tyrosine kinase inhibitor; HGF, hepatocyte growth factor; MTT, thiazolyl blue tetrazolium bromide.

adverse events, including loss of weight, were observed. Tumor volumes on day 39 are shown in Figure 5*A* and *B* (tumor growth curves over time are shown in Supplementary Figure 6, Supplementary Digital Content 6, http://links.lww.com/JTO/A575). Erlotinib markedly inhibited the growth of PC-9/Vec tumors, but TAS-115 inhibited it only modestly (81.7% and 40%, respectively). In PC-9/HGF tumors, erlotinib alone and crizotinib alone inhibited tumor growth only slightly (30% and 31.9%, respectively). Moreover, bevacizumab alone and TAS-115 alone inhibited tumor growth modestly (67% and 76.6%, respectively). Erlotinib plus crizotinib, with or without bevacizumab, inhibited tumor growth markedly (87.1% and 88.3%, respectively). Importantly, erlotinib plus TAS-115 further inhibited tumor growth significantly (93.7%).

Effect of Combined Treatment on Regrowth of HGF-Overexpressing Tumors after Cessation of the Treatment

We further evaluated the effect on regrowth of PC-9/HGF tumors after cessation of drug treatment. After 10 days of cessation, tumors treated with erlotinib plus crizotinib with or without bevacizumab regrew to 4.5 and 3.3 times their initial size at the start of cessation, respectively. Tumors treated with erlotinib plus TAS-115 regrew to only 1.7 times their initial size (Fig. 6*A*). To explore the mechanism of this phenomenon, we again evaluated tumor vascularization on day 49 (10 days after the start of cessation). Consistent with an inhibitory effect against tumor regrowth, vessel density was high (104.6±7.3) and modest (68.6±8.0) in tumors treated with erlotinib plus crizotinib without and with bevacizumab, respectively, whereas vessel density in the tumors treated with erlotinib plus TAS-115 was very low (37.8±3.5; Fig. 6*B*). However, the number of apoptotic cells was low (1.5±0.6), modest (7.3±5.7), and high (22.7±6.4) in the tumors treated with erlotinib plus crizotinib, crizotinib and bevacizumab, and TAS-115, respectively. These results suggest that erlotinib plus TAS-115 prevents tumor regrowth, even after cessation, by means of sustained inhibition of angiogenesis.

DISCUSSION

In the present study, we demonstrated that combined use of erlotinib and TAS-115, a novel angiogenesis inhibitor with Met inhibitory activity, and the use of a triplet of clinically available drugs (such as erlotinib, crizotinib, and bevacizumab) could inhibit the growth of HGF-triggered EGFR-TKI–resistant tumors containing *EGFR* mutations.

