A comparison of epidermal growth factor receptor mutation testing methods in different tissue types in non-small cell lung cancer

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Abstract. The detection of somatic epidermal growth factor receptor (*EGFR*) mutations is valuable when an appropriate therapy, either EGFR-tyrosine kinase inhibitor (TKI) therapy or chemotherapy, for patients with advanced non-small cell lung cancer (NSCLC) needs to be selected. Although it is well-understood that *EGFR* mutation detection is significant for the decision-making regarding treatment, no consensus on the methodology that should be the most preferebale for detecting mutations in clinical practice has been reached. The presence of false positives due to the technique carried out for mutation analysis affects the accurate estimation of response EGFR-TKI therapy. Furthermore, false negatives directly exclude the potential application of an EGFR-TKI. In the present study, we present the results of detecting *EGFR* mutations in individual sample types using three different low- or high-sensitivity techniques. We suggest that the choice of the method used should be made based on the type of the sample. Our results revealed that *EGFR* mutations were less frequently detected in bronchoscopic biopsies, regardless of the method used. However, the amplification refractory mutation system (ARMS) was optimal owing to the small amount of DNA prepared for biopsy. The cytology sample was a valuable alternative to traditional samples, given that a sensitive method for detecting mutations was used. For surgical resections, the testing method may be selected based on the expertise of each laboratory, but direct sequencing is highly recommended. We also suggest that two methods should be used sequentially (the screening and targeted methods) in clinical practice due to the presence of non-neglected discordance between any method from its own benefits and drawbacks.

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Introduction

In the latest guidelines by the National Comprehensive Cancer Network (NCCN), epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) is recommended as first-line therapy for patients with advanced non-small cell lung cancer (NSCLC) harboring activating *EGFR* mutations.

EGFR mutations occur more frequently in patients of Asian ethnicity, females, individuals with no smoking history, and in patients diagnosed with adenocarcinoma. Treatment with EGFR-TKI manifests the greatest efficacy in these patients, with no smoking history being the best predictor of good response to TKI. Mutations are associated with an enhanced sensitivity to an EGFR-TKI located in *EGFR* exons 18-21, which encode the tyrosine kinase domain. In-frame deletions in exon 19 and a point mutation in exon 21 (p.L858R) are the most prevalent *EGFR* mutations. Mutations associated with resistance to TKI include a point mutation (p.T790M) and insertions (e.g., p.D770_N771insNPG) in exon 20, and a point mutation (p.D761Y) in exon 19 (1-3).

Although the clinical relevance of activating *EGFR* mutations with TKI response in advanced NSCLC is well-addressed, a standardized and commonly accepted approach in terms of optimal sensitivity, specificity, reproducibility and accuracy in detecting *EGFR* mutations has not been adopted to date (4-6). A variety of techniques for mutation analysis of the *EGFR* gene exist. These are classified into screening methods that identify all mutations and targeted methods that distinctively detect known and pre-determined mutations.

Among diverse screening methods, the direct sequencing of polymerase chain reaction (PCR) products is still widely used, despite its low sensitivity. Direct sequencing does not require sample batching, while it provides better contamination control since the exact, specific mutation is presented. However, direct sequencing is time-consuming and successful only when viable tumor cells constitute at least 25% of the tissues (7,8). Alternative screening methods include high resolution melting (HRM), pyrosequencing and denaturing high pressure liquid chromatography (dHPLC) analysis. HRM is an in-tube, fast method that detects sequence variation by

monitoring the melting curve of PCR amplicons. HRM is able to detect mutant genes at levels of 1-10% (9-11). Nevertheless, the requirement for sequencing validation increases the turn-around time and reduces the value of high sensitivity.

The limited sensitivity of conventional sequencing necessitates the adoption of more sensitive approaches. Scorpion amplification refractory mutation system (ARMS) falls into the targeting method category and has been successfully used to analyze the *EGFR* mutation status in the phase III Iressa Pan-Asia Study (IPASS) clinical trial (12). ARMS detects mutations in samples with a mutation frequency as low as 0.1 to 1%, but detects known mutations only. Additionally, ARMS requires the batching of samples and the reagents required are expensive.

Samples used for analyzing *EGFR* mutations differ between laboratories. Samples can be obtained either at the stage of diagnosis (biopsy) or at the stage of surgical intervention (resections). Large samples from surgical intervention are preferred, but small biopsy samples are also regularly used. Unfortunately, tissue samples are not always available; therefore, cytological materials, including pleural effusion, bronchial scraping and bronchofiberscopic brushing are being increasingly used. In fact, the mutation detection rate achieved with cytologyical material is comparable with that achieved with tissue samples obtained by biopsy or resection (5).

In the present study, we analyzed the *EGFR* mutation status of 356 patients with advanced NSCLC and systemically compared the mutation detection rate of direct sequencing, the gold standard, with more sensitive methods, i.e., ARMS and HRM, in different tissue types. The overall mutation rate of the *EGFR* gene was 44.10% and the rate of activating *EGFR* mutations was 39.04%. The activating *EGFR* mutations occurred more frequently in females and patients diagnosed with adenocarcinoma. The *EGFR* mutation frequency identified from the bronchoscopic biopsies was lower than that from surgical resections, regardless of the method used. The mutation rate detected from the cytological sample was similar to that achieved with surgical resections and a sensitive method for detecting mutations in the cytological samples was required. Each method has advantages and disadvantages; it was thus suggested that the choice of method in clinical practice should be made based on the sample type. ARMS was recommended when mutations were detected in bronchoscopic biopsies and cytological samples. Direct sequencing was recommended when mutations were identified in surgical resections. However, the lack of *EGFR* mutations tested by direct sequencing is possibly due to the limited sensitivity of the method. The absence of *EGFR* mutations, determined by methods that detect known mutations, such as ARMS, cannot be the exclusion criterion for TKI treatment. To reduce false positives and false negatives caused by the limitations of each method, the combination of direct sequencing and a more sensitive technique, such as ARMS, is recommended for identifying *EGFR* mutations in clinical practice.

