Molecular profiling of key driver genes improves staging accuracy in multifocal non–small cell lung cancer

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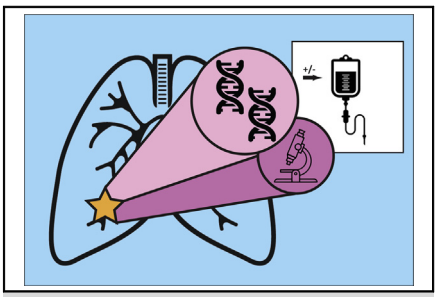
ABSTRACT

Objective: Multifocal non–small cell lung cancer has historically been separated into synchronous primary lung cancers or intrapulmonary metastases with the use of histopathology. We hypothesize that using targeted next-generation sequencing of key driver mutations in multifocal non–small cell lung cancer will improve our ability to differentiate intrapulmonary metastases from synchronous primary lung cancers.

Methods: We identified patients who underwent surgery for non–small cell lung cancer between 2013 and 2018 with multifocal tumors. Archived specimens were reviewed with a 4-gene next-generation sequencing panel identifying mutations of EGFR, KRAS, BRAF, and NRAS. Synchronous primary lung cancers were classified as lesions with different histopathologic subtypes or driver mutations. Tests of hypotheses were performed with the Fisher exact test. Calculations were performed in Stata (v13.0; StataCorp LLC, College Station, Tex).

Results: A total of 18 patients had non–small cell lung cancer tumor specimens (n = 41) available from 2 or more sites. The pathologic diagnosis was predominantly adenocarcinoma (39/41 specimens). We detected a driver mutation in 68.3% (28/41) of all tumors. The most common mutations observed were in *KRAS* (n = 17/41) and *EGFR* (n = 7/41). Eleven patients had synchronous primary lung cancers, and 4 patients had intrapulmonary metastases based on combined histopathologic and molecular profiling results. Three lacked driver mutations in either lesion. Eight synchronous primary lung cancers (8/18, 44%) were downstaged when compared with their original diagnosis ($P = .08$). Of these, 4 patients received adjuvant chemotherapy unnecessarily in hindsight.

Conclusions: Molecular non–small cell lung cancer profiling using a 4-gene next-generation sequencing panel allows for better distinction between synchronous primary lung cancers and intrapulmonary metastases than histopathology alone. Routine use of next-generation sequencing for multifocal lesions prevents unnecessary adjuvant treatment for patients with histologically similar synchronous primary lung cancers. (J Thorac Cardiovasc Surg 2020;160:e71-9)



Molecular profiling of multifocal non–small cell lung cancer lesions decreases overtreatment with adjuvant therapy.

CENTRAL MESSAGE

DNA sequencing of multifocal NSCLC lesions improves staging accuracy compared with histologic review alone, and can prevent overtreatment of synchronous tumors with unindicated adjuvant therapy.

PERSPECTIVE

Multifocal NSCLC lesions pose a diagnostic dilemma for clinicians, because lesions of different clonal origins (SPLCs) are treated differently from intrapulmonary metastases. We show that a small, 4-gene sequencing panel is able to discern differences between lesions that simple histologic review could not, potentially influencing downstream treatment decisions.

See Commentaries on pages e81, e82, and e83.

Multifocal non–small cell lung cancer (NSCLC) presents a unique diagnostic and therapeutic dilemma. Ipsilateral multifocal lesions can be classified as intrapulmonary metastases (IPMs) or synchronous primary lung cancers (SPLCs). IPMs are similar in clonal origin and staged as T3 or T4 lesions according to the AJCC 8th edition of

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Abbreviations and Acronyms

IASLC = International Association for the Study of Lung Cancer
IN = inconclusive
IPM = intrapulmonary metastasis
NGS = next-generation sequencing
NSCLC = non–small cell lung cancer
SPLC = synchronous primary lung cancer

NSCLC staging.[1] In contrast, SPLCs are often treated as separate tumors and are staged according to the characteristics of each individual lesion. These differences are important for both prognostication and determining adjuvant treatment. Patients with SPLCs have decreased survival relative to those with solitary tumors, but improved survival when compared with those with IPMs.[2] Resectable IPMs, being stage II or greater, are an indication for treatment with adjuvant chemotherapy, based on the current standard of care. However, small SPLCs (T1 or T2) without nodal involvement have not been shown to derive any survival benefit from adjuvant therapy.[3]

Martini and Melamed[4] first established diagnostic criteria for differentiating SPLC from IPM in 1975. These criteria, based on observable tumor characteristics alone, required that synchronous tumors must be histologically different, anatomically separate, or originate from known carcinoma in situ to be considered SPLCs. Although histopathologic examination is often sufficient for establishing a diagnosis of multiple primaries, distinguishing SPLCs from IPMs can be challenging when 2 lesions are similar in microscopic appearance.[5] Molecular profiling with next-generation sequencing (NGS), used to identify mutations that direct the selection of precision therapy, has emerged as a helpful adjunct in identifying the relationship between multifocal lesions based on driver mutations in key oncogenes such as KRAS, EGFR, and BRAF. Whole-exome sequencing of early-stage NSCLC tumors has shown that mutations in EGFR, BRAF, and KRAS represented early driver mutations and were almost always clonal.[6] Indeed, molecular profiling has been demonstrated to be able to parse out differences in tumors that histologic assessment cannot.[7,8] As much as 32% of all histologically identified synchronous lung lesions have been found to be misclassified as intrapulmonary metastases when compared with their molecular profiles.[9]

