Detecting the spectrum of multigene mutations in non–small cell lung cancer by Snapshot assay

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Abstract

As molecular targets continue to be identified and more targeted inhibitors are developed for personalized treatment of non–small cell lung cancer (NSCLC), multigene mutation determination will be needed for routine oncology practice and for clinical trials. In this study, we evaluated the sensitivity and specificity of multigene mutation testing by using the Snapshot assay in NSCLC. We retrospectively reviewed a cohort of 110 consecutive NSCLC specimens for which epidermal growth factor receptor (EGFR) mutation testing was performed between November 2011 and December 2011 using Sanger sequencing. Using the Snapshot assay, mutation statuses were detected for EGFR, Kirsten rat sarcoma viral oncogene homolog (KRAS), phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3CA), v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), v-ras neuroblastoma viral oncogene homolog (NRAS), dual specificity mitogen activated protein kinase kinase 1 (MEK1), phosphatase and tensin homolog (PTEN), and human epidermal growth factor receptor 2 (HER2) in patient specimens and cell line DNA. Snapshot data were compared to Sanger sequencing data. Of the 110 samples, 51 (46.4%) harbored at least one mutation. The mutation frequency in adenocarcinoma specimens was 55.6%, and the frequencies of EGFR, KRAS, PIK3CA, PTEN, and MEK1 mutations were 35.5%, 9.1%, 3.6%, 0.9%, and 0.9%, respectively. No mutation was found in the HER2, NRAS, or BRAF genes. Three of the 51 mutant samples harbored double mutations: two PIK3CA mutations coexisted with KRAS or EGFR mutations, and another KRAS mutation coexisted with a PTEN mutation. Among the 110 samples, 47 were surgical specimens, 60 were biopsy specimens, and 3 were cytological specimens; the corresponding mutation frequencies were 51.1%, 41.7%, and 66.7%, respectively (P = 0.532). Compared to Sanger sequencing, Snapshot specificity was 98.4% and sensitivity was 100% (positive predictive value, 97.9%; negative predictive value, 100%). The Snapshot assay is a sensitive and easily customized assay for multigene mutation testing in clinical practice.

Key words: Non–small cell lung cancer, multigene mutation, Snapshot assay, Sanger sequencing
et al.\textsuperscript{[7]} reported that patients with NSCLC harboring KRAS mutations may be resistant to EGFR-TKIs. Ohashi et al.\textsuperscript{[10]} described cells that harbored v-ras neuroblastoma viral oncogene homolog (NRAS) Q61K mutation-mediated resistance to erlotinib via the MEK signaling pathway. However, the NRAS mutation may be associated with sensitivity to MEK inhibitors\textsuperscript{[10]}. As the library of molecular targets expands and targeted inhibitor development continues, identifying multigene mutations will be increasingly important in practice and in clinical trials. Sanger sequencing is traditionally used to detect gene mutations. However, the sensitivity of Sanger sequencing is suboptimal for many clinical tumor samples. Sanger sequencing analysis is also time-consuming for multigene mutation testing. Thus, we developed a sensitive and simple method to routinely and simultaneously detect the mutation statuses of EGFR, Kirsten rate sarcoma viral oncogene homolog (KRAS), NRAS, BRAF, PIK3CA, MEK1, phosphatase and tensin homolog (PTEN), and HER2.

**Methods and Materials**

**Cell lines**

We used three NSCLC cell lines, H1650, H1975, and H460, to detect DNA input information from Snapshot. H1650 cells contain a deletion mutation in exon 19 of the EGFR gene; H1975 cells contain T790M and L858R point mutations in exon 20 and exon 21, respectively, of the EGFR gene; and H460 cells contain an E545Q mutation in PIK3CA. For DNA input detection, we tested a series of concentrations of mutant DNA: 2, 5, 10, 20, and 50 ng/μL.

