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Fine-Needle Aspiration Smears as a Potential Source of DNA for Targeted Next-generation Sequencing of Lung Adenocarcinomas

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Abstract

Background—Diff-Quik stained fine-needle aspiration (FNA) smears and touch preparations from biopsies represent alternative specimens for molecular testing when cell block or biopsy material is insufficient. We describe the use of these samples for targeted next-generation sequencing (NGS) of primary and metastatic lung adenocarcinoma and report DNA quality and success rates of FNA smears compared with other specimens from one year of clinical use.

Methods—A validation set of 10 slides from 9 patients with prior clinical *EGFR* Sanger sequencing and *KRAS* pyrosequencing (5 *KRAS* +/*EGFR*–, 4 *KRAS/EGFR*–) underwent DNA extraction, quality assessment, and targeted NGS. Subseqently, lung adenocarcinoma specimens submitted for NGS solid tumor mutation panel testing in one calendar year (60 biopsies, 57 resections, 33 FNA cell blocks, 12 FNA smears, 10 body fluid cell blocks) were reviewed for specimen adequacy, sequencing success, and DNA quality.

Results—All 10 validation samples met the DNA quality threshold (CT threshold <8, range –2.2 to 4.9) and yielded 0.5 to 22 μg of DNA. *KRAS* and *EGFR* mutation status from FNA smears by NGS were concordant with previous clinical testing for all 10 samples. In the one year review, FNA smears were 100% successful, suggesting performance equivalent to or better than established specimen types, including FNA cell blocks. DNA quality by   CT was significantly better from FNA smears than from biopsies, resections, and FNA cell blocks.

Conclusions—We conclude that FNA smears of lung adenocarcinomas are a high quality alternative specimen for a targeted NGS panel with a high success rate in clinical practice.

Keywords

lung carcinoma; massive parallel sequencing; *EGFR*; *KRAS*; fine-needle aspiration

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INTRODUCTION

Molecular technology is increasingly recognized for its role in patient care. At the same time, molecular laboratories are being asked to evaluate more genes with smaller volumes of tissue. Formalin-fixed paraffin-embedded (FFPE) core needle biopsies, cell blocks from fine-needle aspirations (FNAs), and fresh FNA material in solution are common samples submitted for molecular oncology testing. Though these specimens are often adequate and informative, they may fail for a variety of reasons. Tumor burden or cellularity may be too low and thus DNA input to the assay may be insufficient in amount or in quality. FFPE biopsy tissue is frequently exhausted by immunohistochemical stains needed for diagnosis. The first pass of FNA collection is often prioritized for diagnostic smears and to ensure adequacy of diagnostic material, while subsequent passes used to prepare a cellblock often contain more blood and less tumor tissue, both of which may compromise molecular testing. The material collected for cell block is often processed without direct visualization of tumor content. Given all of these limitations, a significant number of fresh or fixed FNA specimens contain too little tumor for molecular testing.

Knoepp, et al reported a series of 76 endobronchial ultrasound FNAs for which cell blocks were assessed for cellularity. Of those, 37% were acellular and another 20% were sparse or borderline for molecular testing. Even cell blocks created from dedicated passes had a similar failure rate and often represented pooled samples that likely diluted malignant material. (1) The pitfalls associated with testing small samples are particularly problematic for patients with advanced disease who require neoadjuvant treatment prior to resection. If molecular tests on a biopsy or FNA material are unsuccessful, patients may have to undergo an additional procedure to collect more tumor tissue, at additional risk of morbidity and cost.

