MET Increases the Sensitivity of Gefitinib-Resistant Cells to SN-38, an Active Metabolite of Irinotecan, by Up-Regulating the Topoisomerase I Activity

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Introduction: Most non–small-cell lung cancer tumors with epidermal growth factor receptor mutations are responsive to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib, but almost all such tumors ultimately acquire resistance. We previously found that a gefitinib-resistant cell line, PC-9/Met in which MET (MNNG-HOS transforming gene) is amplified, was more sensitive than its parent cell line (PC-9) to 7-ethyl-10-hydroxy-camptothecin (SN-38), an active metabolite of irinotecan. The purpose of this study was to investigate the mechanisms responsible for the increased sensitivity of the gefitinib-resistant cell line to SN-38.

Methods: The sensitivity of PC-9 and PC-9/Met to SN-38 was assessed by performing water soluble tetrazolium salt (WST-1) assays. Topoisomerase I (topo I) activities were determined for the cell lines cultured in the presence of hepatocyte growth factor and for those of which MET expression was knocked down by introducing a MET-specific small interfering RNA.

Results: PC-9/Met exhibited higher topo I activities, and higher topo I gene and protein expression levels than PC-9 did. Suppression of MET expression by a MET-specific small interfering RNA led to a decrease in the topo I protein expression in the PC-9/Met cells. The stimulation of PC-9 with hepatocyte growth factor caused an increase in the topo I protein level via the activation of MET.

Conclusions: The increased sensitivity of PC-9/Met cells to SN-38 compared with that of PC-9 cells was partially because of topo I activities resulting from increased topo I mRNA and protein expression caused by MET signaling.

Key Words: Topoisomerase I, MET, Epidermal growth factor receptor-tyrosine kinase inhibitors resistance.

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The epidermal growth factor receptor (EGFR) is frequently expressed in non–small-cell lung cancer (NSCLC), and elevated EGFR levels are associated with advanced-stage disease, resistance to platinum-based chemotherapy, and a shorter survival time.[1–4] EGFR tyrosine kinase inhibitors (EGFR-TKIs) have a significant impact in NSCLC patients with EGFR mutations.[5–7] Several prospective trials have shown that the tumor response rates to gefitinib therapy are approximately 75% in the patients with tumors harboring EGFR mutations.[8–12] Nevertheless, despite the initial dramatic tumor shrinkage, the tumors of most patients thereafter become resistant to EGFR-TKIs and eventually relapse.

Several mechanisms of acquired resistance to EGFR-TKIs have recently been reported, including a secondary point mutation that substitutes metionine for threonine at position 790 (T790M) in EGFR, and an amplification of the MET proto-oncogene, which are expressed in 44% to 50% and 22% of the patients, respectively.[13–15] Ongoing preclinical and clinical studies have suggested that irreversible EGFR-TKIs overcome the resistance because of the T790M mutation, and that treatment with MET inhibitors, such as MET-TKI, could overcome the resistance because of MET amplification.[16–19] The efficacy of cytotoxic agents after the development of resistance to EGFR-TKI is uncertain.

7-ethyl-10-hydroxy-camptothecin (SN-38), an active metabolite of irinotecan, has been demonstrated to have a broad spectrum of activity against mammalian cancer cells including NSCLC cells, small-cell lung cancer cells, and colorectal cancer cells.[20–23] SN-38 prevents the religation of breaks in single-DNA strands by stabilizing the strands in a DNA-topoisomerase I (topo I) "cleavable complex." SN-38 induces DNA damage and transient S-phase arrest, and these interactions have lasting effects that are correlated with its cytotoxic activity.[23] Topo I activity is important for the initiation and elongation during DNA synthesis.[24] It has been found that the topo I expression level and activity are positively correlated with the cytotoxicity of topo I inhibitors both in vitro and in vivo.[25–28]

The purpose of the present study was to investigate the sensitivity of a gefitinib-resistant cell line, PC-9/Met, to SN-38, and to investigate the mechanism underlying the collateral sensitivity to SN-38 in PC-9/Met cells. In particular, we investigated whether the hepatocyte growth factor (HGF)/MET signaling pathway can regulate the topo I status.

MATERIALS AND METHODS

Drug Formulation and Administration

Gefitinib [N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-{morpholin-4-yl}propoxy)quinazolin-4-amine], cisplatin, etoposide, vincristine, and SN-38 were purchased from Toronto Research Chemicals Inc. (North York, Canada). Both drugs were dissolved in DMSO for use in the in vitro study.

Cell Lines and Cell Culture

The human NSCLC cell line PC-9, which was derived from a previously untreated adenocarcinoma patient, was provided by Professor K. Hayata (Tokyo Medical College, Tokyo, Japan). PC-9 is a gefitinib-sensitive cell line, and we developed the gefitinib-resistant PC-9/Met subline by continuously culturing PC-9 cells in gefitinib and gradually increasing the concentration of the drug in the medium over a period of 1 year.[29] Resistance to gefitinib was confirmed after culture in gefitinib-free conditions for 6 months. We demonstrated MET amplification in the surviving cells that were cloned during gefitinib exposure, and named the subline PC-9/Met. It was confirmed that the PC-9 and PC-9/Met cells were the same as the parental cells (PC-9) by a short tandem repeat–polymerase chain reaction (PCR) analysis (Supplementary Data 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A302). Both cell lines were maintained in RPMI medium 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen) in an incubator at 37°C in a less-than 5% carbon dioxide atmosphere.

