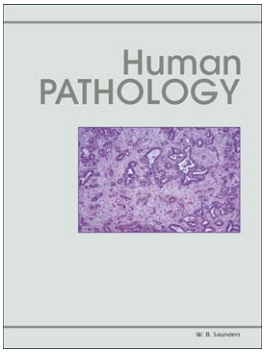

A Single EBUS-TBNA Procedure Can Support a Large Panel of Immunohistochemical Stains, Specific Diagnostic Subtyping, and Multiple Gene Analyses in the Majority of Non-Small Cell Lung Cancer Cases

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A Single EBUS-TBNA Procedure Can Support a Large Panel of Immunohistochemical Stains, Specific Diagnostic Subtyping, and Multiple Gene Analyses in the Majority of Non-Small Cell Lung Cancer Cases

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Abstract

Targeted therapies for pulmonary adenocarcinoma (ACA) necessitate specific subtyping and molecular testing of non-small cell lung carcinomas (NSCLC). However, endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has decreased the tissue available for these assessments. While EBUS-TBNA specimens have previously been reported to, successfully subtype NSCLC, allow immunohistochemistry (IHC), and support molecular diagnostics, no studies have documented the extent to which all objectives are possible in a single sample. Of 107 consecutive EBUS-TBNA specimens that were eligible for molecular testing, 98.8% had enough tissue for IHC, 80.2
% received a definitive subtype, and 71.0% had both sufficient tissue to attempt molecular testing and technical success on multigene next generation sequencing (NGS) and ALK fluorescence in situ hybridization (FISH) assays. Both subtyping and molecular diagnostics were possible in 57.9% of patients. The mean number of immunostains performed did not differ between patients with or without successful molecular testing (4.4 vs. 4.6, $p = 0.88$). Only 40% of patients with insufficient tissue underwent repeat sampling. These findings indicate that a majority of EBUS-TBNA specimens provide sufficient tissue for subtyping pulmonary NSCLC, performing IHC, and completing multiple gene analyses. Although priorities must be assessed for each case individually, performance of IHC does not detract from completion of molecular diagnostics in general. Because most patients never undergo repeat sampling, the tissue yield of EBUS-TBNA should be improved to maximize evaluation for targeted therapies.

Keywords

Cytology; EBUS-TBNA; Non-Small Cell Lung Cancer; Immunohistochemistry; Molecular Diagnostics; Adequacy

Introduction

While separating small-cell lung carcinoma (SCLC) from non-small-cell lung carcinoma (NSCLC) was once the only clinically relevant distinction, distinguishing different NSCLC subtypes now has equal therapeutic significance. In adenocarcinoma (ACA), targeted therapies including gefitinib and erlotinib for EGFR mutations[1, 2] and crizotinib for ALK gene fusions[3] can dramatically prolong survival, making molecular testing imperative. Conversely, in squamous cell carcinoma (SqCC), pemetrexed can decrease survival[4] and bevacizumab can precipitate pulmonary hemorrhage[5], contraindicating these treatments. Because of this differential efficacy and toxicity, the 2011 International Association for the Study of Lung Cancer/ American Thoracic Society/ European Respiratory Society Multidisciplinary Classification of Lung Adenocarcinoma mandated that pathologists subtype NSCLC and perform molecular diagnostics whenever feasible[6]. Consequently, tissue samples must often support both immunohistochemical stains and molecular genetic analysis for ACA[7].

But as evolving expectations for lung cancer diagnosis have increased demands on tissue, changing diagnostic procedures have decreased sample sizes. Because surgery offers no benefit in advanced lung carcinoma, open or video-assisted biopsies have fallen out of favor for the 60 to 70% of patients who present with high stage tumors[8-10]. Instead, endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), a minimally invasive procedure that obtains small cytologic samples, has become the standard of care for diagnosis and staging. EBUS-TBNA has a sensitivity of 80-94% for identifying tumor [11-17], is associated with fewer complications than percutaneous biopsy or mediastinoscopy [15, 18], and is the most cost-effective initial diagnostic approach available [19]. However, the real test for EBUS-TBNA is its ability to provide enough tissue for a complete evaluation despite its limited sample size. EBUS-

TBNA specimens are reported to have sufficient tissue available for IHC studies in 96%-97.5% cases and to allow specific subtyping of NSCLC in 77 to 79.5% of cases[15, 20]. Additionally, EBUS has demonstrated analytic success in 88.8%-98.7% of samples submitted for molecular testing[9, 15, 18, 20, 21], although only 60.1%-77.7% have adequate tissue to attempt such testing[22-24].

