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No Alteration in DNA Topoisomerase I Gene Related to CPT-11 Resistance in Human Lung Cancer

Nobuyuki Ohashi,[1] Yasuhiro Fujiwara,[1,2,4] Naoki Yamaoka,[1] Osamu Katoh,[3] Yukio Satow[3] and Michio Yamakido[1]

[1]Second Department of Internal Medicine and [2]Department of Integrated Medicine, Hiroshima University School of Medicine, and [3]Department of Environment and Mutation Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734

Mutations and reduced expression of DNA topoisomerase I (topo I) gene have been shown to be important in the acquisition of resistance to camptothecin and its analogues *in vitro*, but their significance has not been verified *in vivo*. We investigated possible topo I gene mutations and topo I mRNA expression levels in 127 samples obtained from 56 patients with lung tumors, including patients who had developed clinical resistance to topo I inhibitors. No mutations were detected in any of the samples examined and expression levels did not differ significantly between clinically resistant cases and others. However, the topo I mRNA expression level was significantly higher in small cell lung carcinomas than in non-small cell lung carcinomas ($P<0.05$). These results suggest that topo I mRNA levels may affect CPT-11 sensitivity in human lung cancer. However, topo I gene mutations and reduced topo I mRNA expression may not be the main mechanism of clinically acquired resistance to camptothecin analogues *in vivo*.

Key words: Topoisomerase I — Point mutation — mRNA expression — CPT-11 — Lung cancer

One of the main reasons for the failure of cancer chemotherapy is the development of resistance to antitumor agents. In order to overcome this problem, it is necessary to elucidate the mechanisms of clinical (*in vivo*) drug resistance. CPT-11, a CPT derivative, is a novel antitumor agent that has been shown to have broad spectrum anti-cancer activity in multidrug-resistant tumor models and is a very promising new agent against several human malignancies. DNA topoisomerase I (topo I) is an intracellular target of CPT and its analogues, which inhibit topo I activity by stabilizing the topo I-DNA cleavable complex, eventually causing single-strand DNA breaks, leading to cell death.

Recent studies have clarified several mechanisms of the acquisition of resistance to CPT and its analogues *in vitro*: (a) resistance mediated by a point mutation of the topo I gene, (b) resistance due to a low topo I expression level, (c) reduction of cellular uptake of CPT, and (d) decreased metabolic activation of CPT-11 to SN38.[1,2] However, to our knowledge, no study demonstrating any specific cause of clinical drug-resistance to CPT-11 or other topo I inhibitors has been published. Previous analyses of clinical samples, demonstrating that topo I expression levels in colorectal and prostate, but not kidney, tumor tissues were elevated compared to corresponding normal tissues, suggest that topo I expression levels may reflect tumor sensitivity to topo I inhibitors.[3,4] However, as far as we are aware, the analysis of topo I expression in clinically resistant samples has not been reported. Therefore, in the present study, we analyzed samples obtained from patients with lung cancer, including those showing clinical resistance to CPT analogues, in an attempt to determine whether the tumors expressed topo I and whether point mutations of the topo I gene were present.

MATERIALS AND METHODS

Antitumor agents CPT-11 and topotecan were supplied by Daiichi Seiyaku Co., Ltd. (Tokyo), and Smith Kline Beecham Seiyaku Co., Ltd. (Tokyo), respectively.

Tumor samples and cell lines One-hundred-and-twenty-seven samples were obtained from 56 Japanese lung cancer patients, including three with mesotheliomas, who were admitted to Hiroshima University Hospital from January 1992 to October 1995 (Table I). Seventy-three fresh specimens of primary lung tumors, metastatic lesions and normal tissues (lung or liver) were obtained at autopsy from 33 patients (15 adenocarcinomas, nine small cell carcinomas, five squamous cell carcinomas, three mesotheliomas and one large cell carcinoma). Seven samples of primary lung tumors were obtained during diagnostic fiberoptic bronchoscopy from seven of 33 autopsied patients. Twenty-one tumor samples obtained during fiberoptic bronchoscopy for diagnostic pur-

[4] To whom correspondence should be addressed.

The abbreviations used are: CPT, camptothecin; CPT-11, 11,7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction; SCLC, small cell lung cancer; SSCP, single-strand conformation polymorphism; topo I, DNA topoisomerase I.

