Previous studies indicated enhanced kinase activity and transformation capabilities of EGFR in the presence of L858R or exon 19 deletion mutation.[27,28] Crystal structure analysis of the L858R mutant EGFR showed that this substitution activates the kinase through disruption of autoinhibitory interactions, resulting in receptors with high kinase activity compared with wild-type EGFR.[10,29,30] Consistent with these observations, mutant EGFR was constitutively phosphorylated, while the levels were varied among cell lines used in the present study. Taken together, these results indicate that Aki1 binds constitutively with mutant EGFR because mutant EGFR is constitutively activated.

There is accumulating evidence that scaffold proteins maintain signaling specificity and facilitate the activation of pathway components.[24,31,32] We showed that Aki1 constitutively forms complexes with EGFR, PDK, and Akt in *EGFR* mutant lung cancer cells. As in EGFR non-mutated cancer cells,[25] Aki1 did not bind to IGF-1 R even after stimulation with IGF-1 in *EGFR* mutant cells, indicating that Aki1 is the determinant of receptor signaling selectivity for EGFR. In a phase II clinical trial, anti-IGF-1 R antibody improved the response rate of conventional chemotherapy in non-small-cell lung cancer.[33] However, the phase III trial was terminated because of a trend toward poorer overall survival in the group with anti-IGF-1 R antibody. A preliminary report of toxicity from a phase II trial with the anti-IGF-1 R antibody demonstrated severe adverse events, including hyperglycemia.[34] These findings indicated difficulty of targeting IGF-1 R in cancer. As Aki1 is an EGFR-selective scaffold protein, Aki1 inhibition may have advantage over non-selective inhibition of IGF-1 R or its downstream PI3K/Akt pathway in terms of safety.

*EGFR*-T790M gatekeeper mutation is associated with 50% of cases of acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer.[4,8,9] Recently, mutant-selective EGFR-TKIs were developed, which inhibit EGFR with not only activating mutations, such as exon 19 in-frame deletion and L858R point mutation, but also T790M resistant mutation.[35] As the inhibitors were reported to have less activity for non-mutated EGFR, they may overcome T790M-mediated resistance and reduce adverse events, including skin toxicity. We found not only that Aki1 inhibition further augmented the efficacy of mutant EGFR-selective TKI and irreversible EGFR-TKI, but also that Aki1 constitutively associated with EGFR regardless of treatment with EGFR-TKI. In addition, Aki1 was detected in all tumors with acquired resistance, including tumors with *EGFR* T790M mutation. Our findings indicated the necessity of development of efficient Aki1 inhibitors, and suggested that combined use of Aki1 inhibitors may increase the therapeutic effects and may reduce adverse events concerning EGFR blockade of new generation EGFR-TKIs in *EGFR* mutant lung cancer.

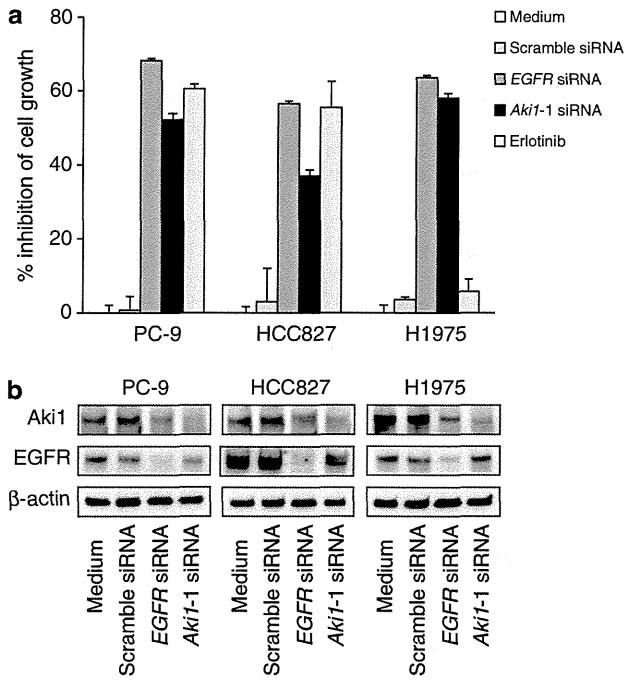


**Figure 3.** Comparison of efficacy between Aki1 knockdown and EGFR inhibition on cell viability. Cells were treated with *Aki1*-1 siRNA, *EGFR* siRNA, control scramble siRNA or erlotinib (1 μM). (**a**) After 72-h incubation, cell viability was determined by MTT assay. (**b**) After 24-h incubation, cells were lysed and the indicated proteins were detected by western blotting.

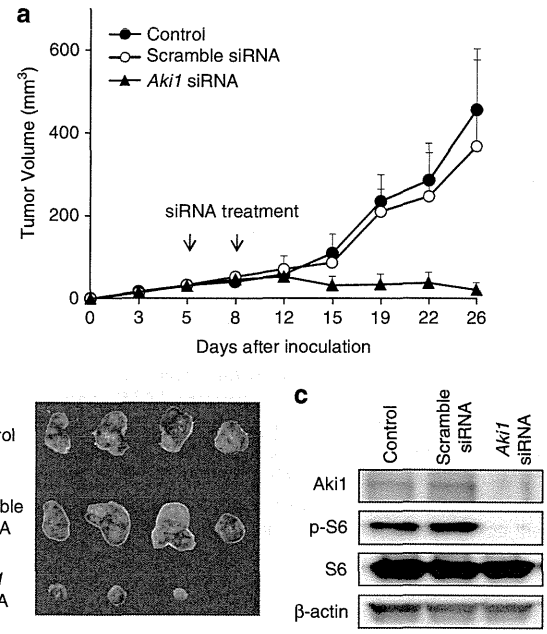


**Figure 4.** Therapeutic effects of *Aki1* knockdown against lung cancer cells with *EGFR* T790M secondary mutation *in vivo*. H1975 cells ($5 \times 10^6$ cells per 100 μl of PBS) were injected subcutaneously into the flanks of 5-week-old male SCID mice. After cell inoculation, 50 μg of either scramble or *Aki1* siRNA complexed with invivofectamine was injected intratumorally on days 5 and 8. (**a**) Tumor size was measured twice a week and tumor volume was calculated as described in Materials and methods. (**b**) Macroscopic appearance of the tumors harvested on day 26. (**c**) The harvested tumors were examined for Aki1, and the inhibition of downstream signaling molecule, S6, in tumors by western blotting.

**Figure 2.** Effects of *Aki1* siRNA on cell viability and apoptosis in EGFR mutant human lung cancer cell lines. Cells were treated with *Aki1*-1 or control scramble siRNA. (**a**) After 72-h incubation, cell viability was determined by MTT assay. (**b**) After 24-h incubation with control scramble siRNA (lanes 1, 3 and 5) or *Aki1*-1 siRNA (lanes 2, 4 and 6), cells were lysed and the indicated proteins were detected by western blotting. (**c**) After 48-h incubation, cell apoptosis was determined with an Annexin V-FITC Apoptosis Detection Kit I. The numbers show percentages of early apoptotic cells.

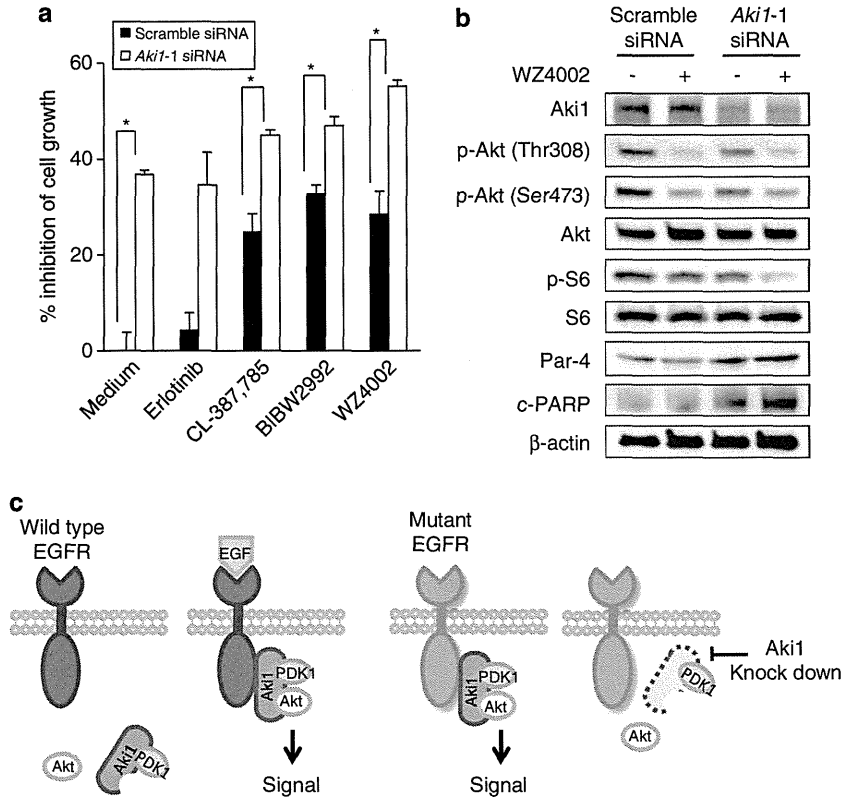


**Figure 5.** Effects of *Aki1* knockdown combined with new generation EGFR TKI in lung cancer with *EGFR* T790M secondary mutation. H1975 cells were treated with *Aki1*-1 or control scramble siRNA in the presence or absence of erlotinib (1 μM), CL-387, 785 (0.3 μM), BIBW2992 (0.1 μM) or WZ4002 (0.1 μM). (**a**) After 72-h incubation, cell viability was determined by MTT assay. *$P<0.01$, one-way ANOVA. (**b**) After 24-h incubation, cells were lysed and the indicated proteins were detected by western blotting. (**c**) Schema showing the role of Aki1 in cells with wild-type EGFR and mutant EGFR.

