ORIGINAL ARTICLE

Next-Generation Sequencing Analysis on Image-Guided Biopsy Samples in Early-Stage Lung Cancer: Feasibility Study and Comparison With Surgical Samples

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ABSTRACT

Introduction: Limited information exists on next-generation sequencing (NGS) success for lung tumors of 30 mm or less. We aimed to compare NGS success rates across biopsy techniques for these tumors, assess DNA sequencing quality, and verify reliability against surgical resection results.

Methods: We used data from the Initiative for Early Lung Cancer Research on Treatment study, including patients with lung tumors measuring 30 mm or less who had surgery and NGS on biopsies since 2016. We collected data on biopsy type, nodule characteristics, complications, sequencing feasibility, clinical actionable variants, surgery type, and TNM classification. We compared NGS feasibility and quality between biopsy methods and, for those with NGS on surgical samples, compared feasibility, quality, and detection of actionable variants.

Results: Among the 654 participants with lung tumors of 30 mm or less who underwent surgery, 70 had NGS on prior biopsies. The median age was 68.5; 51.4% were male individuals, and 75.7% were smokers. The mean diameter of biopsied nodules was 17.7 mm, with 67.1% fine-needle aspiration, 17.1% computed tomography–guided transthoracic core needle biopsies, and 17.1% endobronchial ultrasound–guided transbronchial needle aspiration. DNA sequencing was feasible in 97.1% of biopsy samples; 2.9% had low tumor cellularity. Coverage depth was achieved in 89.7% of biopsies. RNA sequencing was successful in 66.2% of biopsies, especially in core needle biopsies. Actionable alterations were found in 41.4% of patients. Among the participants, 30% had NGS on surgical samples. RNA sequencing was more feasible on surgical samples (95.2% versus 42.9% for biopsies). NGS on surgical samples matched biopsy results in 90% of patients, with 10% showing additional alterations.

Conclusion: DNA sequencing succeeded in 97.1% of biopsies of nodules 30 mm or less, whereas RNA sequencing feasibility was lower. NGS on biopsy samples is generally reliable but requires careful review.

Keywords: NGS feasibility; Early-stage lung cancer; Stage I; Fine needle aspiration; Core needle biopsy; EBUS-TBNA

Introduction

Low-dose computed tomography (CT) lung cancer screening programs can detect at least 80% of lung cancers at stage I, with a long-term survival rate of at least 80%.[1–3] With the emergence of precise adjuvant treatments, we are transitioning into the molecular era for

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distinguishing early-stage NSCLC.[4,5] The first step of early-stage lung cancer diagnosis involves identifying a suspicious nodule on imaging, followed by a biopsy whose primary aim is to confirm malignancy.[6] The collected sample can also be utilized for genomic analyses.

Next-generation sequencing (NGS) of tumor cell-derived DNA and RNA is the preferred choice, with targeted NGS panels covering generally recommended specific gene sets.[7] DNA sequencing serves as the standard method for identifying the most actionable mutation, whereas RNA sequencing is emerging as the standard for detecting fusion genes.[2,8] Samples should ideally possess a neoplastic proportion of 20% or higher, although successful clinical genotyping can be achieved with tumor proportions as low as 5%.[2,9] RNA quality is more likely to be compromised, especially in routine formalin-fixed, paraffin-embedded samples, compared to DNA.[10]

High-throughput NGS is performed to examine multiple genes at once and involves several major steps in sequencing: DNA and RNA fragmentation, library preparation, massively parallel sequencing, bioinformatics analysis, and variant/mutation annotation and interpretation, ending ultimately with a report that clinicians will then use to treat patients. Most NGS protocols start with a random fragmentation of the genome into short fragments, which are then sequenced and aligned.[11] This alignment process extends contiguous sequences by tiling short sequences, necessitating overlaps between different reads to ensure confident alignment. Consequently, higher sequencing depth yields more significant overlaps and results in more robust outcomes.[12] This is especially crucial for varied samples like tumor specimens, as higher coverage allows for variant detection even when the variant is present in only a small fraction of the cells.[13]

Robust genetic testing is vital for identifying or ruling out pathogenic variants, depending on the performance of NGS assays and analytical tools.[14] Quality management of NGS pipelines is crucial, ensuring reports include clear data on test performance and limitations for clinical decisions.[15] Although best practice, specific guidelines for achieving this are lacking, and most test reports provide limited information. Primary metrics for evaluating sequence quality include depth of coverage, base quality, and mapping quality.[16] Insufficient coverage depth often leads to false negatives.[12]

Different methods exist for the image-guided biopsy of lung nodules, including, but not limited to, surgical biopsies, percutaneous CT–guided transthoracic core needle biopsies (CNBs), percutaneous fine-needle aspiration (FNA) and endobronchial ultrasound–guided transbronchial needle aspiration (EBUS-TBNA). The choice of biopsy technique depends on patient characteristics and the size and location of the nodule. The availability of specialists to perform the procedure is also a crucial factor.

