Development of a rapid and practical mutation screening assay for human lung adenocarcinoma

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Abstract. Mortality after initial diagnosis of lung cancer is higher than from any other cancer. Although mutations in several genes, such as *EGFR* and K-*ras*, have been associated with clinical outcome, technical complexity, cost and time have rendered routine screening prohibitive for most lung cancer patients prior to treatment. In this study, using both novel and established technologies, we developed a clinically practical assay to survey the status of three frequently mutated genes in lung cancer (*EGFR*, K-*ras* and *TP53*) and two genes (*BRAF* and *β-catenin*) with known hotspot mutations in many other cancers. A single 96-well plate was designed targeting a total of 14 fragments (16 exons) from *EGFR*, K-*ras*, *TP53*, *BRAF* and *β-catenin*. In 96 lung adenocarcinoma patients, the mutation frequencies of three major genes (*EGFR*, K-*ras* and *TP53*) were between 21-24%. Fifty-six out of 96 (58%) patients had a mutation in at least one of the five genes. K-*ras* mutations positively correlated with smoking pack-years (p=0.035). *EGFR* mutations were frequent in never-smokers (p=0.0007), Asians (p=0.0204) and non-stage I lung cancer (p=0.016). There was also a trend towards an association between the presence of any mutation and improved recurrence-free survival (p=0.070). We demonstrate that our novel multigene mutation assay technology can rapidly and cost-effectively screen for mutations in lung adenocarcinoma. This screening assay can be used in the clinical setting for the large-scale validation of prognosis and/or predicting therapeutic response so that the majority of lung cancer patients can benefit from leveraging up-to-date knowledge on how mutation profiles may influence treatment options.

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Introduction

Lung cancer is the leading cause of cancer mortality in both men and women in the US, taking more lives each year than breast, prostate, colon and pancreatic cancers combined (1). Personalized medicine, or the tailoring of treatments to heterogeneous diseases based on the unique biological profile of each individual, has not yet gained widespread clinical acceptance in lung cancer. Ideally, personalized therapies would be provided to early-stage lung cancer patients in order to dramatically decrease the likelihood of recurrence or for administration of anti-cancer drugs contra-indicated for specific genetic subtypes of lung cancer. Several key genes known to be mutated in lung cancer have been associated with patient prognosis and/or response to clinical therapy. It is already known, for example, that lung adenocarcinoma patients with an *EGFR* mutation usually show better response to tyrosine kinase inhibitors (TKIs), such as gefitinib or erlotinib (2). In contrast to *EGFR*, lung cancer patients with K-*ras* mutations display primary resistance to TKIs or chemotherapy (3). *EGFR* mutations tend to be more common in non-smoker and East-Asian females while K-*ras* mutations are frequently found in smokers (4,5). Thus, an *EGFR* mutation is usually considered a good prognostic marker while a K-*ras* mutation is considered a bad prognostic marker (6). *TP53* mutations are among the most frequently screened in human cancer; however, the prognostic value of *TP53* in lung cancer remains controversial. Certain studies have reported that *TP53* mutations are associated with worse prognosis; however, these findings are inconsistent (7). Although a few studies have screened multiple genes in lung cancer patients, the correlation between the sum of different mutations and patient prognosis remains unclear (8-10).

Despite the known clinical associations of *EGFR* and K-*ras* mutations in lung cancer, and notwithstanding their significance in predicting prognosis or selecting therapeutic regimens, technical complexities, cost and delayed results (often days to weeks) have prevented clinical screening for somatic mutations from becoming routine. Much effort has gone into developing simple and high-throughput mutation screening technology, but the common use of radioactive isotopes and relatively low sensitivity has been reported as problematic (11,12). Next-generation sequencing (NGS) is a promising new technology that can

screen either whole genomes or pre-designed coding exons (exome) and identify all of the somatic mutations in a patient. However, depending on the size of the sequencing target region it usually takes up to several weeks to run a sample and even more time after that to analyze and validate the final somatic mutations. Calculating the composite effects of key mutations using NGS will require both testing and validation so that key or driving mutations can be selected from around 20,000 candidates (13-15). Ideally, key driver genes/mutations would be identified first using different mutation screening technologies so that a low-cost and simple assay for these genes or mutations can be developed for clinical use. Such a fast and reliable technology would rapidly assay commonly found somatic mutations to potentially significantly alter lung cancer prognosis and survival.

