Original Article

EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study

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Background: Although lung adenocarcinoma (LADC) with sensitizing mutations of the epidermal growth factor receptor (*EGFR*) is highly sensitive to EGFR tyrosine kinase inhibitors (EGFR-TKIs), in most cases disease progression inevitably occurs. Our aim was to investigate the predictive and prognostic significance of adjusted tumoral *EGFR* variant allele frequency (EGFR-aVAF) in the above setting.

Methods: Eighty-nine Caucasian advanced-stage LADC patients with known exon-specific *EGFR* mutations undergoing EGFR-TKI treatment were included. The correlations of EGFR-aVAF with clinicopathological variables including progression-free and overall survival (PFS and OS, respectively) were retrospectively analyzed.

Results: Of 89 *EGFR*-mutant LADC patients, 46 (51.7%) had exon 19 deletion, while 41 (46.1%) and 2 (2.2%) patients had exon 21- and exon 18-point mutations, respectively. Tumoral EGFR-aVAF was significantly higher in patients harboring *EGFR* exon 19 mutations than in those with exon 21-mutant tumors (P<0.001). Notably, patients with *EGFR* exon 19 mutant tumors demonstrated significantly improved PFS (P=0.003) and OS (P=0.02) compared to patients with exon 21 mutations. Irrespective of specific exon mutations, a statistically significant positive linear correlation was found between EGFR-aVAF of tumoral tissue and PFS (r=0.319; P=0.002). High (≥70%) EGFR-aVAF was an independent predictor of longer PFS [*vs.* low (<70%) EGFR-aVAF; median PFSs were 52 *vs.* 26 weeks, respectively; P<0.001]. Additionally, patients with high EGFR-aVAF also had significantly improved OS than those with low EGFR-aVAF (P=0.011).

Conclusions: Our study suggests that high (≥70%) EGFR-aVAF of tumoral tissue predicts benefit from

EGFR-TKI treatment in advanced LADC and, moreover, that exon 19 *EGFR* mutation is associated with high EGFR-aVAF and improved survival outcomes.

Keywords: Epidermal growth factor receptor mutation (*EGFR* mutation); lung adenocarcinoma (LADC); variant allele frequency (VAF)

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Introduction

Lung cancer is the most frequently diagnosed malignancy worldwide (11.6% of the total cases) and the leading cause of cancer-related mortality (18.4% of the total cancer deaths) (1). Histologically, non-small cell lung cancer (NSCLC) is the predominant lung cancer subtype and more than 40% of all NSCLCs diagnosed are lung adenocarcinomas (LADCs) (1). However, not all LADCs are the same, and inter-tumoral heterogeneity exists both in terms of pathological and molecular features (2).

Epidermal growth factor receptor (*EGFR*) mutations are the second most common oncogenic driver events in LADC, accounting for approximately 15% of all LADCs in Caucasian patients and about 40% to 50% in Asian patients (3,4). EGFR is a member of the ErbB family of tyrosine kinase receptors that is expressed in some normal epithelial, mesenchymal, and neurogenic tissue with cytoplasmic kinase activity transducing important growth factor signaling (5,6). However, in malignant tumors including LADC, EGFR is often constantly stimulated due to the sustained production in the tumor microenvironment of EGFR ligands, or as a result of a mutation in *EGFR* itself that locks the receptor in a state of continuous activation (7,8). About 90% of activating *EGFR* mutations are short in-frame deletions in exon 19 or point mutations in exon 21 often referred to as "classical" *EGFR* mutations (9,10). Exon 18 mutations are rare and relatively homogenous (compared to other rare mutations such as *EGFR* exon 20 insertions) as they represent about 4% of all *EGFR* mutations (9,10). Importantly, in LADC, these *EGFR*-sensitizing mutations confer sensitivity both to first-, second- and third-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib, erlotinib, dacomitinib, afatinib and osimertinib in patients with advanced-stage disease (11-13).

Over the past decade, the application of EGFR-TKIs have led to a new era in the treatment of LADC. Accordingly, EGFR-TKIs improve both the progression-free survival (PFS) [10.8 *vs.* 5.4 months in the chemotherapy (CHT) group; P<0.001] and overall survival (OS) (30.5 *vs.* 23.6 months in the CHT group; P=0.31) in patients who were selected on the basis of *EGFR*-sensitizing mutations (14). Still, the objective response rate to EGFR-TKIs in patients carrying *EGFR*-sensitizing mutations is only 70% to 80%, and while some patients show clear survival benefit to TKIs others failed to respond properly (15,16). Therefore, in order to assess the effectiveness of current treatment options, it is crucial to understand the intrinsic and extrinsic factors that influence the responsiveness to TKIs in these patients.

Sensitivity to EGFR-TKIs is associated with female sex, never-smoking status and Asian ethnicity, however, such clinical factors are in fact predictors of *EGFR* mutations rather than true treatment-related prognosticators for TKI efficacy (14,15,17,18). Nevertheless, different *EGFR* mutation subtypes and molecular characteristics can also determine different predictive and prognostic features (15). In addition, differences in the proportion of tumor cells (TCs) harboring *EGFR* mutations might also contribute to therapy response, since only a fraction of cancer cells in an individual patient carry heterozygous activating mutations, whereas other cancer cells carry wild-type *EGFR* (19-22). Accordingly, previous studies on Asian patients suggest that higher relative *EGFR* mutational abundance might predict benefit from EGFR-TKI treatment (19,23,24).

