

Detection of epidermal growth factor receptor mutations in lung adenocarcinoma cytological specimens by immunocytochemistry

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Abstract. Tyrosine kinase inhibitors of epidermal growth factor receptor (EGFR) improve the survival of patients with lung adenocarcinoma, and determine the EGFR mutation status before treatment is necessary. In contrast to biopsy samples, cytological specimens are obtained less invasively and are useful for EGFR mutation analyses. Recently, novel antibodies against two major EGFR mutations were developed: SP111, which is specific for the E746-A750 deletion in exon 19; and SP125, which is specific for the L858R mutation. To the best of our knowledge, no study has evaluated cytological specimens using the two novel antibodies, thus their specificity and sensitivity were examined in surgical resection, and cytological lung adenocarcinoma samples in the present study. Previous screening for EGFR mutation status by molecular testing identified delE746-A750 in 3 cases and the L858R mutation in 7 cases; the other cases did not have the L858R or the delE746-A750 mutation. Using a four-grade scoring system (score 0 to 3+), the immunohistochemistry (IHC) and immunocytochemistry (ICC) results were compared with those of molecular testing. Using a score of ≥ 2 as positive, IHC and ICC using SP111 demonstrated sensitivities of 100 and 33.3%, and specificities of 100 and 100%, respectively. IHC and ICC using SP125 revealed sensitivities of 100 and 71.4%, and specificities of 100 and 100%, respectively. Therefore, screening for EGFR mutations by ICC may facilitate therapeutic decision-making, particularly in medical centers that are unable to perform molecular testing.

Introduction

Lung cancer is the leading cause of cancer-related mortality and is responsible for approximately 1.59 million deaths

annually worldwide (1). Non-small cell lung carcinoma (NSCLC) accounts for more than 80% of lung cancer cases, with adenocarcinoma (ADC) being the most common histological subtype. Detection of activating somatic mutations in the epidermal growth factor receptor (*EGFR*) gene in ADC (2-5) has revolutionized diagnosis and treatment and resulted in a shift towards individual targeted therapy (2). Although several mutations can occur in exons 18-21 of the tyrosine kinase domain of EGFR, approximately 90% are the 15 bp (E746-750) in-frame deletion in exon 19 or the L858R point mutation in exon 21 (3,4,6). Mutational status is associated with a clinical response to EGFR-tyrosine kinase inhibitor (TKI) treatment (2-7). EGFR-TKIs are more efficacious than carboplatin-paclitaxel as an initial treatment (8).

Direct DNA sequencing is the classical method used to detect EGFR mutations; however, this method requires a sufficient quantity of DNA. In addition, the high costs of equipment and reagents, and the time-consuming procedure, hamper its application in clinical practice. To overcome these issues, various molecular tests for EGFR mutations have been developed, such as the Scorpion amplified refractory mutation system and polymerase chain reaction single-strand conformation polymorphism (9,10). However, many patients in developing countries do not undergo molecular testing due to the lack of available equipment and trained personnel. Immunohistochemistry (IHC) is an inexpensive and accurate method of identifying EGFR mutations and is available in most pathology laboratories. Antibodies specific for the exon 19 deletion E746-A750 (delE746-A750) and the L858R mutation have been developed to assess tumor EGFR status (11-20). A meta-analysis recommended that IHC but not molecular testing is suitable for detection of EGFR mutations (21).

As approximately 70% of NSCLC patients are diagnosed at an advanced stage and are thus ineligible for surgical resection (22,23), therapeutic decision-making is dependent on the availability of biopsy samples or cytology specimens. Compared with biopsy samples, obtaining cytological specimens is minimally invasive and simple. Several studies have evaluated cytological specimens using immunocytochemistry (ICC) with EGFR mutation-specific antibodies (18,24-26); however, the results were inconsistent (sensitivity 43-100% and specificity 74-100%) (18,24-26). Two novel antibodies against EGFR-mutated proteins were developed recently. SP111 is specific for delE746-A750 and SP125 for the L858R mutation;

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both SP111 and SP125 show an efficacy similar to that of the original clone (27-29). To date, no study has assessed the utility of SP111 or SP125 for cytological specimens. Here, we examined the specificity and sensitivity of IHC and ICC using the SP111 and SP125 antibodies in surgical and cytological samples from patients with lung adenocarcinoma.

