five methods for *EGFR* mutation analysis in clinical studies and diagnostic applications. However, as the concordance rates were not 100% for any one method, we would advocate the selection of a single method for consistent use during a clinical study. With regard to daily practice, the decision to select and adopt a particular technology is at the discretion of individual laboratories and may be influenced by the diagnostic environment in which they reside. Selection factors may include technical expertise of operators, cost, test status (*in vitro* diagnostic versus laboratory-developed test), or availability of instrumentation.

Several factors may have contributed to the discordances between the *EGFR* mutation tests. These factors may have included differences in sensitivity and specificity, different DNA extraction procedures between laboratories, variation in tumor cell content within and across samples, and tumor heterogeneity within an FFPE block [10, 11, 16].

The performance of all five *EGFR* mutation tests was comparable in the analysis of both BB and pleural effusion cytology samples, with 100% success rates. BB cytology samples showed high concordance rates (>93%, excluding samples unsuccessfully analyzed) between pairs of *EGFR* mutation tests and versus FFPE samples by each detection method. Using the PNA-LNA PCR clamp method, analysis of BB cytology samples was successful where the matched FFPE sample failed analysis. Some mutations were detected in cytology samples of low DNA concentrations where matching FFPE samples were assessed as mutation negative. This result suggests that cytology samples can be useful in mutation analysis when tissue samples cannot be used, are in a small quantity, or degradation of FFPE samples is suspected. Pleural effusion cytology samples may be particularly suitable for analysis as they can be obtained easily, non-invasively and repeatedly, and generally contain plenty of cancer cells, relative to other sample types.

To our knowledge, this is the first high-quality comparison study of *EGFR* mutation tests in both FFPE and cytology samples. The results of the current study indicate that cytology-derived DNA is a suitable alternative to FFPE samples for the analysis of *EGFR* mutations and may be useful when FFPE samples are unavailable for molecular analysis. Other studies have also shown that ARMS can be used to detect *EGFR* mutations in cytology samples from transbronchial needle aspirates [17] or pleural effusion [18] and that this technique appeared to be more sensitive than direct sequencing in this sample type. Other methods for *EGFR* mutation testing, including pyrosequencing [19] and high-resolution melting analysis [20], also exist.

In summary, the performance of all five *EGFR* mutation tests was comparable in the analysis of FFPE and cytology samples. Where *EGFR* mutation tests and standard operating procedures are used in a reliable robust way, with trained operators, in a well-developed diagnostic setting, comparable results are obtained across mutation tests and sample types. FFPE specimens are currently the sample of choice for determining *EGFR* mutation status [11]. However, the ability to use cytology samples allows additional patients to be tested for *EGFR* mutations, and therefore, more appropriate treatment of their disease.

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disclosure

KG has received honoraria from Ono Pharmaceutical and Chugai Pharmaceutical and fees for consultancy/advisory boards from Ono Pharmaceutical. MS has received honoraria from Chugai Pharmaceutical and AstraZeneca. KN has received research grants from Daiichi Sankyo, Chugai Pharmaceutical, AstraZeneca, Glaxo SmithKline, and Solasia Pharma KK, research support from Chugai Pharmaceutical, and honoraria from Kyowa Hakko Kirin, Sumitomo Bakelite, Taiho Pharmaceutical, and Qiagen. KH has received patent fees from Mitsubishi Chemical Medience. TM has received honoraria from AstraZeneca and Chugai Pharmaceutical. JW, ED, RM, and TT are employees of AstraZeneca and hold stock in AstraZeneca. GI has declared no conflicts of interest.

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Original Article

Enhancer of Zeste Homolog 2 Is a Novel Prognostic Biomarker in Nonsmall Cell Lung Cancer

Huqun, MD, PhD[1,2]; Rinako Ishikawa, MD[1]; Jialing Zhang, PhD[1,3]; Hitoshi Miyazawa, PhD[4]; Yoshiya Goto, PhD[5]; Yoshihiko Shimizu, MD, PhD[6]; Koichi Hagiwara, MD, PhD[4]; and Nobuyuki Koyama, MD, PhD[1]

BACKGROUND: Enhancer of zeste homolog 2 (EZH2) epigenetically silences many genes through the trimethylation of histone H3 lysine 27 and is implicated in tumor growth, invasion, and metastasis. However, its role in lung cancer has not been well characterized. The objective of the current study was to elucidate the role of EZH2 in nonsmall cell lung cancer (NSCLC) by investigating both clinical samples and cell lines. **METHODS:** An immunohistochemical analysis of EZH2 expression was performed in samples from patients with stage I NSCLC to investigate the association of EZH2 expression levels with clinicopathologic variables. An in vitro cell growth assay and a Matrigel invasion assay also were conducted in the *EZH2*-expressing NSCLC cell lines A549 and H1299 after knocking down *EZH2* expression by using an *EZH2*-specific short-hairpin RNA. **RESULTS:** The immunohistochemical analysis classified stage I NSCLC samples (n = 106) into a negative EZH2 expression group (n = 40; 37.7%) and a positive EZH2 expression group (n = 66; 62.3%). Positive EZH2 expression was associated significantly with larger tumor size ($P = .014$). Kaplan-Meier survival analyses and log-rank tests demonstrated that patients whose samples were classified into the positive EZH2 expression group had a significantly shorter overall survival ($P = .015$). Experiments in the NSCLC cell lines revealed that the knockdown of EZH2 expression reduced the tumor growth rate and invasive activity. **CONCLUSIONS:** The current results indicated that EZH2 promotes progression and invasion of NSCLC, and its expression is a novel prognostic biomarker in NSCLC. *Cancer* 2012;118:1599-606. © *2011 American Cancer Society.*

KEYWORDS: EZH2, nonsmall cell lung cancer, prognostic biomarker, tumor size, tumor progression, tumor invasion.

INTRODUCTION

Lung cancer is a leading cause of cancer death worldwide. Two-thirds of patients with lung cancer are diagnosed with the disease after it has advanced to a stage at which curative resection is not possible. Even early stage disease exhibits a high mortality rate that reflects the frequent emergence of metastatic lesions and local recurrence after resection. Information on the mechanism of cancer progression and metastasis in early stage lung cancers is vital for establishing an efficient treatment strategy that can improve patient outcomes.

Epigenetic silencing of tumor suppressor genes (TSGs), including retinoblastoma (*RB*), *ras*-association domain family 1 isoform A (*RASSF1A*), and semaphorin 3B (*SEMA3B*), is an important mechanism in lung tumorigenesis.[1-3] In addition to promoter hypermethylation,[4-6] modification of the histone structure is another main mechanism of epigenetic silencing of TSGs: Histone deacetylase deacetylates lysine in core histones (H2A, H2B, H3, and H4), strengthens histone-DNA binding, and, thus, silences TSGs.[7]

Polycomb group proteins are transcriptional repressors that form 2 protein complexes: polycomb repressor complex 1 (PRC1) and PRC2.[8-10] PRC2 has 3 core components: suppressor of zeste 12, embryonic ectoderm development, and

Corresponding author: Nobuyuki Koyama, MD, PhD, Department of Respiratory Medicine, Saitama Medical University International Medical Center, 1397-1 Yamane Hidaka-shi Saitama, 350-1298, Japan; Fax: (011) 81-42-984-4790; nkoyama@saitama-med.ac.jp

[1]Department of Respiratory Medicine, Saitama Medical University International Medical Center, Saitama, Japan; [2]Department of Medical Oncology, The Affiliated Hospital of Inner Mongolia Medical College, Hohhot, China; [3]Clinical Research Center, The Affiliated Hospital of Inner Mongolia Medical College, Hohhot, China; [4]Department of Respiratory Medicine, Saitama Medical University, Saitama, Japan; [5]Department of Pathology, Saitama Medical University International Medical Center, Saitama, Japan; [6]Department of Pathology, Saitama Prefectural Cardiopulmonary Center, Saitama, Japan

The first 2 authors contributed equally to this article.

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enhancer of zeste homolog 2 (EZH2). EZH2 trimethylates histone H3 lysine 27 and, thus, epigenetically silences the genes involved in development, differentiation, and growth, as well as TSGs.[11-14] Moreover, EZH2 recruits DNA methyltransferases to their target promoters. Therefore, EZH2 is involved in both DNA methylation and histone methylation, and it also is involved in the suppression of TSGs through multiple mechanisms.[15] Indeed, it has been demonstrated that *EZH2* expression induces the development of aggressive and metastatic phenotypes in early stage cancers.[16-19] However, a seemingly conflicting phenomenon has been observed in malignant lymphoma and myelodysplastic syndrome, in which inactivation of the *EZH2* gene by chromosomal deletion or somatic mutation promotes tumorigenesis.[20,21] EZH2 may silence different sets of genes in different cell types, and the role of EZH2 needs be studied in each type of cancer. Currently, information on the role of EZH2 in nonsmall cell lung cancer (NSCLC) is scarce. The objective of the current study was to elucidate the association between EZH2 expression and NSCLC.

In the current study, we analyzed the association of EZH2 expression in stage I NSCLC samples with clinicopathologic variables. We observed that positive and stronger EZH2 expression is associated with larger tumor size and shorter overall survival (OS). This finding suggests that EZH2 is involved in progression of NSCLC. In vitro experiments produced consistent results: Knocking down *EZH2* expression in A549 and H1299 NSCLC cells inhibited cell growth and invasion. Our observations demonstrated that EZH2 drives malignant behavior of NSCLC, and its expression is a novel prognostic biomarker in early stage NSCLC.

