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 α and ER β 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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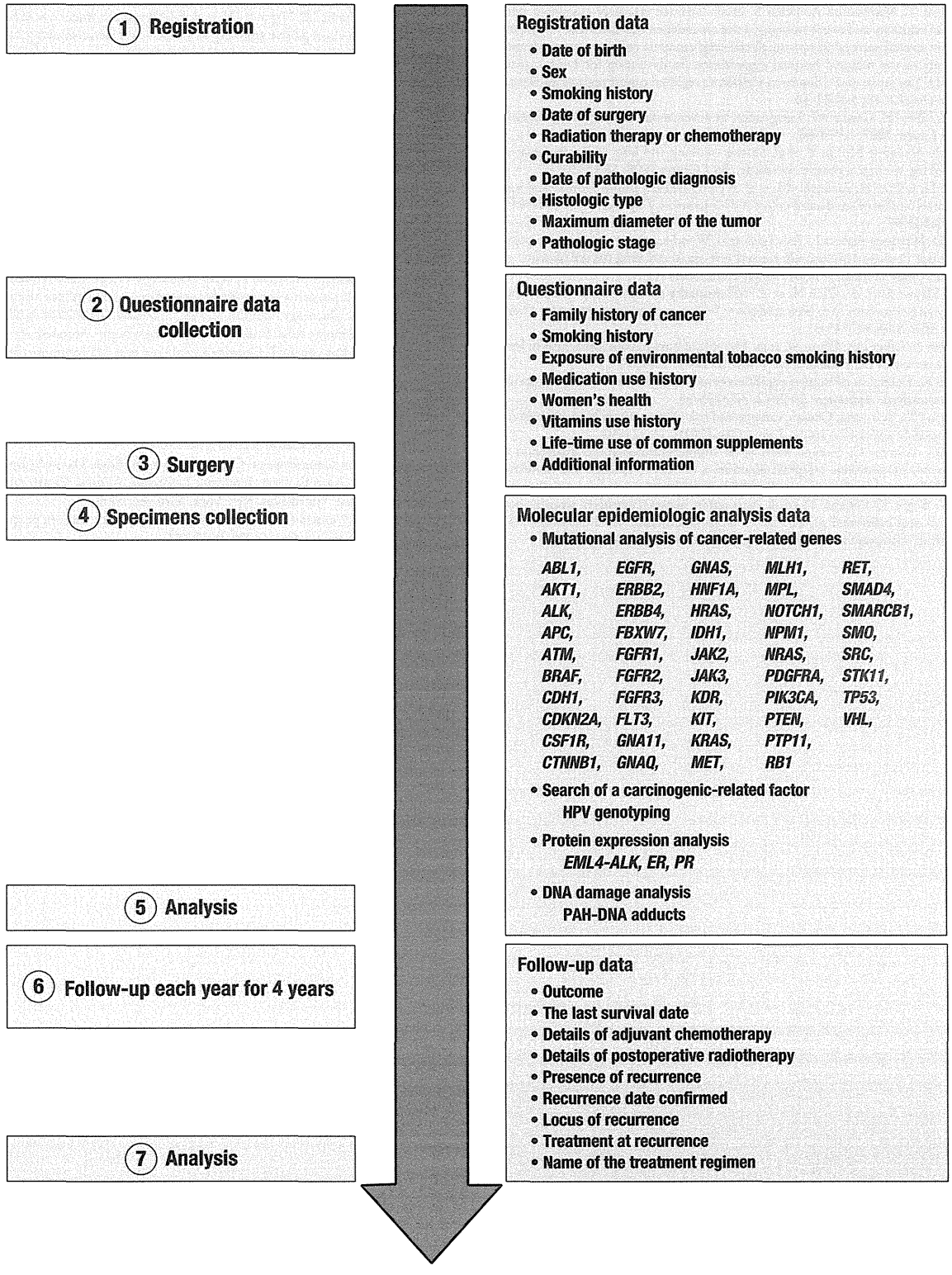
Figure 1 Scheme of the Japan Molecular Epidemiology for Lung Cancer



Disclosure

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

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CLINICAL TRIAL REPORT

Phase II study of concurrent thoracic radiotherapy in combination with weekly paclitaxel plus carboplatin in locally advanced non-small cell lung cancer: LOGIK0401

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Abstract
Objectives Concurrent chemoradiotherapy for regionally advanced stage III non-small cell lung cancer is the standard treatment method. However, the clinical implications of consolidation chemotherapy following chemoradiation have been unclear. Therefore, we conducted a phase II study of concurrent weekly carboplatin plus paclitaxel treatment in combination with radiotherapy followed by vinorelbine monotherapy. The primary endpoint was the 1-year survival rate.
Patients and methods Chemonaive PS 0–1 patients with stage IIIA/B NSCLC were enrolled. During the concurrent chemoradiation phase, patients were treated with weekly paclitaxel 40 mg/m^2 plus carboplatin AUC 2. The primary tumor and involved nodes received 60 Gy in 2-Gy fractions over 6 weeks. During the consolidation phase, vinorelbine 25 mg/m^2 on days 1 and 8 was repeated for three cycles.
Results A total of 40 eligible patients (72.5 % male; median age, 63 years; range 29–74 years) were analyzed for efficacy. Squamous cell carcinoma was the most common histology (47.5 %), and more patients had clinical stage IIIB (55 %) cancer. The average radiation dose was 56.5 Gy, and the average number of carboplatin plus paclitaxel cycles was 4.93. Seventeen patients proceeded to the consolidation chemotherapy phase, and 14 completed three cycles of vinorelbine monotherapy. The objective response rate was 75.0 %, including 1 patient who achieved a complete response. Progression-free survival and overall survival (OS) were 46 weeks [95 % confidence interval (CI) 31–64 weeks] and 110 weeks (95 % CI 90–184 weeks), respectively. The OS rate at 1 and 2 years was 85.0 % (95 % CI 69.6–93.0 %) and 53.9 % (95 % CI 37.1–68.0 %), respectively.
Conclusion Concurrent chemoradiation with weekly carboplatin and paclitaxel followed by vinorelbine

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consolidation is effective for stage III non-small cell lung cancer and shows a generally mild toxicity profile.

Keywords Locally advanced NSCLC · Chemoradiotherapy · Consolidation chemotherapy · Carboplatin · Paclitaxel · Vinorelbine

Introduction

The first randomized trial to demonstrate superiority of the addition of chemotherapy to radiation therapy in locally advanced non-small cell lung cancer (NSCLC) patients was trial CALGB 8433 [1]. Subsequent clinical trials have confirmed that concurrent chemoradiation improves overall survival (OS) compared with sequential combination chemotherapy followed by radiotherapy [2–4]. A meta-analysis conducted by Auperin et al. [5] revealed a significant survival benefit of concurrent therapy with an absolute benefit of 5.7 % at 3 years. Although a decrease in locoregional progression in concurrent chemoradiotherapy due to the radiosensitizing effect of platinum-based chemotherapy has been reported, no difference between concurrent and sequential arms has been noted concerning distant progression, meaning the need for additional chemotherapy. Belani et al. [6] conducted a randomized phase II trial comparing induction and consolidation chemotherapy in combination with concurrent chemoradiation. In their trial, concurrent chemoradiation with carboplatin plus paclitaxel (CP) followed by two cycles of the same regimen showed significantly prolonged survival compared with that in the induction chemotherapy arm. However, the Hoosier Oncology Group reported that consolidation docetaxel after concurrent chemoradiation with cisplatin plus etoposide could not improve survival in inoperable stage III NSCLC [7, 8] patients. These previous trials suggested the contribution of consolidation chemotherapy for improved survival, but the findings were not confirmed.

We previously conducted a phase II trial with concurrent chemoradiation and a weekly CP regimen followed by the same regimen as consolidation therapy after the completion of radiation. In this trial, paclitaxel 45 mg/m^2 plus carboplatin AUC 2 was repeated weekly during the consolidation phase as well as in the chemoradiation phase, but the median number of cycles was only 3, primarily because of myelosuppression and pneumonitis (unpublished data). Since the chemoradiation phase affects efficacy and toxicity during the consolidation phase, various drug combinations are needed to assess the total treatment strategy. Based on our previous trial, we conducted the present phase II trial with a similar chemoradiation strategy, but with a different consolidation chemotherapy comprising vinorelbine alone. Since the introduction of the oral agent vinorelbine to the market, it has attracted attention in the field of lung cancer treatment. In fact, oral vinorelbine has demonstrated a favorable risk–benefit ratio in concurrent chemoradiation therapy [9].

