DOI:10.14753/SE.2022.2687

EVALUATION OF PREDICTIVE AND PROGNOSTIC BIOMARKERS IN THORACIC MALIGNANCIES

PhD Thesis

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Budapest

2022

DOI:10.14753/SE.2022.2687

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List of Abbreviations

aVAF	adjusted variant allele frequency
BM	biomarker
BSC	best supportive care
CHT	chemotherapy
CHT-RT	combined chemoradiotherapy
CI	confidence interval
ECOG	Eastern Cooperative Oncology Group
EGFR	epidermal growth factor receptor
EPP	extrapleural pneumonectomy
FFPE	formalin-fixed paraffin-embedded
HR	hazard ratio
LADC	lung adenocarcinomas
LMR	lymphocyte to monocyte ratio
MICE	multiple imputations by chain equation
MMT	multimodality treatment
MPM	malignant pleural mesothelioma
NCCN	National Comprehensive Cancer Network
NOS	not otherwise specified
NSCLC	non-small cell lung cancer
OS	overall survival
PFS	progression-free survival
PS	performance status
P/D	pleurectomy/decortication
PD-1	programmed cell death 1
PDL-1	programmed death ligand 1
RECIST	response evaluation criteria in solid tumors
RT	radiation therapy
TAM	tumor-associated macrophages
TC	tumor cells
TIL	tumor-infiltrating lymphocytes
TKI	tyrosine kinase inhibitors

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1. Introduction

1.1. Biomarkers

In recent decades, with the accessibility and application of different anti-tumor treatments, the survival of cancer patients has improved. Nevertheless, the overall survival rate in general is still relatively poor. Therefore, in order to improve the therapeutic outcomes through a better patient selection, tumor researchers continuously strive to identify novel prognostic- and predictive biomarkers (BMs).

A BM is a biological “sign” that can predict a clinically relevant endpoint or intermediate outcome. The National Institutes of Health has defined a BM as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[1]. The term "biomarker" has been first used in an article entitled: "A search for porphyrin biomarkers in Nonesuch Shale and extraterrestrial samples" in 1973[2]. Accordingly, BMs ideally help the clinical decision-making in a way that improves the survival of patients. Importantly, the benefits of clinical decisions must outweigh the harms of false-positive or -negative choices. In addition, a BM should minimize damage and costs without increasing cancer mortality in a risk management environment[3].

BMs can be found in several body fluids and secretions such as serum, plasma, urine or sweat. But more invasive techniques that require tumor tissue samples are also widely used for immunohistochemistry and DNA/RNA analysis. Prognostic BMs offer information about a patient's overall cancer outcome regardless of therapy. Therefore, the absence or presence of such prognostic markers may help to select patients for a particular treatment but does not predict the therapeutic response. Prognostic BMs can be divided into two subgroups. They can either provide information on recurrence in patients who receive treatment with curative intent or provide insights into the duration of survival (i.e. overall survival, OS) in patients with metastatic disease.

In contrast, predictive biomarkers provide information about the impact of different therapeutic interventions. Therefore, a predictive BM may also be a therapeutic target. A distinction can be made between pre-treatment and early predictive markers. The first can be used to select patients, whereas the second provides information in the early stages of therapy[4]. Current interest in marker determination is enhanced by discovering

pathological genes that have proven to be of clinical significance, such as epidermal growth factor receptor 1 (EGFR) mutations and programmed cell death 1/programmed death ligand 1 (PD-1/PD-L1) proteins. The aim of our study was to assess the clinicopathological relevance of the aforementioned BMs in thoracic malignancies.

1.2. Lung cancer

Despite novel diagnostic methods, emerging treatment options and personalized therapy, lung cancer remains the leading cause of cancer-related deaths worldwide. More than 2.21 million new lung cancer cases and 1.80 million lung cancer deaths were documented worldwide in 2020.[5] Most patients are still diagnosed at an advanced stage and have a limited prognosis, with an overall 5-year survival rate of 19,4% in the United States[6,7,8]. Of note, Hungary has high mortality rates of lung cancer both in men and women.[9]

Lung cancer is a heterogeneous malignancy with several histological subtypes. Importantly, these subtypes have widely different pathological and clinical features[10]. Histologically, non-small cell lung cancer (NSCLC) is the predominant lung cancer subtype, and more than 40% of all NSCLCs are LADCs[11]. However, not all LADCs are the same, and inter-tumoral heterogeneity exists both in terms of pathological and molecular charachteristics[12]. Therefore, there is an ongoing need to identify specific predictive BMs to facilitate patient selection for targeted therapy.[13] The search for these therapeutically relevant predictive biomarkers has changed the paradigm of lung cancer diagnosis[14].

1.3. Epidermal growth factor receptor mutations in lung adenocarcinomas

EGFR mutations are the second most common oncogenic driver alterations in LADC, accounting for approximately 15% of all LADCs in Caucasian patients and about 40% to 50% in Asian patients[15,16].

EGFR is a member of the ErbB family of tyrosine kinase receptors which is expressed in normal epithelial, mesenchymal, and neurogenic tissue with cytoplasmic kinase activity transducing important growth factors signaling[17,18]. However, in malignant tumors including LADC, EGFR is often constantly stimulated due to the sustained production in the tumor microenvironment of EGFR ligands or a mutation in EGFR itself that locks the

receptor in a state of continuous activation[19,20]. About 90% of activating EGFR mutations are short in-frame deletions in exon 19 or point mutations in exon 21 (often called "classical" EGFR mutations)[21,22]. 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[21,22]. Importantly, in LADC, these EGFR-sensitizing mutations confer sensitivity to first-, and next-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib, erlotinib, dacomitinib, afatinib and osimertinib in patients with advanced-stage disease[23–25]. 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 PFS [10.8 vs. 5.4 months in the chemotherapy (CHT) group; $p<0.001$] and OS (30.5 vs. 23.6 months in the CHT group; $p=0.31$) in patients who were selected based on EGFR-sensitizing mutations[26]. Still, the objective response rate to EGFR-TKIs in patients carrying EGFR-sensitizing mutations is only 70% to 80%, and while some patients show a clear survival benefit to TKIs, others fail to respond properly[27,28]. Therefore, 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 predictors of EGFR mutations rather than true treatment-related prognosticators for TKI efficacy[26,27,29,30]. Nevertheless, different EGFR mutation subtypes and molecular characteristics can also determine various predictive and prognostic features[27]. 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 carry heterozygous activating mutations, whereas other tumor cells have wild-type EGFR[31–34].

1.4. Malignant pleural mesothelioma

Malignant pleural mesothelioma (MPM) is a rare and aggressive malignancy arising from the pleural mesothelium. The OS of MPM patients' is ranging from 10 to 20 months, depending on the stage and histological subtype[35–38]. Platinum-based chemotherapy (CHT) has been used in MPM treatment and remains the backbone for current combination strategies[39]. Recent advances in multidisciplinary therapeutic approaches, including surgery, CHT, and radiation therapy (RT) have improved the OS in highly

selected patients[40–44]. Moreover, recent phase I/II trials have shown some benefit of immunotherapy in MPM. Still, single-agent checkpoint inhibitors were so far not demonstrated to be superior to standard CHT in more extensive phase III trials[45–47]. Nevertheless, a recent phase III study investigating the efficacy of first-line nivolumab plus ipilimumab (vs. platinum doublet CHT) showed promising results regarding OS[48]. Of note, however, the PFS was similar between the treatment arms even in case of combination immunotherapy[48]. Altogether, selecting MPM patients for appropriate therapeutic approaches remains a crucial problem, resulting in an unmet need to identify prognostic BMs which can predict the OS.

1.5. PD-L1 and PD-1 expressions in cancer

In recent years, immunotherapy strategies against cancer have emerged as a powerful tool for the treatment of different tumoral entities. PD-1 plays a crucial role in inhibiting the immune reactions and stimulating self-tolerance by activating antigen-specific T cell apoptosis, inhibiting regulatory T cell apoptosis and modulating T cell activity. Meanwhile, PD-L1 is a transmembrane protein that is considered to be a co-inhibitory factor of the immune response. Accordingly, by combining with PD-1, it can reduce the proliferation of PD-1 positive cells, inhibit their cytokine secretion and induce apoptosis[49]. Besides their potential to predict the efficacy of immunotherapy, PD-L1 and PD-1 expressions have shown conflicting results regarding their prognostic significance[50]. Specifically, high PD-L1 levels were associated with impaired prognosis in renal and gastric cancers, but with favorable outcomes in primary colorectal cancer and thymic carcinoma[50–52]. In lung cancer, immunotherapy is a well-established first-line treatment and its use is primarily based on the predictive role of PD-L1 expression[53]. Although the majority of studies concluded studies reached the same conclusion. Therefore, the prognostic significance of PD-L1 is rather controversial in lung cancer[54–57]. With regards to MPM, currently, only limited data is available on the prevalence and prognostic role of PD-L1 and PD-1 expression. The exact role of these tissue BMs in predicting MPM outcome remains thus controversial[51,58–61].