Despite these promising findings, one question that is not yet resolved is whether EBUS-TBNA can reliably support a full IHC complement and expanded molecular diagnostic panel in a single procedure without repeat sampling or more invasive intervention. Although a few investigators have reported that EBUS-TBNA specimens can simultaneously subtype NSCLC and evaluate for several targetable mutations[15, 20], no studies have fully documented the extent to which each step is possible in a single sample. Moreover, although many authors have advocated for moderation in IHC to preserve specimens for molecular testing[7, 25], no studies have formally evaluated the impact of IHC on subsequent molecular analysis. In this study, we retrospectively evaluated a consecutive series of malignant EBUS-TBNA specimens from a high volume academic institution to more comprehensively determine the limits of tissue availability from EBUS-TBNA in routine clinical practice.

Materials and Methods

The Institutional Review Board at The Johns Hopkins Hospital granted approval for this study. We searched the cytopathology archives for all consecutive EBUS-TBNA specimens with malignant diagnoses between February 1st, 2013 and May 30th, 2014. Figure 1 illustrates our case selection algorithm. Overall, we identified 253 malignant EBUS-TBNA specimens during the fifteen-month study period. From this group we selected all 153 specimens considered

eligible for molecular testing at our institution, including (1) cases with diagnoses of ACA, (2) cases with diagnoses of NSCLC- not otherwise specified (NSCLC-NOS) or (3) cases where clinicians requested molecular testing, regardless of diagnosis. Of these, we included only the 107 specimens that had adequacy assessed for molecular testing in our final analysis. For each case, we retrospectively reviewed patient demographics, procedure details, IHC results, molecular adequacy assessment, molecular testing outcomes, and associated pathologic diagnoses. Statistical analysis, including Fisher Exact Tests and 2-tailed Student t-tests were performed using R (R Foundation, Vienna, Austria).

At the time of original tissue sampling, experienced interventional pulmonologists from a high-volume practice performed or directly supervised all EBUS-TBNA procedures as described elsewhere[26, 27]. In brief, lung tumors or mediastinal and hilar lymph nodes were identified under ultrasound guidance using an Olympus BF UC 160F/BF UC 180F 7.5 MHz convex-probe, linear-array, puncture EBUS bronchoscope, and sampling to obtain cytologic material was performed using an Olympus Vizishot 22-guage needle. Aspirated material was smeared, air-dried, and immediately stained using the Diff-Quik method, and rapid on-site evaluation (ROSE) for adequacy was performed by an experienced cytotechnologist. Additional material was fixed in 100% ethanol for Papanicolaou staining in the cytopreparatory laboratory. If feasible, dedicated passes were performed to obtain material for a cell block; otherwise, the needle rinse was used to make a cell block. This material was centrifuged, formalin fixed and processed histologically to prepare one hematoxylin and eosin (H&E) slide.

At the time of diagnosis, all smears and cell blocks were screened by an experienced cytotechnologist prior to review by a primary board-certified cytopathologist. IHC was performed on cell blocks in a subset of cases at the discretion of that primary pathologist. When

the initial H&E slide was cut, 7 subsequent unstained sections were also cut from the cell block at 5-micron thickness for potential IHC; additional sections were later cut if >7 stains were ordered. Unstained slides were deparaffinized and subjected to antigen retrieval using 10 mM citrate buffer (92°C for 30 minutes). IHC was performed using a wide range of commercially available antibodies on Ventana Benchmark Ultra, Ventana Benchmark XT (Ventana Medical Systems, Inc. Tucson, AZ) Leica BOND-MAX and Leica III (Leica Biosystems, Buffalo Grove, IL) automated staining systems as designated with corresponding Ventana UltraView (Ventana) and Leica BOND-MAX (Leica) detection kits. All staining was performed according to manufacturers' instructions in the presence of appropriate controls.