Patients and methods

Patients

Patients aged 20–74 years with histologically or cytologically confirmed inoperable stage IIIB or IV (UICC TNM staging system version 6) non-small cell lung cancer were eligible for enrollment. Other eligibility criteria included measurable disease, ECOG performance status (PS) of 0–1, life expectancy of at least 12 weeks, and adequate hematologic (absolute leukocyte count >4,000/μL and platelet count >100,000/μL), hepatic (bilirubin <1.5 mg/dL, aspartate transaminase/alanine transaminase <2.0 × ULN), renal (serum creatinine <1.0 × ULN), and pulmonary (PaO_2 > 70 Torr) function. Patients who received prior radiation therapy or systemic chemotherapy were excluded. Patients with active interstitial lung disease, active serious infection, or other serious underlying medical conditions were ineligible. The radiation field was limited to <50 % of the total area of the ipsilateral lung. The study protocol was approved by the institutional review boards that had jurisdiction over the sites where patients were registered for the study. All patients provided informed consent before enrollment.

Pretreatment evaluations included medical history, physical examination, ECOG PS, electrocardiography, radiologic tumor assessment, complete blood count with differential, and serum chemistry (aspartate transaminase, alanine transaminase, bilirubin, alkaline phosphatase, glucose, and creatinine). Imaging studies of brain and bone were performed when clinically indicated. Women with childbearing potential had to test negative for pregnancy via a urine test or serum pregnancy test.

Treatment plan

During the concurrent chemoradiation phase, patients were treated with paclitaxel 40 mg/m^2 plus carboplatin AUC of 2 mg/mL × min weekly. Consolidation chemotherapy was initiated 4 weeks after the completion of concurrent chemoradiotherapy and repeated for three cycles. As consolidation chemotherapy for the patients who completed radiation, vinorelbine 25 mg/m^2 was administered on days 1 and 8 in each cycle as shown in Fig. 1. Eligibility criteria for consolidation chemotherapy included PS 0–2, adequate organ function, and recovery to grade 2 or less non-hematological toxicities.

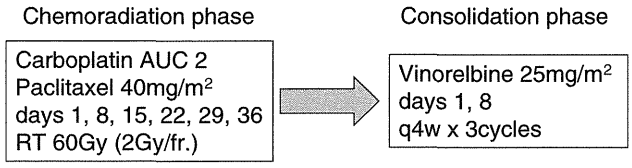

Fig. 1 Treatment schedule. *q4w* every 4 weeks, *RT* radiotherapy, *fr* fraction, *AUC* area under the plasma concentration–time curve

Radiation therapy

All patients were treated with a linear accelerator photon beam of at least 4 MV. The gross tumor volume (GTV) included the primary tumor, ipsilateral hilum, and mediastinal lymph nodes of 1 cm in the shortest diameter. The clinical target volume (CTV) included the GTV plus a 0.5-cm margin and mediastinal nodal areas from the paratracheal to the subcarinal lymph nodes. The supraclavicular lymph area could be treated when the supraclavicular lymph nodes were involved. The primary tumor and involved nodes received 60 Gy in 2-Gy fractions over 6 weeks. The initial 40 Gy was delivered based on planning target volume (PTV) including CTV with an appropriate margin. A margin of 1.5–2.0 cm for GTV and 0.5–1.0 cm for the mediastinal nodal area was added. The contralateral #10 lymph node was included in the PTV, but #11 and #12 lymph nodes were excluded. The remaining 20 Gy was delivered to only the primary tumor and the involved nodes. The spinal cord was excluded from the fields for the remaining 20 Gy by the oblique opposing method. When grade 4 hematologic toxicity, grade 3 or 4 esophagitis or dermatitis, pyrexia >37.5 °C, a worsened ECOG PS of 3 or 4, a decrease in partial arterial oxygen pressure of 10 Torr or more from baseline occurred, thoracic radiation was interrupted.

Assessment of efficacy and safety

Eligible patients who received any form of treatment were considered assessable for efficacy and toxicity. Chest X-ray, complete blood count, and blood chemistry were repeated once a week during the treatment period. Thoracic computed tomography was performed once a month during the treatment period. Other imaging examinations were performed when recurrence was suspected. Responses were assessed using the Response Evaluation Criteria in Solid Tumors version 1.0 guidelines. The overall response rate (ORR) was defined as the percentage of patients achieving CR or PR. In the evaluation of the antitumor effect, extramural review was conducted. Overall survival (OS) was defined as the time from the start of treatment until death from any cause. Progression-free survival (PFS) was defined as the time from the start of treatment until disease progression, death, or last known follow-up. OS, PFS, and the 1-year survival rate were estimated by the Kaplan–Meier method. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria Version 3.

Statistical analysis

The primary efficacy endpoint was the 1-year survival rate. The secondary efficacy endpoints were ORR, PFS, OS, first recurrence site, and toxicity. The sample size was calculated assuming a 5.0 % one-sided type I error and 80 % power. The patient accumulation period was 1.5 years, and the follow-up period was 3 years. In view of the dropouts, the sample size was set at 45 patients based on the assumption of a 1-year survival rate of 50 % as the threshold and 70 % in the experimental regimens [10, 11].

Results

Patient characteristics

Between November 2004 and May 2008, 45 patients were enrolled from 8 institutions. One patient did not undergo any treatment because of deterioration of respiratory status after enrollment. Four patients were found to be ineligible by extramural review. Therefore, efficacy analysis was performed for the 40 remaining eligible patients. Safety analysis was performed for the 44 patients who underwent chemoradiation. Baseline characteristics of patients are summarized in Table 1; 72.5 % of the 40 patients were male, and median age was 63 years (range 29–74 years). Squamous cell carcinoma was the most common histology (47.5 %), and more patients had clinical stage IIIB (55 %). The upper lobe was the primary site in most patients (65 %).

Treatment delivery

Table 2 shows the radiotherapy and chemotherapy delivered to eligible patients. The average radiation dose was 56.5 Gy. Radiation was interrupted in 7 patients due to adverse events (3 cases of fever, 1 neutropenia, and 1 pneumonitis) or the investigator's decision. The average number of chemotherapy (paclitaxel plus carboplatin) cycles in combination with radiation was 4.93. Fourteen patients completed chemoradiation without any treatment interruption, while 11, 8, and 7 patients skipped chemotherapy once, twice, and three times or more, respectively, primarily because hematological toxicity did not meet the criteria for starting chemotherapy. After chemoradiation therapy, 17 patients proceeded to the consolidation chemotherapy

Table 1 Patient characteristics for efficacy ($N = 40$)

	No. of patients	Percentage (%)
Gender		
Male	29	72.5
Female	11	27.5
Age		
Median	63	
Range	29–74	
Performance status		
0	16	40
1	24	60
Clinical stage		
IIIA	18	45
IIIB	22	55
Histology		
Squamous cell carcinoma	19	47.5
Adenocarcinoma	15	37.5
Not other specified	6	15.0
Location of primary site		
Upper lobe	26	65.0
Middle lobe	3	7.5
Lower lobe	10	25.0
Others	1	2.5

Table 2 Treatment delivery

Chemoradiation phase ($N = 40$)	
Radiation dose (average)	56.5 Gy
Reason for radiation interruption	
Adverse events	12.5 %
Others	5.0 %
No. of chemotherapy (average)	4.93
Chemotherapy skip: 1 time	27.5 %
Chemotherapy skip: 2 times	20.0 %
Reason for chemotherapy skip	
Adverse events	17.5 %
Others	7.5 %
Consolidation phase ($N = 17$)	
Rate of completion of 3 cycles	82.4 %

phase, while 14 patients completed three cycles of vinorelbine monotherapy.

Efficacy

The objective response rate was 75.0 %, including 1 patient who achieved a complete response. The disease control rate was 95 %, including 8 patients with stable disease. Only two patients showed progressive disease. The overall median follow-up time for the 40 eligible patients was 103 weeks (15–243 weeks). As shown in Fig. 2a, b, the median PFS and OS was 46 weeks [95 % confidence interval (CI) 31–64 weeks] and 110 weeks (95 % CI 90–184 weeks), respectively. The OS rate at 1 and 2 years was 85.0 % (95 % CI 69.6–93.0 %) and 53.9 % (95 % CI 37.1–68.0 %). The site of the first recurrence was confirmed in 33 patients (82.5 %) with disease progression. Of them, 14 (35.0 %), 11 (27.5 %), and 4 (10.0 %) had distant metastases, intrathoracic local disease, or both, respectively. Intrathoracic disease occurred in the irradiated field. Frequently observed initial distant metastases were in the brain (4 patients), bone (3 patients), and lung (3 patients).

Safety

Table 3 provides a summary of grade 3 and 4 toxicities. Among the hematological toxicities of the chemoradiation phase, grade 3 or higher leucopenia, neutropenia, anemia, and thrombocytopenia were observed in 10 patients (22.7 %), 6 patients (13.6 %), 2 patients (4.5 %), and 1 patient (2.3 %), respectively. Among the non-hematological toxicities, grade 3 infection, esophagitis, and hypersensitivity to the drug was observed in 5 patients (11.4 %), 4 patients (9.1 %), and 2 patients (4.5 %), respectively. No grade 4 non-hematological toxicity or treatment-related death was observed. Seventeen patients proceeded to consolidation chemotherapy, with 7 (41.2 %) exhibiting grade 3 or higher leucopenia and 7 (41.2 %) exhibiting neutropenia. Only 1 patient showed grade 3 phlebitis after vinorelbine administration.

