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Can dose-dense chemotherapy improve outcome in patients with better-prognosis small-cell lung cancer?

GLOSSARY

DOSE INTENSITY
The amount of drug delivered per unit of time, expressed as mg/m^2/week

DOSE-DENSE THERAPY
Treatment where dose intensity is increased by shorter treatment interval rather than by dose escalation

KAPLAN–MEIER ANALYSIS
A conditional probability strategy used for estimation of survival in clinical trials with censored observations

NORTON–SIMON HYPOTHESIS
A mathematical model suggesting that more frequent dosing of chemotherapeutic agents minimizes tumor regrowth between doses; this theory forms the basis for dose-dense and sequential chemotherapy

GOMPERTZIAN PHENOMENON
Where the cell number increases with time, but the relative rate of increase falls exponentially until a 'plateau phase' of very slow actual growth is reached

Original article Lorigan P *et al.* (2005) Randomized phase III trial of dose-dense chemotherapy supported by whole-blood hematopoietic progenitors in better-prognosis small-cell lung cancer. *J Natl Cancer Inst* **97**: 666–674

SYNOPSIS

KEYWORDS dose-dense chemotherapy, small-cell lung cancer, survival

BACKGROUND

For patients with small-cell lung cancer (SCLC), response rates with combined chemotherapy and radiotherapy are high, but long-term survival is poor. The role of dose intensification in the treatment of SCLC is controversial. The main dose-limiting toxicity of ifosfamide, carboplatin and etoposide (ICE) chemotherapy is hematologic, so the authors used filgrastim and autologous whole-blood progenitor cells (WBPC) to allow an increase in the relative DOSE INTENSITY of this chemotherapy regimen.

OBJECTIVES

To compare ICE chemotherapy with a 4-week interval between cycles (standard therapy) with ICE chemotherapy with a 2-week interval between cycles supported by filgrastim and WBPC (DOSE-DENSE THERAPY).

DESIGN AND INTERVENTION

In this phase III trial, patients with pathologically confirmed SCLC with a prognostic score of 0 or 1 were randomized to receive six cycles of ICE chemotherapy (ifosfamide at 5 g/m^2 intravenously for 24 hours on day 1 with mesna at 5 g/m^2, carboplatin at 300 mg/m^2 intravenously on day 1, and etoposide at 180 mg/m^2 intravenously on days 1 and 2). For standard therapy, a 4-week interval was left between cycles, whereas dose-dense therapy cycles were given at 2-week intervals, with subcutaneously administered filgrastim (300 μg for patients weighing <70 kg and 5 μg/kg for patients weighing >70 kg, daily on days 4–14) and autotransfusion of autologous blood.

OUTCOME MEASURES

The primary outcome assessed was survival, estimated using KAPLAN–MEIER ANALYSIS. Response rate, relative dose intensity, time to disease progression and toxicity were studied as secondary endpoints.

RESULTS

Age, sex and performance status were balanced between the groups receiving dose-dense or standard chemotherapy (median age 58 years in both groups). All 318 patients were analyzed for survival on an intention-to-treat basis. Median follow-up was 14 months. Response rates, median time to progression, median survival and 1-year or 2-year survival did not differ significantly between the groups. Overall response to treatment was seen in 129 of 147 patients receiving dose-dense therapy (88%) versus 118 of 148 patients receiving standard therapy (80%) (a nonsignificant difference). Median overall survival was 14.4 months (95% CI 13.1–15.4 months) for dose-dense therapy versus 13.9 months for standard therapy (95% CI 12.1–15.7 months) and 2-year survival rates were 19% (95% CI 14–27%) and 22% (95% CI 16–29%) respectively, with neither difference reaching statistical significance. Median delivered dose intensity was 99% for standard therapy and 182% for dose-dense therapy. More hematologic toxicity was reported in the dose-dense arm than in the standard arm, but the number of cycles complicated by neutropenic sepsis was higher with standard therapy than with dose-dense therapy (15.3% versus 11.6% respectively; 95% CI –2% to 9.6%; $P = 0.03$).

CONCLUSION

Dose-dense ICE chemotherapy has been validated as a safe, acceptable regimen and might be applicable to other tumor types in which dose intensification might improve survival.

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COMMENTARY

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Randomized trials using various dose intensities to treat SCLC have shown inconsistent survival results. Nevertheless, when the same drugs were delivered at the same dose per cycle, the same number of cycles, and the same planned cumulative doses using shorter treatment intervals supported by hematopoietic growth factor, a modest survival benefit was demonstrated in four randomized trials.[1] Dose intensity was 106–134% in these trials versus 100% for standard treatment, a moderate increase. Superior results using dose-intensified chemotherapy could therefore be anticipated in the treatment of SCLC.

Lorigan and colleagues have reported the first randomized trial in which the dose density of ICE chemotherapy was doubled, facilitated by filgrastim and WBPC—an attractive method for accomplishing dose densification effectively and safely[2] in patients with better-prognosis SCLC. Dose-dense chemotherapy using WBPC and filgrastim produced acceptable toxicities, a lower risk of infection and shorter treatment duration than standard chemotherapy.[2] Although more red-cell and platelet transfusions were needed to treat grade 3–4 anemia and thrombocytopenia resulting from dose-dense chemotherapy, 69% of the patients still completed six cycles of dose-dense chemotherapy. Dose-dense chemotherapy with a 2-week interval resulted in a greater relative dose intensity (up to 182%) than standard ICE chemotherapy with a 4-week interval, but could not prolong overall survival.

Why did dose-dense chemotherapy supported by WBPC fail to improve overall survival in this trial? First, this phase III trial was designed to clarify the efficacy of dose-dense chemotherapy with a focus on specific drugs only, and leaving thoracic radiation therapy out of consideration. More patients in the standard-treatment arm than in the dose-dense chemotherapy arm received thoracic radiotherapy, whereas more patients receiving dose-dense treatment achieved objective response than those receiving standard treatment. The use of thoracic radiotherapy, the most powerful treatment for local control in limited-stage SCLC, might have cancelled a dose-dense effect in this trial. Second, the advantage of dose densification is in its ability to improve cure rates. Based on the NORTON–SIMON HYPOTHESIS, an increased chemotherapy dose rate enhances cell kill in *ex vivo* and *in vivo* experiments.[3] The true benefit of dose-dense therapy comes from total tumor-cell kill, because even if repeated therapy induces multiple regressions, tumor-cell regrowth occurs because of GOMPERTZIAN PHENOMENA. Thus, the 2-year survival rate of around 20% is somewhat low to enable a comparison with the cure rates in patients with better-prognosis SCLC. Similarly, dose-dense chemotherapy as adjuvant treatment produces longer survival in primary breast cancer,[4] but not in breast cancer that has metastasized.

A preliminary report of a randomized phase II study with the same design as this study reported significantly better survival, but not time to progression, in the dose-dense treatment arm than in the standard treatment arm. The 2-year survival rate was 62% in the dose-dense treatment arm, but the study size was small and thoracic radiotherapy was not mentioned.[5] We will have to reserve final judgment on dose-dense chemotherapy supported by WBPC in the clinical practice of SCLC.

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Competing interests
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PRACTICE POINT
Dose-dense chemotherapy supported by whole-blood progenitor cells should not be routinely employed in the clinical practice to treat small-cell lung cancer

of chemotherapy in NSCLC, and a considerable number of new RCTs have been completed. The total number of patients randomly assigned has risen to approximately 23,000 patients.

As the aim of the NSCLC Collaborative Group is to provide an up-to-date and reliable review of the role of chemotherapy, both to act as a sound basis for evidence based medicine and to help guide future research, it was decided that an update was timely. A number of new agents and timings have been investigated in all settings, and the update consists of adding trials published since the 1995 analyses and additional follow-up data from trials already included, as well as looking at additional outcomes in certain settings. We are also investigating the effect of chemotherapy in three additional settings (comparisons 2, 5, and 6), bringing the total to seven: (1) surgery versus surgery plus chemotherapy (adjuvant); (2) surgery versus chemotherapy plus surgery (neoadjuvant); (3) surgery plus radiotherapy versus surgery plus radiotherapy plus chemotherapy; (4) radiotherapy versus sequential radiotherapy plus sequential chemotherapy; (5) radiotherapy versus radiotherapy plus concomitant chemotherapy; (6) radiotherapy plus sequential chemotherapy versus radiotherapy plus concomitant chemotherapy; (7) supportive care versus supportive care plus chemotherapy.

For the update of the 1995 meta-analyses, we have identified a total of 22 new RCTs with more than 8,000 patients in the equivalent setting to that which is described by Hotta et al, bringing the total number of trials to 38. If we can include these patients, it would bring the total number of patients in this comparison alone to more than 10,500 patients.

As Piedbois and Buyse point out, IPD meta-analyses are considered the gold standard but need time and funding. This meta-analysis by Hotta et al, is a valuable resource in the absence of other evidence, but the results should be considered with caution until they can be compared with the updated IPD meta-analysis.

