Effect of Ephedra Herb on Erlotinib Resistance in C-Met-Overexpressing Non-Small-Cell Lung Cancer Cell Line, H1993, through Promotion of Endocytosis and Degradation of C-Met

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Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) are used to treat non-small-cell lung cancer (NSCLC), harboring an EGFR-activating mutation. However, acquired resistance to these treatments emerges after a few years. One of causes of resistance to EGFR-TKIs is a high level of c-Met amplification or c-Met protein overexpression/hyperactivation. Therefore, combination therapy with EGFR-TKIs and a c-Met inhibitor is thought to be effective treatment for patients with NSCLC resistance carrying c-Met amplification and/or protein hyperactivation. Ephedra Herb is a crude drug and is used in Japan as a component in many Kampo formulae. We previously reported that Ephedra Herb extract (EHE) inhibits HGF-induced phosphorylation of c-Met by preventing c-Met tyrosine kinase activity. Thus, we investigated the combination effect of EHE and erlotinib, an EGFR-TKI, on growth of H1993 cells, an erlotinib-resistant NSCLC cell line with overexpression of c-Met. The EHE and erlotinib combination proved to be effective in suppression of the growth of H1993 xenograft tumors and on inhibition of proliferation of H1993 cells, suggesting that EHE is effective in rescuing NSCLC cells from erlotinib resistance. Moreover, EHE not only inhibited the phosphorylation of c-Met, but also downregulated the expression of c-Met by facilitating clathrin-mediated endocytosis and lysosomal degradation of c-Met. EHE also promoted downregulation of the expression of EGFR and phosphorylation of EGFR. Ephedrine alkaloids-free Ephedra Herb extract (EFE) had the same effects as EHE, and the 40% MeOH fraction from EFE, which mainly contained the high-molecular mass condensed tannins, decreased the expression levels of c-Met, pMet, EGFR, and pEGFR to almost the same level as EFE. These results suggest that recovery from resistance to erlotinib by EHE is derived from the high-molecular mass condensed tannins and that EHE may be suitable for treatment of c-Met-overexpressing NSCLC with resistance to EGFR-TKIs.

1. Introduction

Lung cancer is the leading cause of cancer-related death in many countries around the world, including Japan. Lung cancer is categorized into small cell lung cancer and non-small-cell lung cancer (NSCLC). NSCLC is accountable for approximately 85% of lung cancers. EGFR-TKIs, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, are used to treat NSCLCs harboring EGFR-activating mutations. Although first-generation reversible EGFR-TKIs, including gefitinib and erlotinib, are highly effective in treating NSCLC cells harboring an EGFR mutation within
codon 858 of exon 21 (L858 R mutation), acquired resistance to these treatments emerges after just a few years [1]. The T790 M mutation, situated in the ATP binding pocket of EGFR exon 20, was detected in up 50% of patients treated with the first-generation EGFR-TKIs. Second-generation EGFR-TKIs such as afatinib bind irreversibly to the T790 M mutant EGFR receptor [1]. Recently, third-generation irreversible EGFR-TKIs, such as osimertinib, were developed, to target EGFR T790 M resistance. The third-generation of EGFR-TKIs were designed to covalently bind only to mutated forms of EGFR, thus lowering the incidence of EGFR-TKI-mediated toxicities at wild-type receptors [2]. Nonetheless, some patients have developed resistance to the first- to third-generation EGFR-TKIs, and these patients demonstrate a high level of c-Met amplification or c-Met protein overexpression/hyperactivation [1, 3]. Therefore, combination therapy with EGFR-TKIs and a c-Met inhibitor is required for patients with resistant NSCLC cells carrying c-Met amplification and/or protein hyperactivation.

Ephedra Herb is a crude drug and is used in Japan as a component in many Kampo formulae, including maoto, kakkonto, and shoiseyuto (http://mpdb.nibiohn.go.jp/stork/). Ephedra Herb is defined in the sixteenth edition of the Japanese Pharmacopoeia (JP) as the terrestrial stem of Ephedra sinica Stapf., Ephedra intermedia Schrenk, and C.A. Meyer or Ephedra equisetina Bunge (Ephedraceae), which have stems with total alkaloids (ephedrine and pseudoephedrine) content greater than 0.7% [4]. We previously reported that Ephedra Herb extract (EHE) has c-Met inhibitory activity, the H2O Fr had no c-Met inhibitory activity. EHE inhibits HGF-induced phosphorylation of c-Met by preventing c-Met tyrosine kinase activity [5–7]. Thus, we investigated the effect of combining EHE and an EGFR-TKI such as erlotinib on erlotinib resistance in NSCLC cells. In the present study, we used H1993 cells [8], an erlotinib resistance NSCLC cell line showing overexpression of c-Met.