2. EGFR variant allele frequency as a potential biomarker in predicting the survival outcomes of EGFR-TKI-treated lung adenocarcinoma patients

2.1. Objectives

In the era of precision and individualized cancer therapy, finding appropriate BMs is crucial[62,63]. Targeting EGFR is a promising strategy for treating LADC patients since numerous studies over the past decade have shown that TKI inhibitors gefitinib and erlotinib are effective in advanced-stage NSCLCs harboring EGFR sensitizing mutations[64,65].

Previous studies on Asian patients suggest that higher relative EGFR mutational abundance might predict the efficacy of EGFR-TKI treatment[31,66,67]. However, the biological and clinical relevance of adjusted tumoral EGFR variant allele frequency (EGFR-aVAF) in disease prognosis and clinical response to EGFR-TKIs is still mostly unclear. Therefore, to improve patient selection and better understand the influence of EGFR-aVAF in this setting regarding therapeutic approaches, we aimed to assess the relationship between EGFR-aVAF and response to EGFR-TKIs in a homogenous patient cohort of Caucasian LADC patients.

2.2. Results

2.2.1. 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 2. All patients had an advanced-stage disease and Caucasian background. The 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. The 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 the 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 according to age ($p=0.93$), gender ($p=0.809$), or smoking history ($p=0.467$).

DOI:10.14753/SE.2022.2687

Table 2. Patient characteristics and adjusted tumoral EGFR VAF in human LADC

Characteristic	Number of Patients (%)	Mean EGFR-aVAF	p value[a]
All patients	89 (100%)		
Age (years)			
<65	36 (40.4%)	63.53%	0.93[b]
≥65	53 (59.6%)	64.6%	
Gender			
male	25 (28.1%)	64.12%	0.809[b]
female	64 (71.9%)	64.19%	
Smoking history			
never smoker	48 (51.7%)	64.46%	
ex-smoker	10 (11.2%)	73.3%	0.467[c]
current smoker	14 (15.7%)	58.5%	
no data	19 (21.3%)	-	
Therapeutic agent			
Gefitinib	58 (65.2%)	61.64%	0.428[b]
Erlotinib	31 (34.8%)	68.9%	
Treatment line			
1st-line	46 (51.7%)	63.35%	0.882[b]
2nd-line	43 (48.3%)	65.05%	
EGFR exon mutation			
exon 18	2 (2.2%)	-	
exon 19	46 (51.7%)	75.04%	**<0.001[b]**
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. (Gieszer B, Megyesfalvi Zs, Dulai Vet al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

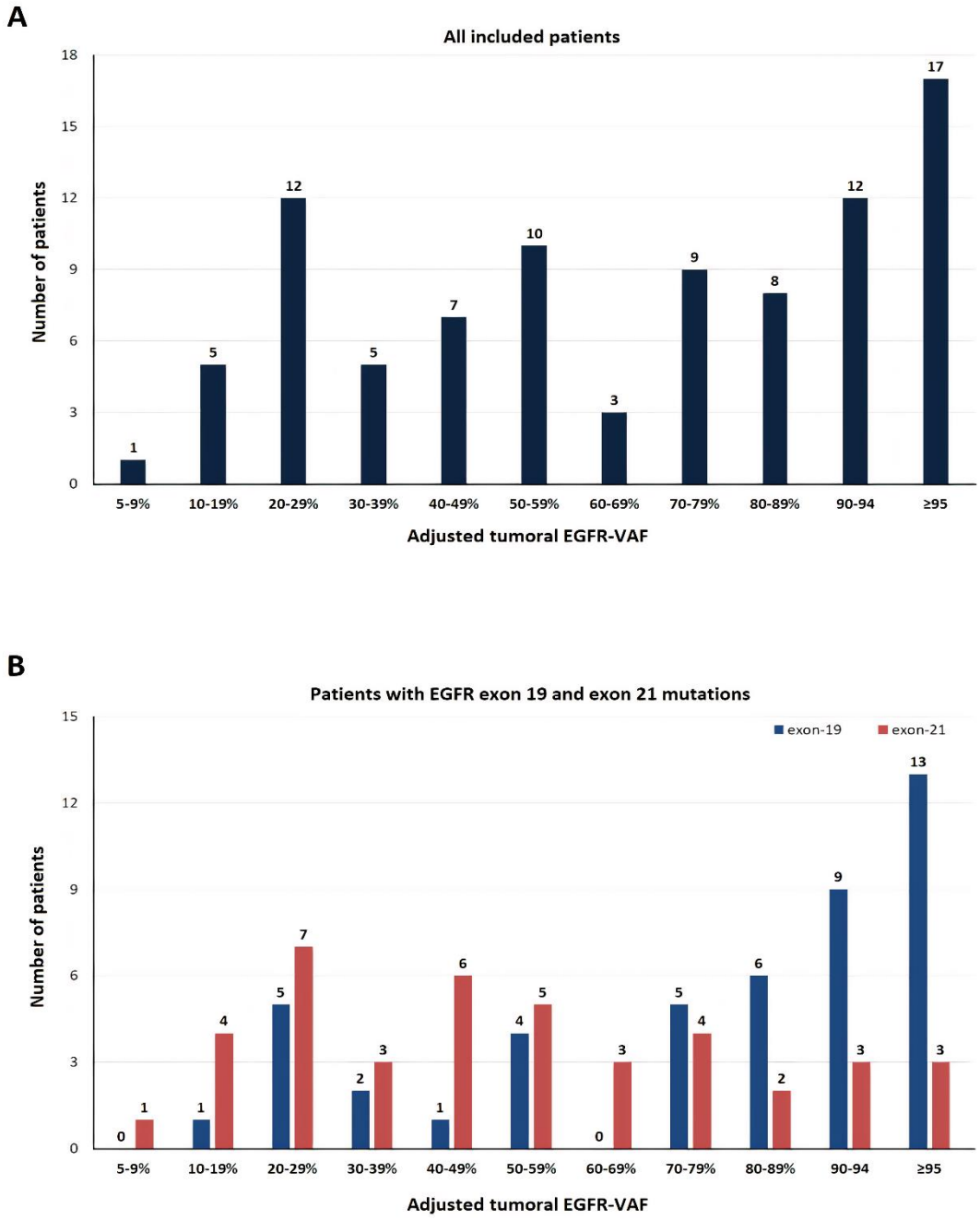

Figure 1 *EGFR-aVAF of tumoral tissue in LADC patients. (A) Bar chart illustrating the distribution of all included LADC patients (n=89), according to tumoral EGFR-aVAF irrespective of specific exon mutations. (B) Distribution of LADC patients diagnosed with EGFR exon 19 and exon 21 mutations (n=46 and n=41, respectively). EGFR, epidermal growth factor receptor; EGFR-aVAF, adjusted EGFR variant allele frequency; LADC, lung adenocarcinoma. (Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)*

2.2.2. EGFR exon 19 mutation associates with superior survival outcomes

The median PFS and OS of the total cohort was 38 and 72 weeks, respectively. At 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 the 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). Regarding the administered therapeutic agents, no significant differences have been observed either in PFS (p=0.654; Figure 2C) or 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 either (Figure 3A, 3B). As for smoking history, there was no significant difference in PFS between never-smoker versus ever-smoker patients (p=0.099; Figure 3C). 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 3D).

