Prognostic Role of the FGFR4-388Arg Variant in Lung Squamous-Cell Carcinoma Patients With Lymph Node Involvement

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Abstract

The identification of prognostic biomarkers and novel therapeutic targets in lung squamous-cell carcinoma (SCC) is crucial. We analyzed the presence of the FGFR4-388Arg variant in 114 SCC patients and related it to increased mitogen-activated protein kinase (MAPK) signaling, and to reduced survival in lymph node–affected patients. These findings suggest that FGFR4 targeting in these patients may be a potential therapeutic strategy.

Background: The identification of prognostic biomarkers for lung squamous-cell carcinoma (SCC) pathology is crucial because of its poor prognosis. A variant of the *FGFR4* (fibroblast growth factor receptor 4) gene, FGFR4-388Arg, has been associated with prognosis and is linked to oncogenesis in vitro in several types of cancer. We analyzed the association of this variant with prognosis and downstream signaling alteration in lung SCC patients. **Methods:** The presence of the FGFR4-388Arg variant was determined in 114 formalin-fixed, paraffin-embedded lung SCC tissue samples by DNA genotyping and was correlated with clinicopathologic data. The activation of the protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways was determined by immunohistochemistry, and its association with the presence of FGFR4-388Arg was analyzed. **Results:** We found that tumor differentiation status and adjuvant chemotherapy administration could be independent prognostic factors for overall survival (OS) in lymph node–affected patients, as expected. The progression-free survival and OS of patients with lymph node involvement (n = 41) and the FGFR4-388Arg genotype were significantly lower than those of patients lacking this variant ($P = .035$ and $P = .042$, respectively). Importantly, multivariate analysis supported the independent prognostic role of the FGFR4-388Arg genotype in OS ($P = .025$). Regarding downstream signaling, the FGFR4-388Arg genotype was not correlated with altered AKT signaling but was associated with increased MAPK activation in the SCC tumor samples ($P = .017$). **Conclusion:** The FGFR4-388Arg variant may represent a promising prognostic biomarker in SCC patients with lymph node involvement. For these patients, FGFR4 may be a potential therapeutic target.

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Introduction

Lung cancer mortality is increasing worldwide[1] and currently accounts for 27% of cancer-related deaths.[2] The pathology of lung cancer is heterogeneous and can be divided into 2 major histologically different groups: small-cell lung cancer and non–small-cell lung cancer (NSCLC), which account for 15% and 85% of lung cancer cases, respectively.[3] Within NSCLC, the most common subhistologic types are adenocarcinoma (ADC) and squamous-cell carcinoma (SCC), accounting for 40% and 30% of NSCLC cases, respectively. The discovery of molecular alterations in driver

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genes with relevance in lung cancer and the development of targeted therapies against them have significantly improved outcomes for lung cancer patients, especially in ADC tumors.[4-6] In contrast, the benefits of tested targeted molecular therapies in SCC patients have thus far been modest.[7] Indeed, lung cancer patients show a 5-year survival rate of 15% to 18% after diagnosis.[2] Thus, the discovery of novel prognostic biomarkers for SCC patients, especially those that offer a therapeutic opportunity, is critical.

Many molecular alterations with relevance in lung cancer occur in receptor tyrosine kinases. Some of them have proven to be of interest in prognosis and targeted therapy design.[8] Among them, fibroblast growth factor receptor 4 (FGFR4) has proven to be relevant in many cancers, such as hepatocellular carcinoma, prostate cancer, and lung cancer.[9] This gene belongs to a family of receptor tyrosine kinases that triggers diverse downstream signaling events, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase and protein kinase B (PI3K/AKT) signaling.[10] Although several molecular alterations have been described for this gene,[11] to date only one has proven to be relevant in NSCLC prognosis. This alteration is the rs351855 variant, which causes the substitution of a glycine residue with an arginine in codon 388 (FGFR4-388Gly to FGFR4-388Arg). However, the implications of this variant in patient prognosis seem to be controversial in studies that jointly analyze multiple histologic subtypes of NSCLC.[12-14] Nonetheless, other studies that analyze the different NSCLC subhistologic types individually have shown that the FGFR4-388Arg variant is related to poorer overall survival (OS) in several cohorts of ADC patients.[15,16] In an SCC patient cohort analysis, however, the correlation between this variant and outcome was not clear.[16] Regarding the downstream signaling promoted by FGFR4-388Arg, it was reported that this variant may affect signaling pathways related to cancer. For instance, higher activation of the MAPK pathway has been reported in prostate cancer cell lines overexpressing FGFR4-388Arg.[17] However, to date, the effect of this variant on downstream signaling associated with FGFR activation and cancer in NSCLC patients is unknown.