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IN REPLY: We appreciate the constructive comments from Burdett et al concerning the importance of meta-analysis using individual patient data (IPD) in patients with all stages of non–small-cell lung cancer (NSCLC). The meta-analysis conducted by the NSCLC Collaborative Group (NSCLC-CG) in 1995 has been a very important and helpful reference for clinicians involved in NSCLC treatment.[1] Its results have also been an important reference for new clinical trials for NSCLC. Despite our great respect for the NSCLC-CG study, we were still interested in whether meta-analysis of trials not included in the NSCLC-CG study would be in accordance with its results because most of the trials in the NSCLC-CG analysis involved outdated regimens no longer used in NSCLC treatment. Therefore, we narrowed our objective to clarifying the role of cytotoxic agents, including platinum or uracil-tegafur, as adjuvant chemotherapy, and limited eligible trials to those we analyzed.[2]

We have no objection to their statement that IPD-based meta-analysis is more ideal than abstracted data–based meta-analysis, in terms of to obtaining answers to more specific clinical questions. We conducted our abstracted data–based analysis to address our aforementioned clinical question because we were not in a position to conduct an IPD-based analysis. We are very pleased to hear that an IPD-based meta-analysis is underway, and we eagerly await the results. We especially look forward to seeing whether the results are consistent with ours.

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Treatment of Secondary Acute Myeloid Leukemia

TO THE EDITOR: In a recent issue, Kern et al reported the experience of the German Cooperative Group on the treatment of therapy-related acute myeloid leukemia (tAML), concluding that these patients should be treated as "de novo" AML. The most important prognostic parameter was the cytogenetic pattern, while being a "therapy related disease" itself does not retain a specific negative prognostic significance.[1]

We want to add the experience of the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) in the treatment of tAML. We analyzed the outcome of patients with tAML in comparison with de novo AML in patients treated with standardized chemotherapy according to four consecutive trials for previously untreated AML conducted during the period January 1987 to January 2001 by the GIMEMA cooperative group. The study population comprised more than 2,000 adult patients with newly diagnosed AML; only patients who had no recurrence of their prior malignancy at the time of tAML were included in the analysis. Thirty-eight patients were treated with chemoradiotherapy and subsequently developed tAML (1.5%). In a case-matched study, three cases of de novo AML, comparable for age, French-American-British criteria, WBC count at AML diagnosis, trial, and time of diagnosis were chosen for each tAML case. Cytogenetic study was available only in a limited number of cases (12 patients), and a comparison was made between tAML patients in whom cytogenetic pattern was known, with de novo AML patients belonging to the same risk category and respective to all the previously reported matching criteria. tAML occurred after a median latency of 38 months from primary malignancy. None of the patients with tAML had a previous myelodysplastic phase. Sixty-six percent of patients with tAML achieved compete response (CR), 16% died in induction, and 18% were resistant. The actuarial Kaplan-Meier projection at 5 years showed a disease-free survival (DFS) of 35%. The median overall survival (OS) for all patients was 11.4 months. The actuarial Kaplan-Meier curve showed an OS of 19% at 5 years, and of 15.2% at 10 years. Comparing the CR rate between 38 tAML and 114 de novo AML patients selected according to the previously reported criteria, no difference was observed (66% *v* 58%; Pearson $\chi^2 = 0.7393$; $P = .390$), and no difference was observed comparing the DFS and the OS between the two groups.

In a recent experience of the European Bone Marrow Transplantation Group the actuarial 2-year survival, DFS, relapse rate, and transplant-related mortality of patients with tAML were not statistically different from those of patients with de novo AML.[2] However, this procedure can really be performed in only a small proportion of patients with tAML, because they are, in the majority of cases, too old for the procedure and are frequently unable to tolerate conventional myeloablative regimens. Recently, Rowe[3] emphasized that the prognosis of the tAML is absolutely similar to that of de novo AML with corresponding cytogenetic risk. This observation was also confirmed by a GIMEMA study on secondary acute promyelocytic leukemia that presented no difference in remission rate when compared with de novo acute promyelocytic leukemia enrolled in the AIDA (All-*trans*-retinoic acid plus Idarubicin) trial.[4]

Furthermore, no specific treatment strategies for tAML demonstrated a higher activity with respect to standard therapy. The lack of cytogenetic data in our patients surely represents a limit of our evaluation; however, in the risk analysis, we separately analyzed the cohort of patients, comparing them with de novo AML cases in which the cytogenetic profile was available with corresponding cytogenetic risk; the CR rate, OS, and DFS did not differ between the two groups. It is noteworthy that in none of our tAML patients was a previous myelodysplasia reported. It is well known that a myelodysplastic phase generally worsens the outcome of AML, above all in tAML. The absence of an myelodysplastic phase could influence the results of treatment in our series. In fact, a recent report by Goldstone et al[5] on a large population of AML patients enrolled in the MRC AML 10-11-12 trials, demonstrated that those patients with tAML had a worse prognosis and were in more than 50% of cases of postmyelodysplastic AML.

The results of this study and the above considerations, together with the data reported by Kern et al,[1] support the evidence that tAML patients usually have a worse prognosis frequently because of older age, lower performance status, and higher comorbidity, frequently associated with unfavorable cytogenetic profile with respect to de novo AML. Therefore, the secondary nature of the disease should not itself be considered an adverse factor, and the therapeutic strategy should be defined considering the conventional risk factor combination, similar to the de novo AML cases.

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The Relationship between Epidermal Growth Factor Receptor Mutations and Clinicopathologic Features in Non–Small Cell Lung Cancers

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ABSTRACT

Purpose: **Recent studies reported that clinical responsiveness to gefitinib was associated with somatic mutation of epidermal growth factor receptor (*EGFR*) gene in non–small cell lung cancers (NSCLC). Here, we investigated the relationship between *EGFR* mutation and clinicopathologic features.**

Experimental Design: EGFR **mutational status of 120 NSCLCs was determined mainly in *EGFR* exons 18 to 21 by direct sequence and correlated with clinicopathologic parameters.**

Results: EGFR **mutations were present in 38 cases (32%) and the majority of mutations were in-frame deletions of exon 19 (19 cases) and a missense mutation in exon 21 (18 cases). *EGFR* mutations were frequently associated with adenocarcinoma ($P < 0.0001$), never smoker ($P < 0.0001$), and female gender ($P = 0.0001$). Of interest, increasing smoke exposure was inversely related to the rate of *EGFR* mutation ($P < 0.0001$). Multivariate analysis showed that smoking and histology were independent variables. Furthermore, gender difference was observed for the mutational location ($P = 0.01$) dominance of exon 19 for males and exon 21 for females. Twenty-one cases were treated with gefitinib and found that *EGFR* mutation was significantly related to gefitinib responsiveness ($P = 0.002$). In addition, median survival times of patients with and without *EGFR* mutations treated with gefitinib were 25.1 and 14.0 months, respectively. Patients with *EGFR* mutations had approximately 2-fold survival advantage; however, the difference was not significant.**

Conclusions: **We show that *EGFR* mutations were significantly related to histology and smoke exposure and were a strong predictive factor for gefitinib responsiveness in NSCLC.**

INTRODUCTION

Lung cancer is one of the major causes of cancer deaths in the world with over 1 million cases diagnosed every year (1). Human lung cancers are classified into two major types, small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC), the latter consisting of several types (2), mainly adenocarcinoma and squamous cell carcinoma. Previously, squamous cell carcinoma was the predominant form of NSCLC, but in the last few decades it has been replaced by adenocarcinoma (3, 4). Tobacco smoking is a widely recognized risk factor for lung cancer, especially for squamous cell carcinoma and SCLC, but smoke exposure seems to be a less potent oncogenic factor for adenocarcinoma.

NSCLC is generally less sensitive to chemotherapy than SCLC and curative intent surgical resection is the treatment of first choice (5). However, chemotherapy and/or radiotherapy are often used for advanced or recurrent cases. With the accumulation of knowledge of molecular biology of lung cancer, several genetic changes including *TP53* mutation were reported to be related to response to chemotherapy (6). Epidermal growth factor receptor (*EGFR*) is a receptor tyrosine kinase identified as being highly expressed in cancer cells including lung cancers (7). EGFR is a transmembrane protein consisting of an extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase (TK) domain and a regulatory region (8). After ligand binding, specific tyrosine residues of the intracellular domain are autophosphorylated, which results in initiation of the intracellular signaling cascade, including the Ras/Raf/MAPK, JAK/STAT and PI3K-Akt pathways, leading to a multitude of effects including cell proliferation, cell differentiation, angiogenesis, metastasis, and antiapoptosis (9). Gefitinib is an orally active EGFR TK inhibitor and has been widely used in clinical trials and is approved for the treatment of advanced NSCLC (10–12).

The mechanism of antitumor effect or drug sensitivity has not been clearly understood (12); recently, however, Lynch et al. and Paez et al. reported that clinical responsiveness to gefitinib was associated with somatic mutations in the TK domain of *EGFR* gene in NSCLCs (13, 14). These mutations occurred near the ATP cleft of the TK domain in which

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Table 1 Patient characteristics and *EGFR* mutation

Variables	No.	*EGFR* mutation (%)	*P*
A. In NSCLCs			
Age (y)			
≤67	60	21 (35%)	
>67	60	17 (28%)	
Gender			
Male	83	17 (20%)	0.0001
Female	37	21 (57%)	
Smoking history			
Never smoker	36	25 (69%)	<0.0001
Ever smoker	84	13 (15%)	
Histology			
Adenocarcinoma	82	37 (45%)	<0.0001*
Squamous cell carcinoma	35	0	
Adenosquamous cell carcinoma	1	1 (100%)	
Large cell carcinoma	2	0	
B. In adenocarcinomas			
Age (y)			
≤67	42	21 (50%)	
>67	40	16 (40%)	
Gender			
Male	52	17 (33%)	0.003
Female	30	20 (67%)	
Smoking history			
Never smoker	33	24 (73%)	<0.0001
≤30 pack-years	16	5 (31%)	
>30 pack-year	33	8 (24%)	
Smoking status			
Former smoker	21	5 (24%)	
Current smoker	28	8 (29%)	

*Histological difference was examined between adenocarcinoma and other types of NSCLCs. *P* values are stated when there were significant differences between groups.

