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Gene Mutation Analysis and Quantitation of DNA Topoisomerase I in Previously Untreated Non-small Cell Lung Carcinomas

Hiroshi Takatani,[1] Mikio Oka,[1,3] Minoru Fukuda,[1] Fumihiko Narasaki,[1] Reiji Nakano,[1] Koki Ikeda,[1] Kenji Terashi,[1] Akitoshi Kinoshita,[1] Hiroshi Soda,[1] Tetsuro Kanda,[1] Erasmus Schneider[2] and Shigeru Kohno[1]

[1]*Second Department of Internal Medicine, Nagasaki University School of Medicine, 7-1-1 Sakamoto, Nagasaki 852 and* [2]*Division of Molecular Medicine, Wadsworth Center for Laboratories and Research, Albany, NY, USA*

To elucidate whether gene alterations of topoisomerase I (topo I) exist in untreated non-small cell lung carcinomas (NSCLC), polymerase chain reaction-single strand conformation polymorphism analysis was performed in forty-four NSCLC tissue samples. Gene alterations of topo I were sought in three regions, near codons 361 and 363, 533, and 722 and 729, where point mutations have been found in resistant tumor cell lines selected by chronic camptothecin exposure. In addition, nuclear topo I contents were determined by immunoblotting. No mobility shifts were observed compared to the pattern observed in a normal control at any of the three regions in any sample, whereas topo I levels showed an approximately 12-fold variation. The variation is remarkably large compared to those seen in previous *in vitro* and *in vivo* studies. The results suggest that mutations of topo I may not contribute to intrinsic resistance of NSCLC to camptothecins, but low topo I levels may account, at least in part, for the resistance.

Key words: Lung cancer — Topoisomerase I — Drug resistance — Camptothecin — Gene mutation

Topoisomerases (topos) are nuclear enzymes controlling the topological states of DNA by catalyzing the concerted breaking and rejoining of DNA strands. The enzymes are involved in many crucial cellular processes including replication, transcription, and recombination.[1–3] Topo I, one of two major types of topo in eukaryotic cells, relaxes DNA supercoiling by making single-strand breaks.[1–3] These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed cleavable complex.[1–3] Increased expression of topo I in tumor cells, compared to normal cells, could provide selective therapeutic cytotoxicity of drugs directed against topo I.[2–4] *In vitro* topo I inhibitors, such as camptothecin and its derivatives, interfere with the breakage-rejoining reaction of topo I by trapping the cleavable complex.[2,5] However, the precise mechanisms of tumor cell death as a result of topo I-DNA complex accumulation are not well understood.[2,3]

Camptothecins alone or in combination with other drugs such as cisplatin are clinically effective against several cancers including lung cancer.[6–11] However, about a half of non-small cell lung carcinomas (NSCLC) intrinsically show resistance to combination chemotherapy with these drugs.[9] Resistance mechanisms to these drugs that have been characterized *in vitro* include reduced topo I levels, drug-resistant forms of topo I due to gene mutations, decreased catalytic activity, decreased intracellular drug accumulation, overexpression of the multidrug resistance gene, lengthened cell cycle time and altered DNA repair functions.[1–3,5,12] These mechanisms may explain acquired resistance in tumor cells, but it is unknown whether they are also responsible for intrinsic resistance. In the present study, we examined whether *topo I* gene mutations, such as have been found in camptothecin-selected resistant tumor cell lines,[13–17] exist in untreated NSCLC. In addition, levels of nuclear topo I were determined.

MATERIALS AND METHODS

Cell line and reagents Cell culture medium RPMI 1640 and fetal calf serum (FCS) were obtained from Gibco BRL (Grand Island, NY). All other chemicals not specifically mentioned were purchased from Sigma (St. Louis, MO). A human lung adenocarcinoma cell line, NCI-H358, was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, and 80 μg/ml kanamycin sulfate in a humidified incubator (5% CO_2 at 37°C).

Tumor tissues Surgical tissue samples from 44 patients with NSCLC, 22 adenocarcinomas, 20 squamous cell carcinomas and two large cell carcinomas, were obtained in Nagasaki University Hospital. The characteristics of these patients are shown in Table I. None of the patients had received preoperative chemotherapy. The samples

[3] To whom correspondence should be addressed.

were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. The identities of all tissues were confirmed microscopically, and all specimens were free of necrosis and damage caused during preparation or storage.

Reverse-transcriptase polymerase chain reaction (RT-PCR) and sequencing Total RNA from cells and tissues was extracted by using a guanidine isothiocyanate method.[18] After reverse transcription of 0.5 μg of total RNA, target sequences of the human *topo I* gene were amplified for 30 cycles with specific primers following a protocol recommended by the manufacturer (RNA PCR Kit; Takara Shuzo, Shiga). A 10 μl aliquot of each PCR product was separated on a 1.5% agarose gel, which was stained with ethidium bromide and photographed. In the case of H358 cells alone as a normal control, the DNA fragments purified from the PCR products were sequenced following the ABI protocol for Taq-dye terminator cycle sequencing on an automated ABI 373A sequencer (Applied Biosystems, Foster City, CA).

