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ORIGINAL PAPER

Analysis of Driver Mutations in Female Non-Smoker Asian Patients with Pulmonary Adenocarcinoma

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Abstract Previous studies have revealed that EGFR mutation and/or EML4–ALK gene fusion rate was higher in the non-smoker Asian females with pulmonary adenocarcinoma. The aim of this study is to determine the distribution of known oncogenic driver mutations in the female non-smoker Asian patients with pulmonary adenocarcinoma. 104 consecutively resected lung adenocarcinomas from 396 non-smoker females (less than 100 cigarettes in a lifetime) at a single institution (Tongji University, Shanghai, China) were analyzed for mutations in EGFR, EML4−ALK, KRAS, HER2, BRAF, and PIK3CA. 73 (70.2 %) tumors harbored EGFR mutations; among these, 28 were deletions in exon 19, 44 were L858R missense changes, and eight were T790M mutations. 10 (9.6 %) harbored EML4−ALK fusions, two harbored KRAS mutations, two harbored BRAF mutations, and two harbored PI3K mutations. A majority of the mutations were mutually exclusive, except two with EGFR mutation and BRAF mutation, one with EML4−ALK fusions and PI3K mutation. Thus, 82.7 % (86 of 104; 95 % CI, 75.4–90.0 %) of lung adenocarcinomas from non-smoker females were found to harbor the well-known oncogenic mutations in five genes. Lung cancer in non-smoking Asian females is a distinct entity, with majority of this subgroup being developed by the oncogenic mutations. The prospective mutation examination in this population will be helpful for devising a targeted therapy for a majority of the patients.

Keywords Pulmonary adenocarcinoma · Mutation driver · Non-smoker · Female · Asian

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Introduction

Lung cancer is the leading cause of cancer-related deaths, with 1.38 million deaths occurring worldwide annually [1]. The major cause of lung cancer has been thought to be tobacco smoke. However, approximately 25 % of lung cancers worldwide occur in lifelong non-smokers (defined as those who smoked fewer than 100 cigarettes in their lifetime) [1]. Smoking, the leading cause of lung cancer, accounts for 80 % of the lung cancer burden in males and at least 50 % of the burden in females, worldwide [2]. In the US, approximately 10 % of patients with lung cancer are non-smokers [3]. In Asia, more than 30 % of patients with lung cancer are non-smokers, and nearly half or more lung cancer cases in women occur in non-smokers [3], suggesting that risk factors other than smoking may play an important role in the development of lung cancer. In addition, non-smoker East Asian female population was highly correlated with adenocarcinoma, which makes Lung cancer in non-smoking Asian females a distinct/unique entity. The association of female non-smoking Asian patients with pulmonary

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adenocarcinoma has first emerged in lung cancer thanks to the subgroup analysis in the Iressa Survival Evaluation in Lung Cancer (ISEL) trial [4]. Comparing with placebo, gefitinib failed to demonstrate a favorable outcome in the whole population; however, subgroup patients of non-smoking Asian females and adenocarcinoma showed a great benefit when they received the treatment of gefitinib. Subsequently, epidermal growth factor receptor (EGFR) kinase domain mutations (i.e., deletions in exon 19 and L858R point mutations in exon 21) were found to be enriched in patients with these clinical features and to be highly associated with increased sensitivity to EGFR tyrosine kinase inhibitors (TKIs) [5]. Furthermore, several Phase III studies [6–9] compared first line EGFR-TKIs such as gefitinib and erlotinib with doublet platinum-based chemotherapy in the advanced non-small cell lung cancer (NSCLC) patients harboring activating EGFR mutations and found that EGFR-TKIs had a significantly higher response rate and longer progression-free survival than traditional chemotherapy in this selected population. As a result, 2011 National Comprehensive Cancer Network (NCCN) guidelines [10] recommend EGFR-TKIs as the first line choice in the advanced NSCLC patients with activating EGFR mutations.

EML4−ALK fusion is another important finding in the development of lung cancer, which inhibits apoptosis and the promotion of cellular proliferation through activation of downstream PI3K/Akt and MAPK signaling pathways [11]. Similar to EGFR mutations, previous results from limited samples found that the frequency of EML4−ALK fusions was increased in people with adenocarcinomas, in young adult patients, and in people who have never smoked or who were light smokers [11]. More importantly, based on the excellent efficacy of crozitinib, an ALK inhibitor, in the advanced NSCLC patients with EML4−ALK fusion [12, 13], FDA (Food and Drug Administration) had approved its use in the clinical practice just recently.

