Almost all evidence available has thus been derived from subset analysis of trials for locally advanced NSCLC. A secondary analysis of RTOG 94-10 revealed a greater survival benefit for concurrent chemotherapy (14). Schild et al. reported no significant difference in tumor regression between younger and older patients in an NCCTG trial (15). Meanwhile, some reports on inoperable NSCLC patients indicate that chemoradiotherapy has survival benefit compared with radiotherapy, but this may not be applicable for those >70 years of age, for whom radiation alone could be most beneficial (16,17).

Therefore, we cannot treat the elderly in the same way as we can younger patients: first, as elderly patients have poorer prognosis than younger patients, they may think that their quality of life is more important than risking radical treatment. Secondly, the elderly tend to be vulnerable to intensive care and toxicities of treatment drugs (18–21). Less toxic therapy may be more effective for the elderly with NSCLC.

Some clinical trials, in which the elderly were not included, showed some efficacy of carboplatin (CBDCA), an analog of CDDP, having no nephrotoxicity, neurotoxicity or ototoxicity and being much less emesis-provoking than CDDP (22–24). Additionally, some investigators found the same radiosensitizing properties of CBDCA (25–28) as also found for CDDP. Therefore, we hypothesized CBDCA to be more acceptable in the treatment of elderly patients. A phase II study has reported the use of radiotherapy and concurrent low-dose daily CBDCA in elderly patients with locally advanced NSCLC (29). For stage III patients, the median survival time (MST) was 15.1 months. Given an MST of ~10 months by radiation alone (5,6,8,9,11,17), this combined chemoradiotherapy seemed promising. Here we performed a randomized study to determine whether this combined chemoradiotherapy has an impact on survival in elderly patients with unresectable locally advanced NSCLC compared with radiotherapy alone.

PATIENTS AND METHODS

PATIENTS

Eligibility criteria for this study were as follows: age ≥71 years; a histologically confirmed non-small cell carcinoma; unresectable disease; stage IIIA except T3N1M0 and IIIB which does not have disease extended to any contralateral hilar nodes or any supraclavicular nodes, atelectasis of the entire lung or malignant pleural effusions; measurable disease; a required radiation field of less than one half of one lung; no previous chemotherapy or radiotherapy; an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; PaO_2 ≥70 torr, white blood cell count ≥4000/μl, hemoglobin level ≥9.5 g/dl, platelet count ≥100 000/μl, serum bilirubin level ≤1.5 mg/dl, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤ twice the upper limit of normal, and serum creatinine level ≤ the upper limit of normal; a life expectancy of at least 3 months; and written informed consent. Exclusion criteria included patients with active infection, interstitial pneumonia or active lung fibrosis, chronic obstructive pulmonary disease (COPD) or uncontrolled heart disease, an active synchronous cancer, or a metachronous cancer within three disease-free years.

Staging was performed by chest radiograph in two directions, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the head, CT scan of the chest, CT scan or ultrasound of the abdomen, and bone scintigraphy.

TREATMENT

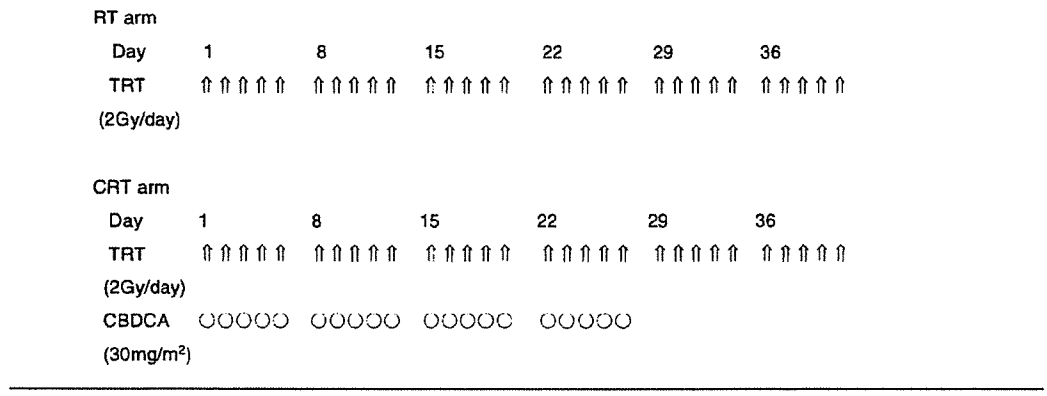
Patients were randomly assigned to the radiotherapy (RT) arm or the chemoradiotherapy (CRT) arm, by the minimization method of balancing PS (0 or 1 versus 2), stage (IIIA versus IIIB) and institution. The RT consisted of 60 Gy in 30 fractions over 6 weeks. In the CRT arm, patients received the same radiotherapy as in the RT arm and concurrent intravenous administration of CBDCA 30 mg/m^2 (30 min infusion) 1 h before every radiation treatment up to the first 20 fractions (Fig. 1).

Radiotherapy was delivered with megavoltage (6–10 MeV photons) equipment using anterior/posterior opposed fields up to 40 Gy including the primary tumor, the metastatic lymph nodes and the regional node. A booster dose of 20 Gy was given to the primary tumor and the metastatic lymph nodes for a total dose of 60 Gy using bilateral oblique fields. The clinical target volume (CTV) for the primary tumor was defined as the gross tumor volume (GTV) plus 1 cm taking account of subclinical extension. CTV and GTV for the metastatic nodes (>1 cm in shortest dimension) were the same. Regional nodes excluding contra-lateral hilar and supraclavicular nodes were included in the CTV; however, lower mediastinal nodes were included only if the primary tumor was located in the lower lobe of the lung. The planning target volumes for the primary tumor, the metastatic lymph nodes and regional nodes were determined as CTVs plus 0.5–1.0 cm margins laterally and 1.0–2.0 cm margins cranio-caudally taking account of set up variations and internal organ motion. Lung heterogeneity corrections were not used.

The criteria for stopping the treatment are pulmonary toxicities, which include the National Cancer Institute-Common Toxicity Criteria (NCI-CTC; version 2.0) grade 2 respiratory distress and <60 torr PaO_2, other than hematopoietic toxicities (leukopenia, neutropenia and thrombocytopenia) or gastrointestinal toxicities (dysphagia).

EVALUATION

To assess the rate of tumor response and toxicity, all patients received a complete blood cell count; blood chemistry, including AST, ALT, lactate dehydrogenase, bilirubin, serum creatinine, blood urea nitrogen, total protein, serum albumin, serum electrolytes and calcium; and weekly chest X-rays during the treatment period. Best overall response was evaluated as tumor response by mono- or bi-dimensional measurement in accordance with the World Health Organization (WHO) criteria (30), and toxicity was evaluated in accordance with the NCI-CTC (version 2.0).



RT, radiotherapy; CRT, chemotherapy; TRT, thoracic radiotherapy; CBDCA, carboplatin.

Figure 1. Treatment schema.

Study Design and Statistical Analysis

This trial was a multi-center randomized phase III study. The study protocol was approved by the JCOG Clinical Trials Review Committee and the institutional review board of each participating institution before the initiation of the study.

The primary end-point was overall survival, which was defined as the interval from randomization to death from any cause. Secondary end-points were response rate, which was the proportion of the patients evaluated as having a complete reponse (CR) or partial response (PR) in best overall response out of all eligible patients; progression-free survival (PFS) defined as the interval from randomization to the diagnosis of progression or death from any cause; sites of progression; and toxicity. The estimate of survival time was performed by the Kaplan–Meier method (31). The trial was designed to have an 80% power to detect 5 months difference in MST (10 months in the RT arm and 15 months in the CRT arm) with a one-sided alpha of 0.05 by log rank test (32). The planned sample size was 190 patients by Shoenfeld and Richter's methods (33) with 1.5 years follow-up after 3 years accrual.

In-house interim monitoring is performed by the JCOG Data Center to ensure data submission, patient eligibility, protocol compliance, safety and on-schedule study progress. The monitoring reports are submitted and reviewed by the JCOG Data and Safety Monitoring Committee (DSMC) twice yearly.

An expedited report was required by the JCOG DSMC to allow rapid identification of any life-threatening adverse events or unexpected toxicities according to the JCOG toxicity reporting system based on the ICH-E2A guidelines.

RESULTS

From November 1999 to February 2001, 46 patients were enrolled in this study: 23 in the RT arm and 23 in the CRT arm. Four treatment-related deaths (TRDs) had been reported, however, before the forty-sixth patient were assigned. Therefore, we suspended the registration and checked the details of all randomized patients to assess the safety of treatment regimens. As a result, it was revealed that three of these deaths were due to pneumonitis. The JCOG DSMC advised consultation with the JCOG Radiotherapy Committee (RC) about the radiotherapy compliance in all patients. The JCOG RC collected each patient's irradiation planning data retrospectively and found poor protocol compliance which was related to TRD. Consequently, we decided to terminate this trial in August 2001 following the recommendation of the JCOG DSMC.

PATIENTS CHARACTERISITICS

Patient characteristics are listed in Table 1. No specific characteristics of patients were found in the elderly patients with locally advanced NSCLC compared with younger patiests and the two treatment arms were well balanced with respect to age and stage.

Toxicity of Treatment

Both hematological and non-hematological toxicities during the treatment and follow-up period were assessed. Table 2 summarizes the hematological toxicity. Patients receiving CBDCA suffered from leukocytopenia, neutropenia and thrombocytopenia more than patients receiving RT alone. There was no grade 4 hematological toxicity in the RT arm. Two (8.7%) and four (17.4%) patients in the CRT arm experienced grade 4 leukocytopenia and neutropenia, respectively.

Non-hematological toxicity observed in this study is listed in Table 3. None of the patients developed grade 3 esophagitis in either treatment arm. In the RT arm, other grade 3/4 toxicities were edema, fatigue, dyspnea and pneumonitis in one patient each. In the CRT arm, other grade 3/4 toxicities were neutropenic fever, dyspnea and pneumonitis. Grade 3/4 (RTOG/EORTC Radiation Toxicity Score) of late lung toxicity was observed in two patients in the RT arm and four patients in the CRT arm. Four TRDs were observed in this study. Three of

Table 1. Patient characteristics

Characteristics	RT arm	CRT arm
No. of eligible patients	23	23
Age (years)		
Median	77	77
Range	72–84	71–83
Male/female	19/4	16/7
Type of tumor		
Adenocarcinoma	6	11
Squamous cell	16	11
Large cell	1	1
PS (ECOG)		
0	3	9
1	19	13
2	1	1
Stage of disease		
IIIA	11	12
IIIB	12	11
Weight loss		
<10%	21	23
≥10%	2	0

RT, radiotherapy; CRT, chemoradiotherapy; PS, performance status.

