original article

Implementing multiplexed genotyping of non-small-cell lung cancers into routine clinical practice

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Background: Personalizing non-small-cell lung cancer (NSCLC) therapy toward oncogene addicted pathway inhibition is effective. Hence, the ability to determine a more comprehensive genotype for each case is becoming essential to optimal cancer care.

Methods: We developed a multiplexed PCR-based assay (SNaPshot) to simultaneously identify >50 mutations in several key NSCLC genes. SNaPshot and FISH for *ALK* translocations were integrated into routine practice as Clinical Laboratory Improvement Amendments-certified tests. Here, we present analyses of the first 589 patients referred for genotyping.

Results: Pathologic prescreening identified 552 (95%) tumors with sufficient tissue for SNaPshot; 51% had ≥1 mutation identified, most commonly in *KRAS* (24%), *EGFR* (13%), *PIK3CA* (4%) and translocations involving *ALK* (5%). Unanticipated mutations were observed at lower frequencies in *IDH* and β-*catenin*. We observed several associations between genotypes and clinical characteristics, including increased *PIK3CA* mutations in squamous cell cancers. Genotyping distinguished multiple primary cancers from metastatic disease and steered 78 (22%) of the 353 patients with advanced disease toward a genotype-directed targeted therapy.

Conclusions: Broad genotyping can be efficiently incorporated into an NSCLC clinic and has great utility in influencing treatment decisions and directing patients toward relevant clinical trials. As more targeted therapies are developed, such multiplexed molecular testing will become a standard part of practice.

Key words: carcinoma, non-small cell, genotype, molecular targeted therapy

introduction

Certain genetically defined cancers are 'oncogene addicted' to activated kinases and are thereby highly sensitive to drugs that selectively inhibit the corresponding kinase. Employing genotype-based therapy has been highly successful in chronic myelogenous leukemia, gastrointestinal stromal tumors, non-small-cell lung cancer (NSCLC) and melanoma, and in many instances, the targeted agent is far more effective than traditional chemotherapy [1–9]. This shifting paradigm has dramatically impacted lung cancer treatments. Until recently, therapeutic options for advanced NSCLC were limited to

chemotherapies that were 'personalized' only by considering the side-effect profiles of a number of similar modestly effective regimens. Response rates were typically 20%–30% and progression-free survival (PFS) was 3–5 months [10–13]. But now, we know that determining NSCLC genotype can inform the most effective personalized therapies. Patients with mutations in the epidermal growth factor receptor (*EGFR*) gene benefit from EGFR tyrosine kinase inhibitors (TKIs) with a response rate of ∼75%, PFS of 9–13 months and improved quality of life compared with chemotherapy [8, 14–16]. Similarly, patients with *EML4-ALK* translocations have a 60% response rate, 9-month PFS and a low degree of toxicity when treated with crizotinib, an ALK TKI [6].

Although these landmark studies have focused on a single or small number of genetic mutations, there is an increasing motivation to develop technologies that can simultaneously determine the mutational status of many genes. Responding to

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the need for real-time, effective, multiple-gene tumor genotype analysis, our group developed a clinical genotyping test (SNaPshot) based on a commercially available platform. SNaPshot is a multiplexed PCR-based assay designed to test >50 hot-spot mutation sites in 14 key cancer genes. The development of the SNaPshot platform focused on capturing somatic events with known or putative implications for molecularly targeted therapy and has previously been described in detail [17]. We began using SNaPshot routinely in our clinic in March 2009. This report constitutes our experience screening 589 patients during the initial 15 months of test availability.

methods

patients

NSCLC patients seen at Massachusetts General Hospital and Mass General/ North Shore Cancer Center (a satellite location) between March 2009 and May 2010 underwent clinical genotype testing at the discretion of their treating physician. The cut-off for this analysis was set to coincide with our site opening the Lung Cancer Mutation Consortium genotype testing (NCT01014286), a USA collaborative genotyping effort. When SNaPshot was initiated, only patients with adenocarcinoma were eligible. In August 2009, as the laboratory became more efficient at handling high throughput, any patient with NSCLC could be tested. All patients signed a clinical consent form and test results were entered into the medical record. Records from patients who had been successful genotyped were reviewed for demographic, clinical and pathological data under an Institutional Review Board-approved protocol. Smoking status was categorized as 'never' if <100 cigarettes were consumed per lifetime, 'former' if >100 cigarettes and smoking cessation was >1 year before lung cancer diagnosis, otherwise 'current'. Pack-years of smoking were calculated as packs per day multiplied by years of smoking. Patients undergoing a repeat biopsy at the time of acquired resistance to EGFR TKIs were excluded from this analysis as they have been reported elsewhere [18].

genotype screening

All specimens submitted for clinical genotyping were prescreened by a pathologist to confirm sufficient tumor in the sample. Genotyping was carried out on DNA derived from formalin-fixed paraffin-embedded (FFPE) tumor specimens using SNaPshot, a targeted mutational analysis assay designed by our group [17]. The SNaPshot platform from Applied Biosystems consists of multiplexed PCR and single-base extension reactions that generate fluorescent labeled probes designed to interrogate hot-spot mutation sites. The SNaPshot products are then resolved and analyzed using capillary electrophoresis. During the first year of this study, tumor genotyping was carried out with our original SNaPshot panel (SNaPshot Version 1, Table 1), as previously described [17]. In April 2010, the assay was expanded to accommodate a broader range of tumor types being genotyped, leading to the addition of some additional tests including IDH1 and HER2 genotyping (SNaPshot Version 2, Table 1), see Supplemental Methods (available at Annals of Oncology online).

