Identification of Genetic Mutations in Human Lung Cancer by Targeted Sequencing

Hongxiang Feng[1], Xiaowei Wang[1], Zhenrong Zhang[1], Chuanning Tang[2], Hua Ye[2], Lindsey Jones[3], Feng Lou[2], Dandan Zhang[2], Shouwen Jiang[2], Hong Sun[2], Haichao Dong[2], Guangchun Zhang[2], Zhiyuan Liu[2], Zhishou Dong[2], Baishuai Guo[2], He Yan[2], Chaowei Yan[2], Lu Wang[2], Ziyi Su[2], Yangyang Li[2], Vijayalakshmi Nandakumar[3], Xue F. Huang[3], Si-Yi Chen[3] and Deruo Liu[1]

[1]Department of Thoracic Surgery, China-Japan Friendship Hospital, Beijing, China. [2]San Valley Biotechnology Inc., Beijing, China. [3]Norris Comprehensive Cancer Center, Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA.

ABSTRACT: Lung cancer remains the most prevalent malignancy and the primary cause of cancer-related deaths worldwide. Unique mutations patterns can be found in lung cancer subtypes, in individual cancers, or within a single tumor, and drugs that target these genetic mutations and signal transduction pathways are often beneficial to patients. In this study, we used the Ion Torrent AmpliSeq Cancer Panel to sequence 737 loci from 45 cancer-related genes and oncogenes to identify genetic mutations in 48 formalin-fixed, paraffin-embedded (FFPE) human lung cancer samples from Chinese patients. We found frequent mutations in EGFR, KRAS, PIK3CA, and TP53 genes. Moreover, we observed that a portion of the lung cancer samples harbored two or more mutations in these key genes. This study demonstrates the feasibility of using the Ion Torrent sequencing to efficiently identify genetic mutations in individual tumors for targeted lung cancer therapy.

KEYWORDS: lung cancer, genetic mutations, targeted sequencing, targeted therapy

CITATION: Feng et al. Identification of Genetic Mutations in Human Lung Cancer by Targeted Sequencing. *Cancer Informatics* 2015:14 83–93 doi: 10.4137/CIN.S22941.

RECEIVED: December 17, 2014. **RESUBMITTED:** January 21, 2015. **ACCEPTED FOR PUBLICATION:** January 22, 2015.

ACADEMIC EDITOR: J.T. Efird, Editor in Chief

TYPE: Original Research

FUNDING: This research was supported by an institutional grant and grants from the Wu Jieping Medical Foundation, the National Institute of Health (R01 CA90427 and R01 I084811 to SYC), the National Natural Science Foundation of China (Grant No. 81372503), and the China-Japan Friendship Hospital Youth Science and Technology Excellence Project (Grant No. 2014-QNYC-B-08). The authors confirm that the funders had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: These authors are employees of San Valley Biotechnology Inc in Beijing, China: CT, HYe, FL, DZ, SJ, HS, HD, GZ, ZL, ZD, BG, HYan, CY, LW, ZS, YL. Other authors disclose no potential conflicts of interest.

CORRESPONDENCE: deruoliu@vip.sina.com

Introduction

Lung cancers develop spontaneously with an accumulation of genetic and epigenetic changes in response to environmental factors such as tobacco smoke and air pollution, but underlying genetic factors may also play a role in disease development and progression.[1–4] While cigarette smoking significantly increases the risk of developing lung cancer, up to 25% of lung cancers arise in never-smokers.[3,5,6] Regardless of causal origin, lung cancers commonly exhibit non-specific symptoms, and many patients are diagnosed with advanced disease or with metastases present.[7] Despite continued efforts for early diagnosis and treatment options for lung cancer patients, the widespread incidence, poor prognosis, and staggering mortality rate remain: lung cancer is the most prevalent malignancy with the highest mortality rate worldwide with an estimated 1.8 million new cases and 1.6 million deaths in 2012.[8] Over 35% of these new cases and deaths were in China alone, where lung cancer prevails as the leading cancer in both men and women.[8]

Small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) are the two major forms of lung cancer. Roughly 85% of all lung cancers are NSCLCs, which comprises three major histologic subtypes: squamous-cell carcinoma (SCC), adenocarcinoma (AC), and large-cell lung cancer. Tobacco smoke, which is strongly associated with SCLC and SCC,[7] contains greater than 60 mutagens capable of binding to and chemically modifying DNA, and these changes leave characteristic mutational patterns seen in lung cancers.[9,10] For example, distinctive point mutation patterns in KRAS and TP53 have been observed in lung cancer patients with a history of smoking versus their non-smoking counterparts.[9,11] Compared to lung cancer in smokers, cases in never-smokers are more likely to be AC and develop in young women.[12,13] Because smoking versus non-smoking lung cancer patients have distinct mutation patterns, certain drug treatments may be more effective in one group versus the other.

The various genetic and environmental factors that contribute to lung cancer vary widely, and the gene mutation profile of each tumor can be entirely unique. As such, the accumulating evidence suggests that generalized treatments for lung cancers are less effective, and individualized therapies targeting specific mutations are critical for effective

treatment. Personalized treatments utilize drugs specifically designed to target particular gene mutations in an individual tumor,[14] and these observed mutations can determine which drug regimen to implement. For example, patients with EGFR mutations, particularly non-smoking women with advanced NSCLC, are commonly treated with erlotinib, which blocks EGFR signaling and slows lung cancer progression.[15,16] Additionally, drugs have been developed to target VEGF mutations and an ALK/EML4 fusion.[17] Clinical trials have also shown that a combination of chemotherapeutics and drugs targeting specific mutations can work synergistically and specifically to provide patient benefits greater than any single treatment.[14,18]

A critical step in directing lung cancer treatments is identifying genetic alterations in the tumor. Currently, different clinical methods are used to detect gene mutations in lung cancer patients, including direct polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), and immunohistochemistry (IHC), none of which has been standardized in clinical diagnostics and each has pros and cons.[19,20] As an alternative to first-generation Sanger sequencing, next-generation sequencing (NGS) has become more popular to sequence the cancer genome of individual tumors, but the instruments and assays are costly with relatively lengthy run times, making these technologies impractical for widespread clinical use. Second- and third-generation sequencing platforms, such as Illumina HiSeq and MiSeq, 454 pyrosequencing, Helicos HeliScope, SOLiD sequencing, and Ion Torrent sequencing,[21–23] are facilitating the advancement of personalized cancer treatments by allowing for cost- and time-effective high-throughput screening and sequencing.[24,25] Specifically, the Ion Torrent platform has further revolutionized NGS through the use of post-light sequencing technology, which utilizes standard DNA polymerase sequencing with unmodified dNTPs and a hypersensitive ion sensor to detect hydrogen ions released as each nucleotide is incorporated into the growing complementary DNA strand.[26] This innovative method circumvents much of the cost and complexity associated with the four-color optical detection system used in the other aforementioned NGS platforms, helping to further make personalized cancer sequencing and treatments a possibility in the near future.[25,27]

To investigate the feasibility of using Ion Torrent sequencing to reliably detect mutations in individual lung cancer samples of different types, we have used Ion Torrent sequencing with the Ion Personal Genome Machine (PGM) and Ion Torrent AmpliSeq Cancer Panel to analyze 48 lung cancer samples from Chinese patients and identify genetic mutations at 737 loci from 45 known cancer-related genes and oncogenes. This study demonstrates the feasibility of using the Ion AmpliSeq Cancer Panel to efficiently identify genetic mutations in individual tumors to potentially direct targeted therapies in lung cancer patients.

