Evaluation of *EGFR* Mutation Status in Cytology Specimens:
An Institutional Experience

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Epidermal growth factor receptor (EGFR) mutation status has been shown to predict response to anti-EGFR tyrosine kinase inhibitors in non-small cell lung cancer (NSCLC). In patients with advanced-stage NSCLC, evaluation of mutational status is increasingly requested on biopsy or fine-needle aspiration specimens, which often have limited material. There are limited data on the suitability of cytology cell blocks (CB) for EGFR mutation testing. In this study, we report our institutional experience with cytology cell block material for EGFR mutation testing. We retrospectively reviewed EGFR mutation analyses performed on 234 surgical (SP) and cytology (CB) from October 2007 to May 2010. One hundred ninety-two SP specimens and 42 CB specimens were evaluated for EGFR mutation. CB specimens were evaluated for overall specimen size based on aggregate cellularity in comparison to small biopsy specimens, and percent tumor. Of the 192 SP and 42 CB specimens, 31 (16.1%) and 11 (26.2%) were positive for EGFR mutation, respectively; there does not appear to be an association between mutation detection rate and the source of the specimen ($P = 0.124$). Limited DNA was obtained from 70.0% (29/42), including 81.8% (9/11) of those which were mutation positive. Additionally, 45.4% (5/11) of mutation positive specimens had extremely low DNA yields. Although 16.6% (7/42) of CB specimens had <10% tumor, all 11 mutation positive CB cases had >10% tumor. These data indicate that CB specimens provide an alternative source for molecular evaluation of NSCLC, and that tumor percentage may be more important than specimen size and/or DNA yield in determining the suitability of these specimens for testing. Diagn. Cytopathol. 2013;41:316–323.

Key Words: EGFR; molecular; mutation; cell block; adequacy

Lung cancer is the most frequent cause of cancer-related death, with an estimated mortality of 1.4 million deaths annually worldwide.[1] Despite the high burden of this disease and extensive ongoing research, the median survival for patients with lung cancer remains low, with 5 year relative survival rates averaging just above 15%.[2]

In the last decade, it has become evident that aberrations of the epidermal growth factor receptor (*EGFR*) signaling pathway are a common event in non-small cell lung cancer (NSCLC).[3] Activating mutations in *EGFR* (*ERBB1, HER1*) are seen in a significant subset of patients with NSCLC, ranging from 10 to 40% of patients, with increased incidence in adenocarcinoma, non-smokers, females, and Asians.[4] EGFR is a receptor tyrosine kinase which, upon activation, triggers a downstream cascade of growth-promoting signals, and activating mutations in *EGFR* are thought play a key role in the molecular pathogenesis of NSCLC.[5] The elucidation of the role of activating *EGFR* mutations in NSCLC carries a significant clinical import, as treatment with the EGFR tyrosine kinase inhibitors gefitinib and erlotinib have been shown to prolong progression free survival in patients harboring an activating mutation.[6] These findings have led to the recommendation to use EGFR tyrosine kinase inhibitors as first-line therapy in patients with advanced stage disease harboring an activating mutation in *EGFR*.[7]

The evaluation of *EGFR* mutation status in patients with advanced stage NSCLC has increasingly moved into routine clinical practice, and is considered by some to be a new standard of care.[8] However, a logistical predicament occurs in this setting, as patients with advanced stage NSCLC are not considered surgical candidates, and thus there is frequently limited lesional tissue on which to perform *EGFR* mutation analysis. The majority of patients with NSCLC presents with advanced stage disease[2,9] and

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Received 3 June 2011; Revision 5 September 2011; Accepted 12 September 2011
DOI 10.1002/dc.21851
Published online 18 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

do not undergo surgical resection; therefore this problem carries broad implications in the widespread implementation of molecular characterization prior to targeted therapy.

