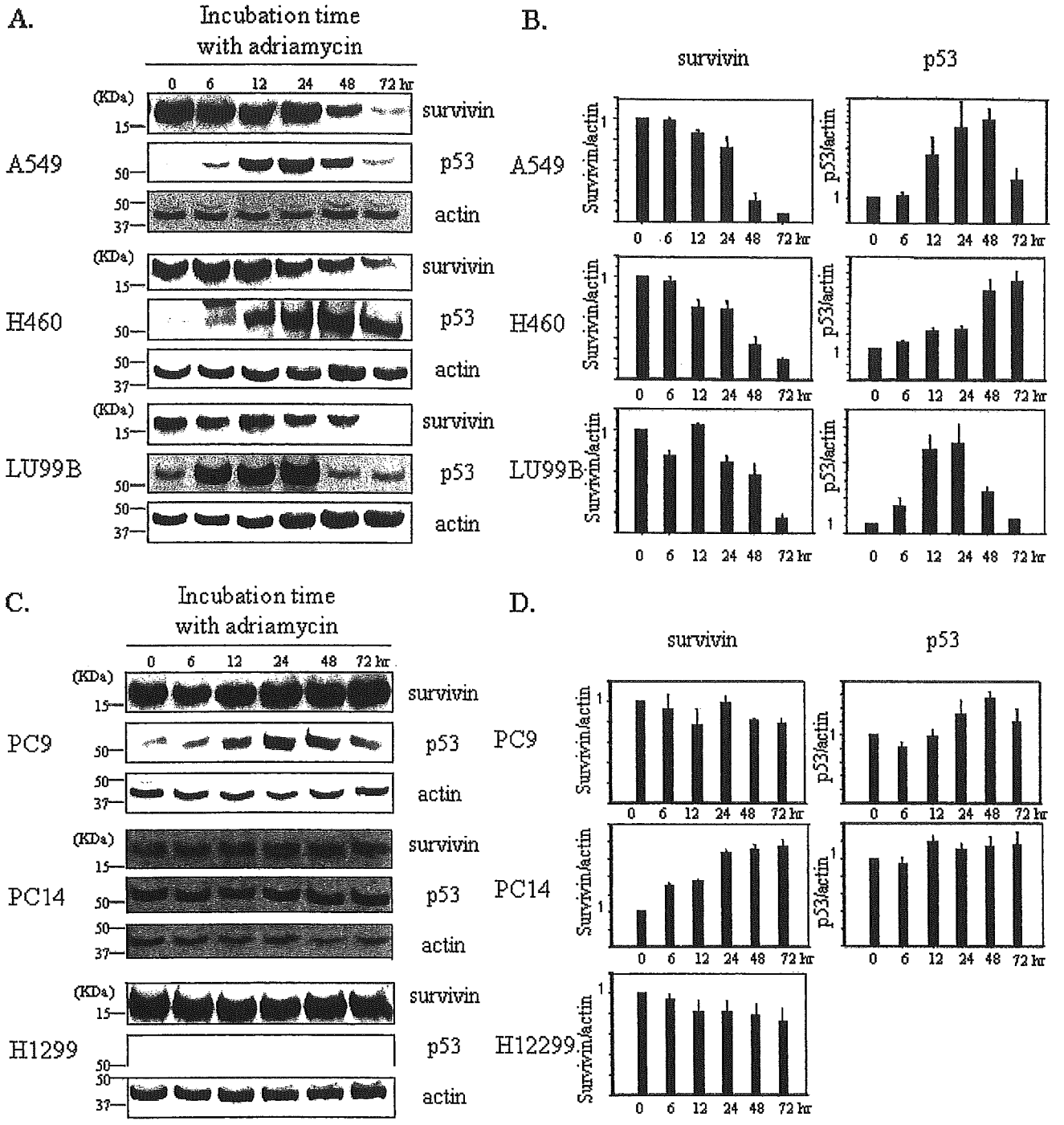

FIGURE 2 – Expression of survivin and p53 protein in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. (*a*) Western-blotting analysis for expression of survivin and p53 in cell lines possessing wild-type p53, including A549, NCI H460 and LU99B.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

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

siRNA transfection

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

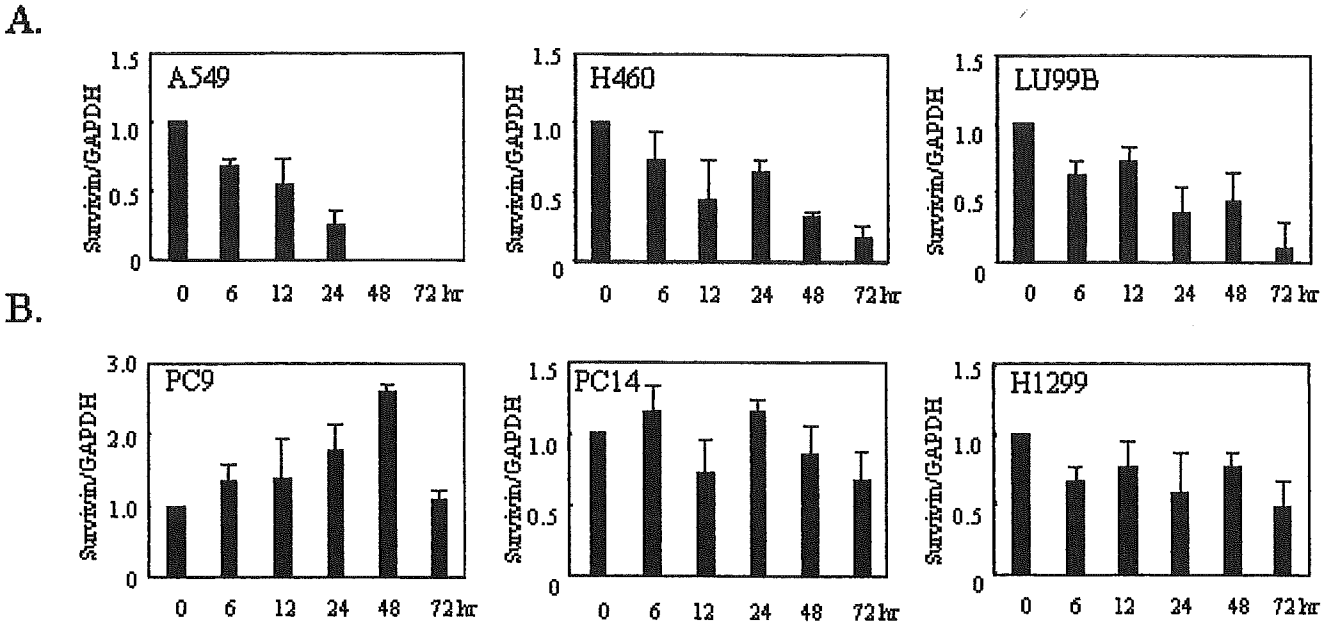

FIGURE 3 – Expression of survivin mRNA in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. Each of the cell lines with wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin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

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

TUNEL assay

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

Cell viability analysis

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

Statistical analysis

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

Results

Survivin mRNA expression in lung cancer cell lines

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

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

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

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

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

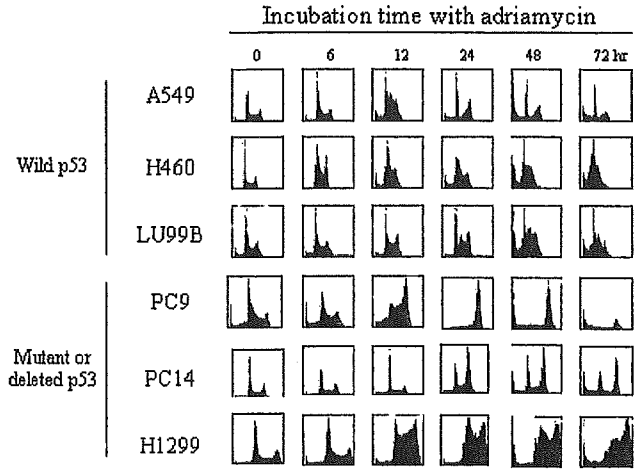

FIGURE 4 – Cell cycle analysis of lung cancer cell lines with different p53 phenotypes after exposure to adriamycin. Each of the cell lines possessing wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for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

