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An evaluation study of *EGFR* mutation tests utilized for non-small-cell lung cancer in the diagnostic setting

K. Goto[1]*, M. Satouchi[2], G. Ishii[3], K. Nishio[4], K. Hagiwara[5], T. Mitsudomi[6], J. Whiteley[7], E. Donald[7], R. McCormack[7] & T. Todo[8]

[1]*Division of Thoracic Oncology, National Cancer Center Hospital East, Chiba;* [2]*Department of Thoracic Oncology, Hyogo Cancer Center, Hyogo;* [3]*Pathology Division, Innovative Medical Research Center, National Cancer Center Hospital East, Chiba;* [4]*Department of Genome Biology, Kinki University School of Medicine, Osaka;* [5]*Department of Respiratory Medicine, Saitama Medical University, Saitama;* [6]*Department of Thoracic Surgery, Aichi Cancer Center Hospital, Aichi, Japan;* [7]*Department of Personalised Healthcare and Biomarkers, AstraZeneca Pharmaceuticals, Macclesfield, UK;* [8]*Department of Research and Development, AstraZeneca KK, Osaka, Japan*

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Background: Epidermal growth factor receptor (*EGFR*) mutation is predictive for the efficacy of EGFR tyrosine kinase inhibitors in advanced non-small-cell lung cancer (NSCLC) treatment. We evaluated the performance, sensitivity, and concordance between five *EGFR* tests.

Materials and methods: DNA admixtures ($n = 34$; 1%–50% mutant plasmid DNA) and samples from NSCLC patients [116 formalin-fixed paraffin-embedded (FFPE) tissue, 29 matched bronchofiberscopic brushing (BB) cytology, and 20 additional pleural effusion (PE) cytology samples] were analyzed. *EGFR* mutation tests were PCR-Invader®, peptide nucleic acid-locked nucleic acid PCR clamp, direct sequencing, Cycleave™, and Scorpion Amplification Refractory Mutation System (ARMS)®. Analysis success, mutation status, and concordance rates were assessed.

Results: All tests except direct sequencing detected four mutation types at ≥1% mutant DNA. Analysis success rates were 91.4%–100% (FFPE) and 100% (BB and PE cytology), respectively. Inter-assay concordance rates of successfully analyzed samples were 94.3%–100% (FFPE; kappa coefficients: 0.88–1.00), 93.1%–100% (BB cytology; 0.86–1.00), and 85.0%–100% (PE cytology; 0.70–1.00), and 93.1%–96.6% (0.86–0.93) between BB cytology and matched FFPE.

Conclusions: All *EGFR* assays carried out comparably in the analysis of FFPE and cytology samples. Cytology-derived DNA is a viable alternative to FFPE samples for analyzing *EGFR* mutations.

Key words: cytology, EGFR mutation, FFPE, NSCLC, PCR

introduction

Epidermal growth factor receptor (*EGFR*) mutation is a key predictive factor for the efficacy of EGFR tyrosine kinase inhibitors in the treatment of patients with advanced non-small-cell lung cancer (NSCLC) [1–3]. *EGFR* mutation testing is necessary to enable the physician to offer the most suitable therapy for a patient with advanced NSCLC.

Four *EGFR* mutation tests, PCR-Invader® [4], peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp [5], PCR direct sequencing [6], and Cycleave PCR™ [7] are used commercially in Japan, with testing generally carried out by centralized contracted laboratories. The Scorpion Amplification Refractory Mutation System (ARMS)® [8] is another sensitive globally available method and in particular was used in the phase III Iressa Pan-Asia Study (IPASS) to determine *EGFR* mutation status [1, 9]. A variety of methods, including direct sequencing, PCR-Invader, PNA-LNA PCR clamp, fragment analysis, and Cycleave PCR, were used in the WJTOG3405 phase III study to select *EGFR* mutation-positive patients [2], and the PNA-LNA PCR clamp method was used in the NEJ002 study [3]. To date, a study to compare the sensitivity and concordance of methods for *EGFR* mutation testing in Japan has not been conducted.

