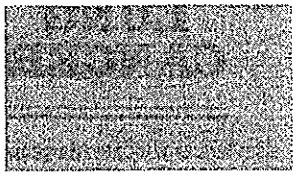

and grade 1 diarrhea and dry skin developed. A chest CT scan on March 3 revealed multiple pulmonary metastases that had decreased in size. A brain MRI on March 4 showed that multiple brain metastases also had decreased in size. Based on RECIST criteria, they had stable disease but radiographic tumor regression was observed.

1. Introduction

1.1. Efficacy of gefitinib

The epidermal growth factor receptor (EGFR) autocrine pathway contributes to a number of processes important to cancer development and progression, including cell proliferation, apotosis, angiogenesis, and metastatic spread [1]. EGFR-tyrosine kinase has become a particularly promising drug targeting for treating non-small cell lung cancer. Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells [2]. Responsiveness characteristics include distinct subgroups of women, patients who have never smoked, patients with adenocarcinoma, and Asians [3–5]. Molecular predictive markers have also been investigated. It is suggested that MAPK is a predictive marker for survival after treatment with gefitinib in chemo-naive patients with bronchioloalveolar carcinoma [6]. Patients with P-Akt-positive tumors who received gefitinib had a better response rate, disease control rate, and time to progression than patients with P-Akt-negative tumors, suggesting that gefitinib may be most effective in patients with basal Akt activation [7]. However, it was not possible to predict gefitinib sensitivity by the level of EGFR overexpression as determined by immunohistochemistry [8] or immunoblotting [9]. Recently it has been reported that somatic mutations in the tyrosine kinase domain of the *EGFR* gene occur in a subset of patients with lung cancer who showed a dramatic response to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib [10–12]. All of these mutations were within exons 18 through 21 of the kinase domain of the *EGFR* gene.

1.2. Drug summary

EKB-569 (Wyeth Research, Collegeville, PA) is a potent, low molecular weight, selective, and irreversible inhibitor of EGFR that is being developed as an anticancer agent. EGFR is a receptor tyrosine kinase that is activated by a variety of growth factors. Upon binding ligands, including epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α), EGFR dimerizes and its intracellular kinase domain is activated, leading to the recruitment and phosphorylation of a number of proteins that ultimately lead to cell growth [13,14]. Several features of EKB-569 may provide certain advantages over other EGFR inhibitors. First, EKB-569 is an orally available, small-molecule EGFR inhibitor, whereas antibody-targeted EGFR inhibitors require intravenous (IV) administration. Second, EKB-569 is an irreversible inhibitor of EGFR, while other small-molecule EGFR inhibitors bind EGFR reversibly [15].

1.3. Effects in humans (Japanese)

A phase 1, open-label, dose-escalation study to assess the safety, tolerability, and pharmacokinetics of EKB-569 was conducted in Japanese patients. EKB-569 was administered orally, once daily, in 28-day cycles, to patients (pts) with advanced-stage malignancies known to overexpress EGFR. Enrollment and treatment are completed; 15 pts (six men, nine women) were treated with 25 mg (3 pts), 35 mg (8 pts), or 50 mg (4 pts) of EKB-569. Their median age was 62 years (range 47–72); ECOG performance status varied: 0 = 4/15 (26.7%) or 1 = 11/15 (73.3%).

The most frequently occurring tumor types included non-small cell lung (10 pts) and breast (2 pts). The remaining tumors were renal, leiomyosarcoma, and malignant thymoma (1 pt each). The most frequently reported EKB-569-related adverse events were diarrhea (86.7%), rash (53.3%), anorexia (40.0%), and dry skin (40.0%). Dose-limiting toxicities were observed at the 50-mg dose level with grade 4 interstitial lung disease and grade 3 diarrhea, stomatitis, and increased blood calcium levels. Thus, the maximum tolerated dose was 35 mg EKB-569 per day.

1.4. Molecular analysis of lung cancer specimens

We obtained appropriate approval from the institution and written informed consent from the patients for the comprehensive use of tumor samples for molecular and pathologic analyses. Surgically resected tumor samples were obtained retrospectively before the patients received

any systemic treatment. All of these tumors were formalin fixed and paraffin embedded by the Department of Pathology. To minimize non-neoplastic tissue contamination, the tumor portion was first selected and marked on an H&E-stained tissue section slide by a pathologist. Only the tumor portion was dissected from the unstained tissue section and sent for DNA extraction.

DNA was extracted from the paraffin section containing a representative portion of each tumor, using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). For mutational analysis of the kinase domain of the *EGFR* coding sequence, exons 19, 20, and 21 were amplified with three pairs of primers (exon 19, F: 5′-TCACAATTGCCAGTTAACGTCT-3′-(this is the convention for writing a primer), R: 5#-cagcaaagcagaaactcacatc; exon 20, F: 5#-tgaaact-caagatcgcattcat, R: 5#-catggcaaactcttgctatcc; exon 21, F: 5#-gagcttcttcccatgatgatct, R: 5#-gaaaatgctggctgacctaaag). The PCR conditions were one cycle at 95 °C for 11 min, 46 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, followed by one cycle at 72 °C for 7 min. PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Forster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and chromatograms were reviewed manually and analyzed by BLAST (basic local alignment search tool). High-quality sequence variations found in both directions were scored as candidate mutations.

2. Clinical cases

Two patients from the Japanese phase 1 study are described in detail.

2.1. Case #1

A 63-year-old man with smoking history (BI: 720) who was treated for hyperlipidemia and hypertension showed an abnormal chest X-ray in February 1996. Further examinations including a chest computed tomography (CT) scan and bronchoscopy revealed an adenocarcinoma of the lung, c-T1N0M0, stage Ia, in the right upper lobe. He had undergone a right upper lobectomy with mediastinal lymph node dissection in July 1996 and was proven to have a well-differentiated adenocarcinoma, p-T1N0M0, stage Ia. After further follow-up, multiple pulmonary metastases in both lungs were found in January 2000. Then he was given first-line chemotherapy of cisplatin and docetaxel beginning in May 2000. After two courses of this regimen, multiple pulmonary metastases had not increased in size by CT scan; however skin metastases were found. He was started on oral gefitinib 250 mg/day on November 2000. After 4 weeks, a CT scan indicated a reduction of multiple pulmonary metastases. During this treatment, grade 2 rash and grade 1 nail changes, AST/ALT elevations, and diarrhea were observed. On June 2002, multiple pulmonary metastases had increased, and this treatment was discontinued. The patient entered a phase I study of a new EGFR tyrosine kinase inhibitor (TAK-165), starting treatment on October 2002. After 2 weeks of treatment, grade 3 anorexia was observed and the therapy was stopped. On February 2003, multiple pulmonary metastases had more increased, and on March 2003, he entered a phase I study of EKB-569, receiving treatment from 4 March 2004. EKB-569 (25 mg) was administered orally, once daily, in 28-day cycles. Because he had no severe adverse events, a total of 10 courses of therapy were completed through December 16. Grade 2 skin rash and ALT elevation, and grade 1 diarrhea and nail changes developed during this therapy. Based on RECIST criteria, the patient had stable disease (SD) but radiographic tumor regression was observed on 4 August 2003 (day 27 in the sixth course) (Fig. 1). The size of multiple pulmonary metastases increase by CT scan on 8 December 2003, and the treatment was stopped on 17 December 2003.

A lung cancer specimen was obtained at surgery and studied by immunohistochemistry. EGFR overexpression was detected. In addition, we found the heterozygous in-frame deletion E746-A750 in exon 19 of the *EGFR* gene by direct sequencing of the specimen.

