Identifying a wide range of actionable variants using capture-based ultra-deep targeted sequencing in treatment-naive patients with primary lung adenocarcinoma

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Abstract: Precision medicine requires accurate multi-gene clinical diagnostics. In current clinical practice, the minimum confidence threshold for variant calling of targeted next-generation sequencing (NGS) on surgical specimens is set to 2%-5%. However, few studies have been conducted to identify a wide range of actionable variants using capture-based ultra-deep targeted sequencing, which has a limit of detection (LOD) of 1%. The AmoyDx® Essential NGS panel for capture-based ultra-deep targeted sequencing (dual-indexed sequencing adapters with UMIs) was performed on 372 surgical specimens obtained from treatment-naive patients with primary lung adenocarcinoma, to detect actionable somatic driver mutations associated with each patient. Single-nucleotide variants, insertion/deletion events, and rearrangements were reported. Amplification-refractory mutation system (ARMS) assay and fluorescence in situ hybridization (FISH) were performed for the validation of hotspot mutations in EGFR and ALK, ROS1, and RET fusions. Potentially actionable variants were identified in 80.5% (352/437) of the nonsynonymous variants that were able to be sequenced, and were most commonly found in EGFR mutations (59.7%, 261/437), followed by KRAS mutations (5.5%, 24/437), PIK3CA mutations (3.7%, 16/437), ALK rearrangements (3.4%, 15/437), BRAF mutations (2.7%, 12/437), ERBB2 mutations (2.5%, 11/437), and RET rearrangements (2.3%, 10/437). A total of 7.2% (28/372) of the samples had multiple actionable mutations. Among the 93 triple-negative cases, which did not harbor mutations in EGFR, KRAS, or BRAF, gene fusions were detected in 26 cases (28%). Of the 328 samples, concordance of EGFR between the ARMS assay and NGS was observed in 318 samples (97.0%), and among 32 samples, concordance between ARMS/FISH test and NGS for ALK/ROS1/RET fusion genes was observed in 30 samples (93.8%). Here, we demonstrated that the capture-based ultra-deep targeted sequencing method, which has a LOD of 1% to profile a wide range of actionable variants in surgical specimens of treatment-naive lung adenocarcinoma patients, highlights the need for treatment-naive patients to undergo genomic profiling.

Keywords: Next-generation sequencing, unique molecular identifiers, amplification-refractory mutation system, lung adenocarcinoma, driver gene alterations

Introduction

Lung cancer is the most common cause of cancer-related deaths in China and many other countries. Targeted therapies directed at tumor cells harboring activating epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) fusions and v-ros UR2 sarcoma virus oncogene homolog 1 (ROS1) fusions have revolutionized the landscape of lung adenocarcinoma treatment [1-4]. In addition to EGFR, BRAF, ALK, and ROS1, there are several other abnormalities that could potentially be treated with drugs already approved for other malignancies or investigational agents, such as ERBB2, MET, and KRAS mutations and RET fusions [5-7].

In current clinical practice, single-gene assays, including, but not limited to, amplification-refractory mutation system (ARMS), Sanger sequencing, and fluorescence in situ hybridiza-
tion (FISH) are often applied in treatment-naïve patients to outline driver mutations, such as ALK and ROS1 fusions and EGFR mutations, to guide treatment decisions [8, 9]. These patients often undergo several single-gene tests to identify driver mutations. However, each of these conventional techniques is associated with its own disadvantages, including limitations in detecting certain types of aberrations, their low-throughput nature, and low sensitivity. Furthermore, serial testing takes time and depletes tumor tissue.

