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Original Article

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 rate 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

Lung cancer is the leading cause of cancer-related deaths worldwide[1]. The subclass non–small cell lung cancer (NSCLC) can be further divided into molecular subsets according to genomic changes that are defined as "driver mutations"[2]. Some of these mutations have been identified as predictive factors of clinical response to targeted therapeutic agents, including epidermal growth factor receptor (*EGFR*) mutation activity to gefitinib or erlotinib and echinoderm microtubule associated protein-like 4-anaplastic lymphoma kinase (*EML4-ALK*) fusion to crizotinib[3-5]. The remarkable effectiveness of these drugs in molecularly selected populations has led to a surge in lung cancer trials to study targeted therapies in other molecularly selected populations. Recently, new tyrosine kinase inhibitors targeting mutations of phosphoinositide-3-kinase catalytic alpha polypeptide (*PIK3CA*), v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*), human epidermal growth factor receptor 2 (*HER2*), and dual specificity mitogen activated protein kinase kinase 1 (*MEK1*) have been developed and tested in clinical trials[6]. Moreover, some investigators have reported mutations in RAS signaling pathway genes associated with acquired resistance to tyrosine kinase inhibitors (EGFR-TKIs) in NSCLC. Campos-Parra

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et al.[7] reported that patients with NSCLC harboring *KRAS* mutations may be resistant to EGFR-TKIs. Ohashi *et al*.[8] 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[9].

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[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, G13C and Q61H of the *NARS* gene; G466V, G469A, L596V, V600G, V600K of the *BRAF* gene; E542K, E545Q, Q546K, Q546R, L1047Y, L1047L, G1049S of *PIK3CA*; Q56P, K58N, and D67N of the *MEK1* gene; R130X, R173C, 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[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[11]. *NRAS* exon 2 primers were 5'-AAGAACCAAATGGAAGGTCACACTA-3' (forward) and 5'-GTAAAGATGATCCGACAAGTGAGAG-3' (reverse). *NRAS* exon 3 primers were 5'-AAATGGGCTTGAATAGTTAGATGCT-3' (forward) and 5'-ACCTCATTTCCCCATAAAGATTCAG-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	*P*	*EGFR* MUT	*P*	*KRAS* MUT	*P*	*PIK3CA* MUT	*P*	*MEK1* MUT	*PTEN* MUT
Sex			0.017		0.01		0.031		0.08		
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			0.028		0.011		0.481		0.127		
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			<0.001		<0.001		0.477		0.252		
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		0		1 (20.0)		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 rate 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.

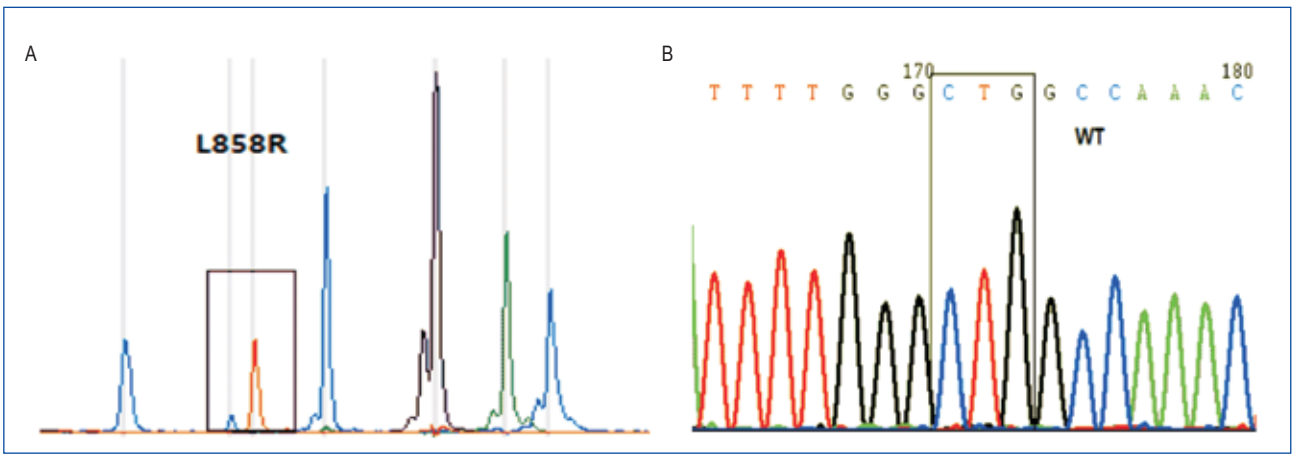

Figure 1. **Snapshot assay is more sensitive than Sanger sequencing.** Snapshot assay result shows epidermal growth factor receptor (*EGFR*) L858R mutation in Case 4340 (A), whereas Sanger sequencing result shows a wild-type *EGFR* (B).

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.

study. 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.

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