2.2. Case #2

A 49-year-old woman with no smoking history, who was treated for Basedow's disease, insomnia, and bronchial asthma, had an abnormal chest X-ray in October 2000. Further examinations including a chest CT scan and bronchoscopy revealed lung cancer in the left upper lobe. She was diagnosed with adenocarcinoma, c-T1N0M0, stage Ia. She had a left-upper lobectomy with mediastinal lymph node dissection, which revealed a well-differentiated adenocarcinoma, p-T4N2M1, stage IV. She was then given first-line chemotherapy of carboplatin and paclitaxel beginning in January 2001. After two courses of therapy, she discontinued treatment because of adverse events. Right supraclavicular lymph node metastases were found on August

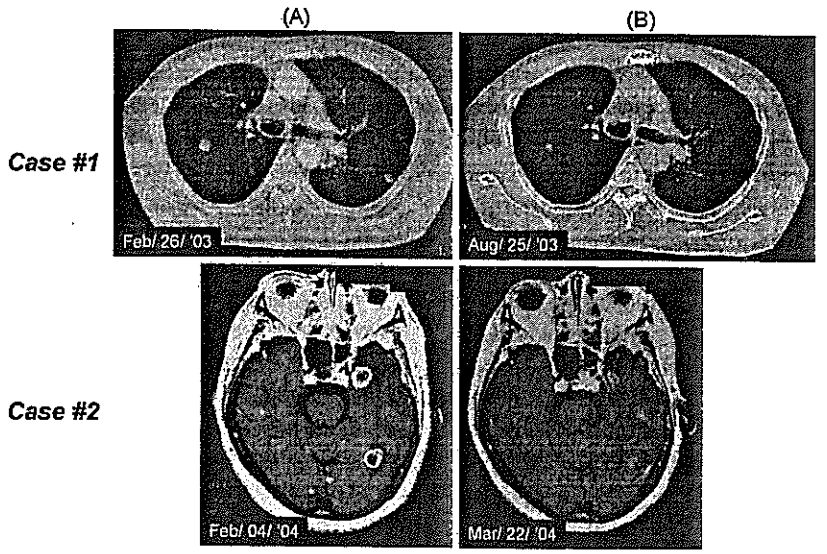

Fig. 1 *Clinical case #1*: a 63-year-old man with adenocarcinoma of lung. CT scan before treatment (A) and after initiation of EKB-569 (B). *Clinical case #2*: a 49-year-old woman with adenocarcinoma of brain metastasis. MRI scan before treatment (A) and after initiation of EKB-569 (B).

2001. Radiotherapy for the metastases (60 Gy/30 fractions) was done, and they decreased in size. On March 2002, right supraclavicular lymph node metastases increased and left clavicular lymph node metastases were found. On April 2002, the patient enrolled in a phase II trial of cisplatin, gemcitabine, and irinotecan for non-small-cell lung cancer. After two courses of therapy, bone metastases were found and pulmonary metastases had grown slowly so the treatment was stopped. She entered a phase I study of a new EGFR tyrosine kinase inhibitor (TAK-165) and started treatment on July 2002. The treatment was stopped after a week later due to grade 3 fatigue. In September 2002, the patient was started on oral gefitinib 250 mg/day. While she was taking 250 mg gefitinib daily for 15 months, the size of multiple pulmonary and bone metastases did not increase by CT scan and she had SD. On December 2003, the patient developed grade 3 oral mucositis and discontinued treatment. On January 2004, the size of multiple pulmonary and bone metastases increase by CT scan. She then entered a phase I study of EKB-569 and received therapy from 9 February 2004. EKB-569 (35 mg) was administered orally, once daily, in 28-day cycles. She received a total of five courses of the therapy until 22 June 2004. Grade 3 nausea and vomiting and grade 1 diarrhea and dry skin developed during the therapy. A chest CT scan on March 3 (day 24 in the first course) revealed multiple pulmonary metastases that had decreased in size. A brain MRI on March 4 (day 25 in the first course) showed that multiple brain metastases also had decreased in size (Fig. 1). The response was SD by RECIST criteria, although tumor regression was observed. The size of bone metastases increase by CT scan on 18 June 2004, and the treatment was stopped on 22 June 2004.

A lung cancer specimen was obtained by surgery and studied by immunohistochemistry. EGFR overexpression was detected. This lung cancer specimen had a heterozygous point mutation in exon 21 (L858R, CTG to CGG) of the *EGFR* gene.

3. Discussion

This is the first case report to describe the effects of EKB-569 on patients with adenocarcinoma of the lung. Case 1 is a 63-year-old man with a smoking history (BI: 720), and case 2 is a 49-year-old woman with no smoking history. Case 1 had an exon 19 deletion of E746-A750, and case 2 had an exon 21-point mutation. These patients underwent surgery and were treated with platinum-based chemotherapy and EGFR tyrosine kinase inhibitors. The treatment with EKB-569 was effective in these two patients after resistance to gefitinib and cytotoxic chemotherapy. These cases suggest that EKB-569 is effective in patients with *EGFR* mutations as has been reported for gefitinib and erlotinib. Despite initial responses to these EGFR inhibitors, patients eventually progress by unknown mechanisms of "acquired" resistance.

Recently, a second mutation in the *EGFR* kinase domain, which is associated with acquired resistance of non-small cell lung cancer to gefitinib or erlotinib, was reported [16,17]. Pao et al. showed that in two of five patients with acquired resistance

to gefitinib or erlotinib, progressing tumors contained, in addition to a primary drug-sensitive mutation in EGFR, a secondary mutation in exon 20. This mutation leads to a substitution of methionine for threonine at position 790 (T790M) in the kinase domain [16]. Kobayashi et al. reported the case of a patient with EGFR-mutant, gefitinib-responsive, advanced non-small cell lung cancer who relapsed after two years of complete remission during treatment with gefitinib. The DNA sequence of the EGFR gene in his tumor biopsy specimen at relapse also revealed the presence of the secondary point mutation, T790M [17]. Kurata et al. reported an interesting case in which acquired resistance to gefitinib could be overcome [18]. In this case, the patient received gefitinib, then a combination of nedaplatin and gemcitabine, and then gefitinib again. The cytotoxic agents may have altered the *EGFR* gene or associated genes to produce acquired sensitivity to gefitinib.

Kobayashi et al. also found that CL-387,785, a specific and irreversible, anilinoquinoline EGFR inhibitor [19], strongly inhibited the EGFR kinase in cells transfected with DNA containing the L747-S752 deletion in the *EGFR* gene or a double mutation with the L747-S753 deletion and the T790M point mutation. They speculated that CL-387,785 inhibited the EGFR kinase of the double mutant because of its altered binding to the kinase domain or its covalent binding to EGFR [17]. Kwak et al. used a bronchoalveolar cancer cell line with an L746-A750 deletion in the *EGFR* gene to isolate gefitinib-resistant clones. These clones had not acquired secondary EGFR mutations but were sensitive to the irreversible, anilinoquinoline EGFR inhibitor EKB-569 [20].

We have shown that EKB-569 had clinical activity in two patients with advanced non-small cell lung cancer with EGFR mutations and acquired gefitinib resistance. Thus, irreversible EGFR inhibitors may be an effective therapy for patients with EGFR-mutant advanced non-small cell lung cancer who have relapsed after treatment with gefitinib.

Acknowledgments

We thank Tetsuya Mitsudomi and Yasushi Yatabe (Aichi Cancer Center Hospital) for technical assistance in molecular analysis of tumors.

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Int. J. Cancer: **118**, 812–820 (2006)

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.

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).

