In our study, 9 patients out of 62 long-term survivors of stage III NSCLC treated with chemo-radiotherapy had a SPC. The relative risk for any SPC (2.8; 95% CI 1.3–5.3) compared with the general population was significantly increased. Instead of many reports examining the risk, these do not provide adequate follow-up information to determine relative risk in the patients with NSCLC. Most studies only show a percent risk per patient per year (5–8). In the current study, the overall rate of developing SPC is estimated at 2.9% per patient per year, which is in agreement with the rates in most surgical series. Ginsberg and Rubinstein (5) reported that SPC occurrence rate was 1.7% per patient per year on 247 patients operated for T1 N0 NSCLC. Other studies showed the rate of 2.8% by Martini et al. (6) and 2.4–3.6% by Thomas and Rubinstein (7). In the current study, we also confirmed the effect of the passage of time on developing SPC. Thomas and Rubinstein (7) reported that the rate of SPC increased from 2.4% for the first 5 years after surgical resection to 3.6% after the fifth year.

We previously studied the relative risk of SPC in the SCLC patient successfully treated with chemotherapy with or without RT (9). Our results showed a similar trend as previous studies (10,11) and demonstrated that the patient had a significantly increased relative risk of 3.6 (95% CI 2.0–5.9) and that the patients who continued to smoke demonstrated a significantly increased risk for a SPC (4.3, 95% CI 1.1–15.9, $P = 0.03$) compared with those who stopped smoking.

Unlike the results of SCLC patients study, the risk of SPC in NSCLC patients was lower, and the impact of continued smoking on developing SPC in the patients was less significant, but the reason for this observation is not completely understood. According to the case–control study from Japan (17), lung cancer risk reduction due to smoking cessation appeared to be greater in SCLC than squamous cell carcinoma or adenocarcinoma, and SCLC seems to be more smoking-related than NSCLC. However, there have been a couple of germline polymorphism as cytochrome P 450 1A1 (CYP1A1) and glutathione *S*-transferase class mu (GSTM1), reported, which is implicated in smoking-related carcinogenesis (18,19). Therefore, SCLC patients are speculated to have a higher potential to develop a SPC, particularly smoking-related cancers.

Among NSCLC patients, there seems to be a special group of roentgenographically occult early stage squamous cell carcinoma of the lung. In this patient group, the rate of occurrence of SPC, particularly SPLC was estimated at 3–4% per patient per year (20,21). The risk for SPLC seemed to be substantially higher than that of 1–2% in the NSCLC patients treated with surgery or RT from the previous study and treated with chemo-radiotherapy from our study. Therefore, the group should be given a special focus and be divided from the general population of NSCLC patients in the research of risk of SPC. Most of the patients can be cured by surgery, photodynamic therapy, brachytherapy and chest RT because of its early clinical stage (22), and are not included in our study. Roentgenographically occult early stage squamous cell carcinoma of the lung is associated with the concept of field cancerization (23), and smoking status seems to be very important to evaluate the risk of SPC, which awaits further examination.

A relatively small sample size and rare events such as SPC in this study resulted in large confidence intervals for the estimates. It is still difficult to conclude the effect of continued smoking on the development of SPC. Cigarette smoking causes not only developing cancers but also cardiovascular and lung damage as well (24,25). It may be speculated that continued smokers died off early when interpreting the results. The cessation of smoking is still warranted among patients with stage III NSCLC treated by chemo-radiotherapy.

In conclusion, stage III NSCLC patients treated with chemo-radiotherapy were at risk of developing SPC and this risk increased with time. A large sample size study in a longer follow-up period may be required in further research to conclude the effect of continued smoking on the development of SPC. SPC in another particular group such as roentgenographically occult early stage squamous cell carcinoma of bronchus also awaits further studies.

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References

1. Ohe Y, Ishizuka N, Tamura T, Sekine I, Nishiwaki Y, Saijo N. Japan Clinical Oncology Group. Long-term follow-up of patients with unresectable locally advanced non-small cell lung cancer treated with chemoradiotherapy: a retrospective analysis of the data from the Japan Clinical Oncology Group trials (JCOG0003A). *Cancer Sci* 2003;94:729–34.
2. Komaki R, Seiferheld W, Ettinger D, Lee JS, Movsas B, Sause W. Randomized phase II chemotherapy and radiotherapy trial for patients with locally advanced inoperable non-small-cell lung cancer: long-term follow-up of RTOG 92-04. *Int J Radiat Oncol Biol Phys* 2002;53:548–57.
3. Jeremic B, Shibamoto Y, Acimovic L, Milicic B, Milisavljevic S, Nikolic N, et al. Hyperfractionated radiation therapy and concurrent low-dose, daily carboplatin/etoposide with or without weekend carboplatin/etoposide chemotherapy in stage III non-small-cell lung cancer: a randomized trial. *Int J Radiat Oncol Biol Phys* 2001;50:19–25.
4. Johnson BE. Second lung cancers in patients after treatment for an initial lung cancer. *J Natl Cancer Inst* 1998;90:1335–45.
5. Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. Lung Cancer Study Group. *Ann Thorac Surg* 1995;60:615–22.
6. Martini N, Bains MS, Burt ME, Zakowski MF, McCormack P, Rusch VW, et al. Incidence of local recurrence and second primary tumors in resected stage I lung cancer. *J Thorac Cardiovasc Surg* 1995;109:120–9.
7. Thomas PA Jr, Rubinstein L. Malignant disease appearing late after operation for T1 N0 non-small-cell lung cancer. *J Thorac Cardiovasc Surg* 1993;106:1053–8.
8. Jeremic B, Shibamoto Y, Acimovic L, Nikolic N, Dagovic A, Aleksandrovic J, et al. Second cancers occurring in patients with early stage non-small-cell lung cancer treated with chest radiation therapy alone. *J Clin Oncol* 2001;19:1056–63.

9. Kawahara M, Ushijima S, Kamimori T, Kodama N, Ogawara M, Matsui K, et al. Second primary tumours in more than 2-year disease-free survivors of small-cell lung cancer in Japan: the role of smoking cessation. *Br J Cancer* 1998;78:409–12.
10. Tucker MA, Murray N, Shaw EG, Ettinger DS, Mabry M, Huber MH, et al. Second primary cancers related to smoking and treatment of small-cell lung cancer. *J Natl Cancer Inst* 1997;89:1782–8.
11. Richardson GE, Tucker MA, Venzon DJ, Linnoila RI, Phelps R, Phares JC, et al. Smoking cessation after successful treatment of small-cell lung cancer is associated with fewer smoking-related second primary cancers. *Ann Intern Med* 1993;119:383–90.
12. Martini N, Melamed MR: Multiple primary lung cancers. *J Thorac Cardiovasc Surg* 1975;70:606–12.
13. The Research Group for Population-based Cancer Registration in Japan. Cancer incidence and incidence rates in Japan in 1998: estimates based on data from 12 population-based cancer registries. *Jpn J Clin Oncol* 2003;33:241–5.
14. Boice J, Lubin J, Preston D. Epidemiologic analysis with a personal computer (EPITOME). NIH Publication (91–380) 1991.
15. SAS Institute SAS/STAT User's Guide, Version6, Vol. 2, 4th edn. Cary, NC: SAS Institute 1989;1070–26.
16. Ng AK, Bernardo MV, Weller E, Backstrand K, Silver B, Mauch PM, et al. Second malignancy after Hodgkin disease treated with radiation therapy with or without chemotherapy: long-term risks and risk factors. *Blood* 2002;100:1989–96.
17. Sobue T, Suzuki T, Fujimoto I, Matsuda M, Doi O, Mori T, et al. Lung cancer risk among exsmokers. *Jpn J Cancer Res* 1991;82:273–279.
18. To-Figueras J, Gene M, Gomez-Catalan J, Galan MC, Fuentes M, Ramon JM, et al. Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) polymorphisms and lung cancer risk among Northwestern Mediterraneans. *Carcinogenesis* 1997;18:1529–33.
19. Kihara M, Kihara M, Noda K. Risk of smoking for squamous and small cell carcinomas of the lung modulated by combinations of CYP1A1 and GSTM1 gene polymorphisms in a Japanese population. *Carcinogenesis* 1995;16:2331–6.
20. Woolner LB, Fontana RS, Cortese DA, Sanderson DR, Bernatz PE, Payne WS, et al. Roentgenographically occult lung cancer: pathologic findings and frequency of multicentricity during a 10 year period. *Mayo Clin Proc* 1984;59:453–66.
21. Saito Y, Nagamoto N, Ota S, Sato M, Sagawa M, Kamma K, et al. Results of surgical treatment for roentgenographically occult bronchogenic squamous cell carcinoma. *J Thorac Cardiovasc Surg* 1992;104:401–7.
22. Kawaguchi T, Yamamoto S, Naka N, Okishio K, Atagi S, Ogawara M, et al. Immunohistochemical analysis of bcl-2 protein in centrally located early stage lung cancer treated with photodynamic therapy. *Br J Cancer* 2000;82:418–23.
23. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* 2003;63:1727–30.
24. Howard G, Wagenknecht LE, Burke GL, Diez-Roux A, Evans GW, McGovern P, et al. Cigarette smoking and progression of atherosclerosis: The Atherosclerosis Risk in Communities (ARIC) Study. *J Am Med Assoc* 1998;279:119–24.
25. Fujisawa T, Iizasa T, Saitoh Y, Sekine Y, Motohashi S, Yasukawa T, et al. Smoking before surgery predicts poor long-term survival in patients with stage I non-small-cell lung carcinomas. *J Clin Oncol* 1999;17:2086–91.