**Patient specimens**

We reviewed a cohort of 110 consecutive lung cancer specimens, for which EGFR mutation testing was performed between November 2011 and December 2011 with Sanger sequencing. Of the 110 specimens, 47 were surgical specimens, 60 were biopsy specimens, and 3 were cytologic specimens. Informed consent was obtained from each patient in Guangdong Lung Cancer Institute.

**DNA extraction**

DNA was extracted from cell lines and patient specimens using a QiAmp DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions and was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Extracted DNA was stored at –20°C until use.

**Snapshot assay and fragment analysis**

The Snapshot assay was used as previously described\textsuperscript{[10]} to detect hot-spot mutations of EGFR, KRAS, NRAS, BRAF, PIK3CA, PTEN, MEK1, and HER2. These mutations included G719X, T790M, L858R, L861Q, deletions in exon 19, and insertions in exon 20 of the EGFR gene; G12D, G12A, G12V, G12S, G12R, G12C, G13D, G13A, G13V, G13S, G13R, G13C of the KRAS gene; G12D, G12A, G12V, G12S, G12R, G12C, G13D, G13A, G13V, G13S, G13R, and G13C of the NRAS gene; G12D, G12A, G12V, G12S, G12R, G12C, G13D, G13A, G13V, G13S, G13R, G13C, G13G, and Q61H of the BRAF gene; E542K, E545Q, Q546K, Q546R, Q546L, L596V, V600E, V600K of the BRAF gene; E542K, E545Q, Q546K, Q546R, Q546L, L596V, V600E, V600K of the PIK3CA gene; G56D, G56S, K58N, and D67N of the MEK1 gene; R130X, R131C, R233X of the PTEN gene; and insertions in exon 20 of the HER2 gene. A total of 36 point mutations of the 8 genes were assigned to 6 panels tested by Snapshot assay, whereas in-frame mutations were tested using fragment analysis. For the Snapshot assay, polymerase chain reaction (PCR) primers and extension primers were pooled into 6 panels in proportion respectively. Thermocycling conditions were as follows: 5 min at 94°C followed by 40 cycles of 94°C for 30 s, 58°C for 20 s, 72°C for 30 s, and then a final incubation at 72°C for 30 s. Then, 2 μL of amplified products were purified with exonuclease I (TaKaRa, Dalian, China) and alkaline phosphatase (shrimp) (TaKaRa, Dalian, China), and purified products were subjected to extension reactions using the Snapshot Multiplex Ready Reaction Mix (Applied Biosystems, Life Technologies, California, USA). The extension products were purified with 1 μL alkaline phosphatase (shrimp) and separated in an ABI 3730 Genetic Analyzer (Applied Biosystems, Life Technologies, California, USA) according to the manufacturer’s instructions. Data were interpreted using ABI GeneMapper (version 4.1).

In-frame mutations in exons 19 and 20 of EGFR and in exon 20 of HER2 were detected using fragment analysis, as described previously\textsuperscript{[10]}. Briefly, PCR was performed using a primer mixture, and the resultant amplicon was separated by using capillary electrophoresis and then analyzed in an ABI 3730 Genetic Analyzer.

**Sanger sequencing**

EGFR, KRAS, and NRAS mutations were detected by Sanger sequencing using a previously published protocol\textsuperscript{[11]}. NRAS exon 2 primers were 5′-AGAGACAGGTAAGTGATCAGCTACACT-3′ (forward) and 5′-ATGGATCGCAAGTGAAG-3′ (reverse). NRAS exon 3 primers were 5′-AACATGGGCTTTAGATGATGATGCTAG-3′ (forward) and 5′-ACCTATTTCCTCATTAAAGAGTACG-3′ (reverse).

