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CPT-11 May Provide Therapeutic Efficacy for Esophageal Squamous Cell Cancer and the Effects Correlate with the Level of DNA Topoisomerase I Protein

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CPT-11 is a potent anti-cancer drug and a specific inhibitor of DNA topoisomerase I (Topo I). In this study, we aim to evaluate the effects of CPT-11 on esophageal squamous cell cancers (ESCC) and to determine the correlation between the effects and the levels of Topo I expression. We examined the growth-inhibitory effect caused by SN-38, an active metabolite of CPT-11, in 14 human ESCC cell lines established from 10 primary and 4 metastatic lesions. CPT-11 was considered effective against 5 cell lines from primary lesions and one from metastatic lesions, and thus may show therapeutic efficacy against both primary and metastatic ESCC tumors. Although Topo I mRNA levels in these 14 ESCC cell lines, as quantitated by northern blot analysis, showed no correlation with the IC_{50} values, Topo I protein levels, as quantitated by western blot analysis, showed an inverse correlation with the IC_{50} values. Topo I protein levels could be an indicator of sensitivity to CPT-11. We also determined Topo I protein levels in 40 ESCC tumors and matched normal mucosae. Thirty-four tumors showed 1.2–22.3-fold increases in Topo I levels. Two patients receiving pre-operative chemotherapy and one receiving radiotherapy exhibited increased Topo I protein levels in their tumor lesions. It appeared that CPT-11 could provide selective therapeutic efficacy against ESCC tumors. CPT-11 may be effective for the treatment of metastatic ESCC tumors and as a second-line anti-cancer drug for ESCC.

Key words: Esophageal squamous cell cancer — CPT-11 — SN-38 — DNA topoisomerase I — Therapeutic efficacy

The prognosis for esophageal squamous cell cancers (ESCC) remains unsatisfactory, despite recent advances in diagnosis and treatment. This is partly because the disease is often advanced and has distant metastasis when diagnosed. Therefore, to improve long-term survival rates for ESCC, an effective combination therapy including surgical treatment, chemotherapy and radiotherapy is necessary. *cis*-Dichlorodiammineplatinum (II) (CDDP)-based systemic combination therapy is commonly performed for ESCC, but the response rate is only about 36%, and the results are unsatisfactory.[1, 2] Moreover, because the progression of cancer is often associated with chemoresistance to the anti-cancer drugs used as the first-line therapy, an effective alternative agent is needed for the treatment for ESCC.

Camptothecin is a naturally occurring anti-cancer drug isolated from the Chinese tree *Camptotheca acuminata*.[3] It is reported to be effective in various experimental animal tumor models.[4] However, its clinical usefulness is restricted by severe side-effects, including hemorrhagic cystitis, diarrhea, and myelosuppression.[5–7] CPT-11 is a semi-synthetic analogue of camptothecin, which has better water solubility and fewer side-effects.[8] Its anti-tumor activity is exerted by SN-38,[9, 10] an active metabolite of CPT-11 generated by carboxylesterase,[11] which selectively inhibits DNA topoisomerase I (Topo I) by stabilizing the Topo I-DNA cleavable complex,[12] resulting in single-strand DNA breaks, which lead to cell death.[13, 14] CPT-11 has potent anti-tumor activity against various experimental and clinical cancers.

In human colorectal cancers, it has been reported that the sensitivity to CPT-11 is related to Topo I levels.[15, 16] Moreover, Topo I levels appear to be elevated in tumor lesions, compared to the normal tissues.[17, 18] Therefore, CPT-11 is considered to be a very effective agent for human colorectal cancers, because increased Topo I expression in tumors, compared to normal tissues, can provide tumor-selective cytotoxicity. On the other hand, in lung cancers, although there are reports that Topo I mRNA expression might affect the sensitivity to CPT-11,[19] others have reported no correlation between the protein expression of Topo I and the sensitivity to CPT-11.[20] Therefore, whether Topo I levels predict sensitivity to CPT-11 is controversial. Moreover, in kidney cancers, it has been reported that there is no difference in Topo I levels between tumors and their counterpart normal tissues.[18] In this study, we aimed to evaluate the therapeutic efficacy of CPT-11 for ESCC and to examine the correlation between the effects and levels of Topo I expression. Differences in Topo I levels between ESCC tumor lesions and normal tissues were also examined.