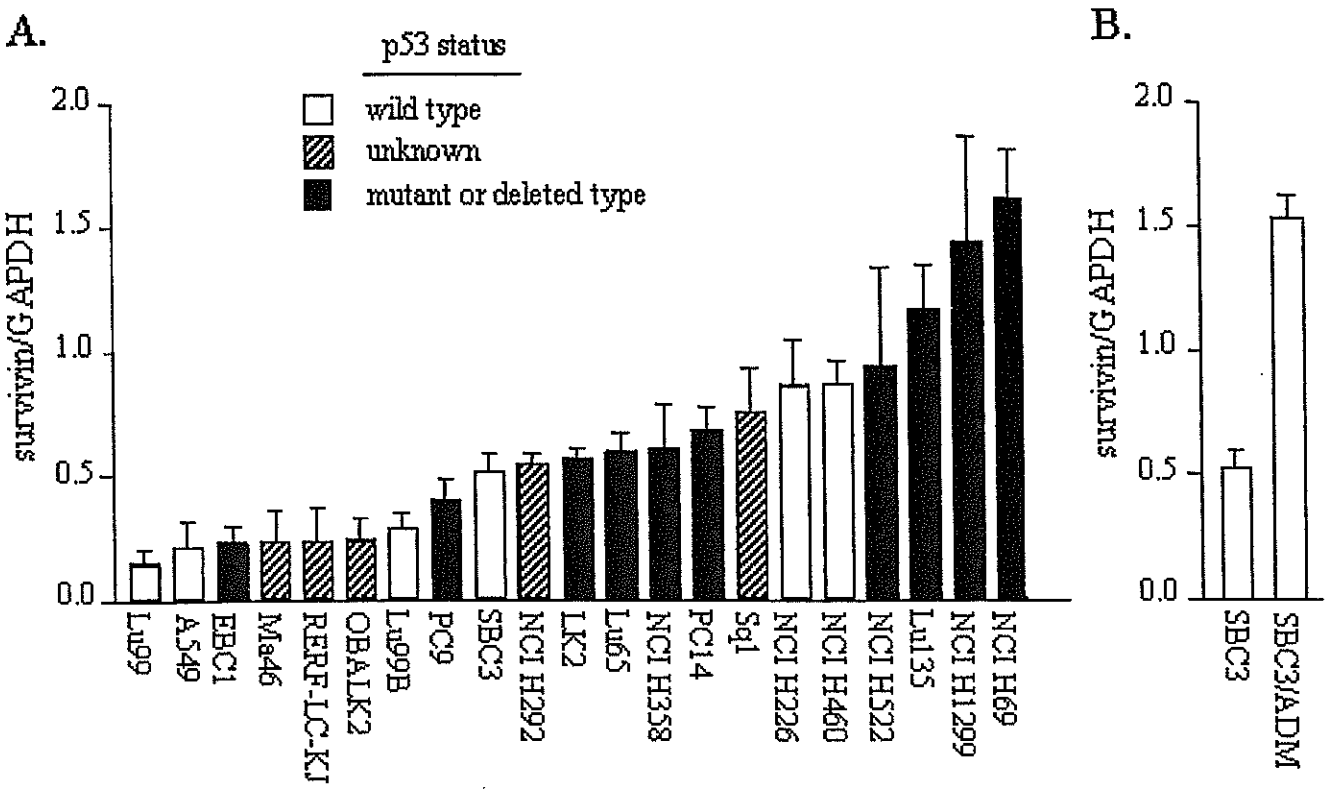

FIGURE 1 – Level of survivin mRNA in 22 lung cancer cell lines. (*a*) Cells were incubated in a 75 cm^2 flask, harvested and analyzed using real-time PCR as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH and are presented 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-

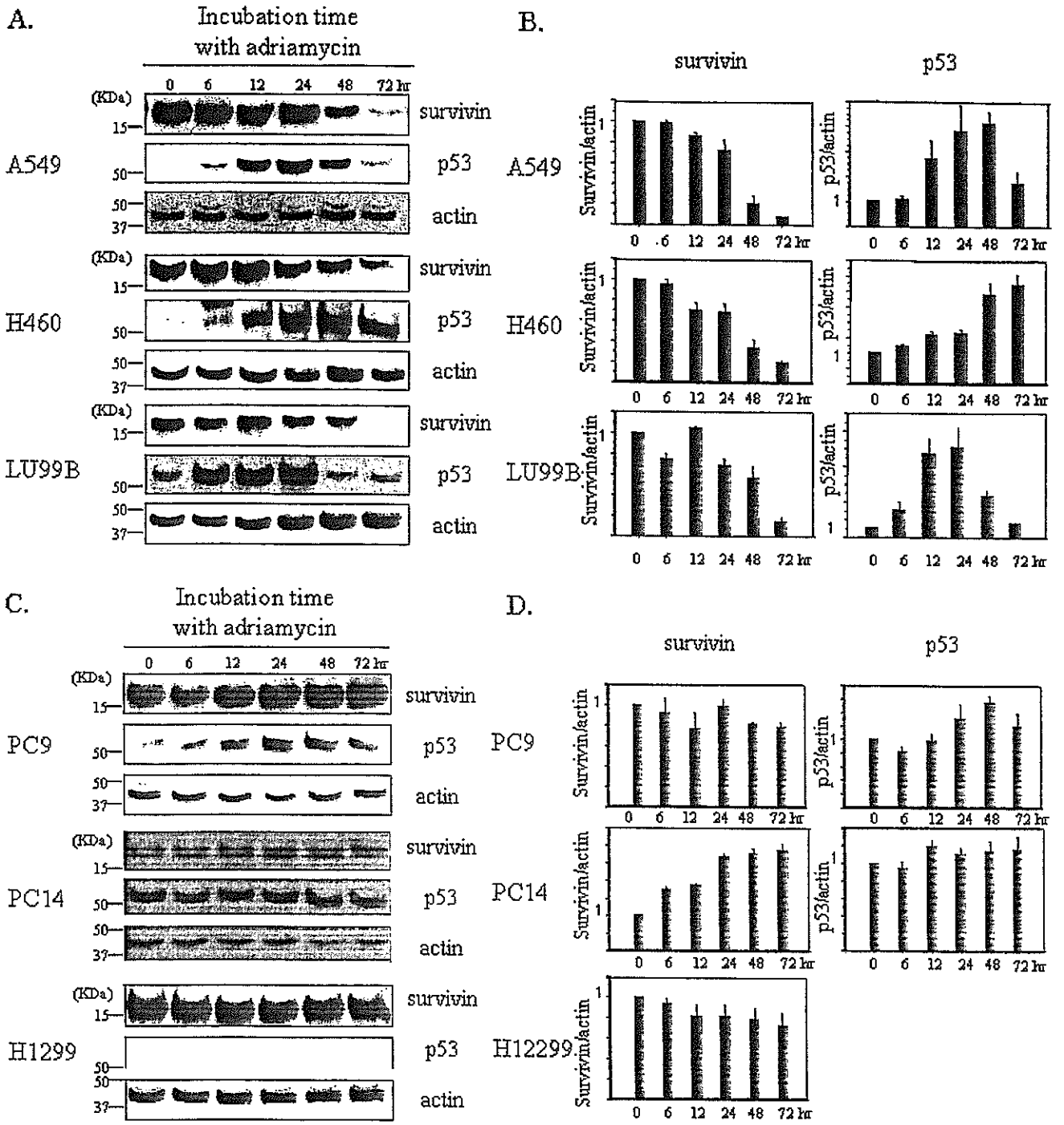

FIGURE 2 – Expression of survivin and p53 protein in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. (*a*) Western-blotting analysis for expression of survivin and p53 in cell lines possessing wild-type p53, including A549, NCI H460 and LU99B. 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