We targeted three of the most frequently mutated genes in lung cancer and two additional genes that have been reported to be widely mutated in many different types of cancer. In lung cancer, the combined frequency of mutations in any of these five genes amounts to more than 50% of cases.

Materials and methods

Patient samples. The Committee on Human Research (CHR) of the University of California San Francisco (UCSF) reviewed and approved the application for the collection of blood, sputum and tissue samples from patients with suspected or biopsy-proven thoracic malignancies (approval number: 10-03352). All samples were collected under the IRB approval granted by the CHR and written informed consents were obtained from all patients in this study.

DNA extraction from frozen lung adenocarcinoma tissues. DNA was extracted from 96 adenocarcinoma patients using either the Qiagen DNA mini kit (Qiagen) or the phenol/chloroform method. For the phenol/chloroform method, the tissue was ground in liquid nitrogen using a mortar and pestle and then incubated in DNA lysis buffer and proteinase K overnight at 56˚C. The incubated solution was mixed with the same volume of phenol-chloroform solution [UltraPure™ phenol:chloroform:isoamyl alcohol (25:24:1, v/v), Invitrogen] and then centrifuged. The top layer of the solution was mixed with the same volume of isopropyl alcohol. After centrifugation, the DNA pellet was washed using 70% ethanol, dried and eluted in distilled water (DW). DNA from formalin-fixed paraffin-embedded (FFPE) tissue samples was extracted using the Epicentre MasterPure FFPE DNA Extraction kit. The starting material was six 10-μm thick slices from a pathology block of FFPE tissue. These samples were incubated at 65˚C in tissue and cell lysis solution with proteinase K and then treated with RNaseA. Nucleic acids were precipitated and purified according to the standard Epicentre protocol and the final elution was into 50 μl of nuclease-free water. The clinical characteristics of the 96 adenocarcinoma patients are shown in Table I.

Development of the mutation assay with five genes. Three of the most frequently mutated genes (K-*ras*, *EGFR* and *TP53*) in lung cancer and two genes reported to be widely mutated in many different types of cancer (*BRAF* and *β-catenin*) were selected for the development of the mutation assay. A total of 14 fragments covering 16 exons of the five genes were designed to be amplified under the same conditions. Primers were designed using the Primer3 software and then tested and optimized. PCR primer sequences are shown in Table II. Amplification was performed at 58˚C using 20 ng of genomic DNA, 10X PCR buffer supplemented with 1.5 mM $MgCl_2$, 10 pmol of each primer, 50 mM of each dNTP, and 0.2 units of Taq polymerase (Qiagen) in a total volume of 25 μl. To prepare the ready-to-use mutation assay, master mixes were prepared for each primer pair and 23 μl were aliquoted into individual wells of a 96-well plate and frozen at -20˚C until use. Pre-made plates stored for up to two weeks did not produce different results from plates that were stored for shorter amounts of time. PCR products were subjected to agarose gel electrophoresis (2% agarose) to confirm amplification. The PCR products were cleaned up using 1 μl of SAP (1 unit/μl), 0.1 μl of *Exo*I enzyme (10 units/ μl), 1 μl of 1X PCR buffer, 2.4 μl of 10X NEB buffer (type 3), 5 μl of the PCR product, and 15 μl of water. For samples with a less intense band on the agarose gel, the amount of PCR product used was increased while the amount of water used was decreased. The reaction was incubated in a thermal cycler for 10 min at 25˚C, 60 min at 37˚C, and 15 min at 95˚C. The cleaned PCR product (5 μl) was added to 1 μl of primer and 9 μl of water and this was then sent to a commercial sequencing company to be sequenced. Bi-directional sequencing was performed using the Taq dideoxy terminator cycle sequencing kit and an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Each plate took approximately 24 h to amplify and sequence, with a mutation report generated for each patient at the end of the 24-h period. Sixteen wells were used for each patient, allowing up to six patients to be simultaneously run on a single 96-well plate (Fig. 1).

