

Clinical Genotyping of Non–Small Cell Lung Cancers Using Targeted Next-Generation Sequencing: Utility of Identifying Rare and Co-mutations in Oncogenic Driver Genes¹



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Abstract

Detection of somatic mutations in non–small cell lung cancers (NSCLCs), especially adenocarcinomas, is important for directing patient care when targeted therapy is available. Here, we present our experience with genotyping NSCLC using the Ion Torrent Personal Genome Machine (PGM) and the AmpliSeq Cancer Hotspot Panel v2. We tested 453 NSCLC samples from 407 individual patients using the 50 gene AmpliSeq Cancer Hotspot Panel v2 from May 2013 to July 2015. Using 10 ng of DNA, up to 11 samples were simultaneously sequenced on the Ion Torrent PGM (316 and 318 chips). We identified variants with the Ion Torrent Variant Caller Plugin, and Golden Helix's SVS software was used for annotation and prediction of the significance of the variants. Three hundred ninety-eight samples were successfully sequenced (12.1% failure rate). In all, 633 variants in 41 genes were detected with a median of 2 (range of 0 to 7) variants per sample. Mutations detected in *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *NRAS*, and *PIK3CA* were considered potentially actionable and were identified in 237 samples, most commonly in *KRAS* (37.9%), *EGFR* (11.1%), *BRAF* (4.8%), and *PIK3CA* (4.3%). In our patient population, all mutations in *EGFR*, *KRAS*, and *BRAF* were mutually exclusive. The Ion Torrent AmpliSeq technology can be utilized on small biopsy and cytology specimens, requires very little input DNA, and can be applied in clinical laboratories for genotyping of NSCLC. This targeted next-generation sequencing approach allows for detection of common and also rare mutations that are clinically actionable in multiple patients simultaneously.

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Introduction

Lung cancers are broadly classified as small cell or non-small cell cancers (NSCLCs), with NSCLCs further subtyped largely on the basis of histologic features and immunohistochemistry profile. NSCLCs include adenocarcinoma (ADC), squamous cell carcinoma (SqCC), large cell carcinoma, and other less common subtypes (e.g., adenosquamous carcinoma and sarcomatoid carcinoma) [1]. The genomic profile of NSCLC is highly variable both across and within histologic subtypes [2,3].

Incorporation of molecular analysis in the pathologic evaluation of nonsquamous NSCLC is now considered the standard of care in clinical practice [4–6]. Once the molecular profile of a tumor is known, the appropriate use of targeted clinical therapies or eligibility for clinical trials can be determined. It is desirable to have the ability to analyze several genes simultaneously to assess for the presence of a known clinically actionable variant in a tumor. In cases without clinically actionable mutations, it is also beneficial to document the genomic profile of a tumor should a targeted therapy be discovered. In addition, immunotherapies may be an alternative therapeutic option for patients who lack known actionable mutations, forming another pathway to targeted therapy.

Next-generation sequencing (NGS) is one testing modality that can detect multiple gene variants simultaneously, allowing for the precise diagnosis of a tumor at the genetic level. The Ion Torrent platform can be used in the clinical laboratory for sequencing of NSCLC, among other cancer types, in an efficient and cost-effective manner. In many instances, only a small biopsy or cytology specimen is available for molecular testing; therefore, the ability to detect known targetable driver mutations from a small amount of input DNA is often required. Here, we present our experience with NGS using the Ion Torrent Personal Genome Machine (PGM) to detect somatic mutations in NSCLC; this assay covers 2855 COSMIC-cited mutations in 50 cancer-related genes.

Methods

All NSCLCs with a diagnosis of ADC or poorly differentiated NSCLC, favor ADC (small biopsy and cytology samples), and adenosquamous carcinoma or those in which adenosquamous carcinoma cannot be excluded are reflexively genotyped at our institution. In May 2013, our laboratory introduced a targeted NGS panel, the Ion AmpliSeq 50-gene Cancer Hotspot Panel v2, for this purpose followed by reflex *ALK* fluorescence *in situ* hybridization testing for tumors that are negative for *EGFR*, *KRAS*, and *BRAF* [4]. Rarely, an NSCLC of other histology (sarcomatoid, SqCC, or large cell neuroendocrine carcinoma) was also tested on a per-request basis. Institutional review board approval, including a waiver of consent, was granted to review our genotyping experience in lung cancer.

