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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Clinical impact of PD-L1 and PD-1 expression in squamous cell cancer of the vulva

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Abstract

Purpose Squamous cell carcinoma of the vulva (SQCV) is the fifth most common cancer in women and accounts for about 5% of all genital cancers in women. The PD-L1 signaling pathway is activated in many malignant neoplasms and its blockade enhances anti-cancer immunity. The aim of our study was to examine the protein expression of PD-L1 and PD-1 in squamous cell cancer of the vulva, its correlations with clinicopathologic features and prognostic value.

Methods Patients with SQCV treated in one institution were used for the analyses. PD-L1 immunohistochemistry was performed on 4 µm-thick section of the respective FFPE tissue blocks using the 28-8 antibody. PD-L1 scoring was performed separately for tumour cells (TC) and tumour associated immune cells. DNA was extracted to determine HPV status. Kaplan–Meier estimates for disease-free-survival and overall-survival were calculated and compared by log-rank test.

Results PD-L1 expression in tumour cells could be observed in 32.9% of the patients. The expression of PD-L1 in peritumoural immune cells was confirmed in 91.4% of the patients. A significant correlation between PD-L1 expression in tumour cells and tumour stage was detected ($p = 0.007$). PD-L1 expression was independent from HPV status. Using the log-rank test we could not prove any significant differences in disease-free survival ($p = 0.434$) and overall survival ($p = 0.858$). Regression analysis showed that nodal status is a predictive factor of survival ($p < 0.001$).

Conclusion The present study showed that a relevant amount of patients with squamous cell cancer of the vulva express PD-L1 in both, tumour cells and tumour-associated immune cells. Furthermore, the significant correlation of PD-L1 expression in TCs with tumour stage indicated the clinical impact of PD-L1 expression during tumour development. These data indicate that SQCV might be amenable to immune checkpoint-inhibition and constitute a rational for the future clinical trials.

Keywords Vulvar carcinoma · PD-L1 · PD-1 · HPV

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Purpose

Squamous cell carcinoma of the vulva (SQCV) is the fifth most common cancer in women and accounts for about 5% of all genital cancers in women with a current incidence of 2–3 in 100,000 women per year (Mahner et al. 2015). It is known to be a disease of the elderly woman (median age 65–70 years), but currently incidence rates in younger women are rising (Beller et al. 2006; Hampl et al. 2008; Mahner et al. 2015). The aetiology of this tumour shows two different subgroups. One group of tumours is linked to an infection with high-risk Human papilloma virus (HPV), cigarette smoking and high-grade squamous intraepithelial lesion (HSIL) (Gargano et al. 2012), whereas the second group of tumours is linked to chronic vulvar inflammatory disease (i.e. lichen sclerosus and lichen planus) and high-grade vulvar intraepithelial neoplasia II/III (VIN II/III) (Del Pino et al. 2013). Besides surgical procedures and radiation therapy, treatment options, in particular for advanced and recurrent disease, are limited.

In both tumour subtypes, however, chronic inflammation seems to be a key driver of SQCV carcinogenesis (Multhoff et al. 2012). In general, tumours of viral etiology are known to induce a strong inflammatory reaction triggering cytolytic T cell responses (Topalian et al. 2016) that render these cancers sensitive to immunotherapy (Topalian et al. 2016), indicating new treatment options with the potential to improve the prognosis of these patients.

As aberrant programmed death ligand 1 (PD-L1) or programmed death ligand 2 (PD-L2) expression may cause local immune-suppression (Schultheis et al. 2015), the so-called immune checkpoint programmed death (PD-1)/PD-L1/PD-L2 pathway is a major target in anti-tumour immunotherapy.

Aberrant activation of such co-inhibitory pathways is a key determent of local immune-suppression, and counteracting PD-1/PD-L1 was demonstrated to yield strong and durable tumour regression in several solid tumours (Schultheis et al. 2015; Topalian et al. 2014; Wolchok et al. 2013).

