Poor Cell Block Adequacy Rate for Molecular Testing Improved With the Addition of Diff-Quik–Stained Smears: Need for Better Cell Block Processing

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BACKGROUND: In the era of personalized medicine, requests for molecular testing of specimens obtained with minimally invasive procedures such as fine-needle aspiration have been increasing. Although cell blocks (CBs) are the recommended specimens for molecular testing, their performance has not been well analyzed. The objective of this study was to assess the frequency and types of samples deemed unsatisfactory for molecular testing (quantity not sufficient [QNS]). **METHODS:** One year after the implementation of careful monitoring of QNS cases, cases submitted for lung cancer molecular testing were analyzed for the QNS rate. When the cases were rejected for the inadequacy of CBs of cytology specimens, air-dried, Diff-Quik (DQ)–stained smears were reviewed and used if they were adequate. The QNS rates were compared across 4 specimen categories: large resection, small biopsy, CB alone, and CB with DQ smears. **RESULTS:** One hundred seventy-six cases were studied, and 45 (25.6%) were unsatisfactory. Only 1 of 73 large resection specimens was rejected because of decalcification. The QNS rate for small biopsy specimens was 35.9% (28 of 78), whereas 64% (16 of 25) of cytology cases ordered on CBs were rejected. In combination with DQ smears, the QNS rate of cytology specimens was 32% (8 of 25), which was a significant improvement over CBs only ($P = .024$) and was not significantly different from the QNS rate for small biopsies ($P = .671$). **CONCLUSIONS:** The utilization of DQ-stained smears for molecular testing improves the adequacy of cytologic samples and provides a minimally invasive alternative to surgical biopsy when molecular analysis of tumor material is necessary. ***Cancer (Cancer Cytopathol)*** **2015;123:480-7.** © *2015 American Cancer Society.*

KEY WORDS: air-dried direct smear; cell block; cytology; Diff-Quik; epidermal growth factor receptor (EGFR); molecular testing.

INTRODUCTION

Molecular testing to detect mutations of driver genes is now becoming a part of the standard of care for patients with many different cancers, including lung adenocarcinoma.[1] As the number of targeted therapeutic agents increase, the clinical demand for ancillary molecular testing of small samples, including cytologic specimens and small biopsies, will continue to grow. This is especially true for lung cancer because many patients with lung cancer present at an advanced stage for which surgical treatment is not an option.[2] Recent guidelines from the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) recommend testing epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*) fusions in all patients with advanced-stage adenocarcinoma.[1] Other commonly tested genes include the Kristen-Rous sarcoma virus (*KRAS*) mutation and *ROS1* gene rearrangement because of their mutation frequency *(KRAS)* and the availability of targeted therapies *(ROS1)*.[3]

Fine-needle aspiration provides a minimally invasive source of tumor cells, and requests for molecular testing of these specimens have become common in the molecular laboratory. According to recently published

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CAP, IASLC, and AMP guidelines, cytologic samples are suitable for *EGFR* testing, and cell blocks (CBs) are the preferred medium.[1]

Although the CB technique has been used for a long time in cytopathology, there was no precise review in the literature dealing with its methodology, utility, and role in ancillary studies until a recent study by Jain et al.[4] Currently, there is a lack of standardization in cytologic specimen preservation as well as CB preparation.[5,6] A recent survey about CBs revealed that 44% of respondents were either unsatisfied or sometimes satisfied with their CB quality, with low cellular yields being the leading cause of dissatisfaction.[5] The reported figures for inadequate cellularity of CBs during the molecular analysis of lung cancer vary from 6.4% to 57%.[7,8]

Alternative specimens are air-dried direct smears, which have been shown to be a robust and valuable specimen source for molecular studies, when CBs exhibit insufficient cellularity.[8,9] The major advantage is that the cells on direct smears are not formalin-fixed, paraffin-embedded (FFPE); therefore, the quality of the DNA is better, and as a result, a 5- to 8-fold decrease in the amount of DNA required for molecular testing has been reported.[10]

Although there are many studies validating any form of cytology specimen as a reliable source for DNA-based testing,[2,4,7–10] in reality, we commonly face the challenge of small biopsies or cytology specimens with limited amounts of tumor cells. If pathologists order molecular testing only for the cases that they think have adequate tumor cellularity, the real rate of cases with insufficient quality or quantity is not known. To monitor the real rate of insufficient quantity or quality for molecular testing, we started assigning an accession number for all cases for which molecular tests were requested, regardless of the quality of the available tissue. The objective of this study was to assess the frequency and types of samples, including both surgical biopsies and cytologic materials, deemed unsatisfactory for molecular testing at a large academic institution.