2. Materials and Methods

2.1. Materials. EHE (Lot. 2091037010) was purchased from Tsumura Co. (Tokyo, Japan). SU11274 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Erlotinib Hydrochloride Salt was purchased from LC Laboratories (Woburn, MA, USA). Recombinant human hepatocyte growth factor (HGF) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). The antibodies (Abs) used were as follows: anti-p-Met (Tyr1234/1235) monoclonal Ab (mAb) (CST#3077), anti-Met mAb (CST#8198), anti-EGFR mAb (CST#4267), anti-p-EGFR (Tyr1068) mAb (CST#3777), anti-Akt mAb (CST#4691), anti-pAkt (Ser473) mAb, anti-extracellular signal related kinases 1 and 2 (ERK1/2) mAb (CST#137F5), anti-p-ERK (Tyr202/Tyr204) mAb (#8544), anti-growth factor receptor-bound protein 2 (GRB2) polyclonal Ab (CST#3972), anti-Grb2-associated-binding protein 1 (Gab1) mAb (CAT#3232), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPD) mAb (CST#2118), and horseradish peroxidase (HRP) labeled anti-rabbit IgG Ab (CST#7074); all were obtained from Cell Signaling Technology Japan, K.K. (Tokyo, Japan).

2.2. Preparation of Ephedrine Alkaloids-Free Ephedra Herb Extract (EFE) and Four Fractions Fractionated from EFE. EFE was prepared as described by Oshima et al. [9]. Ephedra Herb (200 g, E. sinica, Japanese pharmacopoeia grade) was added to water (2000 ml), extracted at 95°C for 1 h, and filtered, after which the residue was washed with water (200 ml). The extract was centrifuged at 1800 g for 10 min, and half of the supernatant was passed through 100 ml of DIAIONTM SK-1B ion-exchange resin (pretreated with 30 ml 1 M HCl/100 ml water and then washed with 100 ml water). The pH of the unadsorbed fraction (1100 ml) was adjusted to pH 5 using 5 % NaHCO3aq. (60 ml), and the solution was then evaporated under reduced pressure to obtain EFE (11.8 g).

The four fractions, H2O Fr, 20% MeOH Fr, 40% MeOH Fr, and MeOH Fr, were prepared as previously described by Yoshimura et al. [10]. EFE was dissolved in H2O and partitioned with EtOAc and n-BuOH to produce the EtOAc, n-BuOH, and H2O extracts. The H2O extract was separated by column chromatography using Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), with aqueous MeOH solvent in stepwise gradient mode to produce four fractions, H2O Fr, 20% MeOH Fr, 40% MeOH Fr, and MeOH Fr. The 20% MeOH Fr, 40% MeOH Fr, and MeOH Fr contained high-molecular mass condensed tannin. While 20% MeOH Fr, 40% MeOH Fr, and MeOH Fr were subsequently demonstrated to show c-Met inhibitory activity, the H2O Fr had no c-Met inhibitory activity. The total recovery rate of the three methanol fractions was approximately 25%, and the recovery rate of H2O Fr was approximately 50%. Thus, the effect of 100 µg/ml EFE was compared with that of 50 µg/ml H2O Fr, 25 µg/ml 20% MeOH Fr, 25 µg/ml 40% MeOH Fr, or 25 µg/ml MeOH Fr.

2.3. Cell Lines and Culture. The human NSCLC cell line H1993 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI 1640 medium (Sigma-Aldrich, Tokyo, Japan) containing 10% fetal calf serum (FCS) (Invitrogen Corp., Carlsbad, CA, USA) at 37°C in an atmosphere containing 5% CO2.

2.4. Animals. Female 6-week-old BALB/c-nu/nu mice were obtained from Charles River Japan (Kanagawa, Japan). All mice were acclimatized for one week prior to the experiments. Animals were kept at constant temperature (25 ± 2°C) and humidity (50 ± 10%) under isolation in a specific pathogen-free (SPF) conditioning room with a 12 h light/12 h dark cycle.

The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of Kitasato University. Experiments were performed in accordance with the Kitasato University guidelines for animal care, handling, and termination, which are in line with international and Japanese guidelines for animal care and welfare.

2.5. Xenograft Model. H1993 cells (2.5 × 106) were inoculated subcutaneously into the left thigh of Balb/c-nu/nu
2.6. Cell Viability. H1993 cells (2 × 10^3 cells/100 µl) were suspended in 100 µl of 10% FCS-RPMI 1640 medium in 96 well plates (AGC Techno Glass Co., Ltd., Shizuoka, Japan). After 24 h, the medium was removed and replaced with 100 µl of 10% FCS-RPMI 1640 medium containing (as appropriate) 2.5, 5, 10 µM erlotinib; 100, 200, 400 µg/ml EHE; a combination of erlotinib and EHE; 1:2, 2:5 µM SU11274; or a combination of erlotinib and SU11274. After 6 days, 10 µl of Cell Counting Kit-8 solution (DOJINDO LABORATORIES, Kumamoto, Japan) was added to each sample, and the resulting mixture was incubated at 37°C for 3 h. Formazan absorbance (450 nm) was quantified using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The 50% inhibitory concentration (IC50) of erlotinib was calculated using GraphPad Prism 7.03 (MDF Limited Company, Tokyo, Japan).