In these cases, the primary pathologist assessed adequacy for molecular testing on original H&E sections using a standard of approximately 200 viable tumor cells. Molecular testing was not attempted on cases that did not meet this minimum threshold. If sufficient material was available, molecular testing was performed for a gene panel including *AKT, BRAF, EGFR, ERBB2, KRAS, NRAS,* and *PIK3CA*. Tumor was macro-dissected from 3-10 unstained slides cut at 5-10 micron thickness using PCR precautions. DNA was isolated using the Pinpoint DNA Isolation System (Zymo Research, Irvine, CA) and purified via the QIAamp DNA Mini Kit (Qiagen, Valencia, CA)[28]. Next generation sequencing (NGS) was performed using the AmpliSeq Cancer Hotspot Panel (v2) as described previously [29]. The Ion AmpliSeq Library Kit 2.0 was used 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 (PGM) for sequencing (Life Technologies, Carlsbad, California). All protocols were executed per manufacturers' instructions. Sequencing data of targeted genes were 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 Viewer (IGV) (http://www.broadinstitute.org/igv/).

If *EGFR* and *KRAS* testing were negative, fluorescent in situ hybridization (FISH) for ALK translocation was also performed on a subset of cases. ALK FISH was performed using a Vysis ALK Break Apart FISH probe (Abbott Molecular, Des Plaines, IL) following manufacturer's instructions. Tumor was indicated by a pathologist and signals were enumerated manually from the corresponding area. Two scorers analyzed a total of 50 nuclei following fluorescence in situ hybridization (FISH) with a dual color probe set for a locus on 2p23. For 50 nuclei, separation of the 5'ALK and 3'ALK signals (showing a red and green fused signal separated into individual red and green signals) in less than 10% of cells was considered negative and in more than 50% was positive. If results fell between 10% and 50%, an additional 50 cells were scored, and a total of ≥ 15% cells with separation were considered positive.

Results

Among 253 malignant lung tumors identified during the study period, there were 107 cases that were both eligible for molecular analysis and assessed for adequacy for molecular testing. These 107 cases represented 103 unique patients, including 54 males and 49 females with a mean age of 63 (range 36-87). 84 (78.5%) patients received their initial diagnosis during the index EBUS procedure, while 23 (21.5%) had a previous lung carcinoma diagnosis. Among patients with such history, 9 (39.1%) had molecular testing performed at a remote time (5 cases) or different site (4 cases) and 9 (39.1%) underwent repeat sampling because of inadequate tissue on earlier samples, including 4 EBUS-TBNA specimens, 4 other FNA specimens, and 1 core

biopsy. The EBUS-TBNA specimens represented both lung masses (21 cases, 19.6%) and hilar or mediastinal lymph nodes (86 cases, 80.4%). When ROSE was performed on corresponding smears, 103 (96.3%) were assessed as adequate and 4 (3.7%) as less than optimal. A mean of 4.4 dedicated passes were made to collect material for a cell block (range 2-12); no cases had accompanying core biopsies. Figure 2 summarizes outcomes for the 107 EBUS-TBNA specimens at each stage of evaluation.

Immunohistochemical staining results

IHC was performed in 80 cases (74.8%) overall. Sufficient tissue was available to perform immunohistochemical studies in 80 of the 81 cases (98.8%) where it was deemed necessary. A total of 50 unique immunostains were utilized in this cohort, the most common of which included TTF-1 (72%), Napsin-A (60%), and P40 (42%) (Table 1). Cases that underwent IHC had a mean of 4.5 stains performed (range 1-18). Overall, 86 cases (80.2%) received definitive subtyping. Of these, no IHC was necessary for definitive diagnosis in 26 cases (24.3%), while IHC supported the morphologic diagnosis in 60 cases (51.3%). The remaining NSCLC-NOS cases included 20 cases (18.7%) with either non-definitive or conflicting morphology and IHC profiles and 1 case (0.9%) with insufficient tissue for IHC. NSCLC-NOS cases had a significantly higher rate of IHC usage compared to subtyped tumors (95.2% vs. 69.4%, p=0.01.) as well as a significantly larger mean number of stains performed per case (3.8 vs 6.5, p=0.03).