In our practice, NGS is not performed on all nodules in tumors with multifocal lesions, because SPLCs and IPMs have traditionally been differentiated on the basis of histopathology alone. However, NGS has been used selectively for at least 1 lesion for multifocal cases to guide selection of adjuvant treatments. We hypothesize that molecular profiling will more accurately differentiate SPLCs from IPMs and, as a result, correctly identify which patients may benefit from adjuvant therapy.

MATERIALS AND METHODS

We retrospectively identified patients who underwent curative resection for NSCLC at our institution between 2013 and 2018 and had multifocal tumors identified on pathology. Archived formalin-fixed, paraffin-embedded specimens were identified from our pathology laboratory. Basic patient demographic information (ie, age, sex, race), pathological tumor characteristics, and clinical outcomes were collected. All multifocal NSCLC lesions in an individual patient were resected simultaneously during the same operation.

Histopathology

Previously archived hematoxylin–eosin slides of multifocal tumors were simultaneously re-reviewed by 2 independent, board-certified pathologists. Sets of lesions were categorized as histologically identical, different, or similar. Lesions were classified as "identical" if they belonged to the same International Association for the Study of Lung Cancer (IASLC) subtype and showed identical cytomorphologic and architectural features. Lesions were classified as "different" if they belonged to different IASLC subtypes. "Similar" lesions shared the same IASLC subtype but differed morphologically in at least 1 way, whether in terms of cytomorphology (nuclear or cytoplasmic features) or the presence of a secondary architectural pattern in 1 tumor but not the other.

Molecular Profiling

Genomic DNA was extracted from qualified specimens of selected patients. To qualify for this study, tumor tissues from all tumor nodules from the same patient must have been available within our archives and contain at least 10% tumor cells for extraction and further analysis. Individual tumor nodules were microdissected, and DNA was extracted for analysis by NGS. The majority of NGS was performed using a custom, small amplicon-based NGS panel developed in the Jefferson Molecular & Genomic Pathology Laboratory that targets EGFR (exons 18-21), KRAS (exons 2-4), NRAS (exons 2-4), and BRAF (exons 11 and 15). These 4 genes were chosen because driver mutations in EGFR and BRAF have been shown to be unique, inciting factors in tumorigenesis that are clonally preserved throughout development of NSCLC.[6] Furthermore, different mutational profiles in EGFR and KRAS have been shown to be largely mutually exclusive and linked to different demographic groups.[10]

This 4-gene panel is able to analyze small biopsy specimens and provide results to clinicians with a short turnaround time of approximately 7 days. The extracted DNA underwent a 2-step polymerase chain reaction amplification for amplicon enrichment and barcoding. The polymerase chain reaction products were normalized and pooled before sequenced on Illumina MiSeq platform (Illumina). In 6 patients, multiple tumor nodules were sequenced at the time of diagnosis with the Illumina TruSeq Amplicon Cancer Panel (Illumina), which contains 48 commonly mutated genes in cancer. Bioinformatics analysis was performed via the TruSeq workflow, which included data collection, base calling, sequence alignment, variant identification and annotation, and filtering with specific criteria. Mutations in targeted regions were identified, and mutations with pathogenic significance were reported.

