Table 2
Summary of 88 Mutations Detected in the Tyrosine Kinase Domain of the *EGFR* Gene

Type of Mutation	Exon	Nucleotide Change	Amino Acid Change	No. of Cases
In-frame deletion	19	del2235-2249	del746-750	14
		del2239-2247, G2248>C	del747-749, A750P	9
		del2236-2250	del746-750	5
		del2240-2254	del747-751	4
		del2237-2254, C2255>T	E746V, del747-752	2
		del2239-2256	del747-752	1
		del2240-2257	L747S, del748-753	1
		del2235-2249 and del2239-2247, G2248>C	del746-750 and del747-749, A750P	2
		del2239-2247, G2248>C and del2236-2250	del747-749, A750P and del746-750	1
		del2239-2247, G2248>C and del2240-2254	del747-749, A750P and del747-751	1
Single-nucleotide substitution	21	T2573>G	L858R	24
		G2575>A	A859T	1
		T2582>G	L861R	1
In-frame deletion and single-nucleotide substitution	19 and 21	del2239-2247, G2248>C and T2573>G	del747-749, A750P and L858R	5
		del2235-2249 and T2573>G	del746-750 and L858R	2
		del2235-2249 and T2582>G	del746-750 and L861R	1
		del2236-2250 and T2573>G	del746-750 and L858R	1

EGFR, epidermal growth factor receptor.

Relationships Between *EGFR* Mutations and Clinicopathologic Features

We analyzed the relationships between the *EGFR* gene status and clinicopathologic factors **Table 3**. *EGFR* mutations were significantly more frequent in women than in men, in never-smokers than in ever-smokers, and in patients with adenocarcinomas than in patients with nonadenocarcinomas (1/23 [4%]) among the 141 NSCLCs. No statistically significant associations were found between the *EGFR* mutation status and age or tumor stage (Table 3). Logistic regression models suggested that adenocarcinoma histologic features ($P = .0047$) and female sex ($P = .0084$) independently affected the incidence of *EGFR* mutations, whereas smoking status ($P = .2385$) did not.

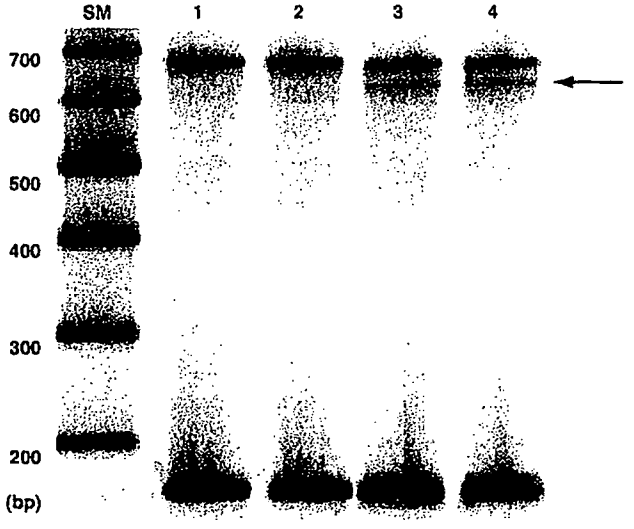

Image 1 Detection of point mutations at codon 858 of the epidermal growth factor receptor gene exon 21. The electrophoretogram after loop-hybrid mobility shift assay to detect the point mutation L858R in the tumor DNA in lung adenocarcinomas. Lanes 1 and 2 show normal sequences; lanes 3 and 4, heterozygous mutations; arrow, mutational bands. The homoduplex bands are at 161 bp. bp, base pair; SM, size marker.

Because adenocarcinoma was the dominant histologic diagnosis for *EGFR* mutations, further analyses were limited to adenocarcinomas. *EGFR* mutations were significantly associated with older age at diagnosis, female sex, never smoking, and histologic features with a nonmucinous BAC component, including pure nonmucinous BACs and invasive adenocarcinoma with a nonmucinous BAC component, compared with younger age at diagnosis, male sex, ever smoking, and a histologic diagnosis other than a nonmucinous BAC subtype (10/36 [28%]), respectively. There was no significant relationship between *EGFR* mutations and tumor stage (Table 3). Logistic regression models showed that a nonmucinous BAC component ($P = .0006$) and female sex ($P = .0083$) were independent variables, whereas smoking status ($P = .9105$) and age at diagnosis ($P = .3083$) were not **Image 2A**.

Expression of EGFR and Phospho-EGFR

Table 4 summarizes the relationships between *EGFR* mutations and EGFR expression among the 118 adenocarcinomas studied. There was no statistically significant difference between *EGFR* mutations and the intensity of EGFR expression ($P = .1799$). When scores of 0 or 1+ were considered negative for EGFR overexpression and scores of 2+ or 3+ were considered positive, no statistically significant association between *EGFR* mutations and EGFR overexpression was found (Table 3; $P = .0631$). Phospho-EGFR expression was observed in 21

Table 3
Patient Characteristics and Frequency of *EGFR* Mutations

Variable	No. of Cases	No. (%) of *EGFR* Mutations	*P*
NSCLC (n = 141)			
Age (y)			NS
≤65	73	36 (49)	
>65	68	39 (57)	
Sex			<.0001
Male	69	21 (30)	
Female	72	54 (75)	
Smoking history			<.0001
Never smoked	64	50 (78)	
Ever smoked	77	25 (32)	
Histologic diagnosis			<.0001*
Adenocarcinoma	118	74 (62.7)	
Adenosquamous carcinoma	2	0 (0)	
Squamous cell carcinoma	21	1 (5)	
Stage			NS
IA and IB	105	59 (56.2)	
II through IV	36	16 (44)	
Adenocarcinoma (n = 118)			
Age (y)			.0321
≤64	60	32 (53)	
>64	58	42 (72)	
Sex			<.0001
Male	51	20 (39)	
Female	67	54 (81)	
Smoking history			.0002
Never smoked	64	50 (78)	
Ever smoked	54	24 (44)	
Histologic diagnosis			<.0001†
Adenocarcinoma with a nonmucinous BAC component‡	82	64 (78)	
Adenocarcinoma without BAC components	27	8 (30)	
Adenocarcinoma with a mucinous BAC component	9	2 (22)	
Stage			NS
IA and IB	93	59 (63)	
II through IV	25	15 (60)	
Overexpression of EGFR			NS
Positive	54	29 (54)	
Negative	64	45 (70)	
Expression of phospho-EGFR			NS
Positive	21	17 (81)	
Negative	97	57 (59)	

BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; NS, not significant; NSCLC, non–small cell lung cancer; phospho-EGFR, phosphorylated EGFR.
* Histologic differences were examined between adenocarcinoma and other types of NSCLCs in 141 NSCLCs.
† Histologic differences were examined between adenocarcinoma with a nonmucinous BAC component and other subtypes of adenocarcinomas in 118 adenocarcinomas.
‡ Adenocarcinoma with a nonmucinous BAC component included pure nonmucinous BAC (15/17 [88%]) and invasive adenocarcinoma with a nonmucinous BAC component (49/65 [75%]).

(17.8%) of 118 adenocarcinomas studied. No statistically significant association between *EGFR* mutations and expression of phospho-EGFR was observed (Table 3; $P = .0806$).

Relationships Between K-*ras* Mutations and Clinicopathologic Features

K-*ras* mutations at codon 12 were present in 10 (8.5%) of 118 adenocarcinomas but not in the other histologic types (0 of 23) examined. The mutations consisted of six 34G>T (G12C), two 35G>A (G12D), and single examples of 35G>C (G12A) and 35G>T (G12V). K-*ras* mutations were significantly more frequent in ever-smokers than in never-smokers, in tumors with histologic features with a mucinous BAC component than in those without a mucinous BAC component (4/109 [3.7%]), and in tumors with the wild-type *EGFR* gene than tumors with mutated *EGFR* among the 118 adenocarcinomas. Neither age at diagnosis nor sex significantly modified the frequency of K-*ras* mutations **Table 5**. Logistic regression analyses demonstrated that a mucinous BAC component ($P = .0002$) was the only significant determinant for K-*ras* mutations, whereas smoking history ($P = .0699$) and *EGFR* mutation status ($P = .1648$) were not **Image 2B**.