FISH was carried out on FFPE tumor sections using a break apart probe to the ALK gene (Vysis; Abbott Molecular, Downers Grove, IL). Samples were classified positive for ALK rearrangement if >15% of scored tumor cells had split ALK 5′ and 3′ probe signals or isolated 3′ signals [6]. Though technically separate tests, ALK FISH analyses and EGFR and HER2 sizing assays are referred to in conjunction with the mutational analyses of SNaPshot collectively as 'SNaPshot' for convenience throughout the manuscript.

All genotyping tests were carried out in our hospital's Clinical Laboratory Improvement Amendments-certified Translational Research Laboratory. Turnaround time (TAT) was calculated as the interval from

Table 1. Summary of findings from SNaPshot assay versions 1 and 2.

Gene	Loci tested, amino acid–nucleotide	Mutations identified, n (%)
AKT1[V2]	E17–49G	—
APC	R1114–3340C	—
	Q1338–4012C	
	R1450–4348C	
	T1556fs-4666_4667insA	
BRAF		9 (100)
	V600–1798G	
	V600–1799T	V600E, 9 (100)
CTNNB1 (β-catenin)		11 (100)[a]
	D32–94G	
	D32–95A	D32A, 1 (9)
	S33–98C	S33Y, 2 (18)
	G34–101G	G34V, 1 (9)
	S37–109T	
	S37–110C	S37C, 2 and S37F, 3; total, 5 (45)
	T41–121A	T41A, 1 (9)
	T41–122C	T41I, 1 (9)
	S45–133T	
	S45–134C	
EGFR		73 (100)
	G719–2155G	G719C, 2 (3)
	T790–2369C	
	L858–2573T	L858R, 24 (33)
	Exon 19 deletions[b]	45 (62)
	Exon 20insert/del[bV2]	2 (3)
ERBB2 (HER2)[V2]		2 (100)
	Exon 20 insertions[b]	2 (100)
FLT3[V1]	D835–2503G	—
IDH1[V2]		1 (100)
	R132–394C	R132C, 1 (100)
	R132–395G	
JAK2[V1]	V617–1849G	—
KIT	D816–2447A	—
KRAS		134 (100)
	G12–34G	G12S, 5; G12R, 4; G12C, 58; total, 67 (50)
	G12–35G	G12V, 26; G12D, 19; G12A, 10; total, 57 (41)
	G13–37G	G13C, 6 (4)
	G13–38G	G13D, 6 (4)
NOTCH1	L1575–4724T	—
	L1601–4802T	
NRAS		6 (100)
	G12–34G	G12S, 1 (17)
	G12–35G	
	G13–37G	
	G13–38G	
	Q61–181C	
	Q61–182A	Q61L, 3; Q61R, 2; total, 5 (83)
	Q61–183A	
PIK3CA		22 (100)
	R88–263G	
	E542–1624G	E542K, 6 (27)

Table 1. (Continued)

Gene	Loci tested, amino acid–nucleotide	Mutations identified, n (%)
	E545–1633G	E545K, 8 (36)
	Q546–1636C	Q546K, 1 (5)
	Q546–1637A	
	H1047–3139C	
	H1047–3140A	H1047R, 6; H1047L, 1; total, 7 (32)
	G1049–3145G	
PTEN	R130–388C	—
	R173–517C	
	R233–697C	
	K267fs–800delA	
TP53		26 (100)
	R175–524G	R175H, 1; R175L, 3; total, 4 (15)
	G245–733G	G245C, 3 (12)
	R248–742C	R248W, 5 (19)
	R248–743G	R248Q, 2; R248L, 3; R248P, 1; total, 6 (23)
	R273–817C	R273C, 3; R273S, 2; total, 5, (19)
	R273–818G	R273L, 2 (8)
	R306–916C	R306X, 1 (4)

Tested loci are listed and differences between versions 1 and 2 are indicated. V1—this assay was included in SNaPshot version 1 only V2—this assay was included in SNaPshot version 2 only. The number and frequency of observed genotype alterations are listed (percent refers to the frequency of a particular mutation among all mutations identified for that gene).
[a]One patient was found to have two separate β-catenin mutations, one at locus S33–98C (S33Y) and one at S37–110C (S37F).
[b]Sizing assays were used to identify these mutations.

genotype requisition to result finalization. Thus, TAT includes the time required to obtain the pathology specimen, which had to be requested from outside institutions in some cases. Also, when the initial genotyping data did not meet clinical quality control standards due to limiting tissue amount or integrity, genotyping was repeated on DNA re-extracted from either the same tumor specimen or from an alternative paraffin block, which significantly prolonged the TAT.

statistical considerations

Summary statistics are provided regarding the demographic characteristics of the 546 patients tested. Demographic and disease characteristics were compared among patients with mutant and wild-type status for each gene using Wilcoxon, χ^2 and Fisher's exact tests as appropriate. In these analyses, the demographics of the patient corresponding with each tested tumor specimen were included; hence, the six patients with two distinct specimens genotyped were accounted for twice. Survival was analyzed via the Kaplan–Meier method and compared between groups with a log-rank test.

results

patients

From March 2009 to May 2010, 1016 patients with NSCLC were seen at Massachusetts General Hospital and 589 were referred for clinical genotyping (supplemental Figure S1,

available at *Annals of Oncology* online). Pathology prereview of the submitted FFPE specimen(s) identified adequate material in 552 (94%) cases, with 37 (6%) having insufficient tissue for genotyping; all cases passing pathology prereview were successfully tested. The 552 genotyped samples were from 546 patients, with 6 patients having two samples tested. The majority of samples ($n = 431$; 78%) were tested with SNaPshot version 1, while those sent after 19 April 2010 ($n = 100$; 18%) were tested with SNaPshot version 2, which included *AKT1*, *HER2* and *IDH1* mutations (Table 1). Nearly all samples ($n = 528$; 96%) also underwent *ALK* FISH testing. A minority of cases ($n = 21$, 4%) had *ALK* analysis only.