Diagnostic practices, and therefore, samples available for *EGFR* mutation analysis, differ between laboratories and countries. Large surgical samples are optimal for *EGFR* mutation analysis but small tissue from a tumor biopsy is the most commonly used and preferred sample type for diagnosis by clinicians [10, 11]. In clinical practice, tissue samples are not always available for diagnosis, and cytology samples, including bronchofiberscopic brushing (BB) cytology and pleural effusion cytology samples, are used in Japan and increasingly globally.

The aim of this study was to evaluate the sensitivity and performance of different *EGFR* mutation tests using artificial DNA admixtures, and clinical samples including formalin-fixed

Correspondence to: Dr K. Goto, Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwanoha, 6-5-1, Kashiwa, Chiba, 277-8577, Japan.
Tel: +81-4-7133-1111; Fax: +81-1-7131-4724; E-mail: kgoto@east.ncc.go.jp

paraffin-embedded (FFPE) tissue, BB cytology, and pleural effusion cytology samples from patients with NSCLC.

materials and methods

This was an observational study using control DNA admixtures and clinical samples. Patients provided written informed consent for samples to be used in research. The study was conducted as a collaborative research of AstraZeneca KK with National Cancer Center Hospital East (NCCHE) and Hyogo Cancer Center (HCC) after protocol approval by each Institutional Review Board and was conducted in accordance with ethical guidelines for epidemiological studies.

samples and DNA extraction

DNA admixtures

Four types of mutant plasmids were prepared including the *EGFR* mutation L858R, T790M, G719S, and E746-A750 deletion (nt del 2234-2249) in the Blue Heron pUC plasmid by Invitrogen Inc. (Tokyo, Japan). The sequence inserted into each plasmid corresponded with the longest sequence requirements spanning the exons across all of the methods to be evaluated, from −300 to +220 bp for exon 18 (for G719S) and from −200 to +200 bp for exons 19, 20, and 21 (for E746-A750 deletion, T790M, and L858R, respectively). Admixtures were prepared at Saitama Medical University Hospital. The plasmid preparations (5.4 ng/μl) were diluted with water and whole-human genomic DNA (12.5 ng/μl) (Promega Inc., Madison, WI) to prepare an admixture containing a 1 : 1 ratio (confirmed by Sanger sequencing) of copies of mutated and wild-type *EGFR* (5.4 fg/μl plasmid DNA, 10 ng/μl genomic DNA; referred to here as a 100% admixture). The 100% admixture solution was then diluted with genomic DNA to provide DNA solutions simulating those isolated from a clinical sample containing *EGFR*-mutated and wild-type cells at ratios of 50 : 50 (50% admixture), 25 : 75 (25%), 10 : 90 (10%), 5 : 95 (5%), 2 : 98 (2%), and 1 : 99 (1%). The samples were divided into aliquots for each laboratory, randomized and assigned an identification code, and 20 μl of each sample sent to the laboratories for mutation testing in a blinded manner. Ten wild-type control samples (from a single stock of genomic DNA) (10 ng/μl) were also distributed for testing.

formalin-fixed paraffin-embedded samples

In total, 120 FFPE NSCLC samples collected at NCCHE ($n = 100$) and HCC ($n = 20$) between December 2005 and October 2009 were used. Twelve consecutive sections (5-μm thickness), prepared by Sanritsu Co. Ltd (Tokyo, Japan) from each FFPE tissue block, were allocated as follows: sections 1 and 12, hematoxylin–eosin (H&E) staining; sections 2 and 7, PCR direct sequencing; sections 3 and 8, Cycleave PCR; sections 4 and 9, PCR-Invader; sections 5 and 10, PNA-LNA PCR clamp; sections 6 and 11, Scorpion ARMS. Samples were randomly assigned an identification code by Sanritsu Co. Ltd, with separate identification codes for the samples for PCR direct sequencing and Cycleave PCR (as they were to be analyzed by the same laboratory). A table of corresponding randomized identification codes was retained by AstraZeneca KK until analysis. H&E-stained sections (Sanritsu Co. Ltd) were reviewed by a single pathologist at NCCHE for histological type, tumor cell content, and tumor dimension in a blinded manner. DNA was extracted at each testing laboratory using their own standard operating procedures (SOPs), all of which utilized the QIAamp kit (QIAGEN Japan, Tokyo, Japan) (see supplemental Methods, available at *Annals of Oncology* online).