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MATERIALS AND METHODS

Anticancer agent SN-38, an active metabolite of CPT-11, was kindly provided by Yakult Co., Ltd. (Tokyo). SN-38 was dissolved in NaOH and stored at −20°C with protection from light.

Cell lines and tissues Twelve human ESCC cell lines of the TE series[21] were kindly provided by Dr. T. Nishihira of the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. EC-GI-10 was purchased from the Riken Gene Bank, and TT was from the Human Science Research Resources Bank. Ten cell lines (TT, TE-1, TE-2, TE-5, TE-8, TE-10, TE-11, TE-12, TE-13, TE-15) were established from primary ESCC lesions, and others were from metastatic lesions.

Forty specimens of ESCC tumors and normal mucosae were obtained from patients who underwent surgery at the Tokyo Medical and Dental University Hospital after informed consent had been obtained. Two of them had received chemotherapy and one had had radiotherapy prior to surgery. All specimens were frozen immediately in liquid nitrogen and stored at −80°C until analyzed.

***In vitro* drug sensitivity assay** The sensitivity to SN-38 was evaluated in terms of the concentration of drugs required for 50% inhibition of cell growth of treated cells compared with control cells (IC_{50}). In brief, suspensions of 2.5×10^4 cells/ml of 14 ESCC cell lines were seeded in 96-well microplates. After incubation at 37°C in a humidified 5% CO_2 atmosphere for 24 h, the cells were exposed to various concentrations of SN-38 for 72 h. The anti-proliferative effects were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[22] The plates were incubated for an additional 4 h with a final concentration of 0.40 mg/ml of MTT, and then the dark blue crystals were dissolved in 150 μl of dimethyl sulfoxide. The absorbance was measured at 540 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA), and the inhibition rate was calculated as follows: Inhibition rate=(1−absorbance of drug treated cells/absorbance of control cells) × 100. The IC_{50} values were determined from the inhibition rates, and expressed as the means of at least 3 independent experiments, each performed in 5 replicates.

Preparation of the cDNA probes The digoxigenin-11-UTP-labeled cDNA probe for Topo I was prepared using PCR DIG Labeling Mix (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions. A human fetal brain cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA) was used as the template DNA, and the sequences of the primers used for the Topo I cDNA probe were as follows.[19] Topo I: sense, 5′-GGAGAGACCTGAAAAGTGCTAA-3′; antisense, 5′-TAAATCTTCTCAATTGGGAC-3′. After predenaturation at 90°C for 10 min, amplification was carried out for 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 60 s, followed by a final incubation at 72°C for 7 min, using a thermal cycler (PCT-200 DNA Engine, MJ Research, Inc., Waltham, MA). The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions.

Northern blot analysis Total RNA from 14 ESCC cell lines was prepared by the acid guanidium-phenol-chroloform method using ISOGEN (Nippon Gene, Toyama). Electrophoresis of the RNA was performed essentially as described previously.[23] Extracted RNA was quantified by measuring the absorbance at 260 nm, and 15 μg of each RNA was denatured at 65°C for 15 min in 50% formamide, 17.5% formaldehyde, 10 m*M* 3-morpholinopropanesulfonic acid (pH 7.0), 40 m*M* CH_3COONa, and 0.5 m*M* EDTA (pH 8.0). Following the addition of formaldehyde gel-loading buffer [50% glycerol, 1 m*M* EDTA (pH 8.0), 0.25% bromophenol blue, and 0.25% xylene cyanol], samples were separated in 1.2% agarose gels containing 2.2 *M* formaldehyde. RNA was transferred onto a nylon membrane using a TURBOBLOTTER (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions, and immobilized with a UV cross-linker (CL-1000, UVP, Inc., Upland, CA) under UV light at 254 nm. Hybridization was performed using Express Hyb (CLONTECH Laboratories, Inc.) according to the manufacturer's instructions. Following prehybridization at 68°C for 30 min, 300 ng/ml digoxigenin-11-UTP-labeled cDNA probe for Topo I was added, and the mixtures were incubated at 68°C for 1 h. The bands for Topo I were developed using a DIG Luminescent Detection Kit (Boehringer Mannheim GmbH Biochemica) according to the manufacturer's instructions and detected using a lumino-image analyzer (LAS-1000, Fuji Film, Tokyo). The probe for Topo I was removed by washing the membrane in 0.1× SSPE [15 m*M* NaCl, 0.865 m*M* NaH_2PO_4, and 0.125 m*M* EDTA (pH 7.4)] and 0.5% sodium dodecyl sulfate (SDS) at 90°C for 30 min, and re-hybridization for glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) was performed as an inner control. The bands related to Topo I and GAPDH were quantified by Image Gauge v.3.01 (Fuji Film). The Topo I mRNA index was calculated as follows: Topo I mRNA Index=(Topo I mRNA level/GAPDH mRNA level) ×100.

