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Concurrent Oncogene Mutation Profile in Chinese Patients With Stage Ib Lung Adenocarcinoma

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Abstract: Molecular characteristics in lung cancer are associated with carcinogenesis, response to targeted therapies, and prognosis. With concurrent oncogene mutations being reported more often, the adjustment of treatment based on the driver gene mutations would improve therapy. We proposed to investigate the distribution of concurrent oncogene mutations in stage Ib lung adenocarcinoma in a Chinese population and find out the correlation between survival outcome and the most frequently mutated genes in EGFR and KRAS in Chinese population. Simultaneously, we tried to validate the Sequenom method by real time fluoresce qualification reverse transcription polymerase chain reaction (RT-PCR) in oncogene detection.

One hundred fifty-six patients who underwent complete surgical resection in our hospital between 1999 and 2007 were retrospectively investigated. Using time-of-flight mass spectrometry, 238 mutation hotspots in 19 oncogenes were examined.

Genetic mutations occurred in 86 of 156 patients (55.13%). EGFR was most frequently gene contained driver mutations, with a rate of 44.23%, followed by KRAS (8.33%), PIK3CA (3.84%), KIT (3.20%), BRAF (2.56%), AKT (1.28%), MET (0.64%), NRAS (0.64%), HRAS (0.64%), and ERBB2 (0.64%). No mutations were found in the RET, PDGFRA, FGFR1, FGFR3, FLT3, ABL, CDK, or JAK2 oncogenes. Thirteen patients (8.3%) were detected in multiple gene mutations. Six patients had PIK3CA mutations in addition to mutations in EGFR and KRAS. EGFR mutations can coexist with mutations in NRAS, KIT, ERBB2, and BRAF. Only one case was found to have a KRAS mutation coexisting with the EGFR T790M mutation. Otherwise, mutations in EGFR and KRAS seem to be mutually exclusive. There is no survival benefit in favor of EGFR/KRAS mutation.

Several concomitant driver gene mutations were observed in our study. None of EFGR/KRAS mutation was demonstrated as a prognostic factor. Polygenic mutation testing by time-of-flight mass spectrometry was validated by RT-PCR, which can be an alternative option to test for multiple mutations and can be widely applied to clinical practice and help to guide treatment.

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Abbreviations: FFPE = formalin-fixed paraffin-embedded, HE = hematoxylin and eosin, MALDI-TOF = matrix-assisted laser desorption ionization time-of-flight mass spectrometer, NSCLC = non-small cell lung cancer, PFS = progress-free survival, RT-PCR = reverse transcription polymerase chain reaction, TKIs = tyrosine kinase inhibitors.

INTRODUCTION

With the discovery of oncogenic driver mutations in non-small cell lung cancer (NSCLC), therapy has been modified to target those mutations. Driver mutations in genes such as KRAS, EGFR, HER2, PIK3CA, ALK, MET, AKT1, MEK1, BRAF, ROS1, RET, and NRAS have been identified in lung adenocarcinoma. According to the molecular alteration, these lung cancers can be classified into diverse subsets related to rationally targeted therapies.[1,2]

Previous studies showed that although targeted therapies for unselected patients with NSCLC resulted in limited improvements in outcomes, the therapy for patients with EGFR mutations resulted in a better response rate (>60%) and a marked improvement in progression-free survival (PFS) in some clinical trials in Asia and Europe.[3] Therefore, the presence of EGFR mutations has been regarded as a predictive marker of good response to tyrosine kinase inhibitors (TKIs).[4,5] However, after a median of approximately 10 months of TKIs treatment, some patients developed resistance. Some studies found that this resistance was associated with acquired mutations such as EGFR T790M, and others in RET.[6,7] Treatment with the ALK-TKI crizotinib showed similar results, with a response rate of >60% and a PFS of 10 months in ALK fusion-positive patients.[8,9] The identification of oncogenic driver mutations that are sensitive to epidermal growth factor receptor and ALK-TKI has resulted in a surge of interest in the search for additional targetable oncogenes. Promising results from clinical trials with other targeted drug therapies against tumors with oncogenic mutations in KRAS, BRAF, PIK3CA, HER2, ROS1, RET, among others, suggest that such therapies will be of great use in the future.[10,11] These data have created a new way to classify NSCLC, which is currently performed based on the mutational profile. Therefore, the integration of multigene mutation testing into NSCLC clinical practice is necessary to direct molecularly targeted treatments. Additional studies on multigene profiling have demonstrated the coexistence of

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mutations in multiple genes, which suggests that those driver mutations may lie molecularly upstream or downstream of one another or may predict different efficacies of targeted therapy.[12–14] Some studies reported that ALK and EGFR can be co-mutated.[15,16] One study included a patient with 5 different genetic alterations.[13] Some studies showed the concurrent genetic alteration rate to be approximately 3–9%.[13,17] These were mostly case reports. Thus, knowing the concurrent oncogene mutation profile can allow better selection of the appropriate targeted therapy.