PD-L1 expression is upregulated in many different cancers and its blockade enhances anti-cancer immunity. Several clinical trials testing anti-PD1 or anti-PDL1 drugs demonstrated promising results with durable responses in different cancers including melanoma, renal, lung, prostate and bladder carcinomas (Bertucci et al. 2015; Brahmer et al. 2012; Powles et al. 2014; Topalian et al. 2012). Especially, immunotherapy with checkpoint inhibitors demonstrated efficacy in advanced NSCLC. In 2015 PD-1 inhibitors nivolumab and pembrolizumab were approved for second-line therapy of NSCLC. In 2016 PD-L1 inhibitor atezolizumab was approved for the same indication. Pembrolizumab also received approval in 2016 for first-line NSCLC treatment in patients with high PD-L1 expressing tumours (Malhotra et al. 2017). Currently, no comprehensive data for PD-L1 and PD-1 protein expression and its clinical impact in SQCV are available. The aim of our study was to examine the protein expression of PD-L1 and PD-1 in squamous cell cancer of the vulva and its potential correlations with clinicopathologic features including its prognostic value in these tumours.

Materials and methods

Patients and tissue sample selection

Seventy patients with squamous cell carcinoma of the vulva who underwent intended curative surgical resection at the University Hospital Cologne in the Department for Gynaecology and Obstetrics were used for the analyses. Representative formalin-fixed, paraffin-embedded tissue blocks of each case were selected from the archives of the Institute of Pathology, University Hospital Cologne, Germany. Patients had signed written informed consent prior to the study and approval by the local ethics committee was granted for the study. Primary diagnoses were independently confirmed on H&E stained slides by two experienced pathologists (A.H.S. and A.M.S.) according to the current WHO recommendations (Kurman 2014).

Immunohistochemistry

PD-L1 immunohistochemistry was performed on 4 µm-thick section of the respective FFPE tissue blocks using EDTA-based antigen-retrieval and primary antibody clone 28-8 at 1:100 dilution (Abcam, Cambridge, UK). The primary antibody is the same clone used in the Dako 28-8 pharmDx assay that has been clinically validated in combination with PD-1 inhibitor nivolumab (Borghaei et al. 2015).

Detection was performed on an automated staining system with polymer-based secondary antibody kit and DAB (Leica Bond Polymer Refine; Leica Biosystems, Wetzlar, Germany). For PD-1, the primary antibody NAT105 (mouse monoclonal, Abcam, Cambridge, UK) was used. Detection was performed using the Bond Polymer Refine Kit (Leica Biosystems Newcastle Ltd, Newcastle, UK).

Scoring was performed independently by two pathologists (A.M.S., A.H.S.), who were blinded to the clinical parameters. PD-L1 scoring was performed separately for tumour cells (TC) and tumour-associated immune cells (IC). The proportions of positive cells were scored and the percentage of positive cells was given.

For the tumour cells this approach is identical to the tumour proportion score established for non-small cell lung cancer (Roach et al. 2016). For the immune cells, macrophages/histiocytes, lymphocytes and dendritic cells were scored in the tumour area, i.e. within the field of view of the tumour cells at 200× magnification.

A case was considered as 'positive' (TC1) if $\geq$ 1% of the respective cells showed a specific staining of any intensity. Furthermore, cases were subdivided into groups with cases showing high expression of PD-L1 protein (TChigh), if $\geq$ 50% of tumour cells expressed PD-L1, and low expression (TClow) if $<$ 50% of tumour cells expressed PD-L1. The expression of PD-L1 in immune cells was classified as positive if at least 10% of immune cells showed expression and was further subclassified as follows: low expression (IClow) if $<$ 10% of immune cells were positive and high expression (IChigh) if $\geq$ 10% showed PD-L1 expression. For analysis of PD1 protein expression, expression in immune cells was scored and the percentage of positive cells was given□□.

DNA extraction and evaluation of HPV status

The tumour area was marked on a haematoxylin–eosin (H&E) stained slide by a pathologist (A.M.S). Six 10 µm-thick sections of the respective tumour blocks were cut and mounted on unstained glass slides. After deparaffinization, tumour areas were macrodissected and tissue was lysed with proteinase K overnight. DNA was purified with the Maxwell® 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega, Mannheim, Germany) on the Maxwell® 16 (Promega, Mannheim, Germany) and DNA was eluted in Tris–HCl (pH 7.6). All extraction procedures were performed following the manufacturers' instructions.