Discussion

During chemoradiation treatment, the role of chemotherapy is improvement of the radiation effect as a radiosensitizer and eradication of micrometastasis by a cytotoxic anticancer agent. Based on previous clinical trials, carboplatin plus paclitaxel combination chemotherapy has already been confirmed to be effective with concurrent radiation [6, 10]. Moreover, a phase III trial in Japan showed that carboplatin plus paclitaxel with concurrent thoracic radiotherapy was equally efficacious and exhibited a more favorable toxicity profile compared with other cisplatin-based regimens [12]. Segawa et al. [13] reported that 49.5 % of stage III patients treated with concurrent chemoradiation using a mitomycin, vindesine, and cisplatin combination experienced initial failure. On the other hand, patients treated with cisplatin plus docetaxel tended to have a lower frequency of distant metastasis (37.4 %). Since no consolidation chemotherapy was performed in either treatment arm, it is considered that the relatively high-intensity chemotherapy using 3rd generation anti-cancer drugs suppresses micrometastases more strongly.

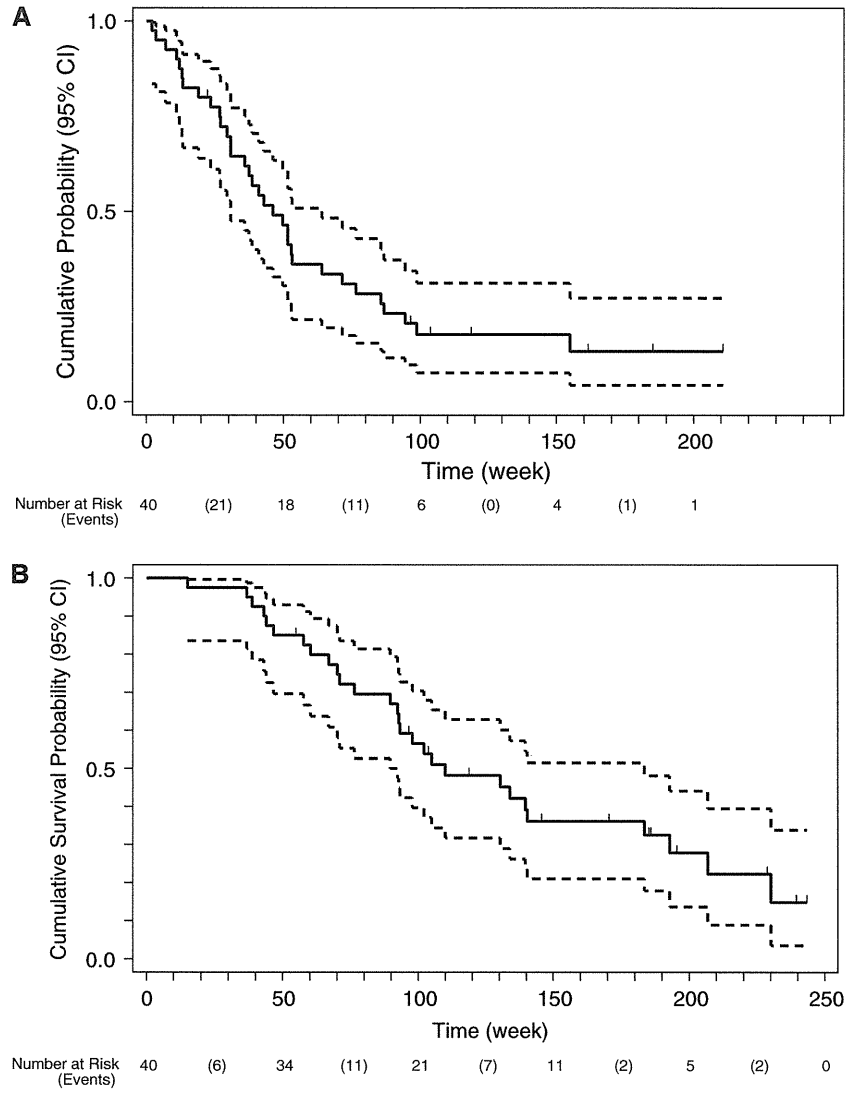

Fig. 2 Progression-free survival and overall survival. PFS (**a**) and OS (**b**) *curve* by Kaplan–Meyer method shown as a *solid line*. The *upper* and *lower limit* of the 95 % confidential interval is shown as a *dashed line* in each curve

Table 3 Hematological and non-hematological toxicities

	Chemoradiation phase ($N = 44$) (%)	Consolidation phase ($N = 17$) (%)
Hematological (G3/4)		
Leukopenia	22.7	41.2
Neutropenia	13.6	41.2
Anemia	4.5	0
Thrombocytopenia	2.3	0
Non-hematological (G3)		
Infection	11.4	0
Esophagitis	9.1	0
Hypersensitivity	4.5	0
Phlebitis	0	5.9

Other recent chemoradiation trials have shown distant metastasis in almost half of the patients [14–16]. This is considered inadequate systemic therapy in a patient population with locally advanced NSCLC. Therefore, there has been a desire to conduct additional cycles of chemotherapy either before or after concurrent therapy. However, induction chemotherapy provides no survival benefit, as reported previously [17], possibly due to reduced delivery of concurrent therapy. A three-arm study compared sequential chemotherapy/radiotherapy, induction chemotherapy followed by concurrent chemoradiation, and concurrent chemoradiation followed by consolidation therapy [6]. Although the study was not designed for direct comparison of the three treatment arms, the consolidation arm had a longer median survival, 16.3 months, compared with other arms. These data support the provision of definitive treatment up front followed by systemically active doses of chemotherapy as the preferred therapeutic approach. Trial S9504 reported by Gandara et al. [11] showed better overall survival with docetaxel consolidation therapy after concurrent

chemoradiation compared with observation, although no significant difference was noted. On the other hand, a phase III trial conducted by the Hoosier Oncology Group failed to demonstrate that consolidation docetaxel following concurrent therapy improves survival [7, 8]. Thus, consolidation therapy after concurrent chemoradiation requires additional studies.

We previously conducted a phase II trial of concurrent chemoradiation with weekly paclitaxel 45 mg/m^2 plus carboplatin AUC 2 followed immediately by the same weekly chemotherapy regimen. However, the median number of cycles of weekly consolidation therapy was 3, possibly because of hematological toxicity (unpublished data). Therefore, in the present study, we reduced the dose of paclitaxel from 45 to 40 mg/m^2 and ensured a non-dosing period of 4 weeks before consolidation chemotherapy. Vinorelbine was used as a consolidation chemotherapy agent because of its preferred toxicity profile, especially the lower pulmonary toxicity for previously untreated or treated lung cancer patients [18, 19]. In fact, vinorelbine is commonly used in chemoradiation therapy and has been confirmed to be safe in this treatment modality [16, 20].

In the present study, of the 30 patients who completed the full dose of radiation, 13 could not proceed to consolidation chemotherapy. The most likely reason was radiation pneumonitis, which did not meet the criteria for starting consolidation chemotherapy. Other reasons included myelosuppression, progressive diseases, and patient decisions. Since the investigators stopped the initiation of consolidation chemotherapy due to relatively mild radiation pneumonitis, the start criteria remain to be considered. The initial relapse sites were the brain, bone, and lung, as reported previously. In particular, we need to be creative when treating brain or bone metastases that are difficult to treat with systemic chemotherapy alone. Prophylactic cranial irradiation or bone-modifying agents such as bisphosphonate or denosumab in combination with chemotherapy may be efficacious.

In conclusion, concurrent chemoradiation with weekly carboplatin and paclitaxel followed by vinorelbine consolidation is effective for stage III non-small cell lung cancer and has a generally mild toxicity profile. However, additional studies are needed to allow more patients consolidation treatment.

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Conflict of interest Koichi Takayama was a principal investigator in Kyushu University Hospital in one funded clinical trial by Bristol-Myers Squibb Co. and one funded clinical trial by Kyowa Hakko Kirin Co.

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Nicotine Induces Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor by α1 Nicotinic Acetylcholine Receptor–Mediated Activation in PC9 Cells

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Introduction: Nicotine, the major component among the 4000 identified chemicals in cigarette smoke, binds to nicotinic acetylcholine receptors (nAChRs) on non–small-cell lung cancer (NSCLC) cells and regulates cellular proliferation by activating mitogen-activated protein kinases [AQ: MAPK has been expanded to mitogen-activated protein kinases. Please approve.]and PI3K/Akt pathways. In patients with smoking-related lung cancer who continue smoking, the anticancer effect of epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) is weaker than that in nonsmokers; however, the precise reason for this difference remains unclear. We investigated the role of α1 nAChR subunit in this phenomenon.

Methods: We screened for α1 nAChR mRNA in three NSCLC cell lines and analyzed the protein in resected primary NSCLC tissues. We used Western blot and RNA interference (siRNA) methodology to confirm the results.

Results: We determined that α1 nAChR plays an essential role in nicotine-induced cell signaling and nicotine-induced resistance to EGFR-TKI. In addition, we showed that silencing of α1 nAChR subunit in NSCLC may suppress the nicotine-induced resistance to EGFR-TKI.