Growth Inhibition Assay

We used the water soluble tetrazolium salt (WST-1) assay (Cell Proliferation Reagent WST-1; Roche, Tokyo, Japan) to evaluate the cytotoxicity of various concentrations of the drugs. A 180-μl volume of a growing cell suspension (2×10^3 cells/well) was seeded into each well of a 96-well microtiter plate, and a 20-μl volume of a solution of each drug at various concentrations was added to each well. After incubation for 72 hours at 37°C in less-than 5% carbon dioxide atmosphere, 10 μl of WST-1 solution was added to each well, and the plates were incubated at 37°C for an additional 90 minutes. The absorbance of these samples was then measured at 450 nm with a microplate enzyme-linked immunosorbent assay reader (Thermo Labosystems, Multiskan JX, Osaka, Japan). Each experiment was carried out independently in triplicate. The IC_{50} value was defined as the concentration required to reduce the absorbance by 50%, and was calculated based on the survival curves for the assessment of growth inhibition.

Preparation of Nuclear Lysates and Whole-Cell Lysates

Nuclear lysates were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL), and whole-cell lysates were prepared using M-PER mammalian protein extraction reagents (PIERCE, Rockford, IL). The protein concentrations were determined using the BCA protein assay reagent (Thermo Scientific).

Fluorescence In Situ Hybridization Analyses

All the PC-9 and PC-9/Met cells that were cultured with 10% FBS were trypsinized. The cell suspension was centrifuged at 1200 rpm for 5 minutes and the pellet was washed two times with phosphate-buffered saline. An unstained slide was made for each cell pellet, and the pellet was subjected to dual color fluorescence in situ hybridization assays using a MET/chromosome 7 centromere (CEP7) probe labeled with SpectrumRed and SpectrumGreen CEP7 (Abbott Molecular Inc., Des Plaines, IL) according to the manufacturer's instructions. The total number of red and green signals counted in the tumor nuclei was recorded, and then ratio of the MET (red) to CEP7 (green) signals for a total of 20 tumor nuclei was calculated. The cells with a MET:CEP7 ratio greater than 2.0 were considered to have MET amplification.

Topoisomerase I Activity Assay

After adjusting the protein concentrations, the topo I activity was measured by DNA relaxation assays that were performed using a topoisomerase assay kit (TopoGEN, Inc., Florida, CA;) according to the manufacturer's instructions.[30,31] The DNA was stained with ethidium bromide, and the bands were visualized by UV transillumination.

Western Blot Analysis

A 20-μl volume of protein per lane was loaded on 4% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Bio-Rad, Tokyo, Japan) and run at a constant current of 90 mV for 80 minutes. The proteins were transferred onto polyvinylidene fluoride microporous membranes (Millipore, Bedford, MA) by electroblotting at a constant current of 190 mA for 80 minutes. The membranes were then blocked by immersion for at least 1 hour in 5% milk (nonfat powdered milk) buffered with Tris-buffered saline. Next, the membranes were incubated overnight with a primary antibody suspended in 5% buffered milk at an adequate dilution. After washing three times with Tris-buffered saline, the membranes were exposed to horseradish peroxidase–labeled secondary antibodies and probed with rabbit polyclonal antibodies against EGFR, phospho-EGFR, Akt, phospho-Akt, Erk phosphor-Erk (Cell Signaling, Beverly, MA), and with a monoclonal antibody against topo I (TopoGEN, Inc., Port Orange, FL). Specific signals were visualized by using the ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd., Amersham, UK). The intensities of the blots for topo I expression were quantified using the Image J software (version 1.45; National Institutes of Health, Bethesda, MD).

Quantitative Reverse Transcription-PCR for the Expression of Topo I mRNA

Total cellular RNA was extracted with the ISOGEN reagents (Nippon Gene, Tokyo, Japan). The quantity of total RNA was measured using a NanoDrop ND1000 fluorospectrometer (NanoDrop Technologies, Tokyo, Japan). cDNA was synthesized from 500 ng of deoxyribonuclease-treated RNA by using a random hexamer primer. To measure the topo I mRNA expression, quantitative reverse transcription-PCR was performed on a LightCycler

(Roche, Tokyo, Japan). The topo I expression levels were determined by using SYBR premix Ex Taq II perfect real-time reagents (Takara, Shiga, Japan) and the primers 5′-CAATGGCCCAGGCAAACTTC-3′ (forward) and 5′-AAGTTCAAGCCACAGACCGAGAG-3′ (reverse) under cycling parameters that consisted of one cycle of 98°C for 2 minutes; 30 cycles of 98°C for 10 seconds, 58°C for 30 seconds, and 72°C for 14 seconds; and followed by one cycle of 72°C for 5 minutes. The amplification of β_2 microglobulin was used for normalization. The relative topo I expression levels were calculated by the delta-delta method.[32,33]

