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ORIGINAL ARTICLE

The Relationship Between Tumor-Infiltrating Lymphocytes, PD-L1 Expression, Driver Mutations and Clinical Outcome Parameters in Non-Small Cell Lung Cancer Adenocarcinoma in Patients with a Limited to no Smoking History

Sacha Mignon[1] · Karen Willard-Gallo[2] · Gert Van den Eynden[2] · Roberto Salgado[2,3] · Lore Decoster[1] · Koen M. Marien[4] · Johan F. Vansteenkiste[5] · Erik Teugels[1] · Jacques De Grève[1]

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Abstract

Tumor infiltrating lymphocytes (TIL), programmed death 1 (PD-1) and programmed death-ligand 1 (PD-L1) expression are important prognostic markers. This study aimed to investigate these markers in lung adenocarcinoma (ADC) biopsies from patients with stage IIIB or IV ADC with little or no smoking history, to investigate their prognostic value and to correlate these results with the presence of driver mutations in the tumors. TIL were retrospectively evaluated on hematoxylin and eosin stained slides from 152 tumor samples. PD-1/PD-L1 expression was retrospectively evaluated with PD-1/PD-L1 immunohistochemistry (IHC) double staining on 74 tumor samples with sufficient residual tissue. PD-L1 expression was analysed on stromal cells of the tumor compartment, the tumor cells and TIL and PD-1 on TIL. Median overall survival (OS) was longer in patients with high stromal TIL infiltration compared to patients with low stromal TIL infiltration (68 weeks vs. 35 weeks respectively; $p = 0.003$). This was observed most prominently in *KRAS* mutant tumors (95 weeks vs. 12 weeks; $p = 0.003$). Only PD-L1 expression on tumor stromal cells influenced OS and indicated a worse prognosis (77 weeks vs 25 weeks; $p = 0.013$). Stromal TIL counts nor PD-1/PD-L1 expression levels were associated with the presence of driver mutations. The results of the current study reinforce the prognostic role of TIL in lung ADC, which is most prominent in *KRAS* mutant cancers. The results of the PD-1/PD-L1 analysis suggest that stromal cells can effectively suppress the anti-tumor immune response via the PD-L1 pathway.

Keywords Lung adenocarcinoma · Tumor infiltrating lymphocytes (TIL) · Programmed death-ligand 1 (PD-L1) · Programmed death-1 (PD-1) · Driver mutations

✉ Sacha Mignon
sacha.mignon@uzbrussel.be

[1] Departement of Medical Oncology, Oncologisch Centrum UZ Brussel, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium

[2] Molecular Immunology Laboratory, Institut Jules Bordet, Boulevard de Waterloo 121, 1000 Brussels, Belgium

[3] Department of Pathology, GasthuisZusters Antwerpen (GZA), Oosterveldlaan 22, 2610, Wilrijk, Antwerp, Belgium

[4] Universiteit Antwerpen, Prinsstraat 13, 2000 Antwerp, Belgium

[5] Department Respiratory Oncology, University Hospital KU Leuven, Herestraat 49, 3000 Leuven, Belgium

Introduction

The role of the immune system in the development of non-small cell lung cancer (NSCLC) has become apparent and immunotherapeutic strategies have been developed, in particular the inhibition of immune checkpoints programmed death-1 (PD-1) and programmed death- ligand 1 (PD-L1) [1].

In a meta-analysis PD-L1 overexpression on tumor cells was associated with worse overall survival (OS) in solid tumors. However, conflicting results exist especially for melanoma and lung cancer [2]. In NSCLC some studies report a negative correlation between high PD-L1 expression and prognosis [3, 4], while other studies demonstrate a positive correlation [5, 6].

The importance of tumor-infiltrating lymphocytes (TIL) is nowadays recognised in immunoediting, especially during the elimination phase [7].

In NSCLC, the presence of infiltrating $CD3^+$ and $CD8^+$ T cells in the tumor biopsy is associated with longer OS and considered as an independent prognostic marker [8, 9]. The presence of TIL in tumor stroma was a stronger prognostic marker for OS than their presence in tumor nests. On the other hand, infiltration of the tumor stroma with $FOXP3^+$ regulatory T cells is associated with poor progression-free survival [9, 10]. A more recent study in large NSCLC cohorts showed that intense TIL infiltration was associated with a longer OS and with longer disease-free survival [11].