bronchofiberscopic brushing cytology samples

Thirty BB cytology samples (with matched FFPE samples available) obtained at NCCHE ($n = 10$) and HCC ($n = 20$) between 2006 and 2009 were used. Samples were collected by exfoliative cytodiagnosis brushing or curette washing in saline solution, without anticoagulant, and stored frozen. The BB cytology samples were randomized and assigned an identification code. The presence of tumor cells and histological type were confirmed by a pathologist at each center. DNA was extracted (QIAamp DNA Mini kit, QIAGEN Japan) at Kinki University of Medicine (Department of Genome Biology) and divided into 22 μl aliquots for analysis by the testing laboratories (direct sequencing was excluded due to the small amount of DNA anticipated, and for Scorpion ARMS, if the DNA concentration was <1 ng/μl, only exon 19 deletions, L858R, and T790M mutations were analyzed—see supplemental Methods, available at *Annals of Oncology* online).

pleural effusion cytology samples

Pleural effusion cytology samples were provided by NCCHE. Twenty pleural effusion cytology samples were collected from patients diagnosed with NSCLC (adenocarcinoma) between February 2009 and February 2010 and confirmed by a pathologist to contain tumor cells. Samples were frozen within 10 and 30 min of sampling and stored at −80°C. Frozen samples were thawed at 37°C and refrozen rapidly three times to disrupt the cells and ensure an even distribution and then divided into five equal aliquots that were sent to each of the testing laboratories. Samples were randomly assigned an identification code as for the FFPE samples. DNA was extracted at each laboratory using their own SOPs, all of which were based on the use of the QIAamp kit (see supplemental Methods, available at *Annals of Oncology* online).

EGFR mutation analysis

Samples were analyzed using five different *EGFR* mutation tests carried out by four different testing laboratories: PCR-Invader [4, 12] by BML Inc. (Tokyo, Japan); PNA-LNA PCR clamp [5] by Mitsubishi Chemical Medience Corp. (Tokyo, Japan); PCR direct sequencing (with the exception of the BB cytology samples, due to the anticipated tumor DNA yield based on published evidence regarding the detection limit of this method [13]) [6] by SRL Inc. (Tokyo, Japan), Cycleave PCR [7] also by SRL Inc., and Scorpion ARMS [14, 15] by Genzyme Analytical Services (Los Angeles, CA). Scorpion ARMS analysis employed the DxS *EGFR* Mutation Test Kit for research use only [QIAGEN Manchester (formerly DxS Ltd), UK] and was carried out according to the manufacturer's instructions with modifications described in the supplemental Methods (available at *Annals of Oncology* online). The other methods were carried out using each of the laboratories' experimental set up, with data analysis and quality control completed according to their own specific protocols (further details in the supplemental Methods, available at *Annals of Oncology* online). Samples were defined as mutation negative where sufficient material was present to generate a result but the presence of a mutation was not observed within the detection limit of the assay. The *EGFR* mutations detected by each *EGFR* mutation test are shown in supplemental Table S1 (available at *Annals of Oncology* online).

Analysis data (positive, negative, not detected, mutation type) and any supplemental information (e.g. failure of PCR amplification) were reported to AstraZeneca KK (Osaka, Japan).

statistical analysis

The correct determination rates (whether or not the positive/negative *EGFR* mutation assessment result was correct) and sensitivity (lowest percentage DNA admixture detected) by *EGFR* mutation type were assessed using DNA admixture samples for each *EGFR* mutation test.

The success and positive rates of each *EGFR* mutation test were determined using FFPE, and BB and pleural effusion cytology samples. The success rate was defined as the proportion of samples successfully analyzed

where it was possible to determine the mutation status. Samples were classified as unsuccessful where it was not possible to determine the mutation status, the PCR amplification failed, or if values of samples exceeded the cut-off value of Scorpion ARMS. The positive rate was defined as the number of samples analyzed as positive by each method as a proportion of the number of samples successfully analyzed. False-positive and false-negative rates were not determined, as no reference or 'gold standard' has been defined for *EGFR* mutation analysis.

