

Keywords: non-small cell lung cancer; somatic mutations; double and triple mutations; multiplex testing; liquid chip technology

Coexistence of *EGFR* with *KRAS*, or *BRAF*, or *PIK3CA* somatic mutations in lung cancer: a comprehensive mutation profiling from 5125 Chinese cohorts

S Li^{*1,8}, L Li^{1,8}, Y Zhu², C Huang¹, Y Qin¹, H Liu¹, L Ren-Heidenreich³, B Shi⁴, H Ren⁵, X Chu⁶, J Kang⁷, W Wang⁷, J Xu³, K Tang³, H Yang³, Y Zheng³, J He³, G Yu³ and N Liang^{*1}

¹Department of Thoracic Surgery, Peking Union Medical College Hospital, Beijing 100730, China; ²Department of Thoracic Surgery, Air Force General Hospital of PLA, Beijing 100036, China; ³SurExam Bio-Tech Co., Guangzhou 510663, China; ⁴Department of Thoracic Surgery, China-Japan Friendship Hospital, Beijing 100029, China; ⁵Department of Thoracic Surgery, General Hospital of Armed Police Forces, Beijing 100039, China; ⁶Department of Thoracic Surgery, Chinese PLA General Hospital, Beijing 100853, China and ⁷China Navy General Hospital, Beijing 100037, China

Background: Determining the somatic mutations of epidermal growth factor receptor (EGFR)-pathway networks is the key to effective treatment for non-small cell lung cancer (NSCLC) with tyrosine kinase inhibitors (TKIs). The somatic mutation frequencies and their association with gender, smoking history and histology was analysed and reported in this study.

Methods: Five thousand one hundred and twenty-five NSCLC patients' pathology samples were collected, and *EGFR*, *KRAS*, *BRAF* and *PIK3CA* mutations were detected by multiplex testing. The mutation status of *EGFR*, *KRAS*, *BRAF* and *PIK3CA* and their association with gender, age, smoking history and histological type were evaluated by appropriate statistical analysis.

Results: *EGFR*, *KRAS*, *BRAF* and *PIK3CA* mutation rates revealed 36.2%, 8.4%, 0.5% and 3.3%, respectively, across the 5125 pathology samples. For the first time, evidence of *KRAS* mutations were detected in two female, non-smoking patients, age 5 and 14, with NSCLC. Furthermore, we identified 153 double and coexisting mutations and 7 triple mutations. Interestingly, the second drug-resistant mutations, T790M or E545K, were found in 44 samples from patients who had never received TKI treatments.

Conclusions: *EGFR* exons 19, 20 and 21, and *BRAF* mutations tend to happen in females and non-smokers, whereas *KRAS* mutations were more inclined to males and smokers. Activating and resistant mutations to EGFR-TKI drugs can coexist and 'second drug-resistant mutations', T790M or E545K, may be primary mutations in some patients. These results will help oncologists to decide candidates for mutation testing and EGFR-TKI treatment.

Lung cancer has the highest incidence among all cancers and is the leading cause of death worldwide (Jemal *et al*, 2011). Non-small cell lung cancer (NSCLC) accounts for the vast majority of lung cancers (~80%) (American Cancer Society, 2011). Treating advanced NSCLC with the small molecule tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, which target the

epidermal growth factor receptor (EGFR), has significantly improved the overall survival rate of patients harbouring activating mutations (Pao *et al*, 2005; Toyooka *et al*, 2005).

This report serves to provide oncologists with patient demographics associated with a higher statistical probability of having somatic mutations of the EGFR signalling pathway networks.

*Correspondence: Dr N Liang; E-mail: happinessliang80@sina.com or Professor S Li; E-mail: doctorlishanqing@sina.com

⁸These authors contributed equally to this work.

Received 20 November 2013; revised 14 March 2014; accepted 22 March 2014; published online 17 April 2014

© 2014 Cancer Research UK. All rights reserved 0007–0920/14

Owing to the large cohort of patients, we were able to analyse the coexistence of double or triple somatic mutations of the relevant genes. Although they are rare, multiple mutations are very critical for the decision of TKI treatment. Importantly, for the first time, we report here detection of *KRAS* somatic mutations in NSCLC samples obtained from non-smoking children, which may be associated with second-hand smoke exposure or some environmental factors.

EGFR, *KRAS*, *BRAF* or *PIK3CA* mutations have been shown to predict clinical response to EGFR-TKIs in NSCLC patients. Mutations of these four genes are associated with gender, smoking history and histology. For example, deletions in *EGFR* exon 19 and the point mutation L858R in exon 21 are the most common activating mutations and have been predominantly found in females, never smokers, adenocarcinomas and Asian patients (Rosell *et al*, 2009; Xu *et al*, 2011). In addition, they have been identified as predictors of responsiveness to both gefitinib and erlotinib (Lynch *et al*, 2004; Pao *et al*, 2004). *KRAS*, *BRAF* or *PIK3CA* mutations are also important indicators for EGFR-TKI therapy (Marchetti *et al*, 2005; Pao *et al*, 2005; Schmid *et al*, 2009; Ludovini *et al*, 2011; Paik *et al*, 2011; Dogan *et al*, 2012). Conversely, *KRAS* mutations are more common in individuals with a history of cigarette use and are associated with resistance to EGFR-TKI (Pao *et al*, 2005; Xu *et al*, 2011; Dogan *et al*, 2012). *KRAS*, mutated to an oncogenic form by introducing amino-acid substitutions at codons 12, 13 and 61, are detected in 25–33% of patients with lung adenocarcinomas from the United States and Europe (Smits *et al*, 2012) and 8% from China (Xu *et al*, 2011). *BRAF*, a serine/threonine kinase, is activated by somatic point mutation V600E in exon 15 in only 3% of lung cancer patients. *BRAF* mutations are associated with resistance to TKI therapy (Pao *et al*, 2005; Lin and Bivona, 2012). *PIK3CA* encodes the p110 α subunit of the mitogenic signalling protein phosphatidylinositol 3-kinase (PI3K). *PIK3CA* mutations in the helical-binding domain and the catalytic subunit of the protein have been associated with tumourigenesis and treatment resistance in various malignancies. Indeed, *PIK3CA* mutations are detected in ~4% of lung cancers and have become an important predictor for drug resistance to EGFR-TKI (Ludovini *et al*, 2011).

Owing to very low frequencies, the clinical significance of coexistence of somatic mutations has never been systematically analysed up until now. Although coexistence of somatic mutations on EGFR pathway networks have been observed in some individual cases (Toyooka *et al*, 2005), the low frequency of this occurrence has presented limitations to determining the clinical implications for these multiple mutation cases. Accordingly, large-scale mutation analysis could identify the frequencies of multiple mutations and expand our understanding of somatic mutations on the EGFR pathway networks.