Materials and Methods

Ethics statement. The study has been approved by the Human Research Ethics Committee of the China-Japan Friendship Hospital, China. For formalin-fixed, paraffin-embedded (FFPE) tumor samples from the tumor tissue bank at the Department of Pathology of the hospital, the Institutional Ethics Committee waived the need for IRB consent as all samples and medical data used in this study have been irreversibly anonymized.

Sample DNA preparation. The 48 lung cancer samples used in the study were collected from the China-Japan Friendship Hospital, China. Paraffin sections (3–5 μm thick) extracted from FFPE samples were deparaffinized in xylene, and then DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) following manufacturer's instructions.

Ion Torrent PGM library preparation and sequencing. The Ion AmpliSeq Library Kit 2.0 (Life Technologies; Part #4475345 Rev. A) was used to construct an Ion Torrent adapter-ligated library as per manufacturer's instructions, and the Ion PGM Sequencing 200 Kit was used for sequencing reactions according to the recommended protocol (Part #4474004 Rev. B), detailed in our previous publications.[28,29]

Variant calling. The Ion Torrent platform-specific pipeline software Torrent Suite was used to initially process data from the PGM runs and generate sequence reads, trim adapter sequences, filter, and remove poor signal profile reads. Torrent Suite Software v3.4 with a plug-in variant caller v3.4 generated initial variant calling from the Ion AmpliSeq sequencing data. Several subsequent filtering steps were used to eliminate erroneous base calling and to generate final variant calling: the first filter was fixed at an average total coverage depth >100, each variant coverage >20, a variant frequency of each sample >5%, and *P*-value <0.01; the second filter utilized visual inspection of the mutations using Integrative Genomics Viewer (IGV) software (http//www.broadinstitute.org/igv) or SAMtools software (http://samtools.sourceforge.net), along with eliminating possible strand-specific errors; the third filter was set as variants within 737 hotspots, as per manufacturer's instructions; and the final filtering step eliminated variants in amplicon AMPL339432 (PIK3CA, exon13, chr3:178938822–178938906), which is not uniquely matched in the human genome. From our sequencing runs using the Ion AmpliSeq Cancer Panel, the JAK2 gene locus generated false deletion data; therefore, the sequencing data from this locus were excluded from further analysis. Supplementary Figure 1 shows this filtering process in more detail.

Somatic mutations. To distinguish somatic and germline mutations, our detected mutations were compared to variants in the 1000 Genomes Project[30] and 6,500 exomes of the National Heart, Lung, and Blood Institute's Exome Sequencing Project.[31]

Bioinformatical and experimental validation. We used COSMIC[32] (version 64), My Cancer Genome database (http://www.mycancergenome.org/), and other publications to assess

reappearing mutations in lung cancer (see Supplementary Table 1). Additionally, the accuracy of the Ion Torrent PGM was compared to the Sanger sequencing method. Because DNA from the 48 experimental samples was limited, we used a trial of an additional 60 negative and 62 positive FFPE lung cancer samples that were obtained from the tumor tissue bank at the Department of Pathology of the China-Japan Friendship Hospital, China.

Statistical analysis. Odds ratios (ORs) of samples with mutations and without mutations for smoking versus non-smoking patients were determined using 2 × 2 contingency tables, and the Fisher's exact test was used to calculate *P*-values in the detected mutated genes and total variants using GraphPad QuickCalcs online calculator for scientists (http://www.graphpad.com/quickcalcshttp://www.webcitation.org/query.php?url=http://www.graphpad.com/quickcalcs/index.cfm&refdoi=10.1186/1756-0500-7-805). All *P*-values are two sided, and statistical significance was defined as $P < 0.05$.

Results and Discussion

Ion Torrent versus Sanger sequencing experimental validation. For experimental validation of the Ion Torrent PGM, additional FFPE lung cancer samples were used, and only common mutations in exons 19 and 21 of EGFR were sequenced. All positive Sanger samples generated positive data from the Ion Torrent PGM, and only one sample generated negative data with Sanger sequencing and positive data from the Ion Torrent PGM for EGFR exon 21 mutations (Supplementary Figure 2 and Supplementary Table 2). This discrepant sample had a variant frequency of 5.59%, indicating that this may have actually been a false negative in Sanger sequencing as opposed to a false positive in Ion Torrent sequencing. Sanger sequencing has been shown to miss mutations when the allele frequency of the mutation is lower than 10%,[33] whereas the Ion Torrent PGM has been shown sensitive enough to detect variant frequencies of 5%.[34] The greater sensitivity has important clinical implications where tumor samples may be a homogenous mixture of normal and cancerous cells.

Sequence coverage in 48 lung cancer samples. The mean read length of each sequence read was 80 bp, and the average sequence per sample was approximately 23 Mb. With normalization to 300,000 reads per specimen, there was an average of 1,639 reads per amplicon (range: 59–3,504) (Fig. 1A), where 181/189 (95.8%) amplicons averaged at least 100 reads, and 171/189 (90.5%) amplicons averaged at least 300 reads (Fig. 1B).

Lung cancer patients. The average age of all 48 lung cancer patients included in the study was 62.7 years, with a range of 42–78 years (SD ±8.6 years). Lung cancer samples were divided into three pathologic subtypes: AC ($n = 22$), SCC ($n = 22$), and other ($n = 4$) (Table 1). Slightly more than half of the AC samples were from females (54.5%) and never-smokers (72.7%), whereas the majority of the SCC samples were from males (86.4%) and heavy smokers (63.6%).

Gene mutations in lung cancer subtypes. From the 45 genes screened in our study, a total of 35 mutations were identified in EGFR, TP53, KRAS, PIK3CA, CDKN2A, and CTNNB1, and these were detected in 26 of the 48 samples (51.2%) (Fig. 2 and Tables 2 and 3). A total of 15 (68.2%) AC samples contained at least one mutation, and 13 (86.7%) of these AC samples with mutations were from never-smokers (OR: 0.115; $P = 0.054$), whereas 9 (40.9%) SCC samples contained at least one mutation, 7 (77.8%) of which were from patients with a history of smoking (OR: 0.292; $P = 0.544$). Additionally, seven of the 48 samples (14.6%) contained combination mutations in two genes (Table 4). Interestingly, combination mutations were only found in AC samples from females, where three samples each contained at least one EGFR mutation and either a mutation in CTNNB1, PIK3CA, or TP53. Three male SCC samples each harbored a PIK3CA mutation and either a KRAS or TP53 mutation.