The primers were designed to amplify three regions of the human *topo I* gene, according to D'Arpa *et al.*[19] The target sequences contained the five mutation sites at codons 361,[13] 363,[14] 533,[15] 722,[16] and 729,[17] that have been found in camptothecin-resistant tumor cells. The names and nucleotide sequences of the primers, and the lengths of the amplified DNA fragments are listed in Table II.

Single strand conformation polymorphism (SSCP) analysis For SSCP analysis the Pharmacia "PhastSystem" was used.[20,21] The above PCR products were mixed with an equal volume of denaturing buffer (98% formamide, 0.05% xylane cyanol, 0.05% bromophenol blue) and denatured at 95°C for 5 min. Then, the denatured products were chilled on ice to stabilize single-stranded DNA and separated on precast polyacrylamide gels (PhastGel homogenous 12.5, 20; Pharmacia Biotech, Uppsala, Sweden) using PhastGel native buffer strips (Pharmacia Biotech) and the PhastSystem according to the conditions recommended by the manufacturer. The gels were transferred to the coloration unit of the PhastSystem and silver-stained following the instructions of the manufacturer (Pharmacia Biotech).

Preparation of nuclear extract Nuclei were isolated according to the method reported by Bender.[22] All steps were performed on ice, and about 500 mg of each tissue sample and the cell pellet of H358 cells were used. One milliliter of TBS (pH 7.4, 137 m*M* NaCl, 25 m*M* Tris-EDTA) containing protease inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, 40 μ*M* pepstatin, 2 m*M* PMSF) was added to the samples in a homogenizer. The homogenized lysate was transferred into a 15 ml conical tube and centrifuged for 5 min at 500*g*. Then, 5 ml of NP-40 lysis buffer (10 m*M* Tris, pH 7.4, 10 m*M* NaCl, 3 m*M* $MgCl_2$, 0.5% NP-40) containing protease inhibitors was added dropwise to the pellet under gentle vortexing. After a 5-min incubation, the cell suspension was re-centrifuged and the pellet resuspended in the NP-40 lysis buffer. The above step was repeated, and the pellet (nuclei) was resuspended in TBS and lysed in 2×sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (125 m*M* Tris-HCl, 4 m*M* EDTA, 30% sucrose, 20% glycerol, 6% SDS, 10% 2-mercaptoethanol). The extracts were denatured by boiling for 10

Table I. Characteristics of Patients

Total no. of patients	44
Sex	
Male	35
Female	9
Median age (range)	67 (43–84)
Histologic type[a]	
adenocarcinoma	22
squamous cell carcinoma	20
large cell carcinoma	2
Pathological stage[a]	
I	24
II	3
IIIa	15
IIIb	2

a) Histologically confirmed lung cancer was classified according to the World Health Organization histologic classification, and the International Union Against Cancer staging system.

Table II. Primer Positions and Sequences

Primer	Position[a]	Sequence
T-3	1145–1166	5′-CAGACGGAAGCTCGAAACAGA-3′
	1459–1438	5′-CCAGGAAACCAGCCAAGTAACC-3′
T-5	1758–1779	5′-CAGAGTTGGATGGTCAGGAATA-3′
	1900–1880	5′-ATCATCCTCGGGCTGCTTGTT-3′
T-7	2238–2259	5′-AGGATGCAAAGACGAAGAAGGT-3′
	2577–2556	5′-GGCTCAGTTTATCCATCTTTCC-3′

a) Numbered according to D'Arpa *et al.* (Ref. 19).

min, and protein concentration was measured by the method of Bradford (Bio-Rad Protein Assay Kit; Bio-Rad, Hercules, CA).

Immunoblotting SDS-PAGE was performed according to the procedure of Laemmli.[23] One hundred micrograms of nuclear extract was loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were incubated in 10% skimmed milk in TBST (20 m*M* TBS with 0.5% Tween 20) for 1 h at room temperature. After blocking, the membranes were incubated with human anti-DNA topo I polyclonal antibody (TopoGEN Inc., Columbus, OH) diluted in TBST for 1 h at room temperature. Then, the membranes were washed with TBST and incubated with alkaline phosphatase-conjugated goat anti-human IgG (BioRad) for 1 h at room temparature. After additional washing, bands were developed using an alkaline phosphatase conjugate substrate kit (BioRad). The density of each band was measured on an image analyzer (MCID Image Analyzer; Fuji Film Co., Kanagawa) and compared to that from H358 cells in each immunoblot. The relative topo I level in each sample was calculated by use of the following formula:

$$\frac{\text{Density of band in sample}}{\text{Density of band in H358 cells}} \times 100$$

