

# Significance of c-MET overexpression in cytotoxic anticancer drug-resistant small-cell lung cancer cells

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## Key words

c-MET inhibitor, c-MET overexpression, drug resistance, gene amplification, lung cancer

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The c-MET receptor tyrosine kinase is the receptor for hepatocyte growth factor. Recently, activation of the c-MET/hepatocyte growth factor signaling pathway was associated with poor prognosis in various solid tumors and was one of the mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib. But the link between c-MET activation and the cytotoxic anticancer drug has not been fully examined. Here, we found that the enhanced expression and activation of c-MET in cytotoxic anticancer agent-resistant small-cell lung cancer cells. Downregulation of c-MET expression by siRNA against the *c-MET* gene or inhibition of c-MET activation by SU11274, a c-MET inhibitor, in the resistant cells altered resistance to the cytotoxic anticancer agent. These results indicated that c-MET overexpression might play an important role in acquired resistance to cytotoxic anticancer drugs. Furthermore, the number of *c-MET* gene loci was increased in the resistant cells compared to the parental cells. In conclusion, increased c-Met expression through an increase in the number of *c-MET* gene loci is one of the mechanisms of acquired resistance to cytotoxic anticancer drugs. Our results add a new strategy, the targeting of c-MET, for overcoming resistance to cytotoxic agents in small-cell lung cancer.

Approximately 12% of all lung cancer patients have SCLC, of whom 75% are staged as having ED at the time of diagnosis.<sup>(1,2)</sup> Several therapeutic agents have been tested during the last three decades in ED-SCLC. With the first-line chemotherapy, usually etoposide or irinotecan plus platinum compound, response rates are 70–80% and median survival time is 9–12 months.<sup>(3,4)</sup> Topotecan or amrubicin are active agents for the treatment of refractory and relapsed SCLC.<sup>(5,6)</sup> But the prognosis of ED-SCLC patients is very poor and less than 5% of them remain alive at 2 years.<sup>(2)</sup> The problem is the rapid development of drug resistance, which results in the failure of the first- or second-line therapy. Therefore, new therapeutic strategies to overcome the drug resistance are urgently needed.

The c-MET receptor tyrosine kinase is the receptor for HGF.<sup>(7)</sup> The extracellular sema domain of c-MET mediates binding to HGF, resulting in stimulating phosphorylation of Y1234/1235 and activating c-MET's autocatalytic domain.<sup>(8)</sup> Activation of c-MET carboxy-terminal binding domain (Y1349/1356) might stimulate many downstream genes in the c-MET pathways, including members of the matrix metalloproteinase family, plasminogen activator, and integrins, all associated with an invasive growth phenotype.<sup>(9–11)</sup> Missense germline mutations in the tyrosine kinase domain lead to constitutive activation of the c-MET protein in hereditary papillary renal carcinomas.<sup>(12)</sup> Activation of the HGF/c-MET signaling pathway was associated with poor prognosis in various solid tumors.<sup>(13–16)</sup> Therefore, agents targeting HGF/c-MET signaling have been developed, which inhibit down-

stream signaling and biological events typical to c-MET activity, such as oncogenesis, cancer metastasis, and drug resistance.<sup>(17)</sup>

Recently, activation of HGF/c-MET signaling was reported to be a new mechanism of acquired resistance to gefitinib (EGFR-TKI). Amplification of the *c-MET* gene leads to gefitinib resistance by transactivation of ERBB3.<sup>(18)</sup> Hepatocyte growth factor-mediated c-MET activation was also a novel mechanism of gefitinib resistance in lung adenocarcinoma with EGFR-activating mutations.<sup>(19)</sup> However, it was not fully clarified whether there were fundamental linkages between HGF/c-MET signaling activation and resistance to the cytotoxic anticancer drugs. c-MET receptor activation by scatter factor/HGF protects certain glioblastoma cells from DNA-damaging agents by activating PI3K-dependent and AKT-dependent antiapoptotic pathways.<sup>(20)</sup> In addition, HGF induced cisplatin resistance through c-MET to activate FAK and downregulate apoptosis-inducing factor expression in lung cancer cells.<sup>(21)</sup> However, HGF-secreting cells did not show altered proliferation rates or survival but were strongly sensitized to death triggered by CDDP and TXL in ovarian cancer.<sup>(22)</sup> c-MET overexpression increased the sensitivity to SN-38, compared through upregulation of topo I activities resulting from increased topo I mRNA and protein expression in non-SLCL.<sup>(23)</sup>

We here found that levels of c-MET expression were significantly increased in cytotoxic anticancer drug-resistant lung cancer cells. These data prompted us to determine whether THE HGF/c-MET signaling pathway has an important role in acquired resistance to cytotoxic anticancer agents. Therefore,

we examined the significance of c-MET overexpression in drug-resistant cells.

## Materials and Methods

**Cell lines and chemicals.** We used the SN-38-, TXL-, and CDDP-resistant cell lines PC-6/SN-38, PC-6/TXL, and PC-6/CDDP that were derived from the human SCLC cell line PC-6.<sup>(20,21)</sup> The human SCLC cell lines NCI-H69 and cells from the TXL-resistant human lung SCLC cell lines NCI-H69/TXL were used as described previously.<sup>(24)</sup> PC-6/SN-38 cells were approximately 4500-fold more resistant to SN-38, PC-6/TXL and NCI-H69/TXL cells were approximately 460-fold and 460-fold more resistant to TXL, respectively, and PC-6/CDDP cells were approximately 1800-fold more resistant to CDDP than each parental cell line (Table 1). SU11274 was purchased from Calbiochem (Darmstadt, Germany), SN-38 from Daiichi-Sankyo (Tokyo, Japan), and CDDP and TXL from Bristol Myers (Tokyo, Japan).