Statistical analysis. Associations between dichotomous and categorical patient clinicopathological characteristics and specific gene mutations were analyzed using the Chi-square test for dichotomous variables and a trend test for categorical variables. Continuous clinicopathological variables (age, tumor size and smoking pack-years) were regressed on the number and type of gene mutation using univariate linear regression and tested using a t-statistic. For all statistical tests, a two-tailed α-value of 0.05 was considered to indicate statistically significant differences. To determine recurrence-free survival, we used a right-censored dataset in which tumor recurrence or patient death were counted as events. Comparison between five-year recurrence-free survival by mutation status was tested using the Wilcoxon (Breslow) test. Hospital records including radiological and pathological reports as well as clinical notes were examined for evidence of recurrence. Vital status was obtained by querying the Social Security Death Master File (http://www.ssdmf.com). Five-year Kaplan-Meier survival curves were plotted for patients with and without mutations. STATA/MP 11.1 was used for all statistical analyses (StataCorp LP, College Station, TX).

Results

Mutations of five genes in the assay. A total of 16 exons from five genes (K-*ras*, *EGFR*, *TP53*, *BRAF* and *β-catenin*) were

Table I. Clinical information summary.

Characteristics	No.	Percentage	Patient no. with information
Total patients	96	100	96
Female	62	65	96
Number of deaths	37	39	96
Smoking history	66	77	86
Bronchioloalveolar carcinoma	19	20	96
Stage I	58	67	86
Stage II	11	13	86
Stage III	14	16	86
Stage IV	3	3	86
Recurrence	35	36	96
Asian ethnicity	13	14	96
	Average	SD	Patient no. with information
Average age in years	67.8	9.9	96
Follow-up months	46.2	25.0	96
Average pack years	48.0	29.2	63
Size in cm	3.25	2.15	83

SD, standard deviation.

Table II. Primer sequences in the assay.

Gene	Exon	Sequences (F/R, 5' to 3')
EGFR	18	CCAAATGAGCTGGCAAGTG/TGGAGTTTCCCAAACACTCAG
	19	CCCCAGCAATATCAGCCTTA/TGTGGAGATGAGCAGGGTCT
	20	CCCTGTGCTAGGTCTTTTGC/CCGTATCTCCCTTCCCTGAT
	21	AGCCATAAGTCCTCGACGTG/ATCCTCCCCTGCATGTGTTA
TP53	3/4	CCCCTCTGAGTCAGGAAACA/GCCAGGCATTGAAGTCTCAT
	5	CTAGCTCGCTAGTGGGTTGC/AACCAGCCCTGTCGTCTCT
	6	GAGAGACGACAGGGCTGGTT/TTGCACATCTCATGGGGTTA
	7	CTTGCCACAGGTCTCCCCAA/AGCAGTAAGGAGATTCCCCG
	8/9	CAAGGGTGGTTGGGAGTAGA/CCCCAATTGCAGGTAAAACA
K-*ras*	2 (codons 12 and 13)	ACGTCTGCAGTCAACTGGAAT/AGAATGGTCCTGCACCAGTAA
	3 (codon 61)	TCAAGTCCTTTGCCCATTTT/TGCATGGCATTAGCAAAGAC
BRAF	11	TGTATCCCTCTCAGGCATAAGG/GAAACTTTTGGAGGAGTCCTGA
	15	AACACATTTCAAGCCCCAAA/AGCATCTCAGGGCCAAAAAT
β-catenin	3	GCTTTTCTTGGCTGTCTTTCA/TCAAAACTGCATTCTGACTTTCA

F, forward primer; R, reverse primer.

simultaneously amplified. The mutation assay is shown in Fig. 1. Three lung cancer cell lines (H1650, A549 and H460) with known mutations were used as the positive controls. The mutation frequencies and the amplified exon information of the five genes are shown in Fig. 2a. The mutation frequencies for *EGFR*, *TP53* and K-*ras* were between 21-24%. Only two mutations were identified in *BRAF* and one mutation was identified in *β-catenin*. In 56 out of the 96 (58%) adenocarcinoma patients, there was a mutation in at least one of the five genes. An *EGFR* L858R mutation was found in 18% (10/56) and *EGFR* exon 19 deletion mutations were found in 9% (5/56) of the total mutations. K-*ras* codon 12 and 13 mutation frequencies were 36% (20/56) and 3% (2/56), respectively (Fig. 2e). Other mutations or variants, such as nonsense mutations and polymorphisms, were also detected in this assay.

