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EGFR and *KRAS* mutation analysis in cytologic samples of lung adenocarcinoma enabled by laser capture microdissection

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The discovery of activating mutations in *EGFR* and *KRAS* in a subset of lung adenocarcinomas was a major advance in our understanding of lung adenocarcinoma biology, and has led to groundbreaking studies that have demonstrated the efficacy of tyrosine kinase inhibitor therapy. Fine-needle aspirates and other cytologic procedures have become increasingly popular for obtaining diagnostic material in lung carcinomas. However, frequently the small amount of material or sparseness of tumor cells obtained from cytologic preparations limit the number of specialized studies, such as mutation analysis, that can be performed. In this study we used laser capture microdissection to isolate small numbers of tumor cells to assess for *EGFR* and *KRAS* mutations from cell block sections of 19 cytology samples from patients with known lung adenocarcinomas. We compared our results with previous molecular assays that had been performed on either surgical or cytology specimens as part of the patient's initial clinical work-up. Not only were we able to detect the identical *EGFR* or *KRAS* mutation that was present in the patient's prior molecular assay in every case, but we were also able to consistently detect the mutation from as few as 50 microdissected tumor cells. Furthermore, isolating a more pure population of tumor cells resulted in increased sensitivity of mutation detection as we were able to detect mutations from laser capture microdissection-enriched cases where the tumor load was low and traditional methods of whole slide scraping failed. Therefore, this method can not only significantly increase the number of lung adenocarcinoma patients that can be screened for *EGFR* and *KRAS* mutations, but can also facilitate the use of cytologic samples in the newly emerging field of molecular-based personalized therapies.
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The emergence of targeted therapeutics in lung adenocarcinoma has revolutionized the field of personalized medicine and established a prognostic and predictive role for molecular analysis in conjunction with morphologic diagnosis in determining clinical outcomes of patients with advanced-stage disease.[1–3] The epidermal growth factor receptor (EGFR) is known to play a role in the development and progression of cancer, and somatic mutations within the tyrosine kinase domain of *EGFR* have been identified in a subset of lung adenocarcinomas. The most common mutations are a point mutation c.2573T>G (L858R) in exon 21 and small in-frame deletions in exon 19, which result in constitutive activation of tyrosine kinase.[4] What has made targeted therapy an exciting and developing field is that several clinical trials using tyrosine kinase inhibitors, gefitinib and erlotinib, have shown that patients with advanced lung adenocarcinomas harboring an *EGFR* mutation have a longer progression-free survival and response to tyrosine kinase inhibitors as a first-line therapy, whereas patients

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without these mutations have better outcomes with chemotherapy.[2,4–10] Approximately 15–30% of lung adenocarcinomas also harbor activating mutations in the downstream GTPase, *KRAS*, most frequently found in codons 12 and 13 of exon 2. Mutations in *EGFR* and *KRAS* are mutually exclusive and recent studies indicate that patients with mutant *KRAS* tumors do not respond to tyrosine kinase inhibitors.[11,12]

A large fraction of lung carcinoma patients are diagnosed by cytology on fine-needle aspirates, pleural fluids, bronchial washes/brushes and bronchoalveolar lavages,[13] and often cytology samples may be the only available material for molecular analysis. The majority of these patients who present with advanced-stage disease due to unresectable tumor or metastatic disease are increasingly being managed by targeted therapy. Minimally invasive procedures like endobronchial ultrasound-guided transbronchial needle aspiration are gaining popularity in the staging of advanced lung carcinoma patients because of the multitude of information that can be obtained from the aspirated material, including pathologic diagnosis and molecular testing.[14,15] These minimally invasive techniques also allow for serial sampling of a patient's tumor to assess therapeutic response as well as identify additional molecular markers of resistance. With this recent paradigm shift in lung cancer diagnosis and management, cytology has moved away from being a screening modality or ancillary technique and has established itself as an independent diagnostic procedure that plays a predictive role in determining clinical management.[13,16]

