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.

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**.

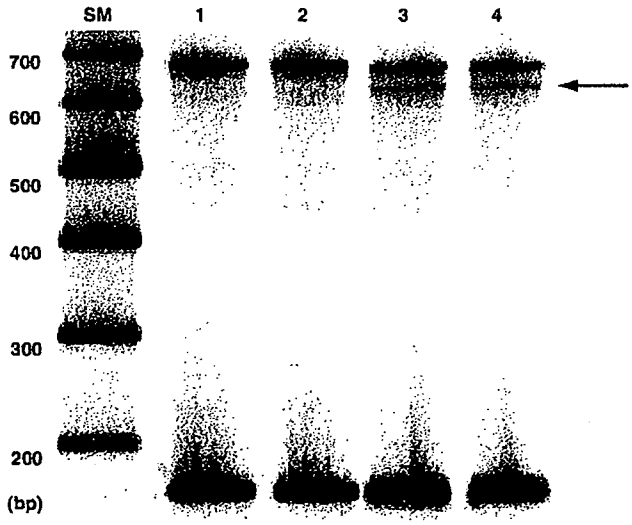

Image 1 Detection of point mutations at codon 858 of the epidermal growth factor receptor gene exon 21. The electrophoretogram after loop-hybrid mobility shift assay to detect the point mutation L858R in the tumor DNA in lung adenocarcinomas. Lanes 1 and 2 show normal sequences; lanes 3 and 4, heterozygous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

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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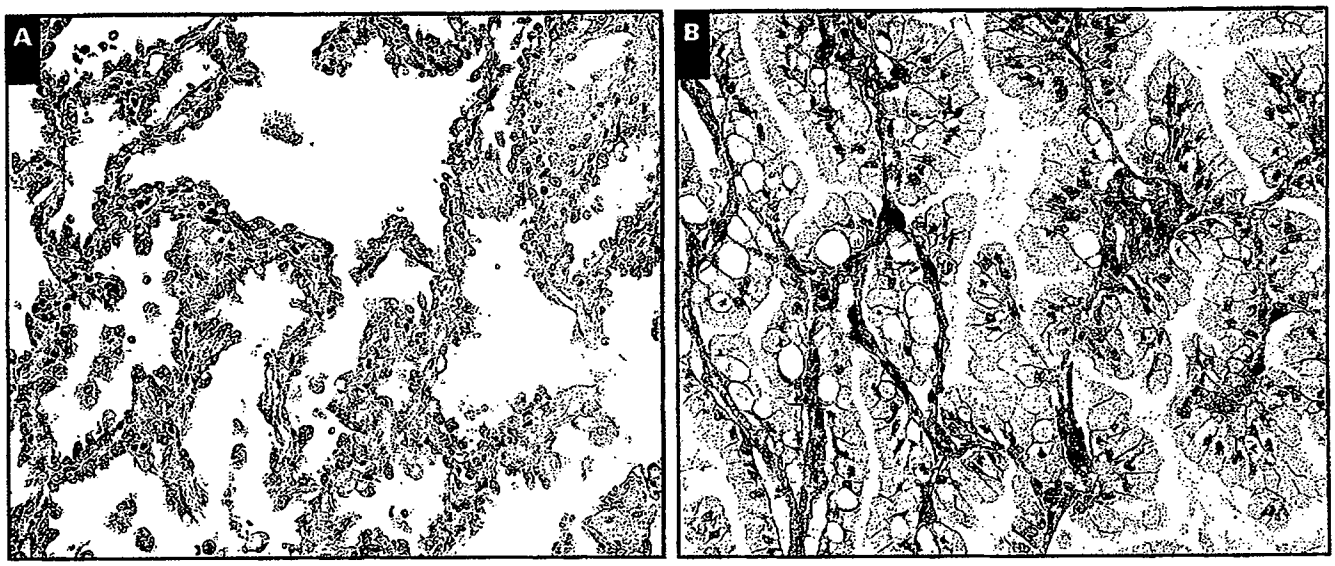

Image 2 Typical histopathologic features of epidermal growth factor receptor (*EGFR*)- and K-*ras*–mutated lung adenocarcinomas. **A**, Nonmucinous bronchioloalveolar carcinoma (BAC) component of an *EGFR*-mutated adenocarcinoma. This tumor has a deletion mutation (del746-750) in exon 19. **B**, Mucinous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