MATERIALS AND METHODS

Cell Cultures

Lung cancer cell lines were obtained from the following sources: NCI-H1299 cells (large cell neuroendocrine carcinoma) were purchased from the American Type Culture Collection (Rockville, Md); PC-7 and PC-9 cells (adenocarcinomas) were purchased from IBL (Takasaki, Japan); RERF-LCAd2, RERF-LC-MS, and PC-3 cells (adenocarcinomas) along with RERF-LC Sq-1 cells (squamous cell carcinomas) were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan); RERF-LC-KJ, LC2/ad, and PC-14 cells (adenocarcinomas) were purchased from the Riken Bioresource Center (Tsukuba, Japan); and A549 cells (adenocarcinoma) along with LK79, LK2, and Sq-1 cells (squamous cell carcinomas) were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). Cells were grown in RPMI-1640 medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) (CELLect GOLD; MP Biomedicals, Eschwege, Germany) in a humidified chamber with air containing 5% CO_2.

Patients

Patients who were included in this study underwent surgical resection for NSCLC at Saitama Medical University Hospital from 2000 to 2006 and were diagnosed with pathologic stage I NSCLC. We retrospectively analyzed clinicopathologic characteristics in a total of 106 patients. OS was calculated from the date of surgery to the date of death or the end of observation period in censored patients, regardless of treatment for recurrent disease.

Immunohistochemical Analysis

Thin sections of 10% formalin-fixed, paraffin-embedded tissue specimens were stained using mouse antihuman EZH2 monoclonal antibody (Cell Signaling Technology, Danvers, MA). The percentage of positively stained cancer cells was determined by investigating more than 4 visual fields at ×400 magnification. Samples were investigated independently by 2 pathologists and classified into 2 groups: negative EZH2 staining (no cells were intensely stained), and positive EZH2 staining (at least some cells were intensely stained). The positive staining group was divided further into 2 groups according to the intensity of staining: high EZH2 staining (>50% of cells were intensely stained) and low EZH2 staining (<50% of cells were intensely stained).

Semiquantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA from each cell line or from normal human lung tissue (Clontech, Mountain View, CA) was reverse transcribed, mixed with SYBR Premix Ex Taq (Takara Inc., Shiga, Japan), and amplified by polymerase chain reaction (1 cycle at 95°C for 120 seconds, 45 cycles at 95°C for 5 seconds and 56°C for 20 seconds) using the Smart Cycler (Cepheid Innovation, Sunnyvale, CA). The following primers were used: for *EZH2*, 5′-AGAAT AATCATGGGCCAGACTG-3′ (forward) and 5′-GTA GCAGATGTCAAGGGATTTC-3′ (reverse); for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′-CC TCAACGACCACTTTGTCA-3′ (forward) and 5′-TTA CTCCTTGGAGGCCATGT-3′ (reverse). The amount

of *EZH2* messenger RNA (mRNA) was normalized by the amount of *GAPDH* mRNA.

Short-Hairpin RNA

Short-hairpin RNAs (shRNAs) against *EZH2* 5′-AAGACTCTGAATGCAGTTGCT-3′ and its scrambled control (SCR) 5′-TCTTAATCGCGTATAAGGC-3′ were integrated into a pSINsi-hU6 vector (Takara Bio Inc., Shiga, Japan). Recombinant retrovirus particles for each shRNA were produced using the Retrovirus Packaging Kit Ampho (Takara Bio Inc.) according to the manufacturer's protocol. A549 and H1299 cells were exposed to each retrovirus for 2 hours and then cultured in Dulbecco Minimal Essential Medium containing neomycin (4 mg/mL) for 1 week; stable transfectants of each shRNA, which we called shEZH and shSCR, were established. Mock-transfected A549 and H1299 cells were used as controls (Cont).

Immunoblot Analysis

The shEZH, shSCR, and control cells were plated into 100-mm dishes and incubated in 10 mL RPMI-1640 medium for 24 hours. Cell lysates were prepared using CelLytic M Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO) and subjected to Western blot analysis. In brief, cell lysates were dissolved in CelLytic M Cell Lysis Reagent, heated at 95°C for 5 minutes, electrophoresed in 5% to 10% Ready Gels J (Bio-Rad Laboratories Inc., Hercules, CA), and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA) with iBlot (Invitrogen, Carlsbad, CA). The membranes were immersed in Tris-buffered saline (TBS), pH 7.6, containing 5% skim milk and 0.1% Tween-20 to block nonspecific binding. The membranes were incubated with mouse antihuman EZH2 monoclonal antibody (Cell Signaling Technology, Danvers, MA) diluted with Immuno-Enhancer Reagent A (Wako, Osaka, Japan) for 1 hour at 25°C, and then with antimouse immunoglobulin G conjugate (Promega, Madison, WI) diluted with Immuno-Enhancer Reagent B (Wako) for 1 hour at 25°C. The membranes were washed with TBS-Tween, then with TBS, and the specific signals were observed with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

In Vitro Cell Growth Assay

The shEZH, shSCR, and control cells were seeded into 96-well dishes (2500 cells per well) containing 100 mL of RPMI-1640 with 2% FBS (day 0). Cell Counting Kit-8 reagent (10 μL; DOJINDO, Kumamoto, Japan) was added to each well for 3 hours, and absorbance at 450/620 nm was measured at 32 hours and at 68 hours. Experiments were done in triplicate for each cell line.

Matrigel Invasion Assay

BD BioCoat Matrigel Invasion Chambers (Bio-Rad Laboratories, Inc.) were used in the Matrigel invasion assay according to the manufacturer's protocol. The chambers were immersed in RPMI-1640 medium at 37°C for 2 hours. After removal of the medium, shEZH, shSCR, or control cells (5×10^4 cells per well) were seeded, and medium with or without 10% FBS was added as an inducer. The chambers were incubated at 37°C for 22 hours. After the removal of noninvading cells, the remaining cells were stained with hematoxylin and eosin, and cell numbers were counted. The invasion rate was calculated as ratio of the number of invaded cells observed in the chamber with 10% FBS to the number of invaded cells observed with 0% FBS. Experiments were done in triplicate for each cell line.

Statistical Analysis

Correlations between EZH2 expression and each clinicopathologic characteristic were analyzed by *t* tests or chi-square tests. Correlations between OS and EZH2 expression were investigated using Kaplan-Meier analysis and log-rank tests. Factors that contributed to OS were assessed with a multivariate Cox proportional hazards model. Differences in the measurements of shRNA-mediated knockdown of *EZH2* expression (ie, the in vitro cell growth assay and the Matrigel invasion assay) were assessed with a median test. *P* values $<.05$ were considered significant.

Ethical Considerations

The current study was approved by the ethical committee of Saitama Medical University. Immunohistochemical analyses were performed after patients provided informed consent.

RESULTS

EZH2 Messenger RNA Expression in Lung Cancer Cell Lines

First, we examined the expression of *EZH2* mRNA in NSCLC cell lines. *EZH2* mRNA was increased in 11 of 14 cell lines and was decreased in 3 cell lines compared with normal human lung tissue (Fig. 1). No significant differences in *EZH2* expression were observed between histologic types. The increased expression in many of the cell lines indicated that EZH2 may significantly contribute toward determining the molecular characteristics of

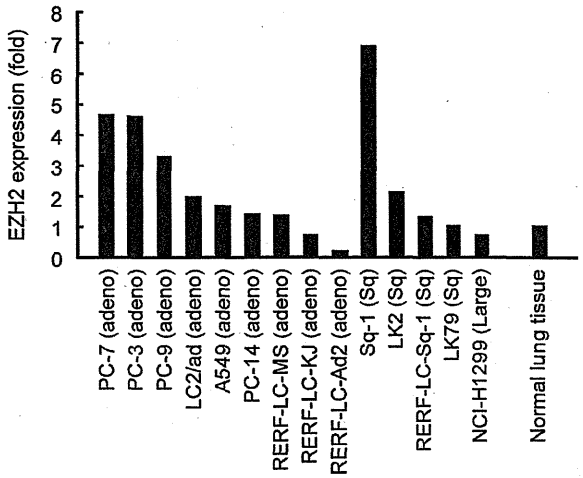

Figure 1. Enhancer of zeste homolog 2 (*EZH2*) messenger RNA expression is illustrated in lung cancer cell lines. Expression levels were measured by real-time polymerase chain reaction and were normalized to the levels in normal lung tissue. Adeno indicates adenocarcinoma; Sq, squamous cell carcinoma; Large, large cell neuroendocrine carcinoma.

NSCLC; thus, we decided to further investigate EZH2 using resected cancer specimens from patients.

