

Programmed Death Ligand-1 (PD-L1) expression is up-regulated and related to the pattern of invasion in FIGO Stage I vulvar squamous cell carcinomas

Hermann Brustmann, MD, PhD¹

Keywords: Vulva, Programmed Death Ligand-1, PD-L1, squamous cell carcinoma, pattern of invasion

Abstract

The immune checkpoint protein programmed death ligand-1 (PD-L1) is expressed in different types of cancer and is a potential prognostic factor as well as therapeutic target.

This study evaluated PD-L1 expression in the neoplastic progression of vulvar epithelia with respect to the pattern of infiltration in FIGO stages I keratinizing squamous cell carcinomas (SCC). Normal squamous vulvar epithelia (n=20), usual type vulvar intraepithelial neoplasia (uVIN, n=23), differentiated VIN (dVIN, n=21) and FIGO stage I SCC (n=35) were immunostained for PD-L1. In SCC a cohesive growth with well-delineated borders was considered as pushing, dissociative growth in small groups or single cells was defined as diffuse pattern of infiltration. Immunostaining was done with a monoclonal anti PD-L1 antibody (clone SP263, Ventana) and scored to determine up-regulation and overexpression (score 0/1+, 0-5% immunoreactive cells; score

2+, >5 to 50% immunoreactive cells; score 3+, >50% immunoreactive cells). PD-L1 immunoexpression was comparable in normal epithelia and VINs (score 0/1+, n=59; score 2+, n=5, in VINs only; score 3+, n=0), was significantly increased (P<0.0001) in SCC (score 0/1+, n=13; score 2+, n=16; score 3+, n=6), and was related to a diffuse pattern of infiltration (P<0.0001). Staining was accentuated at the invasive margins of SCC frequently. PD-L1 expression is up-regulated in the neoplastic cells of vulvar low stage SCC, related to the development of an invasive phenotype reflecting the initiation of cancer immunoediting, and to an aggressive diffuse type of stromal invasion.

¹Department of Pathology, Landeskrankenhaus Baden-Moedling, Baden, Austria

Introduction

Invasive squamous cell carcinoma (SCC) is the most common type of all invasive vulvar cancers. High grade

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Corresponding author: Dr. Hermann Brustmann, Landeskrankenhaus Baden-Moedling, Waltersdorfer Straße 75, A-2500 Baden, Austria. Tel: +43-2252-9004-14700, Fax: +43-2252-9004-49355. preferred e-mail: ddrbrustmann@hotmail.com. professional e-mail: hermann.brustmann@baden.lknoe.at

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vulvar intraepithelial neoplasias (VIN) are precancerous changes, defined as proliferative intraepithelial squamous lesions which display abnormal growth. Basaloid and warty (condylomatous) or usual subtypes of VIN (uVIN) are associated with relatively younger women, and evidence of human papillomavirus (HPV) nucleic acids. The differentiated subtype of VIN (dVIN) is associated with relatively older women and develops independently of HPV. The morphologic and etiologic heterogeneity of VIN is consistent with a dual pathogenesis of vulvar squamous cell carcinomas. Warty or basaloid carcinomas are related to the presence of HPV, genital warts, uVIN, and occur in younger women. In contrast, keratinizing SCC is frequently found in women older than 55 years, is usually unrelated to HPV infection, but may be associated with dVIN and lichen sclerosus. This type of SCC accounts for 65% of vulvar invasive SCC and marks another pathway in the development of these tumors.¹

The recent interest in therapeutic immune checkpoint inhibition targeting programmed cell-death receptor (PD-1) or its ligand programmed death ligand 1 (PD-L1) has resulted in evidence for clinicopathologic relevance of these factors in different cancer types (e.g. non-small cell lung cancer, melanoma, lymphomas, etc.).²⁻⁵ PD-L1 is not expressed or expressed in low levels in most normal cells, but frequently up-regulated in tumor cells, stromal cells including immune cells, or both, and is amenable to immunohistochemical evaluation.^{2,3} PD-L1 expression has been studied in vulvar SCC, too.⁶⁻¹¹ However, the pattern of invasion is well

known to be associated with aggressive clinical behaviour in SCC of the vulva¹², and has not been investigated for PD-L1 expression. This study hypothesized that an aggressive pattern of invasion may be related with PD-L1 overexpression in vulvar SCC. Thus, cohorts of early stage vulvar HPV-independent SCC and dVIN as well as uVIN were analysed to study and compare the epithelial PD-L1 immunoexpression in intraepithelial as well as early invasive neoplastic tissues.