Patients and methods

Clinical samples and DNA analysis. This study involved 17 patients with pulmonary adenocarcinoma from whom surgical and cytological samples were obtained from January 2015 to March 2017 at Osaka University Medical Hospital. Formalin-fixed paraffin-embedded (FFPE) tissue sections (5 μ m) were prepared. DNA extraction from FFPE samples was performed according to the standard procedure of the Cobas DNA Sample Preparation kit (Roche Molecular Systems, Inc., Alameda, CA, USA). Briefly, the samples were incubated with a protease in chaotropic lysis/binding buffer to release nucleic acids and protect genomic DNA from degradation by DNase. The amount of genomic DNA was spectrophotometrically determined (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE, USA) and adjusted to 2 ng/ μ l. DNA (150 ng) was obtained for the Cobas EGFR assay. Target DNA was amplified and detected using the Cobas 4800 Analyzer (Roche Molecular Systems, Inc.) according to the manufacturer's instructions.

The study protocol was approved by the Research Ethics Board of the Osaka University Research Committee and conducted according to Institutional Review Board guidelines (cat. no. 16293).

IHC and ICC of EGFR mutations. FFPE tissue sections (5 μ m) were stained immunohistochemically using the SP111 anti-EGFR delE746-A750 rabbit monoclonal antibody and SP125 anti-EGFR L858R rabbit monoclonal antibody (Ventana Medical Systems, Tucson, AZ, USA) and an automatic staining system (Ventana BenchMark XT; Ventana Medical Systems). Briefly, the sections were incubated with SP111 or SP125 for 16 min at 37°C, and immunoreactions were detected using the Ultraview Universal DAB detection kit. The negative control lacked a primary antibody. The slides were assessed by at least two pathologists blinded to the EGFR mutation status. The IHC staining was scored based on the staining intensity and percentage staining area in the membrane and/or cytoplasm of tumor cells, as follows (18): 0 no staining; 1+, light-yellow staining with no obvious particulates or yellow staining with obvious particulates in <10% of tumor cells; 2+, yellow staining with obvious particulates in >10% of tumor cells or brown staining with obvious particulates in <10% of tumor cells; and 3+, brown staining with obvious particulates in >10% of tumor cells. Scores of 2+ and 3+ were considered positive (18).

A cell-transfer technique was used for ICC. A layer of Malinol medium (Muto Pure Chemicals Co. Ltd., Tokyo, Japan) was spread uniformly over the top of the cellular material. The slide was placed into a 70°C oven for 72 h to harden the coating material and then transferred to a water bath at 50°C for 15 min to soften the Malinol medium. Using a blade, the liquid coverslip and attached cells were slowly peeled off

the slide. The peeled membrane was sectioned into 3-5 pieces. Each section was transferred to another slide, on which ICC was performed using a similar method to that of IHC. Staining was evaluated as for IHC (18).

Statistical analysis. Statistical analyses were performed using R version 3.2.2 (<https://cran.r-project.org/bin/windows/base/>). Antibody performance was assessed by determining the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The agreement between IHC or ICC and molecular testing was calculated using Cohen's kappa score. A κ value of 0.81-1.0 was defined as nearly perfect agreement, 0.61-0.8 as substantial agreement, 0.41-0.60 as moderate agreement, 0.21 to 0.40 as fair agreement, and 0.00-0.20 as slight agreement.

Results

Patient characteristics. Of the 192 patients with pulmonary adenocarcinoma who underwent surgical resection from January 2015 to March 2017 at Osaka University Medical Hospital, cytological specimens were obtained from 17 patients. The clinicopathological characteristics of the patients are shown in Table I. The cytologic specimens comprised exfoliative (bronchial brush/wash/lavage) (n=9), transbronchial fine-needle aspiration (FNA; n=7), and trans-thoracic FNA (n=1) specimens. The age of the patients was 53-77 years (median, 67.2 years). Twelve and five of the patients were male and female, respectively. Six cases were stage IA, seven stage IB, two stage IIA, and two stage IIIA. All the cases had previously undergone screening for EGFR mutations by molecular testing. Exon 19 deletion (delE746-A750) was present in three patients and the L858R mutation in exon 21 in seven patients; the other patients had neither the L858R mutation nor delE746-A750 (Table I).