In the current study, we retrospectively investigated TIL and PD-1/PD-L1 expression in a prospective cohort of lung adenocarcinoma (ADC) patients, preselected for no or limited smoking history in which baseline driver mutations were systematically examined. The investigated variables were correlated with outcome parameters and driver mutation status.

Methods

Patient Selection

From May 2006 till May 2010 patients with stage IIIB and IV lung ADC and no or limited smoking history were included in a Belgian multicenter phase II study (FIELT) investigating the activity of first-line erlotinib in epidermal growth factor receptor (*EGFR*) mutant lung ADC [12].

All patients consented that their tumor tissue could be used for further research. The current retrospective study was approved by the ethics committee of the 'UZ Brussel'.

Mutation Analysis

Patients were tested for the presence of somatic driver mutations in the Laboratory of Medical and Molecular Oncology (LMMO) of the Oncologisch Centrum, UZ Brussel. The genes analyzed in all samples were *EFGR*, *HER2*, *HER3*, *KRAS* and *BRAF* [12].

BRAF, *HER2* and *HER3* mutant cases were excluded for the present analysis because of their small numbers. All driver mutations in this cohort were mutually exclusive.

Whole-Slide Imaging

Whole-slide imaging (WSI) was used to evaluate TIL infiltrates and PD-1/PD-L1 expression. The examined slides, 4 μm thick, were made of formalin-fixed paraffin embedded lung ADC tissue. The slides were scanned with an 'Aperio CS/2™' scanner of Leica Biosystems at 20x magnification, using the software module 'Aperio Scancope™' of Leica Biosystems. Snapshots were taken. More focus points were added if the software was unable to get the slide in focus. The scanned images were managed with 'Spectrum eSlide Manager™' of Leica Biosystems, shared with 'PydIO™' open-source software and viewed with 'Pathomation™' software.

Evaluation of TIL

Image analysis was conducted on digitized haematoxylin and eosin (H&E) and haematoxylin eosin saffron (HES) stained slides.

TIL were scored blindly and independently by three observers (RS, GVDE, SM). Intratumoral TIL (iTIL) and stromal TIL (sTIL) were determined according to the modified recommendations for assessing TIL in solid tumors of the International TIL Working Group [13].

Low and high sTIL were defined as sTIL below or above the median value respectively.

Evaluation of PD-1 and PD-L1 Expression

PD-1/PD-L1 double-staining was performed according to the protocol of Buisseret et al. [14] Examples are represented in Fig. 1.

PD-L1 expression was analyzed by two investigators (RS, GVDE) on stromal cells, tumor cells and on lymphocytes. PD-1 expression was measured on lymphocytes only. The score was expressed as the percentage of cells expressing the selected biomarker. Slides with an expression ≥1% were considered positive.

Statistical Analysis

Statistical analyses were performed using 'SPSS statistics' version 22 (IBM Corporate, Armonk, NY, USA) and with 'R' version 3.2.4 (The R Foundation for Statistical Computing Platform) [15]. The reproducibility of the TIL and the PD-1/PD-L1 scoring was assessed by measuring the interobserver variability with the intraclass correlation coefficient (ICC) by using the ICC function from the irr package in R [16]. Kirkegaard et al. [17] defined an ICC >0.70 as the minimum acceptable, >0.80 as good and > 0.90 as excellent.

The comparison of mean TIL and mean PD-1/PD-L1 expression between different subgroups was performed using a non-parametric Mann-Whitney U test. Mutated tumors were compared to double wild type (WT) tumors. Response to erlotinib treatment in *EGFR* mutant lung ADC was defined as having a complete response or partial response. Clinical benefit was defined as response or stable disease after six months of treatment.