Table 2. Hematological toxicity

	RT arm (n = 23)					CRT arm (n = 23)				
Grade	1	2	3	4	%grade 4	1	2	3	4	%grade 4
Leukocytes	10	2	2	0	0	3	7	11	2	8.7
Neutrophils	4	3	0	0	0	2	8	6	4	17.4
Hemoglobin	5	3	0	0	0	5	8	3	0	0
Platelets	2	0	2	0	0	4	5	8	0	0

RT, radiotherapy; CRT, chemoradiotherapy.

these patients were thought to have died as a result of pneumonitis. The details of these cases are follows. Case 1: a 78-year-old man had stage IIIA (T3N2) squamous cell carcinoma. He was treated with RT alone and died of pneumonitis at 28 days after therapy. Case 2: a 79-year-old man had stage IIIB (T4N2) adenocarcinoma. He was treated with CBDCA + RT and died of bacterial pneumonia at 37 days after therapy and had been taking steroid hormone due to radiation pneumonitis. Case 3: a 73-year-old man had stage IIIA (T3N2) squamous cell carcinoma. He was treated with CBDCA + RT and died of pneumonitis at 80 days after therapy. Case 4: a 80-year-old man had stage IIIB (T4N2) squamous cell carcinoma. He was treated with CBDCA + RT and died of pneumonitis at 54 days after therapy. Thus, three out of four TRDs were in the CRT arm and one was in the RT arm.

Table 3. Non-hematological toxicity

	RT arm (n = 23)					CRT arm (n = 23)				
Grade	1	2	3	4	% grade 4	1	2	3	4	% grade 4
Edema	0	0	0	1	4.5	0	0	0	0	0
Fatigue	1	0	0	1	4.5	7	1	0	0	0
Fever	3	0	0	0	0	1	1	0	0	0
Esophagitis	13	2	0	0	0	10	2	0	0	0
Nausea	0	0	0	–	–	2	2	0	–	–
Vomiting	0	0	0	0	0	1	0	0	0	0
Febrile neutropenia	–	–	0	0	0	–	–	1	0	0
Cough	3	1	0	–	–	6	0	0	–	–
Dyspnea	–	0	0	1	4.5	–	2	1	0	0
Pneumonitis	1	0	0	–	4.5	1	0	1	0	0
Creatinine	1	0	0	0	0	0	0	0	0	0
Hyponatremia	7	–	0	0	0	5	–	1	0	0
Heart	0	0	0	0	0	0	1	0	0	0
Lung	8	4	2	0	0	9	6	1	3	13.0

RT, radiotherapy; CRT, chemoradiotherapy.

Protocol Compliance

In the RT arm, 22 (95.6%) patients received full treatment doses. In the CRT arm, 20 (87.0%) patients completed the treatment. As to the administration of CBDCA, there were few protocol deviations.

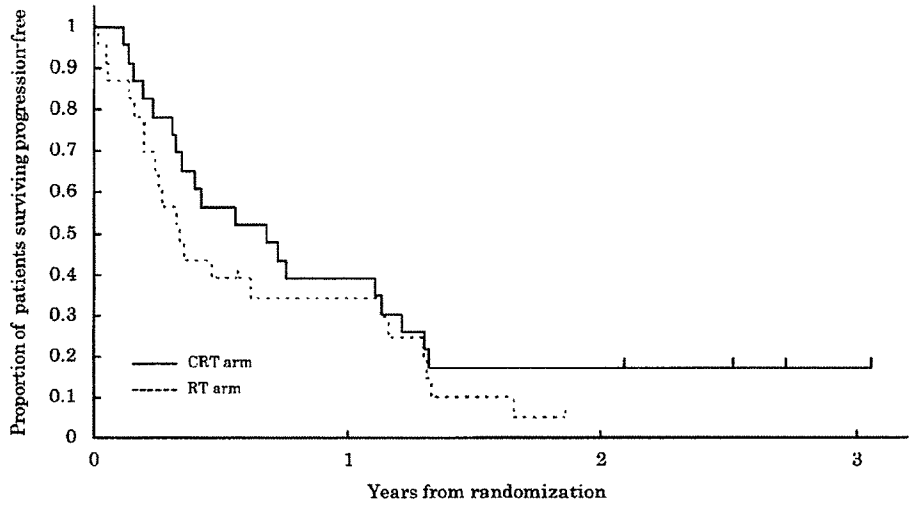
Three of the patients discontinued the protocol treatment: one was due to grade 2 eruption, one was due to cerebral infarction and one was due to insufficient recovery from leukopenia. One patient in the RT arm did not start the treatment due to local progression (Table 4).

Quality Assurance of Radiotherapy

We evaluated the quality of radiotherapy retrospectively based on the collected radiation therapy planning data. The data of 45 patients were reviewed and evaluated for the analysis. Details of this analysis have been reported by Ishikura et al. (34); three cases were revealed to be protocol violation due to normal lung volume constraint defined in the protocol. Unacceptable protocol deviations were identified as follows; 17, 15 and 31 cases on field border placement for the primary tumor, the metastatic lymph nodes and the elective nodal irradiation, respectively. Overall, 27 of 45 cases (60%) had at least one unacceptable deviation. Most cases judged to have protocol violation were primarily due to a smaller radiation field. Only 18 cases (40%) were judged to be protocol compliant.

Response and Survival

The tumor response in each arm is listed in Table 5. No patients achieved a CR in either arm. Of the 23 patients in the RT arm, 12 [52.2%, 95% confidence interval (CI) = 30.6–73.2%] achieved PR and six (26.1%) had stable disease. Of the



RT, radiotherapy; CRT, chemoradiotherapy.

Figure 2. Progression-free survival for patients treated with radiation alone or radiation with concurrent daily CBDCA.

Table 4. Protocol compliance

Pattern	RT arm (n = 23)	CRT arm (n = 23)
Complete protocol treatment	22	20
Progression/relapse*	1	0
Adverse events		
Cerebral infarction	0	1
Eruption	0	1
Leukopenia	0	1
Patient refusal	0	0
Death on protocol	0	0
Other	0	0

*Before starting the radiotherapy.
RT, radiotherapy; CRT, chemoradiotherapy.

Table 5. Response to treatment

Response	RT arm (n = 23)	CRT arm (n = 23)
Complete response	0 (0)	0 (0)
Partial response	12 (52.2)	11 (47.8)
Stable disease	6 (26.1)	7 (30.4)
Progression	4 (17.4)	4 (17.4)
Not evaluable	1 (4.4)	1 (4.4)
Objective response	52.2%	47.8%

RT, radiotherapy; CRT, chemoradiotherapy.

23 patients in the CRT arm, 11 (47.8%, 95% CI = 26.8–69.4%) achieved PR and seven (30.4%) had stable disease.

Seventeen (73.9%) patients in the RT arm and 15 (65.2%) patients in the CRT arm had died at the time of analysis. The median progression-free survival time was 122 days (95% CI = 88–413 days) on the RT arm versus 248 days (95% CI = 127–416 days) on the CRT arm (Fig. 2.). The MST was 428 days (95% CI = 212–680 days) on the RT arm versus 554 days (95% CI = 331 to not estimable) on the CRT arm (Fig. 3.). The 1-year survival rate was 60.9% (95% CI = 40.9–80.8%) on the RT arm versus 65.2% (95% CI = 45.8–84.7%) on the CRT arm.

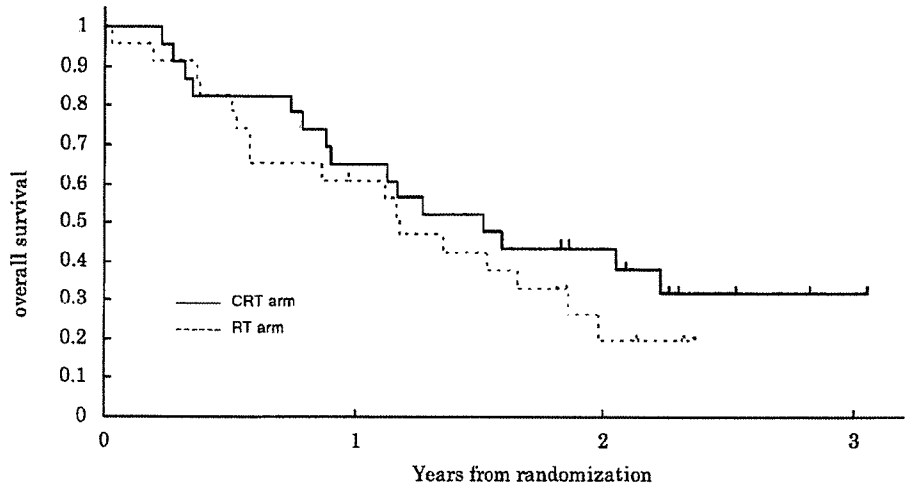
Pattern of Progression/Relapse

The first site of disease progression or relapse is listed in Table 6. Sixteen patients in the RT arm and 13 patients in the CRT arm had relapsed or had disease progression at the time of analysis. Eight patients (out of 16, 50.0%) in the RT arm and seven patients (out of 13, 53.8%) in the CRT arm had relapse or disease progression within the radiation field whether relapse outside the radiation field occurred or not.

DISCUSSION

We conducted this randomized controlled trial to determine whether chemoradiotherapy was superior to radiotherapy alone with respect to overall survival of elderly patients with locally advanced NSCLC. The study was terminated early when 24% of the planned sample size was accrued because of a high proportion of TRDs due to radiation pneumonitis and protocol violation.

Pulmonary toxicities including radiation pneumonitis and fibrosis caused by radiation therapy are, in general, common but not severe. In this study, however, the risk of TRD was 8.7% (four out of 46) and was much higher than in other trials. For instance, Ohe et al. (35) retrospectively analyzed the incidence of TRDs in the treatment of thoracic radiotherapy and/or chemotherapy for patients with locally advanced NSCLC, and reported that seven of 448 patients (1.6%)



RT, radiotherapy; CRT, chemoradiotherapy.

Figure 3. Overall survival for patients treated with radiation alone or radiation with concurrent daily CBDCA.