We aimed to clarify the prognostic role of the FGFR4 Gly388Arg variant in SCC patients and study the downstream signaling pathways potentially involved in the effect of this gene variant on patient outcome.

Material and Methods

Clinical Specimens

The present study involved 114 subjects from the Virgen del Rocio Hospital, Seville, Spain. Patients had undergone surgical resection, and tumor samples were sent to the pathology laboratory for diagnosis and were prepared for storage by formalin fixation and paraffin embedding (FFPE). Inclusion criteria were: 1) confirmed NSCLC diagnosis; 2) access to patient clinical information, including age, gender, smoking status, performance status (as defined by the Eastern Cooperative Oncology Group), lymph node involvement, tumor, node, metastasis classification system (TNM) stage, diagnosis date, date and type of surgery, histologic subtype, differentiation level, chemotherapy/radiotherapy administration, date of relapse, date of the last revision, and status at that time; 3) availability of tumor tissue obtained by surgical resection for immunohistochemistry (IHC). All patients provided written informed consent according to the protocol approved by the local ethics committee (CEI 2013/PI002). Lymph node involvement was assessed and placed in the N1 or N2 categories, according to the international TNM staging system. Typically patients had a follow-up visit plus full blood count and serum biochemistry, and a thoracic upper abdomen computed tomographic scan every 3 months during the first year after surgery, every 4 months during the second year, every 6 months the third year, and annually afterward. Progression-free survival (PFS) was defined as the time elapsed since the diagnosis was performed until pathologic and/or overwhelming radiologic/clinical evidence of tumor recurrence was detected.

Genotyping

DNA was extracted from the tissue sections of FFPE samples using the QIAamp DNA Mini kit (Qiagen, Germantown, MD), and the concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Tech; Thermo Fisher, Waltham, MA). The DNA samples were then preamplified using the Pre-amplification Master Mix (Thermo Fisher) and rs351855 TaqMan Genotyping probe (Thermo Fisher) following the manufacturer's instructions and using an 18-cycle preamplification protocol and a subsequent 1:20 dilution. Genotyping was carried out following the genotyping protocol from TaqMan with 50 amplification cycles using the previously cited rs351855 probe and analyzing using the TaqMan Genotyper software.

Immunohistochemistry

Tumoral areas from FFPE tumor biopsy samples were selected by pathologists after staining with hematoxylin and eosin. Tissue microarrays were established using 1 mm diameter and 3 mm long punches from the selected tumoral areas from each sample. The tissue was protected from oxidation, and the integrity of the samples was maintained throughout the entire process. Deparaffinization and antigenic epitope recovery were performed using the PTLinK kit (Dako, Glostrup, Denmark). Immune detection was carried out with the phosphorylated protein kinase B (pAKT; Ser473; Cell Signaling Technology, Danvers, MA) and phosphorylated extracellular signal–regulated kinase (pERK; Thr202/Tyr204; CST, Danvers, MA) antibodies using horseradish peroxidase–conjugated secondary antibodies. The scoring of IHC staining was based on the criteria described previously[18] and is presented in Supplemental Table 1 in the online version.