Presently, the biological and clinical relevance of adjusted tumoral *EGFR* variant allele frequency (EGFR-aVAF) in terms of prognosis and clinical response to EGFR-TKIs is still mostly unclear. Therefore, in order to improve patient selection and to better understand the influence of EGFR-aVAF in this setting with regards to therapeutic approaches, our aim was to assess the relationship between EGFR-aVAF and response to EGFR-TKIs in a homogenous patient cohort of Caucasian LADC patients.

We present the following article in accordance with the

STROBE reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-814).

Methods

Ethics statement

The present study was directed in accordance with the guidelines of the Helsinki Declaration (revised in 2013) of the World Medical Association. The study was approved by the national level ethics committee (Hungarian Scientific and Research Ethics Committee of the Medical Research Council, ETT-TUKEB, 7214-1/2016/EKU). The need for individual informed consent for this retrospective study was waived. After clinical information was collected, patient identifiers were removed, and subsequently, patients cannot be identified either directly or indirectly.

Study population

Based on our inclusion/exclusion criteria, 89 pathologically confirmed advanced-stage LADC patients were included in this multi-center, retrospective study, who received EGFR-TKI therapy mainly in the following two Hungarian medical centers between 2008 and 2020: Torokbalint County Institute of Pulmonology, Torokbalint; and Department of Pulmonology of the Semmelweis University, Budapest, Hungary. Of note, all ten participating medical centers are enlisted in Table S1. All tumor tissues were tested for *EGFR* mutations required for anti-EGFR therapy and all samples were retrieved from treatment-naïve patients. Based on our inclusion criteria, cytologically or histologically verified unresectable stage IIIb or stage IV patients were included who received either gefitinib or erlotinib as first- or second-line treatment. According to the therapy guidelines of the host institutes, only patients with Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–1 were included. With regards to our exclusion criteria, patients with concomitant mutations in two or more exons, or patients harboring resistance mutations such as T790M substitution in exon 20 were excluded. Additionally, all cases where the exact percentage of neoplastic cells was not available were also subsequently excluded. Patients treated with afatinib were excluded due to the relatively small number of cases. Similarly, patients in whom TKI therapy was suspended by the reason of drug-related toxicities like hepato- and cardiotoxicities, or patients treated with EGFR-TKIs as third-line therapy were also excluded. Finally, patients who received EGFR-TKI therapy for a period less than 4 weeks or the cause of death was not related to lung cancer progression were also excluded. Clinicopathological data regarding gender, age at lung cancer diagnosis, smoking history, type of *EGFR* exon mutation, EGFR-aVAF, treatment and survival data for the included patients were retrospectively collected from medical records and/or records from the National Health Insurance Office or Central Statistical Office.

Treatment

Diagnostic and therapeutic approaches were conducted in accordance with the individual institutional guidelines and with the current National Comprehensive Cancer Network (NCCN) guidelines with no differences across the host institutes (25). All included patients received either gefitinib or erlotinib as first- or second-line systemic therapy on a daily basis (250 and 150 mg, respectively) until disease progression. In case of patients who received first-line CHT before the initiation of TKI therapy, patients were treated with platinum-based standard of care CHT regimens. According to the national treatment financing scheme, all EGFR-TKI-treated patients had to return to the hospital every month for chest X-ray and clinical check-up, and the clinical response to treatment was classified based on follow-up CT scans every 3 months by using the response evaluation criteria in solid tumors (RECIST 1.0) (26).

EGFR mutation analysis

Tissue samples were acquired during diagnostic procedures including wedge resection surgery and bronchoscopic- or transthoracic needle biopsy. The diagnosis of LADC of each case was confirmed on a freshly prepared hematoxylin and eosin stained slide. As this estimate is critical for the study, the exact proportion of neoplastic cells was reassessed by two independent expert histopathologists. All mutational analyses were performed at the 1st Department of Pathology and Experimental Cancer Research of the Semmelweis University. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions, and all samples underwent testing for mutation in *EGFR* codon (in exon 18, 19, 21) using the Therascreen EGFR Pyro Kit (Qiagen, Germany) on a PyroMark™ Q24 (Qiagen) pyrosequencing instrument. The percentage of

Table 1 Patient characteristics and adjusted tumoral EGFR-VAF in human LADC

Characteristics	Number of patients (%)	Mean EGFR-aVAF, %	P value[a]
All patients	89 (100.0)		
Age (years)			0.93[b]
<65	36 (40.4)	63.53	
≥65	53 (59.6)	64.6	
Gender			0.809[b]
Male	25 (28.1)	64.12	
Female	64 (71.9)	64.19	
Smoking history			0.467[c]
Never smoker	48 (51.7)	64.46	
Ex-smoker	10 (11.2)	73.3	
Current smoker	14 (15.7)	58.5	
No data	19 (21.3)	–	
Therapeutic agent			0.428[b]
Gefitinib	58 (65.2)	61.64	
Erlotinib	31 (34.8)	68.9	
Treatment line			0.882[b]
First-line	46 (51.7)	63.35	
Second-line	43 (48.3)	65.05	
EGFR exon mutation			
Exon 18	2 (2.2)	–	<0.001[b]
Exon 19	46 (51.7)	75.04	
Exon 21	41 (46.1)	51.44	

