CME/SAM

Test Feasibility of Next-Generation Sequencing Assays in Clinical Mutation Detection of Small Biopsy and Fine Needle Aspiration Specimens

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ABSTRACT

Objectives: *To evaluate preanalytic factors contributing to failure of next-generation sequencing (NGS) assays.*

Methods: *AmpliSeq Cancer Hotspot Panel was conducted in 1,121 of 1,152 formalin-fixed paraffin-embedded tissues submitted to a clinical laboratory, including 493 small biopsy or fine needle aspiration (FNA) specimens (44%) and 25 metastatic bone specimens (2.2%).*

Results: *Single nucleotide mutations and/or insertion/deletion mutations were detected in 702 specimens. Thirty-eight specimens (3.4%) were reported as "no results" due to NGS assay failure. Higher failure rates were observed in specimens submitted for lung cancer panel and melanoma panel (3.1% and 3.7% vs 1.0% colorectal cancer panel), metastatic bone specimens (36% vs 2.6% nonbone specimens), referred specimens (5.0% vs 1.8% in-house specimens), and small biopsy and FNA specimens (5.8% and 3.1% vs 0.7% resection/excision specimens). Test feasibility was higher in in-house specimens than referred specimens (99.1% vs 96.9% in resection specimens, 94.4% vs 87.3% in small biopsy specimens, and 94.3% vs 58.8% in FNA specimens).*

Conclusions: *NGS assays demonstrated clinical utility in solid tumor specimens, including those taken by biopsy or FNA. Preanalytic factors identified by this study that may contribute to NGS assay failure highlight the need for pathologists to revisit tissue processing protocols in order to better optimize cancer mutational profiling.*

Upon completion of this activity you will be able to:
- describe the clinical utility of next-generation sequencing (NGS) assays in solid tumor specimens, including those taken by biopsy or fine needle aspiration.
- define the preanalytic factors that may contribute to NGS assay failure.
- describe the need for pathologists to revisit tissue processing protocols to better optimize cancer mutational profiling.

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In the past two decades, we have seen major advances in understanding the genetic alterations of cancers and utilizing such information in clinical management. Therapies targeting specific genetic alterations have proven safer and more efficacious compared with traditional chemotherapy. Several kinase inhibitors have been approved by the US Food and Drug Administration for treatment of metastatic solid tumors, including selective BRAF inhibitors (vemurafenib and dabrafenib) and MEK inhibitor (trametinib) for metastatic melanomas with *BRAF* p.V600E mutations,[1-3] anti-EGFR monoclonal antibodies (cetuximab and panitumumab) for metastatic colorectal cancers (CRCs) with neither *KRAS* nor *NRAS* mutations,[4,5] EGFR tyrosine kinase inhibitors (gefitinib and erlotinib) for nonsmall cell lung

cancers (NSCLCs) with certain *EGFR* mutations,[6,7] and ALK tyrosine kinase inhibitor (crizotinib) for NSCLCs with translocations of the *ALK* gene.[8] Prospective testing for target gene mutations is required to identify patients who may benefit from these targeted therapies.[9,10]

Adequate tissue sampling and optimal processing are critical, not only for histopathological interpretation, but also for mutational profiling of cancers. Clinical specimens in the molecular diagnostic setting pose multiple challenges: Tumor cellularity is frequently low, necessitating assays with higher analytic sensitivity;[11-13] core biopsy or fine needle aspiration (FNA) yield limited DNA, requiring multiplex-platform assays to simultaneously test a panel of genes;[11,14] additionally, tissue processing, such as fixation and embedding, may damage DNA.[15-18] A standard operating protocol for tissue processing should be established in histopathology laboratories to extract nucleic acids of sufficient quality and quantity for use in mutational profiling of cancers.

Massively parallel sequencing or next-generation sequencing (NGS) technology has not only led to a revolution in genome discovery but has also changed the approach in clinical molecular diagnostics laboratories from the traditional "one test-one drug" paradigm to a multiplexed genotyping platform.[14] NGS assays have been clinically applied to formalin-fixed, paraffin-embedded (FFPE) and FNA tumor specimens.[19-21] We have previously validated an NGS platform based on the AmpliSeq Cancer Hotspot Panel and Personal Genome Machine in the setting of a Clinical Laboratory Improvement Amendments-certified laboratory.[22] In this retrospective study for quality assessment, we analyzed the performance of our NGS assay on 1,152 clinical FFPE tissue submissions to elucidate factors contributing to assay failure.