Immunoblot analyses Protein extraction and immunoblotting were performed essentially as described previously.[24] For protein extracts from cells, culture dishes with semi-confluent cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), and then harvested. For tissue samples, frozen specimens were homogenized thoroughly in the presence of liquid nitrogen. Cells and tissues were lysed in buffer including 50 μg/ml phenylmethane-

sulfonyl fluoride (PMSF), 5 μl/ml aprotinin, 5 μg/ml leupeptin, 5 μM NaF, and 0.2 μM orthovanadate in NETN [20 m*M* Tris-HCl (pH 8.0), 100 m*M* NaCl, 1 m*M* EDTA, and 0.5% NP-40], and incubated on ice for 30 min. Protein extracts were obtained after centrifugation at 14 000 rpm for 15 min at 4°C, and quantified by using the Bradford assay.

Extracted proteins were boiled with an equal volume of SDS sample buffer [2% SDS, 0.1% bromophenol blue, 100 m*M* dithiothreitol, and 10% glycerol in 50 m*M* Tris-HCl (pH 6.8)], resolved by electrophoresis in 7.5% SDS-polyacrylamide gels, and transferred to PVDF membranes (Hybond-P, Amersham, Buckinghamshire, UK).

The membranes were blocked in 5% powdered milk in TBST [10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.075% Tween 20] for 1 h at room temperature prior to incubation with the primary antibody. The anti-Topo I antibody (clone: T1P1R2, Sigma-Genosys, Ltd., Cambridge, UK) was used at 1:1000 dilution, and the anti-β-actin antibody (clone: AC-15, Sigma, Saint Louis, MO) at 1:5000. Following 4 washes with TBST, the bound antibodies were detected using alkaline phosphatase-conjugated secondary antibodies and developed with the 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP)/p-nitro blue tetrazolium chloride (NBT) substrate. The bands related to Topo I and β-actin were scanned and quantified by NIH Image 1.62. The Topo I protein index was calculated as follows: Topo I Protein Index=(Topo I protein level/β-actin protein level)×100. The Tumor/Normal ratio of Topo I protein level was determined as follows: Tumor/Normal Topo I Protein Ratio=Topo I Protein Index of the tumor/Topo I Protein Index of its matched normal mucosa.

RESULTS

Sensitivity of ESCC cell lines to CPT-11 To evaluate the therapeutic efficacy of CPT-11 for ESCC, growth inhibition assay was performed with 14 ESCC cell lines, and the IC_{50} values of SN-38, an active metabolite of CPT-11, were measured. The IC_{50} values ranged from 4.38 to 258.93 ng/ml with a mean±SD value of 56.35±67.88 ng/ml. We decided the cut-off value for judging sensitivity to CPT-11 as 28 ng/ml, based on one clinical study.[25] Among the IC_{50} values for the 14 ESCC cell lines, 6 (42.9%) were within this cut-off value of SN-38 (Fig. 1). This may suggest that CPT-11 can be an effective agent for ESCC. In addition, 5 of 10 primary tumor cell lines and one of 4 metastatic tumor cell lines were considered susceptible to CPT-11. The mean±SD IC_{50} values of the primary tumor cell lines and the metastatic tumor cell lines were 60.22±80.67 and 46.68±29.68 ng/ml, respectively, and there was no significant difference between these 2 groups by the Mann-Whitney *U* test. Therefore, CPT-11 was considered to be effective for the treatment of both primary and metastatic ESCC tumors.

Relationship between Topo I levels and sensitivity of ESCC cell lines to CPT-11 To examine whether Topo I levels can be an indicator of the sensitivity of ESCC to CPT-11, Topo I mRNA levels and protein levels in the 14 cell lines were quantified. To analyze the levels of Topo I mRNA expression, Northern blot analyses were performed (Fig. 2A) and the densities of the bands related to Topo I and GAPDH were analyzed as described in "Materials and Methods." Topo I mRNA Index values ranged from 19.10 to 85.60 with a mean±SD value of 51.24±17.87. The mean±SD values of primary and metastatic lesions were 55.44±17.80 and 40.76±15.11, respectively, with no significant difference by the Mann-Whitney *U* test. No correlation was observed between the IC_{50} values and Topo I mRNA Index values of the 14 ESCC cell lines (Fig. 2B).