In conclusion, we demonstrated that Aki1 constitutively associates with EGFR activating mutation as well as T790M gatekeeper mutation has important roles as a determinant of receptor selective signaling for mutant EGFR, and mediates the survival signal to Akt. Our data provide a rationale for targeting Aki1 in *EGFR* mutant lung cancer patients, especially in cases with acquired resistance due to EGFR-T790M gatekeeper mutation. We are currently developing a drug delivery system for *Aki1* siRNA and small compounds with Aki1 inhibitory activity.

## MATERIALS AND METHODS

### Cell lines and reagents

The PC-9 and HCC827 human lung adenocarcinoma cell lines with EGFR-activating mutation (deletion in exon 19) were purchased from Immuno-Biological Laboratories (Gunma, Japan) and American Type Culture Collection (Manassas, VA, USA), respectively.[36] The H1975 human lung adenocarcinoma cell line with EGFR-L858R/T790M double mutation[10] was kindly provided by Dr John D Minna (University of Texas Southwestern Medical Center). The A549 human lung adenocarcinoma cell line, which expresses wild-type EGFR, was purchased from American Type Culture Collection. PC14PE6 human lung adenocarcinoma cell line, which expresses wild-type EGFR, was kindly provided by Dr Isaiah J Fidler (MD Anderson Cancer Center, Houston, TX, USA).[37] The human lung embryonic fibroblast MRC-5 (P30-35) and IMR-90 (P20-25) cell lines were obtained from RIKEN Cell Bank (Ibaraki, Japan). H1975, PC-9, HCC827, A549, and PC14PE6 cells were cultured in RPMI 1640, and MRC-5 and IMR-90 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (50 μg/ml), in a humidified $CO_2$ incubator at 37 °C. All experiments were performed in medium supplemented with 10% FBS. Erlotinib hydrochloride was obtained from Roche Pharma AG (Basel, Switzerland). CL-387, 785 was purchased from Calbiochem (San Diego, CA, USA). BIBW2992 and WZ4002 were purchased from Seleck Chemicals (Houston, TX, USA). Human wild-type *Aki1* cDNA in the pFLAG-CMV-2 vector was generated previously.[25] RNAi-resistant *Aki1* cDNA in the pFLAG-CMV-2 vector was generated by mutating CAAACTC of Aki1 siRNA-targeting sequence to TAAGTTA without changing the amino acid sequence.

### Immunoprecipitation and western blotting

Tumor cells were incubated in 10 ml of RPMI 1640 with 10% FBS for 1 h. The cells were then washed twice with phosphate-buffered saline (PBS), harvested in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) and flash-frozen on dry ice. After allowing the cells to thaw, the cell lysates were collected with a rubber scraper, sonicated and centrifuged at 14 000 × *g* (4 °C for 20 min). The total protein concentration was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Aliquots of 400 μg of total proteins were immunoprecipitated with the appropriate antibodies. In some experiments, tumor cells were incubated in 10 ml of RPMI 1640 with 0.1% FBS in the presence or absence of erlotinib (0.3 μM), CL-387, 785 (0.3 μM) for 48 h. In other experiments, tumor cells were incubated in 10 ml of RPMI 1640 with 0.1% FBS in the presence or absence of EGF (50 ng/ml) or IGF-1 (50 ng/ml) for 10 min. In some experiments, tumor cells were transfected with RNAi for 24 h incubation and then incubated in 10 ml of RPMI 1640 with 10% FBS in the presence or absence of WZ4002 (0.1 μM) for 1 h. Immune complexes were recovered with Protein G-Sepharose beads (Zymed Laboratories, San Francisco, CA, USA). For western blotting assay, immunoprecipitates or cell lysates were subjected to SDS–PAGE (Bio-Rad, Hercules, CA, USA) and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature, and the blots were then incubated at 4 °C overnight with anti-phospho-EGFR (Y1068), anti-Akt

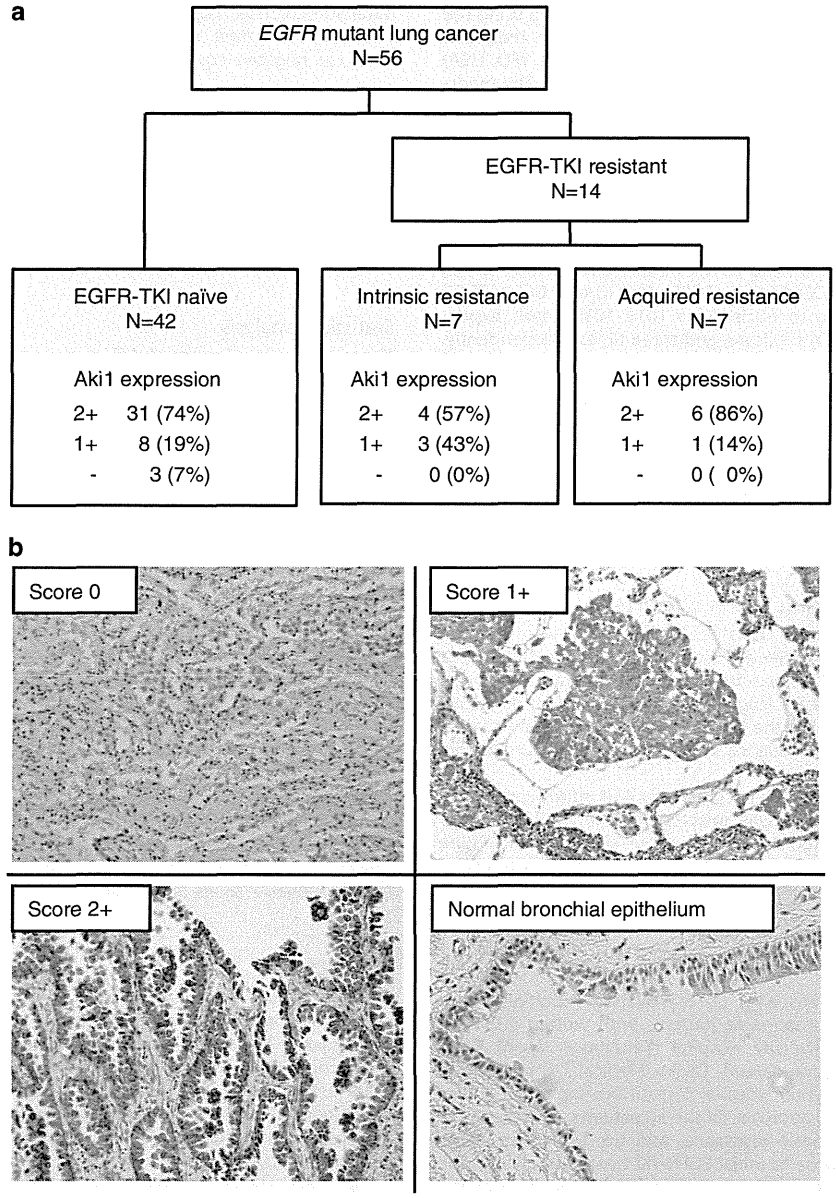


**Figure 6.** Aki1 is frequently expressed in *EGFR* mutant lung cancer. Clinical specimens from EGFR mutant lung cancer patients were stained for Aki1 by immunohistochemistry. (**a**) A total of 56 tumor specimens with EGFR-activating mutations were obtained from 56 lung adenocarcinoma patients. Of the 56 patients, 42 were EGFR-TKI naive, 7 tumors were from patients who showed intrinsic resistance to the EGFR-TKIs, gefitinib or erlotinib. Another seven tumors were from patients who showed acquired resistance to EGFR-TKIs. Of 42 EGFR-TKI naive tumors, the presence of Aki1 protein was scored as 2+ in 31 tumors (74%), 1+ in 8 tumors (19%) and – in 3 tumors (7%). Aki1 protein was detected diffusely in all of seven tumors with intrinsic resistance: 2+ in 4 (57%), 1+ in 3 (43%). Aki1 protein was detected diffusely in all of seven tumors with acquired resistance: 2+ in 6 (86%), 1+ in 1 (14%). (**b**) Representative staining results are shown.

(40D4), anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-Par-4, anti-cleaved PARP (Asp214), anti-phospho-S6 ribosomal protein (Ser235/236), anti-S6 ribosomal protein (5G10), anti-phospho-IGF-1 R (Tyr1131), DYKDDDDK (FLAG) tag antibody or anti-β-actin (13E5) antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-Aki1 (1:1000 dilution; Bethyl Laboratories, Montgomery, TX, USA), anti-PDK1 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-human EGFR (1 μg/ml) or anti-human IGF-1 R (0.1 μg/ml) antibody (R&D Systems, Minneapolis, MN, USA). After washing three times, the membranes were incubated for 1 h at room temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was performed at least three times independently.

### RNAi and proliferation assay *in vitro*

Duplexed Stealth RNAi (Invitrogen) against *Aki1*, *EGFR* and Stealth RNAi Negative Control Low GC Duplex no. 3 (Invitrogen, Carlsbad, CA, USA) were used for RNAi assay. Briefly, aliquots of $1 \times 10^5$ cells in 2 ml of antibiotic-free medium were plated on six-well plates and incubated at 37 °C for 24 h. The cells were then transfected with siRNA (250 pmol) or scramble RNA using Lipofectamine 2000 (5 μl) in accordance with the manufacturer's instructions (Invitrogen). After 24 h, the cells were washed twice with PBS. These partial cells were then used for western blotting and cell apoptosis assay. For proliferation assay, the cells were reseeded at $2 \times 10^3$ per well in 96-well plates, and incubated in antibiotic-containing RPMI 1640 with 10% FBS for 48 h. Otherwise, after 24 h of incubation, erlotinib (1 μM), CL-387, 785 (0.3 and 1 μM), BIBW2992 (0.1 and 0.3 μM) or WZ4002 (0.1, 0.3 μM) was added to each well, and incubation was continued for a further 48 h. These

cells were then used for proliferation assay, which was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) dye reduction method. An aliquot of MTT solution (2 mg/ml; Sigma, St Louis, MO, USA) was added to each well followed by incubation for 2 h at 37 °C. The media were removed and the dark blue crystals in each well were dissolved in 100 μl of dimethyl sulfoxide (DMSO). Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 nm and 630 nm, respectively. The percentage of growth is shown relative to untreated controls.