RESULTS

RT-PCR-SSCP analysis in tumor tissues Gene alterations of topo I were examined by non-radiolabeled PCR-SSCP using the Pharmacia "PhastSystem." H358 cells were used as a normal control, since the direct sequenc-

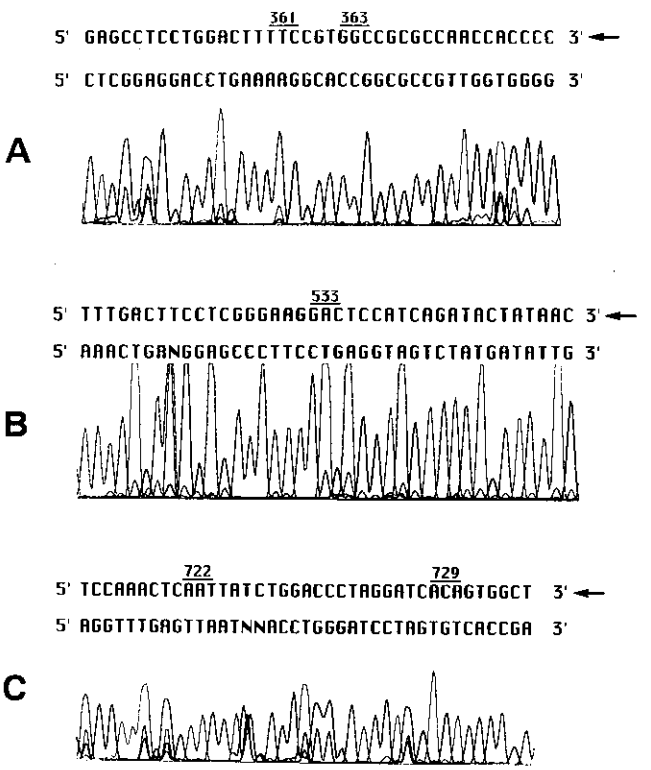

Fig. 1. The sequence of the topoisomerase I gene in H358 cells as a normal control. T-3, T-5, and T-7 primers amplify three regions near codons (A) 361 and 366, (B) 533, and (C) 722 and 729, respectively ("Materials and Methods"). The DNA fragments purified from the PCR products were sequenced using antisense primers, and no mutations were observed at any of the three regions. Arrows, the sequence of the human DNA topoisomerase I gene registered with GenBank (Ref. 19).

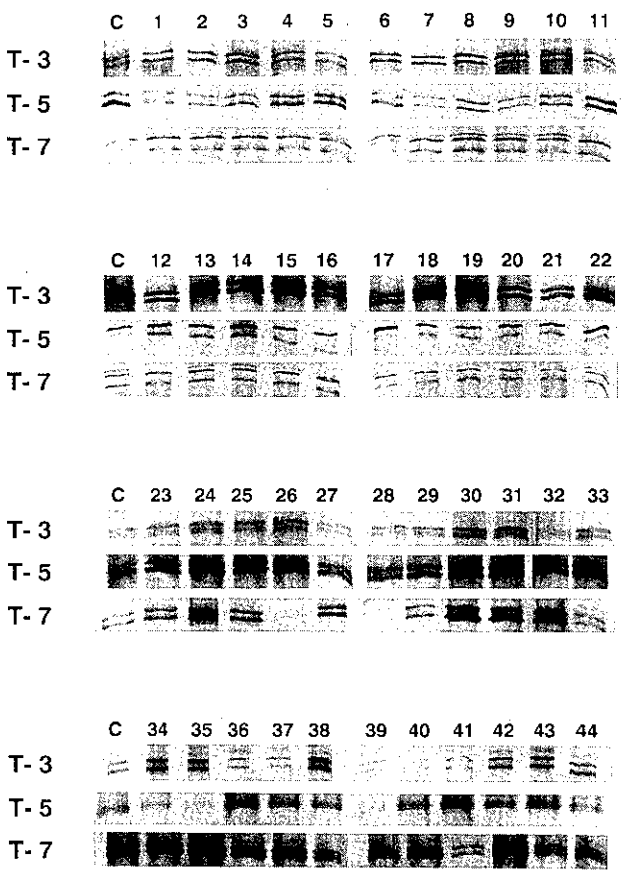

Fig. 2. RT-PCR-SSCP analysis of the topoisomerase I gene in previously untreated non-small cell lung carcinoma tissues. T-3, T-5, and T-7 primers amplify three regions near codons 361 and 366, 533, and 722 and 729, respectively ("Materials and Methods"). No mobility shifts compared to the pattern for H358 cells were observed at any of the three regions in any of the tumor tissues. Lane C, H358 cell (normal control); Lanes 1 to 44, tumor tissues.