MATERIALS AND METHODS

Patients and Test Subjects

We started assigning accession numbers to all patients for whom molecular tests were requested at our institution, regardless of the available tissue quality, on September 1, 2013. When molecular tests were requested for a lung cancer case, we reviewed all the cases available and selected the best specimen. In cases without sufficient tumor cells upon the pathologist's review, a report stating "quantity not sufficient (QNS)" was issued to monitor the true rejection rate. The reported rejection criteria included "not enough tumor cells present on the initial slide," "the percentage of tumor cells is less than 40%," "no tissue or no tumor cells are left in deeper sections," and "DNA quantity after extraction is not enough to perform further testing" (typically less than 0.1 μg of DNA or 1 ng/μL).

One year after this implementation, a retrospective search of the laboratory information system was performed for all cases submitted for lung cancer molecular testing to the Molecular Diagnostic Laboratory of the University of Alabama at Birmingham Hospital during the 1-year period from September 1, 2013 to August 31, 2014. The approval of the institutional review board was obtained for this study. Lung cancer molecular testing included *EGFR* mutation and *ALK1* gene rearrangement analysis for all cases. Although *KRAS* mutation and *ROS1* gene rearrangement analyses were requested for select cases, in this study, we focused on *EGFR* and *ALK1* tests.

A total of 176 cases were included in this study, and the patients' demographics are shown in Table 1. The median age of the patients was 66 years (range, 37-92 years), and there was a slightly male predominance (54.5%). The diagnosis was lung adenocarcinoma in the majority of cases (92.6%), and there were small percentages of cases with a diagnosis of poorly differentiated non–small cell lung carcinoma (NSCLC) or large cell neuroendocrine carcinoma (5.1% and 2.3%, respectively). These cases included both primary and metastatic lesions (59.1% and 40.1%, respectively). The specimens consisted of surgical resections (large specimens), small biopsies, and CBs of cytologic materials. When the cases with CB were rejected according to the criteria described previously, air-dried Diff-Quik (DQ)–stained smears of the respective specimens were reviewed for adequacy for molecular testing. The numbers of specimens deemed unsatisfactory for analysis were compared across 4 specimen categories: large resection, small biopsy, CB alone, and CB with air-dried DQ smears.

CB Preparation

The needle of each fine-needle aspiration pass after the creation of smears was rinsed in BD CytoRich red

TABLE 1. Demographics of the Cases for Which Molecular Tests Were Requested

	Total (n = 176)	Large Specimen (n = 73)	Small Biopsy (n = 78)	Cytology (n = 25)
Age, median (range), y	66 (37-92)	66 (37-83)	65.5 (42-92)	67 (44-86)
Sex, No. (%)				
Male	96 (54.5)	34 (46.5)	46 (59.0)	16 (64.0)[a]
Female	80 (45.5)	39 (53.5)	32 (41.0)	9 (36.0)
Primary or metastasis, No. (%)				
Primary	104 (59.1)	49 (67.1)	52 (66.7)	3 (12.0)[b]
Metastasis	72 (40.1)	24 (32.9)	26 (33.3)	22 (88.0)
Histology, No. (%)				
Adenocarcinoma	163 (92.6)	71 (97.3)	71 (91.0)	21 (84.0)[a]
Non–small cell lung carcinoma	9 (5.1)	1 (1.4)	5 (6.4)	3 (12.0)
Large cell/neuroendocrine	4 (2.3)	1 (1.4)	2 (2.6)	1 (4)

[a] Not significant ($P \geq .05$ for the 3 groups).
[b] $P < 0.05$ versus both large specimens and small biopsies.

preservative fluid or Hanks balanced salt solution. The cells in these solutions were centrifuged, and the pellet was mixed gently with a few drops of molten HistoGel (American Master Tech, Lodi, Calif) at a temperature of approximately 50°C. The HistoGel and the cell pellet were allowed to solidify and then were poured into a nylon-mesh biopsy bag and fixed in formalin for 2 to 12 hours, and then they were embedded in paraffin as a routine histology block.