Molecular Study Results

Sufficient tissue was available to attempt molecular testing in 82 specimens (76.6%). A similar proportion of NSCLC-NOS and subtyped NSCLC (71.4% vs. 77.6%, p=0.57) were assessed as adequate for molecular diagnostics. Of these 82 adequate cases, the NGS panel was

successfully performed in 81 specimens (98.8%), although 4 assays (4.9%) were resulted with a tumor cell percentage less than the 20% limit of detection. The single NGS failure was due to failure of the gene to amplify. ALK FISH was performed in 38 of 43 cases attempted (88.4%). All ALK failures involved an absence of scorable signals. Overall, 76 specimens (71.0%) had both adequate tissue and successful completion of all indicated molecular testing, including all 9 cases (100%) with previous inadequate samples. A mean of 6.6 mutations were evaluated per case (range 1-8). In total, there were 53 mutations identified from 51 patients (62.2%), including 2 patients with 2 mutations each. The most common mutations were *KRAS* (22 cases), *EGFR* (19 cases), and *ALK* (5 cases) (Table 2).

Strikingly, the number of immunohistochemical stains performed did not appear to influence the ability to subsequently perform molecular studies on EBUS-TBNA specimens. IHC was utilized in a higher proportion of cases that were adequate for molecular analysis than those with insufficient tissue (78.0% vs.64.0%, p=0.19), although the difference was not significant. This discrepancy likely reflected a subset of cases with no material in the cell block for either IHC or molecular. Moreover, the mean number of stains completed in cases where IHC was performed did not significantly differ between cases with or without adequate tissue for molecular studies (4.4 vs. 4.6, p = 0.88). Likewise, the rate of tissue adequacy was similar in the subset of cases that had a limited panel of ≤4 immunohistochemical stains compared to those with ≥5 stains (76.4% vs. 77.2%, p=1). Even between specimens that had ≤7 or >7 immunostains performed, the latter requiring trimming the block to cut additional levels, there was not a significantly different rate of tissue availability for molecular studies (72.7% vs. 77.1%, p=0.72). No procedural variables, including tumor site, results of ROSE, and number of dedicated passes for cell block material contributed to adequacy for molecular testing.

Overall, 62 specimens (57.9%) in the final study group received a definitive diagnosis and successfully underwent all indicated molecular testing, while just 7 specimens (6.5%) failed both subtyping and molecular diagnostics. Of the 21 NSCLC-NOS, 6 (28.6%) underwent subsequent tissue sampling that allowed re-evaluation, including 4 excisional biopsies of more accessible distant metastasis, 1 thoracocentesis, and 1 repeat EBUS-TBNA. More definitive NSCLC subtyping was possible in 3 of these additional specimens (14.3%). Among the 25 specimens assessed as insufficient for molecular testing, 10 patients (40%) had repeat tissue sampling, including 4 repeat EBUS-TBNA, 3 core biopsies, 1 other FNA, and 2 surgical resections. The remaining 15 patients (60%) did not undergo additional procedures to obtain material for molecular analysis. Similarly, of the 6 patients with technical failure on molecular diagnostics, only 1 (16.6%) had a core biopsy for repeat testing; 5 (83.3%) did not undergo additional sampling. Eight of the 11 repeat molecular analyses (72.7%) identified mutations.

Discussion

The advent of targeted therapies has revolutionized the management of pulmonary ACA and, with it, recommendations for pathologic diagnosis of lung cancer. Pathologists are now expected to subtype NSCLC whenever possible and order molecular testing in all cases of ACA and most cases of NSCLC-NOS. But because surgical biopsies have fallen out of favor for patients who will not benefit from resection, this immunohistochemical and molecular workup often must be performed on tiny pieces of tissue. EBUS-TBNA is a minimally invasive procedure that has shown considerable promise for subtyping NSCLC and collecting tissue for molecular diagnostics. However, no studies have exhaustively evaluated whether EBUS-TBNA samples collected in routine clinical practice can support all indicated testing without the need

for repeat sampling. In this study we retrospectively evaluated the ability of EBUS-TBNA to facilitate comprehensive IHC and an expanded molecular diagnostic panel in a single procedure.

