compared to normal images. These images show that a larger SD of set-up displacement dulls the edge of dose distribution. Isodose distribution based on 2 beams was not significantly different from that based on 41 beams.

Clinical case

A case involving prostatic cancer is shown in Fig. 3. The prostate and rectum were manually contoured and planned using a 4-field box technique with 10-MV photons. On the assumption that random set-up displacement within 5-mm SD occurred along all three axes, the isodose distribution image and dose-volume histogram were calculated. Although differences were relatively slight, the impact of random set-up displacement was reflected in both the isodose distribution image and dose-volume histogram.

Discussion

Detailed calculations based on dose coverage of the CTV have been given in a previous report.[5] According to this report, if the SD of systematic errors (Σ) and of random errors (ρ) are known for a specific patient group, the margin M to expand the CTV to a safe planning target volume (PTV) may be expressed as $M = 2\Sigma + 0.7\rho$. With this calculated margin, CTV would be covered with a 95% isodose curve. However, to adapt this calculation to clinical use, manual input of tumor contours is essential. Due to the huge volume of CT data, manual input of all tumor contours for all cases is impractical.

The technique introduced in this report is useful for visually comprehending random set-up displacements, and allows the use of commercially available RTP. Using this technique, it is sufficient to take account of only systematic displacement that is constant during therapy and predictable, rather than random. Systematic displacement of an individual patient can be estimated during the first few fractions, and couch corrections can be applied for subsequent irradiations, although random displacements remain unchanged.[1,6-8] More precise and reasonable planning may be achievable using this technique.

Although this study showed that the isodose distribution based on two beams was not significantly different from that based on 41 beams in a homogeneous phantom study, different distributions may be shown in clinical use with electron density correction. There is need to take account of this point, and further examination is recommended.

Whatever is done to minimize geometrical uncertainties, inaccuracies are to some extent unavoidable. Once typical values for a specific group of patients are known, these should be included in treatment planning for individual patients from that group. Patient set-up displacement not only affects dose in the tumor region, but in neighboring, possibly critical, organs as well. However, the typical size of set-up displacement differs in each institution, as methods of patient fixation and set-up verification systems vary. In addition, beam profiles of linear accelerators differ in each institution. Simulations of data for typical set-up displacement therefore need to be performed at each institution. For this reason, availability of a simple, standard technique is crucial, and our technique can be applied to any institution. Using the technique introduced in this paper, the impact of random set-up displacements can be effectively reflected in isodose distribution images.

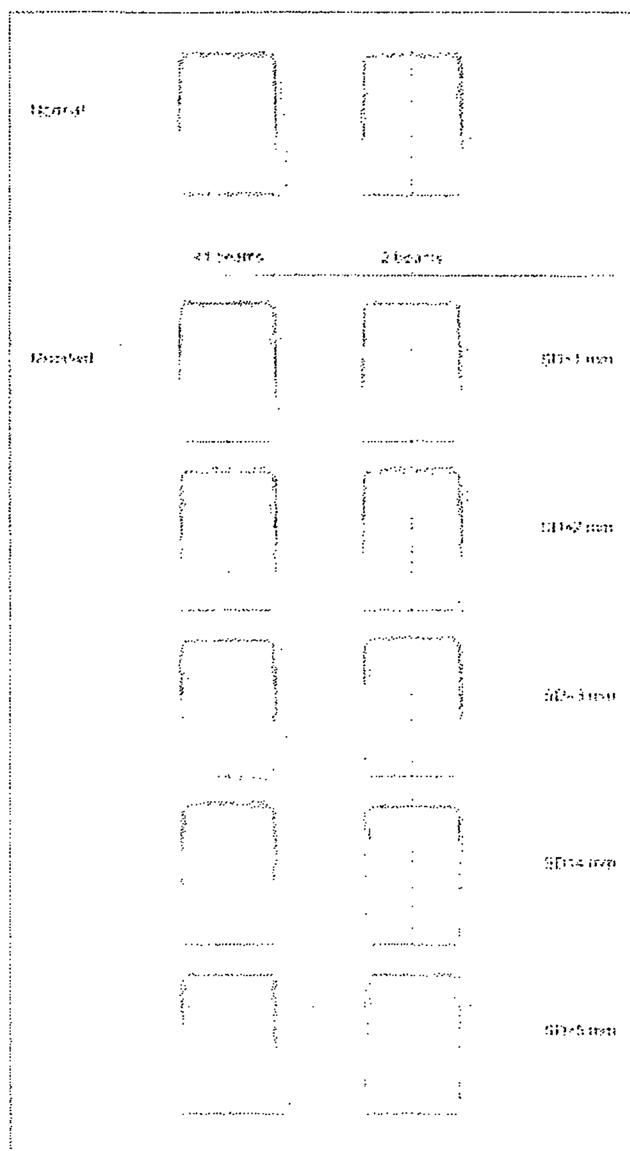
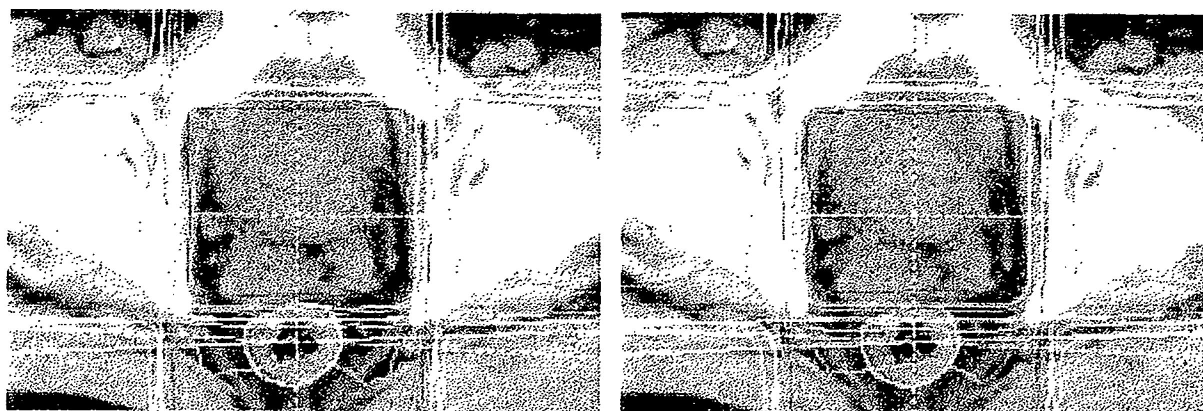
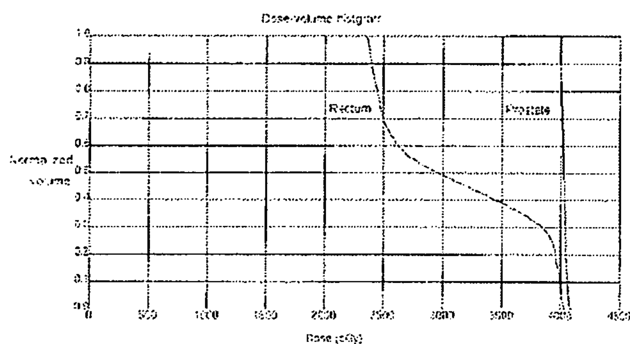


Fig. 2. Modified isodose distributions were calculated from two beams or 41 beams. The portal size was 10 cm×10 cm.





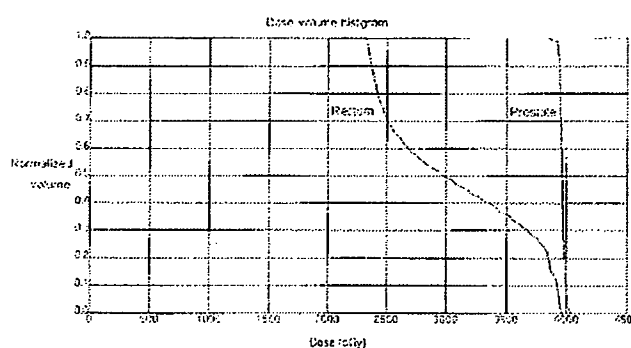


Fig. 3. Edge of the isodose distribution was sharp in normal distribution (A) and dull in modified distribution (B). Dose-volume histograms of the prostate and rectum reflect differences between normal (C) and modified (D) distributions.

A	B
C	D

ACKNOWLEDGEMENTS

This work was supported in part by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and the Ministry of Health, Labour and Welfare in Japan.

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Int. J. Cancer: **118**, 812–820 (2006)
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Small interfering RNA targeting survivin sensitizes lung cancer cell with mutant p53 to adriamycin

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Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is specifically overexpressed in cancer tissues. p53 is one of the tumor suppressor genes; its induction in response to DNA damage causes apoptosis and correlates with drug sensitivity. To investigate the possible regulation of survivin by p53, we examined the level of survivin expression in lung cancer cell lines in response to adriamycin. Levels of survivin mRNA and protein in cell lines with wild-type p53 decreased dramatically after p53 induction, but no such reduction of survivin was observed in cell lines with mutated or null p53. Inhibition of wild-type p53 in A549 cells by small interfering (si) RNA significantly upregulated the expression of survivin. Survivin inhibition by siRNA in PC9 cells with mutated p53 significantly depressed cell proliferation. To investigate the sensitivity of cancer cells to adriamycin after inhibition of survivin, we depressed survivin expression using siRNA, and then added adriamycin at an IC_{50} dose. After a further 48 hr incubation with adriamycin, proliferation was significantly depressed in the cells treated with siRNA targeting survivin, in comparison with siRNA targeting scramble. Furthermore, both TUNEL and pro-caspase3 expression assay showed a significant increase in apoptosis after combined treatment with adriamycin and siRNA targeting survivin. Our results demonstrate that survivin is downregulated by p53, and that siRNA targeting of survivin increases cell sensitivity to adriamycin and promotes apoptosis. siRNA targeting of survivin could be potentially useful for increasing sensitivity to anticancer drugs, especially in drug-resistant cells with mutated p53.
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Key words: Survivin; siRNA; p53; lung cancer; Adriamycin

The success of cancer treatment depends on the response to chemotherapeutic agents. However, malignancies often acquire resistance to drugs if they are used frequently. Inhibition of the apoptosis pathway is one of the factors that may be responsible for such drug resistance.[1] Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is specifically overexpressed in various cancers but not in normal adult tissues.[2] Overexpression of survivin is correlated with poor prognosis in a number of tumor types, including lung cancer,[3] colorectal cancer[4] and gastric cancer.[5] Like other mammalian IAPs (*e.g.*, XIAP, c-IAP-1, c-IAP-2 and livin), survivin binds to caspase-3 and caspase-7.[6] It has been suggested that survivin expression is regulated in a cell cycle-dependent manner.[7] Survivin is maximally expressed in the G2/M phase and physically associates with mitotic spindle microtubules that regulate progression through mitosis. In contrast, survivin is definitively depressed in the G1 phase. p53 is one of the tumor suppressor genes, and it is frequently mutated in cancer tissue/cells.[8] The crucial role of p53 is to maintain genetic stability through its participation in cell cycle checkpoints. After DNA damage induced by various cytotoxic agents, cells with wild-type p53 become preferentially arrested in the G0/G1 phase, after which they choose a path that results in either DNA repair or apoptosis. Apoptosis is closely linked to transcripts that are downregulated by p53. In contrast, mutation or deletion of p53 leads cells away from the apoptosis pathway, causing drug resistance.[9] It is generally accepted that p53 functions as a transcriptional factor and transactivates some genes, resulting in cell growth modulation or death. For example, an elevated level of p21, the first product of p53 transactivation, results in underphosphorylation of the retinoblastoma (Rb) protein, which in turn sequesters the E2F transcription factor; as a result, the cell cycle is blocked in the G1 phase.[10,11] Additionally, some genes, such as stathmin or cdc2, could be negatively regulated by p53.[12,13] Previous reports suggest that p53 also downregulates the expression of survivin in some cell models and cancer cell lines.[14,15] More recent reports have shown that inhibition of survivin by anti-sense oligonucleotide blocks the cell proliferation of myeloid leukemic cells[16] or lung cancer cells,[17] although the mechanism of this transcriptional regulation is not fully understood and requires additional research.