Single-gene assays have been challenged by more efficient next-generation sequencing (NGS) approaches. NGS allows for large-scale parallel sequencing and has been proven to be an accurate and effective tool for the parallel profiling of a large number of gene alterations including substitution mutations, insertion/deletion mutations, fusions, and amplifications [10]. It also allows the identification of novel mutations that cannot be identified by methods such as ARMS. Targeted NGS enables the generation of reliable data with sufficient sequencing depth in the targeted genes of interest [11]. But in addition to sufficient sequencing depth of coverage, it is also imperative to generate specific and sensitive data because NGS can produce erroneous results secondary to formalin-fixation artifacts [12], chemistry sequencing errors [13], or suboptimal coverage, and/or variant calling [14]. In current practice, the minimum confidence threshold for variant calling of targeted NGS on surgical specimens is set to 2%-5% [15-17].

Advances in bioinformatic field support have led to the development of NGS with high sensitivity, such as unique molecular identifiers (UMIs). Typically, PCR duplicates are identified as sequence reads that align to the same genomic coordinates using reference-based alignment. However, identical molecules can be independently generated during library preparation [18]. Incorporation of UMI adapters can improve accuracy and sensitivity by precisely remove bona fide PCR duplicates [18, 19]. By increasing the depth of sequencing and incorporation of UMI adapters, some less common and low frequency mutations can be discovered.

In this study, we performed capture-based ultra-deep targeted sequencing (dual-indexed sequencing adapters with UMIs) on 372 surgical specimens obtained from treatment-naïve patients using the AmoyDx® Essential NGS panel (Amoy Diagnostics, Xiamen, China), which has limit of detection (LOD) of 1%, to identify actionable somatic driver mutations associated with each patient. We report here the sequence findings and validation using an ARMS assay (Amoy Diagnostics, Xiamen, China) and FISH in accordance with the manufacturer’s protocol.

Materials and methods

Patient selection and ethics statement

375 surgical specimens of treatment-naïve patients with primary lung adenocarcinomas were retrospectively registered in this study between January 2015 and January 2017. The present study was authorized by the Hospital Ethics committee and informed consent was obtained from all patients.

Tissue selection for mutation analysis

Clinical slides (paraffin sections) were reviewed to verify specimen adequacy, tumor content, and purity prior to DNA extraction and downstream testing. An estimate of tumor cell content was made by a diagnostic pathologist, with a requirement of ≥10% for the mutational analysis. Tumor morphology was determined by the diagnostic pathologist. If the DNA content and/or quality was too low for NGS-analysis, the case was excluded from the study.

Overview of test

We used the AmoyDx® Essential NGS panel (Amoy Diagnostics, Xiamen, China), which is approved by the Chinese National Medical Products Administration (NMPA) for qualitative detection of gene mutations in patients with non-small cell lung cancer (NSCLC) or colorectal cancer (CRC). The panel enables capture-based ultra-deep targeted sequencing (dual-indexed sequencing adapters with UMIs) for the following driver genes: EGFR, KRAS, BRAF, NRAS, ERBB2, PIK3CA, MET, ALK, ROS1, and RET (Table 1 for gene lists and corresponding capture regions). Clinical target-capture sequencing on an Illumina NextSeq 500 was performed with DNA from formalin-fixed, paraffin-embedded (FFPE) tumor tissue. Single-
nucleotide variants (SNV), insertion/deletion events, and rearrangements were reported. This assay was performed before methods were developed to detect copy number variants (CNV) by NGS.

**DNA and RNA extraction**

DNA and RNA were extracted from scrolls of FFPE tissue comprising ten 10-µm sections using the AmoyDx FFPE DNA/RNA Extraction Kit (Amoy Diagnostics, Xiamen, China). The quality of extracted DNA was assessed using a Nanodrop (Thermo Fisher Scientific, USA) and a Quantus fluorometer (Promega, USA) readings; DNA with the minimum requirements of ≥100 ng total mass by Quantus fluorometry and A260/A280 ratios of 1.7-2.1 was used.

