

***EGFR, ALK, RET, KRAS and BRAF* alterations in never-smokers with non-small cell lung cancer**

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Abstract. Non-small cell lung cancer (NSCLC), caused by various mutations in a spectrum of cancer driver genes, may have distinct pathological characteristics and drug responses. Extensive genetic screening and pathological characterization is required for the design of customized therapies to improve patient outcomes. Notably, NSCLC in never-smokers exhibits distinctive clinicopathological features, which are frequently associated with tumorigenic mutations, and thus may be treated as a unique disease entity. However, to the best of our knowledge, these mutations have not been extensively and accurately characterized in an NSCLC study with a large sample size. Therefore, the present study enrolled a large cohort of NSCLC patients, which consisted of 358 never-smokers, for the screening of genetic alterations in the epidermal growth factor receptor (*EGFR*), ret proto-oncogene (*RET*), anaplastic lymphoma kinase (*ALK*), Kirsten rat sarcoma viral oncogene

homolog (*KRAS*) and B-Raf proto-oncogene serine/threonine kinase (*BRAF*) tumorigenic genes. It was identified that the mutation rate was 47.8, 7.5, 3.6, 1.4 and 0.3% for *EGFR*, *ALK*, *KRAS*, *RET* and *BRAF*, respectively. In addition, clinicopathological features associated with these mutations were characterized. *EGFR* mutations were more frequently observed in female and older patients. By contrast, *KRAS* mutations were more frequently detected in male patients, and *ALK* and *RET* translocations in younger patients. The cancer cells were frequently well-differentiated in carcinoma cases exhibiting *EGFR* mutations, however, were less differentiated in those with *ALK* translocations. In conclusion, the present study determined the frequency of oncogenic alterations and associated clinicopathological features in NSCLC exhibited by never-smokers using a large sample size. The results of the present study may enrich our knowledge of NSCLC in never-smokers and provide useful insights for improvement of the outcome of molecularly targeted therapies for the treatment of NSCLC.

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Abbreviations: *EGFR*, epidermal growth factor receptor; *ALK*, anaplastic lymphoma kinase; *RET*, ret proto-oncogene; *EML4*, echinoderm microtubule-associated protein-like 4; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor; RT-PCR, reverse transcription-polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded

Key words: lung cancer, *EGFR*, *ALK*, *RET*, *KRAS*, *BRAF*, never-smokers

Introduction

Lung cancer is the number one cause of cancer-associated mortality (1). The high mortality rates associated with lung cancer are largely due to the poor outcomes of conventional treatments, including the use of surgical removal combined with adjuvant radiation and chemotherapy (2). Significant improvements have been achieved due to increased efforts to determine the molecular mechanisms underlying tumorigenesis, which has led to the identification of multiple oncogenic alterations, including those observed in epidermal growth factor receptor (*EGFR*) (3), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (4), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) (5), anaplastic lymphoma kinase (*ALK*) (6), ROS proto-oncogene 1 (7) and ret proto-oncogene (*RET*) (7-9). It was previously demonstrated that patients carrying *EGFR* mutations exhibited a significant response to the *EGFR* tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib as a first-line therapy (3,10). By contrast, patients carrying *ALK* fusions exhibited a poor response to these drugs (11), but

responded well to the ALK TKI crizotinib (12). Thus, targeted treatment based on the results of molecular and pathological diagnosis has become a new standard for the treatment of lung cancer (13).

Although the majority of lung cancer cases are associated with an extensive history of cigarette smoking, the prevalence of lung cancer death in non-smokers remains high (14). In the United States, 10-15% of lung cancer cases are diagnosed in patients who are considered never-smokers (15). If listed as a separate category, lung cancer in never-smokers would rank among the top 10 most commonly observed fatal cancer cases in the United States (14,16). This ranking in never-smokers is likely to rise due to increased public awareness of the life-threatening hazards caused by cigarette smoking, resulting in a drop in the population of smokers and thus an increase in the population of never-smokers (17).