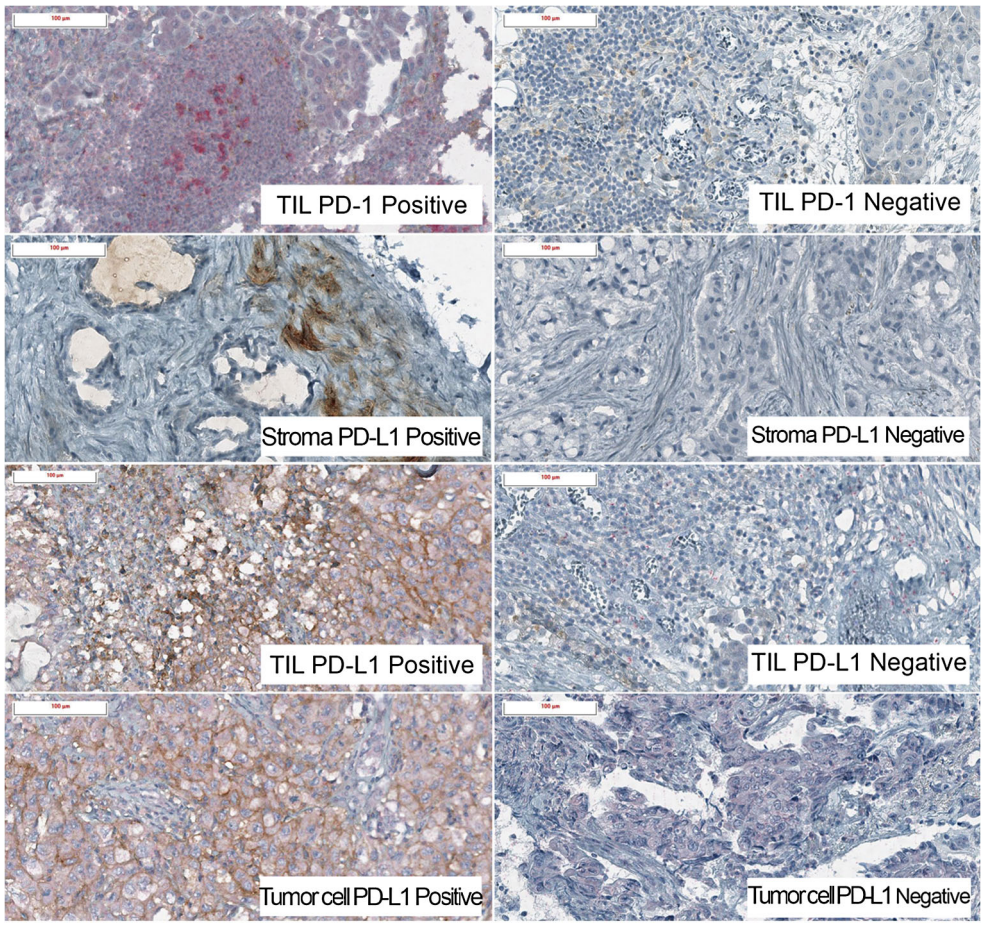

Fig. 1 Examples of the PD-1/PD-L1 double staining with a positive and its corresponding negative staining

The OS between groups was estimated with the Kaplan-Meier method. Log-rank tests were used to compare the survival curves between subgroups.

A p value <0.05 was considered significant.

Results

TIL Evaluation

Eligible Patients

The patient flow chart for inclusion in the TIL analysis is represented in Fig. 2a.

Of the 237 patients included in the FIELT study, 114 were eligible for the TIL analysis and the correlation with outcome parameters.

Interobserver Variability of sTIL and iTIL Analysis

To determine interobserver variability for sTIL an iTIL, 158 of 206 evaluated H&E and HES slides were included. Slides were excluded because of low quality ($n = 31$), only cytology available ($n = 10$) and lymph node biopsies ($n = 7$).

The ICC for sTIL for all three observers was 0.74 (95% CI: 0.64–0.84), which is above the minimal acceptable standard as proposed by Kirkegaard et al. [17].

The ICC for iTIL was 0.16 (95% CI: 0.11–0.23), below the aim of 0.70. Therefore iTIL assessment was considered imprecise and not analyzed further.

sTIL and Mutation Status

There was no significant difference in sTIL between *EGFR* mutant and WT lung ADC (mean 21.3% vs 22.0%; $p = 0.815$), nor between *KRAS* mutant and WT lung ADC (mean 26.9% vs 22.0%; $p = 0.435$).

In addition, sTILs did not differ significantly between *EGFR* mutant and *KRAS* mutant lung ADC ($p = 0.605$).

Stromal TIL and Clinical Outcome Parameters

Median OS (mOS) in the studied population ($n = 114$) was 49 weeks. Median sTIL was 16.67% (standard deviation: 19.06%).