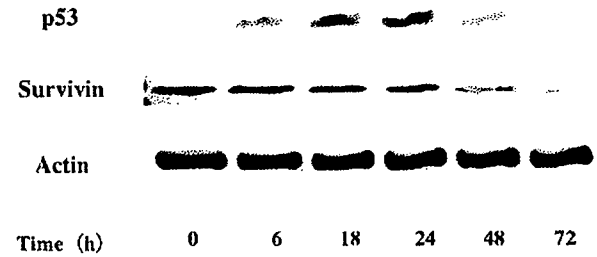

Fig. 2. Effects of UVC on the abundance of p53 and survivin in A549 cells. Total cellular protein extracted at the indicated times after exposure of cells to UVC (30 J/m^2) was subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin (loading control). Data are representative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

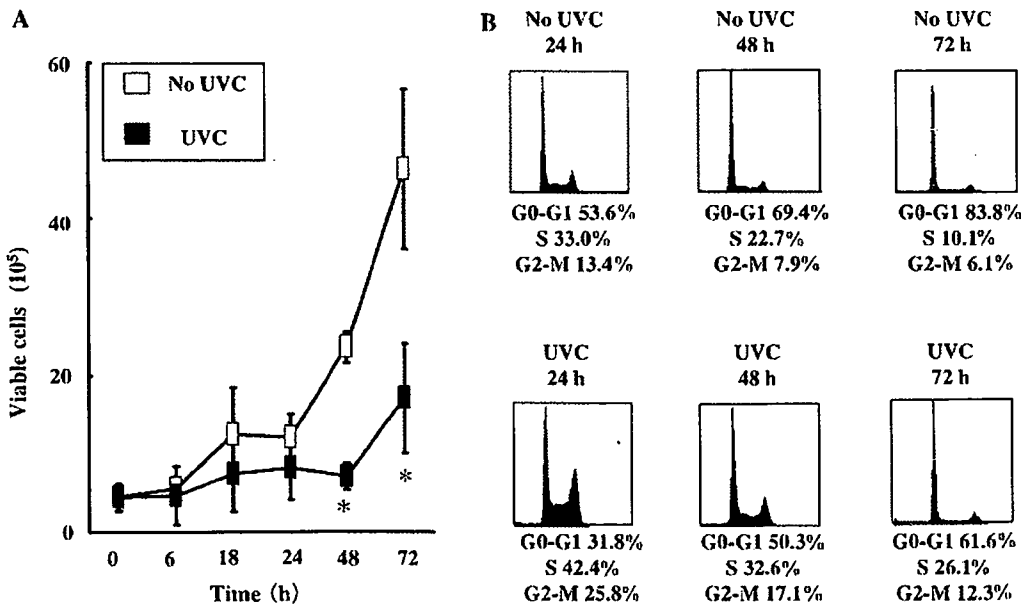

Fig. 1. Effects of UVC on the proliferation and cell cycle distribution of A549 cells. (A) Cell proliferation was evaluated by counting the number of viable cells by trypan blue staining at the indicated times after UVC irradiation (30 J/m^2). Data are means ± SD of values from three independent experiments. *$p < 0.05$ 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.

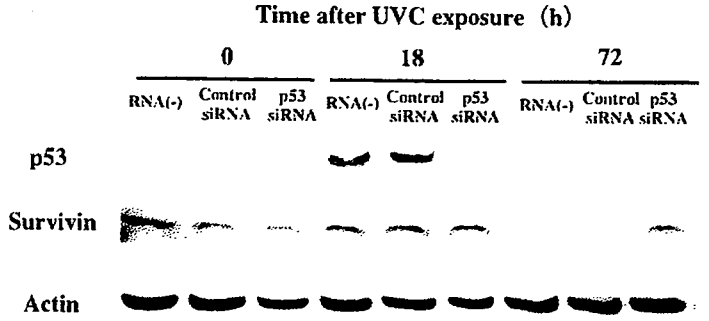

Fig. 3. Effect of UVC on the abundance of survivin in A549 cells depleted of p53 by RNAi. Cells were transfected (or not) with an siRNA specific for p53 mRNA or with a control (scrambled) siRNA, exposed to UVC (30 J/m^2), and subjected to immunoblot analysis with antibodies to p53, to survivin, or to 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