DNA Extraction

Hematoxylin-eosin-stained slides of resection specimens, biopsy, or fine needle aspiration (FNA) cell blocks were reviewed to determine the area of tumor for extraction and the percent tumor content within that area; a minimum of 10% tumor content was considered acceptable for further processing. DNA was extracted from 8 unstained sections (4 μ m) using a manual extraction, the Gentra Puregene Kit (Qiagen Inc., Valencia, CA), or the QIAamp DNA FFPE Tissue Kit automated on the Qiacube Instrument (after August 2015) (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions following deparaffinization with xylene.

DNA quantification was performed using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's directions on a TECAN microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Sequencing

The validation of this targeted panel was previously reported [7]. Briefly, bar-coded libraries were prepared using 10 ng of DNA. Up to 11 samples were simultaneously sequenced on the Ion Torrent PGM (Life Technologies, Rockville, MD) (316 and 318 chips) using the manufacturer's recommended protocol. The Ion Torrent Variant Caller Plugin v4.0 was used to align reads to the reference genome hg19. All variant calls were initially filtered to remove benign polymorphisms and noncoding and synonymous.

Variants. Golden Helix SNP and Variation Suite software v.8.2.1 was used for annotation and prediction of the significance of the variants. A report detailing the detected variants and resultant amino acid changes was included in each patient's medical record.

Quality Assurance Metrics

At various steps in the sequencing process, all samples were subject to six quality assurance measures (Table 1): minimum tumor cellularity, DNA quantification, DNA quality, library quantification, ISP quantification, and data analysis metrics [8].

Results

Four hundred fifty-three (453) samples from 407 individual patients were submitted for sequencing from May 2013 to July 2015. There were a total of 204 females and 203 males. All tumors were diagnosed as NSCLC; the vast majority were ADC or poorly differentiated carcinoma, favor ADC ($n=437$). Additional histologic types included cases where adenosquamous carcinoma could not be excluded ($n=8$), squamous cell carcinoma ($n=4$), sarcomatoid carcinoma ($n=3$), or large cell neuroendocrine carcinoma ($n=1$).

Quantity Not Sufficient (QNS) Cases

Overall, 55 of 453 samples tested (12.1%) from 48 individual patients had an insufficient amount of material for sequencing; all of these were of the ADC subtype. Of these, the lung core biopsies had the highest failure rate comprising 42% (23/55) of all QNS specimens (Figure 1A). Twenty-two of the patients whose sample originally failed processing underwent sequencing with a different sample in which 19 (86.4%) were successfully sequenced on the second attempt. Twenty-six of the patients with QNS samples did not undergo additional testing during the study period. In total, 378 of 407 patients (93%) had sequencing results.

Sequencing Results

Sequenced samples ($n=398$) were from primary and metastatic sites and consisted of the following: lung core biopsies ($n=110$, 28%), regional lymph node (LN) FNA ($n=92$, 23%), excision of the primary lung tumor ($n=59$, 15%), biopsies of metastatic sites (other than regional LN) ($n=51$, 13%), lung FNAs ($n=38$, 9%), cell block of a fluid (pleural/pericardial) ($n=21$, 5%), outside consult cases sent for molecular testing ($n=18$, 4%), and regional LN biopsies or excisions ($n=9$, 2%) (Figure 1B). Metastatic sites included the following: abdominal metastasis (1), adrenal (4), bone (2), brain (22), epidural tumor (1), paraspinal tumor (2), gastroesophageal junction metastasis (1), inguinal LN (2), liver (4), neck LN (3), kidney (3), pleural biopsy (3), skin metastasis (2), and spleen (1). Among the