Mutation analysis, however, has been discouraged in cytology specimens because of scant material,[17] and cytology specimens are often underutilized for decisions regarding targeted cancer therapy.[18] In a recent study from Smouse *et al*[19] over a period of 2 years, from a total of 239 cases tested for *EGFR* mutations, only 12 were cytology material. A similar review by Clark[18] reports only 13 cytology cases from a total of 59 specimens tested for *EGFR* mutations. Nonetheless, as shown in the retrospective study by Smouse *et al*,[19] mutation analysis of cytology cell block material shows similar or higher sensitivity in comparison with surgical specimens and was likely dependent on the proportion of tumor cells present in a given specimen.

Molecular analytical techniques have rapidly gained pace in recent years and high-throughput methods have changed the study of molecular events associated with pathological processes.[20–22] However, molecular diagnosis is limited by the amount of tissue available for analysis and the number of tumor cells present within the sample. Tumor cell heterogeneity presents a challenge for molecular assays, where it is often necessary to isolate subpopulations of cells within a neoplasm to obtain a pure sample of tumor cells for DNA isolation and amplification. Laser capture microdissection provides a simple technique for rapid and accurate selection of pure populations of cells under direct microscopic visualization.[23–26]

In this study, we assessed the feasibility of using laser capture microdissection to enable and facilitate *EGFR* and *KRAS* mutation detection from cytologic samples (formalin-fixed, paraffin-embedded cell blocks), and compared the performance of this technique with results from standard mutational assays that had been previously performed on surgical or cytology materials as part of the routine clinical work-up. Our data indicate that laser capture microdissection-enabled mutation detection on cytologic material provides highly accurate and reproducible data comparable or superior to standard methods, and could be invaluable particularly when the tumor sample is limited.

Materials and methods

Clinical Samples

A total of 19 cytology specimens of either primary or metastatic lung adenocarcinomas from 14 patients were examined, including 9 fine-needle aspirates of the lung, 3 fine-needle aspirates of regional lymph nodes, 1 fine-needle aspirate of a distant metastasis (iliac crest), 1 bronchoalveolar lavage and 5 pleural fluids. All cases had cytomorphological evaluation of direct smears and/or cytospins as well as immunohistochemical studies (TTF-1 and Napsin A positivity) confirming the diagnosis. All specimens also had formalin-fixed, paraffin-embedded cell blocks that were cut into 4 μm sections and stained with hematoxylin and eosin (H&E) without a coverslip. Eight specimens were analyzed for *EGFR* mutations, seven specimens were analyzed for *KRAS* mutations and four specimens were analyzed for both *EGFR* and *KRAS* mutation status.

Laser Capture Microdissection

Laser capture microdissection was performed using an Arcturus XT (Life Technologies, Carlsbad, CA, USA). To improve visualization of the cells at the microscope, a drop of xylene was applied to the section. The tumor cells were identified by morphology, captured using a near-infrared laser pulse and transferred onto a cap (Capsure™ Macro LCM Caps, Life Technologies, catalog number LCM0211). The DNA was extracted from the cap after overnight incubation with proteinase K buffer at 56 °C, using the QIAamp DNA micro kit (Qiagen, Valencia, CA, USA, catalog number 56304). Following proteinase K digestion, the buffer was incubated at 90 °C for 1 h and DNA was isolated and eluted in 20 μl of buffer following the kit manufacturer's instructions.