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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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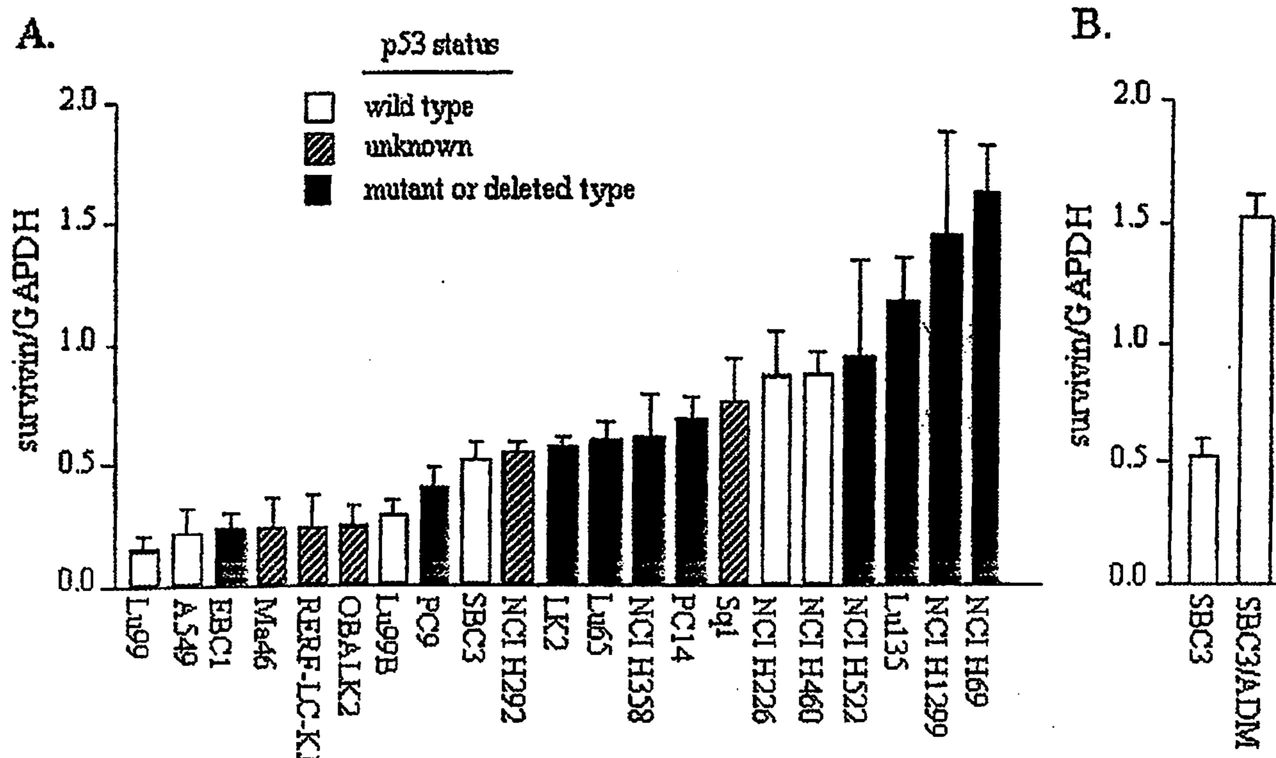

FIGURE 1 – Level of survivin mRNA in 22 lung cancer cell lines. (*a*) Cells were incubated in a 75 cm² flask, harvested and analyzed using real-time PCR as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH and are presented 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-

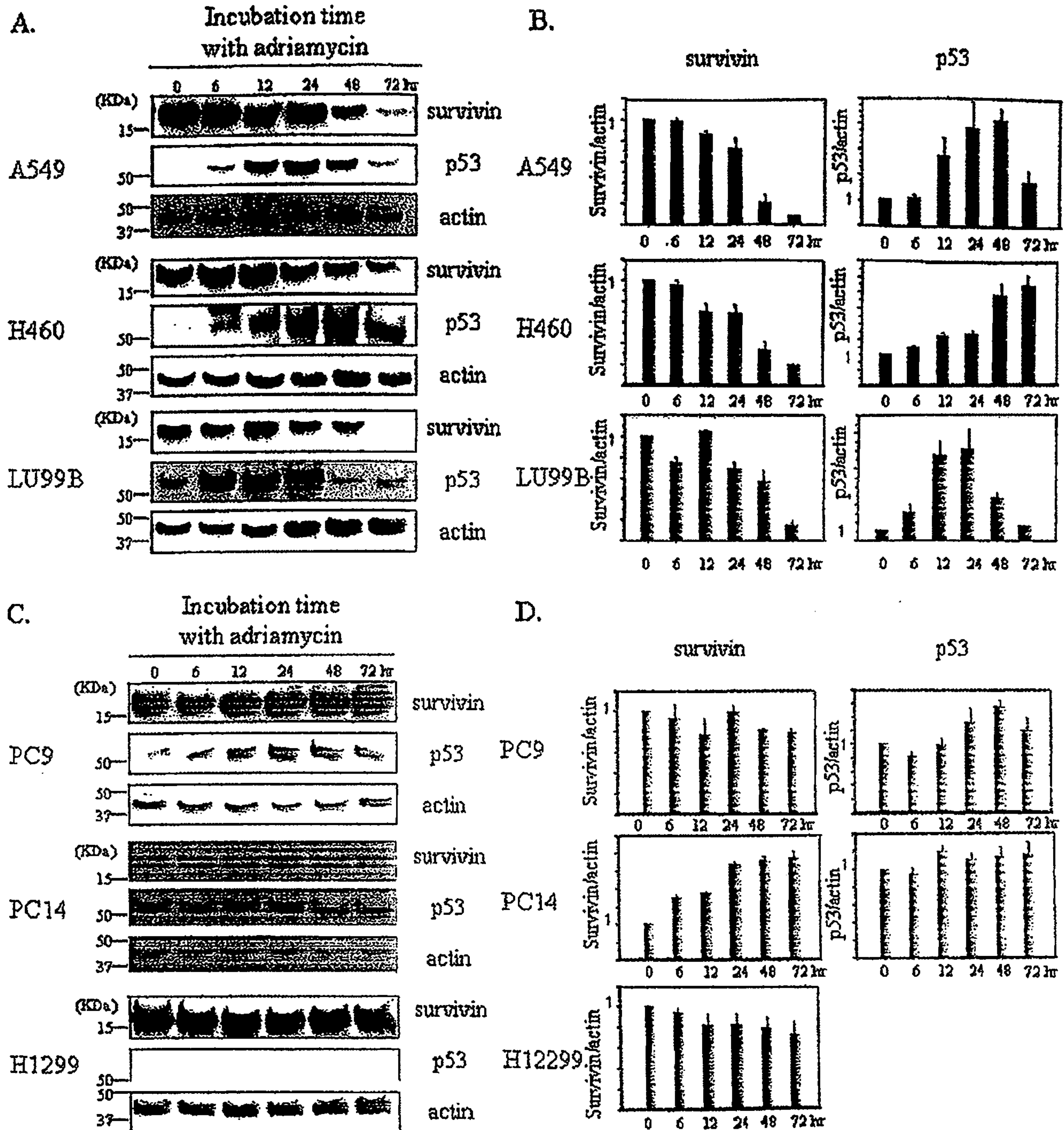

FIGURE 2 – Expression of survivin and p53 protein in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. (*a*) Western-blotting analysis for expression of survivin and p53 in cell lines possessing wild-type p53, including A549, NCI H460 and LU99B.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

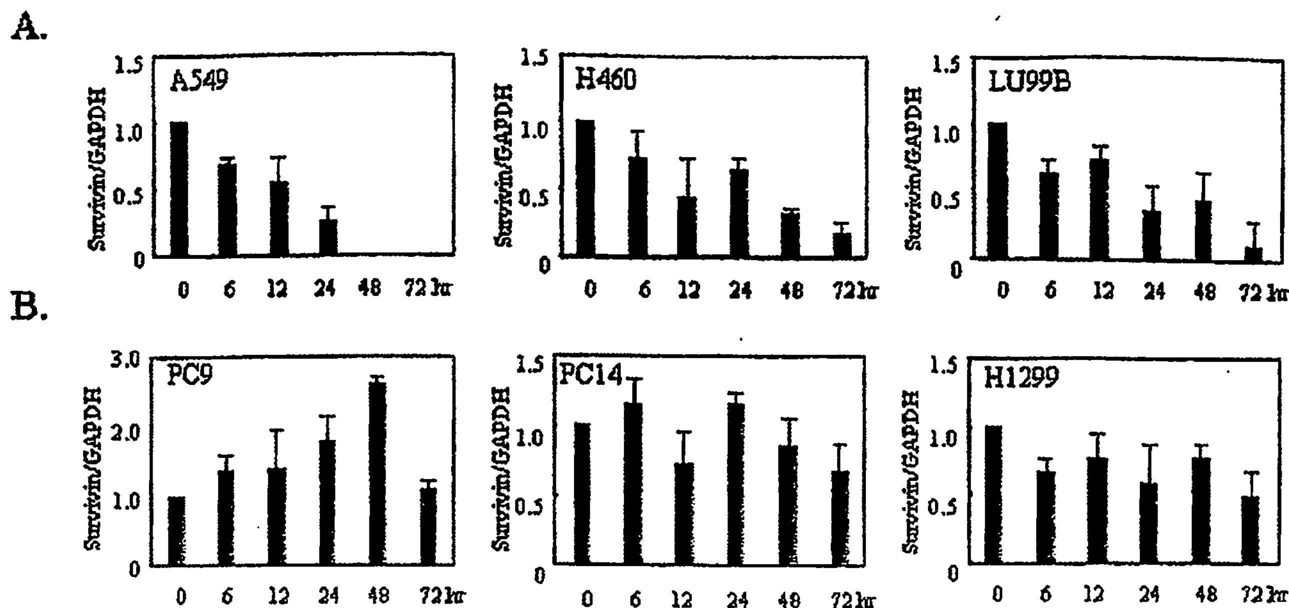

FIGURE 3 – Expression of survivin mRNA in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. Each of the cell lines with wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin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

