The Use of Stained Cytologic Direct Smears for *ALK* Gene Rearrangement Analysis of Lung Adenocarcinoma

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BACKGROUND: Rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene are present in approximately 5% of lung adenocarcinomas. Crizotinib is approved for the treatment of lung adenocarcinomas harboring *ALK* rearrangements. Patients with advanced stage lung cancer are not candidates for surgical resection of their primary tumors. For these patients, cytologic specimens often represent the only diagnostic tissue available. Cell blocks (CBs) are routinely used for molecular studies; however, insufficient CB cellularity can impede the performance of these assays. **METHODS:** Thirty-two cytology cases of lung adenocarcinomas were analyzed by fluorescence in situ hybridization (FISH) for *ALK* rearrangements. Diff-Quik–stained smears were examined to identify tumor cell-enriched areas that were marked using a diamond-tipped scribe. Paired *ALK* rearrangement FISH was performed using smears and CBs in each case. **RESULTS:** An *ALK* rearrangement was detected on direct smears and CB sections in 5 (16%) and 4 (13%), respectively, of the 32 cases studied. Concordant FISH results for smears and CBs were observed in 31 (97%) of 32 cases. In the 1 discordant case, an *ALK* rearrangement was detected on the direct smear but not in the CB. Reverse transcriptase-polymerase chain reaction analysis of this CB revealed the presence of an *EML4-ALK* rearrangement, thereby confirming a false-negative FISH result in the CB. **CONCLUSIONS:** Stained cytologic direct smears can be effectively used for *ALK* rearrangement analysis by FISH. This approach represents a useful safeguard when insufficient CB cellularity is encountered and could prevent delays in treatment in this era of precision medicine. ***Cancer (Cancer Cytopathol)*** **2013;121:489-99.** *© 2013 American Cancer Society.*

KEY WORDS: lung cancer; anaplastic lymphoma kinase (*ALK*) rearrangement; fluorescence in situ hybridization; cytology; direct smear; fine-needle aspiration; non-small cell lung cancer; adenocarcinoma; precision medicine.

INTRODUCTION

Lung cancer represents a leading cause of cancer mortality worldwide.[1] In the United States, an estimated 226,160 individuals will be diagnosed with lung cancer and approximately 160,340 will die of the disease.[2] Histologically, lung cancer is dichotomized into 2 general categories: small cell lung carcinoma and non-small cell lung carcinoma (NSCLC). NSCLCs represent a diverse entity that can be subclassified further into distinct histologic subtypes including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, large cell neuroendocrine carcinoma, anaplastic carcinoma, and giant cell carcinoma.[3] Of these, adenocarcinoma represents the most common subtype of lung cancer.[4]

Lung cancer is associated with a grim overall prognosis because cure is currently achieved in approximately 10% to 15% of patients.[5] Approximately 40% of patients are diagnosed with stage IV disease.[6] Recently,

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rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene were reported in approximately 5% of lung adenocarcinomas.[7,8] Most commonly, these rearrangements are a result of small inversions within the short arm of chromosome 2 that lead to fusion of portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) and *ALK* genes.[5,8] Crizotinib, recently approved by the US Food and Drug Administration for the treatment of NSCLCs with *ALK* rearrangements, is a small-molecule inhibitor of the ALK tyrosine kinase.[9] A recent phase 1 study evaluating 143 patients revealed a response rate of 60.8% and estimated overall survival rates at 6 months and 12 months of 87.9% and 74.8%, respectively.[10]

A large number of patients with lung cancer are diagnosed at a late stage of disease and are not candidates for surgical resection of their primary tumors. For these patients, small biopsies and cytologic specimens, obtained through minimally invasive procedures, often represent the only opportunity to obtain tumor cells and tissue for diagnosis and necessary molecular studies. Thus, pathologists are assuming increasing responsibilities to optimally triage cellular material for these purposes. For exfoliative and aspiration cytology specimens, cell block preparations are traditionally used for molecular studies. Unfortunately, insufficient cell block cellularity can be encountered in a significant percentage of cases, resulting in an obstacle for the performance of these studies.[11] This can result in repeat procedures, for which a satisfactory cell block is not necessarily guaranteed, and consequent delays in treatment.

We recently reported that Diff-Quik–stained direct smears prepared from cytologic samples of lung carcinoma and melanoma represent a rich source of cellular material for epidermal growth factor receptor (*EGFR*) and *KRAS* mutational analysis and *BRAF* mutational analysis, respectively.[12–14] Currently, the prescription of crizotinib requires the use of the Vysis *ALK* break apart fluorescence in situ hybridization (FISH) probe kit (Abbott Molecular, Des Plaines, IL), which has only been approved for use on formalin-fixed, paraffin-embedded (FFPE) sections.[5] Nonetheless, given the main inherent flaw in relying solely on cell blocks for molecular studies (the variable cellularity and insufficient cellularity in a significant percentage of cases[11]), we sought to investigate the application of *ALK* break apart FISH analysis to Diff-Quik–stained cytologic direct smears. The potential advantages of this approach over cytologic cell blocks include the ability to directly assess the smear for specimen adequacy and to score FISH signals in entire nuclei, rather than truncated nuclei present in paraffin sections. Hence, to validate this approach, we tested both stained smears and cell blocks prepared from cytologic samples of adenocarcinoma, in parallel, and correlated the results from the 2 testing platforms.