From another viewpoint, inhibition of survivin might play a role in overcoming acquired drug resistance. It has not been clarified how DNA-damaging agents influence survivin expression and cause cell cycle arrest and apoptosis. One report has suggested that anti-sense targeting of survivin sensitizes lung cancer cells to chemotherapy.[17] However, that study employed only 1 lung cancer cell line containing wild-type p53 and did not address the outcome that would be expected with mutated or deleted p53.

RNA interference (RNAi) is a mechanism whereby double-stranded RNA post-transcriptionally silences a specific gene. It has been reported that synthetic, double-stranded small-interfering RNA (siRNA) can effectively silence a gene through the RNAi mechanism.[18] siRNA can be a novel tool for clarifying gene function in mammalian cells and may be applicable to gene-specific therapeutics.[1] In our study, using siRNA, we aimed to sensitize lung cancer cell line to adriamycin. Our results suggest that siRNA targeting of survivin can inhibit cell growth and produce a combined anti-proliferative effect and apoptosis when combined with adriamycin, especially in cell lines containing mutated p53.

Material and methods

Drugs and cell lines

Adriamycin, obtained from Kyowa Hakko Kogyo Co. (Tokyo, Japan), was dissolved in distilled water and stored at −30°C until use. All cell lines used in our study were derived from patients with lung cancer. Lines NCI H226, H292, H358, H460, H522 and H1299 were obtained from the American Type Culture Collection (Manassas, VA). Lines A549, EBC-1, LK-2, Lu99, Lu99B, OBA-LK-1 and Sq-1 were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). SBC3, Lu65 and RERF-LC-KJ were obtained from the Japan Health Sciences Foundation (Tokyo, Japan). Lines PC9 and PC14 were kindly donated by Prof. Hayata, Tokyo Medical University (Tokyo, Japan). SBC3/ADM,[20]

Abbreviations: dH_2O, distilled H_2O; DW, distilled water; FBS, Fetal Bovine Serum; GAPDH, glyceraldehyde-3- phosphate; IAP, inhibitor of apoptosis protein; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; si RNA, small interfering RNA; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; SD, standard deviation; SE, standard error, TUNEL, TdT mediated dUTP nick end labeling.

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Received 22 September 2004; Accepted after revision 29 April 2005
DOI 10.1002/ijc.21350
Published online 17 August 2005 in Wiley InterScience (www.interscience.wiley.com).

UICC global cancer control — Publication of the International Union Against Cancer

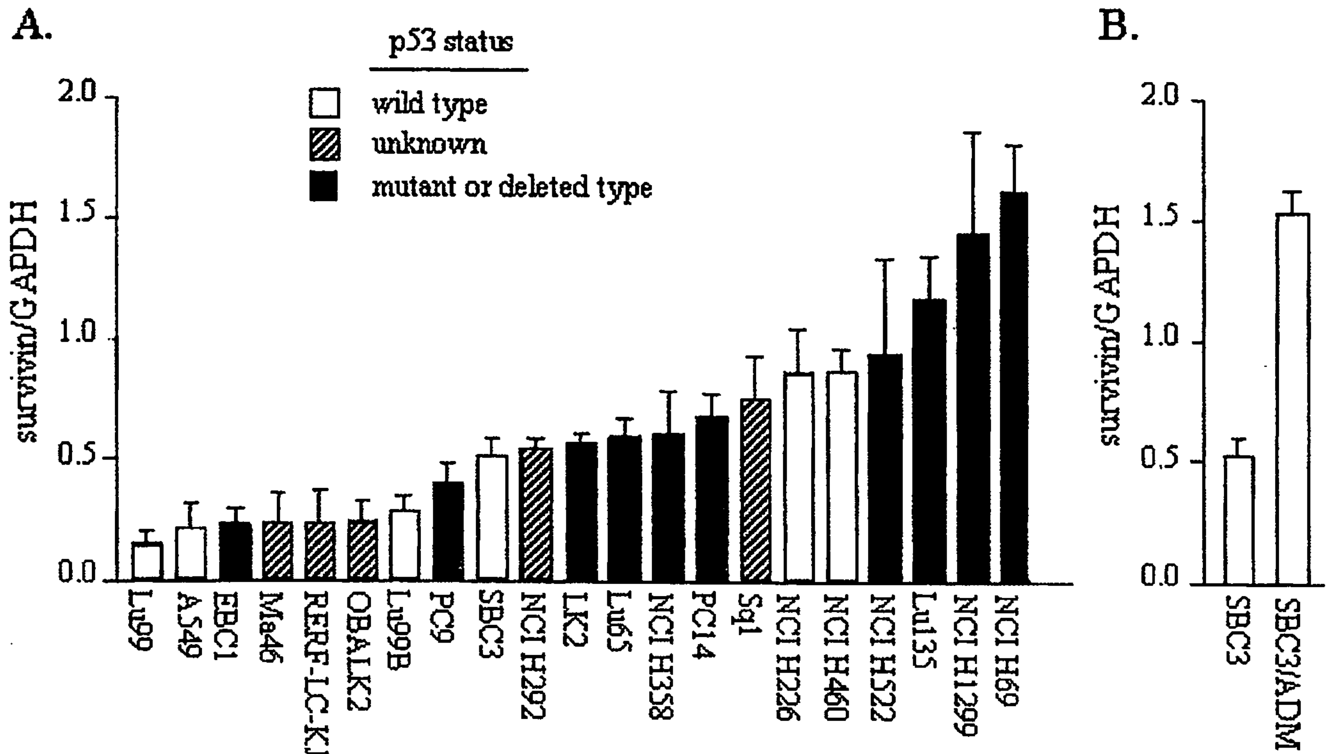

FIGURE 1 – Level of survivin mRNA in 22 lung cancer cell lines. (*a*) Cells were incubated in a 75 cm^2 flask, harvested and analyzed using real-time PCR as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH and are presented as the mean ± SD for at least 3 independent experiments. p53 status is presented. (*b*) Comparison between SBC3 and SBC3/ADM, the adriamycin-resistant subline, is shown.

a subline of SBC3 with approximately 8-fold stronger resistance to the growth-inhibitory effect of adriamycin, as determined by the MTT assay, was provided by Dr. Kiura, Okayama University (Okayama, Japan). Lu135 was provided by Riken Cell Bank (Tokyo, Japan). Ma46 was established in our laboratory from malignant effusion of an NSCLC patient. The cells were cultured in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 and air at 37°C. All cell lines were discarded after 20 generations, and new lines were obtained from frozen stocks. Some cell lines were analyzed for their IC_{50} values using the MTT assay by incubating them with adriamycin for 72 hr.[21] With regard to p53 status, NCI H226, H460, A549, SBC3, SBC3/ADM, Lu99 and Lu99B possess wild-type p53. EBC-1, PC9, LK2, Lu65, NCI H358, H522, H69, PC14, Lu135 and Lu65 possess mutated p53. NCI H1299 has deleted p53.[22–26]

Real-time RT-PCR

Total RNA was extracted from cells treated with adriamycin, siRNA or water using an RNeasy Mini Kit (Qiagen, Inc., Tokyo, Japan). For first-strand cDNA synthesis, 1 µg total RNA from a sample was added to components of the Super Script Preamplification System (Life Technologies, Inc., Gaithersburg, MD), as described in the user's manual. Real-Time PCR was performed using the Gene Amp 5700 Sequence Detection System (Perkin-Elmer), and mRNA expression was quantified. For this purpose, 1 µl cDNA was mixed with commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer Biosystems), following the manufacturer's protocol. Survivin cDNA was amplified using a forward primer consisting of 5′-ATGGGTGCCCCGACGT-3′ and a reverse primer consisting of 5′-AATGTAGAGATGCGGTGG-TCCTT-3′ and detected by a Taqman probe consisting of 5′-CCCCTGCCTGGCAGCCCTTTC-3′, each nucleotide corresponding to positions 50–65, 92–114 and 69–89 of the 1,619 bp survivin mRNA (GenBank NM001168). Relative quantification of gene expression was performed as described previously,[27] using the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) as an internal standard.

Western-blotting analysis

Cells treated with adriamycin, siRNA or water were harvested with trypsin/EDTA, and PBS-washed cell pellets were treated with HEPES lysate buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 10 mM NaCl). Equal amounts of protein extracts were loaded onto sodium dodecyl sulfate-polyacrylamide gels and ran at 200 V for 45 min followed by transfer to nitrocellulose membranes at 100 V for 30 min. at room temperature. The membranes were probed with the following primary antibodies: affinity-purified rabbit anti-survivin antibody (R&D Systems, Inc., Minneapolis, MN), mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-actin affinity isolated antibody (Sigma-Aldrich Co., St. Louis, MO) and mouse monoclonal anti-caspase3 antibody (Santa Cruz Biotechnology) at room temperature for 120 min. As secondary antibodies, goat anti-rabbit labeled with horseradish peroxidase (Amersham Biosciences, England) and sheep anti-mouse labeled with horseradish peroxidase (Santa Cruz Biotechnology) were used. Blots were developed using a chemiluminescence detection system (Perkin Elmer Life Sciences, Boston, MA).[28]

Flow cytometry

Cells were treated with adriamycin, harvested, washed with PBS, fixed with 70% methanol, washed with PBS and stained with propidium iodide solution (0.05 mg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA and 0.05 mg/ml RNase A). Approxi-