Lung cancer, like other cancers, develops through an accumulation of genetic changes that affect different signaling pathways and hinder normal functions, including cell growth, survival, proliferation, and apoptosis. In our study, differences in signaling pathway disruption can be seen between AC and SCC in the EGFR pathway (EGFR, PIK3CA, and KRAS), tumor suppressor pathways (TP53 and CDKN2A), and Wnt pathway (CTNNB1) (Fig. 3). All the genes identified to be mutated in our study have previously been classified as driver mutations,[35] for mutations in these genes can promote or drive tumorigenesis by conferring a selective growth advantage to the cells with these mutations. The number of driver mutations differs by patient and cancer type, where some may have few and some many. Tumors of various cancer types with only one driver mutation tend to have this mutation in an oncogene, while tumors with more driver mutations tend to have a combination of oncogene and tumor suppressor gene mutations.[35] Accordingly, in our study, the majority (72.2%) of samples with one mutation harbored the mutation in an oncogene (CTNNB1, EGFR, KRAS, or PIK3CA) (Table 3), and 37.5% of samples with two or more mutations revealed combination mutations in both oncogenes and the tumor suppressor gene TP53 (Table 4).

EGFR mutations. Mutations in EGFR is one of the most common genetic alterations found in NSCLCs.[36] Roughly 34% of all lung ACs contain EGFR mutations,[37] and these mutations are more common in non-smoking and Asian populations, with some studies reporting a frequency of 50% or higher.[38–40] EGFR mutations are much less common in SCCs, and are found in only 6% of these tumors.[37] Accordingly, we identified 13 AC samples (59.1%) and 1 SCC (4.5%) sample with EGFR mutations, and 3 (13.6%) of these AC samples contained two EGFR mutations. We found EGFR mutations to be significantly associated with AC versus SCC ($P = 0.0002$; OR: 30.3). Additionally, EGFR mutations were only found in samples from never-smokers.

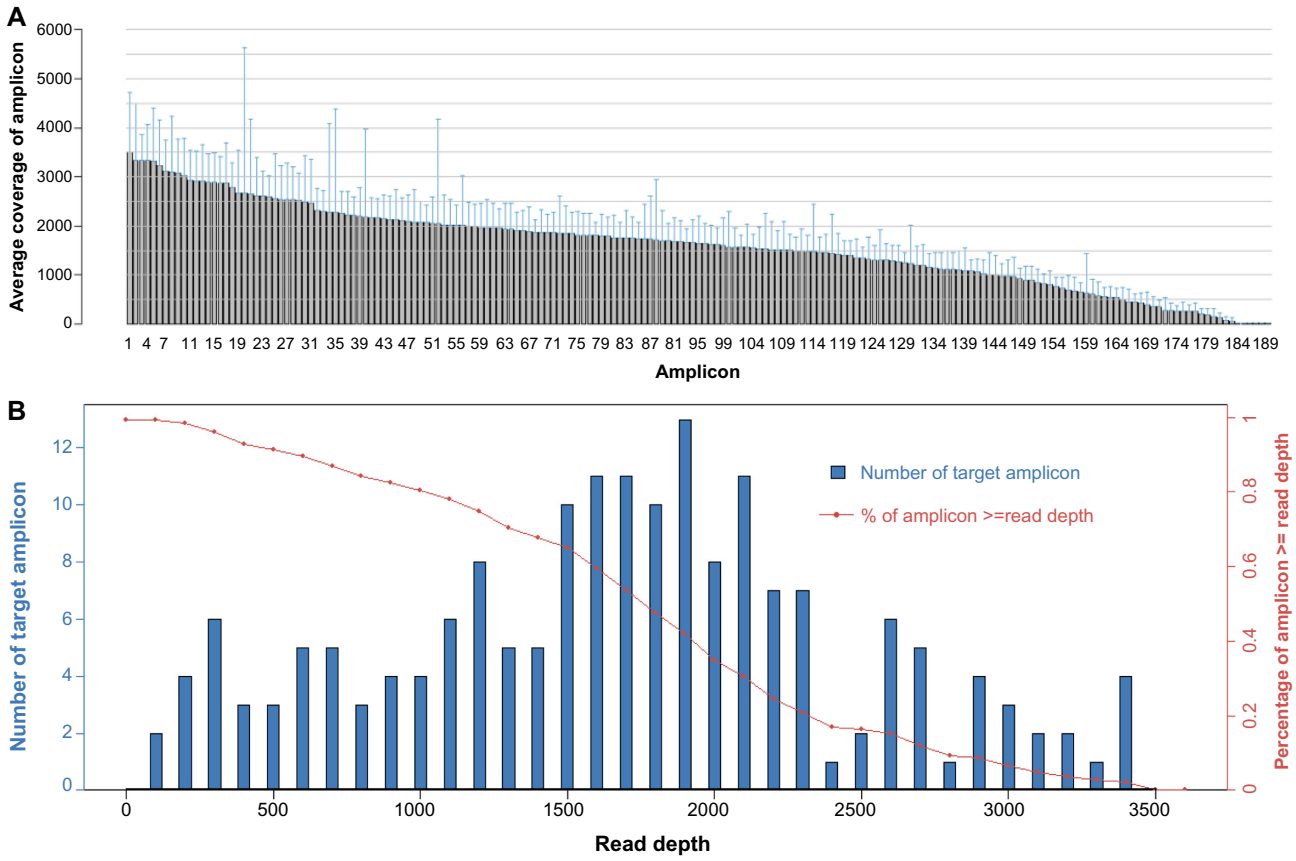

Figure 1. Sequence read distribution across 189 amplicons generated from 48 FFPE specimens, normalized to 300,000 reads per sample. (**A**) Distribution of average coverage of each amplicon. Data are shown as mean ± SD. (**B**) Number of amplicons with a given read depth, sorted in bins of 100 reads. (Blue bars represent number of target amplicons within read depth and the red line represents % of target amplicons ≥ read depth.)

Table 1. Clinical features of 48 lung cancer patients.

CHARACTERISTIC		AC n (% OF 22 SAMPLES)	SCC n (% OF 22 SAMPLES)	OTHER n (% OF 4 SAMPLES)
Age (years)		60.4 ± 9.0	64.8 ± 8.1	63.8 ± 8.3
Sex	Male	10 (45.5%)	19 (86.4%)	3 (75.0%)
	Female	12 (54.5%)	3 (13.6%)	1 (25.0%)
TNM stage	1a	6 (27.3%)	2 (9.1%)	0 (0.0%)
	1b	4 (18.2%)	3 (13.6%)	0 (0.0%)
	2a	8 (36.4%)	3 (13.6%)	1 (25.0%)
	2b	1 (4.5%)	3 (13.6%)	0 (0.0%)
	3a	3 (13.6%)	8 (36.4%)	2 (50.0%)
	3b	0 (0.0%)	3 (13.6%)	0 (0.0%)
	ND	0 (0.0%)	0 (0.0%)	1 (25.0%)
Differentiation	Low	7 (31.8%)	17 (77.3%)	2 (50.0%)
	Middle	10 (45.5%)	5 (22.7%)	0 (0.0%)
	Middle	4 (18.2%)	0 (0.0%)	0 (0.0%)
	ND	1 (4.5%)	0 (0.0%)	2 (50.0%)
Smoking history	Never	16 (72.7%)	3 (13.6%)	1 (25.0%)
	Light	4 (18.2%)	5 (22.7%)	0 (0.0%)
	Heavy	2 (9.1%)	14 (63.6%)	3 (75.0%)

Abbreviations: AC, adenocarcinoma; SCC, squamous-cell carcinoma; ND, not determined.