Material and Methods

Materials

A total of 1,152 consecutive FFPE specimens were submitted to the Molecular Diagnostics Laboratory at the Johns Hopkins Hospital between April 2013 and October 2014 for NGS assays. Cancellations occurred in 31 specimens, comprised of 4 specimens due to redundant ordering where a prior specimen from the same patient had been successfully tested by NGS, 1 melanoma specimen with poor fixation of soft tissues containing abundant fat, and 26 specimens due to exhausted tissues within the blocks, few tumor cells and/or low tumor cellularity likely less than 1% to 5%. These latter 26 specimens consisted of 4 resections, 12 biopsies, and 10 FNAs. Excluding the cancellations, 623 specimens were submitted for a lung cancer panel (*AKT*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *NRAS* and *PIK3CA* genes), 312 specimens for a CRC panel (*BRAF*, *KRAS*, *NRAS* and *PIK3CA* genes), 168 specimens for a melanoma panel (*BRAF*, *KIT*, *NRAS* and *PIK3CA* genes), and 18 specimens for a gastrointestinal stromal tumor (GIST) panel (*KIT* and *PDGFRA* genes). There were 598 resection/excision specimens, 361 biopsy specimens (including core biopsy, endoscopic biopsy and shave biopsy), 132 FNA specimens and 26 aspiration specimens of pleural effusion, pericardial effusion or ascites, 3 bronchoalveolar lavage specimens and 1 endometrial curettage specimen. Twenty-five specimens were of bone metastasis. The majority of specimens were taken and processed at the Johns Hopkins Hospital: 814 in-house specimens vs 307 taken and/or processed at referring hospitals. For FNA specimens and effusion specimens, cell blocks were prepared and then processed using the same procedure as other FFPE specimens. Tissue blocks with 10% or more tumor cellularity were selected by pathologists who made the diagnosis. One H&E slide followed by 5 to 10 unstained slides and one additional H&E slide were prepared with polymerase chain reactin (PCR) precaution. The H&E slide was examined and marked by pathologists for subsequent macrodissection of FFPE neoplastic tissues from 3 to 10 unstained slides of 5 or 10-micron thick sections. DNA was isolated from the area(s) designated by pathologists using the Pinpoint DNA Isolation System (Zymo Research, Irvine, CA), followed by further purification via the QIAamp Mini Kit (Qiagen, Valencia, CA).[23]

Next-Generation Sequencing

NGS was performed using AmpliSeq Cancer Hotspot Panel (v. 2) for targeted multigene amplification as described previously.[22,24] Briefly, we used Ion AmpliSeq Library Kit 2.0 for library preparation, Ion OneTouch 200 Template Kit v2 DL and Ion OneTouch Instrument for emulsion PCR and template preparation, and Ion PGM 200 Sequencing Kit with Ion 318 Chip and Personal Genome Machine as the sequencing platform (Life Technologies, Carlsbad, CA). The DNA input was up to 30 ng measured by Qubit 20 Fluorometer (Life Technologies). Up to 8 specimens were barcoded using Ion Xpress Barcode Adapters (Life Technologies) for each Ion 318 chip. One to three controls (nontemplate control, a normal peripheral blood control from a male and/or positive control specimens) were included in each chip. Sequencing data of the targeted genes was analyzed using Torrent Suite (Life Technologies). Mutations were identified and annotated through both Torrent Variant Caller and direct visual inspection of the binary sequence alignment/map (BAM) file on the Broad Institute's Integrative Genomics ViewerIGV.[25] During our validation of this NGS assay, a background noise threshold

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of 2% was chosen for single nucleotide variations.[22] Provided sufficient DNA input, the limit of detection is dictated by the depth of coverage (or number of sequencing reads) in the target genomic region. Approximately 150 and 500 reads are needed to detect a heterozygous mutation at a 99% confidence in a specimen with 20% and 10% tumor cellularity, respectively.

Statistical Analysis

χ^2 test or Fisher exact test was performed to calculate *P* values.

Results

High Failure Rate in Bone Specimens

Among the 1,121 specimens with NGS assays conducted, there were 702 specimens with one or more mutations detected, 381 specimens reported as "negative for mutation" with or without a note indicating less than 20% estimated tumor cellularity in the specimen, and 38 specimens (3.4%) reported as "no results" due to failure of the NGS assay. Metastatic bone specimens showed a remarkably higher failure rate (9/25, 36% vs 29/1096, 2.6% in nonbone specimens, $P < .001$). Bone specimens were therefore excluded from the rest of the analysis. Specimens submitted for CRC panel (3/311, 1.0%) showed a lower failure rate as compared to those submitted for lung cancer (19/604, 3.1%, $P = .04$) or melanoma panels (6/163, 3.7%, $P = .07$) **Table 1**. One of 18 specimens submitted for GIST panel failed NGS. These 18 specimens were also excluded from the rest of analysis.