To screen for concurrent oncogene mutations in our study, we used 19-gene Sequenom testing (time-of-flight mass spectrometry) that included identification of 238 genetic mutations. Mutation incidence and coincidence in non-small cell cancer differs by ethnicity and the histology of the lung cancer. Therefore, we focused on stage Ib adenocarcinoma in a Chinese population with NSCLC after complete surgical resection. The mutation profile of advanced cancer is well known because these cancers are the major candidates for systemic treatments. The Sequenom testing for a wide range of mutations is not broadly applied, especially in the care of patients with early-resected NSCLC, and the efficacy of this testing for mutation screening is also unclear. In our study, we tried to validate the accuracy and sensitivity of Sequenom testing by real time fluoresce qualification reverse transcription polymerase chain reaction (RT-PCR), and use the Sequenom testing to determine the concurrent oncogene mutation profile in stage Ib adenocarcinoma NSCLC after complete surgical resection. Furthermore, we purposed to identify the correlation between survival outcome and the most frequently mutated genes in EGFR and KRAS in Chinese population.

MATERIALS AND METHODS

The Institute Research Medical Ethics Committee of Sun Yat-sen University Cancer Center (SYSUCC) granted approval for this study (ethics number: B2013-028-01).

Patient Selection

A total of 156 patients with stage Ib (pT2aN0M0) adenocarcinoma NSCLC who underwent radical surgery at SYSUCC from 1999 to 2007 were retrospectively enrolled in this study. Informed consent and clinicopathologic information were obtained from all patients. The disease stages of all patients were classified or reclassified according to the UICC 2009 TNM staging system.

Patients were eligible to be included if they had undergone a curative resection according to the 6th edition of the American Joint Commission on Cancer guidelines and had been diagnosed with stage Ib (pT2aN0M0) adenocarcinoma of NSCLC at our institution.

The exclusion criteria included missing or inadequate tissue blocks (could not procure surgical specimens or specimen paraffin blocks contained <50% tumor cells), death within 30 days of resection, positive margins on pathology and occurrence of a second cancer after surgery, and loss to follow-up. The clinical dates, follow-up results, and cause of death were obtained from a review of medical records and from the follow-up department of the hospital.

Tumor Sample

Tumor tissue came from the postoperative formalin-fixed, paraffin-embedded (FFPE) samples of the 156 stage Ib adenocarcinoma patients diagnosed between October 1999 and July 2007 at SYSUCC. Informed consents were signed by all patients before starting initial treatment for using tissue samples and clinic-pathologic information in future research. All of the paraffin-embedded specimen blocks were evaluated by a pathologist who microscopically reviewed hematoxylin and eosin (HE) stained slides prepared from the blocks. Specimen paraffin blocks with ≥50% tumor cells were used in this study. All 156 samples were obtained from surgical resection, and six 4-μm thick tissue sections were prepared from each.

A matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) that was produced by Sequenom Inc., San Diego was used (a 19-gene expression assay that includes identification of 238 genetic mutations).

DNA Extraction

Based on a review of HE stained sections of each tumor, we choose paraffin blocks with >50% tumor cells, cut 6 sections (4–6 μm), and placed them in 1.5 ml Eppendorf tubes for DNA extraction. DNA was extracted using a QIAamp DNA FFPE Tissue Kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). The quantity and quality of the isolated DNA was tested by using a Nanodrop ND-2000 Spectrophotometer (Thermo Scientific, Niederelbert, Germany). DNA was diluted to a final concentration of 10 ng/μl for analysis.

OncoCarta Assay

Two hundred thirty-eight mutations in a panel of 19 oncogenes were detected in 156 samples by an OncoCarta Panel v. 1.0. (Sequenom Inc., San Diego). This is a set of predesigned and prevalidated assays for sensitive and efficient mutation screening by parallel analysis of 238 mutations across 19 common oncogenes: ABL1, AKT1, AKT2, BRAF, CDK, EGFR, ERBB2, FGFR1, FGFR3, FLT3, HRAS, JAK2, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, and RET.

In brief, 20 ng of DNA was amplified using 24 different OncoCarta PCR primer mixtures, and then an extension reaction using the OncoCarta extension primers was performed. After using a cation exchange resin to remove salts, the products were spotted on a 384-well SpectroChipII using the MassARRAY Nanodispenser RS1000 (Sequenom Inc., San Diego) and analyzed on MALDI-TOF. We used high performance liquid chromatography (HPLC) pure water as the blank and normal human somatic cells as the negative control in each experiment.

Data Analysis

The mutation data were analyzed using MassARRAY TYPER 4.0 software (Sequenom Inc., San Diego) with the cutoff of the mutation frequency set at 1%. An experiment was deemed successful when the standard sample gave a typical result and the blank had no peak [see figure, supplemental content 1 (http://links.lww.com/MD/A105), which illustrates the interpretation for EGFR positive mutation by Sequenom test with blank and negative control].

The assay protocol included the following steps:

Isolate DNA from the sample;
Prepare DNA dilutions;
Perform PCR amplification;
Perform the SAP treatment;
Perform the TypePLEX extend reaction;
Design an OncoCarta Plate;
Dispense samples to a SpectroCHIP II array, continued;

Analyze the SpectroCHIP II array on the MassARRAY Compact Analyzer;
Generate an OncoCarta mutation analysis report.

Validation of Sequenom Method Using RT-PCR

EGFR Contrast Reagent

The EGFR mutation detection was performed by using the Human EGFR Gene 21 Mutations Fluorescence PCR Diagnostic Kit (AmoyDx, Xiamen, China) (Figure 1A).

Twenty cases of EGFR mutation specimens and 20 cases of EGFR wild type specimens were randomly sampling to perform the contrast with Sequenom or RT-PCR kit.