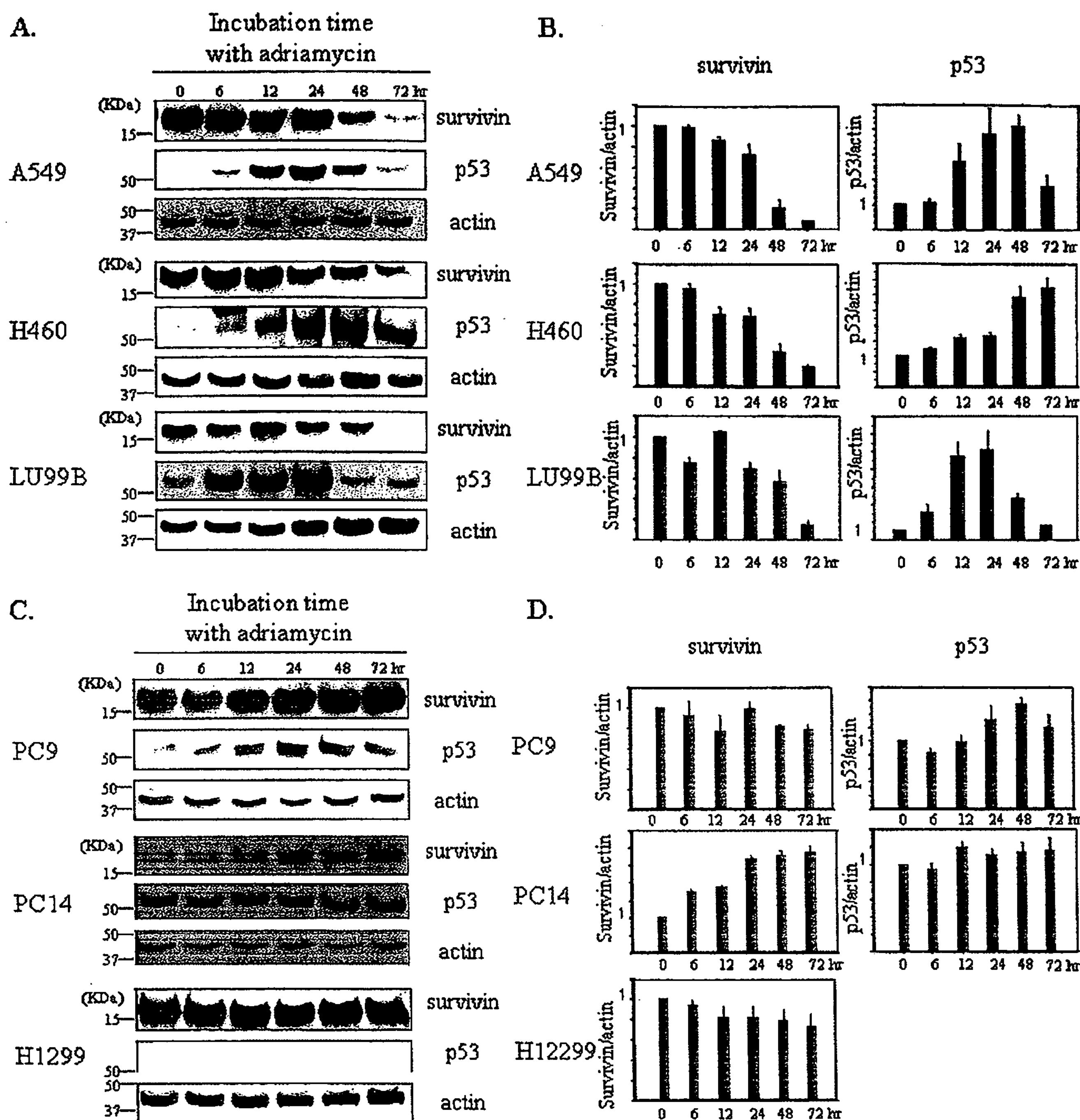

FIGURE 2 – Expression of survivin and p53 protein in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. (*a*) Western-blotting analysis for expression of survivin and p53 in cell lines possessing wild-type p53, including A549, NCI H460 and LU99B. Each of the cell lines was incubated with adriamycin at the IC_{50} dose for the indicated time. Actin served as a control. Treatment, harvest and analysis were repeated 3 times. (*c*) Western-blotting analysis for expression of survivin and p53 in PC9 and PC14, possessing mutated p53, and in NCI H1299, possessing deleted p53. Each of the cell lines was incubated with adriamycin at the IC_{50} dose for the indicated time. Actin served as a control. Treatment, harvest and analysis were repeated 3 times. (*b*,*d*) Protein expression levels were presented as the mean±SD.

mately 1×10^5 stained cells were analyzed by flow cytometry in a Becton Dickinson FACS calibur.[28]

siRNA transfection

The siRNA duplexes for survivin and p53 were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry. The siRNA targeting survivin corresponded to the coding region 206–404 relative to the start codon (GenBank NM001168). The siRNA targeting p53 corresponded to the coding region 775–793. BLAST searches of the human genome database were carried out to ensure the sequences would not target other gene transcripts. Cells in the exponential phase of growth were

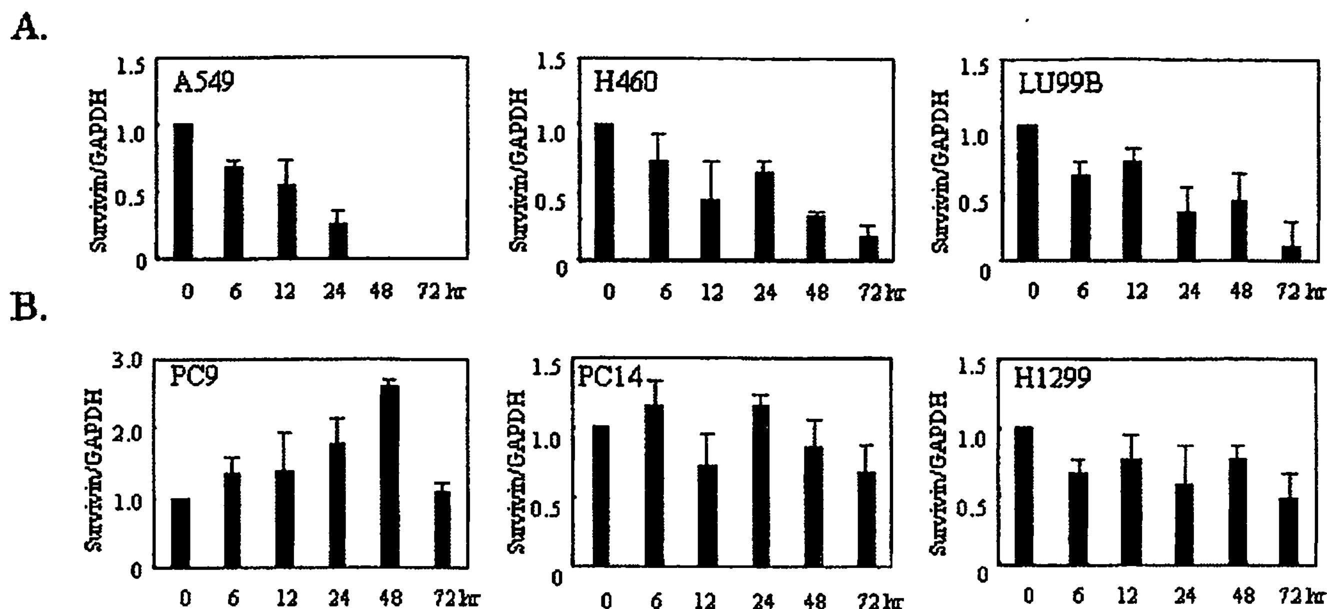

FIGURE 3 – Expression of survivin mRNA in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. Each of the cell lines with wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for the indicated time and analyzed by real-time PCR, as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH, and are presented as the mean ± SD for at least 3 independent experiments.

plated in 12-well tissue culture plate at 4×10^4 cells/well, grown for 24 hr and then transfected with 300 nM siRNA using oligofectamine and OPTI-MEM. Serum media (Invitrogen Life Technologies, Inc., Carlsbad, CA) were reduced according to the manufacturer's protocol. Gene silencing was examined with Western blotting 24–72 hr after transfection. Control cells were treated with siRNA duplex targeting scramble (Dharmacon). These studies were repeated 3 times and the data was presented as mean ± SE.

TUNEL assay

Cells were fixed in 4% paraformaldehyde (pH 7.4) and then stained and analyzed for apoptosis using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). Fixed cells were permeabilized using a mixture containing 0.1% sodium citrate and 0.1% TritonX100 and incubated with TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP at 37°C for 60 min. Flow cytometric analysis using a FACS calibur was done to quantitate apoptosis.[29]

Cell viability analysis

Cells treated with adriamycin or transfected with siRNA duplex were washed with medium once and PBS twice, after staining with trypan blue.

Statistical analysis

All data are presented as mean ± SD or mean ± SE, and statistical analysis was done by Student's 2-tailed *t*-test (Stat View, SAS Institute, Inc.). Differences at $p < 0.05$ were considered significant.

Results

Survivin mRNA expression in lung cancer cell lines

The level of expression of survivin mRNA in the 22 human lung cancer cell lines was analyzed by TaqMan real-time PCR (Fig. 1). Normalization was performed using GAPDH as an internal control. Harvest and analysis of each cell line was repeated at least 3 times, and the mean and standard deviation for each cell lines is shown. All lung cancer cell lines expressed survivin mRNA, although the expression level varied. Among the 22 cell lines, the p53 status of 17 has been reported. The mean survivin expression of cells with wild-type p53, except for SBC3/ADM, tended to be less than that of cells with mutated or deleted p53 ($p = 0.0192$). Moreover SBC3/ADM, which is 8 times more adriamycin-resistant than SBC3 in terms of IC_{50}, expressed about 3 times more survivin mRNA than did SBC3.

Decrease of survivin expression after adriamycin exposure is dependent on functional p53 accumulation

To examine the p53 regulation of survivin expression, we monitored the expression of survivin protein in cells treated continuously with adriamycin at the IC_{50} dose by Western blotting (Fig. 2). Harvest, treatment and analysis of each cell line were repeated 3 times. The p53 phenotype of cell lines A549, NCI H460 and Lu99B has been reported previously as wild-type p53; PC9, PC14 and NCI H1299 possess mutant or deleted p53. In the cells with wild-type p53 (A549, H460 and Lu99B), p53 expression was induced 6 hr after adriamycin exposure and reached a peak level by 24 hr or later. Survivin protein expression was repressed for 72 hr after p53 accumulation (Fig. 2*a*). On the other hand, expression of survivin protein in cells with mutated or deleted p53 (PC9, PC14 and H1299) was not significantly decreased, and in fact appeared to be strongly increased in PC14 (Fig. 2*b*). Additionally, we analyzed survivin mRNA modification after adriamycin exposure using real-time PCR (Fig. 3). As was observed for the protein, the level of survivin mRNA showed a temporal decrease in all cell lines (A549, H460 and LU99B) containing wild-type p53. Repression of survivin mRNA in these cell lines started with accumulation of p53 during the first 6 hr (Fig. 3*a*). In contrast, in cell lines with mutated or deleted p53 (PC9, PC14 and H1299), survivin mRNA did not decrease throughout the period of adriamycin exposure. Furthermore, in cell line PC9, the level of survivin mRNA tended to increase (Fig. 3*b*).

Dependence of altered cell cycle distribution on p53 phenotype following exposure to adriamycin

In each of the cell lines treated with adriamycin, the cell cycle distribution was analyzed by flow cytometry (Fig. 4). It was found that the cell cycle distribution varied markedly depending on the p53 phenotype. That is, following exposure to adriamycin cells possessing wild-type p53 tended to show arrest in G1/S phase, whereas cells with mutated or deleted p53 became arrested in G2 phase. In cells containing wild-type p53, the G2/M peak tended to decline along with repression of survivin protein after 24 hr of adriamycin exposure, and the proportion of apoptotic cells (sub-G1) increased. On the other hand, in cells with mutated or deleted p53, the decline in the G2 peak was delayed in comparison with wild cells possessing wild-type p53, and only a small proportion of the cells became apoptotic after 24 hr of expression to adriamycin (Fig. 4).

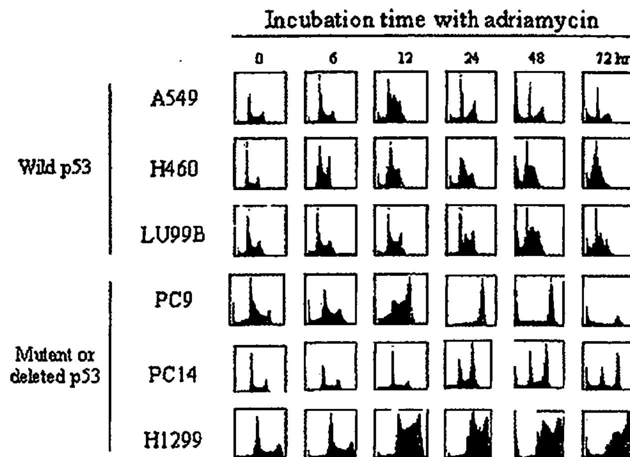

FIGURE 4 – Cell cycle analysis of lung cancer cell lines with different p53 phenotypes after exposure to adriamycin. Each of the cell lines possessing wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for the indicated time and analyzed by flow cytometry as described in Material and methods.

Inhibition of p53 using siRNA duplex, and resulting change in survivin expression

We examined whether wild-type p53 functionally regulates survivin, using the novel siRNA technique, which specifically inhibits p53. The siRNA duplex was designed to target coding region 775–793 after the start codon of p53. A549, a lung cancer cell line possessing wild-type p53, was transfected with siRNA duplex targeting p53, or scramble as a control, and the resulting levels of survivin expression were determined by Western blotting (Fig. 5*a*). All siRNA molecules have some intrinsic effect on treated cells. We compared cells treated with scrambled siRNA and cells treated with distilled water about p53 and survivin expression. In a result, there is not a significant difference between these. The siRNA duplex targeting p53 reduced p53 protein expression to 54% of the control level within 48 hr (Fig. 5*b*), and this was accompanied by an increase of survivin protein by as much as 2 times the control level (Fig. 5*c*).