Patients had a median age of 64 years (range 22–89), and included 58% females, and 92% white patients, reflecting our clinic's racial homogeneity (Table 2). Twenty-four percent were never smokers. Histology was predominantly adenocarcinoma (81%), due to the initial restriction of testing to adenocarcinoma only and the overrepresentation of this tumor type in our clinic.

genotypes

Among the 552 genotyped cases, TAT (defined in 'Methods') was 2.8 weeks (range 1.0–8.9 weeks). Samples with longer TAT were more likely to have required DNA re-extraction to confirm initial test results not meeting quality control standards (7% required re-extraction among cases with TAT ≤ 2.8 weeks compared to 35% among those with TAT > 2.8 weeks; $P < 0.001$).

Mutations in at least one tested gene loci and/or translocations involving *ALK* were identified in 282 (51%) samples, while 270 (49%) had a negative screen (Table 1, Figure 1A). Twenty-five (5%) samples were positive for two mutations while two tumors had three simultaneous mutations (Figure 1B). Overall, we observed 73 (13%) *EGFR* mutations, 134 (24%) *KRAS* mutations, 27 (5%) *ALK* translocations, 26 (5%) *TP53* mutations, 22 (4%) *PIK3CA* mutations, 11 (2%) β-*catenin* mutations, 9 (2%) *BRAF* mutations, 6 (1%) *NRAS* mutations, 2 *HER2* mutations and 1 *IDH1* mutation. Of note, *IDH1* and *HER2* were assessed only in SNaPshot version 2 ($n = 100$) and hence, their frequencies are not necessarily representative.

Examining demographic and other clinical correlations with genotype (Table 2), we observed both expected and novel associations. As anticipated, patients with *EGFR* mutations were significantly more likely to be female, Asians, and have adenocarcinoma than *EGFR* wild-type patients. In our cohort, *KRAS* mutation positivity was associated with white race ($P = 0.02$), adenocarcinoma histology ($P = 0.004$) and earlier stage disease ($P = 0.002$). *ALK* translocations correlated with young age ($P < 0.001$) and possibly more advanced stage ($P = 0.06$), while *PIK3CA* mutations occurred in squamous cell cancers ($P = 0.003$). Smoking history seemed to be one of the most discriminating clinical features (Table 2, Figure 2), as low smoking was strongly associated with *EGFR* mutations ($P < 0.001$), *ALK* translocations ($P < 0.001$) and β-*catenin* mutations ($P = 0.03$), while heavier smoking history was significantly associated with mutations in *KRAS* ($P < 0.001$) and *NRAS* ($P = 0.05$).