Mutations and clinical outcome. The mutation data were compared with the clinicopathological characteristics of our

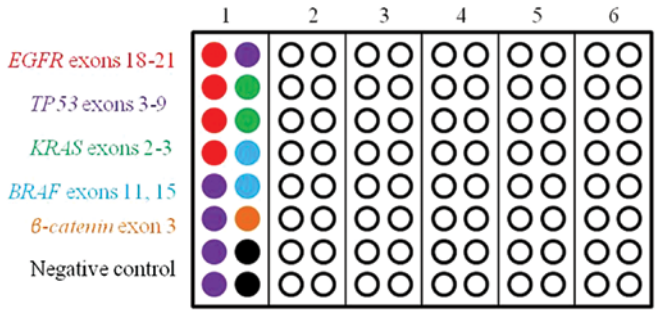

Figure 1. A mutation assay of five frequently mutated genes in lung cancer. Five mutations in lung cancer (*EGFR*, K-*ras*, *TP53*, *BRAF* and *β-catenin*) were designed to be analyzed in one plate under the same conditions. A total of 14 fragments covering 16 exons of five genes were designed in this assay. Short exons, *TP53* exons 3/4 and 8/9, were designed to be amplified together in one fragment. In a regular 96-well plate, six different patient DNAs can be analyzed in one plate, which will provide high-throughput mutation screening in the clinic.

patient cohort (shown in Table III). There was a mutually exclusive correlation between *EGFR* and K-*ras* mutations (p=0.0041). Never-smokers and 6.88-fold higher odds of having an *EGFR* mutation than patients with a smoking history (95% CI 1.89 to 24.96, p=0.0007), while smokers tended towards 6.59-fold higher odds of having a *TP53* mutation than never-smokers (95% CI 0.75 to 57.69, p=0.0624). On average, patients with an *EGFR* mutation had 32.69 fewer pack-years than those without an *EGFR* mutation (95% CI 16.7 to 48.65, p<0.0005), while patients with a *TP53* mutation had 15.7 more pack-years than those without a *TP53* mutation (95% CI -2.18 to 33.6, p=0.084). In addition, patients with a K-*ras* mutation had 18.49 more pack-years than those without a K-*ras* mutation on average (95% CI 1.38 to 35.60, p=0.035). *EGFR* mutations were more frequent (4.04-fold higher odds) in Asians than non-Asians (95% CI 1.13 to 14.51, p=0.0204). *EGFR* mutations were also more frequent in the more progressively advanced stages of lung cancer (p=0.016 for evaluating the trend between stages I, II, III and IV). Patients with bronchioloalveolar carcinoma (BAC) (2.74-fold higher odds, 95% CI 0.88 to 8.51, p=0.0696) and females (2.79-fold higher odds, 95% CI 0.83 to 9.42, p=0.0843) tended to have *EGFR* mutations but had 0.15 lower odds of having a *TP53* mutation (95% CI 0.02 to 1.32, p=0.0492). Females had 6.48-fold higher odds of having BAC (95% CI 1.30 to 32.25, p=0.0086) and non-smokers had 3.78-fold higher odds of having BAC (95% CI 1.11 to 12.88, p=0.0223) (Table III).

Prognosis prediction based on mutation status. There was a trend toward the presence of any mutation and improved prognosis. Patients with any mutation showed improved five-year recurrence-free survival vs. patients without a mutation (p=0.070) (Fig. 3). No single gene mutation was significantly associated with patient outcome.