A previous clinical study demonstrated that targeted therapy in never-smoker lung cancer patients typically produces an improved response compared with that in smokers (18). It has been suggested that the molecular profiles of lung cancer cases are likely to vary between heavy smokers and never-smokers. Accumulating evidence based on molecular and clinicopathological studies has suggested that non-small cell lung cancer (NSCLC) in never-smokers should be considered as a distinct entity (19). Thus, it is critical to determine the mutation state of NSCLC in never-smokers as a unique type of cancer, for the purpose of cancer research and clinical translation. With this aim in mind, the present study performed a large-scale screen for tumorigenic alterations in the oncogenes *EGFR*, *KRAS*, *BRAF*, *ALK* and *RET* in 358 Chinese NSCLC adenocarcinoma patients who were exclusively never-smokers. The clinicopathological characteristics associated with these genetic alterations were additionally determined. The present study may yield a clear picture concerning the molecular profile of NSCLC in never-smokers, thus providing valuable information for cancer research and the improvement of targeted therapies for the treatment of NSCLC.

Materials and methods

Specimen collection. The present study was approved by the Institutional Review Boards of Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China), and Chongqing Cancer Institute (Chongqing, China). All participants underwent lung resection and needle aspiration, and provided written informed consent. Samples were snap-frozen with liquid nitrogen at the time of resection and stored at -80°C until required. All cases were independently reviewed by two pathologists during disease diagnosis. Patients were considered never-smokers if they had never smoked or had smoked <100 cigarettes in their lifetime (15).

Detection of mutations in *EGFR*, *KRAS* and *BRAF*. Genomic DNA was extracted with the QIAamp DNA formalin-fixed paraffin-embedded (FFPE) kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocols. *EGFR*, *KRAS* and *BRAF* mutations were detected by amplification refractory mutation system in multiple quantitative polymerase chain reaction (ARMS-multi-qPCR) analysis with the Human *EGFR* Mutation Detection kit (YuanQi Bio-Pharmaceutical

Co., Ltd., Shanghai, China) and the Human *KRAS* and *BRAF* Mutation Detection kit (YuanQi Bio-Pharmaceutical Co., Ltd.), respectively. The PCR conditions used were as follows: 42°C for 5 min, 94°C for 3 min, followed by 40 cycles at 94°C for 15 sec and 60°C for 1 min on the 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following primers were used: *EGFR-exon (E)18* forward, 5'-CAAGTGCCGTGTCCTGG-3' and reverse, 5'-CCTTACCTTATACACCGTGCC-3'; *EGFR-E19* forward, 5'-CGGTGCATCGCTGGTAACAT-3' and reverse, 5'-ATG GACCCACACAGC-3'; *EGFR-E20* forward, 5'-CTGGCC ACCATGCGAAG-3' and reverse, 5'-TCCTGGCTCCTTATC TCCC-3'; *EGFR-E21* forward, 5'-GCTTCTTCCCATGAT GATCTG-3' and reverse, 5'-CTGGTCCCTGGTGTGACG-3'; *KRAS* forward, 5'-TTTGTATTAAGAGTACTGGTGG-3' and reverse, 5'-CCTCTATTGTTGGATCATATTCG-3'; and *BRAF* forward, 5'-ACTCTTCATAATGCTTGCTCTG-3' and reverse, 5'-TGAATACTGGGAATATGAAAATAC-3'. All PCR products were subjected to direct sequencing to verify mutations in *EGFR*, *KRAS* and *BRAF*. The following probes were used: for *EGFR-E18*, 5'-GGTGACCCTTGTCTCTGT GTTC-3'; *EGFR-E19*, 5'-ATCACTGGGCAGCATGTG-3'; *EGFR-E20*, 5'-CCCTGATTACCTTTGCGAT-3'; *EGFR-E21*, 5'-TGATCTGTCCCTCACAGCAG-3'; *KRAS*, 5'-TGTATT AAAAGGTACTGGTGGAG-3'; and *BRAF* 5'-TGAGAC CTTCAATGACTTTCTAG-3'. All primers and probes were purchased from Sangon Biotech Co., Ltd., Shanghai, China.