Using a liquid chip technology for somatic mutation identification (Wu *et al*, 2010; Li *et al*, 2011), we conducted a large-scale mutation analysis for *EGFR*, *KRAS*, *BRAF*, or *PIK3CA* mutations on 5125 tumour samples from patients with NSCLC, and analysed their associations with gender, smoking and histology. Of these, 160 cases were identified as having multiple mutations. In this study, the clinical significance of these 160 cases has been analysed and is discussed.

MATERIALS AND METHODS

Patients. Between 2009 and 2012, 5125 patients with lung cancer from most major hospitals throughout China were enrolled in this study. Formalin-fixed and paraffin-embedded (FFPE) tumour samples were prepared from primary surgical or biopsy specimens in lung. All samples were identified by pathologists as primary

NSCLC and were provided by the SurExam Clinical Testing Centre. Written informed consent was obtained from all participants.

Mutation analysis of EGFR, KRAS, BRAF and PIK3CA.

Tumour genomic DNA from each FFPE slide was extracted with the Maxwell system (Promega, Madison, WI, USA). The mutation status was analysed with the 70plex liquidchip platform (Surexam, Guangzhou, China) for the 70 alleles (Li *et al*, 2011). The 70plex includes five major steps: (1) multiplex PCR to amplify 70 target genes; (2) exonuclease I and shrimp alkaline phosphatase (EXO-SAP) cleaning to remove excess nucleotides and primers; (3) allele-specific primer extension where EXO-SAP-cleaned PCR products were amplified with 70 specific primers that were linked to 70 universal tags; (4) hybridisation to beads and (5) Luminex analysis and the median fluorescence intensity was read and analysed. The 70plex platform has been validated for clinical use. All of 5125 tests and data analyses were performed at the SurExam Clinical Testing Centre. As part of quality control, samples were randomly sent to independent companies for DNA sequencing analysis.

Statistical analysis. The mutation status of *EGFR*, *KRAS*, *BRAF* and *PIK3CA* and their association with gender, age and smoking history were evaluated using Maximum Likelihood Multivariate Logistic Regression. Variables were selected by the Complete Model. The adjusted odds ratios were calculated. A two-sided *P*-value of <0.05 was considered statistically significant if there was no alpha correction. The phenotypes of gender and smoking were analysed using the Binomial test and then the Bonferroni correction was performed. Smoking refers to current and ever smoking in this study. The significant difference refers to *P*<0.001 between smoking and non-smoking or male and female proportions in comparison with overall samples and phenotypes. All analyses were performed using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Demographic characteristics. The presence of *EGFR*, *KRAS*, *BRAF* and *PIK3CA* mutations was analysed in 5125 lung cancer patients; 2072 of them were female (40.4%) and 3053 male (59.6%). Patient ages ranged from 5–91 years with the median age of 59 years. All specimens were NSCLC. Non-small cell lung cancer forms were identified in all of patients: 4046 (78.9%) samples were adenocarcinomas, whereas only 1079 (21.1%) were squamous cell carcinomas (see Table 1).

Mutation distributions. Two thousand two hundred and eight (2208) NSCLC patients (2208 out of 5125, 43.1%) harboured single mutations of *EGFR*, *KRAS*, *BRAF* or *PIK3CA*, and 160 were found to have multiple mutations (Figure 1, 153 double and coexisting mutations and seven triple mutations). Of the 153 double and coexisting mutations, 58 patients carried *EGFR* + *PIK3CA* mutations; 53, two *EGFR* double mutations; 29, *EGFR* + *KRAS* mutations; 9, *KRAS* + *PIK3CA* mutations; 2, *EGFR* + *BRAF* mutations; and 2, *BRAF* + *PIK3CA* mutations. Of the seven triple mutations, five patients carried 2 \times *EGFR* + 1 \times *PIK3CA* mutations; one patient carried 1 \times *EGFR* + 2 \times *PIK3CA* mutations; and one patient carried 1 \times *EGFR* + 1 \times *KRAS* + 1 \times *PIK3CA* mutations (Figure 1B).

Together, there were 36.2% patients with *EGFR* mutations (1854 out of 5125); 8.4%, *KRAS* mutations (429 out of 5125); 0.5%, *BRAF* mutations (26 out of 5125) and 3.3%, *PIK3CA* mutations (167 out of 5125). The percentage distributions of *EGFR*, *KRAS*, *BRAF* and *PIK3CA* among mutation-positive samples were 74.9%, 17.3%, 1.1% and 6.7%, respectively (Figure 2B).

Table 1. Patient characteristics (n = 5125)

	No. of patients
Median age	59 (5–91) years
Gender	
Male	3053
Female	2072
Histology^a	
ADCs	4046
Squamous	1079
Smoking history	
Smokers ^b	2314
Non-smokers ^c	2493
Unknown	318
Abbreviation: ADCs = adenocarcinomas.	
^a All patients in this study with NSCLC.	
^b Include ever smokers and current smokers.	
^c Never smokers.	

EGFR mutations. EGFR mutations were detected in 36.2% patients (1854 out of 5125), with most of these located in exon 19 (18.0%, 920 out of 5125) or exon 21 (16.9%, 868 out of 5125). The remaining of these were located in exon 20 (1.5%, 75 out of 5125) or exon 18 (1.0%, 49 out of 5125). The percentage distributions in exons 18, 19, 20 and 21 were 3%, 48%, 4% and 45%, respectively, (Figure 2B). Double EGFR mutations were found in 1.1% patients (58 out of 5125) (Figure 2A).

KRAS mutation. KRAS mutation was detected in 8.4% patients (429 out of 5125), with most of these located in exon 2 (8.0%, 408 out of 5125); the remaining ones were detected in exon 3 (0.4%, 21 out of 5125) (Figure 2). In these KRAS mutation specimens, there were 134 G12C mutations and 87 G12V mutations (Figure 2). Notably, there were two patients under or near the age of 15 years, who were found to carry the KRAS mutation. The youngest of these patients, who was 5 years of age, suffered from NSCLC and was also found to carry a KRAS E3 mutation (Q61H). The other of these patients, who was 14 years of age, had a KRAS E2 mutation (G12V). Interestingly, neither of these two patients was a smoker, and both were female.

BRAF mutation. BRAF mutation was detected in 0.5% patients (26 out of 5125). All of them were a V600E mutation (Figure 2).

PIK3CA mutation. PIK3CA mutation was detected in 3.3% patients (167 out of 5125) with 2.1% of these mutations located in exon 9 (109 out of 5125) and 1.1% in exon 20 (58 out of 5125) (Figure 2). There were 62 cases of E545K mutations and 44 cases of E542K (Figure 2). We found one patient who carried two PIK3CA mutations plus one EGFR mutation (E545K + E542K + L861Q) (Figure 1B).