Patients and methods

Subjects. This study was approved by the Institutional Review Board, where samples were collected from and analyzed (the First Affiliated Hospital of Soochow University, Suzhou, China). A total of 356 patients diagnosed with NSCLC were included in the study. The pleural effusion cytological samples were collected from patients diagnosed with NSCLC and confirmed by a pathologist to contain tumor cells.

Sensitivity determination. Two lung cancer cell lines, PC-9 and A549, were used. The A549 cell line was purchased from the Shanghai Institute for Biological Sciences (Shanghai, China). PC-9 cells harbor in-frame deletions in exon 19 of the *EGFR* gene (heterozygous for c.2235_2249del15). A549 cells are wild-type for the *EGFR* gene. Serial dilutions of the *EGFR* mutant PC-9 cells with A549 cells were used to determine the sensitivity of direct sequencing, ARMS and HRM.

Genomic DNA (gDNA) extraction. Before the extraction of gDNA, representative formalin-fixed, paraffin-embedded (FFPE) sections were stained with hematoxylin and eosin (H&E) and diagnosed by pathologists. At least 20% of tumor cells was observed in the FFPE sections. DNA extraction was performed usng the QIAamp™ DNA FFPE Tissue kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. To obtain DNA fromthe cell lines, the cells were harvested by trypsinization when grown to confluence. To obtain DNA from pleural effusion, the cells were collected by centrifugation. DNA was extracted using gDNA isolation kits (Omega BioTek Guangzhou, Ltd., Guangzhou, China) according to the manufacturer's instructions. DNA was quantified using a NanoDrop ND-1000 fluorospectrometer (ThermoFisher Scientific, Shanghai, China), and the A260/280 value was ensured between 1.8-2.0.

Direct sequencing. Mutation screening of *EGFR* exons 18-21 was carried out by PCR amplification as previously described (13). The primers for PCR amplification were as follows: *EGFR* exon 18 forward, GCATGGTGAGGGCTGAGGTGAC and reverse, TATACAGCTTGCAAGGACT CTG; exon 19 forward, GTGCATCGCTGGTAACATCCA and reverse, GGAGAT GAGCAGGGTCTAGAGCA; exon 20 forward, GATCGC ATTCATGCGTCTTCACC and reverse, TTGCTATCCCAGG AGCGCAGACC; exon 21 forward, TCAGAGCCTGGCAT GAACATGACCCTG and reverse, GGTCCCTGGTGTCAGG AAAATGCTGG. PCR reaction was amplified using Platinum Taq DNA polymerase (Invitrogen, Beijing, China) and conducted under the following conditions: 94˚C 5 min, (94˚C, 30 sec, 60˚C, 30 sec, 72˚C, 45 sec) x40 cycles, 72˚C 10 min. The PCR products were checked on 2% agarose gels. PCR products were purified and followed by bi-directional sequencing using an ABI 3730 DNA analyzer (Applied Biosystems, Inc., Beijing, China). Sequencing chromatograms were analyzed using DNA Baser 3.0. Nucleotide changes detected by sequencing were all checked in Sanger's COSMIC database (http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=EGFR#histo), and diagnosed as mutations accordingly.

ARMS assay. The presence of *EGFR* mutations was determined using the AmoyDx™ EGFR 29 Mutations Detection kit, (Amoy Diagnostics Co., Ltd., Xiamen, China). The kit, which has been approved for clinical use by the State Food and Drug Administration (SFDA) in China, detects the most commonly reported 29 somatic mutations (both activating and TKI resistance-related) in the *EGFR* gene: 19 deletions in exon 19,

three insertions in exon 20 and point mutations p.G719X (exon 18), p.S768I and p.T790 M (exon 20), p.L858R and p.L861Q (exon 21). The test detects the presence of these mutations, but does not distinguish between them. The analysis was performed according to the manufacturer's instructions using a LightCycler 480 (Roche Diagnostic, Ltd., Shanghai, China).

HRM assay. HRM assay was performed using an *EGFR* gene mutation detection kit, detecting mutations in *EGFR* exons 18-21 (Suzhou MicroDiag Biomedicine Co., Suzhou, China) on a LightCycler 480. The melting profiles of the amplicons were analyzed using gene scanning software to detect wild-type and mutations. To affirm the gene scanning results, the amplicons were sequenced after HRM assay.

Statistical analysis. The association of the mutation status of the *EGFR* gene with any of the clinicopathological characeristics was evaluated. Frequencies were compared using two-tailed Pearson's Chi-square or Fisher's exact test. The difference was considered significant when the P-value was $P<0.05$. Analyses were performed using the GraphPad Prism 5 program.

Results

Patient characteristics. A total of 356 samples (from August 2010 to December 2012) were collected and successfully evaluated for *EGFR* mutations. The clinical characteristics of the patients, such as age, gender, smoking history and pathological evaluation, are summarized in Table I. A total of 210 patients (58.99%) were male and 146 (41.01%) were female, with a median age of 57.5 years (range, 27-88 years). Of these patients, 162 (45.51%) were current smokers, 173 (48.60%) were non-smokers, and 21 (5.89%) had an unknown smoking history. All samples were confirmed to contain malignant cells and the pathological and cytological diagnosis revealed that 173 (48.60%) of the samples contained adenocarcinoma cells, 56 (15.73%) contained squamous cell carcinoma cells and 127 (35.67%) contained other types of carcinoma cells.