At present a common modality for the diagnosis of NSCLC is through evaluation of cytologic specimens (fine-needle aspiration (FNA), pleural fluids, and bronchial washing and brushings). Therefore, it is of crucial clinical importance to understand whether small samples, such as cytology specimens, are adequate for mutational analysis of tumors. Previous studies have examined the suitability of small specimens for *EGFR* mutational analysis using a variety of approaches and have demonstrated that preparations from cytology material are appropriate for testing.[10] Here, we report our institutional experience at the University of Pennsylvania in evaluating and utilizing cell block preparations of routine cytology material for directed mutational analysis of *EGFR* for therapeutic decision-making in patients with NSCLC.

Methods

Patient Specimens

We retrospectively reviewed a cohort of 234 consecutive specimens with NSCLC on which *EGFR* mutation testing was performed clinically between October 2007 and May 2010 with appropriate institutional approval (IRB protocol 810602). One hundred ninety-two (192) specimens were surgical pathology (SP) specimens, consisting of either biopsy or resection material, and 42 specimens were cytology cell blocks (CB). Cytology CB were chosen for testing for mutational analysis of *EGFR* mutation when no other specimen was available, or paired biopsy material was deemed to be inadequate due to no or low tumor content. The cytology cell block specimens included FNA of primary lung lesions, FNA of metastatic lesions, and exfoliative cytology specimens. The cytology specimens were processed according to routine cytopathology procedures, with a portion of the material utilized for air-dried Diff-Quik ® stained slides, Papanicolaou stained alcohol fixed smears (the smears were made only in FNA specimens), and the remaining aspirated material was rinsed in Normosol ® for cytopsin (made only in cases of exfoliative cytology specimens), ThinPrep®, and cell block preparations and was stored at 4°C if not immediately processed. The CB were prepared within 24 hours of specimen receipt by centrifuging the pooled needle rinse at 1800 rpm for 10-min to form a pellet/sediment which was resuspended in 10 ml of 95% formalin solution. This was followed by another round of centrifugation at 1800 rpm for 10 min; the cell button was gently scraped and transferred to a small filter paper packet for routine paraffin embedding and evaluation by H&E staining. Immunohistochemical stains for TTF-1 (Mouse monoclonal, 1:50 dilution, Dako, Inc., Carpinteria, CA) were performed as part of the routine diagnostic evaluation at the discretion of the cytopathologist. In three cases, a matched pair consisting of resection specimen and FNA specimen was available for evaluation.

Cytology cell block specimens were retrospectively reviewed by three pathologists for assessment of tumor percentage. Tumor percentage was evaluated based on the number of tumor cells compared to all nucleated cells in the specimen, and graded by consensus into five categories of tumor percentage: $<5\%$; >5–10%; >10–25%; >25–50%; $>50\%$. When available, TTF-1 stain of the cell block was utilized to confirm the morphologic determination of the tumor percentage. CB were also assessed for the number of tumor clusters of at least 10 cells per $\times 10$ field (averaged over 5 observed fields in areas of highest cellular concentration). Cell clusters which were greater than 10 cells were counted as multiples of 10 clusters as appropriate (i.e., a cluster of approximately 20 cells counted as two clusters). Additionally, CB were estimated for overall size of tissue present based on a visual aggregation of tissue into four categories: scant; >0.5–2 mm^2; 2–5 mm^2, >5 mm^2–1 cm^2.

Evaluation of EGFR *Mutation Status*

Two major types of *EGFR* mutations, exon 19 in-frame deletions and p.Leu858Arg (L858R), which account for approximately 90% of mutations by prevalence, were evaluated. For SP specimens, formalin-fixed paraffin-embedded tissue was cut into 5 micron sections and manually macrodissected to enrich for areas of tumor based on areas identified by a surgical pathologist. For cell block specimens, 10 micron paraffin curls were cut from the cytology CB, and 6–9 curls of tissue were utilized for DNA extraction, with the exception of one cytology cell block specimen, which showed an area with a high concentration of tumor cells and was manually macrodissected in a manner analogous to a SP specimen. DNA from the specimens was extracted by deparaffinization with xylene followed by proteinase K (Qiagen, Inc.) digestion in Qiagen ATL buffer overnight at 56°C. After an RNase digestion step, the DNA was extracted using QIAmp (Qiagen, Inc.) spin columns according to the manufacturer instructions. Analysis of DNA concentration was performed using spectrometry. Evaluation for *EGFR* mutation status was performed using a polymerase chain reaction (PCR) approach as previously described.[11] Briefly, two separate PCR reactions were performed to amplify regions of interest in exon 19 and exon 21 using fluorescently labeled PCR primers. The products of the exon 19 amplification were directly evaluated for size to determine whether a fragment of reduced size, representing a deletion was present. The products of the exon 21 amplification were subjected to restriction endonuclease digestion with Sau96I, which allows for the detection of the L858R mutation (NM_005228.3:c.2573T>G) by virtue