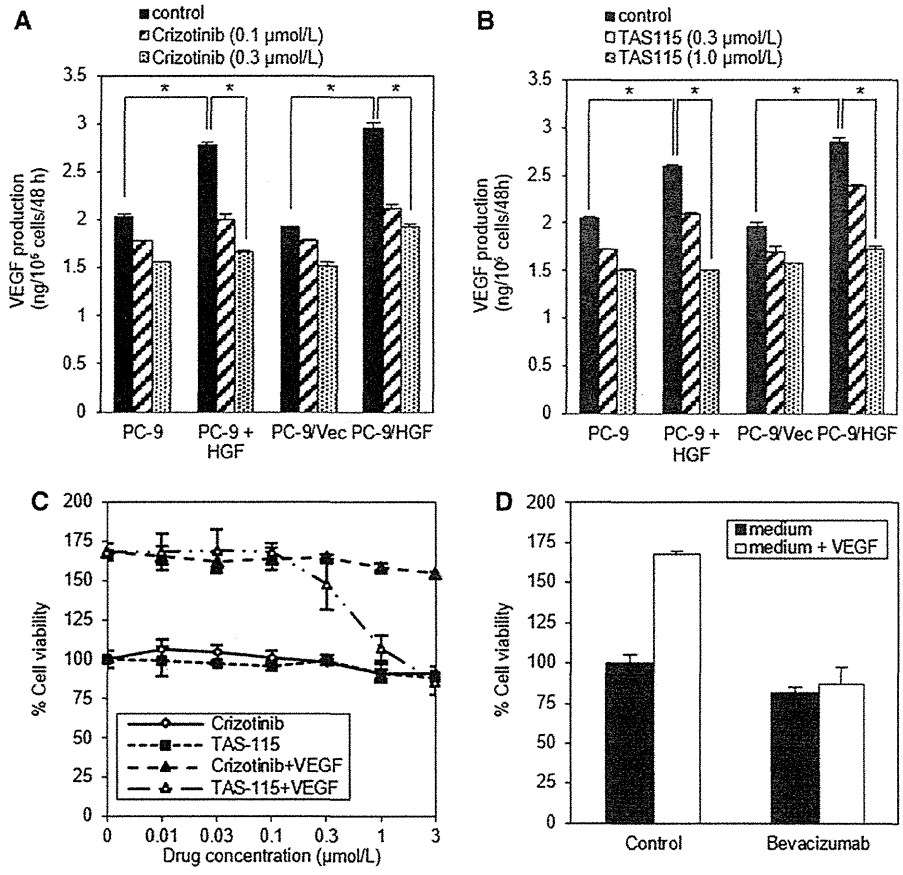

FIGURE 3. Crizotinib and TAS-115 inhibits VEGF production by cancer cells and endothelial proliferation. *A* and *B*, Tumor cells were incubated with or without HGF (50 ng/ml) in the presence of different concentrations of crizotinib or TAS-115 for 48 hours. Thereafter, supernatants were harvested, and the number of tumor cells was counted. VEGF concentration in the supernatants was determined by ELISA. VEGF levels corrected by the tumor cell number are shown. *C* and *D*, HMVECs were incubated in RPMI-1640 medium with 10% FBS (control) or RPMI-1640 medium with 10% FBS in the presence or absence of VEGF (50 ng/ml) with different concentrations of TAS-115, crizotinib, or bevacizumab for 72 hours. Thereafter, cell viability was determined by MTT assay. Bars show SD. The data shown are from three independent experiments with similar results. VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; ELISA, enzyme-linked immunosorbent assay; HMVECs, human dermal microvascular endothelial cells; FBS, fetal bovine serum; MTT, thiazolyl blue tetrazolium bromide.

Moreover, TAS-115 combined with erlotinib remarkably delayed the regrowth of the HGF-triggered EGFR-TKI–resistant tumors.

Because we reported that HGF is a resistance factor to EGFR-TKI in *EGFR*-mutant lung cancer,[9] HGF has been shown to induce resistance to various molecularly targeted drugs in different types of cancers with driver oncogenes. HGF causes resistance to a selective ALK inhibitor[26] and a BRAF inhibitor[27] in lung cancer with *ALK* rearrangement and melanoma with *BRAF* mutation, respectively, by inducing bypass signals that trigger resistance. Moreover, HGF restores angiogenesis associated with Met expression in tumor vascular endothelial cells and thus induces resistance to sunitinib in various types of cancer.[28] These observations indicate that HGF induces resistance to molecularly targeted drugs by multiple mechanisms; therefore, it is an important therapeutic target for circumventing resistance to various molecularly targeted drugs.

HGF and its receptor Met have a close relation with VEGF. Anti-VEGF treatment resulted in a remarkable up-regulation of Met expression in tumors.[29] Hypoxia-stimulated expression of VEGF,[30] Met,[29] and Neuropilin1 (NRP1), a receptor of VEGF, promotes tumor progression.[29,31] Furthermore, it was reported that serum levels of HGF and VEGF were inversely correlated with the clinical response to EGFR-TKIs in lung cancer.[32–34] In addition, a dual inhibitor of VEGFR-2 and Met (XL-184) was shown to have completely suppressed the invasion and metastasis in a pancreatic cancer model in vivo.[29] These studies indicate the rationale for simultaneous inhibition of the HGF-Met and VEGF/VEGFR-2 axes for cancer therapy.

In line with our previous results, we observed that inhibition of both the driver signal (EGFR) and the resistance signal (Met) remarkably suppressed the growth of HGF-triggered EGFR-TKI–resistant tumors in vivo. However, the tumors regrew immediately after the cessation of the dual inhibition, which indicated the presence of cancer cells with proliferating potential that persisted continuously throughout the dual inhibition. Mechanisms of the resistance to dual inhibition should be clarified in the near future.

Additional inhibition of angiogenesis by VEGF neutralization or VEGFR inhibition in addition to dual inhibition (EGFR and Met) could further inhibit growth of HGF-triggered EGFR-TKI–resistant tumor and delay regrowth of the tumors after cessation of the treatment. Bevacizumab in combination with cytotoxic chemotherapy has been shown to prolong progression-free survival in various solid tumors. Our results suggest that the angiogenesis inhibitor in combination with molecularly targeted drugs such as EGFR-TKI and Met-TKI, which directly act on cancer cells, may also delay tumor progression.

It is still controversial whether tumor blood vessels rapidly regrow after cessation of VEGF inhibition. Mancuso et al.[35] reported that tumor vasculature regrew within 7 days of