*Aki1* and *EGFR* knockdown were confirmed by western blotting analysis. The target sequences of siRNAs were as follows: *Aki1*-1, 5′-AGGAGCAGTT CAAACTCTGCATCAA-3′ (corresponding to nucleotides 2125–2168); *Aki1*-2, 5′-AACAAAGACAUCCAGAUCGCCAGGG-3′; *EGFR*, 5′-CGGAATAGGTATTGGT GAATTTAAA-3′ (corresponding to nucleotides 1014–1038). Each experiment was performed at least in triplicate, and three times independently.

### Cell apoptosis assay

Cell apoptosis induced by *Aki1*-1 siRNA, *Aki1*-2 siRNA or Scramble siRNA was detected with an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen, Heidelberg, Germany) in accordance with the manufacturer's protocols as we described previously.[36] The analysis was performed on a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

### Xenograft studies in SCID mice and *in vivo* RNAi

Suspensions of H1975 cells ($5 \times 10^6$ cells per 100 μl of PBS) were injected subcutaneously into the flanks of 5-week-old male SCID mice (Nihon Clea Co., Ltd, Tokyo, Japan). Tumor size was measured using digital calipers and tumor volume was calculated as $0.5 \times \text{length} \times (\text{width})^2$. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

After cell inoculation, 50 μg of either scramble or Aki1 siRNA complexed with Invivofectamine (Invitrogen) was injected intratumorally on days 5 and 8. Tumors were harvested on day 26. siRNA and Invivofectamine complex was prepared in accordance with the manufacturer's instructions (Invitrogen). Aki1 knockdown in tumor tissue was confirmed by western blotting analysis.

### Patients

A total of 56 tumor specimens with EGFR-activating mutations were obtained from 56 lung adenocarcinoma patients with written informed consent at the Kanazawa University Hospital (Kanazawa, Japan), Aichi Cancer Center Hospital (Nagoya, Japan), Osaka Medical Center (Osaka, Japan) and National Cancer Center Hospital East (Chiba, Japan) in studies with Institutional Review Board approval. Of the 56 patients, 42 were EGFR-TKI naive, seven showed intrinsic resistance, and the remaining seven patients showed partial response to initial EGFR-TKI treatment. As intrinsic resistance is not yet clearly defined, in the present study, we defined intrinsic resistant tumors as follows: response to treatment with an EGFR-TKI as defined by either documented stable disease or progressive disease (RECIST). Data for specimens from the seven patients who showed intrinsic resistance were obtained before EGFR-TKI treatment. For the seven patients who showed acquired resistance, tumor specimens were available after the development of acquired resistance to EGFR-TKI. Tumors with acquired resistance were defined as described previously.[38] Four of seven tumors from seven patients who showed acquired resistance had T790M secondary mutation.

### Histology and immunohistochemistry

Immunohistochemical staining was carried out on formalin-fixed, paraffin-embedded tissue sections of lung adenocarcinoma specimens. Sections 4-μm thick were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. After blocking the endogenous peroxidase activity with 3% aqueous $H_2O_2$ solution for 12 min, the sections were treated with 5% normal horse serum. The sections were then reacted with primary antibody (1:100 dilution, rabbit polyclonal anti-CC2D1A antibody; Sigma-Aldrich Corp., St Louis, MO, USA) at 4 °C overnight. After washing with PBS, the sections were treated with biotin-conjugated anti-rabbit IgG (1:200 dilution) for 30 min at room temperature and allowed to react for 30 min with avidin–biotin–peroxidase complex (ABC) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The DAB (3,3′-diaminobenzidine tetrahydrochloride) Liquid System (DakoCytomation, Glostrup, Denmark) was used to detect immunostaining. Omission of primary antibodies served as negative control.

### Evaluation of immunohistochemical results

Aki1 immunoreactivity was evaluated as the percentage of cancer cells with positive cytoplasmic staining (0, <5%; 1+, 5%–50%; 2+, >50%). Positive cells were defined as those with staining intensity that was the same or greater than that of normal bronchial epithelium (Figure 6b). Evaluation was performed independently by two investigators (TY, HU) who were blind to individual clinical information about specimens.

### Statistical analysis

The statistical significance of differences was analyzed by one-way ANOVA performed with GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA, USA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

## CONFLICT OF INTEREST

Seiji Yano received honoraria from Chugai Pharmaceutical Co., Ltd. and AstraZeneca. Seiji Yano received research funding from Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd. and Eisai Co., Ltd.

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# Rationale and Design of the Japan Molecular Epidemiology for Lung Cancer Study

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## Abstract

We present the rationale for the Japan Molecular Epidemiology for Lung Cancer study designed to elucidate molecular mechanisms of carcinogenesis in smokers and never-smokers with non–small-cell lung cancer. This prospective, ongoing, multicenter study is being conducted nationwide in Japan. Although there is no doubt that active smoking is the major cause of lung cancer, the contribution of other possible factors, including environmental tobacco or wood smoke, human papilloma virus, radon, occupational exposures, and genetic susceptibility, is highly likely, based on studies of never-smokers with non–small-cell lung cancer. Because of the predominance of women in the never-smoker subgroup, the role of female hormones in lung cancer development has also been considered. We hypothesize that driver mutations, which are critical for the development of lung cancer, are triggered by the environmental factors with or without the influence of the hormone. The SWOG-led intergroup molecular epidemiology study S0424 was conducted to focus on these issues by using a detailed questionnaire and specimen collection in statistically significant cohorts of smokers and never-smokers from both sexes. The Japan Molecular Epidemiology for Lung Cancer study follows and extends the S0424 molecular epidemiology concept in principle by using a similar approach that will facilitate future comparisons between the studies but with a greater focus on more recently defined driver mutations and broad genomic sequencing.

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**Keywords:** Driver mutations, Molecular epidemiology, Never-smokers, Non–small-cell lung cancer, Smokers

## Introduction

Lung cancer is a leading cause of cancer-related morbidity and mortality in the world. Although the disease is predominantly caused by tobacco smoke, approximately 25% of all lung cancers worldwide are not attributable to this etiology. In fact, approximately 30% of Japanese patients with non–small-cell lung cancer (NSCLC) are never-smokers, as observed in a study that consisted of more than 20,000 patients.[1] Lung cancer in never-smokers differs significantly from that of smokers in clinical characteristics and in the distribution of oncogenic abnormalities, and it has been suggested to be a distinct disease.[2]

Although several possible explanations have been proposed, the cause of lung cancer in never-smokers remains unclear. Explanations include environmental tobacco smoke (ETS) exposure,[3] radon,[4] wood smoke,[5] occupational exposure,[6] oncogenic virus,[7,8] genetic change,[9] and sex hormone.[10,11] A Japan Public Health Center–based prospective study showed that, in Japan, second-hand smoke exposure is clearly related to the development of lung adenocarcinoma in never-smokers.[3] The study identified a statistically significant dose-response relationship between the quantity and the intensity of husbands' smoking and their wives' incidence of lung cancer. Our previous study with a detailed questionnaire in a prospective way enhances this finding that the development of epidermal growth factor receptor (*EGFR*) mutations is significantly associated with the dose of ETS exposure in never-smokers.[12] However, there are con-

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flicting data published on the relationship between ETS and *EGFR* mutations in never-smokers with NSCLC. A study from Korea showed opposite results, in which the development of the mutation was inversely proportional to the ETS[13]; although, in the United States, there was no association between them.[14] A study with a well-designed and standardized questionnaire in a larger sample size is required to conclude this issue.

An oncogenic role for the HPV has been widely investigated in NSCLC.[7] Although all the published reports were retrospective analyses with potentially significant limitations and bias, the systematic review nevertheless suggested that the development of lung cancer in Asia can be attributed to some extent to HPV. Moreover, a different detection rate was observed geographically even within east Asia, with a higher rate in the southern area than in the northern regions. There is a substantial need to confirm these findings by using a standardized HPV detection methodology in a prospective study in Japanese patients.

The association between sex and lung cancer carcinogenesis is also an important consideration. Although studies provide conflicting results on the strength of this association, it has been postulated that women are more vulnerable to tobacco smoke–associated carcinogens than men. The large SWOG study S0424 was originally designed to address this issue by using a detailed questionnaire and NSCLC tissue specimens from smoker and never-smoker men and women with newly diagnosed stage I, stage II, or stage III NSCLC,[15] in which polycyclic aromatic hydrocarbons and aromatic amines of DNA adducts are measured to quantitate levels of DNA damage stratified by sex and the smoking status. Cigarette smoke contains a large number of carcinogens, and polycyclic aromatic hydrocarbons and aromatic amines are among the most important contributors to the carcinogenic process. The Japan Molecular Epidemiology for lung cancer (JME) study follows and extends the concept of S0424 by using a similar approach that will allow for direct comparison of data in the future.

Sex hormones, including estrogen and progesterone, have been suggested to play an important role in lung carcinogenesis. Results of epidemiologic studies showed that women were predominant in number in the never-smoking subpopulation. Further, results of large randomized studies suggest that estrogen plus progestin therapy is associated with an increased risk of lung cancer. The prospective Vitamins and Lifestyle Study followed a cohort of more than 36,000 peri- and postmenopausal women during 6 years of follow-up.[16] After adjusting for smoking and other confounding factors, the incidence of lung cancer was increased for those who used estrogen plus progestin. The risk was proportional to the duration of hormone exposure (hazard ratio 1.48 [95% CI, 1.03-2.12] for those with ≥10 years of exposure to estrogen plus progestin).

