Gene Mutations in Squamous Cell NSCLC: Insignificance of *EGFR*, *KRAS* and *PIK3CA* Mutations in Prediction of EGFR-TKI Treatment Efficacy

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Abstract. *Background: Epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene (KRAS) and phosphatidylinositide-3-kinase catalytic subunit-alpha (PIK3CA) mutations are biomarkers used for the prediction of efficacy of EGFR tyrosine kinase inhibitors (EGFR-TKIs) in advanced non-small cell lung cancer (NSCLC). Patients and Methods: In total, 223 patients with advanced-stage squamous cell NSCLC were tested; 179 patients were treated with EGFR-TKIs. Genetic testing was performed using a combination of denaturing capillary electrophoresis and direct Sanger sequencing. Results: EGFR mutations were detected in 7.2%; KRAS mutations in 7.4% and PIK3CA mutations in 3.8% of patients. No correlation of EGFR or PIK3CA mutation status with progression-free survival (PFS) (p=0.425; p=0.197), nor overall survival (OS) (p=0.673; p=0.687), was observed. KRAS mutations correlated with shorter OS (p=0.039), but not with PFS (p=0.120). Conclusion: We did not observe any role of EGFR, KRAS, PIK3CA mutations in prediction of EGFR-TKIs efficacy in patients with advanced-stage squamous cell NSCLC.*

Squamous cell lung cancer (SLC) is a subtype of non-small cell lung cancer (NSCLC) and it accounts for approximately 25-30% of all lung cancer cases (1). Low-molecular weight tyrosine kinase inhibitors directed at epidermal growth factor receptor (EGFR) represent one of the novel options for the treatment of advanced-stage NSCLC. Erlotinib and gefitinib are two reversible EGFR tyrosine kinase inhibitors (EGFR-TKI) currently used for the treatment of patients with advanced-stage NSCLC. Considerable progress in the field of molecular genetics in recent years enabled for identification of several gene alterations predicting clinical outcome of patients treated with EGFR-TKIs. Activating *EGFR* mutations (predominantly exon 19 deletions or a point mutation in exon 21 termed *L858R*) are currently strong predictors of a good response to EGFR-TKI treatment (2, 3). Activating *EGFR* mutations are frequently found in patients with adenocarcinoma (ADC), never-smokers, women and Asians (4-7). In contrast to the positive predictive role of activating *EGFR* mutations, Kirsten rat sarcoma viral oncogene (*KRAS*) mutations are frequently described as biomarker predicting for resistance to EGFR-TKIs in NSCLC (8-11), however phase-III clinical trials failed to firmly support such a hypothesis (12-16). The predictive role of both *EGFR* and *KRAS* mutations has been well-described, mainly in ADC because of their common occurrence in patients with this histological subtype of NSCLC. On the other hand the predictive role of *EGFR* and *KRAS* mutations in SLC remains unclear because of their scarcity. Aside from *EGFR* and *KRAS*, other genes and their alterations have recently been identified as candidate biomarkers predicting the efficacy of EGFR-TKIs in NSCLC. Phosphatidylinositide-3-kinase (PI3K), an intracellular signal transducing enzyme, plays an important role in mediating several pathways downstream of EGFR activation (17). The *PIK3CA* gene encodes the p110α catalytic subunit of PI3K protein and its mutation leads to constitutive activation of protein kinase B (AKT) signaling (18, 19). *PIK3CA* mutations have been identified in various types of human cancer, including NSCLC (20). For NSCLC, very little clinical data exist on the role of *PIK3CA* mutations in prediction of EGFR-TKI treatment efficacy. Data showing resistance to EGFR-TKIs in patients with NSCLC harboring a *PIK3CA* mutation have been recently

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Key Words: Squamous cell lung cancer, NSCLC, targeted-therapy, *EGFR*, *KRAS*, *PIK3CA*, mutation, EGFR-TKI.