Table I. Characteristics of Patients

No. of patients	56
Sex (male: female)	44 : 12
Age (years)	
Median	64.7
Range	44–82
Clinical stage	
I	4
II	1
IIIA	5
IIIB	8
IV	38
Histological type	
Adenocarcinoma	21
Squamous cell carcinoma	17
Small cell carcinoma	13
Mesothelioma	3
Large cell carcinoma	1
Adenoid cystic carcinoma	1

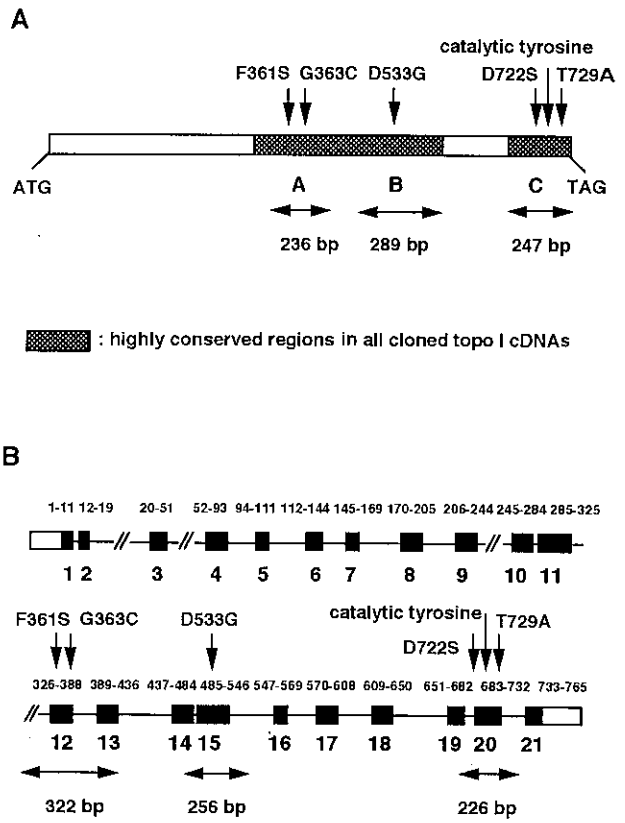

Fig. 1. Regions of topo I cDNA and the topo I gene amplified and subjected to PCR-SSCP analysis. In A, three fragments within the coding regions of topo I cDNA (Fragments A, B, and C) are indicated by bars with arrows at both ends, and their nucleotide lengths are indicated under the bars. ATG and TGA indicate the initiation and termination codons, respectively, of the reading frame of the topo I cDNA. In B, the three regions within the twenty-one exons of topo I genomic DNA (exons 12, 15, and 20) are indicated by bars with arrows at both ends, and their nucleotide lengths are indicated by solid boxes with amino acid positions, while noncoding regions are indicated by open boxes. In both A and B, the locations of point mutations topo I cDNA reported in human camptothecin-resistant cell line are indicated by vertical arrows.

poses from 20 live patients (12 squamous cell carcinomas, four small cell carcinomas, three adenocarcinomas and one adenoid-cystic carcinoma) were also used in this study. Three samples taken by thoracentesis from three adenocarcinomas that were diagnosed cytopathologically as class V were also used. Twenty-three peripheral blood samples that had been taken for routine laboratory testing were used for extracting normal (control) DNA/RNA when we analyzed the samples from fiberoptic bronchoscopy and thoracentesis. All the patients gave their informed consent before tissue samples were taken. Immediately after having been taken, the tissues were frozen in liquid nitrogen and stored at −80°C until analyzed. Hematoxylin and eosin-stained sections of the autopsy specimens and biopsy samples obtained during fiberoptic bronchoscopy were used to identify those samples containing mainly tumor cells.

The chemotherapy regimens prescribed to the patients analyzed in the present study were carboplatin (8 cases), carboplatin-etoposide (7 cases), cisplatin-etoposide (6 cases), cisplatin-CPT-11 (5 cases), cisplatin-vindesine (4 cases), etoposide (3 cases), cyclophosphamide-adriamycin-etoposide (2 cases), CPT-11 (2 cases), taxol (2 cases), topotecan (1 case) and best supportive care (4 cases).