**Library generation, enrichment, and sequencing**

Extracted DNA (100-150 ng) was fragmented using a Covaris M220 Focused ultrasonicator (Thermo Fisher Scientific, USA) and quality control was performed using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), followed by fragment screening, end repair, and sequencing adapter ligation. The adapter-ligated DNA was amplified and fragments of 300-500 bp were selected by beads (Agencourt AMPure XP Kit, Beckman Coulter, USA) followed by hybridization with capture probe bait, hybrid selection with magnetic beads, and PCR amplification. Indexed samples were sequenced on a NextSeq 500 instrument (Illumina, USA) to obtain paired-end 150 bp reads.

**Variant annotation and reporting**

We defined sequencing quality control failure as any base in the specified targeted region sequenced at the unique depth of <500×. As the AmoyDx® Essential NGS panel has a LOD of 1%, variants detected in bases or regions with <500× coverage (unique depth) and/or with variant allele frequency (VAF) <1.0%, were excluded. Potentially actionable variants were classified (Table 2) according to the precision oncology knowledge base of OncoKB [20].

**Mutational validation techniques**

The ARMS assay (Amoy Diagnostics, Xiamen, China) has a LOD of 1% and was used to detect hotspot mutations in EGFR and ALK, ROS1, and RET fusions in the NGS assay validation cohort. ALK FISH analysis was conducted using the Vysis ALK break apart FISH probe (Vysis, Abbot molecular, USA), and RET FISH analysis was conducted using the RET break apart FISH probe (CytoTest, USA) in accordance with the manufacturers’ instructions.

**Statistical analysis**

Descriptive statistics were calculated with Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA). The data were described as the number of mutations.

**Results**

**Patient characteristics**

Three cases were excluded from this study because the DNA content was too low for NGS-analysis. The characteristics of the 372 lung adenocarcinoma patients are presented in Table 3. The median age of patients was 62 years (range, 23-89 years). Overall, 51.1% of patients (190 cases) were male, and 20.7% patients (77/372) were diagnosed in advanced (tumor-node-metastasis III and IV) stages.

**Overview of identified nonsynonymous variants**

Among the 372 adenocarcinoma specimens, 437 nonsynonymous variants were called within the coding regions of the 10 sequenced...
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Table 2. Classification of identified variants according to clinical actionability

<table>
<thead>
<tr>
<th>Variant Level</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FDA-recognized biomarker predictive of response to a FDA approved drug in this indication</td>
</tr>
<tr>
<td>2</td>
<td>Standard care biomarker predictive of response to a FDA approved drug in this indication</td>
</tr>
<tr>
<td>3A</td>
<td>Compelling clinical evidence supports the biomarker as being predictive of response to a drug in this indication</td>
</tr>
<tr>
<td>3B</td>
<td>Compelling clinical evidence supports the biomarker as being predictive of response to a drug in another indication</td>
</tr>
<tr>
<td>4</td>
<td>Compelling biological evidence supports the biomarker as being predictive of response to a drug</td>
</tr>
<tr>
<td>5</td>
<td>Variants with unknown clinical significance/variants that are benign or likely benign</td>
</tr>
</tbody>
</table>

Table 3. Baseline characteristics of all patients (n=372) for which capture-based ultra-deep targeted sequencing was completed

<table>
<thead>
<tr>
<th>Number of patients, n</th>
<th>372</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis, n (%)</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>372 (100%)</td>
</tr>
<tr>
<td>Age at diagnosis, y (S.D.)</td>
<td>62 (11)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>190 (51.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>182 (48.9%)</td>
</tr>
<tr>
<td>Stage at diagnosis</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>235 (63.2%)</td>
</tr>
<tr>
<td>II</td>
<td>44 (11.8%)</td>
</tr>
<tr>
<td>III</td>
<td>60 (16.1%)</td>
</tr>
<tr>
<td>IV</td>
<td>17 (4.6%)</td>
</tr>
<tr>
<td>Unknown/undetermined</td>
<td>16 (4.3%)</td>
</tr>
</tbody>
</table>

Genes by the standard vendor supplied data analysis pipeline. There was a mean of 1.17 nonsynonymous variants per specimen. A total of 90.3% (336/372) of cases had 1-3 called non-synonymous variants (Figure 1A). The non-synonymous variants included 284 substitutions, 61 deletions, 23 insertions, 43 combined insertion/deletions, and 26 rearrangement events (Figure 1B).