Table 6. First site of disease progression

	RT arm (n = 23)	CRT arm (n = 23)
Local	8	5
Distant	8	6
Local + distant	0	2

RT, radiotherapy; CRT, chemoradiotherapy.

died of radiation-induced pneumonitis. The high proportion of pulmonary toxicities in our trial may be due partly to the high age of the patients. Schild et al. (15) reported that they found 6% of elderly (older than 75 years) with NSCLC had grade 4 pneumonitis whereas this was the case in only 1% of younger patients ($P = 0.02$). It was controversial that the four TRDs out of 46 was sufficient reason to terminate the on-going trial; however, we thought it was serious that half of the TRDs (two out of four) were judged to be associated with protocol violation concerning the radiation field, which was to be less than half of one lung. Because the JCOG had not yet established the quality control/assurance system for radiotherapy before this trial, we concluded that we would not be able to control the risk of radiation pnuemonitis due to protocol deviation if we continued this study. What was an issue in this study was not only the high TRD rate, but also the poor protocol compliance of RT. The reasons for the poor protocol compliance are limited participation of radiation oncologists during protocol development, limited educational resources for attending radiation oncologists and no quality control program. Although the retrospective systematic review of radiation planning and protocol compliance of radiotherapy was the first experience in the JCOG, both the Lung Cancer Study Group and the entire JCOG had become aware of the importance of a quality control system for radiotherapy. The JCOG Executive Committee decided to establish the Radiation Therapy Quality Assurance Center (RTQAC) within the JCOG Data Center under the supervision of the JCOG Radiotherapy Committee. The RTQAC started the prospective quality control and quality assurance (QC/QA) program in September 2002 with a new activated phase III study for limited disease of small cell lung cancer, JCOG0202. Up to 2004, the QC/QA program has been expanded to the other group studies, such as esophageal cancer study, breast cancer study, prostate cancer study and brain tumor study. In addition, the JCOG Executive Committee mandates the QC/QA program by the RTQAC for all JCOG trials when protocol treatment includes radiation therapy.

The clinical question raised in this trial has not been answered. The data from the 46 patients enrolled were not considered to be conclusive because of the small sample size. No remarkable difference was found between the arms in terms of safety and efficacy such as tumor response, PFS and overall survival. We considered that it still remained an important clinical question to be investigated whether the daily low-dose CBDCA plus radiotherapy was effective or not. Therefore, we re-planned and started a new phase III trial (JCOG0301), in which the prospective QC/QA program by the RTQAC is added to the identical design to this JCOG9812. The protocol involves initial review of radiation planning and final review of the actual radiation record for all randomized patients. The JCOG0301 was activated in September 2003, and we have achieved very good protocol compliance upto now.

Acknowledgments

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References

1. Annual Statistical Report of National Conditions. Tokyo: Health and Welfare Statistics Association 2002.
2. Choi NC, Doucette JA. Improved survival of patients with unresectable non-small cell bronchogenic carcinoma by an innovated high-dose en-bloc radiotherapeutic approach. *Cancer* 1981;48:101–9.
3. Perez CA, Pajak TF, Rubin P, Simpson JR, Mohiuddin M, Brady LW, et al. Long term observations of the patterns of failure in patients with unresectable non-oat cell carcinoma of the lung treated with definitive radiotherapy. Report by the Radiation Therapy Oncology Group. *Cancer* 1987;59:1874–81.
4. Petrovich Z, Stanley K, Cox JD, Paig C. Radiotherapy in the management of locally advanced lung cancer of all cell types: final report of a randomized trial. *Cancer* 1981;48:1335–40.
5. Dillman RO, Seagren SL, Propert KJ, Guerra J, Eaton WL, Perry MC, et al. A randomized trial of induction chemotherapy plus high-dose radiation versus radiation alone in stage III non-small-cell lung cancer. *N Engl J Med* 1990;323:940–5.
6. Gregor A, Macbeth FR, Paul J, Cram L, Hansen HH. Radical radiotherapy and chemotherapy in localized inoperable non-small-cell lung cancer: a randomized trial. *J Natl Cancer Inst* 1993;85:997–9.
7. Jeremic B, Shibamoto Y, Acimovic L, Milisavljevic S. Hyperfractionated radiation therapy with or without concurrent low-dose daily carboplatin/etoposide for stage III non-small-cell lung cancer: a randomized study. *J Clin Oncol* 1996;14:1065–70.
8. Johnson DH, Einhorn LH, Bartolucci A, Birch R, Omura G, Perez CA, et al. Thoracic radiation therapy dose not prolong survival in patients with locally advanced, unresectable non-small cell lung cancer. *Ann Intern Med* 1990;113:33–8.
9. Le Chevalier T, Arriagada R, Quoix E, Ruffie P, Martin M, Tarayre M, et al. Radiotherapy alone versus combined chemotherapy and radiotherapy in nonresectable non-small-cell lung cancer: first analysis of a randomized trial in 353 patients. *J Natl Cancer Inst* 1991;83:417–23.
10. Schaake-Koning C, van den Bogaert W, Dalesio O, Festen J, Hoogenhout J, van Houtte P, et al. Effects of concomitant cisplatin and radiotherapy on inoperable non-small-cell lung cancer. *N Engl J Med* 1992;326:524–30.
11. Trovó MG, Minatel E, Franchin G, Boccieri MG, Nascimben O, Bolzicco G, et al. Radiotherapy versus radiotherapy enhanced by cisplatin in stage III non-small cell lung cancer. *Int J Radiat Oncol Biol Phys* 1992;24:11–5.
12. Marino P, Preatoni A, Cantoni A. Randomized trials of radiotherapy alone versus combined chemotherapy and radiotherapy in stage IIIa and IIIb nonsmall cell lung cancer. A meta-analysis. *Cancer* 1995;76:593–601.
13. Pritchard RS, Anthony SP. Chemotherapy plus radiotherapy compared with radiotherapy alone in the treatment of locally advanced, unresectable, non-small-cell lung cancer. A meta-analysis. *Ann Intern Med* 1996;125:723–9.
14. Langer CJ, Hsu C, Curran WJ, Komaki R, Lee JS, Byhardt R, et al. Elderly patients with locally advanced non-small cell lung cancer (LA NSCLC) benefit from combined modality therapy, secondary analysis of radiation therapy oncology group (RTOG) 94-10. *Proc Am Soc Clin Oncol* 2002;21:299a (abstract).
15. Schild SE, Stella PJ, Geyer SM, Bonner JA, McGinnis WL, Mailliard JA, et al. The outcome of combined-modality therapy for stage III non-small-cell lung cancer in the elderly. *J Clin Oncol* 2003;21:3201–6.
16. Movsas B, Scott C, Sause W, Byhardt R, Komaki R, Cox J, et al. The benefit of treatment intensification is age and histology-dependent in patients with locally advanced non-small cell lung cancer (NSCLC): a quality-adjusted survival analysis of radiation therapy oncology group (RTOG) chemoradiation studies. *Int J Radiat Oncol Biol Phys* 1999;45:1143–9.
17. Sause W, Kolesar P, Taylor S IV, Johnson D, Livingston R, Komaki R, et al. Final results of phase III trial in regionally advanced unresectable non-small cell lung cancer. Radiation therapy oncology group, Eastern cooperative oncology group, and Southwest oncology group. *Chest* 2000;117:358–64.
18. Mayer RJ, Davis RB, Schiffer CA, Berg DT, Powell BL, Schulman P, et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. *N Engl J Med* 1994;331:896–903.
19. Gomez H, Mas L, Casanova L, Pen DL, Santillana S, Valdivia S, et al. Elderly patients with aggressive non-Hodgkin's lymphoma treated with CHOP chemotherapy plus granulocyte–macrophage colony-stimulating factor: identification of two age subgroups with differing hematologic toxicity. *J Clin Oncol* 1998;16:2352–8.
20. Langer CJ, Manola J, Bernardo P, Kugler JW, Bonomi P, Cella D, et al. Cisplatin-based therapy for elderly patients with advanced non-small-cell lung cancer: inplications of Eastern Cooperative Oncology Group 5592, a randomized trial. *J Natl Cancer Inst* 2002;94:173–81.
21. Kubota K, Fruse K, Kawahara M, Kodama N, Ogawara M, Takada M, et al. Ciplatin-based combination chemotherapy for elderly patients with non-small-cell lung cancer. *Cancer Chemother Pharmacol* 1997;40:469–74.
22. Calvert AH, Harland SJ, Newell DR, Siddik ZH, Jones AC, McElwain TJ, et al. Early clinical studies with cis-diammine 1,1-cyclobutane dicarboxylate platinum II. *Cancer Chemother Pharmacol* 1982;9:140–7.
23. Smith IE, Harland SJ, Robinson BA, Evans BD, Goodhart LC, Calvert AH, et al. Carboplatin: a very active new cisplatin analog in the treatment of small cell lung cancer. *Cancer Treat Rep* 1985;69:43–6.
24. Wiltshaw E. Ovarian trials at the Royal Marsden. *Cancer Treat Rev* 1985;12 (Suppl A):67–71.
25. Begg AC, van der Kolk PJ, Emondt J, Bartelink H. Radiosensitization *in vitro* by cis-diammine (1,1-cyclobutanedicarboxylato) platinum (II) (carboplatin, JM8) and ethlenediammine-malonato-platinum (II) (JM40). *Radiother Oncol* 1987;9:154–65.
26. Eisenberger M, Van Echo D, Aisner J. Carboplatin: the experience in head and neck cancer. *Semin Oncol* 1989;16 (Suppl 5):34–41.
27. Knox RJ, Friedlos F, Lydall DA, Roberts JJ. Mechanism of Cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum (II) and cis-diammine-(1,1-cyclobutanedicarboxylato) platinum (II) differ only in the kinetics of their interaction with DNA. *Cancer Res* 1986;46:1972–9.
28. O'Hara JA, Douple EB, Richmond RC. Enhancement of radiation-induced cell kill by platinum complexes (carboplatin and iproplatin) in V79 cells. *Int J Radiat Oncol Biol Phys* 1986;12:1419–22.
29. Atagi S, Kawahara M, Ogawara M, Matsui K, Masuda N, Kudoh S, et al. Phase II trial of daily low-dose carboplatin and thoracic radiotherapy in elderly patients with locally advanced non-small cell lung cancer. *Jpn J Clin Oncol* 2000;30:59–64.
30. World Health Organization. WHO Handbook for Reporting Results of Cancer Treatment, Vol. 48. WHO Offset Publication, Geneva: World Health Organization 1979.
31. Kaplan ES, Meier P. Non parametric estimation for incomplete observations. *J Am Stat Assoc* 1958;53:557–80.
32. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 1966;50:163–70.
33. Shoenfeld DA, Richter JR. Nomograms for calculating the number of patients needed for a clinical trial with survival as an endpoint. *Biometrics* 1982;38:163–70.
34. Ishikura S, Teshima T, Ikeda H, Hayakawa K, Hiraoka M, Atsgi S, et al. Initial experience of quality assurance in radiotherapy within the Japan Clinical Oncology Group (JCOG). *Radiother Oncol* 2002;64:S224.
35. Ohe Y, Yamamoto S, Suzuki K, Hojo F, Kakinuma R, Matsumoto T, et al. Risk factors of treatment-related death in chemotherapy and thoracic radiotherapy for lung cancer. *Eur J Cancer* 2001;37:54–63.