Three CPT- or CPT analogue-resistant human cancer cell lines and their parental cell lines were used as positive and negative controls, respectively, for topo I gene mutation detection. The CPT-11-resistant cell line PC-7/CPT[5] and its parental cell line PC-7 were kindly provided by Dr. N. Saijo of the National Cancer Center Research Institute. The CPT-resistant cell line CPT/K5[6] and its parental cell line RPMI 8402 were kindly provided by Dr. K. Okada of Hiroshima University. The CPT-resistant cell line CEM/C2[7] and its parental cell line CEM, which were also used as controls for topo I mRNA quantitation, were kindly provided by Dr. W. G. Harker of the University of Utah.

DNA and RNA preparation, reverse transcriptase reaction Genomic DNA and total cellular RNA were prepared from frozen tissues as described previously.[8,9] cDNA was synthesized using random hexamer (Amersham, Tokyo) with Superscript RNase H- reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), as described previously.[10]

PCR-SSCP analysis To detect topo I gene mutations in human samples, we designed and synthesized new intron-based PCR primers, with the assistance of Advanced Gene Technology Inc.,[11] based on the sequences obtained from Genbank.[12] In the present study, we attempted to analyze the mutation sites in exons 12, 15 and 20 which were found in several human cancer cell lines (Fig. 1).[13–17] The sequences of the primers used were:

exon 12,	sense	5′-CAAGAGATAAAATGCATTAG-TAGAGAAC-3′
	antisense	5′-CCAAGTACAATAAAGGACGC-CC-3′
exon 15,	sense	5′-GTCTCTTCCATTCATGCTCAT-C-3′
	antisense	5′-GTCAGACAGTACAGGTACAT-TAAC-3′
exon 20,	sense	5′-GGTACAGTGTGCTCTTGTCT-AG-3′
	antisense	5′-TAAGAGGCCTAACCTTACCA-AG-3′

Genomic DNA was subjected directly to PCR in a reaction mixture (10 μl) comprising 1.5 m*M* $MgCl_2$, 10 m*M* NaCl, 5 m*M* Tris HCl (pH 8.0), 0.8 m*M* deoxynucleotide triphosphate, 1 μCi [γ-^{32}P]dCTP (3000 Ci/mmol, Amersham), 10 pmol of each primer and 0.2 u of Taq DNA polymerase (Promega, Madison, WI). After pre-denaturation at 90°C for 5 min, amplification was carried out for 30 cycles of denaturation (94°C for 30 s), annealing (60°C, 30 s) and extension (72°C for 1 min), using a thermal cycler (GeneAmp PCR System 2400; Perkin Elmer, Norwalk, CO). After the final cycle, the reaction mixture was maintained at 72°C for 7 min. The PCR products with nucleotides 322, 256 and 226 base pairs long corresponded to exons 12, 15 and 20, respectively. A sample (2 μl) of each PCR product was diluted to 40 μl with denaturing loading buffer (95% formamide, 20 m*M* EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), heated at 90°C for 5 min and chilled on ice. Each product was analyzed under four separate sets of conditions, as follows: 2 μl of solution was applied to 5% polyacrylamide (49 : 1 acrylamide to *N,N′*-methylene-*bis*-acrylamide) gel containing 90 m*M* Tris borate (pH 8.3) and 4 m*M* EDTA with or without 10% glycerol and electrophoresed in 0.5× TBE buffer at 40 W for 2 to 6 h at 4°C or 20°C. The gels were dried on filter paper and autoradiographed using Hyperfilm MP film (Amersham) at −80°C for 3 to 24 h. The following cell lines were used as positive controls; CPT/K5 for exon 15 (Fig. 2A, lane 4); PC-7/CPT for exon 20 (Fig. 2B, lane 4); and CEM/C2 for exon 12 (Fig. 3, lane 6). Their respective parental cell lines were used as mutation-negative controls (Fig. 2A, lane 3; Fig. 2B, lane 3; and Fig. 3, lane 5). Abnormal bands with mobility shifts were detected in exons 12 and