The feasibility of performing biopsies on lung tumors is well established, but there is limited information regarding the success of NGS on biopsies on tumors measuring 30 mm or less. A key challenge in this context is whether DNA and RNA of sufficient quality can be extracted from these minimally invasive samples to allow for high-quality sequencing. Successful NGS analysis may be the first crucial step in the introduction of effective treatment.

We aim to compare the success rates of DNA and RNA sequencing across different image-guided biopsy techniques and radiological characteristics, assess DNA sequencing quality, and verify reliability by comparing with results from surgical resections in early-stage NSCLC.

We aimed to explore this in a real-world context using data from the Initiative for Early Lung Cancer Research on Treatment program (IELCART), a prospectively collected cohort dedicated to enhancing treatment for stage I lung cancer.[17]

Methods

Eligible patients were adults included from IELCART in the Mount Sinai Health System since its start in 2016, who underwent surgery for first primary lung tumors of 30 mm or less and had previous NGS analyses on biopsies. There was no fixed time limit between the biopsy and the surgery.

All participants had signed a Health Insurance Portability and Accountability Act–compliant, institutional review board–approved consent for enrollment in IELCART, a cohort undergoing treatment for lung cancer, in North America. The study was conducted according to the ethical principles of the Declaration of Helsinki and the Good Clinical Practice guidelines.

Each participant's demographics, smoking history, height, weight, body mass index, comorbidities of diabetes, chronic obstructive pulmonary disease or emphysema, hypertension, and cardiovascular diseases were self-reported and documented at the time of enrollment before treatment.

We described the characteristics of suspicious radiological nodules in the included patients, the type of image-guided biopsy (CNB, EBUS-TBNA, FNA), the feasibility of DNA and RNA sequencing, the clinically actionable variants identified, the type of surgery performed and the pathological TNM classification documented post-surgery.

Genomic Testing

Genomic testing was performed utilizing a commercially available NGS assay Sema4 tissue-based test.[18] The Sema4 hotspot panel encompasses 161 cancer genes, enabling the detection of structural and copy number

changes in addition to the hotspot and other coding/splice mutations. This NGS assay is designed to comprehensively capture point mutations and small insertions and deletions in all guideline lung cancer driver genes, ensuring complete coverage of all crucial exons. Targeted sequencing is an NGS method that identifies mutations in clinically significant markers by enriching specific regions of interest from the whole genome. In the test we used, quality metrics require that target regions (5827 in total) be sequenced to a minimum sequencing coverage depth of 200x. NGS sequencing depth impacts the reproducibility of variant detection: more aligned sequence reads increase the confidence in base calls at specific positions, whether matching the reference or mutated.[16] Essentially, individual sequencing errors become statistically insignificant when outnumbered by correct reads.[12] This assay is performed using DNA and RNA from tumor tissue only and detects somatic variants.

Variants in somatic conditions are categorized into four tiers on the basis of their clinical impact: tier I, variants with strong clinical significance (level A and B evidence); tier II, variants with potential clinical significance (level C or D evidence); tier III, variants with unknown clinical significance; and tier IV, variants that are benign or likely benign.[19] In this study, we focus only on variants from tier I and II, referred to as significant variants.

DNA and RNA sequencing were considered successful if the NGS assay was able to produce results, regardless of whether the results indicated a molecular alteration with predictive or prognostic value.

For our study, exploratory analysis of DNA sequencing meeting quality standards was on the basis of depth of coverage criteria ensuring that less than 2.5% of targeted regions did not reach 200x coverage. Samples with a higher number of regions that did not achieve the required sequencing depth were considered at risk for false negative results.

Tumor cellularity for each sample was evaluated by a pathologist on a representative slide and quantified by the ratio of tumor cells to non-tumor cells.

Biopsy Versus Surgery Samples

For patients who also underwent NGS analysis on surgical samples, we conducted a comparison of the feasibility of DNA, RNA, and sequencing depth and an empirical comparison between the detection of clinically actionable variants. The results were deemed concordant if they contained the same significant variants and discordant otherwise.

Immunohistochemical tests (TTF1, p40, Napsin A, and PDL1) performed on both samples were also compared.

The time between biopsy and surgery was noted.

Statistical Analysis

Continuous data are shown as means (SD) or medians (interquartile range) and categorical data as frequencies and percentages. The chi-square test or Fisher's exact test was used to evaluate differences among categorical variables between groups, and the Welch *t*-test or Wilcoxon Rank-Sum test was used for continuous variables. Statistical tests were conducted using Medistica. *p*value.io, a Graphic User Interface to the R statistical analysis software for scientific medical publications. Statistical significance was determined by a p-value threshold of less than 0.05.[20]

Results

Among the 654 participants in the IELCART study with lung cancer of 30 or less mm who underwent surgery, 70 participants (10.7%) had NGS performed on prior biopsy samples, and of these, 21 participants (30%) also had NGS performed on their surgical samples. Figure 1 shows the flowchart of patients included in the analysis. The median age was 68.5 years; almost an equal proportion of male and female participants (male individuals: 36/70, 51.4%) was reported, and nearly all were past or current smokers (53/70, 75.7%), with an average pack-year of 20.