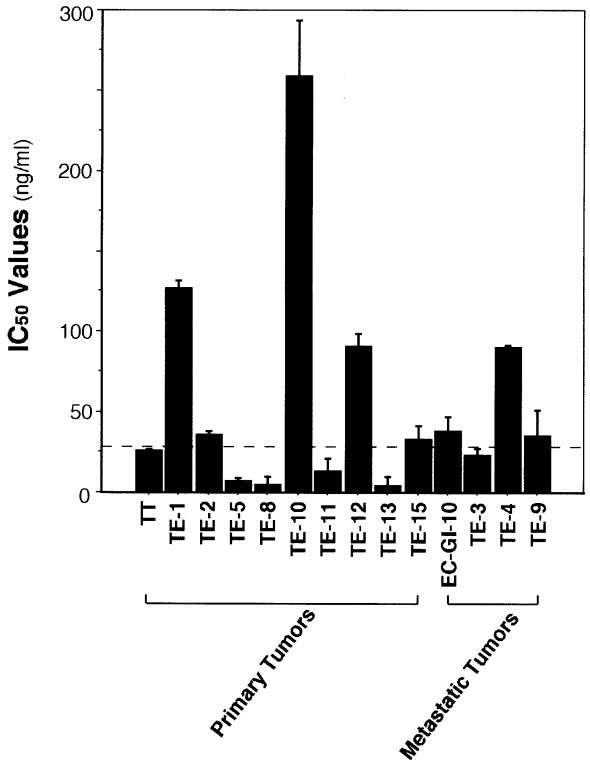

Fig. 1. The IC_{50} values of 14 ESCC cell lines for SN-38, an active metabolite of CPT-11, were measured by growth inhibition assay. Suspensions of 2.5×10^4 cells/ml were exposed to various concentrations of SN-38 for 72 h, and the anti-proliferative effects were analyzed by MTT assay. The IC_{50} values of SN-38 (mean±SD) showed that 10 ESCC cell lines, 5 of 10 established from primary ESCC lesions and one of 4 from metastatic lesions, were within the cut-off value of SN-38 (28 ng/ml, shown with the broken line).

These results suggest that Topo I mRNA expression can not be used as an indicator of sensitivity to CPT-11.

The levels of Topo I protein expression were analyzed by western blot (Fig. 3A), and the densities of the bands due to Topo I and β-actin were analyzed as described in "Materials and Methods." Topo I Protein Index values of the 14 ESCC cell lines ranged from 32.15 to 101.59 with a mean±SD of 59.36±19.40. The mean±SD values of primary and metastatic lesions were 56.42±22.24 and 66.72±6.68, respectively, and showed no significant correlation by the Mann-Whitney *U* test. There was a weak inverse correlation between the IC_{50} values and Topo I Protein Index values (correlation coefficient=−0.556, P=0.0332: Fig. 3B). There was no relationship between Topo I mRNA Index values and Topo I Protein Index values of these ESCC cell lines (data not shown). These