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

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

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

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

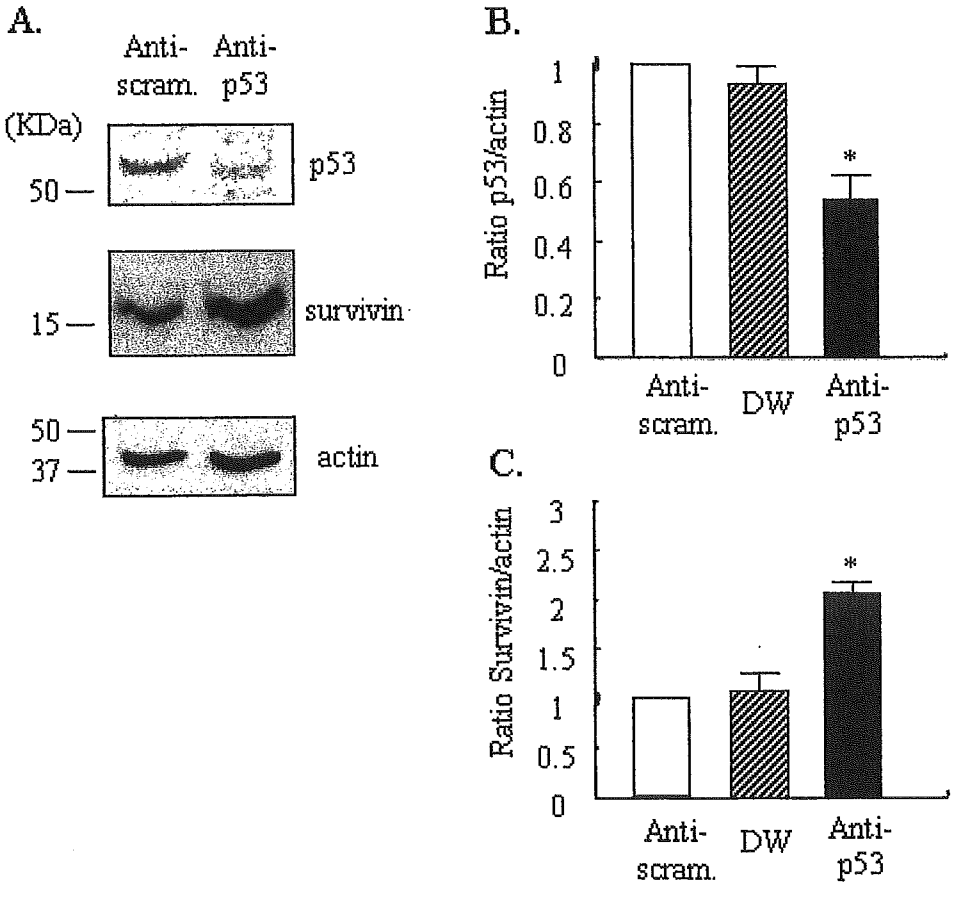

FIGURE 5 – (*a*) Increasing survivin expression in A549 lung cancer cells possessing wild-type p53 as a result of p53 inhibition by siRNA duplex. A549 cells were treated with siRNA duplex targeting p53, scramble or distilled water and then 48 hr later, cell lysates were prepared from the siRNA-treated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

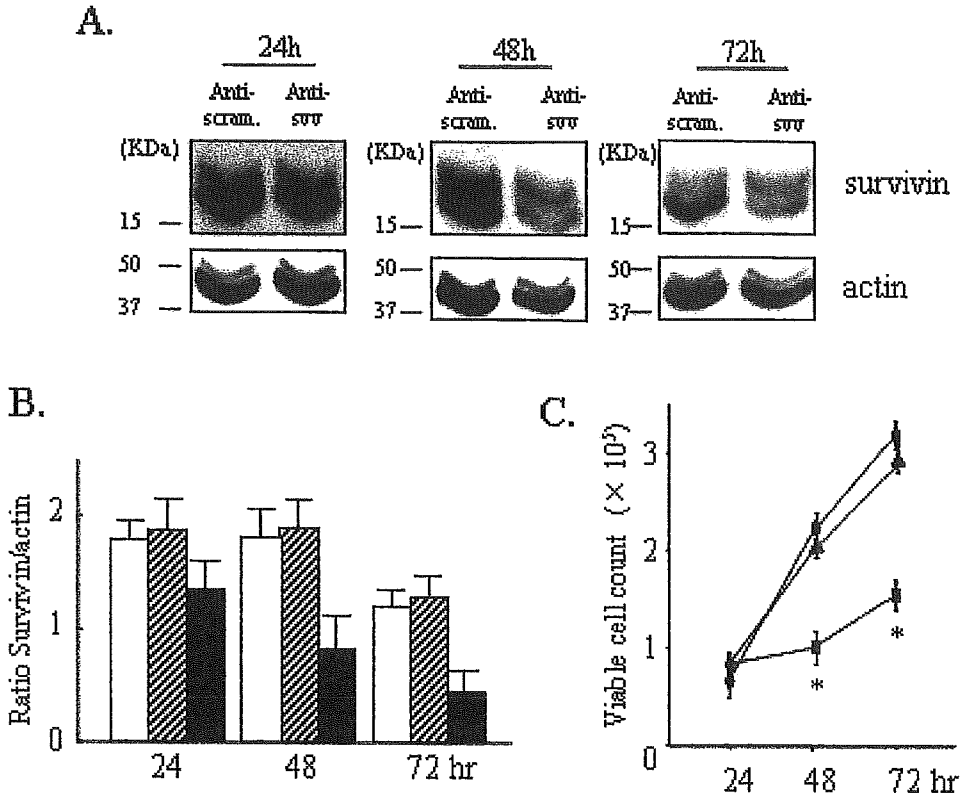

FIGURE 6 – Effects of siRNA targeting survivin on proliferation of PC9 lung cancer cells. PC9 cells were treated with siRNA duplex targeting surviving, scramble or distilled water. At the indicated time, the cells were harvested and assayed using the following procedure. (*a*) Expression of survivin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

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

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

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

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

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

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

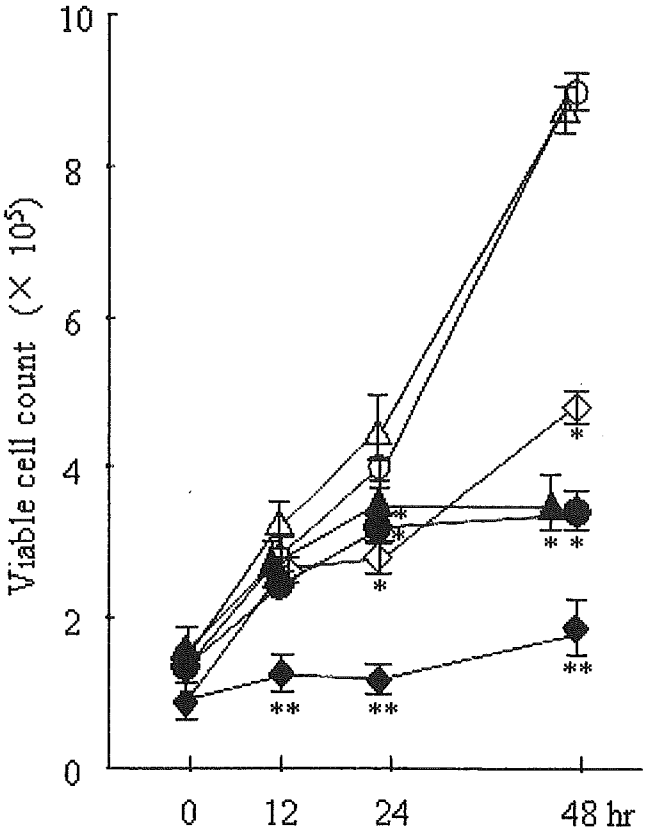

FIGURE 7 – Effects of siRNA duplex targeting of survivin on proliferation of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin or water after 48 hr transfection with siRNA duplex targeting surviving, scramble or distilled water. Open triangle:water after distilled water;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

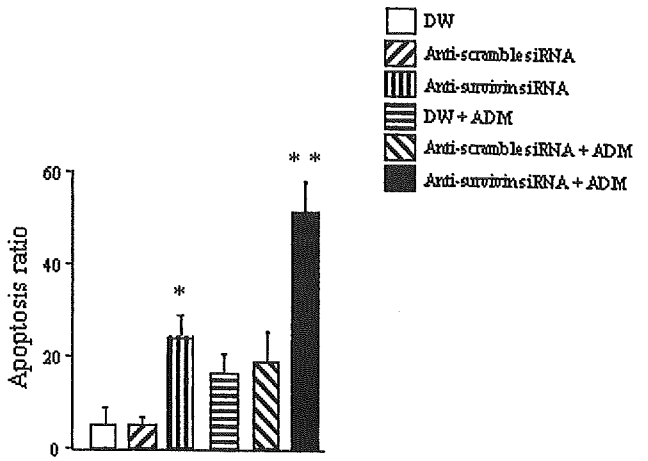

FIGURE 8 – Effects of siRNA targeting survivin on apoptosis of PC9 lung cancer cells treated with adriamycin, evaluated by TUNEL assay. PC9 cells were exposed to adriamycin or water for 24 hr after 48 hr transfection with duplex siRNA targeting surviving, scramble or distilled water. The data are presented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

