Adequacy of small biopsy and cytology specimens for comprehensive genomic profiling of patients with non-small-cell lung cancer to determine eligibility for immune checkpoint inhibitor and targeted therapy

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ABSTRACT

Aims In advanced-stage non-small-cell lung cancer (NSCLC), incomplete genotyping for guideline-recommended genomic biomarkers poses a significant challenge to making informed and timely clinical decisions. We report our institution's experience in assessing the adequacy of small specimens for comprehensive genomic profiling for guideline-recommended lung cancer biomarker testing.

Methods We performed a retrospective evaluation of all image-guided procedures for NSCLC performed in our institution between October 2016 and July 2018, including core needle biopsy (CNB) and fine-needle aspiration (FNA) in patients who had undergone genomic profiling for lung cancer. Lung cancer biomarker adequacy, defined as successful testing of guideline-recommended biomarkers including, epidermal growth factor receptor (*EGFR*); serine/threonine protein kinase B-Raf (*BRAF*); anaplastic lymphoma kinase (*ALK*); proto-oncogene tyrosine protein kinase ROS (*ROS1*); Rearranged during Transfection (*RET*); Tyrosine protein kinase Met (*MET*); and programmed cell death ligand 1 (PD-L1), was evaluated.

Results A total of 865 cases were evaluated in this study, 785 of which included testing of all lung cancer biomarkers. Lung tissue was adequate for biomarker testing in 84% of cases; this rate increased to 87% when biomarker testing was combined with concurrently acquired FNA or CNB specimens. Biomarker testing success correlated strongly with DNA concentration ($p<0.0001$) and the use of 22G needles in endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) procedures ($p=0.0035$). Biomarker testing of CNB specimens showed a significantly higher success rate than did biomarker testing of cytology FNA specimens ($p=0.0005$). The adequacy of EBUS-TBNA samples was not significantly different from that of the transthoracic needle aspiration samples ($p=0.40$). Variables such as age, gender, lesion size, site, diagnosis and number of needle passes showed no significant correlation with success rates in lung cancer biomarker testing.

Conclusion The growing numbers of therapeutic biomarkers in NSCLC requires judicious triage of limited-volume tissue from small specimens. Our study showed that thoracic small tissue specimens can be used successfully to provide prognostic and predictive information for the current guideline-recommended biomarkers for NSCLC in most cases.

INTRODUCTION

In recent years, advances in characterising the genomic landscape of lung cancer, particularly non-small-cell lung cancer (NSCLC), have resulted in significant progress in the development of effective targeted therapies.[1–3] The recent addition of testing for Tyrosine protein kinase Met (*MET*) exon 14 skipping mutation and Rearranged during Transfection (*RET*) fusions to the already well-established testing for epidermal growth factor receptor (*EGFR*) and serine/threonine protein kinase B-Raf (*BRAF*) mutations and anaplastic lymphoma kinase (*ALK*) and proto-oncogene tyrosine protein kinase ROS (*ROS1*) rearrangements has increased the number of 'must test' genes recommended by the National Comprehensive Cancer Network (NCCN) for NSCLC.[4] In addition to biomarkers for targeted therapy, the development of immune checkpoint inhibitor (ICI) therapies that use immune-mediated destruction of tumour cells has led to United States Food and Drug Administration (FDA) approvals of immunomodulatory monoclonal antibodies such as pembrolizumab, nivolumab, atezolizumab and durvalumab for treatment of advanced-stage NSCLC.[5] In contrast to therapy that targets specific molecules in signalling pathways involved in the growth and propagation of cancer cells, immunotherapy uses the host immune system to recognise tumour cells, stimulate the immune system, and relieve the suppression of antitumour activity. Current NCCN guidelines for advanced or metastatic NSCLC therefore recommend programmed cell death ligand 1 (PD-L1) testing to evaluate eligibility for ICI, in addition to routine molecular profiling for *EGFR*, *BRAF*, *ALK*, *ROS1*, *RET* and *MET* exon 14 skipping mutations.[4] In addition, biomarkers such as neurotrophic receptor tyrosine kinase (*NTRK*) have been included in the NCCN guidelines, since the FDA has approved larotrectinib and entrectinib as first-line therapy in *NTRK* fusion-positive patients with any advanced-stage solid organ malignancies. Furthermore, emerging markers such as Erb-B2 receptor tyrosine kinase

(*ERBB2* or *HER2*), high level *MET* amplification, and Kirsten Rat Sarcoma Viral Oncogene (*KRAS*) exon 2 p.G13C are likely to be included in the NSCLC-targeted therapeutic armamentarium in the near future.