The concordance rates and Cohen's kappa coefficients were determined between different methods of detection and between FFPE versus BB cytology sample types for mutation types commonly detectable by all assessed methods. Cohen's kappa coefficient was calculated as: kappa = (Po–Pe)/(1–Pe), where Po is the observed concordance rate and Pe is the expected probability of chance agreement.

results

patient samples

In total, 116 FFPE samples were evaluable for analysis, as four samples were confirmed not to contain NSCLC cells. The majority of samples were of adenocarcinoma histology and had a tumor cell content of at least 50%. Both tissue and tumor dimensions were ≤19 mm in most samples.

Of the 30 BB cytology samples (24 adenocarcinoma, four squamous, one adenosquamous, one large cell), one sample was excluded from the analysis because its matching FFPE sample was not judged as NSCLC. The samples were taken at a mean of 39 days (range 20–70 days) before operation and the mean DNA concentration was 8.73 ng/μl (range 0.2–40.3 ng/μl). All 20 pleural effusion cytology samples were assessable for analysis. Volumes of pleural effusion cytology samples used for each test method were 0.7–0.8 ml.

comparability of five *EGFR* mutation tests

DNA admixtures

PCR-Invader, PNA-LNA PCR clamp, Cycleave PCR, and Scorpion ARMS methods detected each of the *EGFR* mutation types L858R, T790M, G719S, and the in-frame deletion E746-A750 type I at ratios ranging from 1% to 50% of mutant/wild-type allele. PCR direct sequencing detected all types of mutations in samples containing 5%–50% of plasmid DNA but could not detect any of the mutations in the 1% mutant DNA admixture, nor exon 19 deletion or L858R in the 2% mutant DNA admixture. There were no false positives in wild-type samples.

formalin-fixed paraffin-embedded samples

Success rates of all five *EGFR* mutation tests were over 90% in FFPE samples (Table 1). Concordance rates between any two methods ranged from 85.3% to 99.1% including samples unsuccessfully analyzed and from 94.3% to 100% excluding samples unsuccessfully analyzed (supplemental Table S2, available at *Annals of Oncology* online). The rate of type 1 discordance (different mutations detected between the methods) was 3.4% (4/116 samples) and the rate of type 2 discordance (mismatch of mutation status between the methods) was 6.9% (8/116 samples) in FFPE samples (supplemental Table S3, available at *Annals of Oncology* online).

Unsuccessful rates of mutation analyses and discordance rates by tumor/sample characteristics for FFPE samples are shown in supplemental Figure S1 (available at *Annals of Oncology* online). Higher unsuccessful rates were associated with histological subtype [squamous cell carcinoma, 4/7 (57.1%)], older sample age [year of surgery 2006, 9/10 (90.0%)], and larger tumor dimension [20–29 mm, 3/15

Table 1. Success rate and *EGFR* mutation status determined by different *EGFR* mutation tests in FFPE, BB, and pleural effusion samples

Sample type and method	No. of samples successfully analyzed (%)[a]	No. of mutation-positive samples (%)[a]	No. of mutation-negative samples (%)[a]
FFPE samples ($n = 116$)			
Scorpion ARMS	115 (99.1)	65 (56.5)	50 (43.5)
PCR-Invader	116 (100.0)	65 (56.0)	51 (44.0)
PNA-LNA PCR clamp	106 (91.4)	61 (57.5)	45 (42.5)
PCR direct sequencing	110 (94.8)	64 (58.2)	46 (41.8)
Cycleave PCR	116 (100.0)	63 (54.3)	53 (45.7)
BB cytology samples ($n = 29$)			
Scorpion ARMS	29 (100.0)	15 (51.7)	14 (48.3)
PCR-Invader	29 (100.0)	17 (58.6)	12 (41.4)
PNA-LNA PCR clamp	29 (100.0)	17 (58.6)	12 (41.4)
Cycleave PCR	29 (100.0)	16 (55.2)	13 (44.8)
Pleural effusion cytology samples ($n = 20$)			
Scorpion ARMS	20 (100.0)	11 (55.0)	9 (45.0)
PCR-Invader	20 (100.0)	11 (55.0)	9 (45.0)
PNA-LNA PCR clamp	20 (100.0)	10 (50.0)	10 (50.0)
PCR direct sequencing	20 (100.0)	11 (55.0)	9 (45.0)
Cycleave PCR	20 (100.0)	11 (55.0)	9 (45.0)

[a]Percentage calculated based on the number of samples successfully analyzed; *EGFR* mutation status was determined before the study and samples were selected to allow for an ~1 : 1 ratio of mutation-positive : mutation-negative samples.