[a], P values refer to mean EGFR-aVAF between patient subgroups; [b], Mann-Whitney U test; [c], Kruskal-Wallis test; [d], not included in the statistical calculation. EGFR, epidermal growth factor receptor; EGFR-aVAF, adjusted EGFR variant allele frequency; LADC, lung adenocarcinoma.

mutated nucleic acid was calculated with the equipment software (Qiagen PyroMark™), relating the peak of mutated base to that of the wild-type base, which was considered 100%. The obtained VAF for each patient was then normalized to the proportion of neoplastic cells in each specimen using the following formula:

$$\text{Adjusted VAF (aVAF)} = \frac{\text{VAF}}{\text{TC\%}} \times 100 \quad [1]$$

Where VAF represents the percentage of the *EGFR* variant alleles determined by the pyrosequencing assay and TC% is the estimated percentage of neoplastic cells.

Statistical analyses

All statistical analyses were performed using the SPSS Statistics 23.0 package (SPSS Inc., Chicago, IL, USA). Data distribution was verified by the Kolmogorov-Smirnov normality test. EGFR-aVAF of tumoral tissue as a continuous variable was analyzed with regards to dichotomized clinicopathological variables by Mann-Whitney U test and Kruskal–Wallis test. The co-primary endpoints were PFS and OS. PFS was defined as the time from commencement of gefitinib or erlotinib treatment to disease progression according to the aforementioned RECIST 1.0 criteria. OS was defined as the interval between the initiation of medication and death related to progressive disease. Clinical follow-up was closed on the 1st of April, 2020. Survival curves were estimated by Kaplan-Meier plots and the differences between different groups were compared using the log-rank test. The association between EGFR-aVAF as continuous variable and PFS and OS was also evaluated by using the Spearman's correlation coefficient. The value of linear correlation coefficient (r) varies from –1 to 1 both values inclusive. No linear correlation (r=0), weak positive correlation (0< r ≤0.3), moderate positive linear correlation (0.3< r ≤0.7), strong positive linear correlation (0.7< r ≤1) (27). The independent prognostic value of the clinicopathological variables was studied with Cox proportional hazard regression model, which was adjusted for EGFR-aVAF and age (as continuous variables), gender (male versus female), *EGFR* exon mutation (exon 19 versus exon 21), therapeutic agents (gefitinib versus erlotinib) and treatment line (first- versus second-line). All reported P values are two-sided, and a level of 0.05 or less was considered statistically significant.

Results

Patient characteristics and EGFR-aVAF

After applying the exclusion criteria, 89 LADC patients with known *EGFR* gene mutations were enrolled in the study whose clinicopathological characteristics are summarized in *Table 1*. All patients had advanced-stage disease and Caucasian background. Median age of all cases was 67 (range, 34–92) years and patients were predominantly female (71.9%). A total of 46 (51.7%) patients had exon 19 deletion, while 41 (46.1%) and 2 (2.2%) patients had exon

21- and exon 18-point mutations, respectively. Median age was 61, 66 and 70 years in exon mutation subgroups 18, 19 and 21, respectively (with no significant differences in age distribution, P=0.332; data not shown). As for therapeutic approaches, 58 (65.2%) patients received gefitinib, while 31 (34.8%) patients were treated with erlotinib.

In order to study the clinical relevance of mutational percentage of tumoral tissue, we performed comparative statistical analyses of EGFR-aVAF and clinicopathological variables. Out of all 89 cases, 72 cases showed EGFR-aVAF between 5% and 94% and 17 patients exhibited EGFR-aVAF ≥95% (*Figure 1A*). In case of six patients the EGFR-aVAF of tumoral tissue was <20%. Interestingly, the adjusted VAF was significantly higher in patients harboring *EGFR* exon 19 mutations than those with exon 21 mutant tumors (P<0.001; *Table 1*, *Figure 1B*). There were no statistically significant differences in the mean EGFR-aVAF with respect to age (P=0.93), gender (P=0.809), or smoking history (P=0.467).

EGFR exon 19 mutation associates with superior survival outcomes

The median PFS and OS of the full cohort was 38 and 72 weeks, respectively. At the time of the closing date of the clinical follow-up, all patients with *EGFR* exon 18 mutations, 42 patients with exon 19 mutations and 39 patients with exon 21 mutations had experienced disease progression after EGFR-TKI therapy. Due to the small number of patients in *EGFR* exon 18-mutated subgroup, statistical analyses were performed solely by comparing the median PFS and OS of exon subgroups 19 and 21. Accordingly, as shown in *Figure 2A*, LADC patients with tumors harboring *EGFR* exon 19 mutations had significantly improved median PFS than those with exon 21 mutations (median PFSs were 44 *vs.* 25 weeks, respectively; P=0.003). In line with the PFS data, *EGFR* exon 19 mutations were significantly associated with longer OS as well (*vs.* exon 21 mutation, median OSs were 76 *vs.* 57 weeks, respectively; P=0.02; *Figure 2B*). With regards to the administered therapeutic agents, no significant differences have been observed neither in PFS (P=0.654; *Figure 2C*) nor in OS (P=0.665; *Figure 2D*) in patients treated with gefitinib *vs.* erlotinib. Of note, the treatment line of EGFR-TKI did not influence the survival outcomes neither (Figure S1A,B). As for smoking history, there was no significant difference in PFS between never-smoker versus ever-smoker patients (P=0.099; Figure S1C). Interestingly, however, Kaplan-Meyer curves demonstrated significantly longer median OS in never-smoker patients (*vs.* ever-smokers, median OSs were 106 *vs.* 52 weeks, respectively, P=0.007; Figure S1D).