Transfection and Small Interfering RNA Experiments

PC-9 and PC-9/Met cells were seeded in six well culture dishes at a density of 5×10^4 cells/well and allowed to grow overnight in RPMI 1640 medium containing 10% FBS. The cells were transfected with small interfering RNA (siRNA) against MET or with scrambled RNA by using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. The sequence of the siRNA was: MET, 5′-UGAAUUAGGAAACUGAUCUUCUGGA-3′ and 5′-UCCAGAAGAUCAGUUUCCUAAUUCA-3′. After incubation for 24 hours, the cells were washed with fresh medium and incubated for an additional 48 hours, and the cell lysates were then prepared from the cultured cells for the detection of MET expression.

Stimulation of PC-9 and PC-9/Met With HGF

PC-9 and PC-9/Met cells were seeded in cell culture plates at a density of 1×10^5 cells/well, and were starved overnight in RPMI 1640 medium containing 0.1% FBS. The cells were then incubated with 20 ng/ml of recombinant HGF (R&D Systems, Minneapolis, MN). Whole-cell proteins were extracted from the cells at 30 minutes and 60 minutes, whereas nuclear proteins were extracted at 6 hours, 12 hours, and 24 hours after treatment, respectively. In the sensitivity test, the tumor cells were incubated with 0.1% FBS with RPMI 1640 medium overnight, then 20 ng/ml of HGF was added to the cell suspensions. The drug exposure for 24 hours was chosen because the cell viabilities under starvation conditions were too low for longer periods of time.

Statistical Analysis

All data are expressed as the means ± SD and were analyzed by Student's *t* test. *p* values less than 0.05 were considered to be evidence of statistical significance. All statistical analyses were performed by using the Stat Mate IV (ATMS, Tokyo, Japan) software program.

RESULTS

The Growth Inhibitory Effects of Gefitinib and SN-38 on PC-9 and PC-9/Met Cells

We first evaluated the sensitivities of the PC-9 and gefitinib-resistant PC-9/Met cells. The IC_{50} values for gefitinib in these cells were 0.088 ± 0.014 μM and 10.3 ± 5.0 μM, respectively (Fig. 1), confirming that the PC-9/Met cells are resistant

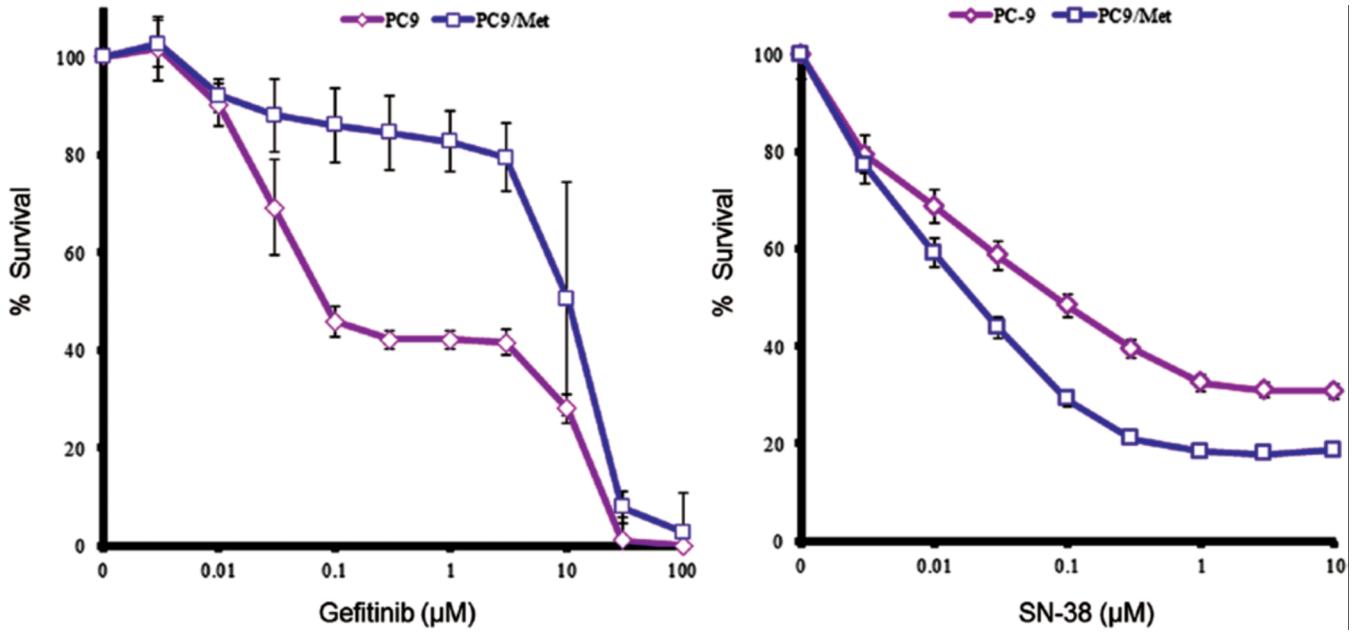

FIGURE 1. The sensitivity of PC-9 and gefitinib-resistant PC-9/Met cells to gefitinib and SN-38. The cells (2×10^3 cells/well) were seeded on 96-well plates, and after preincubation for 24 hours were exposed to various concentrations of each drug. After incubation for 72 hours, the growth inhibition rate was analyzed by a WST-1 assay as described in the Materials and Methods section. The points represent the means of the data generated from at least three experiments performed in triplicate. Bars, SD. PC-9 cells, PC-9/Met cells. SN-38, 7-ethyl-10-hydroxy-camptothecin.