In terms of biologic function, estrogen receptors (ER) are expressed in diverse normal and neoplastic tissues, and mediate growth and maturation of normal tissue. A number of studies have noted expression of ERs in a large portion of lung tumors. In a couple of studies, the development of *EGFR* mutations was significantly associated with expression of ER $\beta$ in NSCLC surgical specimens.[10,11] There have been no studies that systematically evaluated ER expression in lung cancer and its relationship with genetic mutations or environmental and reproductive risk factors.

Identification of driver mutations in NSCLC has been instrumental in improving treatment strategies. *ALK* (anaplastic lymphoma kinase) gene translocations have been demonstrated to be critical targets and biomarkers for crizotinib efficacy,[17] similar to *EGFR* mutations for gefitinib and erlotinib, and the discovery of other mutations for treatment is ongoing. The Lung Cancer Mutational Consortium in the United States[18] and the Lungscape project in the European Union[19] are currently exploring new molecular targets for treatment in lung cancer. Powerful tools for genome-wide characterization have been developed, including next-generation sequencing, which enables comprehensive examination of somatic mutations associated with carcinogenesis. The Cancer Genome Atlas is an ongoing global project that uses this technology to distill essential driver abnormalities from the background noise.[20] A focus of the JME study is to explore new driver mutations by using advanced technologies and approaches now available with regard to sex of the patient and tobacco smoke exposure. The association between oncogenic abnormality profiles and drug sensitivity and prognosis will also be examined.

In addition, the JME study is designed to investigate the relationship between ethnicity and NSCLC carcinogenesis. It is clear that NSCLCs are different in tumor biology between Caucasian and Asian patients. Gandara et al[21] showed that there was a significant difference in survival and toxicities between the US and the Japanese patients treated with carboplatin and paclitaxel in a "common arm" trial, in which the study design, eligibility criteria, and staging were similar. The median overall survival in the metastatic disease was 12 and 14 months for Japanese patients vs. 9 months for US patients ($P = .0006$).[21] As for *EGFR* mutations, the frequencies appear to be highly distinct; the high detection rate in Asia was reported consistently across publications. Different influences of smoking status on the development of NSCLC also was observed between the United States and Japan in population-based prospective studies. In a comparison of the Japanese cohort with US Cancer Prevention Study II during the same period,[22] Japanese never-smokers had an increased risk of lung cancer, whereas Japanese current smokers were at a lower risk of the cancer compared with those in the United States. To elucidate the mechanistic contributions of ethnic differences, there is a need to collaborate in comprehensive and global approaches for examining development of NSCLC as well as the clinical behavior and outcome.

## Objectives

The primary objective of this study is to assess surgical lung specimens from patients with stage I, stage II, stage IIIA, or stage IIIB NSCLC for driver mutations, expression of HER2 and ER $\alpha$ and ER $\beta$, the presence of smoking-associated DNA adducts, and evidence of HPV, and to explore new molecular markers by using next-generation sequencing. By using information collected before surgery on patient demographics, smoking history and occupational exposures, carcinogenic mechanisms will be elucidated in never-smokers and ever-smokers. Secondary objectives are to examine whether the relapse rate, disease-free survival, and overall survival time differ among the patients with different mutational

spectrums, and whether mutational profiles differ between Japanese and Caucasian populations.

## Patients and Methods

Eligible patients are those with pathologically proven NSCLC with stage I and II, IIIA, or IIIB disease[23] who underwent surgery with a curative intent (Figure 1). Patients with prior chemotherapy and/or radiotherapy are excluded, as are patients with other prior malignancies except for adequately treated basal cell or squamous cell skin cancer or in situ cervical cancer. Patients are stratified according to smoking status. Never-smokers are defined as those who smoked fewer than 100 cigarettes during their lifetime, and ever-smoker who smoked 100 or more cigarettes during their lifetime.

Patients are required to complete the questionnaire before surgery for detailed assessment of the following: exposure to active and passive smoke, occupational exposures, reproductive and hormonal risk factors, weight loss, family history of cancer, medication use, and diet and exercise. DNA is extracted from all formalin-fixed paraffin-embedded surgical tissues. *EGFR* and *KRAS* (v-Ki-ras2 Kirsten rat sarcoma) mutations are examined by using real-time polymerase chain reaction and *ALK* by immunohistochemical staining and fluorescence in situ hybridization. HPV genotyping is performed by using a polymerase chain reaction–based microarray system for detection of 23 HPV types, including high-risk (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and low-risk or risk-unknown types (HPV types 6, 11, 30, 34, 40, 42, 53, 54, 61, and 66). In addition, multiplexed targeted deep sequencing is applied to the tumors, including 48 cancer-associated genes, such as *ABL1, AKT1, CSF1R, CTNNB1, IDH1, MET, MLH1, PIC3CA, RET, STK11,* and *TP53*.

Surgical samples are examined for DNA adducts levels, and polycyclic aromatic hydrocarbons and/or aromatic amines–induced DNA damage is assessed by immunohistochemical staining and immunofluorescence. ER $\alpha$ and ER $\beta$ are assessed by immunohistochemistry. Patients will be followed up annually for up to 4 years to capture relapse rate, disease-free survival, and overall survival time. Whether mutational profiles differ between Japanese and Americans will be determined after adjusting for sex, smoking status, and other clinical backgrounds.

## Statistical Consideration

Sample size in this study is 900 patients, which consists of 450 ever-smokers and 450 never-smokers, which was calculated to ensure >80% power for testing all individual hypotheses at the 2-sided .05 significance level. Based on the review article including the published data,[24] the assumed proportion of patients with *EGFR* mutation is expected to be approximately 7% and 45% in smokers and never-smokers, respectively. Mutation of *KRAS* is expected to be 30% to 43% in smokers and 0% to 7% in never-smokers. When several examples are given to detect differences of 30% to 50% in mutation-positive frequency between smokers and never-smokers, the power is >90% in most cases. Less common driver mutations are also considered and calculated based on published data[17]; the assumed proportion of patients with *ALK* fusion is expected to be approximately 3.5% and 9.9% in smokers and never-smokers, respectively. The power is >90% in most cases to detect differences of 5% to 7% in fusion-positive frequency between the 2 groups.

According to our study in never-smokers,[12] more *EGFR* mutations were observed in those who had longer ETS exposure. When the length of ETS is divided by the median of the value, if the *EGFR* detection rate differs by more than 15% between the 2 groups overall, then the power is >90% in most cases.

The meta-analysis on HPV and lung cancer showed that, when using polymerase chain reaction, there were 22% of cases (95% CI, 18%-27%) possibly associated with the virus.[8] The presence of HPV is expected to be observed at least in approximately 160 patients in smokers and never-smokers, and the geographic distribution is also examined.

Based on our previous study, which included approximately 20,000 Japanese patients,[1] it is assumed that 350 female never-smokers, 100 male never-smokers, 120 female ever-smokers and 330 male ever-smokers will be accrued in this study. A possible fluctuation in accrual on sex is expected to be with a range of ±20%. If the detection rate of *EGFR* mutation differs by more than 15% between male and female subjects, then the power is >90% in most cases overall and >80% in most cases within smokers and never-smokers.

The prognostic value of each unique *EGFR* and *KRAS* mutation, along with other abnormalities, will initially be assessed by using multivariable proportional hazards regression when adjusting for strata. Relationships will be graphically displayed for each prognostic group by using Kaplan-Meier curves. The classification and regression tree method will be used to identify prognostic risk groups based on these measures of the mutations combined with other patient demographic and correlative data.

## Conclusion

The JME study is a prospective project sponsored by an independent administrative agency in Japan to use advanced molecular technologies to improve our understanding of the underlying biology of NSCLC in Japanese patients nationwide. The primary focus of this study is on the relationships among tumor carcinogenesis; patterns of biomarkers, including driver mutations; and detailed demographic information. This study is currently ongoing, and successful accrual to date supports the feasibility of the study design. The outcomes of the JME study will have clinical implication with respect to establishing a model for lung cancer carcinogenesis and will provide a wealth of information on driver mutations to better understand the tumor carcinogenic process and to improve therapeutic options for patients with NSCLC.