2.7. Western Blotting. H1993 cells (1 × 10^6 cells) were incubated in 4 ml of 10% FCS-RPMI-1640 for two days. The cells were washed twice with 4 ml RPMI-1640 and then incubated for 4 h at 37°C in 4 ml RPMI-1640 or RPMI-1640 containing 200, 400 µg/ml EHE; 5, 10 µM erlotinib; a combination of erlotinib and EHE; 1:2, 2:5 µM SU11274; or a combination of erlotinib and SU11274. After 15 min. The cells were processed for western analysis as described above. The blotswere incubated with antibodies against c-Met, pMet, EGFR, pEGFR, and GAPDH as described above.

2.8. Clathrin-Mediated Endocytosis. H1993 cells (1 × 10^6 cells) were incubated in 4 ml of 10% FCS-RPMI-1640 for two days. After the cells were washed twice with 4 ml RPMI-1640, they were incubated for 5 min at 37°C in 4 ml RPMI-1640 or RPMI-1640 containing 10 and 20 µM Pitstop-2 (Abcam, Cambridge, UK). Next, 50 µg/ml EHE was added to the samples and the cells were incubated for 15 min. The cells were processed for western analysis as described above. The blots were incubated with antibodies against c-Met, pMet, EGFR, pEGFR, and GAPDH as described above.

2.9. Caveolae-Mediated Endocytosis. H1993 cells (1 × 10^6 cells) were incubated in 4 ml of 10% FCS-RPMI-1640 for two days. After the cells were washed twice with 4 ml RPMI-1640, they were incubated for 30 min at 37°C in 4 ml RPMI-1640 or RPMI-1640 containing 5 or 10 mg/ml methyl-β-cyclodextrin (Sigma-Aldrich, Tokyo, Japan) or RPMI-1640 containing 5 or 10 mg/ml methyl-β-cyclodextrin and 50 µg/ml EHE. The cells were processed for western analysis as described above. The blots were incubated with antibodies against c-Met, pMet, EGFR, pEGFR, and GAPDH as described above.

2.10. Lysosomal Degradation. H1993 cells (1 × 10^6 cells) were incubated in 4 ml of 10% FCS-RPMI-1640 for two days. After the cells were washed twice with 4 ml RPMI-1640, they were incubated for 1 h at 37°C in 4 ml RPMI-1640 or RPMI-1640 containing 50 or 100 µg/ml bafilomycin A1 (AdipoGen Life Sciences, San Diego, CA, USA) or RPMI-1640 50 or 100 µg/ml bafilomycin A1 and 50 µg/ml EHE. The cells were
processed for western analysis as described above. The blots were incubated with antibodies against c-Met, pMet, EGFR, pEGFR, and GAPDH as described above.

2.11. HGF-C-Met Downstream Signaling Molecules. H1993 cells (1 × 10⁶ cells) were incubated in 4 ml of 10% FCS-RPMI-1640 for two days. After the cells were washed twice with 4 ml RPMI-1640, they were incubated for 4 h at 37°C in 4 ml RPMI-1640 containing 50 ng/ml hepatocyte growth factor (HGF) (PeproTech, Rocky Hill, NJ, USA) and 50 µg/ml EHE or for 0, 1, 2, 4, and 6 h at 37°C in 4 ml RPMI-1640 containing 50 ng/ml hepatocyte growth factor (HGF) (PeproTech, Rocky Hill, NJ, USA) or in RPMI-1640 containing 50 ng/ml HGF and 50 µg/ml EHE. The cells were processed for western analysis as described above. The blots were incubated with antibodies against c-Met, pMet, Akt, pAkt, ERK, pERK, GRB2, Gab1, and GAPDH as described above.

2.12. Effects of Ephedrine Alkaloids-Free Ephedra Herb Extract (EFE) and Fractions. H1993 cells (1 × 10⁶ cells) were incubated in 4 ml of 10% FCS-RPMI-1640 for two days. After the cells were washed twice with 4 ml RPMI-1640, they were incubated for 4 h at 37°C in 4 ml RPMI-1640 or RPMI-1640 containing 100 µg/ml EFE, 25 µg/ml Water Fr, 25 µg/ml 20% MeOH Fr, 25 µg/ml 40% MeOH Fr, or 25 µg/ml MeOH Fr. The cells were processed for western analysis as described above. The blots were incubated with antibodies against c-Met, pMet, EGFR, pEGFR, and GAPDH as described above.