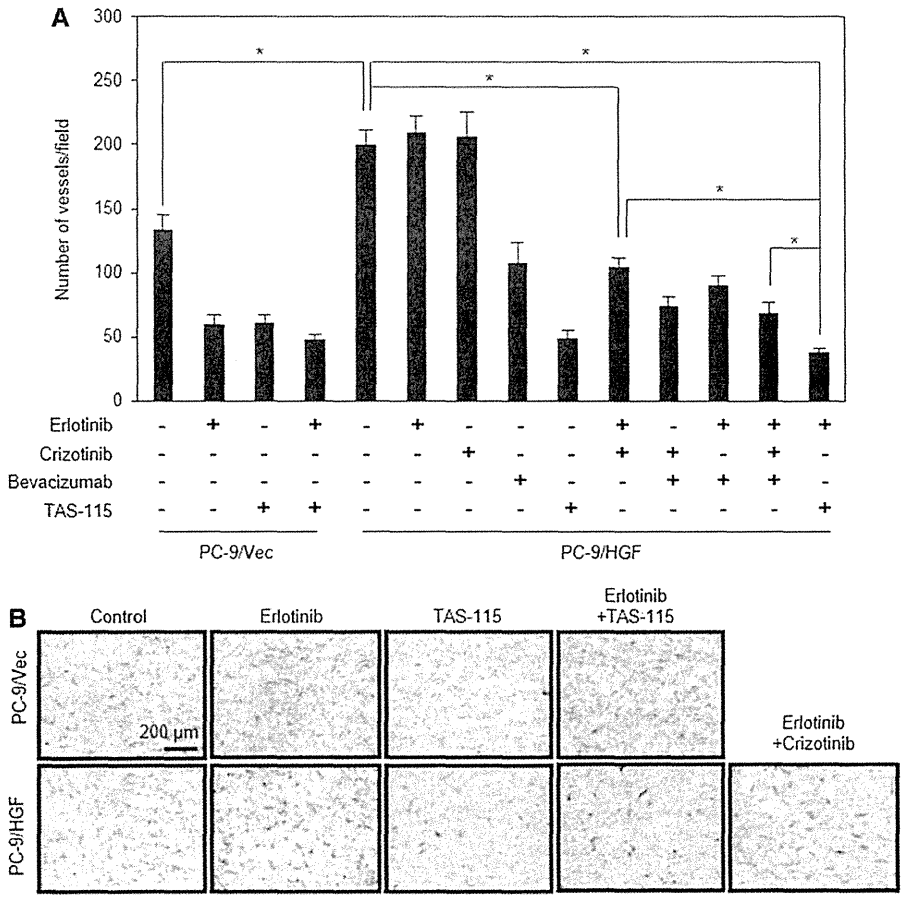

FIGURE 4. Treatment with erlotinib plus TAS-115 inhibits angiogenesis in PC-9/HGF tumors in vivo. Nude mice bearing PC-9/Vec or PC-9/HGF tumors (approximately 100 mm^3 in size) were administered erlotinib and/or crizotinib and/or TAS-115 orally, once daily for 4 days, and/or bevacizumab intraperitoneally only once. *A*, The mice were killed on day 4, and the tumors were harvested. *B*, Numbers of tumor vessels (mean ± SE) determined by CD31 immunohistochemical staining are shown for groups containing five mice each. Representative graphs are shown. The data shown are representative of two independent experiments with similar results. HGF, hepatocyte growth factor.

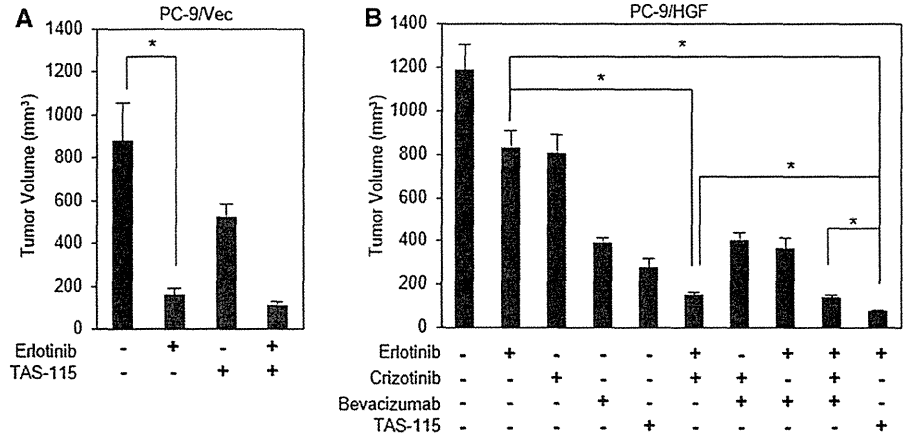

FIGURE 5. Treatment with erlotinib plus TAS-115 inhibits the growth of PC-9/HGF tumors in vivo. *A* and *B*, Nude mice bearing PC-9/Vec or PC-9/HGF tumors (approximately 100 mm^3 in size) were administered erlotinib and/or crizotinib and/or TAS-115 orally once daily and/or bevacizumab intraperitoneally once a week. Tumor volume was measured using calipers. Mean ± SE tumor volumes on day 39 are shown for groups containing five mice each. The data shown are representative of two independent experiments with similar results. HGF, hepatocyte growth factor.

cessation of VEGFR inhibitors (given for 7 days) in the RIP-Tag2 pancreatic cancer model and the Lewis lung carcinoma-xenograft model. Bagri et al.[36] showed that long-term (7 weeks) treatment with an anti-VEGF antibody prevented the regrowth of tumors compared with control or short-term (2 weeks) treatment, but the effect of the long-term treatment on vasculature regrowth after cessation was not well elucidated. In the present study, we demonstrated that regrowth of tumor vasculature was inhibited even after cessation for 10 days of treatment when, before that, continuous treatment (for 39 days) consisted of bevacizumab plus erlotinib and crizotinib or TAS-115 plus erlotinib; and this inhibition was associated with a high number of apoptotic cells in the tumors and delayed tumor regrowth. These effects were more remarkable with TAS-115 plus erlotinib than with the triplet treatment in our experimental conditions. It is unclear why continuous triple inhibition, especially by TAS-115 plus erlotinib, delayed the recovery of tumor angiogenesis. One possible explanation is that continuous treatment with