IHC using SP111 and SP125. IHC using SP111 and SP125 yielded scores of 0 to 3+ (Figs. 1 and 2). Using SP111, 13 of 17 cases showed a score of 0, one a score of 1+, one a score of 2+, and two a score of 3+ (Table I). Using SP125, 7 of 17 cases showed a score of 0, three a score of 1+, four a score of 2+, and three a score of 3+ (Table I). The three and seven cases with a score of 2+ or 3+ using SP111 and SP125, respectively, were evaluated as SP111- and SP125-positive, respectively, as described previously (18) (Table II).

ICC using SP111 and SP125. ICC using SP111 and SP125 yielded four scores (Figs. 3A-C and 4A-D). Using SP111, 13 of 17 cases showed a score of 0, three a score of 1+, and one a score of 2+. Using SP125, 6 of 17 cases showed a score of 0, six a score of 1+, two a score of 2+, and three a score of 3+. The one case with a score of 2+ using SP111 and the five cases with a score of 2+ or 3+ using SP125 were evaluated as SP111- and SP125-positive, respectively (Table III).

EGFR mutations. The seven cases without EGFR mutations were negative by IHC using SP111 and SP125. The three cases with delE746-A750 were positive using SP111 and negative using SP125. Similarly, the seven cases with the L858R mutation were positive using SP125 and negative using SP111.

Table I. Patient characteristics and EGFR mutations status in primary NSCLC samples and comparative analysis of IHC and ICC.

Patient no.	Age, years	Sex	Stage	EGFR mutation status	IHC		ICC		
					SP111	SP125	Sample type	SP111	SP125
1	60	F	IB	L858R	0	3+	BB	0	3+
2	77	M	IB	L858R	0	2+	FNA	0	1+
3	53	F	IIA	L858R	0	2+	FNA	0	3+
4	71	M	IIA	L858R	0	3+	BB	1+	3+
5	59	F	IA	L858R	0	2+	FNA	0	2+
6	76	M	IA	L858R	0	2+	FNA	0	1+
7	61	M	IB	L858R	0	3+	FNA	1+	2+
8	72	M	IA	Exon 19 del	2+	0	BB	0	0
9	70	F	IA	Exon 19 del	3+	1+	FNA	0	0
10	76	M	IB	Exon 19 del	3+	0	FNA	2+	0
11	69	M	IIIA	No mutation	0	0	BB	0	0
12	72	M	IIIA	No mutation	0	0	BB	0	0
13	77	M	IB	No mutation	0	0	FNA	1+	1+
14	67	M	IA	No mutation	1+	0	BB	0	0
15	53	M	IB	No mutation	0	0	BB	0	1+
16	54	F	IA	No mutation	0	1+	BB	0	1+
17	75	M	IB	No mutation	0	1+	BB	0	1+

BB, bronchial brush; FNA, fine-needle aspiration; M, male; F, female; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; ICC, immunocytochemistry; NSCLC, non-small cell lung carcinoma.