4-anilinoquinazoline compounds such as gefitinib compete with ATP for binding (13). In addition, Paez et al. found that the mutations were more frequent in cases of adenocarcinoma histology, female gender, and Japanese origin (14).

In this study, we examined the *EGFR* mutational status in 120 NSCLC specimens including 21 cases treated with gefitinib and analyzed the relationship between the *EGFR* status and clinicopathologic features to investigate the clinical importance of *EGFR* mutation in NSCLCs.

MATERIALS AND METHODS

Clinical Samples. Surgically resected specimens of 120 NSCLCs consisting of 82 adenocarcinomas, 35 squamous cell carcinomas, 2 large cell carcinomas, 1 adenosquamous cell carcinoma, and corresponding 10 nonmalignant peripheral lung tissues were obtained from Okayama University Hospital, Okayama, Japan, after acquiring informed consent from each patient, between 1994 and 2003. Institutional review board permission and informed consent were obtained for all cases. The NSCLC patients consisted of 83 males and 37 females and their median age was 67. Seventy patients had stage I disease, 20 stage II, 24 stage III, and 3 stage IV. Disease stages were not known in 3 patients because systemic dissection for lymph node was not done for these cases. Eighty-four cases were from ever smokers with a median smoke exposure of 45.5 pack-years (50 cases were current smokers and 34 were former smokers) and 36 cases were never smokers. The patient characteristics are shown in Table 1. Twenty-one cases were treated with gefitinib for recurrent disease and clinical responsiveness was recorded. These cases had been previously treated with some chemotherapy that failed to respond to disease. For evaluation of response, all patients underwent complete blood counts, blood chemistry, urinalysis, plain chest radiograph at least weekly for a month from the beginning of gefitinib treatment and at least monthly thereafter, and serum carcinoembryonic antigen monthly; computed tomography scan of the chest (and abdomen if tumor had spread to intraabdominal organs) was taken at least every 3 months for 2 years. Response was assessed using Eastern Cooperative Oncology Group criteria (15). Cases of complete or partial response were considered as responsive cases and that of no change or progressive disease as nonresponsive cases. Clinicopathologic staging was determined according to International Union Against Cancer tumor-node-metastasis classification of malignant tumors (16).

DNA Extraction and Sequencing Analysis. Genomic DNA was isolated by digestion with proteinase K followed by phenol-chloroform (1:1) extraction and ethanol precipitation from frozen specimen (17) and by DEXPAT (TaKaRa, Shiga, Japan) from paraffin embedded tissues following the manufacturer's instructions. *EGFR* mutations were detected using PCR-based direct sequencing of the seven exons of the TK domain (exons 18-24). PCR amplification was done in 20-μL volume containing genomic DNA using HotStarTaq DNA polymerase (Qiagen Inc., Valencia, CA). DNA was amplified for 40 cycles at 94°C for 20 seconds, 56°C to 65°C for 30 seconds, and 72°C for 20 seconds, followed by 7 minutes extension at 72°C. The primers and condition of PCR amplification are shown in Table 2. PCR products were incubated using ExoSAP-IT (Amersham Biosciences Corp., Piscataway, NJ) and sequenced directly using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA) with ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Several samples, whose mutation status were difficult to determine by direct sequence, were amplified and cloned into pBluescript after sequencing with M4 and RV6 primer (Fig. 1).

Statistical Analyses. The rates of *EGFR* mutation between two groups were compared using the χ^2 or Fisher's exact test. Fisher's exact test was done if there were five or fewer observations in a group. Logistic regression models were used to further explore observed differences and to identify baseline factors that may independently predict for *EGFR* mutation. The Cochran-Armitage test for trend was used to examine the dose effect of smoke exposure on *EGFR* mutation. Univariate analysis of overall survival was carried out by the Kaplan-Meier method using the log rank test and generalized Wilcoxon test for the two groups. Probability values <0.05 were defined as being statistically significant. All statistical tests were two-sided.

RESULTS

***EGFR* Mutation in Non–Small Cell Lung Cancers.** Because results of sequencing for *EGFR* TK domain (exons 18-24) in unselected 45 cases indicated that nucleotide change

compared with original sequence (Genebank accession no. AL365274) were limited to the first four exons (exons 18-21), further analyses were limited to these four exons. We examined the *EGFR* status in 120 cases of NSCLCs and found mutations in 38 cases (32%). Mutation was detected in one case of point mutation in exon 18, 19 cases of in-frame deletion in exon 19 involving 3 to 8 codons around the uniformly deleted codons 747 to 749 (Leu-Arg-Glu sequence), and 18 cases of point mutation (L858R) in exon 21. Representative nucleotide sequences of the *EGFR* gene mutation were shown in Fig. 1. There were eight types of deletion variants in exon 19 (Fig. 2) and new types of deletion were variants 4, 5, 7, and 8. In addition, the third letter of codon 787 (CAG) in exon 20 was changed in eight cases homozygously (CAA) and 14 cases heterozygously (CAG and CAA) resulting in no amino acid change. Thus, this change was considered to be a polymorphism or silent mutation. We also examined 10 nonmalignant lung tissues of cases in which the corresponding tumors harbored mutation and found no mutations, indicating that these mutations were somatic in origin.

***EGFR* Mutation and Clinicopathologic Correlations.** We analyzed the relationship between the *EGFR* status and clinicopathologic factors (Table 1, A and B). *EGFR* mutations were frequently present in adenocarcinoma ($P < 0.0001$), never smokers ($P = 0.0001$), and female gender ($P = 0.003$) in NSCLCs (Table 1A). Logistic regression models showed that smoking status ($P = 0.005$) and histology ($P < 0.0001$) were independent variables. Because adenocarcinoma is the dominant histology for mutation, further analyses were limited to adenocarcinoma. *EGFR* mutations were significantly associated with female gender (67%, $P = 0.003$) and never smoker status (73%, $P < 0.0001$) compared with male gender (33%) and ever smoker status (27%), respectively, by univariate analysis (Table 1B). Logistic regression models showed that smoking status was the only independent variable in adenocarcinomas ($P = 0.01$). In addition, the cases were divided into three groups based on smoke dose: (*a*) never smokers ($n = 33$), (*b*) smokers with exposure of ≤30 pack-years ($n = 16$), and (*c*) smokers with exposure of >30 pack-years ($n = 33$). The Cochran-Armitage test for trend was done to examine the dose effect of smoke exposure on *EGFR* mutation. We found that there was an inverse trend between smoking dose and mutation ($P < 0.0001$; Table 1B). We also examined the relationship between *EGFR* mutations and detailed smoking status (current and former smokers); however, there was no significant difference between these two groups (Table 1B). Exon 19 deletions were significantly more frequent in males, and exon 21 mutations were more frequent in females ($P = 0.049$). To exclude the possible effect of smoke exposure on the difference of mutation location and gender, the same analysis was done in the never-smoking adenocarcinoma group showing significant difference as well ($P = 0.008$). *EGFR* mutations were not related to other clinicopathologic factors including disease stage and patient age.

***EGFR* Mutation and Gefitinib Responsiveness.** Among 120 NSCLC patients, 21 cases were treated with gefitinib and clinical responsiveness was recorded. These cases consisted of 8 (38%) cases of females, 8 (38%) of never smokers, and 15 (71%) of adenocarcinomas. Detailed characteristics of these cases are shown in Table 3. In 21 treated cases, mutations were present in 8 of 10 cases with gefitinib responsiveness and in 1 of 11 cases with nonresponsiveness, showing that *EGFR* mutations were significantly more frequent in gefitinib response cases ($P = 0.002$). However, gender, smoke exposure, and

Table 2 Summary of primer sequences and annealing temperatures for direct sequence