MATERIALS AND METHODS

Case Selection

The study was approved by the Institutional Review Board at the University of Michigan. Thirty-two cytology cases of metastatic pulmonary adenocarcinoma for which the cell block exhibited sufficient tumor cellularity, as judged by examination of the initial hematoxylin and eosin (H&E)-stained section, and there existed at least 2 diagnostic Diff-Quik–stained smears were retrieved from the archive. These included 18 fine-needle aspirate specimens (FNAs), 13 pleural fluids, and 1 pericardial effusion (Table 1). Cases that were previously identified as being positive for *ALK* rearrangement were preferentially selected, when possible, to increase their representation in this study. Diff-Quik–stained smears were examined to identify tumor cell-enriched areas. These areas, onto which the *ALK* break apart FISH probe set would be applied, were marked on the underside of the slides with a diamond-tipped scribe. The marked smears were then decoverslipped in xylene at room temperature and subsequently destained via the acid-alcohol technique.[15] Specifically, smears were incubated sequentially in 100% ethanol, 95% ethanol, and 70% ethanol for 2 minutes each. Subsequently, the smears were placed in acid-alcohol (1% hydrochloric acid in 70% ethanol) for 1 hour and then sequentially washed in running water, Scott tap water substitute, and twice in water for 15 minutes, 5 minutes, and 1 minute each, respectively. The smears were then dried and triaged to the Molecular Diagnostics laboratory for *ALK* rearrangement FISH.

FISH for ALK Rearrangement

Air-dried destained smears were pretreated with the Vysis FISH Pretreatment Kit (Abbott Molecular) following the manufacturer's instructions. Hybridization of the Vysis LSI *ALK* Break Apart Rearrangement Probe (Abbott Molecular) and slide washing were then performed

TABLE 1. FISH Analysis of *ALK* Gene Rearrangement on Cytologic Smears and Corresponding Cell Blocks

		Cytologic Smears		Cell Blocks	
Case	Specimen Source	No. of Cells Scored *ALK* Positive/Total No. of Cells Scored	Interpretation	No. of Cells Scored *ALK* Positive/Total No. of Cells Scored	Interpretation
1	R paratracheal LN FNA	2/50 (4%)	Negative	1/50 (2%)	Negative
2	L sixth rib lesion FNA	2/50 (4%)	Negative	1/50 (2%)	Negative
3	L pleural fluid	0/50 (0%)	Negative	2/50 (4%)	Negative
4	R pleural fluid	1/50 (2%)	Negative	3/50 (6%)	Negative
5	L pleural fluid	1/50 (2%)	Negative	3/50 (6%)	Negative
6	L pleural fluid	2/50 (4%)	Negative	2/50 (4%)	Negative
7	R pleural fluid	3/50 (6%)	Negative	3/50 (6%)	Negative
8	R supraclavicular LN FNA	29/50 (58%)	Positive	28/100 (28%)	Positive
9	R pleural fluid	44/50 (88%)	Positive	40/50 (80%)	Positive
10	R pleural fluid	1/50 (2%)	Negative	1/50 (2%)	Negative
11	L neck LN FNA	3/50 (6%)	Negative	1/50 (2%)	Negative
12	Level 7 LN FNA	2/50 (4%)	Negative	3/50 (6%)	Negative
13	L hilar mass FNA	3/50 (6%)	Negative	3/50 (6%)	Negative
14	R pleural fluid	1/50 (2%)	Negative	0/50 (0%)	Negative
15	Level 4L LN FNA	0/50 (0%)	Negative	2/50 (4%)	Negative
16	R lower lobe lung FNA	2/50 (4%)	Negative	1/50 (2%)	Negative
17	L hip mass FNA	4/50 (8%)	Negative	0/50 (0%)	Negative
18	Level 4R LN FNA	1/50 (2%)	Negative	2/50 (4%)	Negative
19	Level 10L LN FNA	1/50 (2%)	Negative	3/50 (6%)	Negative
20	Level 7 LN FNA	26/50 (52%)	Positive	3/50 (6%)	Negative
21	Mediastinal mass FNA	3/50 (6%)	Negative	3/50 (6%)	Negative
22	Pericardial fluid	34/50 (68%)	Positive	36/50 (72%)	Positive
23	Level 7 LN FNA	0/50 (0%)	Negative	2/50 (4%)	Negative
24	Level 2R LN FNA	4/50 (8%)	Negative	2/50 (4%)	Negative
25	Level 4R LN FNA	2/50 (4%)	Negative	2/50 (4%)	Negative
26	L pleural fluid	2/50 (4%)	Negative	0/50 (0%)	Negative
27	R pleural fluid	1/50 (2%)	Negative	3/50 (6%)	Negative
28	L pleural fluid	0/50 (0%)	Negative	0/50 (0%)	Negative
29	R pleural fluid	0/50 (0%)	Negative	1/50 (2%)	Negative
30	Level 7 LN FNA	0/50 (0%)	Negative	1/50 (2%)	Negative
31	Level 7 LN FNA	2/50 (4%)	Negative	3/50 (6%)	Negative
32	R pleural fluid	37/100 (37%)	Positive	34/50 (68%)	Positive

Abbreviations: *ALK*, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; FNA, fine-needle aspiration; L, left; LN, lymph node; R, right.

according to the manufacturer's package insert beginning at the hybridization step. FISH on the cell blocks was performed on 4-micron paraffin sections following the standard procedure included with the *ALK* break apart probe. Scoring for both smears and cell blocks was performed according to standard criteria outlined in the *ALK* break apart probe package insert. According to these standard criteria, 50 tumor nuclei are scored for each case. Orange and green signals that are fused (yellow), touching, or separated by a distance < 2 signal diameters apart are classified as negative for *ALK* rearrangement. Orange and green signals separated by a distance > 2 signal diameters or a single orange signal without a corresponding green signal are considered positive for *ALK* rearrangement. Cases are considered positive for *ALK* rearrangement if > 25 of 50 cells are positive. An additional 50 cells are scored in cases with 5 to 25 positive cells, and are considered positive for *ALK* rearrangement if at least 15 of 100 cells are positive.