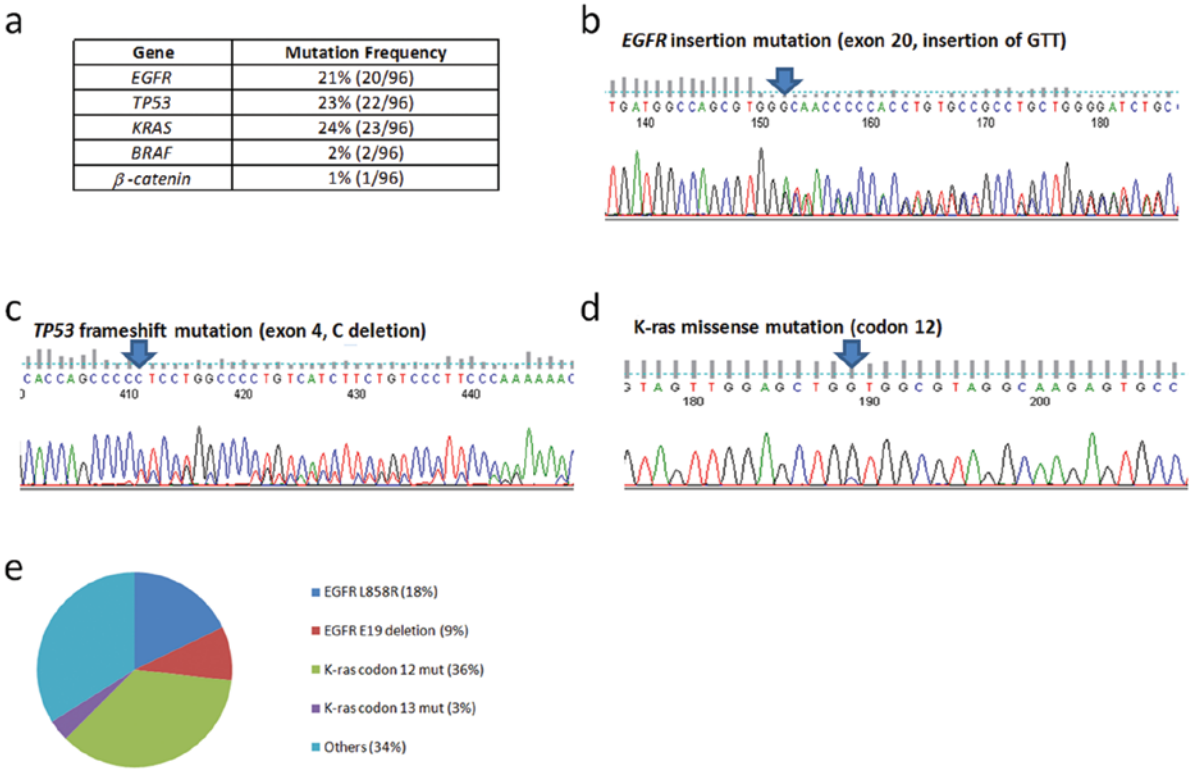

Figure 2. Summary of mutations from an assay with five frequently mutated genes. (a) Mutation frequencies of five genes are shown. Similar mutation frequencies, between 21% and 24%, were identified in *EGFR*, K-*ras* and *TP53*. Only two mutations were identified in *BRAF* and one mutation was identified in *β-catenin*. Each panel (b-d) shows different mutations from different lung adenocarcinoma patients. DNA from approximately 100 lung adenocarcinoma tissues was analyzed using a five gene mutation assay. (b-c) *EGFR* insertion mutation, c.2308_2309insGTT, and *TP53* frameshift mutation, c.267_ delC, are shown. (d) K-*ras* missense mutation at codons 12 (G12A) is shown. (e) The mutation frequencies of the four most frequent mutations (*EGFR* L858R, *EGFR* exon 19 deletion, mutations from K-*ras* codons 12 and 13) are 66% of the total mutations. FS, frameshift mutation.

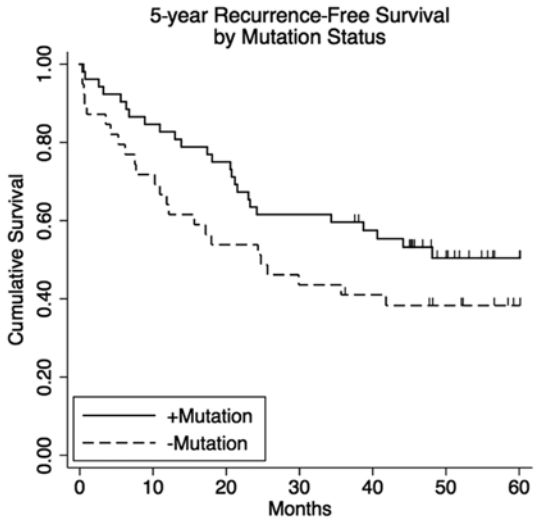

Figure 3. Five-year recurrence-free survival in the lung adenocarcinoma cohort by mutation status. The cohort included 92 flash-frozen tissue samples from patients with stage I-IV lung adenocarcinoma. Five gene mutation statuses were assessed using the sequencing assay described in Materials and methods. Five-year overall survival for the entire cohort categorized by the presence (+ mutation, n=53) and absence (- mutation, n=39) of any mutation is shown [p=0.07 by the Wilcoxon (Breslow) test].