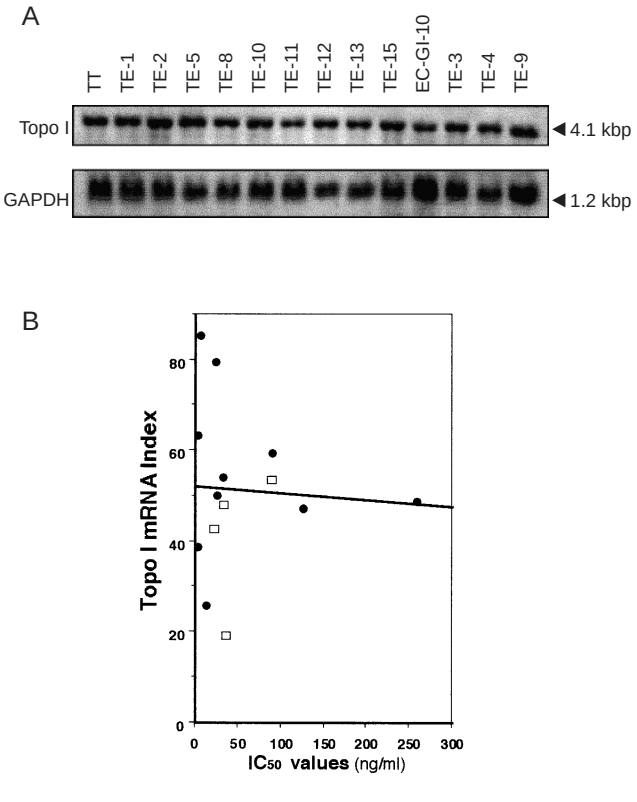

Fig. 2. Northern blotting of Topo I and GAPDH was performed using 14 ESCC cell lines (A). Total RNA (15 μg) extracted from each of 14 ESCC cell lines was separated in 1.2% agarose gels containing formaldehyde, transferred to a nylon membrane, and hybridized. The digoxigenin-11-UTP-labeled cDNA probe for Topo I was prepared as described in "Materials and Methods." Bands of Topo I and GAPDH were detected by chemiluminescence measurement using a lumino-image analyzer. Topo I was detected as a 4.1 kbp band, and GAPDH was detected as a 1.2 kbp band. These bands were analyzed and the Topo I mRNA Index was calculated as the ratio of Topo I to GAPDH mRNA levels (B). Topo I mRNA Index values ranged from 19.10 to 85.60, and the mean values of the Topo I mRNA Index of 10 primary tumor cell lines (●) and 4 metastatic tumor cell lines (□) were 55.44 and 40.76, respectively. These mean values showed no significant difference by the Mann-Whitney *U* test. Topo I mRNA Index values of these 14 cell lines showed no correlation with the IC_{50} values of SN-38.

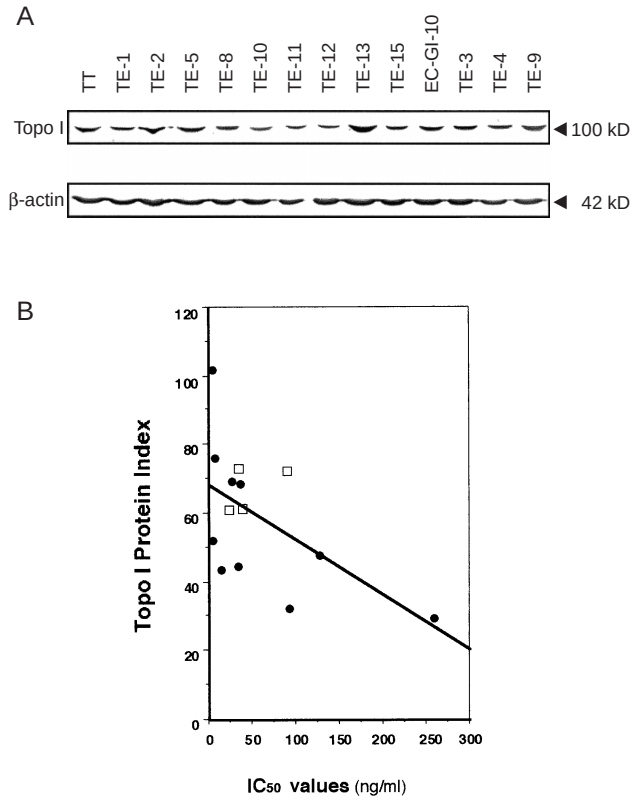

Fig. 3. Western blotting of Topo I and β-actin was performed using 14 ESCC cell lines (A). A 50 μg sample of protein extracted from each of the 14 ESCC cell lines was separated in 7.5% SDS-polyacrylamide gels, and transferred to PVDF membranes. Protein quantification was done by the Bradford assay. Anti-Topo I antibody (clone: T1P1R2, Sigma-Genosys, Ltd.) was used at 1:1000 dilution, and anti-β-actin antibody (clone: AC-15, Sigma) at 1:5000. Bands of Topo I and β-actin were detected by development with alkaline phosphatase. Topo I was detected as a 100 kDa band, and β-actin was detected as a 42 kDa band. These bands were analyzed and the Topo I Protein Index was calculated as the ratio of Topo I to β-actin protein levels (B). Topo I Protein Index values ranged from 32.15 to 101.59, and the mean values of the Topo I Protein Index of 10 primary tumor cell lines (●) and 4 metastatic tumor cell lines (□) were 56.42 and 66.72, respectively. These mean values showed no significant difference by the Mann-Whitney *U* test. Topo I Protein Index values of these 14 cell lines showed a weak inverse correlation with the IC_{50} values of SN-38 (correlation coefficient=−0.556, P=0.0332).

results suggest that elevated Topo I protein expression results in increased DNA damage by CPT-11, and that Topo I protein levels can be an important indicator of sensitivity to CPT-11.