Reverse Transcriptase-Polymerase Chain Reaction for the EML4-ALK *Fusion Transcript*

Ten paraffin scrolls of the cell block measuring 10 microns were deparaffinized and digested in cell lysis buffer (Gentra Puregene; Qiagen, Hilden, Germany) with 125 μg of Proteinase K overnight. RNA was extracted using TRIzol LS reagent (Life Technologies/Invitrogen, Carlsbad, Calif). One-step reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the GeneAmp Gold RNA PCR Core Kit (Life Technologies/Applied Biosystems, Foster City, Calif) on an Applied Biosystems 9700 thermal cycler. Reaction components for the 50 μL reaction were as follows: 150 ng total of

RNA template, 1× RT-PCR buffer, 1.75 mM of magnesium chloride, 0.8 mM of dNTP blend (200 μM each), 0.3 μM of *EML4* exon 13 forward primer (5′TATGGAGCAAAACTACTGTAGAGC3′),[9] 0.3 μM of ALK exon 20 reverse primer (5′CGGAGCTTGC TCAGCTTGTA3′),[16] 10 U of RNase inhibitor, 5.0 mM of dithiothreitol (DTT), 1.25 of μM random hexamers, and 2.5 U of AmpliTaq Gold DNA. The following thermal cycling conditions were used for the RT-PCR: reverse transcription at 42°C for 12 minutes followed by pre-PCR denaturation at 95°C for 10 minutes and then 40 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds, and primer extension at 72°C for 1 minute followed by final extension of amplification products for 7 minutes. The fusion transcript and breakpoint were confirmed by bidirectional Sanger sequencing of RT-PCR products using the BigDye Terminator 1.1 Cycle Sequencing Kit (Life Technologies/Applied Biosystems) and the above-mentioned PCR primers.

RESULTS

A total of 32 cytology cases of pulmonary adenocarcinoma for which the paraffin-embedded cell block was determined to be of sufficient tumor cellularity for satisfactory *ALK* FISH evaluation, as judged by examination of the routinely prepared H&E-stained section, were retrieved from the archive. These cases are outlined in Table 1. This approach was used because the Vysis *ALK* FISH assay is intended to be used on paraffin sections; FISH results obtained using this platform would represent the standard to which the FISH results obtained on cytologic smears would be compared.

FISH for *ALK* rearrangement was performed on paraffin sections from each cell block using a commercially available break apart probe set. FISH was satisfactory for evaluation in all 32 cases. Four cases were interpreted as positive for *ALK* rearrangement (cases 8, 9, 22, and 32 in Table 1). In 3 cases, 50 tumor cell nuclei were scored and 40, 36, and 34 nuclei, respectively, were scored as positive. For the fourth case, 100 tumor cell nuclei were scored and 28 of these were scored as positive.

Corresponding Diff-Quik–stained direct smears for these cases were examined by light microscopy to identify tumor-enriched areas containing at least 200 tumor cells. These areas, which ranged in size from 24 mm^2 to 132 mm^2, were marked by a diamond-tipped scribe. Slides were decoverslipped and destained, and the marked areas were analyzed by FISH. All 32 smears were tested successfully, 5 of which were positive for *ALK* rearrangement (2 FNAs and 3 effusion specimens) (Fig. 1). For the 2 FNA specimens (cases 8 and 20 in Table 1), 50 tumor cell nuclei were scored; 29 and 26 nuclei, respectively, were scored as positive. For the 2 effusion specimens (cases 9 and 22 in Table 1), 44 nuclei and 34 nuclei, respectively, of 50 tumor cell nuclei were scored as positive. For 1 effusion specimen (case 32 in Table 1), 100 tumor cell nuclei were scored and 37 nuclei scored positive. The remaining 27 cases tested negative for *ALK* rearrangement (Fig. 2).

In total, 31 of 32 cases (97%) yielded concordant *ALK* FISH results in paired cell blocks and smears. The single discordant case (case 20 in Table 1) tested positive for *ALK* rearrangement on the smear but negative on the corresponding cell block (Fig. 3). The cell block consisted of dichomatous populations of small lymphocytes and scattered discrete clusters of tumor cells with enlarged nuclei (Fig. 3). Only 3 of 50 tumor nuclei in the cell block section scored positive on FISH, which was insufficient for an interpretation of a positive *ALK* rearrangement result. In contrast, 26 of 50 cells in the corresponding smear scored positive, thereby meeting the positive result criteria. To clarify this discrepant result, we next performed RT-PCR to interrogate for the presence or absence of an EML4-ALK fusion transcript in the cell block preparation for this case. This analysis was positive for the EML4-ALK fusion and identified the specific EML4-ALK transcript as variant 1, which joins exon 13 of *EML4* to exon 20 of *ALK*. Sequencing of the RT-PCR products confirmed the presence of the *EML4-ALK* variant 1 rearrangement (Fig. 3).

The ages of the 5 patients in whom the *ALK* FISH assay was positive for an *ALK* rearrangement ranged from 31 years to 75 years at the time of diagnosis. Three patients were male (aged 31 years, 53 years, and 75 years, respectively) and 2 were female (aged 55 years and 66 years, respectively). Four patients were nonsmokers and 1 reported a 20-year history of smoking cigars. Finally, we observed that the direct smears, previously analyzed by FISH, could be restained with the Diff-Quik stain. The cytomorphologic features of the tumor cells remained well-preserved in the restained smears (Fig. 4).

DISCUSSION

In this era of precision medicine, the discovery of molecular alterations in NSCLC has revolutionized the management

