Reversal of c-MET-mediated Resistance to Cytotoxic Anticancer Drugs by a Novel c-MET Inhibitor TAS-115

EIJI KUNII[1], HIROAKI OZASA[2], TETSUYA OGURI[1], KEN MAENO[1], SATOSHI FUKUDA[1], TAKEHIRO UEMURA[1], OSAMU TAKAKUWA[1], HIROTSUGU OHKUBO[1], MASAYA TAKEMURA[1] and AKIO NIIMI[1]

[1]*Department of Respiratory Medicine, Allergy and Clinical Immunology, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya, Japan;*
[2]*Department of Multidisciplinary Cancer Treatment, Graduate School of Medicine Kyoto University, Sakyo-ku, Kyoto, Japan*

Abstract. *Background: The cellular N-methyl-N'-nitroso-guanidine human osteosarcoma transforming gene (c-MET) protein is the receptor tyrosine kinase for hepatocyte growth factor. We recently found that c-MET protein expression and activation were enhanced in the majority of small cell lung cancer cell lines with cytotoxic anticancer drug resistance, and that down-regulation of c-MET reduced resistance to these drugs. Materials and Methods: Expression of c-MET was studied in three non-small cell lung cancer (NSCLC) cell lines, including six resistant cell strains to cytotoxic anticancer drugs. To assess the effect of c-MET activation on drug resistance, we studied drug sensitivity in the presence of a novel c-MET inhibitor TAS-115. Results: c-MET expression and activation are also enhanced in some cytotoxic anticancer drug-resistant NSCLC cell lines, and inhibition of c-MET activation by TAS-115 reduced resistance of these cell lines to anticancer drugs. Conclusion: The mechanism of cellular resistance to anticancer drugs via hepatocyte growth factor/c-MET signal activation is not restricted to small cell lung cancer cell lines, and TAS-115 might be able to reverse the drug resistance of these cancer cells.*

The cellular N-methyl-N'-nitroso-guanidine human osteosarcoma transforming gene (c-MET) protein is the receptor tyrosine kinase for hepatocyte growth factor (HGF) (1). HGF/c-MET signaling recruits a wide spectrum of transducers and adaptors, including phosphatidylinositol 3-kinase (PI3K), Src, Growth factor receptor-bound protein 2 and Shc (2-5). Enhanced HGF/c-MET signaling is detected in various cancer cells, including lung cancer cells, and is associated with poor prognosis for various solid tumor types (6-8). Activation of the HGF/c-MET signal results in the activation of many downstream genes which are associated with biological events such as oncogenesis, cancer metastasis and drug resistance (9).

The prevalence of HGF/c-MET signal pathway activation in human cancer has influenced anticancer drug development. Multiple agents targeting against this pathway are currently under study, and some agents are in phase III trials (10). These agents may be competitors of HGF/c-MET, monoclonal antibodies directed against HGF or c-MET, or small-molecule tyrosine kinase inhibitors (TKIs). Synthetic small-molecule TKIs outnumber the other classes of compounds.

TAS-115, 4-[2-fluoro-4-[[[(2-phenylacetyl)amino]thioxo-methyl]amino]-phenoxy]-7-methoxy-*N*-methyl-6-quino-linecarboxamide, which is a small-molecule TKI, was identified as a novel dual inhibitor of c-MET and vascular endothelial growth factor receptor (VEGFR). In a previous *in vitro* study, TAS-115 inhibited the kinase activity of both VEGFR2 and MET as strongly as other known VEGFR or MET inhibitors. However, the kinase selectivity of TAS-115 was more specific, and TAS-115 induced less damage in various normal cells compared to other VEGFR inhibitors (11). Since favorable tolerability of TAS-115 was also shown in an *in vivo* study, it is expected that TAS-115 will eventually be used as a drug (11).

Recent studies have demonstrated that enhanced HGF/c-MET signaling is a new mechanism of acquired resistance to epidermal growth factor receptor (EGFR) TKI in lung adenocarcinoma harboring EGFR-activating mutations (12). In addition, we recently showed that c-MET protein

Correspondence to: Tetsuya Oguri, Department of Respiratory Medicine, Allergy and Clinical Immunology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. Tel: +81 528538216, Fax: +81 8520849, e-mail: t-oguri@med.nagoya-cu.ac.jp

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expression and activation were enhanced in the majority of small cell lung cancer (SCLC) cell lines resistant to cytotoxic anticancer drugs, and that c-MET inhibition reduced resistance to such drugs (13). Therefore, c-MET activation is considered to be one mechanism by which SCLC cell lines acquire resistance to cytotoxic anticancer drugs.