**Quantitative real-time PCR.** Total RNA was extracted using an RNeasy mini kit (Qiagen, Chatsworth, CA, USA). Quantitative real-time PCR was carried out with a TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems, Foster City, CA, USA) using the Step One Plus Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The PCR program was carried out as described previously.<sup>(22)</sup> The primer and TaqMan probe sets (TaqMan Gene Expression Assays, Inventoried) for c-MET (Hs00179845\_m1), HGF (Hs0030159\_m1), and GAPDH (Hs99999905\_m1) were purchased from Applied Biosystems (sequences not disclosed).

**Protein extraction and WB analysis.** Protein extraction was carried out as described previously.<sup>(25)</sup> Lysates were electrophoresed on 10% Ready Gel Tris-HCl Gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to Immobilon-P filters (Millipore, Billerica, MA, USA). The filters were first incubated with primary antibodies for 2 h (c-MET, p-MET, p-AKT, p-ERK1/2, and cleaved-PARP) and 1 h ( $\alpha$ -tubulin) at room temperature and then with HRP-conjugated secondary antibodies. The following antibodies were used: c-MET, p-MET (Tyr1234/1235), p-AKT (se473), p-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), cleaved-PARP (BD PharMingen, San Jose, CA, USA),  $\alpha$ -tubulin (Sigma-Aldrich, St. Louis, MO, USA) and HRP-conjugated secondary antibody (GE Healthcare Bioscience, Little Chalfont, UK).  $\alpha$ -Tubulin was used as a loading control. The relative band intensities were determined by densitometry using NIH Image (U. S. National Institutes of Health, Bethesda, MD, USA).

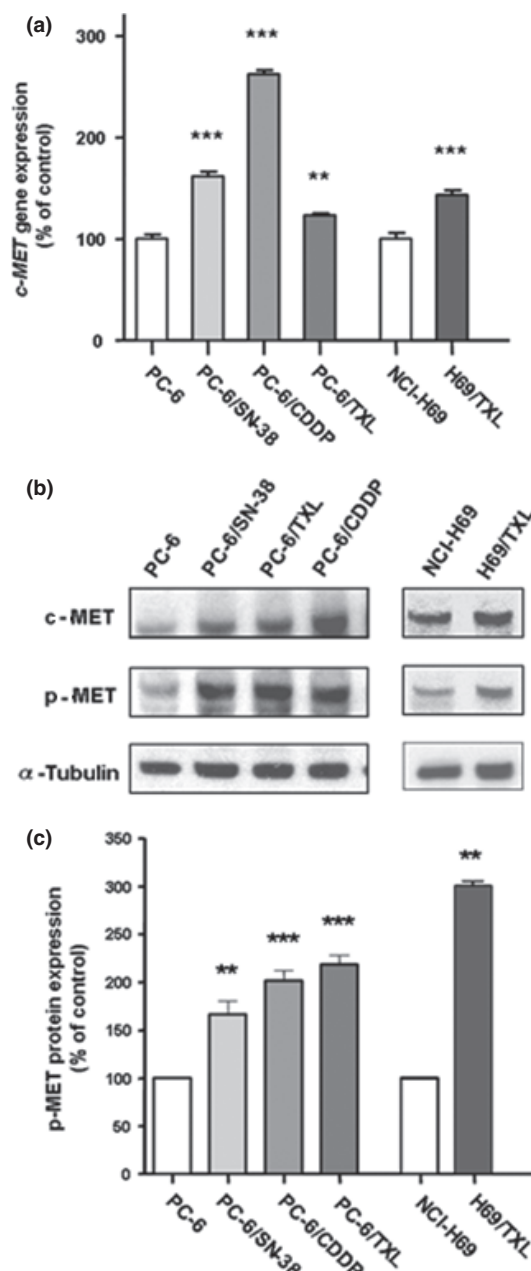
**Cell viability assay.** Cells were cultured at 5000 per well in 96-well tissue culture plates. To assess cell viability, stepwise

**Table 1. Inhibitory concentrations (50%) of 7-ethyl-10-hydroxycamptothecin (SN-38), paclitaxel (TXL), and cisplatin (CDDP) in PC-6 and NCI-H69 small-cell lung cancer cells**

	PC-6	PC-6/SN-38	RR
SN-38	0.98 pM	4.48 nM	4571.42
	PC-6	PC-6/TXL	RR
TXL	23.75 pM	11.05 nM	465.26
	PC-6	PC-6/CDDP	RR
CDDP	8.34 nM	15.02 $\mu$ M	1800.95
	NCI-H69	NCI-H69/TXL	RR
TXL	0.028 pM	13.12 pM	468.57

RR, relative rate.