Immunohistochemical Analysis of EZH2 Expression in Stage I Nonsmall Cell Lung Cancer

There have been several reports that EZH2 is involved in early stage cancers; thus, we focused on stage I NSCLC. First, we investigated whether EZH2 expression was associated with any clinicopathologic variables in 106 specimens of stage I NSCLC to evaluate the role of EZH2 (Table 1). The samples were classified into 2 groups based on EZH2 staining level: a negative EZH2 expression group (n = 40; 37.7%) and a positive EZH2 expression group (n = 66; 62.3%) (Fig. 2A). Then, the association of each clinicopathologic variable with EZH2 expression was studied. The results revealed that tumor size was the only variable with a significant association ($P = .014$) (Table 1). EZH2 expression was more frequent in larger tumors (Fig. 2B). These results suggested that EZH2 may play a role in cancer cell growth.

Progression of NSCLC is determined not only by its speed of cell growth but also by the ability of cancer cells to metastasize. Metastasis status is especially important for surgically resected stage I disease, because, at this stage, it is believed that all visible local lesions are removed successfully. In addition, the rate of metastasis directly influences OS. To further evaluate the role of EZH2 in cancer progression, we investigated the relation between EZH2

Table 1. Patient Characteristics

Characteristic	EZH2 Expression			
	All (n = 106)	Negative (n = 40)	Positive (n = 62)	*P*
Sex				.167
Men	62	20	42	
Women	44	20	24	
Average age, y	64.1	65.1	63.5	.463
Histologic type				.235
Adenocarcinoma	75	31	44	
Squamous cell carcinoma	31	9	22	
Differentiation				.688
Well differentiated	67	27	40	
Moderately differentiated	28	10	18	
Poorly differentiated	11	3	8	
Stage				.157
IA	78	33	45	
IB	28	7	21	
Average greatest tumor dimension, mm	25.8	21.3	28.5	.014[a]
p factor				.103
0	80	34	46	
1	21	6	15	
2	5	0	5	
ly factor				.389
0	96	35	61	
1	9	5	4	
2	1	0	1	
v factor				.987
0	95	36	59	
1	8	3	5	
2	3	1	2	

Abbreviations: EZH2, enhancer of zeste homolog 2; p factor, the grade of tumor invasion into visceral pleura; ly factor, the grade of tumor invasion into lumphatic vessels; v factor, the grade of tumor invasion into veins.
[a] Significant *P* value.

expression and OS using Kaplan-Meier analysis and log-rank tests. The positive EZH2 expression group had a significantly shorter OS ($P = .015$) (Fig. 2C). Moreover, when the positive EZH2 expression group was divided further into a high EZH2 expression group and a low EZH2 expression group (Fig. 2A), the length of OS was in the order of negative EZH expression group>low EZH expression group>high EZH2 expression group (Fig. 2D). This dose-dependent relation between EZH expression level and OS suggests that EZH2 has a role in tumor progression by influencing both the growth and metastasis of cancer cells.

Next, we evaluated the factors that contribute to OS using a multivariate Cox proportional hazards model. The analysis revealed that EZH2 expression and tumor

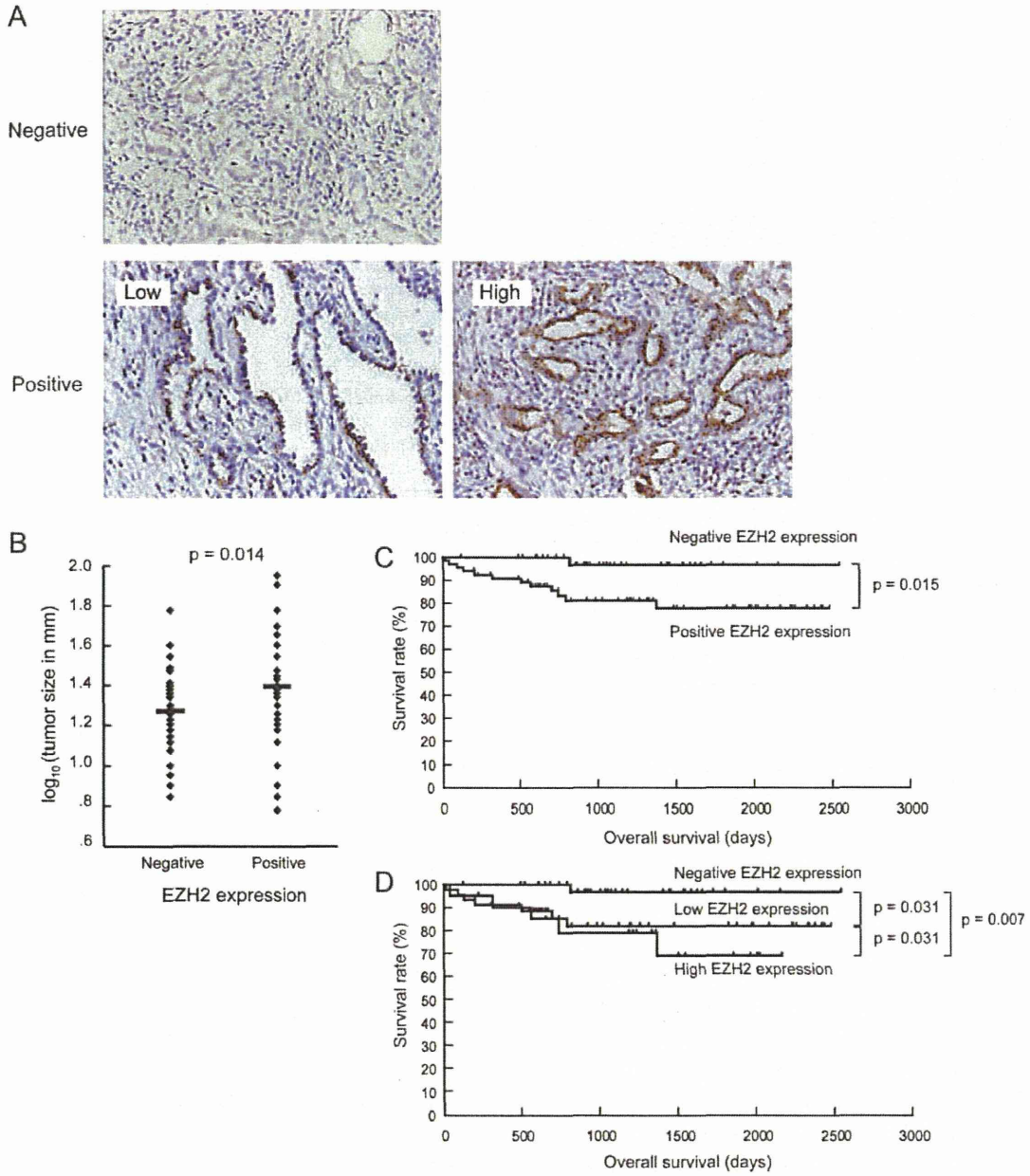

Figure 2. An immunohistologic analysis of enhancer of zeste homolog 2 (EZH2) expression is illustrated. (A) These are representative images of EZH2 expression. (B) This scatter plot demonstrates a significant correlation between EZH2 expression level and tumor size. (C) Kaplan-Meier analysis is shown for 2 EZH2 expression groups. (D) Kaplan-Meier analysis is shown for 3 EZH2 expression groups.

size were correlated independently with OS (EZH2 expression: hazard ratio [HR], 8.30; 95% confidence interval [95% CI], 1.078-63.881; $P = .04$; tumor size: HR, 115.729; 95% CI, 8.955-1495.680; $P = .00,027$) (Table 2). These results suggest that EZH2 has a role in cancer progression by enhancing cell growth and promoting metastasis.

Effects of EZH2 on Lung Cancer Growth and Invasion

We also performed in vitro experiments to investigate the effect of shRNA-mediated knockdown of *EZH2* mRNA using the *EZH2*-expressing NSCLC cell lines A549 and H1299 (Fig. 1). Each cell line in which *EZH2* shRNA had been introduced (shEZH cells) had reduced levels of

Table 2. Lifetime Analysis Using a Cox Proportional Hazards Model

Variable	HR	95% CI	P
EZH2 expression	2.80	1.19-6.59	.018[a]
Age	1.05	0.98-1.13	.150
Sex	0.72	0.17-2.99	.649
Histology	0.94	0.28-3.25	.927
Disease stage	0.91	0.13-6.20	.920
Tumor size	125.8	1.01-14399	.046[a]

Abbreviations: CI, confidence interval; EZH2, enhancer of zeste homolog 2; HR, hazard ratio.
[a] Significant *P* values.

EZH2 mRNA measured by real-time polymerase chain reaction analysis (Fig. 3A) and protein measured by Western blot analysis (Fig. 3B), whereas cell lines in which scrambled shRNA (shSCR cells) had been introduced had expression levels similar to those in mock-transfected cells (control cells). The in vitro cell growth assay using both A549 cells and H1299 cells demonstrated that the growth rate of shEZH cells was reduced significantly compared with the growth rate of shSCR cells and control cells ($P < .01$), indicating that EZH2 has growth-promoting activity in NSCLC cells (Fig. 3C). The Matrigel invasion assay revealed that shEZH significantly attenuated the invasive activity of A549 and H1299 cells ($P < .05$), suggesting that EZH2 may enhance metastasis of NSCLC cells (Fig. 3D). These results support the observations obtained from clinical samples that EZH2 may enhance cancer progression by enhancing cell growth and promoting metastasis.