Patients' characteristics and somatic mutations. Analysis by multivariate logistic regression revealed that females were more likely to have E19/E21 mutations (male vs female: 23.5% vs 51.5%, adjusted OR 0.63, CI 0.52–0.75, $P \leq 0.001$) than males (Table 2). Similar results were also found in BRAF mutations (male vs female: 0.2% vs 1.1%, adjusted OR 0.15, CI 0.04–0.62, $P \leq 0.01$). The KRAS mutation rate was higher in males than in females (male vs female: 11.0% vs 6.1%, adjusted OR 1.38, CI 0.99–1.93, $P = 0.054$), but the difference was not statistically significant. These results suggested a

significant association of EGFR E19/E21 and BRAF mutations with gender.

Non-smokers were more likely to have E19/E21 mutations than smokers (smokers vs non-smokers: 18.1% vs 51.0%, adjusted OR 0.287, CI 0.24–0.34, $P \leq 0.001$) and E20 mutations (smokers vs non-smokers: 0.6% vs 2.5%, adjusted OR 0.39, CI 0.17–0.87, $P = 0.022$). However, an inverse relationship was found in KRAS mutations (smokers vs non-smokers: 6.0% vs 12.3%, adjusted OR 1.83, CI 1.38–2.57, $P \leq 0.001$). There was no significant difference between smoker and non-smoker NSCLC patients for the mutation rates of EGFR E18, BRAF and PIK3CA.

EGFR E19/E21 (adeno vs squamous: 42.0% vs 8.2%, adjusted OR 7.36, CI 5.61–9.57, $P \leq 0.001$), EGFR E20 (adeno vs squamous: 1.9% vs 0.5%, adjusted OR 2.89, CI 1.04–8.04, $P = 0.042$) and KRAS (adeno vs squamous: 10.4% vs 3.6%, adjusted OR 3.26, CI 2.21–4.80, $P \leq 0.001$) mutations were more frequent in adenocarcinomas. However, the PIK3CA mutation rate in adenocarcinomas was lower than in squamous cell carcinoma (adeno vs squamous: 2.9% vs 7.9%, adjusted OR 0.50, CI 0.34–0.73, $P \leq 0.001$). These results suggest a significant association of EGFR E19/E21, EGFR E20, KRAS and PIK3CA mutations with the histology of tumour specimens. Accordingly, adenocarcinomas have a higher likelihood of harbouring EGFR E19/E21, EGFR E20 and KRAS mutations, whereas squamous cell carcinomas have a higher likelihood of harbouring a PIK3CA mutation. Unfortunately, there was no classification information on 3948 NSCLC samples (out of 5125) at time of testing.

In addition, there was no significant difference found for the mutation rates of EGFR, KRAS, BRAF and PIK3CA occurring in between patients over 60 years of age and those 60 years of age or younger. We also conducted statistical analysis by decade of age; there was no significant difference of these four gene mutations in any of the age groups (data not shown).

We conclude, therefore, that non-smoking females with adenocarcinoma lung cancer may have a high likelihood of carrying the EGFR E19/E21 mutation, whereas smokers with adenocarcinoma lung cancer have a high likelihood of carrying KRAS mutations.

Phenotype analysis with smoking history and gender. A significant association was found between smoking history and EGFR pathway gene mutation frequencies (Figure 3): the proportions of smoking vs non-smoking in E746_A750del, L747_A750>P, L747_T751del, L747_P753>S, T790M, L858R and G12C mutations were significantly different with the proportion of smoking vs non-smoking in the overall sample ($P \leq 0.001$). Never smokers showed higher in E746_A750del, L747_A750>P, L747_T751del, L747_P753>S, T790M and L858R mutations. However, there was an inverse relationship in G12C mutations. These results suggest a significant association of mutation phenotypes with smoking history.

Significant associations were also found between gender and mutation phenotypes. The proportions of male vs female in E746_A750del, L747_P753>S, T790M, L858R, L861Q, V600E and G12C mutations were significantly different with the proportion of male vs female in the overall sample ($P \leq 0.001$). E746_A750del, L747_P753>S, T790M, L858R, L861Q and V600E mutations were higher in females than males, whereas G12C was higher in males than in females (Figure 3).

Double mutations. There were 153 patients found to have a double and coexisting mutation profile; 72.5% of them (111 out of 153) were never smokers; 62.1% (95 out of 153) were females (Figure 1A). Interestingly, there were only 13.1% (20 out of 153) of these who were treated with the TKI or TKI-combined therapy before the tests.