Materials and Methods

Tissue specimens

A total of 99 diagnostic vulvar tissues originating from 64 preoperatively untreated patients were included in this retrospective study. Each patient contributed one tissue block only. This study was carried out in accordance with local ethical guidelines and the approval of the local ethics committee of Lower Austria (GS1-EK-4/584-2019). The specimens consisted of vulvar biopsies, local excisions as well as partial and total vulvectomies collected from the files of the author's institution and were obtained between 1998 and 2014. Cases included in this study were largely retrieved from a previous series used for studies on vulvar squamous tissues.^{13,14} Inclusion criteria into the recent study cohort were availability of tissue blocks with sufficient tissue for recutting and immunohistochemical stains which were defined as a diameter of diagnostic tissue of at least 1cm as well as quality of the tissue remaining after previous studies. Tissue blocks not meeting these criteria were excluded and replaced by more recent cases of

appropriate size and quality up to 2014. In ten cases of SCC a lymph node dissection was performed, with no evidence of metastatic deposits. If present in the studied slides, normal epithelia as well as lower grade lesions adjacent to higher grade changes were evaluated, too. The histological diagnoses were as follows: normal epithelia (NE, n=20), uVIN (n=23), dVIN (n=21), SCC (n=35). Six cases of d-VIN were isolated; the others d-VIN cases were associated with SCC. No uVIN was noted in the cases of SCC included in this study. The specimens were fixed in formalin and embedded in paraffin.

Formalin fixation did not exceed 24h. In each case, all original hematoxylin-and-eosin-stained sections as well as the clinical histories were reviewed, and a representative tissue block was chosen for immunohistochemical staining. Information regarding follow-up of patients with SCC was retrieved from the pathological files of the author's institution as well as by contacting the attending gynecologists. Three of the patients with SCC experienced recurrent disease one to 10 years after the primary diagnosis. Due to the small number of these cases a further statistical evaluation was not done.



Figure 1. A case of dVIN showing a widened epithelium, with acanthosis, basal atypia and abnormal maturation of keratinocytes with abundant eosinophilic cytoplasm and abnormal nuclei adjacent to a squamous cell carcinoma with a diffuse pattern of infiltration (arrow). Rete ridges are somewhat elongated, broad and confluent. (H&E, x100).

The classification of VIN as well as the staging of the SCC was performed as described previously.^{1,15} All SCC belonged to the keratinizing subtype, were immunonegative for p16 and diffusely positive for p53 in a strong nuclear staining pattern. Applying the FIGO staging, all cases of SCC were staged as FIGO I. The mean age of patients with SCC was 70.3 (range: 46-90) years. The uVIN (mean age: 43.4

years, range: 28-62 years) lesions covered by this study were p16 positive in a diffuse block-like pattern on immunohistochemistry.¹ DVIN (mean age: 67.8 years, range: 46-90 years) was recognized by defined criteria such as basal atypia and abnormal maturation of keratinocytes with large abundant eosinophilic cytoplasm, abnormal nuclei, and prominent nucleoli (Figure 1).¹



Figure 2. Same case as shown in Fig.1. P53 immunostaining is seen in >90% of the basal/parabasal two to three cell layers. At the left there is normal squamous epithelium with occasional basal cell staining, corresponding to a wild type of immunoreactivity. (p53, x100).

The rete ridges were elongated and branching frequently, with occasional keratin pearls and acanthosis. In the superficial layers, atypia was minimal and maturation was maintained. These lesions have been shown p53 reactive, there was no diagnostic staining for p16. P53 reactivity in dVIN was seen in the

basal/parabasal two to three cell layers in a mutant phenotype represented by strong nuclear staining.^{16,17} (Figure 2) The original tissue blocks and slides (hematoxylin-eosin/H&E as well immunohistochemical stains) were reviewed by the author. Epithelial lesions not matching the above-

mentioned criteria (lichen sclerosus, lichen simplex chronicus, pseudoepitheliomatous hyperplasias, acanthotic lesions with altered differentiation, etc.) were not included in this study.