EGFR mutations status. The presence of mutations in *EGFR* exons 18-21 was analyzed, and the overall mutation rate was 44.10% (157/356). The mutation frequencies in the males and females were 37.61 (79/210) and 53.42% (78/146), respectively. There was a significant difference (P=0.0034) between males and females as regards the *EGFR* mutation frequency (Table II).

The *EGFR* mutations detected were further classified into three types, i.e., activating (TKI-sensitive) mutations, TKI-resistant mutations and mutations that were not associated with TKI response. The frequencies of the three types of mutations were 88.54 (139/157), 3.18 (5/157) and 8.28% (13/157), respectively.

The activating *EGFR* mutations most frequently occurred in exon 19, comprising 57.56% (80/139) of all the activating mutations, followed by 35.25% (49/139) point mutations in exon 21 (p.L858R and p.L861Q). Ten types of deletion/insertion in exon 19 detected in the study are summarized in Table III. Of note, three patients harboring specific mutations of p.H835L and p.H838V in *EGFR* exon 21 were detected. Four patients (2.87%, 4/139) with a point mutation (p.G719S or p.G719A) in exon 18 and three patients (2.16%, 3/139) with double mutations in exons 19 and 21 (p.E746_A750del & p.L858R) were found as activating *EGFR* mutations (Fig. 1 and Table III).

Five TKI-resistant mutations, including p.T790M, p.D770_N771insG, p.H773_V774insPH, p.V774_C775insPR, and double mutations in exons 20 and 21 (p.T790M and p.L858R) were detected. In addition, 13 *EGFR* mutations, which did

Table I. Clinical characteristics of the lung cancer patients.

Characteristics	No. of patients (total, 356)	Frequency (%)
Age (years)		
Median (range)	57.5 (27-88)	
>60	195	54.77
≤60	161	45.23
Gender		
Male	210	58.99
Female	146	41.01
Smoking history		
Current smoker	162	45.51
Non-smoker	173	48.60
Unknown	21	5.89
Histological subtype		
Adenocarcinoma	173	48.60
Squamous	56	15.73
Other	127	35.67

Table II. Mutation rates between male and female NSCLC patients.

Gender	All n (%)	Wild-type n (%)	Mutation n (%)	P-value
Male	210 (58.99)	131 (65.83)	79 (50.32)	0.0034[a]
Female	146 (41.01)	68 (34.17)	78 (49.68)	

[a]Statistically significant. NSCLC, non-small cell lung cancer.

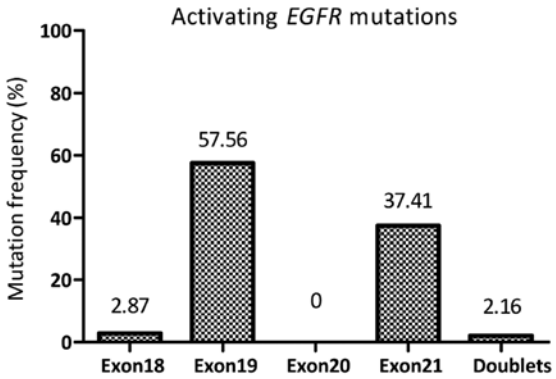

Figure 1. Bar chat showing the distribution of activating mutations in exons 18-21. EGFR, epidermal growth factor receptor.

Table III. Molecular characteristics of the 157 NSCLC patients with *EGFR* mutations.

Exon	Mutation type	Nucleotide change	Amino acid change	No.	TKI response
18	Missense	c.2152_2153CT>TA	L718*	1	U
		c.2155G>A	p.G719S	2	S
		c.2156G>C	p.G719A	2	S
	Deletion/insertion	c.2127_2129delAAC	p.E709_T710del	1	U
19	Missense	c.2186G>A	p.G729E	1	U
		c.2227G>A	p.A743T	1	U
		c.2239_2240TT>CC	p.L747P	1	U
		c.2255C>T	p.S752F	1	U
	Deletion/insertion	c.2235_2249del15	p.E746_A750del	56	S
		c.2236_2250del15	p.E746_A750del	13	S
		c.2237_2253del17insTTGCT	p.E746_T751delinsVA	1	S
		c.2237_2255del19insT	p.E746_S752delinsV	1	S
		c.2238_2248del11insGC	p.E746_A750delinsEP	1	S
		c.2239_2250del12insCCG	P.L747_A750delinsP	1	S
		c.2239_2248del10insC	p.L747_A750delinsP	1	S
		c.2240_2248del9	p.L747_A750del	1	S
		c.2240_2254del15	p.L747_T751del	2	S
		c.2240_2257del18	p.L747_T753del	3	S
20	Missense	c.2230T>A	p.L777Q	1	U
		c.2345_2346CT>AA	p.L782N	1	U
		c.2369C>T	p.T790M	1	R
	Deletion/insertion	c.2310_2311insGGT	p.D770_N771insG	1	R
		c.2319_2320insCCCCAC	p.H773_V774insPH	1	R
		c.2322_2323insCCACGT	p.V774_C775insPR	1	R
21	Missense	c.2483T>A	p.L828*	1	U
		c.2506C>A	p.R836S	1	U
		c.2573T>G	p.L858R	46	S
		c.2582T>A	p.L861Q	3	S
		c.2591C>A	p.A864E	1	U
		c.2597A>T	p.E866V	1	U
		c.2602G>A	p.E868K	1	U
		c.2497T>G & c.2504 A>T	p.H838V and p.H835L	3	S
Doublets		c.2235_2249del15	p.E746_A750del	3	S
		c.2573T>G	p.L858R		
		c.2235_2249del15	p.E746_A750del	1	R
		c.2615G>T	p.T790M		

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer. S, TKI-sensitive; R, TKI-resistant; U, unknown.

not correlate with TKI response, are listed in Table III. These were L718* and p.E709_T710del in exon 18, point mutations (p.G729E, p.A743T, p.L747P and p.S752F) in exon 19, point mutations (p.L777Q and p.L782N) in exon 20, and point mutations (p.L828*, p.R836S, p.A864E, p.E866V and p.E868K) in exon 21.