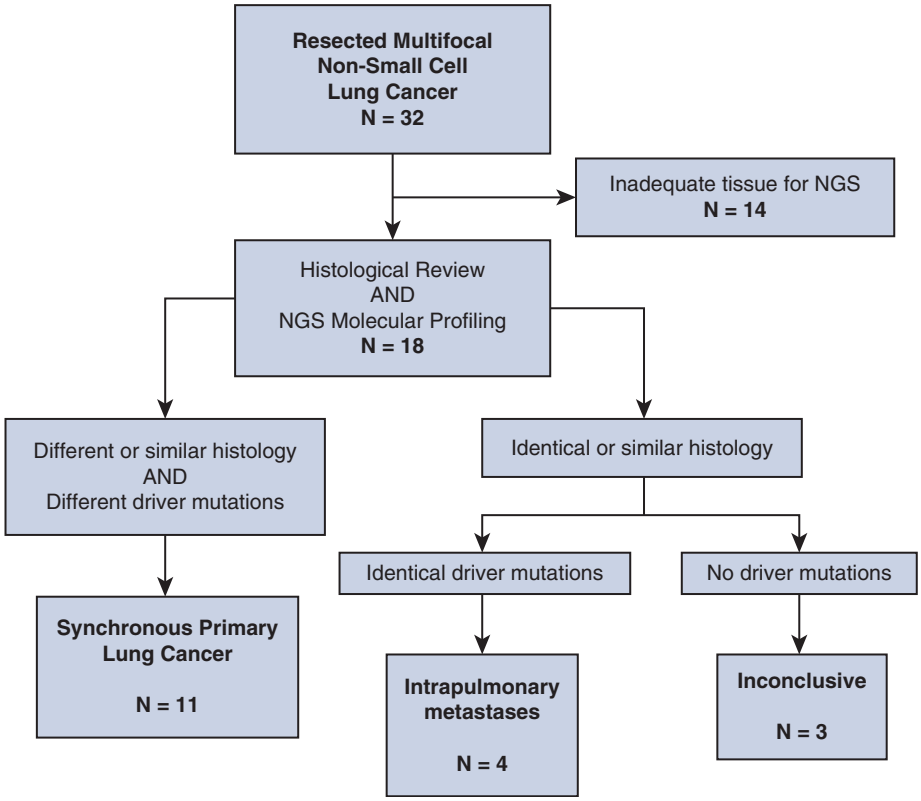


FIGURE 1. Flow diagram for identification of SPLC versus IPM. Criteria for identifying SPLC versus IPM. *NGS*, Next-generation sequencing.

Definitions of Lesion Type

Lesions are categorized into 1 of 3 types according to both histologic similarity and comparison of driver mutations identified by NGS: SPLC, IPM, and inconclusive (IN) (Figure 1). These categories are defined as follows.

Synchronous Primary Lung Cancers

SPLCs were defined as multiple lesions from the same patient harboring different driver mutations or clearly different histologic patterns.

Intrapulmonary Metastases

Intrapulmonary metastases (IPMs) were defined as lesions from the same patient who shared identical driver mutations and the same or similar histologic subtype between lesions.

Inconclusive

IN lesions had no driver mutations identified in either lesion by the NGS panels used and were too histologically similar to differentiate based on histology alone.

Statistical Analysis

McNemar's test was used to compare paired outcomes (receiving adjuvant chemotherapy/radiotherapy, and downstaging after NGS) with regard to lesion type before and after NGS. Overall and disease-free survival analyses were deferred because of low sample size. Calculations of descriptive and inferential statistics were performed in Stata version 13.0 (StataCorp LP, College Station, Tex).

RESULTS

We identified 32 patients who underwent curative surgery for NSCLC and were found to have multifocal tumors on final pathological review. Because of the availability of archived specimens, a total of 41 independent lesions from 18 patients had sufficient materials for histopathologic review and molecular profiling.

Baseline Demographics

Patients had a mean and median age of 69.6 ± 9.5 and 68.5 years, respectively (Table 1). Eleven of 18 patients were female, and 13 were white. There were 3 active smokers (n = 3, 16.7%), 12 former smokers (66.7%), and 3 active smokers at the time of surgery (n = 3, 16.7%). All 3 active smokers were found to have SPLCs. The pathologic diagnosis of resected specimens was adenocarcinoma in 37 of 41 specimens, (90.2%). Most patients underwent video-assisted thoracoscopic resection (15/18, 83.3%). Tumors were found in all pulmonary lobes but were predominantly in the left upper lobe (16/41 specimens, 39.0%) or right upper lobe (16/41 specimens, 39.0%). Only 1 subject underwent neoadjuvant chemotherapy (n = 1, 5.6%).

Histologic Review

The results of the histologic review were documented for each nodule for each patient in Table 2. Hematoxylin–eosin-stained slides of all 18 patients were reviewed by 2 pathologists at our institution. In total, 7 sets of lesions

TABLE 1. Demographics and clinical information by patient

Subject No.	Age (y)	Sex	Race	Smoker	Original TNM* staging	Surgical approach	Adjusted† chemotherapy	Recurred	Died
1	50	F	WHT	Former	T4N0M0	Open RUL/RML lobectomy	Y	N	N
2	77	M	WHT	Former	T1N0M0	Open RML lobectomy	N	Y	N
3	79	F	WHT	Former	T4N0M0	VATS RUL lobectomy, RLL wedge	N	Y	N
4	61	F	BLK	Active	T3N1M0	Robotic-assisted RUL lobectomy	Y	Y	N
5	77	F	WHT	Never	T3N0M0	VATS RUL lobectomy	N	N	N
6	85	M	WHT	Former	T3N0M0	VATS RLL lobectomy, RUL wedge	Y	Y	N
7	66	M	WHT	Former	T3N1M0	VATS RLL lobectomy	Y	Y	Y
8	83	F	WHT	Active	T3N0M0	VATS LUL lobectomy	N	N	N
9	76	M	BLK	Active	T3N0M0	VATS RUL lobectomy	Y	N	N
10	66	F	WHT	Former	T3N0M0	VATS LUL lobectomy	N§	N	N
11	73	M	WHT	Former	T3N0M0	VATS LUL lobectomy	N	N	N
12	79	F	WHT	Former	T3N0M0	VATS RUL lobectomy	N	N	N
13	63	F	WHT	Never	T3N0M0	VATS LLL lobectomy	Y	Y	N
14	62	M	WHT	Former	T4N0M0	VATS RLL lobectomy, RUL wedge	Y	N	N
15	60	F	BLK	Former	T3N2M0	VATS LUL lobectomy	Y	N	N
16	65	F	BLK	Former	T3N0M0	VATS LUL lobectomy	N	N	N
17	60	M	WHT	Never	T3N0M0	VATS LUL lobectomy	Y	Y	Y
18	71	F	BLK	Former	T3N0M0	VATS LUL lobectomy	N	N	N