Inhibition of survivin expression by siRNA duplex inhibits cell proliferation and induces cell death

To evaluate the biological effect of survivin inhibition in lung cancer cell lines, transfection with siRNA duplex was performed. Cell line PC9, with mutated p53, was transfected with siRNA duplex targeting survivin or with that targeting scramble as a con-

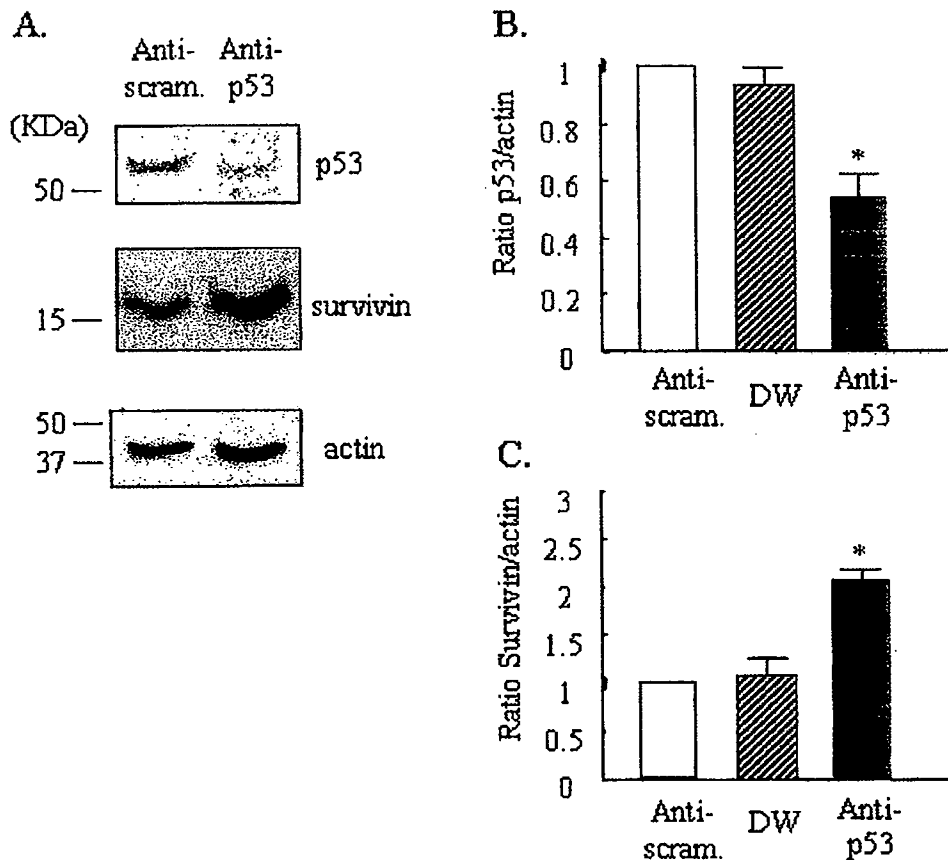

FIGURE 5 – (*a*) Increasing survivin expression in A549 lung cancer cells possessing wild-type p53 as a result of p53 inhibition by siRNA duplex. A549 cells were treated with siRNA duplex targeting p53, scramble or distilled water and then 48 hr later, cell lysates were prepared from the siRNA-treated cells. (*a*) Expressions of p53, survivin and actin were analyzed by Western blotting. (*b*) The expression of p53 protein was analyzed densitometrically using a ChemiImager AlphaImager (ASTEC Co., Japan) and corrected relative to actin. (*c*) The expression of survivin protein was analyzed densitometrically using a ChemiImager AlphaImager and corrected relative to actin. All data are presented as the mean ± SD for at least 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test. $*p < 0.05$ *vs.* cells treated with siRNA duplex targeting scramble.

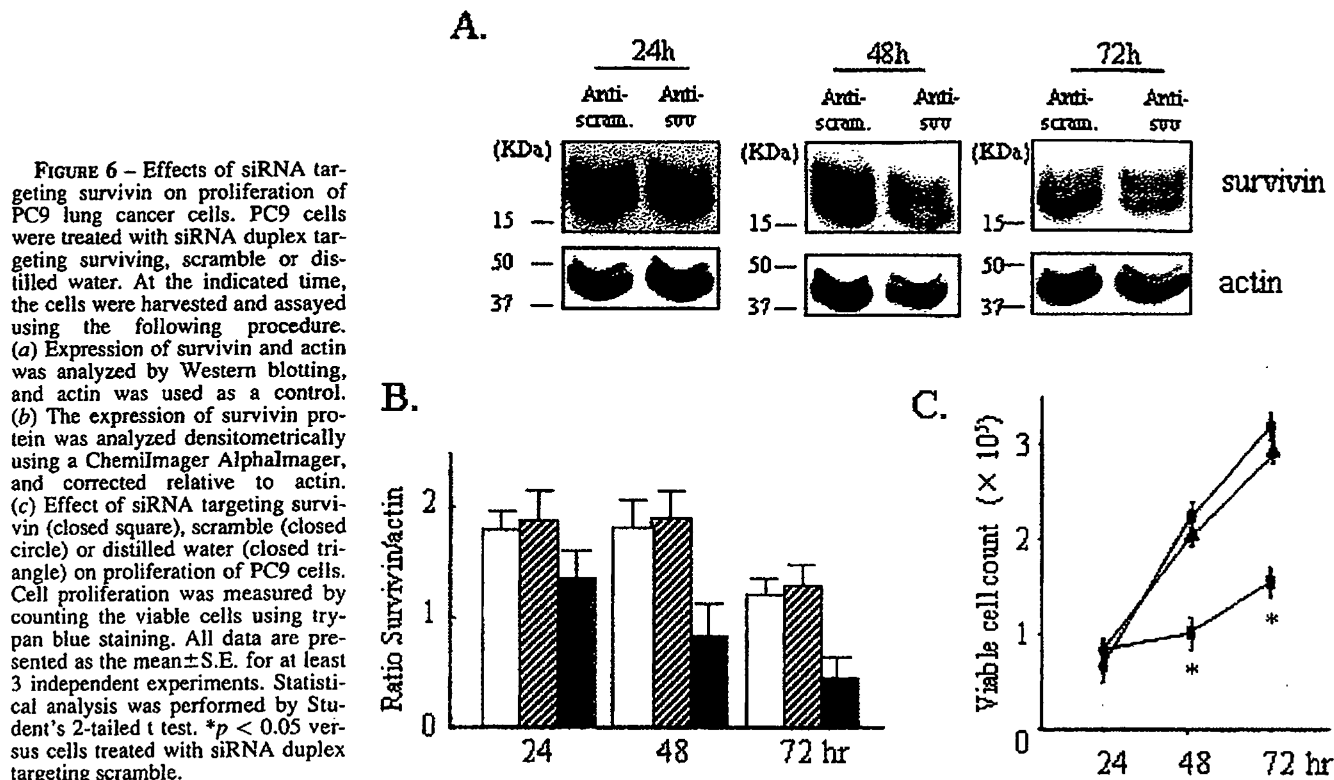

FIGURE 6 – Effects of siRNA targeting survivin on proliferation of PC9 lung cancer cells. PC9 cells were treated with siRNA duplex targeting surviving, scramble or distilled water. At the indicated time, the cells were harvested and assayed using the following procedure. (*a*) Expression of survivin and actin was analyzed by Western blotting, and actin was used as a control. (*b*) The expression of survivin protein was analyzed densitometrically using a ChemiImager AlphaImager, and corrected relative to actin. (*c*) Effect of siRNA targeting survivin (closed square), scramble (closed circle) or distilled water (closed triangle) on proliferation of PC9 cells. Cell proliferation was measured by counting the viable cells using trypan blue staining. All data are presented as the mean±S.E. for at least 3 independent experiments. Statistical analysis was performed by Student's 2-tailed t test. $*p < 0.05$ versus cells treated with siRNA duplex targeting scramble.

trol. Scrambled siRNA did not have unspecific effect on survivin expression compared to distilled water in each point. It was found that expression of survivin protein was significantly repressed after transfection with anti-survivin, compared to the control (Fig. 6*a*,*b*). The level of survivin protein was reduced to 62% of the control within 48 hr and to 45% within 72 hr. We then counted the number of viable cells after siRNA transfection. As shown in Figure 6*c*, the repression of survivin had a direct effect on cell proliferation. At 48 hr post-siRNA, survivin repression significantly reduced the viable cell count to 45% of the scrambled siRNA treated cells ($p < 0.05$) and 47% of the control level at 72 hr ($p < 0.05$). Viable cell count of the scrambled siRNA treated cells was not different from distilled water treated cells in each point. In addition, apoptosis was induced to a greater extent by survivin repression, which is measured by the TUNEL assay (data not shown).

Sensitization of lung cancer cell lines to adriamycin by siRNA targeting survivin

Based on the fact that cell lines with mutated or deleted p53 stably expressed survivin after exposure to adriamycin, we investigated the impact of survivin inhibition on adriamycin sensitivity in cells with mutated p53. Cell line PC9 possessing mutated p53 was transiently transfected with siRNA duplex targeting survivin, or with that targeting scramble as a control, for 48 hr. After the transfection, which significantly inhibited survivin expression, the medium was replaced and adriamycin at the IC_{50} dose, or water, was added. Adriamycin exposure was continued for 48 hr, and the cells were then harvested separately for Western blotting, viable cell assay, TUNEL assay and procaspase 3 assay. It was found that siRNA inhibited the expression of survivin by 57% at the start of adriamycin exposure and that survivin inhibition was weakened to 20% by 48 hr (data not shown). In terms of cell proliferation, anti-survivin siRNA duplex alone, adriamycin alone or a combination of both was significantly more repressive than anti-scramble siRNA followed by water, as a control ($*p < 0.05$, Fig. 7). That is, 48 hr after exposure to adriamycin or water, anti-survivin siRNA alone inhibited cell growth to 55% of the control, adriamycin alone reduced cell growth to 39%, and a combination of the 2r reduced cell growth to 21% of the control. Within 12 hr after exposure to adriamycin or water, exposure to anti-survivin siRNA or adriamycin alone did not significantly inhibit cell proliferation compared to the control; however the combination of the 2 significantly repressed cell proliferation to 44% of the control ($*p < 0.05$), and we compared anti-scrambled siRNA with distilled water followed by adriamycin or not. As a result, the scrambled siRNA effect on cell proliferation was small.

Induction of apoptosis in lung cancer cells by siRNA targeting survivin, and resulting sensitization to adriamycin

Additionally, we performed a TUNEL assay to evaluate apoptosis (Fig. 8). Cells were transfected with anti-scramble, anti-survivin siRNA duplex or distilled water for 48 hr and harvested for the assay 24 hr after exposure to adriamycin or water. Cells treated with water after anti-scramble were 5.1% TUNEL-positive, whereas cells treated with anti-survivin siRNA alone or adriamycin alone were 24.1% and 18.8% TUNEL-positive, respectively. Anti-survivin siRNA duplex induced significantly more apoptosis than that seen in the control ($*p = 0.0298$). Finally, the combination of anti-survivin siRNA duplex and adriamycin exposure resulted in 51.2% TUNEL-positivity, which was a significantly more potent effect than each of the other treatments ($**p < 0.05$). Intrinsic effect of scrambled siRNA on apoptosis was small, compared to cells treated with scrambled siRNA and cells treated with distilled water.