The failure rate of small core biopsies and cell blocks has led to consideration of alternative specimens. The use of FNA material scraped from Giemsa-based (such as Diff-Quik) or Papanicalaou-stained, coverslipped slides has been described in a variety of cancer types, including primary and metastatic lung cancer and melanoma, thyroid cancer, and pancreatic cancer. The extracted DNA was shown to be sufficient for Sanger sequencing, as well as pyrosequencing, PCR amplification with restriction endonuclease fragment analysis, real-time PCR with hybridization probes, allele specific PCR, high resolution melt curve analysis, reverse transcription PCR, and array-based comparative genomic hybridization. (2–17)

More recently, the utility of FNA material for next-generation sequencing (NGS) has been investigated. Both cell blocks and material from FNA smears were shown to be effective for NGS in multiple cancer types on a variety of sequencing platforms when sufficient material was available on the slides. (15, 18–23) NGS testing has the advantage of interrogating multiple genes at once. The ability to visualize malignant cells on the slide and to enrich for malignant cells either by slide selection or by macrodissection allows for higher analytic sensitivity for mutation detection. (15)

FNA cell block material from primary and metastatic lung adenocarcinoma is routinely submitted for molecular testing. Practice guidelines outline recommendations for *EGFR*

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mutation testing in lung cancers to determine eligibility for tyrosine kinase inhibitor (TKI) therapy (24). Although not considered sufficient for TKI therapy decision-making on its own, *KRAS* testing is also clinically informative, as *KRAS* mutation is generally mutually exclusive with *EGFR* mutation and confers resistance to *EGFR*-targeted TKI therapy. Mutations in *KRAS* are also generally mutually exclusive with *ALK, ROS* and *RET* fusions, potentially saving both the expense and the tissue required for FISH or IHC tests for these rare variants.

In 2014, UNC Hospitals implemented an NGS-based solid tumor mutation panel to simultaneously identify mutations in *EGFR, KRAS*, and *BRAF* that are important for molecular classification of lung cancer, along with 23 other genes for which hotspot mutation analysis might assist in selection of experimental therapy. Due to a significant number of samples deemed insufficient for molecular testing secondary to absent or very low tumor cellularity, we sought to determine the utility of FNA smears as a suitable alternative specimen for molecular analysis of lung adenocarcinoma. We report the results of our validation study in which DNA extracted from a single Diff-Quik stained FNA smear or touch prep of primary or metastatic lung adenocarcinoma was analyzed by targeted next-generation sequencing and the results compared with prior clinical testing performed on FFPE specimens by traditional methods. We also report one year's experience of using FNA specimens in routine clinical molecular testing with respect to quality indicators and NGS success rate of FNA smears compared to other specimen types.

MATERIALS AND METHODS

Case selection for validation study

In order to explore the possibility of using cytology smears and touch preparations for NGS, our molecular test database was searched to identify lung adenocarcinoma cases that were previously tested for *KRAS* and *EGFR* mutations by pyrosequencing or Sanger sequencing, respectively, between January 1, 2013 and December 31, 2013 in the UNC Hospitals Molecular Genetics Laboratory. Ten methanol-fixed, Diff-Quik stained FNA smear or touch prep slides from 9 tumors were identified, either from the same diagnostic procedure as the original molecular test or from a separate procedure that also demonstrated tumor. Cases were selected to preferentially include those with and without previously identified mutations, slides that contained greater than 50% tumor cellularity, and slides that represented a range of overall cellularity.

DNA extraction

Diff-Quik stained cytology smears were reviewed by a cytopathologist to determine cellularity and to estimate malignant cell percentage, and were photographed. Coverslips were removed by soaking slides in xylene at 37° C until the coverslip detached (24–48 hours). The slides were rehydrated in 95% ethanol and air-dried. DNA was extracted using a modified *QIAgen Protocol: Purification of genomic DNA from FFPE tissue section* (QIAgen, Valencia, CA). Briefly, material was scraped from the entire slide using a blade and collected in a tube containing Buffer ATL. Proteinase K was added and the samples

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were incubated at 56° C for 2–4 hours. DNA was purified and eluted in 30 μL using a QiaCube (QIAgen, Valencia, CA).