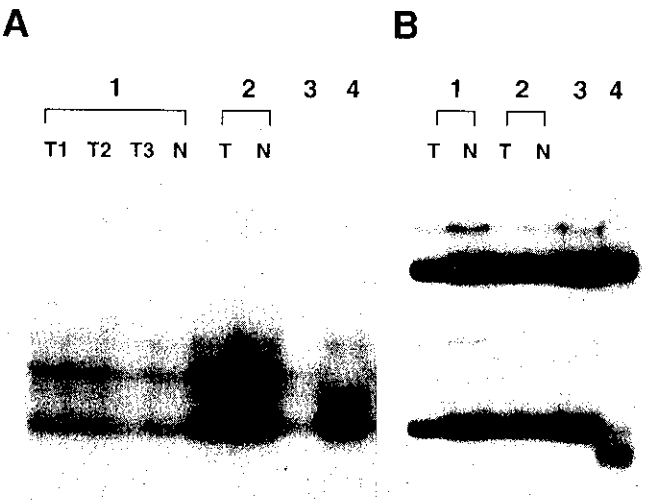

Fig. 2. PCR-SSCP analysis of topo I exon 15 and exon 20 in lung cancers. Genomic DNA from tumor tissues (indicated as T) and normal tissues of the same patient (indicated as N) were analyzed. Radiolabeled PCR products were subjected to SSCP analysis. Electrophoresis was carried out under cooling with a fan at 4°C for exon 15 (A) and at 20°C for exon 20 (B) in 5% polyacrylamide gel (49 : 1) containing 10% glycerol in 0.5×TBE buffer. Detection was by autoradiography. Sources of amplified DNA fragments in A; 6 clinical samples from two patients treated with CPT-11 (lanes 1 and 2 corresponding to cases 1 and 2 in Table II). Human T lymphoblastic leukemia cell line RPMI8402 served as a negative control (lane 3) and a camptothecin-resistant cell line from RPMI8402, CPT/K5, as a positive control (lane 4). T1 in lane 1 was obtained from the primary tumor at the time of diagnostic fiberoptic bronchoscopy, while T2 and T3 in lane 1 were obtained from a recurrent primary tumor and a liver metastatic tumor at autopsy. In B; four clinical samples from two patients treated with CPT-11 (lanes 1 and 2 corresponding to cases 3 and 4 in Table II). Human non-small cell lung cancer cell line, PC-7, served as a negative control (lane 3) and a CPT-11-resistant cell line from PC-7/CPT, as a positive control (lane 4).

15 of the CEM/C2 and CPT/K5 cell lines by electrophoresis at 4°C in a gel containing 10% glycerol. An abnormal band with a mobility shift in exon 20 of the PC-7/CPT cell line was detected by electrophoresis at 20°C in a gel containing 10% glycerol. Under other conditions, however, no abnormal bands were detected in the genomic DNAs extracted from the cell lines with topo I gene mutations. We also performed topo I cDNA analysis using the PCR-SSCP method as described previously.[17]

Direct sequencing Any abnormal bands detected by PCR-SSCP analysis were cut out from the gels and eluted into 1× TE buffer. Some of the normal bands at positions corresponding to the abnormal ones were also cut out and eluted. Each eluted DNA sample (1 μl) was

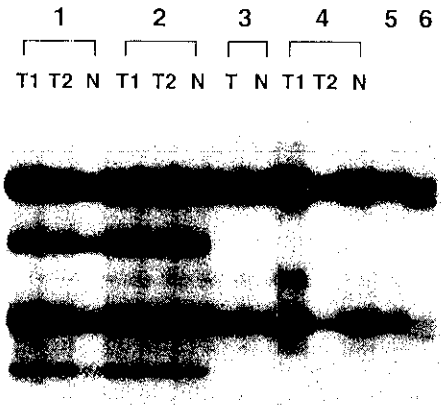

Fig. 3. PCR-SSCP analysis of topo I exon 12 in lung cancers. Genomic DNA from tumor tissues (indicated as T) and normal tissues of the same patient (indicated as N) were analyzed. Radiolabeled PCR products were subjected to SSCP analysis. Electrophoresis was carried out in 5% polyacrylamide gel (49 : 1) containing 10% glycerol in 0.5×TBE buffer at 4°C under cooling with a fan. Sources of amplified DNA fragments: eleven clinical samples from four lung cancers (lanes 1–4), including the two patients treated with CPT-11 or topotecan (lanes 3 and 4, corresponding to cases 5 and 6 in Table II). A human lymphoblastic leukemia cell line, CEM, served as a negative control (lane 5) and a camptothecin-resistant cell line from CEM, CEM/C2, as a positive control (lane 6). T1 and T2 in lane 1 were obtained from the primary tumor of an adenocarcinoma at the time of diagnostic fiberoptic bronchoscopy and at autopsy. T1 and T2 in lane 2 were obtained from the primary tumor and a liver metastatic tumor of a squamous cell carcinoma at autopsy. T1 in lane 4 was obtained from the primary tumor at the time of diagnostic fiberoptic bronchoscopy, while T2 was obtained from the recurrent tumor at autopsy.