Statistical Analysis

Statistical analysis of genotyping, mRNA expression and clinical data was performed by SPSS 19 software (IBM SPSS, Chicago, IL). The relationship between the clinicopathologic features, mRNA expression data, and genotyping results was analyzed using contingency tables, and a P value was obtained by the chi-square test. Kaplan-Meier OS and PFS were calculated, and significant differences were assessed by log-rank analysis. Additionally, multivariate analysis was performed by the Cox proportional hazards method. OS and PFS were defined as the time from diagnosis to exitus and progression, respectively.

Results

Correlation Between FGFR4-388Arg Variant and Clinicopathologic Features

The baseline characteristics of the 114 SCC patient cohort are summarized in Table 1. Most of the patients were men (98.2%) with a median age of 67 years (interquartile range, 54-80 years), with a generally good performance status (Eastern Cooperative Oncology Group performance status 0 or 1). Most of the patients were current or ex-smokers (49.1% and 49.1%, respectively). The differentiation level of tumors was generally poor or moderate (51.8% and 39.5%, respectively). Additionally, 53.5% of patients did not present lymph node involvement, while 36% did. Regarding staging, we found 54.4% of patients with stage I disease, 28.9% with stage II disease, and 16.7% of patients with stage III disease. After radical surgery, 11.4% of patients received adjuvant radiotherapy, and 6.1% were subject to adjuvant chemotherapy; the most frequent treatment regimens were cisplatin–vinorelbine and carboplatin–paclitaxel.

To correlate the rs351855 (FGFR4 Gly388Arg) variant with clinicopathologic characteristics in our cohort, DNA samples were extracted from FFPE tumor tissue from our cohort of lung SCC patients to perform rs351855 genotyping. Seventy-five patients (65.7%) were homozygous for the FGFR4-388Gly variant, and 36 (31.6%) were heterozygous for the FGFR4-388Arg variant; in 3 patients (2.7%), the FGFR4-388Arg variant was detected as homozygous. Because it was previously described that the FGFR4-388Arg variant exerts a dominant effect,[16] patients were split into 2 groups: homozygous FGFR4-388Gly (referred to as Gly) and heterozygote or homozygous FGFR4-388Arg (referred to as Arg). The relationship between the rs351855 variant and clinical characteristics in the cohort was then analyzed. Considering patient characteristics, we found no significant association of the FGFR4-388Arg variant with either age or smoking status. No correlation between the FGFR4 variant and cancer features, such as staging, tumor differentiation, lymph node involvement, or tumor size, was found. Regarding surgery and adjuvant treatment, no association between the variant and administration of adjuvant chemotherapy or radiotherapy was reported (Table 2).

FGFR4-388Arg Variant Is Correlated With Higher MAPK Pathway Activation

It is known that the FGFR receptor family leads to PI3K/AKT and MAPK signaling pathway activation to exert their protumorigenic effects. To assess if the rs351855 pattern is associated with the altered activation of both signaling pathways, we determined their activation in tumor samples from our SCC patient cohort. pAKT and pERK IHC nuclear scores were then related to the rs351855 genotype. In the case of pAKT, a very similar distribution of patients with either low or high pAKT staining could be observed in the FGFR4-388Gly and FGFR4-388Arg genotype groups, so that no significant correlation between the pAKT levels and FGFR4 variant was found ($P = .367$, Table 3, Figure 1). However, in the case of pERK IHC staining, the rs351855 genotype seemed to affect the levels of phosphorylated ERK protein. Patients with low pERK IHC staining accumulated in the FGFR4-388Gly genotype group versus FGFR4-388Arg (69% vs. 31%, respectively), while the