ARMS, Amplification Refractory Mutation System; BB, bronchofiberscopic brushing; FFPE, formalin-fixed paraffin-embedded; PNA-LNA, peptide nucleic acid-locked nucleic acid.

(20.0%)] (supplemental Figure S1, available at *Annals of Oncology* online). Discordance rates tended to be higher in samples with low tumor cell content [0–20%, 2/10 (20.0%); 30–40%, 3/10 (30%)], smaller tumor dimension [0–9 mm, 11/53 (20.8%)], smaller tissue dimension [0–9 mm, 8/33 (24.2%)], and older sample age [year of surgery 2006, 2/10 (20.0%)] (supplemental Figure S1, available at *Annals of Oncology* online).

Concordance rates between five methods for the two major mutation types in FFPE samples were 81.8% (18/22) for exon 19 deletions and 87.2% (34/39) for L858R.

PCR direct sequencing identified rare mutations in six patients that were not detected by any other methods [V689L and E690V, E709V, V834L, I706T D770_N771 (insSVD), H773_V774(insPH)].

bronchofiberscopic brushing cytology samples

Success rates of the four *EGFR* mutation tests utilized for analysis of BB cytology samples were 100% (Table 1) and concordance rates between two methods ranged from 93.1% to 100% (supplemental Table S2, available at *Annals of Oncology* online). Discordances between two methods were found in two (6.9%) samples (supplemental Table S4, available at *Annals of Oncology* online). Both were type 2 discordances (mismatch of mutation status between the methods): in one sample, G719C detected by PCR-Invader and PNA-LNA PCR clamp was assessed as negative by Cycleave PCR (G719X not analyzed by Scorpion ARMS due to insufficient sample). In the remaining sample, L858R detected by PCR-Invader, PNA-LNA PCR clamp, and Cycleave PCR was assessed as negative by Scorpion ARMS.

Concordance rates between analysis of BB cytology and FFPE samples ranged from 65.5% to 96.6% including samples unsuccessfully analyzed and 93.1%–96.6% excluding samples unsuccessfully analyzed (supplemental Table S5, available at *Annals of Oncology* online). Discordances in analysis of BB cytology samples versus FFPE samples by the same detection method (excluding discordances due to unsuccessful mutation analysis of FFPE samples) were observed in three (10.3%) samples (supplemental Table S4, available at *Annals of Oncology* online); all were type 2 discordances (mismatch of mutation status between the methods).

pleural effusion cytology samples

Success rates of all five *EGFR* mutation tests were 100% (Table 1) and concordance rates between two methods ranged from 85.0% to 100% in the pleural effusion samples (supplemental Table S2, available at *Annals of Oncology* online). Discordances between five methods were found in three (15.0%) samples (supplemental Table S6, available at *Annals of Oncology* online). All were type 2 discordances (mismatch of mutation status between the methods): in one sample, an exon 19 deletion was detected by all methods except PCR direct sequencing; in another, an exon 19 deletion was only detected by Scorpion ARMS, Cycleave PCR, and PCR direct sequencing; and in the third sample, L858R was only detected by PCR-Invader. In one of the other 17 samples, PCR direct sequencing detected an additional mutation [exon 18 deletion (E709_T710>D)], which the other methods were not designed to assess.

discussion

Analysis of the control DNA admixture samples showed that the *EGFR* mutation tests had comparable sensitivity, with the exception of direct sequencing. The sensitivity of direct sequencing, although higher than expected and reported elsewhere [15], was lower than the other techniques.