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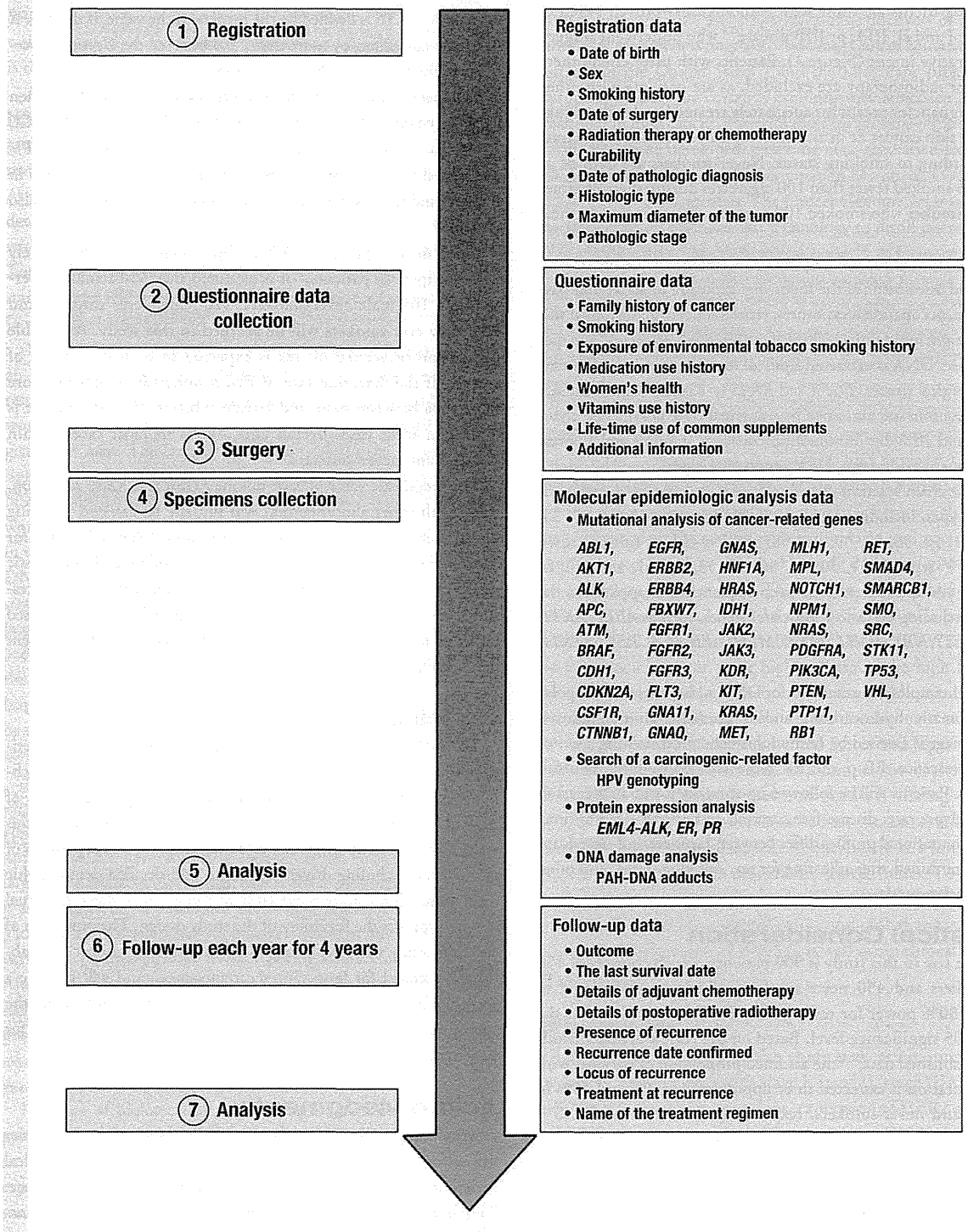
**Figure 1** Scheme of the Japan Molecular Epidemiology for Lung Cancer



## Disclosure

The authors have stated that they have no conflicts of interest.

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# Prognostic factors after complete resection of pN2 non–small cell lung cancer

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**Objectives:** This retrospective, multicenter study aimed to determine prognostic factors of completely resected pathologic N2 stage IIIA non–small cell cancer (NSCLC).

**Methods:** From 25 participating hospitals, 496 patients (325 men and 171 women; median age, 65 years) who underwent complete resection without preoperative treatment for pT1-3 N2 M0, stage IIIA NSCLC between 2000 and 2004 were enrolled. Lobectomy/bilobectomy was performed in 462 patients and pneumonectomy in 34. Some kind of adjuvant chemotherapy was administered to 296 patients. Survivals were calculated using the Kaplan-Meier method, and prognostic factors were determined using the Cox proportional hazards model.

**Results:** Five-year overall survival (OS) and disease-free survival (DFS) were 44.8% and 24.2%, respectively. pT classification (hazard ratio (HR), pT1/pT2/pT3 = 1/1.32/2.03), single or multiple N2 metastases (HR, single/multiple = 1/1.36), and skip or nonskip N2 metastasis (HR, skip/nonskip = 1/1.30) were found to be independent prognostic factors for DFS. Sex (HR, female/male = 1/1.36), performance status (HR, PS-0/PS-1 = 1/1.37), tumor diameter (HR, 1.12 per 1-cm increase), pT-factor (HR, pT1/pT2/pT3 = 1/1.37/2.22), and extent of N2 metastasis (HR, localized/extended = 1/1.39) were shown to be independent prognostic factors for OS.

**Conclusions:** We found that pT classification was a significant prognostic indicator for OS and DFS whereas tumor diameter, performance status, and sex were ones for OS. Single N2 metastasis and skip N2 metastasis were demonstrated as favorable prognostic factors for DFS, limited N2 metastasis was one for OS, and these should be considered as stratification factors for trial on adjuvant therapy. (J Thorac Cardiovasc Surg 2013;146:788-95)

The prognosis of completely resected pathologic (p) N2 stage IIIA non–small cell lung cancer (NSCLC) is still unsatisfactory owing to a high incidence of metastasis or tumor recurrence.[1] A meta-analysis on cisplatin-based adjuvant chemotherapy after initial resection has shown that it improves survival.[2] However, the efficacy of adjuvant chemotherapy in Japanese phase III trials has been controversial.[3,4] Therefore, several phase II and III trials on adjuvant chemotherapy have been planned in Japan.

It is widely known that the prognosis of pN2 stage IIIA NSCLC is heterogeneous, and T and clinical (c) N classifications have been identified as prognostic factors.[5] These factors are considered as stratification criteria in clinical trials. Although pattern of metastasis to N2 regions has also been reported as a prognostic factor.[5,6] Its influence on adjuvant chemotherapy has not yet been clarified; thus, it is not considered as stratification criteria in clinical trials of adjuvant chemotherapy for patients with pN2 stage IIIA NSCLC.

In this study, we retrospectively investigated the outcome of patients with completely resected pN2 stage IIIA NSCLC, who were treated at institutions participating in

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**Abbreviations and Acronyms**

c = clinical
DFS = disease-free survival
ECOG = Eastern Cooperative Oncology Group
EGFR = epidermal growth factor receptor
HR = hazard ratio
IV-CT = intravenous chemotherapy
JMTO = Japan-Multinational Trial Organization
NSCLC = non–small cell lung cancer
OS = operative survival
p = pathologic
PS = performance status
UFT = tegafur-uracil

the clinical trial group Japan-Multinational Trial Organization (JMTO) to determine prognostic factors, particularly prognostic value of pattern of metastasis to N2 regions and adjuvant chemotherapy.

## PATIENTS AND METHODS

### Study Design

In this retrospective study, 25 institutions affiliated with the JMTO (listed in the Acknowledgements section) recruited patients who met the eligibility criteria, which began in January 2008, and all data including follow-up information until either death or December 2010 were assessed in April 2011.

### Eligibility Criteria

Patients with NSCLC who had undergone complete resection by lobectomy, bilobectomy, or pneumonectomy with at least standard ipsilateral hilar/mediastinal lymph node dissection (defined by the General Rule for Clinical and Pathological Record of Lung Cancer[7]) between January 2000 and December 2004, and whose tumors were pathologically proven T1-3 N2 M0, stage IIIA according to the sixth edition of the International System for Staging Lung Cancer,[8] were eligible. The eligibility criteria included the following: no chemotherapy or radiotherapy before surgery; older than 20 years of age; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1; and written informed consent for surgical resection. Patients were excluded if they had 1 or more of the following factors: active double cancer; serious infections; cardiac, hepatic, renal, or psychologic diseases at the time of lung resection; history of preoperative treatment with chemotherapy or radiotherapy; and intraoperative anticancer drug administration in the thorax or pericardium. Patients who underwent sublobar resection such as segmentectomy for their NSCLC were also excluded. The study protocol was approved by the ethics committee of JMTO (JMTO LC 05-01 study) and the institutional ethics committee of each participating institution. From 25 institutions, 512 patients were recruited. Of these, 12 patients received sublobar resection and 4 patients received insufficient lymph node dissection. Finally, 496 patients were eligible in the study (Figure 1). Their characteristics are shown in Table 1. Of these, 2 (0.4%) patients died of postoperative complication within 30 days after surgery.

### Adjuvant Therapy and Follow-up

In the period between January 2000 and December 2004, the significance of induction treatment, adjuvant chemotherapy, or adjuvant radiotherapy was not established. Therefore, indications for resection for cN2 disease and postoperative chemotherapy and/or radiotherapy were dependent on each institution. As adjuvant chemotherapy, oral tegafur-uracil (UFT) or intravenous chemotherapy (IV-CT) was selected. IV-CT regimens that were used included carboplatin and paclitaxel, cisplatin and docetaxel, cisplatin or carboplatin and gemcitabine, cisplatin and 5-fluorouracil, and a combination of cisplatin, mitomycin C, and vindesine. As adjuvant radiotherapy, irradiation with 50 to 60 Gy to hilar and mediastinal areas was performed. Of 496 patients, 122 (24.6%) received IV-CT, 89 (17.9%) received IV-CT and radiotherapy, 60 (12.1%) received UFT, 25 (5.0%) received UFT and radiotherapy, 49 (9.9%) received radiotherapy, and 151 (30.4%) did not receive adjuvant therapy.

The follow-up examination schedule was arranged by each individual institution; most of the patients received medical check-ups and chest x-ray films at least twice per year.

### Data Collection

The collected data included age, sex, ECOG PS, cT and cN classifications, affected lobe of lung, operation date, operation mode, pT classification, maximum tumor size (centimeters), tumor histology in accordance with the World Health Organization classification,[9] station of node(s) with metastasis, adjuvant chemotherapy, adjuvant radiation therapy, presence or absence of recurrent tumor, date of diagnosis of recurrent tumor, and follow-up information until either death or December 2010. These data were obtained from the inpatient and outpatient medical records and imaging tests. These data were accumulated through a web-based registration system established by JMTO.