We additionally assessed procaspase-3 expressed in cells exposed to adriamycin after treatment with anti-scramble, anti-survivin siRNA duplex or distilled water (Fig. 9). It has already been reported that survivin potentially inhibits caspase-3 acti-

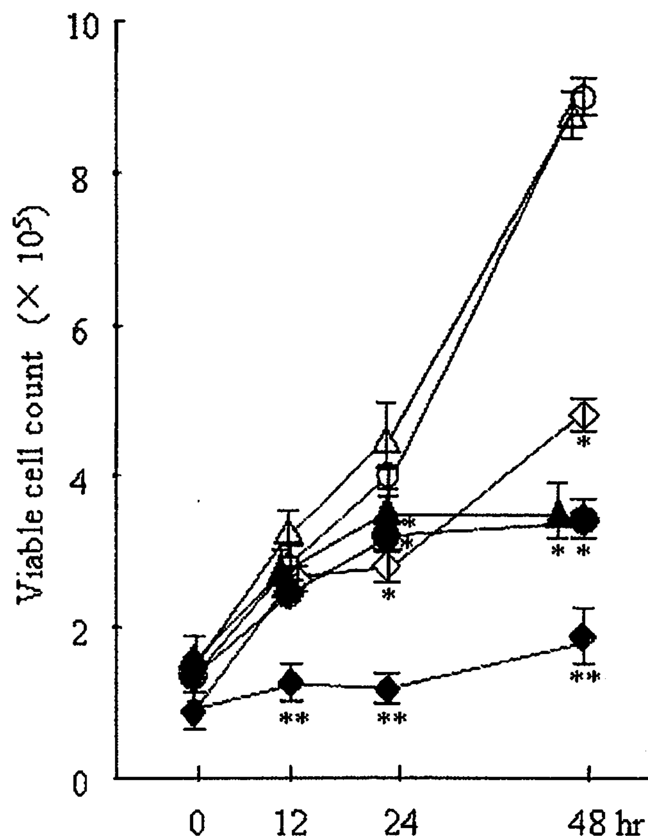

FIGURE 7 – Effects of siRNA duplex targeting of survivin on proliferation of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin or water after 48 hr transfection with siRNA duplex targeting surviving, scramble or distilled water. Open triangle:water after distilled water; open circle: water after transfection with siRNA duplex targeting scramble; open diamond: water after transfection with siRNA duplex targeting survivin; closed triangle: adriamycin after distilled water; closed circle: adriamycin after transfection with siRNA duplex targeting scramble; closed diamond: adriamycin after transfection with siRNA duplex targeting survivin. The data are presented as the mean±S.E. from 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test. $^*p < 0.05$ *vs*. cells treated with water after transfection with siRNA duplex targeting scramble. $^{**}p < 0.05$ vs. other treatments.

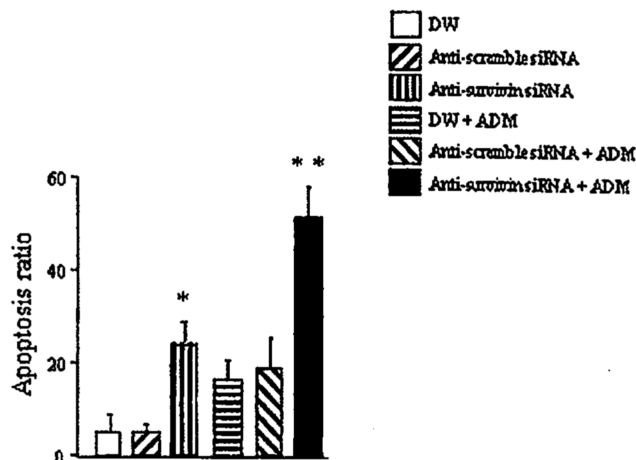

FIGURE 8 – Effects of siRNA targeting survivin on apoptosis of PC9 lung cancer cells treated with adriamycin, evaluated by TUNEL assay. PC9 cells were exposed to adriamycin or water for 24 hr after 48 hr transfection with duplex siRNA targeting surviving, scramble or distilled water. The data are presented as the mean±S.E. for 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test, $^*p<0.05$ *vs*. cells treated with anti-scrambled siRNA. $^{**}p<0.05$ *vs*. cells treated with each of the other treatments.

vation and inhibits apoptosis. The procaspase-3 level in the cells exposed to adriamycin and treated with anti-survivin siRNA decreased to 50% of the level in cells exposed to adriamycin followed by treatment with anti-scramble siRNA duplex. We treated distilled water to replace anti-scramble siRNA, and there is small effect on pro-caspase3 expression in anti-scrambled siRNA.

Discussion

Survivin mRNA is expressed to various degrees in all of the 22 lung cancer cell lines used in our study. It has been reported that survivin mRNA is detectable in 85.5% of NSCLC tissue samples and that its expression level is correlated with poor prognosis.[3] The mean survivin expression in 6 cell lines with wild-type p53, except for SBC3/ADM, tended to be low in comparison with the mean expression in 10 cell lines possessing mutant p53 ($p = 0.019$). There is no relationship between survivin expression and histology or origin of carcinoma (Table I). It has been reported that survivin expression is associated with accumulation of mutant p53 in gastric cancer and pancreatic carcinoma, assayed by immunohistochemical staining.[30–31] These data suggest that p53 might regulate survivin expression. In addition, after exposure to adriamycin, survivin expression show a transcriptional decrease following accumulation of wild-type p53. Adriamycin is generally classified as a topoisomerase II inhibitor that induces DNA double-strand breaks. The cellular response to DNA damage, which includes nuclear accumulation of p53, has been studied extensively using adriamycin. Thus, we used adriamycin in this study. In our study, p53 inhibition by siRNA duplex resulted in downregulation of survivin expression. The dependence of survivin repression on functional p53 has been investigated previously in a number of different cell models and cancer cell lines.[14,15] Although it is generally accepted that p53 activates a number of genes through direct interaction with their promoter DNA, the mechanism whereby p53 regulates survivin expression is still unclear.[8] One possibility is that p53 might directly bind to the promoter of survivin and repress survivin transcription. In fact, a p53-binding motif is reported to exist within the promoter of survivin.[14,15] In contrast, Mirza *et al.*[15] suggested that a p53-binding motif was not required for transcriptional repression of survivin. They suggested that chromatin deacetylation in the survivin promoter could contribute to p53-dependent repression of survivin gene expression. It is also possible that p53 might increase the level of another transcriptional regulator (*e.g.*, p21) and indirectly downregulate survivin elsewhere downstream.[11] In our study, both survivin and p53 expressions were low in 2 cell lines with wild-type p53 treated with adriamycin for 72 hr (Fig. 2*a*). It may be explained by indirect survivin regulation by another transcriptional factor. Z. Wang *et al.*[32] previously showed that survivin post-translationally increased Mdm2 protein, and subsequently ubiquitination of p53, by blocking caspases that could cleave Mdm2 protein. We showed that p53 functionally repressed survivin expression. In our study, there is a possibility that survivin repression followed by adriamycin exposure might affect p53 accumulation in wild-type p53 cell lines. Survivin expression increased after adriamycin treatment in PC14 possessing mutant p53. Wall NR *et al.*[33] also showed survivin protein increase in MCF7 following adriamycin treatment, and they suggested that survivin was phosphorylated by cdc2 and very little degraded by an ubiquitination-dependent mechanism.

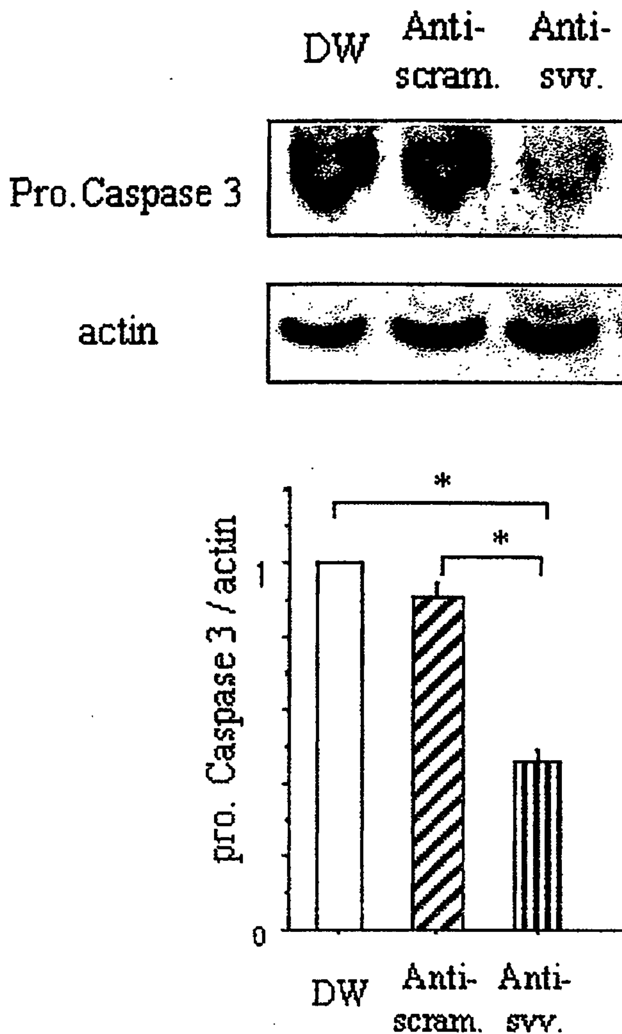

FIGURE 9 – Effects of siRNA targeting survivin on pro-caspase3 expression of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin for 24 hr after 48 hr transfection with duplex siRNA targeting survivin, scramble or distilled water, and each sample was analyzed by Western blotting. The data are presented as the mean ± S.E. for the 3 independent experiments. A representative blot is shown. Statistical analysis was performed by Student's 2-tailed *t*-test, *$p < 0.05$ *vs*. cells treated with other agents.

TABLE I – HISTOLOGY AND ORIGIN OF EACH CELL LINE[1]

Cell Line	Histology	Origin
LU99	La	Prim.
A549	Ad	Prim.
EBC1	Sq	Prim.
MA-46	Sq	Effu.
RERF-LC-KJ	Ad	Prim.
OBALK1	La	Effu.
Lu99B	La	Effu.
PC9	Ad	Prim.
SBC3	Sm	Prim.
NCI-H292	Muc	Prim.
LK-2	Sq	Prim.
LU65	La	Prim.
NCI-H358	Ad	Prim.
PC14	Ad	Prim.
Sq1	Sq	Prim.
NCI-H226	Metho	Effu.
NCI-H460	La	Effu.
NCI-H522	Ad	Prim.
Lu 135	Sm	Prim.
NCI-H1299	La	Lym.
NCI-H69	Sm	Prim.

[1]Ad: adenocarcinoma, Sq: squamous cell carcinoma, La: large cell carcinoma, Sm: small cell carcinoma, Metho: mesothelioma, Muc.: mucoepidermoid carcinoma, Prim.: primary, Lym.: lymph node, Effu.: effusion.

Investigation of cell cycle distribution after exposure to adriamycin has shown that cells possessing wild-type p53 tend to become arrested in G1 phase. In these cell lines, transcriptional p21 activation generally leads to G1 arrest. Additionally, we found G2/M phase repression and apoptosis progression accompanying repression of survivin protein. It has been reported previously that transfection with survivin anti-sense or dominant negative survivin gene resulted in accumulation of apoptotic cells and concomitant loss of G2/M phase cells.[34,35] Li *et al.*[7] showed that cells transfected with a mutant survivin gene or survivin anti-sense appeared to show increased caspase3 activity when synchronized in G2/M phase but not in G1/S phase. We therefore analyzed the cell cycle distribution of cell lines possessing mutated or deleted p53. In contrast to cells with wild-type p53, these cells became arrested in G2/M phase. Thus, survivin retention in cells possessing mutant p53 might make them able to resist apoptosis at the G2/M checkpoint.

One critical point of our study was to investigate differences in the proliferation of cancer cells following survivin repression, with the expectation that survivin inhibition itself would have a potent anti-proliferation effect. In cells possessing mutated or deleted p53, survivin was stably expressed even after adriamycin exposure and cell cycle arrest at the G2/M phase, indicating an anti-apoptotic effect. Survivin inhibition by siRNA downstream of p53 induced cell apoptosis and enhanced the anti-proliferative effect. Survivin associates with microtubules of the mitotic spindle at the beginning of mitosis, and disruption of survivin-microtubule interactions increases caspase-3 activity.[7] In order to inhibit survivin specifically, we used siRNA. This efficiently repressed survivin expression and inhibited cell proliferation in the absence of any cytotoxic stimulus. It has been reported that antisense targeting of survivin induces apoptosis in lung cancer cells. Using TUNEL assay, we also confirmed that anti-survivin siRNA duplex induced apoptosis.