Correlation between EGFR mutation status and smoking history. Among the 210 male patients, 162 were current smokers, 27 were non-smokers, and 21 had an uknown smoking history (Tables I and IV). All 146 female patients were non-smokers. Overall, the rate of *EGFR* mutations was significantly decreased in the smokers (38.27%, 62/162) compared with the non-smokers (50.87%, 88/173, P=0.0141). The mutation rate was significantly higher in the female non-smokers than in the male smokers (53.42%, 78/146 vs. 38.27%, 62/162, P=0.0085). Among the non-smokers, the *EGFR* mutation rates were comparable in the male and female patients (53.42%, 78/146 vs. 37.04%, 10/27, P=0.1439). Among the male patients, no statistically significant difference was observed in the non-smokers, smokers and patients with an unknown smoking history (37.04%, 38.27% and 33.33%; Table IV).

Table IV. Mutation rates between smokers and non-smokers.

	All n (%)	Wild-type n (%)	Mutation n (%)	P-value
Non-smoker	173 (48.60)	88 (56.05)	85 (42.71)	0.0141[a]
Males	27 (7.59)	17 (8.54)	10 (6.37)	
Females	146 (41.01)	68 (34.17)	78 (49.68)	
Smoker	162 (45.51)	62 (39.49)	100 (50.25)	
Males	162 (45.51)	100 (50.25)	62 (39.49)	
Females	0	0	0	
Unknown	21 (5.89)	7 (4.46)	14 (7.04)	
Males	21 (5.89)	14 (7.04)	7 (4.46)	
Females	0	0	0	

[a]Statistically significant.

Table V. Association of activating mutation rates with histological subtypes of NSCLC.

Histological subtype	Total	Frequency (%)	Mutation rate (%)	Mutation
Adenocarcinoma	173	48.60	88	50.87
Squamous cell	56	15.73	14	25.00
Large cell	11	3.09	3	27.27
Adenosquamous	8	2.25	4	50.00
Alveolar cell	6	1.69	5	83.33
Adenocarcinoma and alveolar cell	5	1.40	1	20.00
Squamous and large cell	2	0.56	1	50.00
Neuroendocrine	2	0.56	0	0
Adenocarcinoma and large cell	2	0.56	0	0
Sarcomatodes	1	0.28	0	0
Adenoid cystic	1	0.28	0	0
Poorly differentiated	89	25.00	23	25.84
Total	356	100.00	139	39.04

NSCLC, non-small cell lung cancer.

Correlation between EGFR mutations and histological parameters. The overall rate of activating *EGFR* mutations was 39.04%, with 50.87% (88/173) in the adenocarcinoma and 25.00% (14/56) in the squamous cell carcinoma samples. There was a significant difference in the *EGFR* mutation rate between adenocarcinoma and squamous cell carcinoma (P=0.0004). The prevalence of activating *EGFR* mutation rates in other subtypes of NSCLC are summarized in Table V. It should be noted that the activating *EGFR* mutations often occurred in NSCLC with the adenosquamous carcinoma (50.00%, 4/8) and alveolar cell carcinoma subtypes (83.33%, 5/6). In addition, 25.84% (23/89) of *EGFR* mutations were detected in poorly differentiated NSCLC. Although the difference was not statistically significant, the activating *EGFR* mutations occurred more frequently in females compared to males with the adenocarcinoma subtype (56.18% and 50/89 vs. 45.24% and 38/84, P=0.1722; Table VI).

Table VI. Comparison of the activating mutation rate between males and females with adenocarcinoma.

Adenocarcinoma	All n (%)	Wild-type n (%)	Mutation n (%)	P-value
Males	84 (58.99)	44 (55.00)	38 (50.32)	0.1722
Females	89 (41.01)	36 (45.00)	50 (49.68)	

Between the smoking and non-smoking male patients with the adenocarcinoma subtype, the rate of activating *EGFR* mutations was similar (48.44 vs. 50.00%, P=0.5814). Of note, the activating *EGFR* mutations occurred more frequently in females compared to males with the squamous cell carcinoma subtype (40.00 vs. 23.68%, P=0.4253; Table VII).

Correlation between EGFR mutation status and sample type. Among all the samples analyzed, 112 (31.46%) were bronchoscopic biopsies, 224 (62.93%) were surgical resections and 20 (5.61%) were pleural effusion cytological samples. The pleural effusion cytological samples were collected from patients diagnosed with NSCLC and confirmed by a pathologist to contain tumor cells. The corresponding activating *EGFR* mutation rates were 45.00, 30.36 and 42.86%, respectively (Table VIII). It is important to note that the rate of *EGFR* mutations identified from the bronchoscopic biopsies was lower than that from the surgical resections or pleural effusion, and was also lower than the overall rate. A statistically significant difference in the *EGFR* mutation rate in the bronchoscopic biopsies and surgical resections was observed (P=0.0425).