HPV status of each case was assessed using a standard PCR protocol, followed by an enzyme-linked immunosorbent assay (ELISA) as implemented in the routine diagnostics laboratory of the Department of Pathology of the University Hospital Cologne, Germany (Merkelbach-Bruse et al. 1999).

Statistical analysis was performed using SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). Patient characteristics were described using median [interquartile range (IQR)] or count (percentages), as appropriate.

Fisher's exact test was used to test for associations between PD-L1 expression in TCs and T stage, nodal status and HPV status. Odds ratio (OR), corresponding 95% confidence interval (CI) and p values are presented. Kaplan–Meier estimates for disease-free-survival (DFS) and overall-survival (OS) were calculated and compared by log-rank test. Recurrences divided into local recurrence, lymph nodes metastases and distant metastases were considered as events for DFS. OS was defined as time between diagnosis and death. Patients without an event or who were lost in follow-up were censored.

Results

Patients

Seventy patients with squamous cell carcinoma of the vulva who underwent primary surgical resection were included in the study. Median age of these patients was 59.3 years (range 26–90), whereas median BMI was 27.3 kg/m^2 (range 18.4–44.1). Basic clinicopathologic data are presented in Table 1.

All patients underwent wide tumour excision, and 91.4% of the patients underwent inguinal (sentinel) lymphonodectomy; 25.7% of the patients received adjuvant radiotherapy while 5.1% of the patients underwent adjuvant chemotherapy.

Table 1 Patient characteristics

	Total ($n = 70$)	Missing
Age (years)	61.5 (47.5–71.3)	0 (0)
BMI (kg/m^2)	27.3 (23.5–33.9)	5 (7.1)
Smoker		
Yes	23 (32.9)	10 (14.3)
HPV status		
Positive	11 (15.7)	3 (4.3)
T-stage		
T1	50 (71.4)	0 (0)
T2	16 (22.9)	
T3	4 (5.7)	
N-stage		
N0	47 (67.1)	6 (8.6)
N1	6 (8.6)	
N2	11 (15.7)	
Grading		
G1	1 (1.4)	0 (0)
G2	57 (81.4)	
G3	12 (17.1)	
Radiation therapy		
Yes	18 (25.7)	6 (8.6)
Chemotherapy		
Yes	4 (5.7)	5 (7.1)
Recurrence of disease		
Yes	23 (32.9)	0 (0)
Death		
Yes	10 (14.3)	0 (0)

Data are presented as median (IQR) or n (%), respectively

HPV status and smoking status

Of the patients, 15.7% had a positive HPV status and 32.9% were ever smokers.

PD-L1 expression and PD-1 expression

PD-L1 expression in tumour cells was observed in 32.9% of the patients; 24.3% of the PD-L1 positive patients had a low expression, whereas 8.6% showed a high expression of PD-L1 in tumour cells. Examples of different expression patterns are shown in Fig. 1.

The expression of PD-L1 in peritumoural immune cells was detected in 91.4% of the patients. The prevalence of PD-L1 expression in TCs and ICs is given in Fig. 2.

Simultaneous expression of PD-L1 in TCs and ICs could be observed in 32.9% of patients. 58.6% ($n = 41$) of the patients showed PD-L1 positivity in ICs only. 8.6% ($n = 6$) showed PD-L1 expression in neither TCs nor ICs.

In a representative subgroup ($n = 63$) of patients PD-1 expression in peritumoural immune cells was assessed.

