Establishment and characterization of irinotecan-resistant human non-small cell lung cancer A549 cells

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Abstract. Irinotecan (CTP-11) is a topoisomerase I inhibitor used in the treatment of colorectal cancer and non-small cell lung cancer (NSCLC). Despite an initial response to therapy, resistance to irinotecan reduces its efficacy. We isolated irinotecan-resistant human NSCLC A549 cells, termed A549/CTP-11R cells. A549/CTP-11R cells were resistant to irinotecan, as well as paclitaxel, gemcitabine and carboplatin. Curcumin, a nuclear factor-κB (NF-κB) inhibitor, increased the sensitivity to irinotecan of A549/CTP-11R cells. The expression level of Bcl-X_L and X-linked inhibitor of apoptosis protein, target genes of NF-κB, in A549/CTP-11R cells was higher than that in A549 cells. Our result suggests that the addition of curcumin to irinotecan reverses irinotecan resistance in NSCLC.

Introduction

Lung cancer is the most common cause of cancer death in developed nations. In the United States, an estimated 219,440 new cases and 159,390 deaths are projected for 2009 (1,2). Non-small cell lung cancer (NSCLC) commonly presents as incurable locally advanced or metastatic disease. Despite major research efforts, survival prospects remain dismally small and only 14% of all patients with lung cancer are expected to survive for 5 years after diagnosis (3). Chemotherapy yields response rates of 20-50% in advanced NSCLC and 60-80% in extensive SCLC, but almost all tumors that are not intrinsically resistant rapidly develop acquired resistance, often with broad cross-resistance to other unrelated chemotherapy agents. Alternating chemotherapy agents with differing mechanisms of action does not overcome this resistance (4).

Irinotecan (CPT-11) is a semisynthetic analog of camptothecin, originally isolated from the Chinese/Tibetan ornamental tree *Camptotheca acuminata*. It is a chemotherapy agent that causes S-phase specific cell killing by poisoning topoisomerase I (Topo I) in the cell. It was first discovered and synthesized in Japan in 1983 and has demonstrated potent anti-tumor activity against a wide range of tumors, including colorectal cancer and NSCLC (5,6).

To understand the molecular basis of irinotecan resistance, we isolated irinotecan-resistant cells from NSCLC A549 cell lines.

Materials and methods

Reagents. Paclitaxel, irinotecan hydrochloride, ethyl methanesulfonate and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Gemcitabine hydrochloride was from LKT Laboratories, Inc. (MN, USA). Carboplatin was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Curcumin was from Calbiochem (Darmstadt, Germany). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycine solution (10,000 U/ml penicillin and 10,000 μg/ml streptomycin) were from Hyclone (UT, USA).

Cell culture. The A549 cell line, derived from NSCLC, was maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 humidified atmosphere.

Cell proliferation by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell proliferation *in vitro* was measured by the MTT colorimetric assay in 96-well plates. The cells ($5x10^3$) were inoculated into each well. After overnight incubation (37˚C in 5% CO_2), anti-cancer agents were added to the culture and were then incubated for 3 days. Thereafter, 50 μl of MTT (1 mg/ml) was added to each

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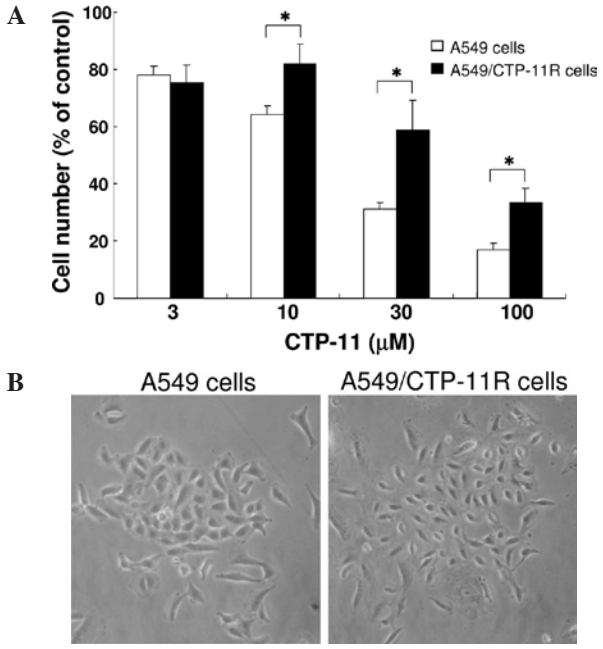