Table III. Association between mutations and clinical outcomes.

	K-*ras* Mut$^+$	K-*ras* Mut$^-$	
EGFR Mut$^+$	0	20	
EGFR Mut$^-$	22	48	p=0.0041
	TP53 Mut$^+$	*TP53* Mut$^-$	
Smokers	17	45	
Never-smokers	1	16	p=0.0624
	EGFR Mut$^+$	*EGFR* Mut$^-$	
Smokers	9	55	
Never-smokers	9	8	p=0.0007
	EGFR Mut$^+$	*EGFR* Mut$^-$	
Asian	6	7	
Non-Asian	14	66	p=0.0204
	EGFR Mut$^+$	*EGFR* Mut	
BAC	7	12	
No BAC	13	61	p=0.0696
	TP53 Mut$^+$	*TP53* Mut$^-$	
BAC	1	18	
No BAC	19	53	p=0.0492

Mut$^+$, mutation-positive; Mut$^-$, mutation-negative (wild-type); BAC, bronchioloalveolar carcinoma.

Sensitivity and specificity assay using additional primary tissues, FFPE samples and serially diluted cell line. We selected an additional 20 tissue samples that were previously analyzed using a conventional sequencing method to examine the sensitivity and specificity of the developed mutation assay. Five samples each containing either a K-*ras*, *EGFR*, or *TP53* mutation plus another five wild-type samples were analyzed in a blind manner. All mutations were clearly detected in the 15 samples containing mutations and no mutation was found in the five wild-type samples (data not shown). We also tested eight different FFPE samples that had mutation data from the corresponding primary tissue in a blind manner. The mutations in all eight FFPE samples were identical to those detected in the primary tissues (Fig. 4). We also serially diluted the H1650 cell line, which contains an *EGFR* mutation, with water and found that the *EGFR* deletion mutation was clearly detected even at the picogram level (Fig. 5).

Discussion

While lung cancer discovered early is potentially curable, patients rarely exhibit clinical symptoms at this stage of their disease. The majority of cases (60-70%) are diagnosed at an advanced, often incurable, stage, desperately underscoring the need for improved clinical outcomes based on individual and personalized genetic information. One example of a personalized approach is the prediction of anti-cancer drug treatment response based on the mutation statuses of *EGFR* and K-*ras* (6). Because *EGFR* and K-*ras* mutation status is predictive of response to chemotherapy, assessing the mutation status of these two key genes in every lung cancer patient to direct the course of major treatment decisions is likely to improve the overall prognosis of lung cancer patients. *TP53* is also frequently mutated in lung cancer; however, the association between *TP53* mutations and clinical outcome has not been clearly established. The unknown significance of *TP53* mutations could be either due to a biological reason or due to unavailability of sufficient research data. *BRAF* is mutually exclusive with K-*ras* and a promising anti-cancer target in many types of cancers (16). The hotspot mutations in the same exon (exon 3) have also been reported in most human cancer types in *β-catenin*. To date it has been impractical to screen for mutations of multiple genes in the clinical setting due to time and cost constraints, highlighting the need for an inexpensive, rapid and reliable mutation screening assay. To this end, we developed a practical multigene mutation assay that may be prognostic of patient outcome. As a pre-designed 'ready-to-use' assay, this assay can be completed from DNA extraction to the final mutation report within 24 h. If any novel key mutation in lung cancer patients is identified in NGS or another analysis, it can easily be added to our developed assay without any major change in the protocol.