Detection of EGFR mutations by different methods. A total of 86 samples was successfully analyzed by PCR amplification followed by direct sequencing. Direct sequencing identified *EGFR* mutations in 34 (39.53%) samples. The *EGFR* mutation rates in pleural effusion, bronchoscopic biopsies and surgical resections were 14.29 (1/7), 30.00 (3/10) and 43.47% (30/69), respectively. Although there was no statistically significant difference, *EGFR* mutations were more frequently identified in the surgical resections than the other two types of samples by direct sequencing.

A total of 120 samples was analyzed by ARMS assay and 43 (35.83%) were identified as *EGFR* mutation-positive. The *EGFR* mutation rates detected by ARMS were 55.56 (5/9), 30.30 (20/66) and 40.00% (18/45) for the individual sample types. A total of 150 samples was analyzed by HRM assay followed by sequencing verification. HRM combined with sequencing verification detected *EGFR* mutations in 62 (41.33%) of the analyzed samples. The *EGFR* mutation rates were 75.00 (3/4), 30.56 (11/36) and 43.64% (48/110) for the three sample types (Table IX). Taken together, for the tissue samples, such as bronchoscopic biopsy and surgical resection, the rate of *EGFR* mutations detected by any of the three methods was comparable. For detecting mutations in pleural

Table VII. Comparison of the activating mutation rate between males and females, and smokers and non-smokers.

	Males			
	Smokers	Non-smokers	Unknown	Females
Adenocarcinoma				
Total	64	15	5	89
Mutation	31	6	1	50
Mutation rate (%)	48.44	50.00	20.00	56.18
Squamous cell carcinoma				
Total	38	3	5	10
Mutation	9	0	1	4
Mutation rate (%)	23.68	0	20.00	40.00

Table VIII. Comparison of the activating mutation rate among different sample types.

	All n (%)	Wild-type n (%)	Mutation n (%)	Mutation rate (%)	P-value
Pleural effusion	20 (5.61)	11 (5.53)	9 (6.47)	45.00	0.8158
Bronchoscopic biopsies	112 (31.46)	71 (35.17)	34 (24.46)	30.36	0.0425[a]
Surgical resections	224 (62.93)	117 (59.30)	96 (69.07)	42.86	0.0618

[a]Statistically significant.

effusion, ARMS and HRM assays were possibly superior to direct sequencing. Nevertheless, it should be noted that no statistically significant difference between direct sequencing and ARMS assay (P=0.1451) or direct sequencing and HRM assay (P=0.0879) with respect to the mutation frequency was obtained due to the limited sample numbers. Regardless of the method used, the *EGFR* mutation rate detected in the bronchoscopic biopsies was the lowest.

Method correlation with sequencing. The *EGFR* mutation status of 114 samples detected by ARMS assay using the AmoyDx™ EGFR 29 Mutations Detection kit (Amoy Diagnostics Co.) was tested again by direct sequencing. We were unable to perform additional sequencing for six samples due to insufficient gDNA. Forty *EGFR* mutation-positive and 44 *EGFR* mutation-negative samples reached a consensus in the two methods (Table X). The concordance rate between the two methods was 73.68%.

Fifteen *EGFR* mutation-positive samples detected by ARMS assay were found to be *EGFR* mutation-negative by sequencing. It is important to note that another 15 *EGFR* mutation-negative samples detected by ARMS assay were found to be *EGFR* mutation-positive by sequencing. Direct sequencing identified 11 rare mutations that were not designed to be detected by ARMS assay, including p.E709_T710del, p.G729E, p.G729V, p.L747P, p.A864E and p.E866V. A discrepancy was observed in another four samples between the two methods. The sensitivity of ARMS assay was 72.73% and the specificity 74.58%.

A total of 150 samples was tested by HRM assay for the detection of mutations in *EGFR* exons 18-21. HRM assay detected more positive samples than sequencing and detected 97 samples as positive for mutations. Among these, 68 samples were confirmed as positive by sequencing and 29 samples were not confirmed, which were possibly false positives. Most of these are likely to be true false positives due to degraded DNA extracted from FFPE specimens. Fifty samples were detected as negatives by both methods used. Three samples that were detected as *EGFR* mutation-negative by HRM were detected as *EGFR* mutation-positive by sequencing (Table XI). The sensitivity and specificity for the samples that were suspected of having mutations by HRM assay was 95.77 and 63.29% when compared to sequencing, with an accuracy rate of 78.67%. For each patient, *EGFR* mutation testing was carried out on the same gDNA, avoiding the inconsistency potentially resulting from intra-tumor heterogeneity. Collectively, the discordance was 26.32% between direct sequencing and ARMS and was 21.33% between direct sequencing and HRM.

Sensitivity testing by direct sequencing, ARMS and HRM. The gDNA of the PC-9 cells was serially diluted into A549 gDNA at ratios of 100, 40, 20, 10 and 2% to yield mutant allele frequencies of 50, 20, 10, 5 and 1%. The relative sensitivity of direct sequencing, ARMS and HRM was evaluated using the diluted DNA. The mutation was detectable (at a low peak) by direct sequencing when the mutant frequency was higher than 10%. However, when the mutation frequency was at 5%, it was only distinguishable from the background.

Table IX. Comparison of the activating mutation rate detected by different methods in different sample types.

	All		Pleural fluid			Bronchoscopic biopsies			Surgical resections		
Method	Total	Rate (%)	Total	Mutation	Rate (%)	Total	Mutation	Rate (%)	Total	Mutation	Rate (%)
Sequencing	86	39.53	7	1	14.29	10	3	30.00	69	30	43.47
ARMS	120	35.83	9	5	55.56	66	20	30.30	45	18	40.00
HRM and Se	150	41.33	4	3	75.00	36	11	30.56	110	48	43.64

ARMS, amplification refractory mutation system; HRM, high resolution melting; HRM and Se, HRM and sequencing.

