2020-03-05

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Cancer Treatment and Research Communications 23 (2020) 100170

Contents lists available at ScienceDirect

Cancer Treatment and Research Communications

journal homepage: www.elsevier.com/locate/ctarc

Diabetes mellitus type 2 is associated with increased tumor expression of programmed death-ligand 1 (PD-L1) in surgically resected non-small cell lung cancer—A matched case-control study

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ARTICLE INFO

Keywords:
PD-L1
Non-small cell lung cancer (NSCLC)
Diabetes
Immunotherapy
Pembrolizumab

ABSTRACT

Objectives: Programmed death-ligand 1 (PD-L1) expression is a biomarker for cancer immunotherapy. Diabetes mellitus type-2 is a comorbid disease associated with adverse outcomes in Non-Small Cell Lung Cancer (NSCLC). We aimed to investigate the differences in PD-L1 expression in diabetics.

Methods: A matched case-control cohort of surgically-resected NSCLC was assembled from an early multicenter study (PMID: 19152440). PD-L1 immunohistochemistry (Clone 22C3) was graded by a tumor positive score (TPS) system (TPS0: no staining; TPS1: <1%; TPS2: 1–49%; TPS3: ≥50%). Variables showing significance at univariate survival analysis were fit in a Cox regression survival model.

Results: Diabetics ($n=40$) and nondiabetics ($n=39$) showed no differences in age, gender, cancer stage, and follow-up. NSCLCs were more likely PD-L1 positive in diabetics but with tumor positivity <50% (TPS0: 7.5 vs. 20.5%, TPS1: 35 vs. 25.6%, TPS2: 45 vs.23.1%, TPS3: 12.5 vs. 30.8%, respectively; $P<0.05$). In diabetics, squamous cell carcinomas (SCC) and adenocarcinomas were mainly TPS2 (65% vs. 20%) and TPS1 (50% vs. 26%), respectively. Peritumoral inflammation correlated with TPS ($r=0.228$), a relationship accentuated in diabetics ($r=0.377$, $P<0.05$) but diminished and non-significant in nondiabetics ($r=0.136$, $P\geq0.05$). This association was stronger in SCC ($r=0.424$). Diabetes was associated with increased tumor recurrence (HR: 3.08; 95%CI: 1.027-9.23).

Conclusion: Diabetes is associated with an increase in peritumoral inflammation, PD-L1 positivity, and recurrence in NSCLC, more pronounced in SCC, suggesting the possibility of metabolic reprogramming and upregulation of PD-L1 by inducible pathways.

Introduction

Lung cancer remains the leading cause of cancer death in women and men worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases with a low 5-year survival rate. For early-stage NSCLC, surgery and adjuvant therapies are available as primary treatment [2]. Hyperglycemia is among the factors associated with a worsened prognosis in NSCLC [3–6]. The prevalence of diabetes mellitus (DM) type 2 in patients with lung cancer varies between 5% and 28% and has been associated with a higher risk of all-cause mortality [4,5]. A large metanalysis revealed that diabetes is a risk factor for death from lung cancer after adjustment for age, sex, smoking status, and body mass index (BMI) [7]. An adverse effect of diabetes on local recurrence in patients with surgically resected NSCLC was demonstrated in a multicenter study and confirmed in a subsequent smaller cohort [8,9]. Overall, this data supports the role of diabetes in

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https://doi.org/10.1016/j.ctarc.2020.100170

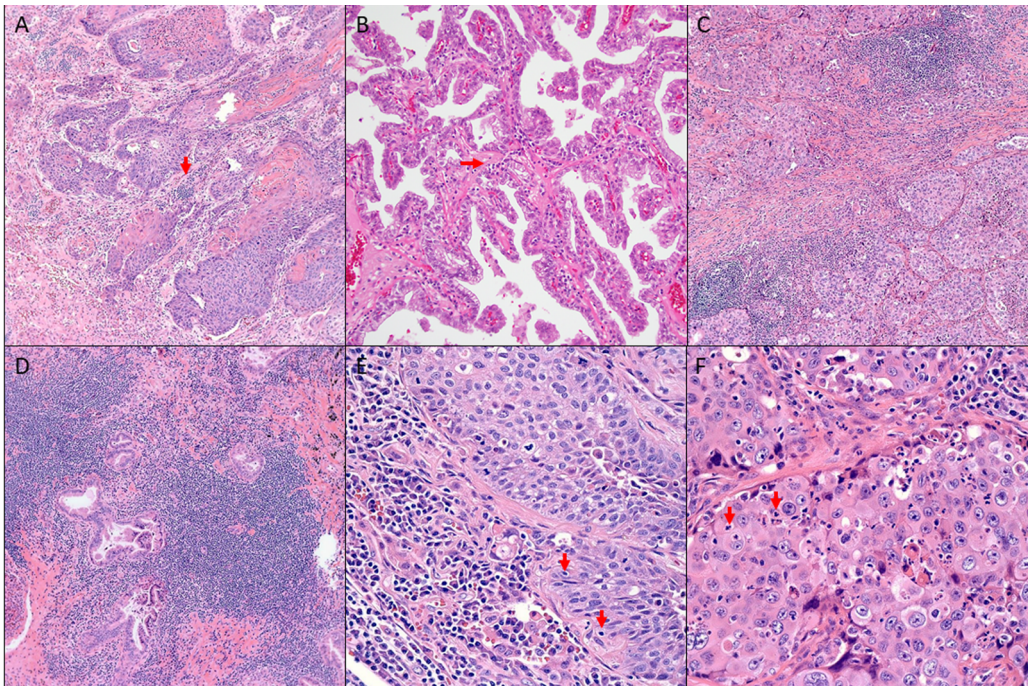

Fig. 1. Examples of peritumoral (A-D) and intraepithelial inflammation (E-F). A. SCC with focal peritumoral lymphocytic aggregates (arrow, 100x). B. ADC with mild peritumoral inflammation (arrow, 200x). C. SCC with moderate peritumoral inflammation (100x). D. ADC with severe peritumoral inflammation (100x). E. SCC with few intraepithelial lymphocytes (arrows) and no neutrophils typical of a case with low neutrophil-to-lymphocyte ratio (400x). F. SCC with numerous intraepithelial neutrophils (arrows) typical of a case with high neutrophil-to-lymphocyte ratio (400x). All pictures correspond to hematoxylin and eosin staining.

facilitating tumor progression in NSCLC.