to gefitinib. The IC_{50} values for SN-38 in the PC-9 and PC-9/Met cells were 0.093 ± 0.049 μM and 0.027 ± 0.014 μM, respectively, showing that PC-9/Met cells are more sensitive than their parental PC-9 cells to SN-38. PC-9/Met cells were equally sensitive to cisplatin and etoposide. Nevertheless, the PC-9/Met cells exhibited a 12-fold decreased sensitivity to vincristine (Supplementary Data 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A303).

Characterization of PC-9 and PC-9/Met Cells

We examined the protein expression levels of the PC-9 and PC-9/Met cells (Fig. 2*A*). As expected, a Western blot analysis revealed higher expression of the MET protein in the PC-9/Met cells than in the PC-9 cells. The MET phosphorylation in PC-9/Met cells was also higher than that in the PC-9 cells. We confirmed that there was MET gene amplification in PC-9/Met cells by a fluorescence in situ hybridization analysis (Fig. 2*B*). The EGFR protein expression and EGFR phosphorylation levels in the PC-9/Met cells were lower than those in the PC-9 cells. In each cell line, gefitinib treatment did not affect the protein expression and phospholylation of MET or topo I (data not shown).

PC-9/Met Cells Have Higher Topo I Activity, and Increased Topo I mRNA and Protein Expression Levels Compared With PC-9 Cells

Our results showed that the topo I expression level in the PC-9/Met cells was significantly higher than that in the PC-9 cells (Fig. 2*A*). We then examined the topo I activity in each cell line, and found that the activity in PC-9/Met cells was approximately fourfold higher compared with that in the PC-9 cells (Fig. 2*C*). The topo I mRNA level of the PC-9/Met