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Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay

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Summary Epidermal growth factor receptor (*EGFR*) gene mutations have been found in a subset of non-small cell lung cancer (NSCLC) with good clinical response to gefitinib therapy. A quick and sensitive method with large throughput is required to utilize the information to determine whether the molecular targeted therapy should be applied for the particular NSCLC patients. Using probes for the 13 different mutations including 11 that have already been reported, we have genotyped the *EGFR* mutation status in 94 NSCLC patients using the TaqMan PCR assay. We have also genotyped the *EGFR* mutations status in additional 182 NSCLC patients, as well as 63 gastric, 95 esophagus and 70 colon carcinoma patients. In 94 NSCLC samples, the result of the TaqMan PCR assay perfectly matched with that of the sequencing excluding one patient. In one sample in which no *EGFR* mutation was detected by direct sequencing, the TaqMan PCR assay detected a mutation. This patient was a gefitinib responder. In a serial dilution study, the assay could detect a mutant sample diluted in 1/10 with a wild-type sample. Of 182 NSCLC samples, 46 mutations were detected. *EGFR* mutation was significantly correlated with gender,

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smoking status, pathological subtypes, and differentiation of lung cancers. There was no mutation detected by the TaqMan PCR assay in gastric, esophagus and colon carcinomas. TaqMan PCR assay is a rapid and sensitive method of detection of *EGFR* mutations with high throughput, and may be useful to determine whether gefitinib should be offered for the treatment of NSCLC patients. The TaqMan PCR assay can offer us a complementary and confirmative test.
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1. Introduction

Lung cancer is the deadliest cancer in many developed countries. Gefitinib (Iressa®, Astra Zeneca, London, UK), an inhibitor of the epidermal growth factor receptor (*EGFR*) tyrosine kinase, has been approved in Japan and the United States for the treatment of non-small cell lung cancer (NSCLC). Recently, erlotinib (Tarceva®, Roche, Basel, Switzerland), another inhibitor of *EGFR* tyrosine kinase, has been approved in the United States and Switzerland for the treatment of NSCLC. Gefitinib caused significant tumor shrinkage in 27.5% of Japanese NSCLC patients but in only 10.4% of Caucasian population [1–4]. Unfortunately, the addition of gefitinib to the traditional chemotherapy did not add any benefit to the patient survival [3], although overexpression of EGFR protein was seen in relatively high frequencies [5]. We and others have shown that the somatic mutation in tyrosine kinase (TK) domain of *EGFR* is associated with sensitivity of NSCLC to gefitinib [6–8]. Gefitinib targets the ATP-binding cleft with the TK domain and the reported mutations are either deletion or single amino acid substitutions in exon 18, 19, or 21 clustered around the ATP-binding pocket of the TK domain.

In vitro, *EGFR* mutations have been reported to confer enhanced tyrosine kinase activity in response to epidermal growth factor (EGF) and increased sensitivity to inhibition by gefitinib [6,7,9,10]. Thus, it is highly likely that *EGFR* mutation is a critical determinant of the patient's response to gefitinib. To determine the *EGFR* gene status may bring important information whether gefitinib is a therapeutic option for the NSCLC patient. If we can avoid unnecessary prescription of gefitinib in patients who are in fact non-responders, we will avoid fatal side effects of the drug and significantly reduce the health care cost. Fluorescent dye-based genotyping technology using the 5′ nuclease assay (TaqMan PCR assay) was developed as a large-scale and highly sensitive method in SNP scoring [11–16]. For SNP genotyping, one pair of TaqMan probes and one pair of PCR primers are used. Two TaqMan probes differ at the polymorphic site, with one probe complementary to the wild-type allele and the other to the variant allele. Recently, this method is being applied for genotyping of insertion/deletion polymorphism as a simple and cost-effective method [17].

We applied this genotyping technique with TaqMan probe to detect *EGFR* somatic mutations. Probes were designed according to the 13 different *EGFR* mutations including 11 that have already been reported. We show in this paper that this method is sensitive enough to detect the mutation in samples contaminated with 9-fold excess of wild-type samples. It is also fast and could be applied in large-scale screening.

2. Materials and methods

2.1. Patients and genomic DNA

NSCLC tissues were obtained by surgical excision between 1997 and 1999 from 67 patients at Nagoya City University Hospital in Japan. NSCLC tissues were also obtained from 27 patients at National Hospital Organization, Kinki-Chuo Chest Medical Center who were subsequently treated with gefitinib. Of 27 gefitinib treated samples, six transbronchial biopsy samples were obtained. These 94 samples were sequenced and also analyzed using TaqMan PCR assay. We have also analyzed additional 182 recent NSCLC cases, as well as 63 gastric, 95 esophagus and 70 colon carcinomas, operated between 2000 and 2003 at Nagoya City University Hospital using TaqMan PCR assay. The research was approved by the Institutional Review Board of each hospital. All the patients consented to the use of their tissues for the present analysis. The tissues were placed in liquid nitrogen immediately after resection or fixed by formalin and paraffin embedded. Genomic DNA was extracted using Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions.

2.2. Genomic DNA PCR and DNA sequencing

All of 27 samples from National Hospital Organization, Kinki-Chuo Chest Medical Center were amplified by PCR reaction for genotyping analysis.

The primers for amplification of exon18, exon19, exon20, and exon21 were designed as previously described [7]. The reaction mixtures were contained with 1 μl of template DNA, 2 μl of 10× LA PCR Buffer II, 2 μl of 25 mM $MgCl_2$, 3.2 μl of 2.5 mM dNTP mixture, 0.4 μl of each primer (250 μM), 0.5 μl of TaKaRa LA Taq (5 U/μl), 10 μl of ddH_2O in a volume of 20 μl. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s; 64 °C for 30 s; and 72 °C for 1 min. The final extension was for 5 min at 72 °C. The PCR products were sequenced by ABI PRISM 3100 Genetic Analyzer® and analyzed by ABI PRISM SeqScape Software Version 2.1.1®.

2.3. Genotyping by the TaqMan PCR assay

The primers and TaqMan® MGB probes were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of the allele-specific probes and primers used in the TaqMan PCR assay are shown in Table 1. TaqMan PCR and genotyping analysis were performed on Applied Biosystems 7500 Real Time PCR System (Applied Biosystems) in the manufacture's instructions. The reaction mixtures were amplified in 1 μl of genomic DNA (10 ng/μl) or 1 μl of 100-fold diluted PCR products, 5 μl of 2× TaqMan® Universal Master Mix (Applied Biosystems), 0.5 μl of 20× primer/probe mix (each final concentration of primer and probe is 9 μM and 2 μM), 3.5 μl of ddH_2O in a volume of 10 μl. PCR cycling conditions were as follows: one cycle at 95 °C for 10 min; and 40 cycle at 95 °C for 15 s and 58 °C for 1 min. The results were analyzed on Applied Biosystems 7500 Real Time PCR System using allelic discrimination assay program.

2.4. *EGFR* DNA amplification

The *EGFR* gene amplification was analyzed for 27 gefitinib treated patients by quantitative real-time PCR, performed on a PRISM 7500 sequence detector (Applied Biosystems) by using a QuantiTect SYBR Green kit (Qiagen Inc., Valencia, CA). We have quantified each tumor DNA by comparing the target locus to the reference *Line-1*, a repetitive element for which copy numbers per haploid genome are similar among all of the human normal and neoplastic cells. Quantification is based on standard curves from a serial dilution of human normal genomic DNA. The relative *EGFR* copy number level was also normalized to normal human genomic DNA as calibrator. Copy number change of *EGFR* gene relative to the *Line-1* and the calibrator were determined by using the formula $(T_{EGFR}/T_{Line\text{-}1})/(C_{EGFR}/C_{Line\text{-}1})$, where T_{EGFR} and $T_{Line\text{-}1}$ are quantity from tumor DNA by using *EGFR* and *Line-1*, and C_{EGFR} and $C_{Line\text{-}1}$ are quantity from calibrator by using *EGFR* and *Line-1*. PCRs for each primer set were performed in at least triplicate, and means were reported. Conditions for quantitative PCR reaction were as follows: one cycle of 50 °C for 2 min; one cycle of 95 °C for 15 min; 40 cycles of 95 °C for 15 s; 56 °C for 30 s; and 72 °C for 34 s. At the end of the PCR reaction, samples were subjected to a melting analysis to confirm specificity of the amplicon. Primers for *EGFR* gene were designed by using Primers 3^{12} to span a 100–150 bp non-repetitive region at exon 28 and were synthesized by Invitrogen (Carlsbad, CA). Primer sequences for *EGFR* gene used in this study are as follows: forward, CCACCAAATTAGCCTGGACA; and reverse, CGCGACCCTTAGGTATTCTG. *EGFR* amplification (increased *EGFR* copy number) was defined as more than five copies.

2.5. Statistical analysis

For comparisons of proportions, the Fisher's Exact test was used. The two-sided significance level was at $P < 0.05$. We did all analyses using a Stat View (version 5, SAS Institute Inc., Cary, NC) software.