Hyperglycemia by itself could explain the adverse effect of DM because it can contribute to a more aggressive cancer phenotype including apoptosis inhibition and chemotherapy resistance [10]. In a similar cohort selected for the present study, tumor recurrence correlated with an increased expression of Epithelial-to-Mesenchymal Transition (EMT) phenotype markers in diabetic compared to non-diabetic patients [11]. Lung adenocarcinomas with an EMT phenotype overexpress Programmed Death-Ligand 1 (PD-L1) and are associated with higher amounts Programmed Cell Death-1 $(PD\text{-}1)^{+}$ tumor infiltrating lymphocytes [12]. Of interest, there is evidence that poor glycemic control in patients with diabetes causes immune dysfunction, a relationship that remains to be proven in NSCLC [13]. It is well-known that intrinsic tumor factors such as tumor immunogenicity and tissue microenvironments can modulate tumor PD-L1 expression [14]; however, the effect of comorbid systemic diseases such as diabetes on tumor PD-L1 expression is mostly unknown. In this study, we aim to investigate whether DM type 2 influences the tumor expression of PDL-1 and tumor recurrence in patients with NSCLC when compared to nondiabetics.

Methods

Study Design

Newly diagnosed, surgically-resected NSCLC cases were selected from a well-characterized cohort of an early multicenter study [8,9,11]. That study evaluated retrospectively the impact of surgical, histopathologic, and some patient-related factors on the risks of local and distant recurrence for patients with mostly early stage NSCLC and no neoadjuvant or adjuvant radiotherapy who were treated between 2000 and 2005. DM was identified as a risk factor for local recurrence in multivariate analysis [8]. For our study, the sample size was calculated given an anticipated incidence for diabetics of 65%, for control (non-diabetics) of 20%, enrollment ratio of 1:1, alpha error of 0.01, and power of 95%. The anticipated incidence were the local recurrence ratios estimated in prior Varlotto *et al.* studies [8,9,11]. This resulted in a case group (diabetics) of N1 = 39 and a control group of N2 = 39 to assess the association of diabetes with PD-L1 expression. The cases meeting the inclusion criteria were patients with DM type 2 and primary adenocarcinoma (ADC) or squamous cell carcinoma (SCC) of the lung without previous malignancies or neoadjuvant or adjuvant chemoradiotherapy. Patients with DM type 1 and cases lacking paraffin-embedded tissue or adequate documentation for the diagnosis of diabetes were excluded. Variables included for the analysis were history of DM type 2, glycosylated hemoglobin (Hb1AC) at the time of excision, BMI (kg/m^2), peripheral blood neutrophil to lymphocyte ratio (NLR), pathologic tumor (pT) stage, local (ipsilateral thorax and pN1-N3 nodes) and distant lymph node metastasis at the time of excision. A similar cohort was evaluated in a recent study correlating the expression of EMT markers with diabetes [11]. Parameters of tumor staging were updated to the AJCC cancer staging system, 8^{th} edition. Additionally, the number of months to recurrence, months to death, number of recurrences (local and distant), and death events associated with cancer progression were compiled.

Histopathologic Evaluation

Tumor hematoxylin and eosin (H&E) glass slides were reviewed to evaluate the NSCLC histologic type, the presence of tumor necrosis and features of tumor inflammation. Tumor necrosis was documented either focal or extensive. Assessment of tumor inflammation included grading of peritumoral inflammation using a 4-tier system: absent (none), mild/focal (< 10% of tumor area), moderate (10-50%), and diffuse (> 50%), and a 2-tier system: cold tumor (absent, mild/focal) and hot tumor (moderate, diffuse) (Fig. 1). Additionally, predominant inflammatory cell (neutrophilic, lymphocytic, lymphoplasmacytic, or mixed inflammation), increased intraepithelial lymphocytes (defined as the presence of more than occasional cells within the neoplastic epithelial component excluding the tumor stroma and in areas away from necrotic debris), increased intraepithelial neutrophils, and intraepithelial NLR were evaluated qualitatively.

PD-L1 Immunohistochemistry

PD-L1 immunohistochemical (IHC) assays were performed in formalin-fixed paraffin-embedded tissue with the maximum amount of viable tumor using the Monoclonal Mouse Anti-PD-L1 (DAKO, Clone