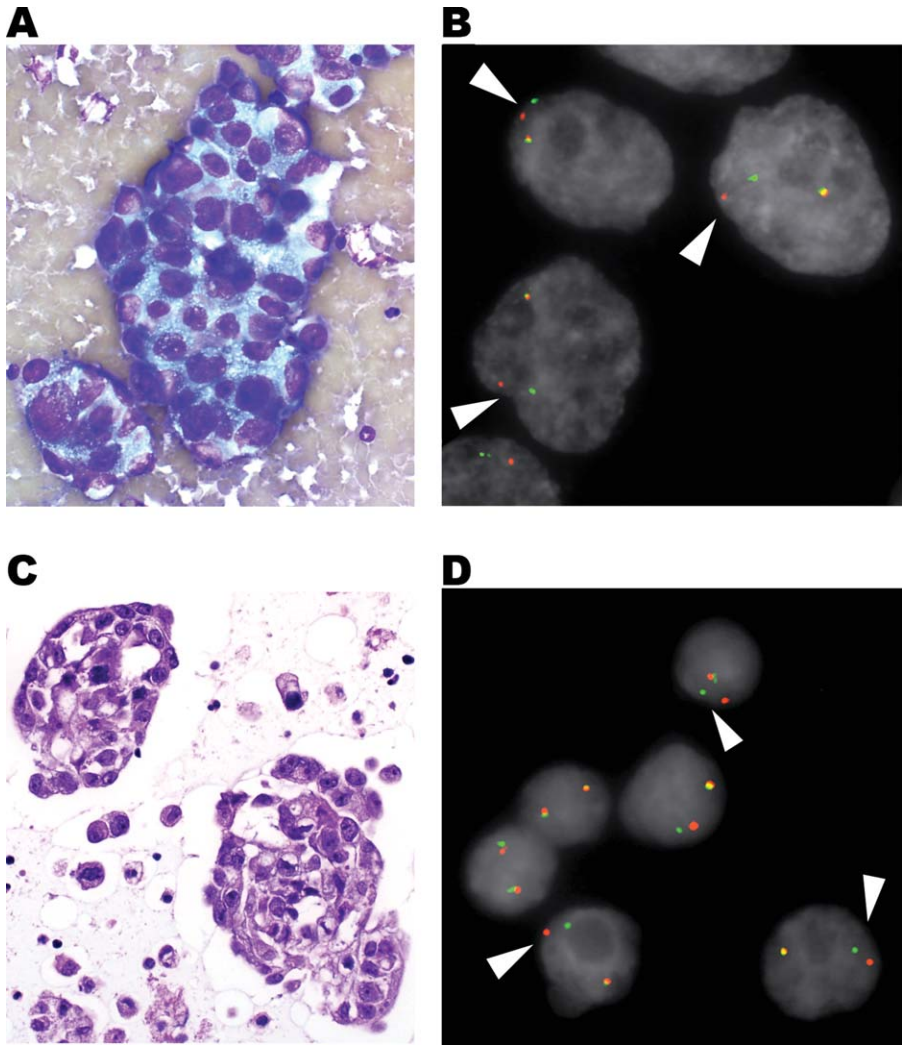

FIGURE 1. Anaplastic lymphoma kinase (*ALK*) rearrangement-positive lung adenocarcinoma is shown. (A) A representative photomicrograph obtained from the Diff-Quik–stained direct smear is shown (× 400). (B) *ALK* rearrangement fluorescence in situ hybridization performed on the direct smear revealed the presence of the probe signal split in tumor cell nuclei, as indicated by the arrowheads (× 1000). (C) Representative photomicrograph obtained from the hematoxylin and eosin-stained section prepared from the corresponding cell block is shown (× 400). (D) *ALK* rearrangement fluorescence in situ hybridization performed on the cell block section revealed the presence of the probe signal split in tumor cell nuclei, as indicated by the arrowheads (× 1000).

of patients with this disease. Patients with NSCLCs harboring *EGFR* mutations are candidates for targeted therapy with geftinib or erlotinib.[12,17–20] Approximately 5% of NSCLCs, especially adenocarcinomas, harbor rearrangements involving *ALK*; patients with these tumors have been shown to benefit from targeted therapy with crizotinib.[10] With the increased use of targeted therapies in patients with advanced stage NSCLC, there is an increasing clinical need to interrogate molecular aberrations in small biopsies and cytologic specimens of primary and metastatic disease. Specimen inadequacy remains a problematic issue in a subset of cases in which molecular testing is requested. This leads to delays in treatment because repeat procedures are necessary to obtain additional diagnostic material.

To our knowledge to date, only a limited number of studies have examined the use of direct smears prepared from cytologic specimens of lung carcinoma for use in molecular analysis. Our group and others have previously demonstrated that direct smears of lung adenocarcinoma represent an effective platform for *EGFR* and *KRAS* mutation testing.[12,21–24] To our knowledge, there are currently no reports in the literature that examine *ALK* rearrangement testing in cytologic smears. Therefore, in the current study, we extended our investigation by applying FISH