Table 1 Characteristics of 114 Patients With Squamous-Cell Carcinoma

Characteristic	Value
Gender	
Male	112 (98.2%)
Female	2 (1.8%)
ECOG Performance Status	
0	76 (66.7%)
1	33 (28.9%)
2	1 (0.9%)
Unknown	4 (3.5%)
Age (y), median (range)	67 (54-80)
Smoking Habits	
Ex-smoker	56 (49.1%)
Current smoker	56 (49.1%)
Never smoker	1 (0.9%)
Unknown	1 (0.9%)
Tumor Differentiation	
Well differentiated	4 (3.5%)
Moderately differentiated	45 (39.5%)
Poorly differentiated	59 (51.8%)
Unknown	6 (5.3%)
Lymph Node Involvement	
N0	61 (53.5%)
N1	28 (24.6%)
N2	13 (11.4%)
Nx/unknown	12 (10.5%)
Stage	
IA	19 (16.7%)
IB	43 (37.7%)
IIA	3 (2.6%)
IIB	30 (26.3%)
IIIA	19 (16.7%)
Adjuvant Radiotherapy	
Yes	13 (11.4%)
No	93 (81.6%)
Unknown	8 (7%)
Adjuvant Chemotherapy	
Yes	7 (6.1%)
No	101 (88.6%)
Unknown	6 (5.3%)
Relapse	
Yes	54 (47.4%)
No	50 (43.9%)
Unknown	10 (8.8%)
Exitus	
Yes	74 (64.9%)
No	30 (26.3%)
Unknown	10 (8.8%)

Continuous variables are expressed as median (interquartile range), and categorical variables are expressed as n (%).
Abbreviation: ECOG = Eastern Cooperative Oncology Group.

Table 2 Correlation of rs351855 Genotype With Clinicopathologic Features

Characteristic	rs351855 Genotype		P[a]
	Gly	Arg	
Age			
<66 years	31 (60%)	21 (40%)	
≥66 years	44 (71%)	18 (29%)	.141
Smoking Status[b]			
Current smoker	32 (57%)	24 (43%)	
Former smoker	38 (75%)	13 (25%)	
Never smoker	4 (80%)	1 (20%)	.132
Stage			
I	41 (66%)	21 (34%)	
II-III	34 (65%)	18 (35%)	.545
Tumor differentiation			
Medium to high	34 (62%)	21 (38%)	
Low	41 (69%)	18 (31%)	.253
Lymph Node Involvement[b]			
No	42 (69%)	19 (31%)	
Yes	26 (63%)	15 (37%)	.359
Tumor size			
<4 cm	30 (61%)	19 (39%)	
≥4 cm	45 (69%)	20 (31%)	.244
Adjuvant Chemotherapy Administration[b]			
No	67 (66%)	34 (34%)	
Yes	5 (71%)	2 (29%)	.571
Adjuvant Radiotherapy Administration[b]			
No	64 (69%)	29 (31%)	
Yes	7 (54%)	6 (46%)	.220

Categorical values are expressed as number of cases (percentage).
[a]*P* values were obtained by chi-square test. *P* values are considered significant when lower than .05.
[b]Information about some cases for these variables was not available, as indicated in Table 1.

Table 3 Correlation of rs351855 Genotype With IHC pAKT and pERK Staining

IHC Variable	Genotype		P[a]
	Gly	Arg	
Nuclear AKT Score[b]			
Grade 3 or less	38 (68%)	18 (32%)	
Grade 3	34 (63%)	20 (37%)	.367
Nuclear ERK Score[b]			
Grade 3 or less	68 (69%)	30 (31%)	
Grade 3	4 (33%)	8 (67%)	.017

Abbreviations: AKT = protein kinase B; ERK = extracellular signal–regulated kinase; IHC = immunohistochemical; pAKT = phosphorylated protein kinase B; pERK = phosphorylated extracellular signal–regulated kinase.
[a]*P* values were obtained by chi-square test. *P* values are considered significant when lower than .05.
[b]Immunohistochemistry was successful in 110 of 114 patient samples.

opposite occurred when considering samples with high pERK levels (33% vs. 67%, respectively), reaching statistical significance ($P = .017$, Table 3, Figure 1).

