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Review

A practical approach for PD-L1 evaluation in gastroesophageal cancer

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Summary
PD-L1 is an established predictive immunohistochemical biomarker of response to immune checkpoint inhibitors. At present, PD-L1 is routinely assessed on biopsy samples of advanced gastroesophageal cancer patients before initiating first-line treatment. However, PD-L1 is still a suboptimal biomarker, due to changing cut-off values and scoring systems, interobserver and interlaboratory variability.
This practical illustrated review discusses the range of staining patterns of PD-L1 and the potential pitfalls and challenges that can be encountered when evaluating PD-L1, focusing on gastric and gastroesophageal adenocarcinoma (G/GEA) and esophageal squamous cell carcinoma (ESCC).

Key words: PD-L1, immunohistochemistry, gastroesophageal adenocarcinoma, esophageal squamous cell carcinoma, immunotherapy

Introduction

The PD-1/PD-L1 axis promotes and maintains immune tolerance within the tumor microenvironment. PD-1 is expressed on tumor-infiltrating immune cells and PD-L1 is expressed on both tumor cells and antigen-presenting cells [1].
Immune checkpoint inhibitors exert their antitumor activity by blocking the PD-1/PD-L1 axis and thus promoting the elimination of tumor cells by the immune system. Antibodies directed against PD-1/PD-L1 have revolutionized the treatment landscape of many cancer types at advanced stages, including melanoma, non-small cell lung cancer, gastrointestinal cancers, breast cancer, kidney cancer and many others [2].
The pattern of expression and potential predictive value of PD-L1 as an immunohistochemical biomarker has been thoroughly investigated in gastrointestinal cancers. According to international guidelines, PD-L1 is currently used as a predictive biomarker in routine clinical practice only

in upper gastrointestinal cancers (gastric and gastroesophageal adenocarcinoma [G/GEA] and esophageal squamous cell carcinoma [ESCC]) [3].

For gastric and gastroesophageal cancers, the expression of PD-L1 should be evaluated using the Combined Positive Score (CPS), which consists in dividing the total number of positive tumor cells, tumor-associated lymphocytes and macrophages, by the total number of viable tumor cells multiplied by 100. An alternative method to evaluate PD-L1 expression is the Tumor Proportion Score (TPS), which is defined as the number of positive tumor cells divided by the total number of viable tumor cells multiplied by 100, thus obtaining a percentage (%). The use of TPS has recently been introduced in ESCC, alongside CPS [4].

$$CPS = \frac{\text{number of positive cells (tumor cells, lymphocytes, macrophages)}}{\text{total number of viable tumor cells}} x100$$

$$TPS = \frac{\text{number of positive tumor cells}}{\text{total number of viable tumor cells}} x100$$

Following the approval by the FDA [5] and ESMO [6] of anti-HER2 and anti-PD-1 agents in combination with chemotherapy in the first-line setting for locally advanced unresectable and metastatic gastric/gastroesophageal cancer patients, the assessment of HER2 and PD-L1 in biopsy samples should be mandatory before the initiation of first-line systemic therapy [7-9]. The assessment of mismatch repair proteins (MMR) by immunohistochemistry (IHC) and Epstein-Barr Virus status by *in situ* hybridization is also recommended, according to tissue availability [10]. Anti-FGFR2b and anti-Claudin 18.2 therapies are showing promising results in phase III clinical trials, which means that FGFR2b and Claudin 18.2 evaluation will soon enter the diagnostic armamentarium [11].

PD-L1 is a very useful, but still suboptimal biomarker. The quantitative nature of PD-L1 scoring and the presence of different scoring systems with changing cut-off thresholds determine high rates of interobserver variability [12,13]. Furthermore, several pitfalls that will be discussed in the following sections may affect PD-L1 IHC evaluation by the pathologist. Moreover, as described for HER2 [14], PD-L1 expression is characterized by a high degree of spatial and temporal intra-tumor heterogeneity. Thus, the assessment of PD-L1 in biopsy samples may not be representative of the real status of the biomarker in the tumor and PD-L1 evaluation in the primary tumor may change in the metastatic samples and/or following neoadjuvant therapy [15]. PD-L1 assessment is also burdened by a certain degree of interlaboratory variability, due to the use of different companion diagnostic assays and antibody clones with different staining patterns [16,17]. PD-L1 protein expression can be influenced by the age of formalin-fixed paraffin-embedded (FFPE) tissue blocks. For this reason, PD-L1 evaluation in tissue blocks older than 5 years should be discouraged [18]. Finally, since only specific inflammatory cells (tumor-associated lymphocytes and macrophages, but not plasma cells and other inflammatory cells) should be included in the CPS numerator, as illustrated below, it is important to evaluate PD-L1 in combination with Haematoxylin and Eosin- and, if necessary, cytokeratin-stained sections, in order to solve any doubts concerning the nature of the immunostained tumor-associated inflammatory cells. Moreover, pre-invasive lesions should be excluded from the formal PD-L1 count.