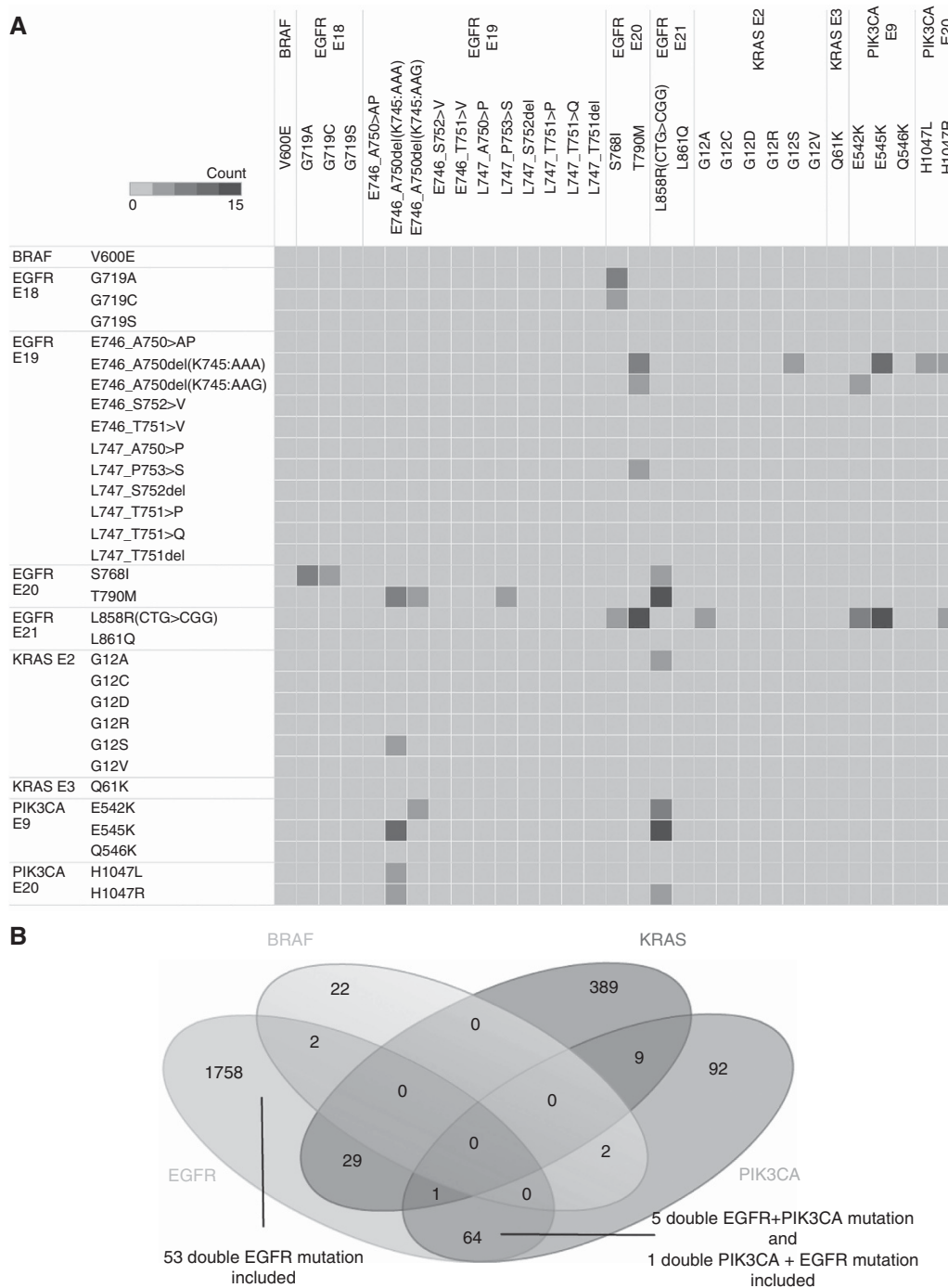


Figure 1. Combinations of multiple mutations. (A) Double mutation sites and case number in 153 patients. Double mutations L858R + T790M showed the highest incidence rate (9.8%, 15 out of 153) followed by L858R + E545K (8.5%, 13 out of 153). (B) Four set venn-diagram of single and multiple mutation panoramagram for the whole study.

There were 30 *EGFR* mutation specimens that also harboured *KRAS* mutations; two had *BRAF* mutations; and 65 had *PIK3CA* mutations. There were 6.0% (10 out of 167) of *KRAS* mutation specimens that also harboured *PIK3CA* mutations. However, *KRAS* and *BRAF* mutations were mutually exclusive.

Double mutations L858R and T790M possessed the highest incidence rate (9.8%, 15 out of 153) followed by L858R + E545K (8.5%, 13 out of 153) (Figure 1A).

It is well known that the *EGFR* E19/E21 mutation is a sensitive or active mutation to TKI therapy, whereas T790M and E545K are resistant mutations. We identified 56 patients who carried both E19/E21 mutations and T790M or E545K; most of these were female (73.2%, 41 out of 56) and never smokers (78.6%, 44 out of

56). Of these, 53.6% (30 out of 56) were detected in patients <60 years of age, and 78.6% (44 out of 56) had never received TKI treatments (data not shown).

Triple mutations. There were seven patients found to carry a triple mutation: four of them were male; two, smokers; age range, 47–76 years. There were five patients who harboured double *EGFR* mutations and one patient (76 years old, female) who had double *PIK3CA* mutations. Three triple-mutation specimens harboured *EGFR* E21, and all *EGFR* E21 mutations in this triplet mutation group were L861Q. The samples with double or triple mutations have been further confirmed by independent DNA sequencing lab (data not shown).

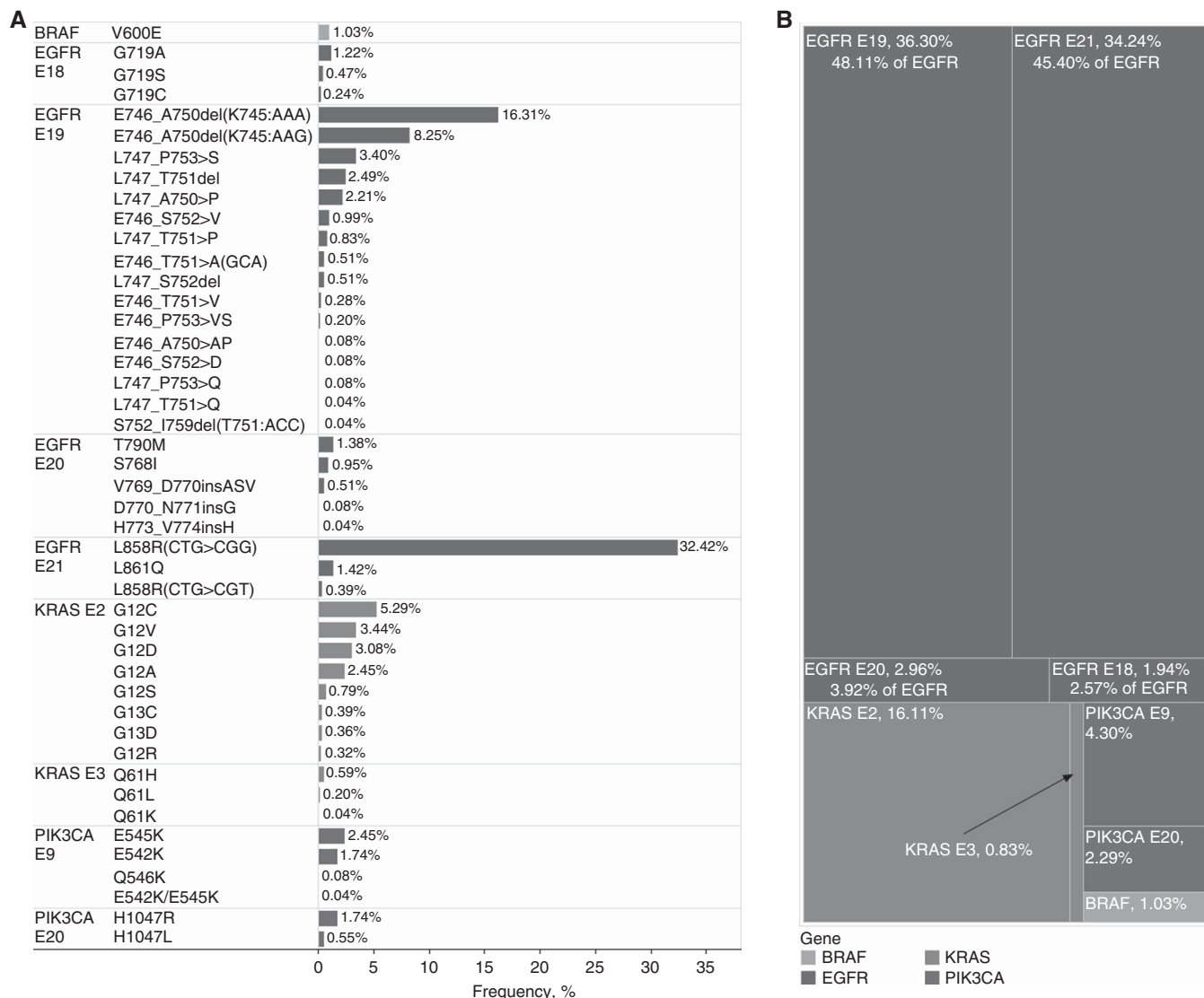


Figure 2. Somatic mutation frequencies of EGFR, KRAS, BRAF and PIK3CA. (A) The mutation sites (abscissas) and frequency (ordinates) of EGFR, KRAS, BRAF and PIK3CA in 5125 patients with NSCLC. (B) Distributions tree-map of EGFR, KRAS, BRAF and PIK3CA mutations in 2368 patients carrying mutations.