PCR Amplification and Mutation Detection

Targeted analysis for *EGFR* mutation hot spots at codons 858, 861 and 863 within exon 21 was performed using pyrosequencing on a PyroMark Q24 instrument (Qiagen), whereas exon 19 deletions were assessed by capillary electrophoresis using a Genetic Analyzer 3130xl (Applied Biosystems). The pyrosequencing assay was designed using PyroMark Assay Design v2.0 (Qiagen). PCR amplification primers for exon 21 are as follows: EGFR-Ex21-FW 5′-biotin-GAGGACCGTCGCTTGGTG-3′ and EGFR-Ex21-REV 5′-TGCCTCCTTCTGCATGGTATTC-3′. Sequencing primer EGFR-Ex21-SEQ 5′-TGCATGGTATT CTTTCTC-3′ was used with nucleotide dispensation order 5′-CTCGCGAGTACATGCATGTAGCATGC-3′ to interrogate the sequence 5′-TTCCGCAYCCAGCNGTT TGGCCNGCCCA-3′. The primers for the exon 19 deletion assay were modified from the design by Pan *et al*[27] to generate a shorter amplicon (154 bps). The primer sequences are as follows: EGFR-Ex19-FW 5′-ACTCTGGATCCCAGAAGGTGAGA-3′ and EGFR-Ex19-REV 5′-fam-AAAGGTGGGCCTGAGGTTCA.

Targeted analysis for *KRAS* mutation hot spots in codons 12 and 13 within exon 2 was performed using the PyroMark Q24 KRAS v2.0 kit (Qiagen), as originally described by Ogino *et al*.[28]

PCR reactions were conducted in a total volume of 25 μl containing genomic DNA template, 200 nM of each forward and reverse primers, 12.5 μl 2 × HotStarTaq Master Mix (Qiagen). PCR cycling conditions for *EGFR* assays were 95 °C, 15 min; 40 × (95 °C, 30 s; 60 °C, 1 min; 72 °C, 1 min), 72 °C, 10 min; 8 °C, hold. COLD-PCR conditions for *KRAS* were 95 °C 15 min, 10 × (95 °C 20 s, 53 °C 30 s, 72 °C 20 s), 72 °C 5 min, 95 °C 2 min, 35 × (95 °C 20 s, 70 °C 8 min, 80 °C 3 s, 53 °C 30 s, 72 °C 20 s), 72 °C 5 min, 8 °C hold. For *EGFR* deletion detection, 1 μl of the 100-fold diluted PCR product was analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer. For the pyrosequencing assays, 10 μl of PCR product was immobilized on streptavidin-coated Sepharose beads (GE Healthcare) and processed according to the manufacturer's instructions.

Results

Patient and Tumor Characteristics

The clinical data for all cases analyzed are summarized in Table 1. Eight patients were male, ranging in age from 32 to 76 years, of which four had adenocarcinomas with gene mutations in either *EGFR* or *KRAS* that had been previously established by molecular assays performed on either cytology or surgical biopsies (Table 3). Six patients were female, ranging in age from 51 to 68 years, and all six cases had previously documented mutations in either *EGFR* or *KRAS* on cytology or surgical pathology material (Table 3). Most patients had advanced-stage lung carcinomas and received multiple modalities of treatment. The clinical management available at the time of our study is also summarized in Table 1.

Estimating the Minimal Number of Cells Required for Mutational Analyses

To assess the minimal number of cells required to successfully run mutation analysis, we microdissected decreasing numbers of tumor cells from cell block sections of four cytology cases and compared the results with the data from the original clinical work-up (Figure 1). For one case we had 300, 250, 200, 150, 100 and 50 cells, and for the remaining three cases we used 300, 100 and 50 cells (Table 2). Two of the cases had *EGFR* mutations in exon 21 (c.2573T > G) and two had *KRAS* mutations in codon 12. The mutations were consistently detected in all the samples tested, including samples containing only 50 cells, and the results were comparable to the original mutation analyses (Table 2). This suggests that mutation analyses for both *EGFR* and *KRAS* could be consistently performed with as few as

Table 1 Clinical characteristics of lung adenocarcinoma patients analyzed for *EGFR* and *KRAS* mutations