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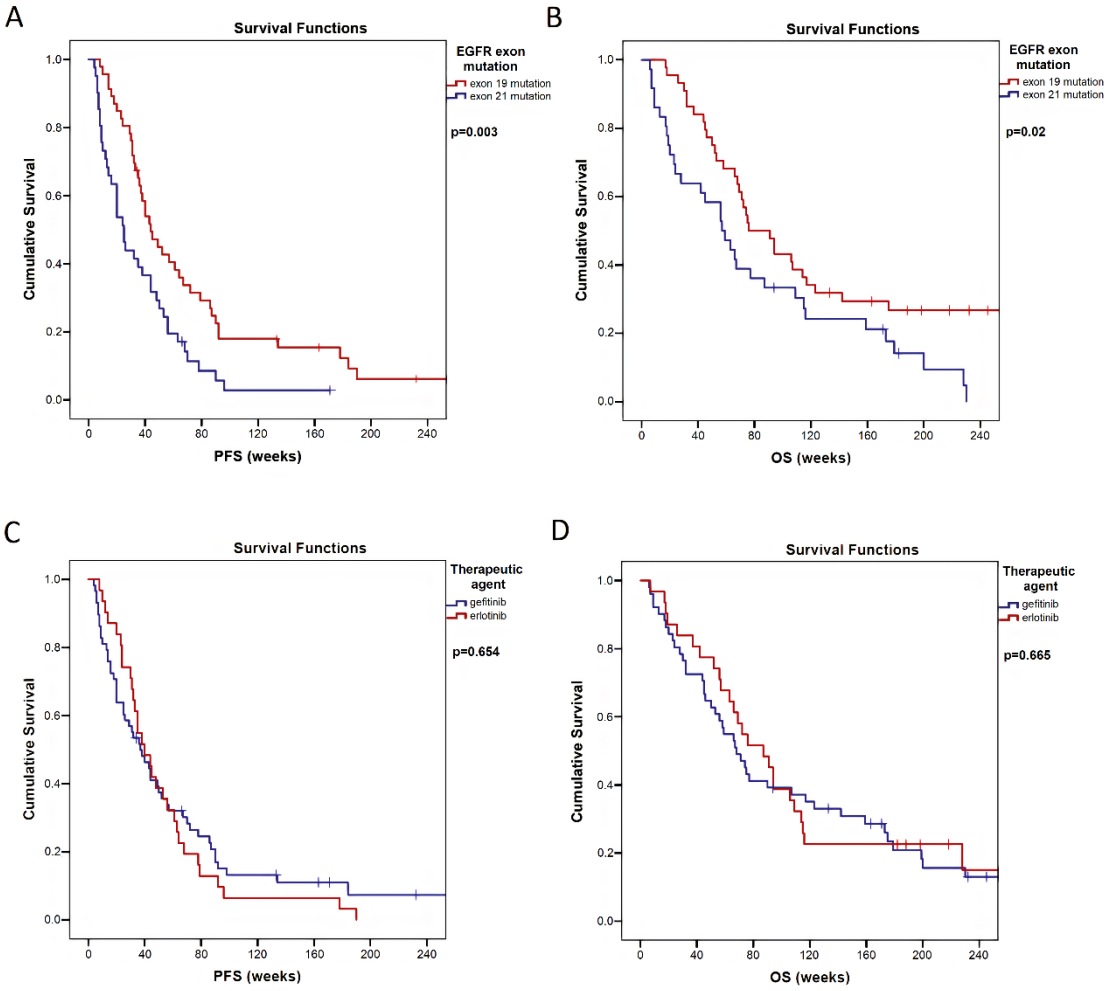

Figure 2 *Kaplan-Meier plots for PFS and OS in patients with LADC according to specific EGFR exon mutations and therapeutic approaches. (A) LADC patients with tumors harboring EGFR exon 21 mutations had a significantly shorter median PFS than those with exon 19 mutations (median PFSs were 25 vs. 44 weeks, 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 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.*
(Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

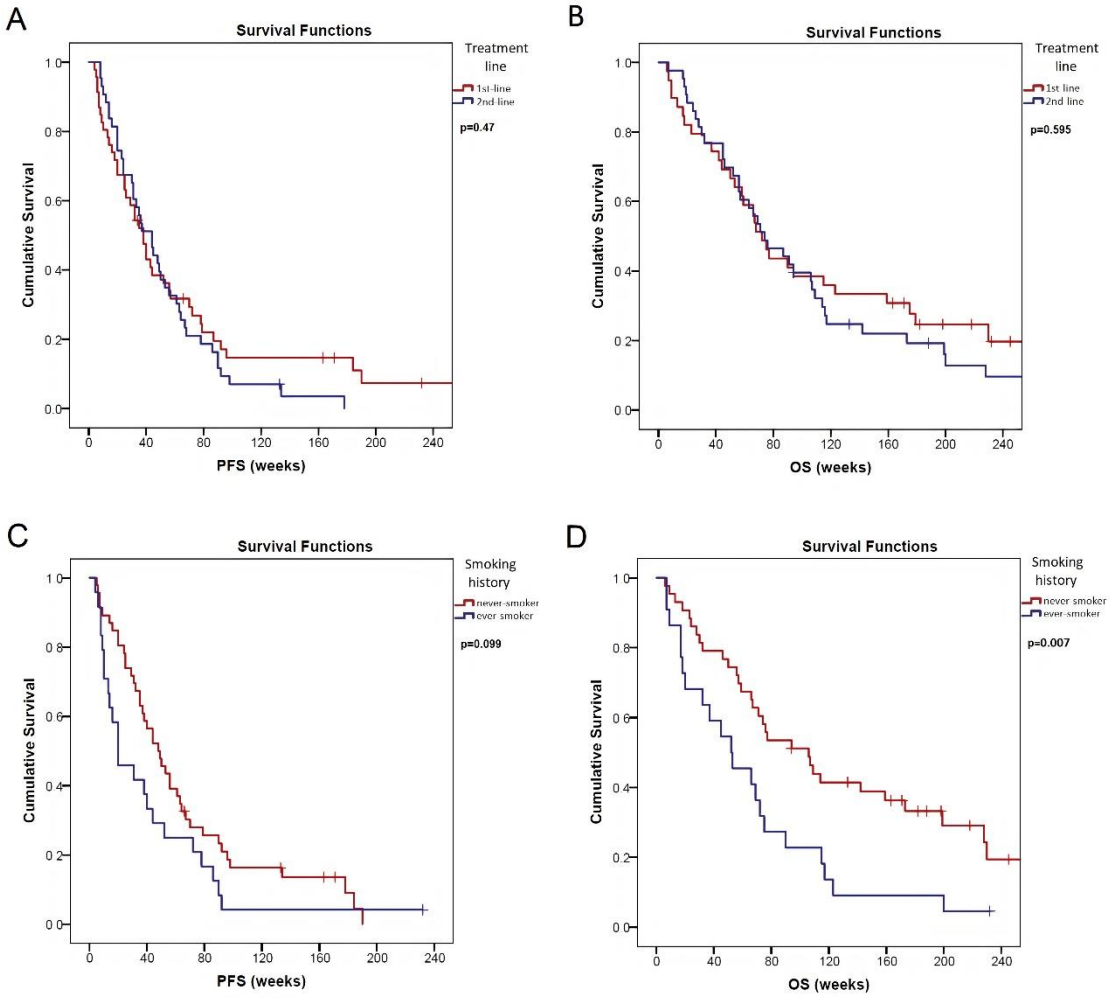

Figure 3 *Comparison of survival outcomes in patients with advanced LADC with regards to treatment line and smoking status. (A) No significant differences in PFS have been observed between patients receiving EGFR-TKI in first- vs. second-line (median PFSs were 38 vs. 44 weeks, respectively; p=0.47, log-rank test). (B) Patients receiving EGFR-TKI in first-line had a similar OS compared to patients receiving EGFR-TKI in second-line (median OSs were 72 vs. 74 weeks, respectively; p=0.595, log rank-test). (C) Statistically non-significant, although the clinically notable difference was found in PFS between never-smoker and ever-smoker patients (median PFSs were 48 vs. 20 weeks, respectively; p=0.099, log-rank test). (D) Never-smoker patients had significantly improved OS (vs. ever-smokers; median OSs were 106 vs. 52 weeks, respectively; p=0.007, log-rank test). LADC, lung adenocarcinoma; PFS, progression-free survival; EGFR, epidermal growth factor receptor; EGFR-TKI, EGFR tyrosine kinase inhibitor; OS, overall survival*

(Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

2.2.3. 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 regarding adjusted tumoral variant allele frequencies. Notably, a statistically significant positive linear correlation was found between EGFR-aVAF and PFS (r=0.319; p=0.002, Spearman's correlation; Figure 4A). In contrast, no significant correlation was found between EGFR-aVAF and OS, although the correlation coefficient was clinically notable (r=0.208; p=0.061, Spearman's correlation; Figure 4B). 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 4C). 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 4D).

In order to assess if the predictive value of tumoral EGFR-aVAF was independent of other clinicopathological factors, we performed a multivariate Cox regression analysis (Table 3). 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 3]. 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.