In the present study, we further investigated whether HGF/c-MET signals are enhanced in anticancer drug-resistant non-SCLC (NSCLC) cell lines, and whether the novel c-MET inhibitor TAS-115 can overcome such resistance of these cells to cytotoxic anticancer drugs.

Materials and Methods

Cell lines and chemicals. We used one human SCLC cell line PC-6 and three NSCLC cell lines NCI-H23, PC-9 and PC-14. The 7-ethyl-10-hydroxycamptothesin (SN-38)-resistant SCLC cells PC-6/SN-38 was derived from PC-6 as described previously (14, 15). PC-6/SN-38 cells were found to be about 600-fold more resistant to SN-38 as compared to the parental cell line. The gemcitabine-resistant NSCLC cells H23/GEM, PC-9/GEM and PC-14/GEM were derived from the human lung adenocarcinoma cell lines NCI-H23, PC-9 and PC-14, respectively. The SN-38-resistant cells H23/SN-38 was derived from NCI-H23. The cisplatin-resistant cells PC-9/CDDP and PC-14/CDDP were derived from PC-9 and PC-14, respectively. Each cell line was maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) in an incubator at 37˚C in an atmosphere that was less than 5% carbon dioxide. TAS-115 was provided by Taiho Pharmaceutical (Tokyo, Japan). SN-38 was purchased from Daiichi-Sankyo (Tokyo, Japan) and gemcitabine was purchased from Eli Lilly Japan (Tokyo, Japan).

Protein extraction and western blotting method. PC-6/SN-38 cells (1×10^6) and PC-9/GEM cells (1×10^6) were plated on tissue culture dishes. After 72 hours, the medium was changed and the cells were treated with DMSO or TAS-115 (0.5, 1, 2, 4 or 8 μM). After a further 2 h, equal amounts of total cell lysates were solubilized in sample buffer [50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 1 mM EDTA, 10% glycerol] supplemented with Complete Mini (Protease Inhibitor Cocktail Tablets) and Phos-STOP (Phosphatase Inhibitor Cocktail Tablets) (both from Roche Diagnostics, Indianapolis, IN, USA). Subsequently, these lysates were electrophoresed on a 7.5% Ready Gel Tris-HCl Gel (Bio-Rad Laboratories, Hercules, CA, USA) and were transferred to Immobilon-P filters (Millipore, Billerica, MA, USA). The filters were first incubated with primary antibodies for 2 h at room temperature against the following proteins: c-MET (monoclonal, mouse, anti-human, #3127; Cell Signaling Technology Inc., Danvers, MA, USA), phosphorylated-MET (p-MET, Tyr1234/1235; monoclonal, rabbit, anti-human, #3129; Cell Signaling Technology Inc.), phosphorylated-Akt (p-Akt, ser473; polyclonal, rabbit, anti-mouse, #9271; Cell Signaling Technology Inc.), phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 (monoclonal, rabbit, anti-human, #4370, Cell Signaling Technology Inc.), poly (ADP-ribose) polymerase (PARP; polyclonal, rabbit, anti-human, #9661; Cell Signaling Technology Inc.) and α-tubulin (monoclonal, mouse, T6199; Sigma-Aldrich, St. Louis, MO, USA) and vinculin (monoclonal, mouse, V9131; Sigma-Aldrich). The filters were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Bioscience, Amersham Place, UK). α-Tubulin and vinculin were used as a loading control.

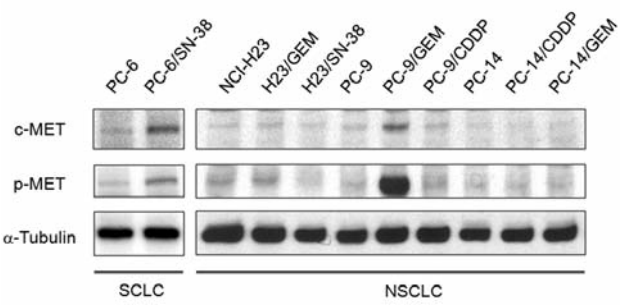

Figure 1. *Increased cellular N-methyl-N'-nitroso-guanidine human osteosarcoma transforming gene (c-MET) protein expression and activation. Western blotting of c-MET protein expression, and of activated, phosphorylated c-MET (p-MET) in drug-resistant and parental small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) cell lines. The drugs to which the cell lines were resistant were: 7-ethyl-10-hydroxycamptothecin (SN-38), gemcitabine (GEM) or cisplatin (CDDP). Tubulin was assayed as a loading control.*