DNA quality

As part of routine clinical testing, DNA quality is checked by real-time PCR on an ABI 7500 instrument (Applied Biosystems, Foster City, CA). Briefly, a proprietary quality control (QC) template or a known volume of patient DNA is mixed with nuclease free water, Power SYBR Green PCR master mix (Life Technologies, Grand Island, NY) and PCR primers, which are specific to either the QC template or a proprietary human target amplicon, respectively (Illumina FFPE QC kit, Illumina, San Diego, CA). DNA is then amplified in triplicate for 40 cycles, with melting at 95 °C, annealing at 60 °C, and extension at 72 °C. Detection of PCR product by SYBR Green fluorescence is used to calculate a CT value by subtracting the average cycle threshold for the human target from the average cycle threshold for the QC template. In clinical practice, samples with a CT value greater than 8 are deemed insufficient. In assay validation work, the concentration of DNA in the input eluate was determined with a NanoDrop instrument (NanoDrop Technologies, Wilmington, DE).

Next-generation sequencing

UNC Hospital's Solid Tumor Mutation Panel, which utilizes Illumina TruSight Tumor Panel reagents on the Illumina MiSeq platform (Illumina, San Diego, CA), was applied to DNA that passed the DNA quality check. This Solid Tumor Mutation Panel targets certain exons in 26 genes related to cancer, including KRAS and EGFR. The assay was previously rigorously validated for FFPE samples, with robust analytic sensitivity to a minor allele frequency of at least 5% demonstrated through mixing studies (data not shown). In the current validation of cytology specimens, based on the CT value, samples were used either diluted or undiluted, per dilution recommendations in the manufacturer's protocol, in a final input volume of 20 μL for library preparation. Up to 12 samples were multiplexed in a single MiSeq run. Results were compared with prior clinical test results for *KRAS* and *EGFR* mutations, as detected by pyrosequencing or Sanger sequencing, to identify discrepancies. Any additional mutations were also noted.

Review of quality indicators and sequencing success from clinical cases

In order to assess performance of various specimen types tested in clinical practice, records from the UNC Hospitals Molecular Genetics Laboratory were reviewed to identify all cases of primary or metastatic lung adenocarcinoma submitted for sequencing between January 2014 and January 2015 (the first year that the test was offered clinically after the previously described validation). In order to capture lung cancer cases in which molecular testing was desired but the specimen was deemed inadequate for sequencing by a pathologist *prior to* submission to our laboratory, a natural language search was also performed in our institution's anatomic pathology laboratory information system, and reports referencing inadequate tumor for molecular testing were identified. Cases were only included if the anatomic pathology specimen was diagnostic of lung adenocarcinoma. Records were then reviewed to determine if cases met quality indicators established for sequencing in our laboratory. Specifically, 1) tumor must represent at least 20% of nucleated cells on glass

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slides used for sequencing and 2) DNA yield must meet an established threshold as measured by real-time PCR (CT < 8).

Submitted cases were grouped by specimen source into one of five categories: surgical resection, small biopsies, FNA cell blocks, body fluid cell blocks, and FNA direct smears. The fraction of cases with successful sequencing was tabulated for each specimen type. Statistical significance of all possible pairwise comparisons was evaluated by Chi-squared analysis.

For all cases that met the 20% tumor threshold, CT were calculated from quantitative PCR (qPCR) assays. Mean CT values were then determined for each specimen type, and all possible pairwise comparisons were performed by a two-tailed student t-test.

Finally, percentage tumor was estimated for all cases, and the mean percentage for each cell type was calculated. All possible pairwise comparisons were evaluated by a two-tailed student t-test.

RESULTS

Case characteristics of validation set

A validation study of Diff-Quik stained direct smear slides was designed in agreement with standards established by the College of American Pathologists and the American College of Medical Genetics guidelines (25–26). As part of the initial validation study, ten Diff-Quik stained slides from nine patient cases of lung adenocarcinoma were identified. Of those, four were FNAs from primary tumors, two were touch preps from core biopsies of primary tumors, and four were FNAs from lymph node metastases (two of which were from the same patient procedure). Since the analytic portion of the assay was already validated for use on FFPE tissues, including demonstration of robust analytic sensitivity at a minor allele frequency of 5%, the current validation attempted only to demonstrate concordance between FFPE samples and cytology smears from the same tumors. Though the minimum tumor percentage for our NGS-based solid tumor mutation panel is 20%, we selected cytology smears with greater than 50% tumor cells to reduce the likelihood of non-concordant results due to inaccurate estimation of tumor cellularity. All tumors previously underwent testing for *EGFR* exon 18–21 mutations by Sanger sequencing and for *KRAS* codon 12, 13, and 61 testing by pyrosequencing. All nine cases were negative for *EGFR* mutations. Six of nine were positive for a *KRAS* mutation. (Table 1)