RUL, Right upper lobectomy; *RML*, right middle lobectomy; *VATS*, video-assisted thoracoscopic surgery; *RLL*, right lower lobectomy; *LUL*, left upper lobectomy; *LLL*, left lower lobectomy. *American Joint Committee on Cancer staging system based upon surgical pathology. †Adjuvant chemoradiotherapy to treat lesions that were surgically resected. §Only subject to receive neoadjuvant therapy.

were deemed to be morphologically similar. Among the 11 identified SPLCs, 4 tumor pairs were similar in histologic appearance (4/11, 36.4%) and 6 (6/11, 54.5%) had clear differences in morphology (1 pair was not available for re-review). Among the 4 IPMs, 3 tumor pairs (3/4, 75%) were histologically identical, and 1 pair (1/4, 25%) was histologically similar. Among the 3 INs, 2 pairs were histologically similar and 1 pair was identical.

Next-Generation Sequencing Results

Table 2 shows the NGS mutation results. A total of 41 specimens from 18 patients were successfully sequenced. One or more driver mutations were found in 68.3% (n = 28/41) of the tumors, including (but not limited to) *KRAS* (n = 17/41, 41.2%); *EGFR* (n = 7/41, 17.1%); *BRAF* (n = 1/41, 2.4%); and *NRAS* (n = 1/41, 2.4%). Additional mutations were found in *TP53* (n = 3) and *APC* (n = 1) among the 6 lesions tested with the TruSeq 48-gene panel. Eleven of 18 tumor pairs (11/18, 61.1%) had discordant driver gene mutations and were determined to be SPLCs. Four tumor pairs (4/18, 22.2%) had IPMs based on their identical molecular and histologic profiles. Three IN lesions (3/18, 16.7%) were found to have no identifiable driver mutation in either lesion (Table 2). Among 7 histologically similar lesions that were all originally deemed to be IPMs, 4 were changed to SPLCs, 1 remained an IPM, and 2 were deemed IN after NGS profiling. In total, conventional histologic examination misdiagnosed 4 sets of lesions (4/18, 22.2%) that were otherwise captured correctly by NGS.

Staging and Treatment Decisions

At the time of initial resection, nodal involvement was found in 3 patients (3/18, 16.7%); all patients with nodal spread were also found to have SPLCs. The majority of patients were originally staged at the time of surgery as 2B (n = 10/18, 55.5%), followed by 3A (n = 7/18, 38.9%) and 1A (n = 1, 5.6%) (Table 3).

Nine patients received adjuvant chemotherapy overall, with 6 SPLCs (6/11, 54.5%, $P = .7$), 1 IPM (1/4, 25%, $P = .08$), and 2 INs (2/3, 66.7%, $P = .02$) receiving adjuvant chemotherapy in each group. Adjuvant chemotherapeutic regimens given to patients included carboplatin/gemcitabine, carboplatin/pemetrexed, cisplatin/pemetrexed, and nivolumab/docetaxel. Three patients received adjuvant radiotherapy in tandem with chemotherapy. Of the patients with SPLCs identified via molecular profiling, 8 patients (8/18, 44.4%) were downstaged from their histologic staging based on NGS ($P = .08$; Figure 2). Of these 8 downstaged patients, 4 (4/8, 50%) received unnecessary adjuvant therapy given their retrospective staging. Four of 11 patients with SPLCs

TABLE 2. Molecular and pathological profiles of multifocal non–small cell lung cancer lesions by patient