Yield of actionable variants

The average coverage of the 372 samples analyzed was 31556× (range 6576×-53080×). The mean unique depth of coverage across the capture region was 4147× (range 525×-7411×). The mean percentage of on-target reads was 70.7% (range, 45.1%-79.3%).

A major objective of NGS in the clinic is to identify samples with potentially actionable variants (defined in Table 2). Because targeted therapies are often chosen based on molecular profiles rather than tumor histology, it may be most relevant to consider potentially actionable variants. By this measure, potentially actionable variants were identified in 80.5% (n=352) of the 437 nonsynonymous variants that were able to be sequenced. The most common genetic alterations were EGFR mutations (59.5%, 260/437), followed by KRAS mutations (5.5%, 24/437), PIK3CA mutations (3.7%, 16/437), ALK rearrangements (3.4%, 15/437), BRAF mutations (2.7%, 12/437), ERBB2 mutations (2.5%, 11/437), and RET rearrangements (2.3%, 10/437) (Figure 1C), revealing the different clinical characteristics of Chinese patients.


Within KRAS, 91.7% (22/24) of actionable mutations were at codon 12, and 72.7% (8/11) of actionable mutations were p.A775_G776insYVMA in ERBB2. Within ALK and RET rearrangements, 86.7% (13/15) and 60% (6/10) of actionable mutations were EML4-ALK and KIF5B-RET fusions, respectively (Table 4).

Cases of simultaneous p.T790M and p.L858R mutation occurrence in EGFR (n=3) showed a similar VAF in each case (17.9%-37.1% VAF versus 21%-41.8% VAF). EGFR p.R776H and p.R776C mutations showed a similar VAF in each case (8.4%-27.4% VAF versus 8.1%-27.3% VAF for activating mutations) [21].

One rationale for using NGS instead of single-gene testing is the possibility that actionable variants are present in more than one gene or...
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A pathway, rendering a multigene approach more efficient [22]. A total of 7.2% (28/372) of the samples had multiple actionable mutations, 27 samples had two actionable variants,

Table 4. Summary of all potentially actionable variants detected

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>372</td>
<td></td>
</tr>
</tbody>
</table>

**EGFR**
- **Exon 18**
  - G719A: 2
  - G719S: 3
  - E709K: 1
- **Exon 19**
  - del/delins: 93
  - A750P: 9
  - D761Y: 1
- **Exon 20**
  - 20-Ins: 13
  - S768I: 3
  - T790M: 3
- **Exon 21**
  - L858R: 125
  - L861Q: 7
  - L853V: 1

**KRAS**
- **Codon 12**
  - G13D: 1
  - Q61H: 1

**PIK3CA**
- **E542K**
  - 4
- **E545K**
  - 7
- **H1047L**
  - 1
- **H1047R**
  - 4

**BRAF**
- **V600E**
  - 7
- **G469A**
  - 1

**ALK fusion**
- EML4-ALK_E6:A20: 5
- EML4-ALK_E13:A20: 8
- HIP1-ALK_H19:A20: 1
- HIP1-ALK_H28:A20: 1

**RET fusion**
- KIF5B-RET_K15:R12: 6
- CCDC6-RET_C1:R12: 3
- CCDC6-RET_C8:R12: 1

**ROS1 fusion**
- CD74-ROS1_C6:R34: 1
- ERBB2
  - A775_G776insYVMA: 8
  - G776delinsVC: 1
  - P780_Y781insGSP: 1
  - L755S: 1

**MET**
- Exon 14 splice: 1

**NRAS**
- **G13R**: 1

The number of lung adenocarcinoma patients is stated in the first row. Subsequent rows indicate number of variants, not patients. Mutations are listed as amino acid changes in HGVS protein nomenclature. Non-actionable variants detected in any case were omitted.
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In 10 samples, these actionable variants were present in more than one gene (Table 5). These are samples in which a single-gene approach might have missed therapeutically relevant information. Eighteen samples had two actionable variants of EGFR gene, and except for one sample, all of them had a similar VAF of two actionable variants (Table 6).