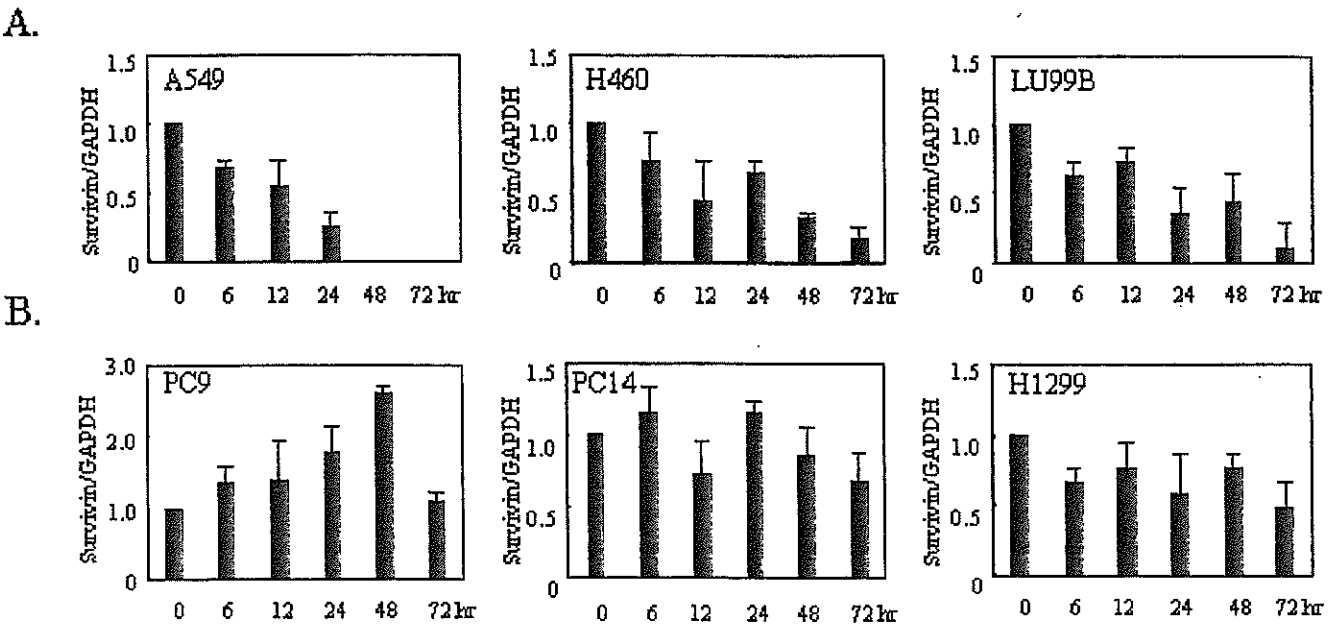

FIGURE 3 – Expression of survivin mRNA in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. Each of the cell lines with wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin 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).

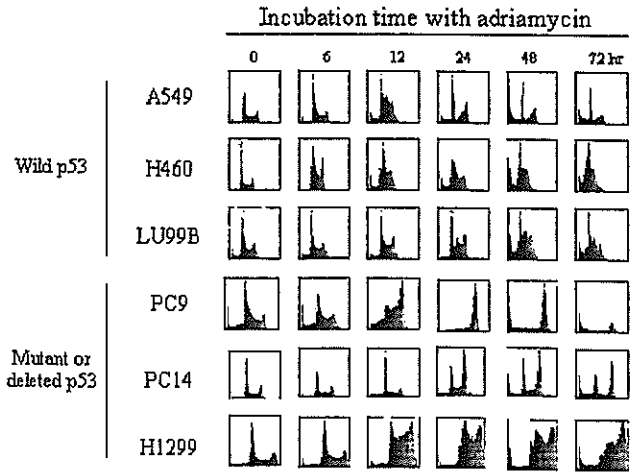

FIGURE 4 – Cell cycle analysis of lung cancer cell lines with different p53 phenotypes after exposure to adriamycin. Each of the cell lines possessing wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for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

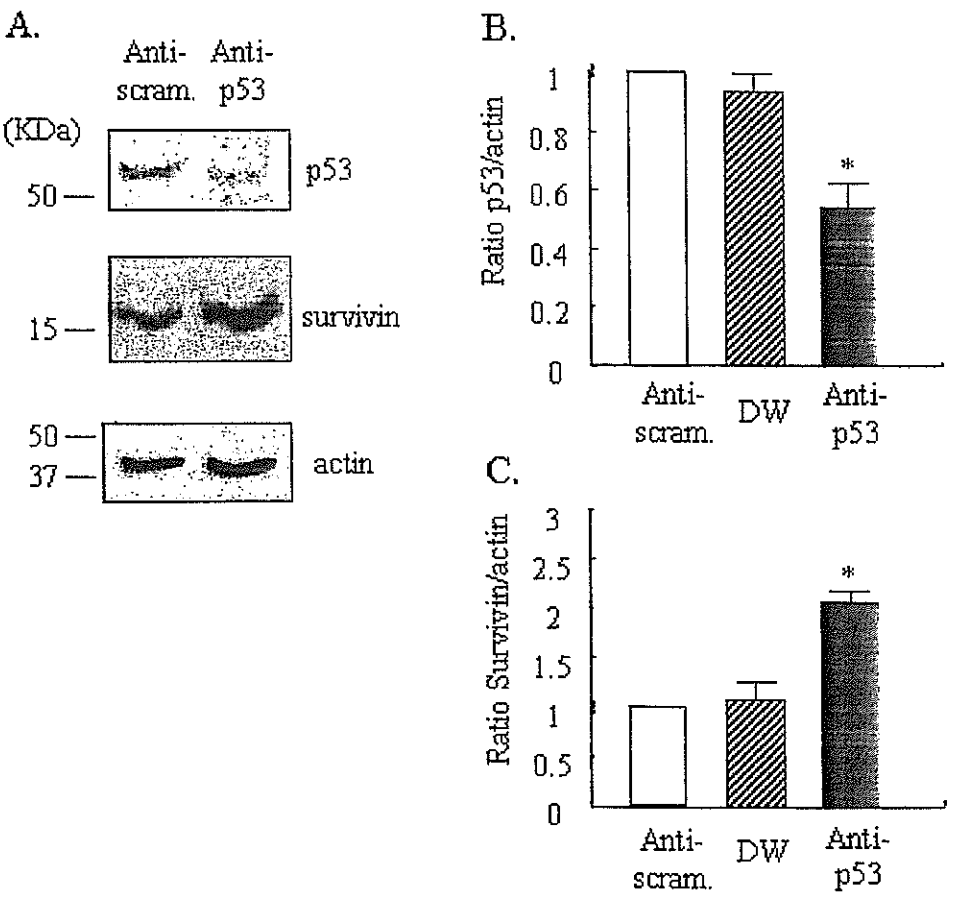

FIGURE 5 – (*a*) Increasing survivin expression in A549 lung cancer cells possessing wild-type p53 as a result of p53 inhibition by siRNA duplex. A549 cells were treated with siRNA duplex targeting p53, scramble or distilled water and then 48 hr later, cell lysates were prepared from the siRNA-treated 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.