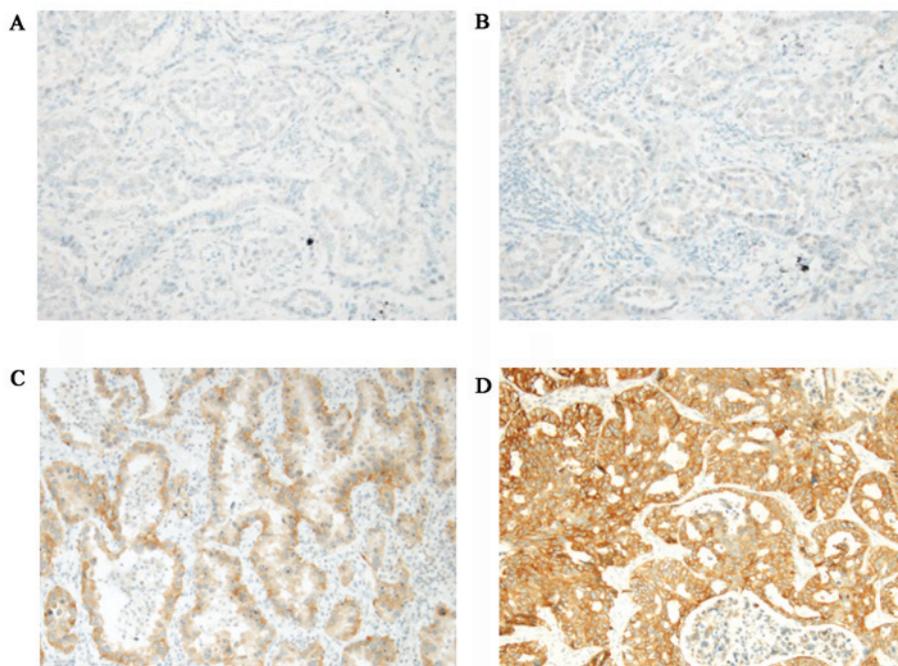


Figure 1. Representative IHC fields using SP111. (A) No significant staining (score 0). (B) Light-yellow staining with no obvious particulates or yellow staining with obvious particulates in <10% of tumor cells (score 1+). (C) Yellow staining with obvious particulates in >10% of tumor cells or brown staining with obvious particulates in <10% of tumor cells. (D) Brown staining with obvious particulates in >10% of tumor cells. Magnification, x200.

Therefore, the sensitivity and specificity of both SP111 and SP125 were 100% (Table II).

The seven cases without EGFR mutations were negative by ICC using SP111 and SP125. Among the three cases with

delE746-A750, one was positive by ICC using SP111. Thus, the sensitivity and specificity of SP111 were 33.3 and 100%, respectively. Five of seven cases with the L858R mutation showed positive staining for SP125; thus, the sensitivity

Table II. Summary of IHC and molecular testing in resection samples.

IHC	Exon19del (n=3) (%)	L858R (n=7) (%)	No mutation (n=7) (%)
SP111-positive	3 (100)	0 (0)	0 (0)
SP111-negative	0 (0)	7 (100)	7 (100)
SP125-positive	0 (0)	7 (100)	0 (0)
SP125-negative	3 (100)	0 (0)	7 (100)

IHC, immunohistochemistry.

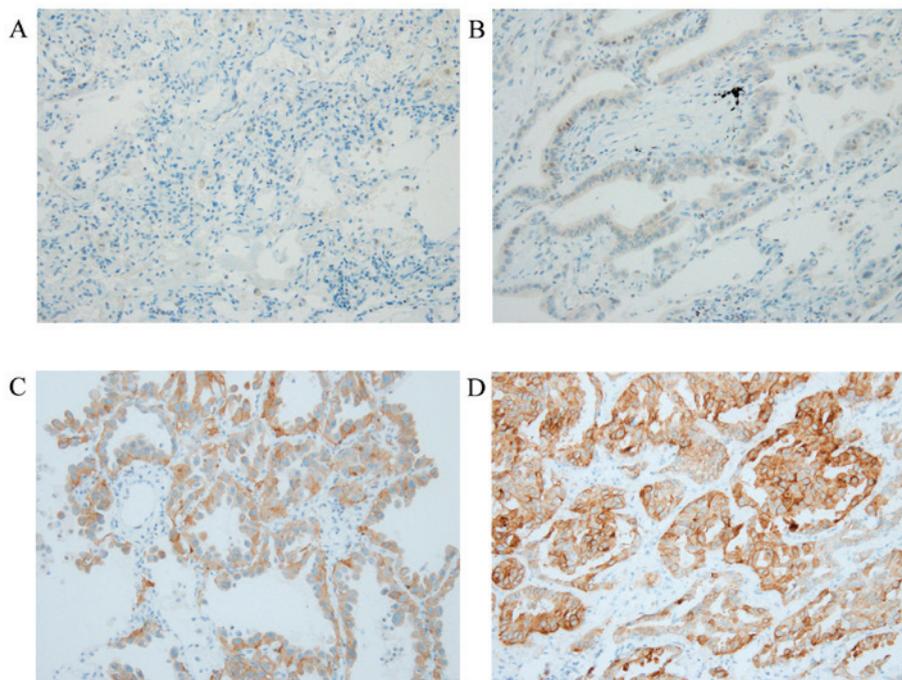


Figure 2. Representative IHC fields using SP125. (A) No significant staining (score 0). (B) Light-yellow staining with no obvious particulates or yellow staining with obvious particulates in <10% of tumor cells (score 1+). (C) Yellow staining with obvious particulates in >10% of tumor cells or brown staining with obvious particulates in <10% of tumor cells. (D) Brown staining with obvious particulates in >10% of tumor cells. Magnification, x200.