Exon	Primer sequences	T_m (°C)	Product size (bp)
For DNA templates from frozen samples			
18	F, 5′-GAGGTGACCCTTGTCT CTGTGT-3′	57	189
	R, 5′-AGCCCAGAGGCCTG TGCCA-3′		
19	F, 5′-CCAGATCACTGGGCAGCAT GTGGCACC-3′	65	265
	R, 5′-AGCAGGGTCTAGAGCAGA GCAGCTGCC-3′		
20	F, 5′-ACTGACGTGCCTCTCC CTCC-3′	57	235
	R, 5′-CCGTATCTCCCTTCCCT GATT-3′		
21	F, 5′-ATCTGTCCCTCACAGCA GGGTC-3′	57	210
	R, 5′-GGCTGACCTAAAGCC ACCT-3′		
22	F, 5′-AATTAGGTCCAGAGTGAG TTAAC-3′	65	251
	R, 5′-ACTTGCATGTCAGAGGAT ATAATG-3′		
23	F, 5′-CATCAAGAAACAGTAACC AGTAATG-3′	65	320
	R, 5′-AAGGCCTCAGCTGTTTG GCTAAG-3′		
24	F, 5′-TTGACTGGAAGTGTCGCA TCACC-3′	65	279
	R, 5′-CATGTGACAGAACACAGT GACATG-3′		
For DNA templates from paraffin-embedded samples			
18a*	F, 5′-GAGGTGACCCTTGTCTCGTGT-3′	56	110
	R, 5′-ATTCAGTTTCCTTCAAGA TCCTC-3′		
18b**	F, 5′-AGTGGAGAAGCTCCCAAC CAAGC-3′	65	131
	R, 5′-AGCCCAGAGGCCTGTGCCA-3′		
19	F, 5′-AACGTCTTCCTTCTCTCTC TGTCAT-3′	56	150
	R, 5′-CCACACAGCAAAGCAGA AACTC-3′		
20a*	F, 5′-ACTGACGTGCCTCTCC CTCC-3′	56	127
	R, 5′-AAGGGCATGAGCTGCG TGA-3′		
20b**	F, 5′-TGCCTCACCTCCACCGT-3′	56	152
	R, 5′-CCGTATCTCCCTTCCCT GATT-3′		
21a*	F, 5′-ATCTGTCCCTCACAGCAG GGTC-3′	56	126
	R, 5′-TGATCTTGACATGCTGCG GTGTT-3′		
21b**	F, 5′-AGCCAGGAACGTACTGGTGA-3′	56	134
	R, 5′-GGCTGACCTAAAGCCACCT-3′		

NOTE. a* and b**, exon 18, 20, and 21 were divided into two parts for PCR amplification and sequenced separately.

Abbreviations: T_m, annealing temperature; *F*, forward primer; *R*, reverse primer.

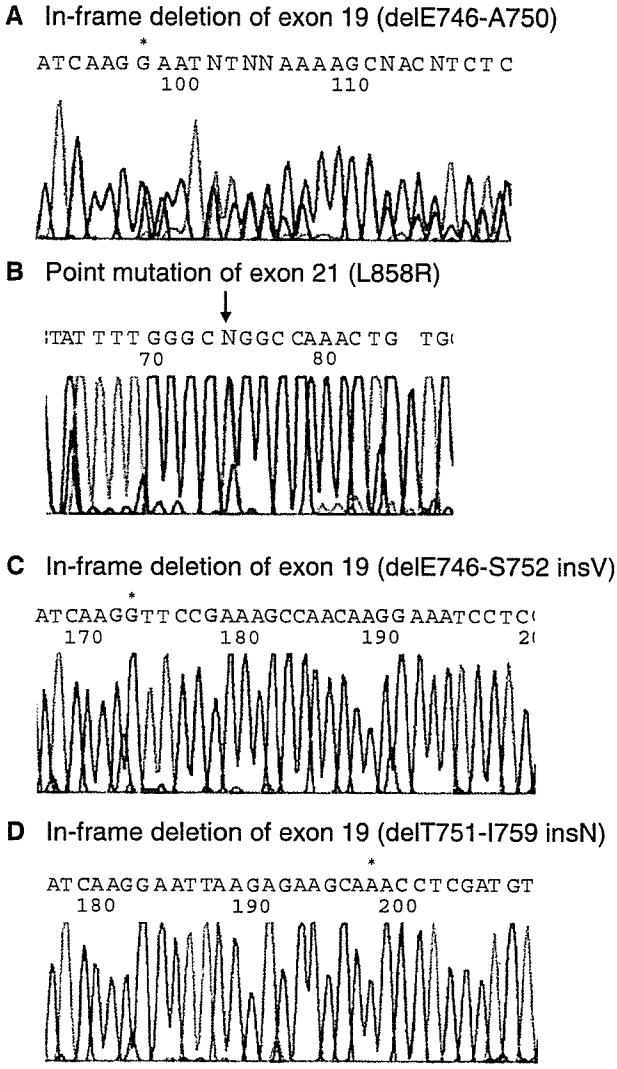

Fig. 1 Representative nucleotide sequence of the *EGFR* gene in tumor specimens *A*, the nucleotide sequence of heterozygous in-frame deletions in exon 19 by direct sequencing (*double peaks*). *B*, heterozygous point mutation in exon 21 (L858R, CTG to CGG). *C* and *D*, the nucleotide sequence of in-frame deletions in exon 19 of cloned sequencing. Amplified sample DNAs were cloned into pBluescript and sequenced. *, the break point of in-frame deletion in exon 19. The vertical arrow indicates the site of point mutation in exon 21.

histology were not related to gefitinib responsiveness. There was no relationship between mutational type and clinical responsiveness to gefitinib therapy in our study. One case with exon 21 mutations (L858R) but no responsiveness to gefitinib was a case of adenosquamous cell carcinoma in a female. In two gefitinib-responsive cases, mutations were not present, and these consisted of an adenocarcinoma and a squamous cell carcinoma in a male. A case of squamous cell carcinoma with complete response was previously reported (18), and the status of partial response has been continuing for 2 years. A case of adenocarcinoma is a 70-year-old Japanese man with partial response and the disease has been controlled for 22 months. Both cases have a smoking history. We also analyzed the relationship between patient survival and *EGFR* mutation in gefitinib-treated cases. Overall, survival curves are shown in Fig. 3 using the Kaplan-Meier method. Survival rate (% ± SE) at 1 and 2 years for patients with mutations were 87.5 ± 11.7% and 87.5 ± 11.7%, and those for patients without mutations 62.5 ± 15.1% and 37.5 ± 16.4%, respectively. Median survival times of patients with and without *EGFR* mutations were 25.1 and 14.0 months, respectively. Although patients with *EGFR* mutations had approximately 2-fold survival advantage, there was no significant difference in the overall survival between two groups (generalized Wilcoxon test, $P = 0.132$; log rank test, $P = 0.153$).

DISCUSSION

In this study, we searched *EGFR* mutations and explored the relationship between *EGFR* mutational status and clinicopathologic features in NSCLCs. The mutations were limited to the three exons (exons 18, 19, and 21) of the TK domain. These affected codons have already been reported to be sites for *EGFR* mutations, although some of the mutations reported herein are novel. All mutation target structures around the ATP binding cleft probably resulted in repositioning of amino acid residues, stabilizing their interaction with both ATP and its competitive inhibitor gefitinib (13). Previous studies indicated that adenocarcinoma histology, never smoker status, and female gender were factors associated with *EGFR* mutations. Our findings confirmed and extended these observations. First, we show that the degree of smoke exposure was inversely related to *EGFR* mutations in adenocarcinoma. Smoke exposure is a well-established risk factor for lung cancer and can cause specific mutational spectrum in *TP53* gene and epigenetic alterations, including the DNA methylation pattern (4, 19). We previously showed that smoking dose was closely related to the rate of methylation in adenocarcinoma (20). Taken together, these facts strongly suggest that the mechanisms for tumorigenesis

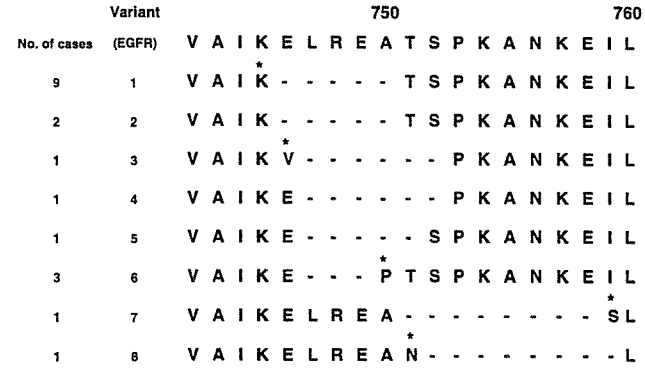

Fig. 2 Summary of in-frame deletion mutations in exon 19. The variation of amino acid residue sequences due to in-frame deletion. The number of cases are described at the left side of each variant. Variants 1 and 2 are the same amino acid sequences; however, nucleotide sequences are different. Deleted nucleotide site of variant 1 is from 2,235 to 2,249; on the other hand, that of variant 2 is from 2,236 to 2,250.