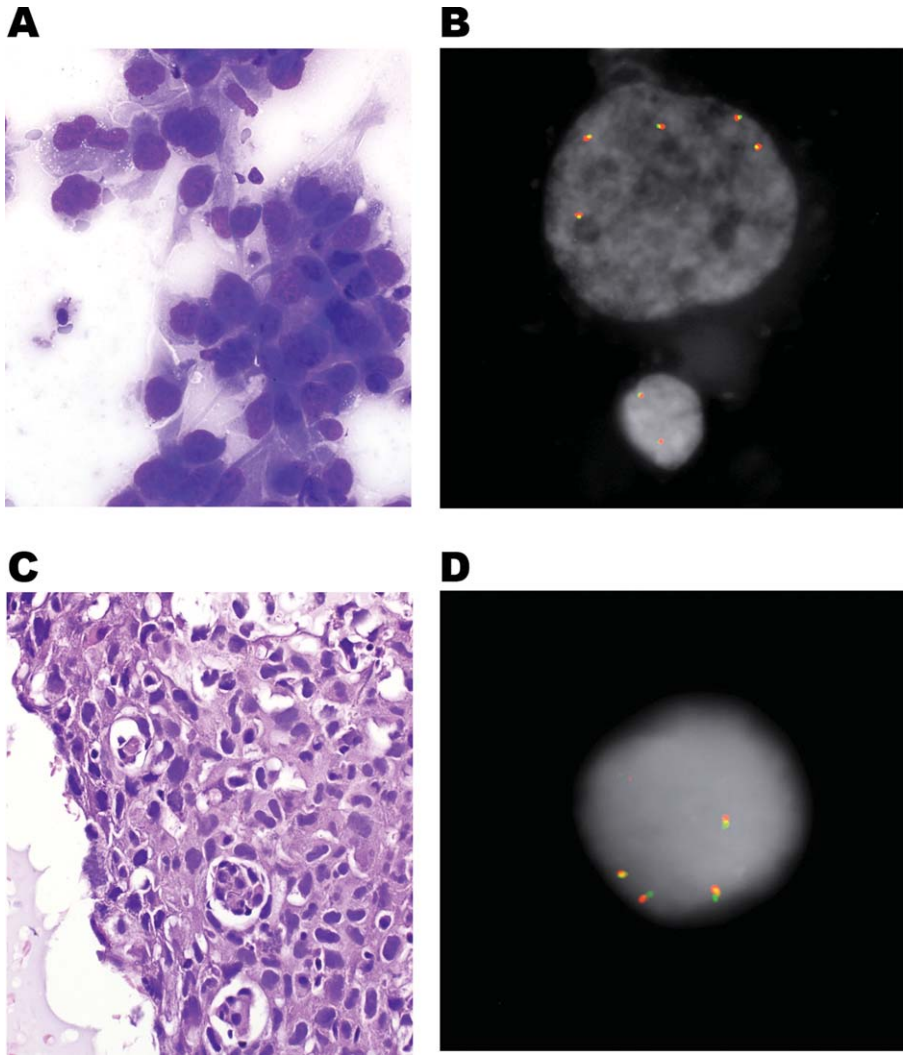

FIGURE 2. Anaplastic lymphoma kinase (*ALK*) rearrangement-negative lung adenocarcinoma is shown. (A) A representative photomicrograph obtained from the Diff-Quik–stained direct smear is shown (× 400). (B) *ALK* rearrangement fluorescence in situ hybridization performed on the direct smear revealed the absence of the probe signal split (× 1000). (C) A representative photomicrograph obtained from the hematoxylin and eosin-stained section prepared from the corresponding cell block is shown (× 400). (D) *ALK* rearrangement fluorescence in situ hybridization performed on the cell block section also revealed the absence of the probe signal split (× 1000).

analysis for *ALK* rearrangements to direct smears of lung adenocarcinoma.

Overall, the use of direct smears for molecular testing of NSCLC, via PCR-based or FISH-based assays, is potentially advantageous over the use of cell blocks for several reasons. First, air-dried smears can be rapidly stained using Diff-Quik and directly examined for tumor cellularity. Especially during on-site assessments of FNA procedures, Diff-Quik–stained smears can be examined to determine whether a cytologic sample of sufficient tumor cellularity has been obtained for cytomorphologic diagnostic evaluation as well as for anticipated molecular studies while the patient is still accessible. Second, the cell block cellularity is not definitively known nor guaranteed at the time of the FNA procedure.[11] Third, the use of smears for FISH-based molecular assays is advantageous because whole nuclei of tumor cells are analyzed, thereby avoiding the nuclear truncation and probe signal loss that is encountered in FISH performed on sections from FFPE specimens.[5]

Currently, the Vysis FISH assay for *ALK* rearrangement has only received FDA approval for use on sections