Advanced-stage lung cancer is commonly diagnosed with the use of minimally invasive techniques such as core needle biopsy (CNB) and/or fine-needle aspiration (FNA). For thoracic lesions, typically an endobronchial/transbronchial approach, which commonly uses ultrasound (US) guidance, or a transthoracic (percutaneous) approach, which uses CT or US guidance, is used to collect these 'small specimens'.[6] The procedural modality of choice depends on multiple factors including the site/size of the lesion, skill and expertise of the proceduralist, availability of resources and institutional/personal preferences. With the rapid pace of genomic discovery and the implementation of molecular-driven personalised care for patients with NSCLC, there is a critical need to perform an increasing number of ancillary studies on these limited-volume small specimens. Several studies have shown the utility and feasibility of performing ancillary testing for genotyping small specimens, including CNB and FNA samples, demonstrating a success rate of about 80%—90% for lung biomarker assays.[7–12] However, the list of lung biomarkers that are needed for patient care is constantly expanding, and incomplete genotyping for guideline-recommended genomic markers poses a significant challenge to making informed and timely clinical decisions.[13] The College of American Pathologists (CAP) recently published guidelines for the collection and handling of thoracic small specimens to improve the adequacy of these limited-volume specimens for lung biomarker studies.[6]

In this study, we evaluated thoracic small specimens (CNB and FNA samples) that were collected in-house for lung cancer biomarker testing, including factors such as patient demographics, lesion size and site, collection modality, tumour type, needle gauge and number of passes, to determine the molecular adequacy and success rate of comprehensive lung biomarker testing on these samples. For the purpose of this study, lung biomarker adequacy was defined as adequate tissue for testing the clinically recommended genes, *EGFR*, *BRAF*, *RET*, *MET*, *ALK*, *ROS1* and PD-L1.

METHODS

Case selection and patient samples

We retrospectively identified consecutive malignant small specimens (CNB and FNA samples) that were collected in-house via image-guided procedures, performed between October 2016 and July 2018, for lung biomarker testing. These included procedures performed by interventional radiologists (IR) under CT or US guidance as well as by interventional pulmonologists using endobronchial US (EBUS) guidance. Only cases with a lung biomarker request that included the molecular markers outlined by current NCCN guidelines, as described above, were included in this study set. Biomarker requests at our institution are usually performed on the CNB sample and reflexed to the concurrent FNA sample if the CNB is inadequate for testing or in cases in which the FNA is the only specimen available for testing (ie, in the absence of a concurrent CNB). The cases were retrospectively reviewed for patient demographics, procedure notes, diagnosis and IHC/molecular adequacy to determine success rates for lung biomarker testing.

All FNA procedures were performed with rapid on-site evaluation (ROSE) for adequacy and a preliminary diagnosis. Direct smears were stained by using both Diff-Quik and rapid Papanicolaou staining as previously described.[14] The FNA rinse collected in RPMI medium was centrifuged (1500 RPM for 10 min) to prepare either a cytospin or a cell block preparation. Details of cell block preparation have been previously described.[15 16] Briefly, the cell pellet was fixed in formalin and embedded in paraffin to generate a formalin-fixed paraffin-embedded (FFPE) block for sectioning into 4–5 micron thick sections that were stained with use of H&E) for morphological evaluation and estimation of tumour fraction. Unstained sections from the cell block were subsequently used for biomarker testing. All CNB samples collected were fixed immediately in 10% neutral buffered formalin and processed according to standard histologic procedures to create an FFPE tissue block that was used for morphological evaluation, diagnosis and subsequent biomarker testing.

Molecular testing for lung biomarkers

All molecular testing for mutation-based assays was performed in-house in our CLIA-certified molecular diagnostic laboratory. Testing for *EGFR*, *BRAF*, *MET*, *KRAS* and *ERBB2* was performed as part of a next-generation sequencing (NGS) multigene panel by using an Ion Torrent platform (ThermoFisher, Waltham, Massachusetts, USA), as previously described.[15 17] Briefly, nucleic acid was extracted with use of AllPrep DNA/RNA Extraction Kit (Qiagen, Germantown, MD) and/or a PicoPure DNA Extraction Kit (ThermoFisher) from direct smear preparations and/or unstained FFPE sections of cytology cell blocks or CNB tissue blocks per the manufacturer's instructions. For the DNA-based NGS assay, 10–20 ng of input DNA was used. In a small subset of cases, if NGS analysis failed, the extracted DNA was used to perform single-gene sequencing assays, such as Sanger sequencing for *EGFR* and pyrosequencing for *BRAF*.[18]

ALK, *ROS1*, *RET* and *MET* genes were evaluated by fluorescence in situ hybridisation (FISH), as previously described.[19] In brief, FISH was performed with use of the LSI *ALK* dual-colour break-apart rearrangement probe (Abbott Molecular, Des Plaines, Illinois, USA); the XT *ROS1* dual-colour, break-apart probe (MetaSystems Group, Newton, Massachusetts, USA); the Clear-View FISH *RET* dual-colour break-apart probe (CymoGen DX, Biocare Medical, Concord, California, USA); and the CymoGen DX MET/CC7 DNA Probe Kit (Biocare Medical), using criteria previously established in our cytogenetics laboratory.[19] A subset of cases were evaluated by an RNA-based NGS fusion assay sequenced with use of a commercially available targeted amplicon-based NGS on the Ion S5XL platform, to amplify a set of targeted fusion sequences in 51 genes that included *ALK*, *ROS1*, *RET*, *MET* and *NTRK* (Oncomine Comprehensive Assay v3M, ThermoFisher). Details of the NGS RNA fusion assay have been previously described.[20]

