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Mutational Profile and New IASLC/ATS/ERS Classification Provide Additional Prognostic Information about Lung Adenocarcinoma: A Study of 125 Patients from Brazil

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Key Words
Lung adenocarcinoma · Mutation · *KRAS* · *EGFR* · ALK-ELM4 · Prognosis · Brazil

Abstract
Aim: To show additional prognostic information about the mutational profile and new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) classification of adenocarcinoma (ADC) in patients without epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor treatments. ***Methods:*** In human lung ADC patients (n = 125), including 24 lepidic, 67 acinar, 23 papillary, and 11 solid predominant subtypes, *EGFR* and *KRAS* were sequenced, and anaplastic lymphoma kinase (ALK) rearrangements were screened using fluorescence in situ hybridization (FISH). ***Results:*** *EGFR* was mutated in 21.6% of patients with 19.57% showing a mean expression. The most frequent *EGFR* mutation was a deletion in exon 19, followed by an L858R amino acid substitution in exon 21. *KRAS* was mutated in 26.4% of patients with 50% displaying mean expression. ALK rearrangement was detected in 6 patients (4.8%). Predominant acinar ADC was strongly associated with *EGFR* and *KRAS* mutation. Clinical stage, lymph node metastases, and *EGFR* mutation in exon 18 showed a significant difference in disease-free and overall survival, but only a trend significance for *EGFR* and *KRAS* mutations. Multivariate analysis revealed that men aged >71 years, with a history of smoking (<72 packs/year), clinical stage I/II, and acinar histologic subtype presented better survival than women aged ≤71 years, with a history of smoking (>72 packs/year), and having a predominant solid ADC and *EGFR* mutation in exon 18. ***Conclusions:*** These results indicate that the mutational profile and new IASLC/ATS/ERS classification provide additional prognostic information about lung ADC.
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Introduction

Lung cancer remains the number one cause of cancer deaths worldwide [1]. In 2014, 27,330 new cases of lung cancer were estimated in Brazil. Globally, the number of

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estimated deaths from lung cancer in 2011 was 1,378,400. Despite improved survival achieved in other forms of cancer in recent years, the 5-year survival rate for male lung cancer patients ranges from 6 to 14%, and for female patients it ranges from 7 to 18%, with a mortality rate that has remained largely unchanged for decades, in part due to limited treatment options [1, 2].

Adenocarcinoma (ADC) is the most common histologic subtype of lung cancer in most countries, accounting for almost half of all lung cancers [3]. Considering that ADCs are a heterogeneous group of tumors with a highly variable prognosis, a new classification of lung ADCs based on the new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) classification system was proposed [4], which has the advantages of providing personalized therapy to patients, a better patient selection, and stratification for clinical trials and molecular studies.

Activating mutations of the epidermal growth factor receptor *(EGFR)* gene are more prevalent in patients with lung ADC who respond to *EGFR* therapy [tyrosine kinase inhibitors (TKIs) gefitinib/Iressa and erlotinib/Tarceva], and the presence of anaplastic lymphoma kinase (ALK) fusion genes predicts response to ALK therapy (crizotinib) [5, 6]. *KRAS* mutations are found in 15–25% of lung cancer patients [7]. *KRAS* is downstream in the *EGFR* tyrosine kinase pathway; therefore, TKI-based treatment with gefitinib and erlotinib is ineffective when *KRAS* is constitutively activated [8, 9].

Although *EGFR* and *KRAS* mutations have been established as predictive and prognostic markers for patients who receive EGFR TKIs [6, 10, 11], results on the prognostic value of these alterations at the genomic level for patients who did not receive EGFR TKIs have rarely been reported. In this study, we aimed to show the comprehensive molecular and clinicopathologic features of lung ADCs, as well as the prognostic value of these characteristics.

Methods

Patients and Tissue Specimens

We analyzed the medical records and archival slides from a population of surgically resected non-small cell lung carcinoma (NSCLC) patients from 2007 to 2012. The cases were selected on the basis of availability of archival slides, tissues, and consecutive surgeries. Of the selected 382 NSCLC cases, 257 cases with a non-ADC histology were excluded. Large-cell carcinoma was differentiated from solid-type ADC by negative mucin staining. A total of 125 ADC cases were selected. The patients underwent pulmonary resection at the São Paulo Cancer Institute (ICESP), AC Camargo Cancer Center, and the Brazilian National Cancer Institute (INCA), three important tertiary Brazilian centers in the field of cancer treatment. This study was approved by the Institutional Review Board of each involved institution. All patients were Caucasian, clinically staged $T_{1-4}N_{0-1}M_0$. Although it is being disputed whether surgery should be done at all in T_4N_0-staged patients (IIIB), these patients were included in the study and considered to have tumors that are partially curable by surgical resection because there was minimal invasion of the pericardium that compressed the heart. Clinical staging comprised bronchoscopy, computerized tomography of the thorax and abdomen, and bone scans. Mediastinoscopy and lymph node biopsy were additionally performed on patients whose lymph nodes had a short axis diameter of >1 cm. The patients did not receive neoadjuvant chemotherapy. However, patients staged T_4N_0, who underwent surgery, received postsurgical chemo- and/or radiotherapy. TNM stages were evaluated in accordance with the seventh edition of the lung cancer staging classification system [12]. Other details of the patients are summarized in table 1.