### Patterns of Metastasis to N2 Regions

Metastasis to the N2 regions was classified as follows: (1) single (metastasis to 1 N2 station) or multiple (metastases to 2 or more N2 stations) N2 metastases; (2) skip (no metastasis to the N1 region) or nonskip (metastasis to the N1 region) N2 metastases; and (3) extent of N2 stations with metastasis, defined as localized or extended N2 metastasis. "Localized N2 metastasis" was defined as metastatic spread of the tumor to right #1, right #2, #3, and/or right #4 N2 station(s) of the right upper lobe; left #1, left #2, #3, left #4, #5, and/or #6 N2 station(s) of the left upper lobe; right #1, right #2, #3, right #4, and/or #7 N2 station(s) of the right middle lobe; #7, right #8, and/or right #9 N2 station(s) of the right lower lobe, and #7, left #8, and/or left #9 N2 station(s) of the left lower lobe. "Extended N2 metastasis" was defined as metastasic spread of the tumor to #7, ipsilateral #8, and/or ipsilateral #9 N2 station(s) of the upper lobe; right #8 and/or right #9 N2 station(s) in the right middle lobe; ipsilateral #1, #2, #3, and/or #4 N2 station(s) in the right lower lobe; and ipsilateral #1, #2, #3, #4, #5, and/or #6 N2 station(s) in the left lower lobe. Under these classifications, 294 (59.3%) patients had single N2 metastasis and 202 (40.7%) had multiple N2 metastasis. An equal frequency of skip and nonskip N2 metastases was observed; both were found in 248 (50.0%) patients. The number of patients with skip N2 metastasis by primary tumor location was 89 (50.3%) of 177 in the right upper lobe, 10 (40.0%) of 25 in the right middle lobe, 57 (47.5%) of 120 in the right lower lobe, 63 (57.3%) of 110 in the left upper lobe, and 29 (45.3%) of 64 in the left lower lobe. Localized N2 metastases were observed in 369 (74.4%) patients, and extended N2 metastases were in 127 (25.6%) patients. The number of patients with extended N2 metastasis by primary tumor location was 34 (19.2%) of 177 in the right upper lobe, 0 (0%) of 25 in the right middle lobe, 50 (41.7%) of 120 in the right lower lobe, 14 (12.7%) of 110 in the left upper lobe, and 29 (45.3%) of 64 in left lower lobe.

### Statistical Considerations

Categorical data were examined using the $\chi^2$ test. Prognostic evaluation was performed by consideration of the disease-free survival (DFS) and overall survival (OS). DFS was defined as time to lung cancer recurrence or non–lung cancer death. The impact of the following clinical-pathologic

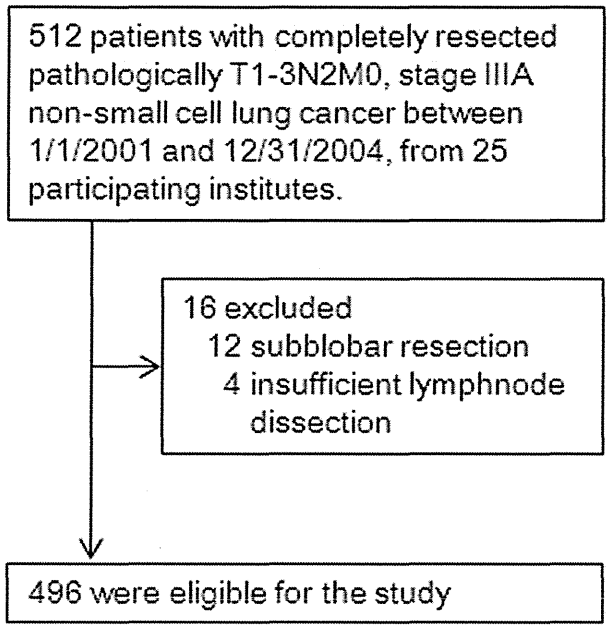


**FIGURE 1.** Patient selection. From 25 institutions, 512 patients who potentially met the study criteria were recruited. Assessment of collected data revealed that 12 patients received sublobar resection and 4 patients received insufficient lymph node dissection. Finally, 496 patients were eligible in the study.

factors on DFS and OS was evaluated: age (≤60, 61-70, or >70 years of age), sex, ECOG PS (PS 0 vs PS 1), tumor size in maximal diameter (centimeters), cT classification, cN classification, tumor location (right vs left), operation mode (pneumonectomy vs lobectomy including bilobectomy and sleeve lobectomy), tumor histology (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, or adenosquamous cell carcinoma), pT classification, adjuvant chemotherapy (none, UFT, or IV-CT for simplification purposes inasmuch as a wide range of intravenous chemotherapy regimens were used), adjuvant radiation therapy, single or multiple N2 metastases, skip or nonskip N2 metastasis, and localized or extended N2 metastasis. Survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. To evaluate the prognostic effect of IV-CT compared with surgery alone, we matched patients on the basis of propensity score. The propensity score for IV-CT was constructed using binary logistic regression including patients' age, sex, ECOG PS, tumor histology, pT classification, operation mode, single or multiple N2 metastases, skip or nonskip N2 metastasis, and localized or extended N2 metastasis. Nearest neighbor matching with a caliper of .005 difference was used to match 128 patients with IV-CT and 128 patients without no adjuvant chemotherapy. Regarding pT classification and pattern of metastasis to N2 region, 5-year DFSs on the basis of IV-CT and radiotherapy were calculated. When significant ($P$ value < .05) and borderline ($P$ value < .10) variables under this univariate method showed linearity and time dependency, they were tested with a multivariate analysis using the Cox proportional hazards model. JMP software version 9.03 (SAS Institute, Inc, Cary, NC) was used to carry out all statistical calculations. All statistical tests were 2-sided. Our statistician (S.T.) reviewed the statistical methods.

**TABLE 1. Patient characteristics**

| Variables | No. of patients (%) |
|---|---|
| Age (y) | Mean 65.0 |
| ≤60 | 152 (30.6%) |
| 61-70 | 175 (35.3%) |
| >71 | 169 (34.1%) |
| Sex | |
| Female | 171 (34.5%) |
| Male | 325 (65.5%) |
| ECOG performance status | |
| PS 0 | 404 (81.5%) |
| PS 1 | 92 (18.5%) |
| Clinical T classification | |
| cT1 | 204 (41.1%) |
| cT2 | 251 (50.6%) |
| cT3/4 | 41 (8.3%) |
| Clinical N classification | |
| cN0 | 311 (62.7%) |
| cN1 | 62 (12.5%) |
| cN2/3 | 123 (24.8%) |
| Clinical stage | |
| Stage I | 295 (59.5%) |
| Stage II | 69 (13.9%) |
| Stage III | 132 (26.6%) |
| Tumor location | |
| Right | 322 (64.9%) |
| Left | 174 (35.1%) |
| Types of resection | |
| Lobectomy | 448 (90.3%) |
| Sleeve lobectomy | 12 (2.4%) |
| Bilobectomy | 2 (0.4%) |
| Pneumonectomy | 34 (6.9%) |
| Pathologic T classification | |
| pT1 | 191 (38.5%) |
| pT2 | 256 (51.6%) |
| pT3 | 49 (9.9%) |
| Tumor histology | |
| Adenocarcinoma | 357 (72.0%) |
| Squamous cell carcinoma | 110 (22.2%) |
| Large cell carcinoma | 17 (3.4%) |
| Adenosquamous cell carcinoma | 12 (2.4%) |
| Adjuvant therapy | |
| None | 151 (30.4%) |
| Intravenous chemotherapy | 122 (24.6%) |
| Intravenous chemotherapy + radiation | 89 (17.9%) |
| Oral tegafur and uracil | 60 (12.1%) |
| Oral tegafur and uracil + radiotherapy | 25 (5.0%) |
| Radiotherapy | 49 (9.9%) |

*ECOG*, Eastern Cooperative Oncology Group; *PS*, performance status.

## RESULTS

### Survival Analyses

The median follow-up period was 57 months. Recurrence occurred in 342 patients during the follow-up period, and of these, 253 patients died of recurrent tumors whereas 89 patients were alive with recurrent tumors. Treatments after recurrence were given according to each institutional protocol. Thirty-six patients died of non–lung cancer–related causes, and 118 patients were alive without tumor recurrence. The 3- and 5-year OSs were 59.5% and 44.8%,

**TABLE 2. Univariate analyses of prognostic factors for OS and DFS**

| Factors | Variables | Five-year OS (%) | *P* value | Five-year DFS (%) | *P* value |
|---|---|---|---|---|---|
| Age | ≤60 | 46.2 | | 22.6 | |
| | 61-70 | 50.2 | .9377 | 25.1 | .8889 |
| | >71 | 37.5 | .0329 | 24.8 | .6914 |
| Sex | Female | 53.8 | | 24.1 | |
| | Male | 40.1 | .0081 | 24.3 | .6939 |
| ECOG performance status | PS 0 | 47.1 | | 25.5 | |
| | PS 1 | 34.1 | .0061 | 18.1 | .1823 |
| Maximum tumor diameter | By 1 cm increase | — | <.0001 | — | .0001 |
| Clinical T classification | cT1 | 54.2 | | 29.8 | |
| | cT2 | 41.1 | .0019 | 21.6 | .0370 |
| | cT3/4 | 21.6 | <.0001 | 12.7 | .0006 |
| Clinical N classification | cN0 | 48.7 | | 25.2 | |
| | cN1 | 41.3 | .2275 | 23.8 | .6084 |
| | cN2/3 | 36.6 | .0095 | 22.1 | .2978 |
| Tumor location | Right | 42.3 | | 24.0 | |
| | Left | 49.2 | .2125 | 24.6 | .6077 |
| Types of resection | Lobectomy* | 46.5 | | 25.2 | |
| | Pneumonectomy | 20.1 | .0011 | 11.8 | .0142 |
| Pathologic T classification | pT1 | 58.3 | | 33.1 | |
| | pT2 | 40.3 | <.0001 | 20.1 | .0016 |
| | pT3 | 14.1 | <.0001 | 11.1 | <.0001 |
| Tumor histology | Adenocarcinoma | 58.3 | | 21.8 | |
| | Squamous cell ca. | 42.4 | .1587 | 30.3 | .4844 |
| | Large cell ca. | 35.3 | .4026 | 29.4 | .7746 |
| | Adenosquamous cell ca. | 46.7 | .9827 | 33.3 | .8825 |
| Adjuvant chemotherapy | None | 41.0 | | 22.2 | |
| | Intravenous chemotherapy | 46.9 | .0743 | 26.6 | .0497 |
| | Oral tegafur and uracil | 48.2 | .1124 | 23.3 | .4579 |
| Adjuvant radiotherapy | None | 45.0 | | 26.5 | |
| | Mediastinal irradiation | 44.3 | .9041 | 19.6 | .3846 |
| Single or multiple N2 metastases | Single | 49.4 | | 30.9 | |
| | Multiple | 38.6 | .0291 | 14.8 | .0002 |
| Skip N2 metastasis | Skip | 47.8 | | 29.4 | |
| | Nonskip | 41.7 | .1680 | 19.0 | .0071 |
| Extent of N2 metastasis | Localized | 48.6 | | 27.5 | |
| | Extended | 34.1 | .0047 | 14.9 | .0074 |

*OS*, Overall survival; *DFS*, disease-free survival; *ECOG*, Eastern Cooperative Oncology Group; *ca*, carcinoma; *PS*, performance status. *Lobectomy included lobectomy, bilobectomy, and lobectomy with bronchoplasty.

respectively, and the 3- and 5-year DFSs were 31.1% and 24.2%, respectively.