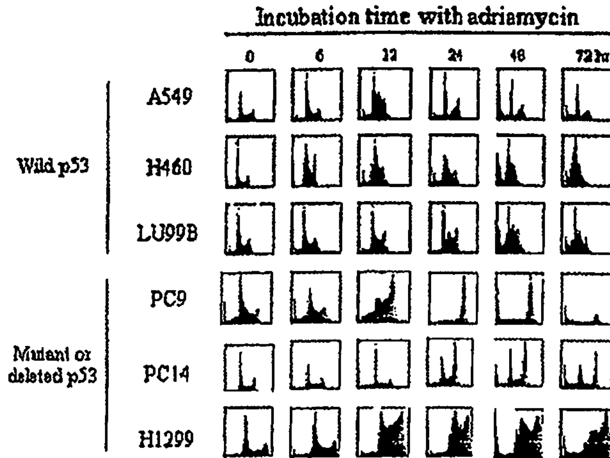

FIGURE 4 – Cell cycle analysis of lung cancer cell lines with different p53 phenotypes after exposure to adriamycin. Each of the cell lines possessing wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for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

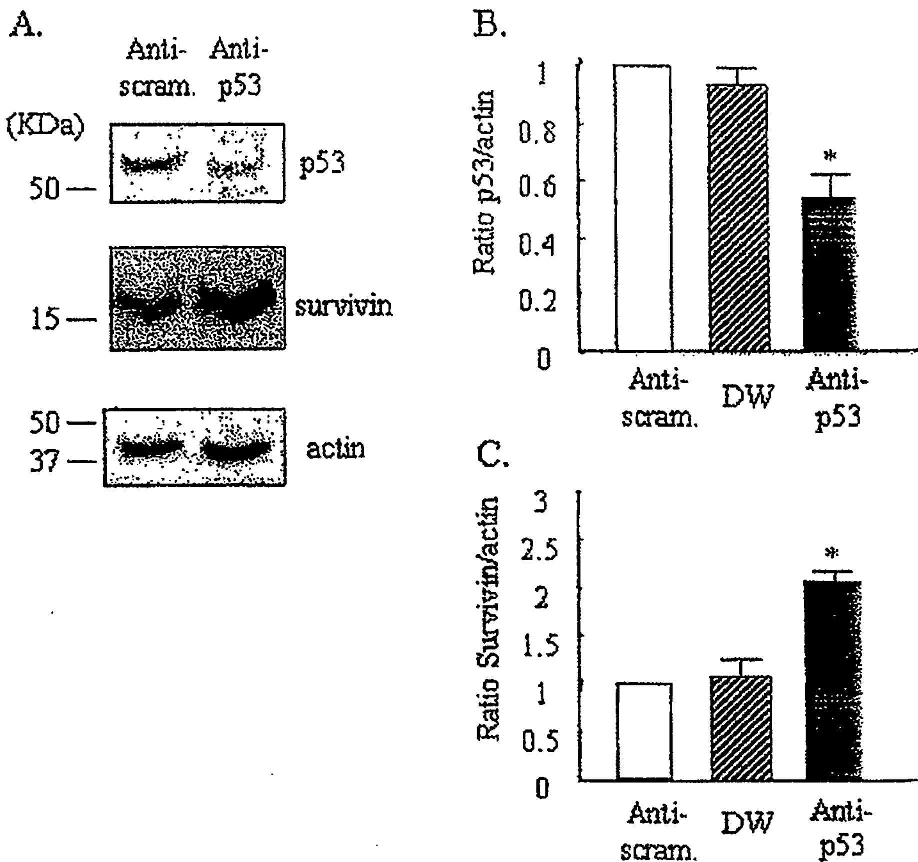

FIGURE 5 – (*a*) Increasing survivin expression in A549 lung cancer cells possessing wild-type p53 as a result of p53 inhibition by siRNA duplex. A549 cells were treated with siRNA duplex targeting p53, scramble or distilled water and then 48 hr later, cell lysates were prepared from the siRNA-treated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

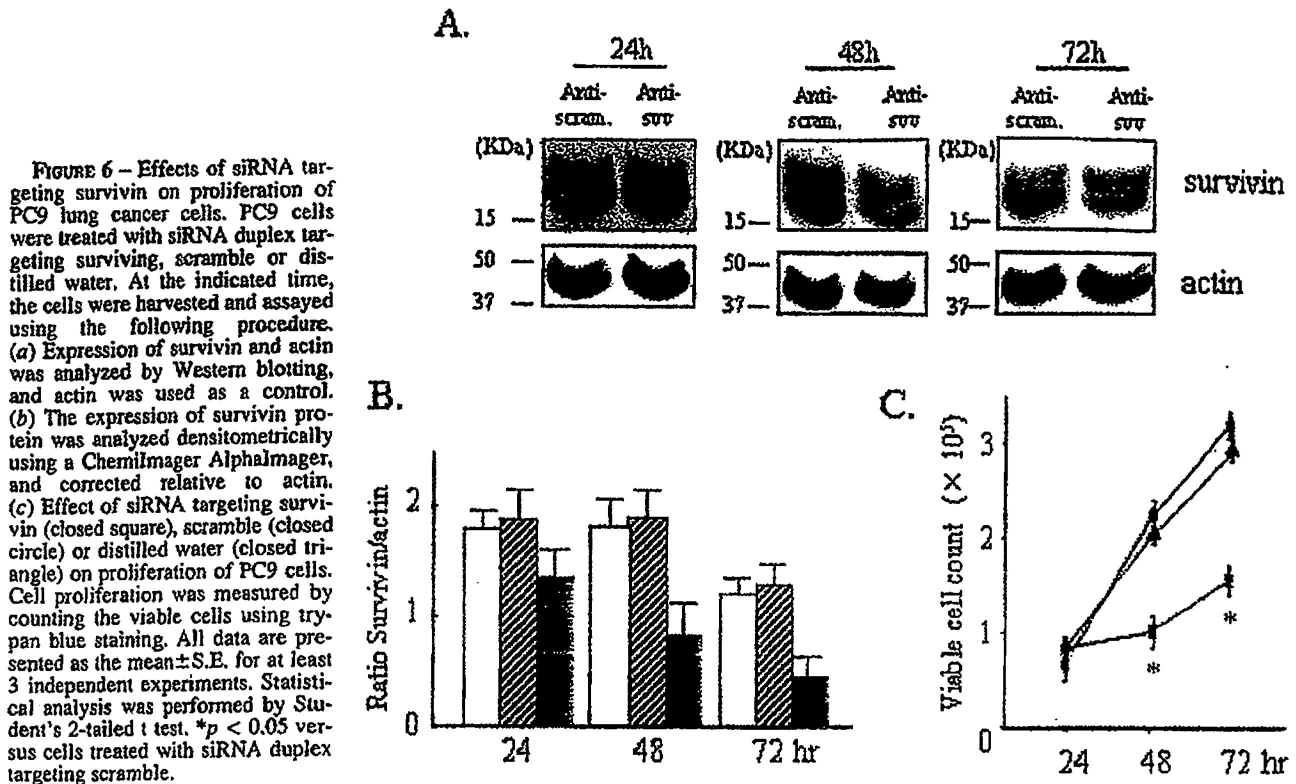

FIGURE 6 – Effects of siRNA targeting survivin on proliferation of PC9 lung cancer cells. PC9 cells were treated with siRNA duplex targeting surviving, scramble or distilled water. At the indicated time, the cells were harvested and assayed using the following procedure. (*a*) Expression of survivin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

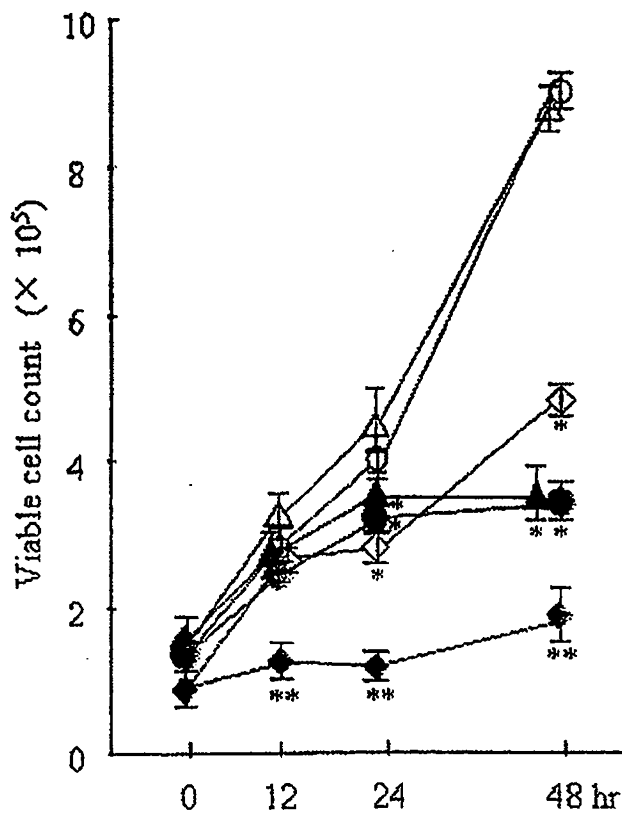

FIGURE 7 – Effects of siRNA duplex targeting of survivin on proliferation of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin or water after 48 hr transfection with siRNA duplex targeting surviving, scramble or distilled water. Open triangle:water after distilled water;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