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

Discussion

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

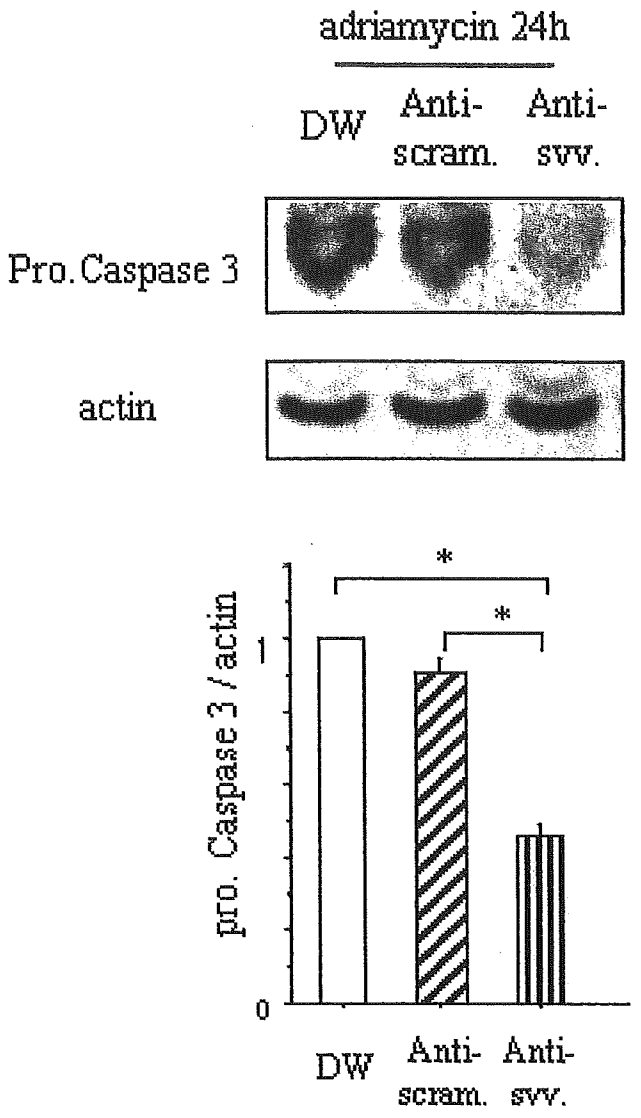

FIGURE 9 – Effects of siRNA targeting survivin on pro-caspase3 expression of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin for 24 hr after 48 hr transfection with duplex siRNA targeting survivin, scramble or distilled water, and each sample was analyzed by Western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

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

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

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

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

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

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

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

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

Acknowledgements

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Overexpression of the Aldo-Keto Reductase Family Protein AKR1B10 Is Highly Correlated with Smokers' Non–Small Cell Lung Carcinomas

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ABSTRACT

Purpose: **Squamous cell carcinoma (SCC) and adenocarcinoma of the lung are currently subject to similar treatment regimens despite distinct differences in histology and epidemiology. The aim of this study is to identify a molecular target with diagnostic and therapeutic values for SCC.**

Experimental Design: **Genes specifically up-regulated in SCC were explored through microarray analysis of 5 SCCs, 5 adenocarcinomas, 10 small cell lung carcinomas, 27 normal tissues, and 40 cancer cell lines. Clinical usefulness of these genes was subsequently examined mainly by immunohistochemical analysis.**

Results: **Seven genes, including aldo-keto reductase family 1, member B10 (*AKR1B10*), were identified as SCC-specific genes. AKR1B10 was further examined by immunohistochemical analysis of 101 non–small cell lung carcinomas (NSCLC) and its overexpression was observed in 27 of 32 (84.4%) SCCs and 19 of 65 (29.2%) adenocarcinomas. Multiple regression analysis showed that smoking was an independent variable responsible for AKR1B10 overexpression in NSCLCs ($P < 0.01$) and adenocarcinomas ($P < 0.01$). AKR1B10 staining was occasionally observed even in squamous metaplasia, a precancerous lesion of SCC.**

Conclusion: **AKR1B10 was overexpressed in most cases with SCC, which is closely associated with smoking, and many adenocarcinoma cases of smokers. These results suggest that AKR1B10 is a potential diagnostic marker specific to smokers' NSCLCs and might be involved in tobacco-related carcinogenesis.**

INTRODUCTION

Lung cancer is the leading cause of cancer death among all types of cancers and continues to increase in frequency worldwide (1). There are two major types of lung cancer, small cell lung carcinoma (SCLC) and non–small cell lung carcinoma (NSCLC), which account for 20% and 80% of all cases (2), respectively. NSCLC is further classified into squamous cell lung carcinoma (SCC) and lung adenocarcinoma. Despite distinct differences in histologic and epidemiologic features, adenocarcinoma and SCC are similarly treated in clinical practice (3) partly because underlying molecular mechanisms are largely unknown. Even the most recent therapeutic innovations for NSCLC have yielded little improvement to prognosis with overall 5-year survival rates still <15% (4).

We reported previously the clinical relevance of expression of G_1-S transition regulatory molecules in prognosis, such as p53, retinoblastoma protein, $p16^{INK4A}$, and p27 in NSCLCs (5–8). We further showed that Ki-67-positive, high-level cyclin E, low-level *N*-acetylgalactosaminyl transferase-3 (GalNAcT3) and low-level *N*-acetylglucosaminyltransferase (GnT-V) are associated with shorter survival in NSCLCs (8–12). However, we did not observe any differences between SCC and adenocarcinoma.

SCC accumulates a series of genetic alterations in the progression from a normal bronchial epithelium, metaplasia, dysplasia, and carcinoma *in situ* to invasive carcinoma (13). Because most SCC develops in smokers and tobacco smoking reversibly induces metaplasia, smoking has been regarded as a major cause of SCCs (14). As diagnostic markers for SCC, SCC antigen and cytokeratin 19 fragment (CYFRA 21.1) have been widely used (15). Despite their usefulness in distinguishing between SCC and adenocarcinoma, these two molecules are hardly adequate for early detection of cancer (15). Moreover, their expression in normal squamous cell suggests that these two

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molecules are not involved in carcinogenesis and inappropriate as therapeutic targets. Thus, search for genes specific to SCC alone will lead to identification of a novel molecular target of SCC, which may help developing both early detection of SCC and personalized therapeutics of SCC.

Microarray analysis has been applied to several aspects of cancer research, including classification, mechanistic elucidation, discovery of therapeutic targets, and development of tumor makers (16–21). For example, we recently explored potential diagnostic or therapeutic markers of hepatocellular carcinoma using microarray analysis and showed that soluble glypican-3 is a novel serologic marker essential for early detection of hepatocellular carcinoma (19). Recent reports on microarray analysis of lung cancer have shown that SCC and adenocarcinoma have different gene expression signatures, suggesting involvement of distinct pathways in carcinogenesis (22, 23). In the present study, we searched for genes specifically overexpressed in SCC through microarray analysis and identified seven genes, including aldo-keto reductase family 1, member B10 (*AKR1B10*). We investigated potential relevance of AKR1B10 in NSCLCs with a newly generated monoclonal antibody and found that it is overexpressed in smokers' NSCLCs, including most cases with SCC.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Forty-five primary lung cancers (15 SCCs, 20 adenocarcinomas, and 10 SCLCs) were obtained with informed consent from patients who underwent lobectomy at Jichi Medical School Hospital (Tochigi, Japan), Cancer Institute Hospital, Japanese Foundation for Cancer Research (Tokyo, Japan), and Hokkaido University Medical Hospital (Hokkaido, Japan). All samples were immediately frozen after resection and stored at −80°C until RNA or protein was extracted. Adenocarcinoma cell lines A549, H23, H522, H1648, and H2347 were purchased from the American Type Culture Collection (Manassas, VA). SCLC cell line Lu130 and SCC cell line H157 were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan).

RNA Extraction and Microarray Analysis. Tissues or cells were directly lysed in Isogen reagent (Nippon Gene, Osaka, Japan) and homogenized. Total RNA was extracted according to manufacturer's instructions. Surgically resected lung tissues and lung cancers, including 5 SCCs, 10 SCLCs, a pooled sample made up of 12 adenocarcinomas, other 5 adenocarcinomas, and 1 normal lung, were analyzed on GeneChip HG U133 oligonucleotide arrays (Affymetrix, Santa Clara, CA) containing probes for ~40,000 human genes. Further information on the source of other RNA from normal tissues analyzed here is provided on request or is available at http://www.lsbm.org/db/index.html. Microarray analysis was done essentially as described previously (24). For global normalization, the average signal in an array was made equal to 100.

Systematic Selection of SCC-Specific Genes Based on Microarray Analysis. We systematically explored SCC-specific genes that were defined as follows: its expression level is (*a*) up-regulated in SCC but minimal in (*b*) normal lung and bronchial epithelia, (*c*) adenocarcinoma and SCLC, and (*d*) normal squamous epithelia, such as skin. Briefly, genes with a median signal score across 5 SCCs of >150 and >10 times that of normal lung were first selected. Among the 136 genes selected, genes with signal score of >150 in skin and small airway epithelial cell were omitted. We subsequently eliminated genes with signal >150 in SCLCs, adenocarcinomas, and most other normal tissues and various primary culture cells. Among 12 genes selected thus far, we additionally eliminated 5 genes that showed low expression throughout all of 40 cancer cell lines, suggesting expression by surrounding stromal cells but not by cancer cells (Table 1).

Quantitative Real-time Reverse Transcription-PCR. After digesting genomic DNA using DNase I (Invitrogen, Carlsbad, CA) cDNA was synthesized from 1 μg total RNA using SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) in 24 μL volume and diluted up to 80 μL. Quantitative real-time PCR for *AKR1B10* were done using an iCycler iQ Detection System (Bio-Rad, Hercules, CA). Reaction mixtures contained SYBR Green I nucleic acid gel stain (BMA, Rockland, ME) and primers 5′-CCCAAAGATGATAAAGGTAATGCCATCGGT-3′ and 5′-CGATCTGGAAGTGGCTGAAATTGGAGA-3′ for *AKR1B10* or 5′-AGAAGGAGATCACTGCCCTGGCACC-3′ and 5′-CCTGCTTGCTGATCCACATCTGCTG-3′ for *β-actin*. PCR condition was 1 cycle of 94°C for 3 minutes followed by 40 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. All the samples were run in triplicate, and the results were averaged. Specific amplification of *AKR1B10* was confirmed by the gel electrophoresis and melting curve analysis after PCR. The expression level of *AKR1B10* was indicated as a relative ratio of its signal to that of *β-actin* to normalize the starting amount of template cDNA. We also did semiquantitative PCR using the same condition for six pair-samples of SCC and corresponding noncancerous lung tissues.