Detection of *ALK* and *RET* fusion variants. Multiplex one-step reverse transcription (RT)-PCR was performed to detect *ALK* fusion gene variants. The Human Lung Cancer Related Fusion Gene Detection kit (YuanQi Bio-Pharmaceutical Co., Ltd.) was used according to the manufacturer's protocols. In brief, the mixture for each reaction contained 3 µl total RNA extracted from the tumor specimen, 12 µl Multiplex RT-PCR buffer, 2.5 µl Multiplex Enzyme mix and 300 nmol/l primers in a total volume of 25 µl. The PCR conditions used were as follows: 42°C for 30 min, 94°C for 5 min, followed by 40 cycles at 94°C for 15 sec and 60°C for 1 min on the 7500 Real Time PCR System. A total of two experiments were performed separately to detect echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK* fusion or alternative *ALK* fusions [transforming growth factor (*TGF*)-*ALK*, kinesin light chain 1 (*KLC1*)-*ALK* and kinesin family member 5B (*KIF5B*)-*ALK*]. The following forward primers were used for detecting *EML4-ALK* variants: *EML4-E2* (V5a and 5b) forward, 5'-GTGGCCTCAGTG AAAAAATC-3'; *EML4-E6* (V3a and 3b) forward, 5'-TAA AGATGTCATCATCAACCAAG-3'; *EML4-E13* (V1 and 6) forward, 5'-CCTGGGAAAGGACCTAAAG-3'; *EML4-E14* (V4b and 7) forward, 5'-GGGAAAGGACCTAAAGGTG-3'; *EML4-E15* (V4a) forward, 5'-TGATGGCTTCCAAATAGA AGTAC-3'; *EML4-E17* (V9) forward, 5'-ACGGGAATGAAC AGCTCTCT-3'; and *EML4-E20* (V2) forward, 5'-CGGGAG ACTATGAAATATTGTACT-3'. The primers for alternative *ALK* fusion variants included: *TGF-E3* forward, 5'-GAGAAC CAGGACCTTCCACC-3'; *KLC1-E9* forward, 5'-ATTCTC ACTCGTGCACATGAAA-3'; *KIF5B-E15* forward, 5'-AAA AGACCTTGCAGAAATAGGAA-3'; *KIF5B-E17* forward, 5'-TCTGTGCGATGCCCTCAGTG-3'; and *KIF5B-E24* forward, 5'-TCAGGTCAAAGAATATGGCCA-3'. The common reverse

primer for all *ALK* fusion variants was 5'-GCTTGACTCAG GGCTCTGC-3'. Multiplex One-step RT-PCR was additionally used to detect *RET* fusion variants, including *KIF5B-RET* and coiled-coil domain containing 6 (*CCDC6*)-*RET*. *RET* Fusion Gene Detection kit (YuanQi Bio-Pharmaceutical Co., Ltd.) was used according to the manufacturer's protocols. All PCR products were subjected to DNA sequencing with the probe 5'-AGCTCCTGGTGCTTCCGGCG-3' for all *ALK* fusion products. The expression of *ALK* tyrosine kinase was examined by immunohistochemistry using the *ALK* (D5F3) CDx Assay kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) containing the rabbit monoclonal antibody against *ALK* (clone D5F3; catalog no., 790-4796; dilution, 1:250), which detected endogenous levels of total *ALK* protein, as well as *ALK* fusion proteins. The experiments were performed on FFPE sections, as described previously (20).

Statistical analysis. P-values were determined by Fisher's exact test or χ^2 test using Prism 6 analysis software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ and $P < 0.01$ were considered to indicate a statistically significant and highly significant difference, respectively.

Results

Clinical characteristics of enrolled patients. Between January 2012 and June 2013, resected lung adenocarcinoma samples were collected from a total of 358 patients who had been diagnosed with NSCLC at Shanghai Chest Hospital or Chongqing Cancer Institute. The cohort consisted of 274 female patients (76.5%) and 84 male patients (23.5%) (Table I). The median age of the patient cohort was 57.1 years. While ~36.3% of patients were 50-59 years old, and 41.6% were ≥ 60 years, only 22.1% of patients were < 50 years old (Table I). All specimens were selected based on the following criteria: i) Re-review confirmed a pathological diagnosis of lung adenocarcinoma; ii) the tumor specimen contained a minimum of 70% tumor cells; iii) sufficient tissue was available for comprehensive analysis; iv) the patient was a never-smoker; and v) the patient did not receive any neoadjuvant treatment. Based on the differentiation level of cancer cells, the specimens could be divided into three groups, which comprised poorly-, moderately- or well-differentiated carcinoma. The number of specimens was similar among these groups, ranging from 32.1 to 35.8% (Table I).