FGFR4-388Arg Variant Correlates With Poorer Outcome in Lymph Node–Affected Lung SCC Patients

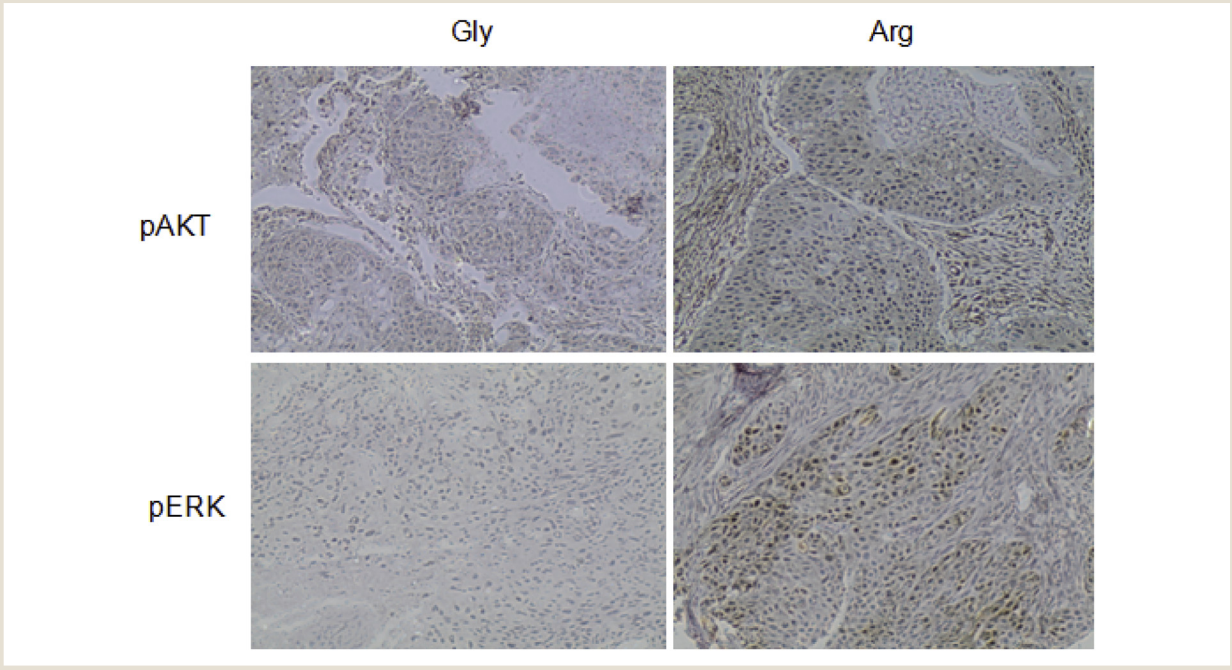
Next, to assess the prognostic potential of the rs351855 genotype, we determined the correlation between patient outcome and the genotyping results. In our cohort, after a median (range) follow-up of 51.5 (21-82) months, 54 relapses and 74 deaths were reported. Multivariate analysis showed that lymph node involvement was an independent prognostic factor of OS, as expected (Table 4). However, in neither the univariate nor the multivariate analysis was an association of the FGFR4-388Arg variant with either OS or PFS found when the whole cohort was studied (Figure 2, Table 4). When only patients with lymph node involvement were considered (n = 41), differentiation and staging were reported as independent prognostic factors of OS in the multivariate analysis, as expected (Table 5). When univariate analysis was performed in this patient subset, the FGFR4-388Arg variant was correlated with PFS, with a median (range) relapse-free survival of 19.9 (0-43.6) months and 55.1 (29.2-120.8) months reported for the FGFR4-388Arg and FGFR4-388Gly groups, respectively ($P = .035$) (Figure 2). Similarly, the FGFR4-388Arg variant was correlated with poorer OS, with a median (range) OS of 25.3 (14.2-36.4) months in the FGFR4-388Arg group and 55.0 (23.8-86.2) months in the FGFR4-388Gly patient subset ($P = .042$) (Figure 2). Furthermore, multivariate analysis confirmed that FGFR4-388Arg was an independent prognostic factor that correlated with poorer OS in patients with lymph node involvement ($P = .025$) (Table 5).