DNA Isolation From FFPE Tissue and DQ Smears

For FFPE tissue, unstained 10-μm-thick slides were sectioned. DNA was macrodissected from the areas indicated on hematoxylin-eosin–stained slides by a pathologist with a Qiagen DNA blood mini kit according to the manufacturer's instructions (Qiagen, Valencia, Calif).[11] DNA was eluted in a final volume of 100 μL of Tris-EDTA (ethylendiaminetetraacetic acid) buffer.

For cytology smear slides, a representative slide was selected, and the coverslip was removed with xylene. The areas containing tumor cells on the slide after air drying were marked. DNA was extracted from these areas with the Pinpoint Slide DNA isolation system (Zymo Research, Irvine, Calif) according to the manufacturer's instructions; the purification step was included.[9] DNA was eluted in a final volume of 25 μL of Tris-EDTA buffer.

Genomic DNA concentrations were measured with the NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, Del).

EGFR Mutation Analysis

Exons 18 to 21 of *EGFR* were evaluated for mutations via polymerase chain reaction amplification with 35 ng of genomic DNA for each reaction and Sanger sequencing. The primer pairs used for polymerase chain reaction included 5′-TGT AAA ACG ACG GCC AGT CTG AGG TGA CCC TTG TCT CTG-3′ (forward) and 5′-CAG GAA ACA GCT ATG ACC CCA AAC ACT CAG TGA AAC-3′ (reverse) for exon 18, 5′-TGT AAA ACG ACG GCC AGT TGC CAG TTA ACG TCT TCC TT-3′ (forward) and 5′-CAG GAA ACA GCT ATG ACC CAG GGT CTA GAG CAG AGC AG-3′ (reverse) for exon 19, 5′-TGT AAA ACG ACG GCC AGT CAT TCA TGC GTC TTC ACC TG-3′ (forward) and 5′-CAG GAA ACA GCT ATG ACC TTA TCT CCC CTC CCC GTA TC-3′ (reverse) for exon 20, and 5′-TGT AAA ACG ACG GCC AGT TGA TCT GTC CCT CAC AGC AG-3′ (forward) and 5′-CAG GAA ACA GCT ATG ACC GGC TGA CCT AAA GCC ACC TC-3′ (reverse) for exon 21.[12] The quantity of the amplified product was analyzed with a fluorometer (Qubit 2.0 fluorometer; Life Technologies, Grand Island, NY). The amplified product was purified, and direct gene sequencing was performed with M13 sequencing primers 5′-TGTAAAACGACGGCCAGT-3′ (forward) and 5′-CAGGAAACAGCTAT GACC-3′ (reverse); it was then separated by capillary electrophoresis. A computer analysis of the sequencing data was performed to determine the presence or absence of mutations. Results were reported as positive or negative for mutations, and the description of the sequence variation was reported according to the Human Genome Variation Society nomenclature recommendations with the expected response to targeted therapeutic agents.

ALK Fluorescence In Situ Hybridization (FISH)

The *ALK* gene status was determined with a Vysis LSI *ALK* dual-color, break-apart rearrangement probe from

TABLE 2. QNS Rates With Different Types of Specimens

	Surgical Pathology		Cytology		
	Large Specimen	Small Biopsy	Cell Block	Cell Block + Smear	Total
Total	73	78	25	25	176
EGFR resulted, No. (%)	72 (98.6)	47 (64.1)	9 (36.0)	17 (68.0)	136 (77.3)
EGFR, limited results, No.		3			3
ALK resulted, No. (%)	72 (98.6)	70 (89.7)	16 (64.0)	16 (64.0)	158 (88.6)
QNS, No. (%)	1 (1.4)	28 (35.9)[a]	16 (64.0)[b]	8 (32.0)[c]	37 (21.0)

Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; QNS, quantity not sufficient.
[a] $P < 0.01$ versus both large specimens and cell blocks.
[b] $P < 0.01$ versus both large specimens and small biopsies.
[c] $P = .024$ versus cell blocks.