Immunohistochemical (IHC) analysis of PD-L1 was performed on FFPE sections from either the cell block preparation or CNB specimens using the 22C3 antibody clone (Agilent Technologies, Santa Clara, California, USA). IHC staining was evaluated by the pathologist and reported as a Tumour Proportion Score based on the percentage of viable tumour cells demonstrating complete or partial membranous staining, as described previously.[21]

Evaluation of molecular adequacy

The tested sample was considered adequate for lung biomarker testing, if *EGFR*, *ALK*, *ROS1*, *BRAF*, *MET*, *RET* and PD-L1 testing was performed successfully. Since the RNA-based NGS assay for fusion detection became available at our institution in early 2018, fusion testing by NGS assay was available in only a small subset of our cases. While most of the recommended lung cancer biomarkers (including *ALK*, *ROS1, RET* and *MET*) were being tested by an alternative assay such as FISH, testing for *NTRK* fusions was only performed in a subset of our cohort with RNA-based NGS fusion assay and therefore were excluded from the statistical analysis of lung biomarker adequacy. Testing for *ALK*, *ROS1* and *RET* was performed by both FISH and NGS fusion assay in a small subset of cases. Testing outcomes were categorised as follows: (1) adequate for biomarker testing for all genes on the same sample; (2) adequate for biomarker testing in combination with another concurrent malignant FNA or CNB sample; (3) partially adequate for some but not all the requested biomarkers and (4) inadequate for all biomarker testing.

Statistical analysis

Wilcoxon rank-sum tests were used to compare the distribution of continuous variables between the groups defined above. Fisher's exact tests were used to compare the distribution of categorical parameters between groups. All statistical analyses were performed using R V.3.6.1. All statistical tests used a significance level of $P<0.005$.

RESULTS

A total of 865 cases were evaluated in this study, including CT-guided CNB specimens (n=679) and FNA samples from EBUS-transbronchial needle aspiration (TBNA) (n=150) and IR (n=36) FNA procedures. The median age of patients was 67 years (range 26–97 years). There was similar gender distribution, with 48% male (n=417) and 52% female (n=448) patients. The site of lesion included lung (n=467), lymph node (n=210), liver (n=44), pleura (n=39), soft tissue (n=36), bone (n=30), adrenal gland (n=29), mediastinum (n=6), kidney (n=3) and stomach (n=1). The diagnosis in most cases was adenocarcinoma (n=643), followed by squamous cell carcinoma (n=87), neuroendocrine carcinomas (including small-cell carcinoma (n=41)), poorly differentiated carcinoma (n=34), non-small-cell carcinoma not otherwise specified (NOS) (n=26), carcinoma NOS (n=26), other malignant neoplasms NOS (n=5) and sarcomatoid neoplasms (n=3). Patient demographics and clinicopathological features are summarised in table 1.

Table 1 Patient demographics and clinicopathological parameters

Clinical parameters	
Patients	**No of patients**
Male	417 (48%)
Female	448 (52%)
Age, median (range)	67 (26–97) years
Procedure modality	**No of cases**
CNB	679
IR-FNA	36
EBUS-TBNA	150
Lesion Site	**No of cases**
Lung	467
Lymph node	210
Liver	44
Pleura	39
Soft tissue	36
Bone	30
Adrenal	29
Mediastinum	6
Kidney	3
Stomach	1
Diagnosis	**No of cases**
Adenocarcinoma	643
Squamous cell carcinoma	87
Neuroendocrine carcinoma	41
Poorly differentiated carcinoma	34
Non-small-cell carcinoma	26
Carcinoma, NOS	26
Malignant neoplasm, NOS	5
Sarcomatoid neoplasm	3

CNB, core needle biopsy; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; IR-FNA, interventional radiology-fine needle aspiration; NOS, not otherwise specified.

Procedural parameters

Of the 679 CNB specimens procured, data for the needle gauge used and the number of core biopsies collected were available in 412 cases. The most commonly used needle gauge was 20G (n=331; 80%), followed by 18G (n=80; 19%) and 19G (n=1; <1%). A coaxial guide needle was used in 366 procedures, including a 19G guide needle (n=308; 84%), a 17G guide needle (n=58; 16%). No guide needle was used in 51 procedures and no data regarding the use of coaxial needle was available for the remaining 262 cases (table 2). Of the 412 CNB procedures that had data on the number of core biopsies collected, in most cases, either 2 (n=162; 39%) or 3 (n=156; 38%) cores were collected. The remaining cases ranged from 1 to 8 core samples that were procured. The median lesion size targeted for CNB specimen collection was 2 cm (range 0.43–9 cm).