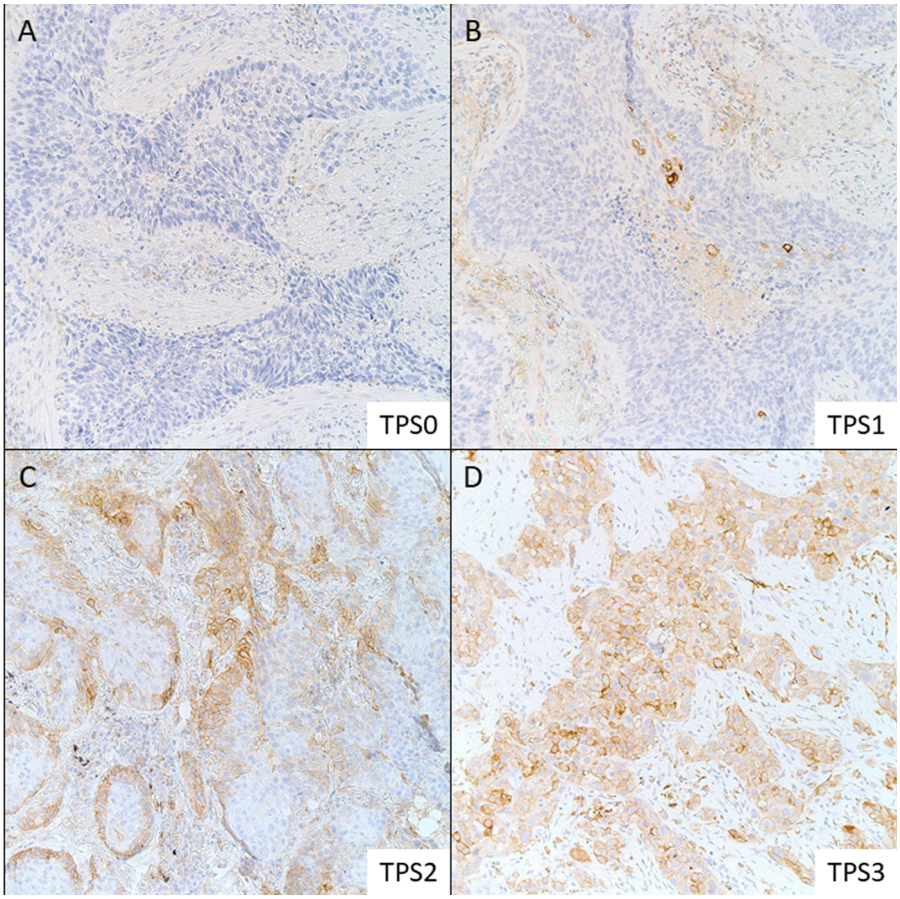

Fig. 2. Examples of PD-L1 expression scores: TPS0 (negative, A), TPS1 (<1%, B), TPS2 (1-49%, C), TPS3 (≥50%, D). All pictures correspond to PD-L1 immunostaining (DAKO, Clone 22C3) at medium magnification (200x).

22C3), DAB+ Substrate-Chromogen revealing system and autostainer Dako ASL48 according to the manufacturer's protocol [15]. Appropriate positive and negative controls were run simultaneously. PD-L1 IHC testing was previously validated at our laboratory.

PD-L1 Expression Scores

A board-certified pathologist (N.R.) assessed all stained slides for PD-L1 expression independently, in a blinded fashion. Tumor Proportion Score (TPS), percentage of viable tumor cells showing partial or complete membrane staining at any intensity, was evaluated based on the 4-tier system of the International Association for the Study of Lung Cancer (IASLC) guideline as follows: TPS0: no staining; TPS1: <1%; TPS2: 1-49%; and TPS3: ≥50% [16] (Fig. 2).

Statistical Analyses

Although the features of the cohort included in this research were explored previously [8], a new independent analysis was performed in order to assure homogeneity between diabetic and nondiabetic patients and to adjust the analysis of recurrence to the new variables incorporated herein. Patient's characteristics were compared using pooled and Satterthwaite *t*-tests for parametric data, and Fischer's Exact and Chi-square tests for nonparametric data in SAS (V.9.0; Cary, NC, USA) system software. For univariate analysis, the survival was estimated using Kaplan-Meier curves and compared with the Log-Rank and Breslow (Generalized Wilcoxon) tests. For multivariate analysis, all variables showing statistical significance at the univariate analysis were fit with a Cox regression (proportional hazard) model. Hazard ratios (HR) with 95% confidence interval (CI) were calculated, and the results under the best fit model are shown. Spearman correlation test was used for categorical variables. The survival and correlation analyses were performed in SPSS (version 22.0; Chicago, Illinois, USA) system software. The graphs were designed in Prism 5 software (version 6; GraphPad Software Inc, La Jolla, CA, USA). Statistical significance was considered with two-sided *P*-values less than 0.05.

Results

Patient's characteristics

A total of 79 patients with NSCLC (ADC: 39, SCC: 40) were selected to assemble a cohort of patients with diabetes ($n=40$; 50.63%) and nondiabetics ($n=39$; 49.37%) for final analysis. No significant difference in the distribution of age, gender, peripheral blood NLR, pT stage, metastasis, cancer histologic type, and tumor necrosis between diabetics and nondiabetics was observed (Table 1). A total of 20 patients with DM type 2 had HbA1C levels within a range of 7.5–7.7% at the time of excision. HbA1C levels were not measured in 14 diabetics and all nondiabetics at the time of excision. Obesity (BMI greater or equal than 30 Kg/m^2) was observed more frequently in diabetics (18; 46.15%) as compared to nondiabetics (9; 23.08%) ($P<0.001$).

Features of the NSCLC-associated inflammation

The entire cohort was relatively homogeneous regarding the features of tumor inflammation (Table 1). NSCLCs in patients with diabetes showed more often moderate peritumoral inflammation (52.5% vs. 33.3%) while mild/focal peritumoral inflammation was more common in tumors of nondiabetics (12% vs. 41%) ($P<0.01$). When the

Table 1
Patient's characteristics and tumor inflammation features of diabetics and nondiabetics with NSCLC selected for this study.