Table 1. Quality Assurance Metrics Applied during the Preanalytic and Analytic Processing and Analysis of the Samples

| QA Measure | Method | Acceptable Criteria |
|---------------------------|---|--|
| Minimum tumor cellularity | Pathologist review | >10% |
| DNA quantification | PicoGreen | 1.7 ng/μl of DNA* |
| DNA quality | Kapa Library Quantification Kit (Kapa Biosystems, Wilmington, MA) | Q129/Q41 > 0.4* |
| Library quantification | qPCR (7500 Fast Real-Time PCR System) | (100 pM each) and Pooled (100 pM) |
| ISP quantification | qPCR (Qubit 2.0 Fluorometer, Life Technologies) | 10% TO 30% (<10%: FAILED E-PCR >30%: POLYCLONAL AMPLIFICATION) |
| Data analysis metrics | Ion Variant Caller Plug-in Golden Helix SVS | Postsequencing metrics were established at the run, sample, and variant levels.† |

* Samples must fail both DNA concentration and KAPA to be deemed quality not sufficient.

† For each run, the following sequencing metrics were verified: chip loading (>70.0%), usable sequences (>55.0%), polyclonality (<35.0%), and low-quality reads (<20.0%). For each individual sample, the metrics assessed were on-target reads (>90.0%), coverage uniformity (>90.0%), and ≥95% amplicons with 500× coverage (to avoid amplicon dropouts and false negatives). And finally, for each variant, the metrics assessed were coverage ≥500×, allelic frequency of ≥5%, and strand bias between 0.40 and 0.59.

successfully sequenced samples, 633 variants in 41 genes were detected (Figure 2) with a median of 2 variants and a range of 0 to 7 variants per case (45 samples with 0 variant [wild type]; 145 samples with 1 variant; 132 samples with 2 variants; 58 samples with 3 variants; 15 samples with 4 variants; 2 samples with 5 variants; and 1 sample [the LCNEC case] with 7 variants).

Mutations detected in *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *NRAS*, and *PIK3CA* were considered potentially actionable. For the purpose of this manuscript, we defined actionable as any variant that either has an FDA-approved therapy assigned to it or for which there is a clinical trial indication. Such actionable mutations were identified in 237 samples, most commonly in *KRAS* ($n=151$, 37.9%), *EGFR* ($n=44$, 11.1%; excluding 6 secondary p. T790M mutations), *BRAF* ($n=19$, 4.8%) and *PIK3CA* ($n=17$, 4.3%). Potentially actionable mutations were detected in 60% of samples (237/398). The breakdown of the specific mutations identified in the genes considered actionable is shown in Figure 3 and Table S1. One hundred sixteen samples had variants detected in other genes that are currently not clinically

actionable, and 45 samples had no mutations detected with the 50 gene panel.

The vast majority of identified potentially actionable mutations were single nucleotide variants (205) followed by insertions and deletions (31) and only 1 frame shift mutation. Within *EGFR*, common and rare mutations were detected in exons 18 to 21 including known activating mutations p. G719C, exon 19 deletions, and p. L858R as well as resistance mutations including p. L747S, p. L761I, exon 20 insertions, p. T790M, and p. L861Q. The 6 samples that had p. T790M mutations also had a coexisting exon 19 deletion, consistent with acquired resistance. Interestingly, within the 1 sample that harbored *EGFR* p. L747S, a described acquired resistance mutation, no other mutations were identified; it is currently not known if this patient was tyrosine kinase inhibitor (EGFR TKI) naive. Four *ERBB2* exon 20 mutations and 2 *NRAS* codon 61 mutations were identified. And in *BRAF*, 19 mutations were identified, 7 of which were p. V600E (37%) with 10 (53%) occurring in exon 11.