A large percentage of the 679 CNB specimens procured had a concurrent FNA sample (n=419; 62%), whereas the remaining samples (n=260; 38%) did not. Adverse events had been recorded in were 417 cases, with pneumothorax noted in 15% of cases requiring chest tubes in a subset (4.5%) of cases; the remaining 85% of cases did not have any adverse events from the procedure (table 2).

Of the 36 IR FNA samples that had biomarker testing requested, 9 were done under US guidance, whereas the remaining 27 were done under CT guidance. Of these FNA cases that were used for biomarker testing, 33 (92%) had a concurrent CNB, 5 of which were negative for tumour. The remaining 28 cases had a concurrent CNB that had inadequate tumour for biomarker analysis, requiring testing to be reflexed to the FNA sample. Data for the needle gauge used for collecting FNA specimens were available for 22 of these cases, all of which were performed with a 22G needle. The median number of needle passes was 2 (range, 1–4 passes). The median lesion size was 2 cm (range 0.8–7.3 cm). Adverse events reported in 19 cases included 2 cases with pneumothorax (both of which had concurrent CNB using 20G needles) and 17 cases had no adverse events noted (table 2).

Of the 150 EBUS-TBNA cases included in this study, only 11 (7%) had a concurrent surgical biopsy. Of the 144 cases that had available data on needle gauge, most procedures used a 22G

Table 2 Procedural parameters for small tissue specimens evaluated for lung cancer biomarker testing

	CNB (N=679)	IR-FNA (N=36)	EBUS-TBNA (N=150)
Needle gauge	**N=412**	**N=22**	**N=144**
	20G: 331 (80%)	22G: 22 (100%)	22G: 133 (92%)
	18G: 80 (19%)		21G: 5 (3%)
	19G: 1 (0.2%)		20G: 6 (4%)
Median no of cores/ passes (range)	**N=412**	**N=22**	**N=144**
	3 (1–8)	2 (1–4)	6 (2–12)
Median lesion size (range)	2 (0.43–9) cm	2 (0.8–7.3) cm	1.7 (0.5–5.4) cm
Concurrent FNA/CNB	N=419 (62%)	N=33 (92%)	N=11 (7%)
Adverse events	63 of 417 (15%)	2 of 19 (11%)	0 of 150 (0%)

CNB, core needle biopsies; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspirations; FNA, fine-needle aspirations; IR, interventional radiology.

needle (n=133; 92%), with a few cases using 21G (n=5; 3%) or 20G (n=6; 4%) needles. All 150 EBUS-TBNAs were performed with the use of a stylet. The median number of needle passes was 6 (range 2–12 passes), and the median lesion size was 1.7 cm (range 0.5–5.4 cm) (table 2). No adverse events were noted in the EBUS-TBNA cases.

Biomarker testing

The median DNA yield for CNB, EBUS-TBNA and IR-FNA specimens was 0.02 µg/µl (range 0.0002–0.2 µg/µL), 0.01 µg/µl (range 0.0002–0.07 µg/µL) and 0.006 µg/µL (range 0.001–0.019 µg/µL), respectively. The adequacy of tissue for lung biomarker testing, for which all clinically recommended genes (*EGFR*, *BRAF*, *RET*, *MET*, *ALK*, *ROS1* and PD-L1) were successfully performed on the requested sample, was 84% (659/785). When testing was performed by using a combination of specimens, that is, combined with a concurrent FNA or CNB, the overall success rate of lung biomarker testing was 87% (679/785). Partial results (for some, but not all, of the lung biomarker tests performed) were obtained in an additional 8% (64/785) of the cases, whereas the overall failure rate for lung biomarker testing was 5% (42/785). A subset of cases (n=80) were excluded from evaluation of biomarker testing success rates, since testing for all of the clinically recommended genes was either not requested or not performed at the discretion of the ordering clinician. Results of biomarker testing are summarised in table 3.

Lung biomarker adequacy correlated strongly with DNA concentration (p<0.0001), with CNB samples demonstrating the highest average DNA yield and IR-FNA samples showing the lowest average DNA yield. Most of the variables we evaluated, including age, gender, lesion size, lesion site and diagnosis, showed no significant correlation with lung biomarker adequacy. There was no evidence that the number of passes or the needle gauge used for obtaining CNB specimens correlated with the adequacy for lung biomarker testing. Similarly, for FNA samples, there was no evidence that the number of passes correlated with adequacy of the sample. There was some evidence that lung biomarker adequacy positively correlated with the use of 22G needles in EBUS-TBNA procedures (p=0.0035); however, the number of EBUS-TBNA procedures using other needles (ie, 21G and 20G) was low (8% of all cases). Lung biomarker testing of CNB specimens showed a significantly higher success rate than did biomarker testing of the cytology FNA specimens (p=0.0005). The adequacy of EBUS-TBNA samples was not significantly different than that of IR-FNA samples (p=0.40); however, the overall sample size of IR-FNA samples was relatively low.