Discussion

Our results show for the first time a clear association between the FGFR4-388Arg genotype and prognosis in lymph node–affected SCC patients. Furthermore, we report higher activation of MAPK signaling in SCC patients with this FGFR4 variant, which may contribute to the reported effect of this variant on prognosis.

When we correlated the rs351855 genotype with prognosis in our cohort of 114 SCC patients, we found no association of the FGFR4 variants with OS or PFS. However, in some of the works relating the rs351855 genotype to prognosis in NSCLC, the prognostic potential of the variants affected only patients with lymph node involvement.[13] For this reason, we repeated the survival analysis only in patients with lymph node involvement in our SCC patient cohort. In this group of patients, the FGFR4-388Arg variant was correlated with poorer OS and PFS. Furthermore, to confirm these results obtained in univariate analysis, we performed multivariate analysis that reinforced the independent prognostic value of the FGFR4-388Arg variant in OS. These data suggest that the FGFR4-388Arg variant is a potential prognostic factor in SCC patients with lymph node involvement. However, these results should be considered carefully because of the small number of patients studied when only those with lymph node involvement were considered, and should be confirmed in a larger cohort of patients presenting this characteristics. Certainly, the FGFR4-388Arg variant has been previously associated with patient outcome in other squamous histology tumor types, such as head and neck SCC.[19-21] These results may be reflecting the similarities between

Figure 1 FGFR4-388Arg Variant Correlates With Higher MAPK Activation. Representative Images of IHC Staining for pAKT and pERK in FGFR4-388Gly (Gly) and -388Arg (Arg) Patients



Abbreviations: IHC = immunohistochemistry; MAPK = mitogen-activated protein kinase; pAKT = phosphorylated protein kinase B; pERK = phosphorylated extracellular signal–regulated kinase.

squamous tumors in different organs and support the results reported in this work. The role of the FGFR4-388Arg variant as a prognostic factor in lung cancer has been previously addressed in the literature, but little work has focused on the role of this variant in SCC patients. In several cohorts jointly analyzing ADC and SCC patients, controversial results have been reported,[12-14] probably as a result of the different ethnicities and clinicopathologic characteristics of the patients included in these study cohorts. Nevertheless, none of these studies analyzed SCC patients independently. ADC and SCC have been demonstrated to be very different pathologies at the molecular level.[22,23] Thus, we considered that the role of the FGFR4-388Arg variant should be addressed in more detail in the SCC patients. In the only study addressing the role of FGFR4-388Arg in SCC survival in a cohort of white patients,[16] no clear association of this variant and prognosis was found. However, in that study, lymph node involvement in patients was not considered, possibly explaining this absence of correlation.