Table 3 Clinicopathological characteristics of 21 NSCLC cases treated with gefitinib

A

No.	Gender	Age (y)	Smoking status	Histology	ECOG performance status	No. of prior chemotherapy	Radiotherapy	Period from surgery to gefitinib therapy (mo)	*EGFR* mutation (exon)	Response to gefitinib
1	F	60	Never	Adenocarcinoma	2	2	+	19.6	19	CR
2	M	64	Never	Adenocarcinoma	1	2	+	5.4	19	PR
3	M	65	Smoker	Adenocarcinoma	2	2	–	28.4	19	PR
4	M	57	Smoker	Adenocarcinoma	1	2	–	43	19	PR
5	M	40	Never	Adenocarcinoma	1	2	+	21.1	19	PR
6	F	59	Never	Adenocarcinoma	1	1	+	21.5	21	PR
7	F	60	Never	Adenocarcinoma	0	3	+	16.7	21	PR
8	F	68	Never	Adenocarcinoma	2	1	–	35.2	21	PR
9	M	70	Smoker	Adenocarcinoma	2	1	–	63.9	—	PR
10	M	77	Smoker	Squamous cell carcinoma	2	2	+	18.8	—	PR
11	F	77	Never	Adenosquamous cell carcinoma	1	1	+	10	21	NC
12	F	75	Never	Adenocarcinoma	0	3	+	51.4	—	NC
13	F	73	Smoker	Squamous cell carcinoma	2	1	–	17.6	—	NC
14	M	77	Smoker	Squamous cell carcinoma	1	2	+	11.8	—	PD
15	M	70	Smoker	Large cell carcinoma	1	3	+	14.3	—	PD
16	M	68	Smoker	Adenocarcinoma	0	1	–	37.8	—	NC
17	M	63	Smoker	Adenocarcinoma	1	3	–	17	—	NC
18	M	58	Smoker	Adenocarcinoma	3	2	+	17.9	—	NC
19	F	55	Smoker	Squamous cell carcinoma	1	3	+	105	—	PD
20	M	49	Smoker	Adenocarcinoma	1	2	+	14.3	—	NC
21	M	62	Smoker	Adenocarcinoma	1	2	–	87.1	—	NC

B

	Responder (n = 10)	Nonresponder (n = 11)
EGFR mutation	8	1
Median age (range), y	62 (40-77)	68 (49-77)
Gender		
Male	6	7
Female	4	4
Smoking history		
Never	6	2
Smoker	4	9
Histology		
Adenocarcinoma	9	6
Squamous cell carcinoma	1	3
Adenosquamous cell carcinoma	0	1
Large cell carcinoma	0	1
ECOG performance status		
0-1	5	9
2	5	1
3	0	1

NOTE. The case that achieved complete response or partial response based on ECOG criteria was considered as responder and no change or progressive disease was considered as no-responder.

Abbreviations: CR, complete response; PR, partial response; NC, no change; PD, progressive disease; ECOG, Eastern Cooperative Oncology Group.

in adenocarcinoma vary according to smoking status. Second, our results show a gender difference based on mutational location/type. The in-frame deletions of exon 19 were significantly more frequent in male gender and point mutations of exon 21 in females. The reasons for these gender differences are not known, but there is a possibility that some factors derived from gender difference would be present for the causes of *EGFR* mutation. Of interest, gender difference has been shown in the *TP53* mutational spectrum in adenocarcinoma arising in never smokers (19). Third, Paez et al. indicated that female gender was one of the factors for *EGFR* mutation in univariate analysis (14). Indeed, it was reported to be one of the predictive factors for gefitinib-responsive cases (11). In our analysis, female gender was also the factor for *EGFR* mutation in univariate analysis; however, it was not an independent factor in multivariate analysis. Our findings are in agreement with those of Miller et al. (21) who showed that female gender was not an independent predictive factor for gefitinib responsive cases, in contrast to those of Fukuoka et al. (11).

We also confirmed previous reports that *EGFR* mutations were significantly associated with gefitinib-responsive cases (13, 14) except some cases. According to Eastern Cooperative Oncology Group criteria (15), one adenosquamous cell carcinoma case with exon 19 mutation was classified as no change. This is the first reported patient with mutation in

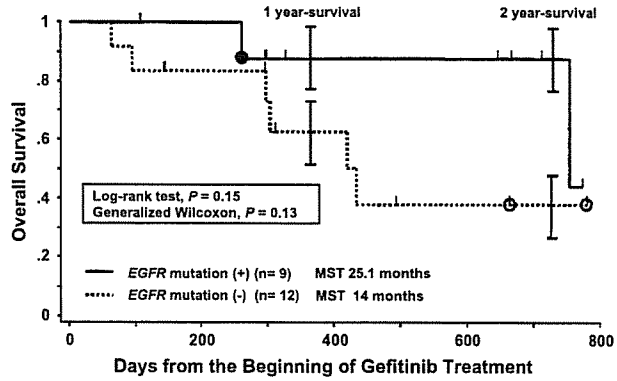

Fig. 3 Correlation of *EGFR* mutational status and patient survival in 21 gefitinib-treated cases by the Kaplan-Meier method. Survival rate (% ± SE) at 1 and 2 years for patients with mutations were 87.5 ± 11.7% and 87.5 ± 11.7%, and those for patients without mutations 62.5 ± 15.1% and 37.5 ± 16.4%, respectively. There was no significant difference in the overall survival between two groups (generalized Wilcoxon test, P = 0.132; log rank test, P = 0.153). Tics indicate at risk. Bars at 1 and 2 years from the beginning of gefitinib treatment indicate standard errors of cumulative survival rate. ●, patients with no change having *EGFR* mutations. O, patients with complete or partial response not having *EGFR* mutations. MST, median survival time.

whom gefitinib did not work. Two cases showed marked clinical responses but lacked mutations, as Lynch et al. reported one of nine responders without *EGFR* mutation. These results suggested that there are other mechanisms to determine gefitinib responsiveness in NSCLCs.

Previous study reported that adenocarcinoma histology, never-smoking status, and female gender were predictive factors for gefitinib responsiveness (11). Our result showed that only *EGFR* mutation was significantly associated with gefitinib responsiveness. This discrepancy may be derived from the small number of our treated cases for analysis. Regarding the response rate, the IDEAL 1 trial reported 27.5% in 102 Japanese and 10.4% in 106 non-Japanese patients (11). Our study showed 48% responsiveness in 21 cases treated with gefitinib. We compared the patient characteristics of 21 treated cases with the total 120 cases. Rates of female cases were 38% and 31%, never smoker cases 38% and 30%, and adenocarcinoma cases 71% and 68% in treated and total cases, respectively. The rates of these factors were slightly higher in the treated population than in the total population with no statistical differences, indicating that the 21 cases represented the total population. Takano et al. (22) reported that response rates in female gender, adenocarcinoma, and never smoker were 53%, 38%, and 63%, respectively, suggesting that our response rate was an acceptable value. Further analysis with additional cases is important to discuss the issue of responsiveness.

The overall survival data showed no significant difference in the limited number of cases. However, we showed better survival in patients having *EGFR* mutations than in those without mutations at 1 and 2 years after the beginning of gefitinib treatment. Data from the IDEAL 1 study, which included Japanese patients, showed the median survival time of gefitinib responders (complete or partial response) was 13.3 months, contrasting with the overall survival of 7.6 months (11). Thus, a survival advantage at 1 and 2 years in patients with *EGFR* mutations seems to be probable. Two gefitinib responders without *EGFR* mutations also live longer. Our present study of survival, which was limited to 21 cases, showed an advantage (although not significant) of gefitinib treatment for *EGFR* mutant cases. Further accumulation of treated cases with gefitinib should be necessary to estimate the effect of *EGFR* mutation gefitinib therapy for patient survival.

Our work confirms and extends the previously reported findings regarding *EGFR* mutations, clinicopathologic features, and response to targeted therapy. In addition, our findings strongly suggest that *EGFR* mutation can be one of the main factors to determine the strategy of chemotherapy and indicate the importance of molecular biological analysis of tumor specimens to establish the appropriate molecular-targeted treatment.

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Identification of Epigenetic Aberrant Promoter Methylation in Serum DNA Is Useful for Early Detection of Lung Cancer

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ABSTRACT

Purpose: **The purpose of this study is to evaluate the usefulness of serum DNA methylation of five tumor suppressor genes for early detection of lung cancer.**

Experimental Design: **Methylation status in serum DNA from 200 patients undergoing bronchofiberscopic examination for abnormal findings on chest radiograph detected by lung cancer screening or surveillance was examined using methylation-specific PCR.**

Results: **Ninety-one patients were given a pathologic diagnosis of lung cancer, 9 other malignant diseases, and 100 nonmalignant pulmonary diseases. In patients with lung cancer, methylation was detected in 18.7% for *MGMT*, 15.4% for $p16^{INK4a}$, 12.1% for *RASSF1A*, 11.0% for *DAPK*, and 6.6% for *RAR-β*, which was higher compared with that in patients with nonmalignant diseases. Age and smoking status seemed to associate with methylation status. Sensitivity, specificity, and predictive value of methylation in at least one gene for diagnosis of lung cancer were 49.5%, 85.0%, and 75.0%, respectively. Adjusted odds ratio (95% confidence interval) for having lung cancer was 5.28 (2.39-11.7) for patients with methylation in one gene and 5.89 (1.53-22.7) for those with methylation in two or more genes. It is of note that methylation was identified in 50.9% of stage I lung cancer patients, whereas serum protein tumor markers were positive in 11.3% of them.**

Conclusions: **These results suggest that identification of promoter methylation of tumor suppressor genes in serum DNA could be useful for early detection of lung cancer.**

INTRODUCTION

Despite intensive treatment, the prognosis of patients with lung cancer is poor. The 5-year survival rate of patients with clinical stage I disease is ~60%, but in those with clinical stage II to IV diseases, the 5-year survival rate ranges from 40% to <5% (1). Thus, the prognosis of lung cancer is strongly correlated to its clinical stage. Over two thirds of lung cancer patients have an advanced disease at the time of initial presentation (2), and lack of efficient diagnostic methods for early detection is considered to be the major reasons for the poor prognosis of lung cancer.

Although lung cancer screening with annual chest radiograph and sputum cytology is currently conducted in many municipalities in Japan (3), the usefulness of mass screening is yet to be fully confirmed. The previous screening trials sponsored by the National Cancer Institute failed to show that screening with sputum cytology and chest radiography reduced mortality from lung cancer (4–7). However, because one of these trials indicated more favorable survival rates associated with the diagnosis of resectable tumors, the American Cancer Society maintains that physicians and patients may decide to have these screening tests on an individual basis (8). Therefore, the development of more useful method in addition to the chest radiograph and sputum cytology for lung cancer screening is urgently required.