Table 2. Demographics of the tested patients

	Overall group, n = 546	EGFR status		KRAS status		Less frequent mutations (only positive columns shown)				
		Positive (n = 73)	Wild type (n = 453)	Positive (n = 134)	Wild type (n = 395)	ALK pos. (n = 27)	B-cat pos. (n = 11)	PIK3CA pos. (n = 22)	BRAF pos. (n = 9)	NRAS pos. (n = 6)
Median age (range)	64 (22–89)	61(39–89)	64 (22–86)	65 (26–83)	63 (22–89)	57 (37–86)	61 (45–85)	62 (44–79)	64 (50–72)	67 (49–85)
Gender										
Male	228 (42)	20 (27)	197 (44)	49 (37)	169 (43)	13 (48)	3 (27)	7 (32)	3 (33)	3 (50)
Female	318 (58)	53 (73)	255 (56)	85 (63)	225 (57)	14 (52)	8 (73)	15 (69)	6 (66)	3 (50)
Race[a]										
White	503 (92)	60 (82)	424 (94)	131 (98)	357 (91)	26 (96)	10 (90)	21 (95)	8 (89)	6 (100)
Black	7 (1)	1 (1)	5 (1)	0	6 (2)	0	0	0	1 (11)	0
Asian	22 (4)	10 (14)	11 (2)	0	20 (5)	1 (4)	0	0	0	0
Smoking[a]										
Never	128 (24)	35 (48)	84 (19)	5 (4)	113 (29)	18 (67)	8 (72)	2 (9)	4 (50)	0
Former	278 (51)	30 (41)	241 (54)	81 (61)	192 (49)	7 (26)	3 (27)	12 (54)	2 (25)	1 (17)
Current	137 (25)	8 (11)	125 (28)	47 (35)	88 (22)	2 (7)	0	8 (36)	2 (25)	5 (83)
Median pack-years[a] (range)	24 (0–180)	1 (0–76)	30 (0–180)	30 (0–158)	20 (0–180)	0 (0–50)	0 (0–80)	40 (0–158)	5 (0–51)	78 (15–163)
Histology										
Adenocarcinoma	440 (81)	66 (90)	357 (79)	120 (90)	306 (77)	23 (85)	11 (100)	11 (50)	8 (89)[c]	4 (67)
Squamous	50 (9)	1 (1)	49 (10)	3 (2)	47 (12)	0	0	6 (27)	0	1 (17)
Adenosquamous	9 (2)	3 (4)	6 (1)	1 (1)	8 (2)	0	0	1 (5)	1 (11)	0
NSC-NOS	47 (9)	3 (4)	41 (9)	10 (7)	34 (9)	4 (15)	0	4 (18)	0	1 (17)
Stage[a]										
IA	107 (20)	16 (22)	89 (20)	38 (28)	70 (18)	2 (7)[b]	2 (18)	5 (23)	2 (22)	2 (33)
IB	58 (11)	10 (14)	47 (10)	14 (10)	42 (11)	1 (4)	1 (9)	3 (14)	1 (11)	0
IIA	11 (2)	0	11 (2)	3 (2)	9 (2)	2 (7)	0	0	0	0
IIB	21 (4)	1 (1)	20 (4)	10 (7)	11 (3)	0	1 (9)	1 (5)	0	0
IIIA	58 (11)	3 (4)	52 (12)	14 (10)	41 (10)	4 (15)	0	2 (9)	1 (11)	2 (33)
IIIB	47 (9)	7 (10)	38 (8)	5 (4)	40 (10)	3 (11)	2 (18)	3 (14)	0	0
IV	241 (44)	36 (49)	193 (43)	50 (37)	179 (46)	15 (56)	5 (45)	8 (36)	5 (56)	2 (33)
Metastatic pattern										
Not metastatic[c]	193 (35)	25 (34)	164 (36)	58 (43)	133 (34)	7 (26)[b]	3 (27)	7 (32)	4 (44)	3 (50)
Lungs only	97 (18)	12 (16)	80 (18)	14 (10)	78 (20)	7 (26)	4 (36)	3 (14)	1 (11)	1 (17)
CNS only	34 (6)	2 (3)	31 (7)	6 (4)	27 (7)	2 (7)	1 (9)	1 (5)	0	2 (33)
Bone only	25 (5)	5 (7)	19 (4)	8 (6)	16 (4)	3 (11)	1 (9)	0	0	0

Characteristics of the entire group (n = 546) and of the patients with tumors testing positive and wild type for each mutation are shown. Note that the overall group (data column one) includes a small number of patients who have two separate tumors accounted for in the other columns of the table. All gene mutations were tested in 552 tumors, while ALK FISH was tested in 549 tumors. Numbers in parentheses indicate percentages. Bolded data indicates that a characteristic varied significantly (P-value ≤ 0.05) among those tested for that genotype comparing the mutated with the wild-type cohorts.

[a]A small number of patients have unknown values for this variable.

[b]A characteristic varied with borderline significance (P-value >0.05 to 0.09) among those tested for that genotype comparing the mutated with the wild-type cohorts.

[c]Not metastatic implies that there was no metastatic disease at baseline nor did it develop during follow-up. All others developed metastatic disease but are listed as a specific pattern only if spread was confined to either lungs only, brain only or bones only.
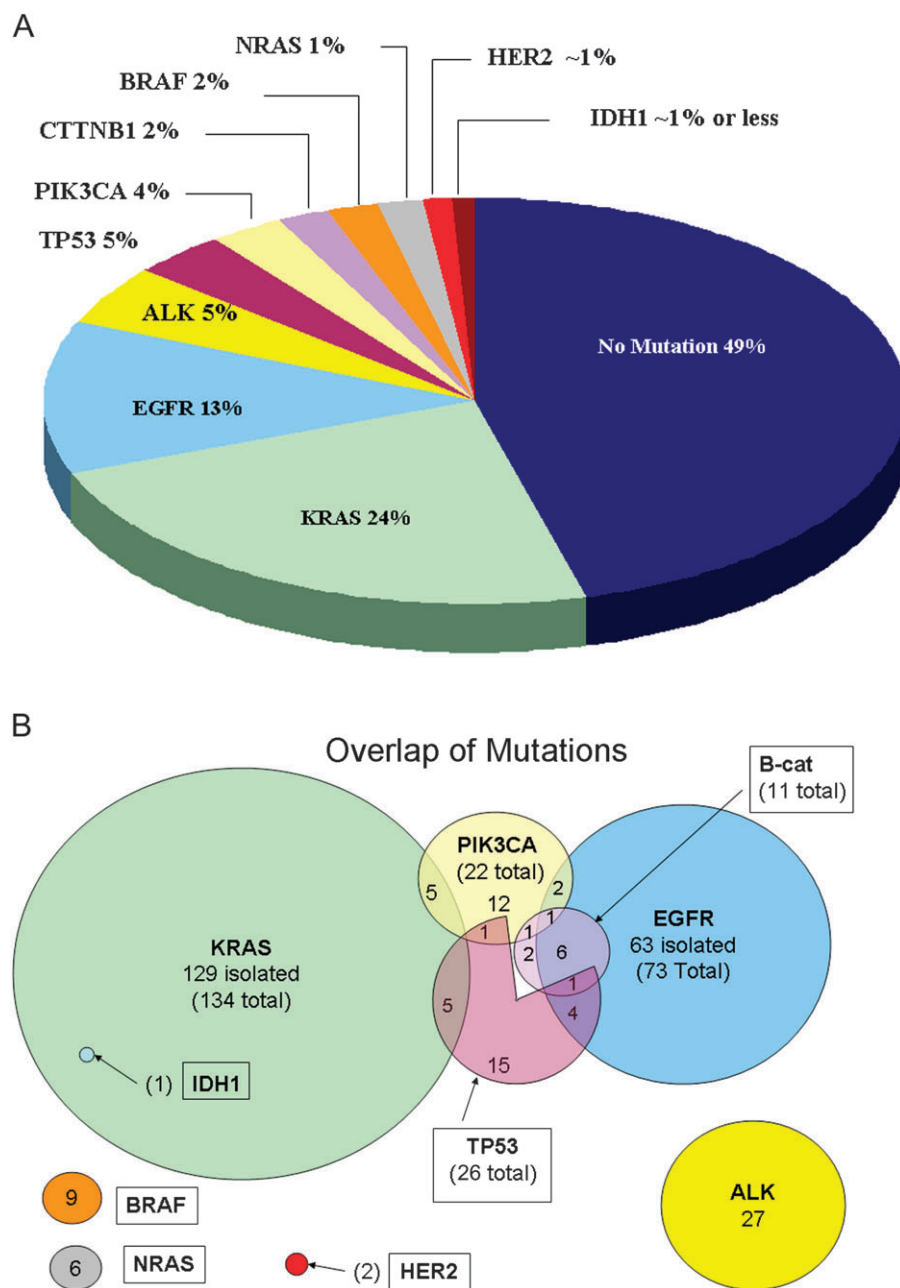
Figure 1. Distribution frequency (A) and overlap (B) of the genotypes observed. Genotypes observed are depicted in a pie chart showing frequency of each mutation with regard to all patients tested (A) as well as in a Venn diagram showing the overlap of patients with more than one mutation (B). Note that only 100 patients were screened for *IDH1* and *HER2* mutations, so the frequency depicted here may not be truly representative. Also note that *TP53* screening only encompassed a minority of the 'hot-spot' mutations described in NSCLC for *TP53*.