Abbott Molecular (Des Plaines, Ill) on FFPE tissue sections. A 4- to 5-μm-thick section obtained from FFPE tissue of a large specimen, small biopsy, or CB was mounted onto positively charged slides, and the area of the tumor was marked by a pathologist. The slides were deparaffinized in xylene, treated with a pretreatment solution followed by a protease solution treatment, and then hybridized with an *ALK* probe mixture for 14 to 24 hours according to the manufacturer's instructions. After washing, a 4',6-diamidino-2-phenylindole counterstain was applied, and each slide was examined under a fluorescent microscope with a proper filter set. Nuclei lacking *ALK* gene rearrangements showed a 2 orange/green fusion signal pattern (2F). Nuclei exhibiting *ALK* gene rearrangements demonstrated a 1 orange/green fusion, 1 orange, and 1 green signal pattern (1O1G1F) or an extra-orange signal (deletion of the green signal) in addition to a fused or broken-apart signal. Evidence of rearrangement in more than 15% was considered to indicate an *ALK*-positive tumor.

Statistical Analysis

The chi-square test and the unpaired Student *t* test were used to evaluate statistical significance. A *P* value equal to or less than .05 was considered statistically significant.

RESULTS

Molecular testing (*EGFR* mutation and *ALK* gene rearrangement analysis) was requested for a total of 176 lung carcinoma cases. The specimens consisted of 73 large surgical resections, 78 small biopsies, and 25 cytologic specimens (Table 1). Cytology specimens had a significantly higher proportion of metastatic disease (88% vs 33% for both large specimens and small biopsies, $P < .05$; Table 1). The vast majority of histologic diagnoses were adenocarcinomas in all 3 groups; there were small proportions of NSCLC and large cell neuroendocrine carcinoma. Although more cases with a diagnosis of NSCLC had small biopsies and cytology specimens, there was no statistically significant difference in the histologic diagnosis distribution (Table 1).

Overall, 45 cases (25.6%) were rejected because of insufficient materials (QNS) for *EGFR* mutation analysis (Table 2). The QNS rate for large resection specimens was significantly lower: only 1 of 73 cases (1.4%) was rejected (Table 2). This large surgical sample was unsatisfactory because the sample was decalcified in an acid solution during processing. The QNS rate for small biopsy specimens was 35.9% (28 of 78), which was significantly higher than the rate for large specimens. The reason for rejection in the majority of the cases was an insufficient number of tumor cells in the deeper sections. Three small biopsy cases yielded partial sequence data, mostly exon 19 only or exons 19 and 21 (limited results), because of a low yield of DNA (0.2-0.3 μg of DNA). Exon 19 has the smallest amplicon, and this explains the better success rate. Only 18 cases (11.4%) were labeled QNS for *ALK* FISH overall: 1 large specimen, 8 small biopsies, and 9 cytology cases. This high success rate was due to the fact that FISH requires only 1 section of tumor with a 4- to 5-μm thickness, whereas sequencing-based analysis requires 5 to 10 unstained slides with a 10-μm thickness for DNA isolation, especially when the area of the tumor is small.