The tumor tissue used in this study was derived from routine formalin-fixed pathologic samples taken from the resected lung specimens after routine pathologic studies had been completed. Two experienced pathologist (V.K.S. and V.L.C.) reviewed the histologic diagnosis, and ADCs were classified according to the IASLC/ATS/ERS international multidisciplinary classification of lung ADC [4].

DNA Isolation

The tumor specimens underwent microdissection prior to DNA extraction from the formalin-fixed embedded tissues. The frozen tissues from the AC Camargo Cancer Center were subjected to histologic analysis by a pathologist who assessed the percentage of malignant tumor tissue. When necessary, samples were manually dissected, and areas containing nonneoplastic tissues, fibrosis, or other contaminants were removed. Only samples with at least 70% of malignant cells were sent to DNA extraction. Genomic DNA from tumor samples was extracted using a standard proteinase K-phenol-chloroform protocol designed by the AC Camargo Cancer Center Biobank.

EGFR and KRAS Mutational Analysis

Exons 18, 19, 20, and 21 of the *EGFR* gene and exon 2 of the *KRAS* gene were investigated using two different approaches: capillary sequencing and pyrosequencing. Capillary sequencing was performed by polymerase chain reaction (PCR) amplification using primers previously described by Shigematsu et al. [13]. PCR was performed in 25-ml reactions containing 100 ng DNA, 100 mM Tris-HCl, 500 mM KCl (pH 8.3), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 mM of each primer, and 1 U platinum Taq polymerase. The procedure was carried out on a PTC-200 MJ Research Thermal Cycler. The initial denaturation at 94°C for 5 min was followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s, with a final extension step of 5 min at 72°C. The amplified DNA was electrophoresed on a 7% polyacrylamide gel. The PCR products were sequenced directly in both directions using the BigDyeH Terminator v3.1 (Applied Biosystems, Foster City, Calif., USA) and sequencing ready reaction kit on the ABI PRISM 3100 Genetic Analyzer (Applied Biosys-

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tems). Subsequent sequence analyses were performed using the Mutation Surveyor v3.9 (SoftGenetics, State College, Pa., USA) and visual inspection. *KRAS* mutations (exon 2 and codons 12 and 13) were also detected using real-time PCR allelic discrimination. The PCR amplification was performed in 5-ml reactions with 5 ng DNA template, 1× TaqMan Universal Master Mix (Applied Biosystems), 1× of each primer and a probe assay (Custom TaqmanH SNP Genotyping assays), and H_2O q.s.p. The thermal cycling was initiated with a denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min on a 7500 Fast Real-Time System (Applied Biosystems).

Procedures for pyrosequencing were performed using the PyroMark *KRAS* Assay® kit and the *EGFR* PyroMark Assay®, referring to the *EGFR* and *KRAS* genes, following the manufacturer's recommendations (QIAGEN®). Accordingly, 70 µl of the 'bead mix' was added to the deep plates, whose composition was 2 µl beads, 40 µl binding buffer, and 28 µl H_2O DEPEC per plate. Of the PCR product, 10 µl were added to each plate except the primer's control board. The control board sample contained bead mix and BR. In the sequel, this material was stirred at 1,400 rpm at 22°C for 10 min. In particular, enzyme cartridge, substrate, and dNTPs (A, C, G, and T) were added according to the manufacturer's (PyroMark Q24®, QIAGEN®) recommendation. In a shallow dish, 24.25 µl buffer and 0.75 µl sequencing primer were added, whereas in the control sample plate, only 25 µl annealing buffer was added. The race or pyrosequencing analysis was started after aspiration of the contents of the sling plate, followed by washing with 70% ethanol and denaturation solution, both for 5 s, and the wash buffer for 10 s. The material was placed in a shallow plate, and subsequently incubated for 2 min at 80°C, before being put in a pyrosequencer.

Fluorescence in situ Hybridization

Fluorescence in situ hybridization (FISH) was performed on formalin-fixed paraffin-embedded tumor samples using a probe specific to the ALK locus (Vysis LSI ALK dual color, break apart rearrangement probe; Abbott Molecular, Des Plaines, Ill., USA) according to the manufacturer's instructions. The FISH results were analyzed under a fluorescence microscope (Zeiss Axio Imager M1, Carl Zeiss AG, Oberkochen, Germany) with the appropriate filters (Chroma Technology GmbH, Fuerstenfeldbruck, Germany) and Metafer 4 software (MetaSystems, Altlussheim, Germany). The slides were analyzed on a fluorescence microscope (BX61; Olympus, Center Valley, Pa., USA). FISH-positive cases were defined as those in which more than 15% of the cells (at least 40 neoplastic cells were counted) presented with split orange and green signals or an isolated orange signal as previously described [14, 15].