subjected to PCR for reamplification using the same primer pairs and conditions as described above. The resulting PCR products were purified using a Gene Clean II kit (BIO 101, Vista, CA) and sequenced using a dsDNA Cycle Sequencing System kit (GIBCO BRL) with [γ-^{32}P]ATP (Amersham). Electrophoresis was performed using 6% polyacrylamide gel containing 7 *M* urea.

PCR for semi-quantitation of topo I mRNA Aliquots (5 μl) of reverse-transcribed cDNA were subjected to PCR amplification with primers based on the topo I and *β-actin* gene (internal control) sequences. After predenaturation at 90°C for 10 min, the cDNA was added to 45 μl of the reaction mixture described above, without [γ-^{32}P]dCTP. The sequences of the primers used were:

topo I, sense 5′-GGAGAGACCTGAAAAGTGCTAA-3′

antisense 5′-TAAATCTTCTCAATTGGGAC-3′

β-actin, sense 5′-TACATGGCTGGGGTGTTGAA-3′

antisense 5′-AAGAGAGGCATCCTCACCCT-3′

Ten to 40 amplification cycles of denaturation (94°C, 30 s), annealing (59°C, 30 s), and extension (72°C, 1 min), followed by a final incubation (72°C, 7 min) were carried out using a thermal cycler, and the optimal numbers of amplification cycles for quantitation of the topo I and *β-actin* gene PCR products were determined to be 24 and 20, respectively. The accuracy of the quantitative PCR was confirmed by titration experiments, as described previously.[18]

Semi-quantitation of PCR products After the required number of PCR amplification cycles, the PCR products were electrophoresed in 2% agarose gel, transferred to nylon membranes (Hybond N$^+$; Amersham) and hybridized with internally labeled topo I and β-actin oligonucleotides using the Multiprime DNA labeling system (Amersham). The associated radioactivity levels were determined by scanning with a Bio-Imaging Analyzer (BAS-2000; Fujix, Tokyo).

Analysis of topo I mRNA expression level The relative radioactivity of every topo I PCR product associated with both normal and tumor tissues was calculated by dividing the radioactivity of the topo I PCR product by that of the corresponding β-actin PCR product (internal control). The relative ratio of topo I mRNA expression in the tumor to normal tissue (topo I index) of each patient was calculated by dividing the relative radioactivity of the tumor tissue by that of the normal tissue. The topo I mRNA expression level in each patient, expressed as the topo I index, was examined in relation to the clinical characteristics.

Statistical analysis The unpaired and two-tailed Mann-Whitney's U-tests were used to compare the mean topo I index in cases exposed to CPT analogues with that in non-exposed cases, and the difference in the mean topo I index among histological types of lung tumors. Here, *P* values of less than 0.05 were considered to be significant. All the statistical analysis was performed using commercial software; StatView (Abacus Concepts Inc., Berkeley, CA).

RESULTS

A total of 127 samples were analyzed to detect topo I mutations. Eighty samples (40 primary tumor, 7 metastatic, 26 normal lung and 7 normal liver tissues) were obtained from 33 autopsied patients. Forty samples (20 primary tumors and 20 peripheral blood) were obtained from 20 patients during diagnostic fiberoptic bronchoscopy before starting treatment. One sample (squamous cell carcinoma) was obtained from a patient during