DISCUSSION

EZH2 is frequently overexpressed in a variety of human cancers and has been associated with aggressiveness, metastasis, and poor outcome of several cancer types. EZH2 mediates histone methylation and recruits DNA methyltransferase in the silencing of a variety of genes. These previous reports prompted us to investigate the role of EZH2 in NSCLC. EZH2 was associated positively with tumor size and was associated negatively with OS in patients with stage I NSCLC. The repression of EZH2 expression in A549 and H1299 NSCLC cells inhibited cancer cell growth and decreased invasive activity. All of these results indicate that EZH2 is involved in cancer progression and is a novel predictive biomarker of poor outcome.

From the data on 6644 resected NSCLC specimens, the greatest difference in the 5-year survival rate between cancer stages was observed between stage IA (pathologic stage; 79.5%) and stage IB (60.1%).[22] This indicates that lung cancers frequently develop subclinical tumor progression or metastasis during stage I of the disease and that molecular changes that occur during stage I disease are important determinants of outcome. The gene that is involved in these changes will be a good marker for prognosis and should be targeted therapeutically to improve outcomes. *EZH2* is one such gene: It is associated with changes in tumor size that occur during stage I disease and is involved in cell growth and invasion. Bachmann et al reported that EZH2 expression was positively correlated with tumor growth or prognosis in several cancers, including melanoma and endometrial, prostate, and breast cancers,[23] and studies of the value of EZH2 both as a molecular marker and as a therapeutic target are warranted.

Prognostic markers may be different in different stages of cancers. Examples are epidermal growth factor receptor (*EGFR*) and *K-Ras* gene mutations, which have been associated with survival in locally advanced or metastatic NSCLCs,[24,25] whereas the association has not been well established in early stage NSCLCs. Whether *EZH2* is a prognostic marker in later stages of NSCLCs, and whether *EZH2* is associated with *EGFR* or *K-Ras* mutation in such stages will be important issues for the elucidation of the role of EZH in the progression of NSCLCs.

EZH2 may change the expression of genes that are related to responsiveness to currently available therapeutic modalities through its ability to silence other genes. Thus, EZH2 may indirectly change the responsiveness of cancer cells to therapies. Recent reports have demonstrated that EZH2 expression is able to predict therapeutic response to chemoradiation in patients with esophageal squamous cell carcinoma, and decreased EZH2 expression is associated with the expression of estrogen receptor and improves responsiveness to tamoxifen therapy in patients with advanced breast cancer.[26,27] Molecular-targeted therapies that use the gene silencing ability of EZH2 will be an interesting approach to cancer therapy. This warrants a clinical trial investigating the efficacy of postoperative chemotherapy for patients with EZH2-positive, stage IA NSCLC.

There have been several reports that EZH2 targets several genes, including p15 (*INK4b*), p16 (*INK4a*), cyclin A, cyclin D1, cyclin-dependent kinase 1C (*CDKN1C*; *p57*), and E-cadherin (*CDH1*).[28-31] These genes, in turn, regulate other genes in a manner specific to each cancer cell type.[32,33] A comprehensive analysis of gene expression changes may be required to clarify the whole picture on the roles of EZH2 in NSCLC, which will be attained only by summarizing studies from many laboratories.

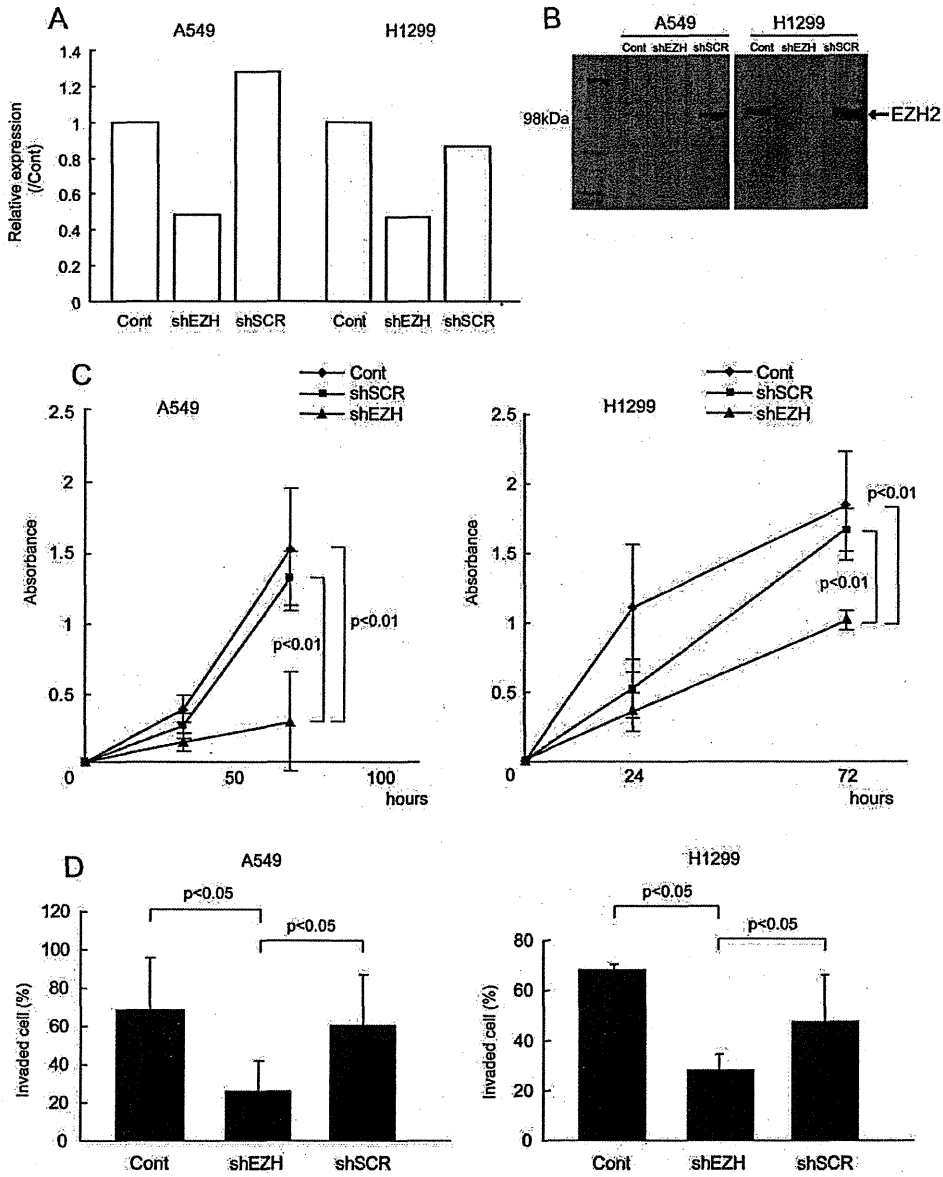

Figure 3. In vitro experiments are illustrated. (A) This chart illustrates the measurement of enhancer of zeste homolog 2 (*EZH2*) messenger RNA (mRNA) by real-time polymerase chain reaction (PCR) after short-hairpin RNA (shRNA)-mediated knockdown of *EZH2* mRNA in (*Left*) A549 cells and (*Right*) and H1299 cells. Cont indicates control; shEZH, short-hairpin enhancer of zeste homolog; shSCR, scrambled control shRNA. (B) Immunoblot analysis reveals *EZH2* expression in the A549 and H1299 cell lines. (C) In vitro cell growth assays are illustrated in A549 and H1299 cells. The growth of shEZH was significantly slower than the growth of shSCR and controls. (D) Matrigel invasion assays are illustrated in A549 and H1299 cells. The invasive activity of shEZH was significantly weaker than the invasive activity of shSCR and controls. Error bars indicate standard deviations.

In conclusion, EZH2 acts as a positive regulator of NSCLC growth and invasion, and its expression may be useful as a novel prognostic biomarker in early stage NSCLC. Studies on EZH should provide a novel perspective on personalized cancer therapies.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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The Peptide Nucleic Acid-Locked Nucleic Acid Polymerase Chain Reaction Clamp-Based Test for Epidermal Growth Factor Receptor Mutations in Bronchoscopic Cytological Specimens of Non-Small Cell Lung Cancer

Noriyuki Yamada[a] Satoshi Oizumi[a] Hajime Asahina[a] Naofumi Shinagawa[a] Eiki Kikuchi[a] Junko Kikuchi[a] Jun Sakakibara-Konishi[a] Tomoaki Tanaka[b] Kunihiko Kobayashi[c] Koichi Hagiwara[b] Masaharu Nishimura[a]

[a]First Department of Medicine, Hokkaido University School of Medicine, Sapporo, [b]Department of Respiratory Medicine, Saitama Medical University, Saitama, and [c]Department of Respiratory Medicine, Saitama Medical University International Medical Center, Hidaka, Japan

Key Words
Non-small cell lung cancer · Epidermal growth factor receptor mutations · Bronchoscopy · Cytological specimens

Abstract
Objectives: Cytological examination of samples obtained by bronchoscopy is a useful method for establishing the diagnosis of non-small cell lung cancer (NSCLC). However, the utility of a highly sensitive method for the detection of epidermal growth factor receptor (EGFR) mutation in the cytological specimens has not been fully evaluated. ***Methods:*** We retrospectively examined the efficacy of the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method for detecting EGFR mutations in 122 bronchoscopic cytological specimens from NSCLC patients. ***Results:*** Overall, 41 specimens (33.6%) were positive for EGFR mutation. Twenty-nine (39.7%) of 73 specimens obtained by using endobronchial ultrasonography with a guide sheath, 7 (33.3%) of 21 specimens obtained under direct vision by using a conventional bronchoscope, 4 (36.4%) of 11 specimens obtained by using an ultrathin bronchoscope, and 1 (5.9%) of 17 specimens obtained by endobronchial ultrasound-guided transbronchial needle aspiration were positive for EGFR mutation. Furthermore, among 22 resected NSCLC cases, the EGFR mutation status obtained from bronchoscopic materials was consistent with the status obtained from surgical samples, with the exception of 1 case. ***Conclusion:*** The detection of EGFR mutation by subjecting bronchoscopic cytological specimens to a PNA-LNA PCR clamp assay proves useful.