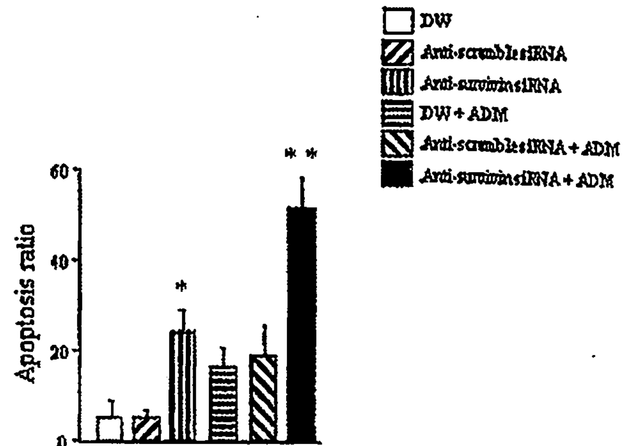

FIGURE 8 – Effects of siRNA targeting survivin on apoptosis of PC9 lung cancer cells treated with adriamycin, evaluated by TUNEL assay. PC9 cells were exposed to adriamycin or water for 24 hr after 48 hr transfection with duplex siRNA targeting surviving, scramble or distilled water. The data are presented 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 1). 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.

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.

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.

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.

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References

1. Brown JM, Bradly G. Wouters. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res 1999:59:1391–9.
2. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997:3:917–21.
3. Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, Martin C, Font A, Barnadas A, Abad A. A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. J Clin Oncol 1999:17:2100–4.
4. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 1998:58:5071–4.
5. Meng H, Lu CD, Sun YL, Dai DJ, Lee SW, Tanigawa N. Expression level of wild-type survivin in gastric cancer is an independent predictor of survival. World J Gastroenterol 2004:10:3245–50.
6. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 1998:58:5315–20.
7. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998:396:580–4.
8. Ferrira CG, Tolis C, Giaconne G. p53 and chemosensitivity. Ann Oncol 1999:10:1011–21.
9. Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T. p53 status and the efficacy of cancer therapy in vivo. Science 1994:266:807–10.
10. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993:75:817–25.
11. Lohr K, Moritz C, Contente A, Dobbelstein M. p21/CDKN1A mediates negative regulation of transcription by p53. J Biol Chem 2003: 29:32507–16.
12. Ahn J, Murphy M, Kratowicz S, Wang A, Levine AJ, George DL. Down-regulation of the stathmin/Op18 and FKBP25 genes following p53 induction. Oncogene 1999:18:5954–8.
13. Yun J, Chae HD, Choy HE, Chung J, Yoo HS, Han MH, Shin DY. p53 negatively regulates cdc2 transcription via the CCAAT-binding NF-Y transcription factor. J Biol Chem 1999:274:29677–82.
14. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002:277:3247–57.
15. Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR, Nielsen LL, Pickett CB. *et al.* Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002:21:2613–22.
16. Carter BZ, Wang RY, Schober WD, Milella M, Chism D, Andreeff M. Targeting Survivin expression induces cell proliferation defect and subsequent cell death involving mitochondrial pathway in myeloid leukemic cells. Cell Cycle 2003:2:488–93.
17. Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, Zangemeister-Wittke U. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000:60:2805–9.
18. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001:411:494–8.
19. Brantl S. Antisense-RNA regulation and RNA interference. Biophysica Acta 2002:1575:15–25.
20. Fukumoto H, Nishio K, Ohta S, Hanai N, Fukuoka K, Ohe Y, Sugihara K, Kodama T, Saija N. *et al.* Effect of chimeric antiganglioside GM2 antibody on ganglioside GM2-expressing human solid tumors in vivo. Int J Cancer 1999:82:759–64.
21. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983:65:55–63.
22. Hensel CH, Xiang RH, Sakaguchi AY, Naylor SL. Use of the Single Strand Conformation Polymorphism Technique and PCR to Detect p53 Gene Mutations in Small Cell Lung Cancer. Oncogene 1991: 6:1067–71.
23. Mitsudomi T, Steinberg SM, Nau MM, Carbone D, Damico D, Bodner S, Oie HK, Linnoila RI, Mulshine JL, Minna JD, Gazdar AF. p53 Gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of *ras* mutations and clinical features. Oncogene 1992:7:171–80.
24. Kashii T, Mizushima Y, Monno S, Nakagawa K, Kobayashi M. Gene analysis of K-ras, H-ras, p53, and retinoblastoma susceptibility genes in human lung cancer cell lines by the polymerase chain Reaction/Single-Strand conformation polymorphism method. J Cancer Res Clin Oncol 1994:120:143–8.
25. Jia LQ, Osada M, Ishioka C, Gamo M, Ikawa S, Suzuki T, Shimodaira H, Niitani T, Kudo T, Akiyama M, Kimura N, Matsuo M, et al. Screening the p53 status of human cell lines using a yeast functional assay. Mol Carcinogen 1997:19:243–53.
26. Fujita T, Kiyama M, Tomizawa Y, Kohno T, Yokota J. Comprehensive analysis of p53 gene mutation characteristics in lung carcinoma with special reference to histological subtypes. Int J Oncol 1999:15: 927–34.
27. Yoshida M, Suzuki T, Komiya T, Hatashita E, Nishio K, Kazuhiko N, Nakagawa K, Fukuoka M. Induction of MRP5 and SMRP mRNA by adriamycin exposure and its overexpression in human lung cancer cells resistant to adriamycin. Int J Cancer 2001:94:432–7.
28. Tamura K, Southwick EC, Kerns J, Rosi K, Carr BI, Wilcox C, Lazo JS. Cdc25 inhibition and cell cycle arrest by a synthetic thioalkyl vitamin K analogue. Cancer Res 2000:60:1317–25.
29. Sgonc R, Gruber J. Apoptosis detection: an overview. Exp Gerontol 1998:33:525–33.
30. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res 1998:58:1808–12.
31. Sarela AI, Verbeke CS, Ramsdale J, Davies CL, Markham AF, Guillou PJ. Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma. Br J Cancer 2002:86:886–92.
32. Wang Z, Fukuda S, Pelus LM. Survivin regulates the p53 tumor suppressor gene family. Oncogene 2004:23:8146–53.
33. Wall NR, O'Connor DS, Plescia J, Pommier Y, Altieri DC. Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. Cancer Res 2003:63:230–5.
34. Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. J Biol Chem 1998:273:11177–82.
35. Grossman D, Kim PJ, Schechner JS, Altieri DC. Inhibition of melanoma tumor growth in vivo by survivin targeting. Proc Natl Acad Sci U S A 2001:98:635–40.
36. Mehdi Mesri, Nathan R. Wall, Jia Li, Richard W. Kim, Dario C. Altieri. Cancer gene therapy using a suvivin mutant adenovirus. J Clin Inv 2001:108:981–90.

Stereotactic Radiosurgery Plus Whole-Brain Radiation Therapy vs Stereotactic Radiosurgery Alone for Treatment of Brain Metastases

A Randomized Controlled Trial

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Context In patients with brain metastases, it is unclear whether adding up-front whole-brain radiation therapy (WBRT) to stereotactic radiosurgery (SRS) has beneficial effects on mortality or neurologic function compared with SRS alone.

Objective To determine if WBRT combined with SRS results in improvements in survival, brain tumor control, functional preservation rate, and frequency of neurologic death.

Design, Setting, and Patients Randomized controlled trial of 132 patients with 1 to 4 brain metastases, each less than 3 cm in diameter, enrolled at 11 hospitals in Japan between October 1999 and December 2003.

Interventions Patients were randomly assigned to receive WBRT plus SRS (65 patients) or SRS alone (67 patients).

Main Outcome Measures The primary end point was overall survival; secondary end points were brain tumor recurrence, salvage brain treatment, functional preservation, toxic effects of radiation, and cause of death.

Results The median survival time and the 1-year actuarial survival rate were 7.5 months and 38.5% (95% confidence interval, 26.7%-50.3%) in the WBRT + SRS group and 8.0 months and 28.4% (95% confidence interval, 17.6%-39.2%) for SRS alone ($P=.42$). The 12-month brain tumor recurrence rate was 46.8% in the WBRT + SRS group and 76.4% for SRS alone group ($P<.001$). Salvage brain treatment was less frequently required in the WBRT + SRS group (n = 10) than with SRS alone (n = 29) ($P<.001$). Death was attributed to neurologic causes in 22.8% of patients in the WBRT + SRS group and in 19.3% of those treated with SRS alone ($P=.64$). There were no significant differences in systemic and neurologic functional preservation and toxic effects of radiation.

Conclusions Compared with SRS alone, the use of WBRT plus SRS did not improve survival for patients with 1 to 4 brain metastases, but intracranial relapse occurred considerably more frequently in those who did not receive WBRT. Consequently, salvage treatment is frequently required when up-front WBRT is not used.

Trial Registration umin.ac.jp/ctr Identifier: C000000412

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BRAIN METASTASES OCCUR IN 20% to 40% of all patients with cancer and are generally associated with a poor prognosis.[1,2] The most common route of metastatic dissemination resulting in brain metastases is hematogenous, and it is therefore presumed that the entire brain is "seeded" with micrometastatic disease, even when only a single intracranial lesion is detected. Consequently, whole-brain radiation therapy (WBRT) has been a mainstay of treatment.[1,2]

Recently, the assumption that the entire brain is seeded with micrometastases in all patients with overt brain metastases has been questioned, prompting

For editorial comment see p 2535.