The most prevalent comorbidity reported at the time of enrollment was hypertension (41/70, 58.6%), hypercholesterolemia (33/70, 47.1%), asthma (16/70, 22.9%), chronic obstructive pulmonary disease or emphysema (11/70, 15.7%) and diabetes (7/70, 10%). At baseline, 15/70 (21.4%) participants had a prior diagnosis of non-lung, non-skin cancer, including five with urologic cancer, three with gastrointestinal cancer, two with breast cancer, one with thyroid cancer, and four with hematologic malignancies. Meanwhile, 22/70 (31.4%) had a family history of lung cancer. The characteristics of the patients are detailed in Table 1.

Nodule Characteristics

The mean maximal diameter of the biopsied nodules was 17.7 mm (SD: 5.91) and the mean maximal diameter of the solid component was 15.5 mm (SD: 7.71). Out of these nodules, 59/70 (77.9%) were solid, 5/70 were part-solid (14.3%), and 6/70 were nonsolid (7.8%).

The biopsies were image-guided, with 46/70 (67.1%) FNA, 12/70 (17.1%) CNB, and 12/70 (17.1%) EBUS-TBNA.

The biopsies diagnosed 64/70 (92.2%) adenocarcinomas, 3/70 (3.9%) squamous carcinomas, and 3/70 (3.9%) cases of NSCLC not otherwise specified.

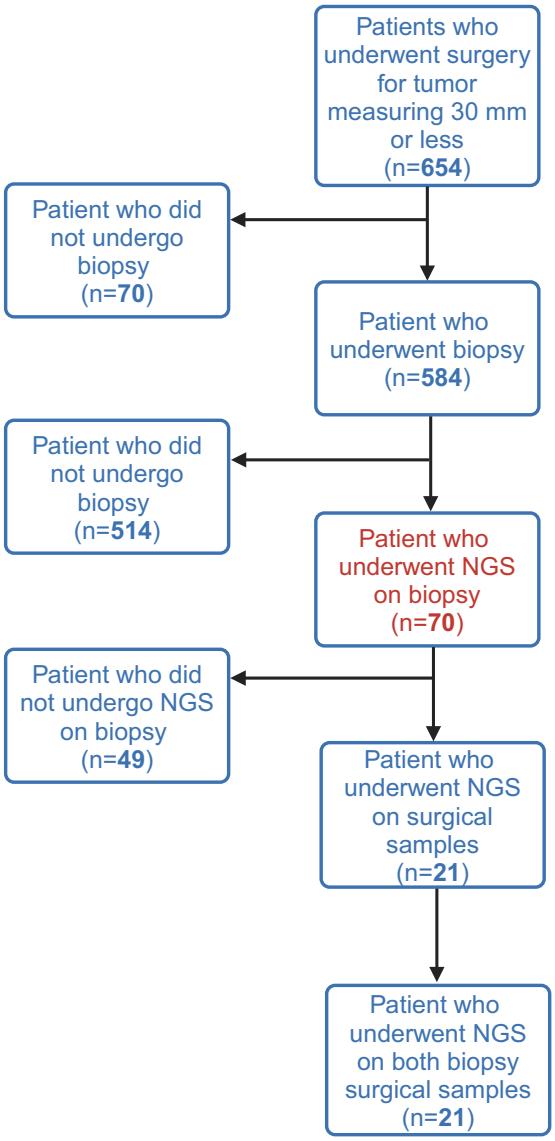

Figure 1. Flowchart of patients included in the analysis. Figure 1 illustrates patient inclusion in our study. Of the 654 IELCART patients with tumors of 30 mm or less who underwent biopsy, 584 had biopsies, and 70 had NGS performed. These patients were included in our study, with 21 also receiving NGS on their surgical specimens. NGS, next-generation sequencing.

None of the patients experienced serious complications after biopsies. Minor complications were reported, with no significant differences observed between biopsy techniques (11/47 (23.4%) for FNA, 5/12 (38.5%) for CNB, 2/12 (20%) for EBUS-TBNA; Fisher's exact test: $p = 0.41$). For both FNA and CNB, these complications consisted of small pneumothoraxes that did not require hospitalization or any other measures. For EBUS-TBNA, minor bleeding was reported in two patients.

Those patients later underwent surgery: 36 had a lobectomy, 32 had a wedge resection(s), and two had a segmentectomy.

Table 1. Demographic Characteristics, Biopsy Methods, Surgical Procedures, and TNM Staging