Figure 1. Sensitivity to irinotecan and morphology of A549 and A549/CTP-11R cells. (A) A549 and A549/CTP-11R cells were treated with various concentrations of irinotecan for 72 h and cell viability was determined using the MTT assay in each cell line. Each column and bar represents the mean ± SD. *P<0.05. (B) Morphology of A549 and A549/CTP-11R cells.

well and the plates were incubated for an additional 4 h. After aspiration of culture medium, the resulting formazan was dissolved with 100 μl of dimethylsulfoxide. The plates were read at 570 nm using a microplate reader.

Chronic irinotecan exposure. Irinotecan-resistant A549/CTP-11R cells were isolated by the A549 cells with increasing concentrations of irinotecan following ethyl methanesulfonate-induced mutagenesis, and then incubated in a selection medium with irinotecan (3-100 μM).

RT-PCR method. Total cellular RNA was extracted by the RNeasy Mini kit (Qiagen Sciences, MD, USA). For RT-PCR,

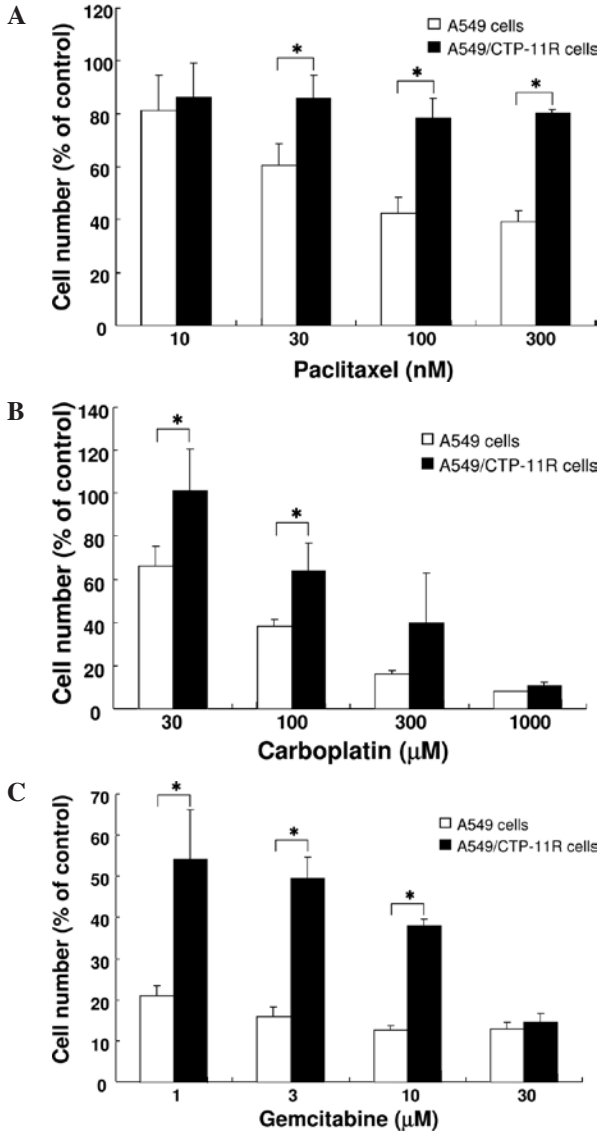

Figure 2. Sensitivity to anti-cancer agents of A549 and A549/CTP-11R cells. A549 and A549/CTP-11R cells were treated with various concentrations of paclitaxel (A), carboplatin (B) and gemcitabine (C) for 72 h, and cell viability was determined using the MTT assay in each cell line. Each column and bar represents the mean ± SD. *P<0.05.