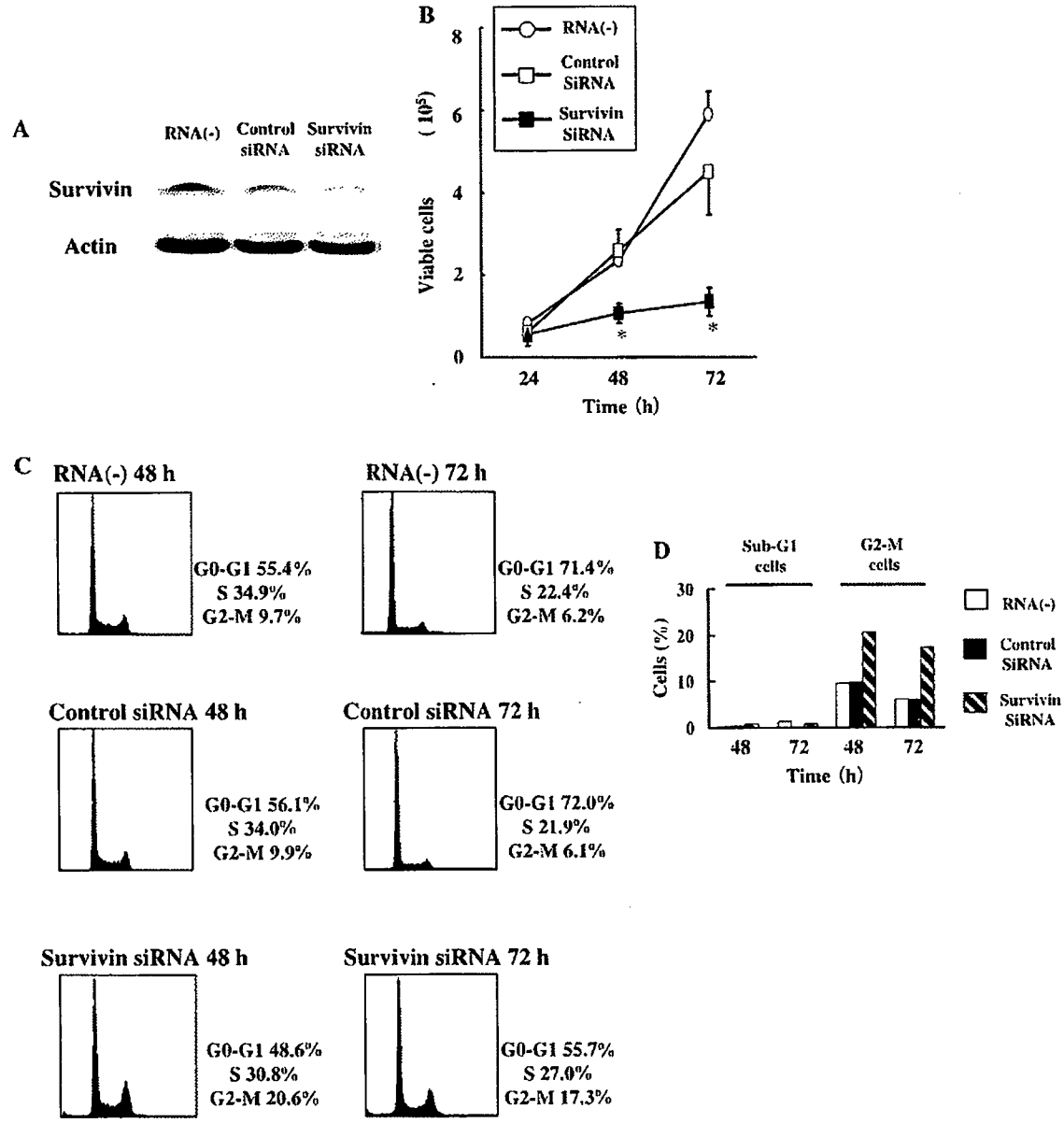

Fig. 4. Effects of survivin depletion by RNAi on the proliferation and cell cycle distribution of A549 cells. (A) Cells were transfected (or not) with a siRNA specific for survivin mRNA or with a control siRNA and were then subjected to immunoblot analysis with antibodies to survivin or to β-actin. Data 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.

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Effectiveness of Concurrent Radiation Therapy with UFT or TS-1 for T2N0 Glottic Cancer in Japan

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Abstract. *The aim of this retrospective study was to compare concurrent radiation therapy (RT) combined with peroral chemotherapy (UFT or TS-1) with conventional RT for T2N0 glottic cancer. Between 1974 and 2005, 153 patients with T2N0 glottic cancer were treated with radiation alone or radiation combined with peroral (UFT or TS-1) or intravenous chemotherapy. All except one patient were treated with 2 Gy per fraction, 5 fractions per week, totaling 60 Gy; and the remaining patient was treated with 1.8 Gy per fraction, 5 fractions per week, totaling 61.2 Gy. Eighty-three patients were concurrently given UFT, 24 were given TS-1, 23 intravenous chemotherapy (mainly cisplatin; the Pt Group), and 23 had no chemotherapy. The 5-year local control rate was 83.4%. Stratified by RT alone (the RT group) and concurrent chemoradiation therapy (the CCRT group), 5-year local control rates of the RT and CCRT groups were 82.7% and 83.4%, respectively (p=NS). Stratified by chemotherapy regimens of the CCRT group, 3-year local control rates of the UFT, TS-1 and Pt groups were 90.1%, 100.0% and 73.4%, respectively. Concurrent chemoradiation therapy using UFT or TS-1 for T2N0 glottic cancer is one of the standard treatments in Japan. Concurrent use of TS-1 could be a breakthrough treatment for T2N0 glottic cancer.*

At diagnosis, about 60% of head and neck cancer is found to be stage III-IV disease, because effective screening examinations to detect early-stage head and neck cancer have not been established yet (1). On the other hand, laryngeal cancer, especially glottic cancer, is mostly detected in the early stage owing to the concomitant symptom of hoarseness. Thus, the strategy for glottic cancer differs from other head and neck carcinomas.