Variable	Diabetics	Nondiabetics	*P*-value
N	40	39	-
Age (years)			
Mean	67	69.07	*0.26*[*,†]
SD	7.50	8.83	
Range	55-82	46-88	
Gender			
Frequency	F:15 (53.85%)	F: 21 (37.50%)	*0.14*[‡]
(Subgroup %)	M:25 (46.15%)	M:18 (62.50%)	*0.17*[§]
HbA1C at diagnosis (%)			
< 6.5	5 (12.82%)	0	*<0.001*[‡§]
≥ 6.5	20 (51.28%)	0	
NA	14 (35.9%)	39 (100%)	
BMI (kg/m^2)			
<30	13 (33.33%)	30 (76.92%)	*<0.001*[‡§]
≥30	18 (46.15%)	9 (23.08%)	
NA	8 (20.51%)	0	
Peripheral blood NLR			
<5	13(32.5%)	10(25.6%)	*0.77*[‡§]
≥5	12 (30%)	11(28.2%)	
NA	15 (37.5%)	18 (46.2%)	
pT stage			
1a	10 (25%)	15 (38.5%)	*0.59*[‡§]
1b	6 (15%)	4 (10.3%)	
2a	17 (42.5%)	12 (30.8%)	
2b	3 (7.5%)	5 (12.8%)	
3	4 (10%)	3 (7.7%)	
Lymph node metastasis			
None	31 (77.5%)	31 (79.49%)	*0.93*[‡]
Local	4 (10%)	3 (7.69%)	*1.00*[§]
Distant	5 (12.5%)	5 (12.82%)	
Cancer histologic type			
Frequency	ADC: 20 (50%)	ADC: 19	*0.90*[‡]
(Subgroup %)	SCC: 20 (50%)	(48.72%)	*1.00*[§]
		SCC: 20 (51.28%)	
Tumor necrosis			
No	18 (46.15%)	20 (52.63%)	*0.56*[‡]
Yes	21 (53.85%)	18 (47.37%)	*0.65*[§]
Peritumoral inflammation			
Frequency (Subgroup %)			
None	7 (17.5%)	1 (2.6%)	*<0.01*[§]
Mild/ Focal	5 (12.5%)	16 (41%)	
Moderate	21 (52.5%)	13 (33.3%)	
Diffuse	7 (17.5%)	9 (23.1%)	
Predominant inflammatory cell Frequency (Subgroup %)			
None	7 (17.5%)	1 (2.6%)	*0.16*[§]
Lymphocyte	10 (25%)	13 (33.3%)	
Neutrophil	0	0	
Lymphoplasmacytic	19 (47.5%)	19 (48.7%)	
Mixed	4 (10%)	6 (15.4%)	
Increased intraepithelial lymphocytes			
No	27 (67.5%)	23 (59%)	*0.48*[‡§]
Yes	13 (32.5%)	16 (41%)	
Increased intraepithelial neutrophils			
No	32 (80%)	29 (74.4%)	*0.55*[‡]
Yes	8 (20%)	10 (25.6%)	*0.60*[§]
Intraepithelial NLR			
<1	37 (92.5%)	32 (82.1%)	*0.16*[‡]
≥1	3 (7.5%)	7 (17.9%)	*0.19*[§]

[*] Pooled *t*- test.
[†] Satterthwaite *t*-test.
[‡] Chi-Square.
[§] Fisher's Exact Test. F: female. M: male. BMI: Body mass index. ADC: adenocarcinoma. SCC: squamous cell carcinoma. NA: not available. NLR: neutrophil to lymphocyte ratio. Local metastasis defined as spread to ipsilateral thoracic lymph nodes.

variable peritumoral inflammation was dichotomized by grouping mild/focal with none in one category ("cold" tumor) and moderate with diffuse in a second category ("hot" tumor), no significant statistical difference between diabetics (cold: 30%; hot: 70%) and nondiabetics (cold: 43.6%; hot: 56.4) was observed ($P = 0.256$). NSCLCs in both groups showed similar percentage distributions according to the variables of predominant inflammatory cell, increased intraepithelial lymphocytes, increased intraepithelial neutrophils, and peripheral blood and intraepithelial NLRs (Table 1).

For the entire cohort, diabetic patients, SCC and ADC cases, peritumoral inflammation (4-tier and 2-tier systems) did not correlate with peripheral blood and intraepithelial NLRs (Fig. 3). However, the proportion of cases with a peripheral blood NLR ≥5 increased in higher grades of peritumoral inflammation in nondiabetics only ($r = 0.368$, $P < 0.05$). There was a weak correlation between peripheral blood and intraepithelial NLRs ($r = 0.129$, $P = 0.344$). Cases with increased intraepithelial (≥1) NLRs were more common in SCC than in ADC (22% vs. 2.6%, $P < 0.05$, Fisher's Exact test). Increased peripheral blood (≥5) NLRs was also more frequent in SCC cases, but it was not statistically significant (51.7% vs. 29.6%, $P \geq 0.05$, Fisher's Exact test). Although the distribution of peritumoral inflammation grades was similar between SCC and ADC ($P = 0.783$, Fisher's Exact test), SCCs presented more frequently than ADCs with increased intraepithelial lymphocytes (50% 20/40 vs. 23.07% 9/39, respectively, $P < 0.05$), and increased intraepithelial neutrophils (42.5% 17/40 vs. 2.5% 1/39, respectively, $P < 0.0001$).

DM type 2 is associated with increased NSCLC recurrence rate

The follow-up time in both cohorts was compared to detect any variation in drop-out rates. There was no significant difference in follow-up time between diabetics and nondiabetics (48.76 ± 46.71 vs. 44.72 ± 34.86 months respectively, *mean ± SD,* $P = 0.664$) (Fig. 4). Furthermore, the distribution of cases on the follow-up timeline was homogenous. Roughly, 50% were followed-up for 3 years or more, 25% for 5 years or more, and 25% for 7 months or less in both cohorts. The average survival in patients with diabetes was 148.3 months versus 104.3 months in nondiabetics, a difference that was not statistically significant ($P = 0.597$, Log-Rank test; $P = 0.666$, Breslow test). Events of tumor recurrence were more frequent in diabetics (12 events) than nondiabetics (6 events). However, this difference was not significant in univariate analysis ($P = 0.128$, Log-Rank test; $P = 0.101$ Breslow test). A multivariate Cox regression analysis adjusted by metastasis and obesity (significant for increased tumor recurrence in univariate analysis), showed that diabetes was associated with increased tumor recurrence (HR: 3.08; 95% CI: 1.027-9.23), (Fig. 4). None of the other variables including tumor necrosis, cancer type, intraepithelial lymphocytes, intraepithelial neutrophils, and peripheral blood and intraepithelial NLRs showed a significant impact on tumor recurrence.