Table 3 Lung cancer biomarker testing adequacy for thoracic small tissue specimens

	CNB	IR-FNA	EBUS-TBNA
Lung BM testing adequacy (all BM)	N=548/610 (90%)	N=26/36 (72%)	N=105/137 (77%)
Partial results	N=43 (7%)	N=4 (11%)	N=17 (12%)
Mutation and fusion testing	4 (<1%)	1 (3%)	3 (2%)
Mutation testing and IHC	4 (<1%)	0 (0%)	5 (4%)
Fusion testing and IHC	21 (3%)	2 (6%)	2 (1%)
Mutation testing only	5 (<1%)	1 (3%)	3 (2%)
Fusion testing only	0 (0%)	0 (0%)	1 (<1%)
IHC only	9 (3%)	0 (0%)	3 (2%)
Lung BM testing inadequate (all BM)	N=19 (3%)	N=6 (17%)	N=17 (12%)
Cases excluded (all BM not requested)	N=69 (10%)	N=0 (0%)	N=11 (7%)

BM, biomarkers; CNB, core needle biopsies; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspirations; FNA, fine-needle aspirations; IHC, immunohistochemistry; IR, interventional radiology.

CNB specimens

All 679 CNB cases had a request for mutation analysis by NGS and PD-L1 IHC analysis. Of these cases, 628 (93%) were adequate for *EGFR*, *BRAF* and *MET* by NGS assay, and 650 (96%) were adequate for PD-L1 IHC analysis. Fusion testing for *ALK*, *ROS1* and *RET* was requested in a subset of cases by FISH and/or RNA-based NGS assay. The sample was adequate for fusion testing in 598 of 634 cases (94%) for *ALK*; 600 of 636 cases (94%) for *ROS1*; and 460 of 485 cases (95%) for *RET*. Fusion testing for *NTRK* was available in 257 cases and was adequate for testing in 90% (n=231) of the cases tested; one case had fusion testing for *NTRK* on a concurrent FNA sample. Lung biomarker testing for the clinically recommended genes (*EGFR*, *BRAF*, *RET*, *MET*, *ALK*, *ROS1* and PD-L1) was successfully performed on 90% (548/610) for the CNB samples (figure 1).

Partial results for a subset of the lung biomarker tests were obtained in an additional 43 (7%) CNB cases. Of these, 21 cases were inadequate for NGS mutation testing but were adequate for fusion testing and PD-L1 IHC analysis; 9 cases were inadequate for NGS and fusion testing with only PD-L1 reported; 5 cases were inadequate for fusion testing and PD-L1 IHC analysis but adequate for NGS; 4 cases were adequate for NGS and fusion testing but inadequate for PD-L1 and 4 cases were adequate for NGS and PD-L1 but inadequate for fusion testing (table 3). In a subset of CNB cases (n=69; 10%), testing for all the clinically recommended genes was not requested by the clinician, and these were excluded from our analysis.

Cytology FNA specimens

For the 150 EBUS-TBNA specimens in our study, a request for mutation analysis by NGS was received in 148 cases and was adequate for *EGFR*, *BRAF* and *MET* analysis in 124 cases (84%), with an additional case for which NGS was performed on a concurrent FNA. All 150 cases had PD-L1 IHC analysis