This practical illustrated review paper discusses the range of staining patterns of PD-L1 and the potential pitfalls and challenges that can be encountered when evaluating PD-L1, focusing on gastric/gastroesophageal adenocarcinoma (G/GEA) and esophageal squamous cell carcinoma (ESCC).

PD-L1 staining patterns of tumor cells

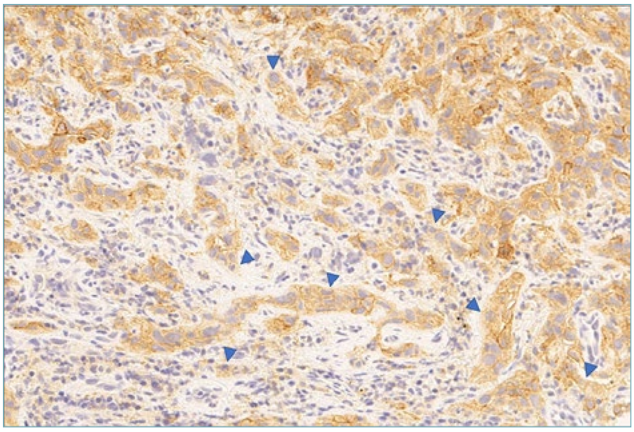

Figure 1. Linear membrane and cytoplasmic staining.

ESCC specimen stained with PD-L1 antibody (Dako 22C3) exhibiting linear membrane and cytoplasmic staining patterns (Fig. 1).

Tumor cells with perceptible and convincing linear membrane staining of tumor cells at any intensity should be included in the CPS and TPS numerator, regardless of the presence of cytoplasmic staining.

Tumor cells exhibiting cytoplasmic staining only (blue arrows) are excluded from the CPS and TPS numerator.

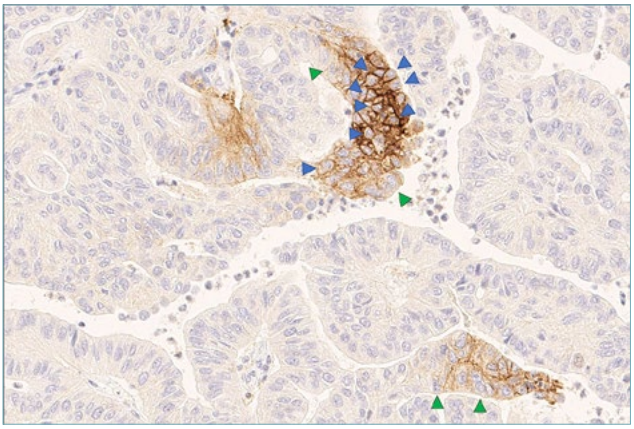

Figure 2. Complete and incomplete membrane staining.

Gastric adenocarcinoma specimen stained with PD-L1 antibody (Dako 22C3) exhibiting complete and incomplete linear membrane staining patterns (Fig. 2). Tumor cells with complete (blue arrows) or incomplete (green arrows) perceptible and convincing linear membrane, regardless of the presence of cytoplasmic staining, should be included in the CPS and TPS numerator. Of note, in a gland-forming neoplasm, staining limited to the luminal border should be regarded as negative.
ESCC specimen stained with PD-L1 antibody (Dako 22C3) exhibiting linear membrane staining at various intensities (Fig. 3).

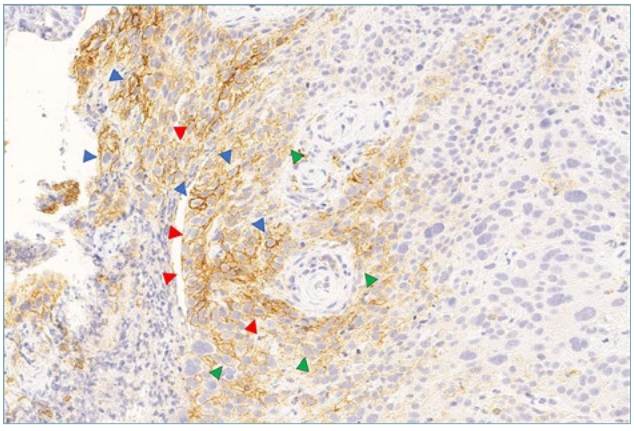

Figure 3. Intense, moderate and weak membrane staining.

Tumor cells with intense (blue arrows), moderate (red arrows), and faint (green arrows), complete or incomplete, linear membrane staining of tumor cells should be included in the CPS and TPS numerator.

PD-L1 staining of immune cells: what to include and exclude from CPS numerator

When calculating the CPS, tumor cells with membrane staining and tumor-associated lymphocytes, as well as macrophages with membrane and cytoplasmatic staining, should be included in the numerator. Neutrophils, eosinophils, plasma cells, stromal cells, necrotic cells, cellular debris and platelets may show significant positivity but should be excluded from the numerator when calculating the CPS.
Gastroesophageal adenocarcinoma specimen stained with PD-L1 antibody (Dako 22C3) exhibiting staining of tumor associated-immune cells (Fig. 4).