Immunohistochemistry

The same paraffin-embedded tissues as those used for the original H&E stained sections were used for immunohistochemistry. They were cut at 3µm. Subsequently, the sections were deparaffinized in xylene and rehydrated via graded ethanol. A standard immunohistochemical technique was performed using a Ventana BenchMark Ultra immunostainer with a prediluted ready-to-use rabbit monoclonal primary antibody to PD-L1 (clone SP263, Ventana Medical Systems, Illkirch Cedex, France). Heat epitope retrieval as provided by the immunostainer was done in a TRIS based buffer supplied by the manufacturer (CC1 cell conditioning solution, Ventana Medical Systems, Illkirch Cedex, France) at pH 7.5 for 64 minutes. The antibody was incubated at 36° Celsius for 16 minutes. The enzymatic reactivity was visualized with 3-3 diaminobenzidine (OptiView DAB IHC Detection Kit, Ventana, Illkirch Cedex, France, incubation time 8 minutes). A human tonsil served as external positive control. For negative controls serial sections of the same specimens were used, omitting the primary antibody from the staining protocol and substituting it by a commercially available nonimmune IgG serum (DAKO). The immunohistochemical slides were evaluated and interpreted by the author (H.B.) without knowledge of the clinical

data in a blinded manner. Necrotic tissue areas were not considered in the interpretation of immunostaining.

Immunoreactivity was scored corresponding to the intensity and membranous pattern of the positive controls. To assess up-regulation and overexpression, sections were scored semi quantitatively as follows: score 0/1, 0-5% immunoreactive cells; score 2+, >5 to 50% immunoreactive cells; score 3+, >50% immunoreactive cells. The slides were screened at low power for any staining; higher magnification (x100) was used to determine immunohistochemical scores. This study investigated the epithelial expression of PD-L1 in the described epithelia; however, tumor-infiltrating immune cells were scored using the same criteria as in neoplastic epithelial tissues.

The patterns of invasion in SCC were scored as pushing or diffuse. A cohesive growth with well delineated borders, sometimes finger-like tumor nests and well demarcation at the tumor-stroma-interface was considered as pushing. Dissociative growth in small groups or single cells ("spray-like") was defined as a diffuse pattern.^{12,18} To avoid statistical cohorts containing a few cases only mixed patterns of infiltration SCC were included into the cases defined as diffusely infiltrative.

Statistical analysis was carried out using GraphPad Prism software (version 4.0, San Diego, CA). The chi-square test was used to analyse the immunoexpression scores of PD-L1. In SCC, scores of epithelial cells and tumor-infiltrating immune cells were compared by Pearson's correlation.

Statistical significance was accepted at the $p < 0.05$ level.

are shown in Table 1. If present, PD-L1 immunostaining was noted in membranous and frequently associated cytoplasmic patterns (Figure 3).

Results

Scores in the different diagnostic groups

Table 1. Immunohistochemical expression of PD-L1 in normal and neoplastic squamous epithelia of the vulva.

Diagnosis	PD-L1 IHS		
	0/1+	2+	3+
NE (n=20)	20	0	0
uVIN (n=23)	20	3	0
dVIN (n=21)	19	2	
SCC (n=35)	13	16	6
SCC pushing pattern of invasion (n=27)	13	14	0
SCC diffuse pattern of invasion (n=8)	0	2	6

IHS, immunohistochemical score; n, number of cases; NE, normal vulvar squamous epithelia; uVIN, high-grade vulvar intraepithelial neoplasia- usual type; dVIN, differentiated vulvar intraepithelial neoplasia; SCC, invasive squamous cell carcinoma of the vulva