Univariate analysis for OS indicated that increased age, male sex, ECOG PS 1, increasing cT classification, increasing cN classification, pneumonectomy, increasing maximum tumor diameter, increasing pT classification, multiple N2 metastases, and extended N2 metastasis were significantly associated with poor OS (Table 2). Univariate analysis also revealed that increasing cT classification, pneumonectomy, increasing maximum tumor diameter, increasing pT classification, multiple N2 metastases, nonskip N2 metastasis, and extended N2 metastasis were significantly associated with poor DFS (Table 2, Figure 2, *A* and *B*). IV-CT had a marginal prognostic advantage on OS and DFS as compared with no adjuvant chemotherapy although UFT or adjuvant radiotherapy did not (Table 2). In the IV-CT group (n = 211), 5-year OS and DFS of patients with radiotherapy (n = 89) were 44.8% and 22.1%, respectively, and were not different from 5-year OS (48.4%) and DFS (29.7%) of those without radiotherapy (n = 122). In 128 matched pairs of patients by propensity score, however, IV-CT had no prognostic advantage on OS and DFS (Figure 2, *C* and *D*).

By multivariate analysis, sex, ECOG PS, maximum tumor diameter, pT classification, and extent of N2 metastasis were revealed to be independent prognostic factors affecting OS (Table 3). Meanwhile, pT classification, single or multiple N2 metastases, and skip or nonskip N2 metastasis were demonstrated to be important prognostic factors for DFS (Table 3).

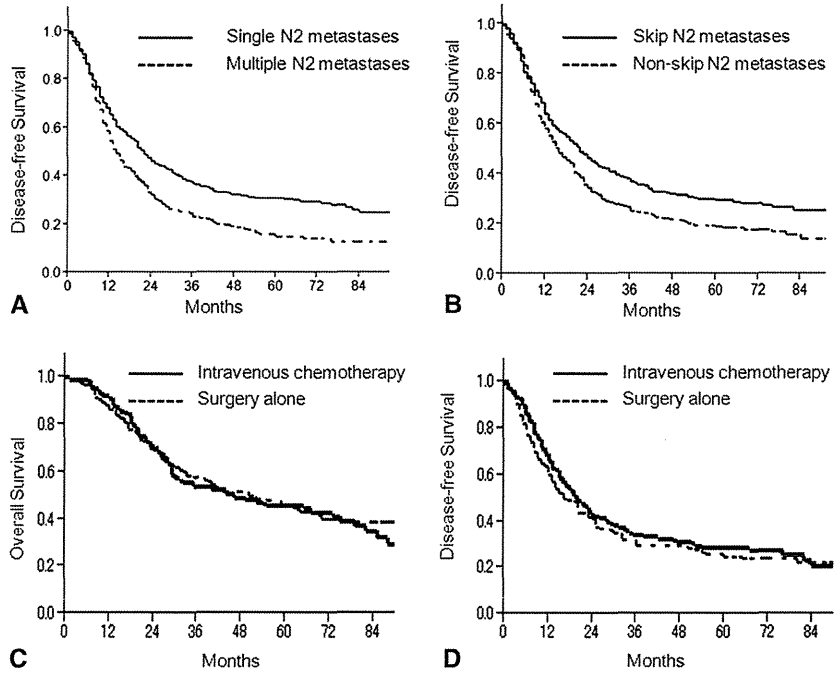


**FIGURE 2.** A, Disease-free survival according to single or multiple N2 metastases. Disease-free survival was significantly better in the single N2 metastasis group than in the multiple N2 metastases group ($P = .0002$, log-rank test). B, Disease-free survival according to skip or nonskip N2 metastasis. Disease-free survival was significantly better in the skip N2 metastasis group than in the nonskip N2 metastasis group ($P = .0071$, log-rank test). C, Propensity score–adjusted overall survival according to intravenous chemotherapy. There was no difference between the intravenous chemotherapy group and surgery alone group ($P = .4583$, log-rank test). D, Propensity score–adjusted disease-free survival according to intravenous chemotherapy. There was no difference between the intravenous chemotherapy group and surgery alone group ($P = .8611$, log-rank test).

### Adjuvant Chemotherapy by pT Classification and Pattern of Metastasis to N2 Regions

Because only IV-CT had a potential prognostic advantage, survival of patients with IV-CT (n = 122) or IV-CT and radiotherapy (n = 89) was compared with that of those with surgery alone (n = 151) according to pT classification and pattern of metastasis to N2 regions. There were no differences of DFS and OS among IV-CT, IV-CT and radiation, and no adjuvant therapy groups according to pT classification, single or multiple N2 metastases, or extent of N2 stations with metastasis. In the skip N2 metastasis group, 5-year DFSs were 44.9% in patients receiving IV-CT (n = 49), 28.7% in patients receiving IV-CT and radiotherapy (n = 46), and 26.1% in patients without adjuvant therapy (n = 68), and an effect of IV-CT on DFS was observed ($P = .0167$, log-rank test). However, in the nonskip N2 metastasis group, 5-year DFSs were 19.3% in patients receiving IV-CT (n = 79), 15.5% in patients receiving IV-CT and radiotherapy (n = 43), and 21.8% in patients without adjuvant therapy (n = 72), with no observed differences among them ($P = .9970$, log-rank test). The percentage of patients receiving adjuvant IV-CT or IV-CT and radiotherapy was not different among the pT classification groups ($P = .5071$, $\chi^2$ test), and not different between single N2 metastasis and multiple N2 metastases groups ($P = .5312$, $\chi^2$ test), skip or nonskip N2 metastasis ($P = .2344$, $\chi^2$ test), or extent of N2 stations with metastasis ($P = .1184$, $\chi^2$ test).

## DISCUSSION

The 5-year OS in the study was 44.8% and was higher than that reported by Goldstraw and associates[10] (24%) under a median follow-up period of 57 months. In our cohort, 89 patients were alive at the end of the study (December 2010), and they provided a higher OS than DFS. This may be due to the exclusion of patients with comorbidity, successful treatment for recurrent diseases including chemotherapy, radiotherapy, and/or molecular targeting drugs,[11] and high percentage of tumors with mutations in the epidermal growth factor receptor (EGFR) gene in Japan,[12-14] which could be a better prognostic factor after recurrence.[14]

We found that pT classification was a strong independent prognostic factor for OS and DFS in patients with completely resected pN2 stage IIIA NSCLC. Increased pT classification signifies enhanced tumor invasiveness and/or tumor burden. Several studies in Japan also showed a prognostic significance of pT classification in cases of completely resected pN2 NSCLC,[5,6,15] and our findings are highly corroborative with theirs.

**TABLE 3. Multivariate analyses of prognostic factors for OS and DFS**

| Factors for overall survival | Variables | Hazard ratio (95% CI) | *P* value |
|---|---|---|---|
| Age | ≤60 | 1 | |
| | 61-70 | 0.86 (0.63-1.18) | .3401 |
| | >71 | 1.19 (0.87-1.62) | .2760 |
| Sex | Female | 1 | |
| | Male | 1.35 (1.05-1.75) | .0190 |
| ECOG Performance status | PS 0 | 1 | |
| | PS 1 | 1.38 (1.02-1.85) | .0395 |
| Maximum tumor diameter (cm) | By 1 cm increase | 1.12 (1.02-1.22) | .0159 |
| Clinical T classification | cT1 | 1 | |
| | cT2 | 0.96 (0.67-1.38) | .8392 |
| | cT3/4 | 1.29 (0.73-2.23) | .3804 |
| Clinical N classification | cN0 | 1 | |
| | cN1 | 0.94 (0.64-1.36) | .7608 |
| | cN2/3 | 1.00 (0.74-1.34) | .9929 |
| Types of resection | Lobectomy* | 1 | |
| | Pneumonectomy | 1.47 (0.93-2.23) | .0950 |
| Pathologic T classification | pT1 | 1 | |
| | pT2 | 1.36 (0.95-1.94) | .0954 |
| | pT3 | 2.09 (1.22-3.51) | .0075 |
| Adjuvant chemotherapy | None | 1 | |
| | Intravenous chemotherapy | 0.79 (0.61-1.03) | .0821 |
| | Oral tegafur and uracil | 0.80 (0.56-1.13) | .2153 |
| Single or multiple N2 metastases | Single | 1 | |
| | Multiple | 1.21 (0.94-1.56) | .1335 |
| Extent of N2 metastasis | Localized | 1 | |
| | Extended | 1.40 (1.06-1.84) | .0184 |
| **Factors for disease-free survival** | **Variables** | **Hazard ratio (95% CI)** | ***P* value** |
| Maximum tumor diameter (cm) | By 1-cm increase | 1.05 (0.96-1.14) | .3037 |
| Clinical T classification | cT1 | 1 | |
| | cT2 | 0.95 (0.71-1.28) | .7564 |
| | cT3/4 | 1.14 (0.69-1.85) | .6024 |
| Types of resection | Lobectomy* | 1 | |
| | Pneumonectomy | 1.40 (0.92-2.05) | .1144 |
| Pathologic T classification | pT1 | 1 | |
| | pT2 | 1.30 (0.96-1.76) | .0927 |
| | pT3 | 2.02 (1.26-3.16) | .0038 |
| Adjuvant chemotherapy | None | 1 | |
| | Intravenous chemotherapy | 0.80 (0.64-1.01) | .0588 |
| | Oral tegafur and uracil | 1.02 (0.75-1.36) | .9211 |
| Single or multiple N2 metastases | Single | 1 | |
| | Multiple | 1.37 (1.10-1.71) | .0049 |
| Skip N2 metastasis | Skip | 1 | |
| | Nonskip | 1.31 (1.06-1.62) | .0109 |
| Extent of N2 metastasis | Localized | 1 | |
| | Extended | 1.14 (0.89-1.46) | .2878 |