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell viability assay. The MTS cell viability assay was performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as described previously (16). The cells were cultured at a density of 5,000 cells per well in 96-well tissue culture plates for 2 hours. After adding TAS-115, the cultures were incubated at 37˚C for 12, 24, 48 or 72 h. At the end of the culture period, 20 μl of MTS solution was added, the cells were incubated for an additional 4 h, and the absorbance was then measured at 490 nm using an enzyme-linked immunosorbent assay plate reader. Mean values were calculated from three independent experiments performed in quadruplicate.

Trypan blue assay. PC-6/SN-38 cells (1×10^6) were plated on tissue culture dishes. After 24 hours, the medium was changed and dimethyl sulfoxide (DMSO) or TAS-115 (2 μM or 4 μM) was added in combination with SN-38 (12.5 nM). After a further 72 h, the cells were suspended and diluted with 5 ml culture medium. Viable cells were counted using trypan blue staining. PC-9/GEM cells were also plated using the same method. After 24 h, the medium was changed and DMSO or TAS-115 (4 μM or 8 μM) was added in combination with gemcitabine (250 nM). After a further 72 h, viable cells were counted using the same method.

Detection of cleaved PARP. PC-6 cells and PC-6/SN-38 cells (1×10^6) were plated on tissue culture dishes. After 24 h, the medium was changed and DMSO, SN-38 (12.5 nM) alone, TAS-115 (4 μM) alone, or both drugs in combination were added. After an additional 72 h, total protein was extracted from each cell type. The protein expression levels of cleaved PARP were compared by western blotting using the above-described method.

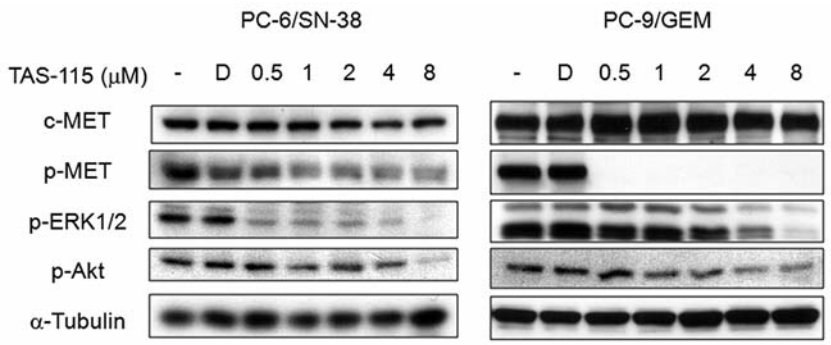

Figure 2. *TAS-115 inhibited downstream signals of the cellular N-methyl-N'-nitroso-guanidine human osteosarcoma transforming gene (c-MET) pathway. Western blotting of downstream signals of the c-MET pathway; phosphorylated-MET, phosphorylated extracellular signal-regulated kinase (ERK) 1/2 and phosphorylated-Akt, following incubation of the drug-resistant cells without (–) and with the indicated concentrations of TAS-115, or with dimethyl sulfoxide (D) as control. Tubulin was used as a loading control.*

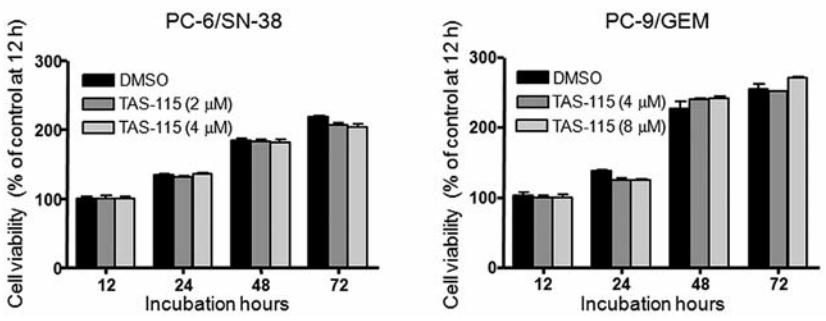

Figure 3. *Treatment with TAS-115 did not inhibit cell growth compared to the untreated control. The effect of incubation for 12, 24, 48 and 72 h with TAS-115 and with dimethyl sulfoxide (DMSO) as the control on growth of the drug-resistant cell lines was analyzed using 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell viability assay. The cell viability is shown by relative value to the control at 12 h. Error bars indicate standard error of the mean. None of the differences were significant.*

Statistical analysis. Differences between samples were evaluated using Student's unpaired *t*-test. The level of significance was set at 5% using two-sided analysis.

