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Author(s)	Yamada, Noriyuki; Oizumi, Satoshi; Asahina, Hajime et al.
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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, MD, PhD[1], Satoshi Oizumi, MD, PhD[1], Hajime Asahina, MD, PhD[1], Naofumi Shinagawa, MD, PhD[1], Eiki Kikuchi, MD, PhD[1], Junko Kikuchi, MD, PhD[1], Jun Sakakibara-Konishi, MD, PhD[1], Tomoaki Tanaka, PhD[2], Kunihiko Kobayashi, MD, PhD[3], Koichi Hagiwara, MD, PhD[2], Masaharu Nishimura, MD, PhD[1]

[1]First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan

[2]Department of Respiratory Medicine, Saitama Medical University, Saitama, Japan

[3]Department of Respiratory Medicine, Saitama Medical University International Medical Center, Hidaka, Japan

This work was performed at Hokkaido University School of Medicine, Sapporo; and Saitama Medical University, Saitama; and Saitama Medical University International Medical Center, Hidaka.

Correspondence to: Satoshi Oizumi, MD, PhD, First Department of Medicine, Hokkaido University School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan

E-mail: soizumi@med.hokudai.ac.jp, TEL: 81-11-709-5911, Fax: 81-11-706-7899

Running head: EGFR mutations in cytological specimens of NSCLC

Key words: non-small cell lung cancer, EGFR mutations, Bronchoscopy, Cytological specimens,

Abstract

Objectives: Cytological examination of samples obtained by bronchoscopy is a useful method of 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 clamp (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 one 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, 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 (TBB) 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 clamp (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 1000-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, IL). Any significant differences among the categorized groups were compared using the chi-square 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.

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 (Figure 1), whereas 80 patients (65.6%) were negative. Three samples that were negative for EGFR mutation contained a very small quantity of DNA; one of these samples was obtained by brushing with EBUS-GS, and the other two 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, one 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 nor the other minor mutation such as G719X and L861Q. The association between EGFR mutation status and clinicopathological characteristics was assessed by the chi-square 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, 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 one 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 *). 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 confirmed for the diagnosis, 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.

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% to 95.7% in EBUS-TBNA [12, 13], 67% to 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 mutation 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 mutation.

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 *). 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 materials 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.

Financial/nonfinancial disclosures: 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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Figure 1

Detection of the EGFR mutation by PNA-LNA PCR clamp

*Three patients (1 patient that underwent brushing with EBUS-GS and 2 patients that underwent the 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	No. of patients (%)
Age	
Median (Range)	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)

Abbreviations: EBUS-GS = endobronchial ultrasonography with a guide sheath; EBUS-TBNA = endobronchial ultrasound guided transbronchial needle aspiration.

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

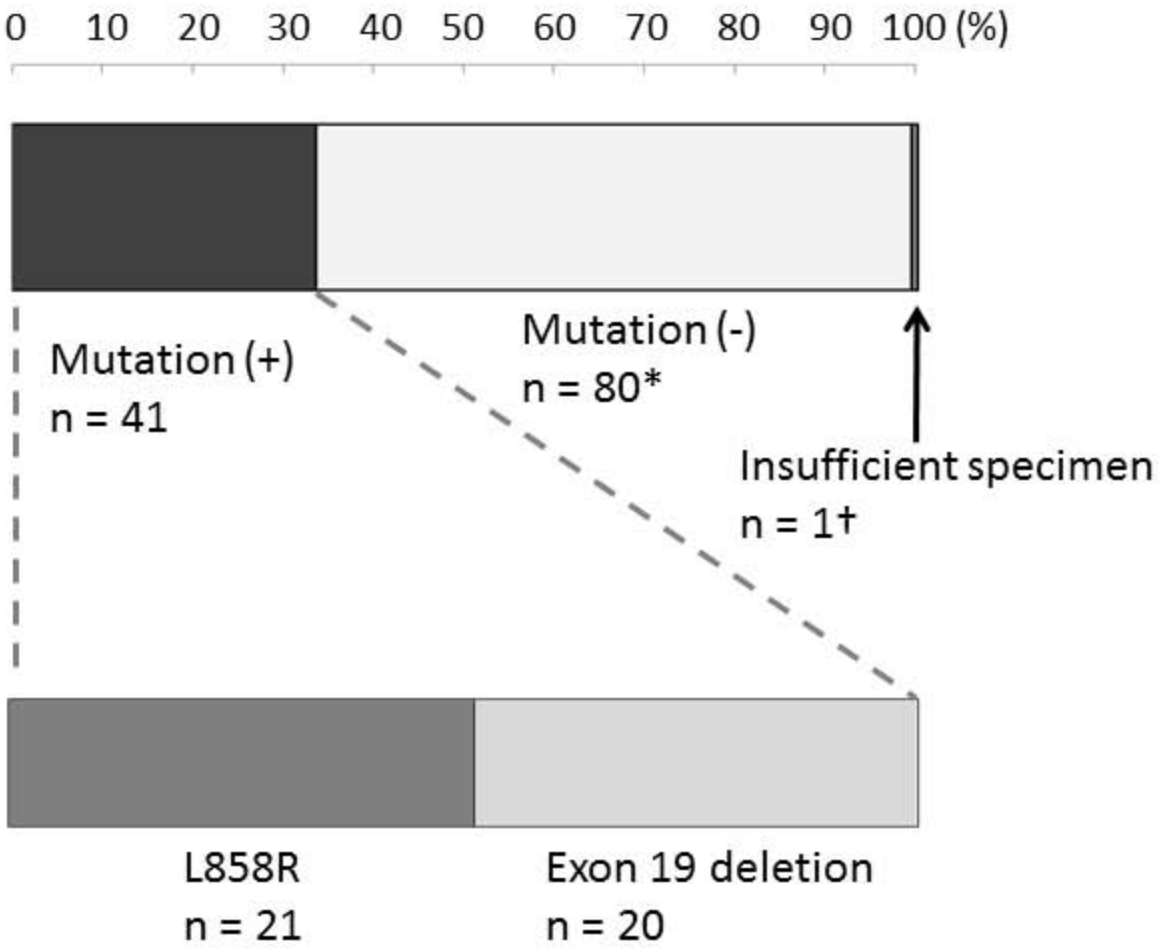
Characteristics		Odds ratio (95% confidence interval)	p-value
Sex	Female vs male	1.67 (0.57-4.92)	0.35
Smoking status	Never smoker vs smoker	2.60 (0.91-7.46)	0.076
Cytology	Adenocarcinoma vs non-adenocarcinoma	9.13 (2.51-33.21)	<0.001

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

Age	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	L747-S752del
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

†Small quantity of DNA

Abbreviations: Ad = adenocarcinoma; Sq = squamous cell carcinoma; NS = non-small cell carcinoma; EBUS-GS = endobronchial ultrasonography with a guide sheath.


0 10 20 30 40 50 60 70 80 90 100 (%)
Mutation (+)
n = 41
Mutation (-)
n = 80*
Insufficient specimen
n = 1†
L858R
n = 21
Exon 19 deletion
n = 20