Conclusions: These results further implicate nicotine in lung carcinogenesis, and suggest that α1 nAChR may be a biomarker for EGFR-TKI treatment and also a personalizing target molecule for patients with smoking-related lung cancer.

Key Words: Nicotinic acetylcholine receptors, Non–small-cell lung cancer, Nicotine, Epidermal growth factor receptor tyrosine kinase inhibitor, Targeting therapy.

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Cigarette smoking is one of the major risk factors associated with non–small-cell lung cancer (NSCLC), which accounts for 80% of all lung cancers.[1–4] Nicotine, the main psychoactive component of cigarette smoke,[5] can induce cell proliferation, angiogenesis, and resistance to apoptosis,[6–9] and may also facilitate the development of lung cancer by sustained activation of growth-promoting pathways.[10–13] These effects of nicotine are mediated by nicotinic acetylcholine receptors (nAChRs) that are widely expressed in the lung as well as the brain and neuromuscular junctions.[5,10,13–15] Nicotine also reduces the effectiveness of cancer chemotherapy[14,16,17]; however, the mechanism by which this occurs remains unclear.

nAChRs are either homopentamers or heteropentamers consisting of 10 α-subunits (α1–α10), four non–α-subunits β (β1–β4), γ, δ, and ε, and are classified into neuronal and muscle forms.[5,18] Although the combinations of nAChRs have not been completely characterized, it is possible that each subtype has distinct pharmacologic properties.[19] In addition, nAChRs stimulate intracellular signaling pathways in a cell type–specific manner, thus cell type–specific oncogenesis occurs in response to different subunits of nAChRs.[13,19] Furthermore, significant smoking-dependent declines in expression of α1, α5, and α7 nAChR have been reported.[13,19] These nAChR subunit genes could be playing roles in nicotine-induced lung cancer. Among the subunit genes, α7 nAChR is well studied[14,20,21]; however, little is known about the others. Further evaluation of the functional roles played by these nAChR subunit genes in nicotine exposure and lung carcinogenesis is needed.

NSCLC is characterized by its poor prognosis and resistance to anticancer drugs.[22] Now, more than ever, clinicians and NSCLC patients are struggling to optimize treatments. Although clinical trials have revealed epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) as the most promising therapeutic agent (e.g., erlotinib, gefitinib) in NSCLC,[23,24] there are still clinical questions to be addressed. Recently, smoking (nicotine) exposure has been shown to have a negative effect on EGFR-TKI therapy in lung cancers,[25] although the mechanisms that contribute to the drug resistance remain unknown. It has been reported that exposure to nicotine increases EGFR expression in lung cells by activating survival pathways.[10,13,26,27] In addition, differential expression of nAChR subunits have been observed between

smokers and nonsmokers.[19] Therefore, we hypothesized that interaction between nicotine and nAChRs may contribute to the process that generates resistance against EGFR-TKI treatment.

In this study, we focused on the functionality of the muscle-type α1 nAChR subunit. We examined the expression of α1 nAChR mRNA and protein in human NSCLC cell lines and human NSCLC tissues. We also analyzed the role of nicotine in activations of ERK and Akt (Ser-473) pathways through the α1 nAChR in PC9 cells. Finally, we studied the ability of nicotine-α1 nAChR signaling to protect NSCLC cells from EGFR-TKI treatment.

MATERIALS AND METHODS

Cell Culture

NSCLC cell lines (A549, H2122, and PC9), HEK293, and BEAS 2B cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium or Roswell Park Memorial Institute media supplemented with 10% fetal bovine serum and incubated at 37°C in 5% carbon dioxide.

Real-Time Reverse Transcription Polymerase Chain Reaction of α1 nAChR

Total RNA was isolated with an RNeasy Mini kit from Qiagen (Valencia, CA), and RNA (1 μg) was reverse-transcribed to generate cDNA with PrimeScript RT reagent kit (Perfect Real Time, Takara, Japan) according to the manufacturer's protocol. The primers and conditions for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) were described elsewhere.[19] The relative quantitation value for α1 nAChR gene compared with the calibrator was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to 18S). A 10-μl aliquot of each reaction was analyzed on 2% agarose gels. glyceraldehyde-3-phosphate dehydrogenase (GADPH) (5′-ACCTACCAAATATGATGACATCA-3′, 5′-CGCTGTTGAAGTCAGAGGA-3′) was used as a positive control for RNA integrity.

Western Blot Analysis

A549, PC9, and H2122 cells were rinsed with ice-cold phosphate-buffered saline and scraped into lysis buffer with protease inhibitors and phosphatase inhibitor. Protein was quantitated with the BCA assay (Thermo Scientific, Pierce). Whole-cell lysates were used for Western blot analysis. In brief, cell lysates adjusted for protein concentration were separated on 10% sodium dodecyl sulfate polyacrylamide gels and electrotransferred to polyvinylidene fluoride membranes. The transferred membranes were blocked with 3% nonfat milk and incubated with appropriate primary antibody (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. Membranes were then washed briefly and incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Inc., Beverly, MA).

Immunohistochemistry for α1 nAChR

For immunohistochemistry (IHC), 43 paraffin-embedded lung cancer samples were obtained from the Department of Pathology in Kyushu University Hospital. The patients' characteristics are summarized in Table 1. Clinicopathological factors, including sex, smoking history, and tumor type were evaluated. The samples included 32 adenocarcinomas, seven squamous cell carcinomas, two large-cell carcinomas, and two small-cell carcinomas. Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm sections. The slides were deparaffinized and hydrated and then pretreated for microwaving. Endogenous peroxidase was quenched with methanol/peroxide. The slides were blocked with rabbit serum, incubated with primary α1 nAChR antibody overnight (Salk Institute by Sigma), and then incubated with secondary antibody (Dako). Immunodetection was performed with an avidin-biotin horseradish peroxidase method and visualized with 3,3′-diaminobenzidine (DAB) as the chromogen. The slides were then counterstained with hematoxylin.

Scoring of α1 nAChR Expression

Immunoreactivity was scored according to the percentage (P) of tumor cells showing characteristic membrane staining (0, undetectable; level 1, few; level 2, <10%; level 3, 10%–50%; and level 4, >50%) and the intensity (I) of staining (1, weak; 2, moderate; and 3, strong). Expression of α1 nAChR in each section was scored by multiplying P by I, the so-called quick score (Q) ($Q = P \times I$; maximum = 12). We classified staining as low-positive ($Q \leq 6$; $n = 16$, [including negative $Q = 0$]) versus high-positive ($Q > 6$; $n = 27$). Assessments were made by two independent observers blinded to histologic diagnoses. The correlation between clinicopathological factors was analyzed using $\div^2$ tests.

RNA Interference

Chemically synthesized double-stranded siRNA specific for nAChR α1 was purchased from Dharmacon Research (Thermo Fisher Scientific, Lafayette, CO). The siRNA was transfected (100 nmol/L) with DharmaFECT reagents according to the manufacturer's instructions. A nontargeting (NT) siRNA sequence (Dharmacon Research) was used as nonspecific control.

Cell Survival after EGFR-TKI Treatment

Intact PC9 cells and PC9 cells pretreated with nicotine (Sigma) were seeded at a density of 5,000 cells per well in a 96-well plate in complete medium overnight. Cells were treated with various concentrations of EGFR TKI (Calbiochem EMD Biosciences, La Jolla, CA) for 48 hours, and then 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega, Madison, WI) was added per well for 1 hour. Survival was determined by comparison of the absorbance at 490 nm with that of the control.

Statistical Analysis

Data are presented as the mean ± SEM for three independent experiments. The $\div^2$ tests were used to compare two groups, and one-way analysis of variance was used to analyze data among groups for significant difference. A p value less than 0.05 indicated a statistically significant difference. JMP version 9 (SAS Institute Inc., Cary, NC) software was used for all analyses.

TABLE 1. Association between α1 nAChR Expression and Clinicopathological Factors in NSCLC

Factors	Total Patients N = 43	α1 nAChR-High Positive $n = 27$	α1 nAChR-Low Positive $n = 16$	p
Sex				
Male	25	18	7	0.2035
Female	18	9	9	
Smoking history				
Smoker	27	13	14	0.0206
Never smoked	16	14	2	
Tissue type				
Adenocarcinoma	32	24	8	0.0138
Nonadenocarcinoma[a]	11	3	8	

[a]Seven squamous cell carcinomas; two large-cell carcinomas; two small-cell carcinomas.
nAChR, nicotinic acetylcholine receptor.

RESULTS

Expression of the α1 nAChR Subunit in NSCLC Cell Lines and in Human NSCLC Tissues

Three NSCLC cell lines (A549, PC9, and H2122) were examined for expression of α1 nAChR subunit mRNA by RT PCR. BEAS 2B cells were used as a positive control,[13,15] and GAPDH was included in each panel as a reference. As shown in Figure 1*A*, all three cell lines expressed α1 nAChR mRNA. Expression of the receptor proteins in the four cell lines was confirmed by Western blot with HEK293 cells as the negative control.[3] All four cell lines expressed detectable levels of the α1 nAChR subunit, as shown in Figure 1B.