Generation of Anti-AKR1B10 Monoclonal Antibodies. Monoclonal antibodies against AKR1B10 were generated as described previously (25). Briefly, glutathione *S*-transferase–fused

Table 1 Genes with SCC-specific overexpression

Symbol	Gene name	UniGene no.	Functional classification
AKR1B10	Aldo-keto reductase family 1, member B10	Hs.116724	Retinal reductase
ELAFIN	Elafin	Hs.112341	Protease inhibitor
AKR1C1	Aldo-keto reductase family 1, member C1	Hs.431175	Detoxification
SPRR3	Small proline-rich protein 3	Hs.139322	Structural component
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	Hs.575	Detoxification
NQO1	NAD(P)H dehydrogenase, quinone 1	Hs.406515	Activation of carcinogens
UGT1A9	UDP glycosyltransferase 1 family, polypeptide A9	Hs.375197	Detoxification

NOTE. Final seven genes selected through microarray analysis are listed. Representative function of each gene is summarized from the literature or National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

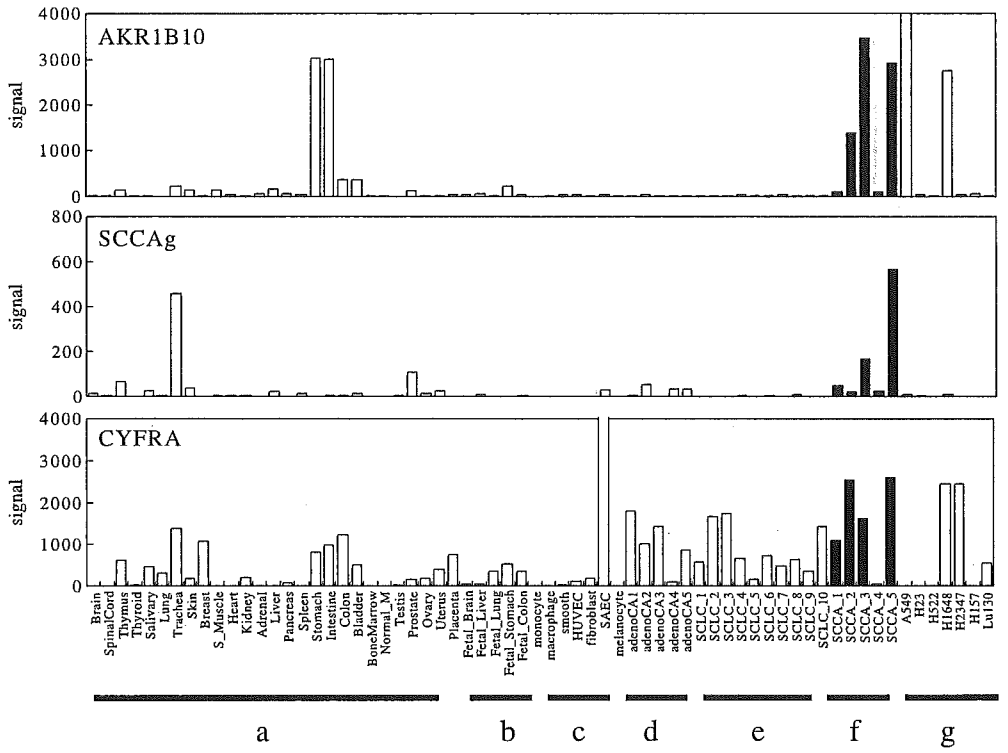

Fig. 1 Expression profiles of AKR1B10. A tissue-wide expression of AKR1B10 was displayed with CYFRA and SCCAg as references. Signal denotes gene expression level obtained from microarray analysis: (*a*) 27 normal tissues, (*b*) 5 fetal tissues, (*c*) 7 cultured normal cells, (*d*) 5 adenocarcinomas, (*e*) 10 SCLCs, (*f*) 5 SCCs, and (*g*) 7 lung cancer cell lines. *Filled columns,* SCC.

full-length AKR1B10 produced in *Escherichia coli* was immunized to female BALB/c mice. Nine clones of monoclonal hybridomas were selected by immunoblotting against recombinant AKR1B10 transiently expressed in COS-7 cells. We selected H4025 as a specific antibody in this study because a single band at around M_r 36,000 was observed only in *AKR1B10*-expressing cell lines as revealed by microarray analysis of 37 cell lines.

Immunoblot Analysis. Immunoblot analysis was done as described previously (25). Briefly, cells or tissues were lysed by 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS with protease inhibitor cocktail (Sigma, St. Louis, MO) at 4°C. H4025 (5 μg/mL) or anti-*β-actin* antibody (0.3 μg/mL, Sigma) was used as primary antibodies.

Immunocytochemistry and Confocal Microscopy Analysis. Immunostaining of culture cells were done after fixation in 4% paraformaldehyde and permeabilization in 0.2% Triton X-100 followed by incubation with 2% nonfat milk in TBS. An antibody H4025 (50 μg/mL) was applied as a primary antibody and incubated in a moist chamber at room temperature for 1 hour. The secondary staining was done with FITC-labeled anti-mouse IgG antibody (Sigma) as secondary antibody at room temperature for 1 hour. Dual-color detection by confocal laser scan microscopy (TCS SP2 system, Leica, Bensheim, Germany) was done after treatment with a 0.5 μmol/L solution of the mitochondrial stain MitoTracker Red CMXRos (Invitrogen) or the intercalator of double-strand nucleic acid stain propidium iodide (Invitrogen).

Immunostaining Analysis. Immunohistochemical analysis for AKR1B10 was done with the formalin-fixed, paraffin-embedded tissue archive at the University of Tokyo. The sections were deparaffinized in xylene, washed in ethanol, and rehydrated in TBS. Antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) at 120°C for 10 minutes following incubation with TBS with 2% nonfat dried milk. Then, H4025 (50 μg/mL) or cytokeratin 5/6 (1:500, DAKO Ltd., Cambridge, United Kingdom) was applied for 1 hour followed by the secondary staining with DAKO Envision+ reagent. All sections were counterstained with Mayer's hematoxylin. We defined AKR1B10 positive if >10% of tumor cells displayed immunoreactivity.

We first examined archival samples of the University of Tokyo to compare expression of AKR1B10 and that of keratin 5/6 in NSCLCs, squamous epithelia of skin and esophagus, alveolar epithelium, and bronchus. We have analyzed previously 217 primary NSCLC specimens for expression of cyclin E, Ki-67, Bcl-2, p53, retinoblastoma protein, p27, GalNAcT3, and GnT-V (8, 9, 11, 12). Among these, we next examined 101 NSCLCs, which were classified into 32 SCCs, 65 adenocarcinomas, and 4 adenosquamous cell carcinomas according to WHO criteria (26). Clinicopathologic features are summarized in Table 3. The postsurgical pathologic tumor-node-metastasis stage was determined according to the guidelines of the American Joint Committee on Cancer (27). The Medical Ethical Committee of Hokkaido University School of Medicine approved this immunohistochemical study.

Statistical Analysis. We analyzed the statistical significance of the relationship between the expression of AKR1B10 and clinicopathologic variables by χ^2 test, Yates χ^2 test, or Fisher's exact test as appropriate. We also analyzed the associations between AKR1B10 expressions and the cyclin E or Ki-67 labeling index (%; refs. 8, 9) by Student's *t* test.