Aberrant methylation of CpG islands, which are in or near the promoter region of various genes, is a common feature in various neoplasms and is associated with the transcriptional silencing of tumor suppressor genes (9–11). In addition, this alteration has been described to occur in the very early stage of carcinogenesis (12). Recent advances in techniques simplified the methods for identification of promoter methylation, among which methylation-specific PCR (MSP) is a simple, sensitive, and specific method to determine the methylation status of any CpG-rich region (13).

Several studies have shown that several genes, including tumor suppressor genes, such as retinoic acid receptor β (*RAR*β; ref. 14) and $p16^{INK4a}$ (15, 16), apoptosis-associated genes, such as death-associated protein kinase (*DAPK*; ref. 17) and *ras* association domain family 1A (*RASSF1A*; refs. 18, 19), and the DNA repair gene O^6-methylguanine DNA methyltransferase (*MGMT*; refs. 20, 21), were frequently methylated in lung cancer cells. Zochbauer-Muller et al. showed that 82% of the non–small cell lung cancer tissues had methylation of at least one gene from eight genes and rarely identified methylation of these genes in nonmalignant lung tissue (20). These findings suggest the potential use of DNA methylation as a marker for lung cancer.

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It has been shown that cancer patients have increased levels of free DNA in their sera, which is thought to be released from cancer cells (22–24). Many investigators have reported that microsatellite alterations and p53 and/or *ras* gene mutations could be identified in the serum and/or plasma DNA of patients with various cancers (25–28). Thus, circulating tumor-derived DNA might be used as a source for tumor detection by PCR analysis, including MSP.

In the present study, we attempted to identify methylated DNA in sera of patients with abnormal findings on their chest radiograph as detected by lung cancer screening or physician surveillance. Although there are some recent reports of DNA methylation analyses carried out with remote medium, including serum, plasma, sputum, and bronchoalveolar lavage fluid or brushing samples, this is the first report that has examined the methylation status of serum DNA on a population basis and showed the usefulness of the approach as a diagnostic tool for early detection of lung cancer.

MATERIALS AND METHODS

Sample Collection and DNA Extraction. In this study, 200 patients undergoing fiberoptic bronchoscopy for abnormal findings on chest radiograph were investigated. The examinations were carried out as part of the lung cancer mass screening program in Okayama Prefecture (3) or through physician surveillance. Diagnosis of these patients was completely blinded to the laboratory researchers. Peripheral blood samples (6 mL) were collected to investigate methylation status of serum DNA with written informed consent. Serum (2 mL) was isolated after centrifugation at 3,000 rpm for 10 minutes and stored at −20°C until use. Serum DNA was extracted using QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We also examined methylation status of the tumor tissues. Of 200 patients evaluated in this study, 30 with lung cancer underwent surgical resection at Department of Cancer and Thoracic Surgery of Okayama University Hospital. Tumor tissues from these patients were investigated with written informed consent. Tumor DNA was extracted from formalin-fixed, paraffin-embedded lung cancer tissues using QIAamp DNA Mini kit (Qiagen) according to manufacturer's instructions.

Methylation-Specific PCR. Sample DNA was treated with sodium bisulfite using the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to the manufacturer's instructions. All bisulfite-modified DNA was resuspended in TE buffer [10 mmol/L Tris-0.1 mmol/L EDTA (pH 7.5)] and used immediately or stored at −20°C until subsequent MSP. Primer sequences for the *RAR*β, *p16^{INK4a}*, *DAPK*, *RASSF1A*, and *MGMT* were as described elsewhere (18, 20). DNA from a small cell lung cancer cell line SBC-3 (29), which has promoter methylation of all tested genes, was used as a positive control for the methylated form and that from serum of normal volunteer for the unmethylated form. The PCR mixture contained 10× PCR buffer [100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 15 mmol/L $MgCl_2$], deoxynucleotide triphosphates (each at 2.5 mmol/L), 0.5 μmol/L of each primer, 0.9 units Taq DNA polymerase (Takara Bio, Shiga, Japan), and 3 μL bisulfite-modified DNA in a final volume of 30 μL. Initial denaturation at 95°C for 5 minutes was followed by 50 cycles of a denaturation step at 95°C for 30 seconds, an annealing step at each specific annealing temperature for 30 seconds, and an extension step at 72°C for 30 seconds and a final extension step at 72°C for 10 minutes was added. After amplification, each PCR product was electrophoresed through a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. All tests were duplicated to confirm the result by blinded two researchers. Methylation-positive cases were defined as the cases whose serum DNA showed a visual band amplified with methylated-specific primers, even if it was faint. Representative results of methylation analysis by MSP are shown in Fig. 1.

Measurement of Serum Protein Tumor Marker Levels. A part of each blood sample was used for examination of conventional serum protein tumor markers, including carcinoembryonic antigen, cytokeratin 19 fragment, and progastrin releasing peptide. The serum levels of carcinoembryonic antigen, cytokeratin 19 fragment, and progastrin releasing peptide were determined by chemiluminescent immunoassay using commercial kits from Abbott Laboratories (Abbott Park, IL), electrochemiluminescent immunoassay from Roche Diagnostics (Basel, Switzerland), and ELISA from Fujirebio (Tokyo, Japan). The cutoff values of these markers were set at 5.0 ng/mL for carcinoembryonic antigen, 3.5 ng/mL for cytokeratin 19 fragment, and 46.0 pg/mL for progastrin releasing peptide according to the manufacturer's instructions.

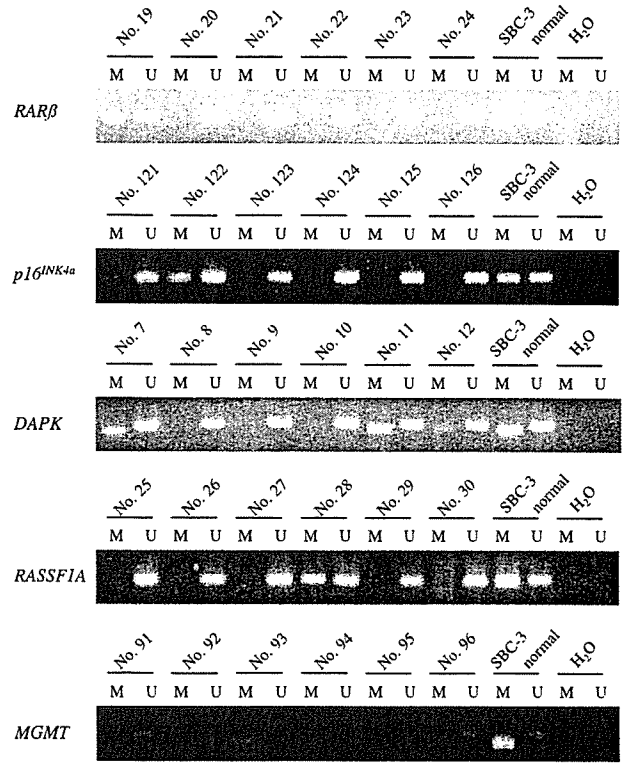

Fig. 1 Representative results showing promoter methylation amplified by MSP. Lanes *M* and *U*, amplified product with primers recognizing methylated and unmethylated sequences, respectively; DNA from SBC-3 cell line and normal volunteer, positive control for methylated and unmethylated forms, respectively.

Statistical Analysis. The methylation status in five genes was scored in each patient as the total number of methylated genes. The methylation score of patients with lung cancer was compared with those with nonmalignant diseases using a *t* test with unequal variance. An unconditional logistic regression model was applied to estimate the odds ratios and its 95% confidence intervals for the occurrence of lung cancer. We used the patients without methylation in every five genes as a reference group and estimated the odds ratios for patients having one methylated gene and those having two or more methylated genes in five genes tested. Crude and multivariate models were examined. The factors adjusted in the multivariate model included age as a continuous variable divided by 10, sex, smoking status divided into quartiles (pack-years calculated by number of packs smoked per day multiplied by the number of years of smoking), and results of three tumor markers (carcinoembryonic antigen, cytokeratin 19 fragment, and progastrin releasing peptide) in a binary variable. χ^2 or Fisher's exact test were applied to examine the distribution in categorical variables. A *t* test was applied to test the continuous variables. The statistical significance was defined as $P < 0.05$. All the statistical analyses were implemented by Stata version 8 (College Station, TX).

RESULTS

Patients Characteristics. Between January 2001 and December 2002, a total of 200 peripheral blood samples were collected from consecutive patients undergoing fiberoptic bronchoscopy. Of these patients, 91 were given a pathologic diagnosis of lung cancer (median age, 71 years; range, 45-92 years; male/female 61:30), 100 nonmalignant diseases (median age, 65.5 years; range, 26-89 years; male/female 64:36), and 9 other malignancies. The histologic subtypes of the lung cancers, based on WHO classification (30), were adenocarcinoma in 64 patients, squamous cell carcinoma in 21 patients, small cell carcinoma in 4 patients, and carcinoid in 2 patients. Clinical stage classifications, based on the International Staging System (1), were as follows: 53 patients had stage I disease, 7 patients stage II, 22 patients stage III, and 9 patients stage IV. Nonmalignant diseases mostly consisted of benign pulmonary diseases, such as tuberculosis, atypical mycobacteriosis, pneumoconiosis, interstitial pneumonia, bronchitis, organizing pneumonia, and bronchiectasis. Malignant diseases other than lung cancer included pulmonary metastasis of laryngeal cancer in 3 patients, invasive thymoma in 2 patients, and non-Hodgkin's lymphoma, thyroid cancer, breast cancer, and rectal cancer in 1 patient each. Elderly patients were more frequently represented among patients with lung cancer than those with nonmalignant diseases (median age, 71 versus 65.5 years; $P < 0.001$). Current smokers were more commonly represented among patients with lung cancer than those with nonmalignant diseases (67% versus 54%; $P = 0.046$).