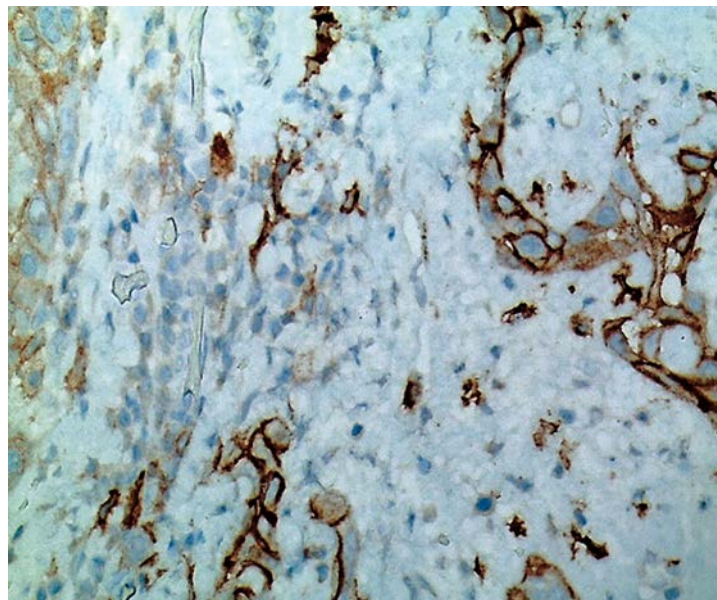


Figure 3. Membranous immunostaining for PD-L1 in a squamous cell carcinoma, diffusely infiltrating type (score 3+, x400).

In NE as well as VIN cases immunoscores were low (score 0/1, n=59; score 2+, n=5, in VINs only, Figures 4 and 5). There was no statistically significant difference between these cohorts ($p=0.265$, chi-

square test). Staining was most frequently localized to the basal and parabasal compartments of these epithelia, with rare reactivities of single cells in the upper epithelial levels.

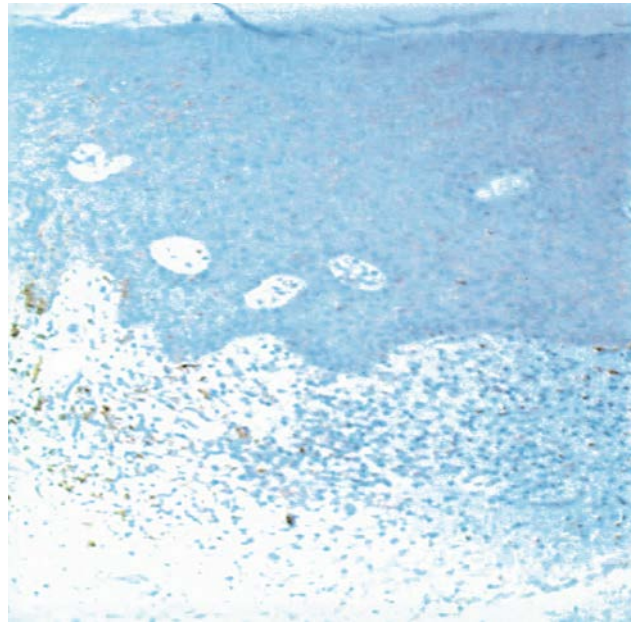


Figure 4. The case of dVIN shown in Fig. 1 with rare cells reactive for PD-L1, scored as group 0/1. Immunostaining is seen in subepithelial immune/stromal cells sometimes (x100).

SCC PD-L1 expression was mainly localized to the tumor invasive front but could be observed in upper epithelial layers and central tumor regions in score 2+ and 3+ cases, respectively, too. (Figures 5 and 6) The latter staining was characteristic for the mixed pattern of infiltration tumors, too, which were

included into the diffuse category. There was a statistically significant increase of immunoscores (score 0/1+, n=13; score 2+, n=16; score 3+, n=6) in this cohort compared to the above described groups of NE and VINs ($p<0.0001$, chi-square test).



Figure 5. A case of uVIN, PD-L1 score group 0/1. (x100).