Patients with high sTIL had a significant longer mOS than patients with low sTIL (68 weeks; 95% CI: 26.40–110.00 versus 35 weeks; 95% CI: 17.73–52.56; $p = 0.003$) (Fig. 3a).

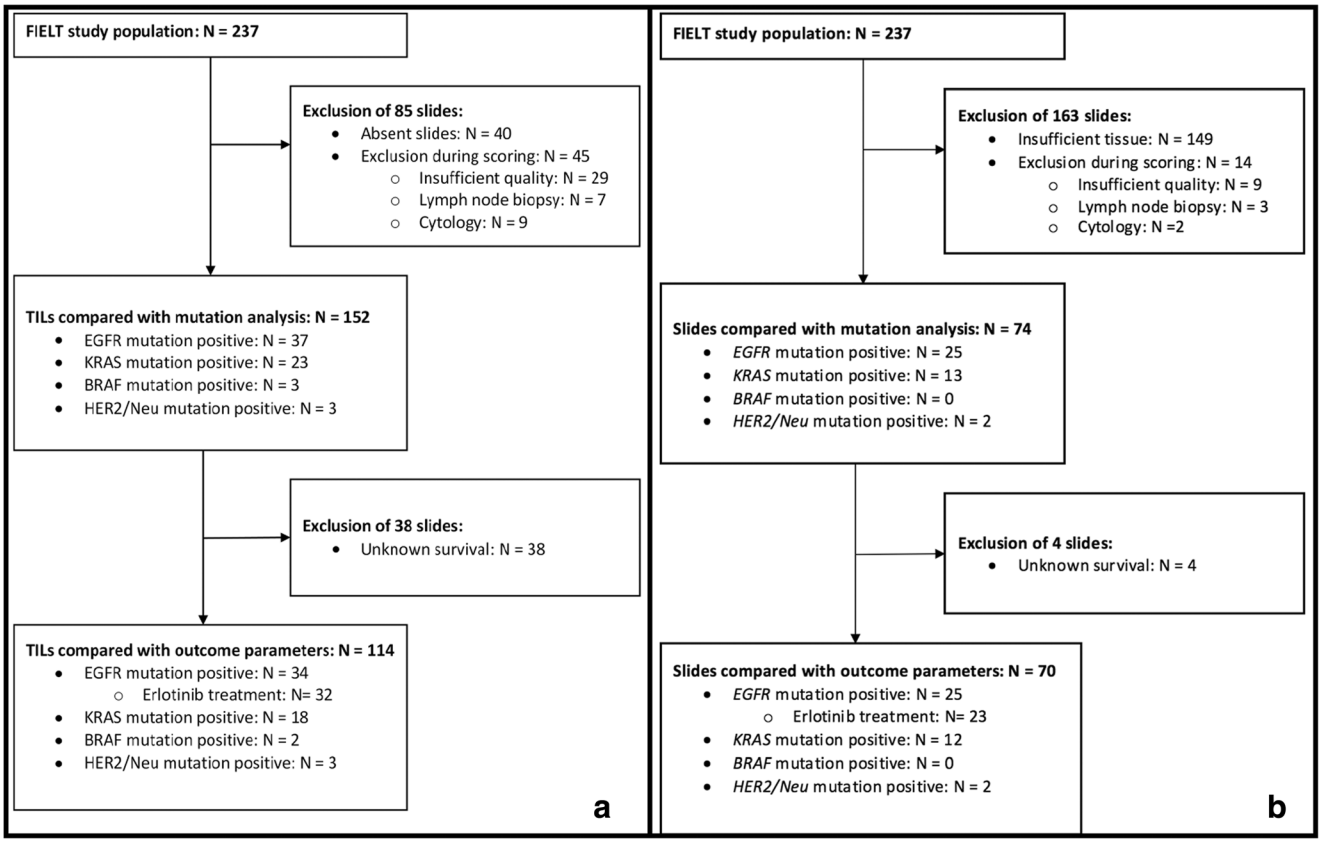

Fig. 2 **a** Schematic representation of the selection algorithm for TIL analysis. **b** Schematic representation of the selection algorithm for PD-1/PD-L1 analysis