PCR was performed to amplify exons 18–21 of EGFR and codons 12, 13, and 61 of KRAS and NRAS. PCR was performed in a 25-μL volume containing 20 ng genomic DNA, 12.5 μL of Premix Ex Taq HotStart version (TaKaRa, Dalian, China), 5 μmol/L of each primer, and 3 μL of nuclease-free water. Then, 4 μL of PCR products were purified with exonuclease I and alkaline phosphatase (shrimp). Next, the purified products were sequenced bidirectionally with BigDye Terminator v3.1 (Applied Biosystems) and an ABI 3730 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s protocol. Sequencing data were analyzed using Sequencing Analysis Software v5.2 (Applied Biosystems).

**Statistical analysis**

Associations between mutations and clinical and biological characteristics were analyzed using a chi-square test or Fisher’s exact test. All data were analyzed using the Statistical Package for
the Social Sciences Version 17.0 Software (SPSS). The two-sided significance level was set at \( P < 0.05 \).

**Results**

**Patient characteristics**

A total of 110 patients were included in this study (median age, 60 years; range, 35–84 years). Table 1 describes patient characteristics.

**Sample input titrations**

To confirm reproducibility and consistency of the methodology, sample input titrations were performed. DNA was extracted from H1975, H1650 and H460 cells and diluted into different concentrations. When DNA input was low to 2 ng/μL, T790M, L858R, deletion, and E542Q mutants could be detected in cell lines using the Snapshot assay.

**Sensitivity and specificity of the Snapshot assay**

For the 110 samples, mutations in all eight genes (EGFR, KRAS, NRAS, MEK1, PIK3CA, BRAF, HER2, and PTEN) were analyzed using the Snapshot assay. As comparison, EGFR, KRAS, and NRAS mutations were detected using Sanger sequencing. Taking Sanger sequencing as the gold standard, Snapshot assay specificity was 98.4% and sensitivity was 100% (positive predictive value, 97.9%; negative predictive value, 100%), as shown in Figure 1.

**Detection of mutations in clinical specimens by Snapshot assay**

Of the 110 samples, almost half harbored at least one mutation. The mutation frequency in adenocarcinoma specimens was 55.6% (Table 1). In contrast, no mutation was found in patients with squamous cell carcinoma. Most mutations were mutually exclusive, except three cases harboring multigene mutations. Table 2 depicts the multigene mutations in the three specimens. Among the 110 samples, 47 were surgical specimens, 60 were biopsy specimens, and 3 were cytologic specimens, and the corresponding mutation frequencies were 51.1%, 41.7%, and 66.7%, respectively (\( P = 0.532 \)). The Snapshot assay was sensitive enough to detect mutations of cytologic and biological samples.

**Discussion**

Determining multiple driver gene mutations in NSCLC patient tumors is important for targeting therapy and for clinical trials. Many high-throughput methods have been used, including sequenom MassArray, as well as targeted and non-targeted next generation sequencing \([12-14]\). Considering limited infrastructure demands and timescales for these techniques, we used the recently developed Snapshot assay to detect multigene mutations in routine clinical practice.

Our data suggested that Snapshot assay, which could robustly discriminate among multigene mutations with lower input DNA, is more sensitive than Sanger sequencing. Tumor cell contains is a critical factor influencing the performance of mutation detection in cancer \([15]\). Enhancing the detection sensitivity might overcome the difficulty of low tumor cellularity \([16]\). Our data indicated that mutation detection frequency did not significantly differ among surgical, biopsy, and cytologic samples (\( P = 0.532 \)), and suggested that Snapshot assay have ability to discriminate mutations from biopsy and cytologic specimens.