Results

c-MET Expression is increased in drug-resistant lung cancer cell lines compared to the parental lines. We evaluated c-MET protein expression levels in several cytotoxic drug-resistant cell lines and their parental cell lines using western blotting (Figure 1). This analysis confirmed that c-MET protein expression level in the cytotoxic drug-resistant SCLC cell line PC-6/SN-38 was increased compared to the parental cell line PC-6, similarly to our previous report (13). We further examined three NSCLC cell lines in this study. A similar result was observed in the gemcitabine-resistant NSCLC cell line PC-9/GEM. Expression levels of the phosphorylated p-MET protein, which reflects c-MET activation, in PC-6/SN-38 cells and PC-9/GEM cells were also significantly increased compared to the parental PC-6 and PC-9 cell lines, respectively.

c-MET Inhibition by TAS-115 reduced resistance to cytotoxic anticancer agents. Inhibition of c-MET by TAS-115 and its effect on cellular resistance to cytotoxic anticancer agents was examined using PC-6/SN-38 cells and PC-9/GEM cells. We first confirmed that TAS-115 inhibited c-MET signaling in these cells. As expected, western blot analysis showed that TAS-115 dose-dependently inhibited c-MET phosphorylation, and phosphorylation of the c-MET downstream signals p-ERK1/2 and p-Akt in PC-6/SN-38 cells (Figure 2). A similar result was observed in PC-9/GEM cells. These results show that TAS-115 strongly inhibits c-MET and its downstream signals.

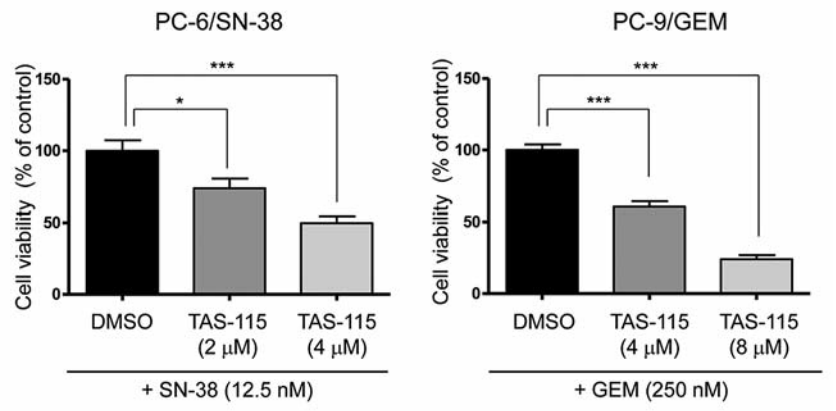

Figure 4. *The cellular N-methyl-N'-nitroso-guanidine human osteosarcoma transforming gene (c-MET) pathway inhibition reduced cellular resistance to cytotoxic anticancer agents. The effect of treatment of PC-6/SN-38 cells with SN-38 (12.5 nM) in the absence (DMSO) and presence of TAS-115 (2 or 4 μM) for 72 h on cell viability was assayed using Trypan Blue staining. PC-9/GEM cells were similarly assayed using 250 nM gemitabine (GEM) instead of SN-38 and 4 or 8 μM TAS-115. Error bars indicate standard error of the mean. *p<0.05 and ***p<0.001.*

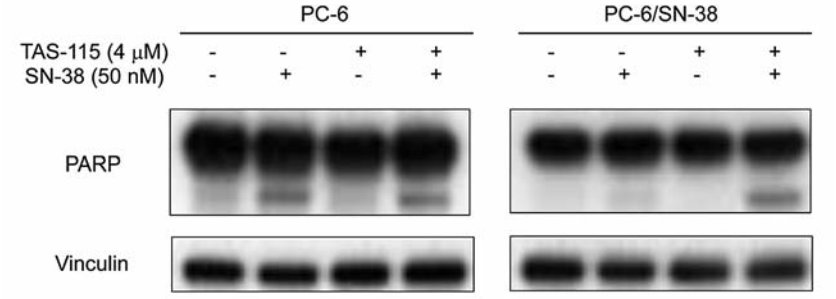

Figure 5. *Treatment with TAS-115 in combination with cytotoxic anticancer agents induces cleavage of PARP. The effect of treatment of PC-6 and PC-6/SN-38 cells with SN-38 with/without TAS-115 on the expression of cleaved poly (ADP-ribose) polymerase (PARP) was examined using western blotting. Vinculin was used as a loading control.*