Introduction

The epidermal growth factor receptor (EGFR) is frequently overexpressed in lung cancer [1], and its kinase domain is a target of tyrosine kinase inhibitors (TKIs) [2,

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Satoshi Oizumi, MD, PhD
First Department of Medicine, Hokkaido University School of Medicine
North 15, West 7, Kita-ku
Sapporo 060-8638 (Japan)
Tel. +81 11 709 5911, E-Mail soizumi@med.hokudai.ac.jp

3]. Recently, activating mutations in the tyrosine kinase domain of EGFR were found to be strongly associated with the clinical response to EGFR-TKIs [4–6]. Since then, many clinical trials have reported that non-small cell lung cancer (NSCLC) harboring EGFR mutation showed a striking response to EGFR-TKIs [7–11]. Thus, testing for EGFR mutations has become an essential step in determining the treatment for NSCLC.

Many advanced lung cancers are diagnosed by using specimens obtained with bronchoscopy. However, many cytological specimens sometimes contain only a small number of cancer cells, and the specimens are comprised of many normal cells. These conditions are disadvantageous for the detection of the EGFR mutation. Therefore, a test that can detect EGFR mutation from a small number of cancer cells is needed.

Recently, new bronchoscopic procedures and devices have become available and have improved the diagnostic yield. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has an advantage in establishing a diagnosis of metastatic disease in the hilar and mediastinal lymph nodes [12, 13]. Transbronchial biopsy using endobronchial ultrasonography with a guide sheath (EBUS-GS) [14, 15] or an ultrathin bronchoscope [16, 17] is useful for the investigation of small peripheral pulmonary lesions. Specimens isolated by using these techniques are most often cytological specimens. Techniques that reliably detect EGFR mutation in these specimens are required.

The peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp is a rapid and sensitive detection procedure for EGFR mutations [18–20]. The method detects mutations in cancer cells present in a background of 100- to 1,000-fold more normal cells [19], thereby enabling the detection of EGFR mutation from cytological specimens.

In the current study, we evaluated the power of the PNA-LNA PCR clamp method for the detection of EGFR mutation in cytological specimens isolated by bronchoscopy. We validated the method by comparing the mutation status determined from the cytological specimens with the mutation status determined from surgically resected tumor tissue.

Materials and Methods

Ethics Committee Approval

This study was approved by the institutional review board of Hokkaido University Hospital, and all patients provided written informed consent.

Patients and Bronchoscopic Procedures to Obtain Cytological Samples

We retrospectively analyzed 122 NSCLC cases diagnosed by using bronchoscopic cytological specimens at Hokkaido University Hospital between February 2006 and November 2009. All patients underwent bronchoscopy under local anesthesia. Bronchoscopically visible tumors were brushed under direct vision by using a flexible fiberoptic bronchoscope (BF-240, Olympus, Tokyo, Japan), while hilar and mediastinal lymph nodes were needle aspirated according to the EBUS-TBNA procedure by using an ultrasonic puncture bronchoscope (BF-UC260F-OL8, Olympus) [12, 13]. Peripheral pulmonary lesions were brushed according to the EBUS-GS procedure [14, 15], and smaller peripheral pulmonary lesions were brushed by using an ultrathin bronchoscope (XP260F, Olympus) [16, 17].

Samples for EGFR Mutation Detection and Cytological Diagnosis

The cytological specimen was suspended in 6 ml of saline and divided into two aliquots. One aliquot was sent to the Pathology Department to investigate the presence of cancer cells. For cytological analysis, the specimen was placed onto a glass slide, spray fixed using ethanol, and Papanicolaou stained. All cytological specimens were confirmed to contain cancer cells, and cytological diagnosis was made by the two clinical cytologists. The diagnosis was based on standard histologic criteria according to the 2004 WHO classification of lung tumors [21]. Staging was based on the sixth edition of the UICC TNM staging system [22].

The other aliquot was centrifuged, and pelleted cells were dissolved in AL buffer (a buffer containing protein denaturant; Qiagen, Hilden, Germany) and stored. Patients who had resectable NSCLC underwent curative pulmonary surgery. Paraffin-embedded specimens of surgically resected tumors were analyzed for EGFR mutation.

EGFR Mutation Analyses with PNA-LNA PCR Clamp

The stored cytologic specimens and/or thin slices of paraffin-embedded specimens of resected tumors were sent and tested for EGFR mutation at the Department of Respiratory Medicine of Saitama Medical University. All analyses of EGFR mutation were performed with the PNA-LNA PCR clamp, as previously described [18–20]. Briefly, genomic DNA fragments from mutation hot spots of the EGFR gene were amplified by PCR in the presence of a peptide nucleic acid clamp. This technique results in the preferential amplification of the mutant sequence, which is then detected by a fluorescent primer that incorporates locked nucleic acids to increase its specificity. This technique detects gefitinib-sensitive mutations (G719C, G719S, G719A, L858R, L861Q, and exon 19 deletions) and a gefitinib-resistant mutation, T790M.

Statistical Analysis

Statistical analyses were performed with SPSS version 11.01 (Chicago, Ill., USA). Any significant differences among the categorized groups were compared using the χ^2 test. The adjusted effects of sex, smoking status, and cytology on EGFR mutation were evaluated by logistic regression analysis. Statistical significance was established at $p < 0.05$. All analyses were two sided.

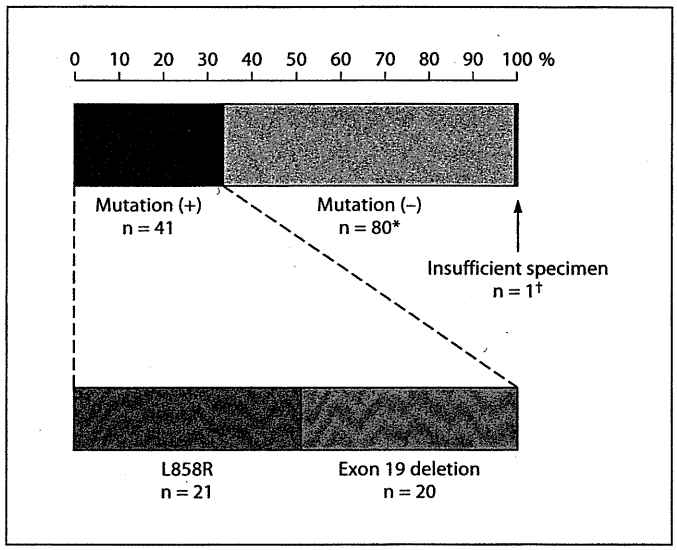

Fig. 1. Detection of the EGFR mutation by PNA-LNA PCR clamp. * Three patients (1 patient who underwent brushing with EBUS-GS and 2 patients who underwent brushing with an ultrathin bronchoscope) had a very small quantity of DNA. † DNA was not obtained from the cytological specimen gathered by brushing under direct vision.

Table 1. Patient characteristics (n = 122)

Characteristics	
Median age (range), years	66 (35–88)
Sex	
Male	59 (48)
Female	63 (52)
Smoking history	
Current	33 (27)
Former	44 (36)
Never	45 (37)
Cytology	
Adenocarcinoma	77 (63)
Squamous cell carcinoma	14 (12)
Non-small cell carcinoma	31 (25)
Clinical stage	
Stage I–II	27 (22)
Stage III–IV	94 (77)
Post-operative	1 (1)
Cytological specimen obtained by	
EBUS-GS	73 (60)
Under direct vision	21 (17)
EBUS-TBNA	17 (14)
Ultrathin bronchoscope	11 (9)

Values are numbers and percentages in parentheses unless otherwise indicated.

Results

Patient Characteristics

Patient characteristics are summarized in table 1. Sixty-three patients (52%) were female, 45 patients (37%) never smoked, and the most common tumor cytology was adenocarcinoma, which occurred in 77 patients (63%). Twenty-two patients had resectable NSCLC and underwent surgical resection.

Frequency of EGFR Mutations

Forty-one patients (33.6%) were positive for an EGFR mutation (fig. 1), whereas 80 patients (65.6%) were negative. Three samples that were negative for EGFR mutation contained a very small quantity of DNA; 1 of these samples was obtained by brushing with EBUS-GS, and the other 2 samples were obtained by brushing with an ultrathin bronchoscope. In these 3 cases, it is possible that the small amount of DNA led to a false-negative result. Additionally, 1 cytological sample obtained by brushing under direct vision failed to be amplified by PCR and was thus considered insufficient for the mutation test.