10-fold dilutions of the anticancer drug were added 2 h after plating, and the cultures were incubated at 37°C for 96 h. At the end of the culture period, 20  $\mu$ L MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added, the cells were incubated for a further 4 h, and the absorbance was measured at 490 nm using an ELISA



**Fig. 1.** Increased c-MET expression and activation in PC-6 human small-cell lung cancer cells resistant to 7-ethyl-10-hydroxycamptothecin (PC-6/SN-38), paclitaxel (PC-6/TXL), and cisplatin (PC-6/CDDP), as well as the NCI-H69 and TXL-resistant NCI-H69 (NCI-H69/TXL) small-cell lung cancer cell lines. (a) c-MET gene expression was examined using quantitative real-time PCR. (b) c-MET and p-MET protein expression was determined by Western blot analysis. (c) Relative band intensities of p-MET protein were determined by densitometry using NIH Imaging. \*\* $P < 0.01$ . \*\*\* $P < 0.001$ .

plate reader. Chemosensitivity is expressed as the drug concentration producing 50% growth inhibition.

**Inhibition of c-MET activation by SU11274.** At 2 h after cells ( $1 \times 10^6$ ) were exposed to DMSO or SU11274 at 0.5 or 2.0  $\mu\text{M}$  concentration, total protein was extracted, or the cells were cultured at 5000 per well in 96-well tissue culture plates for 2 h, and the cultures were incubated at 37°C for 72 h after adding stepwise dilutions of SN-38 or TXL to assess cell viability by the MTS assay.

**Apoptosis.** Cells ( $1 \times 10^6$ ) were cultured for 2 h after exposure to DMSO or SU11274 at 2.0  $\mu\text{M}$  concentration, the cells were incubated at 37°C for 72 h after adding stepwise dilutions of SN-38 or TXL, and total protein was extracted. We examined the levels of cleaved-PARP expression by WB.

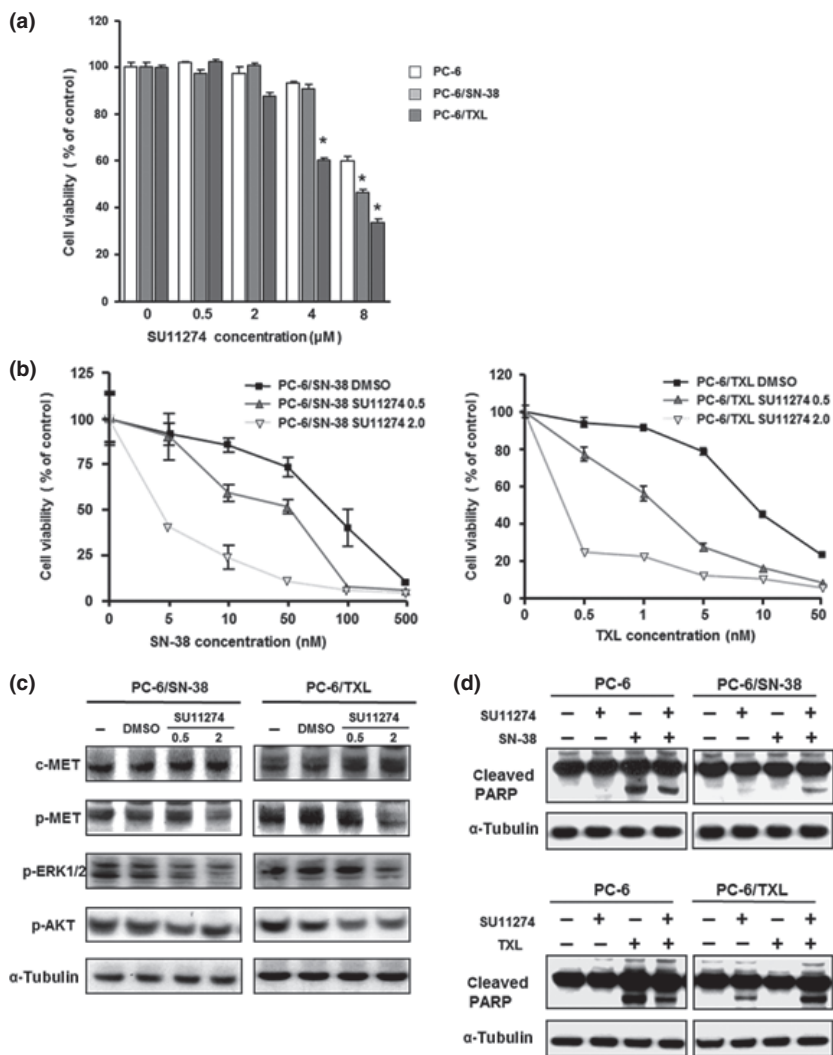
**Transfection and siRNA experiments.** Cells ( $1 \times 10^6$ ) were transfected with siRNA oligonucleotides to result in a final RNA concentration of 30 nM. We used using siPORT NeoFX Transfection Agent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Total RNA or protein at 24 or 48 h after transfection was extracted, or the cells were cultured at 5000 per well in 96-well tissue culture plates for 2 h, and the cultures were incubated at 37°C for 72 h after adding stepwise dilutions of SN-38 or TXL to assess cell viability by MTS assay. The siRNA oligonucleotides for c-MET (silencer

select siRNA, ID s8701) and the negative control siRNA (silencer select siRNA) were purchased from Ambion.

**c-MET copy number assays.** The c-MET gene copy number was analyzed by quantitative real-time PCR, carried out on StepOnePlus (Applied Biosystems) by TaqMan Copy Number Assays (Applied Biosystems), as described previously.<sup>(26–28)</sup> The PCR program was 40 cycles at 95°C for 15 s and 60°C for 1 min. The primer for c-MET (predesigned copy number assays ID, Hs 01432482\_cn) was purchased from Applied Biosystems. We used the ribonuclease P RNA component H1 gene as an endogenous control.