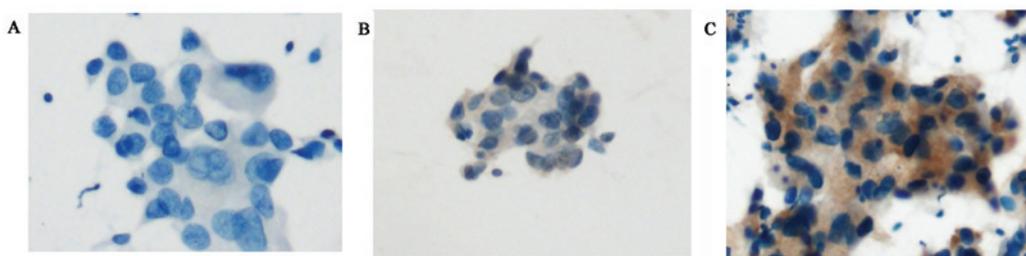


Figure 3. Representative ICC fields using SP111. Similar to IHC, a four-grade scoring system (0 to 3+) was employed. Scores of (A) 0, (B) 1+, and (C) 2+. Magnification, x200.

and specificity of SP125 were 71.4 and 100%, respectively (Table III).

Comparative analyses between IHC and ICC. ICC scores of 2+ and 3+ were considered positive. However, other studies used diverse cut-off values; e.g., Tsai *et al* regarded a score of 1+, 2+, or 3+ as positive (25). We analyzed the concordance of molecular testing and ICC using various cut-off values by

calculating the PPV, NPV, and Cohen's kappa score. If a score of $\geq 1+$ was considered positive, the sensitivity, specificity, PPV, NPV, and the κ values were 100, 57.1, 76.9, 100%, and 0.611, respectively for IHC and 100, 55.6, 66.7, 100%, and 0.239, respectively, for ICC (Table IV).

If a score of $\geq 2+$ was considered positive, the sensitivity, specificity, PPV, NPV, and the κ values were 100, 100, 100, 100%, and 1.0, respectively, for IHC and 60.0, 100, 100, 63.6%,

Table III. Summary of ICC and molecular testing in resection samples.

ICC	Exon19del (n=3) (%)	L858R (n=7) (%)	No mutation (n=7) (%)
SP111-positive	1 (33.3)	0 (0)	0 (0)
SP111-negative	2 (66.7)	7 (100)	7 (100)
SP125-positive	0 (0)	5 (71.4)	0 (0)
SP125-negative	3 (100)	2 (28.6)	7 (100)

ICC, immunocytochemistry.

Table IV. Comparative analyses between IHC and ICC in accordance with grading.

Consideration regarding positive result	Type	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Cohen's Kappa Score
Score ≥ 1	IHC	100	57.1	76.9	100	0.611
	ICC	100	55.6	66.7	100	0.239
Score ≥ 2	IHC	100	100	100	100	1
	ICC	60.0	100	100	63.6	0.553
Score ≥ 3	IHC	50.0	100	100	58.3	0.452
	ICC	30.0	100	100	50.0	0.261

IHC, immunohistochemistry; ICC, immunocytochemistry; PPV, positive predictive value; NPV, negative predictive value.

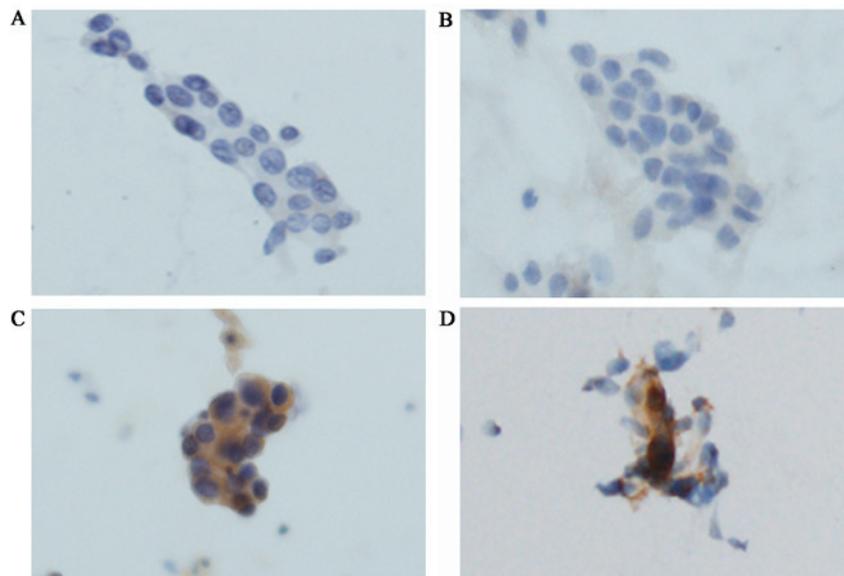


Figure 4. Representative ICC fields using SP125. Similar to IHC, a four-grade score was employed. Scores of (A) 0, (B) 1+, (C) 2+, and (D) 3+. Magnification, x200.

