

High-throughput Molecular Genotyping for Small Biopsy Samples in Advanced Non-small Cell Lung Cancer Patients

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Abstract. *Background:* Despite the key role of mutational analysis in targeted therapy, the difficulty in acquisition of adequate tumor tissues for molecular genotyping in advanced non-small cell lung cancer (NSCLC) has led to the need for a fast and efficient method for detecting genetic alterations for targeted therapy. *Patients and Methods:* We analyzed tissue specimens of advanced NSCLC. A mass spectrometry-based assay was used to investigate 471 oncogenic mutations. All tumor specimens were prepared from fresh-frozen tissues. *Results:* In total, there were 59 hotspot mutations in 67% of the entire patient group (41 out of 61 patients). The most frequent mutation was in TP53 (n=24, 39.3%), followed by EFGR (n=19, 31.1%). Others included MLH1, KRAS, PIK3CA, ERBB2, ABL1 and HRAS. *Conclusion:* Our results suggest that molecular genotyping using high-throughput technology such as OncoMap v4 is feasible, even with small biopsied specimens from patients with advanced NSCLC.

With advances in molecular-targeted therapy, high-throughput genomic profiling and detection of several established

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mutations in tumor tissues are new trends in medical oncology that facilitate identification of specific genetic alterations which will allow for personalized cancer care. Although traditional Sanger sequencing is considered the gold standard for mutation detection, several high-throughput sequencing platforms are now commercially available (1). Nonetheless, the accuracy of the results of these methods should be compared the one of conventional direct sequencing. The major factors that can affect their validity include the quality and quantity of the specimen (1, 2).

Lung cancer, especially non-small cell lung cancer (NSCLC), is one of the most widely studied types of cancer in the targeted therapy era. Because genetic analysis for lung cancer is rapidly moving beyond epidermal growth factor receptor (EGFR) mutations, the acquisition of specimens of good quality and adequate quantity remains challenging. DNA in formalin-fixed paraffin-embedded tissue (FFPE) is thought to be susceptible to degradation over time (1, 2). Acquisition of limited amounts of tissue by bronchoscopic or core needle biopsy makes high-throughput genetic profiling problematic. Due to tumor heterogeneity, various mutations may possibly exist in tumors of identical histological type. Moreover, following genotypic screening at the initial diagnosis, repeated tissue sampling will likely be required because subsequently emerging mutations, which confer resistance to the targeted agents, can differ markedly (3). Because about two-thirds of patients with NSCLC present with unresectable advanced disease at the time of diagnosis (4), molecular diagnosis is achieved in a substantial proportion of patients using small biopsy tissue samples rather than surgical specimens. In particular, pulmonary adenocarcinoma tends to be located at the periphery of the lung; thus, core biopsy is the major diagnostic method for pathological confirmation in most hospitals, unless the patient undergoes curative surgery.

Regarding squamous cell carcinoma, which accounts for ~20-0% of newly-diagnosed lung cancer, identification of the molecular alterations involved is a focus of current research, leading to an increasing need for frequent biopsies (5).

A high-throughput genotyping platform has been used to determine the mutation status in NSCLC. This genotyping platform, OncoMap v4, uses mass spectrometry-based genotyping technology (Sequenom) to identify 471 oncogenic mutations in 41 commonly mutated genes (6, 7). The investigators who first developed OncoMap performed mutation profiling in various types of tumors including aerodigestive and gastrointestinal cancers, with reasonable sensitivity and specificity (6). With tissues obtained from lung cancer, despite the relatively small number of samples, 57.7% of samples were shown to harbor mutations. Based upon this study, we previously performed somatic mutation screening among esophageal squamous cell tumor samples (8). We found mutations in 25% of enrolled patients. Mutations detected by OncoMap included potentially drug-targetable mutations such as *BRAF* V600E. Here, we assessed the mutational status in lung cancer tissues obtained by relative small biopsy of endobronchial ultrasound, bronchoscopic biopsy or radiological intervention-guided core tissue. In terms of *EGFR* and GTPase KRas (*KRAS*) mutations, the results of Sanger sequencing and OncoMap were compared and the feasibility of using OncoMap v4 was assessed.