Early-stage glottic cancer is usually treated with radiation therapy (RT) alone, voice preservation partial-pharyngectomy or altered fractionated RT (1-5). RT alone is effective for T1N0 squamous cell carcinoma of the glottic larynx, is not burdensome for the patients and enables patients to maintain the same voice quality as before treatment. In Japan, RT alone is often used to treat T1N0 glottic cancer. The 5-year overall survival rate and 5-year local control rate of T1N0 glottic cancer has been reported to be 79%-82% and 80%-93% (6-10), respectively (1). However, the treatment outcome of RT alone for T2N0 glottic cancer is unsatisfactory. The 5-year overall survival rate and local control rate of T2N0 glottic cancer has been reported to be 77% and 67%-88% (6-10), respectively (1). Surgery is effective, however, the quality of voice preservation is much worse than radiation therapy (11). Altered fractionated RT may have the possibility to be superior to conventional RT (12-15), although relatively few conclusive reports have been published and acute morbidity might be exacerbated.

Recently, Akimoto *et al.* have demonstrated that concurrent chemoradiotherapy (CCRT) of T2N0 laryngeal cancer had better local control rate than conventional RT alone (16). The 5-year local control rates of CCRT and of RT alone were 89% and 61%, respectively. Nonetheless, there are a few remaining obstacles to overcome in order to apply this strategy to medical practice. The CCRT method of Akimoto *et al.* is a combination of RT and intravenous chemotherapy, which means that patients are treated with CCRT in hospital, increasing the patients' physical, psychological and economical burdens. Most patients prefer peroral rather than intravenous chemotherapy when the treatment outcomes are the same (17). Peroral chemotherapy enables patients to be treated in outpatient clinics.

This study was presented at the 43rd Annual Meeting of the American Society of Clinical Oncology (ASCO), June 1-5, 2007, Chicago, Illinois, U.S.A.

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Key Words: Chemoradiotherapy, T2N0 glottic cancer, peroral chemotherapy, UFT, TS-1.

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The current study were retrospectively reviewed 153 T2N0 glottic cancer patients treated at the Kitasato University Hospital to compare concurrent RT combined with chemotherapy (mostly, peroral anticancer drugs: UFT or TS-1 Taiho Pharmaceutical, Tokyo, Japan, approved by the Japanese government) with conventional RT.

Patients and Methods

From 1974 to December, a total of 153 patients, 151 males and 2 females (median: age 64 years; range: 45-86 years) with T2N0 (according to the 1994 UICC TNM classification) glottic cancer had been treated with RT alone or RT combined with peroral (UFT or TS-1) or intravenous chemotherapy at the Kitasato University Hospital, Kanagawa, Japan. The local control and overall survival rates were retrospectively analyzed and comparisons were made between cases receiving peroral chemotherapy combined with RT and those receiving RT alone.

Regarding the RT protocol, all except one had been treated with 2 Gy per fraction, 5 fractions per week and a total of 60 Gy using 4MV X-ray and lateral paralleled opposed fields, the remaining one patient had been treated with 1.8 Gy per fraction, 5 fractions per week and a total of 61.2 Gy. The reference point for irradiation selected for glottic cancer at the Kitasato University Hospital is the half point of the isocenter and the posterior irradiation field line (the Kitasato Method). However, the usual reference point for glottic cancer is the isocenter. Dose-distribution is superior in the former protocol to the latter one. Paired wedge filters had been used when radiation oncologists considered it to be required for compensation of dose inhomogeneity.

For the chemotherapy protocol 83 patients had concurrently taken UFT, 600-800 mg per day totaling 9,000-30,000 mg (median: 24,000 mg), during RT (the UFT group). Twenty-four patients had taken TS-1, 50-100 mg per day (median: 80 mg per day), totaling between 1,250-2,500 mg (median: 2,000 mg) with a 1- or 2-week break during the RT (the TS-1 group). Twenty-three patients had ungergone intravenous chemotherapy using mainly cisplatin (the Pt group). The remaining 23 patients had undergone no chemotherapy.

The analysis was performed in January 2006. Survival and local control rates were calculated from the first day of RT using the Kaplan-Meier method, and the log-rank sum test was used for comparing variables.