Table I. *Basic clinical characteristics of patients with squamous cell lung cancer.*

Characteristics		Total (n=179)	*EGFR*		*KRAS*		*PIK3CA*	
			M+ (n=16)	WT (n=163)	M+ (n=14)	WT (n=160)	M+ (n=6)	WT (n=164)
Gender, n (%)	Male	147 (82.1)	11 (68.8)	136 (83.4)	9 (64.3)	134 (83.8)	5 (83.3)	134 (81.7)
	Female	32 (17.9)	5 (31.3)	27 (16.6)	5 (35.7)	26 (16.3)	1 (16.7)	30 (18.3)
Age (years)	Median, (range)	63 (48-79)	69 (49-80)	62 (48-78)	56 (33-81)	63 (50-79)	63 (61-66)	63 (48-79)
Smoking, n (%)	Current or former-smoker	163 (94.2)	11 (68.8)	152 (96.8)	12 (85.7)	148 (94.9)	6 (100.0)	150 (93.8)
	Never-smoker	10 (5.8)	5 (31.3)	5 (3.2)	2 (14.3)	8 (5.1)	0 (0)	10 (6.2)
EGFR-TKI, n (%)	Gefitinib	91 (50.8)	11 (68.8)	80 (49.1)	7 (50.0)	79 (49.4)	2 (33.3)	82 (50.0)
	Erlotinib	88 (49.2)	5 (31.3)	83 (50.9)	7 (50.0)	81 (50.6)	4 (66.7)	82 (50.0)
Stage, n (%)	IIIB	67 (37.4)	8 (50.0)	59 (36.2)	4 (28.6)	62 (38.8)	1 (16.7)	63 (38.4)
	IV	112 (62.6)	8 (50.0)	104 (63.8)	10 (71.4)	98 (61.3)	5 (83.3)	101 (61.6)
Treatment line, n (%)	1	24 (13.4)	2 (12.5)	22 (13.5)	0 (0)	4 (2.5)	0 (0)	23 (14.0)
	2	88 (49.2)	13 (81.3)	75 (46.0)	9 (64.3)	106 (66.3)	4 (66.7)	78 (47.6)
	3	65 (36.3)	1 (6.3)	64 (39.3)	5 (35.7)	49 (30.6)	2 (33.3)	62 (37.8)
	4	2 (1.1)	0 (0)	2 (1.2)	0 (0)	1 (0.6)	0 (0)	1 (0.6)
ECOG PS, n (%)	0	4 (2.2)	0 (0)	4 (2.5)	1 (7.1)	22 (13.8)	0 (0)	4 (2.4)
	1	118 (65.9)	12 (75.0)	106 (65.0)	10 (71.4)	74 (46.3)	4 (66.7)	108 (65.9)
	2	56 (31.3)	4 (25.0)	52 (31.9)	3 (21.4)	62 (38.8)	2 (33.3)	51 (31.1)
	3	1 (0.6)	0 (0)	1 (0.6)	0 (0)	2 (1.3)	0 (0)	1 (0.6)

published (21). In the present work, we conducted a retrospective study to evaluate the role of activating *EGFR*, *KRAS* and *PIK3CA* mutations in prediction of EGFR-TKI treatment efficacy in a selected population of patients with advanced-stage SLC.

Patients and Methods

Study design and patients. The initial group consisted of 223 patients with cytologically- or histologically-confirmed advanced-stage (IIIB, IV) NSCLC. Patients were diagnosed and treated at the Department of Tuberculosis and Respiratory Diseases at the University Hospital Pilsen between 2002 and 2012. In total, 223 patients were tested for activating *EGFR* mutation (exon 19 deletion or exon 21 *L858R* mutation), 215 patients were tested for the *KRAS* mutation, 208 patients were tested for *PIK3CA* mutation (exon 9). The subgroup of 179 patients treated with EGFR-TKI (erlotinib or gefitinib) was followed in great detail. The EGFR-TKI treatment was administered at standard approved doses; 88 patients were treated with erlotinib (150 mg per day) and 91 patients were treated with gefitinib (250 mg per day). The treatment was continued until disease progression or development of intolerable toxic effects. In order to analyze the impact of *EGFR*, *KRAS* and *PIK3CA* mutations on clinical benefit from the EGFR-TKI therapy, progression-free survival (PFS) and the overall survival time (OS) were evaluated. The SLC patients' characteristics are summarized in Table I. Subsequently, survival of patients with *EGFR* mutation-positive SLC and that of patients with *EGFR* mutation-positive ADC was compared. The patients' characteristics are summarized in Table II.