We identified two patients with *HER2* and one with an *IDH1* mutation. The *HER2*-mutant tumors were both stage IV adenocarcinomas in never smokers; one a 68-year-old white male and the other a 50-year-old white female. The *IDH1*-mutant tumor also harbored *KRAS* and was a stage IIIA adenocarcinoma in a 77-year-old white male former smoker with a 100 pack-year history.

survival

We examined survival estimates among the 346 patients diagnosed with advanced NSCLC (defined in this analysis as stage III or IV) and divided the analysis by genotype if there were >40 patients in each genotype (mutant and wild type), which in this cohort included *KRAS* and *EGFR*. The median follow-up time was 16.1 months, with 212 deaths observed, and the median overall survival (OS) among all 346 patients was 21.7 months (supplemental Table S1, available at *Annals of Oncology* online). There was a detriment in survival ($P = 0.04$) for those with *KRAS* mutations compared with *KRAS* wild type, with a median OS of 16.4 and 22.5 months for the two groups, respectively (Figure 3A) and an improvement in survival ($P = 0.04$) for those with *EGFR* mutations compared with
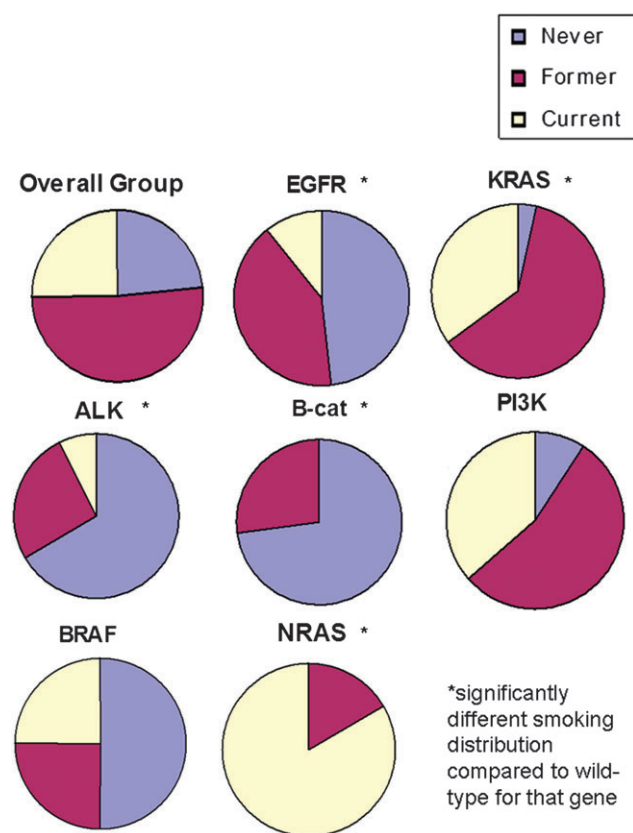
Figure 2. Smoking status distribution by genotype. The proportion of patients that were never, former and current smokers are depicted in separate pie charts representing the overall study cohort and the subset positive for each of the major mutation types.

Figure 3. Survival among patients with stage III and IV non-small-cell lung cancer by *KRAS* mutation status (A) and *EGFR* mutation status (B). Mutant patients are depicted with a solid line and wild-type patients with a dashed line.