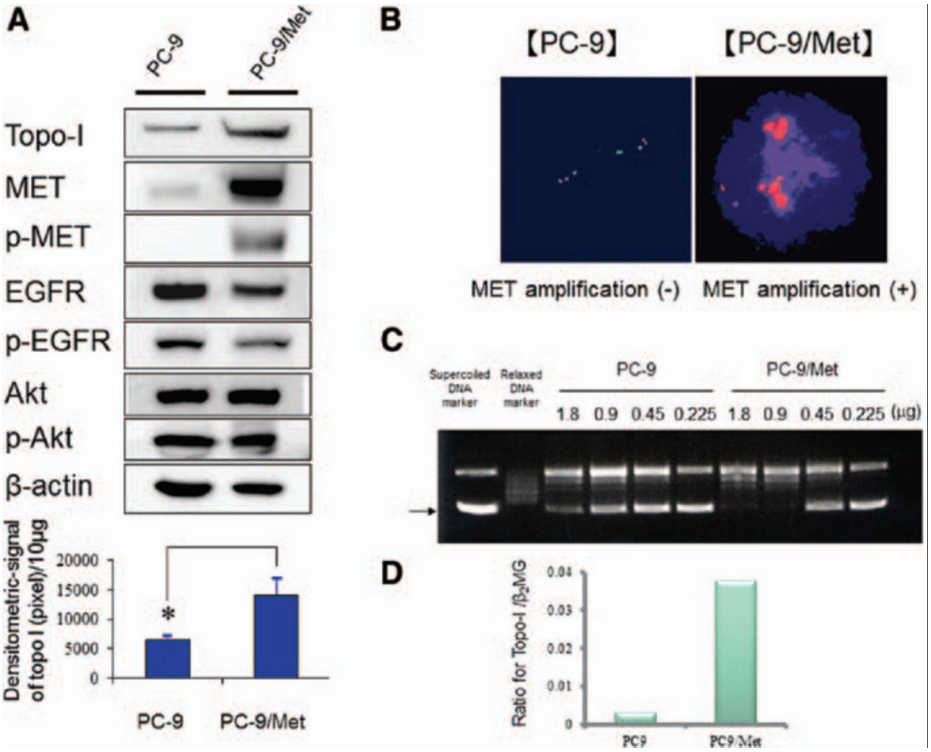

FIGURE 2. *A*, A comparison of the protein expression levels of topo I, MET, p-MET, EGFR, p-EGFR, Akt, and p-Akt in the PC-9 and PC-9/Met cells as determined by a Western blot analysis. Topo I migrated to approximately the 70 kDa position. The data are expressed as the means ± SD from more than three experiments. *$p < 0.05$. *B*. The MET gene amplification status in the PC-9 and PC-9/Met cells was determined by a fluorescence in situ hybridization analysis. The chromosome 7 centromere signals are green, and the MET signals are red. MET amplification was found in the PC-9/Met cells, but not in the PC-9 cells. *C*, Topo I activity in the PC-9 cells, and the gefitinib-resistant PC-9/Met cells. Complete activity is seen when no supercoiled DNA substrate remains, and the presence of some supercoiled DNA indicates partial activity. The upper bands are attributable to DNA nicking by contaminating nucleases. The bottom band, indicated by the arrow, represents supercoiled DNA degraded by topo I. The topo I activity was normalized to the amount of nuclear protein extracted, such as 1.8 μg, 0.9 μg, 0.45 μg, and 0.225 μg, respectively, in both cell lines. In the PC-9/Met cells, the band of supercoiled DNA was eliminated when 0.9 μg of nuclear protein was added to the reaction mixture. However, the band of supercoiled DNA remained when 1.8 μg of nuclear protein from the PC-9 cells was added. These results showed that the topo I activity of the PC-9/Met cells was approximately fourfold higher than that of the PC-9 cells. *D*, Measurement of the topo I mRNA expression in PC-9 and PC-9/Met cells as determined by real-time PCR. The topo I mRNA level of the PC-9/Met cells was approximately 9.2 times higher than that of the PC-9 cells. EGFR, epidermal growth receptor factor; p-EFGR, phospho-EGFR; p-Akt, phospho-Akt; p-MET, phospho-MET; topo I, topoisomerase I; mRNA, messenger RNA; PCR, polymerase chain reaction.

cells was approximately 9.2 times higher than that of the PC-9 cells (Fig. 2*D*).

Knockdown of MET Expression Decreased the Sensitivity of Cells to SN-38 by Reducing the Topo I Activity and Protein Expression Level

The most remarkable feature of the PC-9/Met cells compared with the PC-9 cells was their strong expression of MET and phosphorylation of MET. Therefore, to elucidate the mechanism underlying the high sensitivity of the PC-9/Met cells to SN-38, we focused on the relationship between MET signaling and topo I, and investigated whether knocking down MET expression in PC-9/Met cells would alter the topo I expression and activity. Knocking down MET expression in the PC-9 and PC-9/Met cells using a MET-specific siRNA resulted in down-regulation of the topo I protein expression level and topo I activity in PC-9/Met cells (Fig. 3*A–C*). However, the topo I expression in the PC-9 cells was not affected by the knockdown of MET (Fig. 3*A*). We have also examined the kinetics of topo I expression after the treatment with MET siRNA, and confirmed that the PC-9 -cells were not affected (Fig. 3*B*, and Supplementary Data 3, Supplemental Digital Content 3, http://links.lww.com/JTO/A304). The results of the WST cytotoxicity assays showed that the PC-9/Met cells treated with MET-specific siRNA were less sensitive to SN-38 than the PC-9/Met cells transfected with scrambled RNA were (Fig. 3*D*). Neverthless, the sensitivity of the PC-9/Met cells treated with the MET-specific siRNA was still higher than that of the PC-9 cells. In contrast, the sensitivities to these non-topo I–targeted drugs (cisplatin, etoposide, and vincristine) were not affected by knockdown of MET in either the PC-9 or PC-9/Met cells (Supplementary Data 4, Supplemental Digital Content 4, http://links.lww.com/JTO/A305). We also examined whether topo I expression was down-regulated when EGFR was knocked down. We found that the topo I expression was not affected by the knockdown of the EGFR in either PC-9 or PC-9/Met cells (Supplementary Data 5, Supplemental Digital Content 5, http://links.lww.com/JTO/A306).

HGF Increased the Topo I Activity, Topo I Protein Expression, and Sensitivity to SN-38

As our results suggested that a relationship existed between HGF/MET signaling and the topo I status, we further investigated whether HGF stimulation would increase the level of topo I protein expression in the two cell lines. After HGF stimulation for 60 minutes, the phosphorylation of MET was induced, and after HGF stimulation for 6 hours, 12 hours, and 24 hours, the topo I expression was up-regulated in both cell lines (Fig. 4*A*, *B*). The increase in the topo I level in response to HGF stimulation was significantly higher in the PC-9 cells than in the PC-9/Met cells. We next investigated whether HGF increased the sensitivity to SN-38 in the two cell lines. To exclude the effects of stimulation with other ligands, the cells were cultured overnight in RPMI supplemented with 0.1% FBS under starvation conditions. HGF (20 ng/ml) was then added, and the cells were exposed to SN-38 for 24 hours. Under these conditions, the viability of the PC-9 and PC-9/Met cells was decreased by exposure to SN-38 in a concentration-dependent manner. The sensitivity of PC-9/Met cells was also higher than that of the PC-9 cells cultured under starvation conditions. HGF stimulations increased in the sensitivity of both the PC-9 and PC-9/Met cells to SN-38 (Fig. 4*B*). Although the sensitivity to SN-38 was observed to increase in both cell lines, the PC-9/Met cells without HGF stimulation were still more sensitive than the PC-9 cells subjected to HGF stimulation.