Difference in Topo I protein levels between human ESCC specimens and normal tissues To examine whether Topo I expression is increased in ESCC tumor lesions, Topo I protein levels in ESCC specimens and their normal mucosae were examined (Fig. 4A). For 40 ESCC specimens and their matched normal mucosae, the Tumor/Normal Topo I Protein Ratios were calculated and the values are summarized in Fig. 4B. In 37 ESCC patients without pre-operative therapy, 31 showed 1.2–22.3-fold increases in Topo I protein levels compared to the normal mucosa from the same patient. For 6 patients without pre-operative therapy, Topo I protein levels in the ESCC specimens exhibited 1.5–14.1-fold decreases compared to the corresponding normal esophageal mucosae. In 2 patients who had had pre-operative chemotherapy and one patient who had received pre-operative radiotherapy, the ESCC specimens exhibited increased Topo I protein levels compared to the corresponding normal mucosae. As a result, the majority of the ESCC specimens showed increased Topo I protein levels compared to matched normal mucosae. Because elevated Topo I protein expression could indicate greater potential therapeutic efficacy of CPT-11, these results suggest that CPT-11 may provide effective therapy for ESCC tumors. CPT-11 may be a feasible second line anti-cancer drug for the treatment of ESCC.

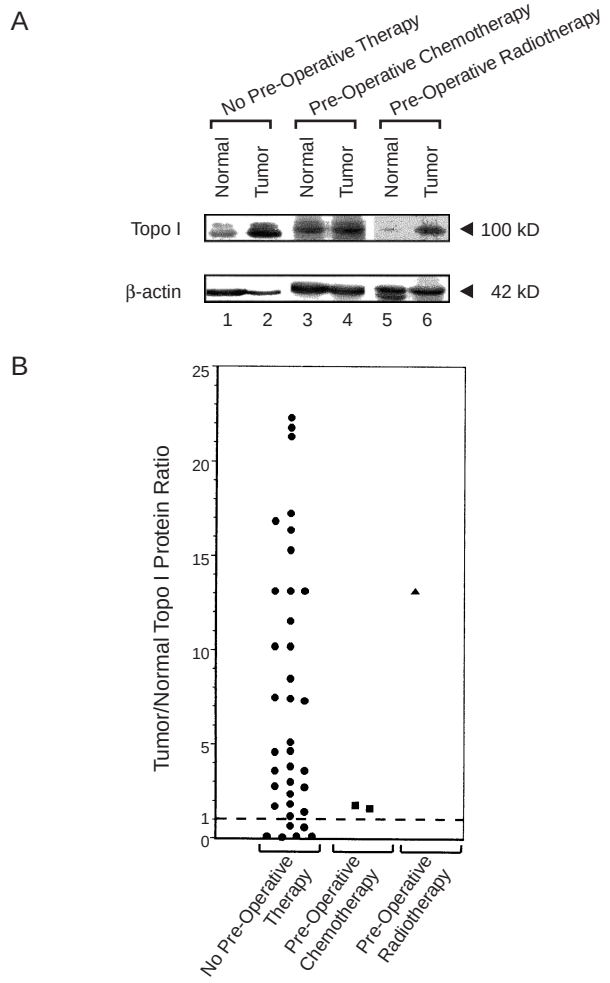

Fig. 4. Analysis of Topo I protein levels in ESCC specimens was performed by western blotting as described before. Forty surgically resected ESCC specimens and matched normal mucosae were analyzed; among the 40 specimens, 2 were from patients who had received pre-operative chemotherapy (lanes 3, 4) and one from a patient who had received radiotherapy (lanes 5, 6). Tumor/Normal Topo I Protein Ratios are the ratio of tumor to normal Topo I Protein Index values (B). In 37 patients without pre-operative therapy (●), 31 showed 1.2–22.3-fold increases and 6 showed 1.5–14.1-fold decreases in Topo I protein levels in the ESCC tumor lesions.