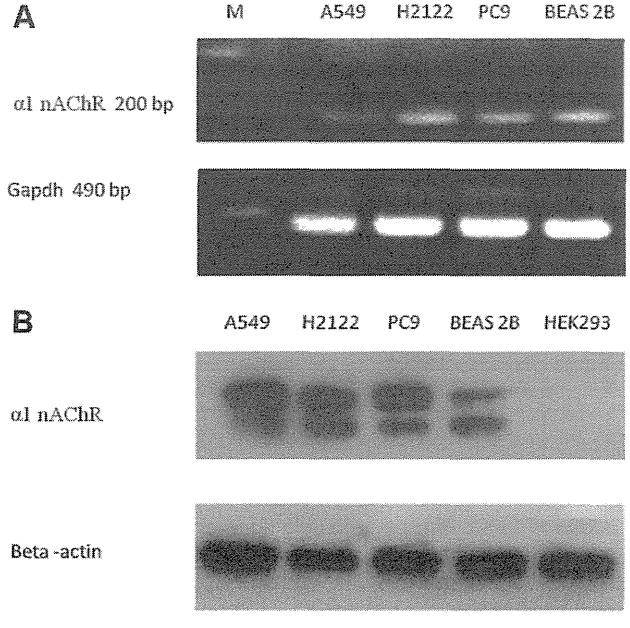

FIGURE 1. *A*, NSCLC cell lines express nAChR mRNA and protein. Detection of reverse-transcriptase polymerase chain reaction products for α1 nAChR subunits in A549, H2122, and PC9 cell lines. The BEAS 2B cell line was a positive control. Products were sequenced and in each case confirmed to be the expected authentic sequence for each subunit. M, 100 bp molecular marker with the brightest band at 600 bp. *B*, Western blot results for α1 nAChR subunits. NSCLC cell lines express nAChR protein. BEAS 2B cell line or HEK293 cell line served as the positive or negative control, respectively. nAChR, nicotine acetylcholine receptors; NSCLC, non–small-cell lung cancer.

We also examined paraffin-embedded specimens of human NSCLC tumors for expression of α1 subunit by IHC. Our series of NSCLC types included 32 adenocarcinomas, seven squamous cell carcinomas, two large-cell carcinomas, and two small-cell carcinomas. Although a certain level of expression of α1 nAChR was shown in almost all types of lung cancer, statistical analysis showed that the adenocarcinoma tissues were stained more strongly than the nonadenocarcinoma tissues (Table 1). Representative photographs of positive immunostaining for each histological type of lung cancer are shown in Figure 2. The results suggested that the α1 nAChR might have a function in lung tumors, especially in adenocarcinoma.

Roles of the α1 nAChR Subunit in Nicotine-Induced Cell-Signaling Pathways of NSCLCs

These studies led to the realization that the α1 nAChR may have functional roles in NSCLC cells. Indeed, given the fact that nicotine could induce cell proliferation, angiogenesis, and growth of tumors,[10–13,28] we next examined the ERK1/2, Akt (Ser-473), and STAT3 pathways induced by nicotine in PC9 cells. In Western blot analysis, we found that phosphorylated ERK1/2 and PI3K/ Akt (Ser-473) increased in a time-dependent manner after treatment with nicotine (Fig. 3*A*). In contrast, phosphorylated STAT3 was not detected at all. To further determine whether α1 nAChR subunit was directly involved in the ERK1/2 and Akt (Ser-473) pathways, PC9 cells were transfected with siRNA targeting α1 nAChR (si-α1) or NT siRNA (si-NT). Markedly down-regulated expression of phosphorylated Akt (Ser-473) and phosphorylated ERK1/2 protein was seen by Western blot (Fig. 3*B*). Taken together, these results suggested that α1 nAChR contributed, at least in part, to nicotine-induced activation of the ERK1/2 and Akt (Ser-473) pathways in PC9 cells.

Effects of the α1 nAChR Subunit in Nicotine-Induced EGFR Signaling Pathways of NSCLCs

The experiments with siRNA targeting α1 nAChR showed that phosphorylation of Akt (Ser-473) and ERK1/2 can be partially blocked in PC9 cells. EGFR has been reported to activate the same downstream pathways as α1 nAChR.[26] Considering the cross-talk between downstream signaling by EGFR and α1 nAChR, it is possible that nicotine activates EGFR through the activation of ERK1/2 and PI3K/Akt (Ser-473). In the studies that followed, we observed that nicotine caused time-dependent phosphorylation of EGFR in PC9 cells (Fig. 3*C*), and that the knockdown of α1 nAChR expression by siRNA could reduce the protein levels of both EGFR and phosphorylated EGFR (Fig. 3*D*). Gene silencing of α1 nAChR by siRNA was validated by real-time RT PCR (Fig. 4*A*, *B*). These results suggest that α1 nAChR might act as an upstream regulator of the EGFR pathway in PC9 cells.

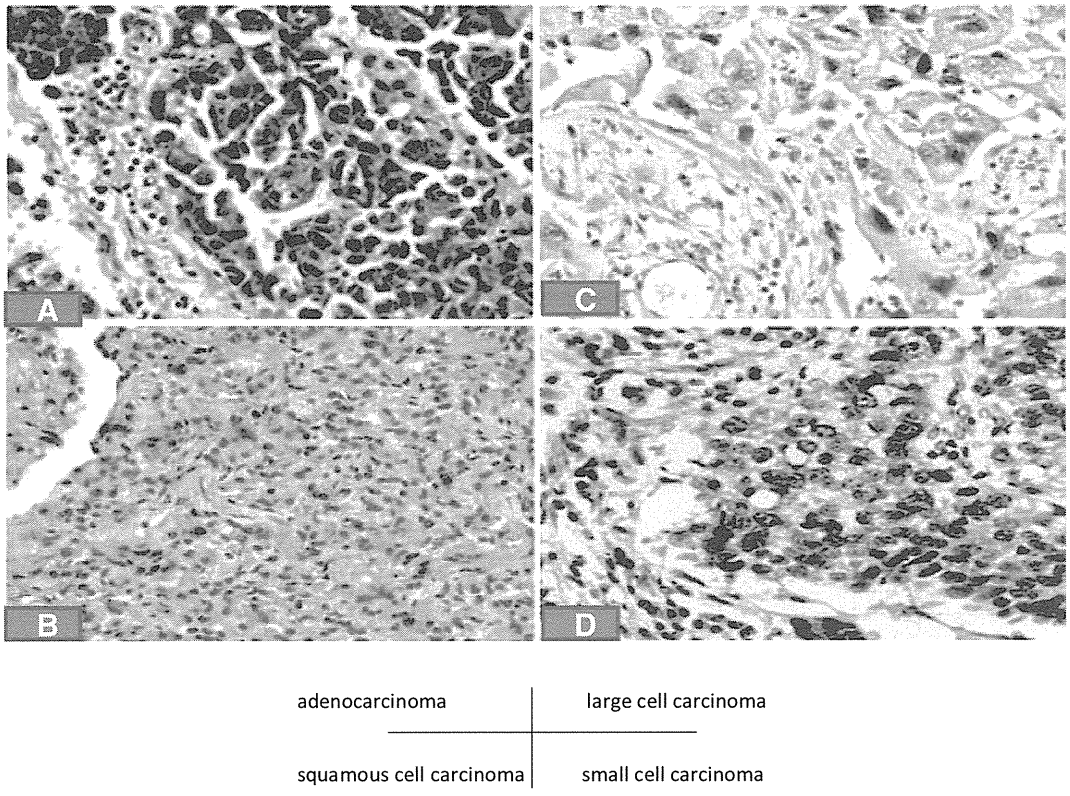

FIGURE 2. Representative photographs of tissue microarrays showing that non–small-cell lung cancer tissue expressed α1 nicotine acetylcholine receptors. Dark-brown staining was indicative of the presence of α1 protein, and tissue was counterstained to indicate nuclei with dark-blue staining. *A*, adenocarcinoma ×400; (*B*), squamous cell carcinoma ×400; (*C*), large-cell carcinoma ×400; and (*D*), small-cell carcinoma ×400.