Table I. Patient Characteristics

	All cases	*Surgical cases*	*Cytology cases*
N	234	192	42
Age (years)			
Range	25–96	25–96	40–80
Median	64	64	67
Gender			
M (%)	88 (38)	75 (39)	13 (31)
F (%)	146 (62)	117 (61)	29 (69)
Smoking status			
Never smoker (%)	62 (26.5)	51 (26.6)	11 (26.2)
Ever smoker (%)	155 (66.2)	126 (65.6)	29 (69.0)
Not available (%)	17 (7.2)	15 (7.8)	2 (4.8)
Race			
Caucasian	158 (67.5)	122 (63.5)	36 (85.7)
African descent	19 (8.1)	17 (8.9)	2 (4.8)
Asian	3 (1.3)	2 (1.0)	1 (2.4)
Hispanic	1 (0.4)	1 (0.5)	0 (0)
Other	5 (2.1)	5 (2.6)	0 (0)
Not available	48 (20.5)	45 (23.4)	3 (7.1)

of the fact that this mutation generates a Sau96I restriction site. Amplification and digestion products were separated by capillary gel electrophoresis on an Applied Biosystems 3130XL according to the manufacturer's protocol. Data analysis was performed with GeneMapper 3.7 software. Interpretive criteria for the presence of mutation were as previously described. The *EGFR* mutation assay was determined to have an analytic sensitivity of $\sim$5% based on dilution studies with an input of 25 ng of DNA.

Statistical Analyses

Statistical analysis was performed using Stata 11 (StataCorp LP, College Station, TX). Comparisons between the surgical and the cytology specimens were made using the

Table II. Specimen Characteristics

No.	*Gender*	*Procedure Type*	*Site*	*Diagnosis*
1	F	FNA	Lung	Adenocarcinoma
2	F	FNA	Lung	Adenocarcinoma
3	M	Fluid	Pleural effusion	Adenocarcinoma
4	M	EBUS-FNA	Lymph node	Adenocarcinoma
5	F	FNA	Bone	Adenocarcinoma
6	F	Fluid	Pleural Effusion	Poorly differentiated carcinoma
7	M	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
8	F	FNA	Bone	Adenocarcinoma
9	M	FNA	Lung	Adenocarcinoma
10	F	Fluid	BAL	Adenocarcinoma
11	F	EBUS-FNA	Lymph node	Adenocarcinoma
12	F	EBUS-FNA	Lymph node	Adenocarcinoma
13	F	FNA	Bone	Adenocarcinoma
14	M	EBUS-FNA	Lung	Poorly differentiated carcinoma
15	F	Fluid	Pleural Effusion	Adenocarcinoma
16	F	FNA	Bone	Adenocarcinoma
17	F	EBUS-FNA	Lymph node	Adenocarcinoma
18	F	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
19	F	Fluid	Pericardial Effusion	Poorly differentiated carcinoma
20	F	FNA	Scapula	Adenocarcinoma
21	F	Fluid	Pleural effusion	Non-small cell lung carcinoma, favor adenocarcinoma
22	F	FNA	Lung	Poorly differentiated carcinoma
23	M	EBUS-FNA	Lymph node	Adenocarcinoma
24	M	EBUS-FNA	Lung	Poorly differentiated carcinoma
25	F	EBUS-FNA	Mass	Non-small cell lung carcinoma, favor adenocarcinoma
26	F	EBUS-FNA	Lymph node	Adenocarcinoma
27	M	Fluid	Pleural	Poorly differentiated carcinoma
28	F	EBUS-FNA	Lung	Adenocarcinoma
29	F	EBUS-FNA	Lung	Adenocarcinoma
30	F	EBUS-FNA	Lymph node	Adenocarcinoma
31	F	EBUS-FNA	Lymph node	Non-small cell lung carcinoma, favor squamous
32	M	FNA	Lung	Adenocarcinoma
33	F	Fluid	Pleural	Adenocarcinoma
34	F	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
35	M	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
36	F	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
37	M	EBUS-FNA	Lymph node	Adenocarcinoma
38	F	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
39	M	EBUS-FNA	Lymph node	Poorly differentiated carcinoma
40	F	FNA	Lung	Poorly differentiated carcinoma
41	F	FNA	Lung	Non-small cell lung carcinoma
42	F	EBUS-FNA	Lymph node	Poorly differentiated carcinoma

