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ORIGINAL REPORT

Lung Adenocarcinoma From East Asian Never-Smokers Is a Disease Largely Defined by Targetable Oncogenic Mutant Kinases

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose
To determine the proportion of lung adenocarcinomas from East Asian never-smokers who harbor known oncogenic driver mutations.

Patients and Methods
In this surgical series, 52 resected lung adenocarcinomas from never-smokers (< 100 cigarettes in a lifetime) at a single institution (Fudan University, Shanghai, China) were analyzed concurrently for mutations in *EGFR*, *KRAS*, *NRAS*, *HRAS*, *HER2*, *BRAF*, *ALK*, *PIK3CA*, *TP53* and *LKB1*.

Results
Forty-one tumors harbored *EGFR* mutations, three harbored *EML4-ALK* fusions, two harbored *HER2* insertions, and one harbored a *KRAS* mutation. All mutations were mutually exclusive. Thus, 90% (47 of 52; 95% CI, 0.7896 to 0.9625) of lung adenocarcinomas from never-smokers were found to harbor well-known oncogenic mutations in just four genes. No *BRAF, NRAS, HRAS,* or *LKB1* mutations were detected, while 15 had *TP53* mutations. Four tumors contained *PIK3CA* mutations, always together with *EGFR* mutations.

Conclusion
To our knowledge, this study represents the first comprehensive and concurrent analysis of major recurrent oncogenic mutations found in a large cohort of lung adenocarcinomas from East Asian never-smokers. Since drugs are now available that target mutant *EGFR*, *HER2*, and *ALK*, respectively, this result indicates that prospective mutation testing in these patients should successfully assign a targeted therapy in the majority of cases.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide.[1] Most cases are associated with a personal history of direct tobacco smoke exposure. However, approximately 10% to 15% of all lung cancers arise in never-smokers, defined as those who smoked fewer than 100 cigarettes in their lifetime.[2] If considered as a separate category, lung cancer in never-smokers would rank among the most common causes of cancer mortality.[3]

The importance of never-smokers has emerged in lung cancer because of recent clinical observations. During the development of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, retrospective studies showed that never-smoking status, along with adenocarcinoma histology and East Asian ethnicity, were associated with a higher likelihood of responding to treatment.[4,5] Subsequently, *EGFR* kinase domain mutations (ie, 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 TKIs.[6-8] Furthermore, lung cancers in patients with minimal and/or a remote history of direct tobacco exposure were found to share some molecular features with their never-smoking counterparts. For example, the likelihood of *EGFR* mutations decreases as the number of pack-years increases.[9] Collectively, all of these observations led to a randomized phase III trial open-label study (Iressa Pan Asian Study [IPASS]), in which previously untreated patients in East Asia who had advanced pulmonary adenocarcinomas and who were never-smokers or former light-smokers (those who had stopped smoking at least 15 years previously and had a total of ≤ 10 pack-years of smoking) were randomly assigned to receive gefitinib or conventional

chemotherapy with carboplatin plus paclitaxel. Patients with *EGFR*-mutant tumors experienced longer progression-free survival with gefitinib, and patients without mutations had longer progression-free survival with chemotherapy.[10] Similar results have been observed with erlotinib in white never-smokers with lung adenocarcinoma.[11]

In IPASS, the objective radiographic response rate in all patients treated with gefitinib was 43%. In contrast, for patients with known *EGFR* mutations, the response rate was 71.2%. A similar high response rate (62.1%) to gefitinib was reported in another recent study, the WJTOG3405 trial, in which only patients with known *EGFR* tumor mutations were randomly assigned to EGFR TKI or chemotherapy.[12] To explore the discrepancy between these results and to better characterize lung adenocarcinomas in never-smokers, we performed a comprehensive analysis of major known driver mutations in 52 East Asian patients from a single institution with resected pulmonary adenocarcinomas and who were never-smokers. Driver mutations occur in genes that encode signaling proteins critical for cellular proliferation and survival. In lung adenocarcinomas, such mutations include *EGFR, HER2, KRAS, BRAF, PIK3CA,* and *EML4-ALK*. We also examined tumors for mutations in other genes (ie, the oncogenes, *HRAS* and *NRAS* and the tumor suppressor genes *TP53* and *LKB1*). Our results have clear therapeutic implications for never-smokers with lung cancer.