**Fluorescence in situ hybridization.** The c-MET probe was labeled with Cy3 by the nick translation method using the RP11-163C9 BAC clone (Chromosome Science Labo, Sapporo, Japan). We used the chromosome 7 centromere probe (CEP7), manufactured by Chromosome Science Labo, as a control. Cells were collected by centrifugation and trypsinization, fixed with methanol and acetic acid (3:1 solution), and expanded on a slide glass. The probe mixture (c-MET and CEP7) was applied to the fixed cell specimens, which were denatured on a hot plate at 70°C for 5 min and hybridized overnight at 37°C. One hundred cells from each fixed cell specimen were analyzed and the number of c-MET and CEP7 signals were determined.

**Hepatocyte growth factor immunoassay.** A total of  $1 \times 10^6$  cells were seeded on 60-mm dishes. The next day, cells were



**Fig. 2.** Inhibition of c-MET activation by SU11274. Growth inhibition induced by DMSO or SU11274 (0.5, 2.0, 4.0, and 8.0  $\mu\text{M}$ ) in small-cell lung cancer PC-6 ( $\square$ ), 7-ethyl-10-hydroxycamptothecin-resistant (PC-6/SN-38) ( $\blacksquare$ ) and paclitaxel-resistant (PC-6/TXL) ( $\blacksquare$ ) cell lines was examined with the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (a). The growth inhibition of SN-38 or TXL to PC-6/SN-38 or PC-6/TXL was examined with the MTS assay (b), the protein levels of c-MET, p-MET, p-ERK1/2, and p-AKT in PC-6/SN-38 and PC-6/TXL were examined (c), and at 72 h after adding none, SN-38 (5 nM), or TXL (10 nM), the levels of cleaved poly (ADP-ribose) polymerase (cleaved-PARP) were examined by Western blotting (d). \* $P < 0.05$

washed twice with PBS and incubated for 48 h with 4 mL Roswell Park Memorial Institute medium (RPMI) medium. Cell culture supernatants were then collected and cleared by centrifugation. The secretion of HGF was quantified by quantitative ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

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

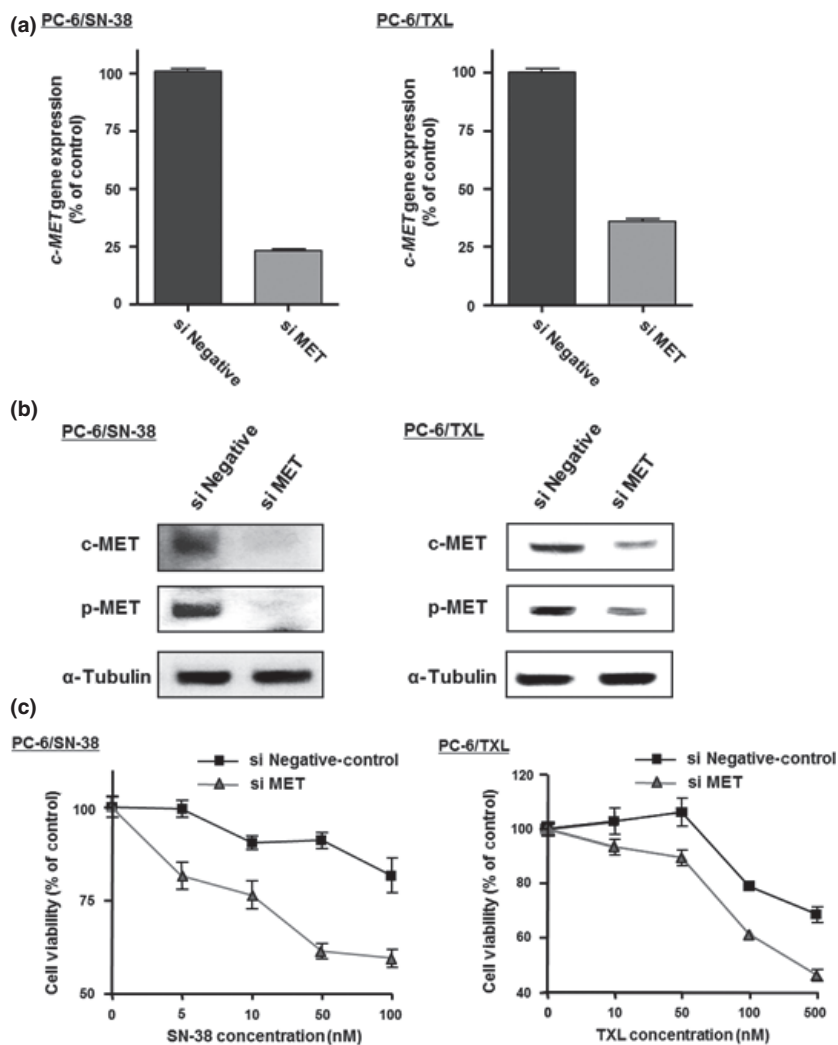
## Results

**Levels of c-MET expression increased in drug-resistant SCLC cells.** The levels of *c-MET* gene expression in PC-6/SN-38, PC-6/CDDP, PC-6/TXL, and NCI-H69/TXL cells were significantly increased relative to the parental cells (Fig. 1a). c-MET protein expression in the drug-resistant cells was also upregulated (Fig. 1b). To investigate activation of c-MET in the drug-resistant cells, we confirmed the levels of p-MET protein. The expression levels of p-MET in these resistant cells were also significantly increased relative to the parental cells (Fig. 1b,c).