EGFR and K-*ras* Mutations in Nonmucinous and Mucinous BAC Subtypes

To date, only 3 studies, including the present study and 2 previous reports,[5,18] subdivided BACs into nonmucinous and mucinous subtypes and examined them for *EGFR* and K-*ras*

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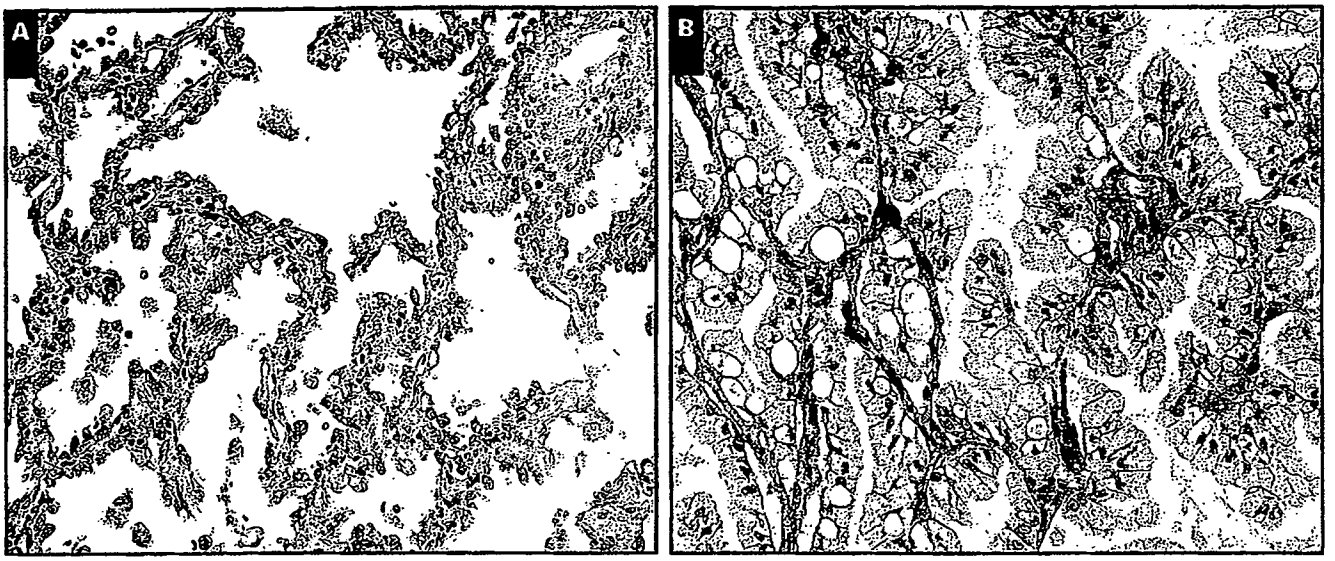

Image 2 Typical histopathologic features of epidermal growth factor receptor (*EGFR*)- and K-*ras*–mutated lung adenocarcinomas. **A**, Nonmucinous bronchioloalveolar carcinoma (BAC) component of an *EGFR*-mutated adenocarcinoma. This tumor has a deletion mutation (del746-750) in exon 19. **B**, Mucinous BAC component of a K-*ras*–mutated adenocarcinoma. This tumor harbors a point mutation (G12V).

mutations. **Table 6** shows a summary of *EGFR* and K-*ras* gene mutational frequencies in the 2 BAC subtypes studied.

Discussion

We have shown that adenocarcinomas with a nonmucinous or mucinous BAC component are significantly associated with *EGFR* or K-*ras* gene mutations, respectively. Some previous reports did not distinguish nonmucinous and mucinous BAC subtypes and did not confirm a significant association between histologic features with a BAC component and

Table 4
Relationship Between *EGFR* Mutations and EGFR Expression in 118 Adenocarcinomas

	EGFR Expression			
***EGFR* Mutation**	**0**	**1+**	**2+**	**3+**
Positive (n = 74)	25	20	11	18
Negative (n = 44)	12	7	12	13

Table 5
Characteristics and Frequency of K-*ras* Mutations in 118 Patients With Adenocarcinoma

Variable	No. of Cases	No. (%) of K-*ras* Mutations	*P*
Age (y)			NS
≤64	60	3 (5)	
>64	58	7 (12)	
Sex			NS
Male	51	7 (14)	
Female	67	3 (4)	
Smoking history			.0053
Never smoked	64	1 (2)	
Ever smoked	54	9 (17)	
Histologic diagnosis			<.0001*
Adenocarcinoma with a nonmucinous BAC component†	82	2 (2)	
Adenocarcinoma without BAC components	27	2 (7)	
Adenocarcinoma with a mucinous BAC component	9	6 (67)	
EGFR mutation			.0055
Mutated	74	2 (3)	
Wild-type	44	8 (18)	

BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; NS, not significant.
* The histologic difference was analyzed between adenocarcinoma with a mucinous BAC component and other subtypes of adenocarcinomas.
† Adenocarcinomas with a nonmucinous BAC component included pure nonmucinous BAC (1/17 [6%]) and invasive adenocarcinoma with a nonmucinous BAC component (1/65 [2%]).

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EGFR mutations.[6,8,9] Our present results clearly demonstrate that BAC histologic features should be further distinctively considered as nonmucinous and mucinous subtypes.

Our present findings, demonstrating mutually exclusive *EGFR* and K-*ras* gene mutations in nonmucinous and mucinous BACs, respectively, totally agree with a previous report by Marchetti et al[5] in Italy on this point, but a significant difference exists. Comparing these 2 studies reveals that the frequency of *EGFR* mutations in pure nonmucinous BACs, ie, peripheral lung adenocarcinomas in situ, was quite different (Table 6). We found 88% *EGFR* mutations in pure nonmucinous BACs, and Marchetti et al[5] reported a frequency of 32%. In addition, a more recent report from Hong Kong demonstrated that 15 (79%) of 19 nonmucinous BAC-type tumors had *EGFR* mutations, although these tumors included pure BACs and focally invasive tumors (Table 6).[18]

Because previous reports clarified that the frequency of *EGFR* mutations in lung adenocarcinomas, including all histologic subtypes in East Asia, was much higher than that in other areas, such as the United States, Italy, and Australia,[1-10] one could speculate that the difference of *EGFR* mutational prevalence, even in a specific histologic subtype, ie, pure nonmucinous BAC, comes from the genetic differences of racial background between East Asians and Italians. Of course, there might be differences of interpretation of diagnostic criteria for nonmucinous BAC by pathologists from different countries, and further studies are needed to clarify the incidence of *EGFR* mutations in pure nonmucinous BACs, especially in areas other than East Asia.

Pure nonmucinous BACs are thought to sequentially progress to invasive adenocarcinomas with a nonmucinous BAC component from the point of clinicopathologic and molecular evidence.[21-23] In the present study, the frequency (49/65 [75%]) of *EGFR* mutations in invasive adenocarcinomas with a nonmucinous BAC component was almost the same as that (15/17 [88%]) in pure nonmucinous BACs. Our results were obtained from simultaneous analyses in 1 institute with the same method and, therefore, strongly support the view of sequential progression from nonmucinous BACs to invasive adenocarcinomas with a nonmucinous BAC component from the *EGFR* gene alteration aspect.

Our results of *EGFR* gene mutation analyses generally confirmed the results obtained in previous studies in relation to the incidence in NSCLCs, by sex, and by smoking status.[1-10] We also demonstrated that 9 (12%) of 75 NSCLCs with *EGFR* mutations had mutations in exons 19 and 21 (Table 2), of which 3 were pure nonmucinous BACs and the others were invasive adenocarcinomas with a nonmucinous BAC component. In addition, 4 (5%) of 75 tumors (3 adenocarcinomas with a nonmucinous BAC component and 1 pure mucinous BAC) had 2 distinct deletion mutations in exon 19. Identical double mutations in exons 19 and 21 of the *EGFR* gene have also been reported in 1 of 28 NSCLC tissues with *EGFR* mutations[9] and in 3 of 19 *EGFR*-mutated NSCLC cell lines.[24] Because all double *EGFR* mutations in the present study and others[9,24] occurred de novo without prior treatments, the *EGFR* gene might be prone to be targeted in a subset of NSCLCs.

Table 6
Summary of *EGFR* and K-*ras* Mutations in BAC Subtypes*

BAC Subtype	No. of Cases	*EGFR* Mutation	K-*ras* Mutation
Marchetti et al[5]			
Nonmucinous	69	22 (32)	10 (14)
Mucinous	17	0 (0)	13 (76)
Tam et al[18]†			
Nonmucinous	19	15 (79)	NA
Mucinous	5	0 (0)	NA
Present study			
Nonmucinous	17	15 (88)	1 (6)
Mucinous‡	9	2 (22)	6 (67)

BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; NA, not available.
* Data are given as number (percentage).
† Tam et al[18] subdivided 215 adenocarcinomas into non–BAC-type (n = 191) and BAC-type (n = 24) tumors. They mentioned that 17 of 24 BAC-type adenocarcinomas did not show invasive growth.
‡ The present study includes 2 pure mucinous BACs and 7 focally invasive adenocarcinomas with a predominant mucinous BAC component.