We screened approximately 100 lung adenocarcinoma patients using this multigene mutation assay. When comparing genetic mutations to the clinicopathological characteristics of our patient cohort, we observed many of the well-established associations previously described in lung cancer research (4,17-19). K-*ras* mutations positively correlated with smoking (p=0.035) while *EGFR* mutations were more common in never-smokers (p=0.0007). These well-established associations validate the compatibility of our new fast multigene mutation assay with conventional single gene mutation screening methods. Different associations between genes and smoking status may suggest that a multigene approach to mutation

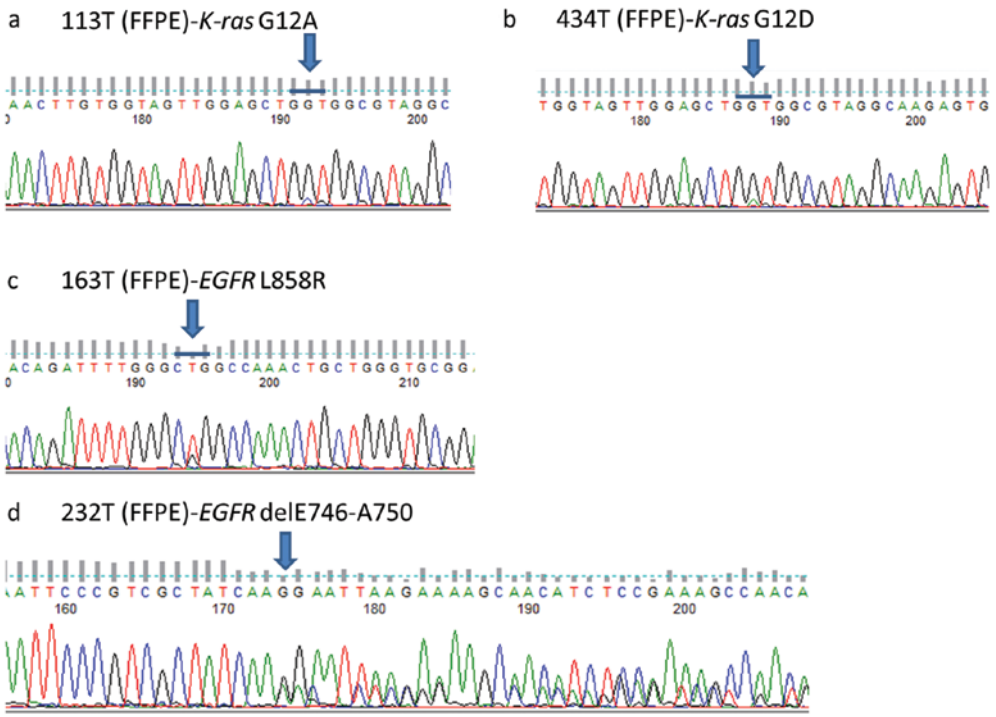

Figure 4. Mutation screening in formalin-fixed paraffin-embedded (FFPE) samples. Eight FFPE samples, whose primary tissues were analyzed with the developed mutation assay and known to have mutations, were analyzed in a blind manner. All mutations were clearly detected in eight FFPE samples. (a and b) Two K-*ras* mutations and (c and d) another two *EGFR* mutations are shown.