and 0.553, respectively, for ICC (Table IV). If a score of $\geq 3+$ was considered positive, the abovementioned values were 50.0, 100, 100, 58.3%, and 0.452, respectively, for IHC and 30.0, 100, 100, 50.0%, and 0.261, respectively, for ICC.

Discussion

Greater understanding of the molecular pathogenesis of lung cancer has led to the development and application of targeted

therapeutic strategies. Activating somatic mutations in EGFR are associated with a clinical response to TKIs (3-5). Thus, molecular testing of EGFR mutations has become routine in clinical practice (9,10). However, such tests are expensive and technically difficult to perform in many laboratories. IHC is an inexpensive and accurate method of identifying EGFR mutations and is available in most pathology laboratories. Several authors evaluated the potential of antibodies specific for the delE746-A750 and L858R mutations for screening for EGFR

mutations using surgically resected and biopsy samples (11-21). Compared with biopsy samples, obtaining cytological specimens is minimally invasive and simple. Several studies evaluated ICC using EGFR mutation-specific antibodies (clone 43B2 and clone 6B6) in cytological specimens (18,24-26). Two novel antibodies specific for EGFR mutations were developed recently: SP111, which is specific for delE746-A750, and SP125, which is specific for the L858R mutation. To date, no report has evaluated the use of SP111 or SP125 with cytological specimens. Thus, we evaluated the potential of these novel EGFR mutation-specific antibodies in ICC.

The present study included 17 cases with surgically resected and cytological samples available. Seven cases were positive for the L858R mutation and three cases for delE746-A750; the remaining seven cases had neither mutation. In ICC, the sensitivity and specificity of SP111 were 33.3 and 100%, respectively. In ICC, the sensitivity and specificity of clone 6B6 for delE746-A750 samples were reportedly 66.7 and 83.3%, 100 and 94%, 88 and 96%, and 72.7 and 100%, respectively (18,24-26). The sensitivity of SP111 was inferior to that of clone 6B6, but its specificity was comparable or superior. The sensitivity and specificity of SP125 were 71.4 and 100%, respectively. Previous studies reported sensitivities and specificities for clone 43B2 of 42.9 and 50%, 100 and 100%, 71 and 86%, and 80 and 93.8%, respectively (18,24-26). Thus, the sensitivity of SP125 is comparable with that of clone 43B2, but its specificity is comparable with or superior to that of clone 43B2.

The sensitivity and specificity of SP111 in ICC were 33.3 and 100%, respectively, and those of SP125 were 71.4 and 100%, respectively; therefore, the combined sensitivity and specificity were 60 and 100%, respectively (Table IV). The combined sensitivity of clones 43B2 and 6B6 in IHC is reportedly higher than that in ICC (79.7 vs. 50% and 85.2 vs. 66.7%, respectively) (18,26). These findings suggest that ICC using antibodies to the L858R and delE746-A750 mutations exhibits low sensitivity. Tumor cells tend to form clusters, which hampers the evaluation of membrane-positive signals. This issue may be resolved by disrupting the tumor cell clusters.

The meta-analysis by Chen *et al* recommended a four-grade IHC scoring system (in which a score of 2+ or 3+ is considered positive), not only to reduce differences among readers, but also to enhance the diagnostic value of mutation-specific antibodies (21). In this study, the PPV, NPV, and Cohens' kappa value were highest when a score of $\geq 2+$ was considered positive. Therefore, a cut-off score of 2+ may be suitable for both IHC and ICC.

Based on the high specificity of ICC using SP111 and SP125, a positive result may eliminate the need for confirmatory molecular testing. Patients with positive cytological samples should immediately start TKI therapy without verification by molecular testing. Screening for EGFR mutations by ICC may facilitate therapeutic decision-making, particularly in medical centers unable to perform molecular testing.

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