Among the patients with EGFR mutations, 21 patients (51.2%) had L858R, and 20 patients (48.8%) had exon 19 deletions. None of the patients had the gefitinib-resistant mutation T790M or other minor mutations, such as G719X and L861Q. The association between EGFR mutation status and clinicopathological characteristics was assessed by the χ^2 test (table 2). Sex (female), smoking status (never smoked), and type of cancer (adenocarcinoma) were significantly associated with the presence of EGFR mutation. In a multivariate logistic regression analysis,

Table 2. Association between positive EGFR mutation and clinicopathological characteristics

Characteristics	Positive	Negative	p value
Sex			
Male	11	47	0.001
Female	30	33	
Smoking status			
Smoker	15	61	<0.001
Never smoker	26	19	
Cytology			
Adenocarcinoma	38	39	<0.001
Non-adenocarcinoma	3	41	

Table 3. Logistic regression analysis for the association between positive EGFR mutation and clinicopathological characteristics

Characteristics	OR (95% CI)	p value
Sex		
Female vs. male	1.67 (0.57–4.92)	0.35
Smoking status		
Never smoker vs. smoker	2.60 (0.91–7.46)	0.076
Cytology		
Adenocarcinoma vs. non-adenocarcinoma	9.13 (2.51–33.21)	<0.001

only cytological subtype (adenocarcinoma) was significantly associated with the presence of EGFR mutation (table 3).

Frequency of EGFR Mutations Isolated by Bronchoscopic Procedures

Cytological specimens were obtained by brushing using EBUS-GS (n = 73), brushing under direct vision (n = 21), brushing using ultrathin bronchoscope (n = 11), and needle aspiration by the EBUS-TBNA procedure (n = 17; table 1). The number of samples positive for EGFR mutation were 23 of 73 (39.7%) for EBUS-GS, 7 of 21 (33.3%) for direct vision, 4 of 11 (36.4%) for ultrathin bronchoscope, and 1 of 17 (5.9%) for EBUS-TBNA.

Validation of EGFR Mutation Detection by Comparing the Mutation Status between Cytological and Resected Samples

To validate the results of EGFR mutation screening with cytologic specimens, we compared the EGFR mutation status between the cytological specimens and surgically resected tumor tissues in 22 patients (table 4). The results matched in 21 patients, including 9 (45.5%) patients who were positive for EGFR mutation. In the 1 case with results that did not match, the sample was isolated by brushing using an ultrathin bronchoscope. The cytological sample was negative for EGFR mutation, whereas the tissue sample was positive (table 4; the patient is marked with an asterisk). The sensitivity, specificity, and accuracy were therefore 90, 100, and 95.5%, respectively.

Discussion

In the current study, we assessed the utility of our EGFR mutation test system, in which cytological specimens obtained by bronchoscopy were tested by a sensitive PNA-LNA PCR clamp-based test. The frequency of EGFR mutations detected by our assay was similar to previously reported frequencies for surgically resected specimens from Asian patients [23, 24].

Several studies have assessed the frequency of EGFR mutations detected in bronchoscopic specimens, although the test for the EGFR mutations was different [19, 25–27]. The frequency of EGFR mutations in these studies varied between 21.1 and 53.3%, probably due to a small number of samples. The current study employed a larger number of samples than that of the reported studies [19, 25–27], and this relatively large study size may be the reason why the frequency of EGFR mutations is comparable with the previously reported frequency for the Japanese population [23, 24].

The concordance rate of EGFR mutations between cytological specimens and tissue samples was 21/22 (95.4%). Nomoto et al. [25] also compared the EGFR mutation status determined in cytological specimens isolated by using bronchoscopy with the mutation status determined in surgically resected specimens from 15 patients. They employed high-resolution melting analysis, which is another highly sensitive method for the detection of EGFR mutation, and demonstrated complete correspondence. Thus, the strategy of using cytological materials in a highly sensitive detection method proves useful for clinical practice. However, we had only 22 NSCLC cases which were surgically resected and in which the diagnosis was confirmed following EGFR mutation test with bronchoscopic examination. The small sample size for the comparison was a limitation of this study.

Until recently, direct sequencing from surgically resected tumor materials had been the mainstay technique for the determination of EGFR mutation status. However, the sensitivity of direct sequencing was approximately 10% [28], indicating that surgically resected or biopsied tissue specimens were required to test for EGFR mutations. However, such specimens are not always available. In many instances, only cytological samples that include samples obtained by bronchoscopic procedures, pleural effusions, or sputum are available. Furthermore, tissue samples require a longer amount of time for the detection of EGFR mutations because more steps are usually needed to prepare DNA from tissue than from cytological samples. When we use the PNA-LNA PCR clamp, the results are obtained within several days, and we usually determine the patient's treatment based on the EGFR mutation status. This sensitive test for EGFR mutation, including the PNA-LNA PCR clamp, is already covered by health insurance in Japan.

Table 4. Comparison of EGFR mutation status between bronchoscopic and surgically resected specimens

Age years	Sex	Smoking status	Cytology	Bronchoscopic procedure	Mutation status of bronchoscopic specimen	Mutation status of surgical specimen
58	F	Current	Ad	EBUS-GS	E746-A750del	E746-A750del
68	F	Never	Ad	Ultrathin	Negative*	L747-T751del
81	F	Never	Ad	EBUS-GS	E746-A750del	E746-A750del
64	M	Never	Ad	EBUS-GS	L747-S752del P753S	L747-S752del P753S
73	F	Never	Ad	Ultrathin	L747-T751del	L747-T751del
77	F	Never	Ad	Ultrathin	E746-A750del	E746-A750del
72	F	Current	Ad	EBUS-GS	L858R	L858R
82	F	Never	Ad	EBUS-GS	L858R	L858R
71	F	Never	Ad	EBUS-GS	L858R	L858R
66	F	Never	Ad	EBUS-GS	L858R	L858R
56	M	Current	Sq	EBUS-GS	Negative	Negative
64	F	Former	Ad	EBUS-GS	Negative	Negative
73	M	Current	NS	EBUS-GS	Negative	Negative
73	F	Never	Ad	EBUS-GS	Negative	Negative
60	M	Current	Ad	EBUS-GS	Negative	Negative
63	F	Former	Sq	EBUS-GS	Negative	Negative
74	F	Never	Ad	EBUS-GS	Negative	Negative
63	F	Former	Sq	EBUS-GS	Negative	Negative
59	M	Former	NS	EBUS-GS	Negative	Negative
68	M	Former	NS	EBUS-GS	Negative	Negative
67	M	Former	NS	EBUS-GS	Negative	Negative
70	M	Former	NS	EBUS-GS	Negative	Negative

Ad = Adenocarcinoma; Sq = squamous cell carcinoma; NS = non-small cell carcinoma. * Small quantity of DNA.

In this study, we mainly assessed the cytological specimens obtained by recently developed bronchoscopic procedures. These new bronchoscopic procedures have improved the diagnostic yield, which is reported to be 94.6–95.7% in EBUS-TBNA [12, 13], 67–77% in EBUS-GS [14, 15], and 65.4% in ultrathin bronchoscope [17]. Thereby, more patients with EGFR mutation could be found using such new procedures. In this study, the frequency of EGFR mutations was somewhat lower in samples from EBUS-TBNA than samples collected with the other procedures. Among the patients who underwent EBUS-TBNA, there were more male patients (13/17, 76.5%), more smokers (15/17, 88.2%), and more non-adenocarcinomas (10/17, 58.8%). Although the number of examined samples for EBUS-TBNA was small, these clinical features might account for the lower frequency of EGFR mutations.

Even with the new procedures, some specimens contained no DNA or had a very small quantity of DNA. One cytological sample obtained by brushing under direct vision failed to be amplified by PCR. We reviewed this cytological slide and found few cancer cells. This might have led to misamplification of DNA. In another case in which the specimen was obtained with an ultrathin bronchoscope, the mutation result was found to be a false negative (table 4; patient marked with an asterisk). We reviewed the cytological slide in this case and found that tumor cells were very sparse. To avoid false-negative results, it is crucial to obtain a sufficient amount of specimen. It is much easier to repeat the specimen collection attempt when using new bronchoscopic procedures as compared to conventional bronchoscopies. The isolation of a sufficient amount of material should always be practiced.

In conclusion, cytological specimens isolated by bronchoscopy and subjected to the PNA-LNA PCR clamp-based test provide clinically useful information regarding the EGFR mutation status. Cytological specimens obtained by bronchoscopy are appropriate for testing EGFR mutation to identify patients with advanced NSCLC who can benefit from EGFR-TKIs.

Disclosure Statement

Dr. Hagiwara has received a patent fee for the PNA-LNA PCR method. The other authors have no conflicts of interest to disclose.