In contrast to surgical specimens, 64% of the cases (16 of 25) ordered on cytology CB specimens were rejected, and this was significantly higher than the rate for both types of surgical specimens (Table 2). Air-dried,

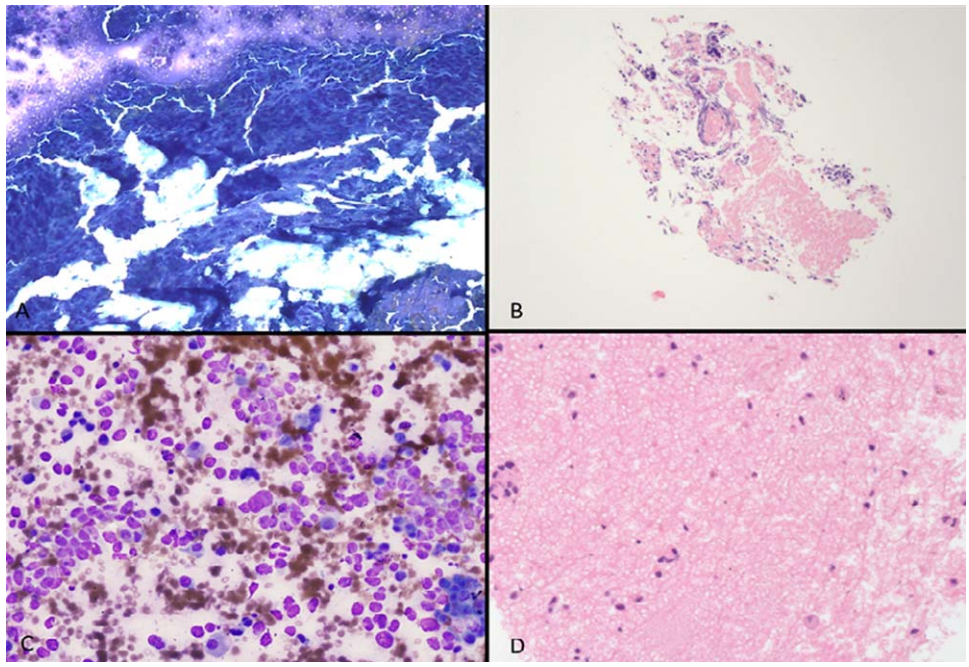

Figure 1. (A,C) Air-dried Diff-Quik smears and (B,D) corresponding cell blocks from endobronchial ultrasound–guided transbronchial needle aspirates from the mediastinal lymph node (low magnification for panels A and B and high magnification for panels C and D).

DQ-stained smears were reviewed for the cases that did not have enough tumor cells in CBs. Eight cases had DQ smears that contained enough tumor cells for *EGFR* mutation analysis (see Fig. 1 for an example). The DNA extracted from these specimens had a total nucleic acid yield of 0.645 ± 0.22 μg (mean ± standard error of the mean) with a corresponding 260/280 ratio of 1.8 ± 0.08 (range, 1.38-2.09). In contrast, the amounts of DNA extracted from large surgical specimens, small biopsies, and CBs that were not rejected were 5.2 ± 0.79, 1.29 ± 0.33, and 1.23 ± 0.26 μg (mean ± standard error of the mean), respectively (Fig. 2A), with 260/280 ratios of 2.10 ± 0.04, 2.29 ± 0.10, and 2.12 ± 0.14, respectively (Fig. 2B). The DNA yield from the large specimens was significantly higher than that from any other specimen type, but there was no significant difference between the remaining specimen types. The 260/280 ratio ranged from 1.8 to 2.0 for the vast majority of the cases; however, the small biopsy group had several cases with a ratio greater than 4.0, but this was probably not reliable because of the low yield of DNA (0.3-0.4 μg). One case isolated from a smear slide had only 0.06 μg of DNA, which was much lower than the typical cutoff for DNA obtained from FFPE tissue (0.1 μg). In this case, the 260/280 ratio was very low (1.38), and this would not be reliable because the concentration of the DNA was so low (2.4 ng/μL). However, all exons were amplified successfully from the smear slides of all 8 cases, and the sequencing electropherograms were of excellent quality and allowed the analysis of exons 18 to 21 for these cases. Therefore, in combination with DQ smears, the QNS rate of cytology specimens was 32% (8 of 25), which was a significant improvement over the rate with CBs only ($P = .024$). The QNS rates for small biopsies (36.71%) and cytologic specimens with DQ smears (32%) were not significantly different ($P = .671$).