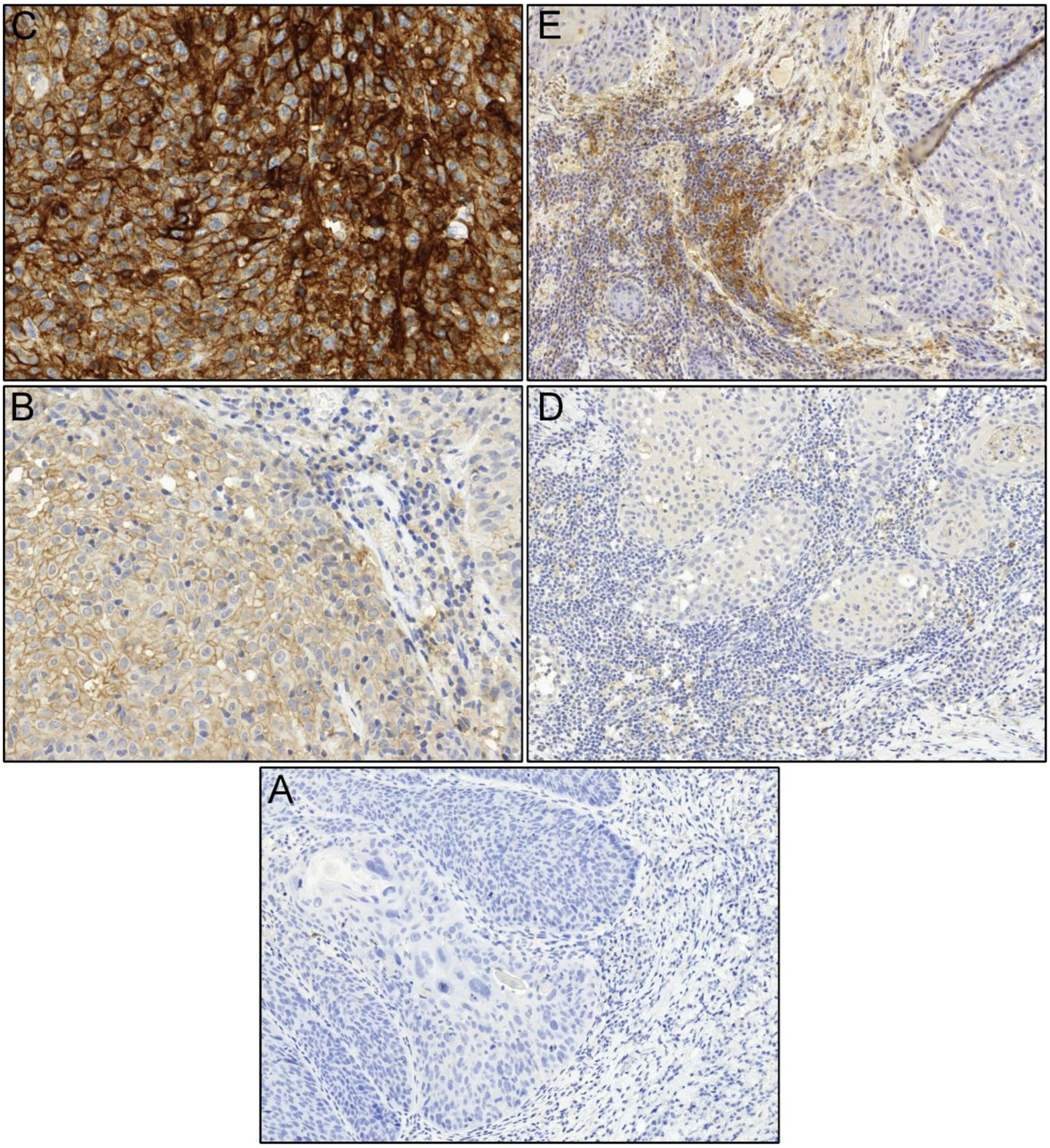

Fig. 1 PD-L1 IHC patterns in vulva-carcinoma. **a** TC and IC negative. **b** TC positive, widespread expression (> 50%), weak staining intensity. **c** TC positive, widespread expression (> 50%), strong staining intensity. **d** IC positive, low proportion; TC negative. **e** IC positive, high proportion; TC negative. *TC* tumor cells, i.e. carcinoma cells, *IC* immune cells, i.e. tumour-associated immune cells, any kind

Among the subgroup 95.2% of the patients showed PD-1 expression in peritumoural immune cells. The expression of PD-L1 and PD-1 is summarised in Table 2.

A strong squamous differentiation could be observed in 14.3% of the study cohort, whereas 44.3% of the study cohort showed a strong inflammation. The morphologic features are summarised in Table 3.

The fisher exact test showed a significant correlation between PD-L1 expression in tumour cells and T-stage ($p = 0.007$) (Fig. 3). Furthermore, a borderline significance could be demonstrated between N-stage and PD-L1 expression in tumour cells ($p = 0.079$).