Clinicopathologic Variables

Clinicopathologic data collected for analyses included age at diagnosis, gender, smoking history, pathologic TNM stage, and histologic subtypes of ADC according to the new IASLC/ATS/ERS multidisciplinary classification of lung ADC. TNM stages were evaluated in accordance with the seventh edition of the lung cancer staging classification system.

Statistical Analysis

The association between the predominant subtype, demographic factors, and molecular status was first compared with Fisher's exact test (when any cell of a contingency table had an expected count <5) and Person's χ^2 tests (when no cell of a contingency table had an expected count <5). The impact of the following variables on the overall survival and disease-free survival rates were evaluated: gender, age, smoking status, pathologic stage, predominant subtype/variant according to the new IASLC/ATS/ERS classification, *EGFR* status, *KRAS* status, and ALK. These clinicopathologic factors were used in univariate and multivariate analyses to determine whether they had a significant effect on overall survival and disease-free survival. Survival analysis was undertaken with stratification of the variables by receiver operating characteristic curves for determining the optimal upper and lower binary cutoff limits using the Kaplan-Meier method and the differences were analyzed by means of the log-rank test. The multivariate analysis was performed by means of the Cox proportional hazard model. Statistical analysis was conducted using SPSS 18.0 (SPSS Inc., Chicago, Ill., USA). All results with a p value ≤0.05 were considered significant.

Table 1. Clinical characteristics of all patients

Patients, n	125
Age, years	71 (44–92)[a]
Gender (F/M)	76/49
Stage	
Ia	25
Ib	42
IIa	7
IIb	15
IIIa	28
IIIb	8
Adenocarcinoma predominant subtypes	
Lepidic	24
Acinar	67
Papillary	23
Solid with mucin	11
Smoking history	
Yes	21
Ex	58
No	46
Packs/year	38 (1–200)[a]
Follow-up, months	72 (1–132)[a]
Censored patients for survival analysis at date of last follow-up	63

[a] Values represent the median with the range in parentheses.

Results

Patient Characteristics

A total of 125 lung ADC patients were included in this study, comprising 76 women and 49 men (average age, 71 years; range, 49–92 years). There were 104 smokers and

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Table 2. Analysis of the frequency, types, and quantification of *EGFR* and *KRAS* mutations and ALK-ELM4 fusion

Mutation/ Type	Frequency		Exon	Quantification, %
	wild	mutated		
EGFR	104 (78.4)	27 (21.6)		19.57 (11.70–32.40)
E746-A750del		6	19	
T710I		1	18	
F795L		1	20	
L858R		3	21	
S695N		1	18	
P753L		1	19	
E749K		2	19	
E866K		1	21	
P694S		1	18	
P733S		1	19	
G719C		1	18	
S768I		1	20	
G796D		1	20	
A859T		1	20	
K745-A750del		2	19	
D770insGT		1	20	
C775Y		1	20	
R776H		1	20	
KRAS	92 (73.6)	33 (26.4)		50 (42.9–57.1)
A18D		2		
G12C		15		42.9
G12C/1		1		
G12D		11		57.1
G13C		3		
G15D		1		
ALK-ELM4	119 (95.2)	6 (4.8)		

Values are given as n (%) or mean (range).

21 nonsmokers, accounting for 83 and 17%, respectively. The number of patients in stages I, II, and III was 67, 22, and 36, respectively. Histologically, ADC was subdivided into 24 cases being lepidic predominant, 67 acinar predominant, 23 papillary predominant, and 11 solid predominant. The median overall survival was 72 months (range, 1–132 months). Sixty-three patients were censored for survival analysis until the last day of follow-up. Detailed information is listed in table 1.

EGFR and KRAS Mutation Status and ALK

Of the 125 lung ADC samples, 21.6% (27/125) were found to harbor *EGFR* kinase domain mutations with 19.57% mean expression (range, 11.70–32.40%). Among these, 12 were deletions in exon 19 (75%) and 3 were L858R missense mutations (25%). Other alterations included 1 insertion in exon 20 (D770insGT) related to resistance and 2 other mutations, 1 of resistance (G719C) in exon 18 and another of sensibility (S768I) in exon 20. In the remaining 15 ADCs (12%), rare variants or mutations never described before were detected (table 2). Four ADCs (0.32%) presented more than one mutation in the tested exons (T710I – exon 18 and F795L – exon 20; S695N – exon 18 and P753L – exon 19; L858R and E866K – exon 21; G719C – exon 18 and S768I – exon 20).

Thirty-tree ADCs (26.4%) presented a *KRAS* mutation in exon 2 with a mean expression of 50% (range, 42.9–57.1%). The majority of them occurred in smokers, with 8 mutations (6.4%) detected in nonsmokers. *KRAS* mutation occurred mainly in codon 12 and 13, except for 2 cases with a mutation in codon 15 and 13, respectively.