	Gender	*Age (years)*	*Stage*	*Smoking history*	*Management*
1	M	55	IB	Yes	Chemo/Rad/Surgery/Erlotinib
2	M	75	IV	Yes	Chemo
3	M	56	IV	No	Surgery/Chemo/Erlotinib
4	F	67	IIIA	Yes	Surgery/Rad/Chemo/Erlotinib
5	F	61	IV	No	Erlotinib
6	F	62	IV	No	Chemo/Erlotinib
7	F	68	IV	Yes	Chemo
8	F	51	IIA	Yes	Chemo/Rad
9	M	63	IV	Yes	Chemo
10	F	58	IV	Yes	Chemo
11	M	37	I	Yes	NA
12	M	76	III	No	NA
13	M	32	IV	No	Chemo/Rad/Erlotinib
14	M	62	IV	Yes	Surgery/Chemo

Abbreviations: M, male; F, female; Chemo, chemotherapy; Rad, radiation; NA, not available.

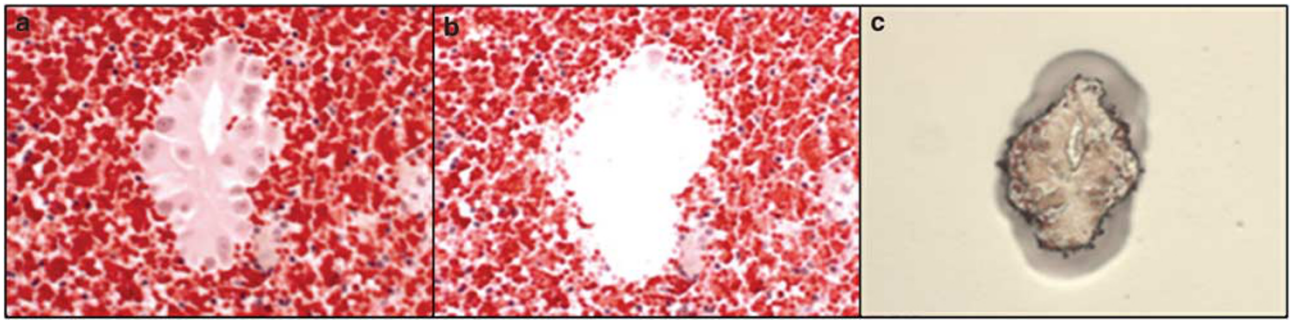

Figure 1 Laser capture microdissection of tumor cells. (**a**) H&E-stained cell block section showing lung adenocarcinoma cells before microdissection. (**b**) Same section after microdissection. (**c**) Tumor cells following microdissection on the cap.

Table 2 Quantitative assay for estimating the minimal number of cells required for mutational analysis

Patient	*LCM source*	*Number of cells*	*Mutation detected*
1	FNA lung	300	EGFR c.2573T>G
		250	EGFR c.2573T>G
		200	EGFR c.2573T>G
		150	EGFR c.2573T>G
		100	EGFR c.2573T>G
		50	EGFR c.2573T>G
6	FNA lymph node	300	EGFR c.2573T>G
		100	EGFR c.2573T>G
		50	EGFR c.2573T>G
7	FNA lymph node	300	KRAS12 c.34_35GG>TT
		100	KRAS12 c.34_35GG>TT
		50	KRAS12 c.34_35GG>TT
8	FNA lung	300	KRAS12 c.34G>T
		100	KRAS12 c.34G>T
		50	KRAS12 c.34G>T

Abbreviations: LCM, laser capture microdissection; FNA, fine-needle aspiration.

50 tumor cells. Below 50 cells there was significant reduction in signal strength; therefore, we did not perform mutation analysis for samples with <50 cells. We subsequently performed our remaining mutation analyses, 3 cases for *EGFR* and 9 cases for *KRAS*, using ~50 tumor cells and successfully amplified DNA (either wild type or mutant), comparable to previous data (Table 3). These results demonstrate that as few as 50 tumor cells, either in groups or as individual cells dispersed within a cell block, are sufficient to detect *EGFR* or *KRAS* mutations.