3. Results

3.1. Genotyping by genomic DNA sequencing

We have already published the *EGFR* genomic DNA sequencing data of 67 NSCLC samples [7,18]. Seventeen cases had a mutated allele and there were six different mutations. Other groups have also reported additional somatic mutations in the same region of the *EGFR* gene [6,8]. We first sequenced some additional samples to find previously unknown mutations. Twenty-seven NSCLC tumor samples from National Hospital Organization, Kinki-Chuo Chest Medical Center were subjected to conventional genomic DNA sequencing in exon 18, 19, 20, and 21. Nine of 27 cases (33.3%) had a mutation. Of these nine cases, two were novel mutations. One patient carried a 24 nucleotide in-frame deletion (2239–2262), removing amino-acid 747 through 754, and three nucleotides insertion at 2270, adding one asparagine (delL747–K754&insK757NK) as shown in Fig. 1A and B. This region overlaps with the other deletion mutations reported previously [6,7]. Another tumor had two mutations: one amino acid substitution in the exon 21: leucine–proline at codon 838 (L838P) (Fig. 1C); and a deletion

Table 1 Sequence of the mutation specific TaqMan probes and PCR primers

Mutation no.	Probe name	Nucleotide	Amino acid	Primer sequence (forward)	TaqMan probe	Primer sequence (reverse)
	WT1				VIC–ATTAAGAGAAGCAACATCT	
1	Del 1a	2235–2249del	E746–A750del		FAM–CGCTATCAAAACATCT	
2	Del 1b	2236–2250del			FAM–CTATCAAGACATCTCC	
3	Del 2	2254–2277del	S752–I759del		FAM–AGAAGCAACACTCGAT	
	WT2			CCCAGAAGGTGAGAAAGTTAAAATTC	VIC–CGAAAGCCAACAAG	CCCACACAGCAAAGCAGAAA
4	Del 3	2239–2247del, 2248G > C	L747–E749del, A750P		FAM–CAAGGAACCAACATC	
5	Del 4	2240–2257del	L747–S752del, P753S		FAM–AAGGAATCGAAAGCC	
6	Del 5	2238–2255del, 2237A > T	L747–S752del, E746V		FAM–CAAGGTTCCGAAAGC	
7	Del 6	2240–2251del	L747–A750del, T747S		FAM–TCAAGGAATCATCTCC	
	WT3				VIC–AAGTGCTGGGCTCC	
8	G719C	2155G > T	G719C	TGAGGATCTTGAAGGAAACTGAATTC	FAM–AAAGTGCTGTGCTCC	TGCCAGGGACCTTACCTTATACA
9	G719S	2155G > A	G719S		FAM–AAAGTGCTGTGCTCC	
	WT4				VIC–TTGGGCTGGCCAAA	
10	L858R	2573T > G	L858R	CCGCAGCATGTCAAGATCAC	FAM–TTGGGCGGGCCAA	TCCTTCTGCATGGTATTCTTTCTCT
	WT5				VIC–CCAAACTGCTGGGTG	
11	L861Q	2582T > A	L861Q		FAM–CCAAACAGCTGGGTG	
	WT6				VIC–ATTAAGAGAAGCAACATCT	
12	Novel Del	2239–2262del	L747–K754del	CCCAGAAGGTGAGAAAGTTAAAATTC	FAM–CTATCAAGGAAGCCAACAA–MGB	CCCACACAGCAAAGCAGAAA
12	Novel Ins	2265–2267 InsCAA	N756Ins		FAM–CCAACAACAAGGAAAT–MGB	
	WT7			GGAGGACCGTCGCTTGGT	VIC–CGCGACCTGGCAG–MGB	CCCAAAATCTGTGATCTTGACATG
13	L838P	2513T > C	L838P		FAM–CGCGACCCGGCAG–MGB	

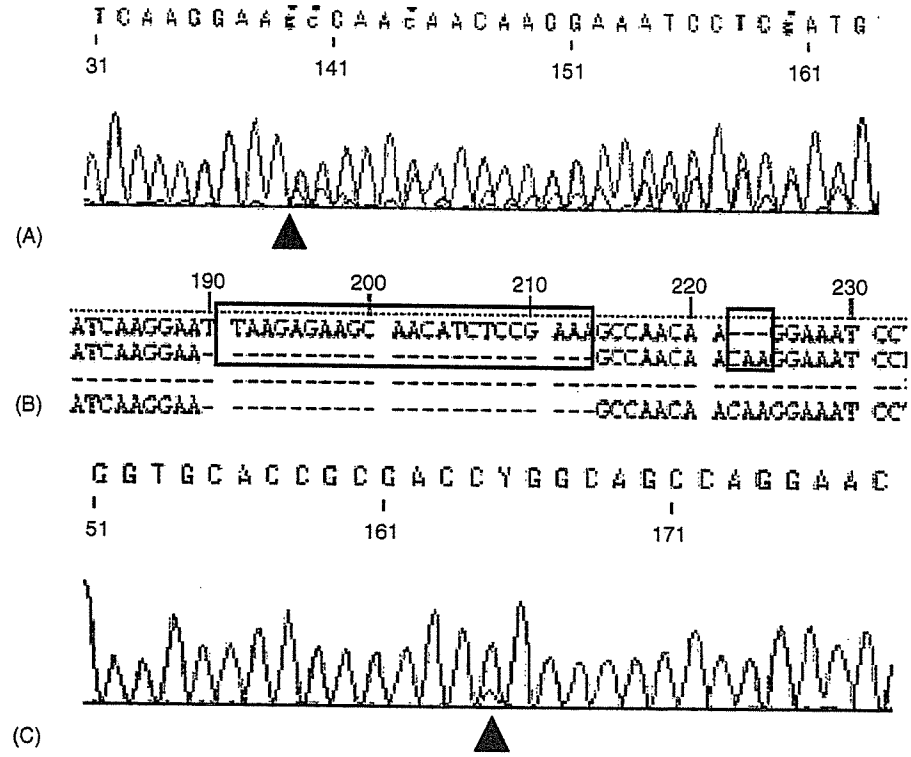

Fig. 1 (A) Data from direct sequencing showing a novel 24 nucleotide in-frame deletion (2239–2262), removing amino-acid 747–754 (arrow head: deletion start). (B) Nucleotide sequence of the novel insertion–deletion mutant as aligned with the wild-type sequence. Three nucleotides (CAA) are inserted at the position 2270 in the exon 19, adding one asparagine (delL747–K754&insK757NK) (box: insertion–deletion sequence, first line: wild-type sequence; second line: mutation type sequence). (C) DNA sequencing of case #6. The other mutation found in case #6. Amino acid substitution: leucine to proline at codon 838 (L838P) due to T–C substitution at position 2513 in exon 21 (arrow head: mutation point).

mutation (Del 5). The substituted amino acid is conserved among all the erbB family members. We designed TaqMan probes for these two novel mutations as described below. We have not determined whether these nine genomic abnormalities found in this cohort are somatic or genomic.

3.2. Analysis using TaqMan PCR assay

Next, we subjected these 94 NSCLC samples to TaqMan PCR assay. To detect all of these mutations specifically, we designed 13 sets of specific TaqMan probes. They targeted 11 previously reported mutations and the two novel mutations that were identified in this paper (Table 1). Each probe has been shown to anneal only to the correct sequence. Using this analysis, 27 cases were detected to have a mutation. In all of the cases except case #1, the results of TaqMan PCR were in complete agreement with the genomic DNA sequencing data (Table 2). Typical results of the TaqMan PCR assay and their corresponding DNA sequence data are shown in Fig. 2. In Table 2, cases #1 to #9 were responders for gefitinib (partial response), and cases #10 to #27 were non-responders.

Case #1 was determined to have a mutation carrying amino acid substitution in exon 21 (L858R) by the TaqMan PCR assay (Fig. 3A). The genomic DNA sequencing could not detect the mutation (Fig. 3B). As the PCR assay has suggested that the sample had a significant contamination with wild-type DNA, we did a serial dilution experiment to determine the relative content of the mutated alleles in this tumor. The standard curve was configured using a titration by 10% steps with the control synthetic oligos harboring the mutant sequence (Fig. 3C), and the approximation curve was calculated (Fig. 3D). In reference to this data, the data for the case #1 suggested that this tumor contained 11% mutated allele and 89% normal allele. The first sequence data was rechecked and a very small peak was found to be present which was compatible with a substitution of G for T at nucleotide 2573 (L858R) (Fig. 3B). The heterogeneous tumor cells or contamination with wild-type DNA from the normal tissue was suspected. The genomic DNA of case #1 was newly