We also identified co-occurrence of some of the most frequently altered and clinically significant genes (Figure 4). Not surprisingly, *TP53* mutations co-occurred with mutations in *KRAS*, *EGFR*, *BRAF*, *PIK3CA*, *NRAS*, and *STK11*. *STK11* mutations were most commonly seen in association with *KRAS* mutations. *PIK3CA* mutations were only rarely identified co-occurring with other driver mutations in *KRAS* or *EGFR*. Similar to prior reports in lung ADC, all mutations in *EGFR*, *KRAS*, and *BRAF* were mutually exclusive in our patient population. Interestingly, we also noticed a mutually exclusive pattern among some additional genes: *SMO*, *SMARCB1*, *SMAD4*, *RET*, *RBI*, *PTPN11*, *PTEN*, *PIK3CA*, and *PDGFRA* (which is currently of uncertain significance).

Patients with Multiple Tumors Tested

Although most of the patients who had testing performed on multiple samples were due to an insufficient quantity of material on the first sample, we did have a cohort of patients who had multiple tumors tested. Four patients had multiple synchronous lung tumors tested: three bilateral and one ipsilateral. In all four cases, divergent mutational profiles were identified (Table 2). Eight patients either had the primary lung tumor and metastatic sites (four patients) or multiple metastatic sites tested. Metastatic sites included LN metastases, pleural fluid or biopsy, adrenal and liver metastases, brain metastases, and a skin metastasis. Among these tumors, there were a consistent mutational profile among primary tumors and metastatic sites and identification of *EGFR* p. T790M resistance mutations in the metastatic tumors of two patients (Table 2).

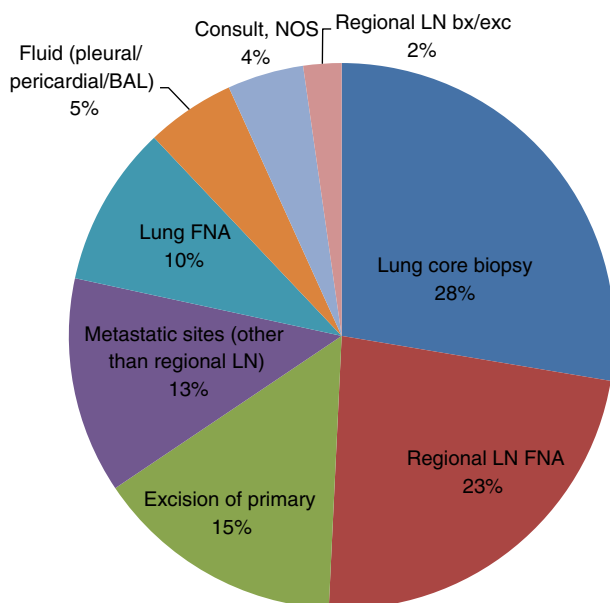


Figure 1. Distribution of sample types that were successfully sequenced ($n=398$). LN – lymph node; FNA – Fine needle aspiration.