Patients Characteristics	All Participants n = 70 (%)
Age (y)	
Median (Q25-75)	68.5 (63.0-78.0)
Female	34 (51)
Male	36 (49)
Race	
African American	19 (27.1)
Asian	6 (8.6)
Other	7 (10)
White	38 (54.3)
Smoking history	
Past smokers	44 (62.9)
Current smokers	9 (12.9)
Never smokers	17 (24.3)
Packyears	
Mean (SD)	20.0 (20.6)
Asbestos exposure	14 (20)
Family history of lung cancer	22 (31.4)
Father	9 (12.9)
Mother	9 (12.9)
Sibling	5 (7.1)
Comorbidities	
BMI	
Mean (SD)	25.4 (6.11)
Diabetes	7 (10)
Asthma	16 (22.9)
Emphysema/COPD	11 (15.7)
Hypercholesterolemia	33 (47.1)
Hypertension	41 (58.6)
Myocardial infarct	4 (5.7)
Previous cancer (except skin)	15 (21.4)
Type of biopsy	
FNA	46 (65.7)
CNB	12 (17.1)
EBUS-TBNA	12 (17.1)
Surgical procedures	
Lobectomy	36 (51.4)
Wedge resection	32 (45.7)
Segmentectomy	2 (2.9)
Clinical TNM eighth edition	
Stage IA	
Tis N0 M0	7 (10)
T1a N0 M0	11 (15.7)
T1b N0 M0	32 (45.7)
T1c N0 M0	19 (27.1)
Stage IIIA	
T1a N2 M0	1 (1.4)

Numbers are self-reported and documented at the time of enrollment. Age reflects the age at the date of the biopsy. Race was reported by the participants. 'Other' includes participants who did not self-define or participants who reported that they were of more than one race. The same question and categories used to determine participants' demographics and co-existing conditions were used for all sites. Percentages for comorbidities were calculated on the basis of participants with available data. The clinical TNM is also reported, on the basis of the eighth edition.

BMI, body mass index; CNB, core needle biopsy; COPD, chronic obstructive pulmonary disease; EBUS-TBNA, endobronchial ultrasound-guided transbronchial fine needle aspiration; FNA, fine needle aspiration.

Characteristics of the nodules, biopsies, and surgeries are detailed in Table 1.

DNA Sequencing Feasibility and Quality Metrics (Depth of Coverage)

NGS was feasible on 68/70 (97.1%) biopsies samples. In two out of the 70 (2.9%) patients, tumor cellularity in biopsy samples—both on part-solid nodules—was too low to conduct the test. One of the samples came from an FNA, and the other from an EBUS-TBNA sample.

Of the remaining 68 samples, the desired pre-defined depth of coverage was successfully achieved in more than 97.5% of targeted regions in 61 samples (89.7%), including 41 of 45 (91.1 %) FNA samples, 10 out of 12 (83.3%) CNB samples, and 10 out of 11 (90.9%) EBUS-TBNA samples. There were no significant differences in success rates between different biopsy methods (Fisher's exact test: $p = 0.83$). Those results are detailed in Table 2.

The maximal diameter of the solid component of the nodule was slightly smaller in patients whose DNA sequencing did not meet the desired pre-defined depth of coverage in more than 2.5% of targeted regions although the difference was not significant (15.5 mm versus 14.7 mm, Welch Two Sample t test: $p = 0.81$). Nodule consistency did not significantly differ between the two groups; nevertheless, samples that did not achieve this quality metric had a higher proportion of part-solid and nonsolid nodules (28.6% versus 13.1%, Fisher's Exact Test: $p = 0.27$).

In addition, tumor cellularity was lower in samples where DNA did not reach the expected depth of coverage in more than 2.5% of targeted regions (48.1% versus 28.6%, Welch Two Sample t test: $p = 0.11$).

RNA Sequencing

In two out of 70 (2.9%) patients, tumor cellularity in biopsy samples—both on part-solid nodules—was too low to conduct the test. Among the remaining 68 samples, RNA sequencing was successfully conducted in 45 out of 68 (66.2%) biopsy samples, including 27 out of the 45 (60%) FNAs, 11 out of the 12 (91.7%) CNBs, and seven out of the 11 (63.6%) EBUS-TBNAs.

The success rate of RNA sequencing was significantly higher in samples obtained from CNBs (Pearson's chi-square test, $p = 0.048$).

The tumor cellularity was lower in samples where RNA sequencing could not be performed, but not significantly (mean tumor cellularity in samples: 49.8 % versus 38.7 %, Wilcoxon rank sum test t test: $p = 0.08$).

We found no significant differences in nodule size, location, and consistency between samples where RNA sequencing was feasible versus unfeasible, though there was a trend towards more part-solid and nonsolid nodules in the infeasible group (21.7% versus 11%). These results are detailed in Table 3.

Table 2. Comparison of Patients by DNA Sequencing Success on Biopsy Samples

Characteristics	Targeted Sequencing Depth Achieved n = 61 (%)	Targeted Sequencing Depth Unachieved n = 7 (%)	*p* Value
Samples characteristics			
Biopsy methods			
FNA (n = 45)	41 (91.1)	4 (8.9)	0.681
CNB (n = 12)	10 (83.3)	2 (16.7)	0.598
EBUS-TBNA (n = 11)	10 (90.1)	1 (9.1)	1
Mean cellularity of the sample	48.1 (25.7)	28.6 (27.0)	0.11
Nodules radiological characteristics			
Mean nodules size (mm)	17.6 (6.11)	18.3 (4.77)	0.72
Solid-component (mm)	15.5 (7.71)	14.7 (8.61)	0.81
Nodule consistency			0.271
Solid (n = 59)	54 (91.5)	5 (8.5)	
Part-solid (n = 5)	4 (80)	1 (20)	
Nonsolid (n = 6)	5 (83.3)	1 (16.7)	
Nodule location			0.43
RUL (n = 28)	26 (92.9)	2 (7.1)	
RML (n = 7)	7 (100)	0	
RLL (n = 14)	11 (78.6)	3 (21.4)	
LUL (n = 14)	12 (85.7)	2 (14.3)	
LLL (n = 7)	7 (100)	0	