Mutations were detected in *EGFR*, *KRAS*, *BRAF*, *EML4-ALK* and *KIF5B-RET*. Out of a total of 358 NSCLC patents, genetic alterations were detected in 217 carcinoma specimens. Among these positive cases, there were 171 patients carrying *EGFR* mutations, accounting for 47.8% of all patients (Fig. 1). A total of 27 patients were detected as exhibiting *EML4-ALK* fusion genes, 13 with *KRAS* mutations, 5 with *KIF5B-RET* fusion variants and 1 with *BRAF* mutations, accounting for 7.5, 3.6, 1.4 and 0.3%, respectively (Fig. 1). No *KIF5B-ALK* or *TFG-ALK* fusion variants were detected in the samples. These results were confirmed by immunohistochemistry. Representative images revealed that *ALK* was absent in *EML4-ALK*-negative carcinoma cases (Fig. 2A), but was highly expressed in *EML4-ALK*-positive samples (Fig. 2B). None of the specimens

Table I. Clinical characteristics of 358 never-smokers with non-small cell lung cancer.

Variable	Patients, n (%)
Gender	
Male	84 (23.5)
Female	274 (76.5)
Age, years	
<40	5 (1.4)
40-49	74 (20.7)
50-59	130 (36.3)
≥ 60	149 (41.6)
Differentiation	
Poorly	115 (32.1)
Moderately	128 (35.8)
Well	115 (32.1)

carried mutations in > 1 gene. However, there was one patient (> 60 years old) who carried two *EGFR* mutations (E19 and E21) in their tumor specimen. These results supported the observation that mutations in the investigated tumorigenic genes are typically mutually exclusive (21).

EGFR mutations were most prevalent in E19, with 102 cases, accounting for 28.5% of all patients and 59.6% of patients exhibiting *EGFR* mutations (Table II). *EGFR* mutations were additionally identified frequently in E21, with 63 cases, accounting for 17.6% of all patients and 36.8% of patients with *EGFR* mutations (Table II). Markedly fewer cases were detected with mutations in E18 (3 cases; 0.8% of all patients) or E20 (4 cases; 1.1% of all patients), accounting for $< 5\%$ of combined patients exhibiting *EGFR* mutations (Table II). Detailed data is listed in Table II according to gender, age, differentiation and histology.

Gender may affect the occurrence of oncogenic mutations. Subgroup analysis was performed in order to uncover clinical features that were associated with the identified genetic mutations in the present study (Table III). In order to determine whether gender affected the frequency of investigated tumorigenic mutations in the present patient cohort, subtype analysis was performed according to patient gender, as shown in Table III. The percentage of patients who possessed *EGFR* mutations was 35.7% (30/84) of male patients and 51.5% (141/274) of female patients. The difference in the number of patients with or without *EGFR* mutations between male (30 vs. 54) and female (141 vs. 133) patients was markedly significant ($P < 0.01$), suggesting that gender is an important factor that may affect *EGFR* mutations in NSCLC in never-smokers. Thus, *EGFR* mutations were more likely to be detected in female patients compared with male patients in the subset of NSCLC exhibited by never-smokers. Using an identical analysis method, a significant difference was observed ($P < 0.05$) in the prevalence of *KRAS* mutations between male and female patients. The frequency of *KRAS* mutations was 7.1% in male patients and 2.6% in female patients. No significant difference was observed in the prevalence of mutations

Table II. Characterization of EGFR mutations in 358 Chinese never-smokers exhibiting non-small cell lung cancer.