The results of this study showed that all five *EGFR* mutation tests had comparable success rates (over 90%) in FFPE samples. These were consistently high success rates given that the fixation of some of the samples was not ideal (e.g. long fixation times). The success rates of direct sequencing were higher than anticipated based on previous studies of clinical samples. For example, in a recent study, ARMS and direct sequencing were used to detect known *EGFR* mutations in NSCLC FFPE samples, and ARMS was found to be a more sensitive and robust technique [13]. However, it should be recognized that even when utilizing the same technologies, differences in reagents, DNA quality, software, and crucially, primer design and amplicon size have a huge influence on direct sequencing success rates and mutation detection potential. Our results show that the processes implemented by the laboratory in this study are highly optimized for the detection of *EGFR* mutations from tumor DNA using direct sequencing. As the testing laboratories also carried out the DNA extraction (with the exception of BB cytology samples), the slight differences in DNA extraction and processes across the different laboratories could also impact on the overall performance of the test methods.

All the FFPE samples were examined by a pathologist and generally found to be of high quality and tumor content. The numbers of samples with different tumor/sample characteristics were too low to make any definitive conclusions regarding unsuccessful and discordance rates by these characteristics. However, sample unsuccessful rates appear to be associated with squamous cell carcinoma, older samples, and samples with long tumor dimension, all of which can make it difficult to extract DNA. In addition, discordance rates appeared higher in older samples or samples of low tumor cell content, short tumor dimension or short tissue dimension, where the quantities of DNA are small.

Concordance rates were generally over 85% (>94%, excluding samples unsuccessfully analyzed) between any two *EGFR* mutation tests in FFPE samples. The lowest concordance rates between the five methods were in comparison with the PNA-LNA PCR clamp method. As the PNA-LNA PCR clamp method also had a higher unsuccessful rate than the other methods, concordance rates were lower in comparison with other methods when including samples unsuccessfully analyzed. However, all kappa statistics were ≥ 0.70, indicating a high concordance of analysis results. Concordance rates for the two major *EGFR* mutation types, exon 19 deletions and L858R, across the five mutation tests in FFPE samples were also high (81.8% and 87.2%, respectively), illustrating the suitability of all

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

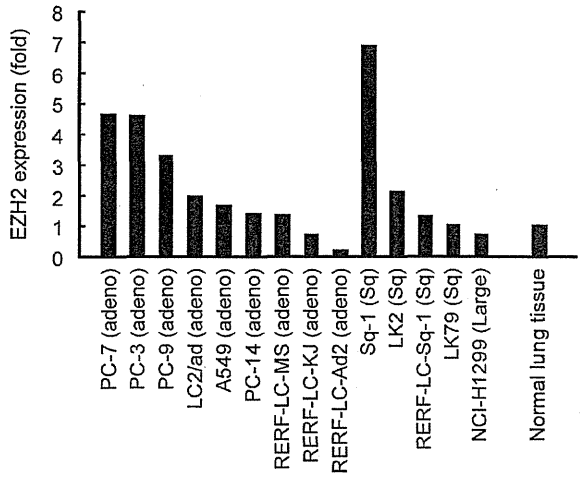

Figure 1. Enhancer of zeste homolog 2 (*EZH2*) messenger RNA expression is illustrated in lung cancer cell lines. Expression levels were measured by real-time polymerase chain reaction and were normalized to the levels in normal lung tissue. Adeno indicates adenocarcinoma; Sq, squamous cell carcinoma;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