Clinical assessment and statistical methodology. Clinical follow-up examinations including physical examination, plain chest x-ray and routine laboratory tests were performed every 3-4 weeks. Computed tomography (CT) or positron emission tomography - CT (PET-CT) controls were performed after 2 or 3 months of treatment with EGFR-TKI. Progression-free survival (PFS) was determined from the date of EGFR-TKI initiation until the date of first documented progression or death. Overall survival (OS) was determined from the date of EGFR-TKI initiation until the date of death. To describe sample characteristics, standard frequency tables and summary statistics (median and percentile range) were used. Fisher's exact test was used to compare occurrence of mutation according to gender and smoking. PFS and OS as well as the estimations of survival probabilities were depicted using Kaplan Meier survival curves; all point estimates were accompanied by 95% confidence intervals. The differences in survival were tested using the log-rank test. As a level of statistical significance, α=0.05 was used.

Mutation analysis. The tumor specimens acquired during initial bronschoscopy examination were evaluated by a senior cytologist using standard giemsa staining. In a few cases, a tumor biopsy was processed into formalin-fixed paraffin-embedded (FFPE) histological sections. The cytology slides or, eventually, the FFPE sections, were submitted for molecular genetic testing, which included detection of somatic mutations in *EGFR* and *KRAS* genes. If necessary, tumor cells were carefully selected and removed from the samples by laser microdissection using a P.A.L.M. microlaser instrument (Carl Zeiss MicroImaging GmbH, Jena, Germany). The microdissected cells were collected directly into the polymerase chain reaction (PCR) buffer and processed without a special DNA extraction step. In all other cases, the DNA was extracted from tissue cells by a standard spin column procedure using JetQuick Tissue DNA Issolation Kit (Genomed GmbH, Loehne, Germany). Mutations in exons 19 and 21 of *EGFR* gene, exon 1 of *KRAS* gene (codons 12 and 13) and mutations in exon

Table II. *Basic clinical characteristics of patients harboring epidermal growth factor receptor mutation (EGFR M+) with adenocarcinoma (ADC) vs. squamous cell lung cancer (SLC).*

Characteristics		ADC (n=43)	SLC (n=16)
Gender, n (%)	Male	9 (20.9)	11 (68.8)
	Female	34 (79.1)	5 (31.3)
Age (years)	Median, (range)	71 (49-84)	69 (49-80)
Smoking, n (%)	Current or former-smoker	15 (34.9)	11 (68.8)
	Never-smoker	28 (65.1)	5 (31.3)
EGFR-TKI, n (%)	Gefitinib	23 (53.4)	11 (68.8)
	Erlotinib	20 (46.5)	5 (31.3)
Stage, n (%)	IIIB	9 (20.9)	8 (50.0)
	IV	34 (79.1)	8 (50.0)
Treatment line, n (%)	1	20 (46.5)	2 (12.5)
	2	18 (41.9)	13 (81.3)
	3	4 (9.3)	1 (6.3)
	4	1 (2.3)	0 (0)
ECOG PS, n (%)	0	1 (2.3)	0 (0)
	1	26 (60.5)	12 (75.0)
	2	12 (27.9)	4 (25.0)
	3	4 (9.3)	0 (0)

9 of *PIK3CA* gene were tested by Genoscan mutation detection kits (Genomac International, Prague, Czech Republic), utilizing a denaturing capillary electrophoresis (DCE) technique on an ABI PRISM 3100 16-capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA). Detected mutations were confirmed by Sanger DNA sequencing using a BigDye v3.0 chemistry (Applied Biosystems, Foster City, CA, USA). In rare cases, where the overall fraction of mutated DNA was below the 20% threshold for DNA sequencing, mutation was identified indirectly after forming only a homoduplex fragment with a given known mutation reference standard.

Results

EGFR mutations. *EGFR* mutations were found in 16 (7.2%) out of 223 patients tested. These included 10 (4.5%) cases of exon 19 deletions and 6 (2.7%) cases of exon 21 *L858R* substitution (Table III). *EGFR* mutations were found in 5 out of 19 (26.3%) never-smokers *vs*. 11 out of 192 (5.7%) current or former-smokers; the difference was statistically significant (p=0.008). The presence of *EGFR* mutations was not in significant correlation with gender (11 out of 180 males *vs*. 5 out of 43 females; p=0.202). Overall, 179 tested patients were treated with EGFR-TKI. The median PFS of patients harboring the *EGFR* mutation (n=16) was 2.9 months *vs*. 1.9 months for patients harboring wild-type *EGFR* gene (n=163); the difference was not statistically significant (p=0.425) (Figure 1A). The median OS of patients harboring *EGFR* mutation (n=16) was 6.8 months *vs*. 7.8 months for patients harboring the wild-type *EGFR* gene (n=163); the difference was not statistically significant (p=0.673) (Figure 1B). The median PFS of patients with SLC harboring *EGFR* mutation (n=16) was 2.9 months *vs*. 10.2 months for patients with ADC harboring *EGFR* mutation (n=43); the difference was highly statistically significant (p<0.001) (Figure 1C). The median OS of patients with SLC harboring *EGFR* mutation (n=16) was 6.8 months *vs*. 17.7 months for patients with ADC harboring the *EGFR* mutation (n=43); the difference was highly statistically significant (p=0.004) (Figure 1D).