Patients and Methods

Patients. The mutational status was assessed in specimens obtained from 61 patients (one specimen per patient). All patients were diagnosed with pathologically confirmed NSCLC between October 2006 and April 2011 at the Samsung Medical Center, Korea, with the exception of one patient who was diagnosed at another site in August 1998. This patient had experienced two episodes of recurrence after curative surgery. A re-biopsy was then performed at our institute for genotypic screening. All tumor samples were fresh-frozen tissues. Samples were collected at the Samsung Medical Center after participants provided informed consent. This study was approved by the Samsung Medical Center Institutional Review Board (#2010-02-058). The requirement for informed consent for the use of archival tissue samples and the respective clinical data was waived. All data were de-identified.

Selection of oncogene mutations and genotyping. OncoMap v4 determines 471 mutations in 41 genes that are associated with cancer (Table I). These are well-known oncogenes and tumor-suppressor genes, based on the Cosmic database and previous reports (6, 7, 9). OncoMap v4 is a recent update of OncoMap v1, which was first introduced by MacConaill *et al.* (6). Genomic DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The detailed methodology was described in a previous report (8). DNA (250 ng) was used for the mutation analysis by OncoMap mass spectrometric genotyping based on the Sequenom MassARRAY platform (Sequenom, Inc., San Diego, CA, USA), as described previously (6, 7). Tumor-derived genomic DNA (100 ng)

Table I. Genes screened for in Oncomap v4.

Gene			
<i>ABL1</i>	<i>ERBB2</i>	<i>IDH2</i>	<i>NRAS</i>
<i>AKT1</i>	<i>FGFR1</i>	<i>JAK2</i>	<i>PDGFRA</i>
<i>AKT2</i>	<i>FGFR2</i>	<i>JAK3</i>	<i>PIK3CA</i>
<i>APC</i>	<i>FGFR3</i>	<i>KIT</i>	<i>PIK3R1</i>
<i>BRAF</i>	<i>FLT3</i>	<i>KRAS</i>	<i>PTEN</i>
<i>CDK4</i>	<i>GNA11</i>	<i>MAP2K1</i>	<i>RB1</i>
<i>CDKN2A</i>	<i>GNAQ</i>	<i>MET</i>	<i>RET</i>
<i>CSF1R</i>	<i>GNAS</i>	<i>MLH1</i>	<i>SRC</i>
<i>CTNNB1</i>	<i>HRAS</i>	<i>MYC</i>	<i>STK11</i>
<i>EGFR</i>	<i>IDH1</i>	<i>NPM1</i>	<i>TP53</i>
			<i>VHL</i>

was prepared for whole-genome amplification (WGA), and then up to 18-multiplexed polymerase chain reaction (PCR) was performed to amplify regions harboring candidate loci. After denaturation of DNA fragments, PCR products were incubated with probes that annealed immediately adjacent to the query nucleotide. Mass spectrometric genotyping, using iPLEX chemistry (Sequenom), was then performed by extending the probes by one base in the presence of chain-terminating di deoxynucleotides that generate allele-specific DNA products. The extension products were spotted onto a specially designed chip and the mutation status assessed by Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on the basis of the difference in mass between the mutant and wild-type bases.

Candidate mutations were then identified using an automated mutation-calling algorithm. Putative mutations were further filtered by manual review and selected for validation using multi-base homogenous mass-extend (hME) chemistry with pooling of a maximum of six assays of the 150-ng DNA remaining of each sample. Primers and probes used for hME validation were designed using the Sequenom MassARRAY Assay Design 4.0 software, with the default multi-base extension parameters.

Only after double-checking by iPLEX followed by confirmation with hME were mutations considered validated. iPLEX candidate mutations not confirmed by hME were considered invalid and are not reported. Additionally, examples of all detected mutations were confirmed by bi-directional Sanger sequencing, which is regarded as the gold standard genetic analysis method (10).

Results

Patients' characteristics. In this study, 61 specimens were collected from a total of 61 patients (one specimen per one patient). Male patients comprised 41 of 61 total patients; 36% of patients, including ex-smokers, had a smoking history. In regard to the type of tumor pathology, adenocarcinoma was the most common and comprised 37% among all patients. There was one patient where tumor was not able to be further defined pathologically due to poor-differentiation and was described as having poorly differentiated carcinoma. Stage IV at initial diagnosis was most common (45.9%). Overall, patients were predominantly

Table II. Baseline characteristics.