Table 2. Multivariate logistic regression of objective gene mutations

Gene mutation	Covariates for logistic regression							
	Gender		Age		Smoking		Histology subtype	
	Male vs female ^a		> 60 vs < 60 ^a		Smokers vs never smokers ^a		ADCs vs SCCs ^a	
	AOR (95% CI)	P-value	AOR (95% CI)	P-value	AOR (95% CI)	P-value	AOR (95% CI)	P-value
EGFR E19/E21	0.628 (0.524–0.754)	<0.0001	1.040 (0.898–1.204)	0.6002	0.287 (0.237–0.347)	<0.0001	7.358 (5.611–9.566)	<0.0001
EGFR E18	0.501 (0.222–1.132)	0.0964	1.509 (0.813–2.799)	0.1922	0.676 (0.284–1.608)	0.3762	1.617 (0.627–4.169)	0.3200
EGFR E20	0.540 (0.273–1.070)	0.0775	0.889 (0.532–1.483)	0.6513	0.387 (0.173–0.870)	0.0216	2.889 (1.039–8.035)	0.0420
KRAS	1.384 (0.995–1.926)	0.0537	1.173 (0.933–1.476)	0.1719	1.833 (1.380–2.568)	<0.0001	3.256 (2.209–4.798)	<0.0001
BRAF	0.147 (0.035–0.623)	0.0093	0.642 (0.251–1.646)	0.3567	1.425 (0.371–5.476)	0.6057	1.053 (0.302–3.675)	0.9352
PIK3CA	1.093 (0.666–1.795)	0.7248	0.840 (0.582–1.212)	0.3512	1.099 (0.682–1.772)	0.6978	0.495 (0.335–0.733)	0.0004

Abbreviations: ADCs = adenocarcinomas; AOR = adjusted odds ratio; CI = confidence interval; SCCs = squamous cell carcinomas.
^aReference variable.

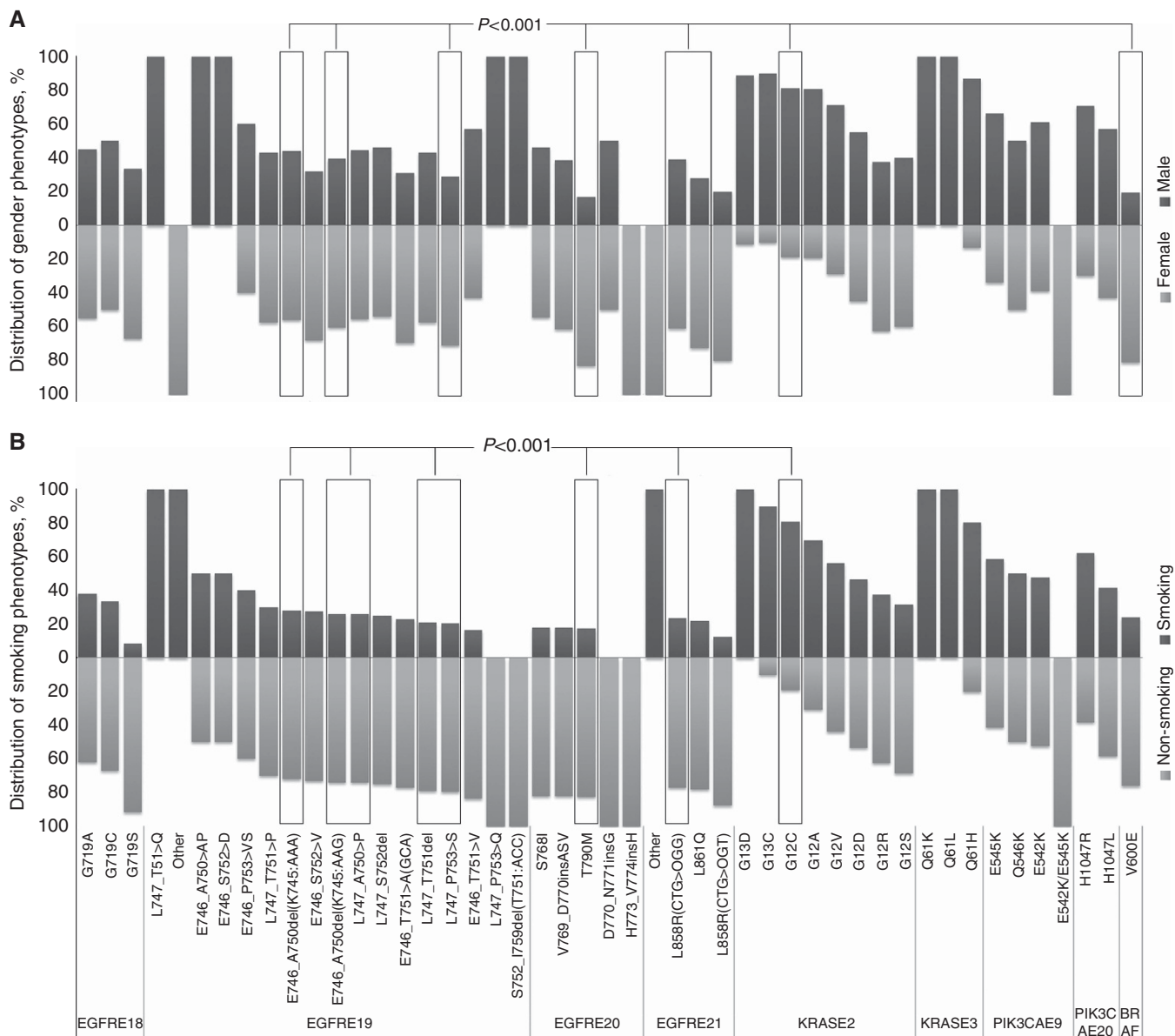


Figure 3. (A) The bars on two sides of the horizontal axis represent the percentage difference of male and female genotypes. The bars above represent male genotypes, and the bars below represent female genotypes. The bars with a rectangle box show the statistical difference of genotypes between male and female by the binomial test ($P < 0.001$). (B) The bars on two sides of horizontal axis represent the percentage difference of smoker and non-smoker genotypes. The above are smokers', and the below, non-smokers'. The bars with a rectangle box show the statistical difference of genotypes in percentage between smokers and non-smokers by the binomial test ($P < 0.001$). Chart (A) and (B) share the same horizontal axis that represents genotypes.