In order to better understand the molecular characteristics of lung cancer in this separate entity, we performed a comprehensive analysis of known major driver mutations in 104 East Asian female patients from a single institution, with resected pulmonary adenocarcinomas and who were non-smokers. Driver mutations occur in genes that encode signaling proteins critical for cellular proliferation and survival. In lung adenocarcinomas, such mutations include EGFR, EML4−ALK, HER2, KRAS, BRAF, and PIK3CA.

Methods and Materials

Specimen Collection

Female NSCLC patients who underwent surgery with a histopathological diagnosis of adenocarcinoma were recruited for this study. Tumor tissues were collected within 0.5 h of resection and stored in paraffin-embedded archival until use. All cases were reviewed by pathologists for confirmation of tumor histology and tumor content. Patients were considered non-smokers in this study if they reported less than 100 cigarettes consumption in their lifetimes. The histology was based on the criteria of the World Health Organization and the TNM (Tumor, Node, and Metastasis) stage was determined according to version 7 of International Association for the Study of Lung Cancer (IASLC) staging system. This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University, Shanghai, China, and a written informed consent was obtained from each participant before the initiation of any study-related procedure.

Mutational Analyses

Genomic DNA or RNA was extracted from lung tumors or distant histologically normal lung as per standard protocols (RNeasy Mini Kit, and QiAamp DNA Mini Kit, Qiagen, Hilden, Germany). Total RNA samples were used for reverse transcription into single-stranded cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). Either genomic DNA or cDNA were used for polymerase chain reaction (PCR) amplification and sequencing. EGFR (exons 18–21), HER2 (exons 18–21), KRAS (exons 2–3), BRAF (exons 11–15), and the exons 9 and 20 of PIK3CAwere PCR amplified using genomic DNA. Cycle sequencing of the purified PCR products was carried out with PCR primers using the commercially available ADx Mutation Detection Kits (Amory, Xiamen, China). The Ct values used to determine if a sample is positive or negative are based on extensive validation.

Detection of EML4−ALK in Clinical Specimens

The EML4−ALK fusion mRNA was readily detected by PCR using ADx EML4−ALK Fusion Gene Diagnostic Kit. In brief, total RNA was extracted with Qiagen RNeasy FFPE Kit (Cat. No.73504), and the mRNA were transcribed to cDNA at 42 °C for 1 h. β-actin was used as the internal control. The PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 95 °C for 25 s, 64 °C for 20 s, 72 °C for 20 s to ensure the specificity; and 31 cycles of 93 °C for 25 s, 60 °C for 35 s, 72 °C for 20 s to perform the data collection and the sensitivity. The qualitative judgement is according to the mutation fluorescence signal.

Statistical Analysis

Statistical analysis was carried out with SPSS version13.0 (SPSS, Inc., Chicago, IL). χ^2 test, or Fisher's exact test was

used to analyze correlations between ALK status and clinical–pathologic variables and EGFR status. Results were considered significantly different if the p values were less than 0.05 in a two-way analysis.

Results

Assembly of Tumor Samples

From Jul 2008 to May 2010, a total of 135 resected female lung adenocarcinomas were consecutively collected at the Shanghai Pulmonary Hospital, Tongji University, in Shanghai, China. All the patients were of Chinese Han Nationality. Of these, 104 cases were included in this study based on the following inclusion criteria: re-review confirmed a pathologic diagnosis of lung adenocarcinoma; tumor specimen contained a minimum of 30 % tumor cells; enough tissue was available for comprehensive analysis; patient was a non-smoker; patient did not receive any previous neo-adjuvant treatment or anti-cancer drugs. Detailed clinical characteristics are listed in Table 1.