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Postprogression survival for first-line chemotherapy of patients with advanced non-small-cell lung cancer

H. Hayashi[1], I. Okamoto[1]*, S. Morita[2], M. Taguri[2] & K. Nakagawa[1]

[1]*Department of Medical Oncology, Kinki University Faculty of Medicine, Osaka-Sayama;* [2]*Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan*

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Background: Given the growing number of drugs available for non-small-cell lung cancer (NSCLC), an effect of first-line chemotherapy on overall survival (OS) might be confounded by subsequent therapies. We examined the relation between postprogression survival (PPS) and OS in phase III trials of first-line chemotherapy for advanced NSCLC.
Patients and methods: A literature search identified 69 trials that were published during the past decade. We partitioned OS into progression-free survival (PFS) and PPS and evaluated the relation between OS and either PFS or PPS. We also examined whether any association might be affected by the year of completion of trial enrollment.
Results: The average PPS was longer in recent trials than in older trials (6.5 versus 4.4 months, $P < 0.0001$). For all trials, PPS was strongly associated with OS ($r = 0.82$), whereas PFS was moderately associated with OS ($r = 0.43$). The correlation between OS and PPS in recent trials was stronger than that in older trials ($r = 0.89$ and 0.66).
Conclusions: Our findings indicate that, especially for recent trials, PPS is highly associated with OS in first-line chemotherapy for advanced NSCLC, whereas PFS is only moderately associated with OS.
Key words: chemotherapy, non-small-cell lung cancer, overall survival, phase III trial, progression-free survival

introduction

Lung cancer remains the leading cause of cancer death worldwide [1, 2], with non-small-cell lung cancer (NSCLC) accounting for ~85% of lung cancer cases. Most individuals with NSCLC have metastatic disease at the time of diagnosis and therefore have a poor prognosis. The standard treatment of advanced NSCLC over the past decade has been platinum-based chemotherapy because of the moderate improvement in survival it confers [3–6]. Although many patients initially achieve clinical remission or disease stabilization with first-line chemotherapy, nearly all subsequently experience disease progression and eventually die of advanced NSCLC.

Overall survival (OS) has been traditionally recognized as the most important therapeutic objective for NSCLC patients. However, in view of the growing number of drugs and combinations thereof that are available for the treatment of such patients, any effect of first-line chemotherapy on OS might be confounded by subsequent therapies [7]. Indeed, an improvement in progression-free survival (PFS) has not necessarily resulted in an improved OS in recent randomized trials in patients with NSCLC [8, 9].

The effect of therapies instituted after disease progression on survival in clinical trials is thus of interest. However, little is known about postprogression survival (PPS) in NSCLC. In the present study, we partitioned OS of phase III trials for chemotherapy-naive patients with NSCLC into PFS and PPS and assessed the association of each with OS.

*Correspondence to: Dr I. Okamoto, Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan.
Tel: +81-72-366-0221; Fax: +81-72-360-5000;
E-mail: chi-okamoto@dotd.med.kindai.ac.jp

methods

search strategy and selection of trials

An independent review of PubMed citations from 1 January 2000 to 31 October 2010 was carried out. Key words included in the search were 'non-small cell lung cancer', 'clinical trial', 'advanced', and 'chemotherapy'. The search was limited to randomized controlled phase III trials and articles published in English. We reviewed each publication, and phase III studies that compared two or more first-line systemic chemotherapies (including treatment with molecularly targeted agents) for advanced or metastatic NSCLC were selected. To find any additional trials, we searched the reference lists of included trials as well as of large systematic reviews. We also checked articles that were in press at leading journals and searched websites listing abstracts from conferences (organized by the American Society of Clinical Oncology or the Federation of European Cancer Societies). We included trials that provided data for both OS and either PFS or time to progression (TTP), whether or not these parameters were explicitly defined. Trials were excluded if they investigated only immunotherapy regimens or hormonal therapies. Trials that were designed to assess combined modality treatments, including radiation therapy and surgery, were also excluded. To avoid bias, two observers (HH and IO) independently abstracted the data from the trials.

data abstraction

We analyzed in detail the primary and secondary efficacy end points, following the definitions of the authors of each trial. When not specifically

stated by the authors, we considered the primary end point to be that used for calculation of sample size. For the sake of simplicity, two end points (PFS and TTP) based on tumor assessment are collectively referred to as PFS in the present study, similar to the approach adopted in a recent report [10]. Median OS and median PFS were extracted from all trials that provided data for each treatment group. Median PPS was defined as median OS minus median PFS for each trial. We also obtained the following information from each report: year of completion of trial enrollment, number of patients randomized, number of patients in each treatment arm, number of treatment arms in each trial, proportion of patients who were male or had adenocarcinoma, and median age of the patients.

data analysis

We summarized the survival data (median OS, median PFS, median PPS, and median PFS/median OS) as the average and standard error (SE) for trial arms. SE was calculated on the basis of previously described models [11]. We also calculated the percentage of OS accounted for by PPS for each trial arm as: 100 − (100 × median PFS/median OS). To assess the relation between median OS and either median PFS or median PPS, we used Spearman's rank correlation coefficient. To account for differences in sample size among trial arms, we weighted all analyses by the number of patients in each arm. In addition, all trials were divided into two groups on the basis of the year in which trial enrollment was completed. Given that the median year for completion of enrollment in the 69 analyzed trials was 2002, we dichotomized at year 2002 (older trials, up to and including 2002; recent trials, 2003 and later) in order to evaluate a possible change in PPS, and we assessed whether the evaluated relations might be dependent on the year of completion of trial enrollment. We examined differences in the survival data between older and recent trials by normal approximation of the average survival data (z test). All reported P-values correspond to two-sided tests, and those of P-values <0.05 were considered statistically significant. Analyses were carried out with SAS for Windows release 9.2 (SAS Institute, Cary, NC).

results

characteristics of the trials

Our search yielded a total of 467 potentially relevant publications. Initially, 366 studies were excluded for at least one of the following reasons: they examined other malignancies or combined modality treatments, they were not randomized, they were phase I or II trials, they were review articles, they represented subgroup analyses, or they were duplicates. The selection process for the randomized controlled trials is shown in Figure 1. Review of the remaining 101 publications yielded 69 trials that were considered to be highly relevant for the present study. The main characteristics of the 69 phase III trials included in the analysis are listed in Table 1. A total of 37 986 patients with advanced NSCLC were enrolled, with a median number of patients per study of 433 (range 153–1725). Most of the trials had a high proportion of male patients and of patients with adenocarcinoma. The average median age of the patients was 62.3 years. Ten trials used an end point based on tumor assessment (PFS or TTP) as the primary end point, whereas OS was assessed as the primary end point in 53 trials. The other six trials used response rate or quality of life as the primary end point.

median OS, PFS, and PPS in all trials and in subgroups based on year of completion of trial enrollment

The survival data for trial arms according to the year in which trial enrollment was completed are shown in Table 2. Although the average median PFS in older (up to and including 2002) trials was the same (4.9 months) as that in recent (2003 and later) trials, the average median PPS was ~50% longer in the

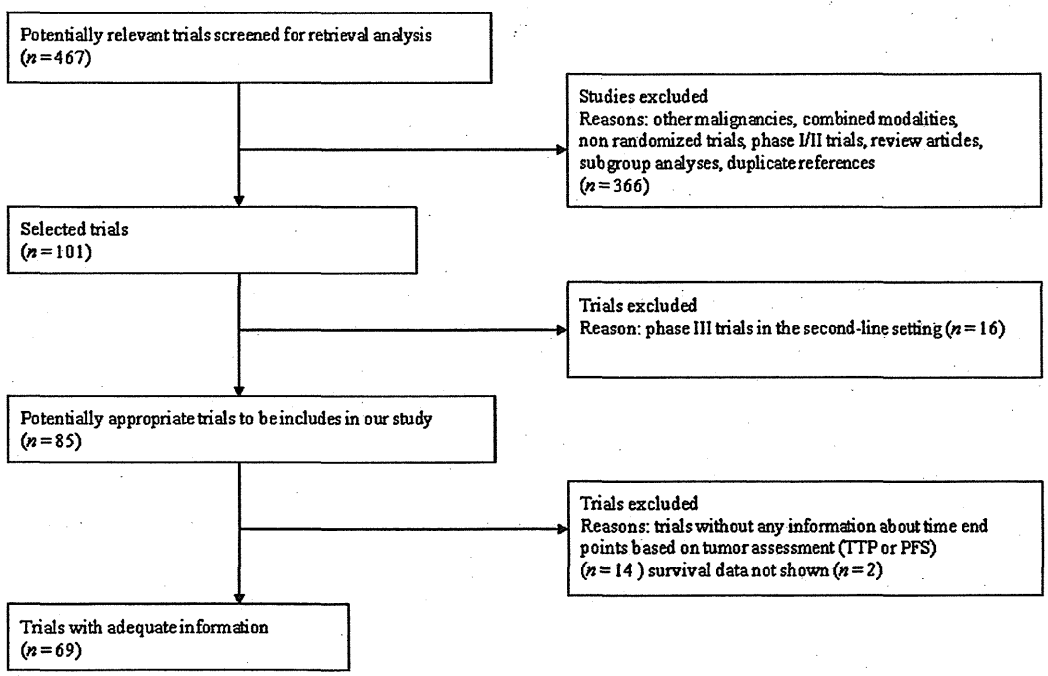

Figure 1. Flow chart showing the progress of trials through the selection process.

recent trials than in the older trials (6.5 and 4.4 months, respectively, $P < 0.0001$). The average proportion of median OS accounted for by median PPS significantly increased from 45.9% in older trials to 54.9% in recent trials ($P < 0.0001$).

relation between OS and either PFS or PPS

The relation between median OS and either median PFS or median PPS for the 151 treatment arms of the 69 trials is shown in Figures 2 and 3, respectively. We found that median PPS was strongly associated with median OS ($r = 0.82$, $P < 0.0001$) on the basis of Spearman's correlation coefficient, whereas median PFS was more moderately correlated with median OS ($r = 0.43$, $P < 0.0001$). The association between median OS and median PPS in recent trials ($r = 0.89$, $P < 0.0001$) was stronger than that in older trials ($r = 0.66$, $P < 0.0001$), whereas the correlation between median OS and median PFS in recent trials ($r = 0.55$, $P < 0.0001$) was similar to that in older trials ($r = 0.44$, $P < 0.0001$).