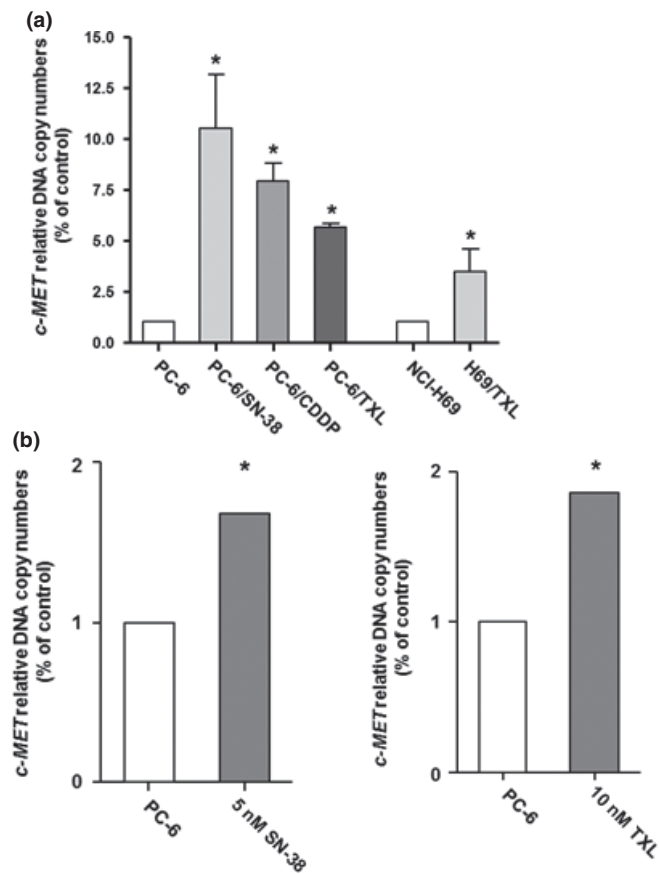
**Inhibition of c-MET activation by c-MET inhibitor (SU11274) improved resistance to cytotoxic anticancer drugs.** We examined the growth inhibition of PC-6, PC-6/SN-38, and PC-6/TXL cells by SU11274, a specific c-MET TKI.<sup>(15,29,30)</sup> SU11274

significantly inhibited the growth of the drug-resistant cells relative to the parental cells (Fig. 2a). Next, we exposed PC-6/SN-38 cells to SN-38 alone, or in combination with DMSO or SU11274 (0.5 or 2  $\mu$ M concentrations). Although PC-6/SN-38 cells were resistant to SN-38 alone or in combination with DMSO, the combined treatment of SN-38 with SU11274 resulted in alteration of cytotoxicity in a dose-dependent manner (Fig. 2b). We obtained the same results in PC-6/TXL cells (Fig. 2b). We then examined downstream in the c-MET pathway. SU11274 inhibited p-MET and p-ERK1/2 in PC-6/SN-38 and PC-6/TXL cells, but not p-AKT at the 2  $\mu$ M concentration (Fig. 2c). We confirmed the protein levels of cleaved-PARP in PC-6, PC-6/SN-38, and PC-6/TXL cells. The levels of cleaved-PARP protein by treatment with SN-38 and SU11274 were increased relative to that by treatment with SN-38 alone in PC-6/SN-38 cells (Fig. 2d). The cleaved-PARP levels in PC-6/SN38 cells with SU11274 alone were higher than PC6 cells (Fig. 2d). We obtained the same results in PC-6/TXL cells (Fig. 2d).

**Downregulation of c-MET expression by siRNA against c-MET gene.** We knocked down c-MET expression by siRNA against the *c-MET* gene, to confirm whether c-MET overexpression activated c-MET signaling resulting in resistance to the cytotoxic anticancer drugs. The levels of *c-MET* gene expression in PC-6/SN-38 or PC-6/TXL cells transfected with siRNA against the *c-MET* gene were significantly decreased by 30%



**Fig. 3.** Knockdown of c-MET expression by siRNA in small-cell lung cancer cells. (a) *c-MET* gene expression was examined using real-time RT-PCR. (b) c-MET and p-MET protein expression was determined using Western blotting. (c) Growth inhibition of stepwise 7-ethyl-10-hydroxycamptothecin (SN-38) or paclitaxel (TXL) concentration in SN-38-resistant (PC-6/SN-38) or TXL-resistant (PC-6/TXL) cells transfected with *c-MET* siRNA (si MET;  $\blacktriangle$ ) or negative-control ( $\blacksquare$ ) was examined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay.



**Fig. 4.** Increased *c-MET* DNA copy numbers in PC-6 human small-cell lung cancer cells resistant to 7-ethyl-10-hydroxycamptothecin (PC-6/SN-38), paclitaxel (PC-6/TXL), and cisplatin (PC-6/CDDP), as well as the NCI-H69 and TXL-resistant NCI-H69 (H69/TXL) small-cell lung cancer cell lines. (a) *c-MET* DNA copy numbers in drug-resistant cells were examined relative to the parental cells by quantitative real-time PCR. (b) *c-MET* DNA copy numbers in PC-6 cells, exposed to 7-ethyl-10-hydroxycamptothecin (SN-38, 5 nM) or paclitaxel (TXL, 10 nM) for 7 days, were examined by quantitative real-time PCR. \* $P < 0.05$ .

or 35% relative to the cells with negative-control (Fig. 3a). The levels of c-MET and p-MET protein in PC-6/SN-38 or PC-6/TXL cells with siRNA against the *c-MET* gene were also downregulated (Fig. 3b). In addition, the growth inhibition of SN-38 in PC-6/SN-38 cells with *c-MET* siRNA was improved relative to the cells with negative-control (Fig. 3c). We also obtained the same result in PC-6/TXL cells with *c-MET* siRNA (Fig. 3c).