KRAS Contrast Reagent

The KRAS mutation detection was performed by using the Human KRAS Gene Mutations Fluorescence PCR Diagnostic Kit (ACCB Biotech Ltd, Beijing, China) (Figure 1B).

We selected the 13 cases of KRAS mutation specimens and randomly sampling 20 cases of KRAS wild type specimens to perform the contrast with Sequenom or RT-PCR kit.

Statistical Analysis

Statistical analysis was performed using SPSS software, version 16.0 (SPSS Inc., Chicago). The relationship between the mutation status and clinical data was assessed using chi-squared, Fisher exact tests or Kruskal–Wallis H test. Kaplan–Meier analysis was used to compare differences in the survival rate between groups, and the correlation between survival outcome and the most frequently mutated genes in EGFR and KRAS in Chinese population. A P-value of <0.05 was considered statistically significant. The clinical follow-up date was updated to January 2013.

RESULTS

Patient Characteristics

The clinicopathologic characteristics of the 156 patients are summarized in Table 1. The patients had a mean age of 58 years (range 32–84 years), and 40.38% were female. Eighty-eight patients (56.41%) had never smoked before (never-smokers).

Results of Validation for Sequenom Test by RT-PCR

Due to the detected mutation hotspots of Sequenom method was more than RT-PCR method, the EGFR positive cases using Sequenom method was 20 other than 18 with real time fluoresce qualification RT-PCR method in the validation experiment, but with no significant difference ($P = 0.500$) [see Table, supplemental content 2 (http://links.lww.com/MD/A106), which illustrates the contrast result between the two kits in the detection of EGFR mutation]. However, comparative results of KRAS detection with RT-PCR and Sequenom test were matched.

Results of Multimutational Profiling

Of the 156 patients in the cohort, 86 (55.13%) were shown to have genetic mutations. EGFR was the most frequent driver mutation (found at a rate of 43.59%), followed by KRAS (8.33%), PIK3CA (3.84%), KIT (3.20%), BRAF (2.56%), AKT (1.28%), MET (0.64%), NRAS (0.64%), HRAS (0.64%), and ERBB2 (0.64%). These findings are summarized in Figure 2. No mutation was found in the RET, PDGFRA, FGFR1, FGFR3, FLT3, ABL, CDK, or JAK2 genes.

Mutations in EGFR and KRAS were usually mutually exclusive, but we found one case with EGFR T790M and KRAS mutations. We found only 2 patients (1.28%) with the EGFR T790M mutation, which produces resistance to EGFR-TKI. Most activating EGFR mutations occur in exons 19 and 21. There were no patients with mutations in EGFR exon 18; 29 had mutations in exon 19; 2 had a mutation in exon 20; 32 had mutations in exon 21; 1 had mutations in exons 18 and 20; 1 had mutations in exons 18 and 21; 1 had mutations in exons 19 and 20; 1 had mutations in exons 19 and 21; and 1 had mutations in exons 18 and 20 (Table 2). The EGFR L858R mutation was the most common amino acid change (28.18%), followed by EGFR E746-T751>A. The frequencies of all mutations are summarized in Table 3.

Concurrent Genetic Alterations

There were 13 patients (8.33%) with multiple mutations. Four of these harbored mutations in three genes (AKT, BRAF, and PIK3CA; PIK3C, EGFR, and KIT; BRAF, EGFR, and KIT; and HRAS, AKT, and KIT) and 9 had mutations in two genes. EGFR mutations were observed in 10 patients as the most common partner for concurrent genetic alterations, indicating that EGFR mutations and other driver genetic alterations were not necessarily mutually exclusive (Tables 4 and 5).

We found that mutations in PIK3CA often accompanied other mutations (4 were found with mutations in EGFR, 1 was found with mutations in KRAS, and 1 was found with mutations in BRAF). This was also observed for KIT and AKT. There were 5 patients who harbored KIT mutations, and all of those mutations coexisted with other mutations in genes such as BRAF, EGFR, PIK3C, HRAS, and AKT. The most common coexisting mutations were in EGFR (4 patients). There were two patients who had AKT mutations, both of which coexisted with other mutations. Therefore, it appears that mutations in PIK3C, AKT and KIT easily coexist with other mutations.

The presence of multiple mutations was not correlated with age, gender, smoking status or differentiation ($P = 0.075$, $P = 767$, $P = 1$, and $P = 1$, respectively; Table 6).

Clinicopathologic Correlations With Genotype

We assessed the relationships between the mutational status and clinical data using the chi squared or Fisher exact test. With respect to smoking status, we found that non-smokers had higher rates of EGFR mutations (53.4%) than smokers (32.4%) ($P = 0.009$). We also observed that in female patients and patients with well differentiated tumors, mutation rates were high. This finding suggested that never-smokers and females could potentially benefit more from treatment with molecularly targeted therapies compared with smokers and males. Mutations in KRAS appear more likely to occur in males and smoking patients (Table 6).