DNA quality results of validation set

All 10 cytology smears met the DNA quality threshold (CT<8, range −2.2 to 4.9). Total recovered DNA ranged from 0.5 to 22 μg. The amount of DNA used for library preparation ranged from 0.3 to 2.2 μg, depending on the sample dilution factor applied based on the CT value. The lowest amount of DNA recovered came from the touch prep samples. (Table 2)

Sequencing results of validation set

In the validation set, *EGFR* and *KRAS* status was concordant with prior test results for all cases (Table 2). However, in Case 1, a different *KRAS* mutation was identified by NGS

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testing than what was interpreted by pyrosequencing. In this case, *KRAS* p.G13C mutation was identified by NGS whereas the pyrosequencing result was interpreted as *KRAS* p.G12V mutation. On review of the pyrosequencing data, this discrepancy was found to be a result of an ambiguous pyrogram that was misinterpreted by visual inspection and by the algorithm used to quantitatively interrogate codons 12 and 13. The clinical impact of this error is negligible. Eleven additional mutations in genes analyzed in the NGS panel were identified in 7 cases, including mutations in *TP53*, *PIK3CA*, *MET*, and/or *STK11*. (Table 2)

Comparison of sequencing success by specimen type in clinical practice

After validation work was deemed sufficient, our laboratory began to accept Diff-Quik stained direct smears for clinical NGS testing in January 2014. In order to evaluate the performance of sequencing assays performed on DNA isolated from these smears relative to other specimen types, we reviewed results of lung adenocarcinoma molecular tests performed as part of clinical care at UNC Hospitals over the following twelve months (ending January 2015). During that interval, in addition to direct cytology smears, accepted specimens included formalin-fixed, paraffin-embedded tissue sections from surgical resections, small biopsies, and cell blocks (generated from either body fluids or FNAs). In our laboratory, several quality indicators must be met prior to sequencing. Specifically, submitted samples must contain at least 20% neoplastic cells, and the DNA yield as measured by quantitative PCR (qPCR) must meet an established threshold (CT < 8). Cases that do not meet both criteria are classified as "quantity not sufficient (QNS)" and are not sequenced.

In order to determine whether the percentage of cases meeting both quality indicators and producing interpretable sequence varies by specimen type, we reviewed all cases of primary and metastatic lung adenocarcinoma collected at our institution and submitted to our laboratory for molecular testing between January 2014 and January 2015. To capture cases in which molecular testing was desired but the specimen was deemed inadequate for sequencing by a pathologist *prior to* submission to our laboratory, cases of lung adenocarcinoma were also included if the anatomic pathology report indicated that inadequate tumor remained for molecular studies.

A total of 172 specimens met criteria for inclusion, comprised of 60 biopsies, 57 surgical resections, 33 FNA cell blocks, 12 FNA direct smears, and 10 body fluid cell blocks. A wide variety of mutations were identified in each group, including four KRAS mutations and one case with a concomitant EGFR exon 19 deletion and resistance mutation in the twelve direct smear specimens. Thirteen additional mutations were identified in 9 of the 12 direct smears in the following genes: *TP53* (seven cases), *PIK3CA*, *STK11*, *MET*, *APC*, *MAP2K*, and *CH1*. The percentage of cases that met all quality indicators and were successfully sequenced varied from 70% (for FNA cell blocks) to 100% (for FNA direct smears), depending on specimen type (Table 3). Although comparison is limited by a small number of cases, in our series, sequencing was more often successful with FNA direct smears than with cell blocks generated from FNAs (p = 0.03) (Table 3). Statistically significant differences were not noted between FNA direct smears and the remaining specimen types, although a non-significant trend was noted towards improved success with smears relative to

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small biopsies (p = 0.09). The percentage of successful cases was also noted to be higher with surgical resections than with either biopsies (p = 0.0004) or FNA cell blocks (p = 0.0003) (Table 3).