Finally, survivin inhibition was found to sensitize PC9 to an anti-cancer agent. Exposure to Adriamycin after repression of survivin by siRNA significantly inhibited cell proliferation compared to cells exposed to either adriamycin alone or anti-survivin siRNA alone. Data obtained by the TUNEL assay confirmed that the difference in cell proliferation was based on apoptosis. *In vitro* binding experiments have indicated that survivin specifically binds to caspase-3 and -7, but not to caspase-8.[6] We also identified repression of procaspase-3 (which means activation of caspase-3) in cells exposed to adriamycin after treatment with anti-survivin siRNA. Activation of caspase-3 by inhibition of survivin may thus promote sensitivity to adriamycin. In our study, the expression of survivin mRNA in SBC3/ADM cells was greater than that in the parental SBC cells (Fig. 1*b*), indicating that survivin expression is related to cell resistance to adriamycin. We identified survivin inhibition by siRNA in cells with mutated p53 sensitized to adriamycin. Combining transfection with a mutant survivin gene with exposure to adriamycin did not enhance apoptosis in HeLa cells and MCF-7 cells, which have wild-type p53, compared to a mutant survivin gene transfection alone or adriamycin alone.[36] The combined effect of the two against apoptosis may be dependent on the character of each cell type, including p53 status or the compound targeting survivin. Additional studies will be needed to

determine the combined effect of survivin inhibition and other drugs on other cell lines.

In conclusion, siRNA targeting survivin could be of potential value for increasing the sensitivity of cancer cells to anti-cancer drugs, especially drug-resistant cells that possess mutated p53.

Acknowledgements

We thank E. Hatashita, Y. Yamada, T. Wada and M. Nagasaka for experimental assistance. This investigation was selected for a Scholar-in-Training Award at the 95th Annual Meeting of the AACR.

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Jpn J Clin Oncol 2005;35(4)195–201
doi:10.1093/jjco/hyi060

Standard Thoracic Radiotherapy With or Without Concurrent Daily Low-dose Carboplatin in Elderly Patients with Locally Advanced Non-small Cell Lung Cancer: a Phase III Trial of the Japan Clinical Oncology Group (JCOG9812)

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Received October 20, 2004; accepted February 14, 2005

Background: The purpose of this study was to evaluate whether radiotherapy with carboplatin would result in longer survival than radiotherapy alone in elderly patients with unresectable stage III non-small cell lung cancer (NSCLC).
Methods: Eligible patients were 71 years of age or older with unresectable stage III NSCLC. Patients were randomly assigned to the radiotherapy alone (RT) arm, irradiation with 60 Gy; or the chemoradiotherapy (CRT) arm, the same radiotherapy and additional concurrent use of carboplatin 30 mg/m^2 per fraction up to the first 20 fractions.
Results: This study was terminated early when 46 patients were registered from November 1999 to February 2001. Four patients (one in the RT arm, three in the CRT arm) were considered to have died due to treatment-related causes. The JCOG Radiotherapy Committee assessed these treatment-related deaths (TRDs) and the compliance with radiotherapy in this trial. They found that 60% of the cases corresponded to protocol deviation and 7% were protocol violation in dose constraint to the normal lung, two of whom died due to radiation pneumonitis. As to the effectiveness for the 46 patients enrolled, the median survival time was 428 days [95% confidence interval (CI) = 212–680 days] in the RT arm versus 554 days (95% CI = 331 to not estimable) in the CRT arm.
Conclusions: Due to the early termination of this study, the effectiveness of concurrent use of carboplatin remains unclear. We re-planned and started a study with an active quality control program which was developed by the JCOG Radiotherapy Committee.

Key words: non-small cell lung cancer – elderly patients – carboplatin – chemoradiotherapy

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the USA, Europe and Japan. In Japan, the number of elderly is increasing dramatically. In 2001, the proportion of Japanese population older than 65 years was 18%; in other words, the number of people older than 65 years exceeded 22 million (1). Lung cancer death rates for men and women aged 75 or more have increased to ~531 and 138 per 100 000 population, respectively (1). To establish the effective treatment for the elderly with lung cancer has thus become of greater importance.

Until recently, the standard treatment for locally advanced non-small cell lung cancer (NSCLC) was radiotherapy alone. However, the 5-year survival rate of patients with stage III remained under 10% (2–4). To improve the survival rates, many clinical trials comparing radiotherapy with chemoradiotherapy have been conducted (5–11). A recent meta-analysis suggested that the combination of chemotherapy containing cisplatin (CDDP) and radiation could improve the survival rate compared with radiotherapy alone (12,13). However, it is still unclear whether the combined chemoradiotherapy is also suitable for elderly patients. This is partly because the elderly had been considered inappropriate as study patients.

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Almost all evidence available has thus been derived from subset analysis of trials for locally advanced NSCLC. A secondary analysis of RTOG 94-10 revealed a greater survival benefit for concurrent chemotherapy (14). Schild et al. reported no significant difference in tumor regression between younger and older patients in an NCCTG trial (15). Meanwhile, some reports on inoperable NSCLC patients indicate that chemoradiotherapy has survival benefit compared with radiotherapy, but this may not be applicable for those >70 years of age, for whom radiation alone could be most beneficial (16,17).

Therefore, we cannot treat the elderly in the same way as we can younger patients: first, as elderly patients have poorer prognosis than younger patients, they may think that their quality of life is more important than risking radical treatment. Secondly, the elderly tend to be vulnerable to intensive care and toxicities of treatment drugs (18–21). Less toxic therapy may be more effective for the elderly with NSCLC.

Some clinical trials, in which the elderly were not included, showed some efficacy of carboplatin (CBDCA), an analog of CDDP, having no nephrotoxicity, neurotoxicity or ototoxicity and being much less emesis-provoking than CDDP (22–24). Additionally, some investigators found the same radiosensitizing properties of CBDCA (25–28) as also found for CDDP. Therefore, we hypothesized CBDCA to be more acceptable in the treatment of elderly patients. A phase II study has reported the use of radiotherapy and concurrent low-dose daily CBDCA in elderly patients with locally advanced NSCLC (29). For stage III patients, the median survival time (MST) was 15.1 months. Given an MST of ~10 months by radiation alone (5,6,8,9,11,17), this combined chemoradiotherapy seemed promising. Here we performed a randomized study to determine whether this combined chemoradiotherapy has an impact on survival in elderly patients with unresectable locally advanced NSCLC compared with radiotherapy alone.

PATIENTS AND METHODS

PATIENTS

Eligibility criteria for this study were as follows: age ≥71 years; a histologically confirmed non-small cell carcinoma; unresectable disease; stage IIIA except T3N1M0 and IIIB which does not have disease extended to any contralateral hilar nodes or any supraclavicular nodes, atelectasis of the entire lung or malignant pleural effusions; measurable disease; a required radiation field of less than one half of one lung; no previous chemotherapy or radiotherapy; an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; PaO_2 ≥70 torr, white blood cell count ≥4000/μl, hemoglobin level ≥9.5 g/dl, platelet count ≥100 000/μl, serum bilirubin level ≤1.5 mg/dl, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤ twice the upper limit of normal, and serum creatinine level ≤ the upper limit of normal; a life expectancy of at least 3 months; and written informed consent. Exclusion criteria included patients with active infection, interstitial pneumonia or active lung fibrosis, chronic obstructive pulmonary disease (COPD) or uncontrolled heart disease, an active synchronous cancer, or a metachronous cancer within three disease-free years.

Staging was performed by chest radiograph in two directions, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the head, CT scan of the chest, CT scan or ultrasound of the abdomen, and bone scintigraphy.

TREATMENT

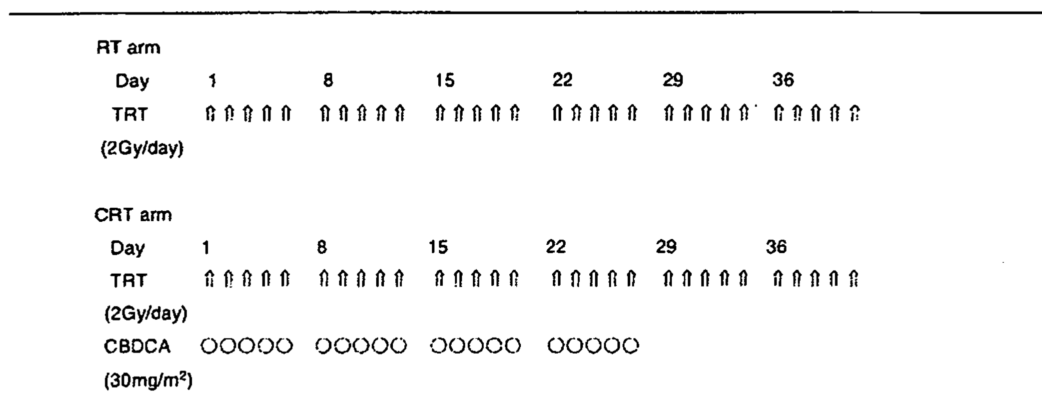
Patients were randomly assigned to the radiotherapy (RT) arm or the chemoradiotherapy (CRT) arm, by the minimization method of balancing PS (0 or 1 versus 2), stage (IIIA versus IIIB) and institution. The RT consisted of 60 Gy in 30 fractions over 6 weeks. In the CRT arm, patients received the same radiotherapy as in the RT arm and concurrent intravenous administration of CBDCA 30 mg/m^2 (30 min infusion) 1 h before every radiation treatment up to the first 20 fractions (Fig. 1).

Radiotherapy was delivered with megavoltage (6–10 MeV photons) equipment using anterior/posterior opposed fields up to 40 Gy including the primary tumor, the metastatic lymph nodes and the regional node. A booster dose of 20 Gy was given to the primary tumor and the metastatic lymph nodes for a total dose of 60 Gy using bilateral oblique fields. The clinical target volume (CTV) for the primary tumor was defined as the gross tumor volume (GTV) plus 1 cm taking account of subclinical extension. CTV and GTV for the metastatic nodes (>1 cm in shortest dimension) were the same. Regional nodes excluding contra-lateral hilar and supraclavicular nodes were included in the CTV; however, lower mediastinal nodes were included only if the primary tumor was located in the lower lobe of the lung. The planning target volumes for the primary tumor, the metastatic lymph nodes and regional nodes were determined as CTVs plus 0.5–1.0 cm margins laterally and 1.0–2.0 cm margins cranio-caudally taking account of set up variations and internal organ motion. Lung heterogeneity corrections were not used.

The criteria for stopping the treatment are pulmonary toxicities, which include the National Cancer Institute-Common Toxicity Criteria (NCI-CTC; version 2.0) grade 2 respiratory distress and <60 torr PaO_2, other than hematopoietic toxicities (leukopenia, neutropenia and thrombocytopenia) or gastrointestinal toxicities (dysphagia).

EVALUATION

To assess the rate of tumor response and toxicity, all patients received a complete blood cell count; blood chemistry, including AST, ALT, lactate dehydrogenase, bilirubin, serum creatinine, blood urea nitrogen, total protein, serum albumin, serum electrolytes and calcium; and weekly chest X-rays during the treatment period. Best overall response was evaluated as tumor response by mono- or bi-dimensional measurement in accordance with the World Health Organization (WHO) criteria (30), and toxicity was evaluated in accordance with the NCI-CTC (version 2.0).



RT, radiotherapy; CRT, chemotherapy; TRT, thoracic radiotherapy; CBDCA, carboplatin.

Figure 1. Treatment schema.

Study Design and Statistical Analysis

This trial was a multi-center randomized phase III study. The study protocol was approved by the JCOG Clinical Trials Review Committee and the institutional review board of each participating institution before the initiation of the study.

The primary end-point was overall survival, which was defined as the interval from randomization to death from any cause. Secondary end-points were response rate, which was the proportion of the patients evaluated as having a complete reponse (CR) or partial response (PR) in best overall response out of all eligible patients; progression-free survival (PFS) defined as the interval from randomization to the diagnosis of progression or death from any cause; sites of progression; and toxicity. The estimate of survival time was performed by the Kaplan–Meier method (31). The trial was designed to have an 80% power to detect 5 months difference in MST (10 months in the RT arm and 15 months in the CRT arm) with a one-sided alpha of 0.05 by log rank test (32). The planned sample size was 190 patients by Shoenfeld and Richter's methods (33) with 1.5 years follow-up after 3 years accrual.