EGFR-aVAF has clinical utility in predicting survival outcomes in LADC patients treated with EGFR-TKIs

Next, we evaluated the survival outcomes of TKI-treated *EGFR*-mutant LADC patients with regards to adjusted tumoral variant allele frequencies. Importantly, a statistically significant positive linear correlation was found between EGFR-aVAF and PFS (r=0.319; P=0.002, Spearman's correlation; *Figure 3A*). In contrast, no significant correlation was found between EGFR-VAF and OS, although the correlation coefficient was found to be clinically notable (r=0.208; P=0.061, Spearman's correlation; *Figure 3B*). In order to rule out the potential confounding effects of Spearman's correlation and to evaluate the survival outcomes with Kaplan-Meier methods, patients were categorized by the median EGFR-aVAF (70%) of tumoral tissue. Therefore, we grouped patients into low (<70%) and high (≥70%) EGFR-aVAF categories and found that patients with high adjusted tumoral EGFR-VAF had significantly longer PFS than those in the low EGFR-aVAF group (median PFSs were 52 *vs.* 26 weeks, respectively; P<0.001, *Figure 3C*). Additionally, patients with high EGFR-aVAF also had significantly improved OS (*vs.* those with low EGFR-aVAF; median OSs were 94 *vs.* 57 weeks, respectively; P=0.011, *Figure 3D*).

In order to assess if the predictive value of tumoral EGFR-aVAF was independent from other clinicopathological factors, we performed a multivariate Cox regression analysis (*Table 2*). The model was adjusted for clinicopathological variables such as EGFR-aVAF, age, gender, *EGFR* exon mutation, therapeutic agents and treatment line. Importantly, we found that EGFR-aVAF of tumoral tissue remained a significant prognostic factor for PFS [continuous variable, hazard ratio (HR): –0.009, 95% confidence interval (CI): 0.982–0.999; P=0.042; *Table 2*]. Besides, Cox regression analysis revealed that the specific exon mutations (nominal variable, HR: 0.284, 95% CI: 1.017–1.735; P=0.037) also influence the PFS independently.

Discussion

In the era of precision and individualized cancer therapy precise definition of tumor type including comprehensive histological classification, and description of clinically