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a contrarian philosophy that in some patients, the intracranial disease is truly limited—the so-called oligometastases situation. For patients who truly have limited intracranial disease, the potential exists that WBRT could be replaced by focal therapeutic options such as resection or stereotactic radiosurgery (SRS), which delivers high-dose, focal radiation.[1-4]

The adverse effects of WBRT require a further examination of its role. Acute adverse effects are generally limited in severity and duration; however, the long-term risks of serious and permanent toxic effects, including cognitive deterioration and cerebellar dysfunction, are poorly understood.[5,6] In the attempt to minimize potential long-term morbidity following WBRT, treatments initially relying on focal therapeutic options are being used with increasing frequency. Although there have been several retrospective reports,[7-14] only 1 prospective randomized study compared the outcome of conventional surgery alone and surgery followed by WBRT.[6] Sneed et al[7] collected raw data on 983 patients from 10 institutions and suggested that there was no survival difference between patients treated with SRS alone and those treated with WBRT plus SRS. Flickinger et al[8] reviewed 116 patients with solitary brain metastases who underwent SRS with or without fractionated large-field radiotherapy and found improved local control, but not improved survival, with the addition of fractionated large-field radiotherapy. Regine et al[9] suggested that SRS alone is associated with an increasingly significant risk of brain tumor recurrence and neurologic deficit with increasing survival time. Pirzkall et al[10] showed a trend for superior local control and survival when SRS was combined with WBRT in 236 patients with 311 brain metastases. Aoyama et al,[11] Chidel et al,[12] and Shirato et al[13] have all shown that omission of WBRT from initial management was not detrimental in terms of overall survival, but brain tumors recurred in more than 50% of patients treated in this manner. Patchell et al[6] have shown that patients with cancer and single metastases to the brain who receive treatment with surgical resection and postoperative WBRT have fewer recurrences of cancer in the brain and are less likely to die of neurologic causes than are similar patients treated with surgical resection alone.

Herein, we report the results of a prospective, multi-institutional, randomized controlled trial comparing WBRT plus SRS vs SRS alone for patients with limited (defined as ≤4) brain metastases. Through a literature search and examination of clinical trial registries, we confirmed that this is the first multi-institutional, prospective, randomized comparison of WBRT plus SRS vs SRS alone.

METHODS

Eligibility Criteria

Patients were eligible who were aged 18 years or older with 1 to 4 brain metastases, each with a maximum diameter of no more than 3 cm on contrast-enhanced magnetic resonance imaging (MRI) scans, derived from a histologically confirmed systemic cancer. Patients with metastases from small cell carcinoma, lymphoma, germinoma, and multiple myeloma were excluded. Eligible patients had a Karnofsky Performance Status (KPS) score of 70 or higher. The protocol was approved by the institutional review boards of Hokkaido University, Sapporo, Japan, and of 10 other institutions that participated in the trial through the Japanese Radiation Oncology Study Group (JROSG 99-1). Written informed consent was obtained from each patient before entry into the study.

Randomization and Treatment

Randomization was performed at the Hokkaido University Hospital Data Center. A permuted-blocks randomization algorithm was used with a block size of 4. A randomization sheet was created for each institution. After written informed consent was obtained, eligible patients were randomly assigned to receive either up-front WBRT combined with SRS or SRS without up-front WBRT. Prior to randomization, the patients were stratified based on number of brain metastases (single vs 2-4), extent of extracranial disease (active vs stable), and primary tumor site (lung vs other sites). Extracranial disease was considered to be stable when the tumor had been clinically controlled for 6 months or longer prior to the detection of brain metastases.

The WBRT dosage schedule was 30 Gy in 10 fractions over 2 to 2.5 weeks. The WBRT treatment visit proceeded to SRS when patients were assigned to the WBRT + SRS group. The SRS dose was prescribed to the tumor margin. Metastases with a maximum diameter of up to 2 cm were treated with doses of 22 to 25 Gy and those larger than 2 cm were treated with doses of 18 to 20 Gy. The dose was reduced by 30% when the treatment was combined with WBRT because the optimal combination of WBRT and SRS had not been studied in well-conducted, prospective, phase 1 dose escalation trials. In the 1990s, the Radiation Therapy Oncology Group (RTOG) initiated a phase 1 dose escalation trial of SRS alone in patients who had previously undergone radiation treatment.[14] This trial was stopped early without reaching the maximum tolerance dose, and tumor size–dependent dose recommendations for SRS alone were described. No phase 1 trial has ever tested the combination of WBRT and SRS doses. Therefore, there is no well-known or scientifically recommended dose for the combination of WBRT and SRS. There are clearly concerns that the combination could be potentially deleterious. Therefore, various studies have adopted different approaches for selection of the dose combinations to be tested. Several retrospective data suggested that the RTOG dose guidelines might be associated with a higher frequency of late radiation toxic effects when used with WBRT.[10,15] Our preexisting experience of SRS with a 30% reduced SRS dose

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combined with WBRT indicated that there is not a significant difference in local tumor control (data not shown) compared with SRS with the dose suggested in the RTOG protocol. Therefore, we decided to use a 30% reduced SRS dose in the WBRT + SRS group in this study.

Follow-up Protocol

We performed clinical evaluations and MRI scans 1 and 3 months after treatment and every 3 months thereafter. In cases in which a recurrence was detected, further treatment was administered at the discretion of the attending physician. The size of the treated lesions was measured in 3 dimensions, and this size, the development of new brain metastases, and the development of leukoencephalopathy associated with radiological findings (according to the National Cancer Institute's Common Toxicity Criteria version 2.0[16]) were scored based on serial MRI scans. Local tumor progression was defined as a radiographic increase of 25% or more in the size of a metastatic lesion (bidimensional product). If an MRI result showed central or heterogeneous low intensity and if the lesion size decreased on serial studies, brain necrosis was scored; positron emission tomography or surgical resection was encouraged as appropriate to confirm MRI findings.

At each visit, functional status and neurologic toxic effects were scored. Systemic functional status was evaluated by using the KPS score. Neurologic function was evaluated according to the criteria listed in TABLE 1.[17] Neurosurgeons or radiation oncologists specializing in neuro-oncology measured the neurologic status as well as the KPS score at the clinic. We did not attempt to blind the investigators with regard to patients' treatment assignments. Systematic functional status and neurologic function were scored by the physicians who treated the patients. An acute toxic effect was identified as an event that arose within 90 days of the initiation of radiotherapy and a late toxic effect was considered as an event that occurred thereafter, according to the central nervous system toxicity criteria listed among the RTOG Late Radiation Morbidity Scoring Criteria.[18] For all patients who died, the cause of death was determined. The cause of death was determined by autopsy in 1 patient and by clinical evaluation based on the definition proposed by Patchell et al[9] in all other patients. Patients were considered to have died of neurologic causes if they had stable systemic disease and

Table 1. Baseline Characteristics*

Characteristics	WBRT + SRS (n = 65)	SRS Alone (n = 67)
Age at diagnosis, mean (range), y	62.5 (36-78)	62.1 (33-86)
<65	32 (49)	34 (51)
≥65	33 (51)	33 (49)
Men	46 (71)	53 (79)
No. of brain metastases		
1	31 (48)	33 (49)
2-4	34 (52)	34 (51)
Primary tumor site		
Breast	6 (9)	3 (4)
Lung	43 (66)	45 (67)
Colorectal	5 (5)	6 (9)
Kidney	5 (6)	5 (7)
Other	6 (9)	8 (12)
Primary tumor status		
Stable	30 (46)	33 (49)
Active	35 (54)	34 (51)
Extracranial metastases		
Stable	41 (63)	38 (57)
Active	24 (37)	29 (43)
RPA		
Class 1 (aged <65 years; no active extracranial disease)	11 (17)	8 (12)
Class 2 (aged ≥65 years; active extracranial disease)	54 (83)	59 (88)
Histological status		
Squamous cell	11 (17)	11 (16)
Adenocarcinoma	43 (66)	43 (64)
Large cell	2 (3)	4 (6)
Other	9 (14)	9 (13)
KPS score†		
70-80	31 (48)	23 (34)
90-100	34 (52)	44 (66)
Neurologic function		
No symptoms (grade 0)	38 (59)	47 (70)
Minor symptoms, fully active without assistance (grade 1)	12 (18)	13 (19)
Moderate symptoms; fully active but requires assistance (grade 2)	8 (12)	4 (6)
Moderate symptoms; less than fully active, requires assistance (grade 3)	7 (11)	3 (5)
Severe symptoms: totally inactive (grade 4)	0	0
Chemotherapy after brain treatment	18 (38)	19 (40)
Maximum diameter of brain metastases, cm		
Mean (SD)	1.53 (0.78)	1.42 (0.79)
Median (range)	1.40 (0.2-3.0)	1.30 (0.2-3.0)
SRS dose at the tumor margin, mean (SD), Gy	16.6 (3.6)	21.9 (2.7)

Abbreviations: KPS, Karnofsky Performance Status; RPA, recursive partition analysis; SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy.
*Data are expressed as No. (%) of participants unless otherwise noted.
†A higher score indicates better performance.

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progressive neurologic dysfunction. Patients with severe neurologic disability who died of intercurrent illness were also included among neurologic deaths, as were patients with both rapidly progressive systemic disease and advancing neurologic dysfunction, because these patients also represent brain treatment failures.