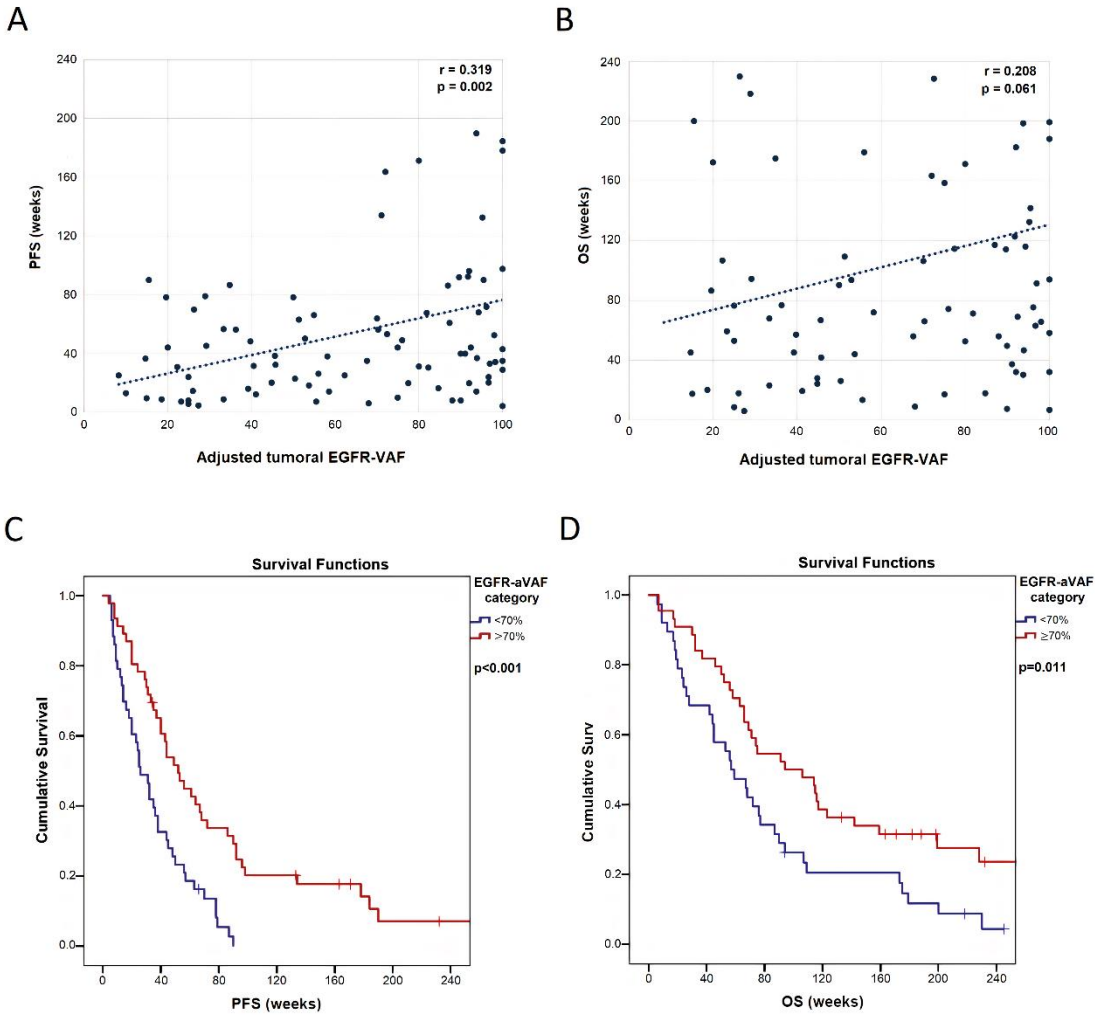

Figure 4 *Scatter plots and Kaplan-Meier estimates for PFS and OS in LADC patients according to EGFR-aVAF. (A) Scatter plot showing a significant positive linear correlation between tumoral EGFR-aVAF and PFS (r=0.319; p=0.002, Spearman's correlation) (each dot represents a single patient, and the dashed 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.*
(Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

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Table 3. Multivariate Cox Regression model for clinicopathological variables influencing the PFS

	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 (1st-line vs. 2nd-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

3. Prognostic impact of PD-1 and PD-L1 expression in malignant pleural mesothelioma

3.1. Objectives

The poor survival outcomes in MPM and the lack of effective therapies require novel therapeutic strategies. Hence, there is an urgent need for identifying specific prognostic and predictive BMs that enable clinicians to allocate patients to appropriate treatment groups.

Currently, only limited data is available on the prevalence and prognostic role of PD-L1 and PD-1 expression in MPM. Previous studies suggest that high PD-L1 expression might be associated with impaired survival outcomes in MPM, yet the prognostic value and clinicopathological significance of both PD-L1 and PD-1 are still controversial[51,60,68].

To further explore the expression and prognostic impact of PD-L1 and PD-1 of TCs and TILs, our multi-institutional study aimed to investigate the expression patterns of these molecules and their relationship with clinicopathologic parameters and long-term outcome in human MPM.

3.2. Results

3.2.1. Correlation of clinicopathological variables with PD-L1/PD-1 expression

203 MPM patients were enrolled in the study whose clinicopathological characteristics are summarized in Table 4 and 5. The full cohort comprised 151 (75%) epithelioid and 39 (19%) non-epithelioid (i.e. biphasic or sarcomatoid) MPMs. Thirteen (6%) cases were classified as MPM not otherwise specified (NOS). The median age of all cases was 64 years (range 27-86) and patients were predominantly male (71.4%). At diagnosis, 63 (31%) and 99 (49%) cases had IMIG/TNM stage I-II and stage III-IV disease, respectively. Twenty-nine (14%) patients received multimodality treatment (MMT), including surgery, while 113 (56%) patients underwent other therapeutic approaches such as CHT, RT, CHT/RT or BSC. In case of 61 patients, treatment-related data was not available. PD-L1 expression was measured in both of the TC and TIL populations. Meanwhile, PD-1 expression was analyzed solely in TILs because we did not observe any positivity on TCs. Out of all 203 cases, 152 (75%) cases did not show any TC PD-

L1 expression. Of the 51 (25%) cases who were categorized as TC/PD-L1 positive (≥1%), the tumor samples of 33 (16%) and 18 (8%) patients were categorized by TC/PD-L1 scores "low" and "high", respectively (Figure 5A). Representative images of PD-L1 expressions of TCs are shown in Figure 5B. Eligible MPM tissue for investigating PD-L1 expression of TILs was available from 165 patients. PD-L1 TIL expression was rarely seen. Positive staining (PD-L1 TIL expression ≥1%) was found in 13 (8%) patients, and only 1 case exhibited a PD-L1 TIL expression >10% (Figure 5C). PD-1 expression of TILs could be measured in 164 patients. TIL PD-1 positivity (i.e. ≥1%) was found in 83 (50%) patients. A higher than 10% TIL PD-1 expression was observed in 39 (24%) patients (Figure 5D).

Next, we studied the correlation between clinicopathological parameters and PD-L1 and PD-1 expression of TCs and TILs. No significant correlation was found between PD-L1 or PD-1 TC or TIL expressions and clinical variables such as age, gender, histological subtype or tumor stage when patients were dichotomized into PD-L1 and PD-1 negative (no staining) vs. positive (≥ 1% staining) categories. Of note, using cut-off values of 10% (Table 4,5) or 50% (data not shown) for PD-L1 or PD-1 expressions did not yield significant associations either. It is also important to mention that we did not find significant associations between TC or TIL PD-L1/PD-1 expressions and histological subtypes or therapeutic modality (Table 4,5).