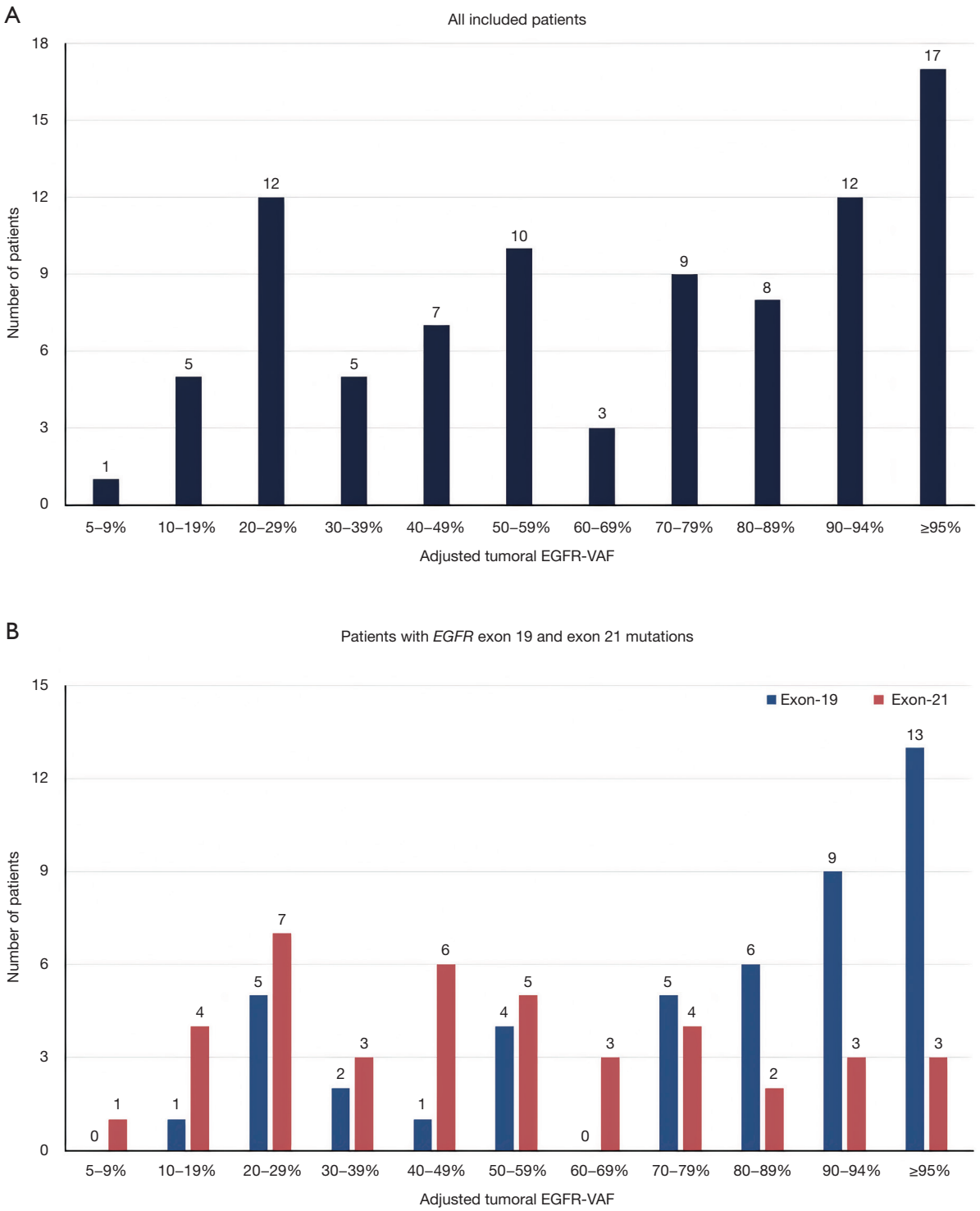

Figure 1 EGFR-aVAF of tumoral tissue in LADC patients. (A) Bar chart illustrating the distribution of all included LADC patients (n=89), according to tumoral EGFR-aVAF irrespective of specific exon mutations. (B) Distribution of LADC patients diagnosed with *EGFR* exon 19 and exon 21 mutations (n=46 and n=41, respectively). EGFR, epidermal growth factor receptor; EGFR-aVAF, adjusted *EGFR* variant allele frequency; LADC, lung adenocarcinoma.