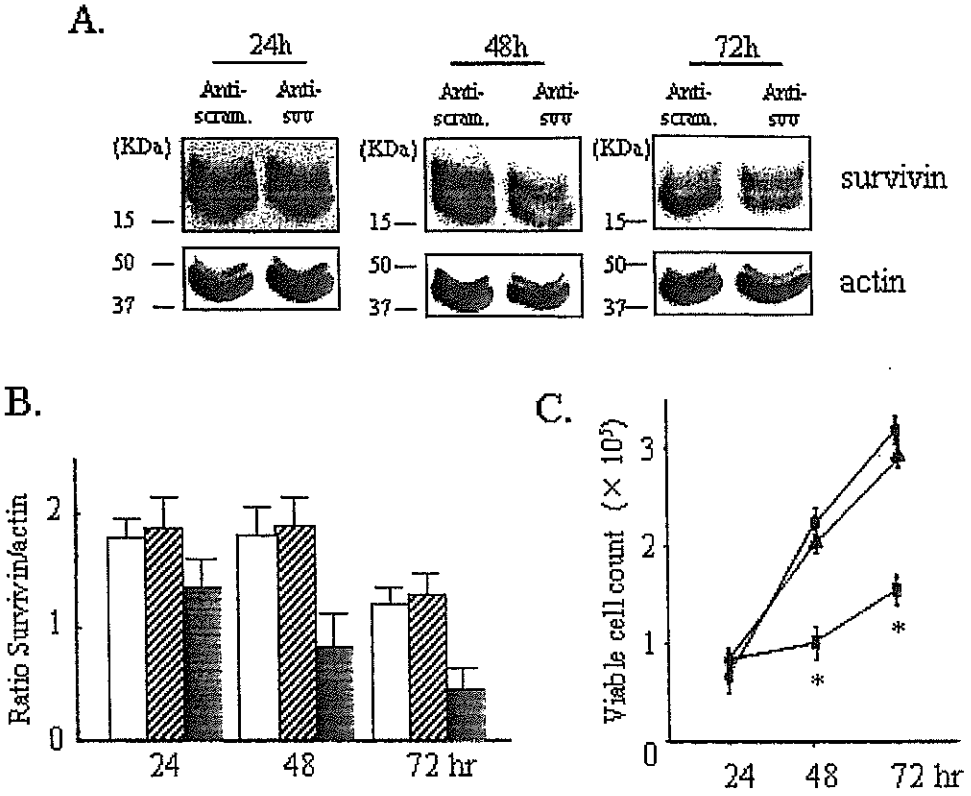

FIGURE 6 – Effects of siRNA targeting survivin on proliferation of PC9 lung cancer cells. PC9 cells were treated with siRNA duplex targeting surviving, scramble or distilled water. At the indicated time, the cells were harvested and assayed using the following procedure. (*a*) Expression of survivin 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-

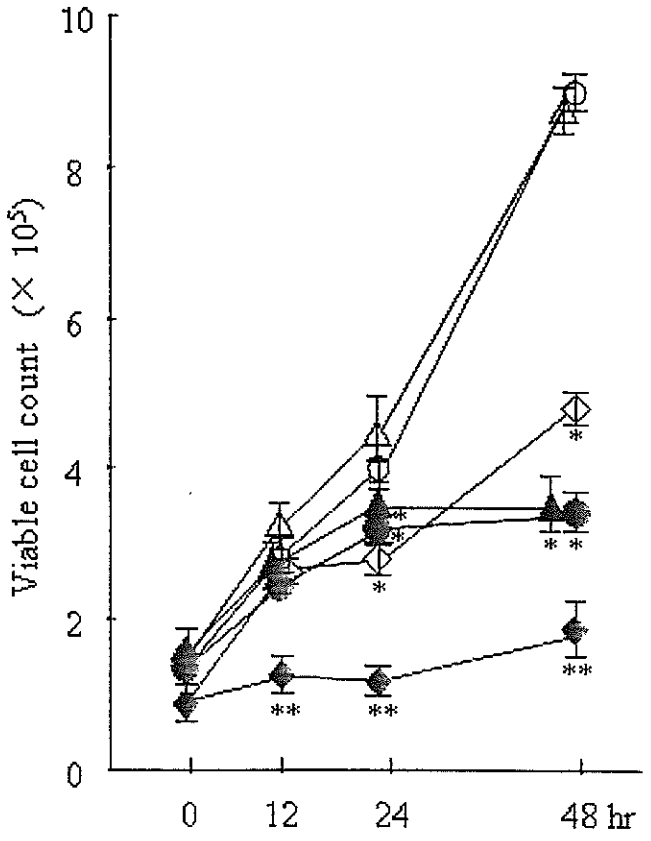

FIGURE 7 – Effects of siRNA duplex targeting of survivin on proliferation of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin or water after 48 hr transfection with siRNA duplex targeting surviving, scramble or distilled water. Open triangle:water after distilled water; 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.

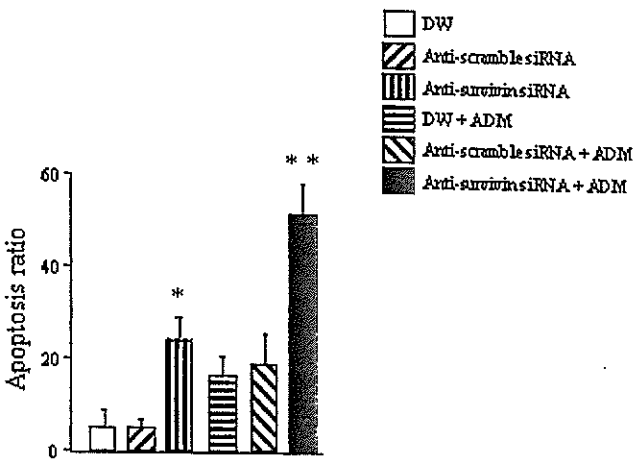

FIGURE 8 – Effects of siRNA targeting survivin on apoptosis of PC9 lung cancer cells treated with adriamycin, evaluated by TUNEL assay. PC9 cells were exposed to adriamycin or water for 24 hr after 48 hr transfection with duplex siRNA targeting surviving, scramble or distilled water. The data are presented 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.

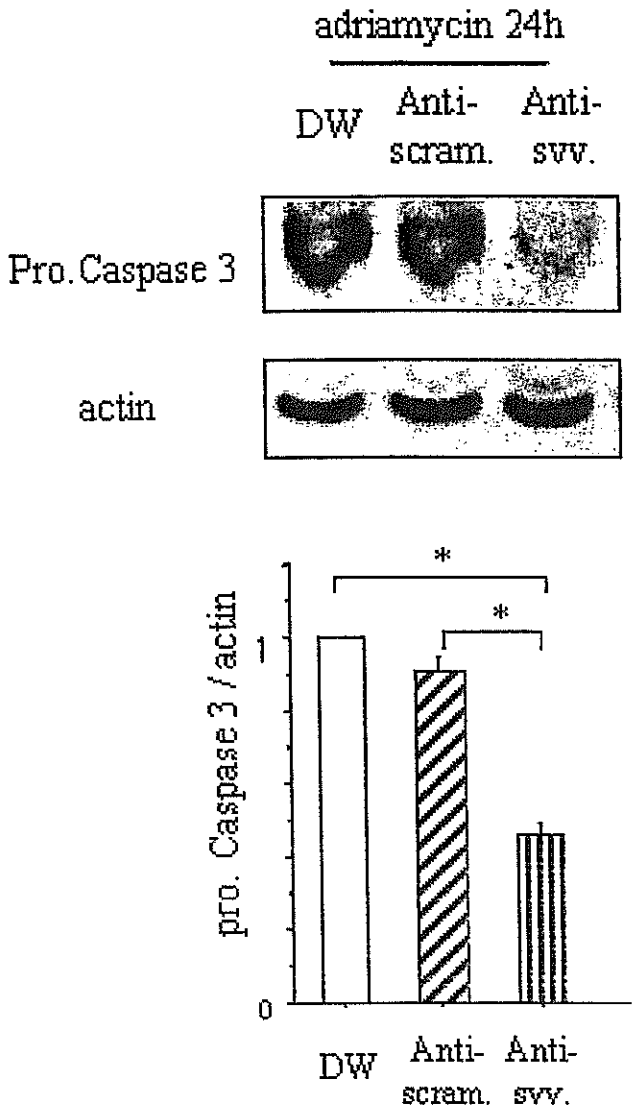

FIGURE 9 – Effects of siRNA targeting survivin on pro-caspase3 expression of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin for 24 hr after 48 hr transfection with duplex siRNA targeting survivin, scramble or distilled water, and each sample was analyzed by Western 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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Reprinted from
Jpn J Clin Oncol 2005;35(4)181–187
doi:10.1093/jjco/hyi057

A Phase I/II Study Comparing Regimen Schedules of Gemcitabine and Docetaxel in Japanese Patients with Stage IIIB/IV Non-small Cell Lung Cancer

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Received October 7, 2004; accepted January 31, 2005

Objective: Gemcitabine and docetaxel are non-platinum agents with activity in non-small cell lung cancer (NSCLC). This study was conducted to determine and evaluate the recommended regimen of gemcitabine–docetaxel and evaluated its efficacy and safety in chemonaive Japanese NSCLC patients.