NSCLC PD-L1 positivity and percentage of PD-L1 expression in diabetic versus nondiabetic patients

In the entire cohort, the expression of PD-L1 exhibited a moderate correlation with peritumoral inflammation ($r = 0.306$, $P < 0.01$) and increased intraepithelial lymphocytes ($r = 0.313$, $P < 0.05$). While negative expression was predominantly observed in tumors with focal/mild inflammation (45.5%), low and moderate expression (TPS1 and TPS2) were associated with moderate inflammation (45.8% and 48.1%, respectively) and high expression (TPS3) with diffuse inflammation (41.2%). Furthermore, high proportions of cold tumors were observed in TPS0 (54.5%), and hot tumors in TPS1, TPS2, and TPS3 (54.2%, 70.4%, and 76.5%, respectively). The relationships between PD-L1

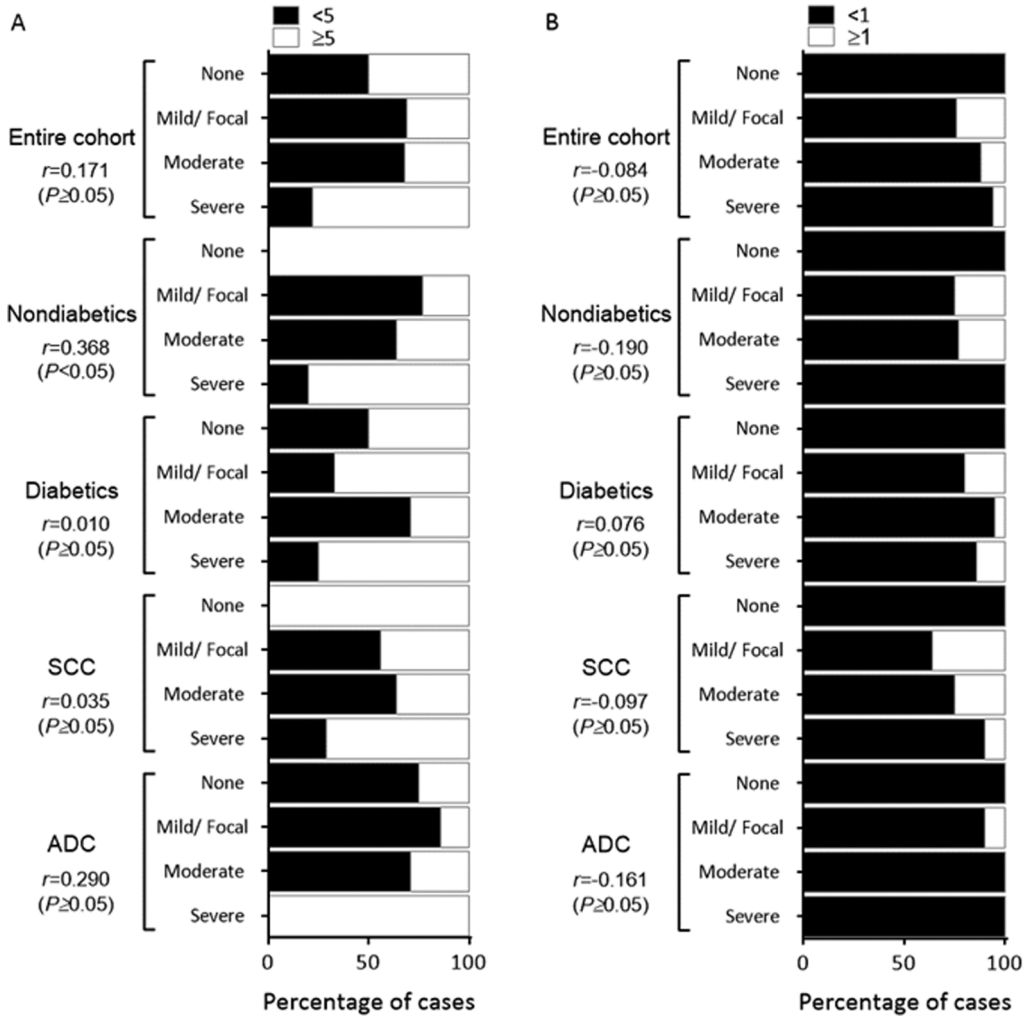

Fig. 3. Percentage frequency distribution of cases according to grade of peritumoral inflammation (none, mild/focal, moderate, and severe) and peripheral blood (A) and intraepithelial (B) neutrophil to lymphocyte ratios in the entire cohort, diabetic and nondiabetic patients, and squamous cell carcinoma (SCC) and adenocarcinoma (ADC) cases.

expression and NLRs were nonsignificant statistically (Table 2). Nonetheless, the category TPS2 (1–49%) was the only one to show an increased number of cases with high peripheral blood NLR (60% vs. 11.1% in TPS0, 36.8% in TPS1, and 37.5% in TPS3).

NSCLC in nondiabetics showed a relatively equal distribution across TPS categories as follows: TPS0 (negative): 20.5% (8/39), TPS1 (<1%):25.6% (10/39), TPS2(1-49%): 23.1% (9/39), and TPS3 (>50%): 30.8% (12/39). Diabetics comprised NSCLC tumors likely to be positive for PD-L1 immunostains, but those tumors largely showed TPS of less than 50%: TPS0 (negative): 7.5% (3/40), TPS1 (<1%):35% (14/40), TPS2 (1–49%): 45% (18/40), and TPS3 (>50%): 12.5% (5/40) (Fig. 5). The difference between TPS categories in diabetics and nondiabetics was statistically significant ($P<0.05$ Chi-square test; $P<0.05$ Fisher's Exact test). Despite there was no significant difference