This table shows that 61 out of 68 patients achieved the targeted sequencing depth (≥200x) in over 97.5% of regions on biopsy samples. There were no significant differences in nodule size, consistency, or location (measured by CT) between samples that did and did not reach this depth.
CT, computed tomography; CNB, core needle biopsy; EBUS-TBNA, endobronchial ultrasound-guided transbronchial fine needle aspiration; FNA, fine needle aspiration; RUL, right upper lobe; RML, right middle lobe; RLL, right lower love; LUL, left upper lobe; LLL left upper lobe.

Table 3. Comparison of Patients on the Basis of RNA Sequencing Feasibility on Biopsy Samples

Characteristics	RNA Sequencing Feasible n = 45 (%)	RNA Sequencing Infeasible n = 23 (%)	*p* Value
Samples characteristics			
Biopsy methods			
FNA (n = 45)	27 (58.7)	18 (41.3)	0.13
CNB (n = 12)	11 (91.7)	1 (8.3)	0.048
EBUS-TBNA (n = 11)	7 (58.3)	4 (41.7)	1
Mean cellularity of the sample %	49.8 (26.8)	38.7 (22.7)	0.084
Nodules radiological characteristics			
Mean nodules size (mm)	17.5 (6.25)	18.1 (5.44)	0.69
Solid-component (mm)	15.8 (7.61)	14.8 (8.13)	0.63
Nodule consistency			
Solid (n = 59)	40 (67.8)	18 (32.2)	0.35
Part-solid (n = 5)	2 (40)	2 (60)	
Non-solid (n = 6)	3 (50)	3 (50)	
Nodule location			0.51
RUL (n = 28)	17 (60.7)	11 (39.3)	
RML (n = 7)	6 (85.7)	1 (14.3)	
RLL (n = 13)	7 (53.8)	6 (46.2)	
LUL (n=13)	9 (69.2)	4 (30.8)	
LLL (n=7)	6 (85.7)	1 (14.3)	

This table shows that 45 out of 68 patients' samples could undergo RNA sequencing. Samples from CNB had a higher success rate than the other techniques. There were no significant differences in nodule size, consistency, or location (measured by CT) between samples that could or could not be sequenced.
CNB, core needle biopsy; CT, computed tomography; EBUS-TBNA, endobronchial ultrasound-guided transbronchial fine needle aspiration; FNA, fine needle aspiration; RUL, right upper lobe; RML, right middle lobe; RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe.

Clinically Actionable Variants

Biopsy results identified actionable oncogenic alterations in 41.4% (29/70) of patients, revealing 19 *EGFR* mutations, five *KRAS* G12C mutations, two *ROS1* fusions, and single instances of *ALK*, *RET*, and *BRAF* V600E mutations. These alterations were notably more common in women (58.8% versus 25%, $p = 0.004$), younger patients (mean age: 67.0 versus 71.6, $p = 0.042$), and never-smokers (82.4% versus 28.3%, $p < 0.001$).

EGFR mutations were identified in 19 of 70 patients, with eight showing deletions in exon 19, six with mutations in exon 21 (L858R), and five with insertions/duplications in exon 20. *EGFR* mutations were predominantly found in never-smokers compared to current or former smokers (64.7% versus 15.1%, $p = 0.0002$).

A total of 26 patients with *KRAS* alterations were identified with a variety of mutations such as the targetable G12C (5/26) and others such as G12A (3/26), G12D (2/26), G12F (1/26), G12S (1/26), G12V (7/26), G13C (1/26), Q61H (3/26), p.T50I (1/26), and amplifications (2/26). These patients were generally older (mean age: 71.6 versus 67.0, $p = 0.042$) and more likely to be current or former smokers (49.1% versus 0%, $p = 0.0003$). Furthermore, 44.3% (31/70) of the patients also had *TP53* mutations.

In addition, two patients had *ROS1* fusions (both *EZR* exon 10 and *ROS1* exon 34), one had an *ALK* fusion (*EML4* exon 6/*ALK* exon 20), and another had a *RET* fusion (*CCDC6* exon 1–*RET* exon 12). Finally, one patient presented with a *BRAF* V600E mutation.

Figure 2 depicts the actionable oncogenic alterations identified in our patients.

Subgroup Comparison: NGS Analysis—Biopsy Versus Surgical Samples

A subset of 21/70 (30%) participants underwent NGS testing on both the image-guided biopsy sample and the surgical sample. The characteristics of this patient subgroup were comparable to those observed in our study population. Among the 21 patients, 11 (52.4%) were female, most were White (10/21, 47.6%), and current or former smokers (14/21, 66.7%), and the mean age was 66.3 years (SD: 9.65).