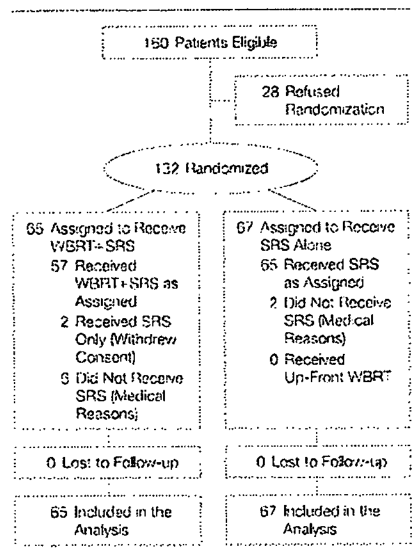

Figure 1. Flow of Study Participants

SRS indicates stereotactic radiosurgery; WBRT, whole-brain radiation therapy.

End Points and Statistical Analysis

The primary end point of the study was overall survival. Secondary end points were cause of death, functional preservation, brain tumor recurrence, salvage treatment, and toxic effects of radiation. All analyses were conducted on an intention-to-treat basis. The study was designed to have 80% power to detect an absolute difference of 30% in the median survival time, with a 2-sided α level of .05. Using an estimated median survival time of 8.7 months for the group receiving SRS alone[11] and a follow-up time of 15 months, the sample size required to detect this difference was 89 patients per group. An interim analysis was planned wherein 50 patients would be assigned to each group to determine whether the sample size was large enough to show a significant difference with a 2-sided α level of .05. End points were measured beginning at the date of randomization. Univariate analyses were carried out by the Kaplan-Meier method.[19] We assumed that the survival rate was always higher in the WBRT + SRS group than in the SRS-alone group based on the suggestions in a retrospective study, and we used the log-rank test to compare differences between the groups. The χ^2 test was used to determine the relationship between 2 categorical variables, and the Fisher exact test was used when small cell sizes were encountered in 2×2 contingency tables. A 2-tailed t test was used to compare the means of continuous variables between the treatment groups. Multivariate analyses were performed to evaluate the factors selected via the univariate analyses ($P<.10$). Stratification in the randomization was taken into account in the statistical analysis. The Cox proportional hazards model was used to calculate hazard ratios and 95% confidence intervals (CIs).[20] A 2-sided P value of .05 or less was considered to reflect statistical significance. Additional covariates were examined as appropriate and are noted in the "Results" section. All statistical analyses were initially performed by a physician (H.A.) using a commercial statistical software package (StatView version 5.0J, SAS Institute Inc, Cary, NC), and all results were verified by a statistician (G.K.) using a different software package (SAS, version 9.1, SAS Institute Japan Ltd, Tokyo, Japan).

RESULTS

Patients and Treatment

The recruitment period was from October 1999 to December 2003. There were

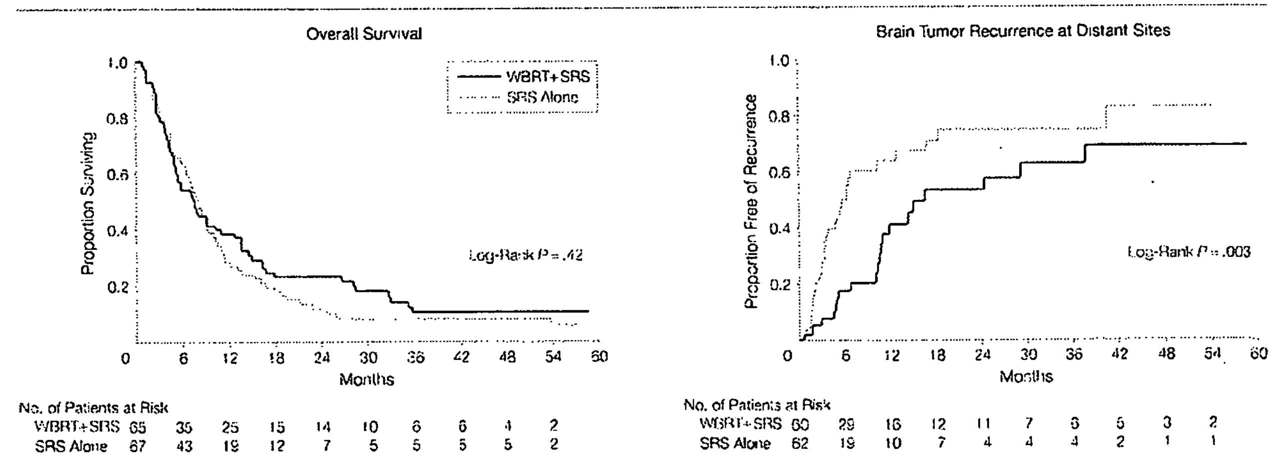

Figure 2. Overall Survival and Brain Tumor Recurrence at Distant Sites

The mean survival time was 7.5 months for patients receiving whole-brain radiation therapy (WBRT) plus stereotactic radiosurgery (SRS) and 8.0 months for patients receiving SRS alone. This difference was not significant ($P=.42$). There was a statistically significant decrease in brain tumor recurrence in the WBRT+SRS group ($P=.003$).

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160 eligible patients, of whom 132 (83%) were randomized (65 to WBRT + SRS and 67 to SRS alone) (FIGURE 1). The date of last follow-up was April 2005. The interim analysis was performed with 122 patients (about 60 in each group), which takes into account the possible number of patients with protocol violations. Patient accrual was terminated before the planned final accrual number had been reached because the results of the interim analyses indicated that at least 805 patients were necessary to detect a significant difference in the primary end points. In addition, the numbers of patients appeared sufficient to detect a significant difference in brain tumor recurrence rates: 31 patients in each group were shown to be enough to detect a 30% difference in the median month of 50% brain tumor recurrence (16.2 months with WBRT + SRS vs 5.5 months with SRS alone).

There was no statistical difference between the groups in the baseline characteristics of the patients (Table 1). The median follow-up time was 7.8 months (range, 0.5-58.7 months) for the entire study and 49.2 months (range, 19.6-58.7 months) for survivors. Ninety-two percent of the patients included in the study completed the assigned treatment (Figure 1).

Survival and Cause of Death

By the time of the last follow-up visit in April 2005, 57 patients in the WBRT + SRS group and 62 patients in the SRS-alone group had died. Death was attributed to neurologic causes in 13 patients (22.8%) in the WBRT + SRS group and in 12 patients (19.3%) in the SRS-alone group ($\chi^2 = 0.21$; $P = .64$). The median survival time was 7.5 months with WBRT + SRS and 8.0 months with SRS alone. The higher median survival time with SRS alone was discordant with the 1-year actuarial survival rates of 38.5% (95% CI, 26.7%-50.3%) for the WBRT + SRS group and 28.4% (95% CI, 17.6%-39.2%) for the SRS-alone group ($P = .42$). FIGURE 2A shows that this discordance was due to the crossing of the 2 survival curves. The results of the univariate and multivariate analyses are shown in TABLE 2 and TABLE 3. The number of patients in each institution was too small to allow for a meaningful comparison among institutions. Recursive partition analysis was not included in the multivariate analysis because it is not independent of age and extracranial metastases. Treatment group was not found to be significant in either analysis.

Posttreatment Neurologic Toxicity

A summary of posttreatment neurologic toxicity is given in TABLE 4. Symptomatic acute neurologic toxicity was observed in 4 patients receiving WBRT + SRS and in 8 patients receiv-

Table 2. Univariate Survival Analysis

	No. of Participants	Survival Time, Median (Range), mo	*P* Value
Treatment group			
WBRT + SRS	65	7.5 (0.8-58.7)	.42
SRS alone	67	8.0 (0.5-57.0)	
Age, y			
<65	66	8.9 (0.9-58.7)	.07
≥65	66	6.5 (0.5-55.6)	
Sex			
Male	99	7.1 (0.5-58.7)	.20
Female	33	10.5 (0.8-57.0)	
No. of brain metastases			
1	68	8.6 (1.4-58.7)	.02
2-4	64	7.3 (0.5-55.6)	
Primary tumor site			
Lung	88	8.1 (0.5-58.7)	.33
Other	44	7.1 (0.9-57.0)	
Primary tumor status			
Stable	69	9.2 (0.9-58.7)	<.001
Active	63	6.5 (0.5-53.8)	
Extracranial metastases			
Stable	79	13.3 (1.1-58.7)	<.001
Active	53	5.1 (0.5-55.6)	
RPA			
Class 1	19	16.0 (0.9-58.7)	<.001
Class 2	113	7.5 (0.5-55.6)	
KPS score			
70-80	54	5.0 (0.5-58.7)	<.001
90-100	78	9.2 (0.8-57.0)	
Chemotherapy after brain treatment			
Yes	37	10.1 (1.3-53.8)	.34
No	95	6.8 (0.5-58.7)	

Abbreviations: KPS, Karnofsky Performance Status; RPA, recursive partition analysis; SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy.

Table 3. Multivariate Survival Analysis

Variables*	Hazard Ratio (95% CI)	*P* Value
Treatment group (WBRT + SRS)	1.37 (0.93-1.98)	.11
Age (<65 y)	1.48 (1.01-2.16)	.04
No. of brain metastases (1)	1.36 (0.94-1.97)	.10
Primary tumor status (stable)	1.62 (1.11-2.36)	.01
Extracranial metastases (stable)	2.35 (1.55-3.55)	<.001
KPS score (90-100)	1.69 (1.16-2.47)	.007

Abbreviations: CI, confidence interval; KPS, Karnofsky Performance Status; SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy.
*Referents appear in parentheses.