DISCUSSION

This study focused on an acquired EGFR-TKI resistant cell line in which MET was amplified. There were two major findings of this study. The first was that a gefitinib-resistant cell line, PC-9/Met, has collateral sensitivity to SN-38, a topo I inhibitor, as a result of an increase in topo I activity. The second major finding was that topo I activity is regulated via MET signaling.

Okabe et al.[34] showed that the addition of S-1 to an EGFR-TKI inhibited the growth of gefitinib-resistant NSCLC with MET amplification by down-regulating thymidylate synthase and the transcription factor E2F-1. A combination of S-1 or SN-38 with an EGFR-TKI also might exert a possible synergistic effect. A phase I/II study showed that a combination of irinotecan and gefitinib was effective in NSCLC patients with gefitinib failure.[35] Although MET amplification was not measured in that study, there is a possibility that MET amplification in the resistant tumors might have made them more sensitive to this combination. An in vivo study by Shimoyama et al.[36] showed the efficacy of a sequential combination of irinotecan and gefitinib in a gefitinib-resistant cell line in which the mechanism of resistance was not reported. The results of our present study indicate that topo I inhibitors, such as irinotecan, might be useful agents for overcoming EGFR-TKI resistance that is mediated by MET amplification.

In our study, PC-9/Met cells showed collateral sensitivity to SN-38 compared with PC-9 cells. To confirm the collateral sensitivity to SN-38 in these cells, we examined the topo I mRNA expression, protein expression, and activity levels in each cell line. In PC-9/Met cells, the mRNA and protein expression, and the activity of topo I were higher than in the PC-9 cells. The results of the topo I activity assays performed in this study are consistent with previous studies showing that the sensitivity to topo I inhibitors correlates with the topo I activity of the tumor cells.[25–28] We next examined the effects of MET-specific siRNA or MET stimulation by HGF on the topo I level in these experiments. The knockdown of MET resulted in the down-regulation of topo I, and HGF stimulation resulted in the up-regulation of topo I in both cell lines. Finally, we examined the sensitivity of the cells to SN-38 after treatment with a MET-specific siRNA or HGF. Down-regulation of the MET amplification in the PC-9/Met cells treated with MET-specific siRNA led to a decrease in their sensitivity to SN-38. However, these cells were still more sensitive than the PC-9 cells without siRNA treatment (Fig. 3*D*). The sensitivity to SN-38 increased when both cell lines were treated with HGF, but the sensitivity of the PC-9 cells stimulated by HGF was still lower than that of the PC-9/Met cells without stimulation. These results suggest that the topo I activity of these cells was partially regulated by HGF-MET signaling.