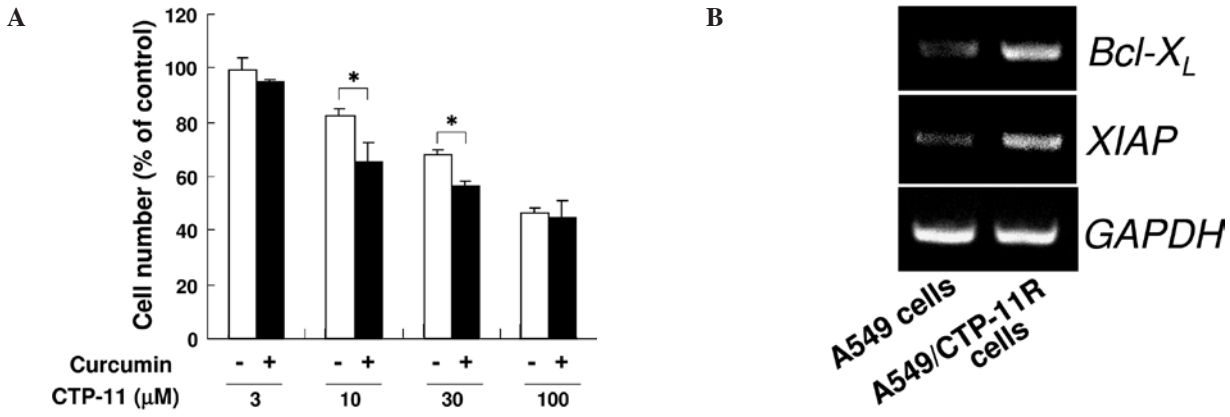

Figure 3. Effect of curcumin on the sensitivity to irinotecan of A549/CTP-11R cells and expression of Bcl-XL and XIAP in A549 and A549/CTP-11R cells. (A) A549/CTP-11R cells were treated with irinotecan in the presence or absence of curcumin (3 μM) for 72 h. Cell viability was determined using the MTT assay in each cell line. Each column and bar represents the mean ± SD. *P<0.05. (B) The expression levels of Bcl-X_L and XIAP in A549 and A549/CTP-11R cells were detected by RT-PCR.

1 μg of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, CA, USA) according to the manufacturer's protocol. The conditions for the RT-PCR were as follows: 5 min at 95˚C and then 28 cycles of amplification in PCR master mix (Promega, WI, USA) at 95˚C for 30 sec, annealing at 52˚C for 30 sec and extension at 72˚C for 1 min. The primers used for this analysis were as follows: GAPDH, forward 5'-gtcttcaccaccatggagaagg-3', reverse 5'-ggcaggtca ggtccaccactga-3'; Bcl-X_L, forward 5-cggtgaatggagccactggcc-3, reverse 5-aagtatcccagccgccgttct-3; XIAP, forward 5-tccatggca gattatgaagca-3, reverse 5-atcaattcttctagttagtga-3.

Statistical analysis. Data are presented as the mean ± SD. Statistical analysis was performed using StatView 5.0 software. (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P<0.05$.

Results

Establishment of irinotecan-resistant non-small cell lung cancer A549 cells (A549/CTP-11R) and morphology in A549 and A549/CTP-11R cells. To isolate irinotecan-resistant A549/CTP-11R cells, A549 cells were cultured in selection medium containing stepwise increases in irinotecan concentration from 3 to 100 μM. The sensitivity to irinotecan of each cell line was examined. A549/CTP-11R cells were more resistant than parental A549 cells, despite retaining a similar microscopic appearance to the parent A549 cells (Fig. 1A and B).

Cross resistance to irinotecan. The drug sensitivity of each cell line was investigated by the MTT assay. Fig. 2 shows the sensitivity to various anti-cancer drugs of the parental and resistant cell lines. Notably, A549/CTP-11R cells were more resistant to paclitaxel, carboplatin and gemcitabine than the A549 cells (Fig. 2A-C).