EGFR wild type, with a median OS of 34.3 and 20.0 months, respectively, (Figure 3B). Due to lack of full information about treatment administration and responses, no multivariable adjusted analyses were carried out.

clinical implications and genotype-directed clinical trials

Six patients had two distinct tumor specimens genotyped; often, the information was useful in establishing the correct stage. Two patients underwent concurrent surgical resections of T1 tumors in different lobes and had each tumor genotyped. Distinct mutations in the resection specimens (*KRAS* G12A and G12C in one case, *KRAS* G12C and *BRAF* V600E in the other) suggested synchronous stage IA primaries (as opposed to metastatic disease) in both patients. Similarly, a third patient with stage I resected NSCLC developed a contralateral lung lesion 2 years later and underwent a biopsy. The initial tumor had *KRAS* G12R, while the subsequent NSCLC was wild type for all tested loci, supporting a second primary, and the patient was treated aggressively. Three additional patients had similar scenarios, but genotyping did not definitively affect clinical care.

Overall, 353 (65%) patients were diagnosed with stage IV NSCLC or recurred during the study follow-up period (through July 2011). Of these, 170 (48%) were found to have
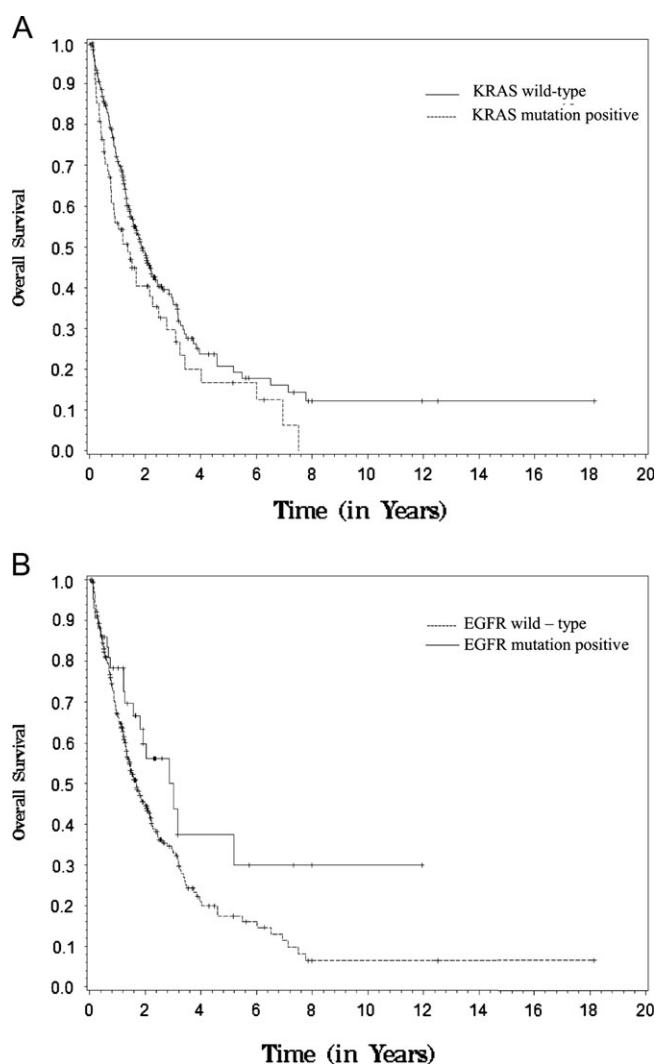
a mutation or translocation in either *EGFR* (*n* = 48), *KRAS* (*n* = 76), *ALK* (*n* = 25), *BRAF* (*n* = 5), *PIK3CA* (*n* = 14) or *HER2* (*n* = 2), which we classified as 'potentially targetable' genotypes since we had appropriate clinical trials open during the study period. Sixty-four (38%) of the patients with a potentially targetable genotype enrolled in at least one study utilizing a targeted therapy (Figure 4). The trials included examined drugs that blocked EGFR, ALK, HER2, BRAF or PI3K, or closely related downstream pathways integral to driver mutation signaling (i.e. MEK inhibitors for *KRAS* mutations). The majority (*n* = 48, 75%) of study accruals resulted directly from genotype results (in most cases, the trials were genotype specific), including 14 *EGFR*, 19 *ALK*, 8 *KRAS*, 3 *BRAF*, 3 *PIK3CA* and 1 *HER2* mutation-positive patients. Furthermore, 30 additional *EGFR*-mutant patients were treated with erlotinib 'off protocol' because of genotyping results, suggesting that a total of 78 (22%) patients (48 on trial, 30 off protocol) with advanced NSCLC
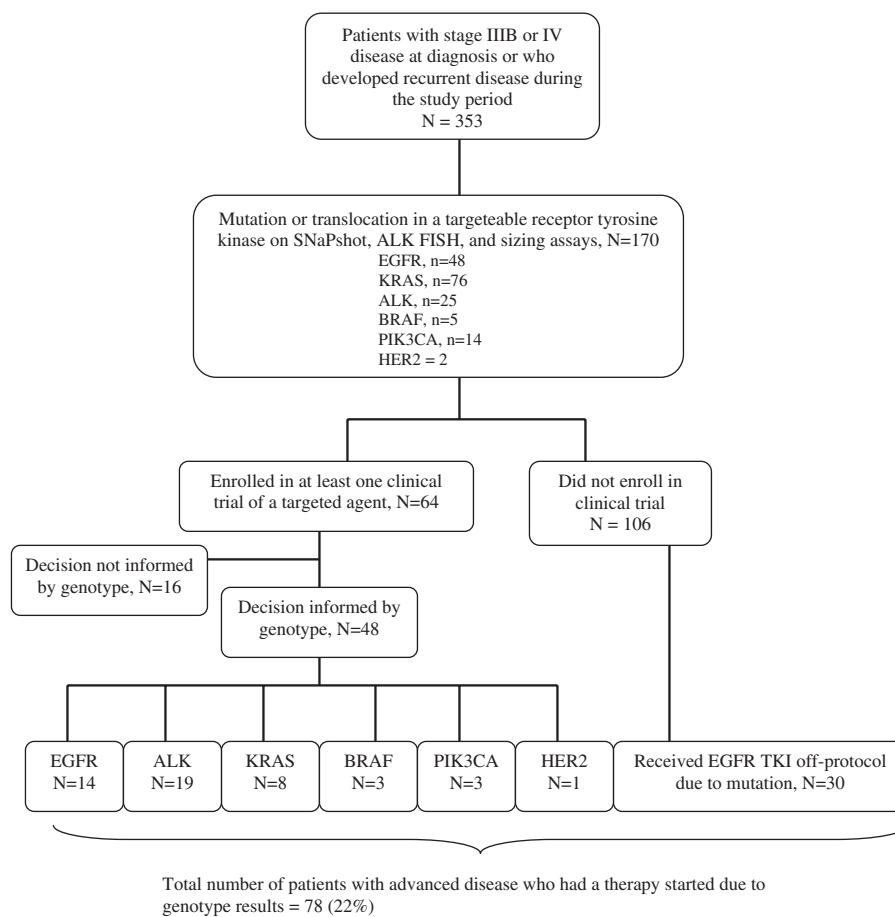
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┌─────────────────────────────┐
│ Patients with stage IIIB or IV │
│ disease at diagnosis or who    │
│ developed recurrent disease during │
│ the study period               │
│ N = 353                        │
└─────────────────────────────┘
           │
┌───────────────────────────────────────┐
│ Mutation or translocation in a targeteable receptor tyrosine │
│ kinase on SNaPshot, ALK FISH, and sizing assays, N=170 │
│ EGFR, n=48                             │
│ KRAS, n=76                             │
│ ALK, n=25                              │
│ BRAF, n=5                              │
│ PIK3CA, n=14                           │
│ HER2 = 2                               │
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Total number of patients with advanced disease who had a therapy started due to genotype results = 78 (22%)