Table III. *The frequency distribution of epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene (KRAS) and phosphatidylinositide-3-kinase catalytic subunit-alpha (PIK3CA) mutations in patients with squamous cell lung cancer.*

	Patients tested	Positive cases
EGFR mutation	223	16 (7.2%)
Exon 19 deletion	223	10 (4.5%)
Exon 21 point mutation L858R	223	6 (2.7%)
KRAS mutation	215	16 (7.4%)
G12C	215	8 (3.7%)
G12D	215	1 (0.5%)
G12V	215	1 (0.5%)
G12A	215	1 (0.5%)
Not specified	215	5 (2.3%)
PIK3CA mutation (exon 9)	208	8 (3.8%)

KRAS mutations. *KRAS* mutations in exon 1 (codon 12 or 13) were found in 16 (7.4%) out of all 215 patients tested. These included eight *G12C* (3.7%) mutations, one *G12D* (0.5%), one (0.5%) *G12V* mutation, one (0.5%) *G12A* mutation and five (2.3%) not otherwise specified *KRAS* mutations (Table III). The presence of *KRAS* mutation was not in significant correlation with smoking status (14 out of 189 current or former-smokers *vs*. 2 out of 19 never-smokers; p=0.645) nor gender (11 out of 173 males *vs*. 5 out of 42 females; p=0.207). Overall, 174 tested patients were treated with EGFR-TKI. The median PFS of patients harboring *KRAS* mutation (n=14) was 1.3 months *vs*. 2.0 months for patients harboring wild-type *KRAS* gene (n=160); the difference was not statistically significant (p=0.120) (Figure 2A). The median OS of patients harboring *KRAS* mutation (n=14) was 5.7 months *vs*. 8.2 months for patients harboring the wild-type *KRAS* gene (n=160); the difference was statistically significant (p=0.039) (Figure 2B).

PIK3CA mutations. *PIK3CA* mutations in exon 9 were found in 8 (3.8%) of all 208 patients tested (Table III). The presence of *PIK3CA* mutations was not in significant correlation with smoking status (6 out of 182 current or former-smokers *vs*. 2 out of 19 never-smokers; p=0.168) nor gender (5 out of 167 males *vs*. 3 out of 41 females; p=0.193). Overall, 170 tested patients were treated with EGFR-TKI. The median PFS of patients