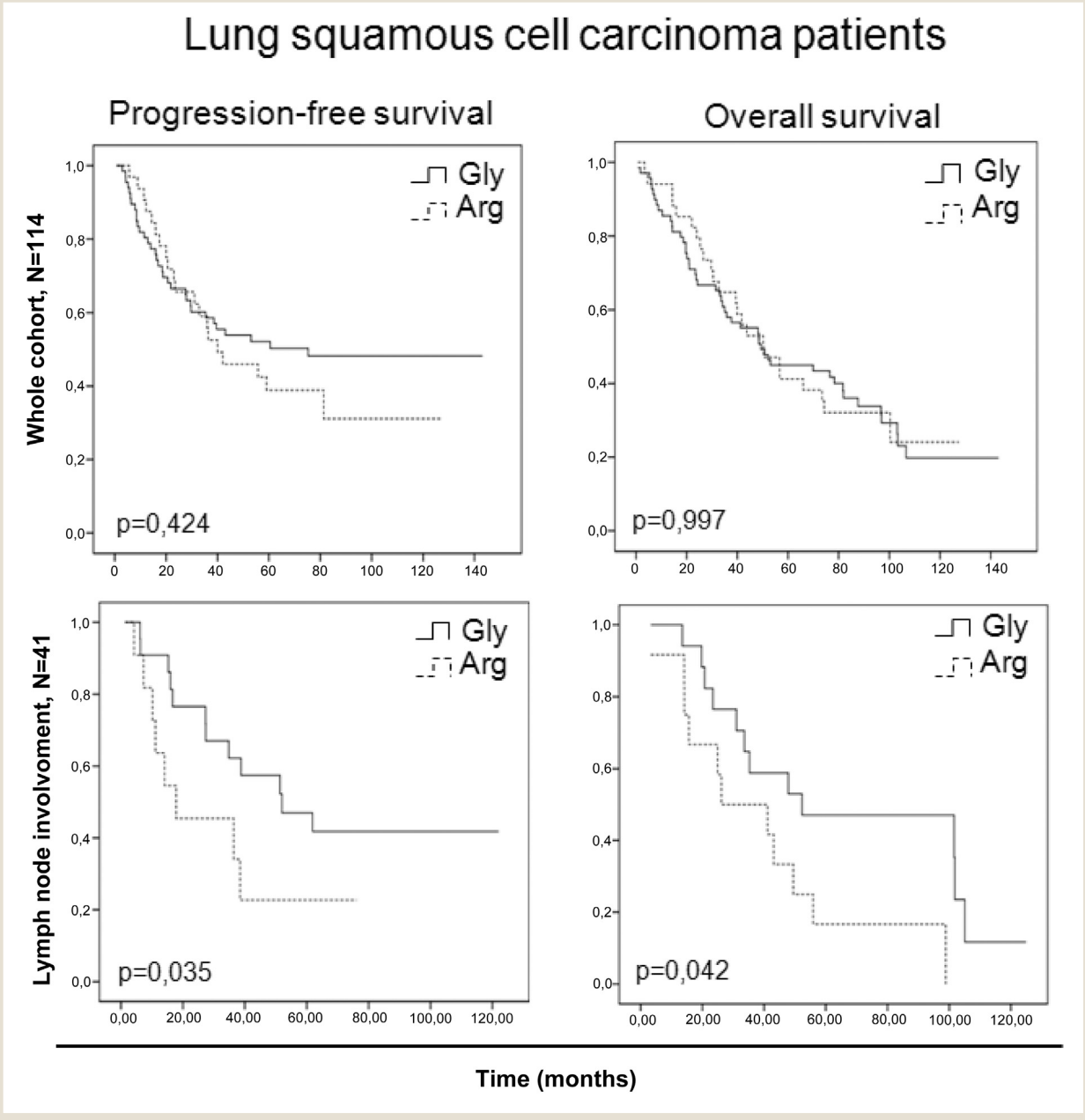
Therefore, to gain mechanistic insight into the reported effect of the FGFR4-388Arg variant in prognosis, we determined the activation of 2 oncogenic signaling pathways, PI3K/AKT and MAPK, by assessing the pAKT and pERK levels by IHC in tissue microarrays with tumor tissue from the same patients. It is known that both signaling pathways are switched on after FGFR4 activation.[24,25] We found that the FGFR4-388Arg variant did not correlate with higher AKT activation. However, there was an association between the FGFR4-388Arg variant and higher pERK levels. It was reported that the overexpression of FGFR4-388Arg induces MAPK activation in in vitro models of prostate cancer,[17] suggesting that MAPK activation by the

Table 4 Multivariate Analysis of Influence of Variables in Patient Outcome in Whole Cohort (N = 114)

	PFS		OS	
Characteristic	**Hazard Ratio (95% CI)**	***P***	**Hazard Ratio (95% CI)**	***P***
Smoking status	0.835 (0.431-1.617)	.593	0.886 (0.507-1.548)	.671
Differentiation	1.535 (0.771-3.052)	.222	1.170 (0.664-2.060)	.587
Stage	1.971 (0.840-4.624)	.119	1.960 (0.950-4.042)	.068
Age	0.962 (0.506-1.827)	.906	1.079 (0.631-1.846)	.780
Adjuvant chemotherapy administration	0.975 (0.752-1.263)	.846	0.951 (0.762-1.187)	.659
Adjuvant radiotherapy administration	0.871 (0.276-2.742)	.813	0.899 (0.332-2.436)	.834
Lymph node involvement	1.002 (0.337-2.980)	.997	1.083 (0.440-2.668)	.862
FGFR4-388 variant	1.107 (0.590-2.076)	.752	1.047 (0.611-1.794)	.867

Abbreviations: CI = confidence interval; OS = overall survival; PFS = progression-free survival.