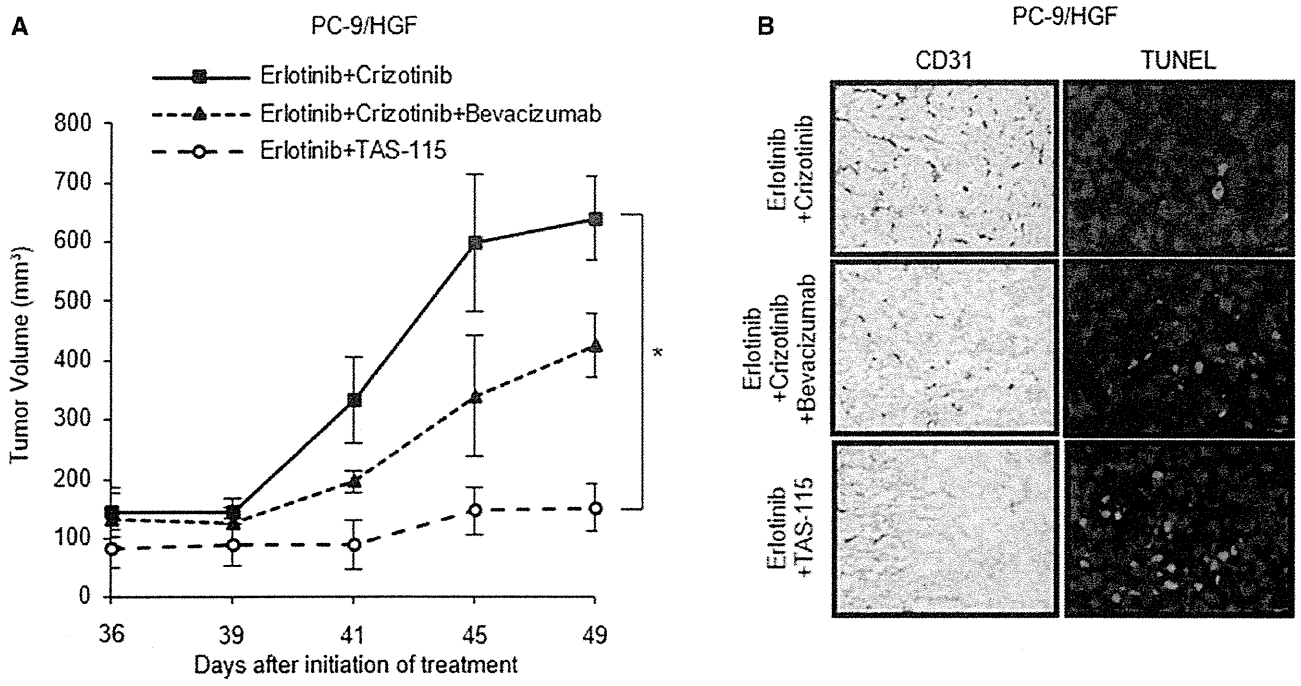

FIGURE 6. Treatment with erlotinib plus TAS-115 most strongly prevented the regrowth of PC-9/HGF tumors even after cessation of treatment. *A*, Nude mice bearing PC-9/HGF tumors were treated as described in Figure 5 until day 39. Thereafter, treatment was terminated, and tumor volume was measured until day 49. *B*, The mice were killed on day 49, and tumors were harvested. Tumor vessels and apoptotic cells were determined by CD31 immunohistochemical and TUNEL staining, respectively. Asterisk indicates $P < 0.01$. HGF, hepatocyte growth factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate-biotin nick end-labeling.

TAS-115 may impair the function of endothelial progenitor cells expressing VEGFR-2. Further studies with longer follow-up and histochemical analysis will be required to determine the exact mechanisms. On the other hand, VEGFR inhibitory activity may be the disadvantage of TAS-115 for specific cases in which EGFR-TKI resistance caused by only MET amplification. Previous study reported that anti-VEGF therapy elicits malignant progression of tumors to increased local invasion and distant metastasis.[37] Therefore, biomarkers for detecting the activities of MET and VEGFRs may be necessary for the optimal use of dual inhibitors for MET and VEGFR.

Inhibition of multiple signaling pathways may cause severe adverse events, especially with continuous admiration of the inhibitors. In our study, 50 mg/kg erlotinib administered daily plus 100 μg/body bevacizumab administered weekly did not show obvious adverse events in nude mice. However, some nude mice treated with daily 50 mg/kg crizotinib plus daily 50 mg/kg erlotinib exhibited severe weight loss and died. Thus, we had to reduce the dose of crizotinib to 25 mg/kg daily when administered along with 50 mg/kg erlotinib. On the other hand, daily administration of 75 mg/kg TAS-115, as expected, inhibited its two targets, Met phosphorylation and angiogenesis, in vivo, and did not show obvious adverse events, including weight loss, even in combination with daily administration of 50 mg/kg erlotinib, suggesting the feasibility of this combined treatment. However, the safety and efficacy of triple inhibition with the triplet of clinically available drugs or with erlotinib plus TAS-115 need to be carefully evaluated in clinical trials.

In conclusion, we demonstrated that triple inhibition of EGFR, Met, and angiogenesis could be achieved by a combination of clinically available drugs (erlotinib, crizotinib, and bevacizumab) or erlotinib and TAS-115 and that the triple inhibition efficiently controlled growth of HGF-triggered, EGFR-TKI–resistant tumors containing *EGFR* mutations. Clinical trials are warranted to evaluate the efficacy and safety of the triple inhibition in *EGFR*-mutant lung cancer patients who acquired EGFR-TKI resistance due to HGF.

ACKNOWLEDGMENTS

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Clinical Predictors of Response to EGFR Tyrosine Kinase Inhibitors in Patients with EGFR-Mutant Non-Small Cell Lung Cancer

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Key Words

Adenocarcinoma · Epidermal growth factor receptor · Erlotinib · Gefitinib · Major mutations · Non-small cell lung cancer