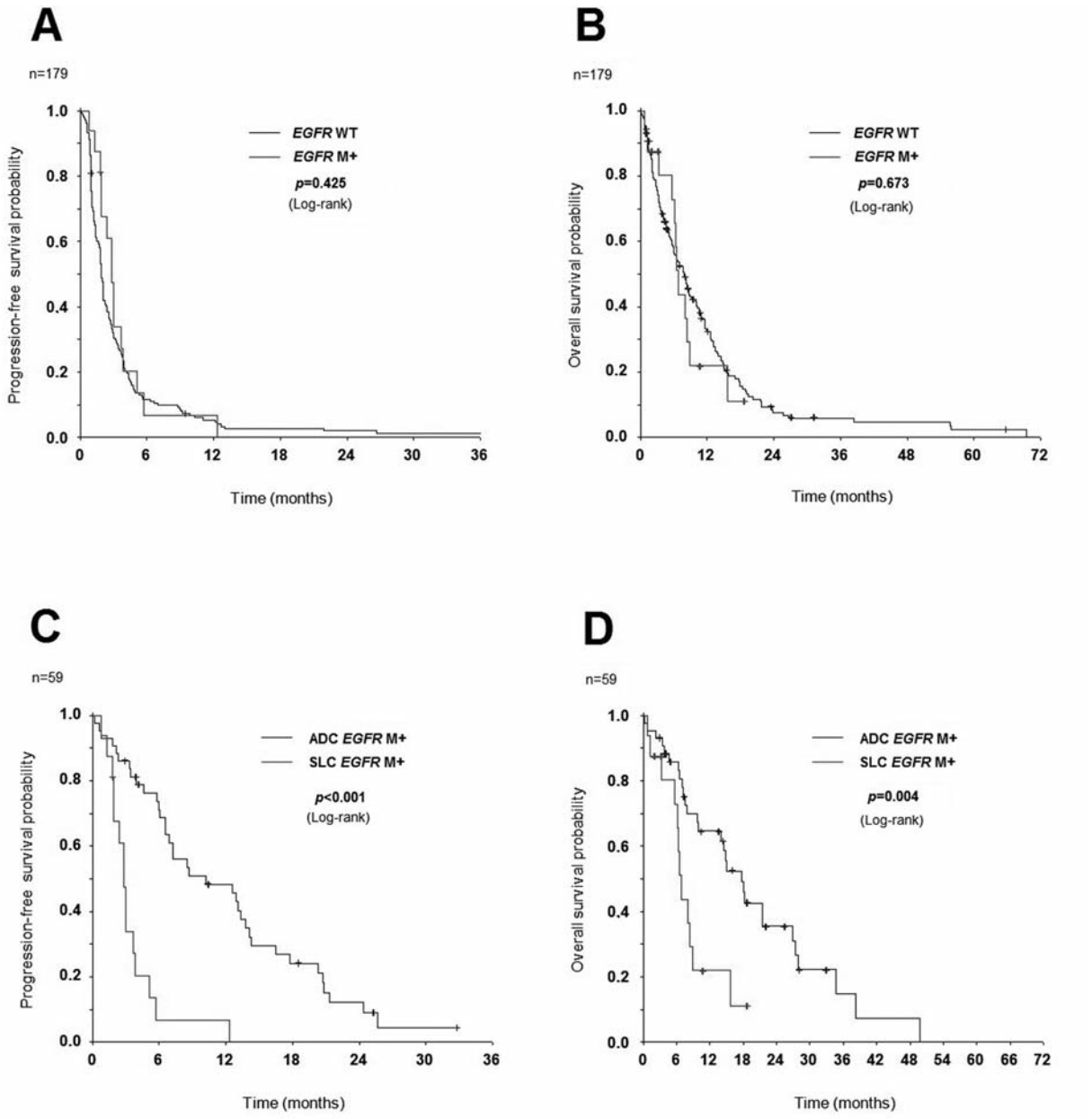

Figure 1. *Kaplan Meier plots showing the comparison of progression-free survival (PFS) (A) and overall survival (OS) (B) between patients with squamous cell lung cancer (SLC) harboring activating epidermal growth factor receptor (EGFR) mutation (M+) and those harboring wild-type EGFR gene (WT); comparison of PFS (C) and OS (D) between patients with SLC harboring activating EGFR mutation and patients with adenocarcinoma (ADC) harboring activating EGFR mutation.*

harboring *PIK3CA* mutation (n=6) was 4.4 months *vs*. 1.9 months for patients harboring the wild-type *PIK3CA* gene (n=164); the difference was not statistically significant (p=0.197) (Figure 2C). The median OS of patients harboring the *PIK3CA* mutation (n=6) was 8.8 months *vs*. 7.6 months for patients harboring the wild-type *PIK3CA* gene (n=164); the difference was not statistically significant (p=0.687) (Figure 2D).

Discussion

There is still a lack of clinical data showing the role of *EGFR*, *KRAS*, *PIK3CA* gene mutations in the prediction of treatment efficacy of EGFR-TKIs in patients with SLC. Both *EGFR* and *KRAS* gene mutations are commonly found in ADC whereas rarely in SLC. In our study, we recorded a slightly higher *EGFR*