2.13. Statistical Analysis. All data are expressed as mean ± standard deviation (S.D.). Data were analyzed by 1-way ANOVA and 2-way ANOVA. Significant differences between the control and treatment groups were determined by Dunnett’s test using GraphPad Prism 7.03 (MDF Co., Ltd., Tokyo, Japan). Results of P < 0.05 were considered statistically significant.

3. Results

3.1. Combination Effects of EHE and Erlotinib on Growth of H1993 Xenograft Tumor. First, we examined the combination effect of EHE and erlotinib on growth of the H1993 xenograft tumor in vivo.

In the vehicle group, tumor sizes increased in a time-dependent manner. In mice administered with a combination of 350 mg/kg/day EHE and 100 mg/kg/day erlotinib, tumor sizes were significantly smaller than the tumor sizes of mice administered with vehicle between the 16th and 22nd days. In mice administered with 350 mg/kg/day EHE or 100 mg/kg/day erlotinib, tumor sizes were significantly smaller than the tumor sizes of mice administered with vehicle on the 22nd day (Figure 1(a)).

The weight of mice administered with 100 mg/kg/day erlotinib was significantly decreased, but administration of 350 mg/kg/day EHE had no effect on weight (Figure 1(b)). Thus, EHE had almost no toxicity by oral administration for 22 days. Mice administered with a combination of 350 mg/kg/day EHE and 100 mg/kg/day erlotinib showed a tendency to recover their weight between the 20th and the 22nd days (Figure 1(b)).

These results show that a combination of EHE and erlotinib is more effective in inhibiting the growth of H1993 xenograft tumor (as compared with either EHE or erlotinib alone) and suggest that EHE contributes to a recovery from resistance to erlotinib in vivo.

3.2. Effects of the Combination of EHE and Erlotinib or the Combination of SU11274 and Erlotinib on H1993 Cells In Vitro. The combination effects of EHE and erlotinib were analyzed in detail in vitro. The inhibitory effects of a combination of EHE (100, 200, and 400 µg/ml) and erlotinib (2.5, 5, and 10 µM) on the viability of H1993 cells were investigated. Depending on the EHE and erlotinib concentrations used, the viability of the cells was significantly decreased (Figure 2(a)). Moreover, the 50% inhibitory concentration (IC₅₀) of erlotinib decreased depending on the concentration of EHE (Table 1). These results are consistent with the proposal that EHE contributes to the recovery from resistance to erlotinib.

The expression levels of c-Met, phosphorylated c-Met (pMet), EGFR, and phosphorylated EGFR (pEGFR) in H1993 cells at 4 h after treatment of EHE, erlotinib, or the combination of EHE and erlotinib were analyzed by western blotting. The control lane shows that EGFR and c-Met were both overexpressed and autophosphorylated in H1993 cells (Figure 3(a)). Erlotinib (5 and 10 µM) had no effect on the expression levels of c-Met and pMet, and inhibited the phosphorylation of EGFR in a dose-dependent manner. Remarkably, erlotinib increased the expression level of EGFR (Figure 3(a)). EHE (200 and 400 µg/ml) both inhibited phosphorylation of c-Met and EGFR and decreased the expression levels of c-Met and EGFR. Likewise, EHE and erlotinib combination treatment both inhibited phosphorylation of c-Met and EGFR and decreased the expression levels of c-Met and EGFR (Figure 3(a)). Surprisingly, these results show that EHE decreased the upregulation of EGFR induced by erlotinib.

SU11274, a c-Met inhibitor, was used as a positive control, and its effect on erlotinib resistance in H1993 cells was investigated. The viability of H1993 cells was significantly decreased by SU11274 (1.25 and 2.5 µM) (Figure 2(b)). The combined effect of SU11274 and erlotinib on the cell viability was not observed. SU11274 (1.25 and 2.5 µM) inhibited phosphorylation of c-Met and EGFR in a dose-dependent manner, and increased the expression level of EGFR (Figure 3(b)). Likewise, SU11274 and erlotinib combination treatment inhibited phosphorylation of c-Met and EGFR, and increased the expression levels of EGFR (Figure 3(b)).

These results indicate that the pharmacological action of EHE is different from the action of typical tyrosine kinase inhibitors such as erlotinib and SU11274. Moreover, EHE not only inhibits the phosphorylation of c-Met and EGFR,
Figure 2: The combined effect of EHE and erlotinib on growth of H1993 xenograft tumor in vivo. Mice were inoculated subcutaneously with H1993 cells. After 7 days, an oral drug administration protocol was initiated with vehicle, 100 mg/kg/day of erlotinib, 350 mg/kg/day of EHE, and a combination of 100 mg/kg/day of erlotinib and 350 mg/kg/day of EHE. The tumor size (a) and weight of mouse were measured twice a week (b). Each error bar represents the average (n = 6) ± S.D. Data were analyzed by 2-way ANOVA (A: DF = 5, F(5, 120) = 34.17, and P < 0.0001; B: DF = 5, F(5, 126) = 4.21, and P = 0.0014). Significant differences were determined by Dunnett’s test; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the vehicle group.