K-*ras* mutations were detected only in adenocarcinomas and were significantly associated with ever smoking, tumors with the wild-type *EGFR* gene, and histologic features with a mucinous BAC component. These results are consistent with those in previous reports.[6,14,18,19] The *EGFR* and K-*ras* gene mutations were generally mutually exclusive of each other in lung adenocarcinomas in the present and reported studies.[4-6,18,25-27] To the best of our knowledge, only 4 lung adenocarcinomas with hot spot mutations in both the *EGFR* and K-*ras* genes have been reported, but histologic characteristics of these tumors were not mentioned.[28,29] Interestingly, Han et al[29] reported that neither of 2 patients with double mutations in *EGFR* and K-*ras* genes responded to gefitinib despite having a gefitinib-sensitive *EGFR* mutation (G719A or deletion in exon 19). In the present study, 2 adenocarcinomas with double mutations had mucinous but not nonmucinous BAC components. Considering these facts, from response to EGFR TKI and morphologic phenotypes, adenocarcinomas with double mutations in *EGFR* and K-*ras* genes might show the same characteristics as K-*ras*–mutated adenocarcinoma but not *EGFR*-mutated adenocarcinoma.

We studied NSCLC tissue samples for mutations of the *EGFR* and K-*ras* genes using LH-MSA technique,[20] which was previously developed by one of us (S.M.). The LH-MSA technique is considered a sensitive and specific, rapid, simple PCR-based method, although in principle, it can only apply to detecting known hot spot mutations. We consider that the high sensitivity and specificity of LH-MSA could be a powerful

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tool for further elucidating the clinicopathologic characteristics of *EGFR*- and K-*ras*–mutated lung adenocarcinomas.

Amplification of the *EGFR* gene has been reported to be an effective molecular predictor for EGFR TKIs efficacy,[30] although curiously, the presence or intensity of EGFR protein expression in pathologic specimens determined by immunohistochemical analysis has been considered not a predictor.[31] We surmised that the phosphorylation (activation) status rather than the expression intensity of EGFR is a good predictor for sensitivity to EGFR TKIs. Although 17 (81%) of 21 adenocarcinomas with phospho-EGFR expression had *EGFR* gene mutations, no statistically significant association between *EGFR* mutations and expression of phospho-EGFR determined by immunohistochemical analysis was observed. Furthermore, no statistically significant association between *EGFR* mutations and overexpression of the protein was confirmed.

We have demonstrated that lung adenocarcinomas with a nonmucinous or mucinous BAC component are significantly correlated with *EGFR* or K-*ras* mutations, respectively. Together with other published studies,[1-5,15,18] our finding that most pure nonmucinous BACs already have *EGFR* mutations leads us to believe that the *EGFR* gene is an addicted oncogene in the pathogenesis of nonmucinous BAC-type lung adenocarcinomas but not mucinous BAC-type tumors. Further clarification of the molecular mechanisms responsible for the progression from nonmucinous BACs to invasive adenocarcinomas should provide new therapeutic targets in addition to EGFR.

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Down-regulation of survivin by ultraviolet C radiation is dependent on p53 and results in G_2–M arrest in A549 cells

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Abstract

Deregulation of survivin expression is implicated in tumorigenesis. To examine the regulation of survivin expression in response to DNA damage, we exposed A549 human lung cancer cells to ultraviolet C (UVC) radiation, which induces DNA single-strand breakage. UVC irradiation induced G_2–M arrest that was accompanied by accumulation of p53 and subsequent down-regulation of survivin. Depletion of p53 by RNA interference prevented the UVC-induced down-regulation of survivin. Furthermore, depletion of survivin resulted in G_2–M arrest, suggesting that down-regulation of survivin by p53 contributes to the p53-dependent G_2–M checkpoint triggered by DNA damage.

Keywords: Survivin; p53; RNA interference; G_2–M arrest; Ultraviolet C

1. Introduction

Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, is thought to play an important role in regulation of both apoptosis and cell division [1,2]. It is present in only small amounts in terminally differentiated normal cells but is overexpressed in almost all types of human malignancy [3–8]. Such overexpression of survivin is associated with poor prognosis in affected individuals, an increased rate of tumor recurrence, and resistance to certain anticancer agents and radiation [9,10].

The expression of survivin is regulated in a cell cycle-dependent manner. The promoter of the survivin gene possesses features typical of genes that are expressed at G_2–M phase of the cell cycle. Indeed, survivin is most abundant in cells at G_2–M and associates with the mitotic spindle of dividing cells [2]. Survivin interacts with Aurora B and inner centromere protein (INCENP), and the complex of Aurora B–INCENP–survivin monitors the integrity of the mitotic spindle [11]. It has been suggested that survivin controls the elimination by apoptosis of cells with an improperly formed mitotic spindle [3,12]. Overexpression of survivin in cancer may overcome cell cycle checkpoints and thereby facilitate aberrant progression of

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transformed cells through mitosis. Although deregulation of survivin expression is an important event in tumorigenesis, the molecular mechanisms of survivin regulation are not fully understood.

The tumor suppressor p53 blocks progression of cells through the cell cycle or induces apoptosis [13,14]. Following its induction in response to DNA damage, p53 up-regulates the expression of various genes that contribute to cell cycle arrest, DNA repair, or apoptosis. It also negatively regulates the expression of a separate set of genes [15–18]. The functional loss of wild-type p53 has been shown to be associated with up-regulation of survivin expression in human cancers [19–21]. We have previously shown that the amounts of survivin mRNA and protein in cell lines positive for wild-type p53 decreased markedly after induction of p53 by adriamycin, which causes DNA double-strand breakage [22]. However, no such down-regulation of survivin was apparent in cell lines with mutated or null p53 alleles. These observations have suggested that p53 negatively regulates the expression of survivin in response to DNA damage.

In the present study, we show that exposure of p53-positive A549 human lung cancer cells to ultraviolet C (UVC) radiation, which induces DNA single-strand breakage, resulted in down-regulation of survivin expression after the induction of p53. Depletion of p53 by RNA interference (RNAi) prevented this down-regulation of survivin in cells exposed to UVC. Furthermore, RNAi-mediated depletion of survivin resulted in growth arrest in G_2–M phase of the cell cycle. These findings suggest that negative regulation of survivin by p53 contributes to the p53-dependent G_2–M checkpoint.

2. Materials and methods

2.1. Cell culture and irradiation

A549 cells were provided by Tohoku University (Miyagi, Japan). The cells were cultured under a humidified atmosphere of 5% CO_2 at 37 °C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Each batch of cells was discarded after 20 generations, and new batches were obtained from frozen stocks. Cells were exposed to UVC (30 J/m^2) with a Hoefer UVC 500 Ultraviolet Crosslinker (Amersham Pharmacia Biotech, Piscataway, NJ).

2.2. Immunoblot analysis

Cells were harvested by exposure to trypsin–EDTA, washed with phosphate-buffered saline (PBS), and lysed in a solution containing 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 lysate protein were fractionated by SDS–polyacrylamide gel electrophoresis at 100 V for 80 min at room temperature. The separated proteins were transferred to a nitrocellulose membrane, which was then probed for 2 h at room temperature with various primary antibodies, including affinity-purified rabbit polyclonal anti-survivin (R&D Systems, Minneapolis, MN), mouse monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and affinity-purified rabbit polyclonal anti-β-actin (Sigma–Aldrich, St. Louis, MO). Immune complexes were detected with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Amersham Biosciences, Little Chalfont, UK) or sheep antibodies to mouse immunoglobulin G (Santa Cruz Biotechnology) and with a chemiluminescence detection system (Perkin-Elmer, Boston, MA).

2.3. Flow cytometry

Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg/ml) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A (0.05 mg/ml). The stained cells ($\sim 1 \times 10^5$) were than analyzed for DNA content with a flow cytometer (FACScaliber; Becton–Dickinson).

2.4. RNAi

Small interfering RNA (siRNA) duplexes specific for survivin or p53 mRNAs were synthesized by Dharmacon Research (Lafayette, CO) with the use of 2′-ACE protection chemistry. The survivin siRNA corresponded to nucleotides 206–224 of the coding region (GenBank Accession No. NM001168), whereas the p53 siRNA corresponded to nucleotides 775–793 of the coding region. BLAST searches of the human genome database were performed to ensure that the siRNA sequences would not target other gene transcripts. Cells in the exponential phase of growth were plated at a density of 3×10^4 cells per well in 12-well culture plates, cultured for 24 h, and then transfected with siRNA (300 nM) with the use of Oligofectamine in OPTI-MEM (Invitrogen, Carlsbad, CA). Control cells were treated with a scrambled siRNA duplex (Dharmacon).

2.5. Statistical analysis

Data are presented as means ± SD and were analyzed by Student's two-tailed t test (Stat View; SAS Institute, Cary, NC). A p value of <0.05 was considered statistically significant.

3. Results

3.1. UVC radiation inhibits A549 cell proliferation and induces G_2–M arrest

To evaluate the effect of UVC on A549 cell proliferation, we counted the number of viable cells at various times after irradiation. UVC treatment resulted in a 70% reduction in the number of viable cells compared with that for untreated cells at 48 h and a 60% reduction at 72 h (Fig. 1A). Flow cytometric analysis of cell cycle distribution revealed that this inhibition of cell proliferation by UVC was accompanied by an approximately twofold increase in the proportion of cells in G_2–M at 24 h (25.8% versus 13.4%), at 48 h (17.1% versus 7.9%) and at 72 h (12.3% versus 6.1%) compared with untreated cells (Fig. 1B), whereas irradiation had no marked effect on the sub-G_1 (apoptotic) population. These data indicated that treatment of A549 cells with UVC results in growth arrest at the G_2–M phase of the cell cycle.