Mean tumor content in these specimens varied from 45% for small biopsies to 69% for body fluid cell blocks (Supplementary Table 1). However, a consistent trend between sequencing success and tumor content was not apparent in our clinical specimens. While there is a trend towards increased percentage tumor on direct smear slides relative to several specimen types, those differences reach significance only when direct smears are compared to small biopsies (p = 0.02). The tumor content in direct smears from clinical specimens ranged from 20 to 90% and all were successfully extracted and sequenced. Moreover, although FNA cell blocks had the highest rate of failure in clinical testing, there were no statistically significant differences in mean percentage of tumor between this and other groups. Though specimens were not quantitatively evaluated for overall cellularity, FNA cell blocks and core biopsies contain the less total cellular material than other specimen types in our experience. These findings reflect the fact that total DNA mass is the most important factor in successful library preparation, while minimum neoplastic cellularity is the most important factor in reducing false negatives (since the limit of detection of our assay is 5% mutant alleles).

Comparison of CT values by specimen type

Given variation in sequencing success, we hypothesized that amplifiable DNA in clinical samples may also vary by specimen type. In our clinical workflow, the concentration of DNA isolated from clinical specimens is not directly measured. Instead, relative amplifiable DNA is inferred by CT values generated by real-time PCR. Specifically, a CT value is calculated by subtracting the cycle threshold for a proprietary human target amplified from patient DNA from the cycle threshold of a proprietary QC amplicon. Lower CT values reflect higher yields of amplifiable DNA. Mean CT values were calculated for each specimen type. Given quality indicators in our laboratory, DNA isolation was attempted only if at least 20% tumor cells were present in the specimen. DNA was not prepared from cases with lower tumor content, and such cases are excluded from this analysis.

Mean CT values for cases submitted for clinical testing between January 2014 and January 2015 varied from 0.9 for FNA direct smears to 4.0 for biopsies (Table 4), corresponding to a slightly greater than 8-fold difference in mean yield of amplifiable DNA. Moreover, the mean CT value for FNA direct smear specimens was lower than that of biopsies (p < 0.0001), FNA cell blocks (p = 0.0007), and even surgical resections (p = 0.03), confirming high DNA yield and quality when direct smears are used as a tissue source (Table 4). No statistically significant difference was noted between FNA direct smears and body fluid cell blocks.

DISCUSSION

This study demonstrates that extraction of DNA from a single cellular cytology smear reliably yields sufficient and high quality DNA for NGS analysis. The CT calculated in the quality control step was consistently within the acceptable range (Table 4), and the amount of DNA recovered from slides in the validation set ranged from 0.5 to 22 μg, or 17–721 ng/

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μL (Table 2). DNA yield from FNA smears has been reported in multiple studies and varies based on the type of staining used, whether malignant material was macrodissected versus the whole slide was scraped, and the type of extraction methods used. (5, 7, 9, 20–22) Thus, it is difficult to do a direct comparison of our findings with those of prior studies, especially since assay input requirements vary depending on the testing method. Some studies have reported up to 300 ng/μL of DNA from smears, though others have reported significantly less. (5, 7, 9, 10, 20–22) The high concentration of DNA recovered from our FNA smears is likely due to the use of the whole slide along with selection of slides with abundant cellularity. Archived slides from stained FNA smears also appear to be a suitable source of DNA. The slides used in our study were up to one year old; effective DNA extraction from slides up to 5 years old was reported in one study and from slides between 11 and 16 years old in another. (5, 7)