Abstract

Background: The presence of EGFR (epidermal growth factor receptor) mutations is a robust predictor of EGFR tyrosine kinase inhibitor (TKI) responsiveness. Predictors of EGFR-TKI responsiveness in EGFR-mutant non-small cell lung cancer (NSCLC) patients, however, have not been well investigated. The purpose of this study is to examine predictors of EGFR-TKI responsiveness in EGFR-mutant NSCLC patients. ***Patients and Methods:*** Seventy EGFR-mutant NSCLC patients who received EGFR-TKIs in our institution between April 2007 and March 2013 were analyzed retrospectively. ***Results:*** The objective response rate was 50.0% (95% confidence interval, CI, 38.6–61.4%) and the disease control rate was 91.4% (95% CI, 82.5–96.0%). The median progression-free survival (PFS) and overall survival were 9.0 (95% CI, 3.92–14.08) and 20.8 months (95% CI, 14.56–27.04), respectively. In multivariate analysis, adenocarcinoma (hazard ratio, HR, 12.25; 95% CI, 37.7–41.10; $p < 0.001$) and major mutations (deletions in exon 19 and L858R point mutation in exon 21; HR, 2.46; 95% CI, 1.14–5.28; $p = 0.022$) were significant predictors of longer PFS. ***Conclusion:*** Major mutations and adenocarcinoma histology were independent predictors of better treatment outcome in EGFR-mutant NSCLC patients who received EGFR-TKIs. Further well-controlled prospective studies are warranted to confirm our findings.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. The identification of EGFR (epidermal growth factor receptor) mutations in non-small cell lung cancer (NSCLC) patients [2, 3] and their association with remarkable response to EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib [2–11], have led to a paradigm shift in the management of advanced NSCLC.

Initially, East-Asian origin, female sex, adenocarcinoma histology and never-smoking history were identified as clinical predictors for a favorable response to EGFR-TKIs in advanced NSCLC patients [12–15]. Subsequent trials revealed that activating mutations of the EGFR gene were predominantly present in patients with the above-

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mentioned clinical predictors [16–19]. It is also worthy of note that the predictive value of the EGFR mutation status is superior to the other clinical predictors for EGFR-TKI responsiveness [20–22]. EGFR mutation is now regarded as the most robust predictor for clinical response to EGFR-TKIs.

Several recent studies have shown that EGFR-TKI responsiveness differs in cases of 'minor mutations' (mutations other than deletions in exon 19 and L858R point mutation in exon 21) or without adenocarcinoma histology from that in cases of 'major classical mutations' [23–26] or adenocarcinoma histology [27–29]. While some reports have shown that EGFR-TKIs are less effective for these cases, the number of EGFR-mutant patients with such characteristics is innately small. Therefore, a direct comparison between these factors and the other aforementioned well-known predictors (e.g. gender/smoking status) of EGFR-TKI responsiveness has not been reported. Furthermore, the predictors of EGFR-TKI responsiveness in EGFR-mutant patients have not been extensively investigated [6, 30]. Identification of the factors which have predominant roles in determining EGFR-TKI responsiveness in EGFR-mutant NSCLC patients would be beneficial for clinicians, because early identification of patients who will be resistant to EGFR-TKIs would be helpful in the choice of treatment.

In this retrospective study, we investigated clinical predictors associated with treatment outcome in EGFR-mutant NSCLC patients treated with EGFR-TKIs.

Methods

Patients and Clinical Characteristics

A total of 378 consecutive patients diagnosed with advanced NSCLC were screened for EGFR mutations between April 2007 and March 2013 at the Tosei General Hospital (Aichi, Japan). EGFR mutations were detected in 122 patients (32.3%), and 80 met the following inclusion criteria: (1) histologically or cytologically confirmed NSCLC at stage IIIB/IV or relapse, (2) history of treatment with either gefitinib or erlotinib, and (3) measurable disease by computed tomography (CT). Ten were excluded from the study: 3 had a history of prior treatment with EGFR-TKI; 7 discontinued EGFR-TKI treatment before the first response evaluation mainly because of symptomatic deterioration in 3 patients, death due to lung cancer in 2 and intolerable toxicity in 2. The remaining 70 patients were selected and assessed in this study. Baseline clinical characteristics were determined by retrospective chart review, including age, gender, tumor histology, EGFR mutation types, smoking history [nonsmoker (<100 cigarettes in a lifetime) vs. former or current smoker], baseline Eastern Cooperative Oncology Group (ECOG) performance status (PS), stage and treatment line (1st or 2nd line vs. ≥3rd line). Lung cancer histology was defined on the basis of the World Health Organization pathology classification. Clinical staging was decided according to the seventh edition of the tumor node metastasis classification of NSCLC. Survival status was monitored until the end of June 2013. This study was approved by the Institutional Review Board of the Tosei General Hospital.

EGFR-TKI Treatment and Response Evaluation

The initial doses of gefitinib and erlotinib were 250 and 150 mg/day, respectively, until disease progression, intolerable toxicity or patient refusal. Pretreatment evaluation before EGFR-TKIs included CT scan of the chest and/or abdomen, bone scintigram and magnetic resonance imaging of the brain. Treatment response was evaluated by CT scan every 4–8 weeks. In accordance with the Response Evaluation Criteria in Solid Tumors, version 1.1 [31], objective clinical responses to EGFR-TKIs were classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). SD was defined as disease control maintained for at least 6 weeks. All responses were confirmed at least 4 weeks after initial assessment. The response rate (RR) and disease control rate were defined as the best tumor responses of CR + PR and CR + PR + SD ≥6 weeks, respectively.