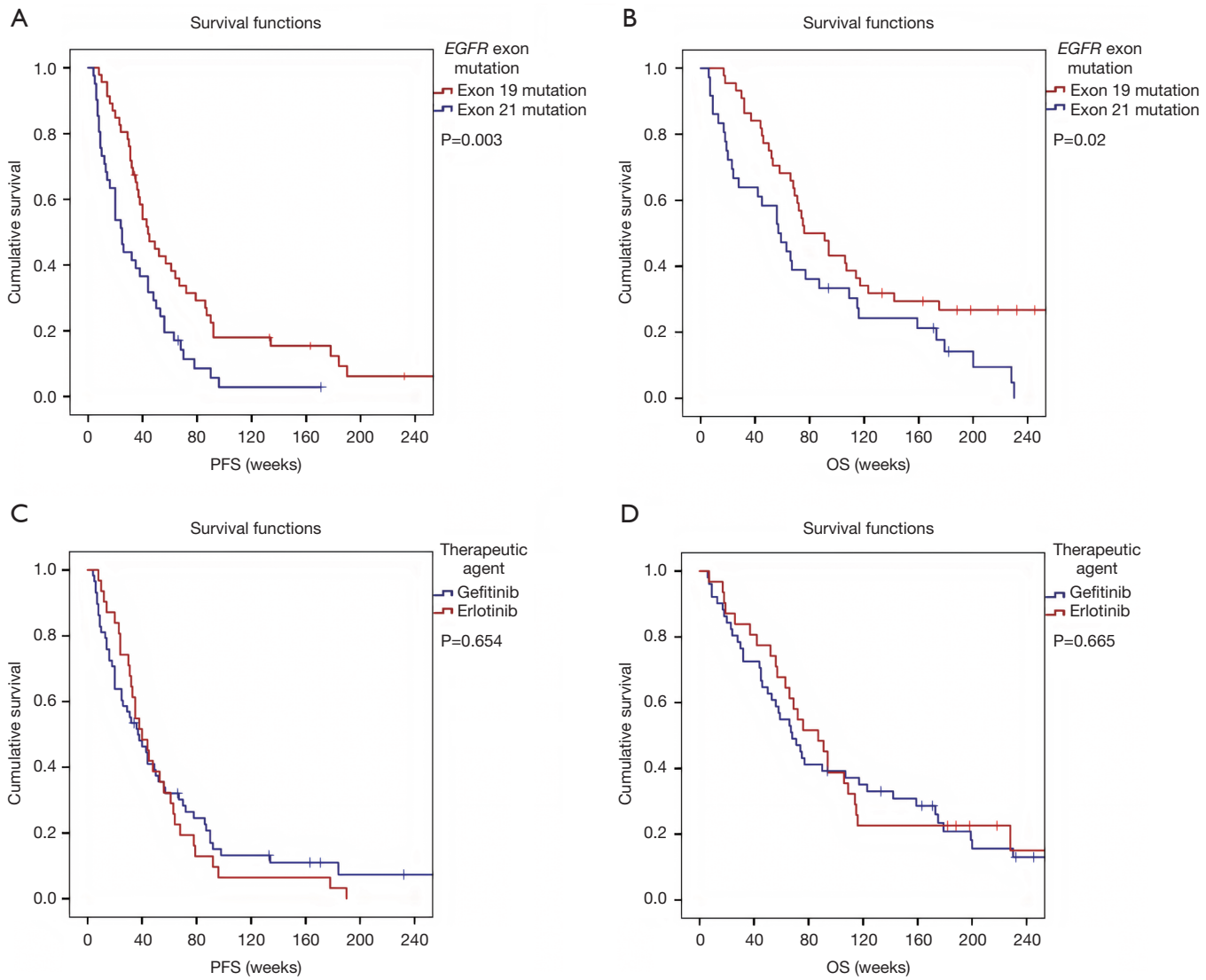

Figure 2 Kaplan-Meier plots for PFS and OS in patients with LADC according to specific *EGFR* exon mutations and therapeutic approaches. (A) LADC patients with tumors harboring *EGFR* exon 21 mutations had significantly shorter median PFS than those with exon 19 mutations (median PFSs were 25 *vs.* 44 weeks, respectively; P=0.003, log-rank test). (B) *EGFR* exon 21 mutation was also associated with significantly shorter OS in these patients (*vs. EGFR* exon 19 mutations, median OSs were 57 *vs.* 76 weeks, respectively; P=0.02, log-rank test). (C) No significant differences in PFS have been observed in patients treated with gefitinib *vs.* erlotinib (median PFSs were 37 *vs.* 40 weeks, respectively; P=0.654, log-rank test). (D) Similarly, the OS also did not differ significantly between the patients treated with gefitinib *vs.* erlotinib (median OSs were 68 *vs.* 87 weeks, respectively; P=0.665, log-rank test). PFS, progression-free survival; OS, overall survival; LADC, lung adenocarcinoma; EGFR, epidermal growth factor receptor.

relevant molecular pathological characteristics is crucial (28,29). Targeting EGFR is a promising strategy for treating LADC patients, since numerous studies over the past decade have shown that the TKI inhibitors gefitinib and erlotinib are effective for advanced-stage NSCLCs harboring *EGFR* sensitizing mutations (30,31). Still, the efficacy of TKIs is not consistent for every patient and not all patients with *EGFR*-activating mutation show similar response rates and PFSs (18). Hence, there is an urgent need for identifying valid predictive and prognostic factors that enable clinicians to effectively select the patients who may benefit more from EGFR-TKI treatment. Early