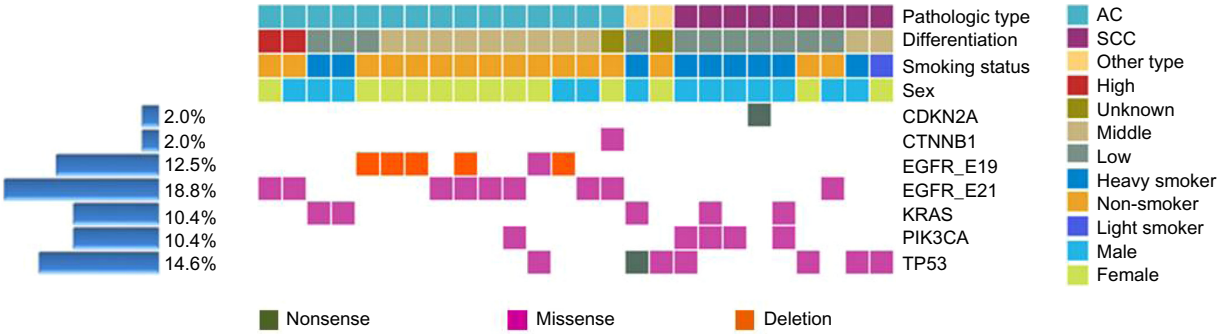

Figure 2. Summary of mutated genes detected in 48 lung cancer samples. A total of 26 samples harbor mutations in EGFR, TP53, KRAS, PIK3CA, CDKN2A, and CTNNB1. Samples are classified by four methods: pathologic type (AC, SCC, others), differentiation (high, middle, low, unknown), smoking history (heavy smoker, light smoker, non-smoker), and sex (male or female). Frequencies of mutations per gene are represented by blue bar graphs.

An EGFR mutation at the tyrosine kinase domain leads to constitutive activation of kinase activity and downstream signaling pathway activation, which results in increased proliferation, angiogenesis, and metastasis and a decrease in apoptosis.[41,42] All the EGFR mutations we identified were in the tyrosine kinase domain localized to exon 19 (E746_A750del, L747_P753>S, L747_A750>P, and A750P) and exon 21 (L858R and L861Q), areas that are known to harbor the majority of mutations. In fact, point mutations L858R and E746_A750del comprise nearly 90% of all EGFR mutations in NSCLCs.[43] Tumors with these two mutations are dependent on EGFR signaling, and are therefore sensitive to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib.[44] Other common EGFR mutations not identified in our study, T790M and insertions in exon 20, have been found to be nonresponsive to these TKIs.[45,46]

The development of EGFR TKIs has significantly improved treatment and prolonged survival for some patients with NSCLCs, and the best responses are seen in those with AC subtype, nonsmokers, younger women, and those of Asian descent.[46,47] However, typical response rates to gefitinib and erlotinib are only about 10% and 12%, respectively,[48,49] and clinical data show that NSCLCs eventually develop drug resistance and progress despite such treatment usually from acquired secondary EGFR mutations or other mechanisms, including KRAS and PIK3CA mutations.[44,46,50] Nevertheless, identifying EGFR mutations is critical in determining the most beneficial treatments for NSCLC patients.

TP53 mutations. TP53 mutations are prevalent genetic alterations found in many lung cancers, with up to 43% of SCCs and 35% of ACs harboring mutations in this gene.[37] Our study detected TP53 mutations in seven samples (14.6%): one AC (4.5%), four SCCs (18.2%), and two in the other lung cancer types. The frequency of TP53 mutations in our study is somewhat lower than others have reported, which may be because of the small sample size and population variations, and also the variant filter process to select for mutations already identified in the COSMIC database (Supplementary Fig. 1). All the identified TP53 mutations were found at known hotspot locations within the DNA-binding domain, including two in exon 5 (V157F and R158L), three in exon 7 (G245V, R248W, R249S), and two in exon 8 (E285K and R306*). Accordingly, most TP53 mutations cluster in the TP53 DNA-binding domain, encompassed by exons 5 through 8, and spans approximately 180 codons.[51] Previous research has shown TP53 mutations in tobacco-associated lung cancers to have distinct profiles that consist of a high proportion of G to T transversions, particularly at codons 157, 158, 179, 248, and 273, and such mutations are rarely found in lung cancers from never-smokers.[52–54] While four of the seven TP53 mutations detected in our study were in fact G to T transversions, including at codons 157 and 158 in samples from smokers, two of these transversion mutations occurred in never-smokers (R219S and G245V) (Table 5). Additionally, the mutation detected at codon 248 was a transition mutation that occurred in a never-smoker with AC.

While many TP53 missense mutations can still result in the formation of a stable protein, the mutated protein lacks DNA-binding specificity and accumulates in the nucleus. Additionally, these mutant proteins lack the ability to transactivate downstream target genes that regulate cell cycle and apoptosis.[55] Some TP53 mutations may lead to gain-of-function (GOF) activities in the mutant protein product, which can actively contribute to tumor progression and metastases, and can also result in increased drug resistance.[56–58] Tumors containing mutant TP53 are more resistant to ionizing radiation than those with the wild-type TP53, and TP53 overexpression in NCLCs has been associated with unresponsiveness to cisplatin-based therapies.[53,59] Overall, TP53 overexpression is associated with increased tumor aggressiveness, poorer patient prognosis, and shorter overall survival in both AC and SCC patients.[53,60,61]

KRAS mutations. Different rates of KRAS mutations have been found in lung cancer subtypes, where an estimated 19% of AC patients harbor KRAS mutations versus only 5% of SCC patients.[37] Our study detected KRAS mutations at equal rates in ACs and SCCs (9.1%), and these samples were all from male patients with a history of smoking. Nearly all (97%) of KRAS mutations are found in the GTP binding domain of exons 2 and 3,[36] and accordingly, all mutations in

Table 2. Mutation frequencies in 48 lung cancer samples based on sex, pathologic type, and smoking history.

GENE	TOTAL NUMBER OF SAMPLES WITH MUTATIONS (% IN 48 SAMPLES)	SEX		PATHOLOGIC TYPE			SMOKING HISTORY		
		MALE SAMPLES WITH MUTATION (% IN 32 SAMPLES)	FEMALE SAMPLES WITH MUTATION (% IN 16 SAMPLES)	AC SAMPLES WITH MUTATION (% IN 22 SAMPLES)	SCC SAMPLES WITH MUTATION (% IN 22 SAMPLES)	OTHER SAMPLES WITH MUTATION (% IN 4 SAMPLES)	NEVER SMOKER SAMPLES WITH MUTATION (% IN 20 SAMPLES)	LIGHT SMOKER SAMPLES WITH MUTATION (% IN 9 SAMPLES)	HEAVY SMOKER SAMPLES WITH MUTATION (% IN 19 SAMPLES)
CDKN2A	1 (2.1%)	1 (3.1%)	0 (0.0%)	0 (0.0%)	1 (4.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)
CTNNB1	1 (2.1%)	0 (0.0%)	1 (6.3%)	1 (4.5%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)
EGFR	14 (29.2%)	4 (12.5%)	10 (62.5%)	13 (59.1%)	1 (4.5%)	0 (0.0%)	14 (70.0%)	0 (0.0%)	0 (0.0%)
KRAS	5 (10.4%)	5 (15.6%)	0 (0.0%)	2 (9.1%)	2 (9.1%)	1 (25.0%)	0 (0.0%)	3 (33.3%)	3 (15.8%)
PIK3CA	5 (10.4%)	4 (12.5%)	1 (6.3%)	1 (4.5%)	4 (18.1%)	0 (0.0%)	1 (5.3%)	2 (22.2%)	2 (10.5%)
TP53	7 (14.6%)	3 (9.4%)	4 (25.0%)	1 (4.5%)	4 (18.1%)	2 (50.0%)	3 (15.8%)	1 (11.1%)	3 (15.8%)