3.2. UVC exposure induces p53 up-regulation followed by survivin down-regulation

Given that p53 mediates cell cycle arrest at the G_2–M transition in response to DNA damage and that we recently showed that down-regulation of survivin expression follows the accumulation of p53 in cells subjected to DNA double-strand breakage [22], we next examined whether survivin and p53 are functionally linked in A549 cells treated with UVC, which induces DNA single-strand breakage. Immunoblot analysis revealed that the abundance of p53 was increased 6 h after UVC exposure, reached a peak at 24 h, and then gradually returned to basal levels by 72 h (Fig. 2). In contrast, the amount of survivin began to decline at 48 h and its down-regulation was more pronounced at 72 h.

To determine whether p53 negatively regulates survivin expression, we examined the effect of UVC radiation on the abundance of survivin in cells depleted of p53 by RNAi. In cells transfected with a control (scrambled) siRNA or in nontransfected cells, the abundance of p53 was increased at 18 h after UVC exposure and the amount of

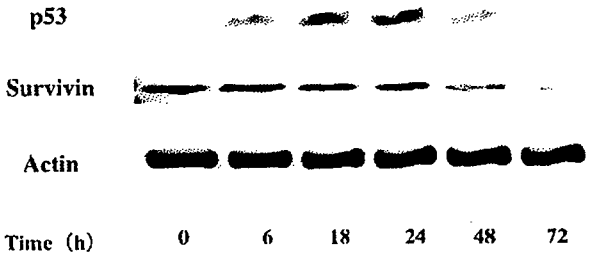

Fig. 2. Effects of UVC on the abundance of p53 and survivin in A549 cells. Total cellular protein extracted at the indicated times after exposure of cells to UVC (30 J/m^2) was subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin (loading control). Data are representative of three independent experiments.

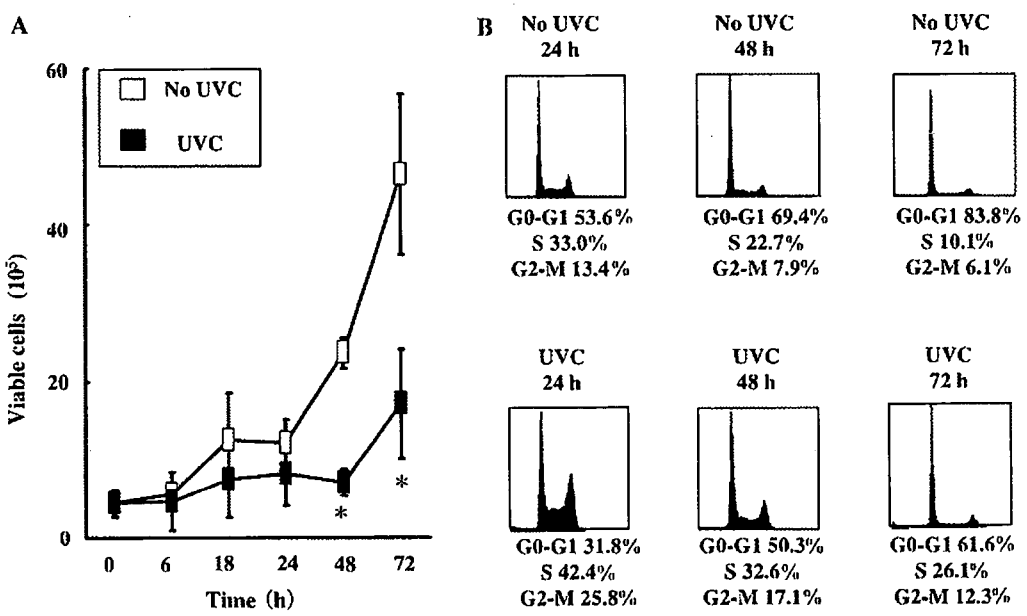

Fig. 1. Effects of UVC on the proliferation and cell cycle distribution of A549 cells. (A) Cell proliferation was evaluated by counting the number of viable cells by trypan blue staining at the indicated times after UVC irradiation (30 J/m^2). Data are means ± SD of values from three independent experiments. *$p < 0.05$ versus the corresponding value for cells not exposed to UVC. (B) Cell cycle distribution was analyzed by propidium iodide staining and flow cytometry at 24, 48 h and 72 h after UVC exposure. The percentages of cells at various stages of the cell cycle are indicated, and the data are representative of three independent experiments.

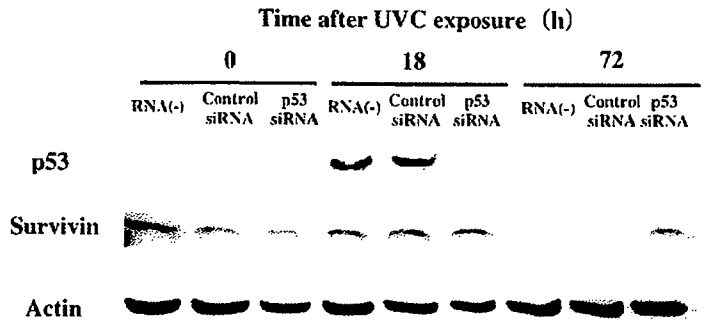

Fig. 3. Effect of UVC on the abundance of survivin in A549 cells depleted of p53 by RNAi. Cells were transfected (or not) with an siRNA specific for p53 mRNA or with a control (scrambled) siRNA, exposed to UVC (30 J/m^2), and subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin at the indicated times after irradiation. Data are representative of three independent experiments.

survivin was decreased at 72 h (Fig. 3). In contrast, in cells transfected with an siRNA specific for p53 mRNA, UVC failed to increase p53 expression and had no effect on the level of survivin. These results thus indicated that induction of p53 by exposure of cells to UVC is necessary for down-regulation of survivin.

3.3. Ablation of survivin inhibits cell proliferation and induces G_2–M arrest

We next examined the effects of UVC irradiation in cells depleted of survivin by RNAi. The abundance of survivin was greatly reduced in cells transfected with an siRNA specific for survivin mRNA compared with that in nontransfected cells or cells transfected with a control (scrambled) siRNA (Fig. 4A). Cell proliferation (as evaluated from viable cell number) was also inhibited by 60% or 70% in cells subjected to transfection with the survivin siRNA for 48 or 72 h, respectively, compared with that apparent in nontransfected cells (Fig. 4B). The viable cell count was not affected by transfection with the control siRNA. Flow cytometry revealed that transfection of A549 cells with the survivin siRNA resulted in a marked increase in the proportion of cells in G_2–M at 48 and 72 h compared with that apparent for nontransfected cells or cells transfected with the control siRNA (Fig. 4C and D). There was no difference in the proportion of sub-G_1 cells among the three treatment groups.

4. Discussion

Several genes whose products play a role in control of the G_2–M transition of the cell cycle, including stathmin, Map4, cyclin B1, Cdc2, and Cdc25c, have been shown to be negatively regulated by p53 [15–18]. Repression of the expression of these genes in response to DNA damage requires wild-type p53 and contributes to a DNA damage-induced G_2–M checkpoint [23,24]. Survivin, a member of the IAP family of proteins, is maximally expressed at G_2–M and physically associates with microtubules of the mitotic spindle [2]. Previous studies have suggested that the expression of survivin is also subject to negative regulation by p53 [25–27], but the mechanism of such regulation has been unclear. We have now shown that exposure of the human lung cancer cell line A549 to UVC, which induces DNA single-strand breakage, resulted in the induction of endogenous p53 and a subsequent decrease in survivin expression. These observations are consistent with those of our previous study showing that survivin expression is repressed subsequent to p53 accumulation in cells treated with adriamycin [22], which induces DNA double-strand breakage. To investigate the possible role of p53 in the down-regulation of survivin induced by DNA damage, we depleted A549 cells of p53 by RNAi. Prevention of endogenous p53 accumulation in cells irradiated with UVC was found to block the repression of survivin expression, providing direct evidence that p53 is required for this effect of UVC. These data thus constitute further support for the notion that the survivin gene is a target of negative regulation by p53 in response to DNA damage.