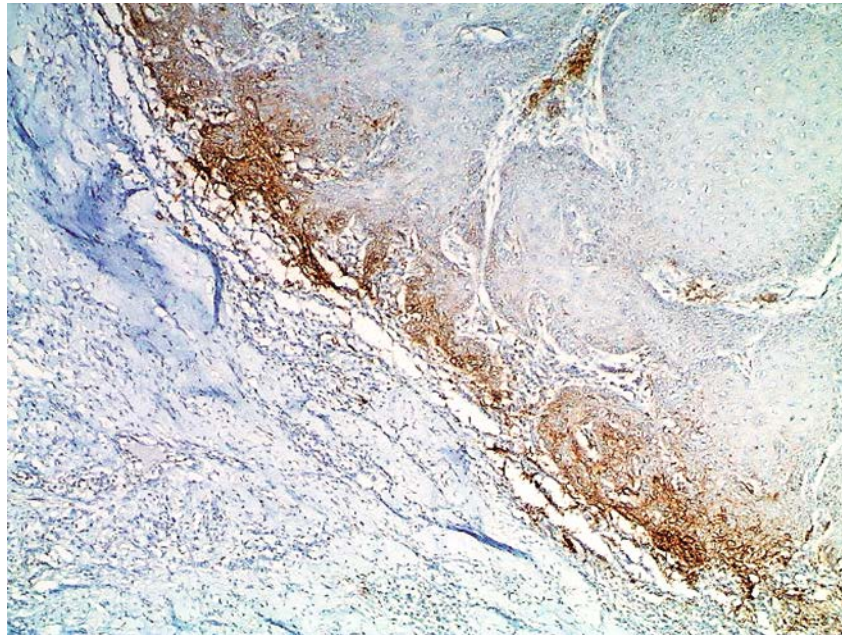


Figure 6. A squamous cell carcinoma showing a pushing type of infiltration. Rare epithelial immunostaining for PD-L1 is limited to cell layers at the infiltrative margin. Frequent PD-L1 reactivity is present in tumor-infiltrating immune cells, too (x100).

A pushing pattern of invasion was recorded in 27 cases of SCC. (Figure 6) In contrast, eight cases of SCC displayed a diffuse manner of invasion into the stroma with dissociative cell spread. (Figure 7) PD-L1 immunoscores were correlated with invasive patterns. They were lower in cases characterized by pushing growth (scores 0/1+, n=13; score 2+, n=14; score 3+, n=0), and

increased in diffusely infiltrating tumors (score 0/1+, n=0; score 2+, n=2; score 3+, n=6). These differences were statistically significant ($p < 0.0001$, chi-square test). Comparing VIN lesions with SCC of pushing type infiltration a strong statistical significance was calculated for an overexpression in the latter cohort ($p = 0.0009$, chi-square test).

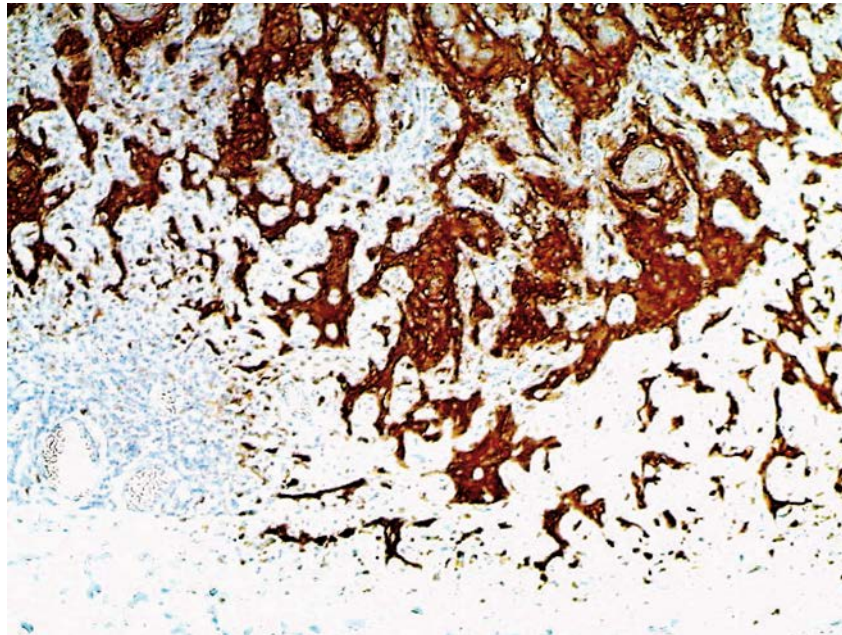


Figure 7. A diffusely infiltrative squamous cell carcinoma, tumor cells are noted in small groups, corresponding to a spray-like pattern scored as 3+ on PD-L1 immunostain (x400).