Correlation Between EGFR and KRAS Mutation and Survival

The Kaplan–Meier survival analysis indicated that the median OS was 104.8 months (95% CI 76.4–133.2) in the EGFR mutation group and the 3-year survival and 5-year survival rate was 85.3% and 66.0%, respectively. The EGFR wild-type group median OS could not be determined, and the 3-year survival and 5-year survival rate was 89.6% and 77.8%,

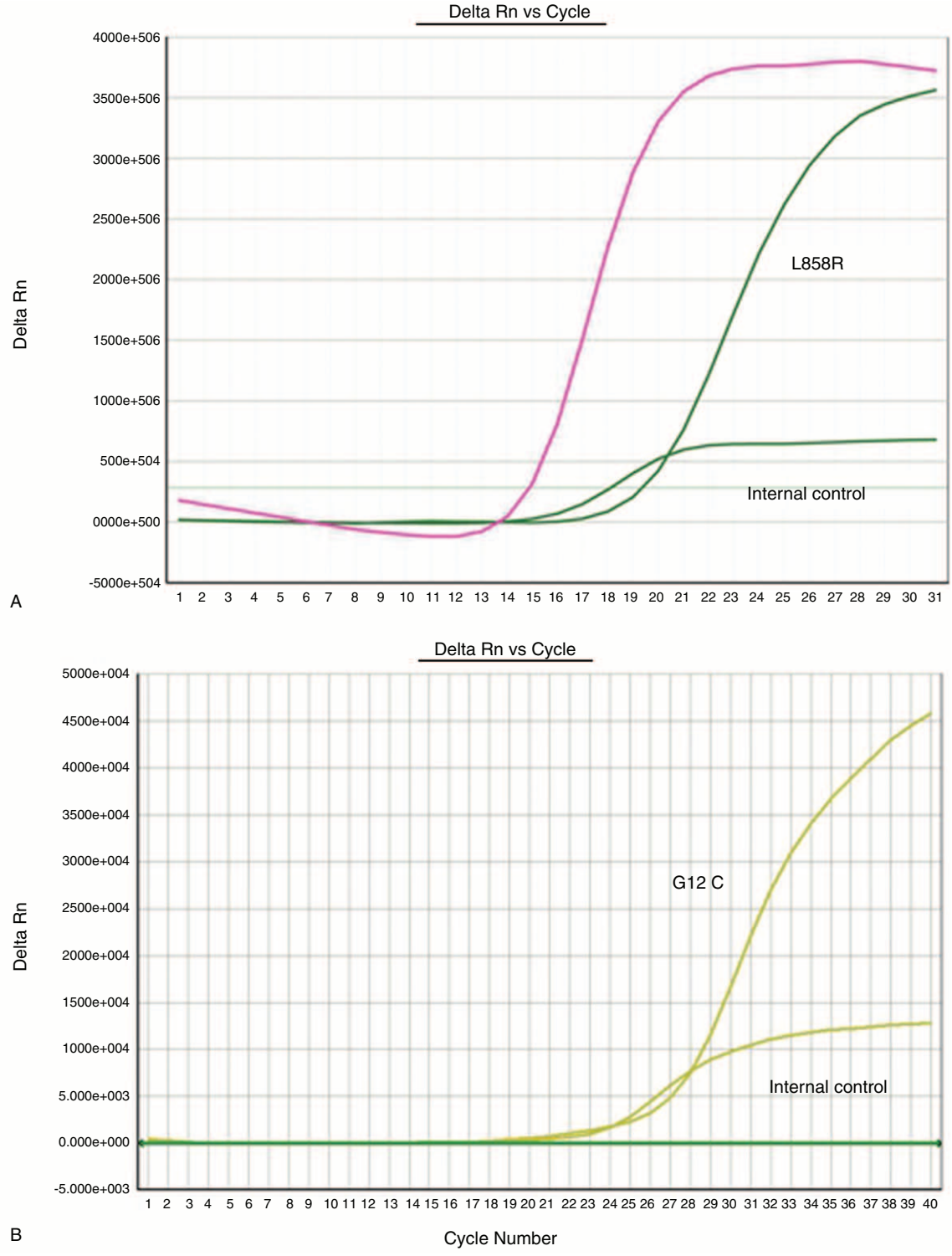

FIGURE 1. Validation of the Sequenom test using RT-PCR. (A) EGFR mutation testing for c.2573T>G. p.(Leu858Arg) mutation, and (B) KRAS mutation testing for c.34G>T. p. (Gly12Cys) mutation.

respectively. However, no significant differences in OS were identified between two groups ($P = 0.298$).

The Kaplan–Meier survival analysis showed that the median DFS was 80.4 months (95% CI 35.6–125.1) in the EGFR mutation group and 96.9 months in wild type group. No significant differences in DFS were observed between groups ($P = 0.602$). However, the OS and DFS survival curves indicated a trend of better survival outcome in EGFR wild-type patients.

TABLE 1. Characteristics of Patients in this Study

Variable	Group	No.	Percentage (%)
Gender	Male	93	59.62
	Female	63	40.38
Age	Mean	58	
	Range	32–84	
Smoking status*	Never	88	56.41
	Former	68	43.59
Differentiation	Well	16	10.26
	Moderately	63	40.38
	Poorly	44	28.20
	Unknown	33	21.15
Follow-up status	Survival	62	37.35
	Death	94	62.65
Visceral pleura invasion	Yes	112	71.79
	No	44	28.21
Bronchia invasion	Yes	24	15.38
	No	133	84.62
Tumor size†	Mean	3.26 cm	
	Range	1.00–5.00 cm	
Adjuvant therapy	Yes	24	15.38
	No	132	84.62
Comorbid conditions‡	Yes	65	41.67
	No	91	58.33
Surgical procedure	Pneumonectomy	2	98.72
	Lobectomy	154	1.28
	Segmentectomy	0	0
Total		156	100

* Smoking status: patients had smoked ≥10 cigarettes per week.
† Tumor size was divided by the largest length.
‡ Comorbid conditions: presence of concomitant disease.