The time course of survivin protein repression following UVC (DNA single-strand breakage)-induced p53 accumulation was almost identical to that observed in the cells having DNA double-strand breakage [22]. These results suggest that p53-dependent survivin suppression in response to these two types of DNA damage may share the common mechanisms at transcriptional level. Hoffmann et al. proposed that direct binding of p53 to a consensus binding site in the survivin gene promoter mediates transcriptional repression of the

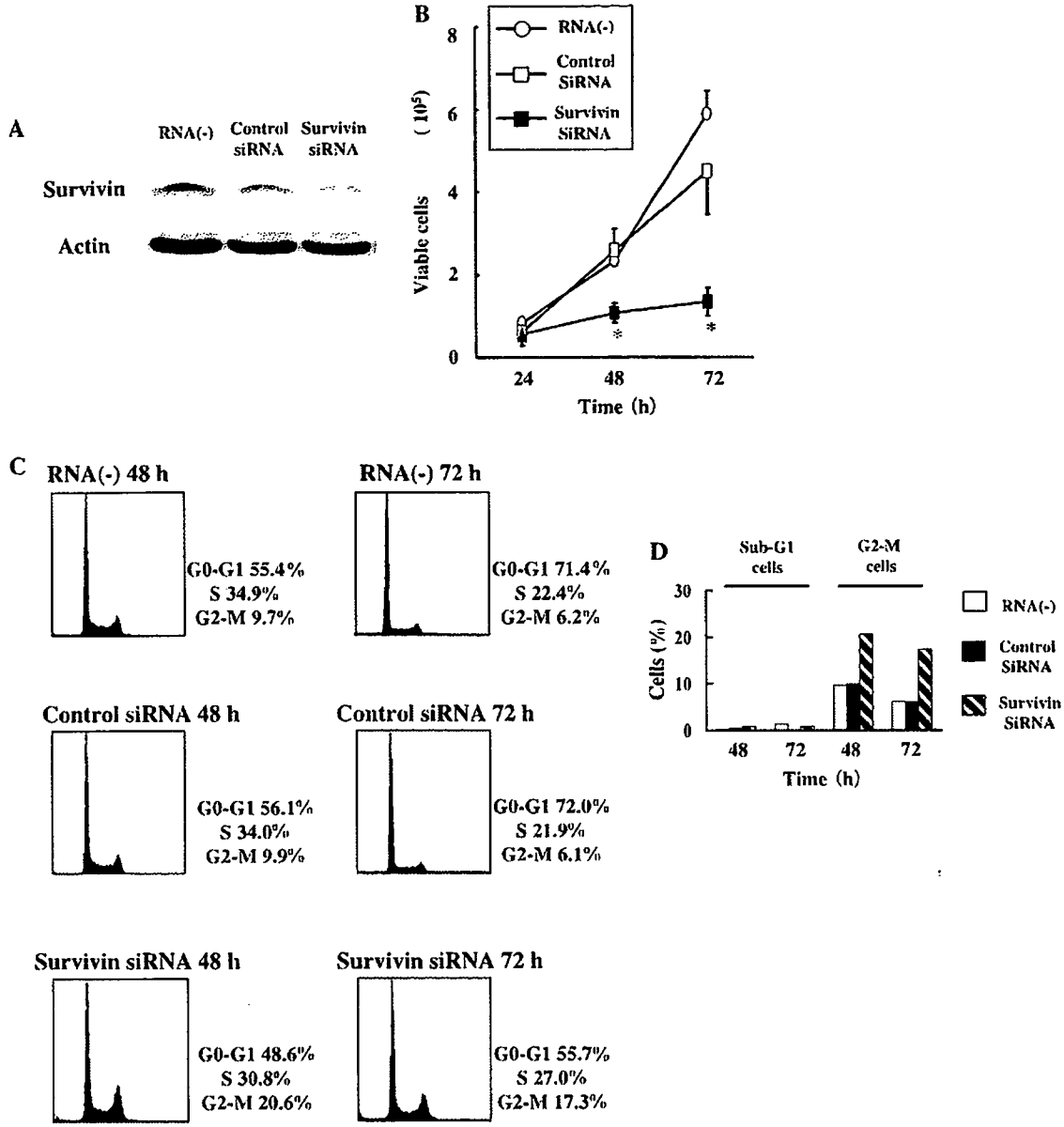

Fig. 4. Effects of survivin depletion by RNAi on the proliferation and cell cycle distribution of A549 cells. (A) Cells were transfected (or not) with a siRNA specific for survivin mRNA or with a control siRNA and were then subjected to immunoblot analysis with antibodies to survivin or to β-actin. Data are representative of three independent experiments. (B) Cells transfected for 24, 48, or 72 h as in (A) were evaluated for cell proliferation by counting the number of viable cells as revealed by staining with trypan blue. Data are means ± SD of values from three independent experiments. $*p < 0.05$ versus the corresponding value for nontransfected cells or cells transfected with the control siRNA. (C) The cell cycle distribution of cells transfected for 48 or 72 h as in (A) was determined by flow cytometry. The percentages of cells at various stages of the cell cycle are indicated. Data are representative of three independent experiments. (D) The percentages of sub-G_1 and G_2–M cells in the experiment shown in (C).

survivin gene by p53 [25]. In contrast, Mirza et al. suggested that chromatin deacetylation in the survivin promoter might contribute to p53-dependent repression of survivin gene expression in the absence of direct binding of p53 to the promoter DNA [26]. In the present study, repression of survivin expression was apparent 24 h after endogenous p53 accumulation, consistent with the results of our previous study [22]. This delay suggests that the mechanism of transcriptional inhibition of the survivin gene by p53 may be indirect. The repression of Cdc2 gene expression by p53 is mediated by a member of the E2F family of transcription factors subsequent to up-regulation of p21 and dephosphorylation of pRB family proteins [17]. However, UV-induced accumulation of p53 and subsequent

down-regulation of survivin have been observed in mouse embryonic fibroblasts derived from p21-null mice [29], suggesting that the ability of p53 to repress survivin gene expression is independent of its ability to up-regulate p21. The molecular mechanism by which p53 induces repression of survivin gene expression in response to DNA damage thus requires further investigation.

To examine the biological consequences of survivin gene repression in cells subjected to DNA damage, we depleted A549 cells of survivin by RNAi. Depletion of survivin resulted in growth arrest in G_2–M phase of the cell cycle, consistent with previous observations [28–31]. Survivin was originally proposed to perform an antiapoptotic function, but this issue remains controversial [29,32]. Indeed, several lines of evidence suggest that survivin plays an important role in regulation of mitotic events [11]. The chromosomal passenger complex (CPC), which consists of Aurora B, INCENP, and survivin, contributes to chromosome segregation and cytokinesis [33]. Depletion or inhibition of survivin or of the other proteins of the CPC thus results in mitotic arrest [30,34]. Furthermore, G_2–M arrest induced by survivin ablation was found to occur in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells, implicating survivin in the p53-dependent G_2–M checkpoint that is essential for maintenance of genomic integrity [29]. Together, these various observations suggest that p53-induced repression of survivin expression in response to DNA damage may lower the threshold for apoptosis in cells in which the p53-dependent G_2–M checkpoint has been activated. Survivin repression following DNA damage may play critical role in deciding if lethal damaged cells die before DNA repair is completed, or if they will have the opportunity to repair and survive. Further characterization of the regulation of survivin in response to DNA damage may provide the basis for potential new approaches to cancer treatment that couple standard cytotoxic DNA-damaging agents with survivin-targeted therapy.

Acknowledgement

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Hypofractionated Stereotactic Radiotherapy (HypoFXSRT) for Stage I Non-small Cell Lung Cancer: Updated Results of 257 Patients in a Japanese Multi-institutional Study

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Introduction: Hypofractionated stereotactic radiotherapy (HypoFXSRT) has recently been used for the treatment of small lung tumors. We retrospectively analyzed the treatment outcome of HypoFXSRT for stage I non-small cell lung cancer (NSCLC) treated in a Japanese multi-institutional study.

Methods: This is a retrospective study to review 257 patients with stage I NSCLC (median age, 74 years: 164 T1N0M0, 93 T2N0M0) were treated with HypoFXSRT alone at 14 institutions. Stereotactic three-dimensional treatment was performed using noncoplanar dynamic arcs or multiple static ports. A total dose of 18 to 75 Gy at the isocenter was administered in one to 22 fractions. The median calculated biological effective dose (BED) was 111 Gy (range, 57–180 Gy) based on $\alpha/\beta = 10$.

Results: During follow-up (median, 38 months), pulmonary complications of above grade 2 arose in 14 patients (5.4%). Local progression occurred in 36 patients (14.0%), and the local recurrence rate was 8.4% for a BED of 100 Gy or more compared with 42.9% for less than 100 Gy ($p < 0.001$). The 5-year overall survival rate of medically operable patients was 70.8% among those treated with a BED of 100 Gy or more compared with 30.2% among those treated with less than 100 Gy ($p < 0.05$).

Conclusions: Although this is a retrospective study, HypoFXSRT with a BED of less than 180 Gy was almost safe for stage I NSCLC, and the local control and overall survival rates in 5 years with a BED of 100 Gy or more were superior to the reported results for conventional radiotherapy. For all treatment methods and schedules, the local control and survival rates were better with a BED of 100 Gy or more compared with less than 100 Gy. HypoFXSRT is feasible for curative treatment of patients with stage I NSCLC.

Key Words: Stereotactic radiotherapy, Non-small cell lung cancer, Stage I, Hypofractionated.