Variable	All EGFR		E18		E19		E20		E21	
	n	%	n	%	n	%	n	%	n	%
Gender										
Male	30	35.7	0	0.0	18	21.4	0	0.0	12	14.3
Female	141	51.5	3	1.1	84 ^a	30.7	4	1.5	51 ^a	18.6
Age, years										
<40	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
40-49	32	43.2	1	1.4	19	25.7	1	1.4	11	14.9
50-59	60	46.2	1	0.8	38	29.2	2	1.5	19	14.6
≥60	79	53.0	1	0.7	45 ^a	30.2	1	0.7	33 ^a	22.1
Diff										
Poorly	56	48.7	2	1.7	36	31.3	1	0.9	17	14.8
Mod	51	39.8	1	0.8	28 ^a	21.9	2	1.6	21 ^a	16.4
Well	64	55.7	0	0.0	38	33.0	1	0.9	25	21.7
Total	171	47.8	3	0.8	102 ^a	28.5	4	1.1	63 ^a	17.6

^aOne patient with double mutations (E19/E21). Diff, differentiation; Mod, moderately; EGFR, epidermal growth factor receptor; E, exon.

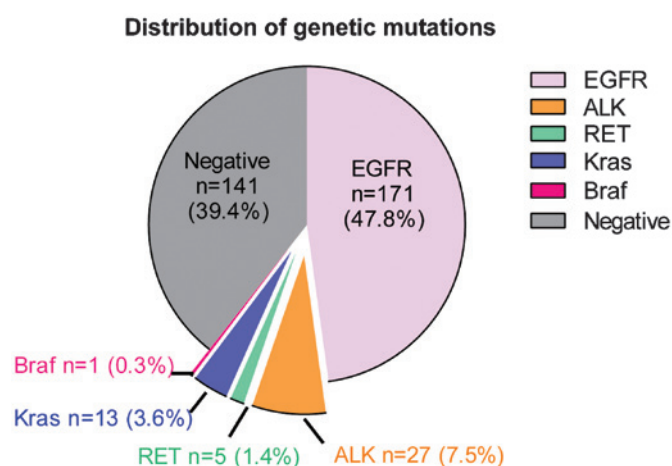


Figure 1. Pie graph showing the frequency of genetic alterations in never-smokers with NSCLC. The frequency of genetic mutations or alterations in EGFR, ALK, RET, KRAS and BRAF was determined in 358 never-smokers with NSCLC. The number and percentage of carcinoma cases harboring each of these genetic mutations is indicated in the graph. EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; RET, ret proto-oncogene; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-Raf proto-oncogene, serine/threonine kinase.

for *ALK*, *RET* and *BRAF* genes between male and female patients, suggesting that gender may have no significant effect on these mutations in NSCLC in never-smokers.

Genetic mutations may be affected by ageing. Subsequently, the effect of ageing on the occurrence of oncogenic mutations was determined. Consistent with previous reports (22,23), in the present study, *EGFR* mutations were more likely to be identified in older patients compared with younger patients. This was determined by comparing the distribution in four age subgroups between patients with and without *EGFR* mutations ($P<0.04$; Table III). By contrast, mutations in *KIF5B-RET*

and *EML4-ALK* were more likely to be detected in younger patients compared with older patients, as there was a significant difference in the distribution of patients in four distinct age groups between wild-type and mutated *KIF5B-RET* or *EML4-ALK*, respectively ($P<0.001$; Table III). The median age was 42.8 ± 1.6 years in *KIF5B-RET*-positive patients or 54.4 ± 0.6 years in *EML4-ALK*-positive patients, compared with 57.9 ± 0.8 years in mutation-negative patients (Table III). These results suggested a potential early onset of the disease in the patients exhibiting *KIF5B-RET* or *EML4-ALK* mutations. No evidence suggested that ageing was a significant factor in the occurrence of mutations in *KRAS* and *BRAF*, as determined by Fisher's exact test (Table III).