Subjectnodule No.	Location	Pathologic diagnosis	Histologic relationship	Original lesion type	NGS mutation	Post-NGS lesion type	Staging change
1-1	RML	Adenocarcinoma	H&E slides missing	IPM	*KRAS p.G12V*	SPLC	**3A → 1A**
1-2	RUL	Adenocarcinoma			*BRAF p.G596R*		
2-1	RML	Adenocarcinoma	Different	SPLC	*KRAS p.G12A*	SPLC	1A → 1A
2-2	RUL	Adenocarcinoma			*None*		
3-1	RUL	Adenocarcinoma	Similar	IPM	*KRAS p.G12C*	IPM	3A → 3A
3-2	RLL	Adenocarcinoma			*KRAS p.G12C*		
4-1	RUL	Adenocarcinoma	Different	IPM	*None*	SPLC	**3A → 2A**
4-2	RUL	Adenocarcinoma			*KRAS p.G12C*		
4-3	RUL	Adenocarcinoma			*KRAS p.G12C*		
5-1	RUL	Adenocarcinoma	Identical	IPM	*EGFR p.L858R*	IPM	2B → 2B
5-2	RUL	Adenocarcinoma			*EGFR p.L858R*		
5-3	RUL	Adenocarcinoma			*EGFR p.L858R*		
6-1	RLL	Adenocarcinoma	Similar	IPM	*KRAS p.G12C*	SPLC	**3A → 2B**
6-2	RUL	Adenocarcinoma			*EGFR p.D770_N771insGL*		
6-3	RUL	Adenocarcinoma			*KRAS p.G12C*		
7-1	RLL	Adenocarcinoma	Different	IPM	*None*	SPLC	**3A → 1A**
7-2	RLL	Adenocarcinoma			*None*		
8-1*	LUL	Adenocarcinoma	Similar	IPM	*TP53 p.M237I*	SPLC	**2B → 1A**
8-2*	LUL	Adenocarcinoma			*KRAS p.G12C*		
9-1*	RUL	Adenocarcinoma	Different	IPM	*TP53 p.R158L*	SPLC	2B → 2B
9-2*	RUL	Combined large cell, squamous			*NRAS p.Q61L* *APC p.S1282** *APC p.S1539**		
10-1	LUL	Adenocarcinoma	Identical	IPM	*KRAS p.G12C*	IPM	2B → 2B
10-2	LUL	Adenocarcinoma			*KRAS p.G12C*		
11-1*	LUL	Adenocarcinoma	Similar	IPM	*KRAS p.G12C*, *TP53 p.R337L*	SPLC	**2B → 1A**
11-2*	LUL	Adenocarcinoma			*None*		
12-1*	RUL	Adenocarcinoma	Similar	IPM	*None*	IN	2B → 2B
12-2*	RUL	Adenocarcinoma			*None*		
13-1	LLL	Adenocarcinoma	Identical	IPM	*None*	IN	2B → 2B
13-2	LLL	Adenocarcinoma			*None*		
14-1*	RLL	Adenocarcinoma	Identical	IPM	*KRAS p.G12C*	IPM	3A → 3A
14-2*	RLL	Adenocarcinoma			*KRAS p.G12C*		
15-1	LUL	Adenocarcinoma	Similar	IPM	*EGFR p.L858R*	SPLC	3A → 3A
15-2	LUL	Adenocarcinoma			*EGFR p.L747_S752del*		
15-3	LUL	Adenocarcinoma			*EGFR p.L747_S752del*		
16-1	LUL	Adenocarcinoma	Different	IPM	*KRASp.G12C*	SPLC	**2B → 1A**
16-2	LUL	Adenocarcinoma			*None*		
17-1	LUL	Large cell	Similar	IPM	*None*	IN	2B → 2B
17-2	LUL	Large cell			*None*		
18-1	LUL	Hyperplasia	Different	IPM	*KRAS p.G12D*	SPLC	**2B → 1A**
18-2	LUL	Adenocarcinoma			*KRAS p.G12C*		
18-3	LUL	Adenocarcinoma			*None*		

Bold values represent instances in which tumor stage was different according to NGS than with histology alone. *NGS*, Next-generation sequencing; *RML*, right middle lobectomy; *H&E*, hematoxylin–eosin; *IPM*, intrapulmonary metastases; *RUL*, right upper lobectomy; *SPLC*, synchronous primary lung cancer; *IN*, inconclusive; *LUL*, left upper lobectomy.
*These lesions were sequenced at the time of resectional surgery with the Illumina TruSeq 48-gene panel.