Nicotine-Induced Resistance to EGFR-TKI

Nicotine-induced activation of pathways related to proliferation (PI3K/Akt, ERK1/2, and EGFR) suggested that exposure to nicotine might protect cancer cells against the cytotoxic effects of EGFR-TKI. Therefore, we investigated whether nicotine exposure caused resistance to EGFR-TKI in PC9 cells (Fig. 5*A*). EGFR-TKI had a dosage-dependent killing effect in PC9 cells as shown in Figure 5*A* (open bar). However, PC9 cells pretreated with nicotine were not killed as effectively by EGFR-TKI. When PC9 cells were pretreated with nicotine for 24 hours, larger numbers survived treatment with 5 or 10 μmol of EGFR-TKI as compared with unexposed PC9 cells. When PC9 cells were pretreated with nicotine for 1 month, the resistance against EGFR-TKI was clear at a concentration of EGFR-TKI as high as 20 μmol. Finally, restoration of sensitivity to EGFR-TKI was examined by α1 nAChR knockdown in nicotine-exposed PC9 cells. PC9 cells were incubated in the presence or absence of 1 μmol of nicotine for 1 month,[19] then transfected with siRNA α1 or NT siRNA for 72 hours. As shown in Figure 5*B*, siRNA against α1 nAChR decreased the resistance to EGFR-TKI induced by nicotine. In contrast, the resistance persisted in cells stimulated by nicotine and transfected with NT siRNA. These results suggested that the enhanced resistance to EGFR-TKI in PC9 cells stimulated by long-term nicotine exposure was, at least in part, dependent on α1 nAChR expression.

DISCUSSION

More than one billion people around the world smoke, and cigarette smoking and second-hand smoking account for nearly 90% of lung cancer deaths.[29] Studies in recent years raise the possibility that exposure to nicotine might lead to increased risk of lung cancer[30]; however, the detailed molecular mechanisms remain largely unknown. The fact that nicotine promotes lung cancer by activating different nAChR subunits, thus leading to the activation of several pathways,[10,12,13] gives reason to believe that the nAChR family may play important roles in lung tumorigenesis.

In the present study, expressions of α1 nAChR mRNA and protein were determined in NSCLC cell lines. The results also proved to be applicable to human NSCLC by analysis of lung cancer human tissues with IHC. Consistent with previous reports,[10,12,19] all these data suggested that α1 nAChR is likely to be involved in the smoking-related pathogenesis of NSCLC.

In accordance with the roles of nicotine in multiple signaling pathways of NSCLC,[10–13] we found that exposure to nicotine stimulated phosphorylation of ERK1/2 and Akt (Ser-473) in a time-dependent manner. The phosphorylation was evident within 5 minutes and reached a peak within 30 minutes, suggesting that the triggering of these pathways could play a role in the early development of nicotine-induced NSCLC (failure of detection of STAT3 phosphorylation may be because of the inappropriate cell line).[28] Because the nicotine-activated

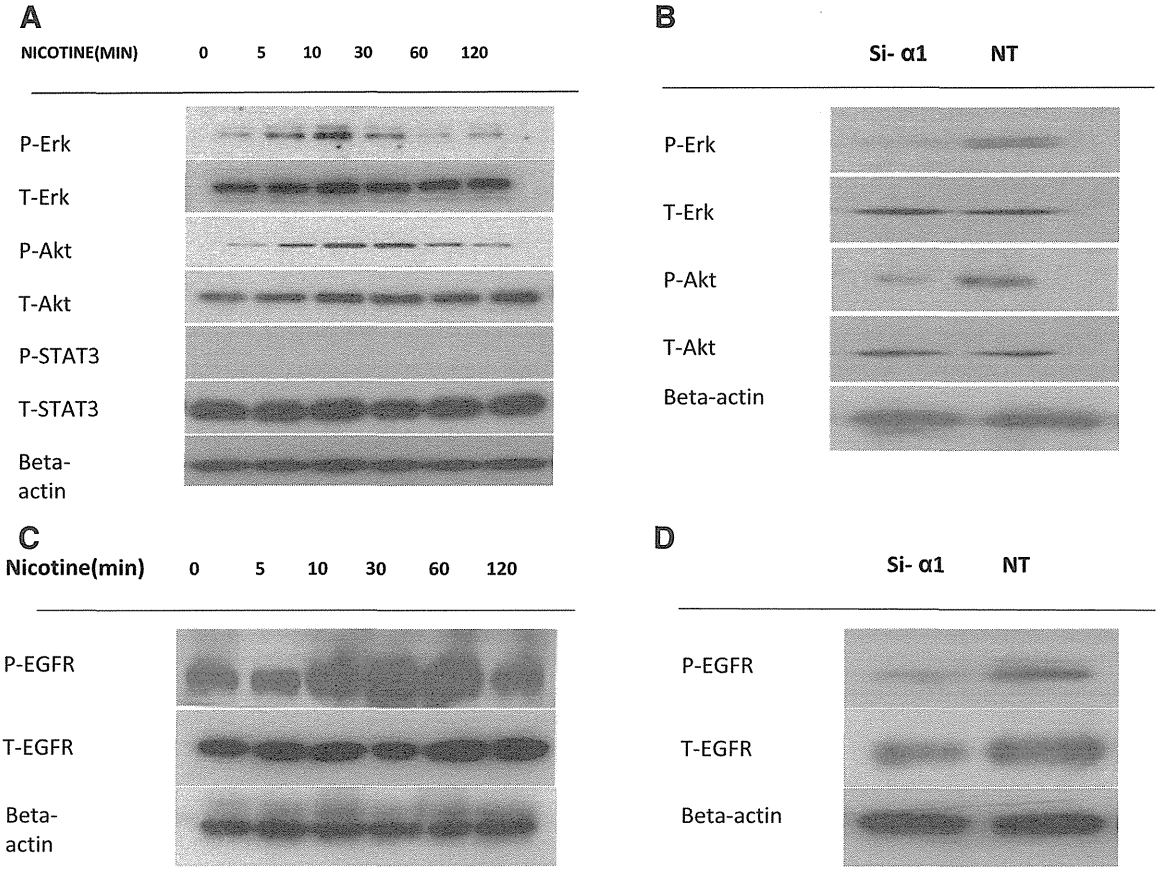

FIGURE 3. *A,* Induction of Erk, Akt (Ser-473) and STAT3 phosphorylation by nicotine. PC9 cells were serum-starved for 24 hours then incubated with 10 μM of nicotine for the number of times indicated (upper panel). Western blot analysis was used to reveal the time-dependent phosphorylation of ERK and Akt (Ser-473); (*B*) Induction of Erk and Akt (Ser-473) phosphorylation was dependent on α1 nAChR. PC9 cells were transfected with siRNA against α1 nAChR subunit (si-α1) or NT siRNA for 72 hours. Western blot analysis revealed the difference in phosphorylated levels of ERK1/2 (p-ERK1/2) and Akt (p-Akt) between cells treated with siRNA α1 and NT; (*C*) Induction of EFGR phosphorylation by nicotine. PC9 cells were serum-starved for 24 hours then incubated with 10 μM of nicotine for the number of times indicated (upper panel). Western blot analysis shows the EGFR and p-EGFR protein levels; (*D*) Role of α1 nAChR subunit in the EFGR signaling pathway. PC9 cells were transfected with siRNA against the α1 nAChR subunits (si-α1) or NT siRNA for 72 hours. Western blot analysis shows EGFR and p-EGFR protein levels. EFGR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; nAChR, nicotinic acetylcholine receptor; NT, nontargeting.

signaling pathways were also thought to be involved with EGFR,[26,31,32] cross-talk between EGFR and nAChR was proposed. In support of this hypothesis, we found in our present study that nicotine caused phosphorylation of EGFR in a time-dependent way. Therefore, we next attempted to identify the specific nAChR subunit responsible for these effects. Although

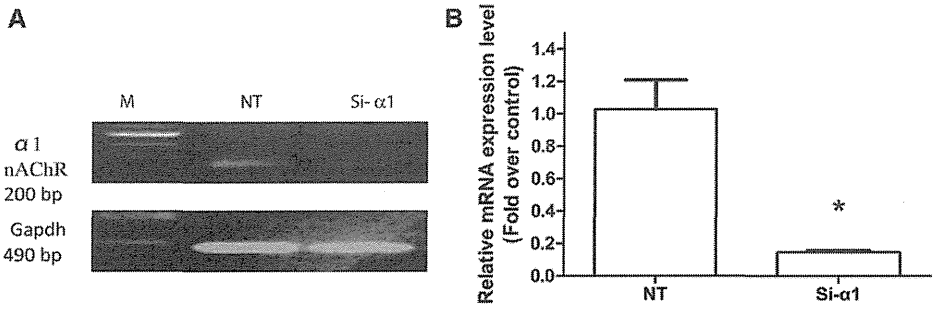

FIGURE 4. *A,* Gene silencing of α1 nAChR was validated by reverse transcription PCR in PC9 cells. α1 nAChR mRNA expression was knocked down by siRNA; (*B*) The relative levels of α1 nAChR mRNA measured by real-time-PCR in control NT and α1 nAChR knockdown (si-α1) PC9 cells. Data are presented as mean ± standard error mean of three independent experiments carried out in triplicate. *$p < 0.05$. nAChR, nicotinic acetylcholine receptor; NT, nontargeting; PCR, polymerase chain reaction.