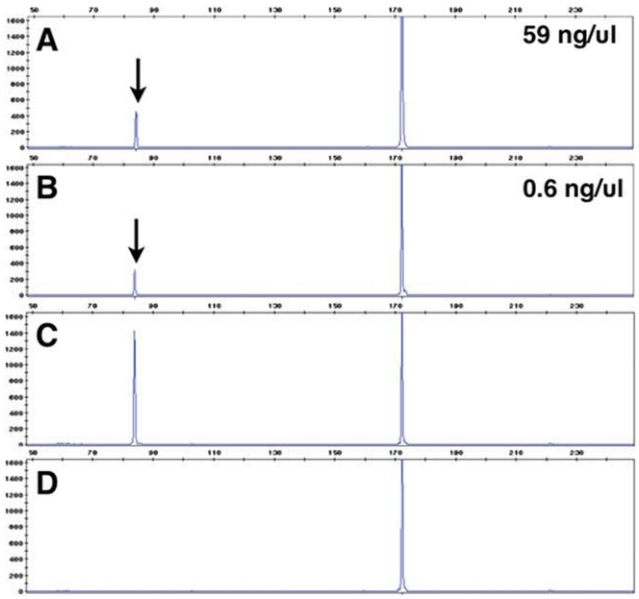

Fig. 1. Electropherograms of matched resection and cytology patient specimen for *EGFR* L858R mutation. Arrow indicates the peak generated from Sau96I digestion of mutant PCR product. **A**: Resection specimen; (**B**) cytology cell block; (**C**) positive control; (**D**) negative control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Wilcoxon rank sum test (age) and the chi-square test (*EGFR* mutation status, race, smoking history, and gender). *P* values are reported as indicated.

Results

Patient and Specimen Characteristics

The demographic and clinical characteristics of the 234 patients included in this study are listed in Table I. Overall, 38% of the patients were male and 62% were female. Smoking history was available in 217 (93%) patients; racial and ethnic data were available in 186 (80%) patients. The distribution of gender, smoking history, and racial/ethnic background were similar in patients for both SP and CB specimen sets ($P = 0.33$, $P = 0.34$, $P = 0.092$, respectively, indicating lack of statistically significant difference in the populations studied). The type and site of each of the cytology specimens is listed in Table II.