Table X. Comparison of the results of the *EGFR* mutation analysis between ARMS and direct sequencing.

Mutation status	Sequencing +	Sequencing -	Total
ARMS			
+	40	15	55
-	15	44	59
Total	55	59	114

EGFR, epidermal growth factor receptor; ARMS, amplification refractory mutation system.

Table XI. Comparison of the results of the *EGFR* mutation analysis between HRM and sequencing.

Mutation status	Sequencing +	Sequencing -	Total
HRM			
+	68	29	97
-	3	50	53
Total	71	79	150

EGFR, epidermal growth factor receptor; HRM, high resolution melting.

When the mutant frequency was below 5%, the mutation was not detectable (Fig. 2A). ARMS assay positively detected the deletions in *EGFR* exon 19 in the sample containing down to 1% mutant allele frequency (Fig. 2B). Using HRM, the melting curve from 1% mutant template sufficiently differed from wild-type template (Fig. 2C), and this distinct melting profile was consistently observed across all other templates measured (5, 10, 20 and 50%). Thus, the sensitivity of direct sequencing, ARMS and HRM was found to be 10, 1 and 1%, respectively.

Discussion

Based on the observations from IPASS and other studies (14,15), the ASCO provisional clinical opinion (PCO) states that 'patients with advanced NSCLC who are being considered for first-line therapy with an EGFR-TKI should have their tumor tested for *EGFR* mutations to determine which is an appropriate therapy: an EGFR-TKI or chemotherapy' (7,16). Therefore, evaluating the *EGFR* mutation status is a matter of urgency in clinical practice, particularly in patients with adenocarcinoma.

As a matter of fact, the selection of patients for EGFR-TKI therapy based on mutation analysis is not an absolute warranty for good response and approximately 20-30% of patients harboring activating *EGFR* mutations do not benefit from TKI treatment (17). The presence of TKI-resistant or increased copy number (amplification) of the *MET* oncogene contributes to resistance to TKI. Furthermore, the low abundance of *EGFR* mutations affects the response to TKI (18). False positives due to the methodology used for mutation detection should not be neglected, particularly when an extremely sensitive test is performed. On the other hand, a proper interpretation of negative results requires a thorough understanding of the technical limitations of the assay and the type of specimen used for mutation detection. One of the possible reasons that patients without activating *EGFR* mutations respond to an EGFR-TKI is the false negatives (13). For example, as previously demonstrated, five out of 50 patients with advanced NSCLC had discrepancies in the results of mutant-enriched PCR, peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR and PCR clamp. All five patients were false-negative as they responded to gefitinib (19).

In the present study, the prevalence of *EGFR* mutations was higher in females than in males and the frequency of activating *EGFR* mutations (39.04%) was similar to that described in earlier studies conducted on patients with advanced NSCLC in an East Asian group (20). Two types of mutations, the short in-frame deletions in exon 19 (particularly p.E746_A750del) and the point mutation in exon 21 (c.2573T>G, p.L858R) comprised up to 90% of mutations. This is also in line with what has been previously described (3,4,21,22). Low-frequency mutations in exon 18 and exon 21, such as p.G719X and p.L861Q, were also found. Of note, three cases carrying the complex mutations of p.L833V and p.H835L in exon 21 were detected in this study. The occurrence frequency of these types of mutation (2.16%, 3/139) was equivalent to that of p.L861Q. A good response to EGFR-TKI therapy has been reported in one patient harboring the p.L833V and p.H835L mutations (23); therefore, this specific type of mutation was considered one of the activating *EGFR* mutations. The correlation of p.L833V and p.H835L mutations with TKI response requires further