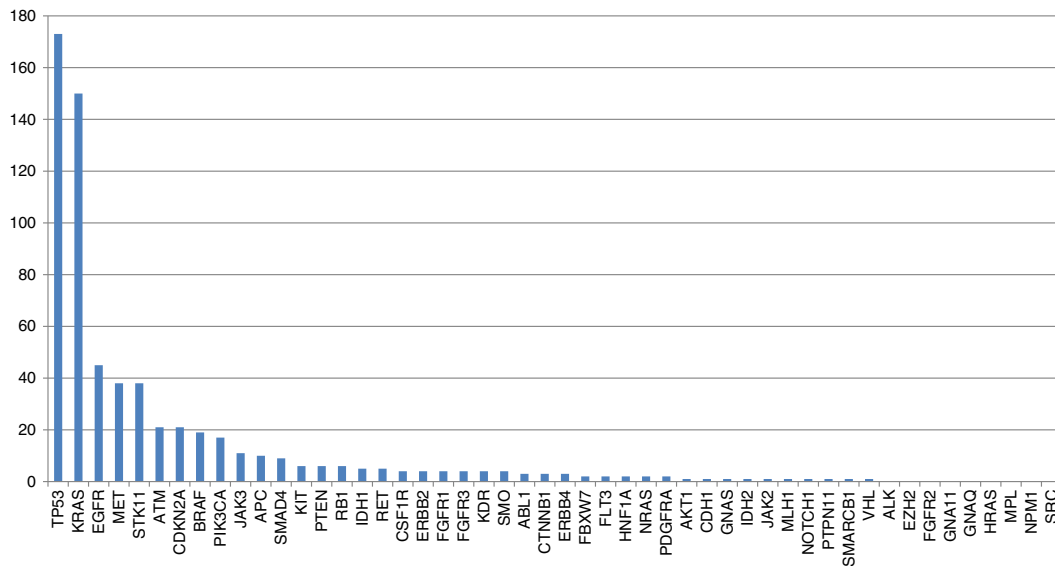


Figure 2. Number of cases with variants detected in the respective 50 genes included in the NGS assay.

Discussion

Next-generation sequencing is a powerful tool that allows for testing multiple targets in multiple genes in multiple patients simultaneously. NGS assays are rapidly being adopted in clinical laboratories for this purpose, replacing individual single gene assays and expanding the testing capacity that can be performed on relatively small amounts of tissue. Here, we report from our experience using the AmpliSeq 50 gene Cancer Hotspot Panel v2 in the clinical testing of a large number of lung adenocarcinomas. The Ion Torrent platform requires very little input DNA (10 ng), thus allowing for successful testing of small biopsy and FNA specimens. As we showed, we had a very high success rate with these samples. Surprisingly, our highest failure rate was with lung core needle biopsies. We speculate that this may in part be due to challenges of getting the fixed tumor cells into solution when associated with a fibrous background/scar as opposed to FNA cell blocks where the cells are already in solution prior to fixation.

Additionally, many of the lung core biopsy procedures at our institution have immediate onsite cytologic assessment performed via touch imprint preparations. Transfer of tumor cells to the cytology slides may result in some decreased tumor cellularity in the formalin-fixed paraffin tissue block. Requesting additional passes, minimizing tissue “rolling,” and preparing unstained slides upfront are measures that we have utilized to increase molecular testing yield.

In our population, we identified a higher percentage of *KRAS* mutations (37.9% vs 28% and 26%) and fewer *EGFR* mutations (11.1% vs 14% and 20%) than reported by Hageman et al. and Dogan et al., respectively [9,10]. This may be due to different prevalence of smoking and ethnicity in the different sample sets or selection bias of the various populations or testing methods (for instance, we tested multiple samples from the same patient in several instances).

Using this targeted NGS panel, we identified clinically actionable rare mutations that have specific therapeutic significance that would