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but also induces downregulation of the overexpression of these receptors.

3.3. Overexpression of c-Met and EGFR Was Downregulated by EHE. We investigated the effects of EHE on overexpression of c-Met and EGFR in detail. The expression levels of c-Met, pMet, EGFR, and pEGFR in H1993 cells at 4 h after treatment with various concentrations of EHE (25–200 µg/ml) were analyzed by western blotting. EHE downregulated the expression levels of c-Met, pMet, EGFR, and pEGFR depending on its concentration (Figure 4(a)). The expression levels of c-Met and pMet were almost completely downregulated by 100 and 200 µg/ml EHE, while the expression levels of EGFR and pEGFR were strongly downregulated by 200 µg/ml EHE, indicating that EHE acts on c-Met at a lower concentration than on EGFR.

The expression levels of c-Met, pMet, EGFR, and pEGFR in H1993 cells from 0 to 24 h after treatment with 100 µg/ml EHE were followed by western blotting. The expression levels of these receptors decreased in a time-dependent manner from 0.25 h to 8 h after treatment with 100 µg/ml EHE, though a tendency for expression levels to recover by 24 h was observed (Figure 4(b)). The downregulation activity of EHE appeared at only 15 min after addition of EHE to H1993 cells, suggesting that EHE does not affect gene expression of c-Met and EGFR. The effect of EHE was reversible.

3.4. Promotion of Clathrin-Mediated Endocytosis of c-Met by EHE. EHE may promote endocytosis of c-Met and EGFR in H1993 cells. To test this proposal, we examined whether inhibitors of endocytosis suppress the EHE-promoted downregulation of c-Met and EGFR. The effect of Pitstop-2, an inhibitor of clathrin-mediated endocytosis, on the EHE-induced downregulation of c-Met was investigated. The expression level of c-Met in H1993 cells treated with 50 µg/ml EHE was increased with Pitstop-2 treatment in a concentration-dependent manner. The expression level of c-Met in cells treated with 50 µg/ml EHE and 20 µg/ml Pitstop-2 was significantly increased when compared with cells treated only with 50 µg/ml EHE (Figures 5(a) and 5(c)). Pitstop-2 had no effects on the expression level of c-Met in untreated H1993 cells. These results show that Pitstop-2 suppressed EHE-promoted downregulation of c-Met and provide evidence that EHE promotes downregulation of c-Met by stimulating clathrin-mediated endocytosis.

Pitstop-2 had no effects on the expression level of pMet in H1993 cells treated with 50 µg/ml EHE (Figures 5(a) and 5(b)). Although the expression level of pMet in untreated H1993 cells was significantly decreased by 20 µg/ml Pitstop-

| Table 1: The 50% inhibitory concentration (IC50) of erlotinib. |
|-------------------------------------------------------------|
| **EHE (µg/mL)** | 0  | 100 | 200 | 400 |
| **IC50 of erlotinib (µM)** | 7.44 | 6.86 | 4.36 | 1.2 |

**Figure 3:** The effect of a combination of erlotinib and EHE (a) or combination of SU11274 and erlotinib (b) on the expression levels of c-Met, pMet, EGFR, and pEGFR. The expression levels of c-Met, pMet, EGFR, and pEGFR in H1993 cells at 4 h after treatment of erlotinib, EHE, SU11274, a combination of EHE and erlotinib, or a combination of SU11274 and erlotinib were analyzed by western blotting.
2, it is possible that this was due to a nonspecific action of Pitstop-2. Pitstop-2 had no effect on the expression levels of EGFR and pEGFR in H1993 cells treated with EHE (Supplemental Figure 2).

The effect of methyl-β-cyclodextrin, an inhibitor of caveolae-mediated endocytosis [11], on the EHE-induced downregulation of c-Met and EGFR, was investigated. Methyl-β-cyclodextrin had no effect on the expression levels of c-Met, pMet, EGFR, and pEGFR in H1993 cells treated with EHE (Figure 6 and Supplemental Figure 3). These results suggest that the EHE had no effect on the caveolae-mediated endocytosis.

3.5. Promotion of Lysosomal Degradation of c-Met by EHE. EHE may promote lysosomal degradation of c-Met and EGFR in H1993 cells. To test this proposal, we examined whether bafilomycin A1, an inhibitor of protein degradation in lysosome [12], suppresses the EHE-promoted downregulation of c-Met and EGFR.