DISCUSSION

We tested 5125 Chinese NSCLC specimens for the somatic mutation frequencies of *EGFR*, *KRAS*, *BRAF* and *PIK3CA*. It was expected that somatic mutation testing from such a large cohort of NSCLC patients would provide more reliable results for interpreting the association of mutation frequencies with patient demographics. In this study, we identified 34.8% *EGFR* E19/E21, 2.2% *EGFR* E18/E20, 8.4% *KRAS*, 0.5% *BRAF* and 3.3% *PIK3CA* mutations within the population (Figure 2B). Age had no significant role in mutations of these four genes. Although most of the *EGFR* mutations were detected in adenocarcinomas, from the unselected Chinese patients with NSCLC, the *EGFR* E19/E21 mutation frequencies were much higher than previously reported in American patients with adenocarcinoma NSCLC (34.8% from

5125 patients vs 19% from 2142 patients) suggesting that racial diversity contributes to occurrence of the E19/E21 mutation (D'Angelo *et al*, 2011). We also observed that *EGFR* E19/E21 mutations were significantly higher in NSCLC from tumours with adenocarcinoma histology (43.6% vs 8.5%, $P \leq 0.001$), in women (51.5% vs 23.5%, $P \leq 0.001$) and in never smokers (51.0% vs 18.1%, $P \leq 0.001$), which are similar to results of previous reports (Xu *et al*, 2011; Dogan *et al*, 2012).

From our 1854 *EGFR*-mutated cases, mutations in exons 18, 19, 20 and 21 occurred in 3%, 48%, 4% and 45%, respectively. These findings are consistent with the frequencies of these mutations reported for an international database by researchers from the Metropolitan Hospital of Athens who collected data reported on 3303 *EGFR* mutations from peer-reviewed publications and concluded that the mutation distribution among these four exons are 4%, 50%, 6% and 40%, respectively (Somatic Mutation in

Epidermal Growth Factor Receptor Database http://www.somatic-mutations-egfr.info/overall_mutation_frequency.html).

Although the *EGFR* exon 20 S768I mutation is considered to be a very rare mutation, we detected a total of 24 (0.5%) NSCLC harbouring this mutation. Interestingly, we found high frequencies of S768I in multiple mutation cases: in two triple-mutation cases (2 out of 7) and in 15 double-mutation cases (15 out of 153), in four cases of L858R/S768I and in 11 cases of G719X/S768I. The function of the S768I mutation in TKI therapy is still uncertain. Masago *et al* (2010) demonstrated that one patient who harboured the S768I mutation showed a good clinical response to EGFR-TKI therapy, whereas Asahina *et al* (2006) reported completely opposite results. The fact that S768I showed high frequencies in multiple mutations suggests a rationale for specific studies designed to explore the role of S768I in NSCLC oncogenesis.

In contrast to *EGFR* mutations, *KRAS* codon 12 and 13 mutations are typically found in smokers, are associated with a poor NSCLC prognosis, are resistant to TKIs and predict lack of a survival benefit from adjuvant chemotherapy. These mutations have been found to have an association with race: ~25% NSCLC in western countries and much less in Asian NSCLC (Rodenhuis *et al*, 1987; Slebos *et al*, 1990; Riely *et al*, 2008). In our study, only 12.3% smokers and 6.0% non-smokers were detected to harbour the *KRAS* mutation in the 5125 NSCLC cases, which is much lower than that previously reported in the United States (34% in smokers and 6% in non-smokers) (Dogan *et al*, 2012). Initially, *KRAS* mutations were thought to be uncommon in NSCLC cases without a history of smoking (Rodenhuis *et al*, 1987). However, we identified 6.0% of *KRAS* mutations in non-smokers as also observed in the United States (Riely *et al*, 2008; Dogan *et al*, 2012), which may lead future studies to explore the impact of factors other than cigarettes, such as genetic, environmental or hormonal factors, on *KRAS* mutations.

Patients with *BRAF* V600E mutations in their tumours had significantly shorter DFS and OS times than those without mutations (Marchetti *et al*, 2011). To our knowledge, this is the largest cohort of V600E tests performed on NSCLC samples obtained from patients of the same race. The frequency of V600E is 0.51% in our study, which is much lower than that reported for Italian (2.8%) (Marchetti *et al*, 2011) and American patients (2.9%), but comparable to Japan patients (0.8%) (Sasaki *et al*, 2006). Therefore, we suspect that like *EGFR* mutations, incidence of V600E mutations may be racial reliant. We also did not see significantly higher V600E mutations in smokers than non-smokers. Similar results were reported by Marchetti *et al* (2011) as well. However, our data indicate that V600E mutations are significantly more prevalent in females than in males ($P \leq 0.05$).

The median age of NSCLC is 71 worldwide (Subramanian *et al*, 2010). However, it is 59 in our study, which is much lower than 71. Although the median age in Asia has been reported as lower than 71 before (Xu *et al*, 2011; Zhang *et al*, 2012), more young patients with NSCLC in China than the western countries may be the reason for the low median age. According to an internet database from 1381 hospitals, there is only 0.7% lung cancer at the age group <40 years (Age Group of Lung Cancer Diagnosed, 2000–2007). However, our patient population included 176 (3.4%) NSCLC patients who were younger than 40 years of age. Lung cancer is extremely rare in children. Surprisingly, for the first time, we discovered *KRAS* mutations in two very young patients: a 5-year old (with a Q61H mutation) and a 14-year old (with a G12V). Both of these patients were non-smoker females. This may be the first report of *KRAS* somatic mutation cases in children with NSCLC. Although it is a widely accepted notion that second-hand smoke (SHS) is linked to lung cancer (Besaratina and Pfeifer, 2008), medical professionals and researchers have not reached a consensus on the extent of the increase in lung cancer risk due to SHS. Although recent data suggested that most *KRAS* mutations in

non-smokers are not due to SHS exposure (Dogan *et al*, 2012), we raised the possibility again that certain subtypes of *KRAS* mutations in children may result from SHS. The epidemiology of young NSCLC in China and association with SHS and environmental factors are currently under investigation.