Methods: In phase I, patients with stage IIIB/IV NSCLC were randomized and received either gemcitabine on days 1 and 8 plus docetaxel on day 1 or gemcitabine on days 1 and 8 plus docetaxel on day 8. The recommended regimen was the dose level preceding the maximum tolerated dose; once determined, patients were enrolled in phase II. Efficacy and toxicity were evaluated in all patients.

Results: Twenty-five patients were enrolled in phase I and six patients were given the recommended regimen; gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8. An additional 34 patients were enrolled into phase II and administered with the recommended regimen. The response rate was 32.2% [95% confidence interval (CI) 20.6–45.6%] overall and 30.0% (95% CI 16.6–46.5%) in patients with the recommended regimen (40 patients). Although grade 3 interstitial pneumonia was observed in two patients (5.0%) who received the recommended regimen, both recovered shortly after steroid treatment. No unexpected events were observed throughout this study.

Conclusions: Gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 has comparable efficacy and more tolerable toxicities than previously reported platinum-based regimens. These results should be verified by a phase III study.

Key words: docetaxel – gemcitabine – non-small cell lung cancer

INTRODUCTION

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors, progresses in a short time period, has a bleak prognosis, and represents the leading cause of cancer death in the world. The number of patients with NSCLC is increasing, and most tumors are inoperable. Despite improvements in the detection and treatment of NSCLC, long-term survival is rare. Therefore, the development of new chemotherapy treatments is essential.

The use of single-agent and combination chemotherapy against NSCLC has been studied. Platinum-based regimens have shown high efficacy but at the cost of severe toxicities (1,2). Therefore, non-platinum agents such as gemcitabine, docetaxel, paclitaxel, irinotecan and vinorelbine have been developed and have proven their efficacies. Among the new agents, the combination of gemcitabine and docetaxel has emerged as one of the most promising, showing equivalent efficacy with, and less toxicity than, cisplatin-based chemotherapies (3).

Gemcitabine (2′-deoxy-2′,2′-difluorocytidine monohydrochloride) is a nucleoside antimetabolite against deoxycytidine. It is intracellularly metabolized to gemcitabine triphosphate,

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which inhibits DNA synthesis, and has shown potent cytocidal activity against solid tumors (4–8).

Docetaxel, an antineoplastic agent that acts on microtubules to promote formation of abnormal microtubule bundles, has also shown cytotoxicity (9–11). Gemcitabine and docetaxel have different mechanisms of action, but by combining them, there is the potential of synergistic antitumor activity (12).

Several studies have been conducted to evaluate the therapeutic benefits of gemcitabine and docetaxel (13–15). The efficacy of gemcitabine–docetaxel is similar to platinum-based regimens, but due to each drug's non-overlapping toxicities, their combination produces toxicities more tolerable than platinum-based regimens. Georgoulias et al. (16) compared gemcitabine 1100 mg/m^2 on days 1 and 8 plus docetaxel 100 mg/m^2 on day 8 with cisplatin 80 mg/m^2 on day 2 plus docetaxel 100 mg/m^2 on day 1 in 441 patients with NSCLC. They reported that the two regimens were equivalent in efficacy, but toxicities were more severe for the combination of docetaxel and cisplatin.

There has been no published report considering both administering dose and schedule for the combination of gemcitabine and docetaxel. Therefore, we conducted a phase I/II study to compare two schedules of gemcitabine–docetaxel in patients with NSCLC and determine the recommended regimen in phase II. We assessed the efficacy and safety in all 59 patients: the efficacy and detailed safety profile were also evaluated in 40 patients who were given the recommended regimen.

SUBJECTS AND METHODS

Eligibility Criteria

Japanese patients with histologically or cytologically confirmed unresectable TNM stage IIIB or IV NSCLC who met the following criteria were eligible for the study: suitable for first-line chemotherapy with no prior chemotherapy; measurable lesions that can be accurately measured in at least one dimension; aged 20–74 years; Eastern Cooperative Oncology Group (ECOG) performance status of 0–1; a life expectancy of at least 3 months; and adequate organ functions as indicated by white blood cell count $\geq 4.0 \times 10^9$/l, absolute neutrophil count $\geq 2.0 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l, hemoglobin ≥ 9.5 g/dl, aspartate aminotransferase/alanine aminotransferase ≤ 2.5 times the upper limit of normal, total bilirubin ≤ 1.5 times the upper limit of normal, serum creatinine $\leq$ the upper limit of normal, PaO_2 in arterial blood ≥ 60 torr. If a patient had received radiotherapy during the 3 weeks before enrollment, the measurable disease had to be outside of the radiation port.

Patients were excluded from the study if they had radiologically and clinically apparent interstitial pneumonia or pulmonary fibrosis, intracavitary fluid retention requiring treatment, or grade 2–4 peripheral neuropathy or edema. Additional exclusion criteria included: superior vena cava syndrome; symptomatic brain metastasis; pregnancy or breast-feeding; active concurrent malignancy; any serious concurrent illness (e.g. uncontrolled diabetes mellitus, hepatopathy, angina pectoris, myocardial infarction within 3 months after onset, severe infection, or fever suggestive of severe infection); history of serious drug allergy; or any condition that, in the opinion of the investigator, disqualified the patient based on safety.

This study was conducted in accordance with the Declaration of Helsinki, Japanese Guidelines for Clinical Evaluation of Antineoplastic Agents (promulgated in February 1991) and good clinical practice. All patients who entered into this study were required to give written informed consent.

Study Design And Treatment

This was a multicenter, open-label, phase I/II study of gemcitabine and docetaxel in Japanese patients with advanced NSCLC.

In the phase I portion of this study, patients were randomized into two arms, each with a different treatment schedule. In both arms (Arm 1 and Arm 2), gemcitabine was administered in a 30-min infusion on days 1 and 8, every 21 days. In Arm 1, docetaxel was administered intravenously over at least 1 h on day 1; in Arm 2, docetaxel was given on day 8. The administration of docetaxel followed an intravenous infusion of dexamethasone 4 mg, and gemcitabine was given immediately after the docetaxel infusion.

Patients were discontinued from the study due to progressive disease; inability to initiate a treatment cycle even at 6 weeks after the start of the previous cycle; recurrence of a dose-limiting toxicity (DLT) after resumption of the study treatment at a reduced dose; occurrence of a serious adverse event or aggravation of a concomitant illness (e.g. interstitial pneumonia, pulmonary fibrosis, or severe infection) which caused rapid aggravation of disease and precluded continuation of the study treatment; patient's request to withdraw from the study; or any event that required discontinuation in the opinion of the investigator.