Results

The median follow-up period was 61 months (range: 3-372 months). The 3- and 5-year local control rates of all patients were 86.8% and 83.4%, respectively (Figure 1). The 3- and 5-year local control rates for RT along (the RT group) and concurrent chemoradiotherapy (the CCRT group) were 82.7% and 82.7%, and 87.7%, and 83.4%, respectively (p=NS) (Figure 2). Stratified by chemotherapy regimens within the CCRT group, the 3-year local control rates of the UFT, TS-1 and Pt groups were 90.1%, 100.0% and 73.4%, respectively (Figure 3).

The 3- and 5-year overall survival rate of all patients was 94.5% and 87.9%, respectively (Figure 4). The 3- and 5-year overall survival rates of the RT and CCRT groups were 82.9% and 78.3%, and 96.1% and 90.1%, respectively (Figure 5). These differences were statistically significant (p=0.04). Furthermore, stratified by chemotherapy regimens within the CCRT group, the 3-year overall survival rates of the UFT, TS-1 and Pt groups were 96.1%, 100.0% and 94.7%, respectively (Figure 6). These differences were not statistically significant.

As for morbidity, 2 patients in the UFT group experienced severe enteritis, so-called "UFT enteritis." However, no patients in the TS-1, Pt or RT groups experienced severe enteritis. No other severe morbidities were recognized in the present study.

Discussion

UFT and TS-1 are 5-fluorouracil (5-FU) prodrugs approved by the Japanese government for head and neck carcinomas and are administered perorally not intravenously. The effectiveness of UFT and TS-1 are reported to be almost the same or somewhat superior to 5-FU in gastric, colon, and lung carcinomas (18-20). The standard treatment of unresectable advanced head and neck carcinoma is concurrent chemoradiation therapy (1). The chemotherapeutic regimens contain platinum drugs, such as cisplatin (21) and nedaplatin (22), and a combination of platinum drugs with 5-FU (21, 22) or docetaxel (23). T2N0 glottic cancer is and early stage cancer usually treated with RT alone or the combination of radiation therapy with platinum drugs, plus 5-FU and docetaxel is regarded as an over-treatment. Therefore, the combination of radiation therapy with UFT or TS-1 for T2N0 glottic cancer was administered in the current study.

Recently, Akimoto *et al.* have reported that the 5-year local control rate of T2N0 laryngeal cancer treated with CCRT was 89% (16). This was the first clinical demonstration of CCRT for T2N0 laryngeal cancer that mostly consisted of glottic cancer. However, their method adopted the chemotherapeutic regimens of cisplatin and/or docetaxel administered in an intravenous infusion. Liu *et al.* reported that patients preferred peroral rather than intravenous chemotherapy when the treatments outcomes were the same (17).

In the current study, the 5-year local control rates were 82.7% and 83.4% for the RT and CCRT groups, respectively. These are nearly the same. However, stratified by the UFT, TS-1 and Pt groups, the 3-year local control rates were 90.1%, 100.0% and 73.4%, respectively, which suggested that peroral chemotherapy was not inferior to intravenous chemotherapy but even somewhat superior. In particular, the 3-year local control rate and overall survival rate of the TS-1 group were both 100%, even though the follow-up period was 4-41 months (median: 19 months).

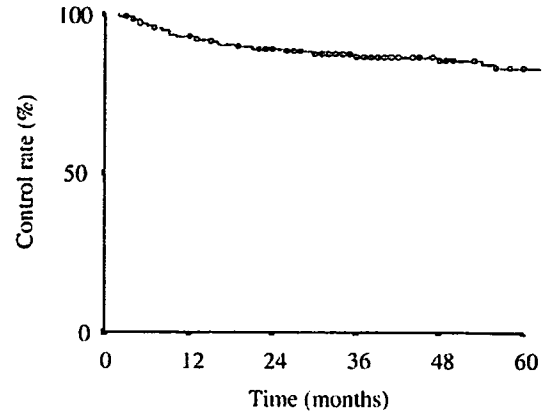

Figure 1. *Local control curve of all patients.*

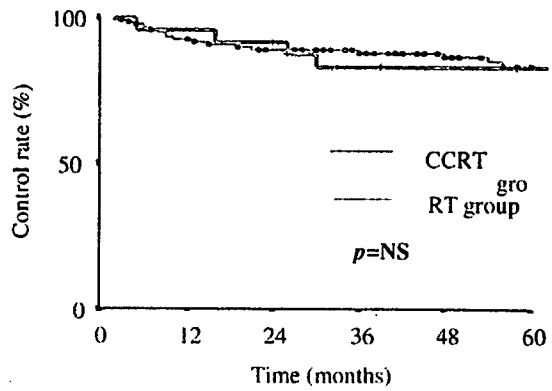

Figure 2. *Local control curves stratified by therapy. RT: radiation therapy alone, CCRT: concurrent chemoradiotherapy.*