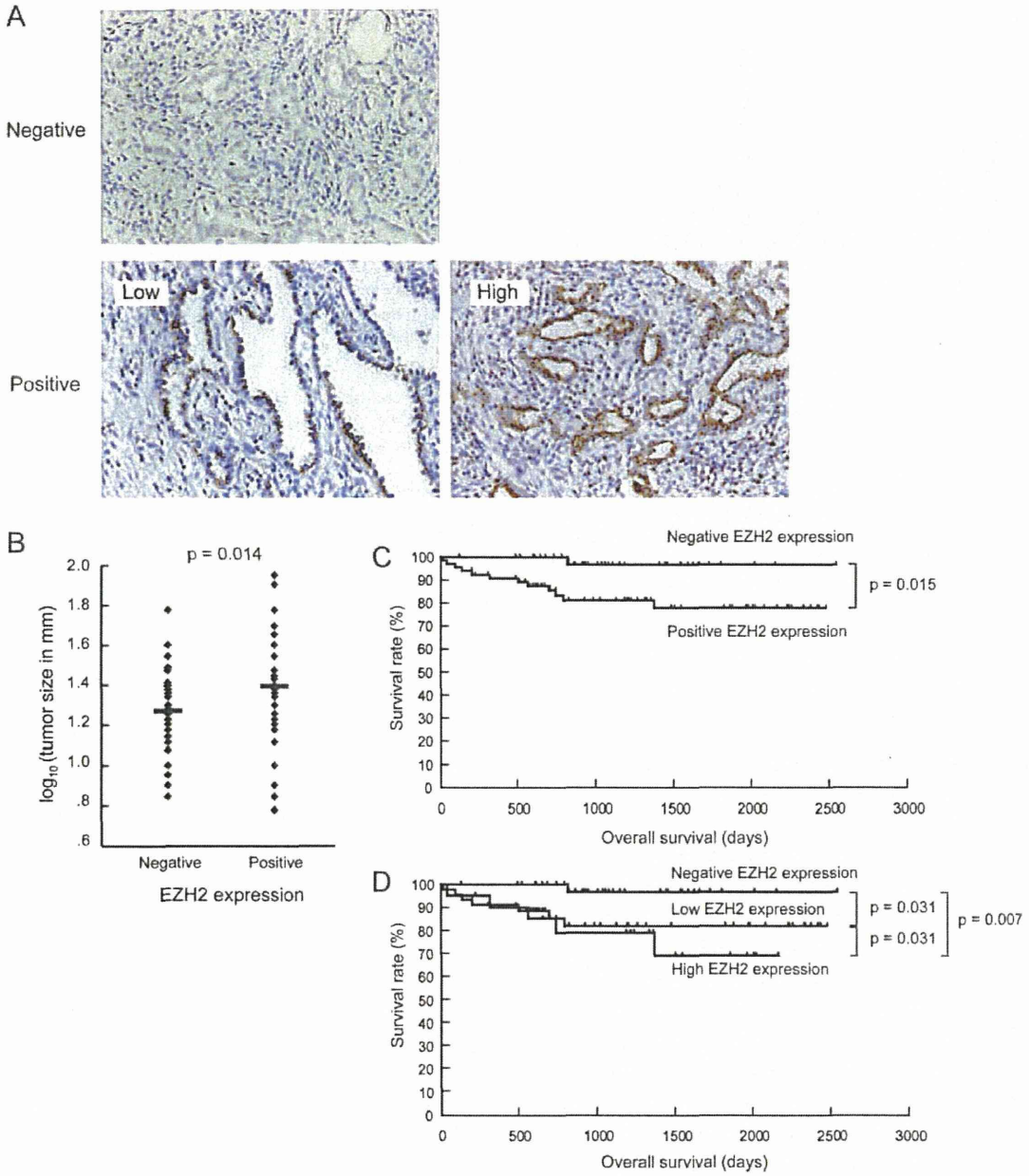

Figure 2. An immunohistologic analysis of enhancer of zeste homolog 2 (EZH2) expression is illustrated. (A) These are representative images of EZH2 expression. (B) This scatter plot demonstrates a significant correlation between EZH2 expression level and tumor size. (C) Kaplan-Meier analysis is shown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