TABLE 3. Summary of demographics and treatments by lesion type

Variable	Total (% total)	SPLC (% SPLC)	IPM (% IPM)	IN (% IN)
Age				
Mean (y) (±SD)	69.6 (9.5)	69.7 (10.5)	71 (8.3)	67.3 (10.2)
Median (y)	68.5	71	71.5	63
Gender				
Male	7 (38.9)	5 (45.5)	1 (25)	1 (33.3)
Female	11 (61.1)	6 (54.5)	3 (75)	2 (66.7)
Race				
White	13 (72.2)	6 (54.5)	4 (100)	3 (100)
African American	5 (27.8)	5 (45.5)	0 (0)	0 (0)
Smoking				
Never	3 (16.7)	0 (0.0)	1 (25)	2 (66.7)
Former	12 (66.7)	8 (72.7)	3 (75)	1 (33.3)
Active	3 (16.7)	3 (27.3)	0 (0)	0 (0)
Surgical approach				
Robotic-assisted	1 (5.6)	1 (9.1)	0 (0)	0 (0)
Open	2 (11.1)	2 (18.2)	0 (0)	0 (0)
VATS	15 (83.3)	8 (72.7)	4 (100)	3 (100)
Resection				
Lobectomy	14 (77.8)	9 (81.8)	2 (50)	3 (100)
Lobectomy/wedge	3 (16.7)	1 (9.1)	2 (50)	0 (0)
Bilobectomy	1 (5.6)	1 (9.1)	0 (0)	0 (0)
Tumor Location				
Left lower lobe	1 (5.6)	0 (0.0)	0 (0)	1 (33.3)
Left upper lobe	7 (38.9)	5 (45.5)	1 (25)	1 (33.3)
Right lower lobe	2 (11.1)	2 (18.2)	0 (0)	0 (0)
Right middle lobe	2 (11.1)	2 (18.2)	0 (0)	0 (0)
Right upper lobe	6 (33.3)	2 (18.2)	3 (75)	1 (33.3)
Nodal staging				
N0	15 (83.3)	8 (72.7)	4 (100)	3 (100)
N1	2 (11.1)	2 (18.2)	0 (0)	0 (0)
N2	1 (5.6)	1 (9.1)	0 (0)	0 (0)
Adjuvant chemotherapy	9 (50)	6 (54.5)	1 (25)	2 (66.7)
Carboplatin/gemcitabine	1 (5.6)	1 (9.1)	0 (0)	0 (0.0)
Carboplatin/pemetrexed	2 (11.1)	2 (18.2)	0 (0)	0 (0.0)
Cisplatin/pemetrexed	5 (27.8)	3 (27.3)	1 (25)	1 (33.3)
Nivolumab/docetaxel	1 (5.6)	0 (0)	0 (0)	1 (33.3)
Adjuvant radiation	3 (16.7)	2 (18.2)	0 (0)	1 (33.3)
Chemotherapy-related complication	4 (22.2)	4 (36.4)	0 (0)	0 (0.0)

SPLC, Synchronous primary lung cancer; *IPM*, intrapulmonary metastases; *IN*, inconclusive; *SD*, standard deviation; *VATS*, video-assisted thoracoscopic surgery.

went on to have complications during their adjuvant chemotherapy, which included intractable nausea and diarrhea (n = 2), symptomatic anemia (n = 1), and pulmonary embolism (n = 1).

DISCUSSION

In 1975, Martini and Melamed[4] first noted that patients with multiple SPLCs have decreased survival relative to those with solitary tumors, but improved survival when compared with those with IPMs. They then also established diagnostic criteria for the differentiation of SPLCs based on histologic tumor characteristics. Since then, other studies have corroborated their findings and sought to better define the relationship between multifocal lung lesions based on secondary features such as nuclear pleomorphism, cell or nucleolar size, acinar formation, mitotic rate, and necrosis.[2,9] We have come to realize that histology is often sufficient to distinguish between lesions, but can fail when lesions share similar but not identical histologic characteristics. For instance, 2 lesions had similar growth patterns, but subtle cytomorphologic differences.[5] More thorough and comprehensive histologic examination has been shown to improve distinction of these lesions over traditional Martini and Melamed criteria,[5] but techniques vary between institutions and reviewers.