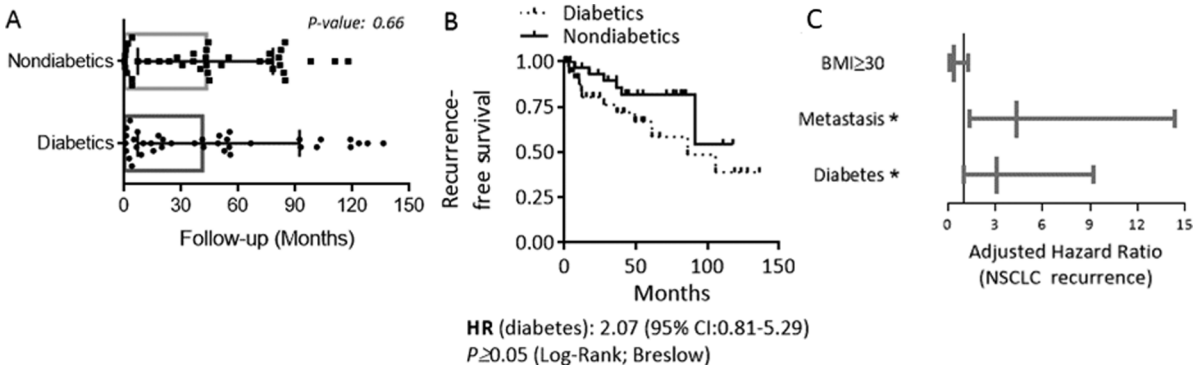

Fig. 4. Overall distribution of follow-up time (A) and Kaplan–Meier estimated curves for the two cohorts, diabetics (dotted line) and nondiabetics (non-dotted line), as a fraction of NSCLC recurrence-free survival (B) with corresponding adjusted hazard ratios (HR) (C, asterisk: $P<0.05$). Follow-up time is shown with lines marking the median and interquartile range in A (*P*-value, *t*-test).

Table 2
Distribution of PD-L1 expression (TPS categories) according to neutrophil to lymphocyte ratios (NLR), increased intraepithelial lymphocytes and neutrophils, and grade of peritumoral inflammation

TPS	Peripheral blood NLR		Intraepithelial NLR		Increased intraepithelial lymphocytes		Increased intraepithelial neutrophils		Peritumoral Inflammation (2-tier)		Peritumoral Inflammation (4-tier)			
	<5	*≥5*	*<1*	*≥1*	*Yes*	*No*	*Yes*	*No*	*Cold*	*Hot*	*None*	*Focal/ Mild*	*Moderate*	*Diffuse*
0 (negative)	**8 (88.9%)**	1 (11.1%)	**10 (90.9%)**	1 (9.1%)	2 (18.2%)	**9 (81.8%)**	2 (18.2%)	**9 (81.8%)**	**6 (54.5%)**	5 (45.5%)	1 (9.1%)	**5 (45.5%)**	4 (36.4%)	1 (9.1%)
1 (<1%)	**12 (63.2%)**	7 (36.8%)	**20 (83.3%)**	4 (16.7%)	7 (29.2%)	**17 (70.8%)**	6 (25%)	**18 (75%)**	11 (45.8%)	**13 (54.2%)**	4 (16.7%)	7 (29.2%)	**11 (45.8%)**	2 (8.3%)
2 (1-49%)	8 (40%)	**12 (60%)**	**23 (85.2%)**	4 (14.8%)	8 (29.6%)	**19 (70.4%)**	6 (22.2%)	**21 (77.8%)**	8 (29.6%)	**19 (70.4%)**	3 (11.1%)	5 (18.5%)	**13 (48.1%)**	6 (22.2%)
3 (>50%)	**5 (62.5%)**	3 (37.5%)	**16 (94.1%)**	1 (5.9%)	**12 (70.6%)**	5 (29.4%)	4 (23.5%)	**13 (76.5%)**	4 (23.5%)	**13 (76.5%)**	0 (0 %)	4 (23.5%)	6 (35.3%)	**7 (41.2%)**
Spearman test (*P*-value)	$r = 0.240$ ($P \geq 0.05$)		$r = -0.057$ ($P \geq 0.05$)		$r = 0.313$ ($P < 0.05$)		$r = 0.015$ ($P \geq 0.05$)		$r = 0.228$ ($P < 0.05$)		$r = 0.306$ ($P < 0.01$)			
Fisher's exact test (*P*-value)	0.08		0.819		0.014		1.000		0.256		0.266			

in PD-L1 expression between ADC and SCC cases ($P = 0.8$ Chi-square test; $P = 0.78$ Fisher's Exact test), the distribution of TPS categories according to diabetes diagnosis differed between cancer histologic types. For instance, SCC in diabetic patients showed higher amounts of cases in the TPS2 category in comparison to nondiabetics (65%, 13/20 vs. 20%, 4/20). On the other hand, ADC in diabetic patients showed a predominant distribution in the TPS1 category (50%, 10/20 vs. 26%, 5/19).

The positive relationship of PD-L1 expression with peritumoral inflammation was more pronounced in patients with diabetes ($r = 0.377$, $P < 0.05$) but diminished and non-significant in nondiabetics ($r = 0.136$, $P \geq 0.05$). Similar correlation analyses applied to the variable's obesity, tumor necrosis, peripheral blood and intraepithelial NLRs, increased intraepithelial lymphocytes and neutrophils, and metastasis failed to reveal a significant link. The association of diabetes with increased proportions of hot tumors in higher TPS categories was also heightened in SCC ($r = 0.424$ vs. $r = 0.181$ (nondiabetics)) and ADC cases ($r = 0.337$ vs. $r = 0.079$ (nondiabetics)). Finally, in univariate and multivariate survival, PD-L1 expression was not a prognostic factor for tumor recurrence (adjusted HR vs. TPS3: 1.260, 95% CI: 0.155-10.21) in the entire cohort. The size of the cohort and amount of PD-L1 negative cases were too small to perform a dichotomized survival analysis of PD-L1 expression (negative versus positive) in diabetics and nondiabetics.