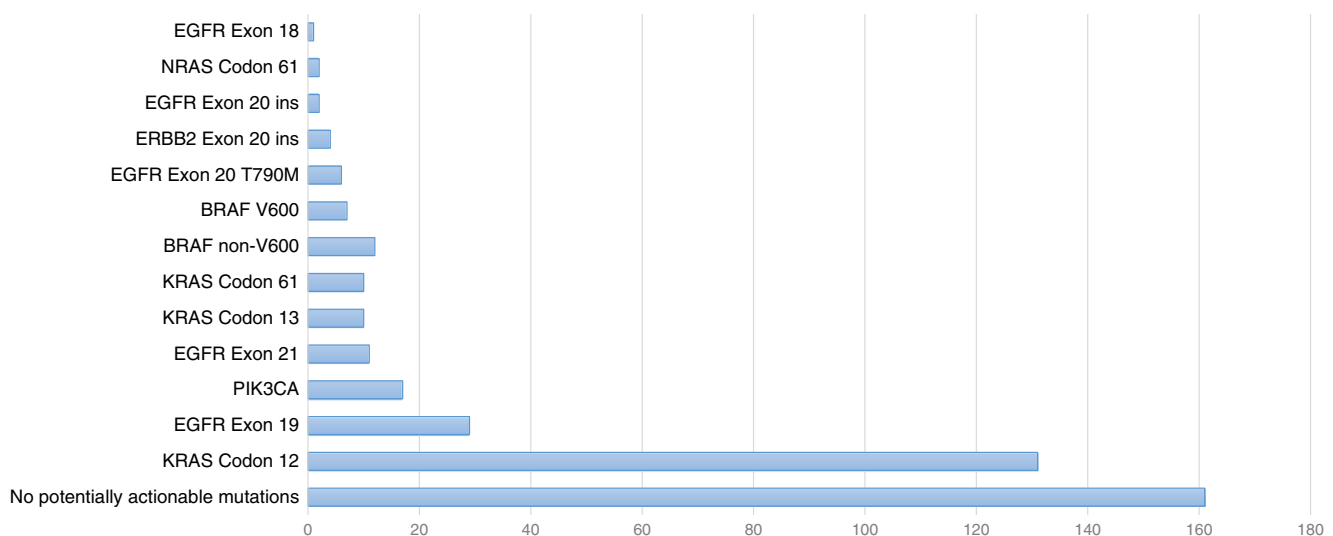


Figure 3. Clinically actionable mutations identified.

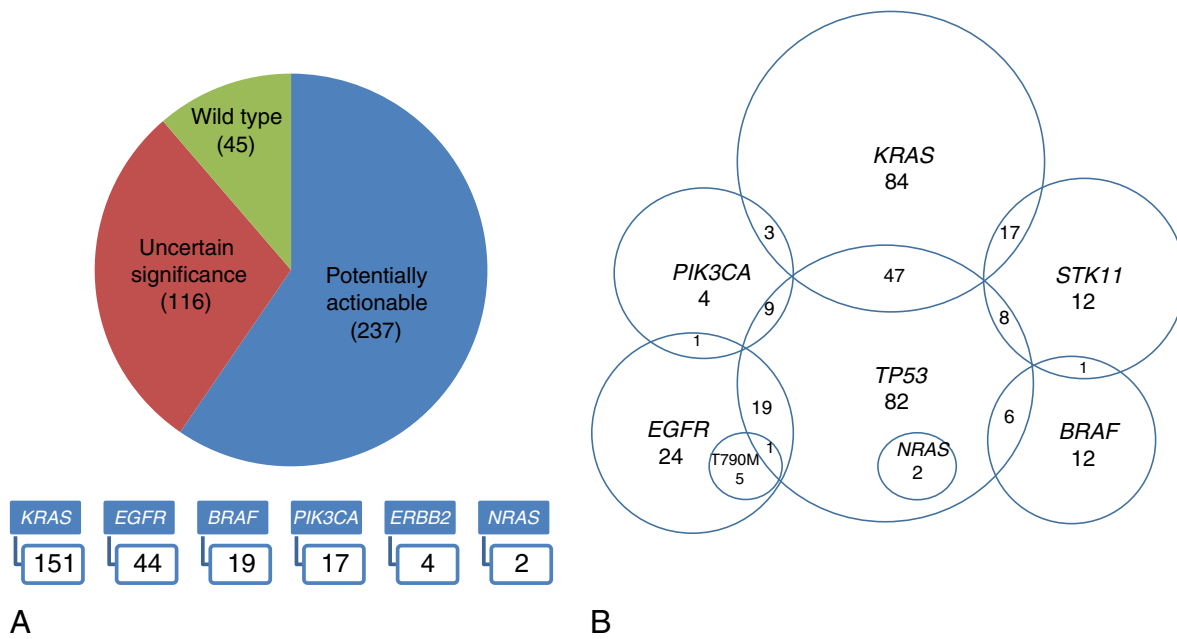


Figure 4. Co-occurrence of clinically actionable mutations.