Higher Failure Rate in Referred Specimens

Referred specimens from other institutes experienced a higher failure rate (14/288, 4.9%) compared with specimens taken and processed at the Johns Hopkins Hospital (14/790, 1.8%; $P < .01$) **Table 2**. The discrepancy was driven predominantly by the lung cancer panel submissions (6.9% referred failure rate vs 2.0% in-house, $P < .01$), particularly lung cancer panel biopsy specimens (9.9% vs 3.8%) and FNA specimens (9.1% vs 2.7%) (Table 2). By contrast, there was no significant difference in failure rate between in-house and referred specimens submitted for the CRC panel (0.8% vs 1.3%) or melanoma panel (3.1% vs 4.5%).

Table 1
Specimens Failed in Next-Generation Sequencing Assays

Assay	Case	Fail (%)	*P* Value[a]
LCP	604	19 (3.1%)	.02
CRCP	311	3 (1.0%)	NC
MP	163	6 (3.7%)	.07
GISTP	18	1 (5.6%)	NC
Total	1,096	29 (2.6%)	

CRCP, colorectal cancer panel; GISTP, gastrointestinal stromal tumor panel; LCP, lung cancer panel; MP, melanoma panel.
[a]Compared with specimens submitted for CRCP. NC, not compared due to a low case number.

Higher Failure Rates in Biopsy Specimens and FNA Specimens

Biopsy specimens (19/342, 5.6%, $P < .001$) and FNA specimens (4/130, 3.1%, $P = .04$) also showed a higher failure rate compared to resection/excision specimens (4/576, 0.7%). Among the 131 biopsy specimens submitted for the lung cancer panel, NGS failed in 3 (4.1%) of 74 of core biopsy specimens, 2 (5.1%) of 39 endobronchial or transbronchial biopsy specimens, and 0 of 18 open biopsy or punch biopsy specimens. Among the 36 biopsy specimens submitted for the melanoma panel, NGS failed in 1 (5.6%) of 18 core biopsy specimens, 1 (6.3%) of 16 shave or punch biopsy specimens, and 0 of 2 open biopsy or endobronchial biopsy specimens. Successful NGS results were obtained from all 26 effusion specimens. Compared with resection specimens, biopsy/FNA specimens specifically showed a higher failure rate in lung cancer or melanoma panel submissions. In the lung cancer panel specimens, 18 (5.2%) of 343 biopsy/FNA specimens failed the NGS assay in contrast to none of 232 resection/excision specimens ($P < .001$). In the melanoma panel, 5 (6.6%) of 76 biopsy/FNA specimens failed the NGS assay in contrast to 1 of 87 (1.1%) resection/excision specimens ($P = .1$). There were higher proportions of biopsy/FNA specimens submitted for the lung cancer panel (343/604, 57%, $P < .001$) and the melanoma panel (76/163, 47%, $P < .001$) compared with the CRC panel (53/311, 17%).

Test Feasibility Assessment of Specimens Submitted for Lung Cancer, CRC and Melanoma Panels

Among 26 specimens cancelled for NGS assay due to insufficient tumor tissues, there were 9 in-house specimens and 17 referred specimens **Table 3**. The referred specimens showed a higher cancellation rate due to insufficient tumor tissues (5.6% vs 1.1%, $P < .001$), particularly in specimens submitted for the lung cancer panel (8.8% vs 1.5%, $P < .001$). A total of 2.9% of in-house specimens and 10% of referred specimens were cancelled or failed NGS assays (Table 2). The overall assay feasibility, defined as the proportion of specimens successfully tested by NGS assay among all submitted specimens, was 97.1% and 90% for in-house specimens and referred specimens, respectively. Assay feasibility was significantly higher for in-house specimens than for referred specimens, specifically for lung cancer panel submissions (96.6% vs 85%, or 3.4% vs 15% cancelled/failed rate, $P < .001$) (Table 2) and for FNA

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Table 2
In-house and Referred Specimens Submitted for Lung Cancer, Colorectal Cancer, and Melanoma Panels