There was either no correlation between PD-L1 in tumour cells and HPV status ($p = 0.330$) nor between PD-L1 in tumour cells and inflammation ($p = 0.264$).

Median follow-up time was 43.5 months (range 0–134.5) for overall survival and 27.8 months for disease-free survival (range 0–134.5). During the follow-up time 22 patients experienced a recurrence of disease and 10 patients died.

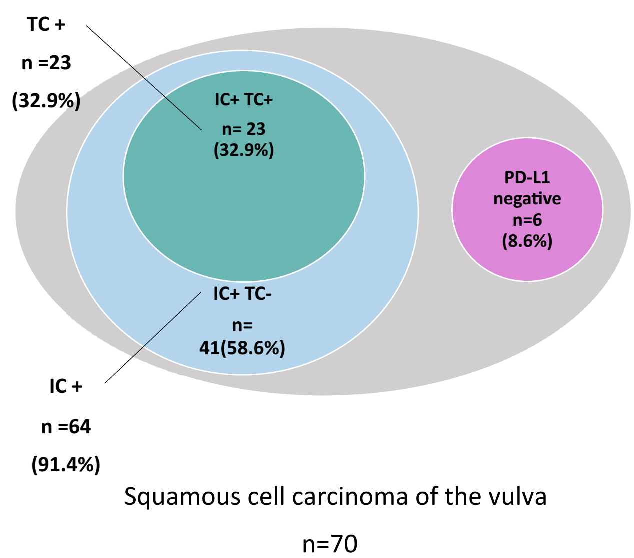

Fig. 2 PD-L1 IHC positive (+) and negative (−) tumour cells (TC) and immune cells (IC) in squamous cell carcinoma of the vulva. PD-L1 positive TCs are frequent and occurs in combination with ICs. ICs may occur isolated or in combination with TCs. Absolute numbers and relative proportions are given for each subgroup

Kaplan–Meier curves of disease-free survival and overall survival are depicted in Figs. 4 and 5. Using the log-rank test we could not prove any significant differences in disease-free survival ($p = 0.434$) and overall survival ($p = 0.858$) according to PD-L1 expression in tumour cells.

Univariate Cox regression analysis showed that N-stage, as expected, is a predictive factor of overall survival ($p < 0.001$).

Discussion

Immunotherapies have been arising as a potential immunological option for tumour therapy in many different malignant neoplasms (Schmidt et al. 2015) with checkpoint inhibitors being already approved in several malignancies (Raju et al. 2018). Currently studies examine efficacy of checkpoint inhibition of the PD-L1/PD-1 pathway in gynaecological cancer (Fan et al. 2018; Saglam and Conejo-Garcia 2018). PD-L1 protein expression on tumour cells has been demonstrated to be a dominant mechanism that is relevant to anti-PD1 drug response (Topalian et al. 2016). Little is known about the profile of PD-L1 and PD-1 expression in SQCV. The few published data on the PD-L1 expression vary extremely. The expression of PD-L1 in tumour cells was reported to be between 32.1 and 72.7% (Choschzick et al. 2018; Sznurkowski et al. 2017). To evaluate checkpoint inhibition as a potential component of multimodal treatment for advanced SQCV, the simple question whether PD-L1/PD-1 is expressed in SQCV has to be answered in more comprehensive patient cohorts. Therefore, we analysed 70 cases of clinically annotated SQCV for protein expression of PD-L1 using the 28-8 antibody.

Our data revealed an expression rate of 32.9% of PD-L1 in tumour cells. While Choschzick et al. used the

Table 2 Squamous cell carcinoma of the vulva

IHC: PD-L1 (Ab: 28-8) ($n = 70$)	
TC	
Negative	47 (67.1)
Positive	23 (32.9)
Low (< 50%)	17 (73.9)
High (≥ 50%)	6 (26.1)
IC	
Negative	6 (8.6)
Positive	64 (91.4)
Low (< 10%)	46 (71.9)
High (> 10%)	18 (28.1)
IHC: PD-1 (Ab: NAT105) ($n = 63$)	
IC	
Negative	3 (4.8)
Positive	60 (95.2)
Low (< 10%)	50 (83.3)
High (> 10%)	10 (16.7)