Analysis of EGFR and Tumor Histology

Tumor specimens were obtained by various methods: transbronchial bronchoscopic lung biopsy, ultrasound or CT-guided needle biopsy, cell blocks of malignant effusion and surgical tissue. Biopsied or surgically resected specimens were fixed with formalin and embedded in paraffin and subjected to an EGFR mutation analysis based on the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method [32]. For this study, deletions in exon 19 and L858R in exon 21 were considered major mutations. Minor mutations were defined as mutations in exons 18 and 20, and unusual mutations occurring in exons 19 and 21. When two or more different mutations were found simultaneously, the mutation types were defined as complex mutations [33]. If one of the contained mutations was a major mutation, they were classified as major mutations according to a previous study that showed gefitinib was as effective for complex mutations containing major mutations as for single major mutations [34]. Paraffin blocks were also used for immunohistochemical staining of cytokeratin 5–6 and thyroid transcription factor 1 after deparaffinization of 3- to 4-μm-thick sections.

Statistical Analyses

We used Pearson χ^2 and Fisher's exact tests to assess the correlations between clinical variables and treatment efficacy, when appropriate. Progression-free survival (PFS) and overall survival (OS) were measured from the date of the initiation of gefitinib or erlotinib until the date of disease progression or death from any cause for PFS and until the date of last follow-up, death or the final follow-up day of the study for OS. The Kaplan-Meier method was applied to estimate PFS and OS, and survival differences between groups were analyzed by the log-rank test. Cox proportional hazard regression models were used to evaluate factors in longer survival. A backward stepwise approach was adopted as our variable selection method for multivariate analyses. All statistical tests were two sided, and values of $p < 0.05$ were considered statistically significant. Statistical analyses were carried out using SPSS version 19.0 (IBM Corporation, Armonk, N.Y., USA).

Table 1. Patient characteristics (n = 70)

Characteristics	Patients	
	n	%
Age, years		
Median	68.1	
Range	43–90	
Gender		
Female	47	67.1
Male	23	32.9
Smoking history		
Former or current smoker	28	40.0
Nonsmoker	42	60.0
ECOG PS		
0	34	48.6
1	23	32.9
2	5	7.1
3	4	5.7
4	4	5.7
Histology		
Adenocarcinoma	66	94.3
Squamous cell carcinoma	3	4.3
Other	1	1.4
Tumor stage		
IIIB	10	14.3
IV	49	70.0
Relapse	11	15.7
Treatment lines		
1st line	12	17.1
2nd line	45	64.3
≥3rd line	13	18.6
EGFR mutation status		
Major mutation	62	88.6
Minor mutation	8	11.4
EGFR-TKIs		
Gefitinib	15	21.4
Erlotinib	55	78.6

Table 2. Univariate analysis of PFS

Characteristics	PFS, months	p value
Age		0.863
Gender		
Female	12.8	0.037
Male	4.0	
Smoking history		
Former or current smoker	6.9	0.080
Nonsmoker	12.8	
ECOG PS		
0–1	11.7	0.112
2–4	8.3	
Histology		
Adenocarcinoma	11.7	<0.001
No adenocarcinoma	2.1	
Tumor stage		
IIIB	7.2	0.553
IV	8.3	
Relapse	17.9	
Treatment lines		
1st or 2nd	11.7	0.038
≥3rd	7.2	
EGFR mutation status		
Major mutation	12.5	0.043
Minor mutation	4.3	
EGFR-TKIs		
Gefitinib	8.7	0.882
Erlotinib	9.7	

Results

Patient Characteristics

Baseline characteristics of the 70 consecutive patients are shown in table 1. Female patients (67.1%), never smokers (60.0%) and patients with PS 0 or 1 (81.5%) were predominant. The most common tumor histology was adenocarcinoma, which was present in 66 patients (94.3%). All patients had advanced or recurrent disease: stage IIIB: 10 patients; IV: 49 patients, and recurrence: 11 patients. Of these patients, 12 received EGFR-TKIs as 1st-line therapy, 45 as 2nd-line therapy, and 13 as ≥3rd-line therapy.

Of the 70 patients harboring EGFR mutation(s), major single mutations were found in 59 patients (deletions in exon 19 in 38 patients and L858R in exon 21 in 21 patients). On the other hand, complex mutations were found in 3 patients (deletions in exon 19 + L858R, G719S + deletions in exon 19 and G719S + L858R in 1 patient each). All 3 of the patients harboring complex EGFR mutations had either of two major mutations (deletions in exon 19 or L858R in exon 21). Therefore, they were classified as major mutations in this study. Finally, 62 patients were regarded as harboring major mutations.

Response to Treatment

Of the 70 patients, 4 had CR, 31 had PR and 29 had SD, yielding an objective RR of 50.0% (95% confidence interval, CI, 38.6–61.4%) and disease control rate of 91.4% (95% CI, 82.5–96.0%).

Analysis of Survival

At the time of analysis, the median follow-up time was 19.7 months (range 1.3–74.0 months). The median PFS was 9.0 months (95% CI, 3.92–14.08 months; fig. 1a), and the median OS was 20.8 months (95% CI, 14.56–27.04 months); 31.4% of the patients were censored at the time