Gailey, et al reportedly found that column purification resulted in a lower DNA concentration than a manual extraction, but that DNA resulting from column purification was of a better quality for NGS applications. (20) We chose an automated column purification method because it promotes streamlined workflow for both FNA smears and FFPE specimen types. Gleeson, et al suggested minimal requirements for downstream NGS tests should include at least 5000 cells, 20% tumor content, less than 50% necrosis, and 5 ng/μL DNA concentration. (21) Our sample requirement for the Solid Tumor Mutation Panel was established at greater than 20% tumor cell percent, and this cutoff for both FNA smears and FFPE in subsequent clinical practice and has led to a 100% success rate thus far.

Although interpretation is limited by small sample size, in our study, 100% of FNA smears submitted for clinical testing were successful, compared with 70% of cell blocks. FFPE resection specimens also had a success rate approaching 100% (Table 3). The CT values for FNA smears were significantly lower, and thus DNA quality was higher than for core biopsies, cell blocks, or surgical resection specimens submitted for clinical testing. The Karnes et al study comparing sequencing results on DNA extracted from cores of FFPE samples and from smears showed that there was no significant difference in total numbers of reads, mapped reads, unique reads, on-target reads, or read depth. (22) Though previous studies have suggested that Giemsa-based stains (e.g. Wright's, Diff-Quik) results in better DNA content than a Papanicolaou stain, there was no significant difference between the two in the Karnes study. (5, 22) These data suggest that when a resection specimen is not available and the cell block and/or core biopsy is acellular or sparse, an FNA smear is a reliable alternative source of DNA. Future, larger studies with direct comparisons between different sample types collected from the same tumor are necessary to determine whether direct smears may be a preferred method for molecular testing.

One of the most important advantages of using FNA smears is the potential to decrease the need for invasive procedures to acquire sufficient tumor for medical decision-making potentially decreasing both morbidity and cost. In our workflow, the cytopathologist reviews the smears and the cell block to determine which to send for molecular tests. If the cell block is considered inadequate and a smear is chosen, the slide is photographed or scanned to document the findings before being sent to the molecular lab. Some pathologists may be uncomfortable with the concept of destroying diagnostic material for molecular tests. In our

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institution, sacrifice of diagnostic material following photographic documentation was considered medically necessary when it was judged that the ancillary test was needed for patient management, potentially avoiding additional invasive procedures. If additional cytologic smears or touch preps are available, the cytopathologist should review them to select which to save or submit for testing.

In the validation set, as well as in clinical practice, the described methods for slide selection, extraction, and downstream testing fit easily into our workflow. We were able to successfully modify the extraction protocol used for FFPE specimens to accommodate FNA smears. FNA smear slides sent for testing are extracted in parallel with other FFPE tissue specimens and undergo the same quality control tests and downstream sequencing. FNA smears have the same approximate turnaround time as other specimen types. The only factor that slows turnaround time is the xylene soak to remove the coverslip. In our hands, this step takes between 24 and 48 hours, and is probably faster when slides were recently coverslipped. A rapid method for removing coverslips by placing slides in a −20 °C freezer for 1 to 2 minutes was described, (27) which could eliminate delay due to the xylene step.

Use of a multi-gene NGS panel allows for detection of additional potentially actionable mutations beyond those identified in single gene hotspot tests. In our validation study, the *EGFR* and *KRAS* status was 100% concordant between the prior pyrosequencing or Sanger methods and the NGS panel. In one case, a different *KRAS* mutation was identified by NGS than what was reported for pyrosequencing, and this case highlights the better resolution of NGS as compared with the sometimes ambiguous results of pyrosequencing, especially in cases with lower tumor content. NGS identified a total 24 additional common and uncommon mutations in various genes other than *KRAS* and *EGFR* in 7 cases from the validation set and 9 of the 12 FNA smears from the one year review, demonstrating the utility of FNA smears beyond single gene targeted analysis. These additional gene mutations are increasingly being used to help select experimental therapy in patients who have failed standard treatments.