***c-MET* gene amplification in cytotoxic anticancer drug-resistant cells.** We examined the *c-MET* DNA copy number in the cytotoxic anticancer drug-resistant cells compared with the parental cells by PCR. The relative *c-MET* DNA copy numbers of the resistant cells was significantly increased compared with the parental cells (Fig. 4a). In addition, we examined the *c-MET* gene amplification in the PC-6 cells at 7 days after exposure to SN-38 (5 nM) or TXL (10 nM). The *c-MET* gene amplification in the PC-6 cells exposed to SN-38 or TXL was significantly increased relative to the cells not exposed (Fig. 4b).

**Chromosomal instability in PC-6/SN-38 cells.** We examined *c-MET* gene amplification or chromosomal instability in PC-6 and PC-6/SN-38 cells by FISH analysis. Diploid cells accounted for 95% (95 cells/100 cells) and tetraploid only 1%

(1 cell/100 cells) of all PC-6 cells (Fig. 5a,c), whereas diploid cells accounted for 35% (35 cells/100 cells), triploid 7% (7 cells/100 cells), and tetraploid 48% (48 cells/100 cells) of all PC-6/SN-38 cells (Fig. 5b,c). In addition, the copy number of the *c-MET* gene increased by one as compared with CEP7 in 6% of PC-6/SN-38 cells relative to PC-6 cells (Fig. 5c).

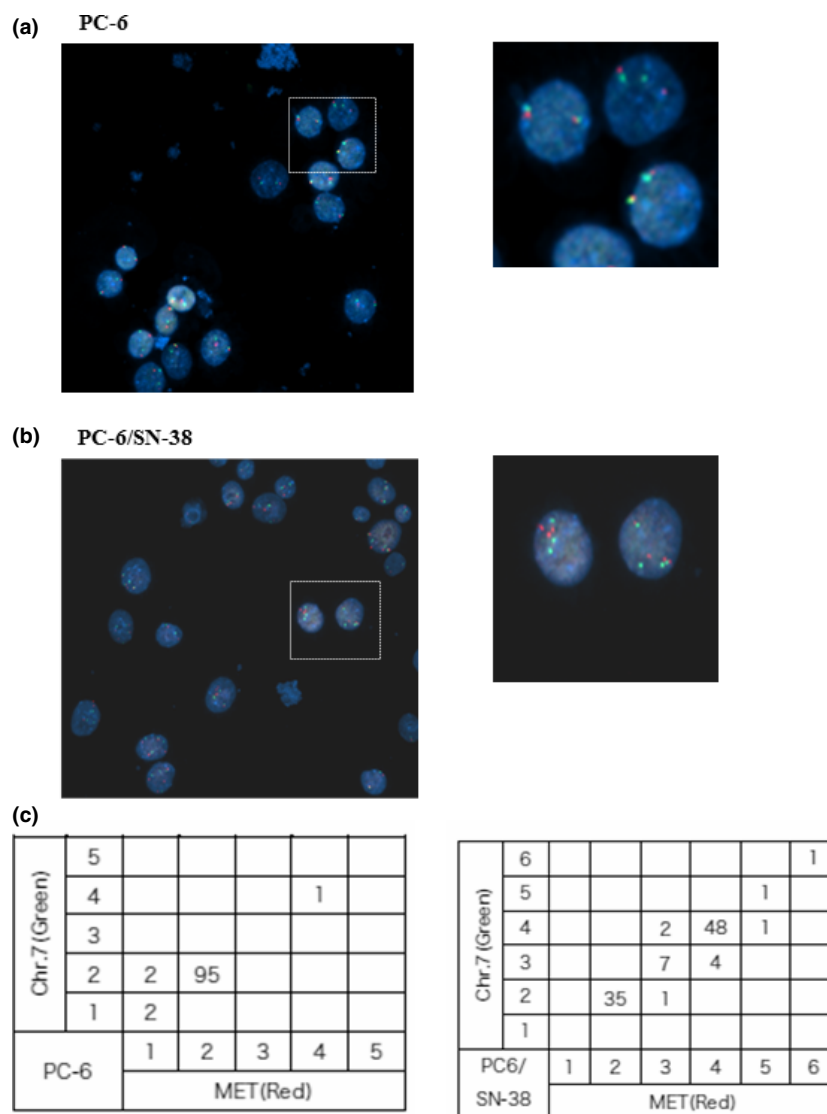
**Levels of HGF expression in drug-resistant cells.** Hepatocyte growth factor-mediated MET activation was a novel mechanism of the acquired resistance to gefitinib in lung adenocarcinoma.<sup>(19,31)</sup> Furthermore, HGF has been shown to accelerate the emergence of c-MET amplification in gefitinib-resistant lung cancer cells.<sup>(31)</sup> In this study, we examined the levels of endogenous HGF expression in PC-6/SN-38, PC-6/TXL, PC-6/CDDP, and NCI-H69/TXL cells by WB. Hepatocyte growth factor protein expression in the resistant cells was upregulated compared with the parental cells (Fig. 6a). The concentration of HGF in cell culture supernatants was significantly higher from PC-6/SN-38, PC-6/TXL, and PC-6/CDDP cells than from PC-6 cells (Fig. 6b). We examined the sensitivity of PC-6 cells to SN-38 after cultured with HGF for 2 weeks. PC-6 cells with HGF were significantly resistant to SN-38 relative to PC-6 cells without HGF (Fig. 6c).