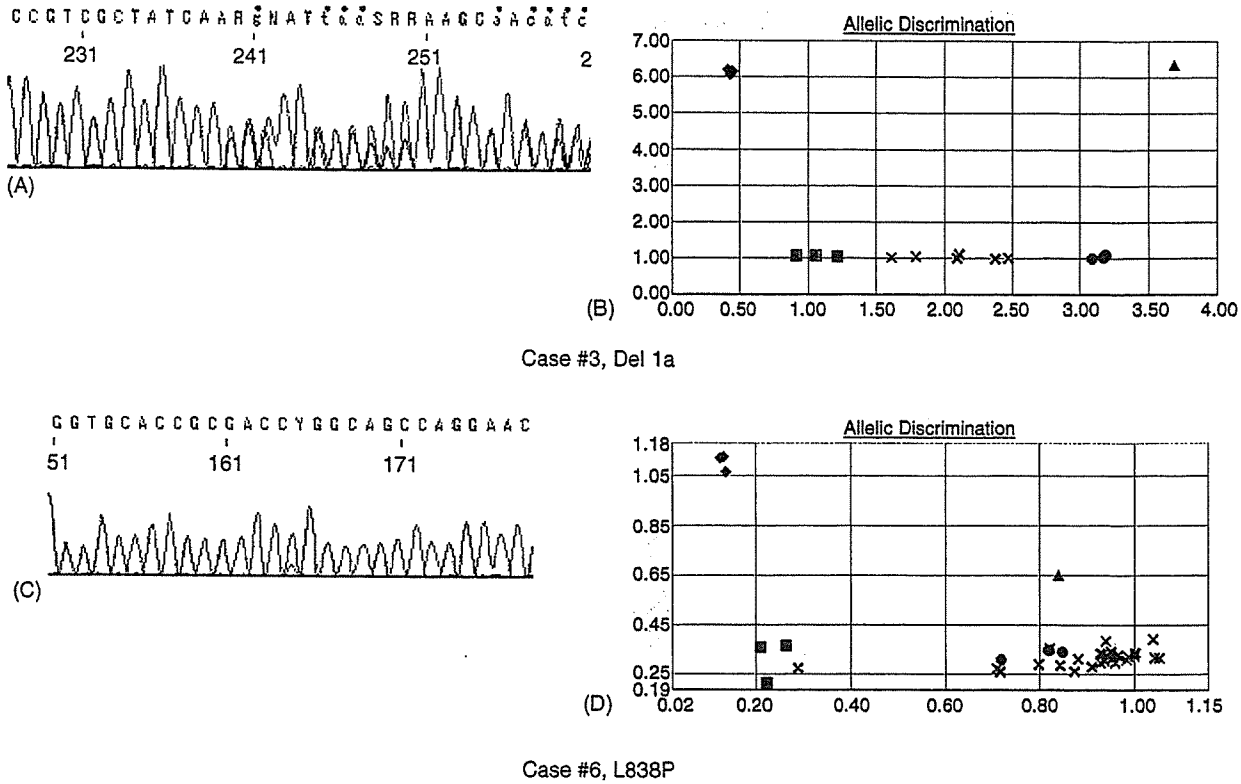

Fig. 2 In the TaqMan PCR assay, triangles indicate the samples with the somatic mutations. Diamonds indicate the mutation controls (artificial template oligo). Circles indicate the wild-type controls (artificial template oligo). Crosses indicate samples that turned out to be wild type. Squares indicate controls without DNA template. Both abscissa and ordinate are fluorescent intensity of each dye. Samples with mutation appear deviated from the abscissa. Heterozygous samples appear deviated from both abscissa and ordinate and are plotted roughly at 45° if the sample contained mutation and wild-type alleles at 1:1 ration. (A) Genotyping by DNA sequencing (case #3, Del 1a). (B) Genotyping by TaqMan PCR assay (case #3, Del 1a). (C) Genotyping by DNA sequencing (case #6, L838P). (D) Genotyping by TaqMan PCR assay (case #6, L838P).

prepared and sequenced again. The sequence data now showed a clear peak of the mutated allele (Fig. 3E). A repeat TaqMan PCR assay of the newly prepared DNA now revealed a proportion of 35% tumor DNA and 65% wild-type DNA (Fig. 3C). In nine gefitinib responders, six patients (66.7%) had *EGFR* mutation from TaqMan PCR assay. Of 27 gefitinib treated samples, six trans-bronchial biopsy samples were also evaluated by TaqMan PCR assay. In these six samples, the result from biopsy samples analysis perfectly matched with that of the surgical removed samples.

3.3. Analysis of *EGFR* DNA copy number

The *EGFR* gene amplification of 27 samples from patients who were treated with gefitinib at National Hospital Organization, Kinki-Chuo Chest Medical Center, was analyzed by quantitative real-time PCR. Four of 27 cases were found to have *EGFR* DNA amplifications (*EGFR* copy number >5) (Table 2). In these four cases, two had *EGFR* mutation. The two cases showed a clinical response to gefitinib. In the 10 cases with *EGFR* mutation in this cohort, only two had *EGFR* amplification. The *EGFR* amplification did not correlate with *EGFR* mutation status ($p=0.6125$). The *EGFR* amplification did not correlate with any of the clinicopathological factors. There was not any statistically significantly correlation between *EGFR* amplification and overall survival (data not shown).

3.4. *EGFR* mutation status in the additional 182 NCSLC samples

In addition to the 67 cases we have already reported and 27 cases we have sequenced in this paper, we genotyped additional 182 recently operated NSCLCs for *EGFR* mutations using TaqMan PCR assay. Forty-six mutations were detected (25.3%):

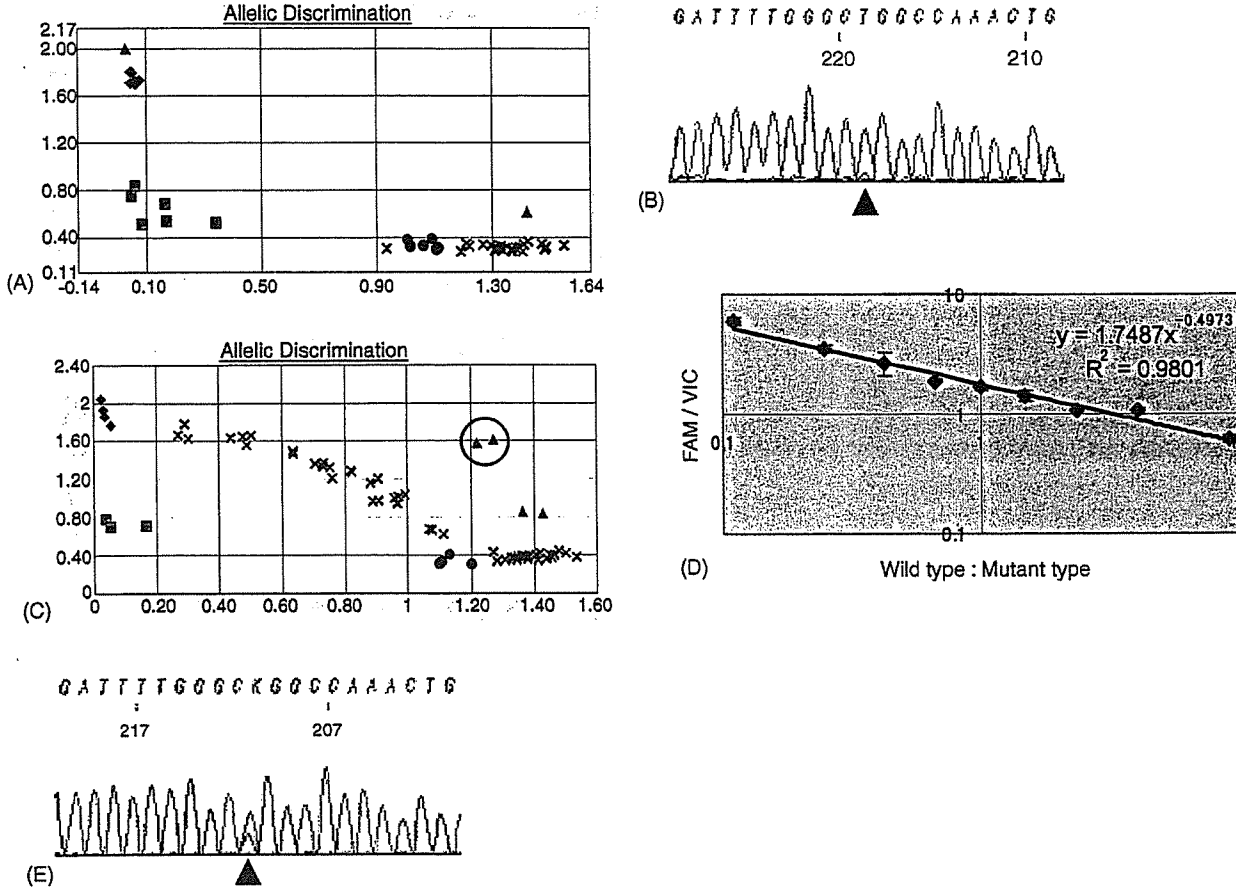

Fig. 3 (A) TaqMan analysis of case #1. Case #1 appeared to have mutation carrying amino acid substitution in exon 21 (L858R) by TaqMan PCR assay. The triangle deviating about 10% from the abscissa is the sample. (B) DNA sequencing of case #1. The first screening data by genomic DNA sequencing of case #1 shows a barely identifiable mutation peak representing a G for T at nucleotide 2573 (arrow head: mutation point). (C) TaqMan PCR assay of titrated samples. The standard curve was configured using a titration by 10% steps of the control synthetic oligos harboring mutated sequence by TaqMan PCR assay. The sample of case #1 is indicated as closed triangles which deviate about 10% from the abscissa. This showed that this tumor contained the mutated allele at the proportion of 11%. Closed triangles in the circle indicate the genomic DNA of case #1 that was newly prepared that was calculated as 35% of total DNA fraction. (D) The approximation curve calculated from the data in Fig. 3C. The *x*-axis indicates the wild type/mutant type ratio, and *y*-axis indicates FAM/VIC which is the degree of deviation from the *x*-axis relative to the 100% mutation control ($y = 1.7487x^{-0.4973}$, $R^2 = 0.9801$). (E) DNA sequencing of the case #1 that has been newly prepared. The sequence showed a clear peak of mutated allele that was substituted G—T at position 2573 (arrow head: mutation point).

20 patients had the L858R mutation; 22 had the deletion mutation in exon 19; two had the G719C mutation; and two patients had the L861Q mutation. Relationship between the *EGFR* mutation and clinical—pathologic factors in additional 182 NSCLC patients is shown in Table 3. Among those with *EGFR* mutation, 17/127 (13.4%) were male and 29/55 (52.7%) were female; 27/46 (58.7%) were never-smokers and 12/113 (10.6%) were ever-smokers; 45/97 (46.4%) were adenocarcinoma and 1/85 (1.2%) was non-adenocarcinoma; 35/80 (43.8%) were well differentiated and 11/85 (12.9%) were moderately or poorly differentiated. The mutation status were significantly correlated with gender (women versus men, $p < 0.0001$), smoking status (never-smokers versus ever-smokers, $p < 0.0001$), pathological subtypes (adenocarcinoma versus non-adenocarcinoma, $p < 0.0001$), and differentiation (well versus moderately or poorly, $p < 0.0001$). In 46 patients with *EGFR* mutations, 43 genomic DNA from matched normal lung tissues were available and were showed to be wild type by TaqMan PCR assay, suggesting that these mutations were somatic. There was no mutation detected by the TaqMan PCR assay in gastric, esophagus and colon carcinomas.

Table 2 Mutation status of the *EGFR* gene and *EGFR* copy number in 27 Gefitinib treated samples

Sample no.	Sequence	TaqMan	Copy number
1	WT	L858R	2.41
2	Del/Ins	Del/Ins	2.78
3	Del 1a	Del 1a	1.6
4	Del 1b	Del 1b	2.19
5	WT	WT	2.1
6	Del 5/L838P	Del 5/L838P	5.97
7	WT	WT	2.03
8	WT	WT	2.08
9	Del 3	Del 3	1.52
10	WT	WT	1.83
11	WT	WT	3.94
12	G719S	G719S	2.79
13	WT	WT	1.14
14	WT	WT	1.67
15	WT	WT	3.13
16	Del 1b	Del 1b	2.31
17	WT	WT	1.35
18	WT	WT	1.15
19	WT	WT	1.42
20	WT	WT	2.34
21	Del 1b	Del 1b	1.28
22	WT	WT	2.26
23	WT	WT	6.79
24	L858R	L858R	9.74
25	WT	WT	5.08
26	WT	WT	1.47
27	WT	WT	1.16

Case #1 to #9: responders; case #10 to #27: non-responders. *EGFR* amplification (increased *EGFR* copy number) was defined as more than five copies.