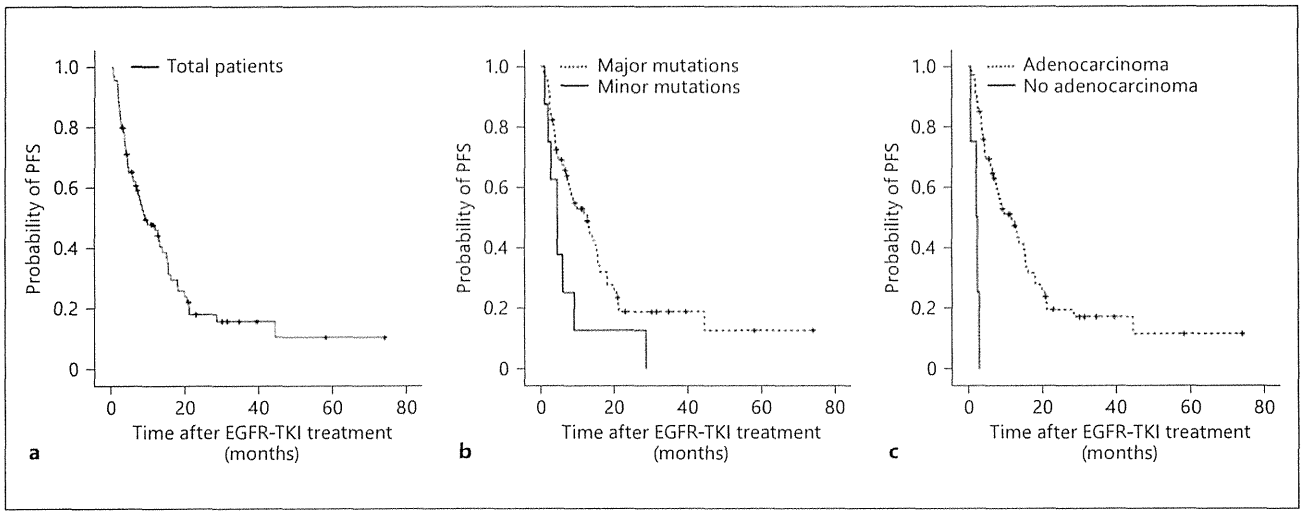

Fig. 1. Kaplan-Meier plot of survival times. **a** PFS in all patients (n = 70): 9 months. **b** PFS of patients classified according to EGFR mutation status: 62 patients with major mutations (12.5 months) and 8 patients with minor mutations (4.3 months; p = 0.043). **c** PFS of patients classified according to tumor histology status: 66 patients with adenocarcinoma (11.7 months) and 4 patients without adenocarcinoma (2.1 months; p < 0.001).

Table 3. Multivariate analysis of PFS

Characteristics	PFS		
	HR	95% CI	p value
Histology, adenocarcinoma	12.25	3.77–41.10	<0.001
EGFR, major mutation	2.46	1.14–5.28	0.022

of data cutoff. Analysis of PFS is shown in table 2. Using univariate analysis, female sex (12.8 vs. 4.0 months, p = 0.037), adenocarcinoma (11.7 vs. 2.1 months, p < 0.001), 1st- or 2nd-line therapy (11.7 vs. 7.2 months, p = 0.038) and major mutations (12.5 vs. 4.3 months, p = 0.043) were suggested to be predictors for longer PFS. There were no significant differences in PFS according to age, smoking history or ECOG PS. In the multivariate analysis using a Cox proportional hazard model, adenocarcinoma (hazard ratio, HR, 12.25; 95% CI, 37.7–41.10; p < 0.001) and major mutations (HR, 2.46; 95% CI, 1.14–5.28; p = 0.022) were significant predictors of longer PFS (table 3). Kaplan-Meier plots stratified according to EGFR mutation patterns or histological patterns are shown in figure 1b, c, respectively. In the log-rank test, the median PFS was significantly longer in patients with major mutations and adenocarcinoma than in those with minor mutations (p = 0.043) and without adenocarcinoma (p < 0.001), respectively. The characteristics of the patients with minor mutations and without adenocarcinoma are shown in tables 4 and 5, respectively.

Discussion

In this report, we retrospectively analyzed the clinical factors associated with PFS in EGFR-mutant NSCLC patients treated with EGFR-TKIs. As mentioned above, we confirmed by means of multivariate analysis that major mutations and adenocarcinoma were independent predictors of longer PFS after EGFR-TKI treatment. This result suggests that minor mutations are independent negative predictors of the efficacy of EGFR-TKI treatment, and that histology is an important additional predictor to consider when applying EGFR-TKI treatment to EGFR-mutant NSCLC patients.

Several previous studies evaluated predictors of EGFR-TKI responsiveness in EGFR-mutant NSCLC patients. For example, good PS and chemotherapy-naïve status [6], presence of deletions in exon 19 compared with L858R [35, 36], and a small number of metastatic sites at baseline [30] are reported predictors of longer survival. However, the number of such studies is still rather small, so that no firm predictors have been established yet.

Previous clinical trials investigating the efficacy of EGFR-TKIs in EGFR-mutant NSCLC patients mainly fo-

Table 4. Characteristics of patients with minor mutations

Age	Gender	Smoking	Histology	PS	Treatment line	EGFR mutation	TKI	PFS, months	Response
76	M	former smoker	Ad	1	1st	G719A	gefitinib	5.9	SD
79	F	nonsmoker	Ad	0	2nd	G719A	erlotinib	4.3	PR
54	M	former smoker	Ad	1	2nd	G719A	erlotinib	2.6	PD
67	M	former smoker	Ad	0	2nd	G719S	erlotinib	1.8	PD
64	M	former smoker	Ad	0	4th	T790M	erlotinib	0.9	PD
63	F	nonsmoker	Ad	0	2nd	L861Q	gefitinib	28.5	PR
79	F	nonsmoker	Ad	0	2nd	L861Q	erlotinib	4.4	PR
84	F	nonsmoker	Ad	3	2nd	L861Q	erlotinib	9.0	SD

M = Male; F = female; Ad = adenocarcinoma; G719A = G719A point mutation in exon 18; G719S = G719S point mutation in exon 18; T790M = T790M point mutation in exon 20; L861Q = L861Q point mutation in exon 21.