Matched Specimen Evaluation

To determine first whether a mutation could be detected in cytology cell block material, we identified cases which met the following criteria: (1) resection and cytology evaluation had been performed on the same site in the same patient, (2) cytology cell block was available, (3) prior positive finding of mutation by directed *EGFR* mutation analysis had been accomplished in the resection material, and (4) all pathologic material was available for testing. The evaluation was restricted to cases in which a prior directed *EGFR* mutation analysis was positive, as a negative mutation evaluation in cytology material in which the resection was negative would not be informative. Three (3) cases in which all criteria were met were identified, one with the L858R mutation in the resection specimen, the other two specimens showing an exon 19 in-frame deletion of 15 base pairs in the resection specimen. Testing of the paired cytology specimen from the same site demonstrated the presence of the same mutation in all three cases (Fig. 1, one representative sample depicted). Of note, the cytology specimen showed a low yield of genomic DNA in one of the three cases, (extracted DNA concentration of 0.6 ng/ul, 130 ng/ul, 25ng/ul in the three specimens); however this low input material did not prevent detection of the mutation. Only the specimen in Figure 1 is included in the subsequent analysis, as evaluation of the *EGFR* mutation status in the remaining two cytology specimens was not undertaken as part of clinical routine, and was instead investigated strictly for concordance with the known *EGFR* mutation status seen in a resection specimen.

Table III. Mutation Frequencies by Specimen Type

	All Cases	*Surgical cases*	*Cytology cases*
N	234	192	42
No. Positive (%)	42 (17.9)	31 (16.1)	11 (26.2)

EGFR *Mutation Status*

As there were limited cases in which evaluation of parallel surgical and resection specimens could be performed, cytology specimens were evaluated for the rate of positive mutation detection as a surrogate mechanism to evaluate adequacy. Only specimens in which the clinical testing of *EGFR* mutation status was performed on the cytology specimen, typically due to lack of availability of a SP specimen showing tumor, were included in the analysis. Of the 42 cytology specimens evaluated, a mutation was detected in 11 (26.2%) while the mutation detection rate for all specimens was 17.9% (42 of 234); and for surgical specimens was 16.1% (31 of 192) (Table III). The mutation detection rates between the surgical specimens and cytology specimens are not statistically different ($P = 0.124$).

Specimen Features

To determine whether specific features of cytology cell block specimens contribute to adequacy for mutational analysis, the specimens were evaluated with regards to several parameters: approximate tumor percentage, estimated total specimen size, number of 10-cell clusters/×10 field, and DNA concentration (Table IV). Based on a minimum specimen DNA concentration of 25 ng/ul (the specimen DNA concentration for which the assay was

Table IV. Cell Block Evaluation

Case no.	*Tumor percentage (%)*	*TTF-1 IHC*	*10× clusters*	*Specimen size*	*DNA concentration (ug/uL)*	*Mutation status*
1	>10–25	Y	4–6	0.5–2 mm^2	0.6	L858R
2	>25–50	Y	4–6	0.5–2 mm^2	1.7	L858R
3	>50	N	>7	2–5 mm^2	139	WT
4	>50	Y	>7	2–5 mm^2	254	Exon 19 deletion
5	>50	Y	>7	2–5 mm^2	79	WT
6	>25–50	Y	4–6	0.5–2 mm^2	0.8	L858R
7	<5	N	1–3	Scant	8	WT[a]
8	>5–10	N	4–6	2–5 mm^2	28	WT
9	>25–50	N	>7	>5 mm^2–1 cm^2	4	WT
10	>10–25	N	4–6	0.5–2 mm^2	6	WT
11	>50	N	>7	>5 mm^2–1 cm^2	37	WT
12	>10–25	Y	>7	0.5–2 mm^2	2	Exon 19 deletion
13	>25–50	N	4–6	0.5–2 mm^2	6	WT
14	>10–25	Y	>7	2–5 mm^2	9	WT
15	>25–50	Y	>7	>5 mm^2–1 cm^2	14	Exon 19 deletion
16	>10–25	Y	N/A[a]	Scant	2	WT
17	<5	N	1–3	0.5–2 mm^2	3	WT
18	>25–50	N	>7	2–5 mm^2	34	Exon 19 deletion
19	<5	Y	N/A[a]	2–5 mm^2	8	WT
20	>25–50	Y	4–6	0.5–2 mm^2	6	WT
21	<5	N	N/A[a]	0.5–2 mm^2	6	WT
22	>25–50	Y	1–3	0.5–2 mm^2	0	WT
23	>5–10	Y	1–3	0.5–2 mm^2	28	WT
24	>50	N	>7	>5 mm^2–1 cm^2	33	WT
25	>10–25	N	1–3	Scant	5	WT
26	>25–50	Y	1–3	Scant	17	WT
27	>50	Y	>7	>5 mm^2–1 cm^2	85	WT
28	>50	Y	4–6	0.5–2 mm^2	19	Exon 19 deletion
29	>25–50	Y	4–6	0.5–2 mm^2	38	WT
30	>25–50	N	1–3	0.5–2 mm^2	14	WT
31	>25–50	Y	1–3	0.5–2 mm^2	18	WT
32	>50	N	>7	2–5 mm^2	23	WT
33	>50	Y	>7	0.5–2 mm^2	23	WT
34	>50	N	>7	0.5–2 mm^2	14	L858R
35	>50	Y	4–6	2–5 mm^2	20	L858R
36	>50	N	>7	2–5 mm^2	17	WT
37	>50	N	>7	0.5–2 mm^2	35	WT
38	>50	Y	>7	2–5 mm^2	35	WT
39	>25–50	N	>7	0.5–2 mm^2	14	WT
40	>5–10	Y	1–3	Scant	1	WT
41	>50	Y	4–6	scant	4	L858R
42	>50	N	>7	2–5 mm^2	60	WT