DOI:10.14753/SE.2022.2687

Table 4. Patient characteristics and PD-L1 expression of TCs in human MPM

Variables	No. of patients (%)	PD-L1 expression		p value[a]
		PD-L1 ≤ 10%	PD-L1 > 10%	
All patients	203	185 (91.1%)	18 (8.9%)	
Age (years)[b]				
<65	104 (51.2%)	95	9	0.245
≥65	99 (48.8%)	90	9	
Gender[c]				
Male	145 (71.4%)	133	12	0.639
Female	58 (28.6%)	52	6	
Histology[c]				
Epithelioid	151 (74.4%)	140	11	0.328
Non-epithelioid	39 (19.2%)	34	5	
No data	13 (6.4%)	11	2	
Treatment[c]				
Multimodality	29 (14.3%)	28	1	0.306
Other[d]	113 (55.7%)	99	14	
No data	61 (30%)	58	3	
Stage[c]				
Early (I/II)	63 (31%)	56	7	0.837
Late (III/IV)	99 (48.8%)	89	10	
No data	41 (20.2%)	40	1	
Medical Center[c]				
#1	42 (20.7%)	38	4	0.285
#2	39 (19.2%)	36	3	
#3	38 (18.7%)	34	4	
#4	46 (22.7%)	45	1	
#5	38 (18.7%)	32	6	

[a], P values refer to PD-L1$_{high}$ versus PD-L1$_{low}$ subgroups; [b], Student's t-test is used in case of continuous variable (age); [c], χ^2 test or Fisher's exact test are used between categorical variables; [d], CHT, RT, CHT/RT or BSC. PD-L1, programmed death ligand 1; TC, tumor cell; MPM, malignant pleural mesothelioma

DOI:10.14753/SE.2022.2687

Table 5. Patient characteristics and PD-1 expression of TILs in human MPM

Variables	No. of Patients (%)	PD-1 expression		p value[a]
		PD-1 ≤ 10%	PD-1 > 10%	
All patients	164	125 (76.2%)	39 (23.8%)	
Age (years)[b]				
<65	92 (56.1%)	69	23	0.754
≥65	72 (43.9%)	56	16	
Gender[c]				
Male	118 (72%)	92	26	0.401
Female	46 (28%)	33	13	
Histology[c]				
Epithelioid	121 (73.3%)	95	26	0.826
Non-epithelioid	30 (18.3%)	23	7	
No data	13 (7.9%)	7	6	
Treatment[c]				
Multimodality	27 (16.5%)	19	8	0.541
Other[d]	76 (46.3%)	58	18	
No data	61 (37.2%)	48	13	
Stage[c]				
Early (I/II)	39 (23.8%)	29	10	0.604
Late (III/IV)	84 (51.2%)	66	18	
No data	41 (25%)	30	11	
Medical Center[c]				
#1	41 (25%)	32	9	0.362
#2	39 (23.8%)	32	7	
#3	38 (23.2%)	25	13	
#4	46 (28%)	36	10	
#5	No data	No data	No data	

[a], P values refer to PD-1$_{high}$ versus PD-1low subgroups; [b] Student's t-test is used in case of continuous variable (age); [c], χ^2 test or Fisher's exact test are used between categorical variables. [d], CHT, RT, CHT/RT or BSC. PD-1, programmed death 1; TILs, tumor-infiltrating lymphocytes

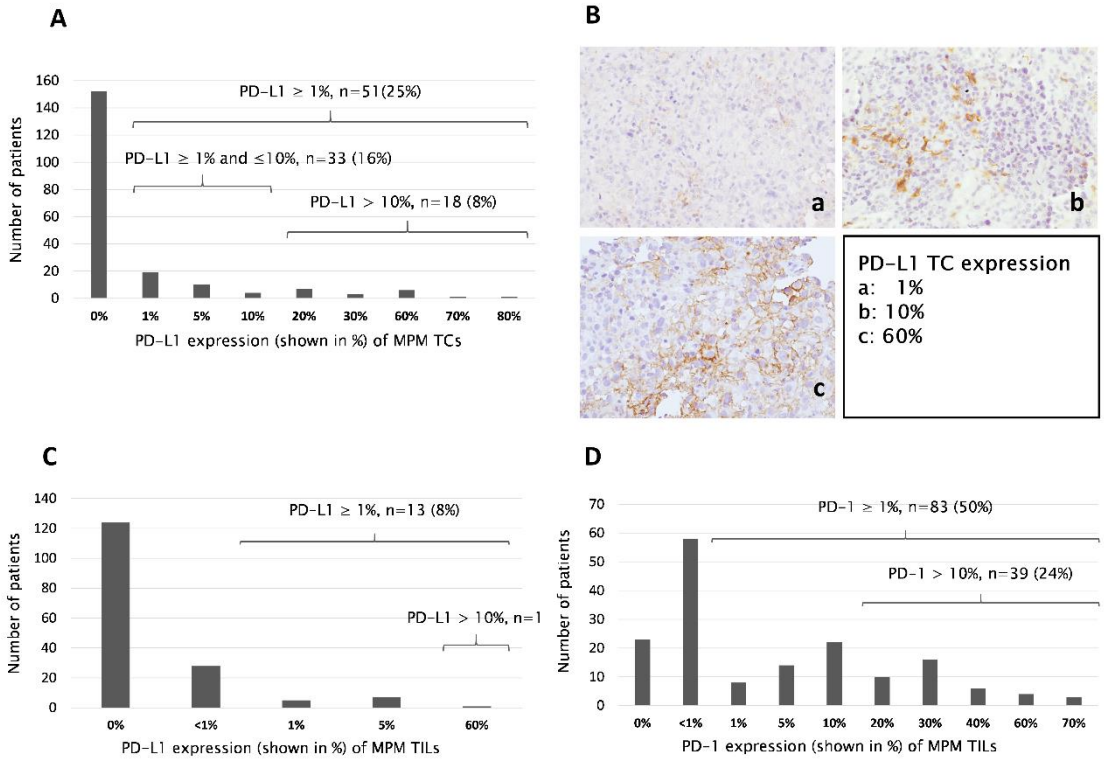

Figure 5 *PD-L1 and PD-1 expression of TCs and TILs in MPM patients. (A) Of all 203 patients, 51 (25%) showed any (≥1%) PD- L1 expression in their TCs. Out of these patients, 18 (8%) were categorized as TC PD-L1 "high". (B) Representative images of PD-L1 expressing TCs in MPM. Immune staining was performed with monoclonal PD-L1 antibodies (Cell Signaling, clone E1L3N, dilution 1:25). All images were captured at a magnification of ×200. (C) No or low (<1%) PD-L1 TIL expression was detected in 152 (92%) patients, while PD-L1 TIL expression of ≥1% was found in 13 (8%) patients. (D) ≥1% PD-1 TIL expression was found in 83 (50%) patients. Of these cases, 39 (24%) patients had a PD-1 TIL expression higher than 10%. PD-L1, programmed death ligand 1; PD-1, programmed cell death 1; TC, tumor cell; TIL, tumor-infiltrating lymphocytes; MPM, malignant pleural mesothelioma.*
(Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

3.2.2. Prognostic parameters and overall survival

The median follow-up time for all 203 patients was 12.8 months. The Median OS of the total cohort was 13.2 months (95% CI 10.6-15.8). First, we performed a univariate survival analysis in order to identify clinical prognostic factors for OS (Table 6). We found that patients with epithelioid histological subtype exhibited significantly improved OS compared to those with non-epithelioid MPM (median OSs were 13.2 vs. 12.7 months, respectively; HR 0.64, p=0.012, Figure 6A). Patients with stage I/II MPM (vs. stage III/IV, respectively, HR 0.66, p=0.01, Table 6 and Figure 6B) and patients receiving multimodality treatment (vs. other therapies, HR 0.32, p<0.001, Table 6 and Figure 6C)

were also associated with significantly improved OS. There were no significant associations between OS and gender (Figure 6D) or age (dichotomized at a cut-off of 65 years, data not shown).

Next, we examined the prognostic value of PD-L1 and PD-1 expression of TCs and TILs (Table 6). Our initial statistical analyses indicated that patients whose TCs did not express PD-L1 (median OS 14 months) had comparable OS to those with PD-L1 TC expressions between 1% and 10% (median OS 16 months, p=0.194, Figure 7A). We grouped patients accordingly into low (≤10%) and high (>10%) PD-L1 TC categories and found that low PD-L1 expression was significantly associated with improved OS (HR 0.39, p<0.001, Table 6 and Figure 7B). PD-L1 was rarely expressed by TILS, and there was no difference in the OS of patients whose tumor samples were categorized by a PD-L1 TIL score <1% (n=152) vs. ≥1% (median OSs were 15.1 vs. 11.8 months, HR 0.82, p=0.508, Table 6 and Figure 7C). Similarly, we could not show prognostic information from the PD-1 expression of TILs when patients were grouped into PD-1 TIL <1% vs. ≥1% and ≤10% vs. >10% categories (Table 6 and Figure 7D).