This study demonstrates that a majority of EBUS-TBNA specimens can successfully support subtyping of NSCLC, IHC, and all indicated molecular testing. In this series, 80.2% of NSCLCs were definitively subtyped. This high success rate was supported by the ability of EBUS-TBNA to provide sufficient material in 98.8% of cases where IHC was indicated. Indeed, IHC was performed in larger numbers and at higher frequency in NSCLC-NOS compared to subtyped NSCLC. As has been discussed in other cytologic specimens[8, 10, 30], such findings suggest a key limitation of subtyping in EBUS-TBNA is not a lack of stainable material but rather the difficulty of interpreting IHC patterns in small samples without supportive morphology. Furthermore, out of 107 cases assessed for molecular diagnostics, 71.0% had both enough material to warrant testing and technical success on indicated NGS sequencing and ALK FISH assays. This composite value underscores that success in molecular testing requires both sufficient tissue and technically adequate assays- a biphasic threshold that is not always emphasized. In contrast to the interpretive difficulties described above, molecular testing in EBUS-TBNA seems more limited by the absolute amount of tissue present. Overall, both subtyping and molecular diagnostics were possible in 57.9% of patients

Unexpectedly, this study also demonstrated that performing IHC did not significantly diminish the ability to perform molecular studies on cell block material. Many authors have recommended limiting IHC to conserve tissue for molecular diagnostics,[7, 25, 31] but there has been little published evidence that success of these ancillary studies is inversely related. And indeed, in this study, the rate of tissue availability for molecular testing was similar in cases with and without extensive IHC. Our institution uses a limited initial panel of TTF-1, Napsin- A, and

P40 to subtype most NSCLCs. Although our laboratory's practice of cutting 7 unstained slides for potential IHC alongside the initial H&E may have facilitated this outcome, there was not a significant decrease in molecular adequacy even when >7 stains were performed. Certainly, we recommend parsimonious utilization of IHC, both because of the high cost and limited utility of excessive staining[32, 33]. Furthermore, testing precedence must be measured against tissue abundance and clinical variables on a case-by-case basis, with prioritization of molecular diagnostics in any specimen where a tissue shortage is possible. However, these findings should reassure pathologists that, when indicated, a few extra immunostains do not negate the possibility of molecular testing

Nevertheless, this study underscores the importance of being able to collect enough material to perform all testing in a single procedure. 60% of patients who had insufficient material for molecular testing in our study did not undergo follow-up procedures to allow molecular diagnostics. Similarly, 83% of patients with technically unsuccessful NGS or ALK FISH assays never underwent tissue sampling to repeat the missing test. While repeat EBUS-TBNA procedures demonstrated 100% success in molecular studies when performed, most patients simply did not undergo additional sampling. These findings highlight a paradox- although the minimally invasive nature of EBUS-TBNA makes it an ideal diagnostic modality for patients with advanced disease, its failure rate for molecular testing may sacrifice the only opportunity to assess medically complex patients for targeted therapies. Despite the overall satisfactory ability to perform molecular testing in EBUS-TBNA specimens, the lack of follow up described here emphasizes the need to further improve tissue yield.

Finally, this study has two main limitations. First, it is unclear how generalizable our results are to other practice settings. Diagnostic yield in EBUS-TBNA has been shown to have

significant heterogeneity between hospitals and a strong association with hospital volume[34]; it is reasonable to expect that rates of successful IHC, subtyping and molecular adequacy might similarly vary. It may be difficult to reproduce our results in centers with a lower volume of EBUS specimens, less experienced interventional pulmonologists, or histology laboratories with less effective procedures for conserving tissue on tiny cell block specimens. Additionally, this study may have been hindered by its relatively small size and inclusion of only malignant sample. Previous studies have highlighted a greater role for ROSE in augmenting tissue yield[14] and better results with higher numbers of passes.[27] Such effects may be better appreciated with more samples and a broader range of diagnoses.