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Table 4. Treatment-Related Neurotoxic Effects*

	No. in WBRT + SRS Group (n = 65)				No. in SRS-Alone Group (n = 67)			
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
Acute toxic effects	2	1	1	0	3	3	2	0
Seizure	0	0	1	0	1	2	1	0
Other	2	1	0	0	2	1	1	0
Late toxic effects	3	0	2	2	1	0	0	2
Radiation necrosis	1	0	0	2	0	0	0	1
Leukoencephalopathy	1	0	2	0	0	0	0	0
Other†	1	0	0	0	1	0	0	1
Radiological leukoencephalopathy	2	3	2	0	1	1	0	0

Abbreviations: SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy.
*From the National Cancer Institute's Common Toxicity Criteria version 2.0.[17]
†Other effects included 1 case of slight lethargy (grade 1) in the WBRT + SRS group and 1 case each of seizure (grade 4) and headache (grade 1) in the SRS-alone group.

Table 5. Univariate Analysis of Development of New Metastases at Distant Brain Sites

	Actuarial Rate, %		Log-Rank
	6 mo	12 mo	*P* Value
Treatment group			
WBRT + SRS	17.5	41.5	.003
SRS alone	49.9	63.7	
Age, y			
<65	34.5	55.9	.65
≥65	33.9	49.0	
Sex			
Male	32.7	51.5	.39
Female	36.3	55.9	
No. of brain metastases			
1	27.3	39.2	.03
2-4	42.4	69.9	
Primary tumor site			
Lung	29.5	52.0	.40
Other	43.1	55.9	
Primary tumor status			
Stable	32.8	44.8	.20
Active	37.1	69.6	
Extracranial metastases			
Stable	29.5	38.4	.02
Active	37.3	69.3	
KPS score			
70-80	43.2	57.4	.05
90-100	29.9	50.8	
Chemotherapy after brain treatment			
Yes	37.1	59.0	.33
No	32.9	50.0	

Abbreviations: KPS, Karnofsky Performance Status; SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy.

ing SRS alone (*P*=.36), including 1 and 2 patients with grade 3 toxicity, respectively, in each group. The symptoms developed a median of 6 days after initiation of treatment (range, 1-64 days) in the WBRT + SRS group and 10 days (range, 1-86 days) in the SRS-alone group. Symptomatic late neurologic radiation toxic effects were observed in 7 patients in the WBRT + SRS group and in 3 patients in the SRS-alone group (*P*=.20). Toxic effects were experienced for a median of 15.6 months (range, 6.7-59.4 months) in the WBRT + SRS group and 6.2 months (range, 5.8-8.1 months) in the SRS-alone group. There were 3 cases of radiation necrosis (grade 1, n=1; grade 4, n=2), 3 cases of leukoencephalopathy (grade 1, n=1; grade 3, n=2), and 1 case of slight lethargy (grade 1) in the WBRT + SRS group. In patients receiving SRS alone, the following effects were observed: 1 case of radiation necrosis (grade 4), 1 of seizure (grade 4), and 1 of headache (grade 1). Radiation necrosis was diagnosed using positron emission tomography or surgical resection in all cases. Radiological findings consistent with leukoencephalopathy were observed in 7 patients in the WBRT + SRS group and in 2 patients in the SRS-alone group (*P*=.09). Three of these 9 patients also experienced symptomatic leukoencephalopathy; the other 6 patients were asymptomatic.

Brain Tumor Recurrence

Brain tumor recurrence at either distant or local sites in the brain was observed in 63 patients (23 in the WBRT + SRS group and 40 in the SRS-alone group). The 12-month actuarial brain tumor recurrence rate was 46.8% (95% CI, 29.7%-63.9%) in the WBRT + SRS group and 76.4% (95% CI, 63.3%-89.5%) in the SRS-alone group (*P*<.001).

Fifty-five patients had new brain metastases at distant sites (21 in the WBRT + SRS group and 34 in the SRS-alone group). The 12-month actuarial rate of developing new brain metastases was 41.5% (95% CI, 24.4%-58.6%) in the WBRT + SRS group and 63.7% (95% CI, 49.0%-78.4%) in the SRS-alone group (*P*=.003) (Figure 2B).

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The multivariate analysis revealed that WBRT + SRS was associated with a reduced risk of recurrence (hazard ratio, 0.32; 95% CI, 0.18-0.58; $P<.001$) (TABLE 5 and TABLE 6).

During the follow-up period, 122 patients (92% of the total patients enrolled) had at least 1 follow-up MRI scan performed. In total, 581 follow-up MRI scans were performed; of these, 87 scans (15%) demonstrated new brain metastases; these 87 "event scans" were obtained in 55 patients. Sixteen percent of these "event scans" (14/87) were associated with neurologic symptoms at the time of the MRI examination.

A total of 247 metastases received initial treatment with SRS (117 in the WBRT + SRS group and 130 in the SRS-alone group). Follow-up MRI was available for 210 metastases (85%). The actuarial local tumor control rate at 12 months was 88.7% (95% CI, 80.1%-97.3%) in the WBRT + SRS group and 72.5% (95% CI, 60.3%-84.7%) in the SRS-alone group ($P=.002$) (FIGURE 3). The histopathological type (adenocarcinoma vs others) was not shown to be a significant factor ($P=.90$). The multivariate analysis also showed significantly better tumor control by WBRT + SRS treatment (hazard ratio, 4.83; 95% CI, 2.00-11.65; $P<.001$).

Salvage treatment for progression of brain tumor was required significantly more frequently in patients receiving SRS alone (29 patients) than in the WBRT + SRS group (10 patients) ($\chi^2=12.33$; $P<.001$). Salvage WBRT was applied in 11 patients in the SRS-alone group but was not used in any patients in the WBRT + SRS group. Salvage SRS was used in 19 patients in the SRS-alone group and in 9 patients in the WBRT + SRS group.

Systemic and Neurologic Functional Preservation

Systemic functional preservation rates (KPS score ≥70) at 12 months were 33.9% (95% CI, 22.2%-45.4%) in the WBRT + SRS group and 26.9% (95% CI, 16.3%-37.5%) in the SRS-alone group ($P=.53$). The decrease in the KPS score to below 70 was attributed to neurologic causes in 17 patients (29%) in the WBRT + SRS group and 14 (22%) in the SRS-alone group.

The actuarial rates of neurologic preservation at 12 months were 72.1% (95% CI, 58.8%-85.4%) with WBRT + SRS and 70.3% (95% CI, 55.6%-85.0%) with SRS alone ($P=.99$) when neurologic preservation was defined as a lack of any worsening of the neurologic grade on follow-up examination, compared with the pretreatment grade. In total, 85 patients (38 in the WBRT + SRS group and 47 in the SRS-alone group) did not have neurologic symptoms when brain metastases were diagnosed (grade 0). Among the 47 patients who had a pretreatment grade of 1 to 3, an improvement in neurologic status was observed at least once in 9 patients and 10 patients in the respective groups ($\chi^2=1.32$; $P=.24$). Deterioration of neurologic function was observed in 43 patients, including 7 who initially experienced improvement after treatment (22 in the WBRT + SRS group and 21 in the SRS-alone group; $\chi^2=0.09$; $P=.75$). This deterioration was attributed to either original or distant brain metastases in 13 patients (59%) in the WBRT + SRS group and 18 patients (86%) in the SRS-alone group ($\chi^2=3.78$; $P=.05$). Late neurologic radiation toxic effects were the cause of deterioration in 4 and 2 patients in each group, respectively. Either meningeal dissemination or spinal cord metastases induced neurologic deterioration in 5 and 1 patient in each group, respectively.

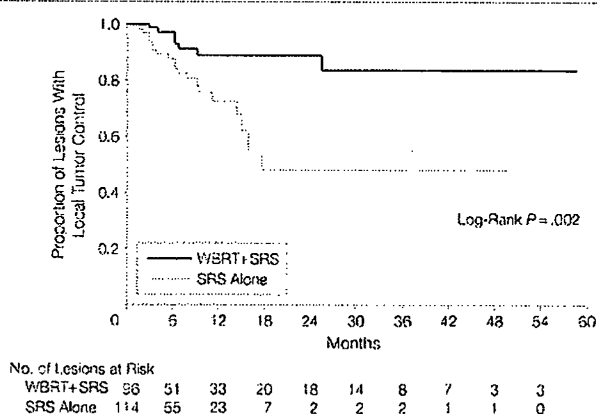
Neurocognitive function was optionally assessed using the Mini-Mental State Examination (MMSE). Among the 44 patients (25 in the WBRT + SRS group and 19 in the SRS-alone group) who lived 12 months or longer, MMSE data were available in 28 patients at least once (16 in the WBRT + SRS group and 12 in the SRS-alone group) at the median follow-up times of 30.5 months (range, 13.7-58.7 months) with WBRT + SRS and 20.7 months (range, 13.3-53.8 months) with SRS alone. The median MMSE pretreatment score was 28.0 (range, 23-30) in the WBRT + SRS

Table 6. Multivariate Analysis of Development of New Metastases at Distant Brain Sites

	Hazard Ratio (95% CI)	P Value
Treatment group (WBRT + SRS)	0.32 (0.18-0.58)	<.001
No. of brain metastases (2-4)	1.69 (0.97-2.93)	.06
Extracranial metastases (active)	2.06 (1.17-3.64)	.01
KPS score (70-80)	2.14 (1.17-3.93)	.01

Abbreviations: CI, confidence interval; KPS, Karnofsky Performance Status; SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy.