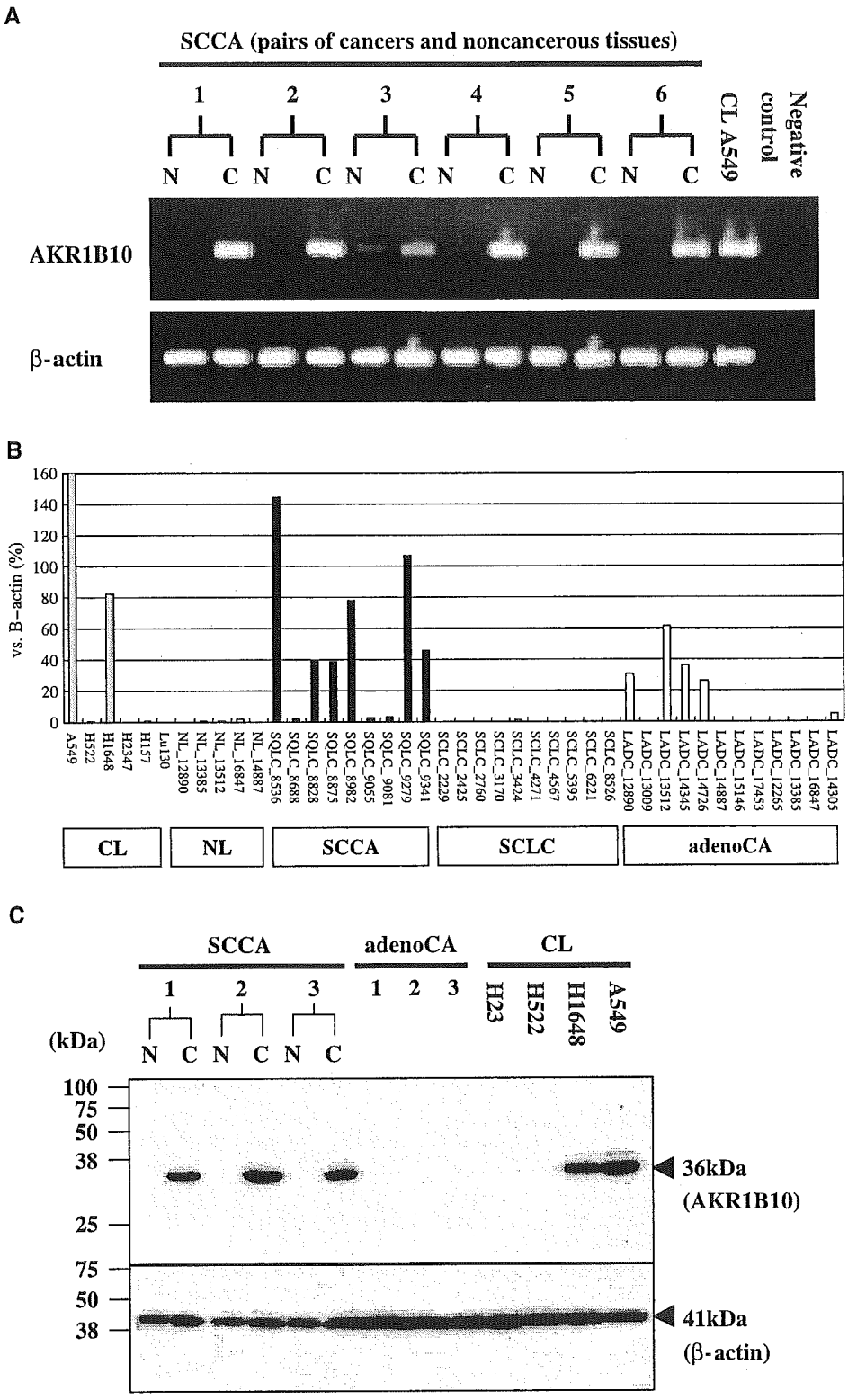

Fig. 2 Overexpression of AKR1B10 in SCC. *A,* semiquantitative PCR using six pairs of SCC and noncancerous lung tissues. Note that *AKR1B10* was up-regulated in all paired samples. *B,* quantitative real-time PCR. Examined samples were 9 SCCs, 12 adenocarcinomas, 10 SCLCs, 6 lung cancer cell lines, and 5 normal lung tissues. Note expression level of *AKR1B10* was remarkably high in 6 SCCs compared with 4 adenocarcinomas. *C,* immunoblot analysis of AKR1B10. A 36-kDa protein was detected in SCCs and AKR1B10-expressing cell lines H1648 and A549. *N,* normal lung tissues; *C,* cancer tissues; *CL,* cell line.

We additionally used multiple regression analysis to extract factors responsible for AKR1B10 expression in NSCLCs and adenocarcinomas alone. Sex, age, smoking history, differentiation, pT classification, pN classification, survival time, histology, cyclin E, Ki-67, GalNAcT3, and GnT-V were used as independent variables and AKR1B10 expression as a dependent variable. Differences were considered significant at $P < 0.05$. We simultaneously examined the correlation coefficient and the partial correlation coefficient between AKR1B10 expression and smoking or sex.

RESULTS

Microarray Analysis Identifies Seven Genes Specifically Up-Regulated in SCC. We selected seven potential SCC-specific genes (see Materials and Methods) using microarray analysis (Table 1). Tissue-wide expression profiles of these genes showed their high specificity compared with two widely used diagnostic markers of SCC, SCC antigen and CYFRA 21.1, suggesting robustness of our selection for SCC-specific genes (Fig. 1; Supplementary Fig. 1). Among these seven genes, *AKR1C1*, *ELAFIN*, *NQO1*, and *UGT1A9* were reported previously as potential target genes for detection or therapy against lung cancer (28–31); *SPRR3* is overexpressed in epidermal SCC (32); and *ALDH3A1* was reported to be involved in metabolism of tobacco carcinogens (33). Overexpression of *AKR1B10* has not been reported previously in lung cancer; then, we investigated whether it represents a good molecular target of SCC.

Overexpression of AKR1B10 in SCC. To confirm array data, we first did semiquantitative PCR. Overexpression of *AKR1B10* in SCC was observed in six pair-samples analysis (Fig. 2*A*). We subsequently investigated expression level of *AKR1B10* across lung cancer tissues by quantitative reverse transcription-PCR. Overexpression of *AKR1B10* was observed in 6 of 9 (67%) SCCs but not in SCLCs or normal lung tissues (Fig. 2*B*). Expression of *AKR1B10* was also observed in 4 of 12 (33%) adenocarcinomas, although its level was not so high as in SCC (Fig. 2*B*).

Next, we investigated expression of AKR1B10 protein by immunoblot analysis with a newly generated monoclonal anti-AKR1B10 antibody, H4025. As for three pair-samples of SCC used in semiquantitative PCR above, AKR1B10 was observed only in cancerous tissues, whereas AKR1B10 was negative in 3 adenocarcinomas (Fig. 2*C*).

Comparison of AKR1B10 with Pan-Squamous Cell Marker Keratin 5/6. As a SCC marker, keratin 5/6 is widely used based on its specificity to squamous cells. Unique feature of AKR1B10 as we identified in our selection is that it is not a merely squamous cell–specific marker unlike keratin 5/6 but a SCC-specific marker. To highlight the difference in "specificity" of these two molecules, we compared their expression in NSCLCs and normal tissues, including squamous epithelia of skin and esophagus, alveolar epithelium, and columnar epithelia of bronchus (Table 2). Keratin 5/6 staining was observed in normal squamous epithelia, columnar epithelia, and 83% of SCCs but not in adenocarcinoma. In contrast, AKR1B10 staining was observed in 64% of SCC and 30% of adenocarcinoma but not in normal epithelia (Table 2).

Distinct Localization of AKR1B10 in SCC and Adenocarcinoma. As we described above, analysis in larger number of samples revealed that AKR1B10 was expressed not only in most cases of SCC but also in a subset of adenocarcinoma. To investigate relevance of AKR1B10 in NSCLCs, we subsequently did immunohistochemical analysis in 101 primary NSCLCs, including 65 adenocarcinoma (Table 3). AKR1B10 staining was observed in 27 of 32 (84.4%) SCCs but also in 19 of 65 (29.2%) adenocarcinomas (Table 3). In adenosquamous cell carcinomas, AKR1B10 staining was observed in 2 of 4 cases and restricted to SCC components of these 2 cases (data not shown). AKR1B10 was preferentially observed in cancer cells with obvious squamous differentiation in SCC (Fig. 3*A-E*), whereas with lower differentiation grade in adenocarcinoma (Fig. 3*G*). Interestingly, we occasionally observed AKR1B10 staining in lesions with metaplasia: squamous metaplasia (Fig. 3*I*) and transitional cell metaplasia (Fig. 3*J*) in noncancerous areas (Fig. 3*K*) of one smoker's SCC specimen. We seldom detected positive staining in noncancerous portion of lung tissue, except two cases in normal bronchial epithelia of smokers (Supplementary Fig. 2A and B).

Table 2 Expression of AKR1B10 and keratin 5/6 in NSCLCs and normal epithelia

		AKR1B10	Keratin 5/6
NSCLCs	SCC ($n = 23$)	Positive (61%)	Positive (83%)
	Adenocarcinoma ($n = 24$)	Positive (33%)	Negative (0%)
Normal epithelia	Pulmonary alveoli ($n = 3$)	Negative	Negative
	Bronchial epithelia ($n = 3$)	Negative	Positive
	Squamous epithelia		
	Skin ($n = 3$)	Negative	Positive
	Esophagus ($n = 3$)	Negative	Positive

AKR1B10 staining was mainly observed in cytoplasm of cancer cells but also in nucleus in a subset of cells (Fig. 3*E* and *F*). Notably, two cases had apparent AKR1B10 staining mainly in nuclei (Fig. 3*M*). These results were essentially confirmed in confocal microscopy analysis of A549 cells. AKR1B10 was generally localized in cytoplasm, neither in nucleus nor in mitochondria in most cells. However, a subset of cells had additional staining in nucleus in 70% confluency (Fig. 3*L* and *M*) but not in full confluency (Fig. 3*N*).

Correlation between AKR1B10 Overexpression and Smoking History in NSCLC and Adenocarcinoma. To clarify the factors that correlate with AKR1B10 immunostaining, we carried out a statistical analysis that examined a variety of clinicopathologic variables and the expression of molecules that we reported previously (refs. 8, 9, 11, 12; Table 3). We observed positive correlations between AKR1B10 overexpression and SCCs (χ^2 test, $P < 0.0001$) and smoking (χ^2 test, $P < 0.0001$) in NSCLCs. AKR1B10 overexpression was observed in 40 of 61 (65.6%) smokers' NSCLCs. The correlation coefficient between AKR1B10 overexpression and smoking was 0.47 in NSCLCs. Partial correlation coefficient was 0.41 even after removing the effect of positive correlation between AKR1B10 overexpression and male ($P < 0.05$). These results indicate the significant correlation between AKR1B10 overexpression and smoking.