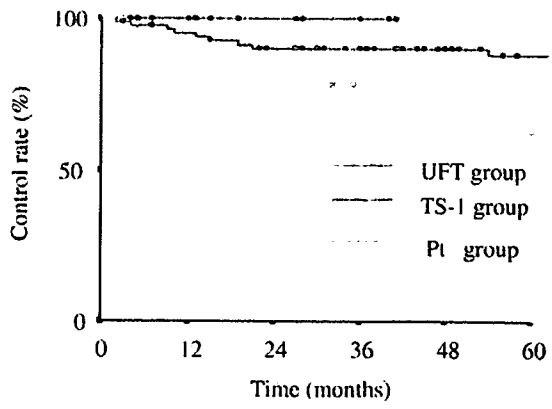

Figure 3. *Local control curves stratified by chemotherapy regime. UFT: peroral UFT, TS-1: peroral TS-1, Pt: intravenous chemotherapy.*

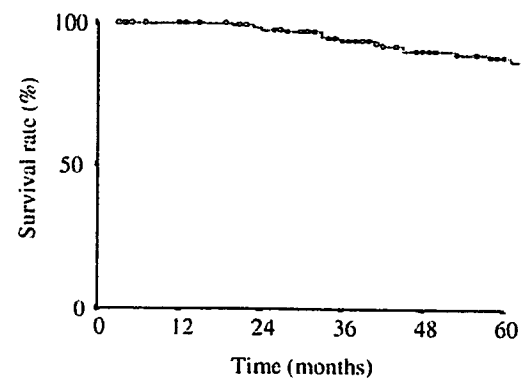

Figure 4. *Overall survival curve of all patients.*

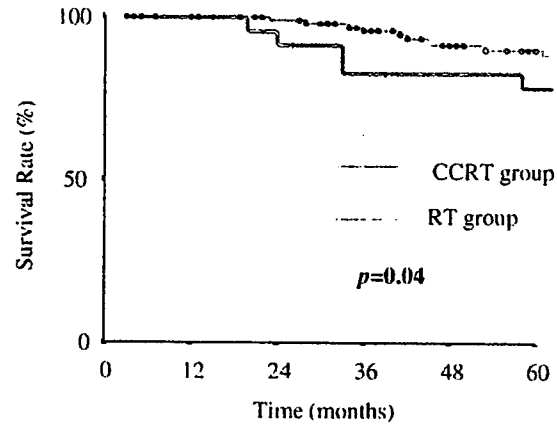

Figure 5. *Overall survival curves stratified by therapy. RT: radiation therapy alone, CCRT: concurrent chemoradiotherapy.*

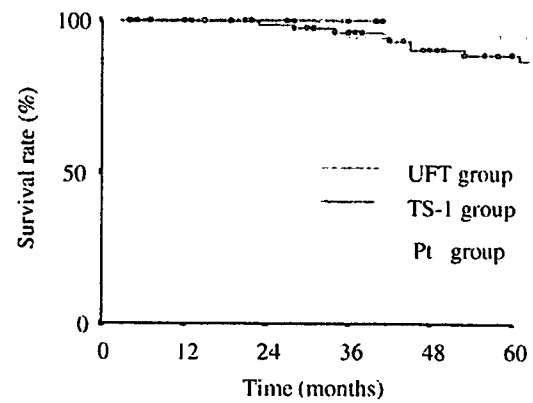

Figure 6. *Overall survival curves stratified by chemotherapy regimen. UFT: peroral UFT, TS-1: peroral TS-1, Pt: intravenous chemotherapy.*

Conclusion

Concurrent radiation therapy combined with chemotherapy could be one of the standard treatment options for T2N0 glottic cancer. Moreover, concurrent radiation therapy combined with TS-1 may prove to be a breakthrough treatment for T2N0 glottic cancer in terms of improving local control rate, overall survival and patients' burdens of treatment.

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Conflict of interest: The authors declare there is no conflict of interest in this study.