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In Japan, due to the routine use of computed tomography (CT), detection of early-stage lung cancer is increasing. For patients with stage I (T1 or 2, N0, M0) non-small cell lung cancer (NSCLC), full lobar or greater surgical resection and regional lymphadenectomy is the standard treatment choice; the local control rates exceed 80% and the overall 5-year survival rates surpass 50%.[1] However, surgical resection is often not feasible or involves a high risk for lung cancer patients with tobacco-related pulmonary illnesses, severe cardiovascular disease, or other medical conditions. Moreover, a small proportion of the patients who are fit for surgery may refuse it for personal reasons.

Radiotherapy (RT) can offer a therapeutic alternative in these cases, but the outcome with conventional RT is unsatisfactory.[2] The reason for the poor survival with conventional RT is thought to be that the dose of conventional RT is too low to control the local tumor. To give a higher dose to the tumor without increasing the adverse effects, hypofractionated high-dose stereotactic RT (HypoFXSRT) has recently been used to treat small cell lung tumors, particularly in Japan.[3–6] Although the optimal treatment technique and

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schedule of HypoFXSRT for stage I NSCLC are unknown, the nationwide number of Japanese patients with stage I NSCLC who are treated with small-volume stereotactic RT (SRT) has increased rapidly.

Therefore, it is meaningful to investigate the results of SRT for stage I NSCLC from many institutions, even in a retrospective manner, despite the large differences in treatment protocols. Previously, we reported the result of a Japanese multi-institutional review of 300 patients with stage I NSCLC treated with SRT.[7] We concluded that SRT with a biological effective dose (BED) of less than 150 Gy is effective for the curative treatment of patients with stage I NSCLC and that the local control and survival rates are better with a BED of 100 Gy or more compared with less than 100 Gy.

The survival rates in selected medically operable patients with a BED of 100 Gy or more were promising and potentially comparable with those of surgery. These results for SRT were encouraging for stage I NSCLC patients; however, the 300 subjects in that report included 17 patients irradiated with comparatively small fractions (<4 Gy) and 26 patients irradiated in combination with conventional RT. This article presents the results for patients irradiated with HypoFXSRT alone in a multi-institutional study. In this study, we compared the reported results for surgery and conventional RT with those for HypoFXSRT.

PATIENTS AND METHODS

Eligibility Criteria

This was a retrospective study to review patients who were treated by HypoFXSRT for their stage I NSCLC in 14 different hospitals in Japan.

All the patients enrolled in this study satisfied the following eligibility criteria: identification of T1N0M0 or T2N0M0 primary lung cancer on chest and abdominal CT, bronchoscopy, bone scintigraphy, or brain magnetic resonance imaging; histological confirmation of NSCLC; performance status of 2 or less according to the World Health Organization (WHO) guidelines; and an inoperable tumor due to a poor medical condition or refusal to undergo surgery.

No restrictions were imposed concerning the locations of eligible tumors, irrespective of whether they were located adjacent to a major bronchus, blood vessel, chest wall, or the esophagus. Patients were informed of the concept, methodology, and rationale of this treatment, which was performed in accordance with the 1983 revision of the Declaration of Helsinki.

Patient Characteristics

The patient pretreatment characteristics are summarized in Table 1. From April 1995 to March 2004, a total of 257 patients with primary NSCLC was treated using high-dose HypoFXSRT in the following 14 institutions: Hokkaido University, Kyoto University, Cancer Institute Hospital, Tokyo Metropolitan Komagome Hospital, Kitasato University, Tohoku University, Hiroshima University, Tokyo Metropolitan Hiroo Hospital, Sapporo Medical University, Institute of Biomedical Research and Innovation, International Medical Center of Japan, Tenri Hospital, Kitami Red Cross Hospital, and University of Yamanashi. Of the 257 patients, 158 were considered medically inoperable mainly because of chronic pulmonary disease, advanced age, or other chronic illness. The remaining 99 patients were considered medically operable, but had refused surgery or had been advised to select HypoFXSRT by medical oncologists.

TABLE 1. Patient Pretreatment Characteristics

Total cases: 257
Age: 39–92 yr (median, 74)
Performance status: PS 0, 109; PS 1, 103; PS 2, 39; PS 3, 6
Pulmonary chronic disease: 168 positive, 89 negative
Histology: 111 squamous cell, 120 adenocarcinoma, 26 other
Stage: 164 IA, 93 IB
Tumor diameter: 7–58 mm (median, 28)
Medical operability: 158 inoperable, 99 operable

Treatment Methods

All the patients were irradiated using stereotactic techniques. For the purposes of this study, all the hypofractionated stereotactic techniques met five requirements: reproducibility of the isocenter of 5 mm or less, as confirmed for every fraction; slice thickness on CT of 3 mm or less for three-dimensional (3-D) treatment planning; irradiation with multiple noncoplanar static ports or dynamic arcs; dose per fraction size more than 4 Gy; and a total treatment period of fewer than 25 days. Details of the techniques and instruments used to achieve SRT in the 14 institutions were summarized in a previous report.[7] The clinical target volume (CTV) marginally exceeded the gross target volume (GTV) by 0 to 5 mm. The planning target volume (PTV) comprised the CTV, a 2- to 5-mm internal margin and a 0–5-mm safety margin. A high dose was concentrated on the tumor-bearing area, while sparing the surrounding normal lung tissues using SRT. The irradiation schedules also differed among the institutions. The number of fractions ranged between 1 and 14, with single doses of 4.4 to 35 Gy. A total dose of 30 to 84 Gy at the isocenter was administered with 6- or 4-MV x-rays within 20% heterogeneity in the PTV dose. No chemotherapy was administered before or during RT.

To compare the effects of various treatment protocols with different fraction sizes and total doses, the BED was used in a linear-quadratic model.[8] Here, the BED was defined as $nd(1 + d/\alpha/\beta)$, with gray units, where n is the fractionation number, d is the daily dose, and α/β is assumed to be 10 for tumors. The BED was not corrected with values for the tumor doubling time or treatment term. In this study, the BED was calculated at the isocenter. The median BED was 111.0 Gy (range, 57.6–180.0). The BED was 100 Gy or more in 215 patients and less than 100 Gy in 42 patients. The median BED for the less than 100 Gy and 100 Gy or more subgroups was 79.6 Gy (range, 57.6–98.6) and 117.0 Gy (range, 100.0–180.0), respectively.

Dose constraints were set for the spinal cord only. The BED limit for the spinal cord was 80 Gy (α/β was assumed to be 2 Gy for chronic spinal cord toxicity).

Evaluation

The objectives of this study were to retrospectively evaluate the toxicity, local control rate, and survival rate according to the BED. All patients underwent follow-up examinations by radiation oncologists. The first examination took place 4 weeks after treatment, and patients were subsequently seen every 1 to 3 months. Tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors by CT.[9] Chest CT (slice thickness, 2–5 mm) was usually obtained every 3 months for the first year and repeated every 4 to 6 months thereafter. A complete response (CR) indicated that the tumor had disappeared completely or was replaced by fibrotic tissue. A partial response (PR) was defined as a 30% or more reduction in the maximum cross-sectional diameter. It was difficult to distinguish between residual tumor tissue and radiation fibrosis. Any suspicious confusing residual density after RT was considered evidence of a PR, so the actual CR rate might have been higher than that given here. Local recurrence was considered to have taken place only when enlargement of the local tumor continued for more than 6 months on follow-up CT. Two radiation oncologists interpreted the CT findings. The absence of local recurrence was defined as locally controlled disease. Lung, esophagus, bone marrow, and skin were evaluated using version 2 of the National Cancer Institute–Common Toxicity Criteria (NCI-CTC).

Statistical Analysis

The local recurrence rates in the two groups were compared with the χ^2 test. The BED among patient groups at each pulmonary toxicity grade was compared using the Kruskal-Wallis test. The cumulative local control and survival curves were calculated and drawn applying the Kaplan-Meier algorithms with day of treatment as the starting point. Subgroups were compared using log-rank statistics. Values of $p < 0.05$ were considered statistically significant. Statistical calculations were conducted using version 5.0 StatView software (SAS Institute, Cary, NC).

RESULTS

All the patients completed the treatment with no particular complaints. The median duration of follow-up for all patients was 38 months (range, 2–128).

Local Tumor Response

Of the 257 patients evaluated using CT, CR was achieved in 66 (25.7%) and PR in 157 (61.1%). The overall response rate (CR + PR) was 86.8%. The overall response rates for tumors with a BED of 100 Gy or more ($n = 215$) or less than 100 Gy ($n = 42$) were 87.5% and 86.7% in 3 years (?), respectively. A typical case of a T1 tumor after HypoFXSRT is shown in Figure 1.