	N (Total=61)	%
Age, median (range), years	36-80	
Gender		
Male (%)	41	0.7
Female (%)	20	0.3
Smoking status		
Never-smoker	25	40.1
Ex-smoker	23	37.7
Current smoker	13	21.2
Histology		
Adenocarcinoma	37	60.7
Squamous cell carcinoma	16	26.2
Non-small cell carcinoma, otherwise unspecified	7	11.5
Poorly-differentiated carcinoma	1	1.6
<i>EGFR</i> mutation by direct sequencing		
Wild-type	25	41.0
L858R	4	6.6
Deletion 19	5	8.2
L861Q	1	1.6
L861G	1	1.6
Unknown	25	41.0
<i>K-RAS</i> mutation by direct sequencing		
Wild-type	35	55.7
Mutation	1	1.6
Unknown	25	42.6
Initial stage		
IA	0	0
IB	4	6.6
IIA	4	6.6
IIB	4	6.6
IIIA	13	21.3
IIIB	8	13.1
IV	28	45.9
Initial treatment		
Surgery	9	14.8
Definitive CCRT	7	11.5
Palliative chemotherapy	24	39.3
Radiotherapy	4	6.6
Neoadjuvant CCRT	6	9.8
Neoadjuvant chemotherapy	3	4.9
None	8	13.1

EGFR: Epidermal growth factor receptor; *KRAS*: GTPase KRas; CCRT: concurrent chemoradiotherapy.

male, with adenocarcinoma, wild-type *EGFR* gene, and advanced stage tumor. The baseline characteristics are summarized in Table II. The median overall survival of the population was 20.0 (0.4-54.6) months, the overall progression-free survival of 38 evaluable patients among the population was 8.0 (1.1-30.6) months.

Mutation analysis. Mutations analyzed by direct sequencing: *EGFR* and *KRAS* mutation analyses shown in Table II were performed by a direct sequencing method. Among 61 patients,

Table III. Frequency of mutations detected by OncoMap v4.

Gene	Amino acid	N	Total (%)
<i>TP53</i>	C176Y	1	24 (39.3%)
	H179R	3	
	R196*	1	
	Y220C	2	
	R273C	1	
	R175C	1	
	G245V	1	
	Q192*	1	
	P190L	1	
	R158H	1	
	V157F	3	
	E271K	2	
	R342*	1	
	P250L	1	
	E298*	1	
<i>EGFR</i>	R280T	1	19 (31.1%)
	G154V	1	
	R249S	1	
	L858R	6	
	E746A	4	
	E746K	3	
	E746S	1	
	L861Q	2	
	V891V	1	
	L747P	1	
<i>MLH1</i>	T790M	1	6 (9.8%)
	V384D	6	
	<i>KRAS</i>	G12C	
G12V		2	
<i>PIK3CA</i>	E545K	1	3 (4.9%)
	E542K	2	
<i>ERBB2</i>	A775G	2	2 (3.3%)
<i>ABL1</i>	G250E	1	1 (1.6%)
<i>HRAS</i>	G12C	1	1 (1.6%)
Total		59	59 (100%)

TP53: Tumor protein 53; *EGFR*: epidermal growth factor receptor; *MLH1*: MutL homolog 1; *KRAS*: GTPase KRas; *PIK3CA*: phosphoinositide-3-kinase, catalytic, alpha polypeptide; *ERBB2*: human epidermal growth factor receptor-2; *ABL1*: abelson murine leukemia viral oncogene homolog-1; *HRAS*: GTPase HRas.

mutational status data were available for 36 patients who underwent testing for *EGFR/KRAS* mutation. Regarding *EGFR* mutations, the wild-type was most common (n=24, 39.3%), followed by L858R (n=4, 6.6%). Only one of the evaluable patients was shown to have a *KRAS* mutation at diagnosis.