Gene Sequencing and Mutation Status

A total of 12 samples were tested for *EGFR* mutation status. Eight cases showed *EGFR* mutations, with seven cases containing a point mutation c.2573T>G (L858R) in exon 21 and one case with an in-frame deletion in exon 19. Four cases were wild type for *EGFR*. All 12 cases had mutation status analyses that had been previously documented on surgical biopsies or cytology material that were in concordance with the current results (Table 3).

For *KRAS* mutation, 11 samples were analyzed. Five cases showed mutations in codon 12, with two cases showing c.35G>A, one case showing c.34G>A, one case showing c.34_35GG>TT and one case showing c.34G>T. Six cases were wild type for *KRAS*. All the cases had mutation status analyses that had been previously documented on surgical biopsies or cytology material that were identical to the current results. Table 3 shows a summary of the mutation analyses performed for *EGFR* and *KRAS* along with the original molecular test data that had been previously performed.

Laser Capture Microdissection Increased Sensitivity of Mutation Detection

To evaluate the effectiveness of selectively using a pure population of tumor cells by laser capture microdissection, three cases (two pleural fluids: cases 4 and 5, and one bronchoalveolar lavage: case 8) with approximately ≤20% tumor cells were selected for mutation analysis (Figure 2a). We compared mutation assays following whole slide scraping of cell block sections (4 slides) with laser capture microdissection-assisted analysis from a single slide (~300 cells). We failed to detect the mutation from the standard method of whole slide scraping; however, the laser capture microdissection-assisted analysis was able to identify the appropriate mutation for all three cases (Figure 2b and c). This suggests that in cases where the tumor load is low and tumor cells are sparse and dispersed, isolating a more pure population of tumor cells can yield a more sensitive method for mutation detection in comparison with standard methods.

Discussion

It is becoming routine clinical practice to perform mutational analyses for prognostic and predictive markers in tumor samples of lung adenocarcinoma patients. The majority of lung cancer patients are

Table 3 Summary of *EGFR* and *KRAS* mutational analysis of laser capture microdissection-assisted cytology specimens in comparison with previously performed assays on surgical or cytology specimens

Patient	*Case*	*LCM source (C)*	*No. of cells*	*LCM mutation*	*Original source*	*Original mutation*
1	1	Lung	300	EGFR c.2573T>G	Lung (S)	EGFR c.2573T>G
	2	Lung	300	EGFR c.2573T>G		
2	9	Lung	300	EGFR WT	Lung (C)	EGFR WT
	13	Lung	50	KRAS 12 c.34G>A		KRAS 12 c.34G>A
3	3	Lung	300	EGFR Ex19del21	Lung (C)	EGFR Ex19del21
4	4	PLFL	300	EGFR c.2573T>G	Lung (S)	EGFR c.2573T>G
5	5	PLFL	300	EGFR c.2573T>G	PLFL (C)	EGFR c.2573T>G
6	6	Lung	300	EGFR c.2573T>G	LN (S)	EGFR c.2573T>G
	7	LN	50	EGFR c.2573T>G		
	8	BAL	300	EGFR c.2573T>G		
7	14	LN	50	KRAS12 c.34_35GG>TT	LN (S)	KRAS12 c.34_35GG>TT
8	15	Lung	50	KRAS12 c.34G>T	Lung (S)	KRAS12 c.34G>T
9	16	LN	50	KRAS 12 c.35G>A	Lung (S)	KRAS 12 c.35G>A
10	12	Bone	50	EGFR WT	Bone (S)	EGFR WT
	17	Bone	50	KRAS 12 c.35G>A		KRAS 12 c.35G>A
11	10	PLFL	50	EGFR WT	Lung (S)	EGFR WT
	20	PLFL	50	KRAS WT		KRAS WT
12	11	Lung	50	EGFR WT	Lung (S)	EGFR WT
	21	Lung	50	KRAS WT		KRAS WT
13	18	Lung	50	KRAS WT	Lung (S)	KRAS WT
	19	PLFL	50	KRAS WT		
	22	PLFL	50	KRAS WT	PLFL (C)	KRAS WT
14	23	Lung	50	KRAS WT	PLFL (C)	KRAS WT