Ultimately, this study demonstrates that EBUS-TBNA does have the potential to accurately subtype NSCLC and perform all indicated molecular testing without repeat sampling or more invasive interventions. In a majority of cases, EBUS-TBNA specimens can produce (1) an H&E cell block section, (2) sufficient immunostains to support subtyping, (3) an expanded NGS molecular panel, and (4) ALK FISH if indicated. Moreover, even if one objective is not completed, almost all cases achieve either specific subtyping or successful molecular diagnostics. This study also suggests that use of IHC does not inherently diminish the ability to perform molecular diagnostics in EBUS-TBNA samples. Regardless, while repeat EBUS-TBNA showed uniform success in molecular testing, the low rate at which such follow-up sampling occurred underscores the need to further improve tissue yield on the first procedure. EBUS-TBNA continues to show promise as a non-invasive technique that can satisfy the many tissue demands of lung cancer diagnosis.

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Figure 1: Out of an initial group of 253 consecutive EBUS specimens with a malignant diagnosis, 153 specimens were selected that were eligible for molecular testing. A further 46 specimens that did not have adequacy assessed were excluded, leaving 107 specimens to be included in the final analysis.

Figure 2: Outcomes of each step in the diagnostic process for EBUS-TBNA specimens, including the cytomorphologic assessment, the performance and success of immunohistochemistry, the ability to assign a definitive subtype, the adequacy for molecular analysis, and the success of attempted molecular studies.