Figure 4. Flow of patients with advanced or recurrent non-small-cell lung cancer onto genotype-directed therapies.

had therapies initiated as a direct result of genotype findings. Note that an additional five patients with early-stage *EGFR*-mutant NSCLC were enrolled on a genotype-specific trial of adjuvant erlotinib. It was not possible to assess how many additional patients were directed 'away' from therapies due to genotype findings (e.g. *KRAS*-mutant patients directed away from erlotinib), though we suspect that this occurred.

discussion

Genotyping for 'driver mutations' is becoming increasingly central to oncology care. Over the course of 15 months, we tested 552 NSCLC tumors for genotype abnormalities using a multiplexed PCR-based SNaPshot assay plus FISH for *ALK* translocations as part of routine clinical practice. To our knowledge, our center was the first in the United States to offer this type of broad screening for NSCLC patients as part of standard care [19]. We found genotype testing to be feasible within the clinical workflow, with a median TAT of 2.8 weeks, which includes the time necessary to acquire FFPE samples from outside hospitals. A full 51% of cancers tested were positive for a driver mutation, most commonly mutations in *KRAS* (24%) and *EGFR* (13%) and translocations involving *ALK* (5%). While widely agreed that it is important to identify patients with *EGFR* and *ALK* given the availability of effective therapeutics, it is also noteworthy that in a short time frame at

a single institution, we identified over 30 patients with less common mutations like *BRAF*, *PIK3CA* and *HER2*, which also have relevant candidate targeted therapies [20]. Among the patients with advanced or recurrent NSCLC seen within these 15 months, 22% began a genotype-specific therapy in response to SNaPshot results. We anticipate that this proportion should increase further in the future, as the scope of genotype-specific clinical trial efforts is rapidly broadening. Furthermore, SNaPshot provided strong evidence of multiple primary cancers in half of patients who had more than one tumor sample screened. This type of testing could significantly affect treatment decisions, especially when considering whether to pursue surgery or other therapy with curative intent versus treatment of metastatic disease. Other groups have similarly described the power of genotyping multiple lesions from the same patient [21]. Overall, we have demonstrated that broad clinical genotyping with SNaPshot can be tightly integrated into clinical practice and we believe it can make a real difference for patients.

A recent study from China examined a research-based genotyping panel in a smaller cohort of early-stage adenocarcinomas from exclusively never-smoking Asian patients [22]. They found that an impressive 90% of patients had a mutation in *EGFR*, *KRAS*, *ALK* or *HER2*. While adenocarcinoma in Asian nonsmokers appears to be almost completely defined by oncogenic driver mutations, it is quite remarkable that 51% of patients in our clinic, made up

primarily of white patients with a positive smoking history, also had mutations defined on SNaPshot. A North American lung cancer genome collaboration reported their sequencing effort of nearly 200 adenocarcinomas and found several recurrent oncogenes and tumor suppressor mutations [23]. Deep sequencing will likely represent the future of clinical genotyping; however, this option is currently neither feasible nor affordable for clinical use.