	In-house Specimen		Referred Specimens		
	Total	Failed	Total	Failed	*P* Value
Lung cancer panel					
Resection	192	0	40	0	
Biopsy	131	5 (3.8%)	91	9 (9.9%)	
FNA	110	3 (2.7%)	11	1 (9.1%)	
Effusion	22	0	4	0	
Other	3	1 (33%)	0		
Total NGS assay	458	9 (2.0%)	146	10 (6.9%)	<.01
Total specimens[a]	465	16 (3.4%)	160	24 (15%)	<.001
Colorectal cancer panel					
Resection	205	2 (1.0%)	52	1 (1.9%)	
Biopsy	26	0	23	0	
FNA	4	0	0		
Effusion	0		0		
Other	1	0	0		
Total NGS assay	236	2 (0.8%)	75	1 (1.3%)	.56
Total specimens[a]	238	4 (1.7%)	76	2 (2.6%)	.63
Melanoma panel					
Resection	55	1 (1.8%)	32	0	
Biopsy	36	2 (5.6%)	35	3 (8.6%)	
FNA	5	0	0		
Effusion	0		0		
Other	0		0		
Total NGS assay	96	3 (3.1%)	67	3 (4.5%)	.69
Total specimens[a]	96	3 (3.1%)	69	5 (7.2%)	.28
Total NGS assay	790	14 (1.8%)	288	14 (4.9%)	<.01
Total specimens[a]	799	23 (2.9%)	305	31 (10%)	<.001

FNA, fine needle aspiration; NGS, next-generation sequencing.
[a]Twenty-six specimens cancelled due to insufficient tumor tissues were included here as failed specimens to assess overall assay feasibility.

Table 3
Specimens Cancelled for NGS Assays Due to Insufficient Tumor Tissues

	In-house Specimens	Referred Specimens	*P* Value
Lung cancer panel	(n = 465)	(n = 160)	
Resection	1[a]	0	
Biopsy	2	8	
FNA	4	6	
Total	7 (1.5%)	14 (8.8%)	<.001
Colorectal cancer panel	(n = 238)	(n = 76)	
Resection	0	1[b]	
Biopsy	2	0	
FNA	0	0	
Total	2 (0.8%)	1 (1.3%)	.57
Melanoma panel	(n = 96)	(n = 69)	
Resection	0	2[c]	
Biopsy	0	0	
FNA	0	0	
Total	0 (0%)	2 (2.9%)	.17
Total	9/799 (1.1%)	17/305 (5.6%)	<.001

FNA, fine needle aspiration; NGS, next-generation sequencing.
[a]Perivascular infiltration of tumor cells in a resection specimen of brain.
[b]No tumor cells were seen on the accompanied H&E slide of a resection specimen of colon.
[c]Microscopic residual and metastatic tumor cells were seen on the original H&E slides of a resected skin specimen and a resected lymph node specimen respectively, but not on the H&E slides prepared along with the unstained slides for DNA extraction.

specimens (94.3% v. 58.8%, $P < .001$) **Table 4**. For in-house specimens, assay feasibility was 99.1% for resection specimens compared with 94.4% and 94.3% for biopsy and FNA specimens, respectively ($P < .001$ and $P = .002$) (Table 4). For referred specimens, assay feasibility was 96.9% for resection specimens compared with 87.3% and 58.8% for biopsy and FNA specimens, respectively ($P = .002$ and $P < .001$) (Table 4).

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Table 4
Assay Feasibility of In-house and Referred Resection, Biopsy, or FNA Specimens Submitted for Lung Cancer, Colorectal Cancer, and Melanoma Panels[a]

	In-house	Referred	*P* Value
Resection	449/453 (99.1%)	123/127 (96.9%)	.07
Biopsy	186/197 (94.4%)	137/157 (87.3%)	.02
FNA	116/123 (94.3%)	10/17 (58.8%)	<.001

FNA, fine needle aspiration.
[a]Both specimens cancelled due to insufficient tumor tissues and specimens that failed next-generation sequencing assay were counted as failure.

Discussion

In this retrospective analysis for quality assessment of clinical mutation detection in FFPE neoplastic tissues, NGS assays were successfully conducted and reported in more than 95% of specimens. Only 3.4% of specimens were reported as "no results." A higher failure rate was observed in bone (vs nonbone) specimens, biopsy/FNA (vs resection) specimens, referred (vs in-house) specimens, and lung cancer and melanoma (vs CRC) panel submitted specimens. The higher failure rate of bone specimens is associated with the decalcification process, which degrades nucleic acids (data not shown). The higher failure rate of biopsy/FNA specimens is most likely due to limited tissues submitted for examination. The higher rate of failure in lung cancer and melanoma panel submissions was partially explained by their higher proportion of biopsy/FNA specimens with an inherently higher overall failure rate.