Among the 125 ADC patients included in this study, 6 (4.8%) ADCs exhibited *ALK-ELM4* rearrangements (numerous in 4 cases and a few rearrangements in the remaining 2).

Associations between EGFR and KRAS Mutations and ALK-ELM4 Fusion and Clinicopathologic Variables

Table 3 shows the results of the associations between mutations and fusion and age, gender, smoking history, histologic subtypes, and lymph node metastases.

Younger patients presented less *EGFR* mutations and more *KRAS* mutations, but this difference did not achieve statistical significance. Among the women, there were significantly more *EGFR* and *KRAS* mutations ($p = 0.05$) as well as ALK-ELM4 fusion ($p = 0.05$). As expected, smokers presented significantly less *EGFR* mutations and more *KRAS* mutations ($p = 0.05$). Predominant acinar ADCs showed a significant increase in *EGFR* ($p = 0.05$) and *KRAS* ($p = 0.04$) mutations and almost the same proportion of ALK-ELM4 fusion. Lymph node metastases were significantly more common in ADCs with an *EGFR* mutation ($p = 0.05$).

Survival Analysis

Tables 4 and 5 show the results of the univariate analysis of overall survival and relapse-free survival of the patients according to the clinicopathologic-biologic variables studied.

Age ≤71 years ($p = 0.001$), female gender ($p = 0.02$), stage III ($p = 0.01$), lymph node metastases ($p = 0.001$), predominant solid ADCs ($p = 0.01$), and mutation in exon 18 of EGFR ($p = 0.04$) were significantly associated with overall survival and relapse-free survival. *EGFR* and *KRAS* mutations presented a statistical trend of associa-

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Table 3. The association between clinicopathologic characteristics and mutational status of *EGFR*, *KRAS*, and ALK-ELM4 in our 125 patients with lung ADC

Variables	EGFR mutation		*KRAS* mutation		ALK-ELM4 fusion	
	wild	mutated	wild	mutated	negative	positive
Age						
≤71 years	54 (43.2)	9 (7.2)	43 (34.4)	20 (16.0)	60 (48.0)	3 (2.4)
>71 years	50 (40)	12 (9.6)	49 (39.2)	13 (10.4)	59 (47.2)	3 (2.4)
Gender						
Female	64 (51.2)	12 (9.6)	56 (44.8)	20 (16.0)[a]	73 (58.4)	5 (4.0)[a]
Male	40 (32)	9 (7.2)	36 (28.8)	13 (10.4)	46 (36.8)	1 (0.8)
Smoking history						
No	19 (15.2)	85 (68)	13 (10.4)	8 (6.4)	20 (16.0)	1(0.8)
Yes	2 (1.6)	19 (15.2)[a]	79 (63.2)	25 (20.0)[a]	56 (44.8)	2 (1.6)
Predominant subtype						
Lepidic	23 (18.4)	1 (8)	21 (16.8)	3 (2.4)	23 (18)	1 (0.8)
Acinar	54 (43.2)	13 (10.4)[a]	49 (39.2)	18 (14.4)[b]	64 (51.2)	3 (2.4)
Papillary	20 (16.0)	3 (2.4)	16 (12.8)	7 (5.6)	23 (18.4)	0 (0.0)
Solid	7 (5.6)	4 (3.2)	6 (4.8)	5 (4.0)	9 (7.2)	2 (1.6)
Metastases						
No	55 (44)	13 (10.4)[a]	50 (40)	18 (14.4)	65 (52)	3 (2.4)
Yes	49 (39.2)	8 (6.4)	42 (33.6)	15 (12.0)	54 (43.2)	3 (2.4)

Figures are n (%).
[a] Linear association (R) = 4.6; p value = 0.05. [b] Linear association (R) = 4.2; p value = 0.04.

tion with relapse-free survival (p = 0.09) and overall survival (p = 0.07) in lung ADC (fig. 1). Multivariate analysis by Cox regression revealed that male patients, with an age of >71 years, smokers of <72 packs/year, in stages I and II, and with acinar predominant ADC presented better survival than women patients, with an age of ≤71 years, smokers of >72 packs/year, with solid predominant ADC mutated for *EGFR* in exon 18 (table 6).

Discussion

In the present study, we investigated the prevalence of the known driver mutations *EGFR* and *KRAS* as well as *ALK* rearrangements in a cohort of 125 lung ADC patients with complete prognostic information from three centers for oncology treatment in Brazil. We found that 21.6, 26.4, and 4.8% of Brazilian patients with ADC harbored *EGFR* and *KRAS* mutations, and *ALK-ELM*4 fusion, respectively. The *EGFR* mutation rate observed in our cohort was higher than that described for Europeans (8–13%) and Americans (10–16%), but still inferior to the rate observed in Asians (30–50%), Latin Americans (33.2%) and in another Brazilian study (30.4%) [13]. The most frequently detected *EGFR* mutation was a deletion in exon 19 (75%) followed by an L858R substitution in exon 21 (25%).