Table II. Cases Treated with CPT-11 or Topotecan

Case	Age	Sex	Histology	Regimen (response)	Sampling times
1	69	F	Sm	CPT-11+Cis (PR), CPA+ADM+VP×7 (PR)	Diagnosis & Autopsy
2	68	M	Sm	Carbo+VP (PR), Cis+VP (NC), CPT-11 (NC)	Autopsy
3	44	F	Ad	Carbo+VP (NC), Chest RT (NC), Topotecan×2 (NC)	Autopsy
4	60	M	Ad	Taxol (NC), CPT-11+Cis×2 (NC)	Autopsy
5	64	M	Sm	CPT-11+Cis×4 (CR)	Diagnosis & Autopsy
6	56	F	Sm	CPT-11+Cis×4 (PR), Carbo+VP (NE)	Diagnosis & Autopsy
7	65	M	Ad	CPT-11+Cis×2 (NC), Taxol (NC), VP (NC)	Autopsy
8	50	M	Meso	CPT-11×2 (NC)	Autopsy

Among 56 patients presented in Table I, eight patients received CPT analogues as anticancer drugs and their patients characteristics are presented here.
Abbreviations: Ad, adenocarcinoma; ADM, adriamycin; Carbo, carboplatin; Cis, cisplatin; CPA, cyclophosphamide; CPT, camptothecin; CR, complete response; NC, no change; NE, not evaluable; PR, partial response; RT, radiation; Sm, small cell carcinoma; VP, etoposide.

fiberoptic bronchoscopy for diagnosing tumor recurrence. Six samples (three tumors and three peripheral blood) were obtained from patients with pleuritis carcinomatosa. We were able to obtain tumor samples during fiberoptic bronchoscopy for both primary and recurrence diagnoses from one patient with small cell carcinoma.

The previously reported mutation sites in the amino acid sequence of human topo I cDNA are 360 Met, 361 Phe, 363 Asp, 533 Asp (CPT/K5), 722 Asn (CEM/C2) and 729 Thr (PC-7/CPT),[13-17] all of which exist in well conserved regions of cloned topo I cDNA in many species (Fig. 1A). CEM/C2 has a 370 Met mutation (T to C), which leads to a morphological change only, and appears to be unrelated to CPT resistance.[13] As shown in Fig. 1B, these mutation sites and catalytic tyrosine residues are present in exons 12, 15 and 20 of topo I genomic DNA.[12] In an attempt to elucidate the molecular pharmacological significance of these mutations, we investigated whether any point mutations existed in these three exons of the topo I gene in humans *in vivo*.

PCR-SSCP analysis No abnormal bands were detected in these exons in samples obtained from patients who were previously untreated or who had been treated with CPT-11, topotecan or other antitumor agents (Table II, Figs. 2 and 3). The patients presented in Table II received CPT analogues as anticancer drugs and became chemotherapy-refractory after several courses of therapy. Bands with abnormal mobility shifts were detected in both normal and tumor tissues from five patients by genomic DNA PCR-SSCP analysis of exon 12 (Fig. 3, lanes 1 and 2). Samples were obtained from two of these five patients during diagnostic examination with a flexible fiberoptic bronchoscope, from two at autopsy and from one during both diagnostic examination and at autopsy. Direct sequencing of topo I genomic DNA revealed an A to T substitution at a position in intron 11, 39 base pairs upstream of the 5′ terminus of exon 12. Genomic

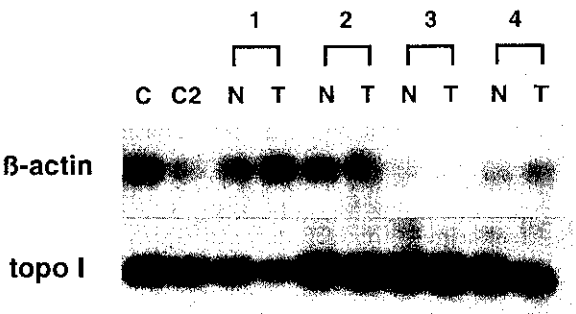

Fig. 4. RT-PCR autoradiographic findings for topo I mRNA (lane 1, CEM; lane 2, CEM/C2; lanes 3–6, cases 2–5 in Table II; N, normal tissue; T, tumor tissue). The PCR products were electrophoresed on 2% agarose gel, transferred to a nylon membrane and hybridized with internally labeled topo I and β-actin oligonucleotides. The associated radioactivity levels were determined by scanning with a Bio-imaging analyzer.