EGFR mutant lung ADC had a longer mOS compared to WT lung ADC, although not significant (98 weeks; 95% CI: 71.41–124.88 versus 33 weeks; 95% CI: 19.89–45.25; $p = 0.066$). In the *EGFR* mutant lung ADC there was a numerical

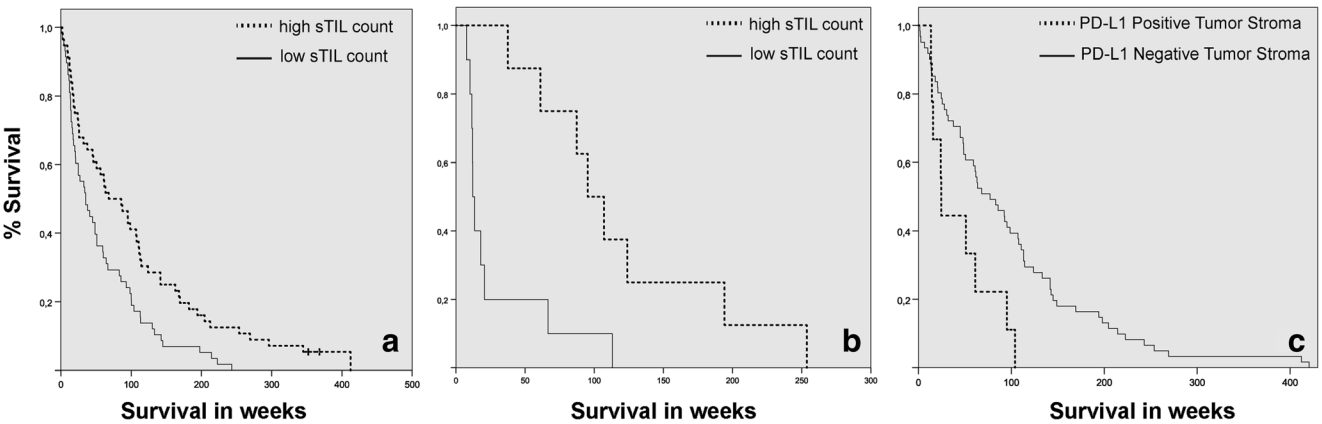

Fig. 3 **a** Kaplan-Meier estimates of the OS of patients with a high sTIL count and a low sTIL count. The median OS of patients with high sTIL counts is 68 weeks (95% CI: 26.40–110.00) compared with a median OS of 35 weeks (95% CI: 17.73–52.56). The difference in OS is statistically significant ($p = 0.003$). A thick mark on the curve indicates censoring of data. **b** Kaplan-Meier estimates for OS in KRAS mutants with high sTIL counts ($n = 10$) and low sTIL counts ($n = 8$). The median OS of KRAS mutants with high sTIL is 95 weeks (95% CI: 67.96–122.61) and 12 weeks in the KRAS mutants with low sTIL (95% CI: 9.93–14.36). The difference in OS is statistically significant ($p = 0.003$). **c** Kaplan-Meier estimates for OS in the overall population for tumor stroma that is PD-L1 positive ($n = 9$) and PD-L1 negative ($n = 61$). The median OS of patients whose tumor stroma is PD-L1 positive is 25 weeks (95%CI: 22.90–26.41) compared with 77 weeks (95%CI: 43–110.58) for patients with PD-L1 negative tumor stroma. The difference in OS is statistically significant ($p = 0.013$). CI: confidence interval. OS: overall survival. sTIL: stromal tumor infiltrating lymphocytes. PD-L1: programmed death-ligand 1

but statistical non-significant higher mOS in patients with high sTIL (111 weeks; 95% CI: 106.97–115.80) versus those with low sTIL (64 weeks; 95% CI: 15.29–113.29) ($p = 0.065$).

There was no significant mOS difference between *KRAS* mutants and WT lung ADC (38 weeks; 95% CI: 29.16–68.27 versus 33 weeks; 95% CI: 19.89–45.25; $p = 0.836$). *KRAS* mutant lung ADC with high sTIL had a significant longer mOS (95 weeks; 95% CI: 67.96–122.6) compared to those with low sTIL (12 weeks; 95% CI: 9.93–14.36) ($p = 0.003$) (Fig. 3b).

In the double WT lung ADC, there was no mOS difference between tumors with high sTIL (25.7 weeks; 95%CI: 0–53.8 weeks) and low sTIL (33.9 weeks; 95%CI: 22.4–45.3 weeks) ($p = 0.248$).