[a]Slides not available for review of this feature.

validated), 29 (69.0%) of 42 cell block specimens would have been classified as inadequate based on DNA concentration. However, of these 29 specimens, 10 had detectable mutation, representing about 91% of all cytology specimens with a detectable mutation and 15 (51.7% of total) of the 29 low yield specimens had DNA concentration that were less than 25% of the ideal input; yet 5 of these had a detectable mutation. The lowest DNA concentration with which a mutation was detectable was 0.6 ng/ul (case no. 1), which is more than 30-fold less than the ideal input for the assay.

Of the 11 cell block specimens with an identifiable mutation, all were estimated to have at least 10% tumor cellularity. This targeted mutation assay was previously determined to have an analytic sensitivity of ~5% based on dilution studies; thus it would be expected that in a population heterozygous for mutation without concurrent gene amplification, 10% tumor cellularity would represent the limit of detection. Of the 42 cell block specimens evaluated, 7 demonstrated less than 10% tumor cellularity, and all of these were negative for detectable mutation. Additionally, the utilization of concurrent immunohistochemistry in evaluation of cell block specimens was felt to be of significant interpretive benefit in pre-testing evaluation of the specimens, as they often highlighted isolated tumor cells.

Evaluation of cytology specimen size and number of 10-cell clusters per ×10 field demonstrated that 9 had low number of 10-cell clusters per ×10 field (1–3 clusters/×10 field), and of these 9 specimens, 5 (55.5%) also had low tumor cellularity. All cases with a low number of 10-cell clusters per ×10 field were negative for detectable mutation. Two specimens could not be evaluated for number of 10-cell clusters per ×10 field as the slides were not available at the time of analysis of cell clusters. Of