Table 5. Characteristics of patients without adenocarcinoma

Age	Gender	Smoking	Histology	PS	Treatment line	EGFR mutation	TKI	PFS, months	Response	Differentiation	CK5–6	TTF-1
75	M	former smoker	Sq	0	3rd	19 del	erlotinib	0.6	PD	well	+	–
72	M	former smoker	Sq	1	2nd	19 del	erlotinib	2.4	SD	well	+	–
54	M	former smoker	Sq	0	3rd	L858R	erlotinib	2.1	SD	well	+	–
61	M	former smoker	NSCLC	1	3rd	19 del	erlotinib	2.8	SD	poor	–	+

CK5–6 = Cytokeratin 5–6; TTF-1 = thyroid transcription factor 1; M = male; Sq = squamous cell carcinoma; NSCLC = NSCLC not otherwise specified; 19 del = deletions in exon 19; L858R = L858R point mutation in exon 21; + = immunopositive; – = immunonegative.

cused on patients harboring two major EGFR mutations (deletions in exon 19 and L858R point mutation in exon 21) [8–11, 18–22], while other minor mutations were excluded from the study cohorts. According to these trials, minor mutations are calculated to account for only about 5–20% of all EGFR mutations. The clinical prevalence and significance of minor mutations have not been completely ascertained. Recently, several studies have described EGFR-TKI responses in patients with minor mutations [24, 25]. From the results it is becoming increasingly clear that not all EGFR mutations confer sensitivity to EGFR-TKIs, and that the clinical associations of minor mutations with EGFR-TKI responsiveness are heterogeneous.

We identified minor mutations in 8 patients (11.4%) in our series, consisting of 4 G719X point mutations in exon 18, 3 L861Q point mutations in exon 21 and 1 T790M insertion in exon 20. The median PFS was 4.3 months, which was significantly worse than that of patients with major mutations. In a recent study of EGFR-mutant NSCLC patients, Wu et al. [24] investigated the clinical features of 'uncommon' EGFR mutations. They demonstrated that patients with G719X or L861Q mutations had better survival than patients with other uncommon mutations. However, compared with major mutations, survival of patients with those mutations was shorter (nonsignificant). On the other hand, T790M mutation before treatment is known as a resistance mutation to EGFR-TKI treatment [37, 38]. In the light of our results and those of recently published studies, EGFR-TKI treatment is less effective in patients with minor mutations than in those with major mutations.

Many large-scale trials have revealed adenocarcinoma histology as one of the independent predictors of outcome in NSCLC patients treated with EGFR-TKIs. However, when limited to EGFR-mutant NSCLC patients, the prognostic value of histology has not been well established. Recent studies have described the incidence of EGFR mutations in patients without adenocarcinoma to be rare, ranging from 0 to 20% [6, 10, 29, 39]. EGFR-TKI seems to be generally less effective in such patients than in EGFR-mutant adenocarcinoma patients. On the basis of a pooled analysis of 15 publications, Shukuya et al. [27] suggested

that 27 EGFR-mutant squamous cell carcinoma patients had diminished EGFR-TKI responsiveness, with a RR and median PFS of 38% and 3.1 months, respectively. Hata et al. [29] found that the incidence of EGFR mutations in patients with squamous cell carcinoma was 13.3% (33 of 249 patients). The RR and median PFS after EGFR-TKI treatment were 25% and 1.4 months, respectively. These results are clearly inferior to pivotal data for EGFR-mutant adenocarcinoma, for which, in general, the RR and median PFS have been reported to be 70–80% and 9–11 months, respectively [6–10]. Cho et al. [39] analyzed clinical outcome in EGFR-mutant NSCLC patients without adenocarcinoma who received EGFR-TKIs and compared the results with those of EGFR-mutant adenocarcinoma patients. The incidence of EGFR mutation was 8.4% (21 of 250 patients). The median PFS was 3.7 months, which was significantly lower than that of adenocarcinoma patients. In the present study, 4 of 70 patients were without adenocarcinoma (5.6%), an incidence which is in agreement with the cited studies. The median PFS in those studies was 2.1 months. This median PFS was significantly worse than that of adenocarcinoma patients. Similar to the results from recently published studies, absence of adenocarcinoma is also a significant negative predictor of EGFR-TKI responsiveness in our study.

It is noteworthy that female sex and absence of a smoking history did not show any significance in predicting longer PFS in multivariate analysis in our study, although they are well-known clinical factors for better PFS after EGFR-TKI treatment. We suppose that this is because our cohort includes only EGFR-mutant NSCLC patients. Previous studies reported the advantage of EGFR mutations in predicting the outcome of gefitinib treatment compared with gender and smoking status [20–22]. Given the superiority of the presence of EGFR mutation in predicting EGFR-TKI responsiveness, it is reasonable that these clinical factors were not retained as independent predictors exclusively in EGFR-mutant NSCLC patients.

Several limitations of this study should be mentioned. First of all, it is a retrospective, nonrandomized study in a single institution. Secondly, this study included only Japanese patients. East-Asian ethnicity is one of the well-known clinical predictors of the efficacy of EGFR-TKIs in NSCLC patients. For that reason, whether or not our results can be applied to other ethnicities should also be investigated. Thirdly, our sample size was small and the associations reported as statistically significant need validation in larger patient cohorts in the future.

In conclusion, major mutations and adenocarcinoma histology were independent predictors of better treatment outcome in EGFR-mutant NSCLC patients who received EGFR-TKIs. While our current findings provide new insights, further well-controlled prospective studies are warranted.

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