Discussion

The primary goal of our investigation was to obtain comparable cohorts for evaluating the effect of DM type 2 on PD-L1 tumor expression in NSCLC. Accordingly, the groups of patients with diabetes ($n = 40$) and nondiabetics ($n = 39$) selected for this study were homogenous with matched age, gender, cancer stage, histologic type, tumor inflammation, NLRs and presence of tumor necrosis. Overall, diabetes appears to have a positive effect on PD-L1 expression in NSCLC. The expression of PD-L1 is under controlled of several biological processes [17]. Briefly, there is a constitutive expression under the control of genetic mechanisms and an inducible expression triggered by the presence of some T-cell subsets. Constitutive PD-L1 surface tumor expression is usually diffuse (>50%), while in those with inducible expression, the location of PD-L1+ cancer cells correlates with peritumoral lymphocytic aggregates and tends to be focal (<50%) [17]. Diabetes was associated with increased tumor PD-L1 positivity but at less than 50%, and the fact that the amount of heavily inflamed NSCLC increased proportionately in higher TPS categories, suggest that inducible expression of PD-L1 was upregulated. It is unclear why this relationship did not extend to the TPS3 category (>50%). Specifically, 30.8% of non-diabetics had a PD-L1 tumor proportion score higher than 50% vs. only 12.5% of patient with diabetes. Perhaps, the growth of tumors with constitutive PD-L1 expression is not favored in diabetic patients, and the positive effect is restricted to some NSCLC phenotypes. We postulate that by reducing blood glucose levels, the inflammatory milieu of tumors can be reduced and the production of PD-L1 could decrease.

Diabetes may promote a neoplastic process by several mechanisms including hyperinsulinemia stimulating tumor growth following the activation of insulin-like growth factor (IGF) receptors, and hyperglycemia feeding cancer cells with aerobic glycolysis (the Warburg effect). Elevated levels of inflammatory molecules that enhance cancer cell survival, invasion or suppressing anti-tumor immunity also plays a role [18]. Indeed, PD-L1 expression correlates with high glucose metabolism in clinical and experimental models. In an *in vitro* cell line model of NSCLC, higher PD-L1 expression was accompanied by increased glucose uptake, suggesting the existence of a potential pathway of immunosuppression induced by increased tumor glucose catabolism [19]. Moreover, NSCLCs with higher glucose uptake measured by 8F-fluorodeoxyglucose PET/CT display higher rates of PD-L1 positivity [20]. In a mouse sarcoma model, the blockage of PD-L1 with antibodies

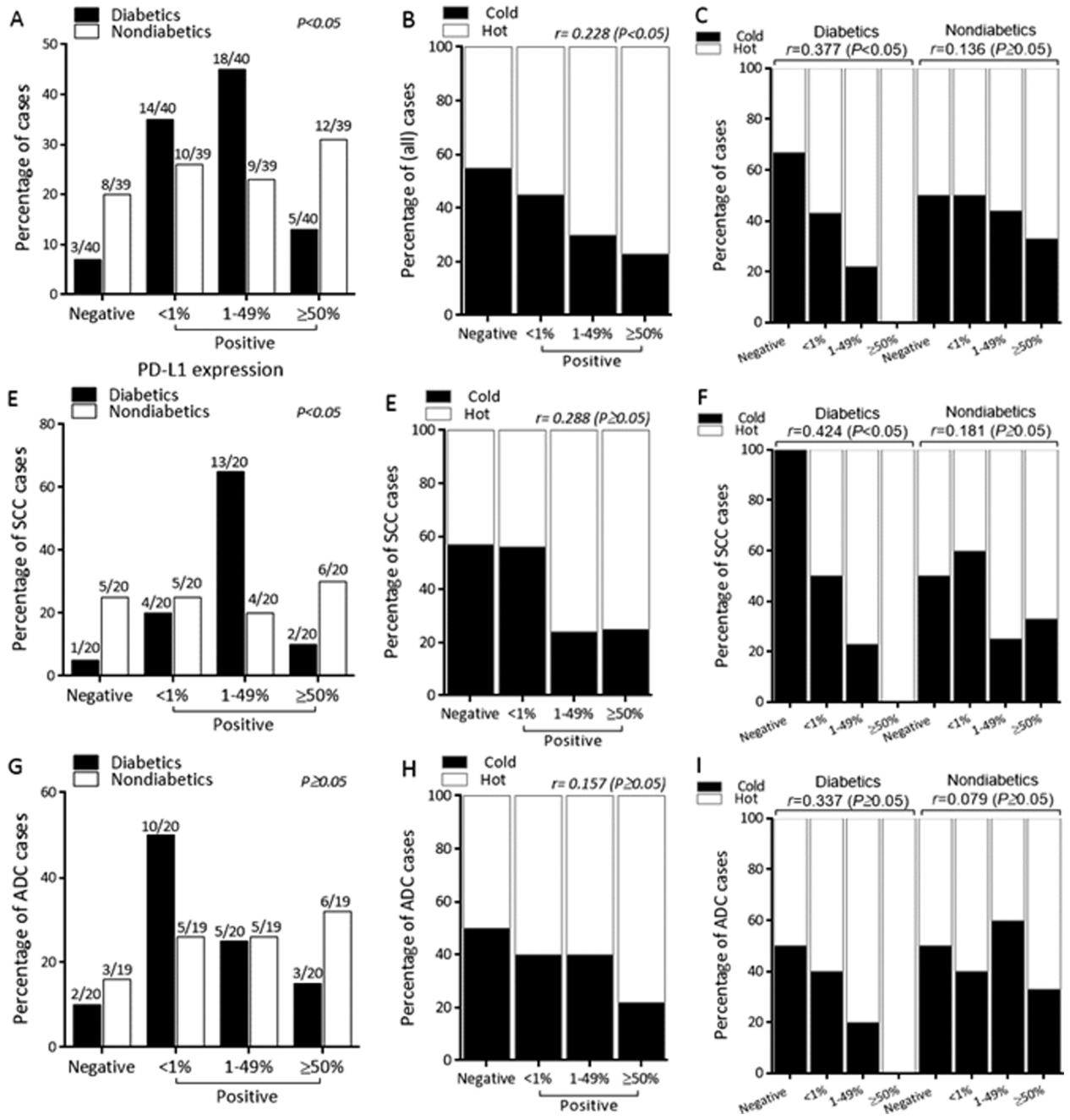

Fig. 5. Percentage frequency distribution of PD-L1 expression (DAKO, Clone 22C3) according to the diagnosis of diabetes mellitus type 2 and peritumoral inflammation in the entire cohort (A-C), squamous cell carcinoma (SCC) cases (D-F), and adenocarcinoma (ADC) cases (G-I).