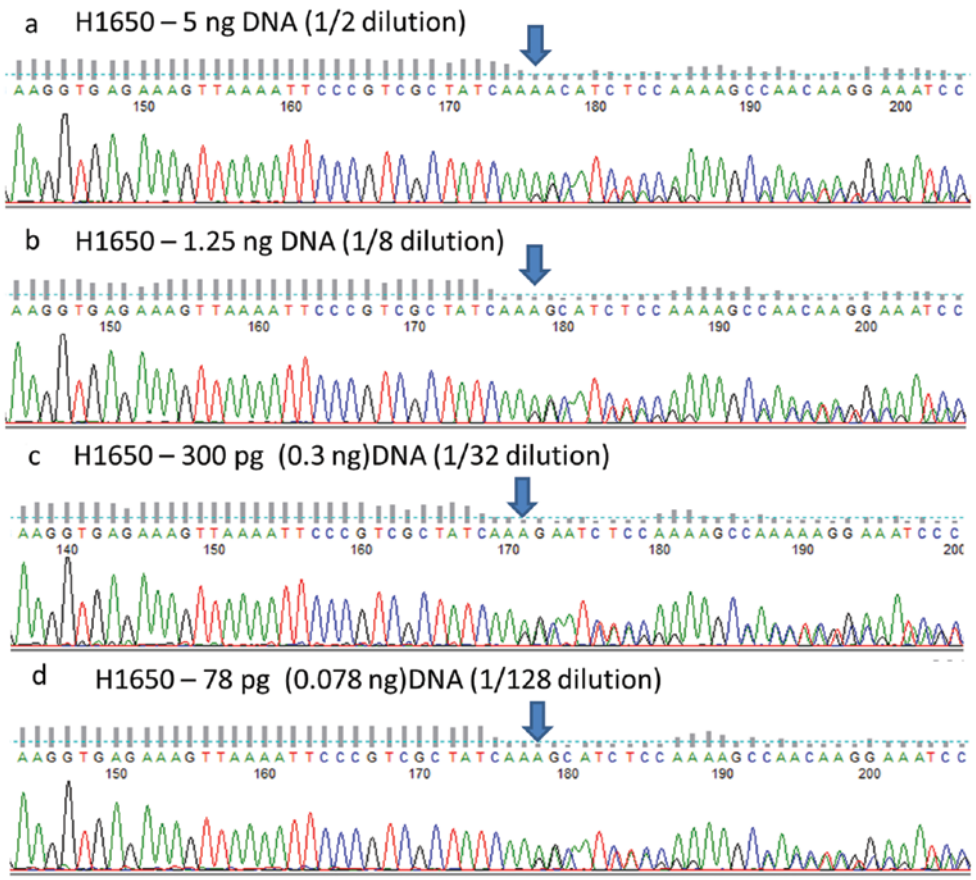

Figure 5. Mutation analysis of the serially diluted H1650 cell line DNA. The H1650 cell line with *EGFR* mutation was serially diluted with water to examine the limits of DNA detection with the sensitivity and specificity assay. The H1650 cell line was diluted from ½ (5 ng) to 1/128 (0.078 ng). Almost identical mutation patterns were observed in all tested samples, which suggests that the developed mutation assay can stably detect a very small amount of DNA. (a) ½ dilution (5 ng), (b) 1/8 dilution (1.25 ng), (c) 1/32 dilution (0.3 ng) and (d) 1/128 dilution (0.078 ng).

screening rather than a single-gene approach is desirable to investigate the association between genes and and clinical findings, although further study with a larger sample cohort is required to investigate this issue.

As FFPE tissue is the most readily available tissue substrate in clinical practice, we also tested DNA extracted from eight FFPE specimens, whose primary tissues were analyzed and for which mutation information was available, and were able to validate the stability of the assay in DNA extracted from FFPE specimens (Fig. 4). Moreover, in the serial dilution analysis of H1650, even picogram amounts of DNA could be used for the mutation screening (Fig. 5). Including *TP53* mutations, approximately 60% of the patients had a mutation in any of the three genes (*EGFR*, K-*ras* and *TP53*). Hotspot mutations of *EGFR* (L858R and exon 19 deletion mutations) and K-*ras* codon 12 and 13 mutations composed 66% (37/56) of the total mutations or 39% (37/96) of the patients. A quick and simple mutation screening for these four different mutations can give us approximately 40% sensitivity in early-stage lung cancer patients. Although K-*ras* mutations have been reported to be associated with bad prognosis and *EGFR* mutations with good prognosis, no single gene was significantly associated with disease or recurrence-free survival (6). Our data suggest that a mutation in any of the five genes is associated with better recurrence-free survival ($p=0.07$, Fig. 3). It is not clear how mutant tumor cells rather than non-mutant tumor cells contribute to improved patient recurrence-free survival. Although it is still controversial, *TP53* mutations have been reported to be associated with worse prognosis. However, this is not clear as the mutation effect of *TP53* is complex and there are several different types of mutations in *TP53*, such as dominant-negative effect or gain-of-function mutations (20). Although the sample size in our study was small and further validation should be performed to validate this association, our results suggest that the combination or the sum of mutations from multiple key genes, not a single gene, could be used for markers of not only early detection, but also patient prognosis. We believe the sum of mutations or polymorphisms, rather than a single gene or variation, may ultimately prove to be most useful in accurately predicting clinical outcomes. Taken together, we developed a practical multigene mutation screening assay containing five genes for lung adenocarcinoma patients. An interesting trend towards increased recurrence-free survival with at least one gene mutation was also noted. As this assay is designed for the analysis of a large number of patients within 24 h, this assay may ultimately be used on biopsy specimens of cancer patients before major treatment decisions or for non-invasive screening in the clinical setting.

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