Table 1. Characteristics of the 69 phase III trials for advanced non-small-cell lung cancer included in the present analysis

Trial characteristic	
Median no. of patients per trial (range)	433 (153–1725)
Percentage of male patients (median)[a]	70.2
Percentage of adenocarcinoma patients[b]	51.2
Average of median age (years)[c]	62.3
Primary end point (no. of trials)	
OS	53
PFS or TTP	10
Response rate	3
Quality of life or toxicity	3
End point based on tumor assessment	
TTP	39
PFS	30
No. of treatment arms	
2	58
3	9
4	2

[a]One trial was excluded (data were not shown).
[b]Five trials were excluded (data were not shown).
[c]One trial was excluded (data were not shown).
OS, overall survival; PFS, progression-free survival; TTP, time to progression.

discussion

In the present study, we defined median PPS as median OS minus median PFS for each treatment arm of phase III trials for chemotherapy-naive patients with advanced NSCLC, as previously described [10, 12]. We also investigated the relation between median OS and either median PPS or median PFS by correlation analysis and found that median OS was more strongly associated with median PPS than with median PFS. Moreover, we also found that the correlation between median PPS and median OS was more pronounced in recent trials than in older trials and that median PPS was longer in recent trials than in older trials. This recent prolongation of PPS is likely the result of the increasing number of active compounds, such as docetaxel, pemetrexed, and epidermal growth factor receptor–tyrosine kinase inhibitors (EGFR-TKIs), which are available for second- or third-line chemotherapy in advanced NSCLC. One trial from a decade ago, when pemetrexed and EGFR-TKIs were not available, reported that only ~20% of patients received second-line chemotherapy [13]. In contrast, in the AVAiL trial, a recent large phase III trial that investigated the efficacy of cisplatin–gemcitabine with or without bevacizumab, second-line chemotherapy was administered in >60% of patients [8, 9]. Clinical trials of chemotherapy for patients with refractory NSCLC yielded a median OS of 5–8 months [14–17], which is similar to the median PPS for recent trials in our analysis. The recent widespread use of active second- and third-line therapies thus appears to have contributed to a prolongation of PPS in patients with advanced NSCLC.

Broglio and Berry [12] recently focused on PPS, which they termed survival postprogression (SPP) and defined as OS minus PFS, in a hypothetical clinical trial setting under the assumption that there was a treatment difference in PFS but not in PPS [12]. As the median PPS increased, the probability of detecting a statistically significant difference in OS decreased substantially. Even for a trial with an observed P value for improvement in PFS of 0.001, whereas there was a >90% probability for statistical significance of the difference in OS if the median PPS was 2 months, this probability decreased to only ~50% if the median PPS was 6 months. In the present study, we found that median PPS constituted more than half of median OS and that median PPS was >6 months in recent trials for NSCLC.

Table 2. Average median PFS, OS, and PPS as well as the average proportion of OS accounted for by PPS for trial arms in all trials or in trials according to year of completion of trial enrollment

Trials	No. of trial arms	No. of patients	Average (median) PFS (months)	Average (median) OS (months)	Average (median) PPS (months)	Average PPS/OS (%)
All	151	37 986	4.9 (0.09)	10.3 (0.24)	5.4 (0.22)	50.1 (1.00)
Recent (2003 and later)	69	19 334	4.9 (0.13)	11.3 (0.42)	6.5 (0.37)	54.9 (1.31)
Older (up to and including 2002)	82	18 652	4.9 (0.13)	9.4[a] (0.17)	4.4[a] (0.16)	45.9[a] (1.33)

Values in brackets are standard errors.
[a]$P < 0.0001$ versus the corresponding value for recent trials (z test).
OS, overall survival; PFS, progression-free survival; PPS, postprogression survival; TTP, time to progression.

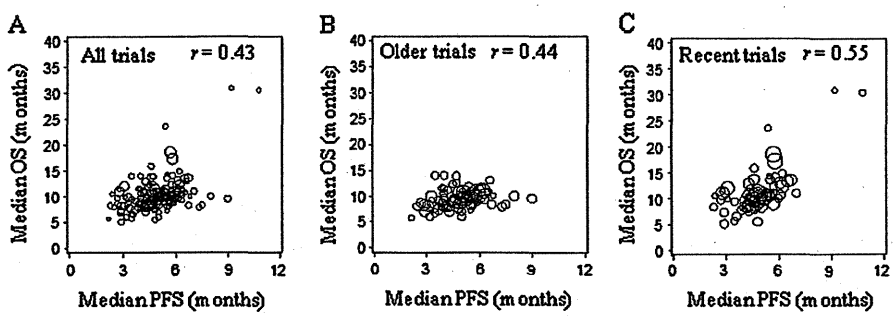

Figure 2. Relation between median overall survival (OS) and median progression-free survival (PFS) for 151 arms of 69 phase III trials for advanced non-small-cell lung cancer. (A) All trials. (B) Older trials (trial enrollment finished between 1996 and 2002). (C) Recent trials (trial enrollment finished between 2003 and 2006). The area of each circle is proportional to the number of patients in each trial arm. The *r* values represent Spearman's rank correlation coefficient.

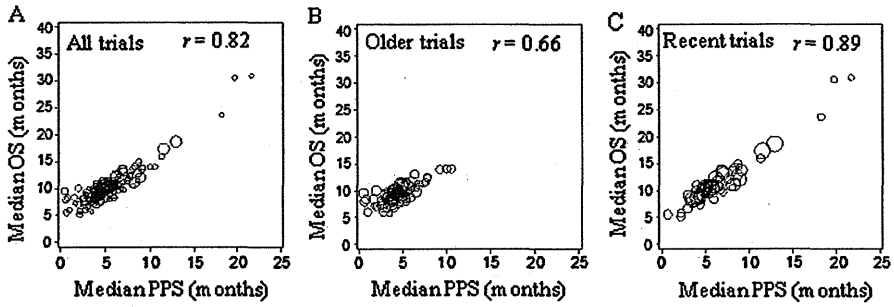

Figure 3. Relation between median overall survival (OS) and median progression-free survival (PPS) for 151 arms of 69 phase III trials for advanced non-small-cell lung cancer. (A) All trials. (B) Older trials (trial enrollment finished between 1996 and 2002). (C) Recent trials (trial enrollment finished between 2003 and 2006). The area of each circle is proportional to the number of patients in each trial arm. The *r* values represent Spearman's rank correlation coefficient.

Surrogacy of PFS for OS has often been assessed by quantifying the strength of the association between these end points at the individual level (referred to as individual-level surrogacy) and of that between the effects of treatment on these end points (trial-level surrogacy) [18–21]. Our examination of the correlation between PFS and OS was not an exercise in surrogate validation because of the lack of investigation into the correlation between the effects of chemotherapy on these end points. However, the present study has yielded the key finding that PPS, not PFS, is highly associated with OS.

The present study has several limitations. First, our analysis was based on abstracted data. The use of individual patient data might be expected to allow a better characterization of the relation between OS and other end points based on tumor assessment, including PFS and TTP. However, such an approach would restrict the analysis to a small number of trials and would hinder its replication by independent researchers. Second, the results of our study potentially have several confounders due to selection of many heterogeneous trials for analysis. The results are generally unaccountable without appropriate adjustment for patient characteristics dependent on differences in predefined eligibility criteria for enrollment in the clinical trials. Third, the assessment of disease progression is potentially subject to measurement error and bias in individual patients, and the quality of measurement for end points based on tumor assessment can vary between centers and trials. Finally, two end points (PFS and TTP) based on tumor assessment are considered as the same parameter, following the example of a previous report for advanced breast cancer [10]. PFS is defined as the time from randomization to tumor progression or death, whereas TTP is defined similarly but considers death as a time point when censoring occurs. TTP is the same as PFS if death does not occur during treatment. Given that death rarely occurs before disease progression in advanced NSCLC, we reasonably considered PFS to be the same as TTP for our analysis. Indeed, we separately analyzed clinical trials providing PFS ($n = 63$ arms) or TTP ($n = 88$ arms), and we found a consistent association between OS and PPS (data not shown). These data thus support our approach in which these two end points (PFS and TTP) are collectively referred to as PFS in the present analysis.

As far as we are aware, our study is the first to analyze PPS in advanced NSCLC. Our findings indicate that, especially for recent trials, PPS is highly associated with OS for first-line chemotherapy in patients with advanced NSCLC, whereas PFS is only moderately associated with OS. Therefore, OS remains an appropriate end point of clinical trials for chemotherapy-naive patients with advanced NSCLC. Given the great effect of PPS on OS, we propose a precise assessment of clinical course after disease progression in each clinical trial.