During study enrollment, the current approved maximum dosage of gemcitabine and docetaxel as single agents in Japan was 1000 mg/m^2 and 60 mg/m^2, respectively. In phase I, the sample size was determined to be six per cohort based on the conventional design of phase I clinical studies of antineoplastic agents. In this study, both arms were randomized according to a predetermined schedule, enrolled patients in cohorts of six, and were initially treated at dose level 1 (gemcitabine 1000 mg/m^2 and docetaxel 50 mg/m^2). For the first cycle of treatment, patients were treated on an inpatient basis; if their condition permitted, patients were treated on an outpatient basis thereafter. If fewer than 50% of the patients in dose level 1 experienced DLTs, patients were enrolled at dose level 2 (gemcitabine 1000 mg/m^2 and docetaxel 60 mg/m^2). If 50% or more of the patients in dose level 1 experienced DLTs, patients were enrolled at dose level 0 (gemcitabine 800 mg/m^2 and docetaxel 50 mg/m^2) (Fig. 1). The maximum tolerated dose (MTD) was defined as the dose level that produced any of the following DLTs (per the National Cancer Institute–Common

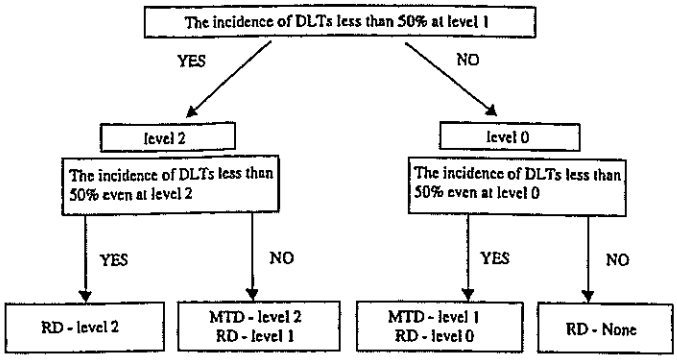

Figure 1. Recommended dosages in each arm. DLT, dose-limiting toxicity; RD, recommended dosage; MTD, maximum tolerated dose.

Toxicity Criteria scale) in 50% or more of patients during the first treatment cycle: grade 4 leukopenia or neutropenia persisting for at least 4 days; grade 3/4 neutropenia associated with a fever ≥38.0°C or infection; thrombocytopenia (<20 × 10^9/l) or need of a platelet transfusion; or grade 3/4 non-hematological toxicities (excluding nausea/vomiting, anorexia, fatigue and hypersensitivity). G-CSFs were administrated for the treatment of grade 4 neutropenia or grade 3 neutropenic fever. A DLT was also reported if any day-8 doses were omitted and dosing requirements were not satisfied until after day 15, or if the second cycle was delayed until after day 29 because the dosing requirements were not satisfied.

The recommended dose for phase II had to be determined from the arm that reached the highest dose level. If at dose level 2 the incidence of DLTs was less than 50%, the recommended dose was defined as dose level 2. The arm that reached the higher dose level reflected the recommended regimen for phase II. If the recommended dose level for the two arms was identical, the recommended regimen would be decided according to the following steps: (i) if frequency of DLTs was 0% in one arm and 33.3% or more in the other arm, the former was selected. If this did not occur, then (ii) if the dose intensity for evaluable patients in one arm was higher by 10% or more than the other arm, the arm with the higher dose intensity was selected. If this did not occur, then (iii) the arm with the fewer day-8 dose omissions in first and second cycles was selected. If the recommended dosage regimen still could not be decided, the sponsor (Aventis Pharma Japan and Eli Lilly Japan K.K.) and the coordinating investigator determined the recommended phase II regimen. If the MTD was dose level 0 in both arms, the study was terminated (Fig. 1).

The sample size for the recommended regimen was determined as follows. The response rate of this regimen and gemcitabine single agent was assumed to be 35 and 20%, respectively, in view of the response rates previously achieved (9,10,17,18). If the sample size of the recommended regimen was set as 40 patients, the probability for the one-sided 90% lower limit of response rate to exceed 20% was 82%. Thus, the target sample size in the recommended regimen including six patients in phase I was set at 40 patients.

The phase II study was conducted with 34 patients. Forty patients who were given the recommended regimen were evaluated for the efficacy and detailed safety profile: these patients consisted of six and 34 patients who entered into the study at phase I and II, respectively.

In this phase I/II study, patients received a minimum of two cycles of gemcitabine–docetaxel and up to four additional cycles.

Dose Modifications

During a cycle, dose modifications were not allowed. If not all of the following requirements were satisfied on either the day of treatment or the previous day, administrations of gemcitabine and docetaxel were delayed until the patient completely recovered. For gemcitabine and docetaxel doses administered on day 1 of Arm 1 or gemcitabine on day 1 of Arm 2, delays occurred for patients with an absolute neutrophil count <1.5 × 10^9/l, a platelet count <70 × 10^9/l, any grade 3/4 non-hematologic toxicities (except PaO_2), or PaO_2 <60 torr. When gemcitabine was given on day 8 of Arm 1, exceptions included leukopenia <2.0 × 10^9/l and an absolute neutrophil count <1.0 × 10^9/l, a platelet count <70 × 10^9/l, any grade 3/4 non-hematological toxicities. When gemcitabine was given on day 8 of Arm 2, exceptions included an absolute neutrophil count <1.5 × 10^9/l, a platelet count <70 × 10^9/l, any grade 3/4 non-hematological toxicities. If a patient developed a DLT, the subsequent doses were cancelled, and in the next cycle the patient could resume the study treatment at the next lower dose level. If a patient developed a DLT at dose level 0, gemcitabine 800 mg/m^2 and docetaxel 40 mg/m^2 were administered in the next cycle.

Baseline and Treatment Assessment

Assessments at baseline included tumor measurements by X-ray and computed tomography (CT) scan within 4 weeks before the day of starting the study treatment. Equally, grading performance status and physical examination were performed within a week; hematology, blood chemistries, urinalysis, arterial blood gas analysis and electrocardiogram were observed within 2 weeks.

After the start of treatment, tumor measurements were obtained every 2 weeks via X-ray and 4 weeks via CT scan. Tumor response was assessed with the World Health Organization (WHO) criteria. Safety assessments, including performance status, hematology, blood chemistries and urinalysis, were obtained weekly. Physical examination, arterial blood gas analysis and electrocardiogram were performed at any time. Adverse events were estimated according to National Cancer Institute–Common Toxicity Criteria version 2.0. All patients were assessed for efficacy and safety. An additional response rate was recorded for patients who received the recommended regimen in phase I and all phase II patients.

RESULTS

PATIENT CHARACTERISTICS

Between July 2000 and July 2002, 59 chemonaive patients (43 male, 16 female) with NSCLC were enrolled in phase I and II portions from the five hospitals after approval by the IRB. Twenty-five patients were enrolled in the phase I portion of the study, and 34 patients were enrolled in phase II. Baseline patient characteristics for all patients and patients who received the recommended regimen are summarized in Table 1.

PHASE I

Twenty-five patients were enrolled into the phase I portion of the study. The number of patients treated and the DLTs observed in the first cycle at each dose level of gemcitabine and docetaxel are shown in Table 2.

In Arm 1, 50% of patients had DLTs at dose level 1 and dose level 0, therefore Arm 1 could not be the recommended regimen: there were 2/6 and 3/6 patients who achieved partial response (PR) at dose level 1 and 0 in Arm 1, respectively.