have gone undetected using single gene assays previously used in our laboratory. Examples include detection of *EGFR* and *ERBB2* exon 20 insertions, *EGFR* p. T790M secondary resistance mutations, as well as additional variants within *EGFR*, *KRAS*, and *BRAF* (Table S1 and Figure 3) [11]. Recently, the efficacy of afatinib, a second-generation irreversible TKI, was reviewed in 75 patients with uncommon *EGFR* mutations that had been enrolled in the LUX-lung trials 2, 3, and 6. Whereas afatinib was active in patients with uncommon mutations including p. L861Q (which is typically resistant to first-generation TKIs) and p. G719X, the response rate was low in tumors with exon 20 insertions [12]. *EGFR* exon 20 insertions are biologically different than common *EGFR* mutations because they do not affect the ATP-binding pocket of the kinase domain but rather the c-helix domain. This results in increased kinase activity but decreased affinity to TKIs [12,13]. Alternative therapies for these patients are needed. Currently, a phase 2 clinical trial of a heat shock protein 90 inhibitor (AUY922) is under way in patients with *EGFR* exon 20 insertion mutations (NCT01854034). Another *EGFR* mutation we detected,

an exon 7 p. A289V, was classified as a variant of uncertain significance; this mutation in the *EGFR* extracellular domain is commonly seen in glioblastomas, but the significance is uncertain in NSCLC [14].

ERBB2 exon 20 insertion mutations are identified in a small proportion of ADCs (approximately 2%) and also define a distinct subset of tumors. The most common mutation is a 12-bp insertion (p. A775 G776insYVMA) found in 50% to 80% of *ERBB2* mutant lung cancers [15,16]. *ERBB2* exon 20 mutations are mutually exclusive with common *EGFR* and *KRAS* mutations and are distinct from tumors showing *ERBB2* amplification [17]. *ERBB2* targeted therapies for this indication are available including lapatinib, dacomitinib, and afatinib (NCT02369484) [17].

In *BRAF*, we identified 19 mutations; however, only 7 of these (37%) were p. V600E and 10 (53%) occurred in exon 11. These findings are consistent with those in a recent report by Carter et al. that demonstrated a broad spectrum of unique *BRAF* mutations in lung cancers with a high prevalence of mutations located in exon 11

Table 2. In a Select Number of Patients, Multiple Tumors Were Tested: Mutations Identified in Four Cases with Synchronous Tumors and Eight Cases with Metastatic Tumors

| Synchronous Tumors | Site | Mutations Identified | Site | Mutations Identified | Site | Mutations Identified |
|--------------------------|----------------|--|-------------------------|---|------------------|--|
| Patient S1 | Lung, left | <i>EGFR</i> L858R | Lung, right | <i>TP53</i> H61R | | |
| Patient S2 | Lung, left | <i>KRAS</i> G12 V; <i>ATM</i> G2869R | Lung, right | <i>KRAS</i> G12C; <i>CCNTB1</i> S37C | | |
| Patient S3 | Lung, left | <i>KRAS</i> G12 V; <i>CDKN2A</i> c.151-2 A > T | Lung, right | <i>STK11</i> D194Y | | |
| Patient S4 | Lung, right | <i>KRAS</i> G12C; <i>TP53</i> R158L | Lung, right | <i>PIK3CA</i> R335S; <i>TP53</i> G245C | | |
| Metastatic tumors | | | | | | |
| Patient M1 | LN | <i>EGFR</i> ex19 del | Pleural fluid | <i>EGFR</i> ex 19 del; <i>EGFR</i> T790 M | Pleural fluid | <i>EGFR</i> ex 19 del; <i>EGFR</i> T790 M |
| Patient M2 | Lung, right | <i>EGFR</i> ex 19 del | Pleural fluid | <i>EGFR</i> ex 19 del | | |
| Patient M3 | Pleural fluid | <i>EGFR</i> ex 19 del; <i>TP53</i> H193Y | Adrenal metastasis | <i>EGFR</i> ex 19 del; <i>TP53</i> H193Y | Liver metastasis | <i>EGFR</i> ex 19 del; <i>EGFR</i> T790 M; <i>TP53</i> H193Y |
| Patient M4 | Lung | None detected | Subcutaneous metastasis | None detected | | |
| Patient M5 | Lung | <i>ERBB2</i> ex 20 ins; <i>TP53</i> Y88C | Brain metastasis | <i>ERBB2</i> ex 20 ins; <i>TP53</i> Y88C | | |
| Patient M6 | Lung | <i>FGFR1</i> A268P; <i>TP53</i> A159P | LN | <i>FGFR1</i> A268P; <i>TP53</i> A159P | | |
| Patient M7 | Pleural biopsy | <i>TP53</i> P190L | LN | <i>TP53</i> P190L; <i>PTEN</i> Y16* | | |
| Patient M8 | LN | <i>KRAS</i> G12C; <i>IDH1</i> R132L | Brain metastasis | <i>KRAS</i> G12C; <i>IDH1</i> R132L | | |