In-house interim monitoring is performed by the JCOG Data Center to ensure data submission, patient eligibility, protocol compliance, safety and on-schedule study progress. The monitoring reports are submitted and reviewed by the JCOG Data and Safety Monitoring Committee (DSMC) twice yearly.

An expedited report was required by the JCOG DSMC to allow rapid identification of any life-threatening adverse events or unexpected toxicities according to the JCOG toxicity reporting system based on the ICH-E2A guidelines.

RESULTS

From November 1999 to February 2001, 46 patients were enrolled in this study: 23 in the RT arm and 23 in the CRT arm. Four treatment-related deaths (TRDs) had been reported, however, before the forty-sixth patient were assigned. Therefore, we suspended the registration and checked the details of all randomized patients to assess the safety of treatment regimens. As a result, it was revealed that three of these deaths were due to pneumonitis. The JCOG DSMC advised consultation with the JCOG Radiotherapy Committee (RC) about the radiotherapy compliance in all patients. The JCOG RC collected each patient's irradiation planning data retrospectively and found poor protocol compliance which was related to TRD. Consequently, we decided to terminate this trial in August 2001 following the recommendation of the JCOG DSMC.

Patients Charactersitics

Patient characteristics are listed in Table 1. No specific characteristics of patients were found in the elderly patients with locally advanced NSCLC compared with younger patiests and the two treatment arms were well balanced with respect to age and stage.

Toxicity of Treatment

Both hematological and non-hematological toxicities during the treatment and follow-up period were assessed. Table 2 summarizes the hematological toxicity. Patients receiving CBDCA suffered from leukocytopenia, neutropenia and thrombocytopenia more than patients receiving RT alone. There was no grade 4 hematological toxicity in the RT arm. Two (8.7%) and four (17.4%) patients in the CRT arm experienced grade 4 leukocytopenia and neutropenia, respectively.

Non-hematological toxicity observed in this study is listed in Table 3. None of the patients developed grade 3 esophagitis in either treatment arm. In the RT arm, other grade 3/4 toxicities were edema, fatigue, dyspnea and pneumonitis in one patient each. In the CRT arm, other grade 3/4 toxicities were neutropenic fever, dyspnea and pneumonitis. Grade 3/4 (RTOG/EORTC Radiation Toxicity Score) of late lung toxicity was observed in two patients in the RT arm and four patients in the CRT arm. Four TRDs were observed in this study. Three of

Table 1. Patient characteristics

Characteristics	RT arm	CRT arm
No. of eligible patients	23	23
Age (years)		
Median	77	77
Range	72–84	71–83
Male/female	19/4	16/7
Type of tumor		
Adenocarcinoma	6	11
Squamous cell	16	11
Large cell	1	1
PS (ECOG)		
0	3	9
1	19	13
2	1	1
Stage of disease		
IIIA	11	12
IIIB	12	11
Weight loss		
<10%	21	23
≥10%	2	0

RT, radiotherapy; CRT, chemoradiotherapy; PS, performance status.

Table 2. Hematological toxicity

	RT arm (n = 23)					CRT arm (n = 23)				
Grade	1	2	3	4	%grade 4	1	2	3	4	%grade 4
Leukocytes	10	2	2	0	0	3	7	11	2	8.7
Neutrophils	4	3	0	0	0	2	8	6	4	17.4
Hemoglobin	5	3	0	0	0	5	8	3	0	0
Platelets	2	0	2	0	0	4	5	8	0	0

RT, radiotherapy; CRT, chemoradiotherapy.

these patients were thought to have died as a result of pneumonitis. The details of these cases are follows. Case 1: a 78-year-old man had stage IIIA (T3N2) squamous cell carcinoma. He was treated with RT alone and died of pneumonitis at 28 days after therapy. Case 2: a 79-year-old man had stage IIIB (T4N2) adenocarcinoma. He was treated with CBDCA + RT and died of bacterial pneumonia at 37 days after therapy and had been taking steroid hormone due to radiation pneumonitis. Case 3: a 73-year-old man had stage IIIA (T3N2) squamous cell carcinoma. He was treated with CBDCA + RT and died of pneumonitis at 80 days after therapy. Case 4: a 80-year-old man had stage IIIB (T4N2) squamous cell carcinoma. He was treated with CBDCA + RT and died of pneumonitis at 54 days after therapy. Thus, three out of four TRDs were in the CRT arm and one was in the RT arm.

Table 3. Non-hematological toxicity

	RT arm (n = 23)					CRT arm (n = 23)				
Grade	1	2	3	4	% grade 4	1	2	3	4	% grade 4
Edema	0	0	0	1	4.5	0	0	0	0	0
Fatigue	1	0	0	1	4.5	7	1	0	0	0
Fever	3	0	0	0	0	1	1	0	0	0
Esophagitis	13	2	0	0	0	10	2	0	0	0
Nausea	0	0	0	–	–	2	2	0	–	–
Vomiting	0	0	0	0	0	1	0	0	0	0
Febrile neutropenia	–	–	0	0	0	–	–	1	0	0
Cough	3	1	0	–	–	6	0	0	–	–
Dyspnea	–	0	0	1	4.5	–	2	1	0	0
Pneumonitis	1	0	0	–	4.5	1	0	1	0	0
Creatinine	1	0	0	0	0	0	0	0	0	0
Hyponatremia	7	–	0	0	0	5	–	1	0	0
Heart	0	0	0	0	0	0	1	0	0	0
Lung	8	4	2	0	0	9	6	1	3	13.0

RT, radiotherapy; CRT, chemoradiotherapy.

Protocol Compliance

In the RT arm, 22 (95.6%) patients received full treatment doses. In the CRT arm, 20 (87.0%) patients completed the treatment. As to the administration of CBDCA, there were few protocol deviations.

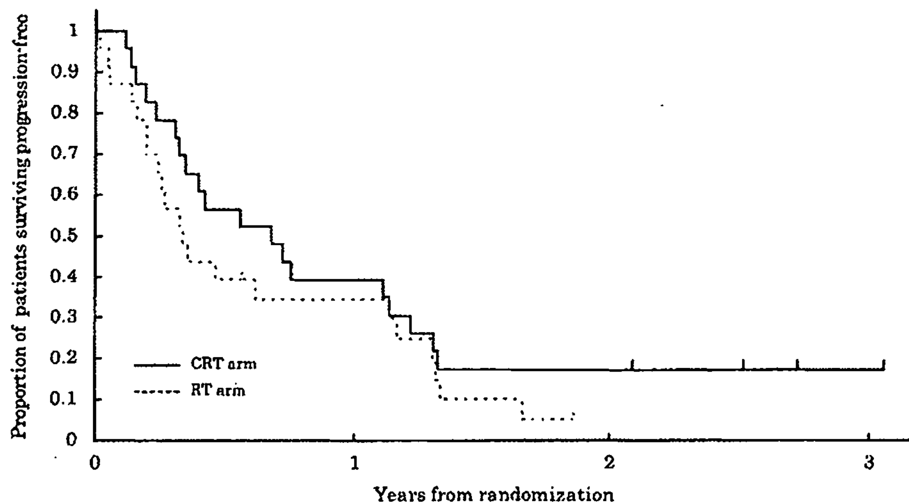
Three of the patients discontinued the protocol treatment: one was due to grade 2 eruption, one was due to cerebral infarction and one was due to insufficient recovery from leukopenia. One patient in the RT arm did not start the treatment due to local progression (Table 4).

Quality Assurance of Radiotherapy

We evaluated the quality of radiotherapy retrospectively based on the collected radiation therapy planning data. The data of 45 patients were reviewed and evaluated for the analysis. Details of this analysis have been reported by Ishikura et al. (34); three cases were revealed to be protocol violation due to normal lung volume constraint defined in the protocol. Unacceptable protocol deviations were identified as follows; 17, 15 and 31 cases on field border placement for the primary tumor, the metastatic lymph nodes and the elective nodal irradiation, respectively. Overall, 27 of 45 cases (60%) had at least one unacceptable deviation. Most cases judged to have protocol violation were primarily due to a smaller radiation field. Only 18 cases (40%) were judged to be protocol compliant.

Response and Survival

The tumor response in each arm is listed in Table 5. No patients achieved a CR in either arm. Of the 23 patients in the RT arm, 12 [52.2%, 95% confidence interval (CI) = 30.6–73.2%] achieved PR and six (26.1%) had stable disease. Of the



RT, radiotherapy; CRT, chemoradiotherapy.

Figure 2. Progression-free survival for patients treated with radiation alone or radiation with concurrent daily CBDCA.

Table 4. Protocol compliance

Pattern	RT arm (n = 23)	CRT arm (n = 23)
Complete protocol treatment	22	20
Progression/relapse*	1	0
Adverse events		
Cerebral infarction	0	1
Eruption	0	1
Leukopenia	0	1
Patient refusal	0	0
Death on protocol	0	0
Other	0	0

*Before starting the radiotherapy.
RT, radiotherapy; CRT, chemoradiotherapy.

23 patients in the CRT arm, 11 (47.8%, 95% CI = 26.8–69.4%) achieved PR and seven (30.4%) had stable disease.

Seventeen (73.9%) patients in the RT arm and 15 (65.2%) patients in the CRT arm had died at the time of analysis. The median progression-free survival time was 122 days (95% CI = 88–413 days) on the RT arm versus 248 days (95% CI = 127–416 days) on the CRT arm (Fig. 2.). The MST was 428 days (95% CI = 212–680 days) on the RT arm versus 554 days (95% CI = 331 to not estimable) on the CRT arm (Fig. 3.). The 1-year survival rate was 60.9% (95% CI = 40.9–80.8%) on the RT arm versus 65.2% (95% CI = 45.8–84.7%) on the CRT arm.

Pattern of Progression/Relapse

The first site of disease progression or relapse is listed in Table 6. Sixteen patients in the RT arm and 13 patients in the CRT arm had relapsed or had disease progression at the time of analysis. Eight patients (out of 16, 50.0%) in the RT arm and seven patients (out of 13, 53.8%) in the CRT arm had relapse or disease progression within the radiation field whether relapse outside the radiation field occurred or not.

Table 5. Response to treatment

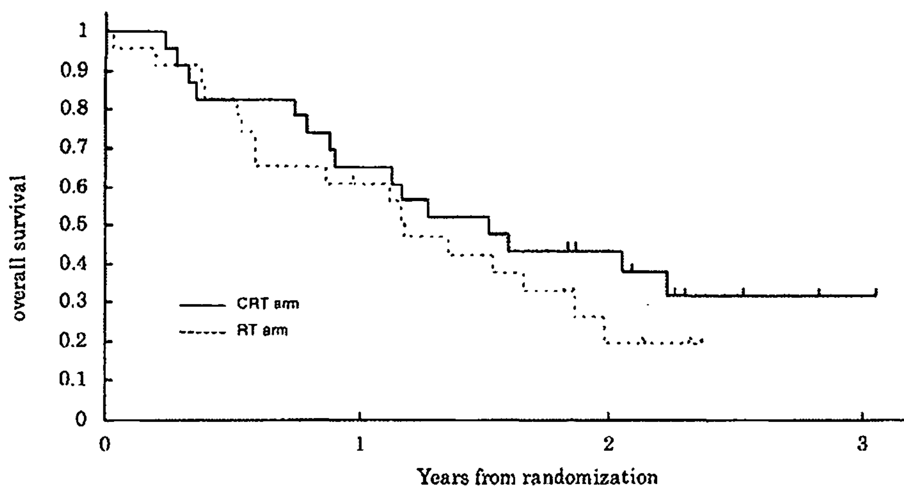
Response	RT arm (n = 23)	CRT arm (n = 23)
Complete response	0 (0)	0 (0)
Partial response	12 (52.2)	11 (47.8)
Stable disease	6 (26.1)	7 (30.4)
Progression	4 (17.4)	4 (17.4)
Not evaluable	1 (4.4)	1 (4.4)
Objective response	52.2%	47.8%

RT, radiotherapy; CRT, chemoradiotherapy.