The mean maximal diameter of the biopsied nodules was 17.1 mm (SD: 6.1), and the average maximal diameter of the solid component was 14.3 (SD 7.68) mm. Out of these nodules, 16/21 (77.3%) were solid, 3/21 (13%) were part-solid, and 2/21 (9.5%) were nonsolid. Biopsy methods were 17 (73.9%) FNA, two (17.4%) CNB, and two EBUS-TBNA (8.7%). All these 21 biopsy samples were identified as adenocarcinomas.

The median biopsy-to-surgery time was 48 days. 12 participants underwent lobectomies, seven had wedge resections, and two had segmentectomy. All 21 surgical samples were also identified as adenocarcinomas. The median size of the tumor was 19.8 mm (SD 7.81).

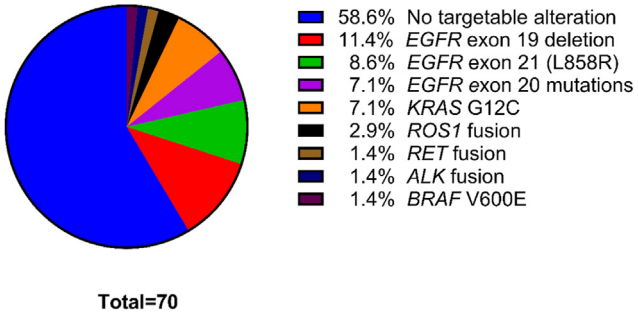

Figure 2. Actionable oncogenic alterations identified in our patients. Figure 2 shows the results of the NGS, on the basis of the analysis of 70 image-guided biopsies, identifying actionable oncogenic alterations in 29 out of 70 patients. Among these, 8/70 had *EGFR* exon 19 deletion, 6/70 had *EGFR* exon 21 (L858R), 5/70 had *EGFR* Exon 20 mutations, 5/70 had *KRAS* G12C mutations, 2/70 had *ROS1* fusions, 1/70 had a *RET* fusion, 1/70 had an *ALK* fusion, and 1/70 had a *BRAF* fusion.

Among these 21 biopsy samples, 20/21 (95.7%) DNA sequencing was feasible; one of the 21 patients lacked cellularity, and no sequencing could be performed. Among the remaining 20 samples, RNA sequencing was feasible in 9/20 (45%).

Among these 21 surgery samples, 21/21 (100%) DNA sequencing was feasible, and 20/21 (95.7%) RNA sequencing was feasible. In two out of the 21 samples (9.5%), a higher-than-expected number of regions did not reach the expected 200x coverage.

The comparison of the reliability of biopsy versus surgery shows that DNA sequencing was feasible at the same rate in both biopsy and surgical samples. RNA sequencing was significantly less feasible on biopsies (42.9% versus 95.2%, $p < 0.001$), and achieving the targeted quality metric depth was also significantly lower in biopsies (61.9% versus 90.5%, $p < 0.05$).

Regarding the exploratory analysis of sequencing quality, among the 20 biopsy samples that underwent sequencing, 35% (seven out of 20) exhibited a higher than anticipated number of regions failing to reach the targeted depth of coverage, compared to 9.5% (two out of 21) of the surgical samples. For these 20 samples, the average coverage was 1537x, with an SD of 583. In addition, a median of 1.9% (with a range of 1.35% to 3.10%) of the 5827 target regions failed to attain a coverage of 200x. For the 21 surgery samples, the mean coverage was 1609x (SD: 267). A median of 1.8% (1.40%–2.10%) of the 5827 target regions did not reach 200x coverage.

These results are detailed in Table 4.

Nevertheless, in 18/20 (90%) patients for whom NGS was feasible, biopsy NGS results remained consistent with surgical NGS results. Clinically actionable variants were found in 12 out of these 18 patients (63.2%): seven *EGFR* mutations, two *KRAS* G12C mutations, one *BRAF* V600E mutation, one *ROS1* fusion, and one *RET* fusion.

For the other 2/20 (10%) patients, NGS on biopsy samples differed from the surgical sample. For these two patients, DNA sequencing fell below quality standards. One other patient biopsy sample lacked sufficient cellularity for any sequencing. NGS analysis of surgical samples from these three patients identified additional alterations, including *CDKN2A*, *EGFR* exon 19 deletion, and *RET* fusion, respectively.

In addition, immunohistochemical test results reported no significant differences between biopsies and surgery samples: TTF1 (biopsy 100%, surgery 100%), Napsin A (biopsy 100%, surgery 80%), and PDL1 (biopsy 66.7%, surgery 50%).