EGFR mutant cancers were prospectively treated with erlotinib [12]. Of the 32 *EGFR* mutants who received erlotinib, 16 had an objective response and 23 experienced clinical benefit. There was no significant sTIL difference between responders and non-responders ($p = 0.160$) or patients experiencing clinical benefit or not ($p = 0.621$).

PD-1/PD-L1 Evaluation

Eligible Patients

The patient flow chart for inclusion in the PD-1/PD-L1 analysis is represented in Fig. 2b. Of the 237 patients included in the FIELT study, 70 were eligible for PD-1/PD-L1 analysis according to clinical outcome parameters.

Interobserver Variability of the PD-L1 and PD-1 Expression

The ICCs were calculated for the two observers for the scores of 77 slides and were good to excellent. For the PD-L1 expression on stromal cells the ICC was 0.958 (95% CI: 0.934–0.973), for PD-L1 expression on tumor cells the ICC was 0.900 (95% CI: 0.827–0.940) and for PD-L1 expression on TIL the ICC was 0.845 (95% CI: 0.738–0.906). The ICC for the PD-1 expression on TIL was 0.843 (95% CI: 0.761–0.898).

PD-1/PD-L1 Expression Versus Mutation Status

The mean expression of PD-L1 was not significantly different in *EGFR* or *KRAS* mutants compared with WT tumors on stromal cells, tumor cells and TIL, nor was there any difference in the expression when *EGFR* mutants were compared with *KRAS* mutants. Also, we observed no significant difference in PD-1 expression on TIL according to the mutation status (Table 1).

PD-1/PD-L1 Expression Versus sTIL Counts

A total of 68 slides were analyzed for both PD-1/PD-L1 expression and the presence of sTIL. The median sTIL count was 18.33% in this population.

There was a significant higher PD-L1 expression on tumor cells and TIL for patients with high sTIL versus low sTIL ($p = 0.017$ and 0.001 respectively). However, there was no significant difference in PD-L1 expression on stromal cells ($p = 0.552$) or PD-1 expression on TIL ($p = 0.070$) between these groups.

PD-1/PD-L1 Expression and Survival Parameters

The mOS of patients in the studied population ($n = 70$) was 63 weeks.

Patients with PD-L1 positive tumor stroma had a significant shorter mOS than patients with PD-L1 negative tumor stroma (25 weeks; 95%CI: 22.90–26.41 versus 77 weeks; 95%CI: 43–110.58; $p = 0.013$) (Fig. 3c). PD-1 positivity was not associated with a difference in mOS. In the double WT, *EGFR* and *KRAS* mutant subpopulation neither PD-1 nor PD-L1 expression seemed to influence the mOS (Table 2).

In the group eligible for PD-1/PD-L1 analysis 23 patients with an EGFR mutant lung ADC were treated with erlotinib of whom 11 showed a response and 16 a clinical benefit. There was no correlation between PD-1 and PD-L1 expression and response rates or clinical benefit rates after erlotinib treatment (Table 3).

Discussion

The present study describes TIL infiltration and PD-L1 expression in a cohort of lung adenocarcinoma patients, preselected for no or limited smoking history and correlates this expression to outcome parameters and mutational status.

We evaluated TIL on H&E stained slides, according to the method developed by Salgado et al. [13] in breast cancer. Our results indicate that this method is also applicable in lung ADC. The method is considered reproducible with an overall ICC of 0.74. A similar assessment method is also proposed for other solid tumors, including lung tumors, by the International TIL Working Group [18].

At present this method is not suitable to measure iTIL, since the results were not reproducible between observers, possibly because of the small numbers of iTIL and the difficulty of detecting them without IHC. These difficulties were also taken into consideration by Salgado et al. in breast cancer research [13]. The absence of valid data for iTIL will probably not affect our results since sTIL have already been found a superior prognostic marker for OS than iTIL [10].