The expression level of c-Met in H1993 cells treated with 50 µg/ml EHE was increased by bafilomycin A1 in a concentration-dependent manner. The expression level of c-Met in cells treated with both 100 µg/ml bafilomycin A1 and 50 µg/ml EHE was significantly increased compared to cells treated only with 50 µg/ml EHE (Figures 8(a) and 8(c)). Bafilomycin A1 had no effects on the expression level of c-Met in untreated H1993 cells. These results suggest that EHE promotes downregulation of c-Met by stimulating lysosomal degradation. In contrast, bafilomycin A1 had no effects on the expression level of pMet in H1993 cells treated with 50 µg/ml EHE or in untreated H1993 cells (Figures 7(a) and 7(b)). Moreover, bafilomycin A1 had no effect on the expression levels of EGFR and pEGFR in H1993 cells treated with EHE or in untreated H1993 cells (Supplemental Figure 4).

3.6. Effects of EHE on HGF-c-Met Downstream Signaling Molecules. Next, we investigated the effects of EHE on HGF-c-Met downstream signaling molecules (Akt, ERK, GRB2, and Gab1) in H1993 cells. First, the effect of EHE on the expression of pMet and c-Met in the presence of 50 ng/ml HGF was examined. EHE decreased the expression levels of pMet and c-Met in a concentration-dependent manner (Figure 8(a)). The expression levels of Akt, ERK, GRB2, and Gab1, from 0 to 6 h after treatment with 50 µg/ml EHE and 50 ng/ml HGF, were analyzed by western blotting. The expression levels of c-Met, pMet, and Gab1 in H1993 cells treated with 50 ng/ml HGF and 50 µg/ml EHE were decreased in a time-dependent manner, while the expression levels of c-Met, pMet, and Gab1 in H1993 cells treated only with 50 ng/ml HGF (control) were essentially unchanged (Figure 8(b)). While Akt was phosphorylated after 1 h to 6 h treatment with 50 ng/ml HGF (control), the phosphorylation level of Akt decreased in a time-dependent manner in cells treated with 50 µg/ml EHE and 50 ng/ml HGF. ERK was phosphorylated after 1 h to 2 h treatment with 50 ng/ml HGF (control), though the phosphorylation level subsequently decreased. In contrast, the phosphorylation levels of ERK in cells after 1 h to 2 h treatment with 50 µg/ml EHE and 50 ng/ml HGF were lower than those of control (Figure 8(b)). These results suggest that EHE suppressed the HGF-c-Met downstream signal via downregulation of c-Met, pMet, and Gab1.

3.7. Effects of EFE and EFE Fractions on the Expression of c-Met, pMet, EGFR, and pEGFR. The effects of EFE and the four EFE fractions (Water Fr, 20% MeOH Fr, 40% MeOH Fr, and MeOH Fr) on the expression levels of c-Met, pMet, EGFR, and pEGFR were examined. The expression levels of these receptors at 4 h after treatment of EFE or each EFE fraction were analyzed by western blotting. EFE (100 µg/ml) decreased the expression levels of c-Met, pMet, EGFR, and pEGFR, and components other than ephedrine alkaloids in EHE may contribute to these effects (Figure 9). Water Fr (50 µg/ml) had no effect on the expression levels of these receptors. 20% MeOH Fr (25 µg/ml) and MeOH Fr (25 µg/ml) decreased the expression levels of c-Met, pMet, and pEGFR and slightly decreased the expression level of EGFR. In contrast, 40% MeOH Fr (25 µg/ml) decreased the expression levels of c-Met, pMet, EGFR, and pEGFR to almost
4. Discussion

Erlotinib resistance in H1993 cells is thought to be the leading cause of c-Met overexpression (Supplemental Figure 1) [8]. EHE was expected to contribute to recovery from erlotinib resistance in these cells, because it inhibits the phosphorylation of c-Met [5]. We initially evaluated the combination effect of oral administration of EHE and erlotinib on growth of a H1993 xenograft tumor. It was important to investigate the effect of oral administration of EHE, because EHE is a component of Kampo formula, and only an oral route of administration of this traditional medicine is observed in Japan. A combination of EHE and erlotinib proved to be a more effective treatment for suppression of the growth of the H1993 xenograft tumor than either EHE or erlotinib alone (Figure 1(a)). Furthermore, the oral administration of EHE for 22 days showed little toxicity (Figure 1(b)). These results suggest that EHE is effective in promoting recovery from resistance to erlotinib.

Next, we compared the combination effect of EHE and erlotinib on the growth of H1993 cells with the combination effect of a c-Met inhibitor, SU11274, and erlotinib in vitro.
The IC50 of erlotinib was decreased depending on the concentration of EHE (Table 1), suggesting that EHE contributes to the recovery from resistance to erlotinib. SU11274 (1.25–2.5 µM) suppressed the viability of H1993 cells and this c-Met inhibitor did not show a combination effect with erlotinib (Figure 2(b)). Moreover, because the c-Met inhibitory effect of 1.25–2.5 µM SU11274 was weak (Figure 3(b)), the antiproliferative effect of SU11274 was likely to be independent of this activity.