Mutations analyzed by high-throughput platform: A mutation analysis was performed with specimens obtained from the same population by a high-throughput genotyping tool, OncoMap v4, and the results compared with each other. In total, 67% of the group (41 out of 61 patients) were found to have mutations (Table III). There were, in total, 59 hotspot mutations among the 61 specimens tested. The

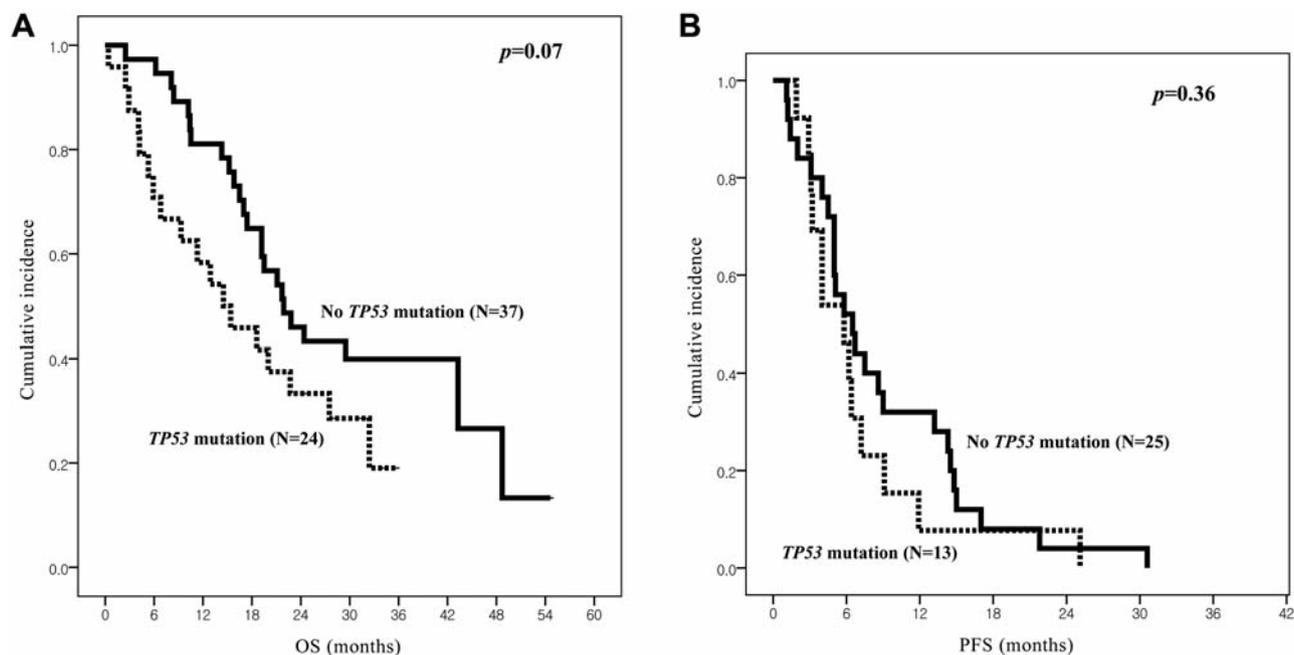


Figure 2. Survival analysis according to *TP53* mutation. A: Overall survival. B: progression-free survival.

screening of the mutation status of a panel of cancer oncogenes using small biopsy specimens obtained by bronchoscopy or core needle biopsy, because only 100 ng of DNA was required for the test.

The first study using OncoMap was reported by MacConaill *et al.* (6). They reported the sensitivity and specificity of OncoMap to be 93.8% and 100%, respectively (6). It is of note that FFPE tissue can be used with this method. In that study, only 26 lung cancer specimens were tested. In this study, a total of 61 patients' specimens were obtained and investigated with the OncoMap v4 platform, which can detect 471 mutations in 41 genes. The most frequently detected mutation was *TP53* (39.3%), which is one of the most common mutation in all solid types of cancer (14). p53, the tumor suppressor protein encoded by *TP53* exerts diverse biological effects, including control of cell cycle, apoptosis, and DNA repair against cellular stress and genetic damage (15, 16). Loss-of-function mutation of *TP53* tumor-suppressor gene plays a major role in cancer development. Given that more than 75% of *TP53* mutations result from missense mutations, the OncoMap platform would be more useful to detect such point mutations. It is known that a substantial proportion of all mutations of *TP53* tend to be located in several major 'hotspot' codons (codons 175, 245, 248, 249, 273, 282) in almost types of cancer (15, 17). Some investigators have reported lung cancer-specific hotspots of *TP53* mutation such as in codons 157, 158, 179, 245, 248, 249, 273 and 282, and the repertoires vary (18-21).