Figure 2 Kaplan-Meier Survival Plots for Overall and Progression-Free Survival. FGFR4-388Arg Variant Correlates With Poorer Overall and Progression-free Survival in SCC Patients With Lymph Node Involvement. Kaplan-Meier Survival Plots Are Shown for Overall and Progression-free Survival in Entire SCC Cohort and in SCC Patients With Lymph Node Involvement



Abbreviations: Arg = FGFR4-388Arg; Gly = FGFR4-388Gly; SCC = squamous-cell carcinoma.

FGFR4-388Arg variant may be relevant as well in other tumors. Furthermore, this increased pERK signaling in SCC tumors harboring this FGFR4 variant indicates that the effects reported in prognosis could be due to the overactivation of the MAPK signaling pathway by this variant at the molecular level. However, these results should be confirmed in independent cohorts and with different techniques, especially considering that phospho-specific antibodies can render false-negative staining in IHC techniques as a result of inaccessibility of antigen to antibody or low sensitivity.[26,27] In any case, further studies would be needed to prove the MAPK pathway as being responsible for these effects. These results also suggest that targeting FGFR4 pharmacologically in these patients may be a feasible therapeutic approach.

Conclusion

Our findings indicate that the FGFR4-388Arg variant may play an important role in lung SCC, which may be mediated by the overactivation of the MAPK pathway. These results suggest the potential use as a prognostic biomarker for the determination of this variant in

Table 5 Multivariate Analysis of Influence of Variables in Patient Outcome in Squamous-Cell Carcinoma With Lymph Node Involvement (N = 41)

Characteristic	PFS		OS	
	Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI)	*P*
Smoking status	0.399 (0.144-1.105)	.077	0.375 (0.156-1.203)	.218
Differentiation	1.969 (0.647-1.969)	.233	4.422 (1.385-11.546)	.018
Stage	3.219 (0.773-13.397)	.108	3.076 (0.998-9.485)	.050
Age	0.755 (0.263-2.166)	.601	0.717 (0.297-1.732)	.460
Adjuvant chemotherapy administration	1.038 (0.774-1.392)	.804	0.951 (0.723-1.251)	.720
Adjuvant radiotherapy administration	0.563 (0.126-2.524)	.453	0.504 (0.145-1.760)	.504
FGFR4-388 variant	1.091 (0.411-2.895)	.861	4.225 (1.589-12.157)	.025

Abbreviations: CI = confidence interval; OS = overall survival; PFS = progression-free survival.

lymph node–affected patients. These results also suggest that FGFR4 could be an interesting therapeutic target in this subset of patients.

Clinical Practice Points

- The FGFR4-388Arg variant has been linked to oncogenesis and poor prognosis in different types of cancer, including lung ADC. However, up until now, no association of this variant in lung SCC has been reported.
- We describe for the first time an association of this FGFR4 variant with higher oncogenic signaling (ie, increased MAPK pathway activation) and poorer outcome in SCC patients with lymph node involvement.
- Our findings suggest that FGFR4-388Arg may be a potential therapeutic target for lung SCC tumors and open the door to further studies addressing the efficacy of FGFR4 inhibition in SCC tumors harboring this variant.

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Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

Supplemental table accompanying this article can be found in the online version at http://dx.doi.org/10.1016/j.cllc.2017.05.008.

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Supplemental Table 1	Scoring Criteria of Immunohistochemical Staining	
Intensity	**Percentage**	**Score**
0	<10%	0
1	≥10%	1
2/3	<10%	2
2/3	≥10%	3