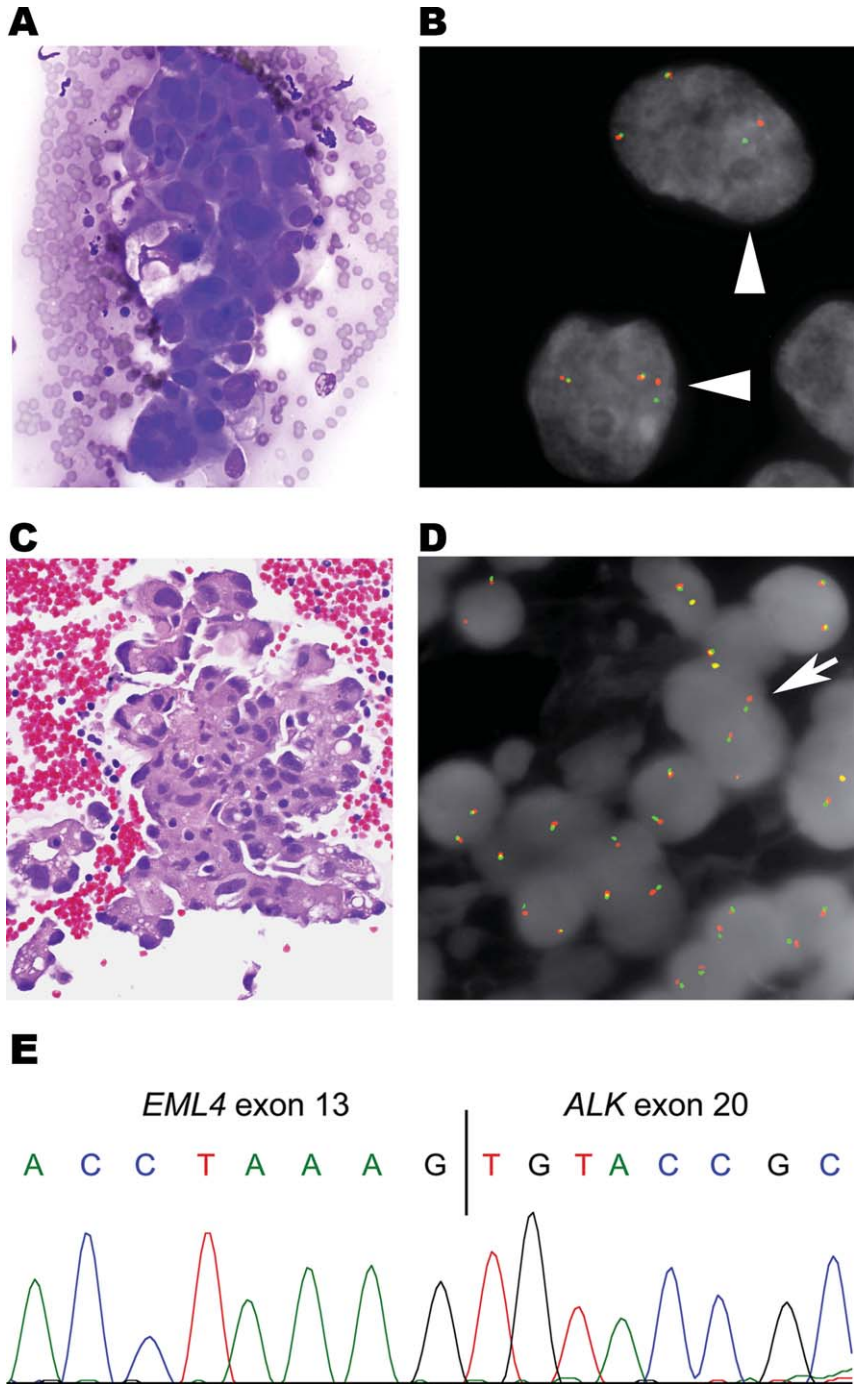

FIGURE 3. A discordant case of lung adenocarcinoma is shown in which the anaplastic lymphoma kinase (*ALK*) rearrangement was scored as positive on the cytologic smear but not on the cell block. (A) A representative photomicrograph obtained from the Diff-Quik–stained direct smear is shown (× 400). (B) *ALK* rearrangement fluorescence in situ hybridization performed on the direct smear revealed the presence of the probe signal split, as indicated by the arrowheads (× 1000). (C) A representative photomicrograph obtained from the hematoxylin and eosin-stained section prepared from the corresponding cell block is shown (× 400). (D) *ALK* rearrangement fluorescence in situ hybridization performed on the cell block section revealed the presence of subtle probe signal splits (indicated by the arrow) that were of insufficient width to be scored as positive (× 1000). (E) Reverse transcriptase-polymerase chain reaction was performed using RNA isolated from the cell block to interrogate for the presence or absence of the echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion transcript. The polymerase chain reaction product was purified and analyzed by Sanger sequencing, which confirmed the presence of the *EML4-ALK* variant 1 rearrangement that joins exon 13 of *EML4* to exon 20 of *ALK*.

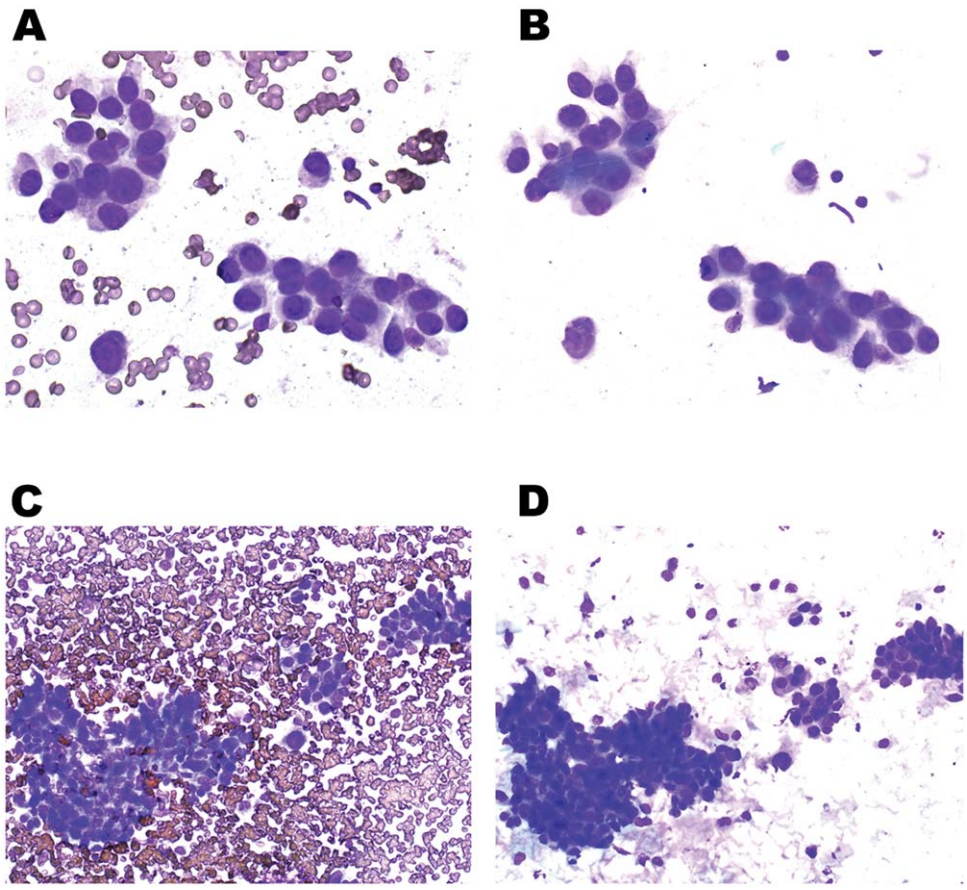

FIGURE 4. Diff-Quik–stained cytologic smears are shown before and after fluorescence in situ hybridization (FISH) analysis. Photomicrographs obtained from Diff-Quik–stained direct smears from 2 cases are shown before anaplastic lymphoma kinase (*ALK*) rearrangement FISH (A and C, × 600 and × 200, respectively). After destaining the smears and subsequent FISH analysis, the smears were restained with Diff-Quik stain. Photomicrographs of these restained smears are shown (B and D, × 600 and × 200, respectively).