4. Discussion

Gefitinib was developed as an inhibitor of *EGFR* tyrosine kinase that is often over-expressed in many cancers. It showed a promising effect on a few cancers in phase I trial [1]. Subsequently, however, in phase II randomized trials in which the drug was used in combination with other traditional chemotherapy, the effect was marginal in patients with NSCLC [7]. Last year, our group and others have reported identification of genetic mutations in the *EGFR* kinase domain [6,7]. The mutation was seen in a subset of NSCLC with a good response to gefitinib. These reports triggered further studies on the *EGFR* mutation and the tumor's response to gefitinib and erlotinib [6–8]. All the groups identified recurrent mutations in the same region around the ATP binding pocket in *EGFR* tyrosine kinase domain. In vitro studies have reported that the kinase activity of *EGFR* or the sensitivity to gefitinib showed a strong association with *EGFR* gene mutation [7,9]. In our analysis, 6/9 (66.7%) gefitinib-responders had *EGFR* mutations. Thus, some of gefitinib-responders might have other mechanism besides *EGFR* mutations.

In this paper, we were unable to show any differences in *EGFR* amplification between tumors carrying the wild-type *EGFR* sequence and tumors carrying the mutant *EGFR* sequence, which is not surprising as it has been convincingly shown that *EGFR* mutation and not expression levels is responsible for the clinical response to *EGFR* tyrosine kinase inhibitors [6,7,19]. Hirsch et al. [20] reported that *EGFR* gene copy number correlated with EGFR protein expression, but not with prognosis in a cohort of patients not treated with gefitinib. Cappuzzo et al. [21] reported that high *EGFR* gene copy number was associated with better survival. However, there was not any statistically significantly correlation between *EGFR* amplification and overall survival in our analysis. Further study will be needed to delineate the relationships among *EGFR* mutation, *EGFR* gene copy number, *EGFR* mRNA expression, and gefitinib sensitivity.

Over the past three decades, the incidence of lung adenocarcinoma has increased worldwide. Most individuals with lung adenocarcinoma, especially women, are nonsmokers, a population that is sensitive to gefitinib. Reported risk factors for the development of lung adenocarcinoma include cigarette smoking, exposure to cooking fumes, air pollution, second-hand smoke, asbestos, and radon; nutritional status; genetic susceptibility; immunologic dysfunction; tuberculosis infection; asthma; and human papilloma virus [22]. In our analysis of recently operated 182 cases, most of the *EGFR* mutations were present in adenocarcinomas except one case. Mutations were more prevalent in females than in males and in nonsmokers than in smokers, confirming and extending the results of previous reports [6,8,9,23]. More recently, it has been reported that all of adenocarcinomas carrying *EGFR* mutations were well to moderately differentiated [24]. These data were comparable with those obtained in our analysis.

In this report, we used the TaqMan PCR assay based on allele specific probe. This method combines the amplification and detection step, and does not require any post-PCR processing. This makes the TaqMan PCR assay easy-to-use and allows high throughput operation. Furthermore, this method was highly sensitive to detect *EGFR* mutations. One gefitinib responded case with a base-substitution mutation could be detected by the TaqMan PCR assay, although it was undetectable at the first conventional genomic sequencing. When the mutated allele consisted only about 10% of total

Table 3 Relationship between the *EGFR* mutation and clinical–pathologic factors

Factors	Patients with *EGFR* mutation	Patients without *EGFR* mutation	*p*-Value
Gender			
Male	17 (13.4%)	110	<0.0001
Female	29 (52.7%)	26	
Age			
≦64	26 (31.7%)	56	0.0868
>64	20 (20%)	80	
Smoking status			
Never-smokers	27 (58.7%)	19	<0.0001
Ever-smokers	12 (10.6%)	101	
Lymph node metastasis			
N0	34 (27.9%)	88	0.3625
N+	12 (20.7%)	46	
Differentiation			
Well	35 (43.8%)	45	<0.0001
Moderately/or poorly	11 (12.9%)	74	
Pathological subtypes			
Adeno	45 (46.4%)	52	<0.0001
Non-adeno	1 (1.2%)	84	
Stage			
I	33 (31.1%)	73	0.0555
II–IV	13 (17.6%)	61	

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

genomic DNA content, it was not detected by the sequencing. It is alleged that the detection limit in genomic sequencing is about 25% content in general. Because, in the clinical settings, it is not always possible to obtain samples carrying containing homogeneous tumor cells, this highly sensitive method is preferable to the conventional sequencing. Previous reports might underestimate the *EGFR* mutations. The present method can be most effectively used with 5–10 ng of DNA but can analyze as few as 1 ng of tumor DNA. To investigate the correlation between *EGFR* mutations and the response to drugs, large-scale statistical analysis is needed. In these clinical research areas, we believe that this TaqMan PCR assay with high throughput is one of the powerful tools.

In trade off to its accuracy, this method is effective only to the mutations that are already known. The probes cannot be designed to the sequences of unknown mutations. Thus, we must take into account that there is always at a false negative risk in TaqMan PCR assay. However, with the high pace of research [23–25], most of the mutations in *EGFR* gene will be identified in a few years. The good news is the presence of predominant mutations (L858R and exon 19 deletions) which comprise 75–90% of all the *EGFR* mutations. The TaqMan PCR assay presented in this paper can offer us a complementary and confirmative test with sequencing. In our 94 sequenced samples, 24/27 (88.9%) were these predominant mutations. With highly significant correlation between the clinical response to gefitinib (and erlotinib) and *EGFR* mutation, many future clinical trials may first need *EGFR* mutation data using high throughput assays like the one described in this paper.

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References

[1] Baselga J, Rischin D, Ranson M, Calvert H, Raymond E, Kieback DG, et al. Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epi-

dermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. J Clin Oncol 2002;20:4292–302.
[2] Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
[3] Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. J Clin Oncol 2004;22:777–84.
[4] Kris MG, Natale RB, Herbst RS, Lynch Jr TJ, Prager D, Belani CP, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149–58.
[5] Rusch V, Baselga J, Cordon-Cardo C, Orazem J, Zaman M, Hoda S, et al. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. Cancer Res 1993;53:2379–85.
[6] Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. New Eng J Med 2004;350:2129–39.
[7] Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
[8] Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutation are common in lung cancer from "never smokers" and are associated with sensitivity of tumors to gefitinib and elrotinib. Proc Natl Acad Sci USA 2004;101:13306–11.
[9] Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing *EGFR* mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
[10] Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Jänne PA. Gefitinib induces apoptosis in the *EGFR* L858R non-small cell lung cancer cell line H3255. Cancer Res 2004;64:7241–4.
[11] Hampe J, Wollstein A, Lu T, Frevel HJ, Will M, Manaster C, et al. An integrated system for high throughput TaqMan based SNP genotyping. Bioinformatics 2001;17:654–5.
[12] Mc Guigan FE, Ralston SH. Single nucleotide polymorphism detection: allelic discrimination using TaqMan. Psychiatr Genet 2002;12:133–6.
[13] Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, Olivier M, et al. High-throughput genotyping with single nucleotide polymorphisms. Genome Res 2001;11:1262–8.
[14] Shi MM, Bleavins MR, de la iglesia FA. Technologies for detecting genetic polymorphisms in pharmacogenomics. Mol Diagn 1999;4:343–51.
[15] Shi MM. Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. Clin Chem 2001;47:164–72.
[16] Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. Hypertens Res 2003;26:301–6.
[17] Robledo R, Beggs W, Bender P. A simple and cost-effective method for rapid genotyping of insertion/deletion polymorphisms. Genomics 2003;82:580–2.
[18] Sasaki H, Endo K, Konishi A, Takada M, Kawahara M, Iuchi K, et al. *EGFR* mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler. Clin Cancer Res 2005;11:2924–9.
[19] Jänne PA, Gurubhagavatula S, Yeap BY, Lucca J, Ostler P, Skarin AT, et al. Outcomes of patients with advanced non-small cell lung cancer treated with gefitinib (ZD1839, "Iressa") on an expanded access study. Lung Cancer 2004;44:221–30.
[20] Hirsch FR, Varella-Garcia M, Bunn Jr PA, Di Maria MV, Veve R, Bremmes RM, et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol 2003;21:3798–807.
[21] Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643–55.
[22] Chen YC, Chen JH, Richard K, Chen PY, Christiani DC. Lung adenocarcinoma and human papillomavirus infection. Cancer 2004;15:1428–36.
[23] Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004;64:8919–23.
[24] Huang S-F, Liu HP, Li LH, Ku YC, Fu YN, Tsai HY, et al. High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. Clin Cancer Res 2004;10:8195–203.
[25] Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K. Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 2004;64:9101–4.

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Smoking history before surgery and prognosis in patients with stage IA non-small-cell lung cancer—a multicenter study

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Summary The prognosis of lung cancer patients with surgically resected non-small-cell lung cancer (NSCLC) can be predicted generally from age, sex, histologic type, stage at diagnosis, and additional treatment. Nine studies have reported that a history of smoking before diagnosis influences the prognosis of the disease in lung cancer patients. In this study, a total of 3082 patients who underwent surgery and were diagnosed with primary pathological stage IA NSCLC at 36 national hospitals from 1982 to 1997 were analyzed for the effect of smoking on survival. Smoking history and other factors influencing either the overall survival or the disease-specific survival rates of patients were estimated with the Cox proportional hazards model. Multivariate analysis demonstrated significant associations between overall

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survival and age ($P<0.0001$), sex ($P=0.0002$), and performance status (PS) ($P<0.0001$). Disease-specific survival was associated with age ($P=0.0063$), sex (0.00161), and PS ($P=0.0029$). In males, disease-specific survival was associated with age ($P=0.0120$), PS ($P=0.0022$), and pack-years (number of cigarette packs per day, and years of smoking) ($P=0.0463$). These results indicate that smoking history (pack-years) is important clinical prognostic factor in estimating disease-specific survival, in male patients with stage IA primary NSCLC that has been surgically resected.
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1. Introduction

The worldwide incidence and mortality from lung cancer have increased rapidly in recent decades [1]. Non-small-cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers [2]. Even after 30 years of improvements in therapeutic approaches, the 5-year mortality rate of all lung cancer remains an alarmingly high 85% [3]. The 5-year survival rate, even in the optimum surgical stage IA (T1N0M0), is 67% [4]. These poor survival rates are due primarily to recurrences [5] and second lung cancers [6].