Abbreviations: AC, adenocarcinoma; SCC, squamous-cell carcinoma.

our study were located in exon 2, codon 12 (G12A and G12C). The intrinsic GTPase activity of RAS is impaired by these mutations, and resistance is conferred to GTPase activators; this causes accumulation of RAS in its active GTP-bound state, thereby sustaining the activation of RAS signaling and a disconnection from upstream EGFR signaling.[36,62]

Because KRAS is part of the EGFR signaling pathway, constitutive activation of KRAS leads to resistance to EGFR TKIs.[63] Patients with KRAS-mutant NSCLC also lack benefits from adjuvant chemotherapy in early stages of the disease and have shown poorer clinical outcomes when treated with erlotinib and chemotherapy.[61,64] Overall, NSCLC patients with KRAS mutations have worse overall survival than those with wild-type KRAS,[18] regardless of the treatment method. As they are fairly prevalent, detecting KRAS mutations in lung cancer patients prior to treatment may prevent unnecessary drug toxicities from certain drug regimens.

PIK3CA mutations. Phosphatidylinositol-3-kinases (PI3Ks), including PIK3CA that encodes the p110α catalytic subunit, are lipid kinases critical in regulating signaling pathways and cellular functions, including cell proliferation and survival. PIK3CA mutations result in constitutive activation in EGFR signaling, and subsequent activation of downstream Akt signaling caused by these mutations interferes with other signaling pathways and contributes to oncogenicity.[65,66]

Commonly found in many cancer types, PIK3CA mutations are present in roughly 6% of SCCs and 4% of ACs.[37,65] In our study, 5 of the 48 samples (10.4%) harbored a PIK3CA mutation either in exon 1 (R88Q), in the helical domain of exon 9 (E542K and E545K), or in the kinase domain of exon 20 (H1047L). Four of these mutations occurred in male SCC patients with a history of smoking, whereas one mutation (H1047L) was from a female never-smoker with AC and a co-occurring EGFR mutation, which was most likely the driver mutation. Accordingly, others have found a higher rate of PIK3CA mutations in SCCs, and approximately two-thirds of all PIK3CA mutations are found primarily at codons 542, 545, and 1047.[66,67]

PIK3CA mutations have been associated with faster disease progression and worse overall survival, and some clinical studies have found PIK3CA mutations to lead to acquired resistance to EGFR TKIs.[68,69] While PIK3CA mutations are found in a smaller subset of lung cancers compared to other genes and are therefore not routinely tested for, detection of these mutations may help guide patient treatment.

Less frequent mutations. One SCC sample from a male with a history of heavy smoking contained a mutation (E69*) in the tumor suppressor gene CDKN2A, which plays a critical role in regulating cell cycle and downstream TP53.[70] Between 7% and 8% of lung ACs and SCCs have been found to have CDKN2A mutations, but this specific point mutation is much less common.[37] As in the sample in our study, previous studies have found a positive relationship between smoking and CDKN2A mutations.[71,72]

Table 3. Single mutations and patient characteristics from 48 lung cancer samples.

GENE	MUTATION	AGE	SEX	PATHOLOGIC TYPE	DIFFERENTIATION	TNM STAGE	SMOKING HISTORY
CDKN2A	E69*	62	M	SCC	Low	2a	Heavy
EGFR	L861Q	49	M	AC	High	1a	Never
EGFR	E746_A750del	48	M	AC	Middle	2a	Never
EGFR	E746_A750del	71	F	AC	Middle	2b	Never
EGFR	L858R	67	M	AC	Middle	2a	Never
EGFR	L858R	73	M	SCC	Low	3b	Never
EGFR	E746_A750del	65	F	AC	Middle	1a	Never
EGFR	L858R	66	F	AC	Middle	1a	Never
EGFR	L858R	62	F	AC	High	2a	Never
EGFR	L747_P753>S	52	F	AC	Low	3a	Never
EGFR	L858R	50	F	AC	Middle	2a	Never
KRAS	G12C	53	M	AC	Low	2a	Light
KRAS	G12A	57	M	AC	Low	2a	Heavy
PIK3CA	E542K	66	M	SCC	Low	3b	Heavy
TP53	R158L	53	M	SCC	Middle	3a	Heavy
TP53	R249S	65	F	other	Unknown	ND	Never
TP53	V157F	68	F	SCC	Middle	1b	Light
TP53	G245V	78	F	SCC	Low	2a	Never

Note: *Nonsense mutation resulting in a stop codon.
Abbreviations: SCC, squamous-cell carcinoma; AC, adenocarcinoma; ND, not defined.

One AC sample contained a mutation in CTNNB1 (S33F), which was co-occurring with an EGFR mutation. While roughly 3% of ACs and 1% of SCCs harbor mutations in this gene, this specific point mutation in lung cancers is extremely rare.[37] The CTNNB1 gene encodes for β-catenin, a ubiquitous intracellular protein that plays a vital role in the Wnt signaling pathway. Mutations in CTNNB1 can cause accumulation of β-catenin in the nucleus and downstream target gene activation that hinders cell growth regulation and contributes to tumorigenesis.[73] Studies have found that since both Wnt and EGFR signaling can act on β-catenin, these two signaling pathways work synergistically in the process of tumorigenesis.[74] Drugs targeting CTNNB1 are currently under testing,[75] which may work in conjunction with EGFR TKIs to enhance treatment in patients with such combination mutations.

Conclusion

In the present study, we used Ion Torrent AmpliSeq Cancer Panel to sequence 737 loci from 45 cancer-related genes, mainly oncogenes and tumor suppressor genes, in 48 lung cancer samples of different pathologic types. We identified frequent mutations in EGFR, TP53, KRAS, and PIK3CA and mutations in CDKN2A and CTNNB1 at lower frequencies,

Table 4. Combination mutations and patient characteristics from 48 lung cancer samples.

GENE 1	MUTATION 1	GENE 2	MUTATION 2	GENE 3	MUTATION 3	AGE	SEX	PATHOLOGIC TYPE	DIFFERENTIATION	TNM STAGE	SMOKING HISTORY
CTNNB1	S33F	EGFR	L858R	-	-	72	F	AC	ND	1b	Never
EGFR	E746_A750del	EGFR	L858R	-	-	56	F	AC	Middle	3a	Never
PIK3CA	R88Q	KRAS	G12C	-	-	69	M	SCC	Low	3a	Light
PIK3CA	R88Q	KRAS	G12C	-	-	56	M	SCC	Low	3b	Light
PIK3CA	E545K	TP53	E285K	-	-	68	M	SCC	Low	2b	Heavy
PIK3CA	H1047L	EGFR	L858R	-	-	73	F	AC	Middle	3a	Never
TP53	R306*	KRAS	G12C	-	-	58	M	other	Low	3a	Heavy
EGFR	L747_A750>P	EGFR	A750P	TP53	R248W	59	F	AC	Middle	1b	Never

Note:*Nonsense mutation resulting in a stop codon.
Abbreviations: SCC, squamous-cell carcinoma; AC, adenocarcinoma; ND, not defined.