The major limitation of the current study is a relatively small sample size, particularly in the review of clinical specimens (Tables 3 and 4). Between the validation set (n = 10) and the 12-month clinical study (n = 12), a total of 22 direct cytology smear specimens had been successfully sequenced by the end of 2014. While confirmation by larger studies is necessary, sample sizes were large enough to produce statistically significant differences in sequencing success and CT values for several groups. As such, these results suggest that direct smears may be equivalent, if not superior, specimens for NGS assays.

In conclusion, Diff-Quik stained FNA smears from primary and metastatic lung adenocarcinoma are suitable specimens for targeted NGS panels because they are a source of high quality DNA. The extraction process was amenable to our existing FFPE workflow. The availability of this high quality alternative specimen type can potentially prevent additional invasive procedures, potentially saving time, expense, and risk to the patient.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of slides used in validation study

Case #	Specimen	*KRAS* status by pyrosequencing	*EGFR* status by Sanger sequencing	Estimated tumor %
1	TP from lymph node	Positive	Negative	70
2a*	Lymph node FNA	Positive	Negative	70
2b*	Lymph node FNA	Positive	Negative	50
3	FNA of primary	Positive	Negative	80
4	TP from lymph node	Positive	Negative	80
5	FNA of primary	Positive	Negative	80
6	FNA of primary	Negative	Negative	90
7	Lymph node FNA	Negative	Negative	100
8	FNA of primary	Negative	Negative	95
9	Lymph node FNA	Negative	Negative	100

*Cases 2a and 2b are from the same patient procedure and were both tested due to variable cellularity. TP=touch prep, FNA= fine-needle aspirate

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Table 2

DNA quality characteristics and sequencing results

Slide #	CT	Total DNA recovered (µg)	*KRAS* pyrosequencing	*KRAS* NGS result	*EGFR* NGS result	Genes containing additional mutations
1	4.9	0.5	**G12V**	**G12C**	Negative	None
2a*	0.7	6.5	G12V	G12V	Negative	TP53
2b*	2.9	1.7	G12V	G12V	Negative	TP53
3	2.6	3.0	G12A	G12A	Negative	PIK3CA, TP53
4	4.5	0.8	G12Y	G12Y	Negative	MET, STK11, TP53
5	2.4	3.0	G12V	G12V	Negative	TP53
6	0.3	6.3	Negative	Negative	Negative	TP53
7	0.9	2.4	Negative	Negative	Negative	TP53
8	−2.2	22	Negative	Negative	Negative	STK11
9	0.5	5.0	Negative	Negative	Negative	None

A discrepancy between the pyrosequencing results and the NGS results is bolded.

*Cases 2a and 2b are from the same patient procedure and were both tested due to variable cellularity.

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Table 3

Sequencing success by clinical specimen type

Specimen type	Successful cases	Total cases	Percent successful (%)
FNA direct smears	12	12	100[*]
Surgical resections	55	57	96[†‡]
Body fluid cell blocks	9	10	90
Small biopsies	48	60	80[†]
FNA cell blocks	23	33	70[*‡]

[*] Statistically significant difference between FNA direct smears and FNA cell blocks (p = 0.03).

[†] Statistically significant differences between surgical resections and small biopsies (p = 0.0004).

[‡] Statistically significant difference between surgical resections and FNA cell blocks (p = 0.0003).

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Table 4

DNA quality by clinical specimen type

Specimen type	Mean CT	Versus FNA direct smears (p-value)	Versus surgical resections (p-value)	Versus fluid cell blocks (p-value)	Versus FNA cell blocks (p-value)	Versus small biopsies (p-value)
FNA direct smears	0.9 +/−2.2	–	0.03 *	0.21	0.0007 *	<0.0001 *
Surgical resections	2.2 +/−1.7		–	0.80	0.003 *	<0.0001 *
Body fluid cell blocks	2.4 +/−2.9			–	0.19	<0.04 *
FNA cell blocks	3.4 +/−1.7				–	0.24
Small biopsies	4.0 +/−2.1					–

* Statistically significant