The investigation of oncogenic driver mutations in lung ADCs has significantly encouraged personalized treatment and improvement of targeted drugs [8, 14, 16]. It has been established that *EFGR* and *KRAS* mutations are predictive and prognostic markers for patients who receive *EGFR* TKIs [6, 10, 11]. However, additional prognostic information of the mutational profile and the new IASLC/ATS/ERS classification of ADC in patients not treated with *EGFR* TKIs has rarely been elucidated.

Our results demonstrate a significant association between overall survival and the acinar predominant subtype according to the new IASLC/ATS/ERS classification [4] in a cohort of patients with lung ADC. A survival advantage in acinar predominant tumors versus nonacinar predominant tumors was observed after adjustment for mutation status. We endorse that *EGFR*- and *KRAS*-mutant ADCs were more likely to be acinar predominant

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Table 4. Survival analysis with stratification of the variables in optimal upper and lower binary cutoff limits by the Kaplan-Meier method and the differences by means of the log-rank test

Variables	Overall survival, months		χ^2 (log-rank)	p value
	mean	standard error		
Age				
≤71 years	75.9	5.7		
>71 years	100.3	5.7	10.10	0.001
Gender				
Female	94.4	5.1		
Male	77.1	6.9	4.86	0.02
Smoking history				
No	96.1	10.3		
Yes	86.4	4.5	1.06	0.30
Stage				
I + II	94.8	4.4		
III	69.3	8.9	6.11	0.01
Metastases				
No	100.8	4.8		
Yes	72.0	6.5	10.61	0.001
Predominant subtype				
Lepidic	81.5	10.0		
Acinar	93.6	5.7		
Papillary	91.0	8.8	5.13	0.01
Solid	62.2	10.2		
EGFR				
Wild	85.9	4.6		
Mutated	97.8	9.3	0.89	0.34
EGFR expression				
≤19.6%	68.5	4.5		
>19.6%	63.0	0.0	2.00	0.15
EGFR mutation type				
Exon 19				
No	87.7	4.4	0.05	0.09
Yes	91.0	10.3		
Exon 20				
No	108		2.46	0.07
Yes	132			
Exon 18				
No	88.4	4.2	4.10	
Yes	60.0	0.0		0.04
KRAS mutation				
Wild	88.9	4.9		
Mutated	85.0	7.6	0.44	0.07
KRAS mutation type				
G12C				
No	88.9	4.5		
Yes	77.7	10.7	0.81	0.36
G12D				
No	88.9	4.4	0.94	0.33
Yes	66.4	7.5		
KRAS expression				
≤31.5%	64.8	1.7	0.28	0.59
>31.5%	62.3	7.1		
ALK-ELM4 fusion				
No	86.9	4.2	0.81	
Yes	108.2	13.0		

subtypes. Several studies [4, 17–20] investigated associations between *EGFR* and *KRAS* mutations and the histologic pattern before the publication of the new IASLC/ATS/ERS classification. In fact, significant associations between papillary and micropapillary patterns and *EGFR* mutations were reported by Motoi et al. [20] and Ninomiya et al. [17]. However, De Oliveira et al. [18] reported that in a series of 15 ADC patients with a micropapillary pattern, a high percentage of mutations was present when compared to the results of all histologic subtypes as previously published by Dacic et al. [19].

Our results showed that *EGFR* mutations were more frequent in acinar predominant subtypes (10.4%). With the advent of the new IASLC/ATS/ERS classification, the association between *EGFR* and *KRAS* mutations and the predominant subtype has previously been analyzed in Korean and Chinese patients (table 7). Shim et al. [21] found 50.5% of *EGFR* mutations in resected lung ADCs with significant associations between *EGFR* mutations and micropapillary predominant tumors and the presence of any amount of a lepidic pattern. However, *EGFR* mutations were reported to be more frequent in micropapillary predominant tumors in the studies by Shim et al. [21], Zhang et al. [22], and Song et al. [23]. Sun et al. [24] found *EGFR* mutations in 55.4% of patients with resected lung ADC, 40% being acinar predominant, 8.5% papillary predominant, and 0.8% micropapillary predominant tumors, but the association between acinar and micropapillary predominant tumors and *EGFR* mutations did not reach statistical significance. Zhang et al.

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Table 5. Association between clinical and molecular factors and disease relapse

Factors	Relapse survival		χ^2 p value
	relapsed	relapse-free	
Clinical stage			
I	17 (13.6)	50 (40)	
II	14 (11.2)	8 (6.4)	0.01
III	15 (12)	21 (16.8)	
EGFR status			
Mutated	6 (4.8)	15 (12.0)	0.08
Wild type	40 (32.0)	64 (51.20)	
EGFR mutation status			
Exon 19			
Yes	4 (3.2)	6 (4.8)	
No	42 (33.6)	73 (58.4)	0.09
Exon 20			
Yes	0 (0.0)	46 (36.8)	
No	46 (36.8)	76 (60.8)	0.03
Exon 18			
Yes	2 (1.6)	15 (12.0)	0.07
No	44 (35.2)	64 (51.2)	
KRAS status			
Mutated	35 (28.0)	57 (45.6)	0.07
Wild type	11 (8.8)	22 (12.6)	
Lymph node metastasis			
Positive	28 (22.4)	29 (23.2)	0.009
Negative	18 (14.4)	50 (40.0)	

Figures in parentheses indicate percentages.