Similarly, KRAS mutations showed no differences in terms of OS ($P = 0.651$) or DFS ($P = 0.654$). In KRAS wild-type group, the median OS was 124.3 months, and the OS of 3- and 5-year were 88.7% and 72.9%, respectively. However, in KRAS mutation group, the median OS could not be obtained, but the 3-year and 5-year OS was 83.3% and 75.0%, respectively. The median DFS was 96.6 months (95% CI 63.8–130.0) in the KRAS wild-type group and 60.2 months (95% CI 8.0–112.4) in the KRAS mutation group ($P = 0.654$) (Figure 3).

DISCUSSION

This study investigated the mutation profile of 19 driver genes in Chinese patients with early stage adenocarcinoma NSCLC. The incidence of driver mutations was similar to that

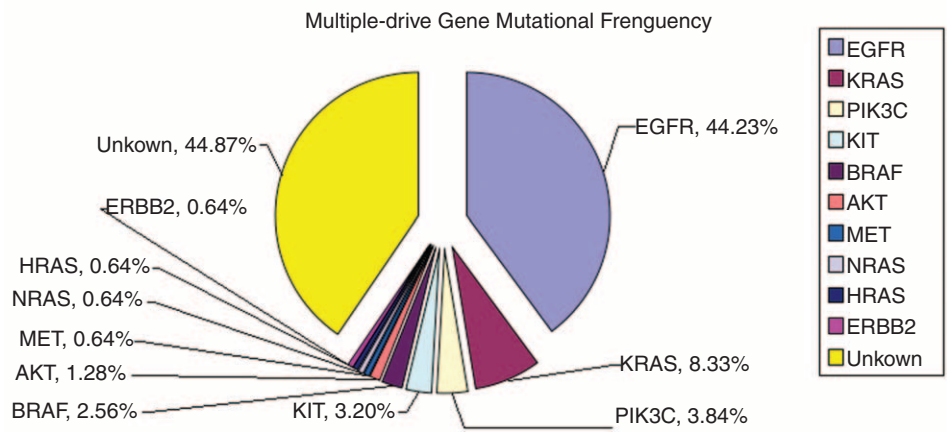

FIGURE 2. Multiple-drive gene mutational frequency.

TABLE 2. The Distribution of EGFR Exon Types

Mutation Type	No.	Percentage (%)
EGFR exon 18	0	0
EGFR exon 19	29	42.0
EGFR exon 20	2	2.9
EGFR exon 21	32	46.4
EGFR exon 18 + 19	1	1.4
EGFR exon 18 + 20	1	1.4
EGFR exon 19 + 20	1	1.4
EGFR exon 18 + 21	1	1.4
EGFR exon 19 + 20	1	1.4
EGFR exon other	1	1.4
Total	69	100

reported in other studies of Asian adenocarcinoma NSCLC patients. Dearden study reported an EGFR mutation rate in Asian adenocarcinoma patients is 47.9% (versus 44.3% in this study), a KRAS mutation rate of 11.2% (versus 8.3% in this study), and a PIK3CA mutation rate of 1.7% (versus 3.8% in this study).[14] Other studies have reported differing results due to differences in patient ethnicity, tumor histology, and smoking status.[18,19] Some patients had multiple mutations in the EGFR gene, such as simultaneous mutations in exons 19 and 21. In our study, there were a total of five patients with these simultaneous mutations. This also occurred with BRAF mutations (BRAF G464E and BRAF L597S). Similar findings are seldom reported in other studies (a 697 patient study on BRAF did not reveal similar mutations).[20]

MALDI-TOF MS was used to detect gene mutation in this study, which was quite different from the generally used RT-PCR method. Oberholzer et al showed that RAS mutations by MALDI-TOF MS are more frequent in cutaneous squamous cell tumor patients treated with RAF inhibitors than in those not so treated.[21] Bar et al[22] also demonstrated the Sequenom-based mutation screen is feasible using FFPE samples in NSCLC. In the same way, Su et al detected EGFRT790M mutations in patients with non-small-cell lung cancer, which detected and quantified the mutations highly sensitively.[23]

In our study, the Sequenom method was validated by the RT-PCR and the results were almost consistent with the conventional RT-PCR and what is more the Sequenom-based mutation screen can better detect more hotspots. Also the Sequenom method in our laboratory had passed the quality control of EMQN EGFR gene mutation detection in 2014. So using this technology we can detect multiple gene mutations with high sensitivity and accuracy.

Because our testing covers a wide range of mutations, we can more effectively find concurrent oncogene mutations. In our study, we found 13 patients had multiple mutations (8.3%).