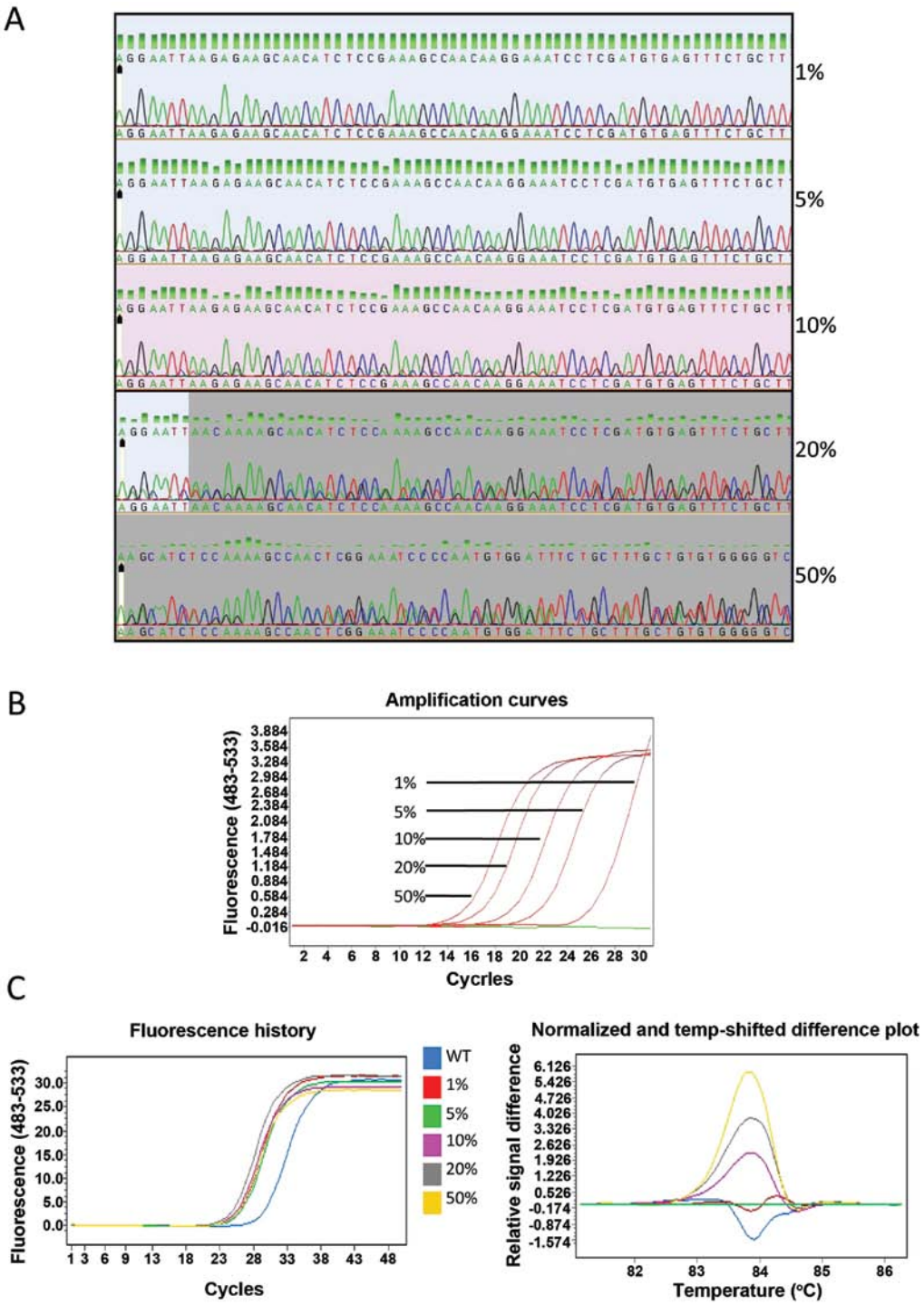

Figure 2. Sensitivity testing for epidermal growth factor receptor (*EGFR*) mutations using serial dilutions of PC-9/A549 DNA. (A) Direct sequencing. At least 10% mutant DNA was necessary to detect *EGFR* mutations. (B) Amplification refractory mutation system (ARMS). One percent mutant DNA was ready to be indentified from wild-type DNA. (C) High resolution melting (HRM). 1% mutant DNA was ready to be plotted differently from wild-type DNA.

clinical investigation. In addition, 13 mutations that were not associated with TKI response were detected in treatment-free samples.

All the females with advanced NSCLC in this study were non-smokers and the *EGFR* mutation rate was significantly higher in the non-smokers than in the smokers, which is in line with previous studies reporting that *EGFR* mutations were more frequently detected in patients without a smoking history (5,15). A recent meta-analysis demonstrated that non-smokers with NSCLC show a statistically significant increase in the prevalence of somatic *EGFR* mutations (20). The activating *EGFR* mutations were more often detected in patients with adenocarcinoma than in patients with squamous cell carcinoma. Among the adenocarcinoma patients, the prevalence of activating *EGFR* mutations was modestly higher in females than in males; however, this difference was not statistically significant. Importantly, among the male patients with adenocarcinoma, no difference was observed in the muta-

tion rate between smokers and non-smokers. Almost all the male patients diagnosed with squamous cell carcinoma were smokers, and the frequency of activating *EGFR* mutations was much higher in the females (non-smokers) than in the male smokers. Only three males with squamous cell carcinoma were non-smokers and were unable to be analyzed statistically. Thus, we suggest that adenocarcinoma, particularly in females, is a valuable predictive factor for the occurrence of *EGFR* mutations. A smoking history largely affected the *EGFR* mutation occurrence in patients with squamous cell carcinoma rather than adenocarcinoma.

In order to carry out a routine *EGFR* mutation screening in clinical practice, good quality DNA in sufficient quantity, including tumor content (particularly for cytological material), and the most reliable method in terms of sensitivity and specificity are an absolute requirement. Resected tumor tissues are preferred, but they are not always available. Small biopsy samples and cytological material, including that obtained from pleural effusion, are increasingly used in clinical practice. In the present study, three types of samples, i.e., surgical resections, bronchoscopic biopsies and pleural effusion, were tested for *EGFR* mutations. We found that the lowest frequency of activating *EGFR* mutations was observed in the bronchoscopic biopsies (30.36%). In clinical practice, it is not possible to obtain both bronchoscopic biopsies and surgical sections from the same patient. In the present study, the bronchoscopic biopsies are obtained from advanced NSCLC patients with unresectable tumors, while the surgical sections were obtained from NSCLC patients, at early clinical stage, who had received surgical therapy. However, the clinical features, including pathological type and gender (data not shown), that significantly affected the *EGFR* mutation frequency in the sample sets from the bronchoscopic biopsies and surgical sections were comparable. The mutation rate in pleural effusion (45.00%) was similar to that in surgical resections (42.86%; Table VIII). Our observations are in accordance with those from previous studies on the detection of *EGFR* mutations in cytological samples (5,24). Five methods, including PCR-Invader, PNA-LNA PCR clamp, direct sequencing, cycleave PCR and ARMS, show a comparable performance in the assessment of tissue and cytology samples. Cytology-derived DNA is a suitable alternative to FFPE samples and very useful when FFPE samples are unavailable for molecular analysis (5,24).