## Discussion

We here found that increased c-Met expression through an increase in the number of *c-MET* gene loci is one of the mechanisms acquired resistance to cytotoxic anticancer drugs. Our results add a new strategy for overcoming the resistance to cytotoxic agents in SCLC.

Activation of the c-MET/HGF signaling pathway through the overexpression of HGF and/or c-MET is identified in many lung cancer patient samples.<sup>(15,16,32)</sup> The functional mutations of c-MET were identified within the semaphoring domain and the juxtamembrane domain of SCLC.<sup>(33)</sup> In addition, a functional link between downstream signaling of the c-MET/HGF pathway and tumor invasion was shown by phosphoantibody-based immunohistochemical analysis of SCLC tumor tissue and microarray.<sup>(15)</sup> Enhanced therapeutic efficacy could potentially be achieved through inhibiting c-MET activation and its known downstream signaling intermediates in SCLC.<sup>(17)</sup> Therefore, c-MET would be an attractive therapeutic target to be inhibited in SCLC to expand the therapeutic armamentarium. In this study, we showed that downregulation of c-MET expression by siRNA against the *c-MET* gene or inhibition of c-MET activation by SU11274 in anticancer-resistant SCLC cells altered resistance to the cytotoxic anticancer agent. These results showed that c-MET overexpression might play an important role in acquired resistance to the cytotoxic anticancer drugs. Therefore, c-MET inhibitor in combination with cytotoxic anticancer agents could be attractive treatments for patients with SCLC, to overcome the rapid development of drug resistance.

There are two general mechanisms of resistance of human tumors to anticancer agents. The impaired ability of drugs to penetrate tumor tissue and to reach all of the tumor cells in a potentially lethal concentration has an important role in resistance to anticancer agents.<sup>(34,35)</sup> Another important determinant of anticancer drug resistance is most often ascribed to epigenetic alternations that affect the expression of genes encoding proteins that influence the uptake, metabolism, and export of drugs from the tumor cell itself.<sup>(36)</sup> Recently, amplification of the *c-MET* gene was induced by clonal selection after exposure to gefitinib, which caused gefitinib resistance by driving ERBB3-dependent activation of PI3K, a pathway thought to be

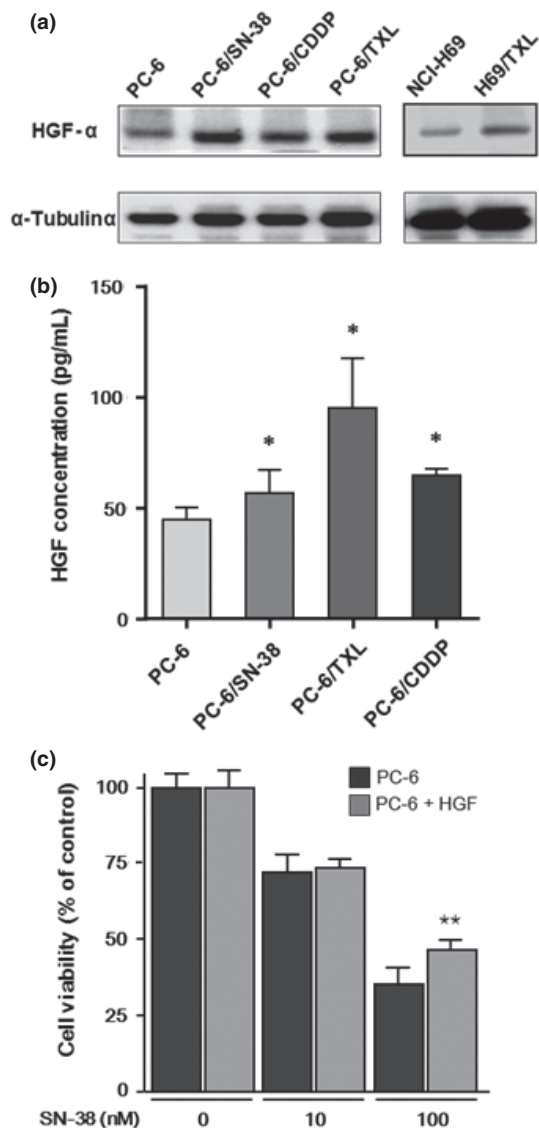


**Fig. 5.** Chromosomal instability in PC-6 small-cell lung cancer cells and 7-ethyl-10-hydroxycamptothecin-resistant (PC-6/SN-38) cells. Chromosomal instability was detected in PC-6/SN-38 and compared to that in PC-6. Dual-color FISH (CEP7 [green]/*c-MET* [red]) was carried out on fixed cell specimens from PC-6 (a) and PC-6/SN-38 (b) cells. The number of CEP7 and *c-MET* signals in 100 cells was listed (c). 20 $\times$  magnification.