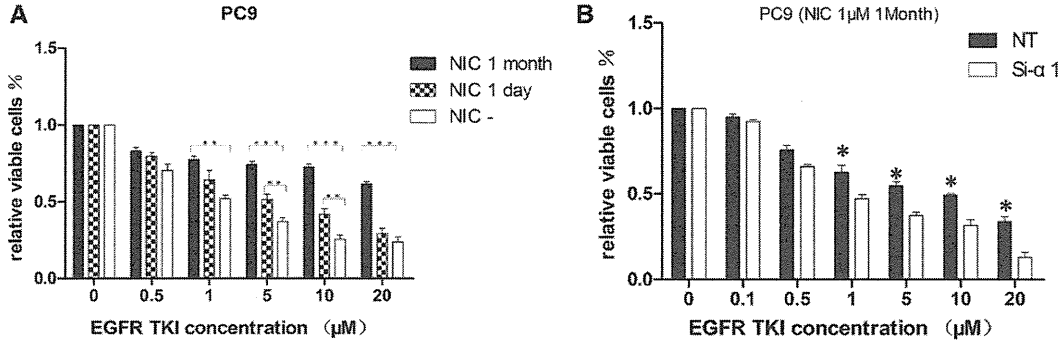

FIGURE 5. *A*, Nicotine protects NSCLC cells from cytotoxicity induced by EGFR-TKI. NSCLC PC9 cells were pretreated with 1 μM of nicotine for 1 month or 10 μM of nicotine for 1 day. Cells were then treated with EGFR-TKI for 48 hours. Cell survival was measured with the MTS assay and was expressed as a percentage of the untreated control. The concentrations of EGFR-TKI were increased in increments as indicated. Data are presented as mean ± standard error mean of three independent experiments carried out in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. *B*, α1 nAChR subunit mediated EGFR-TKI resistance induced by long-term nicotine exposure. PC9 cells were pretreated with 1μM of nicotine for 1 month, then transfected with siRNA against α1 nAChR subunit (si-α1) or nontargeting siRNA NT for 72 hours. Cell survival was measured with the MTS assay and is expressed as a percentage of the untreated control. The concentrations of EGFR-TKI were increased in increments as indicated. Data are presented as mean ± standard error mean of three independent experiments carried out in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. EFGR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; NSCLC, non–small-cell lung cancer; NT, nontargeting.

several functional subunits of nAChRs have been reported recently,[20,33] our IHC results suggest the possibility that nicotine could act through a pathway from α1 nAChR to EGFR as well.

Consistent with these findings, our blocking experiments (siRNA) and Western blot analysis proved that α1 subunit was responsible for EGFR release, and that siRNA down-regulated phosphorylation of EGFR by reducing phospho-ERK and phospho-Akt (Ser-473). Thus, we provide the following new information about nicotine-induced signaling pathways in PC9 NSCLC cells. First, nicotine was suggested as inducing EGFR-signaling pathways. Second, nicotine increased expression and phosphorylation of EGFR, which was coupled with increased ERK and AKT phosphorylation. These findings suggest that in PC9 cells, nicotine-stimulated cell proliferation and survival may be partially dependent on EGFR signals transmitted through AKT and ERK pathways. Third, the phosphorylation of EGFR, ERK, and Akt (Ser-473) were down-regulated by treatment with si–α1 nAChR, suggesting that α1 nAChR may act as an upstream regulator of the EGFR pathway in PC9 cells and may be responsible for mediating the proliferative and apoptotic effects caused by nicotine exposure. Here we identified a novel nicotine-stimulated survival signaling pathway mediated by α1 nAChR through EGFR phosphorylation.

It has been shown that never-smokers with lung cancer are more likely to respond to EGFR-TKI treatment than smokers.[34] Although one major mediator of responsiveness to EGFR-TKI is the mutation status of EGFR that is often shown in never-smokers,[35] we conjectured that nicotine–α1 nAChR signaling pathways may contribute to differences in responsiveness to EGFR-TKI therapy between never-smokers and active smokers or exsmokers who use nicotine replacement therapy. Our results from the MTS assay showed that both short-term (10 μm of nicotine for 1 day) and chronic nicotine stimulation (1 μm of nicotine for 1 month) did confer protection from EGFR-TKI-induced cytotoxicity in PC9 cells. Notably, under conditions of chronic stimulation by nicotine, the resistance to EGFR-TKI was significantly abrogated by silencing α1 nAChR (siRNA). These observations suggested that in addition to nicotine exposure or smoking status, continued or chronic exposure to nicotine (usually taken to be >10 days of continuous exposure to nicotine)[19] increased the resistance to EGFR-TKI compared with immediate or short-term exposure to nicotine; furthermore, the α1 nAChR subunit was responsible for mediating the resistance of EGFR-TKI induced by chronic nicotine exposure in PC9 cells. Therefore, elimination of α1 nAChR subunit or treatment with an α1 nAChR–specific inhibitor could provide highly tailored treatments for smoking NSCLC patients.

In summary, the results of our report, together with previously published reports suggest that the EGFR system shares significant cross-talk with the nAChR system in NSCLC cell lines. Our results indicate that the α1 nAChR subunit mediates resistance to EGFR-TKI therapy, induced by chronic nicotine exposure, through activation of the ERK and Akt (Ser-473) pathways. Thus, our findings might provide a mechanistic basis for the resistance to EGFR-TKI therapy, observed in patients who continue to smoke. A major problem of current NSCLC therapy is the fact that this cancer expresses many different signaling pathways. Inhibition of the activities of ERK, AKT, or EGFR pathways and other angiogenic regulators are therefore, currently targeted therapies that require treatment with multiple agents. Given the potential role of α1 muscle-type nAChR in several of the important pathways, targeted inhibition of α1 nAChR might become a novel treatment in NSCLC. In addition, the notion that α1 nAChR functions to promote lung carcinogenesis raises questions regarding the safety and appropriateness of nicotine-replacement therapies. Also, as smoking cessation is the most effective way to prevent nicotine-induced EGKR-TKI resistance, the study gives us a substantial reason to advocate smoking cessation for the cancer patients who smoke.

Finally, future exploration of nicotine–α1 nAChR-induced lung carcinogenesis should address the question of whether lung cancer risk is directly influenced by α1 nAChR or indirectly influenced by smoking behaviors; the effects of cigarette exposure on the pharmacokinetics involved in the EGFR-TKI resistance actions[36] should also be raised. Moreover, other nAChR subunit genes in chronic nicotine exposure warrant further evaluation.

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Phase II Trial of Erlotinib for Japanese Patients With Previously Treated Non-small-cell Lung Cancer Harboring *EGFR* Mutations: Results of Lung Oncology Group in Kyushu (LOGiK0803)

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Objective: Erlotinib has been reported to be useful for treatment of non-small-cell lung cancer harboring mutation of the epidermal growth factor receptor gene *EGFR-mt*. However, no prospective trial has yet assessed the utility of erlotinib in Japanese patients.
Methods: Patients with *EGFR-mt* (exon 19/21) non-small-cell lung cancer who had previously received one to two chemotherapy regimens were enrolled in this trial. Erlotinib was initially administered at a dose of 150 mg/day orally until disease progression or unacceptable toxicities occurred. The primary endpoint was the objective response rate.
Results: Twenty-six patients were enrolled between February 2009 and January 2011. Objective response was observed in 14 patients (53.8%, 95% confidence interval: 33.4–73.4%), and the disease control rate reached 80.8% (95% confidence interval: 60.7–93.5%). After a median follow-up time of 17.3 months (range: 5.8–29.5 months), the median progression-free survival was 9.3 months (95% confidence interval: 7.6–11.6 months). The median survival time is yet to be determined. Major toxicities were skin disorder and liver dysfunction; most episodes were grade 2 or less, and all were tolerable. Only one patient with grade 3 skin rash discontinued the study. No patients developed interstitial lung disease, and there were no treatment-related deaths.
Conclusions: This prospective study is the first to have investigated the usefulness of erlotinib in Japanese patients with previously treated *EGFR-mt* non-small-cell lung cancer. Although this trial could not meet the primary endpoint, erlotinib was well tolerated and showed clinical benefit such as promising disease control rate or progression-free survival in this population, similar to gefitinib.

Key words: erlotinib – EGFR – mutation – non-small-cell lung cancer

INTRODUCTION

Lung cancer, of which non-small-cell lung cancer (NSCLC) is the most common form, remains the leading cause of cancer death worldwide (1). A decade ago, the standard first-line treatment for all patients with advanced NSCLC was platinum-doublet chemotherapy. Although the development and widespread adoption of cytotoxic (i.e. third-generation) agents such as docetaxel, paclitaxel, irinotecan, gemcitabine or vinorelbine has greatly improved the management of NSCLC patients, the optimal response rate and median survival time still remain only about 30–40% and 10–12 months, respectively (2,3). Accordingly, the therapeutic outcome for standard platinum-doublet chemotherapy appears to have reached a plateau.

Therefore, research on NSCLC has become increasingly focused on molecular biology to identify specific molecules playing a key role in cancer behavior, such as tumor growth, proliferation, migration, survival or angiogenesis as potential targets of new therapeutic agents. As the mechanisms of these pathways have become clarified, some molecular-targeting agents have been developed in the last few years. The most promising oncologically relevant molecular alteration discovered to date is somatic mutation of the tyrosine kinase domain of the epidermal growth factor receptor gene (*EGFR*) (4,5). The association between *EGFR* mutation status and the clinical effectiveness of the EGFR tyrosine kinase inhibitors (EGFR-TKIs), gefitinib or erlotinib, has been studied extensively (6–13), and EGFR-TKIs are now recognized to be the most important agents for NSCLC patients harboring *EGFR* mutations.