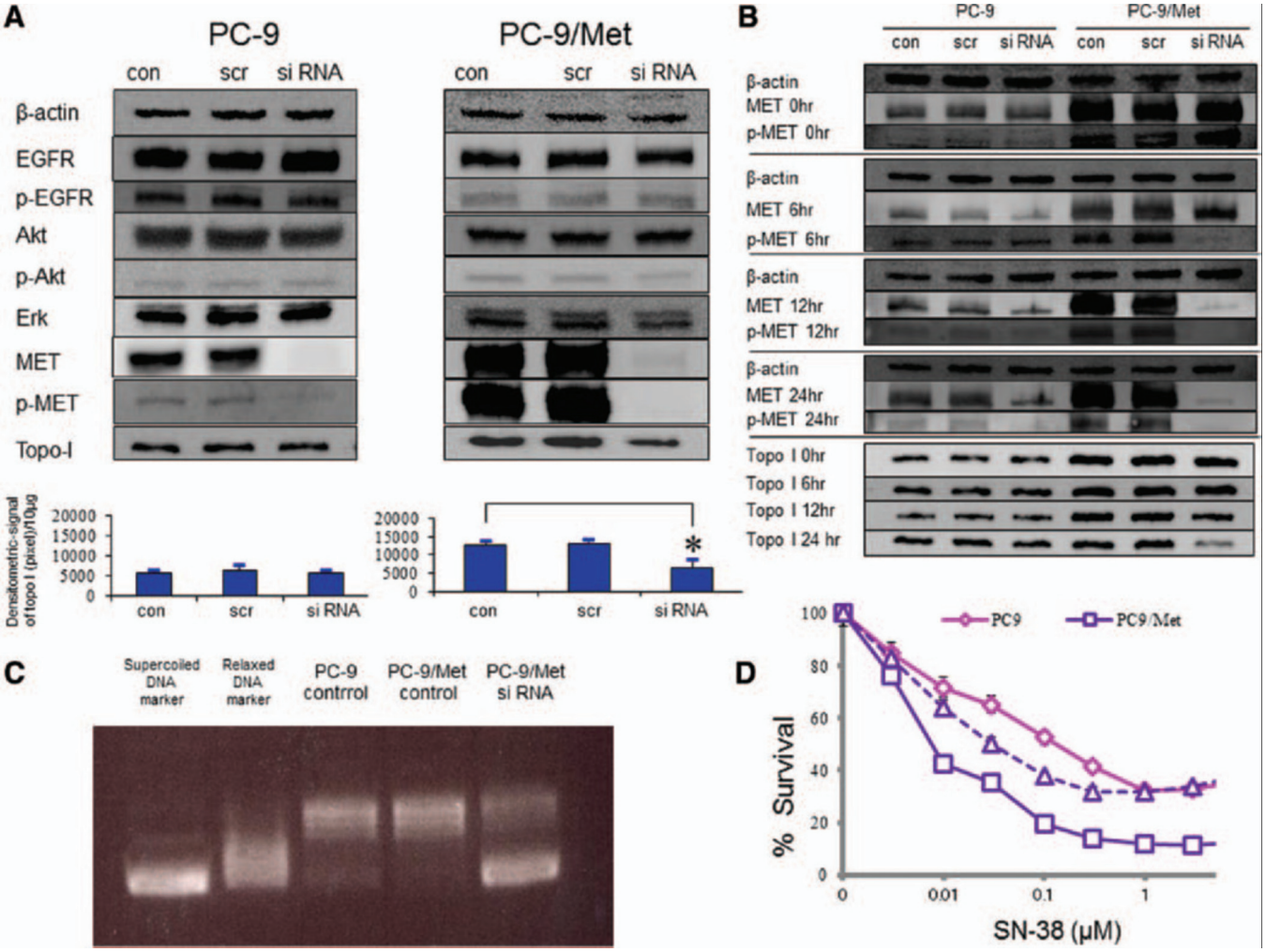

FIGURE 3. Specific down-regulation of MET affects the level of topo I protein expression and the enzyme activity, while also decreasing the sensitivity of cells to SN-38. Control and MET-specific siRNA were introduced into the PC-9 and PC-9/Met cell lines. *A.* Whole-cell proteins and nuclear proteins were extracted 48 hours later, and a Western blot analysis was performed. To detect the MET protein, a 20-μg protein sample was loaded per lane for the PC-9 cells and PC-9/Met cells. To measure topo I protein expression, 10-μg samples of nuclear protein were loaded for both the PC-9 and PC-9/Met cell lines. The data are expressed as the means ± SD from more than three experiments, $*p < 0.05$ versus control. *B.* The relationship between topo I expression and MET expression by a Western blotting analysis at 0 hour, 6 hours, 12 hours, and 24 hours after siRNA addition. *C,* The effect of the specific down-regulation of MET on the topo I activity of the PC-9/Met cells. MET-specific siRNA was introduced into PC-9/Met cells, and nuclear enzymes were extracted from the PC-9/Met cells that contained topo I. The PC-9/Met cells in which MET had been down-regulated had lower topo I activity than did the untreated PC-9/Met cells. *D,* The results of the WST-1 assay of cell-growth inhibition by SN-38. The PC-9/Met cells were more sensitive to SN-38 than the PC-9 cells, and the sensitivity of cells treated with MET siRNA was reduced. The points represent the means of data generated from at least three experiments performed in triplicate. Bars, SD. PC-9, PC-9/Met, PC-9/Met siRNA. topo I, topoisomerase I; siRNA, small interfering RNA, SN-38, 7-ethyl-10-hydroxy-camptothecin; con, control; scr, scramble.

This is the first report to provide evidence that MET signaling plays a role in regulating topo I activity. MET, a high affinity tyrosine kinase receptor for HGF, is a disulfide-linked heterodimer composed of a 45-kDa α-subunit and a 145-kDa β-subunit.[37,38] When MET is activated by HGF, it transduces strong signals to various pathways, including the phosphatidylinositol 3-kinases/Akt pathway.[39,40] HGF and c-Met have been found to be frequently overexpressed in many types of human solid tumors[41–44] and in associated metastases, and the degree of MET expression correlates with the patients' survival.[45] Our data led us to hypothesize that treatment strategies that target topo I may therefore be effective against tumors with enhanced MET signaling. However, as no factors that regulate topo I have ever been identified, the relationship between HGF/MET and topo I is unclear. To support our findings, it will be necessary to identify the link between topo I activity and MET signaling. Further studies are called for to shed light on the mechanism(s) underlying the regulation of topo I activity by MET signaling.