Table 6. Cox proportional hazard model analysis of survival time

	Coefficient (B)	SE	p value	Exp(B) (95% CI)
Gender				
Male	–0.60	0.29	0.04	0.54 (0.30, 0.97)
Age				
≤71 years	1.06	0.31	0.00	2.89 (1.55, 5.38)
Subtypes				
Solid			0.14	
Lepidic	–0.32	0.49	0.50	0.72 (0.27, 1.90)
Acinar	–0.89	0.43	0.03	0.40 (0.17, 0.95)
Papillary	–0.84	0.51	0.10	0.43 (0.15, 1.17)
Stage				
I + II	–0.66	0.33	0.04	0.51 (0.26, 0.99)
Smoking history				
≤72 packs/year	–0.39	0.29	0.01	0.67 (0.37, 1.20)
Mutation				
Exon 18	1.60	0.83	0.04	1.54 (0.10, 2.82)

SE = Error standard; Exp(B) = risk for coefficient (B); CI = confidence interval. $\chi^2 = 23.549$, p = 0.003.

[25] explored 349 never-smoking female lung ADC patients and found *EGFR* mutations in 76.2%, including 43.5% acinar predominant tumors, with a significant association between *EGFR* mutations and acinar predominant histology. The different outcome between *EGFR* mutations and histologic subtypes may be related to the study sample size and ethnic difference. Overall, the results from these three Asian groups in terms of predominant subtypes are relatively similar to our own; however, we found *EGFR* mutations in 21.6% of tumors. These data suggest that in Caucasian populations, the predominant subtype increases for specific genotypes.

We also found that *KRAS* mutations were significantly associated with the acinar predominant subtype (14.4%). The significant association between acinar predominant histology and *KRAS* mutations is in conflict with the results of Zhang et al. [25], who reported a significant association between *KRAS* mutations and invasive mucinous ADCs, and the results of Motoi et al. [20], who did not find any significant associations between histologic subtype and *KRAS* mutations. However, our findings are similar to the results from a North American cohort of 82 resected lung ADCs, in which 33% of the tumors harbored *KRAS* mutations, with 19% of these mutations showing a solid predominant histology in comparison to 13.4% of the *KRAS*-mutant tumors with a nonsolid predominant histology [26]. Our findings also coincide with an Australian study [27] which found *EGFR* and *KRAS* mutations in 29 and 22% of tumors, respectively. In their study, *EGFR* mutations were most often detected in acinar (15.9%) and micropapillary predominant tumors (7.3%). In Latin America, Arrieta et al. [28] found that the frequency of *EGFR* mutations in NSCLCs was 33.2% (Argentina 19.3%, Colombia 24.8%, Mexico 31.2%, and Peru 67%). The same group reported that the frequency of *KRAS* mutations was 16.6%. In Brazil, Bacchi et al. [29] identified a frequency of 30.4% with *EGFR* mutations and 14.6% with *KRAS* mutations in NSCLCs. It appears that the frequency of *KRAS* and *EGFR* mutations in solid predominant subtypes indeed differs between Asian and Caucasian populations. This finding is supported by reports of Asian patients showing a significant number of *EGFR* mutations in solid predominant