EGFR Mutation Status

EGFR kinase domain mutations were noticed in 70.2 % of the tumors (73 of 104; 95 % CI, 61.4–79.0 %) (Fig. 1). Among these, 28 were deletions in exon 19, 44 were L858R missense changes, and eight were T790M mutations (Table 2). The remaining alterations included L861Q and S768I mutation status. Twelve patients (12 of 104; 95 % CI, 5.4–17.6 %) had multiple mutations (details in Table 2). The EGFR mutation status had no significant association with clinical characteristics in terms of age, performance, stage, and differentiation.

Table 1 Clinical characteristics of non-smokers with female lung adenocarcinoma ($N = 104$)

Characteristics	Total
No. of patients	104
Age (years)	59.5 (27–78)
Performance status (ECOG) (%)	
0–1	89 (85.6 %)
2	15 (14.4 %)
Clinical stage (%)	
I	51 (49.0 %)
II	14 (13.5 %)
III	24 (23.1 %)
IV	15 (14.4 %)
Differentiation (%)	
Well	39 (37.5 %)
Moderate	34 (32.7 %)
Poor	31 (29.8 %)

EML4−ALK Genes Fusion Status

EML4−ALK gene fusions were observed in 9.6 % of tumors (10 of 104; 95 % CI, 3.95–15.28 %) (Fig. 1). Among them, nine patients have pure EML4−ALK fusion, and one patient with this gene fusion was accompanied by PI3K mutation. The median age of the patients having ALK translocations was 54 years, younger than 65.1 years old in the patients without ALK translocations. ALK translocations have no statistical relationship with the other clinical characteristics such as performance status, staging, and differentiation (See Table 3).

Spectrum of Mutations in HER2, KRAS, BRAF, and PI3K

KRAS G12V mutations were seen in 1.9 % (2 of 104; 95 %CI, 0.0032–0.1372) of the samples (Fig. 1). Similarly, 1.9 % (2 of 104; 95 % CI, 0.0032–0.1372) of the samples had BRAF V600E mutation (Fig. 1). 1.9 % (two of 104; 95 % CI, 0.0032–0.1372) of the samples had a PI3K R1047R mutation (Fig. 1). No mutations were found in HER2. Strikingly, only 17.3 % (eighteen of 104; 95 % CI, 3.75–21.04 %) of the samples did not harbor any mutations in EGFR, KRAS, BRAF, PI3K, HER2 or EML4−ALK (Fig. 1). Among the 82.7 % of tumors with known mutations, most were mutually exclusive, except two cases harboring both EGFR and BRAF mutation and 1 case harboring both PI3K mutation and EML4−ALK fusion (See Table 4).

Discussion

Over the last decade, a wealth of data from genomic [14], expression [15], mutational [16], and proteomic [17] profiling studies, as well as from various mouse lung tumor models, has led to the identification of additional molecular driver mutations in lung cancer. These driver mutations are suggested to have a role in oncogenesis or in the cancer phenotype from passenger mutations that accumulate through DNA replication, but are irrelevant for tumor development [18]. Major recurrent mutations in lung adenocarcinoma have been found to occur in EGFR, EML4−ALK, KRAS, HER2, BRAF, and PIK3CA genes. Currently, non-smoking-related adenocarcinoma of lung has been established as a distinct entity because of its peculiar epidemiologic, clinical, and biological characteristics; this study comprehensively analyzed the major