DNA-based PCR-SSCP analysis of exon 12 showed no other alterations in these five patients, nor was any abnormally shifted band detected using cDNA-based PCR-SSCP analysis (fragment A in Fig. 1A). Therefore, these results suggest that this structural alteration represents polymorphism. The histological types of tumor observed in these five patients were two squamous cell carcinomas, two adenocarcinomas and one mesothelioma.

Analysis of topo I mRNA expression level We used the quantitative RT-PCR procedure to compare the relative amounts of topo I mRNA in tumor and normal tissues, with β-actin mRNA as an internal control. The relative radioactivity associated with topo I mRNA expression level was quantified in a total of 27 tumor tissues obtained at autopsy from 27 out of 56 patients, as described

previously. The histological types of these tumors were 16 NSCLC (12 adenocarcinomas, 3 squamous cell carcinomas and 1 large cell carcinoma), 9 SCLC and 2 mesotheliomas. Seven of these 27 patients were clinically resistant to CPT-11 or topotecan (Table II). The PCR products with nucleotides 247 and 218 base pairs long, corresponding to topo I and β-actin cDNA, respectively, were quantified after Southern blot hybridization (Fig. 4). The ratio of topo I expression in the CEM/C2 cell line relative to that in the parental CEM cell line was 41%, which is in good accordance with the reported reduction in the topo I expression level in CEM/C2 compared with CEM found by northern blot hybridization.[7, 13] The relative ratio of the topo I expression level in tumor to normal tissues (topo I index) for all 27 patients ranged from 25 to 260% (mean, 110.4%). The mean topo I indices according to histological type were 94.6% in NSCLCs (85.8% in adenocarcinomas, 130.3% in squamous cell carcinomas and 92% in large cell carcinoma), 151.1% in SCLCs and 53.5% in mesotheliomas. We analyzed the differences in the topo I index between SCLCs ($n=9$) and NSCLCs ($n=16$), and the clinical profiles of the 7 clinically CPT-11- (or topotecan-) resistant patients (Table II), as well as the others who had not received CPT-11-based chemotherapy. The topo I indices according to the classification of NSCLC and SCLC ranged from 37 to 201% and 93 to 260%, respectively, and those according to clinical resistance ranged from 73 to 201% (mean, 124.3%), and 25 to 260% (mean, 104.5%) for CPT-exposed and other patients, respectively. The mean topo I index for SCLC was significantly higher than that for NSCLC ($P<0.05$, Fig. 5A), but the topo I index did not differ significantly between CPT-exposed and other patients (Fig. 5B). There was also no significant correlation between the topo I index and other clinical parameters; sex, age, prognosis, and total dose of administered antitumor agents (cisplatin, carboplatin, etoposide, and CPT-11).

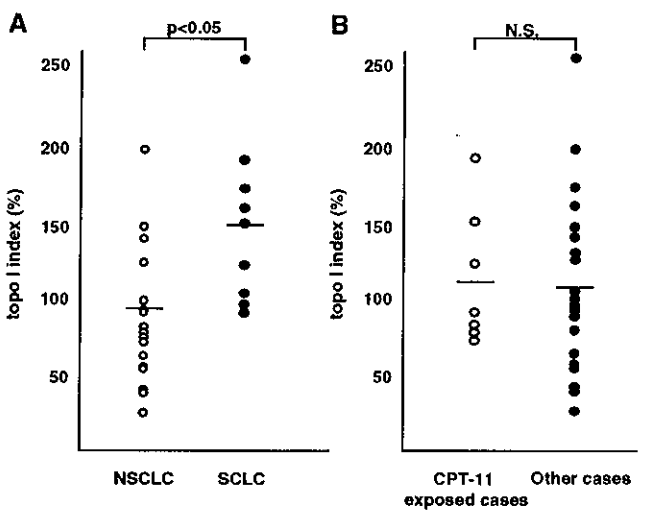

Fig. 5. The correlation of topo I mRNA levels and clinical profile. Topo I mRNA levels are expressed as topo I indices, which were estimated as the relative radioactivities of topo I cDNA compared to β-actin cDNA as an internal control in human tumors divided by those of matched normal samples. Topo I indices of NSCLC were compared with those of SCLC (A), and topo I indices of CPT-11-exposed cases (including one topotecan-exposed case) were compared with those of other cases (B). Bars denote mean topo I indices.