In addition to the genotypes well associated with NSCLC, we made the novel observation of an *IDH1* mutation in one patient. *IDH1* mutations have been mainly associated with glioblastoma, lower grade gliomas and acute myeloid leukemia and will likely have possible therapeutic implications in the near future [24–26]. According to compiled data from published reports, IDH1 mutations appear to be rare in NSCLC [27]. We added *IDH1* genotyping to our panel when we moved from SNaPshot version 1 to 2 primarily for its predicted utility in glioma patients, but since we utilize a single genotyping assay for all tumor types at our hospital, we were able to serendipitously observe an *IDH1* mutation in one lung cancer specimen. We also observed β-*catenin* mutations in 2% of patients, commonly in conjunction with *EGFR* mutations. β-*Catenin* has been associated with lung tumorigenesis and pulmonary blastomas, but to our knowledge has not been related to *EGFR*-mutant NSCLC [28, 29]. We found that *PIK3CA* mutations also tended to be found in combination with other driver mutations, confirming other reports [30].

As with other mutation-specific assays, SNaPshot testing is most suitable for genotyping oncogenes, which are usually affected at a very limited number of loci. Tumor suppressor genotyping is more challenging. While the SNaPshot panel was designed to capture the most commonly mutated sites in *TP53*, these represent only a fraction of the many variants reported to occur in this tumor suppressor. Thus, the 5% incidence of *TP53* mutants detected in our cohort is far lower than the reported frequency expected in NSCLC [31, 32].

A point of discussion recently has been the utility of clinical characteristics in referring patients for genotyping [33]. Our patient cohort is in line with prior literature showing that many genotypes have associated clinical features. Smoking history was one of the more discriminating demographics with low smoking correlated with *EGFR* and *ALK*, while heavier smoking was associated with *KRAS*, consistent with prior literature [22, 34–36]. We made the novel observations that low smoking is correlated with β-*catenin* mutations and heavy smoking with *NRAS*. Unlike Riely et al., who identified *KRAS* mutations in 15% of never smokers with adenocarcinoma, we saw *KRAS* in only 5 of 128 (4%) never-smoking patients [37]. We also observed known histology associations, such as adenocarcinoma among *EGFR* and *KRAS* mutants and squamous cell among *PIK3CA* mutants [38–41]. *ALK* translocations were associated with younger age and more advanced stage, while *KRAS* mutations were seen preferentially in early-stage cancers [35]. However, given the growing panel of relevant genotypes in NSCLC, clinical characteristics are no longer an efficient method for selecting which patients to test. The ability to order a single comprehensive genotyping panel, rather than specific tests *á la carte*, is crucial since clinical

features do not correlate perfectly with genotypes and trends for clinical associations often diverge for different gene mutations. Furthermore, as clinicians become more adept at incorporating genotype information into treatment-making algorithms, they may wish to know not only what genotypes are positive but also what mutations are absent. For example, we know that EGFR TKIs are most active in *EGFR* mutation-positive patients, but there is growing evidence that *KRAS* mutations predict for non-benefit from EGFR TKIs; hence, many clinicians are becoming hesitant to administer erlotinib-known *KRAS* mutants [39, 42].

The results of our study should be interpreted within the context of the retrospective observational study design, and its limitations acknowledged, including selection biases introduced by the population of patients seeking care at our institution and those in which SNaPshot was ordered. We saw interesting survival differences among stage III and IV patients by *EGFR* and *KRAS* genotype, though this analysis is crude and not corrected for other prognostic factors or treatment information. In addition, while SNaPshot provided an improvement in molecular testing over conventional molecular strategies (which have typically focused on *EGFR* and *KRAS* sequencing only), it still required a 2- to 3-week turnaround, which in some cases was prolonged by the need to retest or identify an alternative sample because the initial specimen was of poor quality. Moving forward toward a more comprehensive genetic picture of these tumors may involve expansion of the SNaPshot panels to include additional hot-spot sites and the adoption of further complementary platforms to capture not only a myriad of point mutations but also translocation events and copy number changes.

In summary, in our experience, SNaPshot tumor genotyping is a viable clinically feasible approach to support diagnostic and treatment decisions and to facilitate clinical trial enrollment. It is uncovering new therapeutic opportunities for a growing number of patients and advancing NSCLC management at our institution.

disclosure

Lecia Sequist has consulted for Clovis Oncology, Merrimack Pharmaceuticals, Daiichi-Sankyo and Celgene. Alice Shaw has consulted for Pfizer, Ariad and Chugai. Tom Lynch is a joint holder for a patent for *EGFR* mutation testing. John Iafrate has consulted for Pfizer and Abbott Molecular. Jeff Engelman has consulted for Agios. Leif Ellisen, Darrell Borger, John Iafrate and Dora Dias-Santagata are consultants for Bioreference Laboratories, which has licensed the SNaPshot technology. John Iafrate and Dora Dias-Santagata submitted a patent for the SNaPshot tumor genotyping assay (pending).

None for all other authors have anything relevant to declare, which includes RSH, PF, RR, JST, ITL, SD, BAW, EB, ST, LM, AMuz, SBG, JG, CLC, JCW, HG, DMD, AMun, CW, HW, DJM, NCC, JB, ML, and MM-K.

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