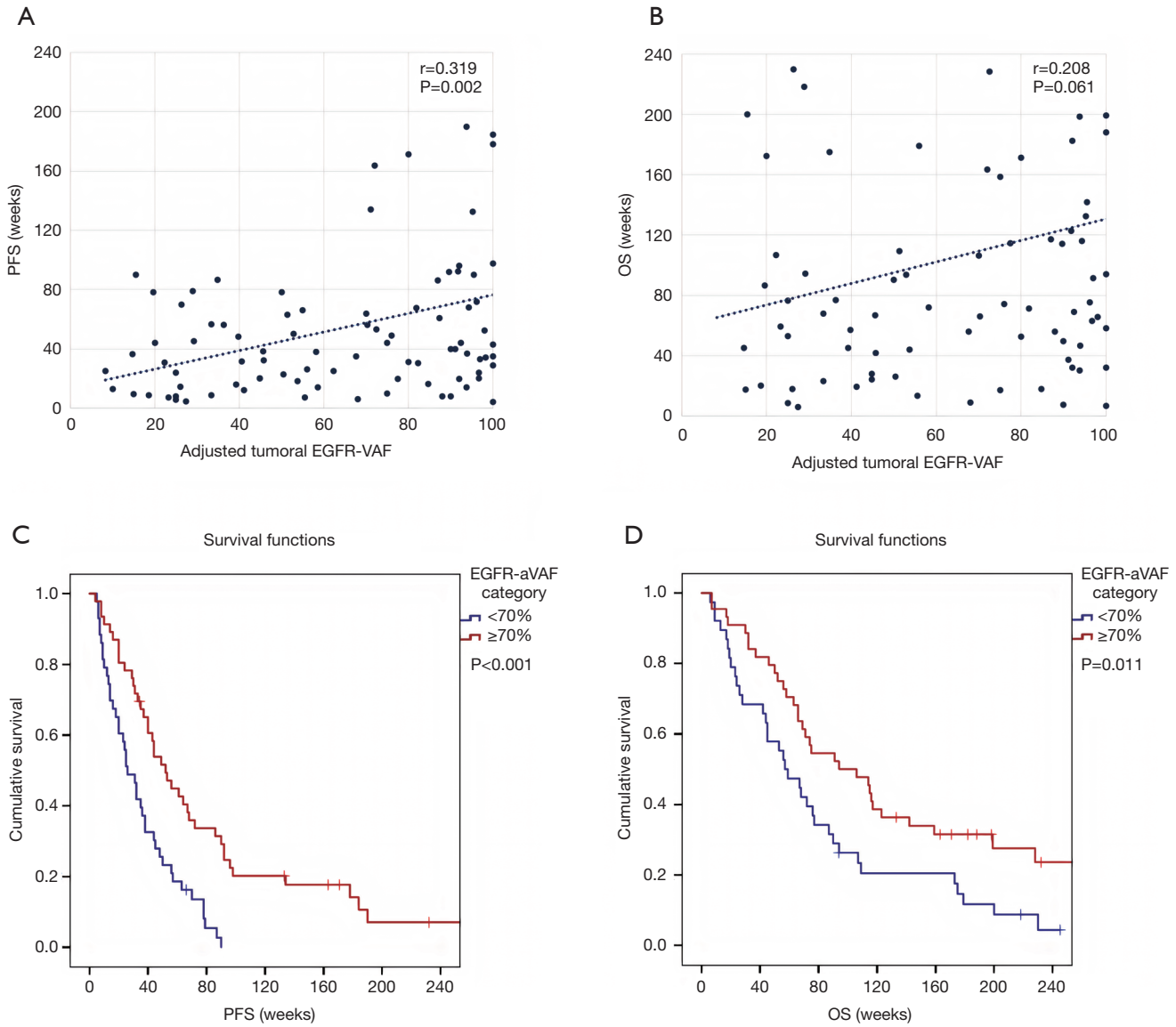

Figure 3 Scatter plots and Kaplan-Meier estimates for PFS and OS in LADC patients according to EGFR-aVAF. (A) Scatter plot showing significant positive linear correlation between tumoral EGFR-aVAF and PFS (r=0.319; P=0.002, Spearman's correlation) (each dot represents a single patient, and the dashed line shows the linear trendline). (B) Statistically non-significant, although clinically notable correlation was found between EGFR-VAF and OS (r=0.208; P=0.061, Spearman's correlation). (C) Patients with tumoral EGFR-aVAF ≥70% had significantly longer PFS than those in the EGFR-aVAF low (<70%) group (median PFSs were 52 *vs.* 26 weeks, respectively; P<0.001, log-rank test). (D) Similarly, the median OS was also significantly increased in patients with high (≥70%) EGFR-aVAF [*vs.* those with low (<70%) EGFR-aVAF, median OSs were 94 *vs.* 57 weeks, respectively; P=0.011, log-rank test]. PFS, progression-free survival; OS, overall survival; LADC, lung adenocarcinoma; EGFR, epidermal growth factor receptor; EGFR-aVAF, adjusted *EGFR* variant allele frequency.

in 2011, Zhou *et al.* reported that the relative *EGFR* mutational abundance might predict the therapy response to gefitinib in advanced-stage Asian NSCLC patients, yet the predictive value and clinicopathological significance of EGFR-aVAF is still controversial, especially in Caucasian patients (19). Therefore, the aim of this study was to assess the clinicopathological significance of EGFR-aVAF and to evaluate its predictive and prognostic relevance in a

Table 2 Multivariate Cox regression model for clinicopathological variables influencing the PFS

Clinicopathological parameters	PFS
EGFR-aVAF (continuous)	
HR	–0.009
95% CI	(0.982–0.999)
P	0.042
EGFR exon mutation (exon 19 *vs.* exon 21)	
HR	0.284
95% CI	(1.017–1.735)
P	0.037
Age (continuous)	
HR	–0.021
95% CI	(0.958–1.001)
P	0.06
Gender (male *vs.* female)	
HR	0.460
95% CI	(0.913–2.747)
P	0.102
Therapeutic agent (gefitinib *vs.* erlotinib)	
HR	–0.032
95% CI	(0.595–1.579)
P	0.899
Treatment line (first- *vs.* second-line)	
HR	–0.013
95% CI	(0.607–1.603)
P	0.957

PFS, progression-free survival; EGFR, epidermal growth factor receptor; EGFR-aVAF, adjusted *EGFR* variant allele frequency; HR, hazard ratio; CI, confidence interval.

homogenous cohort of Hungarian LADC patients treated with EGFR-TKIs.

First, we analyzed the association of major clinicopathological characteristics and tumoral EGFR-aVAF. Our results revealed that a considerable proportion of LADCs contain a heterogeneous population of both *EGFR* mutated and non-mutated cancer cells since the majority of all included cases showed an EGFR-aVAF between 5% and 94% and only 17 patients exhibited EGFR-aVAF ≥95%. This finding is in line with previously published data also suggesting that only a certain percentage of TCs carry heterozygous activating mutations in NSCLC patients, while other TCs carry wild-type *EGFR* (21,22). Accordingly, this might explain the controversial response rates seen in EGFR-TKI-treated patients. In the current study, 2.2% of patients carried exon 18 *EGFR* mutations, therefore the incidence rate is similar to other studies. However, due to the small number of patients harboring exon 18 mutations, subgroup specific statistical calculations were performed without these patients (15). Importantly, we found that the aVAF of the tumoral tissue was significantly higher in patients harboring *EGFR* exon 19 mutations than those with exon 21 mutated tumors. This ratio is in line with a previously published Asian study, however, to the best of our knowledge, ours is the first detailed evaluation of tumoral EGFR-aVAF with regards to specific *EGFR* exon mutations in Caucasian patients (23).

Next, in order to assess the clinical relevance of this heterogeneity in EGFR-aVAF between the patients harboring exon 19 *vs.* exon 21 mutations, we investigated the prognostic and predictive relevance of the aforementioned *EGFR* exon alterations. As expected, patients harboring *EGFR* exon 19 mutations indeed had significantly longer PFS than those with *EGFR* exon 21 mutations. These findings are in line with previously published data also suggesting a significant advantage in PFS for patients carrying exon 19 deletions in comparison with those carrying *EGFR* exon 21 mutations (32-35). In addition, based on a recent study on 55 metastatic NSCLC patients, exon 19-mutated patients tend to have better survival outcomes than patients with exon 18 point-mutations as well (15). To date, the mechanism underlying the different sensitivities to EGFR-TKI treatment between exon 19 and exon 21 mutated tumors remains to be elucidated (34). Based on our results a possible explanation might be that EGFR-aVAF of tumoral tissue is significantly higher in *EGFR* exon 19 mutated patients compared to patients harboring exon 21 mutations and thus EGFR-TKIs might be more effective in these patients. Meanwhile, others suggest that the better survival outcomes with *EGFR* exon 19 than exon 21 mutations might be due to differential inhibition of downstream signals, since EGFR-TKIs inhibit the phosphorylation of EGFR, Akt, and Erk to a greater degree in exon 19 deletion cells than in exon 21 mutated cells (36). Furthermore, an additional explanation might be that exon 19 deletions and 21 mutations present different intrinsic sensitivities to the EGFR-TKIs (34,37). Importantly, different mutations in the same exon might