Methylation Status of Five Genes. As shown in Table 1, serum methylated DNA was detected in 18.7% for *MGMT*, 15.4% for *p16*^{INK4a}, 12.1% for *RASSF1A*, 11.0% for *DAPK*, and 6.6% for *RAR*β in lung cancer patients. When analyzed individually, the proportions of patients with methylated serum DNA were higher in patients with lung cancer than those with nonmalignant diseases in every five genes. Difference is especially evident for *p16*^{INK4a} ($P = 0.003$) and *MGMT* ($P < 0.001$). Of 91 lung cancer patients, 45 (49.5%) had methylation of at least one gene. When methylation of at least one gene was assessed as positive, specificity and predictive values of methylation were 85.0% and 75.0%, respectively. The total number of methylations in five genes per patient was 0.64 in patients with lung cancer, which was higher than that in patients with nonmalignant diseases (0.19; $P < 0.0001$; Table 2). Of 9 patients with malignant diseases other than lung cancer, 6 (66.7%) had at least one methylation in five genes (data not shown). These 9 cases were excluded from subsequent analyses.

Twenty-three of 30 (77%) tumor tissues obtained from lung cancer patients showed methylation of at least one gene (Fig. 2). Sixteen of 18 (89%) tissues from patients with serum DNA methylation also had methylated genes. Methylation of *RASSF1A* gene was identified in one serum sample and *MGMT* gene in two samples, but they were not identified in the corresponding tumor.

Association with Clinicopathologic Features. We analyzed the correlations between methylation status in serum DNA and clinicopathologic variables of the patients. There was no correlation between methylation status and sex or histology in this study. In addition, frequency of serum DNA methylation between smokers and nonsmokers was not significantly different in both patients with lung cancer (48.3% in nonsmokers, 45.8% in <40 pack-years smokers, and 52.6% in ≥40 pack-years smokers; $P = 0.863$) and those with nonmalignant diseases (Table 3). These findings were also observed when analyzed in individual gene. Although serum DNA methylation in ≥40 pack-years smokers with nonmalignant diseases tended to be more frequent than that in <40 pack-years smokers, this trend was particularly obvious in *DAPK* and *RASSF1A* genes. In control group, we found significant correlation between methylation and age (Table 3).

Methylation Status and Risk of Lung Cancer. Table 4 shows the results of a crude and adjusted logistic regression analyses evaluating correlation between number of methylated genes and risk of lung cancer. In the crude model, the patients with one methylated gene showed 5.08 (95% confidence interval, 2.28-11.3) times higher probability of having lung cancer compared with patients without any methylated genes. The odds ratio was higher in patients with two or more methylated genes. To consider the imbalance in baseline characteristics, we conducted similar analysis adjusting for age,

Table 1 Frequency of methylation in five genes

	Patients, *n* (%)		
	Lung cancer (*n* = 100)	Nonmalignancy (*n* = 100)	Total (*n* = 191)
MGMT	17 (18.7)	2 (2.0)	19 (9.9)
p16^{INK4a}	14 (15.4)	3 (3.0)	17 (8.9)
RASSF1A	11 (12.1)	8 (8.0)	19 (9.9)
DAPK	10 (11.0)	5 (5.0)	15 (7.9)
*RAR*β	6 (6.6)	1 (1.0)	7 (3.7)

Table 2 Comparison of methylation status between lung cancer and nonmalignant cases

	Mean*	SE	SD	95% Confidence interval	
Nonmalignancy	0.19	0.0506	0.0506	0.09-0.29	
Lung cancer	0.64	0.0774	0.738	0.48-0.79	$P < 0.0001$†

*Total number of methylations in five genes per patient.
†*t* test with unequal variance.

sex, smoking status, and protein tumor marker results. The patients with methylation in at least one gene and two or more of five genes showed 5.28 (2.39-11.7; $P < 0.001$) and 5.89 (1.53-22.7; $P = 0.010$) times higher probability of having lung cancer, respectively.

Frequencies of Methylation According to Clinical Stage of Lung Cancer. We investigated the correlation between clinical stage and methylation status in five genes or conventional serum protein tumor markers (Table 5). Of 53 patients with stage I disease, 27 (50.9%) patients had methylated serum DNA in at least one gene, whereas only 6 (11.3%) patients showed elevation of at least one serum protein tumor marker. In patients with stage II, III, or IV diseases, the difference was not evident.

DISCUSSION

This study shows that identification of serum DNA methylation is a potentially useful approach to detect lung cancer patients from subjects screened by chest radiograph. Serum DNA methylation was more frequently observed in patients with lung cancer than those with nonmalignant diseases. Although the sensitivity for the diagnosis of lung cancer was only 49.5% when analyzed by a combination of five genes, the relatively high specificity (85.0%) indicates the usefulness for subjects screened by with chest radiograph. The odds ratio for diagnosis of lung cancer was >5.0 in patients with at least one methylated gene even after statistical adjustment by other clinicopathologic risk factors, such as smoking, age, sex, and results of tumor marker tests. Of note, serum DNA methylation could be identified even in patients in the early stages of lung cancer, whereas conventional serum protein tumor markers were rarely elevated, indicating that this DNA-based method is more sensitive than protein-based method for diagnosis of lung cancer in early stage.

In former studies, methylation in tumor tissues was detected in 40% to 43% of non–small cell lung cancer patients for *RARβ*, 25% to 41% for $p16^{INK4a}$, 16% to 44% for *DAPK*, 30% to 40% for *RASSF1A*, and 16% to 27% for *MGMT* (31). These results were consistent with our data in 30 tumor tissue samples. In our experiment, the frequency of detecting methylated genes in serum was about half to two thirds compared with that in tumor tissues. However, when we consider that tumor-derived DNA in blood is generally detectable in less than half of cancer patients (32), the frequency of methylation in serum DNA in our study may be reasonable. Laird reviewed the studies examining methylation status of serum/plasma DNA in patients with various neoplasms and indicated that clinical sensitivity of DNA methylation was ~50% (33). Esteller et al. did methylation analysis in serum DNA from patients with non–small cell lung cancer for multiple genes and showed 33% to 80% of clinical sensitivity by combination analysis of these genes (34). Our results are consistent with the results of these studies, indicating that similar sensitivity is achievable even after mass screening.

Among various techniques used for methylation analysis, we adopted a simple method of qualitative MSP analysis. The specificity of the primers we used in this study had been verified using genomic sequencing and/or restriction analysis in previous reports (13, 35). Recently, several studies showed improved detection rates of methylation status using a nested PCR approach or a quantitative real-time PCR technique (36–38). Particularly, sensitivity of the Taqman method was reported to be 10-fold higher than conventional qualitative MSP (39). To apply DNA methylation as tumor marker for detection of lung cancer, the use of these improved methods is an attractive strategy.

Although promoter methylation was observed predominantly in lung cancer patients, 1% to 8% of patients with nonmalignant disease were methylation positive for each gene in this study. In addition, three lung cancer patients with serum DNA methylation did not show same alteration in the corresponding tumor tissues. We considered the following as possible explanation of these positive results. Firstly, the methylated serum DNA might be derived from undetected precancerous lesions in these cases. According to the previous reports, aberrant promoter methylation is detectable in precancerous lesions, such as dysplasia and nonmalignant lung tissues of patients with lung cancer (20, 37). Methylation-positive nonmalignant patients may develop malignant diseases in the near future. Secondly, aberrant methylation might be caused by environmental factors, such as smoking (12, 40). In

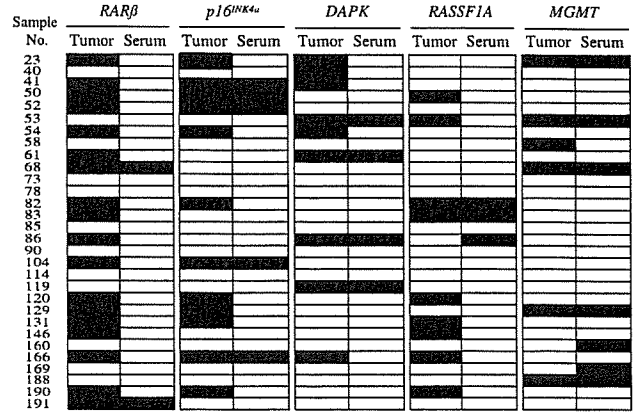

Fig. 2 Summary of methylation of *RARβ*, $p16^{INK4a}$, *DAPK*, *RASSF1A*, and *MGMT* in 30 corresponding tissue and serum samples. *Black boxes,* methylated samples; *white boxes,* unmethylated samples.