### Table 5. Cases with more than one actionable variant of two genes

<table>
<thead>
<tr>
<th>Case</th>
<th>EGFR (VAF)</th>
<th>KRAS (VAF)</th>
<th>PIK3CA (VAF)</th>
<th>BRAF (VAF)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>71</td>
<td>E746_A750del (45.2%)</td>
<td>E545K (1.2%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>E746_A750del (53.7%)</td>
<td>E545K (48.4%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>E746_A750del (19.1%)</td>
<td>H1047R (1.0%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>234</td>
<td>E746_A750del (46.7%)</td>
<td>E545K (26.3%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>L858R (15.3%)</td>
<td>E542K (16.2%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>L858R (22.4%)</td>
<td>E545K (1.3%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>L851Q (43.5%)</td>
<td>E545K (18.2%)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>249</td>
<td>T751_I759delinsN (17.2%)</td>
<td>E542K (2.5%)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H1047R (17.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>256</td>
<td></td>
<td>E542K (1.7%)</td>
<td>N581S (33.9%)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>340</td>
<td></td>
<td>E542K (2.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The actionable variants are listed as amino acid changes in HGVS protein nomenclature (VAF, variant allele frequency).

### Table 6. Cases with two actionable variants of EGFR gene

<table>
<thead>
<tr>
<th>Case</th>
<th>EGFR mutation 1 (VAF)</th>
<th>EGFR mutation 2 (VAF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>L747_A750delinsP (43.4%)</td>
<td>A750P (51.8%)</td>
</tr>
<tr>
<td>32</td>
<td>L747_A750delinsP (22.1%)</td>
<td>A750P (25.6%)</td>
</tr>
<tr>
<td>50</td>
<td>L747_A750delinsP (4.9%)</td>
<td>A750P (15.6%)</td>
</tr>
<tr>
<td>189</td>
<td>L747_A750delinsP (14.1%)</td>
<td>A750P (15.8%)</td>
</tr>
<tr>
<td>191</td>
<td>L747_A750delinsP (33.8%)</td>
<td>A750P (35.5%)</td>
</tr>
<tr>
<td>245</td>
<td>L747_A750delinsP (10.6%)</td>
<td>A750P (11.3%)</td>
</tr>
<tr>
<td>288</td>
<td>L747_A750delinsP (15.8%)</td>
<td>A750P (15.7%)</td>
</tr>
<tr>
<td>346</td>
<td>L747_A750delinsP (6.1%)</td>
<td>A750P (5.8%)</td>
</tr>
<tr>
<td>17</td>
<td>L747_E749del (19.1%)</td>
<td>A750P (19.0%)</td>
</tr>
<tr>
<td>352</td>
<td>L858R (17.0%)</td>
<td>D761Y (17.1%)</td>
</tr>
<tr>
<td>39</td>
<td>L858R (42.1%)</td>
<td>L833V (41.8%)</td>
</tr>
<tr>
<td>371</td>
<td>L858R (21.5%)</td>
<td>S768I (21.9%)</td>
</tr>
<tr>
<td>308</td>
<td>L858R (17.7%)</td>
<td>S768I (16.7%)</td>
</tr>
<tr>
<td>132</td>
<td>L858R (31.3%)</td>
<td>T790M (32.3%)</td>
</tr>
<tr>
<td>149</td>
<td>L858R (37.1%)</td>
<td>T790M (41.8%)</td>
</tr>
<tr>
<td>272</td>
<td>L858R (17.9%)</td>
<td>T790M (21.0%)</td>
</tr>
<tr>
<td>179</td>
<td>L861R (8.3%)</td>
<td>G719A (8.2%)</td>
</tr>
<tr>
<td>253</td>
<td>E709K (20.2%)</td>
<td>G719S (19.7%)</td>
</tr>
</tbody>
</table>

The actionable variants are listed as amino acid changes in HGVS protein nomenclature (VAF, variant allele frequency).