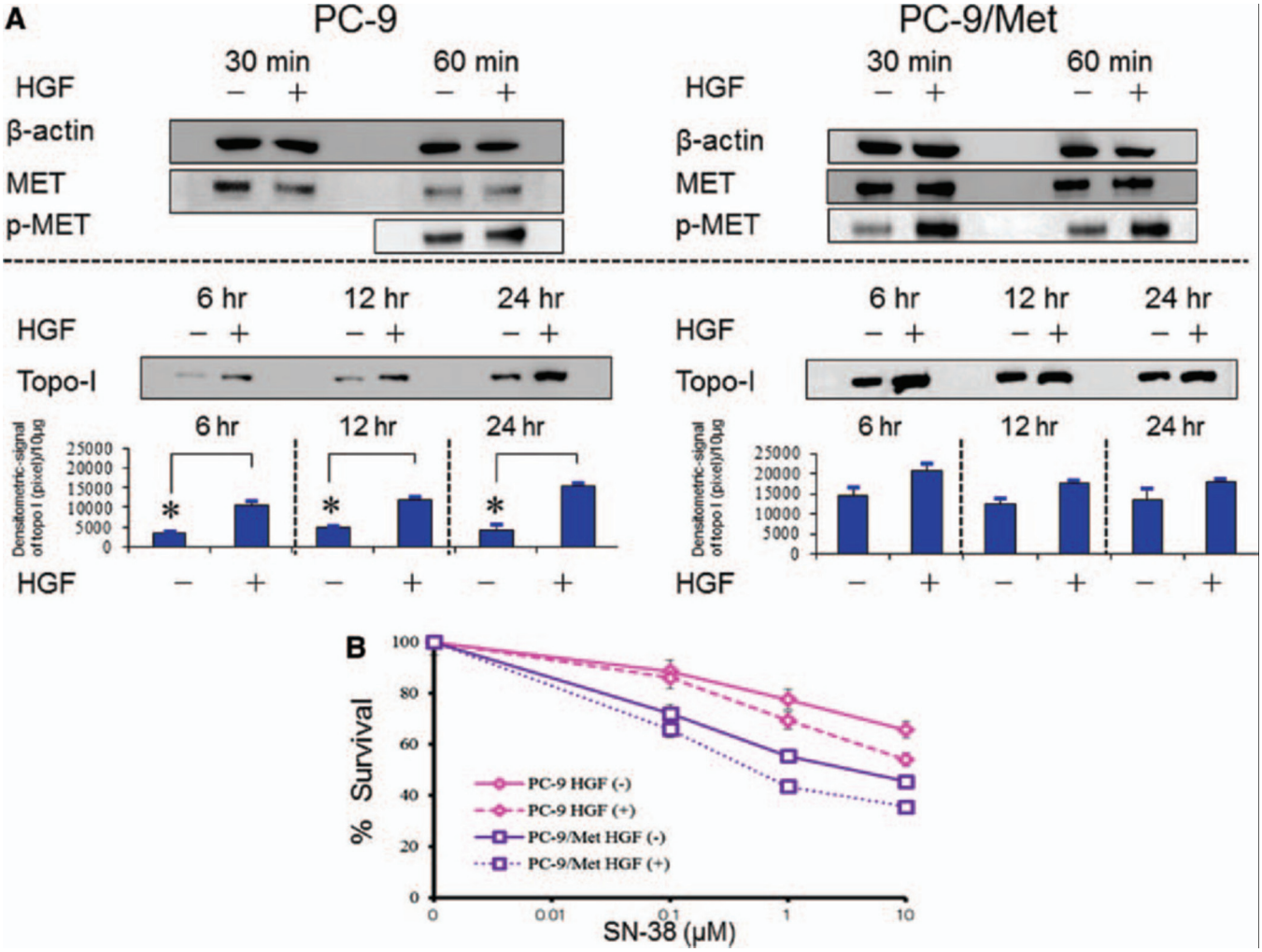

FIGURE 4. *A*, Cell extracts from PC-9 cells and PC-9/Met cells were prepared after the cells were cultured with HGF (20 ng/ml) for 30 minutes and 60 minutes, after serum starvation for 24 hours. HGF stimulation induced MET phosphorylation in both cell lines. Nuclear extracts containing the topo I enzyme were prepared after 6 hours, 12 hours, and 24 hours of HGF stimulation, and the topo I protein expression level in both PC-9 and PC-9/Met cells was increased after HGF stimulation. The data are expressed as the means ± SD from three experiments, $*p < 0.05$ versus control. *B*, HGF increased the sensitivity of PC-9 and PC-9/Met cells to SN-38. Tumor cells at concentration of 2×10^4 cells/well were incubated in RPMI medium supplemented with 0.1% fetal bovine serum overnight. HGF (20 ng/ml) was added to the cultures of tumor cells for 4 hours. Increasing concentrations of SN-38 were added to the each well, and the incubation was continued for a further 24 hours. Then, the cell growth was determined by the water soluble tetrazolium salt-1 assay. HGF, hepatocyte growth factor; topo I, topoisomerase 1.

The major limitation of this study is that it did not examine whether gefitinib-resistant cells whose resistance is mediated by other mechanisms besides MET amplification (for example, by the T790M mutation or insulin-like growth factor receptor expression), have increased sensitivity to SN-38, similar to the PC-9/Met cells with the MET amplification. To our knowledge, no investigators have ever assessed the sensitivity of cell lines with a T790M mutation or IGF-R amplification to SN-38. Furthermore, no other cytotoxic agents were assessed, except in the preliminary experiments. Additional studies of such agents will be needed to determine whether there are similar effects, or whether the increased sensitivity is specific to SN-38.

In conclusion, the results of this study showed that the increased topo I activity induced by MET signaling in a gefitinib-resistant cell line resulted in increased sensitivity of human cancer cells to SN-38. In addition to treatment approaches with involving MET-TKIs, we believe that topo I inhibitors have the potential to overcome MET-amplification–mediated resistance to EGFR-TKIs.

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