PD-L1 stained tumor infiltrating immune cells were noted in different scores in VIN cases; since epithelial scores were generally low in these lesions, no correlation for epithelial and tumor-infiltrating immune cell scores was calculated. In the SCC cohort scores were frequently low (score 0/1+, n=22;

score 2, n=9; score 3, n=4). There was no correlation between epithelial and tumor-infiltrating cell scores in the group of SCCs (Pearson correlation, $p = 0.8943$, Pearson $r = 0.0233$).

Discussion

PD-L1 which is expressed in many cancer cells, prevents tumor death by blocking T cell activity and has been shown a predictive biomarker in cancer immunotherapy.^{8,19} This study assessed the PD-L1 immunoreactivity in vulvar epithelial neoplasia by evaluating squamous intraepithelial neoplasia (VIN of different subtypes) in comparison to early invasive non-HPV associated keratinizing subtype SCC of FIGO stage I and different patterns of invasion of SCC. Up-regulation of PD-L1 was observed between VIN and SCC as well as for a diffuse pattern of stromal invasion in the latter.

The pattern of invasion has been shown an important factor in the aggressivity of female lower genital tract SCC. For cervical SCC, Horn et al. defined a closed pattern of infiltration as cohesive growth with well-delineated pushing borders, a finger-like pattern of tumor growth in solid cords and trabecles, and a highly dissociative growth in small groups or single cells as a spray-like pattern with the highest degree of tumor cell dissociation.¹⁸ They noted that the spray-like pattern was associated with advanced tumor stages, increased rate of recurrence and a reduced overall survival. For the purpose of the study at hand with its limited number of FIGO stage I SCC the latter pattern was defined as diffuse pattern. Others reported for vulvar cancers that tumors with an infiltrative pattern of invasion and fibromyxoid stroma were associated with worse outcomes and the development of epithelial-mesenchymal transition than tumors with pushing or nested patterns.¹² In the study by

Holthoff et al. an infiltrative pattern of invasion was defined as cords or individual tumor cells infiltrating the surrounding stroma in a spray-like pattern.¹² Generally, the pattern of invasion in cancers in its relation to PD-L1 expression is studied rarely. It has been shown that PD-L1 was significantly overexpressed in the budding areas in patients with localized colon cancer and that its levels correlated with a mesenchymal transition profile. The authors pointed to the importance of including budding areas among the samples used for biomarker evaluation.²⁰ Tumor budding corresponds with the category of diffuse infiltration in this study morphologically. To the best of the author's knowledge the study at hand is the first one to describe PD-L1 immunoeexpression in this setting in vulvar SCC.

The group of SCC in this study was HPV-negative. However, the VIN lesions covered both HPV dependent uVIN as well as HPV independent dVIN. There was no significant difference of PD-L1 expression between these two cohorts. Interestingly, Mezache et al reported that about 95% of their cervical intraepithelial lesions (CIN), which are known to be HPV dependent, expressed PD-L1 and concluded that PD-L1 is a solid biomarker of productive HPV infection of the cervix.²¹ Others noted that PD-L1 overexpression is detectable in a substantial proportion of vulvar carcinomas in all stages and is independent of HPV.^{7,8} Chinn et al. found that the majority of intraepithelial lesions of the cervix and vulva were entirely negative for PD-L1, which is largely in agreement with the data reported in this study with respect to

dVIN and uVIN.¹¹ They showed no difference of PD-L1 expression in dVIN associated versus HPV-associated vulvar squamous cell carcinomas. However, there are controversies concerning the use of different PD-L1 antibodies in different studies, and the use of specific and validated antibodies is mandatory.^{11,22} Other reasons for differences include interpretative variability and antibody titration.¹¹ Using a different scoring system Hecking et al. found membranous PD-L1 expression in a minority of their HPV-negative vulvar cancers.²³ They described a geographical correlation of PD-L1 with immunocyte-rich regions of cancer islets, which was an independent prognostic factor for poor outcome. However, an analysis of the pattern of infiltration was not done there. The study at hand focused on epithelial expression of PD-L1. Thus, the scoring method differs from other studies using for instance the combined positive score (CPS) which includes tumor infiltrating immune cells in the final score complicating the direct comparison between different papers.¹¹