Surgical pathologists play an important and multifaceted role in the molecular diagnosis of solid tumors. Besides making histologic diagnoses, they request mandatory molecular tests, select appropriate neoplastic tissues, designate areas for DNA extraction, estimate tumor cell percentage to ensure conformity within the analytic sensitivity of a requested assay, and integrate test results into pathology reports. Thus, it should be considered a pathologist's responsibility to be intimately involved with and guide tissue processing so that adequate quantity and quality of DNA can be recovered from tissue blocks.

A variety of tissue-processing protocols have been developed in histopathology laboratories to ensure adequate histomorphology and immunohistochemistry stains over the past few decades. Recent advances of molecular technology have led to a revolution in genome discovery and rapid expansion of targeted therapeutics.[14] Detection of mutations in pathology tissue blocks has become a prerequisite to selection of patients who may benefit from targeted therapeutics. Therefore, revision and standardization of tissue processing protocols aimed at preserving not only histomorphology but also quality of nucleic acids is crucial in the era of personalized cancer medicine. Several studies have shown that DNA may degrade during the process of fixation, paraffin embedding and storage.[17,18] Fixatives containing acids or heavy metals are notorious for their damage of nucleic acids and inhibition of PCR.[15,16]

A guideline for tissue processing of lung cancer specimens has been proposed by the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology.[26] They recommend that surgical pathology specimens or cell pellet specimens from FNA or effusion should be fixed in 10% neutral-buffered formalin for 6 to 48 hours before processing. Since longer duration of fixations may adversely affect the quality of nucleic acids,[15] 6 to 12 hours of fixation for small biopsy samples may give the best results according to the guideline proposed for tissue processing of lung cancer specimens.[26] In our retrospective analysis, in-house specimens experienced a significantly higher rate of success in the NGS assay than did referred specimens, presumably due in part to a standardized tissue processing protocol applied to in-house specimens.

Communication between oncologists, pathologists, histopathology laboratories, and molecular diagnostics laboratories is also critical, and establishing an optimal tissue processing workflow for molecular testing requires multidisciplinary collaboration to implement the necessary technical steps. In this study, a higher incidence of cancellation was seen in referred specimens, especially biopsy and FNA specimens. Assay feasibility might be improved in this setting by limiting block trimming when preparing slides and by avoiding extensive immunohistochemical workups to preserve sufficient tissues for molecular diagnosis.

A variety of traditional "one test-one drug" assays have been validated for clinical mutation detection of solid tumors. These include Sanger sequencing, pyrosequencing, real-time PCR-based assays, allele-specific PCR, and high-resolution melting analysis and primer extension-based assays, among others.[13,27-30] In the clinical diagnostics setting, specimens from unselected patients often have a low tumor cellularity and therefore require assays with high analytic sensitivity to avoid false-negative results.[11,13,24] This is particularly true if assays with lower analytic sensitivity, such as Sanger sequencing, are used. Other one test-one

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drug assays, though showing an analytic sensitivity of 5% mutant alleles or less, are only capable of targeting one or a few specific mutations per reaction. Thus, comprehensive mutation profiling may not be possible in small biopsy specimens or FNA specimens using these assays. We and others have demonstrated a high analytic sensitivity and a broad reportable range with NGS assays in clinical diagnostic laboratories.[11,31] More importantly, NGS has the capability to provide comprehensive mutational profiling using 10 to 30 ng of DNA extracted from small biopsy specimens or FNA specimens,[19-21] which accounted for 44% of specimens performed for the NGS assay and 57% of specimens performed for the lung cancer panel.

In this retrospective analysis for quality assessment, we demonstrated the clinical utility of NGS assays in the mutation detection of solid tumors, including small biopsy specimens and FNA specimens. We also identified preanalytic factors that potentially contribute to failure of the NGS assay and emphasized the crucial role of surgical pathologists in tissue processing within pathology laboratories for molecular diagnosis of cancers. However, this study has inherent weakness due to its retrospective nature, including potential selection bias of the referred specimens. Further prospective analyses are needed to test the effects of preanalytic variables on the analytic performance characteristics of NGS assays.

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