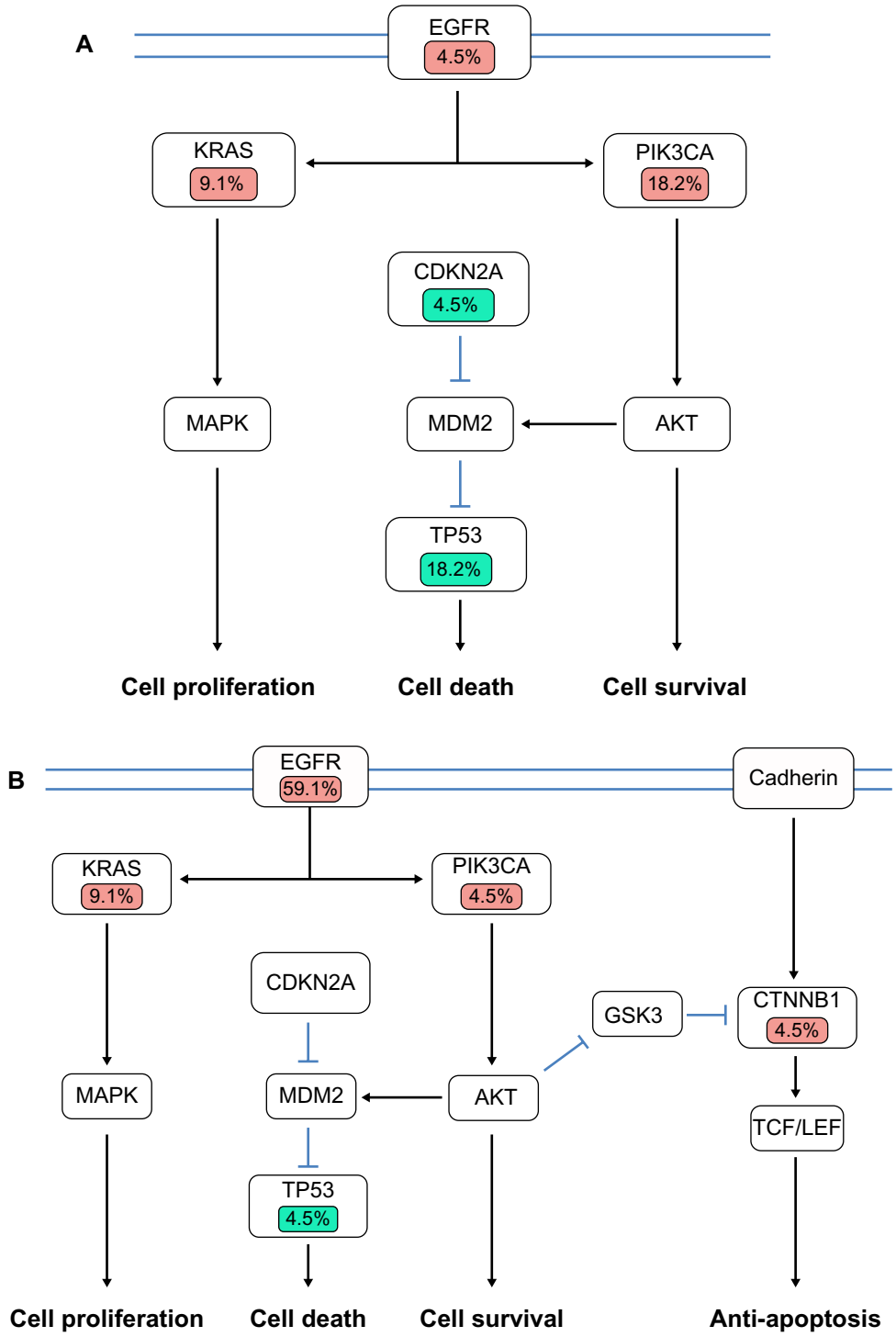

Figure 3. Mutated signaling pathways in SCC (**A**) and AC (**B**). Genetic alterations in lung cancers primarily occur in genes of the EGFR (MAPK and PI3K), tumor suppressors (TP53 and CDKN2A), and Wnt (CTNNB1) signaling pathways. Alterations in oncogenes are indicated in pink and those in cancer suppressor genes are shown in green.

and unique mutation patterns in AC versus SCC samples can be seen. While this supports previous research that different lung cancer types have distinct molecular profiles, and thus potentially different prognoses and patient outcomes, our limited sample size and low TP53 mutation rate suggest that supplementary studies with larger sample sets may be beneficial. Additionally, because lung cancers may exhibit intratumor heterogeneity,[76,77] additional studies utilizing multiregion sequencing may help to more intricately define the mutation profile for these cancers and for each patient. Fortunately, the affordable cost and time efficiency of Ion Torrent sequencing may facilitate such follow-up studies and increase the availability of personalized cancer sequencing and targeted therapies in the near future.

Table 5. Transversion versus transition mutations in TP53.

MUTATION	SUBSTITUTION	MUTATION TYPE	LUNG CANCER TYPE	SMOKING HISTORY
V157F	G>T	transversion	SCC	Light
R158 L	G>T	transversion	SCC	Heavy
G245V	G>T	transversion	SCC	Never
R248W	C>T	transition	AC	Never
R249S	G>T	transversion	other	Never
E285K	G>A	transition	SCC	Heavy
R306*	C>T	transition	other	Heavy

Acknowledgments

We would like to thank Rong Shi at the Wu Jieping Medical Foundation, and Haibo Wang, Ying Li, and other members of San Valley Biotechnology Inc., Beijing for their assistance in sample and data collection. We would also like to thank the staffs at the China-Japan Friendship Hospital and Beijing Military Hospital for their generous support for DNA sequencing and data collection.

Author Contributions

Conceived and designed the experiments: HF, XW, ZZ, SYC, DL. Analyzed the data: CT, HY, FL, DZ, SJ, HS, HD, GZ, ZL, ZD, BG, HY, CY, LW, ZS, YL. Wrote the first draft of the manuscript: LJ, VN. Contributed to the writing of the manuscript: CT, LJ, VN, XFH, SYC. Agreed with manuscript results and conclusions: HF, XW, ZZ, CT, HY, LJ, VN, FL, DZ, SJ, HS, HD, GZ, ZL, ZD, BG, HY, CY, LW, ZS, YL, XFH, SYC, DL. Jointly developed the structure and arguments for the paper: HF, XW, ZZ, CT, LJ, SYC, DL. Made critical revisions and approved the final version: CT, LJ, VN, XFH, SYC. All authors reviewed and approved the final manuscript.