prepared from FFPE blocks.[5] In cases for which existing cytologic preparations represent the only available specimen, safeguards are needed to ensure that these can be used to their fullest potential before subjecting patients to repeat procedures solely for the purpose of obtaining an adequate cell or tissue block, an outcome that is not necessarily guaranteed after repeat sampling. In this respect, the results of the current study demonstrate that cytologic smears can be effectively used for *ALK* rearrangement testing. We found the quality of *ALK* FISH on Diff-Quik–stained smears to be satisfactory in terms of probe signal intensity and probe signal-to-noise ratios. Concordance in *ALK* FISH results between paired smears and cell blocks was high (31 of 32 cases); however, a single case (case 20 in Table 1) tested positive for *ALK* rearrangement in the smear but negative on the cell block section. We noted that the orange and green probe signal splits in the direct smear of this case were 2 to 3 signal diameters apart, which only minimally met the 2-signal diameter split criteria for a positive result (Fig. 3). In contrast, the signal splits observed in the corresponding cell block were smaller, between 1 to 2 signal diameters apart, and therefore did not meet the criteria for positive scoring. The reason for the enhanced signal width separation in the direct smear of this case compared with the cell block is likely the result of the larger size of tumor nuclei in the former; cells on air-dried smears are expected to be larger than cells exposed to formalin, a fixation agent that causes cellular shrinkage. Consistent with this, increased nuclear size in smear preparations compared with corresponding cell blocks was a general feature we observed throughout this study (Figs. 1–3). Thus, using direct smears may provide

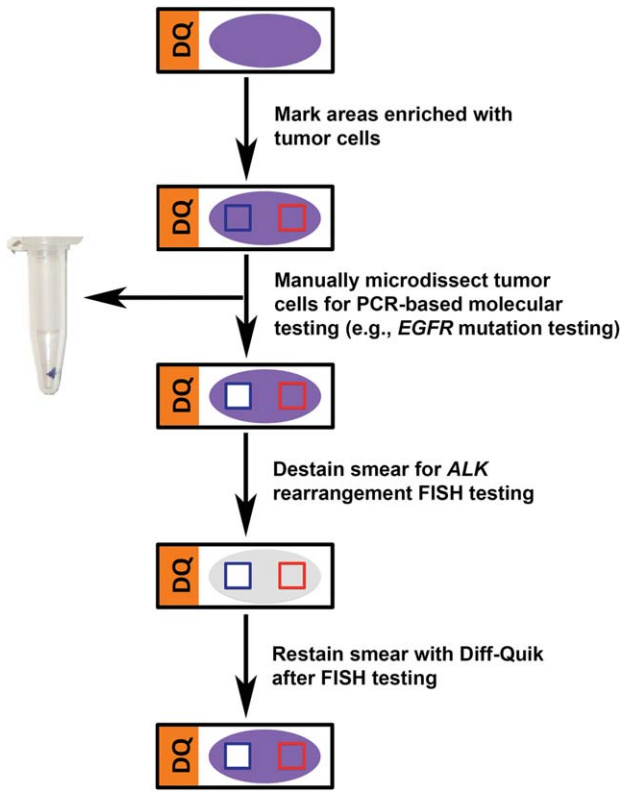

FIGURE 5. Workflow for the use of cytologic direct smears for molecular testing in patients with non-small cell lung cancer is shown. A Diff-Quik (DQ)–stained smear can be examined to identify areas that are enriched with tumor cells. Two areas can be marked on the underside of the slide with a diamond-tipped scribe. Manual microdissection of tumor cells can be performed from 1 area for DNA isolation and polymerase chain reaction (PCR)-based molecular diagnostic assays (eg, epidermal growth factor receptor [*EGFR*] and/or *KRAS* mutation analysis). The slide stained using DQ can then be destained and the anaplastic lymphoma kinase (*ALK*) break apart probe set can be applied to the second area for fluorescence in situ hybridization (FISH) testing. After scoring for the presence or absence of *ALK* rearrangements, the smear can then be restained with DQ and coverslipped, thereby allowing for inclusion in the diagnostic archive. This slide does not necessarily need to be sacrificed after molecular testing.

increased sensitivity for detecting *ALK* rearrangements compared with cell blocks. It is interesting to note that testing smears with their comparatively larger nuclei did not appear to affect the specificity of the *ALK* FISH test because we did not observe false-positive results in these specimens. The subtle splitting of *ALK* break apart probe signals has been previously reported in NSCLCs with *ALK* rearrangements and represents a challenge to case interpretation using the break apart FISH probe strategy.[5,7,16,25] The cause of this pattern is related to the structural nature of *ALK* rearrangements in NSCLC, which most frequently involve fusion of *EML4* to *ALK*. These genes are normally separated by a short distance on chromosome 2p. Fusion results from a small inversion that may also include deletion of intervening sequences.[8] The effect of this small chromosome 2 inversion is that the 2 *ALK* probes become separated by only a short distance, which can lead to subtle probe splits in cases with *EML4-ALK* rearrangements. This contrasts with wide probe splits that occur in cases with the less common interchromosomal *ALK* rearrangements involving the *TFG* or *KIF5B* partner genes.

Detection of the EML4-ALK fusion transcript by RT-PCR in the cell block for case 20 (Table 1) confirmed the false-negative *ALK* FISH result in this cell block. The specific EML4-ALK fusion transcript identified was variant 1 (*EML4* exon 13 to *ALK* exon 20), which is the most common fusion in NSCLC.[9] Consistent with these findings, others have reported difficulties in detecting *ALK* rearrangements by FISH in cases with variant 1 fusions because of small FISH signal splits.[16] In this respect, the increased nuclear size, lack of nuclear truncation, and enhanced cellularity of direct smear preparations compared with FFPE sections may increase the ability to detect *ALK* rearrangements by break apart FISH in NSCLC. Additional studies will be necessary to confirm this. It is interesting to note that thorough destaining in acid-alcohol was particularly important to reduce autofluorescence from residual Diff-Quik staining that can interfere with FISH probe signal visualization. Smears that contained a large amount of blood sometimes exhibited higher background fluorescence in scattered areas of the slide, but this did not affect the ability to find an adequate number of cells to successfully score these cases.