Table 1 Overview of mean PD-1/PD-L1 expression according to mutation analysis. The *p* values are the result of comparison of mean expression levels between mutants and double WT tumors with the non-parametric Mann-Whitney U test. The last column reflects the p value of the comparison of expression levels between *EGFR* and *KRAS* mutants. PD-1: programmed death-1. PD-L1: programmed death-ligand 1. WT: wild type

Mean expression	Studied population	*EGFR* mutants			*KRAS* mutants			*EGFR* vs. *KRAS* mutants
		Mutant	WT	*p* value	Mutant	WT	*p* value	*p* value
PD-L1 stroma	1.2%	0.2%	1.7%	0.134	1.9%	1.7%	0.972	0.211
PD-L1 tumor	4.4%	1.9%	5.3%	0.075	6.7%	5.3%	0.883	0.211
PD-L1 TIL	3.6%	3.1%	4.0%	0.260	3.7%	4.0%	0.978	0.388
PD-1 TIL	0.7%	1.4%	0.5%	0.182	0%	0.5%	0.391	0.133

Our study evaluated the correlation between driver mutations and sTIL and noticed no difference between *EGFR* mutants, *KRAS* mutants and WT lung ADC. In addition, no evidence was found that high sTIL were predictive for response or clinical benefit in *EGFR* mutants treated with erlotinib.

We also evaluated the correlation between sTIL and OS and observed a significant longer mOS in patients with high sTIL especially in the KRAS mutant subgroup, suggesting that this is a prognostic marker. These results indicate that lung ADC with high sTIL might respond better to immune checkpoint blockade because of an enhanced baseline anti-tumor immune response. This effect might even be greater in *KRAS* mutated lung ADC patients, but the subgroup is too small ($n = 18$) to draw solid conclusions and these observations are thus hypothesis generating. No significant differences for OS were observed for the *EGFR* mutants according to sTIL numbers nor for the double WT subpopulation. Still there was a trend towards a longer OS in *EGFR* mutants with high sTIL infiltration. This raises the hypothesis that the immune infiltrate in *EGFR* mutants might have a different constitution than the immune infiltrate in *KRAS* mutants and the *EGFR* WT tumors, possibly with a shift towards tumor-promoting immunity in *EGFR* mutants. This presumption gets enforced by recently published evidence that the immune infiltrate in *KRAS* mutants constitutes of more CD4+ and CD8+ T-helper cells than *EGFR* mutants and is characterized by markers of strong activity, while *EGFR* mutant tumors were characterized by inactive TIL [19]. Furthermore, *EGFR* mutant NSCLC tend to benefit less from checkpoint inhibitors compared to WT tumors [20].

Our results indicate that sTIL possess clinical validity since they can distinguish patient groups with different prognosis.

Table 2 Overview of survival parameters and p values for PD-L1 and PD-1 expression in the studied population, the double wild type population and the mutant subpopulations. CI: confidence interval. mOS: median overall survival. PD-1: programmed death-1. PD-L1: programmed death-ligand 1

		N (%)	mOS (weeks)	*p* value	*N* (%)	mOS (weeks)	*p* value
		Studied population			Double wild type subpopulation		
PD-L1 stroma	negatif	61 (87%)	77; 95% CI: 43–111	0.013	27 (82%)	48; 95% CI: 20–77	0.107
	positif	9 (13%)	25; 95% CI: 23–26		6 (18%)	24; 95% CI: 13–35	
PD-L1 tumor	negatif	50 (71%)	63; 95% CI: 15–112	0.321	21 (64%)	48; 95% CI: 21–76	0.242
	positif	20 (29%)	61; 95% CI: 56–66		12 (36%)	45; 95% CI: 0–90	
PD-L1 TIL	negatif	52 (74%)	63; 95% CI: 24–102	0.714	23 (70%)	48; 95% CI: 19–77	0.194
	positif	18 (26%)	62; 95% CI: 26–98		10 (30%)	45; 95% CI: 0–103	
PD-1 TIL	negatif	64 (91%)	61; 95% CI: 42–80	0.310	31 (94%)	45; 95% CI: 22–68	0.874
	positif	6 (9%)	86; 95% CI: 52–119		2 (6%)	83; 95% CI: /	
		EGFR mutants			*KRAS* mutants		
PD-L1 stroma	negatif	24 (96%)	93; 95% CI: 36–150	0.726	10 (83%)	95; 95% CI: 0–203	0.224
	positif	1 (4%)	104; 95% CI: 104–104		2 (17%)	13; 95 CI: /	
PD-L1 tumor	negatif	21 (84%)	104; 95% CI: 54–154	0.232	8 (67%)	21; 95% CI: 0–54	0.209
	positif	4 (16%)	59; 95% CI: 1–118		4 (33%)	107; 95% CI: 56–157	
PD-L1 TIL	negatif	21 (84%)	93; 95% CI: 32–154	0.540	8 (67%)	61; 95% CI: 0–141	0.616
	positif	4 (16%)	108; 95% CI: 54–154		4 (33%)	21; 95% CI: 0–112	
PD-1 TIL	negatif	21 (84%)	93; 95% CI: 32–154	0.513	12 (100%)	61; 95% CI: 0–159	/
	positif	4 (16%)	111; 95% CI: 21–200		0 (0%)	/	