impaired the tumor ability to burn glucose by decreasing the expression of glycolytic enzymes. PD-L1 was a direct regulator of tumor glucose utilization, and both PD-L1 expression and glycolytic activity were necessary to mediate T-cell hyporesponsiveness [21]. These results support the concept that cellular phenotypes characterized by increased glycolytic metabolism and concomitant high PD-L1 expression are part of coordinated immunosuppressive and pro-survival responses during cancer progression, which are likely to be potentiated in patients with diabetes.

Previously, we showed that the same cohort of NSCLCs included in the presented study demonstrated significantly higher expression of IGF-1 receptor, transforming growth factor-beta (TGF-β), vimentin, and N-cadherin, while the expression of HtrA1 and E-cadherin were decreased in diabetic patients [11]. This immunoprofile is characteristic of NSCLC cells with EMT phenotype. Cancer cells undergoing EMT may be the same as those with PD-L1 overexpression and high glycolytic activity, possibly comprising a set of clones with a more aggressive biologic behavior that is favored in patients with diabetes. Tumor EMT is enhanced by Warburg effect-type metabolism and can be prevented by disrupting either glucose uptake or glycolytic pathways [22,23]. Furthermore, PD-L1 overexpression in a mouse model of cervical cancer cells correlated with an increase in glucose uptake mediated by the promotion of EMT pathways [24]. PD-L1 has also been shown to mediate the induction of EMT in the human esophageal cancer cell line Eca-109 [25]. A recent study revealed that PD-L1 positivity was significantly higher in lung adenocarcinomas with “mesenchymal” or EMT phenotype [12]. This evidence supports that PD-L1 is involved in the acquisition of EMT characteristics along with abnormal glucose metabolism.

The association of diabetes with tumor PD-L1 expression was

stronger in SCC than ADC (Fig. 5). As compared to ADCs, SCCs have been associated with a higher glycemic index, preferential elevation of the GLUT-1 transporter (increased glucose uptake and glycolytic dependency), diabetes, and diets high in saturated fats [26–28]. SCCs in diabetic patients exhibited a skewed distribution of PD-L1 expression to the TPS2 (1–49%) category (65% of all SCCs) while ADCs were to the TPS1 (<1%) category (50% of all ADCs). In the entire cohort, SCCs showed a significant increase in intraepithelial lymphocytes and neutrophils, and peripheral blood and intraepithelial NLRs. Interestingly, TPS2 tumors displayed higher grades of peritumoral inflammation and relatively higher proportions of increased NLRs (Table 2). A retrospective review of surgical specimens suggests that SCCs can evolve with $CD8^+$ tumor-infiltrating lymphocytes within cancer nests, and such infiltrates are associated with a survival benefit at early stages [29,30]. It is possible that hyperglycemia in diabetic patients intensified the expression of PD-L1 in SCCs with conspicuous tumor-associated inflammation and increased NLR, promoting rapid tumor progression by inducing immune dysfunction.

Our findings have potential therapeutic implications. Blockage of the PD-1 receptor or its ligand PD-L1 with monoclonal antibodies is a major strategy for promoting T-lymphocyte-mediated anti-tumoral immune responses, improving the survival as monotherapy or in combination [31,32]. Although there is no universal prognostic biomarker to predict clinical response to anti-PD-1/PD-L1 therapies, PD-L1 positivity on IHC assays correlates with an excellent clinical benefit[33]. Increased tumor PD-L1 expression in patients with diabetes type 2 suggests that this NSCLC subgroup may greatly benefit from immune checkpoint inhibitors. Upon review of the literature, no clinical studies have addressed whether the efficacy of PD-L1 inhibitors changes in diabetic patients with either NSCLC or any other form of cancer. Nevertheless, a recent study proved, across multiple species and tumor models, that obesity causes PD-1-mediated T cell dysfunction and remarkably left tumors markedly more responsive to checkpoint blockage [34]. It is conceivable that diabetes type 2, like obesity, can promote tumor growth and recurrence by immune dysregulation, but paradoxically, this could make checkpoint inhibitors more effective.

Conclusion

In summary, diabetes mellitus type 2 is an extra-tumoral (extrinsic) independent variable that was associated with higher tumor PD-L1 positivity and increased tumor recurrence in NSCLC. Our results indicate that patients with diabetes type 2 may be candidates for PD-L1 inhibitors, especially in those with SCC. The positive effect of diabetes on PD-L1 expression is more pronounced in NCSLC with significant peritumoral inflammation. Taking into consideration prior observations of increased EMT in the same cohort of diabetic patients with NSCLC, it is possible that diabetes can boost the survival and growth of tumor cells skilled in metabolizing glucose through the Warburg pathway as part of a tumor metabolic reprogramming, inducing cancer cell EMT and creating an immunosuppressive microenvironment by upregulation of PD-L1 by inducible pathways. The precise molecular mechanism involved in altered PD-L1 tumor expression in patients with diabetes remains to be determined.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

None to declare.

CRediT authorship contribution statement

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Acknowledgment

None to disclose.

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