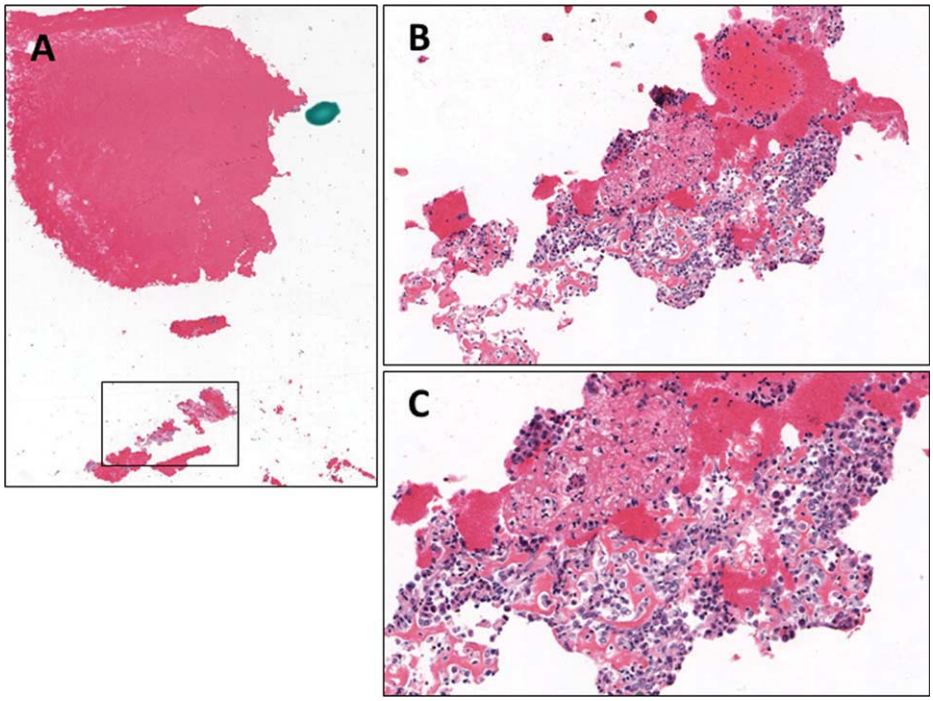

Fig. 2. Representative example of cytology cell block specimen with small size, low overall cellularity, and high tumor cellularity (**A**) scanning power (3×); (**B**) 70× (inset box); (**C**) 100× (inset box). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the 11 specimens positive for mutation, 8 (72.7%) showed an estimated total specimen size of 0.5–2 mm^2. There was a trend towards correlation between DNA yield and visually estimated size of specimen, with larger specimens tending to have higher DNA yields (not evaluated statistically), however of the 29 specimens with low DNA yields, 7 were from specimens of size >2 mm^2. Additionally, there was trend of low number of 10-cell clusters/×10 field with low DNA yields, with 8 of 9 such specimens showing low yields.

Of the 42 cell block specimens evaluated, 25 (59.5%) had a size of <2 mm^2. Twenty of these specimens demonstrated tumor cellularity of >10%, and 15 demonstrated tumor cellularity of >25%. Overall, 29 (69.0%) of all specimens had a tumor cellularity of >25%. An example of a specimen with low specimen size with high tumor cellularity is shown in Figure 2.

Discussion

Evaluation of mutation status in NSCLC is becoming increasingly important, as therapeutic options rest on the genotypic characterization of the tumor. Recent large clinical trials have conclusively demonstrated the benefit of EGFR tyrosine kinase inhibitor therapy, and have further demonstrated the association of response with the presence of mutation in *EGFR*.[6,12] However, as patients frequently present with advanced disease, it is common for the initial diagnosis to be established using cytopathology, with no intention for surgical resection of the primary lesion. In this setting, it is critical to use existing material from cytologic specimens to genotypically characterize the lesion for potential beneficial therapeutic options. Several studies have demonstrated that extremely small specimens, such as cytology CB, can be utilized for directed *EGFR* mutation analysis.[10,13–23] The finding that a mutation is detectable in small specimens with extremely low yield of genomic DNA indicates that the size and/or yield of the specimen is not necessarily a limiting factor for mutational analysis. However, as the analytic methodology was validated using an input level of nucleic acid that is above the yield for many small specimens, for those specimens which did not meet this input requirement and had a negative result, we have routinely included a disclaimer in the molecular pathology report to indicate that the analytic sensitivity of the assay may be compromised by reduced nucleic acid input. Additionally, it is critical to anticipate that in the era of "personalized medicine," as the number of analytes routinely requested per specimen rises, our ability to comprehensively accommodate such requests may be limited. Therefore it is critical to either design a test which simultaneously evaluates multiple analytes using small quantities of input material, or recognize that clinical prioritization of testing is likely to be necessary.