Toxicity

Symptomatic radiation-induced pulmonary complications (NCI-CTC criteria grade >1) were noted in 28 patients (10.9%). Pulmonary fibrosis or emphysema before treatment was observed in 25 (89%) of the 28 patients with pulmonary complications above grade 1. Pulmonary complications of NCI-CTC criteria above grade 2 were noted in only 14

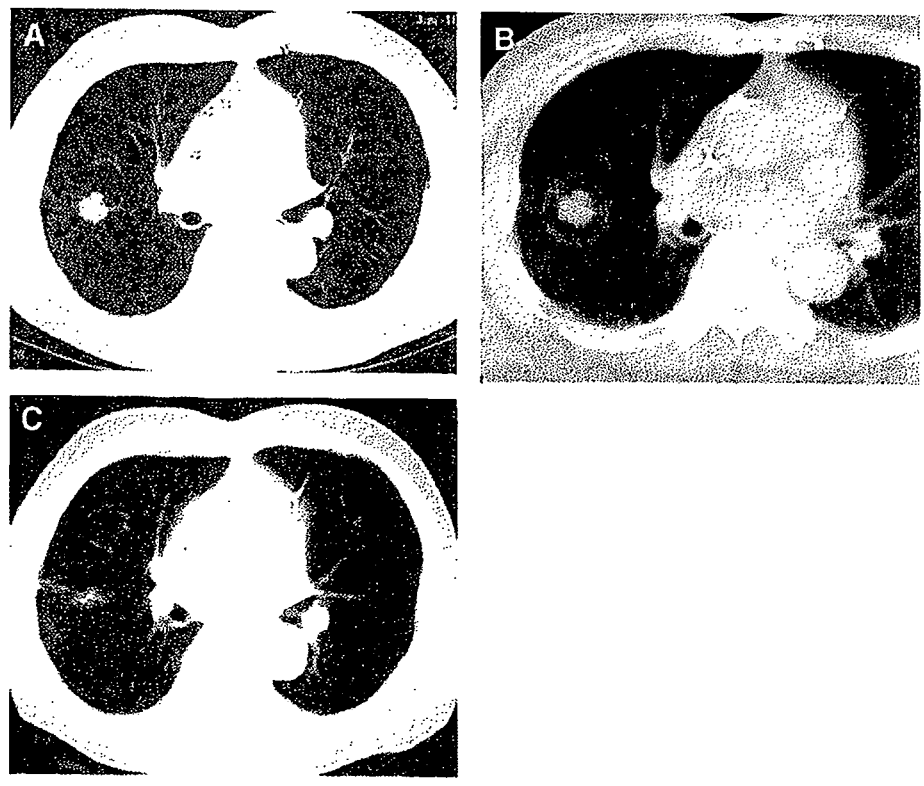

FIGURE 1. A typical example involving SRT for a 76-year-old man with T1N0 adenocarcinoma. He was treated with HypoFXSRT. (*A*) Before hypofractionated stereotactic radiotherapy (HypoFXSRT). (*B*) The calculated dose distribution. The isocenter dose was 75 Gy/10 fractions/5 days, and the tumor was fully enclosed with the 90% dose line. (*C*) Twelve months after HypoFXSRT, a scarred tumor is rated as a partial response.

TABLE 2. Recurrence Rate According to the BED and Stage

	Total cases	BED <100 Gy	BED ≥100 Gy	p	Stage IA	Stage IB	p
Local tumor	36/257 (14.0%)	18/42 (42.9%)	18/215 (8.4%)	<0.01	20/164 (12.2%)	16/93 (17.2%)	0.21
Regional nodal metastasis	29/257 (11.3%)	9/42 (21.4%)	20/215 (9.3%)	<0.05	17/164 (10.4%)	12/93 (12.9%)	0.54
Distant metastasis	51/257 (19.8%)	11/42 (26.2%)	40/215 (18.6%)	0.3	32/164 (19.5%)	19/93 (20.4%)	0.87

BED, biological effective dose.

patients (5.4%). The pulmonary symptoms resolved in most patients without steroid therapy, but six patients who had very poor respiratory function or severe pulmonary fibrosis before irradiation needed continuous oxygen. Chronic segmental bronchitis and wall thickening causing atelectasis in the peripheral lung was observed in one patient (0.4%). Transient grade 3 esophagitis was observed in two patients (0.8%) with tumors adjacent to the esophagus. Grade 3 or 4 dermatitis was observed in three patients (1.2%) with tumors adjacent to the chest wall. Rib fracture adjacent to the tumor was found in four patients (1.6%). No vascular, cardiac, or bone marrow complications had been encountered as of the last follow-up.

Recurrence

The recurrence rates of local, regional nodal, and distant lesions according to the BED and stage are listed in Table 2. The local recurrence rate was significantly lower for a BED of 100 Gy or more compared with a BED of less than 100 Gy (8.4 versus 42.9%, $p < 0.01$). For greater BED subgroups, the local recurrence rate was 11.8% for a BED of 120 Gy or more ($n = 93$) and 8.1% for a BED of 140 Gy or more ($n = 37$). The local recurrence rates for adenocarcinoma and squamous cell carcinoma were 13.3% (16/120) and 17.1% (19/111), respectively in 3 years. The cumulative local control rate curves according to BED subgroup are shown in Figure 2. The 5 (3? according to Table 2)-year local control rates of the BED of 100 Gy or more and less than 100 Gy subgroups were 84.2% (95% confidence interval [CI]: 77.7%–90.8%) and 36.5% (95% CI: 10.4%–62.6%), respectively. According to subgroup analysis, stage IB patients had a significantly higher rate of local recurrence than stage IA patients. The nodal and distant recurrence rates were almost identical in the stage IA and IB subgroups.

In the patients with regional nodal recurrence, nodal failures overlapped local failure in 3.1%, distant metastases in 3.9%, or both in 0.8% of the patients. Isolated local, nodal, and distant recurrences were observed in 8.6%, 5.1%, and 13.6% of the patients, respectively.

Survival

The overall 3- and 5-year survival rates for all patients were 56.8% (95% CI: 50.2%–63.5%) and 47.2% (95% CI: 38.7%–53.5%), respectively. The cause-specific 3- and 5-year survival rates were 76.9% (95% CI: 70.6%–83.2%) and 73.2% (95% CI: 66.1%–80.2%), respectively. The overall survival rates differed significantly according to medical operability, with intercurrent death in 36.8% of inoperable patients and 10.3% of operable patients. The overall 5-year survival rates of medically operable and inoperable patients (Figure 3) were 64.8% (95% CI: 53.6%–75.9%) and 35.0% (95% CI: 25.9%–44.1%), respectively. The overall survival rates according to the BED in all patients differed significantly between the BED of less than 100 Gy and 100 Gy or more subgroups. The overall 5-year survival rates of the BED 100 Gy or more and less than 100 Gy subgroups were 53.9% (95% CI: 46.0%–61.8%) and 19.7% (95% CI: 5.9%–33.4%), respectively. For the subgroup of medically operable patients with a BED of 100 Gy or more, the 3- and 5-year overall survival rates were 80.4% (95% CI: 71.0%–89.7%) and 70.8% (95% CI: 59.3%–82.2%), respectively (Figure 2). The overall 5-year survival rate according to stage in the operable patients irradiated with a BED of 100 Gy or more was 72.3%

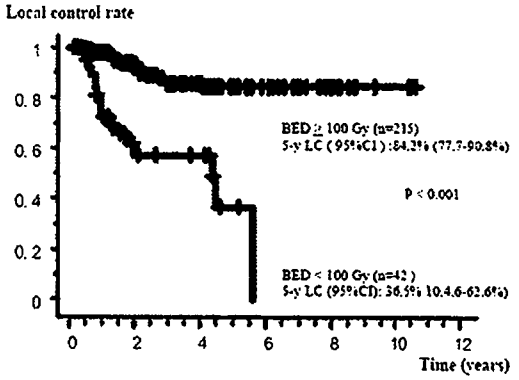

FIGURE 2. Cumulative local control rate according to the biological effective dose (BED). LC, local control rate; CI, confidence interval.

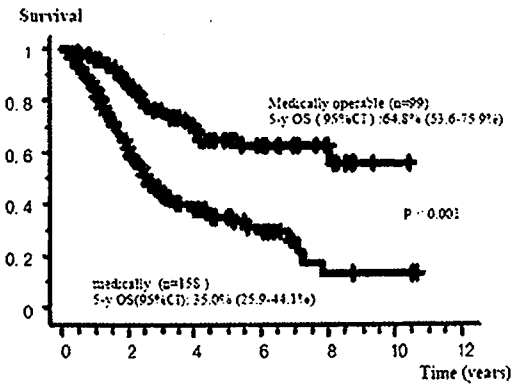

FIGURE 3. Overall survival rate according to medical operability. OS, overall survival rate; CI, confidence interval.