In this study, *TP53* mutations were detected in codons 157, 158, 175, 179, 190, 192, 196, 220, 245, 250, 271, 273, 280, 298, and 342 which are partly consistent with previous results (18-21). Although a limited number of patients was analyzed for survival according to *TP53* status, overall and progression-free survival tended to be shorter in the *TP53*-mutated group compared with the wild-type *TP53* group.

The second most common mutation was *EGFR*, accounting for 31.1%. Compared with the 12% *EGFR* mutation rate reported in a previous OncoMap study performed in a Western population (6), the high rate of mutations in these Korean patients with NSCLC confirms that the rate of mutation differs according to ethnicity. OncoMap v4 detected various point-mutations in *EGFR*, including L858R in 31% of patients, all of which were not identified by direct sequencing (two patients). In contrast, exon 19 deletions in five patients detected by direct sequencing were not detected by OncoMap v4. Somatic mutations of *EGFR* identified by OncoMap v4 in this study included E746A, E746K, E746S, E747P, and V891V. Unlike in-frame deletion of exon 19, T790M in exon 20, L858R or L861Q in exon 21, which are related to sensitivity to TKI, clinical implication of these somatic alterations have not been fully defined (22). Because exon 19 deletions comprise 45-50% of *EGFR* mutations, this may be a limitation for the OncoMap v4 platform (23).

Mutation of *MLH1*, which was detected in 9.8% of this study population, is a germline mutation, which results in hereditary non-polyposis colon cancer (24). Although

alteration of *MLH1* expression and the presence of single-nucleotide polymorphisms, such as the -93A>G variation among never-smokers, have been noted with lung cancer (25, 26), no relationship between *MLH1* mutation and lung cancer has been confirmed to date. *PIK3CA* mutation has clinical implication in terms of druggable target. The PI3K pathway is involved in cell proliferation and survival and is hyperactivated in various human cancer types by *PIK3CA* mutations, particularly in squamous cell cancer of the lung. Consistent with this, PI3K inhibitors have shown promising efficacy in pre-clinical models and are being assessed in clinical trials (27). It is of note that two patients were found to have both *EGFR* and *PIK3CA* mutations. They had metastatic disease at the time of initial diagnosis and were treated by platinum-based palliative chemotherapy. Response durations of first-line chemotherapy were 6.7 and 5.0 months, respectively. One patient died of lung cancer (overall survival=29.5 months), while the other was alive at the time of this analysis, with 27.8 months of follow-up.

KRAS mutations were detected in only three patients with NSCLC patients. According to an epidemiological study, Eastern Asian patients with NSCLC compared with Western populations tend to have a lower incidence of *KRAS* mutations, although there has been no confirmatory study of this (28).

The interaction between erlotinib and *ERBB2* (HER2), as well as *EGFR* (HER1), was studied *in vitro* (29). Although only two patients with adenocarcinoma harboring *ERBB2* mutation in this study were treated with erlotinib, no patients responded to erlotinib and their diseases progressed rapidly. Thus, agents targeting *ERBB2* mutations, such as Herceptin, should be further investigated: indeed, a clinical trial is in progress (ClinicalTrials.gov: NCT00758134).

Although the OncoMap platform provides rapid and highly sensitive detection of various mutations even in small biopsy specimens obtained from FFPE tissues, it has some limitations. Firstly OncoMap can detect only known mutations. Another limitation is its ability to detect only point mutations; other types of mutation, including deletions or gene rearrangements, are not detected. Rapid advances in various technologies, such as next-generation sequencing, may facilitate rapid and accurate analysis of a greater number of types of genetic alteration (30, 31). However, the usefulness of next-generation sequencing in clinical practice is limited due to its high costs and the need for relatively large amounts of tissue, which particularly for whole-genome or exome sequencing may need to be fresh, in order to detect unknown driver genomic alterations. In that sense, OncoMap v4 platform, as a high-throughput genetic analysis, is useful for the assay of repeated small biopsies in clinical practice.

In conclusion, molecular genotyping using high-throughput technology, such as OncoMap v4, is thought to be feasible even with small biopsied specimens from patients

with advanced NSCLC. This platform can be useful for clinicians to make treatment decisions based on molecular genotyping in daily clinical practice.

Conflicts of Interest

None.

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