Unexpectedly, EHE not only inhibited the phosphorylation of c-Met, but also downregulated the expression of c-Met. In addition, EHE inhibited the phosphorylation of EGFR and downregulated the expression of EGFR (Figure 3(a)). In contrast, erlotinib induced the
overexpression of EGFR (Figures 3(a) and 3(b)). Generally, growth factor receptors are downregulated after activation by phosphorylation [13]. It is possible that downregulation of EGFR was arrested via inhibition of EGFR phosphorylation by erlotinib and that EGFR accumulation occurred as a consequence. Surprisingly, EHE downregulated the overexpression of EGFR induced by erlotinib (Figure 3(a)). SU11274 did not downregulate the expression of c-Met and EGFR, and it induced overexpression of EGFR (Figure 3(b)). These results indicate that EHE activity is different from that of a typical c-Met tyrosine kinase inhibitor such as SU11274. EHE decreased the expression and phosphorylation levels of c-Met and EGFR in a concentration-dependent manner (Figure 4(a)). Moreover, the expression and phosphorylation levels of c-Met and EGFR were decreased in a time-dependent manner from 0 h to 8 h after treatment of EHE, though the expression and phosphorylation levels showed a tendency to recover after 24 h (Figure 4(b)).
results reveal that EHE promotes downregulation of non-phosphorylated c-Met and EGFR and that these effects are reversible.

Previously, we reported that EHE stimulates HGF-induced MET and p-MET endocytosis by the early/late endocytic pathways [14]. We investigated the effect of EHE on endocytosis of these receptors using inhibitors such as Pitstop2 and methyl-β-cyclodextrin. According to the results, EHE facilitated the clathrin-mediated endocytosis of c-Met (Figures 5 and 6). In line with these results, c-Met was previously reported to be internalized by clathrin-dependent endocytosis [15].

Using a lysosomal enzyme inhibitor, bafilomycin A1, EHE was also shown to promote the lysosomal degradation of c-Met. It is significant that EHE not only facilitates the clathrin-mediated endocytosis of c-Met, but also promotes the lysosomal degradation of c-Met. If EHE only induced the clathrin-mediated endocytosis of c-Met, it may not suppress c-Met signaling as effectively. Joffre et al. reported that c-Met mutants increased endocytosis/recycling activity and decreased levels of degradation, leading to c-Met accumulation in endosomes. The endosome represents a platform for integrating signaling pathways both temporally and spatially [16]. It should be noted that the expression level of pMet was almost unchanged after treatment with Pitstop2 or bafilomycin A1 (Figures 5−7), suggesting that the observed reduction in the expression level of pMet was affected via inhibition of tyrosine phosphorylation by EHE.

The expression level of EGFR was almost unchanged after treatment with these inhibitors (Supplemental Figures 2−4), and it is unclear how EHE promotes the downregulation of EGFR. Because these inhibitors show cytotoxicity, it was necessary to shorten their incubation times with H1993 cells. The expression level of c-Met was downregulated at 15 min after treatment with EHE. However, the expression level of EGFR was unchanged at 15 to 30 min, and EGFR downregulation was only observed after 2 hr of treatment (Figure 4(b)). Therefore, it was difficult to reveal the mechanism of action of EHE on EGFR using these inhibitors. EGFR was previously reported to be
downregulated via clathrin-mediated endocytosis [13, 17]. It is possible that EHE facilitates clathrin-mediated endocytosis of EGFR. In the future, we plan to investigate the effect of EHE on the expression of EGFR using clathrin knockdown cells.

Next, we analyzed the effects of EHE on HGF-c-Met downstream signaling molecules. EHE decreased the expression levels of pMet, c-Met, pAkt, pERK, and Gab1. The expression level of Gab1 was correlated with the expression levels of pMet and c-Met. Binding of HGF leads to dimerization of c-Met, autophosphorylation of tyrosine residues (Y1234 and Y1235) in the kinase domain, and transautophosphorylation of tyrosine residues (Y1349 and Y1356) in the C-terminal region of c-Met. Gab1 binds to activated c-Met on either Y1349 or Y1356 through its specific c-Met binding site or indirectly through the adaptor Grb2. c-Met activation leads to downstream activation of multiple signaling molecules, including Akt and ERK, through the adaptors Gab1 and Grb2 [15]. It is possible that HGF-c-Met signaling was suppressed via downregulation of pMet, c-Met, and Gab1 by EHE. It is also possible that Gab1 bound to activated c-Met is digested along with c-Met in the lysosome.

We previously demonstrated that the c-Met inhibitory activity of EHE is independent of ephedrine alkaloids, which are the principle components of EHE. Indeed, EFE inhibited the HGF-induced phosphorylation of c-Met at the same level as EHE [7]. In the present study, EFE promoted the downregulation of c-Met, pMet, EGFR, and pEGFR. Moreover, the 40% MeOH Fr fractionated from EFE was equally effective at promoting the downregulation of these receptors (Figure 9). Recently, we have shown that the 40% MeOH Fr contained mainly high-molecular mass condensed tannins. These tannins were procyandrin B-type and partly procyandrin A-type, including pyrogallol- and catechol-type flavan-3-ols as extension and terminal units, and the weight-average molecular weight of the fraction was >45,000 [10].