Table 3 Correlation between clinical features and methylation of the different genes in nonmalignant cases

	% of Cases					
	RARβ	*p16^{INK4a}*	*DAPK*	*RASSF1A*	*MGMT*	Total
Smoking status						
Nonsmoker	0	4.4	2.2	6.5	2.2	13.0
Pack-years <40	0	0	4.8	4.8	0	9.5
Pack-years ≥40	3	3	9.1	12.1	3.0	21.2
P	0.359	0.626	0.379	0.550	0.736	0.442
Age						
Quartile 1*	3.5	0	6.9	0	0	10.3
Quartile 2	0	0	0	5.6	0	5.6
Quartile 3	0	5.0	5.0	20.0	5.0	25.0
Quartile 4	0	13.3	13.3	13.3	6.7	33.3
P	0.480	0.051	0.230	0.063	0.271	0.036

*Age quartile was defined as follows: 1, <59; 2, ≥59 and <69; 3, ≥69 and <73; and 4, ≥73.

published series, controversial correlations between smoking and methylation have been reported (20, 41–44). We did not find any statistically significant correlation between smoking history and methylation; however, heavy smokers had at least one serum DNA methylation more frequently than mild to moderate smokers in patients with nonmalignant disease. The third explanation is possible occurrence of other occult malignancies. Indeed, we observed methylated serum DNA in 66.7% of the patients who were diagnosed as having another malignant disease. The final explanation is the possibility of detecting age-related methylation in control group.

To use this serum DNA methylation as a marker in lung cancer mass screening, several issues must be considered. The fairy good specificity even in patients screened by chest radiograph suggests the advantage of this approach. On the other hand, poor sensitivity may compromise the advantage of specificity. Improving sensitivity as a mass screening test might be achieved by two approaches. One is to increase sensitivity of DNA methylation itself by using large number of tested genes or applying a quantitative methylation assay. The other is to combine the methylation with highly sensitive screening method such as low-dose spiral computed tomography (45–47). Because one of the serious limitations of low-dose spiral computed tomography is its poor specificity (48), combination with the serum DNA methylation may overcome the limitation. Indeed, physicians often experience difficulty in sampling tumor specimen from small legions detected by computed tomography scans for pathologic diagnosis by invasive procedures, such as fiberoptic bronchoscopic examination or computed tomography–guided fine needle aspiration. Accordingly, in consideration of relatively higher frequency of serum DNA methylation in early-stage disease (50.9% in stage I) and serious complications after invasive procedures (49), serum DNA methylation may be a test to be conducted before invasive procedure. Although further evaluation is essential, the results in this study indicate the substantial usefulness for detection of lung cancer.

In conclusion, we examined the aberrant promoter methylation status in serum DNA and showed the usefulness of this approach as a tool for detection of lung cancer in patients screened by chest radiograph. Further studies are warranted to confirm the efficiency of the procedure and search for best combination of genes for methylation analysis. Moreover, it is important to investigate prospectively whether methylation-positive noncancer cases will have malignancies in the near future.

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Table 4 Methylation status and risk of lung cancer

			Model 1†			Model 2†		
	Cases*	Controls*	Odds ratio	95% Confidence interval	*P*	Odds ratio	95% Confidence interval	*P*
No. methylations								
0	46	85	1.00			1.00		
1	33	12	5.08	2.28-11.3	<0.0001	5.28	2.39-11.7	<0.001
≥2	12	3	7.39	1.87-29.2	0.0008	5.89	1.53-22.7	0.010
Age (10-y increase)			—			1.03	1.00-1.07	0.023
Sex (male relative to female)			—			1.49	0.61-3.64	0.386
Pack-years (one level increase quartile)			—			1.00	0.99-1.00	0.207
Tumor marker positive			—			3.08	1.32-7.17	0.009

*Cases, lung cancers; controls, nonmalignant diseases.

†Model 1 included number of methylation only. Model 2 included age, sex, pack-years of smoking, and tumor marker result in addition to number of methylation.

Table 5 Frequency of DNA methylation and elevation of conventional serum protein tumor markers according to clinical stage

	Patients, *n* (%)			
Clinical stage	I	II	III	IV
Methylation	27 (50.9)	3 (42.9)	10 (45.5)	5 (55.6)
MGMT	10 (18.9)	1 (14.3)	4 (18.2)	2 (22.2)
p16^{INK4a}	7 (13.2)	0 (0.0)	5 (22.7)	2 (22.2)
RASSF1A	7 (13.2)	1 (14.3)	3 (13.6)	0 (0.0)
DAPK	5 (5.7)	1 (14.3)	4 (18.2)	0 (0.0)
RARβ	3 (5.7)	0 (0.0)	1 (4.5)	2 (22.2)
Tumor marker	6 (11.3)	3 (42.9)	10 (45.5)	7 (77.8)
Carcinoembryonic antigen	5 (9.4)	1 (14.3)	9 (40.9)	5 (55.6)
Cytokeratin 19 fragment	0 (0.0)	0 (0.0)	2 (9.1)	2 (22.2)
Progastrin releasing peptide	1 (1.9)	2 (28.6)	2 (9.1)	1 (11.1)
Total (n = 91)	53	7	22	9

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They Also Serve Who Only Stand and Wait: Do Individual Clinical Researchers, Too? Hoping for Individual Patient Data As Public Domain

TO THE EDITOR: We have presented two meta-analyses regarding chemotherapy for non–small-cell lung cancer (NSCLC) based on the abstracted data (AD)[1,2] and received an editorial comment about them from Dr Piedbois and Dr Buyse.[3] We have no objection to their comments that state individual patient data (IPD)-based meta-analysis is more ideal than AD-based meta-analysis to draw inference, as they have cautioned for a long time.[4] With all due respect to them, we have to say that we are still dissatisfied with their comments and would like to express our opinions.

"Which treatment is better at present, adjuvant chemotherapy or surgery alone?" This is one of the simple questions that all clinicians involved in NSCLC treatment still encounter in their daily practices, and this question was the reason that motivated us to attempt the analysis.[2] Although we had realized the result of an earlier meta-analysis published in 1995,[5] we did not think that the issue had been concluded when we planned the analysis. Approximately half of the studies included in our analysis still had continued patient enrollment even after 1995, indicating the evidence by IPD-based analysis was not convincing enough to stop these trials. The clinical researchers involved should not have intended to conduct the demonstrational trials for proven efficacy of adjuvant chemotherapy for NSCLC; therefore, we are certain that our topic has some degree of positive significance, and provides justification for us to carry out an AD-based meta-analysis. We assume that the *Journal of Clinical Oncology* published our article with the same point of view, and the indication in the editorial, that our analysis is a retread of a proven issue, is beside the point. Editorialists also indicated that the analyses in each individual trial reported might be biased and were far from reliable to summarize by AD-based analysis. Frankly speaking, this indication is quite puzzling for us, considering it is from the editorialists at a medical journal. The medical articles in the journals are one of the most important sources for clinicians in their daily practices, and it is essential for us to see clear evidence for this indication so that we can fully take advantage of it.

Another subject we have to be attentive to is how much of the precise conclusion would be drawn by IPD-based analysis compared with AD-based analysis to produce the main outcome of interest. Several articles are available relating to this issue that define AD-based analysis as less reliable[6,7]; however, the serious problem in the comparison is that the studies examined in each method are all different. Conclusions should be made based on the direct comparison between these two methods using exactly the same studies. For example, Hamada et al[8] recently presented the IPD-based meta-analysis for adjuvant chemotherapy using tegafur plus uracil for NSCLC. They used six studies, and five of them were included in our analysis.[2] When we analyzed with additional abstracted data of this nonoverlapped study,[9] the summary hazard ratio for overall survival with tegafur plus uracil adjuvant chemotherapy is 0.76 (95% CI, 0.64 to 0.89; $P = .001$), whereas that in their intent-to-treat analysis is 0.76 (95% CI, 0.64 to 0.91; $P = .0025$). Although we still cannot rule out the potential bias in AD-based analysis, the main conclusions are the same in both analyses. Therefore, one may hypothesize that both methods would give similar estimates for the main outcome when exactly the same studies are examined, and this hypothesis must be evaluated in the future study from the scientific point of view. We have lately heard that another IPD-based analysis on adjuvant chemotherapy in NSLCS is underway, and we eagerly await the results to examine this issue. Considering the huge resource burden for IPD-based analysis (as mentioned in the editorials), we must say it is extreme to conclude that AD-based analysis is unreliable and to limit its role to simple hypothesis generation at present.

Lastly, as the editorialists mentioned, how an outlier study is dealt with is important in meta-analysis. Coldtiz et al[10] pointed out the potential danger of study exclusion by source of heterogeneity. If study exclusion aims to make studies homogeneous under the pretext of removing random errors, it may lead to a serious bias. We do not believe that homogeneity of the results across the studies is a prerequisite for meta-analysis; however, if that kind of homogeneity exists, there should be no reason to conduct meta-analysis except to obtain a statistically significant result. This is a common issue for both IPD- and AD-based meta-analyses, and should be considered seriously.

We believe, despite several limitations, that AD-based meta-analysis still has a substantial role in the medical science until an ideal situation for gold standard analysis is established. Having faith in the strength of IPD-based meta-analysis, we sincerely hope that all the primary investigators in the clinical trial groups seriously consider establishing the ideal situation for smooth research as public domain, which enables independent clinical researchers to examine IPD with their new hypothesis, instead of limiting its use to the authorities.