Table 3 Overview of response rates and clinical benefit rates of *EGFR* mutants, treated with erlotinib, in accordance with their mean PD-1/PD-L1 expression. PD-1: programmed death-1. PD-L1: programmed death-ligand 1

Mean expression	Response to erlotinib			Clinical benefit to erlotinib		
	Expression in responders	Expression in non-responders	*p* value	Expression in clinical benefit	Expression in progressors	*p* value
PD-L1 stroma	0.4%	0.0%	0.478	0.3%	0.0%	1.000
PD-L1 tumor	3.2%	1.0%	0.590	2.2%	2.8%	0.462
PD-L1 TIL	3.0%	3.8%	1.000	3.0%	4.3%	1.000
PD-1 TIL	1.5%	1.7%	0.453	1.0%	2.9%	1.000

The next step is to verify if sTIL in lung ADC possess clinical utility, meaning whether sTIL can be used to treat patients differently, which requires prospective studies.

Our results concerning PD-1/PD-L1 expression suggest that mutation status plays no role in the level of expression. In the literature, research focused primarily on PD-L1 expression on tumor cells and led to contradictory results. One study in murine models showed higher PD-L1 expression in *EGFR* mutant tumor cells [21] and this is supported by a second study in human lung tumors [22]. However, a retrospective study did not show any association between *EGFR* mutation and PD-L1 expression, which is consistent with the results of our research [5]. It is important to note that our study population for PD-1/PD-L1 expression was rather small and that statistically significant differences might have been missed, especially concerning PD-L1 expression on tumor cells in *EGFR* mutants where there was a statistically non-significant lower expression ($p = 0.075$).

Little research has been done towards PD-L1 expression on the other tumor compartments and towards PD-1 expression on sTIL. Similarly, little research has been done towards PD-1/PD-L1 expression on *KRAS* tumors.

PD-L1 expression on tumor cells in NSCLC is generally accepted to be a bad prognostic marker [4, 23]. This study could not show a survival correlation for PD-L1 expression on tumor cells, nor on sTIL. The contradictive results in literature could be related to differences in technology such as different antibodies [23]. Another hypothesis states that PD-L1 might instead be a marker of an ongoing anti-tumor immune response [4]. Indeed, PD-L1 expression is known to be dynamic and inducible by IFN-γ, an important cytokine in the immune response, secreted by activated lymphocytes [24]. This might in turn explain the finding that PD-L1 expression on tumor cells and TIL is significantly higher when high sTIL levels are present. Interestingly, PD-L1 expression on stromal cells, which is a bad prognostic sign in this study, is not affected by the levels of sTIL present in the tumor tissue. Therefore stromal PD-L1 expression should be evaluated as a predictive biomarker for immune-therapies.

The results in this study also suggest that PD-L1 expression or PD-1 expression on TIL cannot be used as a predictive biomarker in *EGFR* therapy. There is literature stating that PD-L1 expression on tumor cells does have predictive properties in TKI therapy [25]. However, these differences could again be ascribed to different techniques, as mentioned above.

An important limitation of our study is the absence of smokers in the study population, which is not representative of the whole lung ADC population and thus might influence our results. However, two meta-analyses have shown that there is no association between PD-L1 expression and smoker status [4, 23].

In conclusion, our study suggests that sTIL and PD-L1 expression on stromal cells are prognostic markers in lung ADC, indicating the important role of the stromal tumor compartment.

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Compliance with Ethical Standards

Conflict of Interest Koen Marien is an employee of HistoGeneX NV, a company that offers biomarker services to pharmaceutical companies. There are no potential conflicts of interest for any of the other authors.

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