PATIENTS AND METHODS

Specimen Collection

This study was approved by the institutional review board of the Shanghai Cancer Hospital, Fudan University, Shanghai, China. All participants underwent surgery and provided written informed consent. Samples were snap-frozen in liquid nitrogen at the time of resection and stored at −80°C until use. All cases were rereviewed by pathologists for confirmation of tumor histology and tumor content. Patients were considered never-smokers in this study if they reported never smoking any cigarettes in their lifetimes.

Mutational Analyses

All mutational analyses were performed in China. 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 reverse transcribed 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 18-22), *HER2* (exons 18 to 21), *KRAS* (exons 2 to 3), and *BRAF* (exons 11 to 15) were PCR amplified using cDNA for further sequencing. All exons of *TP53* and *LKB1,* and exons 9 and 20 of *PIK3CA,* were sequenced using genomic DNA.

For detection of *EML4-ALK* fusions, primers were designed to amplify all known fusion variants using cDNA. The forward primers include *EML4* E2F (5′-TGATGTTTTGAGGCGTCTTG−3′), *EML4* E13F (5′-AGATCGCCTGTCAGCTCTTG−3′), *EML4* E18F (5′-TTAGCATTCTTGGGGAATGG−3′), and the reverse primer *ALK* E20R (5′-TGCCAGCAAAGCAGTAGTTG−3′). Multiplex PCR analysis was done with KOD plus DNA polymerase (Toyobo, Osaka, Japan), with the following program: 94°C 5 minutes; 94°C 30 seconds, 60°C 30 seconds, 68°C 1 minute, 35 cycles; 68°C 10 minutes. PCR products were directly sequenced in forward and reverse directions. All mutations were verified by analysis of an independent PCR isolate.

Statistical Analysis

χ^2 and Fisher's exact tests were used to analyze the association of mutations with clinical characteristics, using SPSS 16.0 (SPSS Institute, Chicago, IL). All *P* values were based on a two-sided hypothesis.

RESULTS

Assembly of Tumor Samples

From January 2008 to June 2009, we collected consecutively a total of 168 resected lung adenocarcinomas at the Shanghai Cancer Hospital, Fudan University, in Shanghai, China. All of the patients (100%) were Chinese. Of these, 52 cases were included in this study based on the following criteria: re-review confirmed a pathologic diagnosis of lung adenocarcinoma; tumor specimen contained a minimum of 70% tumor cells; enough tissue was available for comprehensive analysis; patient was a never-smoker; patient did not receive any neoadjuvant treatment; and corresponding normal tissue was also available for analysis. Eleven cases were from males, and 41 from females. Detailed clinical characteristics are listed in Table 1.

EGFR *Mutation Status*

Seventy-eight point eight percent of tumors (41 of 52; 95% CI, 0.6580 to 0.8792) were found to harbor *EGFR* kinase domain mutations (Fig 1). Among these, 21 were deletions in exon 19 and 18 were L858R missense changes (Table 2). The remaining alterations included an exon 20 insertion and a double mutation involving exon 18 (G719S) and exon 21 (L861Q). We did not detect any T790M mutations, which are found more commonly in patients with acquired resistance to EGFR TKIs[13] and rarely in never-smoking patients with lung cancer as a rare germline variant.[14] Eighty-two point nine percent of tumors (34 of 41) from female never-smokers harbored an *EGFR* kinase domain mutation, while 63.6% of tumors (seven of 11) from male never-smokers did (Table 2).

Spectrum of Mutations in HER2, KRAS, BRAF, *and* EML4-ALK

Five point eight percent (three of 52; 95% CI, 0.0138 to 0.1625) of samples were found to harbor *EML4-ALK* fusions (Fig 1). The three variants included E13;A20, E20;A20, and E6a/b;A20.[15] Three point eight percent (two of 52; 95% CI, 0.0032 to 0.1372) of samples had *HER2* kinase domain mutations (Fig 1). One sample (1.9%; 95% CI, < 0.0001 to 0.1107) had a *KRAS* G12V mutation (Fig 1). No mutations were found in *BRAF* (95% CI, 0.0000 to 0.0822).