An association is present between genetic mutations and the level of differentiation in cancer. The differentiation level of carcinoma cases was examined, and it was identified that those expressing *EML4-ALK* were more likely to be poorly- or moderately-differentiated, but not well-differentiated, compared with mutation-negative patients ($P<0.01$; Table III). By comparing the differentiation level between tumors with and without *EGFR* mutations, the cancer cells were more likely to be well-differentiated in carcinoma exhibiting *EGFR* mutations compared with wild-type carcinoma ($P<0.05$; Table III). The differentiation level of carcinoma carrying alternative types of gene mutation was not significantly different from the remaining patients. These results suggested that *ALK* fusions activated cancer types that were less differentiated, but that possessed a more rapid rate of growth and were more resistant to conventional treatment. By contrast, *EGFR* mutations demonstrated the opposite properties for these aspects. The tumors were well-differentiated, with a slower rate of growth and possessed an improved response to conventional treatment (3,10). Therefore, screening for genetic mutations and determining cell differentiation levels may be important

Table III. Characteristics of genetic mutations in 358 never-smokers with non-small cell lung cancer.

Variable	EGFR			ALK			KRAS			RET			BRAF		
	-, n (%)	+, n (%)	P-value	-, n (%)	+, n (%)	P-value	-, n (%)	+, n (%)	P-value	-, n (%)	+, n (%)	P-value	-, n (%)	+, n (%)	P-value
Frequency	187 (52.5)	171 (47.8)		331 (92.5)	27 (7.5)		345 (96.4)	13 (3.6)		353 (98.6)	5 (1.4)		357 (99.7)	1 (0.3)	
Gender															
Male	54 (64.3)	30 (35.7)	0.01	78 (92.9)	6 (7.1)	0.87	78 (92.9)	6 (7.1)	0.05	84 (100.0)	0 (0.0)	0.21	84 (100.0)	0 (0.0)	0.58
Female	133 (48.5)	141 (51.5)		253 (92.3)	21 (7.7)		267 (97.4)	7 (2.6)		269 (98.2)	5 (1.8)		273 (99.6)	1 (0.4)	
Age, years															
<40	5 (100.0)	0 (0.0)	0.04	3 (60.0)	2 (40.0)	0.0002	5 (100.0)	0 (0.0)	0.35	4 (80.0)	1 (20.0)	0.0001	5 (100.0)	0 (0.0)	0.82
40-49	42 (56.8)	32 (43.2)		63 (85.1)	11 (14.9)		73 (98.6)	1 (1.4)		70 (94.6)	4 (5.4)		74 (100.0)	0 (0.0)	
50-59	70 (53.8)	60 (46.2)		121 (93.1)	9 (6.9)		124 (95.4)	6 (4.6)		130 (100.0)	0 (0.0)		129 (99.2)	1 (0.8)	
≥60	70 (47.0)	79 (53.0)		144 (96.6)	5 (3.4)		143 (96.0)	6 (4.6)		149 (100.0)	0 (0.0)		149 (100.0)	0 (0.0)	
Diff															
Poorly	59 (51.3)	56 (48.7)	0.05	105 (89.7)	12 (10.3)	0.004	112 (95.7)	5 (4.3)	0.75	114 (97.4)	3 (2.6)	0.24	116 (99.1)	1 (0.9)	0.36
Mod	77 (60.2)	51 (39.8)		110 (88.7)	14 (11.3)		119 (96.0)	5 (4.0)		122 (98.4)	2 (1.6)		124 (100.0)	0 (0.0)	
Well	51 (44.3)	64 (55.7)		116 (99.1)	1 (0.9)		114 (97.4)	3 (2.6)		117 (100.0)	0 (0.0)		117 (100.0)	0 (0.0)	
Avg. age, years		57.7			54.4			59.3			42.8			58.0	

Diff, differentiation; Mod, moderate; Avg., average; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; KRAS, Kirsten rat sarcoma viral oncogene homolog; RET, ret proto-oncogene; BRAF, B-Raf proto-oncogene, serine/threonine kinase.