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EGFR exon 20 insertion mutation in Japanese lung cancer

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Summary Mutations of the epidermal growth factor receptor (*EGFR*) gene have been reported in non-small cell lung cancer (NSCLC), especially in female, never smoker patients with adenocarcinoma. Some common somatic mutations in *EGFR*, including deletion mutations in exon 19 and leucine to arginine substitution at amino acid position 858 (L858R) in exon 21, have been examined for their ability to predict sensitivity to gefitinib or erlotinib. On the other hand, previous report has shown that the insertion mutation at exon 20 is related to gefitinib resistance. We investigated the exon 20 *EGFR* mutation statuses in 322 surgically treated non-small cell lung cancer cases. Two hundred and five adenocarcinoma cases were included. The presence or absence of *EGFR* mutations of kinase domains was analyzed by direct sequences. *EGFR* insertion mutations at exon 20 were found from 7 of 322 (2.17%) lung cancer patients. We also detected the 18 deletion type mutations in exon 19, and 25 L858R type mutations in exon 21. There was a tendency towards higher exon 20 insertion ratio in never smoker (never smoker 4.4% versus smoker 1.3%, $p=0.0996$) and female (female 4.5% versus male 1.3%, $p=0.0917$). Two exon 20 insertion cases were treated with gefitinib and failed to response.

EGFR insertion mutation in exon 20 could not be ignored from Japanese lung cancers.

1. Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy [1]. There are much accumulated evidences that epidermal growth fac-

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tor receptor (*EGFR*) and its family members are strongly implicated in the development and progression of numerous human tumors, including lung cancer [2,3]. The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small cell lung cancer (NSCLC) since 2002. Original two reports showed that *EGFR* mutations statuses at ATP binding pockets in NSCLC patients were correlated with the clinico-pathological features related to good response to gefitinib [4,5]. These *EGFR* mutations are predominantly found in Japanese lung cancer patients (about 25–40%) [4,6–9] when compared to USA patients (about 8–10%) [4,5,7,10] or European patients [7,11]. Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro* [4,5,10]. Although many *EGFR* mutations have been reported, not all have been associated with responsiveness to gefitinib. The two most common *EGFR* mutations that have been identified, representing 85–90% of *EGFR* mutations, are the *EGFR* exon 19 deletion that eliminates a leucine–arginine–glutamate–alanine motif in the tyrosine kinase domain of *EGFR* and a thymine to guanine transversion that results in an arginine for leucine substitution at amino acid 858 (L858R). These two mutants responded significantly better for gefitinib therapy than other types of mutants [12,13]. However, Greulich et al. showed transformation by an exon 20 insertion, made cells resistant to gefitinib or erlotinib [14]. To determine the *EGFR* mutation status and correlation with clinico-pathological features in Japanese lung carcinoma, we investigated exon 20 insertion mutation status by direct sequences. The findings were compared to the clinico-pathological features of lung cancer.

2. Material and methods

2.1. Patients

The study group included 295 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1994 and 2005. We have also investigated *EGFR* mutation status for 27 lung cancer patients who had undergone surgery followed by treated with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. Gefitinib was used after lung cancer recurrence, and clinical outcome was shown in reference [9]. The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan [15]. All tumor samples were immediately frozen and stored at −80 °C until assayed. Written informed consent was obtained from the patients, and the institutional ethics committee of the Nagoya City University Medical School approved the study.

2.2. PCR assays for *EGFR* mutations

Genomic DNA was extracted using Wizard SV Genomic DNA Purification Systems (Promega) according to the manufacturers' instructions. The primers for exon 20 sequencing were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of the primer sets used in the assay are: forward ACTTCACAGCCCTGCGTAAAC, and reverse: ATGGGACAGGCACTGATTTGT. The sequence results of exon 20 about 131 of 322 cases were already reported [4,16]. The cycling conditions were as follows: initial denaturation at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 60 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review form forward and reverse, both side.

2.3. Statistical analysis

Statistical analyses were done using the Mann–Whitney *U*-test for unpaired samples and Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2 test. The overall survival of lung cancer patients was examined by the Kaplan–Meier methods, and differences were examined by the Log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA), and was considered significant when the p-value was less than 0.05.

3. Results

3.1. *EGFR* gene mutation status in Japanese lung cancer patients

The clinical and pathological characteristics of the 322 lung cancer patients are as follows: 234 (72.7%) were males and 88 were females. Two hundred and five (63.7%) were diagnosed as adenocarcinoma, and 117 were diagnosed as other types of carcinoma. Two hundred and thirty-one (71.7%) were smokers and 90 were non-smokers (one unknown). Of 295 lung cancer patients from Nagoya City University, 167 (56.6%) were stage I.

Most of the sequencing results about exon 18, 19 and 21 were already reported [4,16,17]. In exon 19, 18 patients had the deletion type mutation. In exon 18 or exon 21, 29 patients had the missense point mutations (2 G719S, 1 G719C, 25 L858R and 1 L861Q). Of these 47 patients, 17 were males and 30 were females. Thirty were non-smokers and 17 were smokers. Forty-three patients had adenocarcinoma, one had squamous cell carcinoma and three had adenosquamous cell carcinoma. Thus *EGFR* mutation status at exon 18, 19 or 21 was significantly correlated with gender ($p < 0.0001$), tobacco-smoking ($p < 0.0001$) and pathological subtypes (adenocarcinoma versus non-adenocarcinoma, $p < 0.0001$).