Abbreviations: LCM, laser capture microdissection; C, cytology specimen; S, surgical biopsy; PLFL, pleural fluid; LN, lymph node; BAL, bronchoalveolar lavage; WT, wild type.

diagnosed at later stages that preclude surgical resection, and in advanced-stage lung cancer, platinum-based combined chemotherapy is effective in ~30% of cases.[29] Detecting activating mutations in *EGFR* plays an essential role in determining responsiveness to tyrosine kinase inhibitors for targeted therapeutics. Clinical decisions regarding the use of different agents in patients with lung adenocarcinomas might be improved by pretreatment mutational profiling of both *EGFR* and *KRAS*.

The diagnosis of lung cancer is often based solely on cytology specimens.[16,30] As often the number of tumor cells is limited on cytology specimens, it is critical to develop sensitive assays for mutation detection from small samples. Previous studies have shown that direct sequencing cannot consistently detect mutant DNA in specimens with <50% tumor cells and can rarely detect mutations in specimens with <25% tumor cells.[19] For mutation analysis, enrichment of tumor cells is important to avoid diluting tumor DNA with the nonmutated DNA of benign cells, which in most cases represents the larger proportion of the cell population. Manual microdissection has been previously used on cytology specimens to extract DNA for *EGFR* mutation analysis;[31] however, samples with lower numbers of tumor cells were rejected or reported as inconclusive because of technical difficulties of manually isolating a pure population of malignant cells. Laser microdissection has been successfully employed by other groups using a laser pressure catapulting system[32,33] on Papanicolaou-stained cytology material or paraffin-embedded samples with very small groups of tumor cells. In our hands, using direct lysates of tumor cells interfered with the consistent sensitivity of the mutation assay, and incorporating the DNA extraction step resulted in better DNA quality (data not shown), suggesting that the DNA extraction step is important in removing potential PCR inhibitors.

In this study we validated a method for mutation analysis in cytology cell block samples containing as few as 50 tumor cells using laser capture microdissection. Laser capture microdissection can be successfully used to selectively isolate tumor cells, even in cases where the stochastic distribution of tumor and benign cells precludes more traditional methods of enrichment such as scraping whole samples or manual microdissection with a needle. There have been arguments against using extremely small amounts of DNA for PCR-based mutational analysis as artifactual mutations have been described especially in DNA extracted from paraffin-embedded tissue.[34–37] However, improved methods using DNA extraction kits specially adapted for laser capture microdissected specimens from paraffin-embedded tissues, and more sensitive and reliable detection methods such as pyrosequencing and using small PCR amplicons (150–200 bp) have improved downstream analysis for samples with small amounts of DNA. To ensure we did not interpret false-positive results as true mutations, all our assays were performed in duplicate. Our method not only detected mutations consistently