An MTS cell viability assay was performed to determine whether treatment with TAS-115 at specific concentrations inhibits the cell growth of PC-6/SN-38 and PC-9/GEM cells (Figure 3). Cell growth of PC-6/SN-38 cells was not affected by treatment with DMSO or TAS-115 (2 or 4 μM) for 12, 24, 48 or 72 h. Similar results were observed for PC-9/GEM cells that were treated with DMSO or TAS-115 (4 or 8 μM). These results indicate that TAS-115 treatment alone at these concentrations was not sufficient to inhibit the growth of these cells. However, a trypan blue assay of cell viability showed that treatment with TAS-115 at the same concentrations in combination with SN-38 enhanced the sensitivity of PC-6/SN-38 cells to SN-38 (Figure 4). Therefore, although the concentration of SN-38 to which the cells were exposed was the same, cellular sensitivity to SN-38 was enhanced by TAS-115 in a dose-dependent manner. We obtained similar results for PC-9/GEM cells. These results suggest that inhibition of c-MET reduces the resistance to cytotoxic anticancer drug of drug-resistant cell lines harboring activated c-MET.

Treatment with TAS-115 in combination with cytotoxic anticancer agents induced cleavage of PARP. The protein expression levels of PARP were then examined using western blotting to determine whether treatment with a cytotoxic anticancer agent induces cell death as a result of reduced cellular drug resistance mediated by TAS-115 (Figure 5). Cleaved PARP was not detected in PC-6 or PC-6/SN-38 cells that were not treated with SN-38, regardless of the presence of TAS-115 (4 μM). This means that TAS-115 alone, at a concentration of 4 μM, could not induce cell death in either cell line. After treatment with SN-38, cleaved PARP was detected in PC-6 cells regardless of the presence of TAS-115. In contrast, cleaved PARP was detected in PC-

6/SN-38 cells when treated with SN-38 in combination with TAS-115, but not in PC-6/SN-38 cells treated with SN-38 alone. This result is consistent with the possibility that TAS-115 induces cell death in these cells as a result of TAS-115-mediated reduced cellular drug resistance.

Discussion

In the present study, we showed that HGF/c-MET signaling is also enhanced in some cytotoxic drug-resistant NSCLC cell lines, and that c-MET inhibition by TAS-115 reverses their resistance to cytotoxic drugs. These results indicate that HGF/c-MET signal activation induces acquired resistance against cytotoxic drugs not only in SCLC but also in NSCLC cells, and that TAS-115 might be able to overcome such cellular resistance.

The *c-MET* oncogene was isolated from a human osteogenic sarcoma cell line that had been chemically mutagenized *in vitro*. Isolation of the full-length *c-MET* gene coding sequence revealed structural features of a membrane spanning receptor TK. In normal tissues, the *c-MET* gene is up-regulated after kidney, liver, or heart injury, suggestive of a general homeostatic mechanism of protection of tissue repair and regeneration. In contrast, it is also known that c-MET signaling is enhanced in a wide variety of human malignancies (5). This enhanced c-MET signaling activates a program of cell dissociation and motility that is closely associated with tumor metastasis (17). c-MET has become of increasing interest in recent years for its universal involvement in tumor cell survival, growth, metastasis and multi-treatment resistance (9). In addition, the c-MET pathway in vascular cells stimulates tumor angiogenesis, facilitating tumor growth for cancer that is growth-limited by hypoxia, and promoting tumor metastasis (18). Indeed, a prognostic significance of c-MET expression has been reported in various cancer types including SCLC and NSCLC (8, 19). Therefore, the c-MET signaling pathway is considered an attractive therapeutic target, and a number of c-MET targeted inhibitors have been developed (10).