An *EGFR* mutation previously reported to correlate with responsiveness or resistance to EGFR tyrosine kinase inhibitor therapies was found in 11.5% of the cases (16 of 139), which included 1 case tested with a CB and 1 case tested with a DQ smear. A deletion mutation in exon 19 was found in 8 cases, missense mutations in exon 21 (L858R or L861R) were found in 7 cases, and a missense mutation in exon 18 was found in 1 case. *ALK* rearrangement was found in 9% of the cases (15 of 156). The frequency of alteration of *EGFR* was similar to the reported frequency in non-Asian populations (10%-15%).[1,13–15] The frequency of *ALK* gene rearrangement ranges from 2% to 13%.[16,17] Therefore, our results are similar to the gene alteration rates in a similar population.

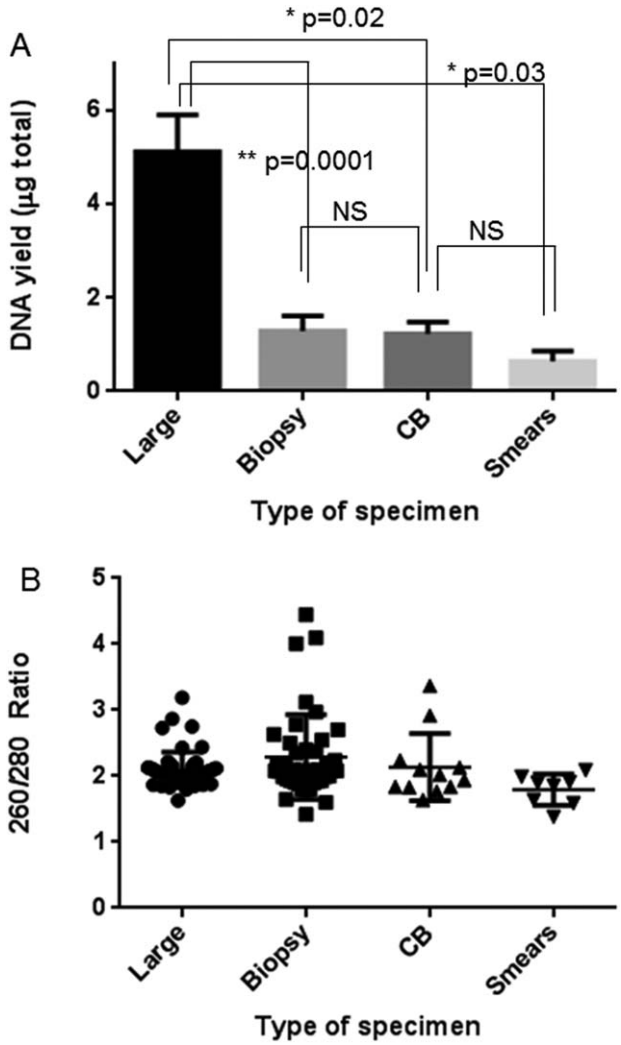

Figure 2. (A) DNA yield and (B) 260/280 ratio for different types of specimens determined by NanoDrop. *$P < .05$ for large resections versus CBs; **$P < .01$ for large resections versus small biopsies. CB indicates cell block; NS, not significant ($P \geq .05$ for small biopsies, CBs, and smears).

DISCUSSION

We have noticed that the clinical demand for ancillary molecular testing of small samples, including cytologic specimens and small biopsies, has been growing continuously. This is especially true for lung cancer cases because many patients with lung cancer present at an advanced stage for which surgery is not a treatment option.[2] We are frequently challenged to perform molecular testing on relatively scant samples. However, evaluating the real rate of cases with insufficient quantity or quality for molecular testing is not easy in a normal clinical setting. This is mainly because the cases with few tumor cells or small tissue samples are rejected by the pathologist who reviews the slides, and these cases are not sent to molecular diagnostic laboratories. We started to place orders for all patients for whom molecular tests were requested, regardless of the available tissue quality. We reviewed all cases available for the patients and selected the best specimens. If no adequate tissue was available, we issued a report stating that there was insufficient quantity or quality (ie, QNS).