Univariate analysis in NSCLCs also showed that AKR1B10 was overexpressed in tumors with high pT classification ($P < 0.05$). Additionally, AKR1B10-positive cases had a higher Ki-67 expression ($P < 0.001$), higher cyclin E expression ($P < 0.01$), lower GalNAcT3 expression ($P < 0.01$), and lower GnT-V expression ($P < 0.05$) than negative cases in NSCLCs. Student's t test revealed that there was a significant difference between AKR1B10 expression and Ki-67 expression ($P < 0.005$) and cyclin E expression ($P < 0.05$) in NSCLCs.

Multiple regression analysis showed that smoking ($P < 0.01$), SCC ($P < 0.01$), and lower GalNAcT3 ($P < 0.05$) were important independent variables responsible for AKR1B10 overexpression in NSCLCs (Table 4). We subsequently analyzed only adenocarcinomas ($n = 65$) because most SCCs were AKR1B10 positive (84.4%) and smokers (96.9%). Interestingly, there was still a remarkable correlation (χ^2 test, $P < 0.01$) between AKR1B10 overexpression and smoking in adenocarcinomas (Table 3). Moreover, it was also shown that smoking was the only important independent variable responsible for AKR1B10 expression in adenocarcinomas ($P < 0.01$; Table 4).

DISCUSSION

Aldo-keto reductases are NAD(P)H-dependent oxidoreductases that catalyze the reduction of a variety of carbonyl compounds (34). AKR1B10 is a member of this superfamily and reduces aromatic and aliphatic aldehyde substrates (34). Reportedly, AKR1B10 mRNA shows expression in adrenal gland, small intestine, and colon, consistent with its putative physiologic roles in steroid metabolism or detoxification of reactive aldehydes in the digested food in intestinal tract (34–36).

Initial goal of our study was to identify SCC-specific molecules, distinct from currently used SCC markers that are specific to squamous cell in general. We eliminated these squamous cell marker genes through our selection and identified *AKR1B10* as a gene highly specific to SCC but not to squamous cells in general. AKR1B10 was expressed in as many as 90% of SCC of the lung but not in normal bronchial epithelium and squamous epithelium from skin and esophagus. This unique feature of AKR1B10 is highlighted when we compared the results of immunohistochemical analysis using AKR1B10 and keratin 5/6 (Table 2). AKR1B10 was highly specific to SCC when SCC and normal epithelia were analyzed by immunohistochemistry, although its specificity and sensitivity for SCC among NSCLCs were lower than those of keratin 5/6.

In the present study, we showed that AKR1B10 is overexpressed in SCC, which is closely associated with smoking. Additionally, we found AKR1B10 expression even in metaplasia, which is also associated with smoking and regarded as precancerous lesions of SCC (37, 38). Unexpectedly, nearly one third of the cases of adenocarcinomas expressed AKR1B10, but it was revealed by multiple regression analysis that smoking was the most important determinant of AKR1B10 expression in adenocarcinomas. Adenocarcinomas can be clustered into several subclasses based on reported expression profiling (22, 23). Together with recent reports that ~40% of adenocarcinomas occur in

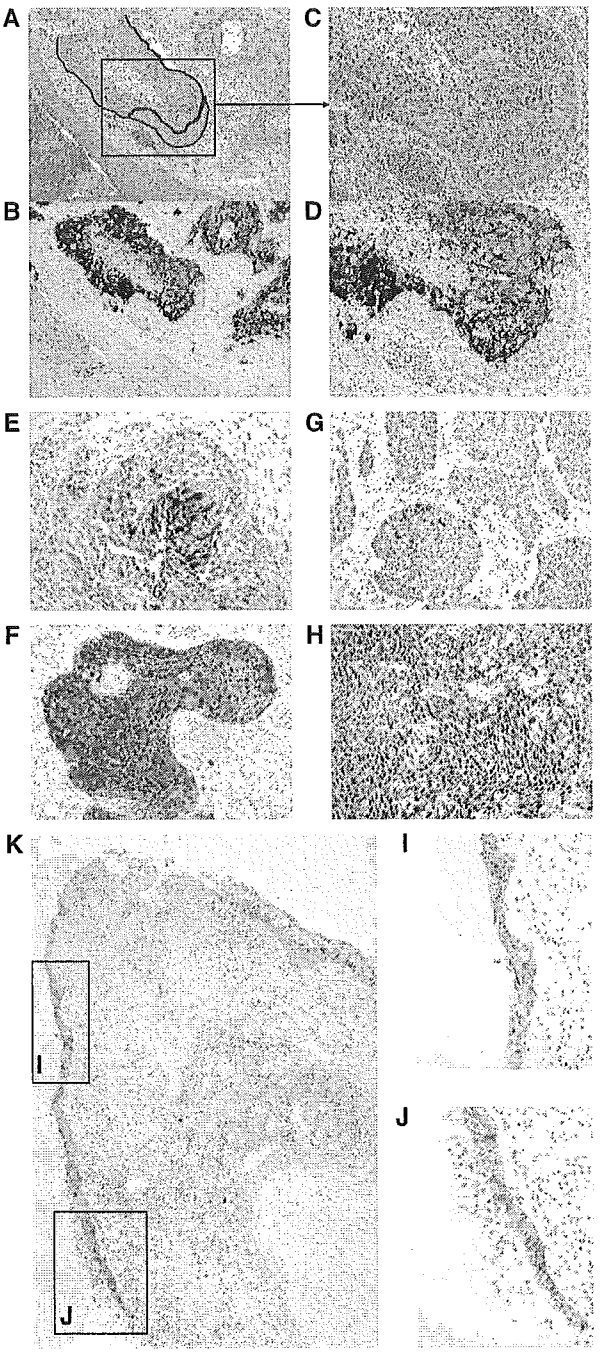

Fig. 3 Immunohistochemical analysis of AKR1B10. *A-E,* two representative cases in SCC. H&E staining, (*A*) ×20 and (*C*) ×100. Cancerous regions with obvious (*red line*) and no (*blue line*) squamous differentiation. Corresponding staining of the same sample (*B* and *D*) and another sample (*E*) by H4025. Note that AKR1B10 is stained in regions with squamous differentiation. *F* and *G,* two representative cases in adenocarcinoma. Homogenous staining was observed in some cases (*F*), whereas preferential staining in regions with lower differentiation was observed in most cases (*G*). *H,* typical case with nuclear staining in SCC (×100). *I-K,* AKR1B10 staining in metaplasia of a smoker. *I,* squamous metaplasia: (*left*) ×20 and (*right*) ×100. *J,* transitional cell metaplasia: (*left*) ×20 and (*right*) ×100. Note that these metaplastic regions are observed successively in noncancerous regions of a case with SCC (*K*).

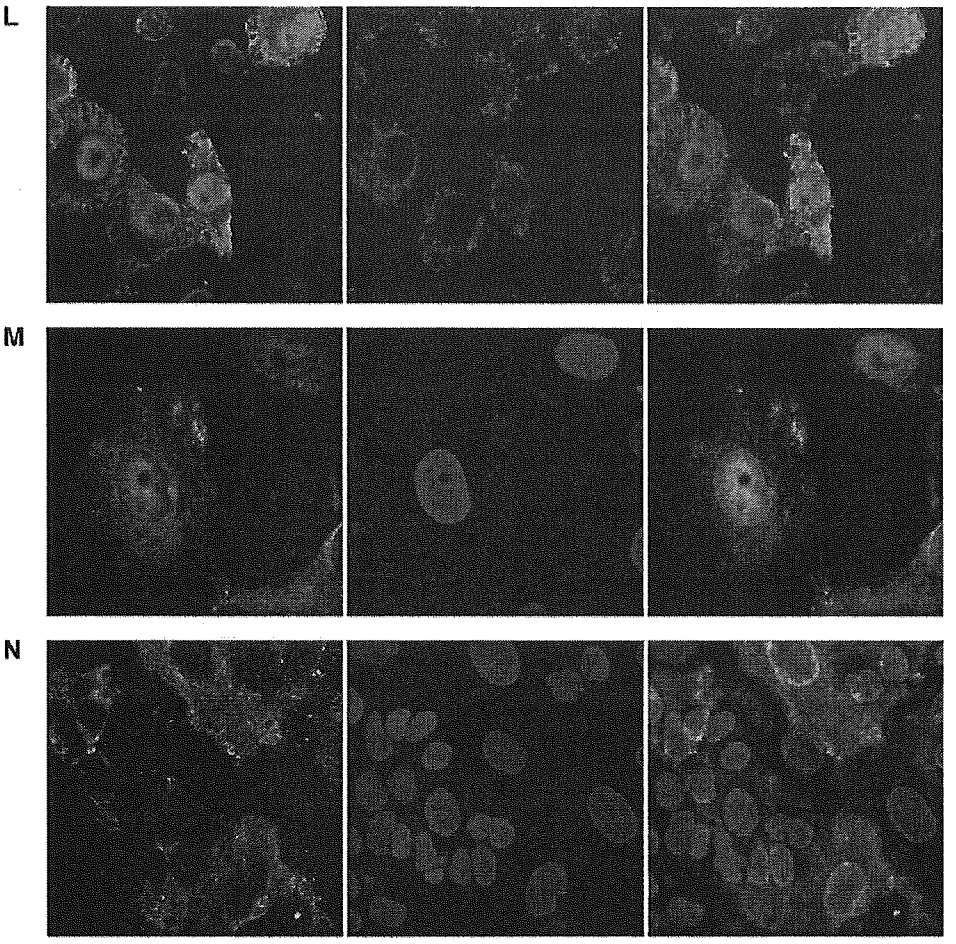

Fig. 3 Continued L-N, subcellular localization of endogenous AKR1B10 in A549 cells in 70% (*L* and *M*) and 100% (*N*) confluency. *Left,* AKR1B10; *middle,* MitoTracker (*L*) or propidium iodide (*M* and *N*); *right,* merged image. Note nuclear staining (*L* and *M*) has disappeared in 100% confluency (*N*).

smokers (39), there is a possibility that AKR1B10 could characterize a subset of adenocarcinoma associated with smoking. Based on our results, AKR1B10 immunostaining could be applied to the early detection of cancer cells or atypical cells in sputum, especially in heavy smokers.