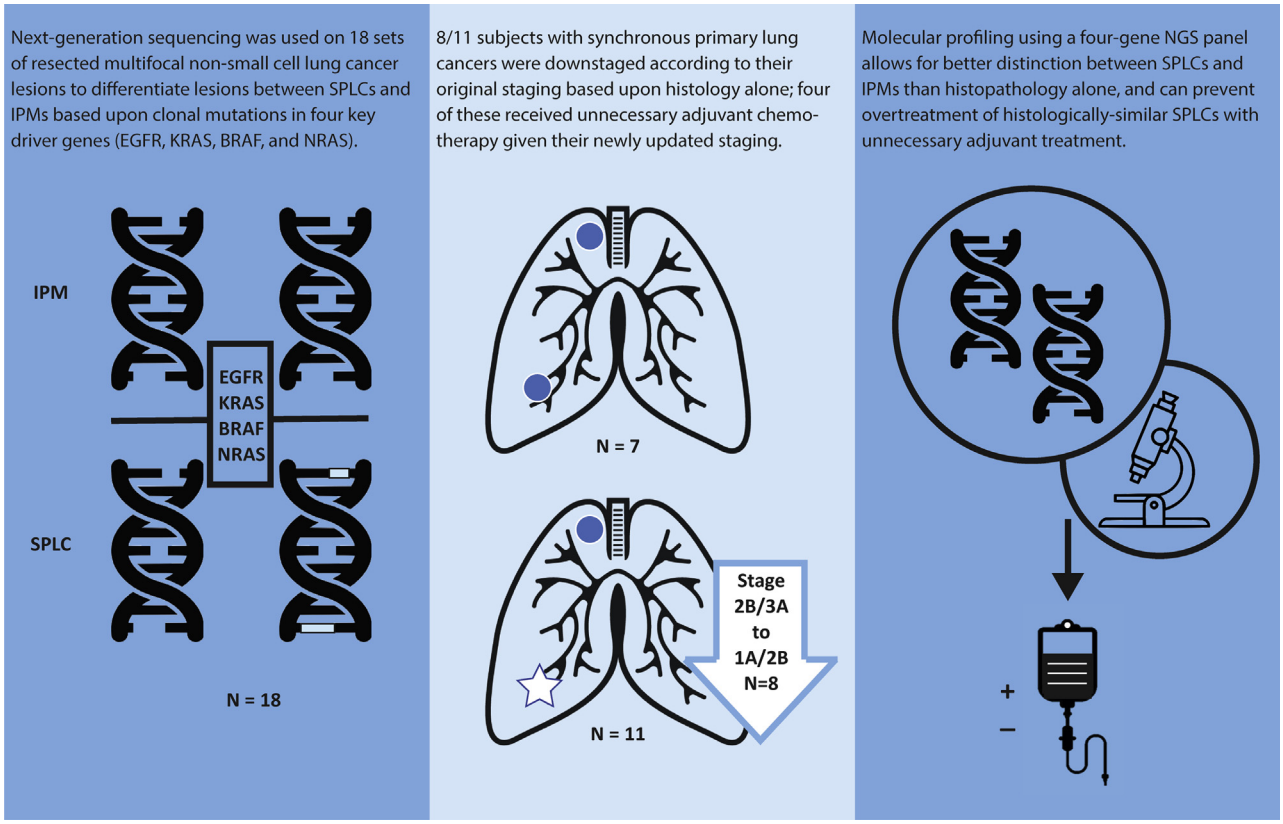


FIGURE 2. The design and outcomes of our study are highlighted. From *left* to *right*: (1) NGS was applied to sets of lesions from 18 subjects with multifocal NSCLC. (2) This allows for improved distinction of SPLCs versus histology alone; some of the patients (n = 8) who were erroneously considered to be a higher stage at initial histologic review were downstaged on the basis of their molecular profiles. (3) Molecular profiling with a 4-gene panel allows for better distinction between SPLCs and IPMs than histopathology alone and can prevent overtreatment of histologically similar SPLCs with unnecessary adjuvant treatment. *SPLC*, Synchronous primary lung cancer; *IPM*, intrapulmonary metastasis.

NGS is a technique that can be implemented in routine clinical practice to provide predictive and prognostic insight into the clinical course of NSCLC. It is highly sensitive and can even provide genomic data on specimens with low relative or absolute tumor cell counts.[11] NGS is now routinely used to identify molecular targets for tailored therapy and simultaneously distinguish lesions on the basis of genomic findings. It is now commonly accepted that cancers with different driver mutations in oncogenes such as EGFR and KRAS have different clonal origins.[12,13] For instance, EGFR and KRAS mutations, the most common mutations seen in our study, have been observed in up to 80% of all SPLCs[5] and yet are still generally considered to be mutually exclusive of one another (with concomitant EGFR/KRAS mutations being rare in NSCLC).[14] Other evidence has also suggested that these mutational profiles have different underlying risk factors, with smokers more likely to have mutations in KRAS and never-smokers more likely to have mutations in EGFR.[10]

Our results show that NGS is able to discern differences in histologically similar lesions and improve the staging accuracy of multifocal NSCLC, capturing an additional 22% of lesions as SPLCs that histopathologic assessment alone failed to identify correctly. Despite sharing the same IASLC subtype, the 7 lesions that were deemed to be similar based on histology alone were shown to actually be 4 SPLCs, 1 IPM, and 2 IN lesions; clearly, there is a significant portion of multifocal lesions in our study group that are difficult to distinguish with histology alone; there also does not seem to be any apparent bias in classifying these as SPLCs or IPMs. This suggests that histologic examination lacks the necessary resolution to classify certain NSCLC tumors into either category and that even the most experienced reviewing pathologists may impart some subjectivity into their analysis. A recent systematic review carried out by an IASLC Staging and Prognostic Factors subcommittee[15] came to the consensus that "few features are sufficiently reliable by themselves" in separating primary tumors; we would agree and add that a comprehensive review of both histology and mutational tumor analysis will provide the best estimate of clonality among lung tumors.

Other studies have corroborated the diagnostic accuracy of molecular profiling for distinguishing SPLCs from

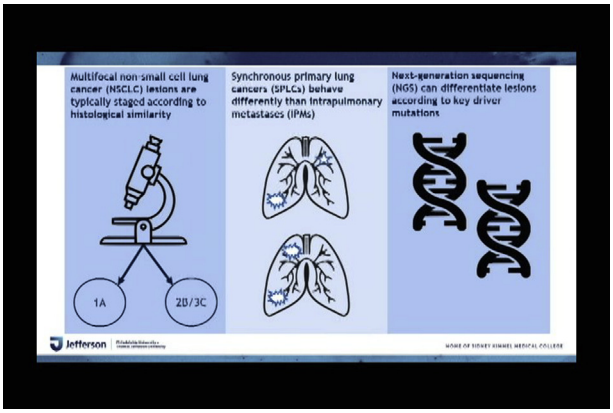
IPMs when compared with histology alone. Without additional diagnostic measures, tumor misclassification may occur in 9% to 32% of all histologically identified synchronous cancers.[8,16] Genomic signatures add value to identifying the relationship between multiple foci of cancer in more than 70% of cases in a small series.[17] The combination of these observed discrepancies between histologic and molecular diagnosis and the recent availability of molecularly targeted therapies such as EGFR-targeted tyrosine kinase inhibitors have led the College of American Pathologists to recommend testing of all multifocal NSCLC lesions for EGFR and ALK mutations.[18]