DISCUSSION

CPT-11, a new anti-cancer drug derived from camptothecin, is reported to be effective for treating various cancers both experimentally and clinically. For ESCC, one clinical trial using CPT-11 and CDDP has been performed, and the treatment was reported to be effective.[26] Herein, we examined the therapeutic efficacy of CPT-11 for ESCC experimentally. Evaluating the results of the growth inhibition assay, we determined the cut-off value of SN-38 as 28 ng/ml. A clinical study showed that the peak plasma concentration (C_{MAX}) of SN-38 reaches 50 ng/ml and the area under the curve (AUC) is 667 ng·h/ml, when CPT-11 is administered at a dose of 165 mg/m^2.[25] It was reported that the cytocidal effect of SN-38 was manifested soon after administration and reached a plateau level at 24 h.[27] Therefore, we consider this cut-off value suitable.

For chemotherapy of ESCC, CDDP-based systemic combination therapy is commonly performed, and CDDP is a major anti-cancer drug for the treatment of ESCC. However, the response rate is only 6–37%.[28–30] Growth inhibition assay showed that CPT-11 was effective against 6 (42.9%) ESCC cell lines, which included not only primary tumors, but also metastatic tumors. Therefore, CPT-11 could be an effective anti-cancer drug for ESCC.

Topo I protein level could be one indicator of sensitivity of ESCC to CPT-11, since tumors with high Topo I protein levels appeared to be more sensitive to CPT-11 treatment. SN-38, an active metabolite of CPT-11, selectively inhibits Topo I by stabilizing the Topo I-DNA cleavable complex,[12] which results in cell death. The cytotoxicities of camptothecin and its derivatives are S-phase-specific[31] and time-dependent.[13, 14] This is because the cleavable

complex must remain stable until the broken single-strand DNA is converted into a DNA double-strand break upon collision with the replication fork. The reason why SN-38 shows strong cytotoxic potency compared to camptothecin is because the SN-38-induced cleavable complex is more stable and shows a slower rate of reversal as the drug concentration decreases during the course of the metabolism and excretion.[32] Therefore, elevated Topo I protein levels can increase the number of cleavable complexes and cause increased DNA damage.[33] In colorectal cancers, elevated Topo I expression has been reported to be related to increased sensitivity to CPT-11.[15, 16] The results presented here suggest that the same could be true for ESCC.

There is a good relationship between the Topo I protein level and catalytic activity,[18] and the Topo I protein level reflects the catalytic activity to break and rejoin single DNA strands. On the other hand, it has been reported that the levels of Topo I mRNA rise when growth-arrested HeLa 299 cells are stimulated by serum addition,[34] or when HeLa cells are infected with adenovirus-5.[35] However, the Topo I protein levels and catalytic activities remain constant. These reports show that increases in Topo I mRNA levels are not always accompanied by corresponding increases in Topo I protein and catalytic activity. This is because Topo I protein synthesis can be regulated at the levels of both transcription and translation. In this study, there was no relationship between Topo I mRNA levels and protein levels, and Topo I mRNA levels showed no correlation with the sensitivity to CPT-11. Therefore, evaluation of Topo I protein levels may be suitable for examination of the expression and activity of Topo I.

In ESCC patients, Topo I protein levels in the tumor lesions were higher than in normal tissues of the same patients. As a result, CPT-11 could provide selective therapeutic efficacy against ESCC tumors experimentally. Moreover, increased Topo I protein levels were observed in tumors after chemotherapy or radiotherapy. In this study, the number of patients who received pre-operative therapy was small. However, the results suggest that CPT-11 could be useful as a second line anti-cancer drug, and further study would be worthwhile.

Our results suggest that CPT-11 could be an effective anti-cancer drug for both primary and metastatic ESCC tumors. Moreover, by comparing the Topo I protein expression levels of ESCC tumors and ESCC cell lines, the sensitivities of ESCC patients to CPT-11 could be predicted before treatment. However, these results were obtained only from the viewpoint of the effect on Topo I. Naturally, sensitivity to CPT-11 is also regulated by various other factors. More indicators of the sensitivity to CPT-11 should be examined. The use of combinations of anti-cancer drugs with CPT-11 may make it possible to improve the poor prognosis of ESCC.

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