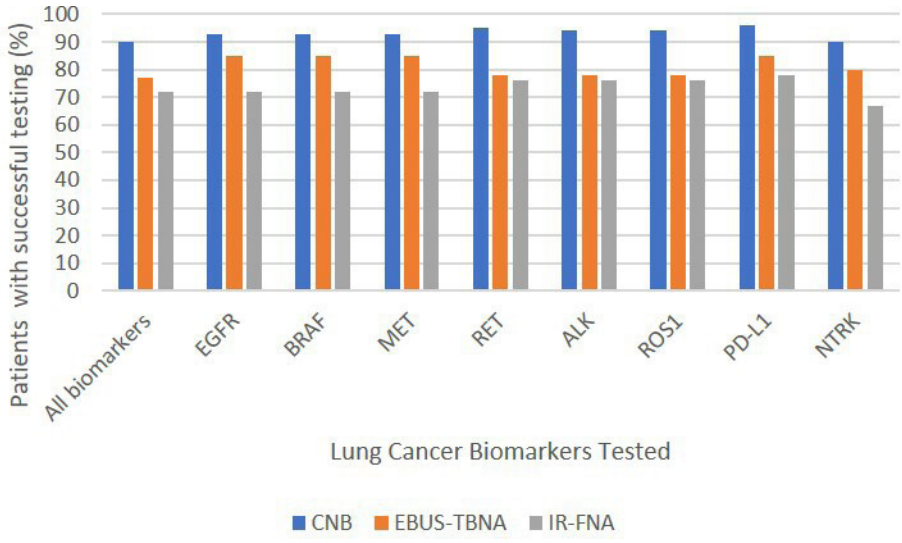

Figure 1 Percentage of patients with completed lung cancer biomarker testing for the guideline recommended biomarkers including *EGFR, ALK, ROS1, RET*, *BRAF*, *MET* and PD-L1. Testing for *EGFR, BRAF* and *MET* was performed by a broad next-generation sequencing (NGS) mutational profiling; *ALK, ROS1* and *RET* fusions were tested by fluorescence in situ hybridisation and/or an RNA-based NGS assay; PD-L1 expression was evaluated by an immunohistochemical assay. Testing for *NTRK* fusions were performed in a subset of cases as part of the RNA-based NGS assay. Success rates of testing are stratified by procedure type and include core needle biopsies (CNB), endobronchial ultrasound-guided transbronchial needle aspirations (EBUS-TBNA), and interventional radiology fine needle aspirations (IR-FNA). While the overall success rates of lung cancer biomarker testing are similar for EBUS-TBNA and IR-FNA specimens, the adequacy of CNB specimens for testing lung cancer biomarkers shows significantly higher success rates than that of fna specimens. ALK, anaplastic lymphoma kinase; BRAF, B-Raf proto-oncogene, serine/threonine kinase; EGFR, epidermal growth factor receptor; MET, MET proto-oncogene, receptor tyrosine kinase; NGS, next-generation sequencing; NTRK, neurotrophic receptor tyrosine kinase; PD-L1, programmed cell death ligand 1; ROS1, ROS proto-oncogene 1.

requested, which was adequate in 119 cases (79%), with an additional seven cases that had adequate material on the concurrent CNB to report PD-L1. Fusion testing for *ALK*, *ROS1* and *RET* was requested in 138 cases and was adequate in 103 cases (75%), with an additional five cases for which fusion testing was reported on a concurrent FNA/CNB specimen. Fusion testing by NGS assay, including *NTRK*, was requested in 46 EBUS TBNA cases and was adequate for testing in 37 cases (80%). Lung biomarker testing for the clinically recommended genes was successfully performed on 71% of the EBUS-TBNA samples (97/137). An additional eight cases were adequate for providing clinically recommended genomic information when testing was performed in combination with a concurrent FNA/CNB specimen (figure 1).

Partial results were obtained in 12% of the cases (17/137). Of these, five cases were adequate for NGS and PD-L1 but inadequate for fusion testing for *ALK*, *ROS1*, and *RET*; three cases were adequate for NGS and fusion testing but inadequate for PD-L1, whereas three cases were inadequate for NGS and fusion testing with only PD-L1 reported; three cases were inadequate for fusion testing and PD-L1 IHC but adequate for NGS; two cases were inadequate for NGS but adequate for PD-L1 IHC analysis and fusion testing and one case adequate for only fusion testing but inadequate for NGS and PD-L1 (table 3). A subset of cases (n=11), for which testing for all the clinically recommended genes was either not requested or not performed at the discretion of the ordering clinician, were excluded from the analysis.

IR-FNA samples made up the smallest group of specimens sent for lung biomarker testing, as molecular test requests at our institution are typically reflexed to the concurrent CNB specimen. Of the 36 FNA cases, all specimens had a request for mutation analysis and PD-L1 and were adequate for *EGFR*, *BRAF*, and *MET* mutation analysis by NGS in 26 cases (72%) and for PD-L1 IHC in 16 cases (44%); however, an additional 12 cases (33%) had adequate material on the concurrent CNB to report PD-L1. Fusion testing for *ALK*, *ROS1*, and *RET* were requested in 33 cases by FISH and/or RNA-based NGS assay; the sample was adequate for testing in 17 (52%) of the 33 cases, with an additional 8 cases for which fusion testing was reported on the concurrent CNB. Nine cases were tested for *NTRK* fusions by an RNA-based NGS assay, of which 6 cases (67%) were adequate for testing. Lung biomarker testing was successfully performed on 39% of the specimens (14/36); the adequacy increased to 72% (26/36) when testing was performed in combination with a concurrent FNA/CNB sample (figure 1). Of these, 10 cases were adequate for NGS, but fusion testing and/or PD-L1 IHC analysis was performed on the concurrent CNB; the remaining two cases were adequate for fusion testing and PD-L1 IHC, but NGS was performed on the concurrent specimen.

Partial results were obtained in four cases, in which one case was adequate for NGS and fusion testing but inadequate for PD-L1, 1 case was adequate for only NGS but inadequate for both fusions and PD-L1 testing, and the remaining two cases had fusion and PD-L1 testing on a concurrent CNB but were inadequate for NGS (table 3).