### ALK, RET, and ROS1 gene fusion analysis

Gene fusion analysis was performed on DNA from lung adenocarcinomas in the 372 samples. Among the 93 triple-negative cases, which did not harbor mutations in EGFR, KRAS or BRAF, gene fusions were detected in 26 cases (28%), with 15 (16.1%) ALK gene fusions (eight EML4-ALK_E13:A20 fusions, five EML4-ALK_E6:A20 fusions, one HIP1-ALK_H19:A20, and one HIP1-ALK_H28:A20 fusion), 10 (10.8%) RET fusions (six KIF5B-RET_K15:R12 fusions, three CCDC6-RET_C1:R12 fusions, and one CCDC6-RET_C8:R12 fusion), and one (1.1%) CD74-ROS1_C6:R34 fusion (Table 4). A total of 28% cases of the 93 analyzed triple-negative lung adenocarcinomas harbored gene fusions, which was higher than the literature had reported [17, 23].

### Mutational validation by ARMS and FISH

For the cases with ≥10% tumor cell content detected by pathological assessment, using capture-based ultra-deep targeted sequencing, we were able to detect and validate known activating driver mutations in EGFR, ALK, ROS1, and RET below 1% VAF by orthogonal methods. Of the 328 samples, concordance of EGFR between the ARMS assay and capture-based ultra-deep targeted sequencing was observed from 318 samples (97.0%) (Table 7). Among the 212 EGFR mutation-positive cases detected by the NGS assay, 202 cases (95.3%) yielded concordant results with the ARMS assay because the ARMS assay
does not cover these mutation sites. Thus, exclusion of these variants implied a concor-
dance of 100% between the NGS and ARMS assays.

Among the six ALK/ROS1/RET fusion-negative cases detected by the NGS assay, six cases
yielded concordant results with the ARMS assay. Of the 32 samples, concordance of
ALK/ROS1/RET fusion genes between the ARMS/FISH tests and the NGS assay was
observed for 30 samples (93.8%) (Table 7). Among all the 26 ALK/ROS1/RET fusion-posi-
tive cases detected by the NGS assay, 24 cases (92.3%) yielded concordant results with
ARMS (n=22), or FISH (n=2). Two discordant calls were due to a variant detected by the NGS
assay but were not analyzed by the ARMS assay. Exclusion of these variants implied a
concordance of 100%.

Discussion

In the assay described herein, only SNVs, dele-
tions, insertions, and rearrangements were
called by NGS, yet CNVs involving ERBB2 and
MET, among others, are important in lung ade-
nocarcinoma. This test focuses on potentially
actionable variants because validated progno-
stic and predictive variants fall into this category
at present. This work underlines the impor-
tance of reviewing submitted samples by
pathologists at the time of intake.

In this study, we investigated a cohort of
372 treatment-naive lung adenocarcinoma
patients using the AmoyDx® Essential NGS
panel, which has limit of detection (LOD) of 1%,
and performed a validation by ARMS and FISH.

Using the panel and Amoy Diagnostics vendor-
supplied bioinformatics pipeline, we were able
to detect and validate known activating driver
mutations in EGFR and ALK, ROS1, and RET
fusion genes below 1% VAF. This study showed
that when the LOD is 2%, approximately 1.9% (n=7)
of patients (VAF range, 1.03%-1.68%) may miss
their treatment opportunity, and when the LOD is 5%, about 7.5% (n=28) patients
(VAF range, 1.03%-5.0%) may miss their treat-
ment opportunity.