For exon 20, 7 patients had the insertion mutations (Table 1). These mutations were exclusively associated with other *EGFR* mutation. Three were males and four were females. Four were non-smokers and three were smokers. Six patients had adenocarcinoma and one had squamous

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Table 1 Clinico-pathological features of 322 lung cancer patients

Factors	*EGFR* exon 20 mutations		
	Mutation patients	Wild type patients	*p*-Value
Mean age (65.5 ± 9.3; years)	7	315	
Age			
≦60	1 (1.1%)	94 (98.9%)	0.6783
>60	6 (2.6%)	221 (97.4%)	
Gender			
Male	3 (1.3%)	231 (98.7%)	0.0917
Female	4 (4.5%)	84 (95.5%)	
Pathological subtypes			
Adeno	6 (3.0%)	197 (97.0%)	0.2666
Non-adeno	1 (0.8%)	118 (99.2%)	
Differentiation			
Well	4 (3.5%)	111 (96.5%)	0.4236
Moderately or poorly	2 (1.5%)	128 (98.5%)	
Lymph node metastasis			
N0	4 (1.9%)	205 (98.1%)	>0.9999
N+	2 (2.3%)	84 (97.7%)	
Smoking status			
Smoker	3 (1.3%)	228 (98.7%)	0.0996
Non-smoker	4 (4.5%)	86 (95.5%)	
Pathological stages			
I	4 (2.4%)	164 (97.6%)	0.7025
II–IV	2 (1.6%)	125 (98.4%)	

Adeno, adenocarcinoma; N+, lymph node metastasis positive.

cell carcinoma. Two were moderately differentiated, and four were well differentiated (one unclassified). There was a tendency towards higher exon 20 insertion mutation ratio in never smoker (never smoker 4.4% versus smoker 1.3%, $p=0.0996$) and female (female 4.5% versus male 1.3%, $p=0.0917$). Two female patients had 774_776 insertion NPH (2320-2328 insertion AACCCCCAC) mutations reported as D7 mutation by Shigematsu et al. (Fig. 1) [7]. A female patient had 770_772 insertion ASV (2308-2316 insertion GCCAGCGTG) mutation reported as D1 mutation by Shigematsu et al. [7]. A male patient had 771_773 insertion SVD (2311-19 insertion GCGTGGACA) mutation reported by Sonobe et al. [18]. A male patient had 772_773 insertion V (2312-14 insertion GGT) reported by Thomas et al. [19]. Two patients had 772_773insertion N (2312-14 insertion AAC) mutations (Fig. 1).

3.2. Relationship between clinical course of patients with lung cancer and *EGFR* mutations

The overall survival of 322 lung cancer patients with follow-up through December 30, 2006, was studied in reference to the *EGFR* mutation status. The prognosis from patients with exon 20 insertion mutation ($n=7$, 2 were dead) and the patient without exon 20 insertion mutation *EGFR* ($n=315$, 102 were dead) was not significantly different (Log-rank test, $p=0.7186$, Breslow–Gehan–Wilcoxon test, $p=0.8593$) (Fig. 2). Eighteen patients received adjuvant chemotherapy (five were with cisplatin base, seven were with calboplatin base and six were with Uracil-Ftegafur). Even if the 18 patients were excluded for survival analysis, the prognosis from patients with exon 20 mutation and without mutation was not significantly different ($p=0.7215$).

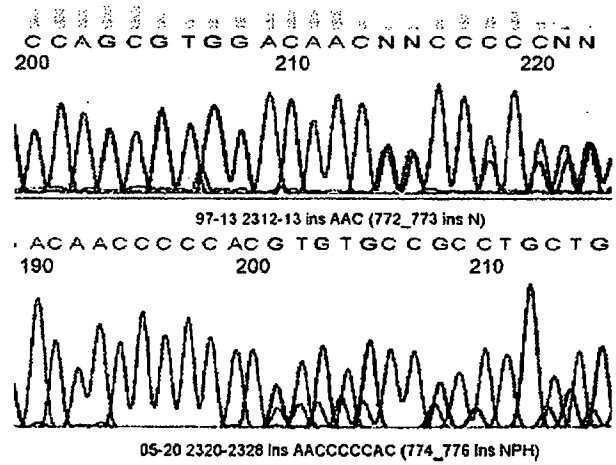

Fig. 1 Two patients had 774_776 insertion NPH (2320-2328 insertion AACCCCCAC) mutations reported as D7 mutation (upper). Two patients had 772_773insertion N (2312-14 insertion AAC) mutations (below).

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