Table 1. Clinical Characteristics of Never Smokers With Lung Adenocarcinoma (N = 52)

Characteristic	Total	Sex		*P*
		Male	Female	
No. of patients	52	11	41	
Age, years	59.04	60.73	58.59	.491
SD	9.04	8.09	9.32	
Clinical stage				
I	24	2	20	.205
II	6	3	5	
III	20	6	14	
IV	2	0	2	
Differentiation				
Well	13	2	11	.113
Moderate	28	4	24	
Poor	11	5	6	

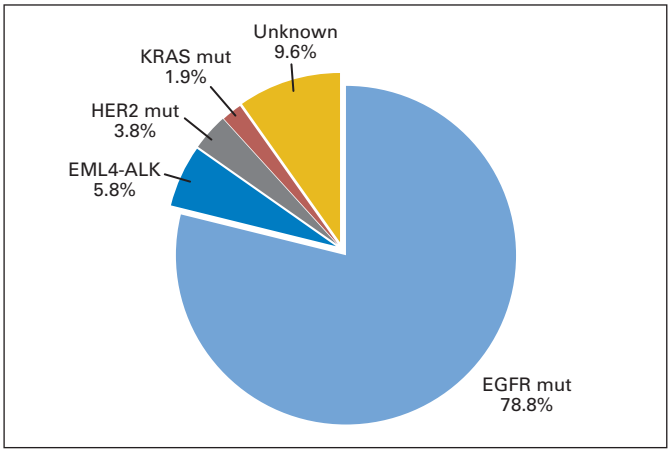

Fig 1. Oncogenic driver mutations in East Asian never-smokers with lung adenocarcinomas. In tumors from 52 patients, 78.8% (41 of 52) harbored *EGFR* kinase domain mutations, 5.8% (three of 52) harbored *EML4-ALK* fusions, 3.8% (two of 52) harbored *HER2* mutations, and 1.9% (one of 52) harbored *KRAS* mutations. Only 9.6% (five of 52) of tumors did not harbor any of these known oncogenic driver mutations.

Strikingly, only 9.6% (five of 52; 95% CI, 0.0375 to 0.2104) of samples did not harbor any mutations in *EGFR, HER2, KRAS, BRAF,* or *EML4-ALK* (Fig 1). All five of these cases were from females. Among the 11 male never-smokers, seven had an *EGFR* mutation, one had a *KRAS* mutation, one had a *HER2* insertion, and two had an *EML4-ALK* fusion. Among the 90.4% of tumors with known mutations, tumors with an alteration in one gene did not harbor a mutation in any of the other genes.

Other Mutations

Four samples (7.7%; 95% CI, 0.0253 to 0.1868) harbored *PIK3CA* mutations, two occurring in exon 9, and two in exon 20 (Table 3). All the *PIK3CA* mutations coexisted with *EGFR* mutations (Table 3). Three were in females. We also searched for mutations in the *RAS*-related genes, *HRAS* and *NRAS,* and in the tumor suppressor genes, *LKB1* and *TP53*. No mutations were found in *HRAS*, *NRAS,* or *LKB1*. Fifteen samples had a *TP53* mutation.

Table 2. Association of *EGFR* Kinase Mutations and Clinical Characteristics in Lung Adenocarcinomas From Never-Smokers

Characteristic	*EGFR* Mutation		
	Total	Male	Female
Patient No.	41	7	34
Age, years	59.10	60.14	58.88
SD	9.58	10.19	9.58
Clinical stage			
I	22	2	20
II	2	1	1
III	15	4	11
IV	2	0	2
Differentiation			
Well	11	2	9
Moderate	23	2	21
Poor	7	3	4
Mutation type			
L858R	18	4	14
Exon 19 deletion	21	3	18
Other *EGFR* mutation	2	0	2

Table 3. Association of *PIK3CA* Mutations With *EGFR* Kinase Domain Mutations in Lung Adenocarcinomas From Never-Smokers

No.	Sex	Age (years)	Stage	*PIK3CA* Mutation	*EGFR* Mutation
168	Female	43	IIIA	E545K	E746-A750del
202	Female	51	IA	H1047R	L747-S752del P753S
338	Male	56	IIIA	H1047R	L858R
411	Female	57	IA	E545K	L747-S752del P753S

DISCUSSION

During the past decade, a wealth of data from genomic,[16] expression,[17] mutational,[18] and proteomic profiling studies[19] have led to the identification of multiple molecularly distinct subsets of lung cancer. Based on such discoveries, one of the most promising treatment strategies now involves the subdivision of non–small-cell lung cancer histologies into clinically relevant molecular subsets, using a classification schema based on specific driver mutations. Major recurrent mutations in lung adenocarcinoma have been found to occur in *EGFR*, *KRAS*, *HER2*, *BRAF*, *ALK*, and *PIK3CA*.