DISCUSSION

We conducted this randomized controlled trial to determine whether chemoradiotherapy was superior to radiotherapy alone with respect to overall survival of elderly patients with locally advanced NSCLC. The study was terminated early when 24% of the planned sample size was accrued because of a high proportion of TRDs due to radiation pneumonitis and protocol violation.

Pulmonary toxicities including radiation pneumonitis and fibrosis caused by radiation therapy are, in general, common but not severe. In this study, however, the risk of TRD was 8.7% (four out of 46) and was much higher than in other trials. For instance, Ohe et al. (35) retrospectively analyzed the incidence of TRDs in the treatment of thoracic radiotherapy and/or chemotherapy for patients with locally advanced NSCLC, and reported that seven of 448 patients (1.6%)



RT, radiotherapy; CRT, chemoradiotherapy.

Figure 3. Overall survival for patients treated with radiation alone or radiation with concurrent daily CBDCA.

Table 6. First site of disease progression

	RT arm (n = 23)	CRT arm (n = 23)
Local	8	5
Distant	8	6
Local + distant	0	2

RT, radiotherapy; CRT, chemoradiotherapy.

died of radiation-induced pneumonitis. The high proportion of pulmonary toxicities in our trial may be due partly to the high age of the patients. Schild et al. (15) reported that they found 6% of elderly (older than 75 years) with NSCLC had grade 4 pneumonitis whereas this was the case in only 1% of younger patients ($P = 0.02$). It was controversial that the four TRDs out of 46 was sufficient reason to terminate the on-going trial; however, we thought it was serious that half of the TRDs (two out of four) were judged to be associated with protocol violation concerning the radiation field, which was to be less than half of one lung. Because the JCOG had not yet established the quality control/assurance system for radiotherapy before this trial, we concluded that we would not be able to control the risk of radiation pnuemonitis due to protocol deviation if we continued this study. What was an issue in this study was not only the high TRD rate, but also the poor protocol compliance of RT. The reasons for the poor protocol compliance are limited participation of radiation oncologists during protocol development, limited educational resources for attending radiation oncologists and no quality control program. Although the retrospective systematic review of radiation planning and protocol compliance of radiotherapy was the first experience in the JCOG, both the Lung Cancer Study Group and the entire JCOG had become aware of the importance of a quality control system for radiotherapy. The JCOG Executive Committee decided to establish the Radiation Therapy Quality Assurance Center (RTQAC) within the JCOG Data Center under the supervision of the JCOG Radiotherapy Committee. The RTQAC started the prospective quality control and quality assurance (QC/QA) program in September 2002 with a new activated phase III study for limited disease of small cell lung cancer, JCOG0202. Up to 2004, the QC/QA program has been expanded to the other group studies, such as esophageal cancer study, breast cancer study, prostate cancer study and brain tumor study. In addition, the JCOG Executive Committee mandates the QC/QA program by the RTQAC for all JCOG trials when protocol treatment includes radiation therapy.

The clinical question raised in this trial has not been answered. The data from the 46 patients enrolled were not considered to be conclusive because of the small sample size. No remarkable difference was found between the arms in terms of safety and efficacy such as tumor response, PFS and overall survival. We considered that it still remained an important clinical question to be investigated whether the daily low-dose CBDCA plus radiotherapy was effective or not. Therefore, we re-planned and started a new phase III trial (JCOG0301), in which the prospective QC/QA program by the RTQAC is added to the identical design to this JCOG9812. The protocol involves initial review of radiation planning and final review of the actual radiation record for all randomized patients. The JCOG0301 was activated in September 2003, and we have achieved very good protocol compliance upto now.

Acknowledgments

We thank Ms M. Imai and Dr M. Niimi for their data management, and Dr H. Fukuda for his direction of the JCOG Data Center and oversight of management of the study. This study

was supported in part by Grants-in-Aid for Cancer Research (11S-2, 11S-4, 14S-2, 14S-4) and for Second Term Comprehensive 10-year Strategy for Cancer Control (H12-Gan-012) from the Ministry of Health, Labour and Welfare.

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Int J Clin Oncol (2005) 10:153–156
DOI 10.1007/s10147-005-0491-z

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FROM THE ASCO-JSCO JOINT SYMPOSIUM

Nagahiro Saijo · Yuji Nimura

Summary of the ASCO–JSCO Joint Symposium

Received: March 30, 2005

Key words ASCO–JSCO joint symposium

The American Society of Clinical Oncology (ASCO) is now rapidly expanding as an international society for clinical oncology. The ASCO mission statement is as follows: "As a nonprofit organization, ASCO is dedicated to achieving its charitable mission outlined by the organization's founders in 1964. ASCO strongly supports all types of cancer research, but in particular, patient-oriented clinical research." To realize the ASCO mission statement, ASCO makes strategic plans, and the new strategic plan is titled "Cancer Prevention and New Control." Because there now are more than 20000 ASCO members, the choice of meeting places is limited. ASCO 2005 will be held in Orlando, Florida, USA, May 14–17, and ASCO 2006 will be held in Atlanta. Thereafter, all meetings are scheduled to be held in Chicago because of the number of flights to the city, hotel accommodations, and the size of the convention center. ASCO has many scientific activities in addition to the annual meeting. For example, the Gastrointestinal Council Symposium and the Multidisciplinary Prostate Cancer Program will be conducted in Miami and Orlando, respectively. In addition, the "Best of ASCO" meetings are scheduled not only in the United States but also in Japan as an advanced course organized by the Japanese Society of Medical Oncology (JSMO), to be held June 11 and 12, 2005. ASCO also publishes materials such as educational curricula and self-assessment tools.

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The ASCO-JSCO Joint Symposium was held in Kyoto, Japan, on October 29, 2004.

The *Journal of Clinical Oncology* (JCO), published by ASCO, is widely read and has an impact factor above 10. In 2005, publication of review article issues began. Membership in ASCO grew from 66 in 1964 to 21837 at the end of 2003. Some 23000 investigators attend the annual meeting every year. Members' board certifications show more than half in medical oncology, with 15% in hematology/oncology, followed by pediatric oncology, radiation oncology, and others. The distribution is similar to that of the Japanese Society of Medical Oncology (JSMO) and is quite different from that of the Japanese Society of Clinical Oncology (JSCO). Domestic membership is 73% and international membership is 27%. By world region, about 50% of the international members are in Europe, 19% are from Asia, 8% from Latin America, with Canada and Mexico accounting for 15%. After the United States, the top 10 countries for ASCO membership are Japan (No. 1 at 591), followed by Canada, Germany, Italy, France, the United Kingdom, Spain, Brazil, and Switzerland. Thanks to the efforts of the International Committee (Nagahiro Saijo, chairman) of JSCO, reciprocal membership application became available to JSCO members, making it possible to avoid complicated application procedures to become an ASCO member. JSCO members are encouraged to use this system and to apply for active membership in ASCO.

The ASCO International Affairs Committee organizes various joint symposiums and workshops. For example, with FRASCA, ASCO held a joint international symposium in 2003 and an Australia/Asia–Pacific clinical research development workshop in 2004. Every year ASCO and AACR have a joint workshop on clinical trials in Vail, Colorado, USA. Japanese oncologists, in addition to facing language barriers, still do not have enough scientific knowledge to attend this meeting. ASCO and the European Society of Medical Oncology (ESMO) approved the core curriculum of medical oncology, which has been published in the *Journal of Clinical Oncology* and the *Annals of Oncology*. The JSMO has almost completed the translation of the curriculum, which will be accredited by ASCO. JSMO provides educational seminars twice a year based on that

Table 1. Japanese contribution to ASCO

1. Description	Total
International members	27%
US members	73%
Total ASCO members	21 800
2. Number of international members by country	
Japan 591, Canada 575, Germany 444, Italy 423, France 337, UK 289	
3. Japanese ASCO presentation	
Original papers (oral, poster discussion, poster)	
1994, 17; 1997, 37; 2000, 64; 2003, 85; 2004, 92	
Participants in poster discussions – N. Saijo (2003)	
Educational sessions	
International symposium	N. Saijo (2002), M. Sasako (2003)
Meet the professor	N. Saijo (2003), M. Tsuboi (2004)
Educational symposium	H. Wada (2005)
4. Committee member in ASCO	
International affairs committee (Director: Paula T. Rieger)	
2001–2003	Nagahiro Saijo
2003–2005	Yasuhiro Fujiwara
2004–2006	Masahiro Fukuoka
5. Endorsement for	
ASCO-JSCO joint symposium (2002, 2003, 2004, 2005)	
ASCO-JSMO joint symposium (2004, 2005, 2006)	
6. ASCO Board member	Nagahiro Saijo (2004–2007)

curriculum. ASCO has established a new international seat on the ASCO Board of Directors, and I (Nagahiro Saijo) was elected as a 2004–2007 ASCO Board member, as was Dr. José Baselga from Spain for 2003–2006.

Japan is the top country in terms of the number of active international members of ASCO (Table 1). The number of Japanese attendees at the ASCO annual meeting increased to 900 in 2004. The acceptance ratio for Japanese abstracts is improving; for posters, poster discussions, and oral presentations it is nearly 50%, and the numbers of presented abstracts are about 80–90 every year. The ASCO-JSCO joint symposium started in 2002 in Tokyo, followed by the one in Sapporo last year. Because we could not attract a large enough audience for those meetings, the program for this year shifted to topics of surgery. Dr. Nimura, a moderator of the symposium, and Drs. Kato, Sasako, and Blumgart are surgeons; Dr. Ajani and I are medical oncologists.

Dr. Kato, a professor of Tokyo Medical College, spoke on adjuvant chemotherapy of early-stage lung cancer. Uracil-tegafur (UFT) is a chemotherapy drug that most American oncologists do not recognize because it has not been approved for use in the United States, although limited numbers of clinical trials of the drug have been conducted against gastrointestinal tumors there. UFT has been widely used in Japan against various tumor types and has been approved for use in many countries. Uracil-tegafur, a prodrug of 5FU, is one of the oral fluorinated pyrimidine drugs that has been synthesized mainly by pharmaceutical companies in Japan. The main purpose of oral fluorinated pyrimidine is to improve the delivery of low-dose 5FU over time, mimicking a continuous infusion of 5FU. The beneficial effect of tegafur is believed to derive from its slow conversion to 5FU through the cytochrome P450 pathway. The released 5FU from the prodrug tegafur competes with uracil for catabolism by the rate-limiting enzyme dihydropyrimidine dehydrogenase. The presence of excess uracil is believed to decrease the degradation of 5FU, maintaining a continuous drug level. Although it has been widely used in various diseases, there have been no large confirmatory randomized controlled trials. In the treatment of non-small cell lung cancer (NSCLC), a small phase II study showed that the response rate of UFT was less than 10%. Surgeons in Japan still prefer to use it after surgery, however, because of its mild adverse effect and because of oral administration. In a previous preliminary phase III trial of adjuvant chemotherapy after resection of NSCLC, UFT taken orally was shown to prolong survival, especially in pathological stage I adenocarcinoma. Based on these data, the Taiho Pharmaceutical Company organized the Japan Lung Cancer Research Group on Postsurgical Adjuvant Chemotherapy and conducted a randomized controlled trial against pathological stage I adenocarcinoma. Patients were randomly assigned to UFT (250mg) for 2 years or to no treatment. From January 1994 through March 1997, 999 patients were enrolled. Twenty patients were found to be ineligible and were excluded from the analysis after randomization, 491 patients were assigned to receive UFT, and 488 were assigned to observation. The median duration of follow-up for surviving patients was 73 months. The difference in overall survival between the two groups was statistically significant in favor of the UFT group ($P = 0.04$ by stratified log-rank test). Grade 3 toxic effects occurred in 10 of the 482 patients (2%) who received UFT.

So far, six randomized trials, including the present one, have been conducted that compare surgery alone with adjuvant UFT chemotherapy. Among them, three trials have shown a survival benefit from treatment with UFT. A meta-analysis of those six trials showed that adjuvant chemotherapy with UFT improved overall survival (hazard ratio for death, 0.77; 95% confidence interval, 0.63–0.94; $P =$