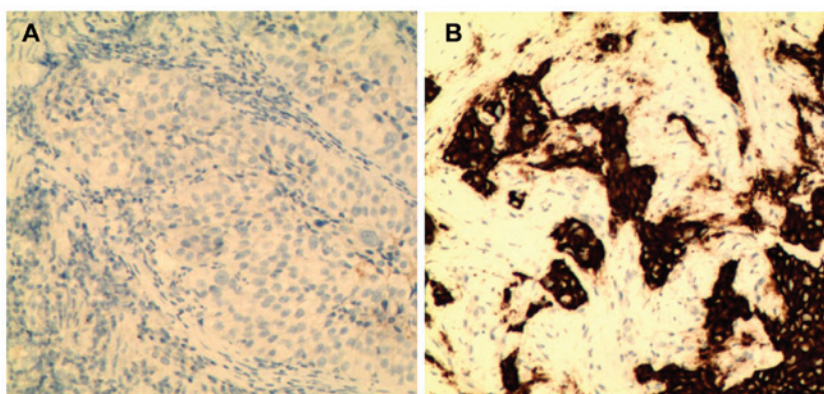


Figure 2. Representative images showing ALK expression in lung cancer. Immunohistochemistry was performed using an antibody against ALK on formalin-fixed paraffin-embedded tissue sections collected from non-small cell lung cancer patients who were never-smokers. Hematoxylin and eosin staining was additionally performed on the sections. (A) ALK protein was absent in *EML4-ALK*-negative carcinoma. (B) ALK protein was aberrantly expressed in *EML4-ALK*-positive carcinoma. ALK, anaplastic lymphoma kinase; EML4, echinoderm microtubule-associated protein-like 4.

steps for improving the efficiency of targeted treatments in NSCLC.

Discussion

Novel discoveries in the molecular genetics of cancer have revolutionized the treatment of the disease by replacing traditional methods with customized therapies based on the clinical pathology and molecular diagnosis of genetic mutations (13). Thus, it is crucial to accurately determine tumorigenic alterations for the success of subsequent treatments. NSCLC in never-smokers has been proposed to be a distinct disease entity due to its unique molecular and clinical properties (19). Although the frequency of oncogenic mutations in never-smokers has been investigated in a large number of studies, the results have been varied and unclear, as never-smoker patients are typically included as a small proportion of the investigated subjects together with a relatively larger amount of smoker patients. In addition, only one mutation at a time has been traditionally investigated in the majority of these previous studies, thus avoiding a direct comparison between various mutations among the same group of patients, which may vary compared with other groups of patients in molecular and clinical characteristics due to differences in race, region, economy and environment. In order to overcome these problems, a comprehensive study was performed to determine the frequency of genetic alterations in five known oncogenes and their associated clinical features in 358 NSCLC patients who were exclusively never-smokers. Using this large-scale screen, a precise molecular profile was generated concerning tumorigenic alterations in NSCLC adenocarcinoma in never-smokers as a distinct disease entity.

EGFR and *KRAS* represent the two most frequently mutated genes in lung cancer, with a frequency of >10% in each case (21). However, the results of the present study indicated that only *EGFR* was frequently mutated in never-smoker patients, while *KRAS* and all other investigated genes were infrequently (<10%) altered in this patient cohort. This supported the idea that NSCLC in never-smokers is a distinct entity in NSCLC, at least with regard to the state of oncogenic mutations. While the causes of these *de novo* mutations

remain to be elucidated, it is agreed that various mutations may have specific impacts on signaling pathways that regulate cellular proliferation and survival (24). For research and clinical purposes, types of cancer with infrequent tumorigenic mutations, particularly those demonstrating poor responses to *EGFR* TKIs and other targeted therapies, may be classified as rare diseases requiring different attention and treatments. An improved classification and diagnosis of lung cancer should consider molecular profiling and pathological characteristics in patients.

The present study reported that 47.8% of patients (171/358) in the present cohort exhibited *EGFR* mutation(s), which was similar to the results of a previous study reporting a frequency of 49.8% in never-smoker patients (25). In the present study, there were 102 patients exhibiting *EGFR* E19 microdeletions and 63 patients demonstrating E21 mutations, including 58 cases with L858R point mutations. These numbers were consistent with previous studies demonstrating that the two most common mutations were located in E19 and E21 (3,10). Notably, one patient exhibited two *EGFR* mutations simultaneously, E19 (E746-A750 DEL) and E21 (L858R). This patient was a female >60 years old who exhibited adenocarcinoma with moderately-differentiated cells. Subgroup analyses suggested that the incidence of *EGFR* mutations was significantly affected by gender and age. In particular, mutations were more likely to occur in female patients compared with male patients and in older patients compared with younger patients. In addition, it was observed that *EGFR* mutations were identified more frequently in well-differentiated tumor cells, which were typically less aggressive and grew more slowly compared with less-differentiated cancer cells.