Data are presented as n (%)

Table 3 Summary of morphologic characteristics

	Total ($n=70$)	Missing
Squamous		
None	16 (22.9)	8 (11.4)
Weak	23 (32.9)	
Intermediate	13 (18.6)	
Strong	10 (14.3)	
Basolid		
Yes	9 (12.9)	8 (11.4)
Solid		
Yes	38 (54.3)	8 (11.4)
Nests		
Yes	58 (82.9)	8 (11.4)
Buds		
Yes	34 (48.6)	9 (12.9)
Single cells		
Yes	9 (12.9)	8 (11.4)
Inflammation		
Weak	8 (11.4)	9 (12.9)
Intermediate	22 (31.4)	
Strong	31 (44.3)	

Data are presented as *n* (%)

clone E1L3N (Cell Signaling Technology, Inc: 1:100), Sznurkowski et al. used a mouse anti-human monoclonal antibody against PD-L1 (clone 22C3, cat. No M3653) obtained from Dako Inc. (Choschzick et al. 2018; Sznurkowski et al. 2017) giving a possible explanation for discrepancies. Furthermore, cut-off values for PD-L1 positivity were either ≥ 1% or ≥ 5%. About 36.9% of patients with SQCV have been reported to be positive for PD-L1 expression in ICs and negative for PD-L1 expression in TCs. According to our analysis the rate was higher with 58.6%, probably due to different cut-off values used.

In the recent years a significant correlation has been reported between T-stage and PD-L1 expression in primary and metastatic sited clear cell renal cancer and gastric cancer (Callea et al. 2015; Qing et al. 2015). According to our current analyses we were able to confirm these results in SQCV. Additionally, we could see a trend between N-stage and PD-L1 expression in TCs.

Both, tumour cells and associated stromal cells are able to express immune checkpoints, turning off T-effector cells (Dong et al. 2002). In our series, a subset of macrophages express PD-L1 which can be induced by activated lymphocytes, endothelial cells and other non-malignant cell types in the inflammatory microenvironment as a part of a physiological process to down-modulate ongoing host immune responses in peripheral tissues (Dong et al. 1999, 2002; Mazanet and Hughes 2002; Topalian et al. 2016). Whereas a subset of SQCV is linked to a chronic vulvar inflammatory disease, a further subset is associated with an infection with HPV (Del Pino et al. 2013; Gargano et al. 2012).

Whether the HPV status is associated with PD-L1 expression in cancer has been discussed controversially in the past (Topalian et al. 2016). In virus-associated tumours, the protein product of expressed genes represents non-self antigen and hence encode potential T-cell epitopes. Products of viral oncogenes are known to drive tumorigenesis and are, therefore, not detected by the immune system or silenced as a part of immune evasion (Topalian et al. 2016). PD-L1 expression in oropharyngeal squamous cell carcinoma, which is known to be associated with HPV infection in a subset of cases, is not correlated to HPV status ($p=0.274$) (Kim et al. 2015). Our results confirm these findings; however, it has to be taken into account that the amount of patients with HPV-positive tumours in the present cohort is low with