In this study, all specimens which had a detectable mutation showed a tumor cellularity of at least 10%, which is concordant with the analytic sensitivity of the

method utilized. Though based on our limited cohort it is not possible to definitively conclude that 10% tumor cellularity is the requirement for specimen adequacy using this testing methodology, our data strongly suggest this is the case. It is important to note that this pre-analytic evaluation is dependent on the methodology utilized for pre-analytic processing and mutation analysis. Utilization of a different methodology, such as Sanger sequencing, would likely require that a greater proportion of tumor be present to confidently detect mutation, or lack thereof. Similarly, a change in pre-analytic processing (such as microdissection in which tumor cells are selectively isolated microscopically, either through manual microdissection, or laser capture microdissection) could impact the threshold which is used to judge the suitability of specimens for testing. Although a side-by-side evaluation of different pre-analytic processing or mutation detection methodologies was not attempted in this study, knowledge of the analytic sensitivity of the specific assay methodology performed by a testing laboratory is key in establishing criteria for acceptability of specimens. This also underscores the necessity for morphologic evaluation of specimens which are tested for *EGFR* mutation, as testing a specimen without such evaluation may result in a false negative due to low tumor cellularity in a specimen. Immunohistochemical stains such as TTF-1 can help to confirm a morphologic impression of tumor cell content in a specimen; however, caution must be exercised to not over interpret staining of normal or reactive cells within the specimen. This approach was found by these authors to be especially useful in the setting of specimens from anatomic locations in which staining of background cells would not be anticipated (i.e., lymph node aspirate).

These findings are consistent with previous studies which have demonstrated that *EGFR* mutations can be detected utilizing a variety of methodologies on cytopathologic specimens, such as those obtained from endobronchial ultrasound guided fine-needle aspiration (EBUS-FNA) or other aspiration or exfoliative cytology techniques.[10,13–23] These data further this observation by demonstrating that *EGFR* mutation testing can be accommodated as part of the routine evaluation of specimens from patients with NSCLC.

One potential confounding factor in this approach to *EGFR* mutational analysis is the potential for misleading results due to tumor heterogeneity or discordance between primary and metastasis. As cytopathology specimens represent a small sampling of a larger lesion, a negative result may reflect sampling bias. Similarly, data suggest that the *EGFR* mutation status may be discordant between primary and metastasis in a proportion of cases; thus evaluation of a cytopathology specimen from a primary may not reflect the status of a metastasis, or vice versa.[24,25] In addition, given the observation that 40–50% of NSCLC harbor *EGFR* copy number gains,[26–28] the possibility that some of the evaluated specimens have concurrent *EGFR* amplification may also account for the successful detection of mutation in a subset of patients. Analysis for *EGFR* amplification was not performed in this study to determine the relationship between successful mutation detection and gene copy number.

As *EGFR* mutation testing becomes more common, additional options are likely to be available and useful in the evaluation of small specimens, especially cytopathologic preparations. One option for this evaluation includes the direct testing of cytopathology smears. A recent study demonstrated the feasibility of using material directly from smeared slides for genomic DNA based applications, with some slides having been archived for many years, and in which the quality of the extracted nucleic acid is high.[29] Several protocols in which tumor cells are selectively dissected from a cytology smear are available,[30,31] and this approach may provide an avenue for testing in patients in whom a cell block preparation was not generated as part of the cytopathology work-up. This novel approach is likely to have applications in clinical practice, especially in the setting of cases in which there is insufficient material for both smear and cell block preparation. However, there are obstacles which may limit widespread adoption of this methodology. One major obstacle is the need to "sacrifice" a smeared slide for this approach, which has potential ramifications in future consultation review of pathology material, and requires a clear understanding of the policies which could allow for such utilization. Additionally, as a novel approach for acquiring a substrate for molecular testing, laboratories must validate this methodology to accept specimens in this manner, which requires time and resources to complete. In conclusion, cytopathology CB are frequently adequate for *EGFR* mutation testing, and present a viable option for obtaining this important information in advanced stage NSCLC patients. Evaluation of the cytology specimen should be paired with knowledge of the mutation testing methodology, and tumor enrichment methods to allow for assessment of sample adequacy for testing.

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