We hypothesize that the high-molecular mass condensed tannins play an important role in the activity of EHE and EFE. The high-molecular mass condensed tannins may bind to the extracellular domains of c-Met and EGFR, and these receptors may be cross-linked by the tannins and thus downregulated (Figure 10).

Sym004 is a novel synergistic anti-EGFR Ab mixture. The two Abs in Sym004 are directed against distinct epitopes on the EGFR extracellular domain III [18], which is the EGF biding site [19]. Sym004 stimulated EGFR internalization and degradation in cancer cells. EGFR internalization is thought to be induced by receptor cross-linking with Sym004 [18]. It is possible that EHE and EFE act on c-Met and EGFR in a similar manner to Sym004. However, EHE and EFE differ from Sym004 in inducing the downregulation of not only EGFR but also c-Met. Furthermore, EHE-induced downregulation of c-Met was faster than that of EGFR. The downregulation of c-Met started at 15 min after treatment with EHE, but the downregulation of EGFR only started at 2 h after treatment with EHE (Figure 4(b)). These results suggest that EHE shows specificity towards c-Met over EGFR. In the future, we plan to compare the binding affinity between EHE and c-Met with the binding affinity between EHE and EGFR using the Biacore surface plasmon resonance detector.

EHE is applicable to the treatment of c-Met-overexpressing NSCLC with resistance to EGFR-TKIs. EFE may
be better suited for clinical treatment than EHE, because ephedrine alkaloids may cause side-effects. Recently, we reported that EFE treatment has none of the adverse side-effects, such as excitation, insomnia, and arrhythmia, observed with ephedrine alkaloids [20], and we revealed the clinical safety of EFE in a double-blind, randomized, crossover comparative study [21]. In the future, we hope to obtain licensing approval for the therapeutic use of EFE. Because the tannins are unstable, isolated high-molecular mass condensed tannins in EFE are unsuitable for the development of drugs. However, different components in EFE may protect the high-molecular mass condensed tannins in this preparation.

Data Availability

(1) Previously reported preparation and characterization of EFE data were used to support this study and are available at DOI: 10.1007/s11418-016-0977-1 and DOI: 10.1007/s11418-016-0979-z. These prior studies are cited at relevant places within the text as [7, 9]. (2) The preparation and characterization of fractions from EFE used to support the findings of this study are available at M. Yoshimura et al., “Quality Evaluation and Characterization of Fractions with Biological Activity from Ephedra Herb Extract and Ephedrine Alkaloids-Free Ephedra Herb Extract,” Chem. Pharm. Bull., 68, 140–149, 2020. The paper is cited at relevant places within the text as [10]. (3) The effect of erlotinib on the viability of erlotinib-resistant H1993 cells used to support the findings of this study is included in Supplemental Figure 1 within the Supplementary information file. (4) Expression of EGFR and pEGFR in H1993 cells after treatment with Pitstop-2 and EHE used to support the findings of this study is included in Supplemental Figure 2 within the Supplementary information file. (5) Expression of EGFR and pEGFR in H1993 cells after treatment with methyl-β-cyclodextrin and EHE used to support the findings of this study is included in Supplemental Figure 3 within the Supplementary information file. (6) Expression of EGFR and pEGFR in H1993 cells after treatment with bafilomycin A1 and EHE used to support the findings of this study is included in Supplemental Figure 4 within the Supplementary information file.

Ethical Approval

The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of Kitasato University. Experiments were performed in accordance with the Kitasato University guidelines for animal care, handling, and termination, which are in line with international and Japanese guidelines for animal care and welfare.

Conflicts of Interest

Oriental Medicine Research Center of Kitasato University received donations from Tsumura Co., Ltd. Sumiko Hyuga, Hiroshi Odaguchi, and Toshihiko Hanawa received research funds from KYUSHIN PHARMACEUTICAL Co., LTD. Sumiko Hyuga, Hiroshi Odaguchi, and Yoshinori Kobayashi received research funds from Tsumura Co., Ltd. Kitasato University, National Institute of Health Sciences, Matsuyama University, Tokiya Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd. are coapplicants for the relevant patent.

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Supplementary Materials

Supplemental Figure 1: the effect of erlotinib on the viability of erlotinib-resistant H1993 cells and erlotinib-sensitive HCC827 cells. Supplemental Figure 2: expression of EGFR and pEGFR in H1993 cells after treatment with Pitstop-2 and EHE. Supplemental Figure 3: expression of EGFR and pEGFR in H1993 cells after treatment with methyl-β-cyclodextrin and EHE. Supplemental Figure 4: expression of EGFR and pEGFR in H1993 cells after treatment with bafilomycin A1 and EHE. (Supplementary Materials)

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