Mutations in *KRAS* are identified in a wide range of types of cancer, including cancer of the pancreas, large intestine and lungs (26). Although 15-25% of NSCLC patients have been observed to exhibit *KRAS* mutations in previous studies, only 3.6% of patients in the present study cohort possessed *KRAS* mutations, which was consistent with the idea that the occurrence of this mutation may be associated with smoking (27-29). In the present study, it was additionally identified that *KRAS* mutations were more frequently detected in male patients compared with female patients, suggesting the effect of

gender on the incidence of *KRAS* mutations. The majority of the mutations have been identified in E12 and less frequently in E13 (30), and mutations in these exons are able to cause sustained activation of RAS signaling leading to tumorigenesis (21,31). However, despite a large sample size in the present study, mutations were only identified in E12 at positions 12 and 13, and not in E13, suggesting that the mutation rate in E13 may be low or undetectable in NSCLC in never-smokers. It was additionally identified that *EGFR* and *KRAS* mutations were mutually exclusive, as has been reported previously (21). As patients exhibiting *KRAS* mutations typically demonstrate a worse response to EGFR TKIs, including gefitinib or erlotinib, compared to those with wild-type *KRAS* (28), *KRAS* mutations may be utilized as a negative predictive marker for responses to EGFR TKI-based therapy (28).

Somatic point mutations in *BRAF* occur more frequently in melanoma and thyroid, colon and ovarian cancer (5). The V600E substitution, which disrupts an inhibitory interaction between the P-loop and the activation segment, and leads to constitutive kinase activation, accounts for ~90% of *BRAF* missense mutations identified in human tumors (32,33). Previous studies have reported that the frequency of *BRAF* mutation in lung cancer is typically low, normally below or around 1% (34,35). Similarly, the present study detected a V600E point mutation in one patient, accounting for 0.3% of NSCLC in never-smokers in the present patient cohort.

The *ALK* gene encodes a receptor tyrosine kinase, which has been identified in a number of fusion proteins in cancer (6,36-39). Adenocarcinoma appears to be the major NSCLC cell type to exhibit *EML4-ALK* fusions. Previous studies, primarily involving East Asian patients, have reported that 3-7% of lung tumors exhibit *EML4-ALK* fusions (6,40-43). A total of 27 cases (7.5%) in the present study demonstrated *EML4-ALK* fusions. No other types of *ALK* fusions were detected in the current patient cohort. The results of the present study suggested that the onset age of lung cancer is likely to be younger in patients possessing *ALK* fusions compared to those without. Furthermore, the carcinoma cases were less differentiated in patients exhibiting *ALK* fusions compared to those without *ALK* fusions. These features may be at least in part responsible for an observed poor response to EGFR TKIs associated with *ALK* fusion (11), but an improved response to *ALK* TKI crizotinib (12). In addition to *ALK* fusion, *KIF5B-RET* fusion was detected in 1.4% of patients; however, no *CCDC6-RET* fusions were observed in the present study. Similar to *EML4-ALK* fusion, *KIF5B-RET* fusion is likely to be associated with early disease onset, as it was identified more frequently in younger patients. No other clinical features were identified as being significantly associated with *RET* mutations. However, this may have been due to the low frequency of this genetic alteration, which may have required a larger sample size to reach a statistically significant level.

In conclusion, the present study screened the genetic mutations in multiple oncogenes and determined clinical features associated with these mutations in a large cohort of NSCLC patients who were exclusively never-smokers. It was identified that *EGFR* mutations, but not other mutations, frequently occurred in NSCLC in never-smokers. It was additionally identified that gender may be associated with mutations in *EGFR* and *KRAS*, differentiation level with *EGFR* and *ALK*, and

ageing with *EGFR*, *ALK* and *RET*. The results of the present study may provide valuable insights for the enhancement of our knowledge of lung cancer and facilitate the advancement of tailored therapies that are targeted to tumorigenic mutations.

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