A genetic basis for PD-L1 expression in SCC of the vulva has been described recently. Recurrent copy number gain of the genes encoding the PD-1 ligands provided evidence for PD-L1 expression in a subset of cervical and vulvar cancers.⁹ These data were interpreted as a hint towards a class of patients that are rational candidates for therapies targeting PD-1. Indeed, pembrolizumab, a humanized monoclonal antibody against PD-1, has been shown a safe and effective chemotherapeutic agent to treat recurrent vulvar carcinoma cases.¹⁰ Xing et al. reported on different

mutational profiles and PD-L1 expression in some cases of primary and metastatic vulvar SCC and emphasized the importance of PD-L1 expression in different tumor locations to avoid false negative information provided for immunotherapy.⁶

The clinical response to therapies targeting the PD-1/PD-L1 pathway is impressive in some patients; however, this approach has not proved uniformly effective.¹¹ As discussed above there are different possible multifactorial reasons for this including technical issues, immunohistochemical antibody variability, intratumoral heterogeneity, as well as complex phenomena in the immune response to cancer.¹¹ Clinicopathologic and histological features associated with PD-L1 expression are still poorly described. One study concluded that there was no association of PD-L1 expression to clinicopathologic parameters like histologic tumor type, tumor and nodal stage, grading, and overall survival in vulvar carcinomas; there was no reference to the pattern of invasion.⁸ Chinn et al. noted that PD-L1 expression is common, but typically only focal among vulvar SCC with and without HPV association, and suggested that anti-PD-1/PD-L1 inhibitors may work only in select cases.¹¹ Including patterns of infiltration which are known factors of aggressive tumor behavior and epithelial-mesenchymal transition may help to discuss appropriate therapies on the background of PD-L1 expression.

The pattern of invasion in vulvar SCC may be characterized by immunohistochemical features. Holthoff et al. investigated a cohort of these

cancers by similar criteria as used in this study, namely pushing versus infiltrative (“spray-like”).¹² They showed that an infiltrative pattern is more likely to exhibit features reflective of an epithelial-mesenchymal transition. Nuclear beta-catenin and presence of vimentin were significantly more likely to occur in tumors with an infiltrative pattern of invasion or a fibromyxoid stromal response, loss of E-cadherin was just associated significantly with an infiltrative pattern. They concluded that loss of cell-to-cell junction may also be an important factor in the development of the spray-like appearance of the infiltrative invasive pattern. Thus, PD-L1 overexpression in diffuse type invasive vulvar SCC shown in this study may be compatible with other molecular features of tumor aggressivity, with relevance to the tumor immunology.

PD-L1 immunoexpression needs to be scored for membranous reactivity.¹¹ However, additional cytoplasmic staining was observed in many cases in this study as well as by others.²⁴ Although cytoplasmic staining is not a specific pattern of reactivity, this may indicate a constitutive overexpression of PD-L1 by tumor cells, consistent with the recurrent copy number gain of the genes encoding the PD-1 ligands in some of these cancers.^{9,24}

This study is limited by a relatively small number of cases. Based on the low stage cancers investigated it does not answer questions on tumor recurrence and survival. Since there is an emphasis on morphological features of invasion of the epithelial neoplastic cells the PD-L1 status of tumor infiltrating immune cells was not evaluated in depth. However,

there was no correlation of immunoscores between epithelial and tumor-infiltrating immune cells in SCC. Further studies on larger case cohorts including PD-L1 staining and characterisation of immune cells, high stage cancers and data on recurrence/survival of patients are necessary to confirm the observations reported in this study.

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