Coexistence of the *PIK3CA* mutation and other activating gene mutations in the *EGFR* signalling pathway were previously reported (Chaft *et al*, 2012). Double mutations of *KRAS* and *PIK3CA* exon 9 (but not exon 20) have also been reported in colorectal cancer (De Roock *et al*, 2010). However, in lung cancer, *EGFR* gene mutations are reported as mutually exclusive with *KRAS* or *BRAF* gene mutations (Marchetti *et al*, 2005). For the first time, we found 30 cases of coexistence of *EGFR* and *KRAS* mutations in FFPE samples of NSCLCs. In other words, a tumour can simultaneously harbour an activating *EGFR* mutation as well as mutations downstream of the pathway in the *KRAS* gene, which means that upstream inhibition of *EGFR* with TKIs will have no therapeutic effect in these cases. This finding also suggests that the *EGFR* mutation test alone may not be sufficient for the decision of whether or not the patient is a candidate for TKI therapy.

Interestingly, our data showed that *KRAS* and *BRAF* V600E mutations are mutually exclusive, which agrees with Rajagopalan *et al* (2002) that the *BRAF* mutation occurs only in tumours that do not carry *KRAS* mutations, as *KRAS* and *BRAF* provide an equivalent or at least redundant oncogenic stimulus in cancer pathogenesis. This phenomenon has also been observed in colorectal cancer (De Roock *et al*, 2010). However, we did find for the first time two cases of *EGFR* and *BRAF* coexisting mutations, which have been previously considered as mutually exclusive mutations, as well. Again, we submit that *KRAS* and *BRAF* mutation tests should be run concurrently with the *EGFR* mutation test.

EGFR-TKIs, such as gefitinib and erlotinib, have been successfully used to treat advanced NSCLCs with *EGFR*-activating mutations, exon 19 deletions or L858R. However, over 6–12 months of treatment, most tumours acquire resistance to EGFR-TKIs. Furthermore, it was discovered that the secondary T790M mutation occurs in 50% of *EGFR*-mutated patients with TKI resistance (Oxnard *et al*, 2011; Ohashi *et al*, 2012). Both *EGFR* T790M and *PIK3CA* E545K are considered as acquired resistance mutations to TKI treatment (Engelman *et al*, 2006; Engelman and Janne, 2008), and are considered as biomarkers for poor clinical prognosis. Among our 160 cases of multiple mutations, an *EGFR*-activating mutation plus a T790M point mutation was accounted for in 32 cases (20%), and 24 cases (15%) also included E545K. Twelve of them (12, T790M and 0, E545K) might be the results of TKI therapy. However, it was very interesting to find that there were 44 cases (20, T790M and 24, E545K) where patients had never received any TKI therapy but already harboured T790M or E545K secondary mutations. This may explain why some NSCLCs who harbour *EGFR*-activating mutations still fail the TKI therapy (Kobayashi *et al*, 2005). The same double mutations have been previously described in two patients who showed very poor responses to TKI treatment (Toyooka *et al*, 2005). Moreover, data have shown that continued activation of PI3K signalling by the *PIK3CA* oncogenic mutant, p110 α E545K, is sufficient to abrogate gefitinib-induced apoptosis (Subramanian *et al*, 2010). Together, our data confirmed the coexistence of the *EGFR*-activating mutation of exon 19 deletions or L858R, and the resistant point mutation of T790M or E545K before the TKI treatment. This finding provides evidence that double mutations with a second resistant mutation are important factors conferring resistance to TKI therapy in NSCLC carrying *EGFR*-sensitive mutations, thus lending insight to further considerations for designing clinical trials, or drugs, for more effective strategies against specific subtypes of NSCLC with both EGFR-TKIs and anti-T790M therapies. In the future, a more sensitive technology

should be able to identify whether these mutations are in the same cellule or not.

Together, our data present a clear panoramagram of mutation frequencies of *EGFR*, *KRAS*, *BRAF* and *PIK3CA* from 5125 NSCLC cases and their associations with gender, smoking history and histology. For the first time, 153 double and seven triple mutation cases were identified in NSCLC. These data will lead further investigations to explore the associations between multiple mutations and clinical outcomes. Finally, we also reported here, for the first time, the detection of *KRAS* mutations in children with NSCLC.