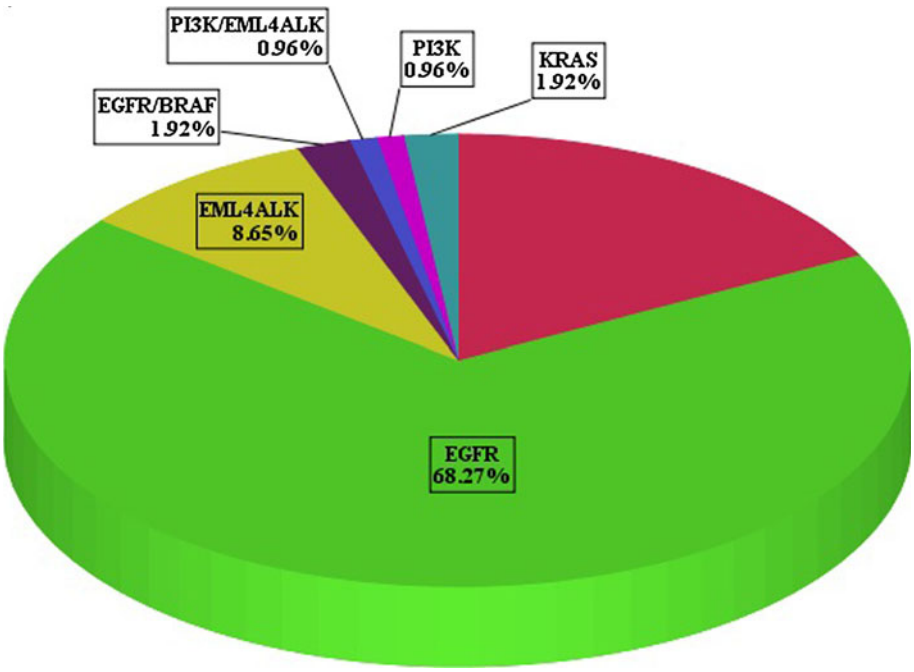

Fig. 1 Oncogenic driver mutations in East Asian non-smokers female with lung adenocarcinomas. In tumors from 104 patients, 70.2 % (73 of 104) harbored EGFR kinase domain mutations, 9.6 % (10 of 104) harbored EML4−ALK fusions, and 1.9 % (2 of 104) harbored KRAS, BRAF, and PI3K mutations, respectively. Only 17.3 % (18 of 104) of tumors did not harbor any of these known oncogenic driver mutations

Table 2 Association of EGFR kinase mutations and clinical characteristics in 104 female non-smoker Asian patients with pulmonary adenocarcinoma

Characteristics	EGFR mutation	
	Positive	Negative (no mutation detected)
No. of patients (%)	73 (70.2 %)	31 (29.8 %)
Age, years	60.0 (32–78)	59.3 (27–76)
Performance status (ECOG) (%)		
0–1	66 (63.5 %)	23 (22.1 %)
2	7 (6.7 %)	8 (7.7 %)
Clinical stage (%)		
I	35 (33.7 %)	16 (15.4 %)
II	10 (9.6 %)	4 (3.8 %)
III	18 (17.3 %)	6 (5.8 %)
IV	10 (9.6 %)	5 (4.8 %)
Differentiation (%)		
Well	28 (26.9 %)	11 (10.6 %)
Moderate	21 (20.2 %)	13 (12.5 %)
Poor	24 (23.1 %)	7 (6.7 %)
Mutation type (%)		
Exon 21 L858R[a]	44 (42.3 %)	N/A
Exon 19 deletion[a]	28 (26.9 %)	N/A
Exon 20 T790M[a]	8 (7.7 %)	N/A
Other[a,b]	8 (7.7 %)	N/A

[a] Twele patients had multiple mutations and are counted once for each type of mutation they had: two patients with exon 19 deletions and exon 21 L858R; five patients with exon 21, L858R, and exon 20 T790M mutations; one patient with exon 19 deletions and exon 20 T790M mutations; one patient with exon 21 L858R, exon 20 S768I, and exon 21 L861Q; two patients with exon 19 deletions, exon 20 S768I, and exon 21 L861Q; one patient with exon 20 S768I and exon 21 L861Q

[b] Other mutations were four patients with exon 20 S768I and four patients with exon 21 L861Q

known driver mutations in 104 female non-smoker Asian patients with pulmonary adenocarcinoma

To our knowledge, this study represents the first comprehensive and concurrent analysis of the known recurrent driver mutations in a large cohort of lung adenocarcinomas from non-smoking Asian females. The main finding of this study is that the majority of lung adenocarcinomas from East Asian Female non-smokers can be defined molecularly by targetable oncogenic mutant kinases. 82.7 % of the 104 samples examined in this study were found to harbor the well-known oncogenic alterations in EGFR, ALK, KRAS, BRAF, or PI3K. Conversely, only approximately 17.3 % of

Table 3 Association of EML4−ALK genes fusion status and clinical characteristics in 104 female non-smoker Asian patients with pulmonary adenocarcinoma