Then, what could be potential roles of AKR1B10 in multistep carcinogenesis of SCCs? There are two possibilities as follows: one is that AKR1B10 may be related to cell proliferation. There was a positive correlation between AKR1B10 expression and putative poor prognosis factors, such as high Ki-67, high cyclin E, low GalNAcT3, and low GnT-V in NSCLCs (8, 9, 11, 12). Moreover, AKR1B10 was localized in nucleus in a fraction of cancer cells in subconfluent culture conditions, which disappeared under confluent culture, suggesting that AKR1B10 translocates during cell cycle and is involved in the regulation of cell cycle in a fashion yet identified.

Another possibility is that AKR1B10 promotes carcinogenesis of SCC through its enzymatic activity that counteracts the conversion of β-carotene to retinoic acid (40). Retinoic acid induces potent differentiation and growth-suppressive effects in diverse premalignant and malignant cells (41). In lung, deficiencies of retinoids are reported to cause hyperplasia and squamous metaplasia of airway epithelium (42) that can be suppressed by retinoic acid (43). Through the analysis of many cancer samples, we noticed positive staining of AKR1B10 even in some cases with metaplasia, precancerous lesion of SCC. Because the number of samples that contained metaplasia was small in the present study, this result was further investigated by another study focusing on idiopathic pulmonary fibrosis, which showed that squamous metaplasia was positive for AKR1B10 in 23 cases of 56 squamous metaplasia lesions.[10] These results strongly suggest that AKR1B10 expression is positive in precancerous lesions and may down-regulate retinoic acid, which could lead to carcinogenesis of SCC. Considering that AKR1B10 is an enzyme related to detoxification and that some smokers' bronchial epithelia without metaplasia were positive for AKR1B10 staining, AKR1B10 may be directly induced by some chemical compounds in tobacco, which should be further investigated. Interestingly, we also observed frequent overexpression of AKR1B10 in SCC of the laryngopharynx and esophagus that is closely associated with smoking and occasional overexpression of esophageal dysplasia and

[10] Fukayama et al., in preparation.

hyperplasia.[10] Remarkably high frequency of its up-regulation specific to SCC warrants further investigation of AKR1B10 in carcinogenesis of SCC.

Various retinoids, including β-carotene, have been shown previously effective for the treatment and prevention of several cancers, including carcinoma of the breast, skin, and kidney (44–49). However, clinical chemoprevention trials of lung cancer by β-carotene have failed to show its effectiveness. Moreover, administration of β-carotene unexpectedly promoted tumorigenesis in smokers (50, 51). Molecular mechanism underlying these adverse effects is currently unknown, but up-regulation of AKR1B10 in precancerous lesions in the bronchial epithelium of smokers may partly explain ineffectiveness of β-carotene observed in the lung.

AKR1B10 was also overexpressed in adenocarcinoma of smokers. Its staining was observed in undifferentiated region in contrast to SCC with staining in differentiated region. Together with its overexpression in hepatocellular carcinomas (34, 36), AKR1B10 may be related to another carcinogenic pathway distinct from that of SCC.

Table 3 Clinicopathologic features correlated to AKR1B10 overexpression

		AKR1B10				AKR1B10		
Characteristics	NSCLC	Positive	Negative	*P*, χ^2 test	Adenocarcinoma	Positive	Negative	*P*, χ^2 test or Fisher test
Age (y)								
Median (range)	63 (31-85)							
<65	58	28	30	NS	37	9	28	NS
≥65	43	20	23		28	10	18	
Sex								
Male	63	36	27	<0.05	30	10	20	NS
Female	38	12	26		35	9	26	
Histology								
Squamous	32	27	5	<0.0001				
Adenocarcinoma	65	19	46					
Adenosquamous	4	2	2					
Differentiation (SCC)								
Poor	14	9	5	<0.05	10	2	8	NS
Moderate/well	15	15	0		49	15	34	
Smoking								
Smoker	61	40	21	<0.0001	30	14	16	<0.01
Nonsmoker	36	6	30		35	5	30	
pT classification								
T_1	33	11	22	<0.05	21	4	17	NS
T_2-T_3	68	37	31		44	15	29	
pN classification								
N_0	56	28	28	NS	36	13	23	NS
N_1-N_3	45	20	25		29	6	23	
Ki-67								
High labeling index*	51	33	18	<0.001	22	11	11	<0.05
Low labeling index†	48	14	34		41	7	34	
Cyclin E								
Positive	76	42	34	<0.01	41	13	28	NS
Negative	24	5	19		23	5	18	
Bcl-2								
Positive	14	8	6	NS	6	2	4	NS
Negative	29	9	20		22	3	19	
p27								
Positive	87	42	45	NS	53	14	39	NS
Negative	9	4	5		8	3	5	
p53								
Positive	28	13	15	NS	16	4	12	NS
Negative	17	5	12		13	1	12	
Retinoblastoma protein								
Positive	31	15	16	NS	18	5	13	NS
Negative	12	2	10		11	1	10	
GalNAcT3								
Positive	63	21	42	<0.01	49	11	38	NS
Negative	34	24	10		14	7	7	
GnT-V								
High‡	47	17	30	<0.05	39	10	29	NS
Low§	52	31	21		24	9	15	

NOTE. NS, not significant.
*≥30% of cancer cells stained.
†<30% of cancer cells stained.
‡50% of cancer cells stained.
§<50% of cancer cells stained.

Table 4 Multiple regression analysis for AKR1B10

	NSCLCs			Adenocarcinomas		
Characteristics	Regression coefficient	*P*	95% Confidence interval	Regression coefficient	*P*	95% Confidence interval
Age	0	0.432	−0.15 to 0.006	−0.01	0.34	−0.02 to 0.007
Sex	−0.19	0.191	−0.474 to 0.096	−0.23	0.13	−0.534 to 0.071
Histology	−0.31	0.004	−0.518 to −0.1			
Differentiation	−0.05	0.694	−0.286 to 0.191	−0.01	0.959	−0.285 to 0.271
Smoking	0.425	0.004	0.143 to 0.707	0.387	0.01	0.0962 to 0.677
pT classification	−0.02	0.763	−0.167 to 0.123	−0.15	0.095	−0.322 to 0.026
pN classification	0.022	0.727	−0.101 to 0.144	0.145	0.088	−0.022 to 0.313
Ki-67	2E−04	0.929	−0.004 to 0.004	−0.01	0.077	−0.011 to 6E−04
Cyclin E	0	0.652	−0.004 to 0.003	0.002	0.424	−0.003 to 0.006
GalNAcT3	−0.23	0.032	−0.483 to −0.02	−0.111	0.507	−0.423 to 0.212
GnT-V	−0.03	0.483	−0.111 to 0.053	0.007	0.889	−0.095 to 0.11
Survival time	2E−06	0.948	−7E−05 to 8E−05	4E−05	0.383	−6E−05 to 1E−04

In summary, we showed that AKR1B10 is overexpressed in most SCCs and in adenocarcinomas that developed in the lung of smokers. Considering its involvement in retinoic acid metabolic pathway, AKR1B10 could be not a mere surrogate marker but a molecule relevant in smoking-related NSCLCs. Elucidation of its roles in carcinogenesis will be required to evaluate AKR1B10 as a therapeutic target in addition to a potential marker of SCC for diagnosis as shown in this study.

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Analysis of the Response and Toxicity to Gefitinib of Non-small Cell Lung Cancer

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Abstract. *Background: Gefitinib is an oral agent that inhibits the tyrosine kinase of epidermal growth factor receptor (EGFR), which had antitumor activity in patients with previously treated non-small cell lung cancer (NSCLC). We analyzed the efficacy, toxicity and overall survival time of gefitinib treatment in patients with NSCLC. Patients and Methods: One hundred and twenty-two patients with NSCLC, who received gefitinib between 2002 and 2004 in our institutes, were evaluated retrospectively. Results: The objective response rate was 24.6%. The variables identified as significant in univariate analysis included gender and smoking habit. The median overall survival time was 14.4 months. Significant variables associated with improved survival included good performance status (PS), female, adenocarcinoma and never smoked status, while never smoked status and good PS were independent prognostic factors in multivariate analysis. Four patients (3.3%) developed interstitial pneumonitis associated with gefitinib. Conclusion: Gefitinib showed favorable anti-tumor activity in females, never smokers and adenocarcinoma.*

Lung cancer is one of the most common fatal malignancies, with an increasing worldwide incidence (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. The current standard of care for patients with locally advanced or metastatic NSCLC is systemic chemotherapy with a two-drug combination regimen that includes a platinum-based chemotherapy (2). Although platinum-based chemotherapy offers modest efficacy and survival advantage over best supportive care (BSC) alone for chemotherapy-naive patients with advanced NSCLC, the overall 5-year survival rate after diagnosis remains at 10-15% due to frequent recurrence and metastasis of the NSCLC (1-4). Docetaxel is currently approved as the second-line chemotherapy on the basis of randomized trials in patients who failed on previous chemotherapy (5, 6). The objective response rates of docetaxel are only 5-10%, associated with a modest survival improvement. No agent has produced a tumor response in more than 5% of patients in the third-line treatment setting. In addition, elderly patients with poor performance status (PS) or patients with complications can not receive standard chemotherapy due to the toxicity associated with anticancer drugs. Thus, there is need for new therapies with novel mechanisms of action that are well tolerated, effective and convenient.