specific to EGFR/ERBB family receptors in lung adenocarcinoma with EGFR-activating mutations.<sup>(18,31)</sup> In this study, we found that *c-MET* gene amplification in the resistant cells was significantly increased compared with the parental cells, by PCR. In addition, *c-MET* gene amplification in the PC-6 cells exposed to SN-38 or TXL was significantly increased relative to the cells not exposed. However, although the number of cells in which *c-MET* gene amplification was observed among PC-6/SN-38 cells was increased by only 6%, FISH analysis revealed that the number of polyploidy cells was markedly higher in PC-6/SN-38 cells than in PC-6 cells. A clinical study previously reported that chromosomal instability was a risk factor for poor prognosis in patients with lung adenocarcinoma.<sup>(37)</sup> Furthermore, MDR-associated protein overexpression in non-SCLC may be a consequence of the reassortment of chromosome 16, which is known to be catalyzed by aneuploidy.<sup>(38)</sup> Chromosomal instability including polyploidy has been associated with cytotoxic anti-cancer-drug resistance including TXL and CDDP.<sup>(39–41)</sup> Our results are in agreement with the findings of these previous studies. Differences in the experimental results obtained between FISH analysis and quantitative real-time PCR may be explained by differences in the controls used. We used

CEP7 as a control in the FISH analysis, and the ribonuclease P RNA component H1 gene mapped to chromosome 14q just below the centromere as a control in PCR. We speculate that the mechanisms responsible for the overexpression of *c-MET* in drug-resistant cells may be induced by an increase in *c-MET* DNA copy numbers and/or polyploidy in the resistant cells.

Hepatocyte growth factor was first identified as a mitogenic protein for hepatocytes and enhanced pleiotropic biological phenomena in a wide variety of cells, including antiapoptotic activities.<sup>(42)</sup> Tumor cell-derived HGF could induce gefitinib resistance of lung adenocarcinoma cells with EGFR-activating mutations.<sup>(19)</sup> In addition, HGF accelerates the emergence of *c-MET* amplification in gefitinib-resistant lung cancer cells.<sup>(31)</sup> In this study, we showed that endogenous HGF protein expression in cytotoxic anticancer drug-resistant SCLC cells was increased relative to the parental cells. PC-6 cells cultured with HGF were resistant to SN-38 relative to cells without HGF. Therefore, increased endogenous HGF expression in the resistant cells might be needed to obtain antiapoptotic activation and induce the selection of cells with a higher number of *c-MET* gene loci in the process of obtaining resistance to the cytotoxic anticancer drugs.



**Fig. 6.** Hepatocyte growth factor (HGF) protein expression in cytotoxic anticancer drug-resistant small-cell lung cancer cells, including PC-6 and NCI-H69 parental cells and those resistant to 7-ethyl-10-hydroxycamptothecin (PC-6/SN-38), paclitaxel (PC-6/TXL and H69/TXL), and cisplatin (PC-6/CDDP). (a) Levels of HGF protein expression in cytotoxic anticancer drug-resistant cells were examined by Western blotting. (b) Secretion of HGF in resistant cells was quantified by quantitative ELISA and compared to that in parental cells. (c) Growth inhibition of SN-38 to PC-6 cultured with or without HGF (50 ng/mL) for 2 weeks was examined with the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay. \* $P < 0.05$ ; \*\* $P < 0.01$ .

Downstream signaling of the c-MET/HGF pathway is activated in a variety of manners, including activating PI3K/AKT, ERK1/2, and FAK signaling, which resistant to the cytotoxic anticancer drugs.<sup>(20,21,43)</sup> In this study, SU11274 at 2  $\mu$ M concentration inhibited ERK1/2 activities in PC-6/SN-38 and PC-6/TXL, but not AKT activities. Inhibition of ERK1/2 activities enhances a stimulation of PARP cleavage, a programmed cell death by apoptosis and chemosensitivity.<sup>(44-47)</sup> Therefore, HGF/c-MET signaling activation may protect SCLC cells from cytotoxic anticancer drugs by activating the ERK1/2-dependent antiapoptotic pathway. In addition, c-MET activation by gene amplification and HGF can independently

rescue not only PI3K/AKT but also ERK signaling in the presence of gefitinib and lead to drug resistance.<sup>(18,19,31)</sup> c-MET activation might induce cross-resistance between the cytotoxic anticancer drugs and EGFR-TKI.

Our current findings provide insight into future therapeutic strategies for the treatment of SCLC. Recent progress in the understanding of the biology of SCLC has led to the identification of crucial signaling pathways and the subsequent development of targeted therapies.<sup>(48)</sup> This is the first report that activation of the c-MET signaling pathway through an increase in the number of c-MET gene loci is one of the novel mechanisms contributing to resistance to cytotoxic anticancer drugs. c-MET inhibitor may overcome the rapid development of drug resistance, which results in poor prognosis for ED-SCLC patients. Therefore, c-MET inhibitor alone or in combination with cytotoxic anticancer agents could be attractive treatments for patients with SCLC.

### Disclosure Statement

The authors have no conflicts of interest.

### Abbreviations

AKT	serine/threonine protein kinase
CDDP	cisplatin
ED	extensive disease
EGFR-TKI	epidermal growth factor receptor tyrosine kinase inhibitor
FAK	Focal Adhesion Kinase
HGF	hepatocyte growth factor
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
p-	phosphorylated
PARP	poly(ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
SCLC	small-cell lung cancer
SN-38	7-ethyl-10-hydroxycamptothecin
TXL	paclitaxel
WB	Western blot

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