TABLE 3. Mutation Characteristics of Detected Oncogenes

Gene	Exon/Domain	N	Main Activated Mutation or Amino Acid Changes
EGFR	Total	69	
	Exon 19	29	E746-T751del,E746-A751del,E746-A750del,E746-A752del,E746-A753del, E747-S752del,L747-T751del,L747-S752del,L747-E749del
	Exon 20	2	T790M,N771-P772>SVDNR
	Exon 21	32	L858R,L861Q,L861R
	Exon 18 + 20	1	Concurrent with EGFR G719S and T790M
	Exon 19 + 20	1	Concurrent with EGFR E746-A750del and H773-V774ins and R108K
	Exon 18 + 21	1	Concurrent with EGFR G719S and L861Q
	Exon 18 + 19	1	Concurrent with EGFR S725I/F and E746-T751del and S752F
	Exon 19 + 21	1	Concurrent with EGFR E746-A750del and L858R
	Other	1	A289V
KRAS	Total	13	
	Codon 12	8	G12C,G12D,G12V,G12A
	Others	5	A59T,G13D
AKT	Total	2	Q43X,rs11555431
BRAF	Total	4	
	Exon 15	4	L597S,V600R,V600E
	Exon 11	1	G464E
PIK3CA	Total	6	
	Exon others	4	E545K,R88Q
	Exon 20	2	H1047Y
KIT	Total	5	
	Exon 11	4	L576P,V559I,K558-E562del
	Exon 1	1	D52M
MET	Total	1	R970C
NRAS	Total	1	
	Condon 13	1	G13D
HRAS	Total	1	
	Condon 13	1	G13S
ERBB2	Total	1	
	Exon 20	1	G776S

TABLE 4. Individual Patient Characteristics, Concurrent Oncogene Mutation

Patient	Age	Gender	Smoking Status	Differentiation	Mutation 1	Mutation 2	Mutation 3	Mutation 4
1	45	Female	Never	Well	PIK3C H1047Y	EGFR L858R	KIT V559I	
2	50	Female	Never	Moderately	AKT rs11555431	BRAF L597S	PIK3CA R88Q	
3	51	Male	Former	Poorly	HRAS G13S	AKT Q43X	KIT V559I	
4	52	Male	Former	Moderately	BRAF G464E	BRAF L597S	EGFR E746∼T751>A	KIT D52M
5	59	Male	Former	Poorly	PIK3C E545K	EGFR G719S	EGFR L861Q	
6	51	Female	Never	Well	NRAS G13D	EGFR L858R		
7	53	Male	Former	Unknown	KRAS G12V	EGFR T790M		
8	57	Female	Never	Poorly	KRAS A59T	PIK3CA E545K		
9	57	Male	Never	Moderately	EGFR E746∼T751>A	KIT L576P		
10	42	Female	Never	Moderately	EGFR E746∼T751>A	PIK3CA R88Q		
11	50	Male	Former	Well	EGFR L858R	PIK3CA H1047Y		
12	62	Male	Former	Moderately	EGFR E746∼S752>A	KIT K558-E562del	EGFR S725I/F	
13	63	Female	Never	Well	EGFR E746∼T751>A	ERBB2 G776S		

Concurrent genetic alterations have been reported in 3% to 9% of lung adenocarcinoma patients by other groups.[13,17] Compared with those studies, we found a fairly high concurrent mutation rate. Our investigation revealed 4 patients with three types of driver mutations and 9 patients with two types of driver mutations. Ten of the 13 patients with multiple mutations harbored an EGFR mutation, which means that EGFR mutations and other driver gene alterations were not necessarily mutually exclusive.

We found that mutations in PIK3CA often accompany EGFR/KRAS mutations (4 patients had EGFR mutations, 1 had a KRAS mutation, 1 had a BRAF mutation). Chen haiquan study showed that PIK3CA mutations frequently coexist with EGFR/KRAS mutations in NSCLC and are associated with poor prognosis in the EGFR/KRAS wild type subgroup.[24] The exact molecular mechanism of this effect on prognosis warrants further study. In colorectal cancer, some studies reported that the use of aspirin was associated with longer survival among patients who harbored PIK3CA mutations.[25,26] We can therefore speculate that in lung cancers with mutated PIK3CA, the use of aspirin plus EGFR-TKI could be effective.

We also found that mutations in KIT and AKT often accompany mutations in other genes. EGFR was the gene most often found to have concomitant mutations with KIT (4 patients). This has rarely been reported in other studies and therefore needs to be studied further.

One patient had the EGFR T790M mutation and a mutation in KRAS, but we found no other EGFR mutations associated with KRAS. Previous studies reported that EGFR and KRAS mutations were generally mutually exclusive, but for the EGFR T790M mutation (which frequently occurs in patients who are resistant to EGFR-TKI) this appears not to be the case.[14,27]

The observation of overlap mutations in driver genes reveals the complexity of individualized therapy in lung cancer. EGFR and KRAS have been found to be the two most important genes by many researchers. Our study found that EGFR mutation could overlap with mutations in other genes including PIK3C, BRAF, and KIT. In contrast, KRAS mutations were rarely found with other mutations. Other rare oncogene mutations in KIT, AKT, NRAS, and HRAS often seem to occur with other driver gene alterations. We believe more overlapping of driver gene mutations will be reported in the future and will necessitate testing for multiple oncogene mutations in the clinic to determine the best therapy.