Effect of curcumin on the sensitivity to irinotecan of A549/CTP-11R cells. Activated nuclear factor-κB (NF-κB) suppresses the apoptotic cascade induced by chemotherapy agents, particularly irinotecan (7,8). Inhibition of NF-κB activation augments irinotecan-induced apoptosis (7). Previous studies have shown that curcumin suppresses a number of key elements in cellular signal transduction pathways, including NF-κB (9-11). Therefore, we examined the effect of curcumin on the sensitivity to irinotecan of A549/CTP-11R cells using the MTT assay. Curcumin treatment at 3 μM resensitized resistant cells to irinotecan (Fig. 3A).

Expression levels of Bcl-X_L and XIAP in A549 cells and A549/CTP11R cells. As shown in Fig. 3A, curcumin, an inhibitor of NF-κB signaling, enhanced the sensitivity to irinotecan of the A549/CTP-11R cells. The release of NF-κB from IκB results in its translocation into the nucleus, where it binds to specific sequences in the promoter regions of target genes, such as Bcl-X_L and X-linked inhibitor of apoptosis protein (XIAP) (12,13). Therefore, we examined the expression levels of Bcl-X_L and XIAP in A549/CTP-11R cells by RT-PCR. Compared to A549 cells, the expression levels of Bcl-X_L and XIAP were increased in A549/CTP-11R cells (Fig. 3B).

Discussion

Irinotecan (CTP-11) is a semisynthetic analog of camptotecin, originally isolated from the Chinese/Tibetan ornamental tree *Camptotheca acuminate*. It is a chemotherapy agent that causes S-phase-specific cell killing by poisoning Topo I in the cell. Although it has demonstrated potent anti-tumor activity against a wide range of tumors (6), resistance to irinotecan reduces its efficacy (5). To investigate the molecular basis for resistance of irinotecan, we isolated irinotecan-resistant human NSCLC A549/CTP-11R cells. The A549/CTP-11R cells were also resistant to paclitaxel, gemcitabine and carboplatin.

Irinotecan exposure frequently results in increased production of TNF-α, interleukin-1, phorbol esters and lipopolysaccharides, as well as in the activation of NF-κB (14). Activation of NF-κB leads to the inhibition of apoptosis. NF-κB is a heterodimer consisting of two proteins, p65 and p50. In unstimulated cells, NF-κB is located in the cytoplasm and is bound to IκBα and IκBβ, which prevents it from entering the nuclei. The external stimuli modulate signal transduction pathways leading to IκB phosphorylation, causing its rapid degradation by proteasomes. The release of NF-κB from IκB results in its translocation into the nucleus, where it binds to specific sequences in the promoter regions of target genes, such as Bcl-X_L and XIAP (12,13). Activated NF-κB also antagonizes p53 function, possibly though cross-competition for transcription coactivators. The inhibition of chemotherapy-induced stabilization and activation of p53 by NF-κB results in resistance to chemotherapy (15,16). Curcumin is one of the agents that suppresses NF-κB, which is implicated in proliferation, survival, angiogenesis and chemoresistance (9,17). Therefore, we examined the expression of Bcl-X_L and XIAP and the effect of curcumin, a known inhibitor NF-κB, on the sensitivity to irinotecan of A549/CTP-11R cells. The expression of Bcl-X_L and XIAP in the A549/CTP-11R cells was increased as compared to that of A549 cells, and curcumin enhanced the sensitivity to irinotecan of A549/CTP11R cells.

It has been reported that NF-κB is activated in response to irinotecan, carboplatin, paclitaxel and gemcitabine. Acquisition of resistance to anti-cancer agents has emerged as a significant impediment to effective cancer treatment (13,18-21). Our result suggests that the combination of curcumin and irinotecan has potential as an anti-cancer treatment combination, and that curcuramin may reverse irinotecan resisitance. Clinical investigation of this combination may be warranted.

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