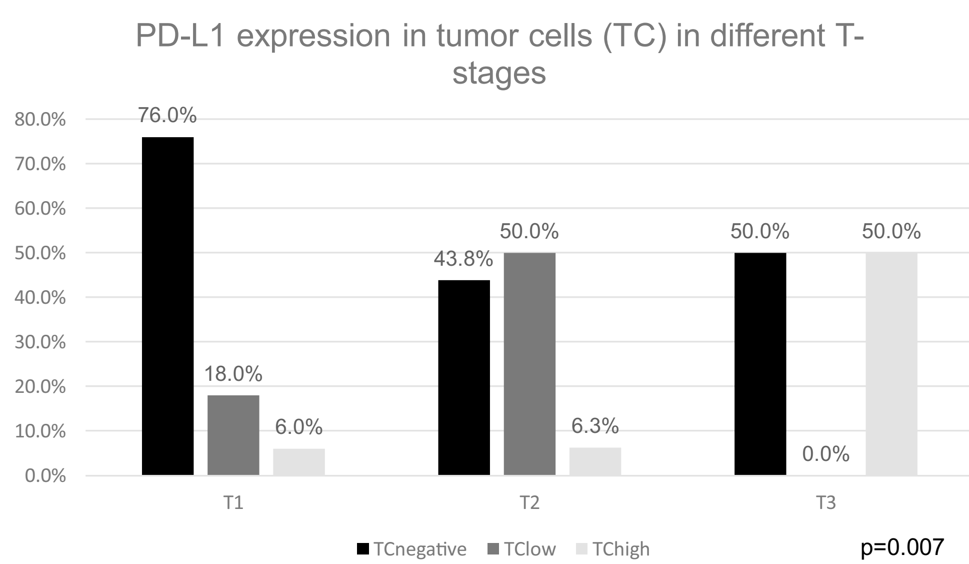

Fig. 3 PD-L1 expression in tumour cells (TCs) in different T-stages. *TCnegative* negative expression, *TClow* low expression (< 50%), *TChigh* high expression (≥ 50%)

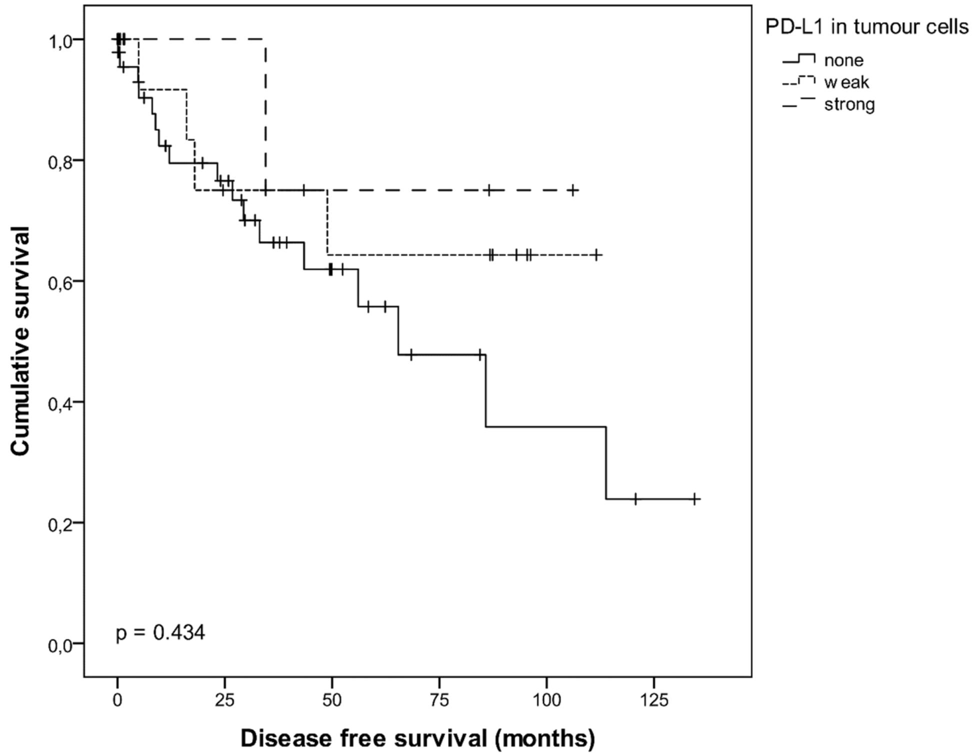

Fig. 4 Disease-free survival (DFS) dependent from expression of PD-L1 in tumour cells (0 = none, 1 = weak and 2 = strong) ($p = 0.434$)