Potentially actionable variants were identified
in 80.5% of the 437 nonsynonymous variants
(Figure 1). In addition to detecting well-known
actionable variants, assays based on complete
gene sequencing have the potential to reveal
noncanonical variants that may potentially be
actionable. Our study demonstrated that tar-
geted sequencing (dual-indexed sequencing
adapters with UMIs) allows for large-scale par-
allel sequencing to accurately detect a wide
range of actionable variants. We identified
numerous well-established driver mutations
that are not covered by commercially available
single-gene testing kits, such as ERBB2 (exon
20 insertions, p.L755S), EGFR (c.2571_2573delinsTCG: p.L858R, c.2573_2574delinsGA:
14 splice mutation), and BRAF (p.D594N, p.
the traditional ARMS method is commonly uti-
lized to detect mutations in treatment-naive
patients, all of the above mutations are not cov-
ered. If tested with commercially available sin-
gle-gene testing kits, patients harboring such
mutations may miss treatment opportunities.
Therefore, when multiple genes need to be

<table>
<thead>
<tr>
<th>NGS</th>
<th>EGFR mut</th>
<th>EGFR wt</th>
<th>ALK mut</th>
<th>ALK wt</th>
<th>ROS1 mut</th>
<th>ROS1 wt</th>
<th>RET mut</th>
<th>RET wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR mut</td>
<td>202</td>
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<td>6**</td>
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</tbody>
</table>

*: detected variant not analyzed by ARMS or FISH assay. **: six same ALK/ROS1/RET fusion-negative cases. Wt: no mutation
detected at investigated loci. Mut: mutant.
Identifying variants using targeted sequencing in lung adenocarcinomas

Table 8. Placement of patients on matched therapy (16/372 patients placed on matched therapy)

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Mutation</th>
<th>Matched therapy</th>
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<tbody>
<tr>
<td>19</td>
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<td>EGFR L747_T751del</td>
<td>Gefitinib</td>
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<tr>
<td>27</td>
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<td>EGFR L858R</td>
<td>Gefitinib</td>
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<td>EGFR L747_P753delinsS</td>
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<td>Crizotinib</td>
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<td>287</td>
<td>lung adenocarcinoma</td>
<td>EGFR L747_T751del</td>
<td>Gefitinib</td>
</tr>
</tbody>
</table>

The actionable variants are listed as amino acid changes in HGVS protein nomenclature.

This test has not been available for long enough to allow for comprehensive clinical follow-up, but it will be important to confirm that treatment decisions have been made based on NGS results in the future. In this study, the patients were receiving routine clinical care (Table 8). Together with several recent reports [15, 22, 25], the clinical validation of commercial capture-based ultra-deep targeted sequencing detected all known insertion/deletions, rearrangements, SNVs, and wild-type loci detected by orthogonal methods (n=360) (Table 7), indicating 100% concordance with known variants.

Although capture-based targeted sequencing has been widely regarded as a powerful tool to accurately detect a wide range of actionable variants, it still has limits [33, 34]. First, the accuracy of new platforms may be lower than conventional methods for the identification of specific mutation types. Second, the capture-based targeted sequencing protocol is commonly longer when compared with conventional methods such as FISH and ARMS. However, we should keep in mind that NGS will continue to be improved and optimized with respect to these disadvantages.

In conclusion, we demonstrate a preferable approach with a LOD of 1% to profile a wide range of actionable variants in surgical specimens of treatment-naive lung adenocarcinoma patients. This approach could further guide
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more precise targeted therapy and help lung adenocarcinoma patients to achieve better clinical benefit.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

NGS, next generation sequencing; UMI, unique molecular identifier; ARMS, amplification-refractory mutation system; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ hybridization; LOD, limit of detection; VAF, variant allele frequency.

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References


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