Taking our results and overall experience into consideration, we propose a complementary approach to the molecular analysis of NSCLCs that provides a safeguard by eliminating the sole reliance on block preparations for molecular testing (Fig. 5). Typically, paired direct smears, one of which is stained using Diff-Quik and the other stained using the Papanicolaou method, are prepared from a given FNA pass and the remaining contents are rinsed in a liquid solution for the preparation of a cell block. We have previously highlighted another viable option in which contents expelled from a single needle pass can be distributed over 3 or more smears.[11] This allows for the preparation of an extra Diff-Quik–stained

smear that can be immediately triaged for molecular testing after it is directly assessed to identify and mark distinct tumor cell-enriched areas (Fig. 5). Tumor cells can be manually microdissected from 1 area for DNA isolation and PCR-based molecular assays such as *EGFR* mutation testing.[12] After microdissection, the smear can be destained and the *ALK* break apart probe set can be hybridized to the second tumor cell-enriched area for FISH analysis. It is interesting to note that sacrifice of smears used for molecular testing represents a potential disadvantage of this approach.[26,27] In the current study, we demonstrated that the smears can be restained after FISH analysis. This allows at least for partial preservation of the slide for inclusion in the diagnostic archive. If the aforementioned strategy of preparing and triaging extra Diff-Quik–stained smears was not used at the time of an FNA procedure, a previously coverslipped Diff-Quik–stained smear could be decoverslipped in xylene and used for molecular testing.[12–14] This overall approach is flexible and forgiving, and provides an effective safeguard in the molecular testing of cytologic samples of NSCLCs.

Cytologic direct smears provide a feasible and effective platform for the molecular diagnostic analysis of NSCLC. Given the ability to ensure cellular adequacy to immediately triage smear preparations for molecular assays, the approach described in the current study represents a useful alternative to relying on paraffin-embedded cell blocks for molecular testing. This model has the potential to facilitate the expeditious management of patients with NSCLC in this era of precision medicine.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.
3. International Agency for Research on Cancer, Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, eds. World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Lung, Pleura, Thymus, and Heart. Lyon, France: IARC Press; 2004.
4. Ladanyi M, Pao W. Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. *Mod Pathol*. 2008;21(suppl 2):S16-S22.
5. Thunnissen E, Bubendorf L, Dietel M, et al. EML4-ALK testing in non-small cell carcinomas of the lung: a review with recommendations. *Virchows Arch*. 2012;461:245-257.
6. Azzoli CG, Baker S Jr, Temin S, et al; American Society of Clinical Oncology. American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. *J Clin Oncol*. 2009;27:6251-6266.
7. Rodig SJ, Mino-Kenudson M, Dacic S, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res*. 2009;15:5216-5223.
8. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448:561-566.
9. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
10. Camidge DR, Bang YJ, Kwak EL, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. *Lancet Oncol*. 2012;13:1011-1019.
11. Knoepp SM, Roh MH. Ancillary techniques on direct-smear aspirate slides: a significant evolution for cytopathology techniques [published online ahead of print July 11, 2012]. *Cancer (Cancer Cytopathol)*. doi: 10.1002/cncy.21214.
12. Betz BL, Roh MH, Weigelin HC, et al. The application of molecular diagnostic studies interrogating EGFR and KRAS mutations to stained cytologic smears of lung carcinoma. *Am J Clin Pathol*. 2011;136:564-571.
13. Hookim K, Roh MH, Willman J, et al. Application of immunocytochemistry and BRAF mutational analysis to direct smears of metastatic melanoma. *Cancer (Cancer Cytopathol)*. 2012;120:52-61.
14. Bernacki KD, Betz BL, Weigelin HC, et al. Molecular diagnostics of melanoma fine-needle aspirates: a cytology-histology correlation study. *Am J Clin Pathol*. 2012;138:670-677.
15. Zhou L, Smith D, Wittwer C. Rare allele enrichment and detection by allele-specific PCR, competitive probe blocking and melting analysis. In: *BioTechniques Protocol Guide*. New York, NY: Informa; 2012:387-391. doi:10.2144/000113783.
16. Wallander ML, Geiersbach KB, Tripp SR, Layfield LJ. Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non-small cell lung carcinoma: implications for optimal clinical testing. *Arch Pathol Lab Med*. 2012;136:796-803.
17. Eberhard DA, Johnson BE, Amler LC, et al. Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J Clin Oncol*. 2005;23:5900-5909.
18. Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. *Cancer Sci*. 2007;98:1817-1824.
19. Mok TS, Wu YL, Yu CJ, et al. Randomized, placebo-controlled, phase II study of sequential erlotinib and chemotherapy as first-line treatment for advanced non-small-cell lung cancer. *J Clin Oncol*. 2009;27:5080-5087.
20. Takano T, Fukui T, Ohe Y, et al. EGFR mutations predict survival benefit from gefitinib in patients with advanced lung adenocarcinoma: a historical comparison of patients treated before and after gefitinib approval in Japan. *J Clin Oncol*. 2008;26:5589-5595.
21. Boldrini L, Gisfredi S, Ursino S, et al. Mutational analysis in cytological specimens of advanced lung adenocarcinoma: a sensitive method for molecular diagnosis. *J Thorac Oncol*. 2007;2:1086-1090.

22. Nomoto K, Tsuta K, Takano T, et al. Detection of EGFR mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. *Am J Clin Pathol*. 2006;126:608-615.
23. Smith GD, Chadwick BE, Willmore-Payne C, Bentz JS. Detection of epidermal growth factor receptor gene mutations in cytology specimens from patients with non-small cell lung cancer utilising high-resolution melting amplicon analysis. *J Clin Pathol*. 2008;61:487-493.
24. van Eijk R, Licht J, Schrumpf M, et al. Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. *PLoS One*. 2011;6:e17791.
25. Sasaki T, Rodig SJ, Chirieac LR, Janne PA. The biology and treatment of EML4-ALK non-small cell lung cancer. *Eur J Cancer*. 2010;46:1773-1780.
26. Aisner DL, Marshall CB. Molecular pathology of non-small cell lung cancer: a practical guide. *Am J Clin Pathol*. 2012;138:332-346.
27. Aisner DL, Sams SB. The role of cytology specimens in molecular testing of solid tumors: techniques, limitations, and opportunities. *Diagn Cytopathol*. 2012;40:511-524.