Supplementary Materials

Supplementary Table 1. Frequencies of recurrent mutations in lung cancer assessed with COSMIC (version 64), My Cancer Genome database (http://www.mycancergenome.org/), and additional publications.

Supplementary Table 2. Performance validation of Ion Torrent PGM compared to Sanger sequencing in 62 positive and 60 negative lung cancer samples.

Supplementary Figure 1. Filter process of variants. Note: (**A**) Strand-biased variants were eliminated using IGV software (http//www.broadinstitute.org/igv). (**B**) Variants in AMPL339432 should be eliminated, because this amplicon is not uniquely matched to PIK3CA in human genome. (**C**) To avoid missing report of the deletions, manually check whether the consistent deletions reported in allele counts file are necessary. (**D**) All of our statistical analysis was based on the data in the blue box.

Supplementary Figure 2. The accuracy trial of 60 negative samples and 62 positive lung cancer samples with Ion Torrent PGM and Sanger method for common mutations in EGFR exons 19 and 21. (**A**) A summary table of the performance of Ion Torrent PGM and Sanger method. (**B**) Figures of Sanger result and sequencing result for one inconsistent sample. (**C**) A table of variant calling result of PGM sequencing for one inconsistent sample.

REFERENCES

1. Chen H, Goldberg MS, Villeneuve PJ. A systematic review of the relation between long-term exposure to ambient air pollution and chronic diseases. *Rev Environ Health*. 2008;23(4):243–97.
2. Clapp RW, Jacobs MM, Loechler EL. Environmental and occupational causes of cancer: new evidence 2005–2007. *Rev Environ Health*. 2008;23(1):1–37.
3. Thun MJ, Hannan LM, Adams-Campbell LL, et al. Lung cancer occurrence in never-smokers: an analysis of 13 cohorts and 22 cancer registry studies. *PLoS Med*. 2008;5(9):e185.
4. Schmid K, Kuwert T, Drexler H. Radon in indoor spaces: an underestimated risk factor for lung cancer in environmental medicine. *Dtsch Arztebl Int*. 2010;107(11):181–6.
5. Sun S, Schiller JH, Gazdar AF. Lung cancer in never smokers – a different disease. *Nat Rev Cancer*. 2007;7(10):778–90.
6. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nat Rev Cancer*. 2003;3(10):733–44.
7. Herbst RS, Heymach JV, Lippman SM. Lung cancer. *N Engl J Med*. 2008;359(13):1367–80.
8. Ferlay J, Soerjomataram I, Ervik M, et al. *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]*. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr. Accessed May 5, 2014.
9. Pleasance ED, Stephens PJ, O'Meara S, et al. A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature*. 2010;463(7278):184–90.
10. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. *Oncogene*. 2002;21(48):7435–51.
11. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. *Mutat Res*. 2004;567(2–3):447–74.
12. Rudin CM, Avila-Tang E, Harris CC, et al. Lung cancer in never smokers: molecular profiles and therapeutic implications. *Clin Cancer Res*. 2009;15(18):5646–61.
13. Lan Q, Hsiung CA, Matsuo K, et al. Genome-wide association analysis identifies new lung cancer susceptibility loci in never-smoking women in Asia. *Nat Genet*. 2012;44(12):1330–5.
14. Dempke WCM, Suto T, Reck M. Targeted therapies for non-small cell lung cancer. *Lung Cancer*. 2010;67(3):257–74.
15. Hannay DR. The evidence-based primary care handbook. *BMJ*. 2000;321(7260):576.
16. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al; National Cancer Institute of Canada Clinical Trials Group. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med*. 2005;353(2):123–32.
17. Sun Y, Nowak KA, Zaorsky NG, et al. ALK Inhibitor PF02341066 (Crizotinib) increases sensitivity to radiation in non-small cell lung cancer expressing EML4-ALK. *Mol Cancer Ther*. 2013;12(5):696–704.
18. Winton T, Livingston R, Johnson D, et al; National Cancer Institute of Canada Clinical Trials Group, National Cancer Institute of the United States Intergroup JBR.10 Trial Investigators. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. *N Engl J Med*. 2005;352(25):2589–97.