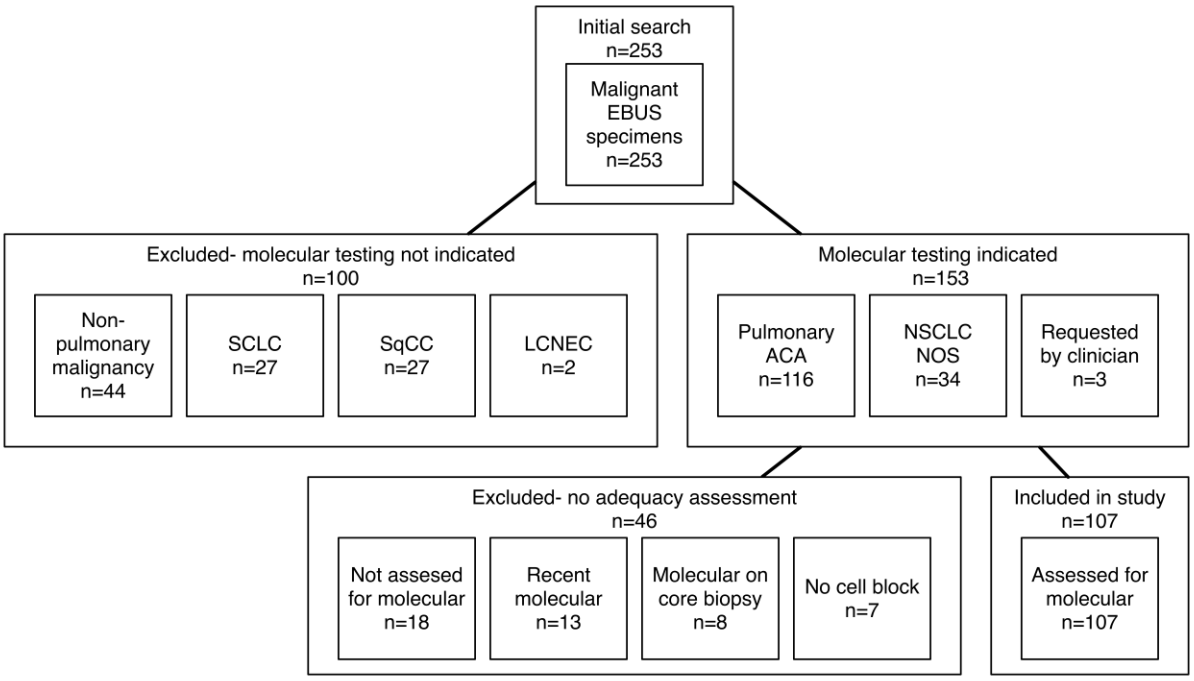

Fig. 1

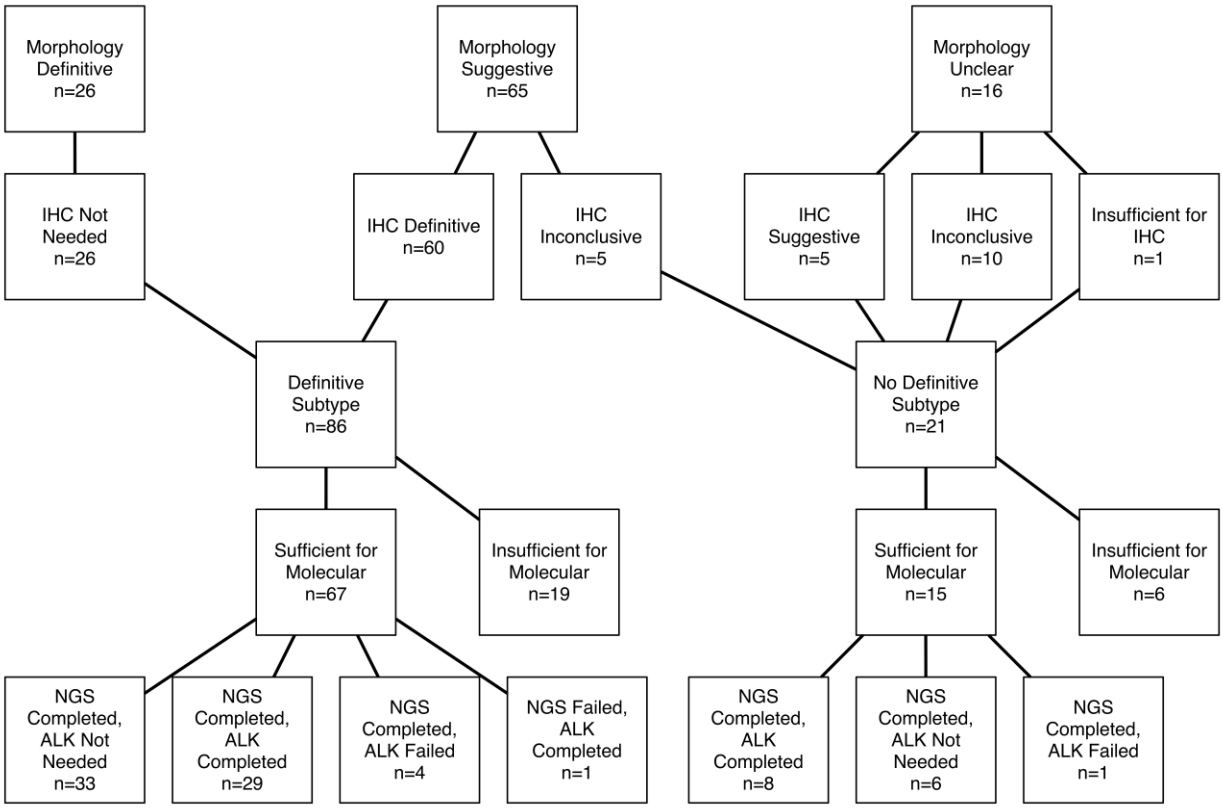

Fig. 2

Table 2: Molecular Testing Results

Mutation	n	%
KRAS	22	26.80%
EGFR	19	23.20%
ALK	5	6.10%
BRAF	3	3.70%
PIK3CA	3	3.70%
NRAS	1	1.20%
AKT	0	0.00%
ERBB2	0	0.00%
None detected	31	37.80%

Table 1: Most Common Stains Performed

TTF-1	77
Napsin A	64
p40	45
CK7	21
CK20	20
Mucicarmine	14
AE1/AE3	9
GATA3	8
Chromogranin	7
Synaptophysin	7
CDX2	6