also indicate different predictive roles since non-L747 to E749 (LRE) deletions has a worse response to TKIs than LRE deletions but we had no data on the type of deletions in exon 19 (38). Altogether, the biology that lies behind the responsiveness to EGFR-TKIs with regards to *EGFR* mutational subtypes is yet to be elucidated, however, our findings might provide background for future studies. In line with the PFS data, *EGFR* exon 19 mutations were also associated with improved OS compared to exon 21 mutations. As for treatment-related data, no significant differences were observed in PFS or OS regarding treatment line and therapeutic agents, which is in line with the findings of others (39-42).

Finally, we investigated the predictive and prognostic relevance of tumoral EGFR-aVAF and a statistically significant moderate positive linear correlation was found between EGFR-aVAF and PFS. Notably, we also found that high (≥70%) tumoral EGFR-aVAF was associated both with improved median PFS and OS, with a clinically relevant difference between low and high subgroups of 26 and 37 weeks, respectively. It should be noted, however, that the patients were divided into low and high EGFR-aVAF subgroups based on the median value in our dataset, therefore, until further validation, caution is needed when using it as a cut-off value in future studies. Our results are of high clinical importance because previous studies have only focused on whether the mutation is positive, and only a few investigated the predictive role of the relative *EGFR* mutational abundance (19,23,24). Yet, to our knowledge, our study is the first investigating the predictive and prognostic relevance of the exact value of EGFR-aVAF in Caucasian patients and, moreover, the first suggesting a clinically relevant threshold for predicting treatment response in these patients. In support of this, multivariate Cox regression analysis also revealed that EGFR-aVAF at diagnosis influenced PFS independently from age, gender, therapeutic agent, treatment line, and type of *EGFR* exon mutation. These results might partly explain why the efficacy of TKIs is not consistent for every patient harboring a certain type of *EGFR* mutation. Accordingly, quantitative diagnosis methods of EGFR-aVAF may help to select patients who are most or least likely to benefit from EGFR-TKIs. Importantly, however, current clinical treatment protocols with regards to EFGR-TKI are still primarily based on the absence or presence of activating *EGFR* mutations (25). Accordingly, until future validation, the clinicians should choose the most appropriate treatment for their patients regardless of EGFR-aVAF status. Nevertheless, changes in EGFR-aVAF might also occur during cancer progression and therapy. For instance, a recent study suggests that cancer genome in colorectal cancer patients adapts dynamically to pulsatile drug schedules and the abundance of resistance mutations could increase after long-time targeted therapies (43). Therefore, dynamic monitoring of EGFR-aVAF during therapy is also warranted.

There are several limitations in our study. Despite the fact that our cohort was homogenous the final number of patients harboring *EGFR* mutations was relatively small due to our strict inclusion/exclusion criteria. Nevertheless, our cohort provided the opportunity to draw some conclusions that evidently need to be validated in additional studies. Another limitation of our study is its retrospective nature with given limitations in interpreting the results. Thus, some of our results need to be confirmed in a prospective setting. Loss of heterozygosity and *EGFR* amplification occurs frequently in LADC patients harboring *EGFR* activating mutations and could serve as an indicator for better response from EGFR-TKI treatment (44-46). Accordingly, both of the aforementioned genetic alterations might also correlate with higher aVAF values, yet we did not investigate the presence of these alterations since they are not part of the routine mutational analyses in Hungary. Finally, all included patients were treated with first-generation EGFR-TKI erlotinib and gefitinib, yet these inhibitors are being slowly replaced by second- and third-generation EGFR-TKIs in the clinical practice. All in all, taken into account all the aforementioned potential study limitations, caution is needed when interpreting the results of the present study and further analyses are warranted to clarify the exact predictive role of EGFR-aVAF in EGFR-TKI-treated LADC patients.

Conclusions

To conclude, our study suggests that EGFR-aVAF of tumoral tissue predicts the extent of benefit from EGFR-TKI treatment. Moreover, in regards with exon specific mutations, the average EGFR-aVAF is higher among patients with exon 19 deletions thus confirming the longer PFS and OS of these patients. Our results might as well explain why the duration of response of some *EGFR* mutant patients was not as long as expected when no resistance related abnormality was found. Altogether, by shedding light on the predictive and prognostic relevance of EGFR-aVAF, our results might help to improve patient selection

and treatment in advanced-stage LADC patients harboring *EGFR*-sensitizing mutations.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was directed in accordance with the guidelines of the Helsinki Declaration (revised in 2013) of the World Medical Association. The study was approved by the national level ethics committee (Hungarian Scientific and Research Ethics Committee of the Medical Research Council, ETT-TUKEB, 7214-1/2016/EKU). The need for individual informed consent for this retrospective study was waived. After clinical information was collected, patient identifiers were removed, and subsequently, patients cannot be identified either directly or indirectly.

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