Our study showed that 1.4% of large surgical specimens and 36% of small surgical biopsies were deemed unsatisfactory for analysis. In contrast, 64% of cytology CB specimens were rejected, and this was significantly higher than the rate for surgical specimens. Among those cases, approximately half had enough tumor cells in air-dried, DQ-stained smears, and this reduced the unsatisfactory rate to 32%, which was significantly lower than the rate for CBs only and slightly lower than the rate for small surgical biopsies. There was no significant difference between small biopsy and CB plus DQ specimens. We note that the number of cytology specimens was small in this study; however, our data indicate that although CBs appear to be unsatisfactory specimens, the utilization of DQ-stained smears for molecular testing has improved the adequacy of cytologic samples, and this provides a minimally invasive alternative to surgical biopsy when the molecular analysis of tumor material is necessary. Further evaluation with an increased number of samples is warranted.

Cytology CBs have been used routinely for more than a century. CB sections offer advantages over conventional cytological smears with respect to cellular architecture and archival storage as well as readiness for special stains, immunophenotypic analysis, and molecular tests.[4] According to recently published CAP, IASLC, and AMP guidelines, cytologic samples are suitable for *EGFR* testing, and CBs are the preferred medium.[1] However, the yield of tumor cells in CBs appears somewhat inconsistent, and variability in CB cellularity represents the most significant limitation. The figures reported for inadequate cellularity in the molecular analysis of lung cancer vary.[7,8] Knoepp and Roh[8] reported that up to 57% of CBs either were acellular or had sparse/borderline cellularity.

There are several different techniques for preparing CBs. The most popularly used methodologies are plasma thrombin and agar techniques.[4] Although each method has its own advantages and disadvantages, a valid comparison of the efficacy of all described methods is a difficult

task because of the lack of a uniform methodology and differences in published technical details.[4,5] Recently, Yung et al[18] reported improvements of cellularity on CB preparations with the tissue coagulum clot method during endobronchial ultrasound–guided transbronchial fine-needle aspiration. Our institution uses an agar embedding method with HistoGel (American Master Tech, Lodi, Calif). Our results indicate that improved CB processing is needed. Dedicated needle passes for CBs, rather than a rinse of the residual specimen after the smear is made, would also help to improve the cellularity of the specimen.[8]

In contrast, direct smears (especially air-dried, DQ-stained smears) for ancillary studies appear to be quite useful and should be more appreciated. With the Zymo Pinpoint Slide DNA isolation system, the areas of interest are easily marked, and DNA in the area is extracted relatively easily.[9,11] DNA extracted from DQ smears has good quality because they are not paraffin-embedded. Unlike CBs, direct smears can be evaluated on site for adequacy. Our data indicate that the utilization of DQ-stained smears for molecular testing has improved the adequacy of cytologic samples, and it provides a minimally invasive alternative to surgical biopsy when the molecular analysis of tumor material is necessary. We recommend the use of DQ-stained smears for molecular analysis when CB material is inadequate.

Interestingly, we noticed that the cytologic specimens had a significantly higher percentage of metastases in comparison with surgical specimens (Table 1). Our previously described workflow ensures that there are not alternative specimens for such patients. This indicates that in many circumstances, for cases with metastatic disease, the only available specimen type is a cytologic specimen because of the less invasive nature of the procedure. This emphasizes that we need to have a better success rate with cytologic specimens.

The methodology of molecular oncology testing has been shifting from single-gene testing to panel testing with next-generation sequencing. Cytological specimens have validated next-generation sequencing oncology panel analysis.[10] No matter what methodology is used to analyze genetic alterations present in cancer, the key initial step is sufficient DNA quality and quantity, which are always necessary for a successful analysis. Improvements in this end of the assay workflow are important despite advances in the genomic test technique.

In summary, our study demonstrated that in combination with DQ smears, the QNS rate for cytology specimens was improved significantly from 64% to 32%, which was not significantly different from the QNS rate for small biopsies. The utilization of DQ-stained smears for molecular testing has improved the adequacy of cytologic samples, and it provides a minimally invasive alternative to surgical biopsy when the molecular analysis of tumor material is necessary. At the same time, better CB processing techniques and standardized quality controls are necessary for successful molecular testing.

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The authors made no disclosures.

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