DISCUSSION

In the current genomic landscape of NSCLC, there is a critical need for the evaluation of biomarkers in patients with advanced-stage disease to determine eligibility for targeted therapy and immunotherapy.[22] Since most NSCLC is diagnosed from small specimens obtained via minimally invasive techniques, the need for optimising tissue acquisition procedures and specimen processing techniques to ensure the adequacy of these

limited-volume samples for biomarker testing is critical.[23 24] A recent CAP guideline provides recommendations for the collection and handling of thoracic small tissue specimens in an effort to ensure adequacy of these small specimens for ancillary studies.[6] Although several studies have evaluated the success rates of thoracic specimens for molecular testing, the literature is limited on the adequacy and success rates of comprehensive biomarker testing for targeted therapy and immunotherapy as per current NCCN guidelines. Therefore, in this study, we retrospectively reviewed consecutive NSCLC core biopsies and FNA specimens that were performed in-house under image guidance and evaluated a variety of parameters including lesion size and site, collection modality, tumour type, needle gauge and number of passes, to determine the adequacy and success rate of lung biomarker testing on these samples.

The results from our retrospective review of lung biomarker adequacy of thoracic small tissue specimens for clinically recommended genes (*EGFR*, *BRAF*, *RET*, *MET*, *ALK*, *ROS1* and PD-L1) showed a success rate of 84%, which increased to 87% when a concurrent FNA or CNB sample was combined to complete the testing. These results were comparable to those published in the literature for lung biomarker testing for smaller panels involving *EGFR*, *ALK* and *ROS1*. The overall failure rate for lung biomarker testing was 5%, with partial results for some of the required lung biomarker tests obtained in 8% of cases. Biomarker testing of CNB specimens showed significantly higher success rates than did biomarker testing of cytology FNA specimens ($p=0.0005$). This may be largely due to the availability of a FFPE tissue block for CNB specimens that can be used for obtaining multiple sections for all of the requested biomarker testing.

At our institution, all CNB specimens are initially evaluated by using 10 levels (2 H&E and 8 unstained sections in between), followed by additional 15 unstained sections recut from the block when the biomarker test request is received. Cytology specimens frequently lack adequate tumour on the cell block preparation for performing biomarker testing. Several studies have reported suboptimal cell block preparation, despite good aspirates as demonstrated by adequate smear preparations.[15 25–28] The reasons are typically multifactorial and include the inability to collect additional dedicated passes into the needle rinse; dilution of a needle pass with good tumour cellularity with subsequent hemodilute passes; and loss of cellular material during cell block processing. NGS testing of cytology specimens at our institution does not rely solely on FFPE cell block preparations, and a large percentage of cases are tested with use of direct smears alone or in combination with cell block sections. As noted in prior studies from our group, the use of smear preparations for molecular testing has significantly improved our adequacy of cytology samples for both DNA and RNA-based NGS assays.[15 20] However, our study showed that the overall adequacy of cytology samples compared with their histologic counterparts significantly decreased with the addition of FISH- and IHC-based biomarker studies, suggesting that lung biomarker adequacy of cytology specimens is often limited by suboptimal cell block preparation. These results underscore the need for collecting an adequate sample for cell block preparation during EBUS TBNA procedures, as highlighted in the recent CAP recommendations.[6]

Although our results demonstrated that both EBUS-TBNA and IR-FNA specimens have similar overall success rates for lung biomarker testing, the adequacy of NGS testing is lower in the latter (85% vs 72%, respectively). This is likely a reflection of the vastly different procurement processes for the two modalities. For EBUS-TBNA procedures, the bronchoscopist relies largely on ROSE and performs on average six needle passes to collect an adequate sample for diagnosis and subsequent biomarker testing. Furthermore, EBUS procedures are largely dependent on acquisition of only an FNA sample, with only 7% of cases in this cohort having a concurrent surgical biopsy specimen. In contrast, IR procedures, especially transthoracic procedures, focus primarily on collecting CNB samples, and typically 2 FNA needle passes are performed during the procedure. In this study, 92% of all IR-FNA samples had a concurrent CNB specimen collected. As previously mentioned, lung biomarker test requests at our institution are reflexed to the CNB samples, and FNA specimens are usually used to supplement the CNB sample or in cases in which the biopsy has insufficient tumour. This is reflected in the significantly lower numbers of IR-FNA samples used for biomarker testing seen in this cohort. Therefore, unlike EBUS TBNA specimens, the IR-FNA samples usually have limited material on both smears and cell block preparation, as reflected in the significantly lower adequacy rates for both NGS-based testing (72% adequate) as well as FISH (76% adequate) and IHC (78% adequate) assays.