To our knowledge, this study represents the first comprehensive and concurrent analysis of these known recurrent driver mutations in a large cohort of lung adenocarcinomas from never-smokers. Previous studies have examined lung adenocarcinomas from never-smokers for multiple mutations, but did not include *ALK* fusions, which had just been reported.[18,20] Other studies on *ALK* fusions have examined tumors for *EGFR* and *KRAS* mutations, but not other driver mutations.[21,22] Many studies have examined lung adenocarcinomas from never-smokers and smokers, but this study focused only on lung cancers from patients who never smoked cigarettes.

The main finding of this study is that the majority of lung adenocarcinomas from East Asian never-smokers can be defined molecularly by targetable oncogenic mutant kinases. Eighty-eight percent of the 52 samples examined in this study were found to harbor well-known oncogenic alterations in *EGFR*, *HER2,* or *ALK*. Conversely, only approximately 10% of tumors did not have an identifiable mutation, including in *BRAF, HRAS,* or *NRAS.* Erlotinib/gefitinib are already available to target mutant EGFR; a recent clinical trial showed that the EGFR/HER2 inhibitor, BIBW2992, induced partial responses in three of three patients with *HER2* mutant lung tumors;[23] and the ALK inhibitor, crizotinib (PF-02341066), has demonstrated remarkable efficacy against ALK fusion–positive lung cancers.[24] Thus, our molecular data in conjunction with the emerging clinical data indicates that prospective genotyping of lung adenocarcinomas from never-smokers for these genetic alterations could lead to rationally chosen targeted therapy in the overwhelming majority of cases.

PIK3CA mutations have been reported to be less common in lung adenocarcinomas than squamous cell carcinomas.[25-27] Interestingly, we found *PIK3CA* mutations in 7.7% (four of 52) of lung adenocarcinomas from never-smokers, which is higher than previous reports.[25-27] *PIK3CA* alterations were also more commonly found in

younger patients. Since the total number of cases was low, in future studies, we plan to clarify in additional samples if these observations remain constant.

Otherwise, our data confirm and extend a number of other published observations. For example, we found only 1 *KRAS* and no *LKB1* mutations; these are rare in never-smokers, especially from East Asia.[28,29] The *TP53* mutation rate in this group was 28.8%, similar to previous studies.[30] 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 *EGFR* wild-type tumors (n = 11), three (28%) harbored *EML4-ALK* fusions. Consistent with this, others recently reported that 33% of adenocarcinomas negative for *EGFR* mutations were positive for *ALK* fusions.[21]

Our results can help explain the radiographic response rates observed with gefitinib in the recent IPASS trial. In all never/light smokers in that study, the collective response rate to the EGFR TKI was only 43%. In our study of only never-smokers, 41 of 52 patients had *EGFR* kinase domain mutations. However, among the 41 mutated cases, one harbored an exon 20 insertion mutation, which has been associated with drug resistance.[31] Another four tumors had concurrent *PIK3CA* mutations, which have been associated with resistance to EGFR TKIs at least in vitro.[32] Thus, only 36 (69%) of the 52 tumors would be predicted to be sensitive to TKI treatment. Since *EGFR* mutant tumors in general display a 70% response rate to EGFR TKIs, 70% of 69% would result in a 48% true response rate, which is very close to the response rate seen in IPASS.

We are now actively trying to identify the driver mutations in the five tumors with no detectable gene alterations. It is possible that they harbor *ALK* fusions that do not involve *EML4*; for example, recently, lung tumors have been found to harbor *ALK* alterations including *KIF5B-ALK*.[33] However, we thus far have no evidence of other fusion events involving *ALK* in these tumors. Using Affymetrix HuEX-1.0 exon array analysis (Affymetrix, Santa Clara, CA), these samples did not display disparate levels of expression between exons at the 5′ and 3′ ends of *ALK* (data not shown), which is suggestive of fusions events.[34] By contrast, the three samples with *EML4-ALK* fusions did. Other candidate drivers include *ROS* fusion or *PDGFRα* amplification;[19] however, our preliminary analysis of both exon array and single nucleotide polymorphism array data have not found evidence for these types of events. More comprehensive genomic analysis and deep sequencing may need to be performed on these pan–wild-type samples, so that the disease is defined completely at the molecular level.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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