In order to assess if the prognostic value of PD-L1 TC expression was independent from significant clinical prognostic factors, we performed a multivariate Cox regression analysis with available data from 126 (62%) patients (Table 7). The model was adjusted for clinical factors such as age, gender, histological subtype, tumor stage at diagnosis and treatment. We found that PD-L1 TC expression at a 10% cut-off remained a significant prognostic factor for OS (low vs. high expression; HR 0.405, p=0.005; Table 7). Histological subtype (epithelioid vs. non-epithelioid; HR 0.504, p=0.009), tumor stage (I-II vs. III-IV; HR 0.545, p=0.007) and treatment (MMT vs. other therapies, HR 0.351, p<0.001) also independently influenced OS. As 126 (62%) patients only had completely available data for the multivariate model, we performed an exploratory multivariate Cox regression analysis, using a dataset after multiple imputations by MICE approach, including all 203 cases, in order to avoid the omission of data. In this exploratory analysis, PD-L1 TC expression remained as a significant prognostic factor for OS (HR 0.443, p=0.004), independent from age, gender, histologic subtype, stage and treatment (data not shown).

Table 6. Univariate survival analysis for 203 MPM patients from 5 European centers

Variables	Subgroups	median OS (mo)	p[c]	HR	95% CI
Age	< 65a	12.8	0.164	1.23	0.92 - 1.65
	≥ 65a	14.4			
Gender	female	11.2	0.725	1.06	0.77 - 1-45
	male	15.1			
Histology	epitheloid	13.2	0.012	0.64	0.39 - 0.89
	non-epitheloid	12.7			
Treatment	MMT	28.7	<0.001	0.32	0.22 - 0.47
	other	11.8			
Stage	I/II	18.6	0.01	0.66	0.47 - 0.92
	III/IV	11.3			
PD-L1 TCs	≤ 10%	15.1	< 0.001	0.39	0.18 - 0.86
	> 10%	6.3			
PD-L1 TILs[a]	< 1%	15.1	0.508	0.82	0.43 - 1.56
	≥ 1%	11.8			
PD-1 TILs[b]	< 1%	15.0	0.703	1.04	0.77 - 1.47
	≥ 1% and ≤ 10%	15.6		0.87	0.59 - 1.33
	> 10%	12.7			

[a] performed in 165 cases, [b] performed in 164 cases, [c] p-value was calculated with the log-rank test. OS, overall survival; mo, months; HR, hazard ratio; CI confidence interval; MMT, multimodality treatment including surgery; TCs, tumor cells; TILs, tumor infiltrating lymphocytes.

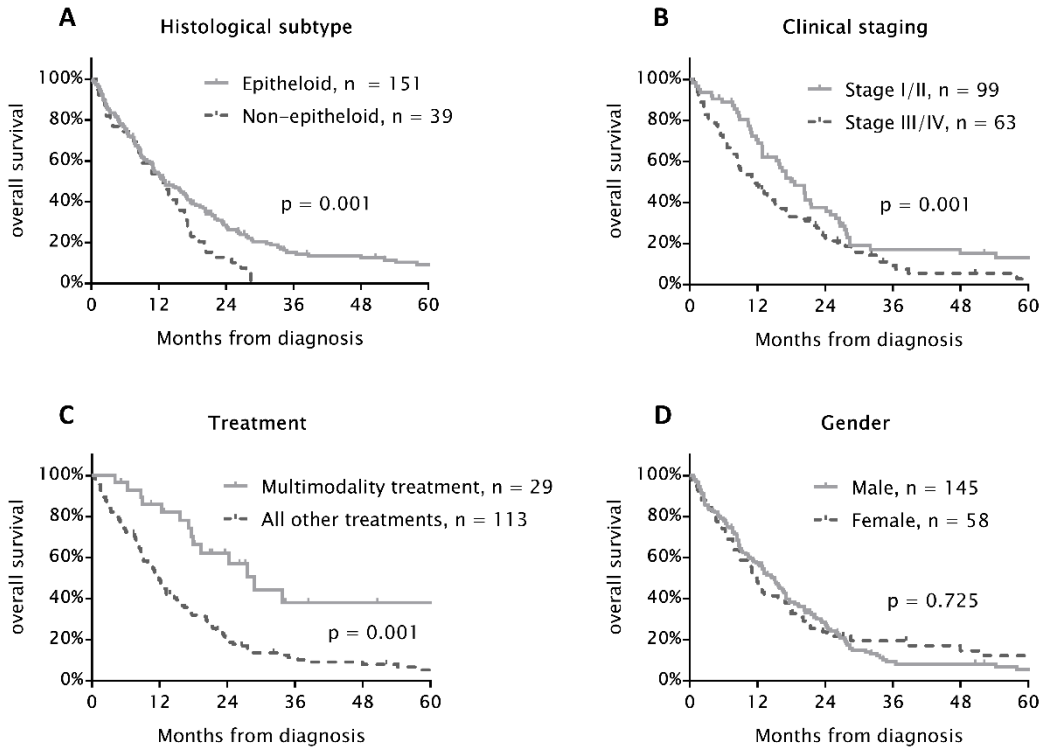

Figure 6 *Kaplan-Meier estimates for OS in patients with MPM according to clinicopathological parameters. (A) Patients with epithelioid subtype exhibited significantly superior OS compared to those with other non-epithelioid histotypes (i.e., sarcomatoid or biphasic) (median OSs were 13.2 vs. 12.7 months, respectively; HR 0.64, p=0.012). (B) Early stage MPM (I and II) at diagnosis conferred significantly longer OS (vs. stages III/IV; median OSs were 18.6 vs. 11.3 months, respectively; HR 0.66, p=0.014). (C) Patients treated with MMT, including surgery, had significantly improved OS (vs. those receiving other treatments; median OSs were 28.7 vs. 11.8 months, respectively; HR 0.32, p<0.001). (D) No significant differences in OS have been observed between male and female patients (median OSs were 15.1 vs. 11.2 months, respectively; HR 106, p=0.725). OS, overall survival; MPM, malignant pleural mesothelioma; HR, hazard ratio.*
(Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