Variables such as age, gender, lesion size, site and diagnosis showed no significant correlation with success rates of lung biomarker testing. As expected, biomarker adequacy correlated strongly with DNA concentration. The use of 22G needles in EBUS-TBNA procedures positively correlated with lung biomarker adequacy but showed no correlation in CNB or IR-FNA samples. In addition, there was no evidence that the number of passes for obtaining CNB and FNA samples correlated with the adequacy for lung biomarker testing.

With the increasing number of clinically relevant lung biomarkers needed for patient care decisions, there is a critical need for optimal collection and processing of thoracic small specimens to judiciously triage and perform all of the necessary testing. Although lung molecular testing guidelines do not outline specific assays for relevant biomarker testing, there is a definite shift from performing single gene testing to expanded multiplexed assays capable of interrogating multiple genes simultaneously.[22] This primarily stems from the need to do more with less, as limited-volume specimens frequently fall short in providing all the relevant genomic information required to guide patient treatment. For example, current NCCN guidelines list several methodologies for testing *EGFR* and *BRAF* (real-time PCR, Sanger sequencing preferably with tumour enrichment, NGS), *ALK* and *ROS1* (FISH, IHC, NGS, targeted real-time PCR), *RET* (FISH, RT-PCR, NGS) and *MET* (NGS, RT-PCR). Testing all of these biomarkers by single gene sequencing assays would require a minimum of 20–30 FFPE sections from a moderately cellular biopsy and/or FNA specimen (table 4). If using direct smears, 10–13 moderately cellular smears would be needed for sequencing and FISH assays alone. The ability to combine biomarker testing with use of multiplexed assays such as NGS has provided the opportunity to perform these assays on multiple genes using limited material. In fact, current NCCN guidelines recommend broad molecular profiling by using either an FDA-approved or laboratory-developed platform to test at a minimum the guideline-recommended biomarkers (*EGFR*, *BRAF*, *ALK*, *ROS1*, *RET*, *MET* and *NTRK*) with an option to test for emerging biomarkers including high-level *MET* amplification, *ERBB2* mutations and tumour mutational burden (TMB).

One limitation of our study was the retrospective nature spanning a period of 2 years, during which lung biomarker evaluation was performed by using different testing methodologies. Whereas mutation testing was performed primarily using a

Table 4 Pathology requirements for tissue-based lung cancer biomarker assays by testing modality

Biomarkers	Single gene-based testing approach			Multiplexed molecular profiling approach
	Sequencing based	FISH based	IHC based	
EGFR	5 sections or 2 smears			10–15 sections or 3–5 smears
BRAF	5 sections or 2 smears			
ALK	5 sections or 2 smears	OR 1 section or 1 smear	OR 1 section or 1 smear	
ROS1	5 sections or 2 smears	OR 1 section or 1 smear	OR 1 section or 1 smear	
RET	5 sections or 2 smears	OR 1 section or 1 smear		
MET	5 sections or 2 smears			
PD-L1			1 section or 1 smear	1 section or 1 smear
No of slides	19–31 sections or 10–13 smears			11–16 sections or 4–6 smears

ALK, anaplastic lymphoma kinase; BRAF, B-Raf proto-oncogene, serine/threonine kinase; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry; PD-L1, programmed Cell death ligand 1; RET, RET proto-oncogene; ROS1, ROS proto-oncogene 1.

DNA-based NGS assay for all samples, fusion testing was done using FISH, NGS, or a combination of both. The FISH assay evaluates *ALK*, *ROS1* and *RET* fusions, but the NGS multiplexed fusion assay includes several other genes including *NTRK*. Although the subset of cases evaluated by the NGS fusion assay was small (n=312), the overall success rate of fusion testing by NGS (*ALK*, *ROS1*, *RET*, *MET*, *NTRK*) was 88%. Hence, if an appropriately designed NGS testing assay is used, with which DNA and RNA are simultaneously extracted, lung biomarker testing could be performed by using as few as 10–15 FFPE sections or 4–6 direct smears (table 4). With use of such an assay, in addition to the current guideline-recommended genes, the combined DNA and RNA NGS assay would cover *ERBB2* and *KRAS* mutations, *MET* amplifications and TMB.

In summary, this study showed that thoracic small tissue specimens can be used successfully to provide prognostic and predictive information for the current guideline-recommended biomarkers for NSCLC in most cases. The availability of a tissue block and concurrently acquired CNB and FNA samples increases the chances of adequacy for lung biomarker testing. The use of multiplexed expanded molecular profiling assays such as NGS, which judiciously uses limited amounts of tissue for testing, can provide all the clinically required biomarker testing needed to guide the care of patients with NSCLC.

Take home messages

- ⇒ Guideline-recommended biomarker testing is critical in non-small-cell lung cancer.
- ⇒ Incomplete genotyping due to inadequate tissue poses significant challenges in small tissue specimens.
- ⇒ If appropriately collected and handled, majority of thoracic small tissue specimens are adequate for determining therapeutic eligibility.
- ⇒ Genomic profiling of small tissue specimens can be used in making clinical decisions.

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