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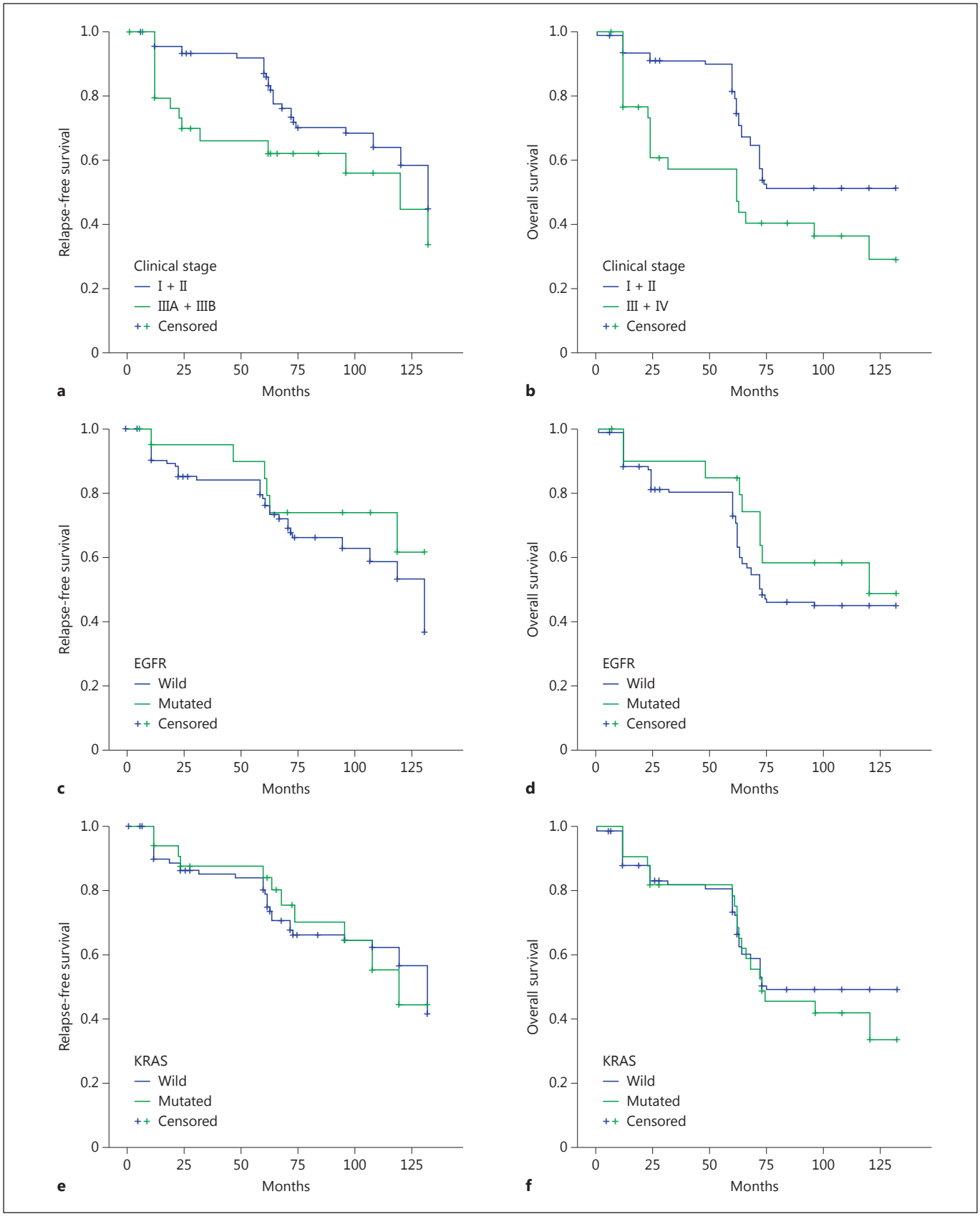

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tumors including 57.2% in a series by Zhang et al. [25] and 14% in a series by Sholl et al. [30], who screened 65 never-smoking Taiwanese women with resected lung ADC.

In the presented study, the *EGFR* and *KRAS* mutations showed a difference in both relapse-free and overall survival. Our data indicated that the IASLC/ATS/ERS histologic subtypes of lung ADC could predict the prognosis of patients. *EGFR*-mutated tumors were more likely to be of the acinar predominant subtype and were less frequent in the lepidic predominant subtype. The prognostic impact of the new classification on recurrence has been validated in several studies [31, 32]. Yoshizawa et al. [31] reported that the IASLC/ATS/ERS histologic classification was predictive of prognosis in stage I ADC. Their data revealed that lepidic predominant, papillary predominant, and acinar predominant subtypes had a 90.0, 83.0, and 84.0% 5-year disease-free survival, respectively. In the study by Hung et al. [32], the lepidic predominant ADCs had a lower risk of recurrence, whereas

Table 7. Details of six studies correlating *EGFR* and *KRAS* mutation status with predominant subtype

	Shim et al. [21], 2011	Sun et al. [22], 2012	Zhang et al. [23], 2012	Ang et al. [24], 2010	Russell et al. [25], 2013	Wang et al. [38], 2014
Patients, n	107	249	349	82	69	332
Stage	I = 42 II = 21 III = 44	NS	I = 206 II = 33 III = 99 IV = 11	NS	IIIA = 68 IIIB = 1	IA = 57 IB = 19 IIA = 14 IIB = 10 IIIA = 85 IIIB = 22 IV = 117 Unknown = 8
EGFR positivity	54 (50.5)	138 (55.4)	266 (72.6)	17 (21)	17 (29)	149 (44.9)
Predominant subtype	(1) MP = 10/12 (2) Any lepidic = 5/8	(1) Acinar = 98/173 (2) Papillary = 21/30 (3) MP = 2/4	(1) Acinar = 152/183	(1) Nonsolid subtypes	(1) Acinar = 11/25 (2) Micropapillary = 5/13	(1) Lepidic = 7 (2) Acinar = 77 (3) Papillary = 47 (4) MP = 6 (5) Solid = 12 (6) IMA = 0 (7) Colloid variant = 0
p value	0.02 0.02	0.06	0.003	N/S	0.009	0.008
KRAS positivity	N/A	N/A	7 (2)	27(33)	12(22)	23/332
Predominant subtype	N/A	N/A	IMA	Solid with mucin = 16/25	Solid = 9/21	(1) Lepidic = 1 (2) Acinar = 11 (3) Papillary = 8 (4) MP = 1 (5) Solid = 2 (6) IMA = 1 (7) Colloid = 0
p value	N/A	N/A	0.028	0.0002	0.016	0.619

Figures in parentheses are percentages. Survival analysis was performed only by Russel et al. [25]. IMA = Invasive mucinous ADC; MP = micropapillary predominant ADC; N/A = not applicable; N/S = not stated.