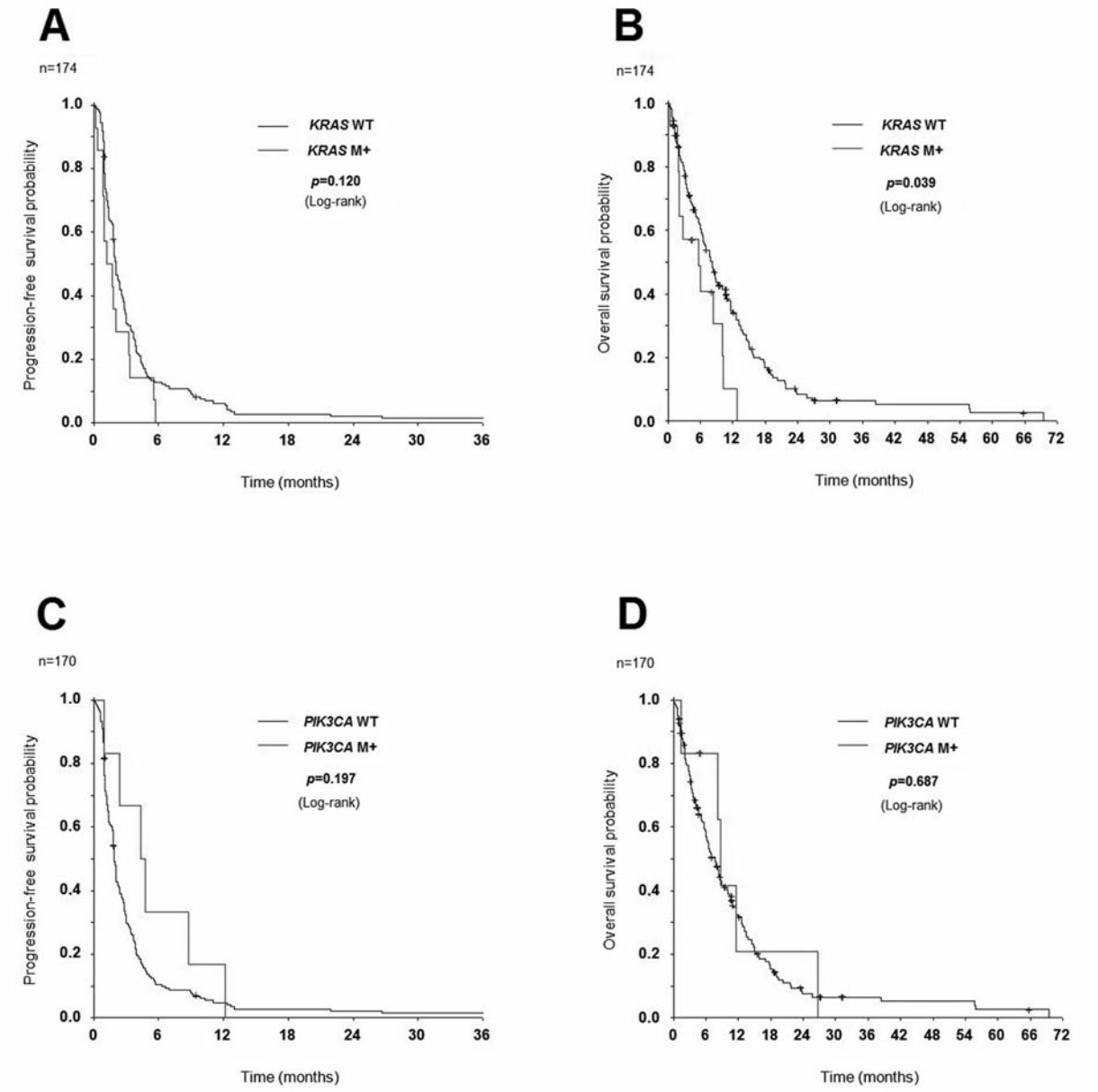

Figure 2. *Kaplan-Meier plots showing the comparison of progression-free survival (PFS) (A) and overall survival (OS) (B) between patients with squamous cell lung cancer (SLC) harboring Kirsten rat sarcoma viral oncogene (KRAS) mutation and those harboring wild-type KRAS gene (WT); comparison of PFS (C) and OS (D) between patients with SLC harboring phosphatidylinositide-3-kinase catalytic subunit alpha (PIK3CA) mutations and patients harboring wild-type PIK3CA gene (WT).*

mutation rate (7.2%) and also a higher *KRAS* mutation rate (7.4%) than have been described in the literature (22-24). As expected, *EGFR* mutations were found more frequently in never-smokers compared to current or former-smokers (p=0.008), a fact previously reported by others (2, 23). On the other hand, we did not observe a significant correlation between *EGFR* mutation and gender, which was reported for ADC (25). Nor did we observe any significant correlation between *KRAS* mutation and smoking status or gender, which was also reported for ADC (25).