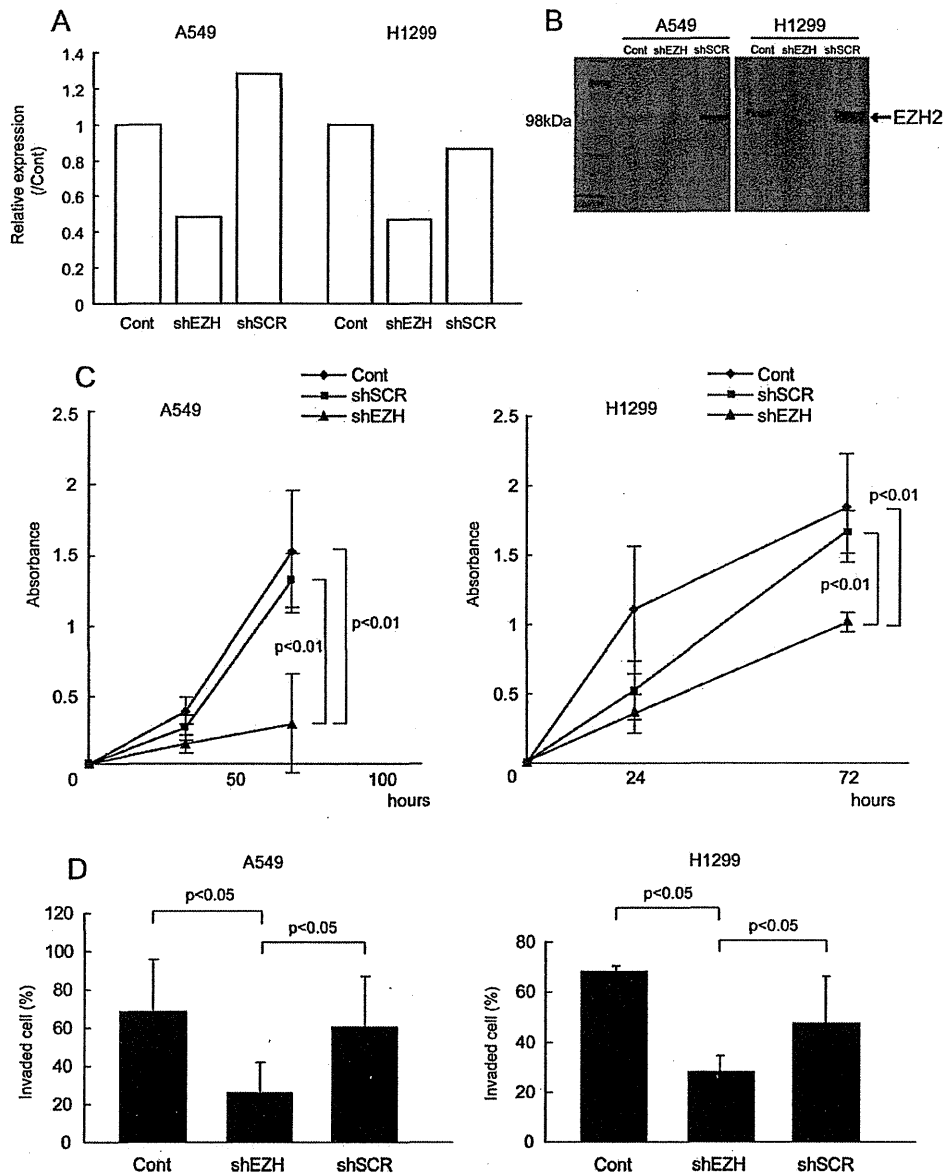

Figure 3. In vitro experiments are illustrated. (A) This chart illustrates the measurement of enhancer of zeste homolog 2 (*EZH2*) messenger RNA (mRNA) by real-time polymerase chain reaction (PCR) after short-hairpin RNA (shRNA)-mediated knockdown of *EZH2* mRNA in (*Left*) A549 cells and (*Right*) and 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.

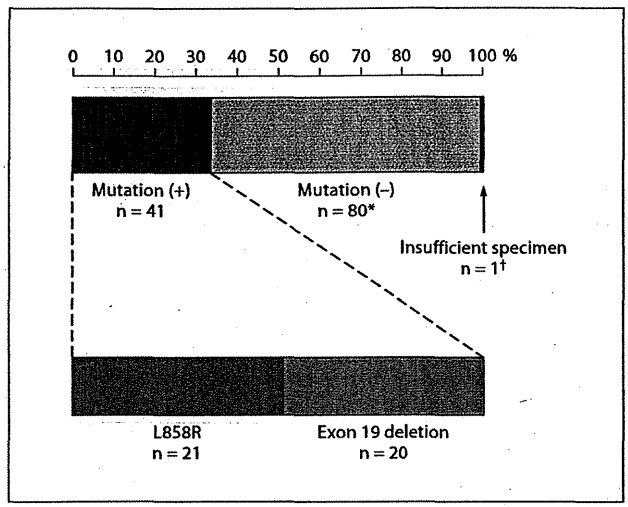

Fig. 1. Detection of the EGFR mutation by PNA-LNA PCR clamp. * Three patients (1 patient who underwent brushing with EBUS-GS and 2 patients who underwent brushing with an ultrathin bronchoscope) had a very small quantity of DNA. † DNA was not obtained from the cytological specimen gathered by brushing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