Figure 3. Local Tumor Control



No. of Lesions at Risk

	0	6	12	18	24	30	36	42	48	54
WBRT+SRS	96	51	33	20	18	14	8	7	3	3
SRS Alone	114	55	23	7	2	2	2	1	1	0

There was a statistically significant increase in local tumor control in patients receiving whole-brain radiation therapy (WBRT) plus stereotactic radiosurgery (SRS) ($P=.002$).

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group and 27.0 (range, 23-30) in the SRS-alone group. The median score at the final follow-up was 27.0 (range, 21-30) in the WBRT + SRS group and 28.0 (range, 18-30) in the SRS-alone group.

COMMENT

Stereotactic radiosurgery is a method of delivering high doses of focal radiation to a tumor while minimizing irradiation of the adjacent normal tissue. This approach was originally developed by the Swedish neurosurgeon Lars Leksell as a substitute for direct surgical intervention.[21] Stereotactic radiosurgery is now available worldwide, and it is increasingly used to treat brain metastases because it is less invasive compared with direct surgical intervention, although a direct randomized comparison of the 2 modes has not been performed to date.

Whole-brain radiation therapy has been a standard treatment for brain metastases for several decades.[1-4,6,7,17] In more recent years, the importance of focal aggressive therapy combined with WBRT has been increasingly recognized.[3,4,22-24] Andrews et al[3] recently reported the results from RTOG 9508, a multi-institutional phase 3 trial of 333 patients with 1 to 3 brain metastases who received WBRT with or without SRS boost. A statistically significant improvement in median survival with the addition of SRS was seen in patients with a single brain metastasis.

To reduce the risk of late radiation effects,[1,2,5] WBRT is increasingly being omitted from the initial management strategy.[6-13] There is not yet a general consensus regarding the risks and benefits of omitting up-front WBRT. One study showed a trend toward improved survival among patients who received SRS alone,[12] whereas another study showed a trend toward worse survival among patients who received SRS alone.[10] A retrospective multi-institutional review of SRS alone vs SRS with WBRT in 569 patients failed to show any difference in survival between the 2 groups.[7] In a single-institution prospective randomized trial comparing WBRT with observation in patients who underwent conventional surgery,[6] a large increase in intracranial relapse and a concomitant increase in death due to neurologic causes were identified in the non-WBRT group; however, no survival difference was identified in that study. In the present study, no significant survival difference was observed between the groups receiving WBRT + SRS and SRS alone, although the number of patients was not large enough to allow detection of any differences that were smaller than we had assumed. In addition, no significant difference in the frequency of death due to neurologic causes was observed. Moreover, these results were obtained in spite of the rather large increase in intracranial failure when WBRT was omitted. A further observation of note from the present trial was the significant increase in local failure with SRS alone, even though the radiation dose in these patients was considerably higher than that administered to patients receiving WBRT + SRS. We have adapted the 30% reduced dose of SRS in the WBRT + SRS group, which could have lowered local control of the brain metastasis in the WBRT + SRS group. However, we have observed opposite results in this study; the local control rate was significantly higher in the WBRT + SRS group than in the SRS-alone group. This observation lends merit to the value of fractionation, which might help overcome some radiation resistance mechanisms, such as hypoxia.

Also of concern in this context is that higher brain recurrence rates are associated with neurologic deterioration.[9] In a previous randomized study of surgery with or without WBRT,[6] the time to neurologic deterioration was dramatically longer in the WBRT group, although no difference in functional independence was observed. In the current study, no significant difference in the preservation of neurologic function was observed. However, the present study might have less ability to detect small differences, and the present assessment of neurologic function was not conducted with sophisticated measures that might have detected differences between patient groups.

Although surgery and SRS are both focal treatments, SRS is less invasive and may be repeated more often than surgical intervention.[11] The optimal timing of these interventions is an issue that remains open for debate. Our results suggest that the early detection of a brain recurrence and early salvage brain treatment may prevent neurologic deterioration and neurologic death, even when WBRT is not included in the initial treatment. However, study participants more frequently undergo physical and radiological examinations than do patients in the community. Given that the majority of new brain metastases were initially detected in asymptomatic patients, studies assessing the benefits of scheduled imaging should be conducted in the future.

In conclusion, our findings demonstrated that SRS alone without up-front WBRT was associated with increased brain tumor recurrence; however, it did not result in either worsened neurologic function or increased risk of neurologic death. With respect to patient survival, the control of systemic cancer might outweigh the frequent recurrence of brain tumors. Therefore, SRS alone could be a treatment option, provided that frequent monitoring of brain tumor status is conducted.

Author Contributions: Dr Aoyama had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Patchell RA. The management of brain metastases. *Cancer Treat Rev.* 2003;29:533-540.
2. Bradley KA, Mehta MP. Management of brain metastases. *Semin Oncol.* 2004;31:693-701.
3. Kondziolka D, Patel A, Lunsford LD, Kassam A, Flickinger JC. Stereotactic radiosurgery plus whole brain radiotherapy versus radiotherapy alone for patients with multiple brain metastases. *Int J Radiat Oncol Biol Phys.* 1999;45:427-434.
4. Andrews DW, Scott CB, Sperduto PW, et al. Whole brain radiation therapy with or without stereotactic radiosurgery boost for patients with one to three brain metastases: phase III results of the RTOG 9508 randomised trial. *Lancet.* 2004;363:1665-1672.
5. DeAngelis LM, Delattre JY, Posner JB. Radiation-induced dementia in patients cured of brain metastases. *Neurology.* 1989;39:789-796.
6. Patchell RA, Tibbs PA, Regine WF, et al. Postoperative radiotherapy in the treatment of single metastases to the brain. *JAMA.* 1998;280:1485-1489.
7. Sneed PK, Suh JH, Goetsch SJ, et al. A multi-institutional review of radiosurgery alone vs radiosurgery with whole brain radiotherapy as the initial management of brain metastases. *Int J Radiat Oncol Biol Phys.* 2002;53:519-526.
8. Flickinger JC, Kondziolka D, Lunsford LD, et al. A multi-institutional experience with stereotactic radiosurgery for solitary brain metastasis. *Int J Radiat Oncol Biol Phys.* 1994;28:797-802.
9. Regine WF, Huhn JL, Patchell RA, et al. Risk of symptomatic brain tumor recurrence and neurologic deficit after radiosurgery alone in patients with newly diagnosed brain metastases: results and implications. *Int J Radiat Oncol Biol Phys.* 2002;52:333-338.
10. Pirzkall A, Debus J, Lohr F, et al. Radiosurgery alone or in combination with whole-brain radiotherapy for brain metastases. *J Clin Oncol.* 1998;16:3563-3569.
11. Aoyama H, Shirato H, Onimaru R, et al. Hypofractionated stereotactic radiotherapy alone without whole brain irradiation for patients with solitary and oligo brain metastasis using non-invasive fixation of the skull. *Int J Radiat Oncol Biol Phys.* 2003;56:793-800.
12. Chidel MA, Suh JH, Reddy CA, Chao ST, Lundbeck MF, Barnett GH. Application of recursive partitioning analysis and evaluation of the use of whole brain radiation among patients treated with stereotactic radiosurgery for newly diagnosed brain metastases. *Int J Radiat Oncol Biol Phys.* 2000;47:993-999.
13. Shirato H, Takamura A, Tomita M, et al. Stereotactic irradiation without whole-brain irradiation for single brain metastasis. *Int J Radiat Oncol Biol Phys.* 1997;37:385-391.
14. Shaw E, Scott C, Souhami L, et al. Radiosurgery for the treatment of previously irradiated recurrent primary brain tumors and brain metastases: initial report of Radiation Therapy Oncology Group protocol (90-05). *Int J Radiat Oncol Biol Phys.* 1996;34:647-654.
15. Joseph J, Adler JR, Cox RS, Hancock SL. Linear accelerator-based stereotaxic radiosurgery for brain metastases: the influence of number of lesions on survival. *J Clin Oncol.* 1996;14:1085-1092.
16. Trotti A, Byhardt R, Stetz J, et al. Common Toxicity Criteria version 2.0: an improved reference for grading the acute effects of cancer treatment: impact on radiotherapy. *Int J Radiat Oncol Biol Phys.* 2000;47:13-47.
17. Gaspar L, Scott C, Rotman M, et al. Recursive partition analysis (RPA) of prognostic factors in three Radiation Therapy Oncology Group (RTOG) brain metastases trials. *Int J Radiat Oncol Biol Phys.* 1997;37:745-751.
18. Radiation Therapy Oncology Group. RTOG/EORTC late radiation morbidity scoring schema. http://www.rtog.org/members/toxicity/late.html. Accessed June 15, 1999.
19. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc.* 1958;53:457-481.
20. Cox D. Regression models and life tables. *J R Stat Soc [Ser A].* 1972;34:187-220.
21. Leksell L. The stereotaxic method and radiosurgery of the brain. *Acta Chir Scand.* 1951;102:316-319.
22. Patchell RA, Tibbs PA, Walsh JW, et al. A randomized trial of surgery in the treatment of single metastases to the brain. *N Engl J Med.* 1990;322:494-500.
23. Mintz AH, Kestle J, Rathbone MP, et al. A randomized trial to assess the efficacy of surgery in addition to radiotherapy in patients with single cerebral metastasis. *Cancer.* 1996;78:1470-1476.
24. Vecht CJ, Haaxma-Reiche H, Noordijk EM, et al. Treatment of single brain metastasis: radiotherapy alone or combined with neurosurgery. *Ann Neurol.* 1993;33:583-590.

> The only true hope for civilization—the conviction of the individual that his inner life can affect outward events and that, whether or not he does so, he is responsible for them.
> —Stephen Spender (1909-1995)

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