Consistent with published literatures \([17-19]\), EGFR mutations were more frequent in females, adenocarcinomas, and nonsmokers in this

### Table 1. Patient characteristics and frequencies of multigene mutations

| Characteristic | No. of patients | Multigene MUT | EGFR MUT | KRAS MUT | PIK3CA MUT | PTEN MUT | MEK1 MUT |
|---------------|----------------|---------------|----------|----------|------------|----------|----------|
| Sex           |                |               |          |          |            |          |          |
| Male          | 77 (70.0)      | 30 (39.0)     | 20 (26.0)| 10 (13.0)| 1 (1.3)    | 1 (1.3)  | 1 (1.3)  |
| Female        | 33 (30.0)      | 21 (63.6)     | 19 (57.6)| 0        | 3 (9.1)    | 0        | 0        |
| Smoking status|                |               |          |          |            |          |          |
| Nonsmoker     | 60 (54.5)      | 34 (56.7)     | 28 (46.7)| 4 (6.7)  | 4 (6.7)    | 0        | 0        |
| Smoker        | 48 (43.6)      | 17 (35.4)     | 11 (22.9)| 6 (12.5) | 0          | 1 (2.1)  | 1 (2.1)  |
| Histology     |                |               |          |          |            |          |          |
| AC            | 90 (81.8)      | 50 (55.6)     | 39 (43.3)| 10 (11.1)| 3 (3.3)    | 1 (1.1)  | 1 (1.1)  |
| SCC           | 15 (13.6)      | 0             | 0        | 0        | 0          | 0        | 0        |
| LCC           | 5 (4.5)        | 1 (20.0)      | 0        | 1 (20.0) | 0          | 0        | 0        |
| Total         | 110            | 51 (46.4)     | 39 (35.5)| 10 (9.1) | 4 (3.6)    | 1 (0.9)  | 1 (0.9)  |

All values are presented as number of patients with percentage in parentheses. MUT, mutation; EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma viral oncogene homolog; PIK3CA, phosphoinositide-3-kinase catalytic alpha polypeptide; MEK1, dual specificity mitogen activated protein kinase kinase 1; PTEN, phosphatase and tensin homolog; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma.
Regarding ethnicity, EGFR mutations occurred more frequently in Asian than in Western patients [20]. Similarly, KRAS mutations differed with ethnicity, occurring less than 10% in Asian patients compared with 30% in Caucasian patients [21,22].

To our knowledge, mutations in HER2, NRAS, and MEK1 are rarely reported in Chinese NSCLC patients. Among the 110 patients studied, only one mutation (Q56P) of MEK1 was observed, and no mutation was found in HER2 and NRAS genes. Despite the small sample size, these results suggested that mutation frequencies of HER2, NRAS, and MEK1 are low in Chinese patients with NSCLC.

Interestingly, we found that 3 of the 51 mutant samples harbored concomitant mutations. Two PIK3CA mutations coexisted with a KRAS mutation and an EGFR mutation respectively, and another KRAS mutation coexisted with a PTEN mutation. According to previously published literature [12,23-25], the reported mutation rate of PIK3CA is ~1%–4%, but PIK3CA mutations were often concurrent with mutations in other genes with coincidence rates of 30%–70%.

KRAS was the most common partner oncogene [12]. Because our sample size was limited, future research is needed to identify the exact frequency of compound mutations and biological behaviors of these tumors.

The Snapshot assay was designed to detect known “hot-spot” mutations in specific oncogenes based on the published literature, so this method may miss unknown mutations.

In summary, Snapshot assay is sensitive and easy to conduct for parallel investigation of gene mutation panels. This method may conserve DNA for multigene testing compared to Sanger sequencing. Considering the availability of genetic analyzer platforms in China, Snapshot assay could offer another option for multigene mutation testing in routine clinical practice.

### Table 2. Molecular and clinical characteristics of patients with multigene mutations

| Sample ID | Sex   | Age (years) | Smoking status | Histology | EGFR | KRAS | PI3K | PTEN |
|-----------|-------|-------------|----------------|-----------|------|------|------|------|
| 4321      | Male  | 84          | Smoking        | AC        | WT   | G13C | WT   | R130X|
| 4327      | Female| 44          | Nonsmoking     | AC        | DEL  | WT   | E542K| WT   |
| 4288      | Male  | 78          | Nonsmoking     | AC        | WT   | G12D | E542K| WT   |

WT, wild type; DEL, deletion. Other footnotes as in Table 1.

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