Fig. 1. Relapse-free survival and overall survival in patients with ADC. **a**, **b** Clinical stage was significantly associated with relapse-free survival (p = 0.01) or overall survival (p = 0.01). **c–f** EGFR mutations in exons 18 and 20 (**c**, **d**) and KRAS mutation status (**e**, **f**) were associated with relapse-free survival and overall survival with marginal significance (p = 0.09 and 0.07, respectively) in lung ADC.

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micropapillary and solid predominant ADCs had a higher risk of recurrence. Russell et al. [27] reported that patients with acinar predominant tumors had significantly improved overall survival compared with those with nonacinar predominant tumors, which remained significant after adjustment for *EGFR* status, tumor stage, sex, and age. Patients with *EGFR*-mutant micropapillary predominant tumors had similar survival to those with *EGFR*-mutant acinar predominant tumors. In our study, the acinar predominant ADCs were shown to have a better prognosis compared with other subtypes. The micropapillary and solid predominant ADCs had the worst prognosis, which is similar to the results from the study by Hung et al. [32]. Our findings contrast those of Li et al. [33], who found that *EGFR* mutation, *KRAS* mutation, and *EGFR*/*KRAS* wild-type groups showed no significant difference in both disease-free and overall survival. They suggest that specific driver mutations of the *EGFR* or *KRAS* gene do not predict a survival advantage or disadvantage when these patients did not receive specific target therapies.

In the current study, we also investigated the frequency of the *ALK* rearrangements and their association with the *EGFR* and *KRAS* status as well as the correlation of the *ALK* rearrangements with clinicopathologic characteristics using FISH in a cohort of 125 randomly selected patients with primary lung ADC. The tumors from these 125 patients were tested by FISH, of which 4.8% were *ALK*-positive. As a group, *ALK*-positive patients were of a similar age as *EGFR* wild-type patients. Compared with *ALK*-negative patients, women presented more *ALK* rearrangements than male patients. There were no differences between the *ALK* rearrangement incidence and smoking status, disease stage, lymph node metastases, histologic subtypes, and survival. Previous studies have reported that the frequency of the *ALK* rearrangements in NSCLCs using FISH ranged from 3 to 13% [34–38]. Values of 3–11% *ALK* positivity were found for the Chinese population in several studies [22, 39–42], with the inconsistency reported resulting from factors including the methodology used to detect the *ALK* rearrangements and the patients enrolled in the studies. Our results for *ALK* rearrangements are consistent with these previous studies.

In our study, according to the IASLC/ATS/ERS classification of lung ADC, the frequency of *ALK* rearrangements in lepidic, acinar, papillary, and solid subtypes was 0.8, 2.4, 0.0, and 1.6%, respectively. Statistical analysis showed that there was no significant difference in the incidence of *ALK* rearrangements between any of the four subtypes. Few studies reported a correlation of *ALK* rearrangements with ADC subtypes evaluated by the IASLC/ATS/ERS classification. The results were not consistent with those in the previous study of Rodig et al. [43] in which a solid histology with signet ring cells according to WHO criteria was positively associated with *ALK*-positive status. The reason for this discrepancy may be that the number of certain subtypes in our study was not very large and thus may have influenced the results. No *ALK* rearrangement was observed in the micropapillary predominant subtype. These results may be related to the fewer number of cases of the above subtypes or a lower frequency of *ALK* rearrangements in these subtypes. These findings should be further investigated in a larger patient cohort. We found that 60% of the tumors manifested the pattern of split green and orange probes, whereas 40% had only the isolated orange probe signals. This positivity is similar to the study of Camidge et al. [44] in which 54.4% had a split pattern and 36.7% had a single red pattern in 90 *ALK*-positive patients.

In conclusion, we report a significant relationship between the predominant subtype of the primary tumor, as defined by the new IASLC/ATS/ERS classification of lung ADC, and overall survival in 125 patients with resected lung ADC. *EGFR* mutations occurred more frequently in acinar predominant tumors, and an improvement in survival was detected depending on the mutation status. These findings indicate that the predominant subtype of the primary tumor determines the outcome of pN1 disease and the mutation status. Subtyping tumors based on the new IASLC/ATS/ERS classification provides important prognostic information and potentially mutational correlates. Subtyping could be used to improve personalized therapy for subgroups of patients that are most probable to benefit from it, an approach that certainly needs examination in prospective studies.

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