The prognosis of lung cancer patients with surgically resected NSCLC can be predicted generally from age, sex, histologic type, stage at diagnosis, and additional treatment [4,7].

The impact of smoking history on survival is controversial. Nine studies have reported that smoking history is a negative prognostic factor in lung cancer [8–16]; whereas, others studies did not find an association [7,17–20].

Recently, Fujisawa et al. have reported that preoperative smoking history is an important clinical postoperative prognostic factor in estimating overall long-term survival in patients with primary resected stage I NSCLC [14].

The aim of this study was to evaluate the effect of smoking history on survival in patients with primary resected stage IA NSCLC.

2. Patients and methods

2.1. Patients

A Central registry for all lung cancer patients has been established in which 33,161 cases have been registered at 36 national hospitals that belong to the Japan National Chest Hospital Study Group for Lung Cancer from 1982 to 1997. We used the central registry data of surgical patients with NSCLC who had been newly diagnosed and undergone surgery. The study group comprised 3217 patients who underwent complete resection and were pathologically confirmed stage IA NSCLC. Ninety-one patients who were lack of smoking history or follow-up interval were excluded from survival analysis. In order to focus on long-term survival, 44 patients (11 with squamous cell carcinoma, 32 with adenocarcinoma, and 1 with large cell carcinoma; a total of 25 men and 19 women) who died within 1 month after surgery were excluded from the survival analyses [21]. Finally, 3082 patients were analyzed for survival analysis. The cancer histologic types included 840 squamous cell carcinomas, 2161 adenocarcinomas, and 81 large cell carcinomas. The patient group consisted of 1221 women and 1861 men who ranged in age from 22 to 89 years (mean age, 64.4 years). Histologic type and TNM classification were classified according to the criteria of World Health Organization. Performance status (PS) was classified according to the criteria of Eastern Cooperative Oncology group (ECOG). The data on smoking history (pack-years, number of packs per day, and years of smoking) were obtained from hospital records. Cause of death was reported by the doctor who followed the patient. At the last follow-up, for overall survival curves, an observation was censored if the patient was alive; for disease-specific curves, data were censored if the patient was alive or had died from a cause other than NSCLC.

2.2. Survival rate and statistical analysis

Overall survival was defined as the time between surgery and death or last follow-up evaluation. Disease-specific survival was defined as the time between surgery and cancer death or last follow-up evaluation.

Bivariate analysis was performed with Fisher's exact test. The difference in age between the two groups was analyzed with the Student's *t*-test. Overall survival and disease-specific survival were calculated with the Kaplan–Meier method, and the difference between survival curves was analyzed with the log-rank test. Variables in this study consist of age, sex, histologic type, tumor classification, and cigarette smoking before surgery. Multivariate analysis was performed with the Cox proportional

hazards model. All statistical analysis in this study was performed with StatView statistical software (StatView version 5.0 for Macintosh; SAS institute Inc., Cary, NC, USA). Statistical significant *P*-values were considered to be less than 0.05.

3. Results

3.1. Association between clinical features and smoking pack-years

Clinical features, including age, sex, PS, and histology, were evaluated according to smoking pack-years (Table 1). The heavy smokers group also had significantly higher population of older age, male patients, poor PS, and squamous cell carcinomas than smokers with less than 40 pack-years or non-smokers.

3.2. Cause of death

Forty-four patients died within 1 month after surgery (1.4% of 3126 patients). After a median follow-up of 3.9 years, of 3082 patients used for survival analysis, 491 patients died from recurrent or second lung cancer, and 159 patients died from non-recurrent diseases. Non-recurrent causes consisted of 27 second primary malignancies.

3.3. Overall survival and disease-specific survival

The overall and disease-specific 5- and 10-year survival curves are shown in Fig. 1. Fig. 2 demonstrates the overall survival and disease-specific survival curves according to cigarette smoking,

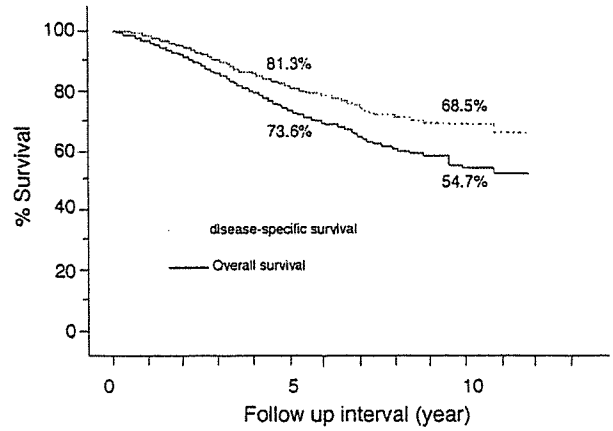

Fig. 1 Overall survival and disease-specific survival curves in patients with primary, surgically resected stage IA NSCLC.

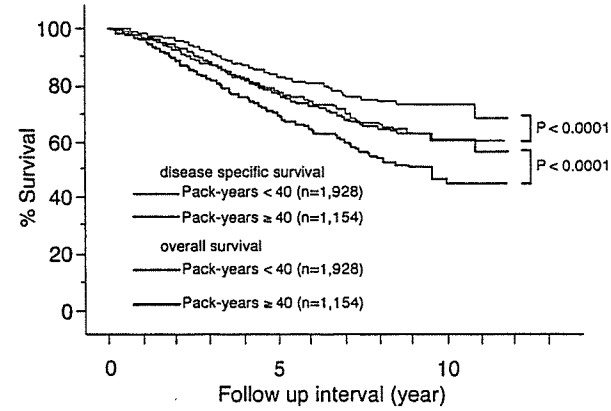

Fig. 2 Overall survival and disease-specific survival curves in patients with primary, surgically resected stage IA NSCLC, evaluated by pack-years.

Table 1 Distribution of clinical features, according to smoking pack-years

Clinical feature	Pack-years		*P*[a]
	<40	≥40	
Age (mean ± S.D.)	62.8 ± 10.3	66.9 ± 8.2	<0.0001
Sex			
Male	770	1091	<0.0001
Female	1158	63	
PS			
0	1612	855	<0.0001
≥1	306	289	
Histology[b]			
Nonsquamous cell carcinoma	1660	582	<0.0001
Squamous cell carcinoma	268	572	

[a] *P*-value for age are by Student's *t*-test and for the remainder are for Fisher exact test.
[b] Nonsquamous cell carcinoma is comprised of adenocarcinoma and large cell carcinoma.

Table 2 Overall survival and disease-specific survival, according to clinical prognostic factors

Clinical feature	No. of patients	Overall survival (%)			Disease-specific survival (%)		
		5 years	10 years	P[a]	5 years	10 years	P[a]
Age (years)							
<70	2115	77.3	60.6	<0.0001	83.1	70.9	0.0003
≥70	961	64.4	36.3		76.1	60.7	
Sex							
Male	1861	70.7	47.3	<0.0001	79.2	63.0	<0.0001
Female	1221	78.1	66.7		84.5	76.7	
Histology[b]							
Squamous cell carcinoma	840	69.8	49.8	0.0041	79.8	61.5	0.0831
Nonsquamous cell carcinoma	2242	75.1	56.6		81.8	71.2	
Performance status							
0	2467	76.8	58.1	<0.0001	82.3	69.9	<0.0001
≥1	595	61.0	41.8		76.5	62.0	
Pack-years							
<40	1928	76.5	60.5	<0.0001	83.4	73.6	<0.0001
≥40	1154	69.0	45.7		77.8	60.3	

[a] P-value for the log-rank test.
[b] Nonsquamous cell carcinoma is comprised of adenocarcinoma and large cell carcinoma.

and the 5- and 10-year survival rates between heavy smokers (pack-years ≥ 40) and light smokers (pack-years < 40) are both significantly different ($P < 0.0001$).

Table 2 shows the overall and disease-specific 5- and 10-year survival rates according to several variables. Significant differences in overall survival were demonstrated with age ($P < 0.0001$), sex ($P < 0.0001$), histologic type ($P = 0.0041$), PS ($P < 0.0001$), and pack-years ($P < 0.0001$). But no significant difference in disease-specific survival was found with histologic type ($P = 0.0831$). With regard to cigarette smoking, the difference between heavy smokers and light smokers was statistically significant ($P < 0.0001$) in the both overall survival and disease-specific survival.

3.4. Multivariate analysis

Multivariate analysis was conducted with the Cox proportional hazards model with the five variables. Multivariate analysis demonstrated a significant association between overall survival and age ($P < 0.0001$), sex ($P = 0.0002$), and PS ($P < 0.0001$), but no association was observed with histologic type ($P = 0.3807$) or pack-years ($P = 0.1742$) (Table 3).

Next, multivariate analysis for disease-specific survival was performed with the five variables. Multivariate analysis demonstrated a significant association of disease-specific survival with age ($P = 0.0063$), sex ($P = 0.0161$), and PS ($P = 0.0029$), and no significant association with histologic type ($P = 0.3935$) or pack-years ($P = 0.0741$) (Table 4).

We conducted a subgroup analysis for overall survival and disease-specific survival according to sex. In a subgroup analysis (Tables 3 and 4), disease-specific survival demonstrated a significant association with age ($P = 0.0120$), PS ($P = 0.0022$), and pack-years ($P = 0.0463$), and no significant correlation with histologic type ($P = 0.1971$). Similar trends were observed for overall survival among males, but pack-years was not a significant prognostic factor ($P = 0.1410$). On the other hand, the analyses for females, a considerably small proportion of heavy smokers (5.1%) gave an unstable odds ratio estimation (Tables 1, 3 and 4).

4. Discussion

In this study, more than 3000 patients with stage IA primary NSCLC that has been surgically resected were analyzed, and we found that older age, poor PS, male, and smoking history, in male, were significant unfavorable prognostic factors. We demonstrated the significant inverse correlation between cigarette smoking and long-term disease-specific survival in stage IA NSCLC patients using multivariate analysis. Even if a curative surgery has been underwent in a very early stage NSCLC, previous smoking history still was disadvantage.

The impact of smoking history on survival is still confusing. Earlier studies found no associ-