TAS-115 was identified as a novel dual inhibitor of c-MET and VEGFR2. In a previous *in vitro* study, the inhibitory activity of TAS-115 against c-MET was shown to be approximately equal to that of crizotinib and its selectivity was shown to be higher than that of sorafenib (11). Indeed, in the present study TAS-115 markedly inhibited c-MET phosphorylation in PC-6/SN-38 and PC-9/GEM cells at concentrations higher than 1 or 0.5 μM. The inhibitory activity of TAS-115 thus seems to be stronger than that of SU11274, which we showed in our previous study inhibited c-MET phosphorylation in PC-6/SN-38 cells at a concentration greater than 2 μM (13). Furthermore, TAS-115 was shown to be well tolerated *in vivo* because of its marked kinase selectivity (11). c-MET inhibitor monotherapy was shown to be useful in a clinical study, but its efficacy is not sufficient (20).

The results of the present study suggest the efficacy of combining c-MET inhibition with conventional cytotoxic anticancer drugs. However, combination chemotherapy sometimes requires dose reduction because of worsened tolerability. The strong kinase inhibitory activity of TAS-115 together with its favorable tolerability may contribute to increasing the possibility of its combination therapy with other conventional anticancer drugs.

TAS-115 is known to inhibit the kinase activity of VEGFR2 in addition to c-MET. VEGF is a key factor involved in tumor vascularization (21). An association between the VEGF expression level and patient prognosis has been shown for many types of carcinomas (22-24). Therefore VEGF signaling is also considered to be another attractive molecular target for cancer treatment. However, a previous study showed that VEGF blockage not only inhibits angiogenesis but also causes vascular pruning (25). Such vascular pruning can cause intra-tumoral hypoxia, and it is known that hypoxia can induce c-MET activation (26). Based on this information and our results, it is possible that first-line chemotherapy involving VEGF blockage can cause poor drug sensitivity after second-line chemotherapy *via* c-MET activation. On the other hand, another report suggested that exogenous and endogenous HGF stimulated VEGF production in the PC-9 cancer cell line (27). Additionally, a recent report showed that TAS-115 strongly inhibits vascularization compared to bevacizumab, a monoclonal antibody to VEGF, in combination with erlotinib in a xenograft model bearing *HGF* gene-transfected PC-9 cells (28). For these reasons, the combination of activated c-MET and VEGF may contribute to poor prognosis in cancer chemotherapy. Therefore, dual inhibition of c-MET and VEGF is a logical combination for therapy to overcome drug resistance of cancer cells.

Previous studies of clinical samples of lung cancer, reported that c-MET expression is detected in 47.8% of adenocarcinomas and 41.4% of squamous cell carcinomas, and that these expression levels are related to prognosis (8). In SCLC, it was reported that c-MET expression is detectable in 66% of the tumors, based on tumor tissue microarray analysis (7). It was recently demonstrated that enhanced HGF/c-MET signaling is a novel mechanism of acquired resistance to EGFR-TKI in lung adenocarcinoma harboring EGFR-activating mutations (12). Therefore HGF/c-MET signaling has also become an attractive therapeutic target in lung cancer. In addition, we reported that enhanced HGF/c-MET pathway signaling is involved in the resistance of several cytotoxic drug-resistant SCLC cell lines, and that c-MET inhibition by SU11274 reduces the resistance of these cells to anticancer drugs (13). In the present study, we obtained similar results using TAS-115, not only for cytotoxic anticancer drug-resistant SCLC cell lines but also for some drug-resistant NSCLC cell lines, such as PC-9/GEM. These

results suggest that resistance to cytotoxic anticancer drugs *via* c-MET/HGF signal activation is not restricted to SCLC cell lines, but may be a mechanism of acquired resistance in a wide range of cancer types. Thus, the combination of c-MET inhibitor and conventional cytotoxic anticancer drugs might become a new therapeutic strategy.

A limitation of our study is that the detailed mechanism linking the activation of HGF/c-MET signaling pathway and resistance to cytotoxic anticancer drugs remains to be verified. It was not possible to carry-out such studies due to a contractual restriction of this study. Additional studies are needed to clarify the molecular mechanism through which c-MET/HGF signaling induces acquired resistance to cytotoxic anticancer drugs.

In conclusion, we confirmed that activated c-MET is one of the mechanisms by which cells acquire resistance to cytotoxic anticancer drugs. TAS-115 is an attractive candidate drug for c-MET inhibition that has the potential to overcome cytotoxic anticancer drug resistance.

Conflicts of Interest

This study was funded by a grant from Taiho Pharmaceutical, 1-27 Kandanishiki-cho, Chiyoda-ku, Tokyo 101-8444, Japan.

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