When we compared the relationship between clinical characteristics and genotype, we found that EGFR mutations are more common in never-smokers or light smokers, women, and in patients with well-differentiated tumors, and that

TABLE 5. Co-Mutation Identity

	Single Mutation	EGFR	KRAS	PIK3C	KIT	BRAF	AKT	MET	NRAS	HRAS	ERBB2	Total
EGFR	59 (85.5)	4 (5.8)	1 (1.4)	4 (5.8)	4 (5.8)	1 (1.4)	0	0	1 (1.4)	0	1 (1.4)	69
KRAS	11 (84.6)	1 (7.7)	0	1 (7.7)	0	0	0	0	0	0	0	13
PIK3C	0	4 (66.7)	1 (16.7)	0	0	1 (16.7)	1 (16.7)	0	0	0	0	6
KIT	0	4 (80)	0	1 (20)	0	2 (40)	1 (20)	0	0	1 (20)	0	5
BRAF	2 (50)	1 (25)	0	1 (25)	1 (25)	0	1 (25)	0	0	0	0	4
AKT	0	0	0	1 (50)	1 (50)	1 (50)	0	0	0	1 (50)	0	2
MET	1 (100)	0	0	0	0	0	0	0	0	0	0	1
NRAS	0	1 (100)	0	0	0	0	0	0	0	0	0	1
HRAS	0	0	0	0	1 (100)	0	1 (100)	0	0	0	0	1
ERBB2	0	1 (100)	0	0	0	0	0	0	0	0	0	1

In each row of this table, the total number of mutations in all genes is given in the rightmost column. Each column presents the number of cases (%) with mutations found in common with the mutation indicated for the relevant row.

TABLE 6. Correlations Between Mutations and Clinicopathologic Characteristics

Variable	Concurrent Mutation			EGFR Mutation			KRAS Mutation		
	Concurrent	Single	*P*-Value	Mutant	Wild Type	*P*-Value	Mutant	Wild Type	*P*-Value
Age			0.075			0.935			0.156
≥60	2 (3.1%)	63 (96.9%)		29 (44.6%)	36 (55.4%)		3 (4.6%)	62 (95.4%)	
<60	11 (12.1%)	80 (87.9%)		40 (44.0%)	51 (56.0%)		10 (11.0%)	81 (89.0%)	
Gender			0.767			0.005			0.002
Male	7 (7.3%)	89 (92.7%)		34 (35.4%)	62 (54.6%)		13 (13.5%)	83 (86.5%)	
Female	6 (10%)	54 (90%)		35 (58.3%)	25 (41.7%)		0 (0%)	60 (100%)	
Smoking status			1.000			0.009			0.017
Yes	6 (8.8%)	62 (91.2%)		22 (32.4%)	46 (67.6%)		10 (14.7%)	58 (85.3%)	
No	7 (8.0%)	81 (92.0%)		47 (53.4%)	41 (46.6%)		3 (3.4%)	85 (96.6%)	
Differentiation			0.870			0.001			0.164
Well	3 (7.5%)	37 (72.5%)		22 (55%)	18 (45%)		3 (7.5%)	37 (92.5%)	
Moderately	4 (5.7%)	66 (94.3%)		38 (54.3%)	32 (45%)		3 (4.3%)	67 (95.7%)	
Poorly	3 (6.5%)	43 (93.5%)		9 (19.6%)	37 (81.4%)		7 (15.2%)	39 (84.8%)	