The epidermal growth factor receptor (EGFR) is a type I family of transmembrane glycoproteins and regulates the growth of both epithelial and non-epithelial cell types. EGFR is highly expressed in NSCLC and the association between EGFR expression and poor patient prognosis has been extensively documented (7, 8). Because activation of EGFR enhances the processes responsible for tumor growth and progression, including the promotion of proliferation,

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Key Words: Non-small cell lung cancer, gefitinib.

angiogenesis, and invasion/metastasis and inhibition of apoptosis, EGFR is a rational molecular target for antitumor therapy and many strategies which are currently being developed (9, 10).

Gefitinib (IressaR, ZD1839; AstraZeneca, Wilmington, DE, USA) is an oral inhibitor of the intracellular tyrosine kinase domain of EGFR, which blocks signal transduction pathways implicated in the proliferation and survival of cancer cells (11, 12). In phase I trials, gefitinib was generally tolerated and confirmed partial responses or survival prolongation were seen in some patients (13, 14). Subsequently, two phase II trials, IDEAL 1 and IDEAL 2, confirmed that response occurred in 12% to 18% in previously treated NSCLC patients receiving 250 mg/day gefitinib, and that gefitinib showed significant antitumor activity (15, 16). However, some patients who received gefitinib developed severe acute interstitial pneumonitis as an adverse event and more detailed investigation is thought to be required (17).

In the present study, we retrospectively analyzed response rates, survival time and toxicity in patients with NSCLC at our institutes who were given 250 mg/day gefitinib. In addition, we compared the relationship between response or survival time and clinical features.

Patients and Methods

Patients and treatment. Between July 2002 and March 2004, 122 patients (66 males and 56 females) with histologically or cytologically confirmed NSCLC received 250 mg/day gefitinib orally at our institutes (Hokkaido University Hospital, National Hospital Organization Hokkaido Cancer Center, Sapporo City General Hospital and Fukushima Medical University Hospital, Japan). Those records were analyzed retrospectively. The histopathological classification was based on WHO criteria for histopathological classification (18) and Tumor-Node-Metastasis (TNM) was determined based on the American Joint Committee on Cancer guidelines (19). Patients, who had recurrent or refractory disease after surgery, radiotherapy or prior systemic chemotherapy, or who could not tolerate standard systemic chemotherapy as judged by a physician, were included. Patients continued treatment until disease progression, no efficacy, intolerable toxicity, or withdrawal of consent. Patients received no systemic anticancer treatment during treatment with gefitinib.

Efficacy. We assessed objective tumor response as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) by repeat radiographic imaging in accordance with the Response Evaluation Criteria in Solid Tumors Group (RECIST) guidelines (20). Disease control was defined as CR, PR, or SD. Patient responses were defined as Unknown if they died of progressive disease or they stopped gefitinib due to adverse events within 10 days. The responses of patients in whom efficacy could not be assessed, due to short duration of observation, were also defined as Unknown. Tumor dimensions were assessed every 4 weeks by radiographic examinations. PR was sustained for 4 weeks or longer and SD was sustained for 8 weeks or longer.

Table I. *Patient characteristics.*

Characteristics	No. of patients	%
Number of patients	122	
Gender		
Male	66	54
Female	56	46
Age, Years		
Median	64	
Range	31-84	
Performance status		
0	15	12.3
1	70	57.4
2	23	18.8
3	8	6.6
4	6	4.9
Stage		
I~II	4	3.3
IIIA	6	4.9
IIIB	15	12.3
IV	76	62.3
Recurrence after surgery	21	17.2
Tumor histology		
Adenocaricinoma[a]	97	79.5
Squamous cell carcinoma	18	14.8
Large cell carcinoma	3	2.5
Other NSCLC	4	3.2
Previous chemotherapy		
0 regimen	20	16.4
1 regimen	40	32.8
2 regimens	39	31.9
More than 3 regimens	23	18.9
Surgery	31	25.4
Radiation	15	12.3
Smoking habit		
Never	48	39.3
Current	23	18.9
Former	51	41.8

[a]One patient with bronchiolar carcinoma was included in this group.

Toxicity. Toxicity was evaluated based on the National Cancer Institute Common Toxicity Criteria, version 2, every 2 weeks or 4 weeks.

Overall survival. Overall survival was defined as the period from the date of start of gefitinib to the date of tumor-related death. Living patients at data cut-off were censored at the last date that they were confirmed to be alive.

Statistical analysis. The associations between response and patient characteristics were statistically analyzed using Fisher's exact test as appropriate using Statview software (version 5.0; SAS Institute, CA, USA). Those variables that were significant in the univariate analysis were included in a multivariable logistic regression analysis. The progression-free survival curves and survival curves were estimated by the Kaplan-Meier method for estimation and the log-rank test for comparing the characteristics of patients. Those variables that were significant in the univariate analysis were

Table II. *Best overall objective responses.*

Response	Number of patients	(%)
Complete response (CR)	0	0
Partial response (PR)	30	24.6
Stable disease (SD)	35	28.7
Progressive disease (PD)	47	38.5
Unknown[a]	10	8.2

[a]No conclusion was achieved about best overall tumor response due to the short duration of observation, death from progressive disease within 10 days, or cessation of gefitinib by adverse events within 10 days.

included in a multivariable Cox proportional hazard model. Values of $p<0.05$ were considered to indicate statistical significance and all tests were two-tailed.

Results

Patients. We assessed 122 patients who received 250 mg/day gefitinib. Table I shows the baseline patient characteristics. The most common tumor histology was adenocarcinoma, including 1 patient with bronchioloalveolar carcinoma. The median PS of all patients was 1 (range 0-4), the median number of chemotherapy regimens was 2 (range, 0-4), and the median duration of treatment for all 122 patients who consented to taking gefitinib was 2.6 months (range, 0-21.7 months).

Efficacy. The best overall tumor responses are shown in Table II. PR was observed in 30 patients (24.6%), SD in 35 patients (28.7%) and PD in 47 patients (38.5%). No CR was achieved. In 10 patients, tumor response could not be determined. Three patients died of progressive diseases within 10 days of gefitinib administration, 2 patients ceased gefitinib due to adverse events (diarrhea and nausea) within 10 days, and in 5 patients efficacy could not be defined due to the short duration of observation. The objective response rate was 24.6% and disease control rate was 53.3%.

Female patients showed a higher response rate (37.5%) than male patients (13.6%) ($p<0.01$) and those who had never smoked showed a higher response rate (41.7%) than current or former smokers (13.5%) ($p<0.01$) (Table III). Although the tumor response in patients with adenocarcinoma tended to be higher than that in patients with other histological types, in the univariate analysis, no statistical difference was observed (27.8% *vs.* 12%, $p=0.1$). Furthermore, no statistical differences were observed between tumor response and gender or smoking habit in the multivariate analysis, although the never smoked group tended to show favorable tumor response (odds ratio of 2.5, 95% CI, 0.89 to 7.21; $p=0.08$) (data not shown). On the other hand, there were 20 patients who had no previous chemotherapy before treatment with gefitinib. PR was observed in 5 patients (25.0%), SD was observed in 7 patients (35.0%), and PD was observed in 6 patients (30.0%) (Table III).

Table III. *Univariate analysis of characteristics associated with response to gefitinib.*

Characteristics	Number of patients[a]	(%)	*P*
Gender			
Male	9/66	13.6	<0.01[b]
Female	21/56	37.5	
Age			
75<	24/102	23.5	
75≥	6/20	30	0.54[c]
Tumor histology			
Adenocarcinoma	27/97	27.8	
Non-adenocarcinoma	3/25	12	0.1[b]
Smoking habit			
Never	20/48	41.7	
Current / Former	10/74	13.5	<0.01[b]
Number of previous chemotherapy regimens			
0	5/20	25.0	
1	12/40	30	
2-4	13/62	21	0.63[b]
Performance status			
0-2	28/108	27.5	
3-4	2/14	14.3	0.34[b]

[a]The number of patients who showed PR/ the number of total patients. [b]Fisher's exact test and [c]*t*-test

The duration of treatment for patients with PR or SD who consented to take gefitinib was 7.6 months (range, 1 ~ 21.7 months) and 18 patients were treated with gefitinib for more than 10 months (data not shown). Although 5 out of 18 patients with long duration of treatment stopped gefitinib due to progression of disease and one patient stopped gefitinib due to adverse event, the remaining patients still had PR or SD at the data cut-off and treatment with gefitinib was continued.

Survival. In all 122 patients, the median overall survival was 14.4 months. The 1-year survival rate was 50% (Figure 1). Female patients had significantly better survival compared to male patients ($p<0.01$) (Figure 2A). Survival of those who had never smoked was significantly longer than that of current or former smokers ($p<0.01$) (Figure 2B). Patients with adenocarcinoma had significantly better survival than