Recently, five phase III trials have compared gefitinib or erlotinib with standard platinum-doublet chemotherapy in patients with advanced or recurrent NSCLC. In the IPASS trial (7) that investigated adenocarcinoma in patients with a non-smoking or light smoking history independently of *EGFR* mutation status, only patients harboring *EGFR* mutations were found to benefit from gefitinib (median progression-free survival (PFS) 9.5 vs 6.3 months, hazard ratio (HR) 0.48, 95% confidence interval (CI) 0.36–0.64). The NEJ002 trial (10) enrolled only patients with *EGFR* mutation-positive (*EGFR-mt*) disease and randomized them to a gefitinib group or a carboplatin plus paclitaxel group. Patients in the gefitinib group showed a longer PFS than those in the standard chemotherapy group (median PFS 10.8 vs 5.4 months, HR 0.30, 95% CI 0.22–0.41). In the WJTOG3405 trial (11), gefitinib alone was found to be superior to cisplatin plus docetaxel in participants selected on the basis of *EGFR* mutations (median PFS 9.2 vs 6.3 months, HR 0.49, 95% CI 0.34–0.71). With regard to erlotinib, the OPTIMAL trial (12) enrolled only individuals with *EGFR-mt* and randomly allocated patients to receive erlotinib or carboplatin plus gemcitabine. Participants in the erlotinib group had a longer PFS than patients who received carboplatin plus gemcitabine (median PFS 13.1 vs 4.6 months, HR 0.16, 95% CI 0.10–0.26). Results from the EURTAC trial (13), comparing erlotinib with standard chemotherapy in patients with *EGFR-mt* tumors, showed a median PFS of 9.7 months for patients receiving erlotinib, compared with 5.2 months for those receiving the standard chemotherapy (HR 0.37, 95% CI 0.25–0.54).

Among these trials, the WJTOG3405 and NEJ002 trials involved Japanese patients, and the IPASS trial involved Asian patients, among whom some were Japanese. Therefore, it can be safely concluded that the clinical benefit of gefitinib for Japanese patients with NSCLC harboring *EGFR* mutations has been definitively confirmed. On the other hand, the two erlotinib trials involved European patients and Chinese patients, respectively. Although *EGFR* mutations occur more frequently in Asian patients, including Japanese, than in Caucasian patients (14), only one phase III trial has investigated the utility of erlotinib in Asian patients with *EGFR-mt* tumors. Moreover, no prospective trial of erlotinib in Japanese patients with *EGFR-mt* tumors has been reported so far. Therefore, we conducted the present trial to evaluate the efficacy and safety of erlotinib in Japanese patients with NSCLC harboring *EGFR* mutations.

MATERIALS AND METHODS

Study Design

This multicenter phase II trial was conducted to evaluate the efficacy and safety of erlotinib in Japanese patients who had been previously treated for NSCLC harboring *EGFR* mutations. *EGFR* mutations were analyzed as described below at each institution beforehand, and only patients with *EGFR-mt* tumors were enrolled. The primary endpoint was the objective response rate (ORR). Secondary endpoints were the disease control rate (DCR), PFS, overall survival (OS) and safety.

Eligibility

Patients with histologically or cytologically confirmed advanced NSCLC were eligible for this study. Each patient was required to meet the following criteria: (i) clinical stage IIIB, IV, or postoperative recurrence; (ii) measurable tumor sites; (iii) Eastern Cooperative Oncology Group performance status of 0–2; (iv) tumors with *EGFR* mutations (exon 19 or 21); (v) history of one to two prior systemic chemotherapies but no prior EGFR-TKI therapy; (vi) appropriate organ functions (lung: $SpO_2 \geq 90\%$, heart: normal 12-lead ECG, bone marrow: hemoglobin ≥ 9.0 g/dl, white blood cells $\geq 3\,000/mm^3$, neutrophils $\geq 1500/mm^3$, platelets $\geq 100\,000/mm^3$, liver: AST/ALT ≤ 2 times upper normal limit, total bilirubin ≤ 1.5 mg/dl, kidney: serum creatinine ≤ 1.5 times upper normal limit) and (vii) life expectancy of at least 3 months.

This study followed the ethical principles of the Declaration of Helsinki, and the study protocol was approved by the institutional review board at each participating institution. All the patients provided written informed consent before study-related procedures were performed.

Study Treatment

Patients received 150 mg of erlotinib orally per day until the occurrence of progressive disease (PD) or unacceptable toxicity. In the event of treatment-related toxicity, two dose reductions were permitted per patient (first reduction to 100 mg/day, second reduction to 50 mg/day), and dosing could be interrupted for up to 14 days. No dose escalations were permitted. For grade 3 or intolerable grade 2 skin disorder or stomatitis, treatment was discontinued until improvement to grade 1 or less, and then a lower dose of erlotinib was started. For any other grade 3 treatment-related toxicities, treatment was interrupted until improvement to grade 1 or less, and then the same dose was restarted. For interstitial lung disease (ILD) of any grade, or grade 4 toxicity, treatment was permanently discontinued. No systemic anticancer treatment, radiotherapy or pleurodesis was permitted during the trial. Salvage regimens were not restricted for patients with PD or those leaving the protocol.

All the patients underwent comprehensive baseline assessments, including clinical laboratory tests and imaging studies. Patients also received follow-up assessments and monitoring at regular intervals. Toxicity evaluations were based on the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0 (v3.0).

Assessment of Tumor *EGFR* Mutation Status

EGFR mutations were analyzed at contracted laboratory of each institution beforehand. Polymerase chain reaction (PCR) invader assay (15), direct sequencing or peptide nucleic acid-locked nucleic acid PCR clamp assay (16) was used for analysis of *EGFR* mutations. Deletions in or near E746-A750 in exon 19 and L858R in exon 21 were regarded as mutation-positive for the purposes of this trial.

Evaluation of Efficacy

Tumor response was evaluated every 4 weeks according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline, and it was confirmed by extramural review. PFS was defined as the period from enrollment until the date of confirmation of PD or the date of death from any cause, whichever was earlier. Overall survival was defined as the period from registration until death due to any cause. Patients for whom there was no information about mortality or disease progression were censored at the date of the last progression-free assessment.

Statistical Analysis

All patients who received at least one dose of the study treatment were included in the safety and efficacy analysis. A one-stage design using the binominal probability was used to determine the sample size. Assuming that a response rate of 80% would be expected, whereas a rate of 50% would be the lower limit of interest, and $\alpha = 0.05$ (two-sided) and $\beta = 0.1$, the estimated number of patients accrued was 23. After assuming an inevaluability rate of $<10\%$, we planned an accrual of 26 patients.

Survival analysis was conducted on the full analysis set (FAS) using follow-up data available as of 31 January 2012. The survival curves were estimated using the Kaplan–Meier method, and differences in survival were compared by the log-rank test.

This study is registered with University Hospital Medical Information Network (UMIN) in Japan, number UMIN000003270.

RESULTS

Patient Characteristics

A total of 26 patients were enrolled between January 2009 and January 2011 at 15 institutes throughout Japan (Table 1). Fifteen (58%) of the patients were female. Median patient age was 68 years (range, 51–79 years). Most of the patients were non-smokers with adenocarcinoma; however, two patients with squamous cell carcinoma (one non-smoker and one former smoker) were also enrolled. EGFR mutations were detected in exon 19 in 19 patients (73%) and in exon 21 in seven patients (23%). Nine of 11 males and five of six smokers had *EGFR* mutation in exon 19.

Treatment Delivery

Median treatment duration was 9.3 months with a range of 0.9–19.2 months. Interruption of erlotinib treatment was necessary in eight patients, mainly due to skin disorder and liver dysfunction. The median duration of treatment interruption was 7 days (range, 3–13 days). During the study period, the dose of erlotinib was reduced to 100 mg/day in eight patients (30.8%) mainly for the above-mentioned reasons, and a further second reduction (to 50 mg/day) was required in three of those patients (11.5%) because of liver dysfunction, fatigue and anorexia, and at the patient's own request, respectively. At the time of data analysis, the treatment had been discontinued in 23 patients because of disease progression in 22 and skin disorder in one.

Response and Survival

Tumor response rates for the FAS are shown in Table 2. Fourteen patients were assessed as having partial response (PR) and seven as having stable disease (SD). The ORR was 53.8% (95% CI 33.4–73.4%) and the DCR was 80.8% (95% CI 60.7–93.5%). Although seven patients were assessed as having SD, all of them showed tumor shrinkage by an average of 20.4% (range: 5.4–29.0%). Patients with exon 21 mutations tended to have a higher RR and DCR than those with exon 19 mutations, although the difference was not significant (Fisher's exact test, $P = 0.39$ for RR, $P = 0.28$ for