Discussion

In our study, we assessed the feasibility and quality of NGS analysis of lung cancer nodules of 30 mm or less obtained through image-guided biopsy and compared them with surgical resection using data from IELCART, a prospectively collected cohort dedicated to enhancing treatment for stage I lung cancer.[21] The main challenge was that NGS analysis on biopsy nodules is neither mandatory nor standardized in early-stage lung cancer and molecular testing protocols have evolved with the discovery of new targets and methods, leading to significant variability in genetic test results. Out of 654 patients, only 70 underwent NGS analysis on biopsy

Table 4. Comparison of Feasibility: NGS Analysis—Biopsy Versus Surgical Samples

Findings	Biopsy Samples n = 21 (%)	Surgery Samples n = 21 (%)	*p* Value
Median cellularity of the sample [IQR]	45.0 [20.0; 60.0]	65.0 [40.0; 70.0]	0.084
DNA sequencing feasibility (n = 21)	20 (95.2)	21 (100)	1
RNA sequencing feasibility (n = 21)	9 (42.9)	20 (95.2)	0.0002
Targeted Sequencing Depth Achieved (n = 21)	13 (61.9)	19 (90.5)	0.03

This table presents data on 21 patients who underwent NGS on both biopsy and surgical samples. The surgical samples demonstrated a significantly higher feasibility rate for RNA sequencing and a better rate of achieving the targeted sequencing depth.
IQR, interquartile range; NGS, next-generation sequencing.

samples of nodules of less than 30 mm. As it is not universally recommended by current guidelines, this highlights the varied practices among doctors.[6]

Previous studies have focused on the feasibility of genetic analysis on lung biopsies, with varying sample sizes and gene panels but to our knowledge, none have focused on small tumors (≤30 mm). A significant study by Johns Hopkins[22] analyzed 1121 specimens, including 343 lung biopsies or FNA using a lung cancer panel of seven genes with a 94.8% success rate for the lung biopsies, but without specifying tumor sizes. In a smaller study involving 22 NSCLC specimens, 21 out of 22 samples were adequate for full DNA sequencing, mostly from metastatic tumors.[23] Another study examined 50 genes in 162 patients, 90% of whom were at stage IV, and detected mutations in 161 patients.[23] A study investigating the feasibility of using EBUS-TBNA for DNA NGS analysis found 86% success in 115 samples submitted for NGS.[24] The size and stage of the lung cancer were not specified.[24]

Continuing this line of inquiry, our research revealed a 97.4% feasibility rate for DNA sequencing on image-guided biopsy samples of lung cancer nodules of 30 mm or less, with only two biopsy samples where NGS analysis could not be performed. Nevertheless, a reduced rate of 61 out of 70 biopsy samples (87.1%) achieved the required sequencing depth in more than 97.5% of the targeted regions, and RNA sequencing was successfully conducted in only 66.2% of the patients. DNA sequencing did not seem to be influenced by biopsy methods, but RNA sequencing was more successful in samples obtained from CNBs.

We have thus demonstrated that DNA sequencing is possible on lung cancer nodules of 30 mm or less, though the quality is not always optimal, and that RNA sequencing is more challenging. RNA quality is likely to be compromised in routine samples,[25] nevertheless RNA sequencing is essential for the diagnosis of fusion genes.[26] Omitting RNA sequencing appears safe only when a clear oncogenic driver is identified in DNA sequencing.[27] It is also established that standardizing tissue collection, fixation, and processing minimizes nucleic acid degradation, thereby improving the quality of molecular analyses.[28]

Our results align with a comprehensive study comparing CT-guided CNB, EBUS-TBNA, and transbronchial biopsy (TBB).[29] This study analyzed 107 samples from 67 patients, obtained from thoracic tumors or metastatic sites, using NGS. The DNA analysis was successful in 80% of CNB, 100% of EBUS-TBNA, 82% of TBB, and 93% of surgical samples. RNA analysis was slightly less successful (100% of CTNB, 82% of EBUS-TBNA, 73% of TBB, and 95% of surgical samples).[29] The authors concluded that NGS on biopsy samples is feasible.[29] Nevertheless, our study is the first to include a large number of FNA samples. This might explain our slightly lower RNA sequencing success rate, which nonetheless aligns with other studies results—a Japanese study on biopsies from 223 lung cancer patients, nearly half with stage IVB—reported a 76.5% success rate for RNA-based NGS analyses.[30]

We went a step further by comparing the actual NGS results from a subgroup of 21 patients who underwent both biopsy and surgical sample analysis. DNA sequencing feasibility was similar between surgical and biopsy samples; nevertheless, DNA sequencing quality metrics and RNA sequencing feasibility were significantly lower in biopsy samples than in surgical samples. One hypothesis is that lung biopsy samples frequently contain only minimal amounts of primary carcinoma.[31] Our results indicate that, whenever possible, NGS on surgical samples should be prioritized as they provide higher-quality results. This is not always possible, especially for inoperable patients or those with multiple tumors, where biopsies are essential for differentiating between multiple primary tumors and intrapulmonary metastases.[32] In addition, NGS analysis on biopsies enabled faster results.

Nevertheless, in our study, NGS results on biopsies were consistent with surgical samples in 90% of patients. For the remaining 10%, surgical NGS revealed additional information. In these patients, the quality of NGS sequencing on biopsy was suboptimal, and the depth of coverage was not achieved for a higher-than-usual number of targeted regions. Insufficient coverage depth frequently results in false negatives. Although it is not the only quality metric to evaluate an NGS analysis, it is practical because it usually appears directly on the reports that clinicians have access to. Clinicians should be aware of NGS limitations and, in the absence of a targetable alteration, consider repeating the analysis, especially if biopsy NGS results do not meet quality standards or if RNA sequencing cannot be done.