S, synchronous tumors; M, metastatic tumors; LN, lymph node.

(41%) [18]. The response to selective BRAF inhibitors in patients with non-codon 600 mutations is unclear, although some report that tumors with exon 11 or mutations impairing the kinase activity are predicted to be unresponsive to current BRAF inhibitors [18,19]. However, preclinical studies suggest that dasatinib and MEK inhibitors with or without a BRAF inhibitor may have efficacy in such patients [18,20]. Presently, patients with non-V600 mutations are also eligible for an NCI-Match trial with the MEK inhibitor trametinib (NCT02465060). We also detected mutations in *KIT*, *IDH1*, *IDH2*, *PTEN*, *CDKN2A*, and *JAK2* that are well characterized in other tumor types and may allow patients to meet eligibility criteria for novel clinical trials. Patients with mutations that not known to be actionable are referred to the Norris Cotton Cancer Center Molecular Tumor Board [21].

The detection of multiple, co-occurring, potentially actionable mutations in an individual tumor represents an advance in molecular pathology with possible significant clinical, therapeutic, and research implications based on the different combinations of mutations. Eng et al. recently published their data on the impact of concurrent *PIK3CA* mutations with other oncogenic driver mutations on response to therapy. Overall, they found that a concurrent *PIK3CA* mutation was a poor prognostic factor in *EGFR* or *KRAS* mutant lung ADC, although it did not significantly alter the benefit of EGFR TKI therapy in the *EGFR* mutant patients [22]. However, co-occurrence with *KRAS*, as was identified in three cases in our study, may in fact be a contraindication for targeted therapies as mutations in both of these genes effect the PI3K-AKT pathway and alternative therapies such as immunotherapies may be more effective. Overall, among *EGFR* and *KRAS* mutant lung cancers, those with a concurrent *PIK3CA* mutation are unique with prognostic and predictive implications.

STK11 mutations, commonly identified in lung ADC, often coexist with *KRAS* mutations and likely also have a confounding prognostic significance. Pécuchet et al., who examined a cohort of 567 resected nonsquamous NSCLC patients and validated their findings in 2 publically available datasets, found that patients with *STK11* mutations, specifically in exons 1 and 2, had a significantly worse prognosis than wild-type tumors or tumors with exon 3 to 9 mutations [23]. Recent reports also suggest that co-occurring genomic alterations can define heterogeneous subsets of *KRAS*-mutant lung ADC with distinct clinical implications [24]. Skoulidis et al. describe three robust subsets of *KRAS*-mutant ADC dominated by co-occurring mutations in *STK11*, *TP53*, and *CDKN2A/B* inactivation. They identified differences in drug sensitivity patterns including expression of PD-L1 and susceptibility to HSP90-inhibitor therapy, significant findings given the challenges to date of therapeutically targeting *KRAS*-mutant lung ADC [24].

In our study, we have shown the utility of the Ion Torrent Ampliseq technology for clinical genotyping of NSCLC which requires very little input DNA and can successfully be performed on small biopsy and cytology specimens. This targeted NGS approach allows for detection of common and also rare clinically actionable mutations and profiles of co-mutations in multiple patients simultaneously.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2016.07.010>.

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