Multiple sensitive techniques are employed as alternatives to direct sequencing. Using cell lines with heterozygous *EGFR* mutations, we found that the sensitivities of direct sequencing, ARMS and HRM in our experimental setting were 10, 1 and 1%, respectively. The overall mutation rate detected by ARMS assay was the lowest (35.83%), and the mutation rate detected by direct sequencing (39.53%) was similar to that detected by HRM assay (41.33%; Table IX). The clinical characteristics of the samples tested by the three methods, which significantly affected the *EGFR* mutation frequency, including pathological type and gender (data not shown), were comparable. In the 224 surgical resections, the difference observed in the mutation rates (43.47, 40.00 and 43.64%) detected by the three methods (sequencing, ARMS, and HRM and sequencing together, respectively) was not considerable. In the 112 bronchoscopic biopsies, the mutation rates detected by the three methods (30.00, 30.30 and 30.56%) were almost the same and clearly lower than those detected in surgical resections. In the 20 pleural effusion samples, the mutation rate detected by direct sequencing was the lowest (14.29%), and the mutation rates detected by ARMS (55.56%) and HRM assay (75.00%) were even higher than the average rate of all samples (39.04%) (Table IX). Collectively, sensitive methods, i.e., ARMS and HRM, were not superior to direct sequencing in surgical resections and bronchoscopic biopsies in terms of mutation detection frequency in this study. The possible reasons for this were the quantity control of tumor content (>20%) in FFPE sections by H&E staining and improved sensitivity (10%) of direct sequencing by optimizing all reaction conditions. It is also important to note that the prevalence of *EGFR* mutations detected in bronchoscopic biopsies using any of the three methods was the lowest. Bronchoscopic biopsies usually contain smaller amounts of tissue than surgical resections due to the limited tissue size; they also provide relatively inadequate information a molecular evaluation due to tumor heterogeneity. The amount of DNA extracted from small biopsy specimens varies significantly, depending on the size of the material, the tumor viability, etc. The minimum amount of DNA extracted from FFPE samples for direct sequencing, ARMS and HRM assay are 300, 100 and 150 ng, respectively. Therefore, ARMS assay is preferred when the sample DNA is extremely low (4,13).

In addition, it was necessary to detect *EGFR* mutations in pleural effusion, using a sensitive technique, such as ARMS or HRM. The findings of the present study were consistent with those of several other studies. ARMS assay is more sensitive in detecting *EGFR* mutations than direct sequencing in cytological samples from transbronchial needle aspirates or pleural effusion (25,26). Other methods, including pyrosequencing and HRM, have been reported (27,28). A recent review evaluating 33 studies using cytological samples for *EGFR* mutation testing suggested that the use of sensitive methods is warranted when cytological samples with low-tumor content are used (5).

In the present study, *EGFR* mutations were identified in the same gDNA using two methods with low and high sensitivity concomitantly. The concordance rate between direct sequencing and ARMS assay was 73.68%. The discordance found in the mutation status in direct sequencing and ARMS may be explained by the different degree of of sensitivity, particularly for identifying a low abundance of mutations. A total of 11 (out of 15) mutations detected by direct sequencing were not detected by ARMS, as the ARMS assay used was not designed to detect these rare *EGFR* mutations. Clinical data on less common mutations are being increasingly gathered. However, further research on the analysis of predictable outcomes on TKI response is required (23,29). An analysis of another four mutations, which were detected only via direct sequencing, was not carried out using ARMS due to insufficient materials. The observations of the present study were in accordance with those of other studies that compare direct sequencing with ARMS. Compared with direct sequencing, 10-20% of mutations are missed by ARMS, but 20% of mutations detected by ARMS at low levels are missed by direct sequencing (8,30,31). In another study, 32% of tumors carrying activating *EGFR* mutations detected by direct sequencing are missed by the commercially available ARMS kit. The percentage of missed mutations is too

high to recommend the use of ARMS for diagnostic application (32).

The sensitivity of HRM in the present study was 95.77%, which is similar to that found in other studies (10,33,34). The concordance rate between HRM and sequencing was 78.67%. The difference in sensitivity was one of the reasons for the discrepancy. In addition, any DNA alteration due to the interference of single nucleotide polymorphism (SNP) or formalin fixation may produce an abnormal melting curve (35). The high rate of false positives in FFPE samples indicates that an additional sequencing should be performed.

Given the respective limitations of the currently available testing methodologies, several laboratories tend to use a combination of methodologies (5,30). In a previous study, we also proposed a sequential detection workflow using ARMS assay and/or direct sequencing (13). Thus, each method compensates for the disadvantages of the other and reduces the frequency of false negatives.

In conclusion, we recommend that the choice of method should be made based on the sample type. An analysis of samples obtained at the diagnostic stage, e.g., bronchoscopic biopsies, should be performed using the ARMS assay for the detection of mutations due to the limited amount of DNA extracted from small biopsy specimens. A sensitive method, such as ARMS, is necessary when mutations in cytological samples, such as those obtained from pleural effusion, need to be detected. The choice of method used for mutation detection in samples from surgical resections is largely based on the expertise of the laboratory, but direct sequencing is highly recommended. However, the low detection rate of *EGFR* mutations by direct sequencing is possibly due to limited sensitivity. The absence of *EGFR* mutations, determined by methods that detect known mutations, such as ARMS, cannot be the exclusion criterion for EGFR-TKI usage. Therefore, we suggest performing two methods (direct sequencing and a sensitive method) sequentially in clinical practice, due to the presence of non-neglected discordance between any method from its own benefits and drawbacks. In the future, we may be able to benefit from the incorporation of next-generation sequencing into daily clinical practice.

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