ACKNOWLEDGEMENTS

We wish to thank Zhiping Cai, Xiaoxia Qin, Huijuan Qin, Xuqiu Qin, Keyin Luo, Zhangmin Liang, Xinsheng Li, Yuting Yu and Qingpeng Zhong for their technical assistance. This research was supported by the Subtask of National Science and Technology Major Projects of China 'Key New Drug Creation and Manufacturing Program' (grant number 2012ZX09506001-003); by a grant from the Chinese Ministry of Education, the Key Project Grant (grant number 311037; PI: SL); and by the Young Investigator Award from Peking Union Medical College Hospital (grant number 2010116, PI: NL).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Age Group of Lung Cancer Diagnosed (2000–2007) <http://www.aboutcancer.com/lung1b.htm>.
- American Cancer Society (2011) *Cancer Facts and Figures 2009*. <http://www2.cancer.org/downloads/STT/500809web.pdf>.
- Asahina H, Yamazaki K, Kinoshita I, Yokouchi H, Dosaka-Akita H, Nishimura M (2006) Non-responsiveness to gefitinib in a patient with lung adenocarcinoma having rare EGFR mutations S768I and V769L. *Lung Cancer* **54**(3): 419–422.
- Besaratina A, Pfeifer GP (2008) Second-hand smoke and human lung cancer. *Lancet Oncol* **9**(7): 657–666.
- Chaff JE, Arcila ME, Paik PK, Lau C, Riely GJ, Pietanza MC, Zakowski MF, Rusch V, Sima CS, Ladanyi M, Kris MG (2012) Coexistence of PIK3CA and other oncogene mutations in lung adenocarcinoma—rationale for comprehensive mutation profiling. *Mol Cancer Ther* **11**(2): 485–491.
- D'Angelo SP, Pietanza MC, Johnson ML, Riely GJ, Miller VA, Sima CS, Zakowski MF, Rusch VW, Ladanyi M, Kris MG (2011) Incidence of EGFR exon 19 deletions and L858R in tumor specimens from men and cigarette smokers with lung adenocarcinomas. *J Clin Oncol* **29**(15): 2066–2070.
- De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeris KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Taberero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* **11**(8): 753–762.
- Dogan S, Shen R, Ang DC, Johnson ML, D'Angelo SP, Paik PK, Brzostowski EB, Riely GJ, Kris MG, Zakowski MF, Ladanyi M (2012) Molecular epidemiology of EGFR and KRAS mutations in 3026 lung adenocarcinomas: higher susceptibility of women to smoking-related KRAS-mutant cancers. *Clin Cancer Res* **18**(22): 6169–6177.
- Engelman JA, Janne PA (2008) Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* **14**(10): 2895–2899.
- Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borrás AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J, Tracy S, Zhao X, Heymach JV, Johnson BE, Cantley LC, Janne PA (2006) Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. *J Clin Invest* **116**(10): 2695–2706.
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *CA Cancer J Clin* **61**(2): 69–90.
- Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* **352**(8): 786–792.
- Li G, Luo X, He J, Zhu Z, Yu G, Qin H, Zeng T, Liu Z, Wu S, Xu J, Ren-Heidenreich L (2011) A novel liquidchip platform for simultaneous detection of 70 alleles of DNA somatic mutations on EGFR, KRAS, BRAF and PIK3CA from formalin-fixed and paraffin-embedded slides containing tumor tissue. *Clin Chem Lab Med* **49**(2): 191–195.
- Lin L, Bivona TG (2012) Mechanisms of resistance to epidermal growth factor receptor inhibitors and novel therapeutic strategies to overcome resistance in NSCLC patients. *Chemother Res Pract* **2012**: 817297.
- Ludovini V, Bianconi F, Pistola L, Chiari R, Minotti V, Colella R, Giuffrida D, Tofanetti FR, Siggillino A, Flacco A, Baldelli E, Iacono D, Mameli MG, Cavaliere A, Crinò L (2011) 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* **6**(4): 707–715.
- Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**(21): 2129–2139.
- Marchetti A, Felicioni L, Malatesta S, Grazia Sciarrotta M, Guetti L, Chella A, Viola P, Pullara C, Mucilli F, Buttitta F (2011) Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations. *J Clin Oncol* **29**(26): 3574–3579.
- Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Campese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* **23**(4): 857–865.
- Masago K, Fujita S, Irisa K, Kim YH, Ichikawa M, Mio T, Mishima M (2010) Good clinical response to gefitinib in a non-small cell lung cancer patient harboring a rare somatic epidermal growth factor gene point mutation; codon 768 AGC>ATC in exon 20 (S768I). *Jpn J Clin Oncol* **40**(11): 1105–1109.
- Ohashi K, Sequist LV, Arcila ME, Moran T, Chmielecki J, Lin YL, Pan Y, Wang L, de Stanchina E, Shien K, Aoe K, Toyooka S, Kiura K, Fernandez-Cuesta L, Fidas P, Yang JC, Miller VA, Riely GJ, Kris MG, Engelman JA, Vnencak-Jones CL, Dias-Santagata D, Ladanyi M, Pao W (2012) Lung cancers with acquired resistance to EGFR inhibitors occasionally harbor BRAF gene mutations but lack mutations in KRAS, NRAS, or MEK1. *Proc Natl Acad Sci USA* **109**(31): E2127–E2133.
- Oxnard GR, Arcila ME, Chmielecki J, Ladanyi M, Miller VA, Pao W (2011) New strategies in overcoming acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer. *Clin Cancer Res* **17**(17): 5530–5537.
- Paik PK, Arcila ME, Fara M, Sima CS, Miller VA, Kris MG, Ladanyi M, Riely GJ (2011) Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. *J Clin Oncol* **29**(15): 2046–2051.
- Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* **101**(36): 13306–13311.
- Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, Zakowski MF, Heelan RT, Kris MG, Varmus HE (2005) KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* **2**(1): e17.
- Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE (2002) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* **418**(6901): 934.
- Riely GJ, Kris MG, Rosenbaum D, Marks J, Li A, Chitale DA, Nafa K, Riedel ER, Hsu M, Pao W, Miller VA, Ladanyi M (2008) Frequency and distinctive

- spectrum of KRAS mutations in never smokers with lung adenocarcinoma. *Clin Cancer Res* **14**(18): 5731–5734.
- Rodenhuis S, van de Wetering ML, Mooi WJ, Evers SG, van Zandwijk N, Bos JL (1987) Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung. *N Engl J Med* **317**(15): 929–935.
- Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, Majem M, Lopez-Vivanco G, Isla D, Provencio M, Insa A, Massuti B, Gonzalez-Larriba JL, Paz-Ares L, Bover I, Garcia-Campelo R, Moreno MA, Catot S, Rolfo C, Reguart N, Palmero R, Sánchez JM, Bastus R, Mayo C, Bertran-Alamillo J, Molina MA, Sanchez JJ, Taron M. Spanish Lung Cancer Group (2009) Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* **361**(10): 958–967.
- Sasaki H, Kawano O, Endo K, Suzuki E, Haneda H, Yukiue H, Kobayashi Y, Yano M, Fujii Y (2006) Uncommon V599E BRAF mutations in Japanese patients with lung cancer. *J Surg Res* **133**(2): 203–206.
- Schmid K, Oehl N, Wrba F, Pirker R, Pirker C, Filipits M (2009) EGFR/KRAS/BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. *Clin Cancer Res* **15**(14): 4554–4560.
- Slebos RJ, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, Wagenaar SS, Vanderschueren RG, van Zandwijk N, Mooi WJ, Bos JL, Rodenhuis S (1990) K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* **323**(9): 561–565.
- Smits AJ, Kummer JA, Hinrichs JW, Herder GJ, Scheidel-Jacobse KC, Jiwa NM, Ruijter TE, Nooijen PT, Looijen-Salamon MG, Ligtenberg MJ, Thunnissen FB, Heideman DA, de Weger RA, Vink A (2012) EGFR and KRAS mutations in lung carcinomas in the Dutch population: increased EGFR mutation frequency in malignant pleural effusion of lung adenocarcinoma. *Cell Oncol (Dordr)* **35**(3): 189–196.
- Subramanian J, Morgensztern D, Goodgame B, Baggstrom MQ, Gao F, Piccirillo J, Govindan R (2010) Distinctive characteristics of non-small cell lung cancer (NSCLC) in the young: a surveillance, epidemiology, and end results (SEER) analysis. *J Thorac Oncol* **5**(1): 23–28.
- Toyooka S, Kiura K, Mitsudomi T (2005) EGFR mutation and response of lung cancer to gefitinib. *N Engl J Med* **352**(20): 2136.
- Wu S, Zhu Z, He J, Luo X, Xu J, Ren-Heidenreich L (2010) A novel mutant-enriched liquidchip technology for the qualitative detection of somatic mutations in KRAS gene from both serum and tissue samples. *Clin Chem Lab Med* **48**(8): 1103–1106.
- Xu J, He J, Yang H, Luo X, Liang Z, Chen J, Cai Z, Xu J, Ren-Heidenreich L (2011) Somatic mutation analysis of EGFR, KRAS, BRAF and PIK3CA in 861 patients with non-small cell lung cancer. *Cancer Biomark* **10**(2): 63–69.
- Zhang L, Yang H, Zhao Y, Liu W, Wu S, He J, Luo X, Zhu Z, Xu J, Zhou Q, Ren-Heidenreich L (2012) Detection of EGFR somatic mutations in non-small cell lung cancer (NSCLC) using a novel mutant-enriched liquidchip (MEL) technology. *Curr Drug Metab* **13**(7): 1007–1011.

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.