Characteristics	EML4−ALK genes fusion	
	Positive	Negative
No. of patients (%)[a]	10 (9.6 %)	94 (90.4 %)
Age, years	54.0 (47–69)	65.1 (27–78)
Performance status (ECOG) (%)[a]		
0–1	7 (6.7 %)	82 (78.8 %)
2	3 (2.9 %)	12 (11.5 %)
Clinical stage (%)[a]		
I	5 (4.8 %)	46 (44.2 %)
II	1 (<1 %)	13 (12.5 %)
III	3 (2.9 %)	21 (20.2 %)
IV	1 (<1 %)	14 (13.5 %)
Differentiation (%)[a]		
Well	3 (2.9 %)	36 (34.6 %)
Moderate	4 (3.8 %)	30 (28.8 %)
Poor	3 (2.9 %)	28 (26.9 %)

[a] Percentage calculated using the total number of patients as denominator

Table 4 The distribution of driver Mutations in 104 female non-smoker Asian patients with pulmonary adenocarcinoma

Items	Frequency	Percent (%)
EGFR	71	68.27
EML4ALK	9	8.70
EGFR/BRAF	2	1.90
PI3K/EML4ALK	1	1.00
PI3K	1	1.00
KRAS	2	1.90
Unknown	18	17.30
Total	104	100.00

tumors did not have an identifiable mutation, including HER2. Erlotinib/gefitinib is already available to target mutant EGFR [6–9]; a recent clinical trial showed that the BRAF inhibitor, PLX4032, induced partial responses in the melanoma tumors with BRAF mutation [19]; the ALK inhibitor, crizotinib (PF-02341066), has demonstrated remarkable efficacy against ALK fusion-positive lung cancers [18]. The dual PI3K/mTOR inhibitor presented promising results in the preliminary study [20] which is ongoing to test its effect in the patients with KRAS or PI3K mutation [21]. Thus, our molecular data in conjunction with the emerging clinical data indicates that prospective genotyping of lung adenocarcinomas from non-smokers for these genetic alterations could lead to rationally chosen targeted therapy in the overwhelming majority of cases.

T790M mutation, regarded as a secondary mutation in the EGFR gene, is thought to underlie up to 50 % of all cases of EGFR-TKI acquired resistance [22]. It remains unclear how tumor cells harboring the T790M mutation arise in patients receiving gefitinib/erlotinib. Clones containing the mutation could arise during treatment [23]; however, inconsistent with the previous studies, our study found 7.7 % of the patients harboring the EGFR T790M mutation at the diagnosis of lung cancer. Similar results were also found in the Biomarker Analyses of the IPASS study [24]. As we know, different methods have different sensitivities to detect the mutations of EGFR [25]. The ARMs method, used in our study together with the IPASS study [6], may account for the different results observed in the above studies.

Overall, our data confirm and extend a number of other published observations. For example, we found only two KRAS, two BRAF and two PI3K mutations; these are rare in non-smokers, especially from East Asia [26, 27]. We also found a high proportion of tumors to harbor EGFR mutations, the majority of which included deletions in exon 19 and the L858R point mutation. Among the EGFR wild-type tumors ($n = 31$), 10 (32.3 %) harbored EML4−ALK fusions. Consistent with this, it was recently [11, 26] reported that 28–33 % of adenocarcinomas that were negative for EGFR mutations were positive for ALK fusions. As in the previous reports [27, 28], the EGFR, KRAS, and ALK mutations were mutually excluded in our study.

In conclusion, the majority of lung adenocarcinomas from East Asian Female non-smokers can be defined molecularly by targetable oncogenic mutant kinases. These observations have led to changes in the overall treatment strategies for lung cancer. Therefore, a genetic testing before the treatment is considered essential for lung cancer in non-smokers to select the appropriate treatment option according to the patient's molecular characteristics. First, EGFR mutations should be tested, since nearly 70 % of tumors in this subgroup harbor such alterations. If a tumor is positive for an EGFR mutation, then no further molecular testing is required. Treatment recommendations would be EGFR-TKIs such as gefitinib or erlotinib or irreversible TKIs such as afatinib in case of T790M mutation. If the tumor bears wild-type EGFR, then it would be tested for ALK translocations, which are found in approximately 30 % of remaining tumors. If positive, treatment would then include an ALK inhibitor. If negative, then these rarer mutations (e.g., in BRAF, KRAS, HER2, PIK3CA, and so on) should be considered for analysis and/or intensive research to identify new driver mutations.

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Conflict of interest None.

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