DISCUSSION

In the present study, no topo I gene mutations considered to be important for CPT resistance *in vitro* were detected in the investigated regions of topo I genomic DNA or cDNA. The relative expression level of the topo I mRNA did not differ significantly between CPT-11-resistant cases and other cases, and was significantly elevated in SCLC compared to NSCLC. This is, to our knowledge, the first report describing the significance of topo I gene mutations under clinical conditions and topo I mRNA expression in human lung cancer.

Point mutations of the topo I gene seem to play an important part in the acquisition of resistance to CPT and its analogues, as recombinant proteins with several kinds of base substitutions were found to be catalytically active and resistant to these drugs *in vitro*.[13–16] Moreover, all the reported mutation sites in CPT- and CPT analogue-resistant cell lines exist in highly conserved regions in different species. It has been hypothesized that the domains around these mutated sites form a pocket structure, which interacts with CPT and its analogues and forms part of the catalytic site for strand passage and/or religation reaction.[19] Therefore, the existence of a topo I mutation that causes conformational change of the topo I enzyme and prevents the drug binding to it seems a likely mechanism for conferring drug resistance *in vivo*. However, we detected no similar mutations in clinical samples, which suggests that topo I gene mutation may not represent the main mechanism or initial step in the development of clinical resistance to CPT and its analogues in the human body.

It is possible that our study failed to detect mutations (false-negative cases). The sensitivity of SSCP analysis can be changed easily by altering several factors in the experimental conditions. However, we used several different sets of conditions in order to reduce the false-negative rate.[20] Moreover, we sequenced all the topo I genomic DNAs extracted from samples from the clinically CPT analogue-resistant patients (Table II) and detected no mutations (data not shown).

Several previous reports have shown that topo I mRNA expression is one of the factors regulating *in vitro* CPT resistance. We analyzed the relative topo I mRNA expression levels in tumor samples, using the RT-PCR method, to elucidate the relationship between topo I expression levels and the clinical profile of each patient. Levels in CPT-11-resistant and other patients did not differ significantly, suggesting that resistance due to reduced topo I mRNA expression levels in tumor cells may not reflect clinically acquired resistance to CPT-11 in clinical samples from patients with lung cancer. However, tumor samples obtained before, during, and after CPT-11-based chemotherapy must be analyzed to monitor the changes in topo I expression level in order to establish whether there is a relationship between clinical drug resistance and mechanisms of CPT-resistance *in vitro*.

It is noteworthy that the topo I expression levels in the samples analyzed varied widely and that the mean level in SCLCs was significantly higher than that in NSCLCs. This suggests that increased topo I mRNA expression levels may be one of the reasons why SCLC are more sensitive to CPT-11 than NSCLCs, as has been demonstrated with colorectal and prostate tumors,[3,4] although the cytotoxic thresholds of topo I poisons have not been evaluated and it is possible that the low topo I mRNA expression in NSCLC tissue may be due to contamination of tumor tissue with stromal cells, which are scarce in SCLC tissue. Moreover, the widely varying ranges of topo I expression levels in both SCLCs and NSCLCs may be due to intrinsic resistance or responsiveness of human lung cancer to topo I poisons, in accordance with the suggestion that the responsiveness of tumor cells to CPT-11 depends on the steady-state level of topo I mRNA in the cells.[21] The estimation of topo I expression levels in tumor biopsy specimens before chemotherapy may be a useful means of predicting the sensitivities of lung cancers to CPT-11 and related agents. The estimation of these levels by the RT-PCR method used in the present study should be useful for analyzing small samples, such as lung tumor biopsy specimens obtained during diagnostic fiberoptic bronchoscopy.

Our results show the importance of research on clinical samples, since experimentally important factors are not necessarily also important in clinical situations. In addition, we should be cautious in extrapolating basic research data obtained using established drug-resistant cell lines *in vitro* to clinical conditions *in vivo*. In order to extend the present study, we are performing clinical pharmacological evaluations[22] with the aim of elucidating other mechanisms of clinical resistance to CPT analogues.

We found an unreported polymorphism of the topo I gene (A to T substitution) in intron 11, 39 nucleotides upstream of the 5′ terminus of exon 12, in five out of 56 patients with lung tumors. It would be worthwhile to analyze samples from healthy Japanese subjects to establish the incidence of this polymorphism in the normal population.

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