*OS*, Overall survival; *DFS*, disease-free survival; *CI*, confidence interval; *ECOG*, Eastern Cooperative Oncology Group; *PS*, performance status. *Lobectomy included lobectomy, bilobectomy, and lobectomy with bronchoplasty.

Extent of N2 metastasis was also revealed to be an independent prognostic factor affecting OS in our study. Okada and associates[16] reported that survival was prolonged in patients in whom mediastinal lymph node metastasis was limited to the upper mediastinal nodes from upper lobe tumors or to lower mediastinal nodes from lower lobe tumors. Uehara and colleagues[17] also reported that metastasis to upper mediastinal lymph nodes from lower lobe tumors was indicative of a poor prognosis. These reports imply that patients with localized N2 metastasis as defined in our study had better OS than did those with extended N2 metastasis. In patients with extended N2 metastasis, tumor cells

metastasize to lymph nodes far from the original tumor and/or through atypical lymphatic drainage routes; thus, the cancer cells may have strong metastatic potential leading to a poor prognosis. Our findings also showed that single N2 metastasis and skip N2 metastasis are better independent prognostic factors for DFS. The finding of better DFS in patients with single N2 metastasis corroborated previous studies that reported that the number of N2 stations with metastasis was a good indicator for prognosis of completely resected N2 NSCLC.[5,18-22] Involvement of multiple N2 stations implies increased tumor burden in the lymphatic flow and opportunity of systemic spread of tumor cells, which can lead to early recurrence of tumors. Skip N2 metastasis is discontinuous spread of tumors to mediastinal lymph nodes without involvement of the hilar lymph nodes. The incidence of lymph node skipping has been reported to be approximately 30% (22%-50%),[5,6,15,17-21,23] and that of our study (50.0%) was slightly higher than these previous reports, which was likely due to a bias resulting from the fact that 60% of our patients had cN0 tumors. Several studies have reported that patients with skip N2 metastasis showed better prognosis than those with nonskip N2 metastasis[5,6,15,19]; however, other reports with contradictory findings also exist.[20,21] In tumors with skip N2 metastasis, the total number of lymph nodes involved in tumor metastasis to both N1 and N2 stations is usually less than in those with nonskip N2 metastasis. Therefore, inasmuch as the total tumor burden in the lymphatic flow in skip N2 metastasis is presumed to be smaller than in nonskip N2 metastasis, this may lead to better DFS.[22] In patients with skip N2 metastasis and who received adjuvant IV-CT, better DFS was observed, although DFS with regard to adjuvant chemotherapy was not affected by single or multiple N2 metastases and extent of N2 metastasis. The effect of adjuvant chemotherapy on the basis of pattern of metastasis to N2 regions remains unclear. Our data indicate that adjuvant IV-CT may be more effective in patients with smaller tumor burden in the lymphatic flow. In clinical trials of adjuvant chemotherapy for N2 NSCLC, pattern of metastasis to N2 regions should be taken into consideration as part of the stratification criteria of patients for further investigation.

Sex and ECOG PS were demonstrated to be independent prognostic factors for OS, but not for DFS. Female patients may gain more benefit from treatment with tyrosine kinase inhibitors for EGFR because many adenocarcinomas in female nonsmoker patients harbor EGFR gene mutations.[12-14] Further, patients with PS 0 can receive more intensive and long-lasting chemotherapy than those with PS 1 after recurrence. Clearly, these endogenous factors have a great influence on OS. However, tumor recurrence depends strongly on biological features of the tumor itself and is largely independent of patient-specific factors such as age, sex, or PS.

In our report, 123 patients were clinically classified as having N2 or N3 (cN2/3) disease and were diagnosed with pN2 NSCLC. This subset of patients had a 5-year OS of 36.6% with surgery alone (n = 58; 5-year OS, 31.8%) or with surgery and adjuvant chemotherapy (n = 65; 5-year OS, 40.8%) and are currently considered ideal for induction chemoradiotherapy followed by surgery in the clinical trial setting.[24] Because patients who were not suitable for initial complete resection might have been excluded in the study, a simple comparison between our results on cN2/pN2 patients and trials on induction chemoradiotherapy would not be valid. However, in some trials on induction chemoradiotherapy followed by surgery, the lung cancer was considered to be potentially technically resectable[25]; thus, findings from future clinical trials on induction chemoradiotherapy followed by surgery for pathologically proven cN2 stage IIIA NSCLC can be noted and compared with our OS results for validation.

This study has several limitations owing to the retrospective aspect of the research. Tumors of enrolled patients were evaluated as resectable without induction treatment, which may represent a bias toward good prognosis. Survival benefit of adjuvant chemotherapy or chemoradiotherapy and detailed evaluation of treatment effects of adjuvant chemotherapy on the basis of selected regimens and of postoperative radiation therapy could not be performed because of differences in treatment protocols at each individual institution and a selection bias in which adjuvant therapy may be applied to patients with more advanced NSCLC. Therefore, our result could not conclude whether adjuvant therapy was effective in the Japanese cohort. Well-designed trials on adjuvant chemotherapy or chemoradiotherapy for completely resected stage IIIA NSCLC should be carried out to establish the significance of adjuvant therapy in the Japanese cohort.

In conclusion, our study showed that single or multiple N2 metastases, skip or nonskip N2 metastasis, and pT classification were independent prognostic factors for DFS, whereas extent of N2 metastasis, pT classification, tumor diameter, ECOG PS, and sex were significant prognostic indicators for OS in patients with completely resected pN2 stage IIIA NSCLC. Because the survival benefit of adjuvant chemotherapy or chemoradiotherapy remains unclear, well-designed trials on adjuvant therapy for completely resected stage IIIA NSCLC should be carried out in the Japanese cohort, and aforementioned factors, especially pattern of metastasis to N2 regions, should be considered as stratification factors in these trials.

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# *Association of cigarette smoking with the expression of nuclear survivin in pathological Stage IA lung adenocarcinomas*

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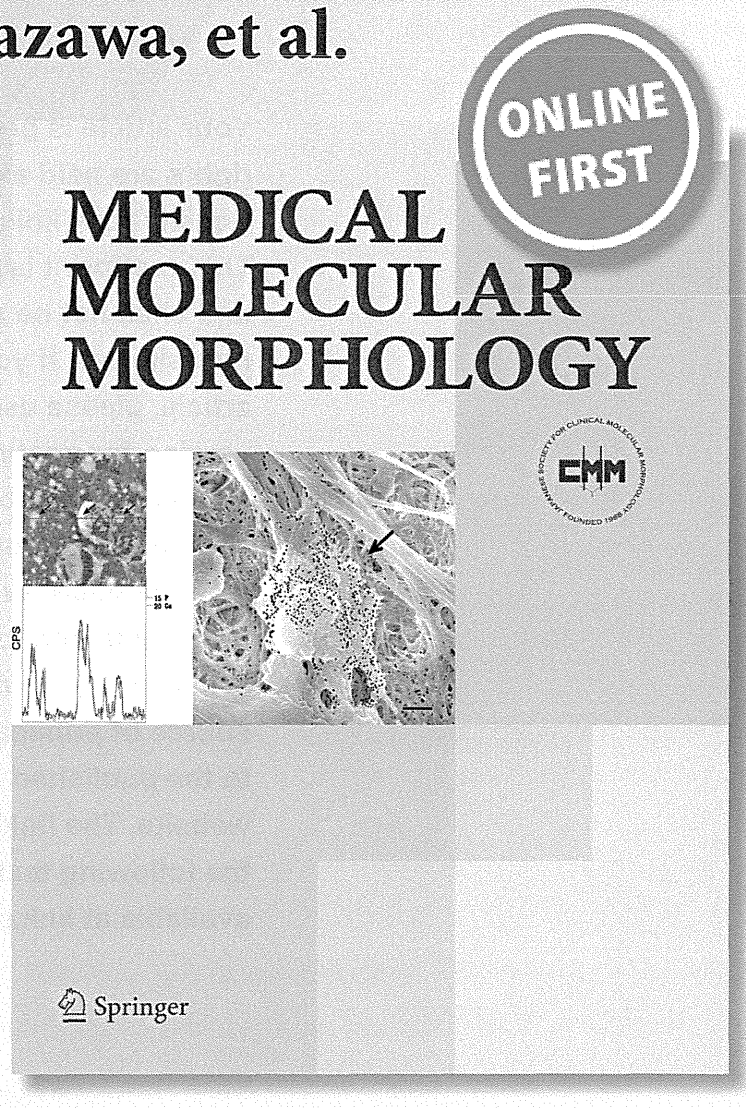


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