19. Dubinski W, Leighl NB, Tsao M-S, Hwang DM. Ancillary testing in lung cancer diagnosis. *Pulm Med*. 2012;2012:249082.
20. John T, Liu G, Tsao MS. Overview of molecular testing in non-small-cell lung cancer: mutational analysis, gene copy number, protein expression and other biomarkers of EGFR for the prediction of response to tyrosine kinase inhibitors. *Oncogene*. 2009;28(suppl 1):S14–23.
21. Schuster SC. Next-generation sequencing transforms today's biology. *Nat Methods*. 2008;5(1):16–8.
22. Voelkerding KV, Dames SA, Durtschi JD. Next-generation sequencing: from basic research to diagnostics. *Clin Chem*. 2009;55(4):641–58.
23. Meyerson M, Gabriel S, Getz G. Advances in understanding cancer genomes through second-generation sequencing. *Nat Rev Genet*. 2010;11(10):685–96.
24. Roukos DH. Trastuzumab and beyond: sequencing cancer genomes and predicting molecular networks. *Pharmacogenomics J*. 2011;11(2):81–92.
25. Glenn TC. Field guide to next-generation DNA sequencers. *Mol Ecol Resour*. 2011;11(5):759–69.
26. Pourmand N, Karhanek M, Persson HHJ, et al. Direct electrical detection of DNA synthesis. *Proc Natl Acad Sci USA*. 2006;103(17):6466–70.
27. Hadd AG, Houghton J, Choudhary A, et al. Targeted, high-depth, next-generation sequencing of cancer genes in formalin-fixed, paraffin-embedded and fine-needle aspiration tumor specimens. *J Mol Diagn*. 2013;15(2):234–47.
28. Xu Z, Huo X, Tang C, et al. Frequent KIT mutations in human gastrointestinal stromal tumors. *Sci Rep*. 2014;4:5907.
29. Cai X, Sheng J, Tang C, et al. Frequent mutations in EGFR, KRAS and TP53 genes in human lung cancer tumors detected by ion torrent DNA sequencing. *PLoS One*. 2014;9(4):e95228.
30. Consortium GP. A map of human genome variation from population-scale sequencing. *Nature*. 2010;467(7319):1061–73.
31. Server EV. *NHLBI Go Exome Sequencing Project (ESP)*. Seattle, WA: 2013. Available at http://evs.gs.washington.edu/EVS/. Accessed March 19.
32. Bamford S, Dawson E, Forbes S, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer*. 2004;91(2):355–8.
33. Meldrum C, Doyle MA, Tothill RW. Next-generation sequencing for cancer diagnostics: a practical perspective. *Clin Biochem Rev*. 2011;32(4):177–95.
34. Malapelle U, Vigliar E, Sgariglia R, et al. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. *J Clin Pathol*. 2015;68(1):64–8.
35. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. *Science*. 2013;339(6127):1546–58.
36. Karachaliou N, Mayo C, Costa C, et al. KRAS mutations in lung cancer. *Clin Lung Cancer*. 2013;14(3):205–14.
37. Forbes SA, Bhamra G, Bamford S, et al. The Catalogue of Somatic Mutations in Cancer (COSMIC). *Current Protocols in Human Genetics*. 2008;57:Chapter 10, Unit 10.11.
38. Shi Y, Au JS, Thongprasert S, et al. A prospective, molecular epidemiology study of EGFR mutations in Asian patients with advanced non-small-cell lung cancer of adenocarcinoma histology (PIONEER). *J Thorac Oncol*. 2014;9(2):154–62.
39. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst*. 2005;97(5):339–46.
40. Li Z, Zhang LJ, Wang WP, Guo K, Shao JY, Rong TH. [Correlation between EGFR gene mutation and high copy number and their association with the clinicopathological features in Chinese patients with non-small cell lung cancer]. *Zhonghua Zhong Liu Za Zhi*. 2011;33(9):666–70.
41. da Cunha Santos G, Shepherd FA, Tsao MS. EGFR mutations and lung cancer. *Annu Rev Pathol*. 2011;6:49–69.
42. Yun CH, Boggon TJ, Li Y, et al. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell*. 2007;11(3):217–27.
43. Sakurada A, Shepherd FA, Tsao M-S. Epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer: impact of primary or secondary mutations. *Clin Lung Cancer*. 2006;7(suppl 4):S138–44.
44. Dienstmann R, De Dosso S, Felip E, Tabernero J. Drug development to overcome resistance to EGFR inhibitors in lung and colorectal cancer. *Mol Oncol*. 2012;6(1):15–26.
45. West H, Lilenbaum R, Harpole D, Wozniak A, Sequist L. Molecular analysis-based treatment strategies for the management of non-small cell lung cancer. *J Thorac Oncol*. 2009;4(9 suppl 2):S1029–39. ; quiz S1041–22.
46. Siegelin MD, Borczuk AC. Epidermal growth factor receptor mutations in lung adenocarcinoma. *Lab Invest*. 2014;94(2):129–37.
47. Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. *Nat Rev Cancer*. 2010;10(11):760–74.
48. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol*. 2003;21(12):2237–46.
49. Pérez-Soler R, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non – small-cell lung cancer. *J Clin Oncol*. 2004;22(16):3238–47.
50. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2005;352(8):786–92.
51. Pfeifer G, Besaratinia A. Mutational spectra of human cancer. *Hum Genet*. 2009;125(5–6):493–506.
52. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. *Science*. 1996;274(5286):430–2.
53. Mogi A, Kuwano H. TP53 mutations in nonsmall cell lung cancer. *J Biomed Biotechnol*. 2011;2011:583929.
54. Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature*. 2008;455(7216):1069–75.
55. Ory KLY, Auguin C, Soussi T. Analysis of the most representative tumour-derived p53 mutants reveals that changes in protein conformation are not correlated with loss of transactivation or inhibition of cell proliferation. *EMBO J*. 1994;13(15):3496–504.
56. Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res*. 2000;60(24):6788–93.
57. Peart MJ, Prives C. Mutant p53 gain of function: the NF-Y connection. *Cancer Cell*. 2006;10(3):173–4.
58. Petitjean A, Achatz MIW, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene*. 0000;26(15):2157–65.
59. Kawasaki M, Nakanishi Y, Kuwano K, Yatsunami J, Takayama K, Hara N. The utility of p53 immunostaining of transbronchial biopsy specimens of lung cancer: p53 overexpression predicts poor prognosis and chemoresistance in advanced non-small cell lung cancer. *Clin Cancer Res*. 1997;3(7):1195–200.
60. Steels E, Paesmans M, Berghmans T, et al. Role of p53 as a prognostic factor for survival in lung cancer: a systematic review of the literature with a meta-analysis. *Eur Respir J*. 2001;18(4):705–19.
61. Tsao MS, Aviel-Ronen S, Ding K, et al. Prognostic and predictive importance of p53 and RAS for adjuvant chemotherapy in non small-cell lung cancer. *J Clin Oncol*. 2007;25(33):5240–7.
62. Suda K, Tomizawa K, Mitsudomi T. Biological and clinical significance of KRAS mutations in lung cancer: an oncogenic driver that contrasts with EGFR mutation. *Cancer Metastasis Rev*. 2010;29(1):49–60.
63. Pao W, Wang TY, Riely GJ, et al. *KRAS* mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med*. 2005;2(1):e17.
64. Eberhard DA, Johnson BE, Amler LC, et al. Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J Clin Oncol*. 2005;23(25):5900–9.
65. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science*. 2004;304(5670):554.
66. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci USA*. 2005;102(3):802–7.
67. Kawano O, Sasaki H, Endo K, et al. PIK3CA mutation status in Japanese lung cancer patients. *Lung Cancer*. 2006;54(2):209–15.
68. Sequist LV, Waltman BA, Dias-Santagata D, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med*. 2011;3(75):75ra26.
69. Ludovini V, Bianconi F, Pistola L, et al. Phosphoinositide-3-kinase catalytic alpha and KRAS mutations are important predictors of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in patients with advanced non-small cell lung cancer. *J Thorac Oncol*. 2011;6(4):707–15.
70. Hara E, Smith R, Parry D, Tahara H, Stone S, Peters G. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol Cell Biol*. 1996;16(3):859–67.
71. Kraunz KS, Nelson HH, Lemos M, Godleski JJ, Wiencke JK, Kelsey KT. Homozygous deletion of p16INK4a and tobacco carcinogen exposure in nonsmall cell lung cancer. *Int J Cancer*. 2006;118(6):1364–9.
72. Rose-James A, TT S. Molecular markers with predictive and prognostic relevance in lung cancer. *Lung Cancer Int*. 2012;2012:12.
73. Shigemitsu K, Sekido Y, Usami N, et al. Genetic alteration of the beta-catenin gene (CTNNB1) in human lung cancer and malignant mesothelioma and identification of a new 3p21.3 homozygous deletion. *Oncogene*. 2001;20(31):4249–57.
74. Hu T, Li C. Convergence between Wnt-beta-catenin and EGFR signaling in cancer. *Mol Cancer*. 2010;9:236.
75. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer*. 2013;13(1):11–26.
76. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366(10):883–92.
77. Zhang J, Fujimoto J, Zhang J, et al. Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. *Science*. 2014;346(6206):256–9.

78. Hamada K, Kohno T, Kawanishi M, Ohwada S, Yokota J. Association of CDKN2A (p16)/CDKN2B (p15) alterations and homozygous chromosome arm 9p deletions in human lung carcinoma. *Genes Chromosomes Cancer*. 1998; 22(3):232–40.
79. Sunaga N, Kohno T, Kolligs FT, Fearon ER, Saito R, Yokota J. Constitutive activation of the Wnt signaling pathway by CTNNB1 (β-catenin) mutations in a subset of human lung adenocarcinoma. *Genes Chromosomes Cancer*. 2001;30(3):316–21.
80. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304(5676):1497–500.
81. Mitsudomi T, Yatabe Y. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. *FEBS J*. 2010;277(2):301–8.
82. Brose MS, Volpe P, Feldman M, et al. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res*. 2002;62(23):6997–7000.
83. Suzuki H, Takahashi T, Kuroishi T, et al. p53 mutations in non-small cell lung cancer in Japan: association between mutations and smoking. *Cancer Res*. 1992;52(3):734–6.