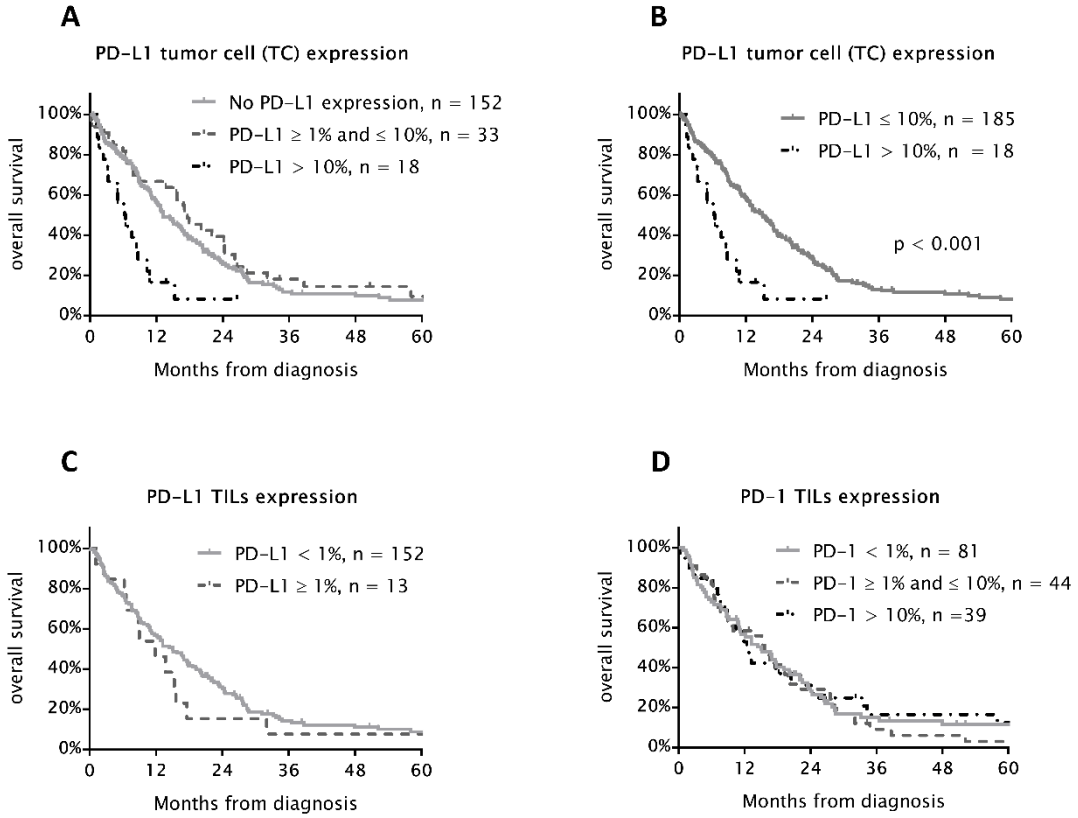

Figure 7 *Kaplan-Meier estimates for OS according to PD-L1 and PD-1 expression of TCs and TILs in human MPM. (A) OS of patients with no vs. ≥1% and ≤10% PD-L1 TC expression was similar, whereas OS in patients with high (>10%) PD-L1 TC expression was significantly worse. (B) Patients with PD-L1 TC expression ≤10% had significantly longer OS than those in the PD-L1 TC high (>10%) group (median OSs were 15.1 vs. 6.2 months, respectively, p<0.001, log-rank test). (C) Patients with a positive PD-L1 TILs staining (≥1%) had a similar OS compared to patients without PD-L1 TILs expression (median OS 15.1 vs. 11.8 months, HR 0.82, p=0.508). (D) PD-1 expression on TILs did not impact OS, as OS was similar among three groups of different expression levels (<1% vs. ≥1% and ≤10% vs. >10%; p=0.703). OS, overall survival; PD-L1, programmed death ligand 1; PD-1, programmed cell death 1; TC, tumor cell; TIL, tumor-infiltrating lymphocytes; MPM, malignant pleural mesothelioma; HR, hazard ratio.*
(Gieszer B, Megyesfalvi Zs, Dulai V et al. EGFR variant allele frequency predicts EGFR-TKI efficacy in lung adenocarcinoma: a multicenter study. Transl Lung Cancer Res. 2021)

Table 7. Multivariate Cox regression model for OS adjusted for clinicopathological variables (n=126)

Variables	**Number**
Age (continuous)	
HR	1.008
95% CI	(0.987-1.028)
p	0.472
Gender (female vs. male)	
HR	0.855
95% CI	(0.546-1.340)
p	0.495
Histology (epithelioid vs. non-epithelioid)	
HR	0.504
95% CI	(0.301-0.843)
p	0.009
IMIG clinical stage (I+II vs. III+IV)	
HR	0.545
95% CI	(0.352-0.844)
p	0.007
Treatment (MMT vs. other)	
HR	0.351
95% CI	(0.194-0.633)
p	<0.001
PD-L1 expression of TCs (PD-L1 >10% vs. ≤10%)	
HR	0.405
95% CI	(0.216-0.759)
p	0.005

OS: overall survival; PD-L1: Programmed Death Ligand 1; HR, hazard ratio; CI, confidence interval; MMT: multimodality treatment

4. Discussion

4.1. Adjusted EGFR-VAF as a potential BM in predicting the survival outcomes of EGFR-TKI-treated LADC patients

In the age of precision and individualized cancer therapy, it is essential to accurately determine the type of tumor, including a comprehensive histological classification and a description of clinically relevant molecular pathological features[62,63]. Targeting EGFR in LADC patients proves to be a promising strategy, as several studies have shown that TKI inhibitors gefitinib and erlotinib are effective in advanced NSCLCs with EGFR-sensitizing mutations[64,65]. Still, the efficacy of EGFR-TKIs is not consistent for every patient, and not all patients with EGFR-activating mutation show similar response rates and PFSs[30]. Hence, there is an urgent need for identifying valid predictive and prognostic BMs that enable clinicians to effectively select the patients who may benefit more from EGFR-TKI therapy. Early 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[31]. Therefore, this study aimed to assess the clinicopathological relevance of EGFR-aVAF and evaluate its predictive and prognostic relevance as a BM in a 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 study results. However, due to the small number of patients harboring exon 18 mutations, subgroup specific statistical calculations were performed without these patients[27]. 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 regarding specific EGFR exon mutations in Caucasian patients[66]. Next, 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 different 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 align with previously published data, suggesting a significant advantage in PFS for patients carrying exon 19 deletions compared to those carrying EGFR exon 21 mutations[69–72]. 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[27]. To date, the mechanism underlying the different sensitivities to EGFR-TKI treatment between exon 19 and exon 21 mutated tumors remains to be elucidated[71]. Based on our results, a possible explanation might be that EGFR-aVAF of tumor tissue is significantly higher in EGFR exon 19 mutated patients than 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 the differential inhibition of downstream signals since EGFR-TKIs inhibits the phosphorylation of EGFR, Akt, and Erk to a greater degree in exon 19 deletion cells than in exon 21 mutated cells[73]. Furthermore, an additional explanation might be that exon 19 deletions and 21 mutations present different intrinsic sensitivities to the EGFR-TKIs[71,74]. Importantly, different mutations in the same exon might also indicate different predictive roles since non-L747 to E749 (LRE) deletions have a worse response to TKIs than LRE deletions, but we had no data on the type of deletions in exon 19[75]. 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 lines and therapeutic agents, which is in line with the findings of others[76–79]. 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 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 present or not, and only a few investigated the predictive role of the relative EGFR mutational abundance[31,66,67]. 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 regarding EFGR-TKI are still primarily based on the absence or presence of activating EGFR mutations[53]. 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 the cancer genome in colorectal cancer patients adapts dynamically to pulsatile drug schedules, and the abundance of resistance mutations could increase after long-time targeted therapie[80]. Therefore, dynamic monitoring of EGFR-aVAF during treatment is also warranted.

There are several limitations in our study. Even though 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 allowed us 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. In addition, loss of heterozygosity and EGFR amplification frequently occurs in LADC patients harboring EGFR activating mutations. Therefore, it could serve as an indicator for a better response from EGFR-TKI treatment[81–83]. 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 slowly replaced by second- and third-generation EGFR-TKIs in the clinical practice. All in all, considering 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.

4.2. Prognostic impact of PD-1 and PD-L1 expression in MPM

Previous studies suggest that high PD-L1 expression might be associated with impaired survival outcomes in MPM, yet the prognostic value and clinicopathological significance of both PD-L1 and PD-1 are still controversial[51,60,68]. Therefore, this study aimed to evaluate the expression of PD-L1 and its receptor PD-1 in MPM and to correlate their expression patterns with clinicopathological parameters and long-term outcomes by analyzing a large patient cohort in a multicenter setting.

The majority of MPM cases are caused by prior exposure to asbestos, leading to increased local infiltrating immune cells and malignant transformation of mesothelial cells[58,84,85]. High numbers of TILs have been associated with a better prognosis, whereas high numbers of tumor-associated macrophages (TAMs) and low lymphocyte to monocyte ratio (LMR) in peripheral blood or tissue have a negative impact on prognosis[58,86–89]. The PD-L1/PD-1 pathway plays a pivotal role in normal immune system regulation but also in tumor immune escape control since the interaction of TC PD-L1with T-cell PD-1 reduces the effector functions of T cells[90]. Accordingly, immunogenic tumors can easily bypass the anti-tumor responses of the organism by overexpressing PD-L1 and thus escaping the immune surveillance[90]. On the other hand, by blocking the PD-L1/PD-1 pathway with therapeutic antibodies, a durable anti-tumor activity and favorable response rates can be achieved in multiple tumor types, including skin melanoma, lung cancer, and partly MPM as well[50,91].

Our international multicenter study found that 25% of cases were categorized as positive (≥1%) for TC PD-L1 expression. These results are in line with two recent MPM studies reporting that 18% to 24% of the patients had PD-L1 expressing tumors[60,92]. Additionally,