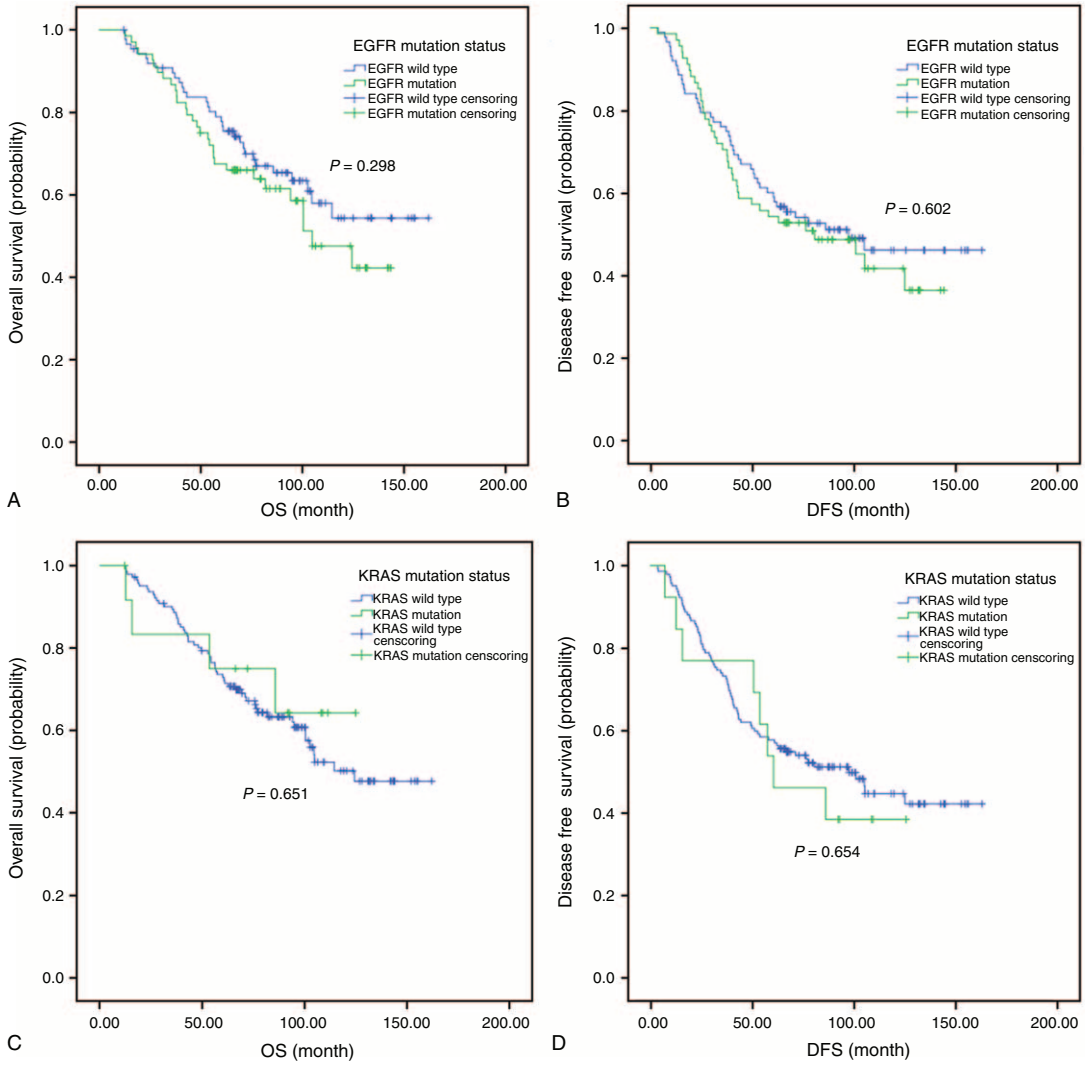

FIGURE 3. Overall survival and disease-free survival analysis regarding EGFR and KRAS gene status. (A) OS in EGFR mutation status, (B) DFS in EGFR mutation status, (C) OS in KRAS mutation status, and (D) DFS in KRAS mutation status.

TABLE 7. Summary of Oncogene Mutations in NSCLC

Gene	Mutation Status in This Study	Ratio (95% Confidence Interval)	Mutation Status in Previous Studies	Ratio (95% Confidence Interval)
EGFR	69/156 (44.2%)	36.4%–52.0%	1492/3117 (47.9%)[14,*]	46.1%–49.6%
KRAS	13/156 (8.3%)	4.0%–12.7%	236/2114 (11.2%)[14,*]	9.8%–12.5%
PIK3CA	6/156 (3.8%)	0.8%–6.9%	22/807 (2.7%)[24,*]	1.6%–3.9%
ERBB2	1/156 (0.7%)	−0.6%–1.9%	20/712 (2.8%)[14,*]	1.6%–4.0%
BRAF	4/156 (2.6%)	0.1%–5.0%	5/321 (1.6%)[14,*]	0.2%–2.9%
AKT	2/156 (1.3%)	−0.5%–3.1%	Rare[1,†]	
KIT	5/156 (3.2%)	0.4%–6.0%	7.10%[†]	
NRAS	1/156 (0.7%)	−0.6%–1.9%	0.70%[27,‡]	
HRAS	1/156 (0.7%)	−0.6%–1.9%	1/141 (0.9%)[‡]	−0.7%–2.1%
MET	1/156 (0.7%)	−0.6%–1.9%	9/411 (2.2%)[13,†]	0.8%–3.6%
RET	0/156		11/633 (1.7%)[†]	0.7%–2.8%
PDGFRA	0/156		No report	
FGFR1	0/156		About 1%[1,‡]	
FGFR3	0/156		No report	
FLT3	0/156		No report	
ABL	0/156		No report	
CDK	0/156		No report	
JAK2	0/156		No report	

[*] Sample came from adenocarcinoma in Asian patients.
[†] Sample came from adenocarcinoma, and race was not specified.
[‡] Sample was not limited with respect to specific histologic type or race.

mutations in KRAS are more common in smokers and men. Previous studies have reported the same observations. Other mutations were too rare to assess statistical significance. These results are consistent with many previous studies.[1,28] The patients with multiple mutations were not correlated with age, gender, smoking status, and differentiation. So we cannot find out the potential patients that existed concurrent mutation according to these clinical characteristics.

In our cohort, majority of patients received no adjuvant therapy after radical operation, except 7 patients accepted EGFR-TKI-targeted therapy after recurrence or metastasis. After a follow-up of >5 years, our data analysis showed no survival difference between EGFR/KRAS mutation and wild type patients in stage Ib, indicating that EGFR/KRAS is not a prognostic factor for lung adenocarcinoma , unlike previous study reported.[29]

The Sequenom method can detect a total of 238 somatic mutations in 19 different oncogenes that are commonly associated with cancer and have been described in the past. Unlike previous studies that have used various tumor tissues, our study used FFPE samples. The similarity in the incidence of mutations between this study and previous studies validates the use of FFPE samples (Table 7) in such research. This is an alternative to high-throughput sequencing methods and should be considered as a routine clinical tool for the screening of oncogene mutations.

The disadvantages of this approach include the inability to detect ALK rearrangements, which are associated with marked sensitivity to the TKI crizotinib, and the need for the vendor (Sequenom) to modify and update the list of genes with targeted mutations. In addition, our sample size was small compared with large-scale genetic screening studies, and the number of concurrent mutation patients was limited.

In summary, for stage Ib adenocarcinoma NSCLC in China, there did coexist concomitant multiple driver gene mutations that may affect the strategies for individual treatments in lung adenocarcinoma. Both of EGFR and KRAS mutation showed as non-prognostic factors for survival analysis. Our study also demonstrated the accuracy and sensitivity of Sequenom test for testing multiple mutations compared with generally used RT-PCR method. It should be widely applied to clinical practice and guidance for treatment.

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