Table 1. Baseline characteristics

Patient characteristics	All patients (n = 59), n (%)	Patients who received the recommended regimen (n = 40), n (%)
Gender		
Male	43 (72.9%)	26 (65.0%)
Female	16 (27.1%)	14 (35.0%)
Age		
Median	62	64
Range	38–74	38–74
ECOG performance status		
0	5 (8.5%)	2 (5.0%)
1	54 (91.5%)	38 (95.0%)
Stage		
IIIB	14 (23.7%)	8 (20.0%)
IV	33 (55.9%)	23 (57.5%)
Postsurgical recurrence	12 (20.3%)	9 (22.5%)
Histological type		
Adenocarcinoma	34 (57.6%)	25 (62.5%)
Squamous cell carcinoma	19 (32.2%)	14 (35.0%)
Large cell carcinoma	5 (8.5%)	1 (2.5%)
Other	1 (1.7%)	0 (0%)
Prior therapy		
None	45 (76.3%)	29 (72.5%)
Surgery	13 (22.0%)	11 (27.5%)
Radiotherapy	0 (0%)	0 (0%)
Radiotherapy and surgery	1 (1.7%)	0 (0%)

ECOG, Eastern Cooperative Oncology Group.

In Arm 2, no DLT was observed at dose level 1: 3/6 patients achieved PR. At dose level 2, one patient discontinued due to progressive disease; therefore, one patient was added. However, another patient discontinued due to grade 3 hypersensitivity (not a DLT). In this regimen, two DLTs had already been observed in five other patients, but the sponsors (Aventis Pharma Japan and Eli Lilly Japan K.K.) and investigators decided not to add one more patient to dose level 2 in Arm 2 in consideration of patients' safety. PRs were observed in 2/7 patients at dose level 2 of Arm 2.

Therefore, the recommended regimen was determined as gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 due to the incidence of DLT.

DOSE ADMINISTRATION

In Arm 1, a total of 49 cycles were accomplished. One case delayed the date of administration on day 1 (defined as more than 8 days) as a matter of convenience; seven and four cases delayed their dates of administration on day 8 (defined as more than 1 day) because of adverse events and non-medical reasons, respectively; and four cases could not be treated on day 8 because of adverse events. In Arm 2, including phase I and II portions, a total of 145 cycles were accomplished. Four and five cases delayed their dates of administration on day 1 because of adverse events and non-medical reasons, respectively; 21 and nine cases delayed their dates of administration on day 8 because of adverse events and non-medical reasons, respectively; and two cases could not be treated on day 8 because of

Table 2. Phase I dose-limiting toxicities

Dose level	GEM/DOC (mg/m^2)	Arm 1	Arm 2
0	800/50	3/6 patients: • G3 ALT increased • G1 fever, G3 neutropenia • G2 infection, G3 neutropenia	N/A
1	1000/50	3/6 patients: • G3 infection, G3 neutropenia • G4 neutropenia, G1 fever, G3 infection • G3 neutropenia, G2 infection, G3 arrhythmia, G3 diarrhea	0/6 patients
2	1000/60	N/A	2/5 patients: • G3 ALT increased • G2 fever, G3 neutropenia

GEM, gemcitabine; DOC, docetaxel; G, grade; ALT, alanine aminotransferase; N/A, not applicable.

adverse events. The most common adverse event for a dose delay was neutropenia.

EFFICACY

All 59 patients were involved in the analysis for efficacy, and 19 of 59 patients achieved PR for an overall response rate of 32.2% [95% confidence interval (CI) 20.6–45.6%]. Of the 40 patients who received the recommended regimen in either phase I or phase II, 12 patients achieved PRs for a response rate of 30.0% (95% CI 16.6–46.5%).

The median time to progressive disease in all 59 patients was 111 days (95% CI 71–154 days). Median survival time was 11.9 months (95% CI 7.0–15.0 months), with 1-year survival rate at 47.1% (95% CI 34.0–60.2%).

SAFETY

All 59 patients were evaluable for safety. Grade 3 and 4 drug-related toxicities observed in all 59 patients are shown in Table 3. Grade 3 and 4 drug-related toxicities observed in 40 patients who received the recommended regimen are also shown in Table 4.

In all 59 patients, grade 3 and 4 neutropenia were observed in 19 (32.2%) and 20 (33.9%) patients, respectively. Grade 3 and 4 leukopenia were observed in 24 (40.7%) and four (6.8%) patients, respectively. Grade 3 non-hematological toxicities included infection in four patients (6.8%), anorexia in four patients (6.8%), and nausea, diarrhea, rash and constipation in three patients (5.1%) each. After starting docetaxel administration, grade 3 interstitial pneumonia was reported in three patients (5.1%), all of whom recovered shortly after steroid treatment; grade 4 anaphylaxis was reported in two patients (3.4%). There were no toxic deaths.

DISCUSSION

In this phase I/II study, we examined the activity and tolerability of gemcitabine and docetaxel. In phase I, the recommended regimen was determined as gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8. The response rate of all 59 patients was 32.2% (95% CI 20.6–45.6%). When re-evaluated in the 40 patients who received the recommended regimen, the response rate was 30.0% (95% CI 16.6–46.5%). Although the number of patients was limited, Arm 1 (docetaxel on day 1) had a numerically better response: for the 12 patients in Arm 1, five PRs were recorded for a response rate of 42%. However, Arm 1 had more toxicities than the docetaxel on day-8 schedule.

Overall, the toxicity associated with the gemcitabine–docetaxel regimen was manageable. In Arm 1, five patients (42%) had grade 3/4 neutropenia supervened with infection or fever, while only one patient (9%) had grade 3 neutropenia with infection or fever in Arm 2. This indicated that docetaxel was better tolerated on day 8 than on day 1 in a 21-day cycle. It is speculated that the influence of time to nadir of neutropenia is different in each agent: 14–20 days with gemcitabine and 9 days with docetaxel. The time to recover from nadir is 7–8 days with gemcitabine and 8 days with docetaxel. This could explain why docetaxel on day 8 was better tolerated.

Table 3. NCI–CTC grade 3/4 toxicities (n = 59)

Toxicities	Grade 3		Grade 4	
	n	%	n	%
Hematological toxicities				
Leukopenia	24	40.7	4	6.8
Neutropenia	19	32.2	20	33.9
Lymphopenia	10	16.9	0	0.0
Hemoglobin decreased	4	6.8	0	0.0
Thrombocytopenia	1	1.7	0	0.0
Thrombocytosis	1	1.7	0	0.0
Non-hematological toxicities				
ALT increased	5	8.5	0	0.0
Infection	4	6.8	0	0.0
Anorexia	4	6.8	0	0.0
Nausea	4	6.8	0	0.0
Diarrhea	3	5.1	0	0.0
Interstitial pneumonia	3	5.1	0	0.0
Rash	3	5.1	0	0.0
Constipation	3	5.1	0	0.0
AST increased	2	3.4	0	0.0
Fatigue	2	3.4	0	0.0
Vomiting	2	3.4	0	0.0
Hyperglycemia	1	1.7	0	0.0
Hyponatremia	1	1.7	0	0.0
Allergic reaction	1	1.7	0	0.0
Vasovagal reaction	1	1.7	0	0.0
Body temperature decrease	1	1.7	0	0.0
Weight increase	1	1.7	0	0.0
Hypotension	1	1.7	0	0.0
Pneumonia	1	1.7	0	0.0
Arrhythmia	1	1.7	0	0.0
Edema	1	1.7	0	0.0
Neuropathy peripheral	1	1.7	0	0.0
Anaphylaxis	0	0.0	2	3.4

NCI–CTC, National Cancer Institute–Common Toxicity Criteria version 2.0; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Meta-analysis studies have reported that cisplatin-based regimens produce a significant survival benefit in NSCLC (20–23), improve median survival time by 6–8 weeks and 1-year survival rate from 15% to 25% when compared with the best supportive care (24). But studies with platinum-based combinations have also reported severe toxicities, so the deterioration of patients' quality of life is a major problem to be solved (3).

New effective non-platinum-based therapies have been used in various combinations in recent years, and the combination of gemcitabine and docetaxel has been established as one of the