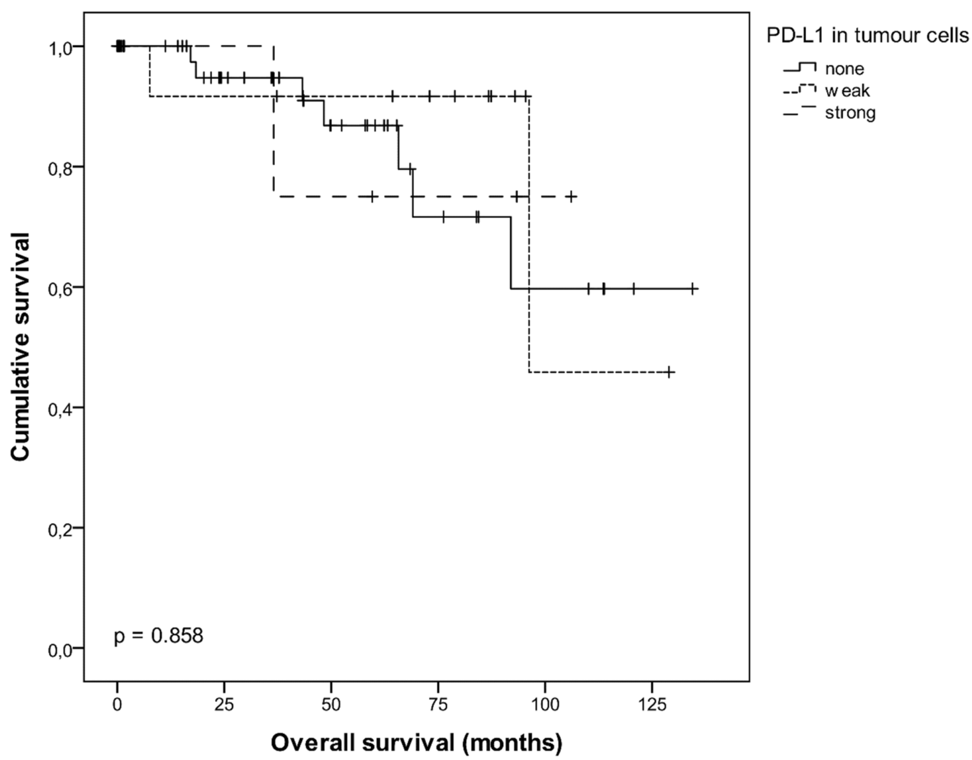

Fig. 5 Overall survival (OS) dependent from expression of PD-L1 in tumour cells (0 = none, 1 = weak and 2 = strong) ($p = 0.858$)

15.7%, which could have influenced the results. Prognostic factors in vulvar cancer are important for therapy planning. Lymph node status is the most important prognostic factor in patients with SQCV (Kowalewska et al. 2012; Raspagliesi et al. 2006). In univariate Cox regression analysis we could confirm the significance influence of N-stage on overall survival ($p < 0.001$).

To our knowledge this is one of the few comprehensive studies investigating PD-L1 expression in SQCV. Together with previously published data, our analysis indicates that PD-L1 expression in TCs of SQCV might serve as a predictive factor for response to checkpoint inhibitors and that at least one-third of patients with SQCV could profit from checkpoint inhibition. Of note, therapy efficacy in other tumours could also be shown in PD-L1 negative tumours (Gong et al. 2018). Therefore, subsequent analyses, such as the investigation of the mutational load or the analysis of the tumour microenvironment are needed to identify additional predictive factors of therapy response in patients with PD-L1 negative SQCV to not miss any SQCV patients with advanced stage tumours that might benefit from immune therapy (Carbone et al. 2017; Gibney et al. 2016; Hellmann et al. 2018; Rizvi et al. 2015, 2018).

Conclusions

In the present study we show that a significant number of patients with squamous cell carcinoma of the vulva express PD-L1 in both, tumour cells and tumour-associated immune cells indicating the possibility of offering patients at need for additional treatment options checkpoint inhibition. In addition, the high prevalence of PD-1 expression in the tumour environment together with the immunogenic profile (virus-driven versus driven by chronic inflammation) indicates the special immunogenicity of this tumour entity. Hence, further comprehensive analyses are warranted to identify more patients with negative PD-L1 status that might benefit from checkpoint inhibitors.

Compliance with ethical standards

Conflict of interest AMS, CA und BH are supported by Roche Pharma AG and Kölner Krebsstiftung. JP was supported by the Else Kröner-Fresenius Stiftung (EKFS-2014-A06 and 2016 Kolleg.19). AHS and RB have participated in advisory boards for BMS, MSD and F. Hoffmann-La Roche AG, pharmaceutical division. FT declares that she has no conflict of interest. BM declares that he has no conflict of interest. CP declares that she has no conflict of interest. LMS declares that he has no conflict of interest. PM declares that he has no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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