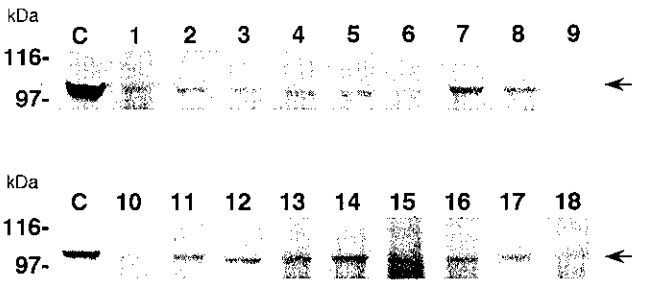

Fig. 3. Immunoblots of topoisomerase I in previously untreated non-small cell lung carcinoma tissues. The sample numbers do not match those in Fig. 2. Nuclear extract (100 μg) from H358 cells (control) and tumor tissues was separated in a 7.5% SDS-polyacrylamide gel. The membranes were incubated with human anti-DNA topoisomerase I polyclonal antibody. A 100 kDa topoisomerase I (arrows) was detected in all samples, and the relative levels were calculated compared to the density in the case of H358 in each blot ("Materials and Methods"). Lane C, H358 cells; lanes 1 to 18, tumor tissues.

ing of their PCR products revealed no mutation at any of the three regions analyzed here (Fig. 1). In all 44 tissue samples no mobility shifts compared to the pattern observed in the normal control were found at any of the three regions, though some metastable bands were seen, as shown in Fig. 2. The mobility patterns observed under these specific SSCP conditions were highly reproducible.

Relative topo I levels of tumor tissues A 100 kDa topo I was detected by immunoblotting in the tissue samples, as shown in Fig. 3. The relative topo I levels were heterogenous, with a mean$\pm$SD (median [25%, 75%]) of 23.6$\pm$14.8 (23.6 [13.1, 39.8]) for all samples ($n=44$), among which the levels showed approximately 12-fold variation. The level was 27.1$\pm$13.6 (29.3 [12.1, 39.3]) in 22 adenocarcinomas and 28.3$\pm$17.6 (20.5 [12.4, 43.2]) in 20 squamous cell carcinomas, the difference being statistically not significant.

DISCUSSION

Camptothecins are promising anticancer drugs for lung cancer chemotherapy.[6,7,9] However, resistance of cancer cells still remains an obstacle. Previous *in vitro* studies have revealed several point mutations of the *topo I* gene related to acquisition of resistance,[13–17] but few studies have analyzed gene alterations in clinical samples.[24,25] The present study demonstrated that 44 untreated NSCLC tissues had no *topo I* gene mutations and that the tumor tissues expressed various levels of topo I contents with an approximately 12-fold variation among them. These results suggest that *topo I* gene alterations may not be involved in the intrinsic resistance of NSCLC to camptothecins, whereas topo I levels may be important.

To date, relatively little is known about *in vivo* gene alterations in targets for anticancer drugs, although alterations of oncogenes or tumor suppressor genes are involved in human carcinogenesis. Recently, a point mutation in the *topo IIα* gene was reported in a tumor sample from a patient with small cell lung cancer treated with etoposide.[26] Topo I undergoes a conformation change which produces a pocket into which camptothecins can fit during formation of the cleavable complex.[1–3] *Topo I* gene mutations induced by *in vitro* chronic camptothecin exposure have been observed in the well-conserved domains analyzed here;[3] they prevent pocket formation and consequently result in drug resistance.[3] These mutations, therefore, might be expected to be present in tumors following chemotherapy with camptothecins, but so far they have not been found.[25] Also, in previously untreated lung cancer, no large deletions or rearrangements in ten NSCLC,[24] and no alterations in 17 NSCLC patients,[25] were found. The present results using a larger number of samples are very similar to those in the previous studies. Thus, topo I gene alterations probably do not contribute to the intrinsic resistance of NSCLC to camptothecins. However, it remains possible that gene alterations in regions other than the three regions we analyzed may be responsible for intrinsic drug resistance.

Using various types of tumor cells, a number of *in vitro* studies have revealed several resistance mechanisms,[1–3,5] other than *topo I* gene alterations. Resistance, however, can not always be explained by a single mechanism only,[27,28] suggesting that in practice, resistance is multifactorial. Recently, camptothecin-induced cleavable complexes rather than topo I levels have been suggested to predict sensitivity to camptothecins in colon cancer cells.[28] Among the NSCLC samples we analyzed, topo I levels showed a high variation compared to those in several previous *in vitro* and *vivo* studies.[28–31] This high variation suggests that topo I levels may account, at least in part, for the clinical resistance of NSCLC to camptothecins. Further *in vivo* studies are needed to elucidate the exact mechanisms of clinical resistance to camptothecins.

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