we found that only a small number of patients (n=18; 8%) had a PD-L1 TC expression higher than 10%. Previous studies have shown that multiple components of the tumor microenvironment can express PD-L1. Therefore, we also investigated PD-L1 expression by TILs[60]. Of note, however, PD-L1 was rarely expressed by TILs in our cohort. These results are only partly in line with the findings of Herbst and colleagues, who studied 732 different tumor types and observed PD-L1 positivity on both TCs and immune cells[93]. A possible explanation for the relatively low number of cases with PD-L1 expressing TILs might be that TIL PD-L1 positivity is usually seen in sarcomatoid MPM, whereas the majority of patients included in our study had epithelioid type MPM[94]. So far, two major studies have investigated the detailed expression pattern of PD-1 in MPM[94,95]. In our study, PD-1 expression of TILs could be measured in 164 patients, whereas we did not observe any PD-1 positive TCs. Our results are in line with the findings of Marcq and colleagues, who demonstrated that PD-1 is expressed to a great extent on immune cells in MPM[94]. They also showed that PD-1 positive TCs are rarely seen in these patients (only 4 of 54 patients had PD-1 positive TCs in their study)[94]. Activated lymphocytes primarily express PD-1, and upon triggering by its ligands (PD-L1 and PD-L2), it can repress Th1 cytotoxic immune responses[50,96]. Notably, half of our patients have been categorized as positive for TIL PD-1 expression, and 24% of them had high (>10%) PD-1 expression. Interestingly, the significance of PD-1-expressing tumor infiltrating CD8+ T cells in predicting the anti-PD-1 therapeutic response in MPM is still unclear[97]. Of note, in case of other solid tumors, such as skin melanoma, it is suspected that the presence of activated PD-1+ CD8+ T cells might be associated with therapeutic efficacy[97,98]. As mentioned before, previous studies have reported higher PD-L1 expression in non-epithelioid (especially sarcomatoid) MPM compared to other histological subtypes[94,95]. We did not find a significant association between PD-L1 or PD-1 expression and histological subtype. Therefore, our results are in contrast to these previous studies[59,60,95]. A possible explanation for this discordance might be related to different cut-off values. In our study, "PD-L1/PD-1 high" patients were defined as those with PD-L1/PD-1 expression >10%. Meanwhile, others used alternative threshold values or grouped the patients solely based on positivity irrespective of the expression percentage. Additionally, the relatively low ratio of patients with non-epithelioid MPM in our study might also explain these divergent results. To date, no threshold expression level of PD-L1 has been

determined to predict treatment response or survival probability in MPM[51]. In contrast to previous studies applying cut-off levels of 1% or 5%[51,60,99,100], in the present study, we investigated the correlation between PD-L1 expression and OS by using cut-off levels of both 1% and 10%. PD-L1 and PD-1 expression have been shown to correlate with survival in several tumor types including hepatocellular, breast, esophageal and thymic carcinomas [50,52,101–103]. As for MPM, the small number of available studies has yielded conflicting results partly due to different threshold values[51,60,92,99,100]. Our study found that high (>10%) TC PD-L1 expression was associated with impaired median OS, with a clinically relevant difference of 8.8 months between low and high subgroups. In addition, by performing a multivariate analysis, we also found that high (>10%) PD-L1 expression was significantly associated with shorter OS regardless of histology, stage or treatment. Of note, the similar survival probabilities between PD-L1 negative patients (<1%) and those with PD-L1 expression between 1-10% might suggest the need for higher cut-off values compared to previous studies. PD-L1 protein expression was previously shown to correlate with tumor aggressiveness and may be a critical factor to promote tumor growth and metastases[60,68,103–105]. Accordingly, the worse OS is related to higher PD-L1 TC expression levels may be partly explained by PD-L1 acting as a surrogate marker for unfavorable tumor behavior. As for the prognostic impact of PD-1 expression by TILs, previous studies suggest that PD-1 expression by immune cells correlate with increased OS in patients with triple-negative breast cancer, gastric cancer or skin melanoma[97,106,107]. Meanwhile, no such association was found in case of other solid tumors (ex. nasopharyngeal carcinoma or in oral squamous cell carcinoma)[108]. To the best of our knowledge, ours is the so far most extensive study investigating the prognostic relevance of PD-1 expression by TILs in MPM patients. Although Marcq et al. also examined the prognostic importance of PD-1 on immune cells, their study included only 54 patients[94]. In this study, we were unable to detect a statistically or clinically relevant difference in the OS according to PD-1 TIL expression. Accordingly, our results suggest that PD-1 TIL expression may not serve as a suitable prognostic biomarker in MPM. The present study is partly limited by its retrospective nature and the lack of a validation set. Consequently, our results have to be interpreted with caution. Additionally, the use of PD-L1 expression as a prognostic BM can be confounded by multiple unresolved issues, including variability in antibody characteristics, tissue processing and expression threshold values.

In this study, we used the commercially available E1L3N antibody for PD-L1 staining. Importantly, however, not all antibody clones show a similar staining pattern and positivity[109]. Therefore, our results should preferentially be considered when using the E1L3N antibody clone. Finally, our results should be interpreted with the caveat that both PD-L1 and PD-1 expressions are variable over time, and although the majority of included patients were CHT-naïve at biopsy, the administration of CHT prior to tissue sampling can also influence the expression patterns[94,110,111]. However, this study examined a relatively large number of patients in a multicenter setting and we used multiple cut-off values to get a clearer insight into the expression pattern and prognostic impact of PD-L1 and PD-1 in MPM.

5. Conclusion

We found that high tumoral (≥70%) EGFR-aVAF can be used as a positive predictive BM for PFS in EGFR-TKI-treated LADC patients, and high (>10%) TC PD-L1 expression is an independent negative prognostic BM for OS in MPM. Moreover, our first study also proposes that EGFR-aVAF is considerably higher among patients with exon 19 deletions, thus confirming these patients' longer PFS and OS. These results might explain why the duration of response in some patients with EGFR-sensitizing mutations is not as long as expected when no resistance related abnormality is detected. 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. In our second study, besides confirming the prognostic role of TC PD-L1 expression, we also found that both TCs and TILs uniformly express PD-L1 in MPM. Furthermore, this was the most extensive study that comprehensively evaluated the prognostic value of PD-1 by TILs in a multicenter cohort of MPM patients. Consequently, our results concerning PD-1 and PD-L1 expression in MPM might as well contribute to the development of new therapeutic and follow-up strategies in this devastating disease.

6. Summary

The current thesis is based on two different studies. In the first study, we aimed to investigate the prognostic and predictive role of EGFR-aVAF of tumoral tissue in EGFR-TKI treated advanced LADC patients. Meanwhile, in the second part, we aimed to assess the prognostic relevance and expression pattern of PD-1 and PD-L1 in MPM.

The first study included 89 advanced-stage Caucasian LADC patients with known EGFR mutations. All patients were treated with EGFR-TKIs. The correlations of EGFR-aVAF with clinicopathological variables including PFS and OS were retrospectively analyzed. We found that 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. The tumoral EGFR-aVAF was significantly higher in patients harboring EGFR exon 19 mutations than in those with exon 21-mutant tumors ($p<0.001$). Remarkably, 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 ($p=0.011$).

In the second international study, FFPE tumor samples were collected from 203 MPM patients who received standard treatment. TCs and TILs PD-L1 and PD-1 expression were measured by immunohistochemistry and correlated with clinical parameters and long-term outcomes. High (>10%) PD-L1 TC and PD-1 TIL expressions were found in 18 (8%) and 39 (24%) patients, respectively. PD-L1 was rarely expressed by TILs [≥1%, n=13 (8%); >10%, n=1]. No significant associations were found between the PD-L1 or PD-1 expression of TCs or TILs and clinicopathological parameters such as stage or histological subtype. Remarkably, patients with high (>10%) TC-specific PD-L1 expression exhibited significantly worse median OS (6.3 vs. 15.1 months of those with low TC PD-L1 expression; HR: 2.51, $p<0.001$). In multivariate Cox regression analysis adjusted for clinical parameters, high TC PD-L1 expression (>10%) proved to be an independent negative prognostic factor for OS (HR: 2.486, $p=0.005$). There was no significant correlation between PD-L1 or PD-1 expression of TILs and OS.

7. References

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DOI:10.14753/SE.2022.2687

9. Acknowledgements

I will be always grateful to Ferenc Rényi-Vámos for offering me several opportunities to improve my surgical skills in thoracic cancer surgery and lung transplantation. Besides, he also helped me to gain insights into clinical and translational research, and to obtain countless scholarships.

I owe special thanks to Balázs Döme and Zsolt Megyesfalvi who have always helped and guided me in the field of clinical and translational research. This dissertation would not have been possible without them.

I would like to thank all of my colleagues at the Department of Thoracic Surgery of the National Institute of Oncology, Budapest. It is a privilege for me to work together with such a talented and creative Team. I would like to express special gratitude to László Agócs, Ákos Kocsis, Jenő Elek, Klára Török and Levente Bogyó and to all who were my mentors in surgical training. I would also like to thank all my co-authors from different pulmonary and surgical centers across Europe for helping our research team to grow and evolve.

And finally, I am grateful to my family for their encouragement, understanding and love, without which I would be lost. Therefore, I would like to say special thanks to

my wife, Júlia Győri for her support and encouragement;

my kids, Levente, Lilla and Boroka for enduring that they had to thrive so many times without Dad;

my parents, Anna Máttyus and Richard Gieszer for supporting me on this path.

Last but not least, I would like to thank all the people who have helped me to get to this point in life.