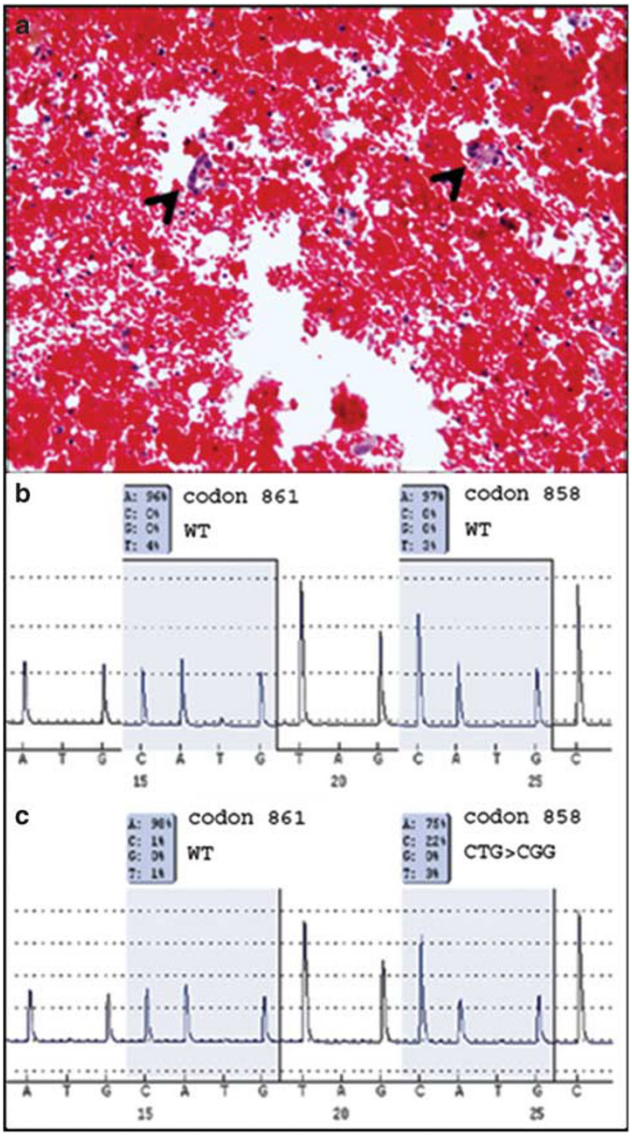

Figure 2 Laser capture microdissection increases sensitivity of mutation detection. (**a**) H&E-stained cell block section showing scattered tumor cells (arrow head) in a background of red blood cells, lymphocytes and mesothelial cells. (**b**) Pyrogram from *EGFR* mutation assay for exon 21 from a whole slide scrape of the same case showing wild type. (**c**) Pyrogram from *EGFR* mutation assay for exon 21 following laser capture microdissection showing detection of c.2573 T>G (L858R) mutation.

from 50 tumor cells, but also detected the exact same mutation that had been previously identified in each of these patients. It is important to note that number of tumor cells does not necessarily equate to number of copies of the gene target, particularly for EGFR for which mutations often occur in an amplified allele.[38] In our experience, selecting and microdissecting a tumor population of only 50 tumor cells is fast, reproducible, specific and quite feasible in the clinical set-up. Laser capture microdissection increased the sensitivity of mutation detection in specimens with limited number of tumor cells, and therefore could significantly increase the number of patients who can be screened for both *EGFR* and *KRAS* mutations.

New diagnostic modalities like endobronchial ultrasound-guided transbronchial needle aspiration are increasingly being used to stage lung cancer through evaluation of mediastinal and hilar lymph nodes for metastasis.[39] These new minimally invasive sampling procedures provide opportunities, but to fully take advantage of their promise, more sensitive analytic approaches capable of interrogating very small amounts of tissue must be developed. As the field moves from simple mutational analysis of one or a few genes to more challenging assays like whole pathway DNA mutation testing and complex expression-based assays using proteomic or transcriptomic measurements, the need for simple and reliable methods of isolating relatively pure cell populations will become essential,[40] and the use of microdissection is likely to be an integral component of such approaches.

In conclusion, our study demonstrates that laser capture microdissection-assisted *EGFR* and *KRAS* mutation analysis from cytology cell block samples provides results that match those obtained from whole histology/cytology slide scrapes typically used for clinical molecular diagnostic testing. We have shown that *EGFR* and *KRAS* mutation analysis can be performed consistently and reproducibly with as few as 50 tumor cells for both assays, and that tumor cell enrichment attainable through the use of laser capture microdissection allows for more sensitive mutation detection. Highly sensitive and reproducible approaches to mutation analysis, such as the method described here, should facilitate the use of cytologic materials for the molecular testing that underpins the newly emerging molecular-based personalized therapies.

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Disclosure/conflict of interest

Michael Emmert-Buck is an inventor on all NIH-held patents covering laser capture microdissection technology and receives royalty payments through the NIH technology transfer program.

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