The presence of *EGFR* mutation has been considered the best biomarker predicting for good response to EGFR-TKI treatment, being the only biomarker used in common clinical practice. The predictive role of *EGFR* mutations in SLC seems to be unclear. Shukuya *et al*. previously published a pooled analysis conducted to clarify the efficacy of gefitinib for patients with non-ADC NSCLC harboring activating *EGFR* mutations. This work demonstrated that the treatment efficacy of gefitinib was significantly inferior in patients with non-ADC NSCLC

harboring *EGFR* mutations compared to patients with ADC harboring activating *EGFR* mutations (26). In our work, we did not observe any significant difference in survival between patients harboring activating *EGFR* mutation and those harboring a wild-type *EGFR* gene (PFS=2.9 *vs*. 1.9 months, p=0.425; OS=6.8 *vs*. 7.8 months, p=0.673). In order to show the different predictive role of activating *EGFR* mutations in SLC and ADC, we compared the survival of patients with *EGFR* mutation-positive SLC to those with *EGFR* mutation-positive ADC. Here we found a highly significant difference in PFS (2.9 *vs*. 10.2 months, p<0.001) and in OS (6.8 months *vs*. 17.7 months, p=0.004). Despite intensive research in recent years, the role of KRAS mutations in prediction of treatment efficacy of EGFR-TKIs is still not fully-understood. In this work, we observed no significant difference in PFS between patients harboring *KRAS* mutation and these harboring a wild-type *KRAS* gene (1.3 *vs*. 2.0 months, p=0.120), but we recorded a significant difference in OS (5.7 *vs*. 8.2 months, p=0.039). This finding indicates a negative prognostic role of *KRAS* mutation, however, its predictive role was not confirmed. In our previous studies, we presented similar data showing the insignificance of *KRAS* mutation status for prediction of treatment efficacy of EGFR-TKIs in a histologically-unselected population of patients with NSCLC (27, 28) and similar results were also reported in phase-III clinical trials, as mentioned above (12-16). Due to the relatively limited number of patients, we were unable to reliably test the predictive role of specific *KRAS* mutation types that we reported recently (28). Most *PIK3CA* mutations cluster to two regions including exon 9 and exon 20, encoding the helical and kinase domains of the protein, respectively (18). *PIK3CA* exon 9 was reported as a hot-spot mutation for oral squamous cell carcinoma (29). Kawano *et al*. studied *PIK3CA* mutations in lung cancer. The study included 235 lung cancer specimens, and exon 9 and exon 20 *PIK3CA* mutations were analyzed. As a result, eight (3.4%) *PIK3CA* mutations in exon 9 were found, no mutations were found in exon 20 (30). According to these results and the fact that we focused on SLC in our study, we decided to analyze only exon 9 mutations. We found a similar mutation rate (3.8%) to the one of Kawano *et al*. (30). The role of *PIK3CA* mutations in prediction of EGFR-TKIs efficacy was previously studied by Ludovini *et al*., who reported shorter PFS and OS of six patients harboring *PIK3CA* mutation (21). This finding contrasts with the results of our study, where we did not find any significant difference in survival between patients harboring *PIK3CA* mutation and these harboring the wild-type *PIK3CA* gene (PFS=4.4 *vs*. 1.9 months, p=0.197; OS=8.8 *vs*. 7.6 months, p=0.687); moreover, we recorded an approximately two-fold higher median PFS for patients harboring *PIK3CA* mutation, although the difference was not statistically significant. When comparing these contradictory results, it should be mentioned that the study by Ludovini *et al*. included unselected patients with NSCLC, predominantly with non-squamous histology (21) in contrast, our study was focused strictly on patients with SLC. The question arises whether there is a different predictive role of *PIK3CA* mutations between ADC and SLC, similarly as in the case of activating *EGFR* mutations that we have observed in this study. In conclusion, we present data of one of the largest studies focusing on the role of *EGFR*, *KRAS* and *PIK3CA* gene mutations in prediction of efficacy of EGFR-TKIs in patients with advanced-stage SLC. Our findings indicate that activating *EGFR* mutations are not predictive in patients with SLC, contrasting with their strong predictive role in patients with ADC. Thus, patients with advanced-stage SLC should not be selected for EGFR-TKI treatment according to *EGFR* mutation status. We did not find any predictive role of *KRAS* or *PIK3CA* mutations. Patients with SLC harboring these mutations could benefit from targeted treatment and should not be excluded from treatment with EGFR-TKIs.

Conflicts of Interest

All Authors declare that they have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence this work.

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