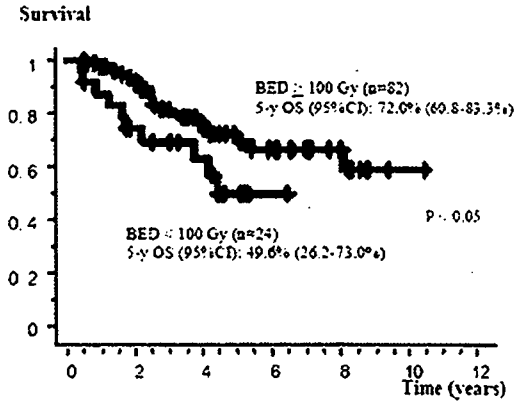

FIGURE 4. Overall survival rate in operable patients according to the biological effective dose (BED). OS, overall survival rate; CI, confidence interval.

(95% CI: 59.1%–85.6%) for stage IA and 65.9% (95% CI: 43.0%–88.9%) for stage IB patients (Figure 4).

Reproducibility of the Data Among Institutions

Table 3 compares the irradiation method and results for three major institutions enrolled in this study. These institutions used a BED of 100 Gy or more. The local control and 3-year survival rates were almost identical.

DISCUSSION

At present, surgery is the standard treatment for stage I NSCLC. RT is offered to patients who are unsuitable for surgery because of medical problems and to patients who refuse surgery. Most information on the results of RT for stage I NSCLC is based on retrospective studies of RT-treated inoperable NSCLC cases. Therefore, the role of RT for stage I NSCLC, as a curative modality, has not yet been established.

Qiao et al. summarized 18 papers on stage I NSCLC treated with conventional RT alone published between 1988 and 2000.[10] Local recurrence was the most common reason for treatment failure of stage I NSCLC with conventional RT, but the frequency of recurrence varied considerably according the report (between 6.4% and 70%). The 3-year recurrence rate was approximately 60%,[11–13] with a median time to relapse that ranged from 21 to 30 months.[12,14,15] Generally, smaller tumor size, low T stage, and increased dose had a favorable impact on local control, and increased local control was followed by increased survival.[14,16] However, the overall treatment results were disappointing. The median survival in these studies ranged from 18 to 33 months. The 3- and 5-year overall survival rates were 34 ± 9% and 21 ± 8% (mean ± 1 SE), respectively. The cause-specific survival rates at 3 and 5 years were 39 ± 10% and 25 ± 9% (mean ± 1 SE), respectively. Regarding treatment toxicity, severe (grade 3 or above) radiation esophagitis[14] and pneumonitis[11] occurred in 4.1% and 6.1% of the cases, respectively. Better local control may be achieved when the total dose is increased,[15,16] and a trend has been growing toward seeking better local control by increasing the BED[13–15] for a relatively limited span of doses (BED 59–76 Gy). Dose escalation has been the focus of developmental therapeutic strategies for inoperable stage I NSCLC to improve local control and survival.

Mehta et al.[17] provided a detailed theoretical analysis regarding the responses of NSCLC to RT and a rationale for dose escalation. They concluded that a greater BED irradiated during a short period must be given to gain local control of lung cancers. Giving a higher dose to the tumor without increasing the adverse effects was shown to be possible using the SRT technique; this is now feasible due to the technological progress that allows increasing the accuracy of localization to the tumor-bearing area using various imaging tools. SRT can also reduce the overall treatment time substantially, from several weeks for conventional RT to a few days, offering an important advantage to the patient.

After Uematsu et al.[18] reported a landmark study on SRT for stage I NSCLC using a CT-linac system, SRT has been actively investigated for stage I NSCLC in Japan and the United States. In the reports listed in Table 4,[3–6,19–21] the local control rates of primary lung cancer with SRT ranged from 87% to 97% when the BED exceeded 100 Gy. Uematsu et al.[3] showed excellent survival rates for medically operable patients, approximating those for full lobar surgical resection; however, they studied only a few patients, and it is not known whether the result is reproducible. Table 5 compares the results of Uematsu et al.[3] with the HypoFXSRT results presented here. These results suggest that the local control and survival rates of HypoFXSRT for stage I NSCLC are promising and reproducible when the BED exceeds 100 Gy.

In Japan, we consider a BED greater than 100 Gy to be a satisfactory dose for HypoFXSRT of stage I NSCLC, with a local control rate better than 85%, and a further dose escalation study is not necessary for tumors smaller than 4 cm in diameter. Conversely, in the United States, Timmerman et al.[22] concluded that 60 Gy in three fractions (BED = 180 Gy) is the proper dose, and they adopted this dose and fraction protocol for their prospective study. We need to observe the

TABLE 3. Comparison of the Irradiation Methods and Results for Three Major Institutions

Institution	No. of Patients	Total Isocenter Dose (Gy)	Single Isocenter Dose (Gy)	BED (Gy)	Median Follow-up (mo)	Local Failure, %	5-yr Overall Survival, %
Kyoto	42	48	12	106	40	3	64
Cancer Institute	30	50–62.5	10–12.5	100–141	25	4	77
Kitami	27	50–60	7.5–10	100–105	71	4	63

BED, biologically effective dose (α/β = 10) recalculated at the isocenter.

TABLE 4. Reports of Stereotactic Radiotherapy for Stage I Non-small Cell Lung Cancer

Author (ref.)	No. of Patients	Total Dose* (Gy)	Single Dose* (Gy)	BED† (Gy)	Median Follow-up (mo)	Local Progression, %	3-yr Overall Survival, %
Uematsu et al.[3]	50	72	7.2	124	60	6	66
Nagata et al.[4]	42	48	12	106	52	3	82
Fukumoto et al.[5]	17	48–60	6–7.5	99–137	24	6	NA
Onishi et al.[6]	28	72	7.2	124	24	8	75
Hof et al.[19]	10	19–26	19–26	55–94	15	20	NA
McGarry et al.[20]	47	75	25	263	15	13	NA
Wulf et al.[21]	12	26–57	19–26	94–165	11	5%	NA

BED, biologically effective dose; NA, not assessed
*Stereotactic radiotherapy dose is calculated at the isocenter
†BED (α/β = 10) recalculated at the isocenter

results of ongoing phase II studies on SRT for stage I NSCLC conducted in Japan (12 Gy × 4 = 48 Gy prescribed to the isocenter) and the United States (20 Gy × 3 = 60 Gy prescribed to cover 95% of the PTV).

The 5-year overall survival rate for medically operable patients with HypoFXSRT is encouraging (Table 6). Representative 5-year overall survival rates for clinical stage IA and IB with surgery range from 61% to 72% and 40% to 50%, respectively.[23–25] According to our data, the survival rate for SRT was not worse than that for large surgical series. Furthermore, concerning toxicity, approximately 3% of patients died as a result of surgery, and chronic morbidity occurs in 10% to 15% of patients after surgery.[26] HypoFXSRT is much less invasive than surgery, and it is postulated that SRT will become a major treatment choice for stage I NSCLC, at least for medically inoperable patients.

TABLE 5. Comparison of the Results between the Multi-institutional Study and the Uematsu et al. Study

	Uematsu et al.[3]	Multi-institutional
Total no. of cases	50	215
T1N0M0	24	141
T2N0M0	26	75
Follow-up duration, mo (median)	22–66 (36)	2–128 (38)
Local control, %	94	90
Regional lymph nodes metastases, %	4	7
Distant metastases, %	14	19
Grade ≥3 toxicity, %	0	3
3-yr overall survival rate, %	66	64
3-yr cause-specific survival rate, %	88	83
5-yr overall survival rate, %	55	55
5-yr cause-specific survival rate, %	81	77
3-yr overall survival rate in operable patients, %	86	82
5-yr overall survival rate in operable patients, %	77	72

TABLE 6. Comparison of 5-Year Overall Survival Rate between Stereotactic Radiotherapy and Surgery

Stage	Author			
	Mountain[23]*	Naruke et al.[24]*	Shirakusa and Koybayashi[25]*	Onishi†
IA	61%	71%	72%	72%
IB	40%	44%	50%	66%

*Surgery.
†HypoFXSRT presented here.

Although the differences in techniques and schedules of the institutions enrolled in this study may be large, it is meaningful that a safe, effective BED was suggested because the optimal dose-fraction schedule of SRT for stage I NSCLC is not known. Furthermore, this is the only report that gives the results of SRT for a large number of medically operable stage I NSCLC patients. Based on our excellent SRT results, it is arguable that a phase III study comparing surgery and SRT for medically operable patients is warranted. However, it is very difficult to perform a phase III study because most patients will opt for less invasive therapy such as SRT. We need much more experience and must study more patients with a longer follow-up duration to establish a safe, effective irradiation method that will instill both medical and social confidence in SRT for treatment of stage I NSCLC.

When we compare the results of conventional RT and surgery with those of HypoFXSRT, we conclude that HypoFXSRT has the following benefits for stage I NSCLC. First, HypoFXSRT is a safe and promising treatment modality. Second, the local control and survival rates are superior to those of conventional RT. Third, HypoFXSRT should be a standard of care for medically inoperable patients. Fourth, HypoFXSRT should be randomly compared with surgery for medically operable patients. Finally, we need additional experience with a longer follow-up duration to conclusively validate these points.

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