Our study used a routinely used clinical NGS panel with only 4 key driver genes that can be easily adopted to determine the driver gene genotype of all tumors from multifocal lesions.[19] Although other studies have highlighted the use of NGS to differentiate multifocal lung cancers, ours is the first to show the consequences of diagnostic errors that occur when NGS is not preemptively used, errors that have significant therapeutic implications with regard to selection of adjuvant treatment. The NSCLCs that were retroactively downstaged to stage I lesions according to NGS had been originally treated as IPMs and given unnecessary adjuvant chemotherapy according to current NCCN guidelines.[3] Chemotherapy for NSCLC, which uses predominantly platinum-based combination regimens, is costly and commonly leads to complications such as nausea, vomiting, diarrhea, and neuropathy, with potential for development of more severe symptoms such as nephrotoxicity, ototoxicity, or cardiotoxicity.[20]

Additionally, NGS analysis of lung cancers is increasingly performed to discover suitable mutations for molecularly targeted gene therapies such as BRAF inhibitors or EGFR tyrosine kinase inhibitors.[21] These mutations can sensitize lesions to be treated with tyrosine kinase inhibitors or confer resistance. In our study, the 4-gene panel would have been as effective in identifying differences in driver gene mutations as the Illumina 48-gene panel (Illumina) because mutations in TP53 gene and APC gene identified by the 48-gene panel did not add additional power to classify these nodules (Table 2).

Study Limitations

Our study is limited by the retrospective nature of data collection, along with its small sample size and inconsistent length of follow-up between patients. The small sample size of each type of lesion impairs our ability to match groups and, more important, to make meaningful conclusions about overall and disease-free survival. For this reason, survival analyses were omitted from our analysis. Others have already described the survival curves of SPLCs relative to IPMs in more detail.[2-4]



VIDEO 1. Presentation from Western Thoracic Society Meeting 2019. Video available at: https://www.jtcvs.org/article/ S0022-5223(19)39883-6/fulltext.

The subset of 3 tumor pairs labeled as IN lesions in this study also represents a unique challenge in terms of making treatment decisions. A recent similar study by Patel and colleagues[22] demonstrated that they were able to identify mutations in 41 of 42 specimens by using a 50-gene Ion AmpliSeq Hotspot Cancer Panel (Thermo Fisher Scientific, Waltham, Mass), which is genetically similar to the 48-gene panel by Illumina that was used on a small subset of patients in our study. Additionally, they were able to compare these NGS results with traditional AJCC staging by histologic examination alone and showed that traditional histopathology may mischaracterize a significant proportion of multifocal lesions (n = 3/19, 15.8%).[22] In the original histopathologic review of the lesions in our study, 5 tumor pairs were initially considered to be IPMs despite our pathologists finding upon re-review that they were actually histologically different; this speaks to the highly variable and observer-dependent nature of histologic review for identification of such lesions. We have demonstrated that a focused 4-gene panel that covers key driver oncogenes in lung cancer is effective to differentiate 83.3% (15/18 patients) of multifocal NSCLC lesions in our study cohort. The advantages of this 4-gene panel are rapidity, cost-effectiveness, and, more important, the power to detect mutations from tumors and biopsy specimens of small sizes. When no mutations can be identified by this 4-gene panel, a large NGS gene panel (on the order of several hundred genes) should be reflexively ordered to minimize tumors in the inclusive group and precisely identify multifocal lesions as SPLCs or IPMs. However, the increase in the amount of DNA required for this process may preclude the analysis of smaller multifocal lesions.

Additionally, other driver mutations such as *PIK3CA* or pathogenic mutations in tumor suppressor genes like *TP53* also vary between multifocal lung lesions.[23] Additional techniques, such as X-chromosome inactivation analysis and analysis of micro-RNA expression profiles, have also been explored as methods of differentiating

between primary and metastatic lesions.[24,25] The current utility of these novel tests is unclear. Further study of some of these novel techniques is necessary before applying them to clinical practice, but opportunities abound for improving the diagnosis of synchronous lesions.

CONCLUSIONS

DNA sequencing of all tumors of multifocal NSCLC lesions with a small NGS panel of driver genes improves staging accuracy when compared with histopathology alone and can simultaneously identify targets for therapy in a reliable and objective fashion. Multifocal lesions evaluated via histologic review alone can lead to overtreatment of synchronous primary lesions with unnecessary adjuvant therapy. This problem can be prevented if all multifocal NSCLC lesions are routinely profiled with NGS, regardless of their histologic similarity. A larger sample size and length of follow-up are required to understand the effect of these unnecessary treatments on overall and disease-free survival. This demonstrates the clinical utility of thorough analysis of NSCLC with multiple tumor nodules to provide evidence for accurate staging and evaluation for further therapy (Video 1).

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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