In the future, an alternative could be a liquid biopsy, enabling the detection of key biomarkers such as circulating tumor DNA, cell-free DNA, micro-RNAs, and DNA methylation signatures.[33] Although commonly used in advanced lung cancer to reduce repeated biopsies, it shows promise for noninvasive screening, though large-scale studies are needed to confirm its effectiveness and refine biomarker panels.[34]

Regarding the actual genomic results, it is noteworthy that 41.1% of the participants presented molecular lesions targetable by drugs, aligning with data from the literature.[35] Mostly found in women, younger patient and never-smokers, consistent with what has been previously described. Specifically, 27.1 % had *EGFR* mutations, a frequency similar to that found in the Asian

population, highlighting the heterogeneity of our population. *KRAS* mutations, which are strongly associated with cigarette smoking, were frequently observed in our population (37.1%).

Our study has some limitations. Despite a large number of participants, owing to the lack of systematic NGS sampling, we could include only a relatively small number of patients. There was no available data explaining why specific biopsy samples were sent for NGS analysis and not others, aside from local practice, test availability, and reimbursement policy. Therefore, this may limit the generalization of our findings. We likewise also had no indication of why the test was repeated on the surgical samples of a subgroup of 21 patients. The quality metric we used was chosen somewhat arbitrarily, on the basis of the routine laboratory practice of incorporating coverage summary statements in NGS reports available to clinicians, as seen in other studies.[29] Insufficient coverage depth frequently results in false negatives but they alone may not guarantee accuracy. False negatives can arise despite adequate coverage if reads are misaligned, and coverage depth offers limited utility in minimizing false positive errors. Nonetheless, we believe that because it is generally the metric available to clinicians, it was appropriate to utilize it as a quality metric. Finally, despite the prospective nature of data collection in IELCART, we studied them retrospectively.

In summary, we observed that NGS sequencing was feasible on image-guided biopsies of nodules of 30 mm or less. DNA sequencing reported a higher feasibility rate compared with RNA sequencing. The success of RNA sequencing was higher in CNB biopsy samples than in FNAs and EBUS-TNBA. Achieving the target depth of coverage appears to be associated with the size and characteristics of the nodules and the higher cellularity of the samples. The NGS results on biopsies seem similar to those from surgical samples, though false negatives remain a concern. Caution is needed with results lacking the required depth of coverage.

To the best of our knowledge, this is the first study on the feasibility of NGS analysis on nodules of 30 mm or less, providing additional insights into DNA and RNA sequencing possibilities and comparison with surgical samples.

Conclusion

DNA sequencing succeeded in 97.1% of image-guided biopsies of nodules of 30 mm or less. Nodule solid component size and tumor cellularity correlated with sequencing quality. RNA-based NGS feasibility was lower, especially in FNA samples. In a subgroup analysis, DNA sequencing was concordant with the surgically resected samples in 90% of biopsy samples. Additional actionable mutations were detected in surgical samples for 9.5% of patients and were reported in 44% of patients overall. These findings indicate that NGS on biopsy samples is a reliable diagnostic tool, delivering results comparable to surgical samples and supporting informed treatment decisions. If no driver mutation is detected in the biopsy samples, repeating the analysis on the surgical sample should be considered.

CRediT Authorship Contribution Statement

Louis Gros: Conceptualization, Design, Data collection and assembly, Data analysis and interpretation, Manuscript writing, Final approval of manuscript.

Rowena Yip: Conceptualization, Design, Administrative support, Provision of study materials or patients, Data collection and assembly, Data analysis and interpretation, Manuscript writing, Final approval of manuscript.

Arel Golombeck: Data collection and assembly, Data analysis and interpretation, Manuscript writing, Final approval of manuscript.

David F. Yankelevitz: Conceptualization, Design, Administrative support, Provision of study materials or patients, Data collection and assembly, Data analysis and interpretation, Manuscript writing, Final approval of manuscript.

Claudia I. Henschke: Conceptualization, Design, Administrative support, Provision of study materials or patients, Data collection and assembly, Data analysis and interpretation, Manuscript writing, Final approval of manuscript.

Disclosure

Dr. Yankelevitz is a named inventor on a number of patents and patent applications related to the evaluation of chest diseases including measurements of chest nodules. He has received financial compensation for the licensing of these patents. He is a consultant and co-owner of Accumetra, a private company developing tools to improve the quality of computed tomography imaging. He is on the advisory board and owns equity in HeartLung, a company that develops software related to computed tomography scans of the chest. He is on the medical advisory board of Median Technology which is developing technology related to analyzing pulmonary nodules and is on the medical advisory board of Carestream, a company that develops radiography equipment; and is on the advisory board for LungLife AI. Dr. Claudia Henschke is also an inventor of the patents and pending patents owned by the Cornell Research Foundation (CRF); as of April 2009, she has divested herself

of all royalties and other interests arising from these; and is on the medical advisory board for LungLife AI. The remaining authors declare no conflict of interest.

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