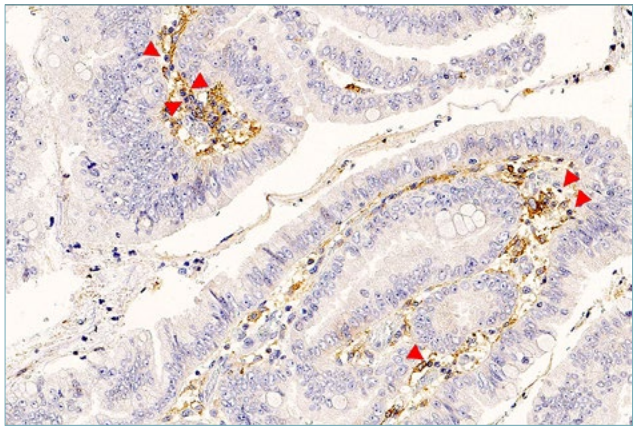

Figure 4. Positive lymphocytes.

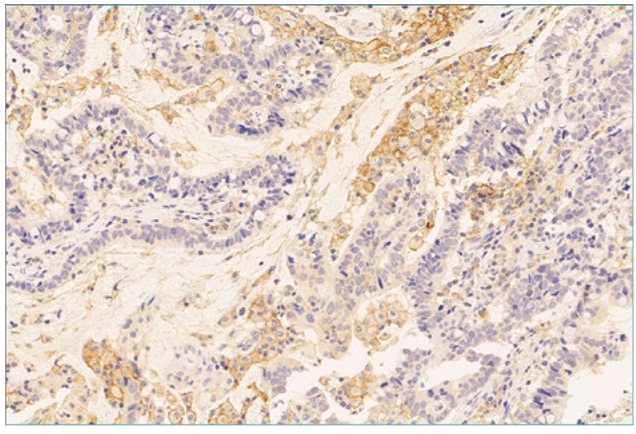

Figure 5. Positive macrophages.

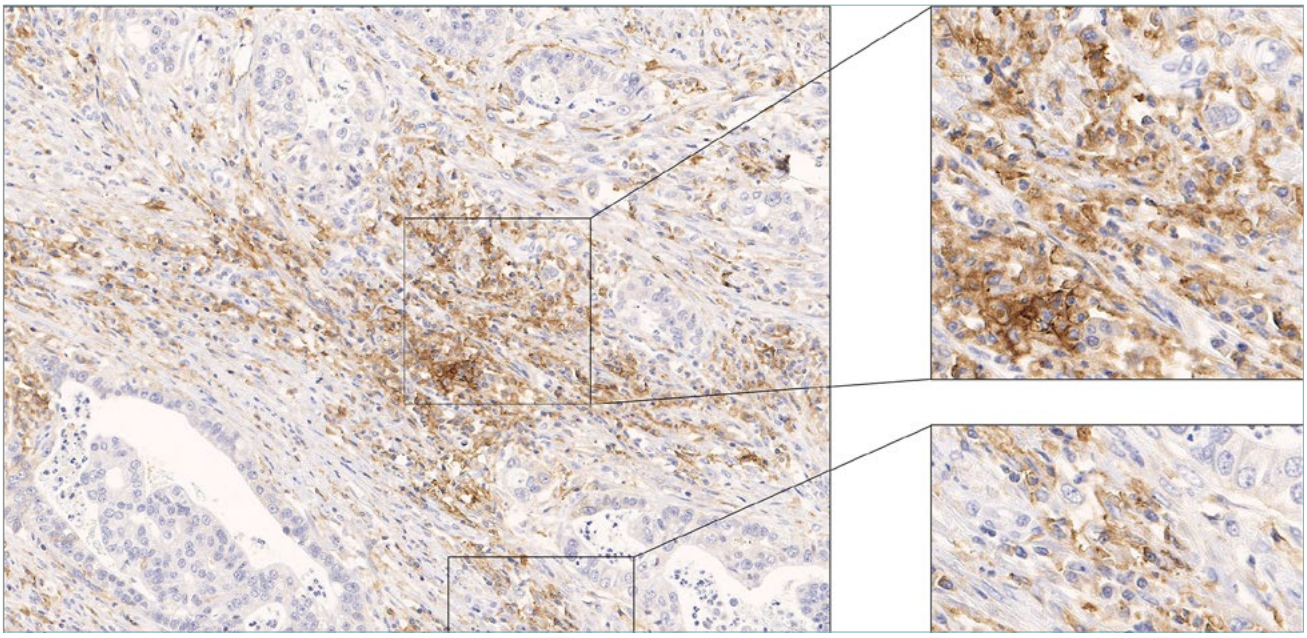

Figure 6. Positive plasma cells.

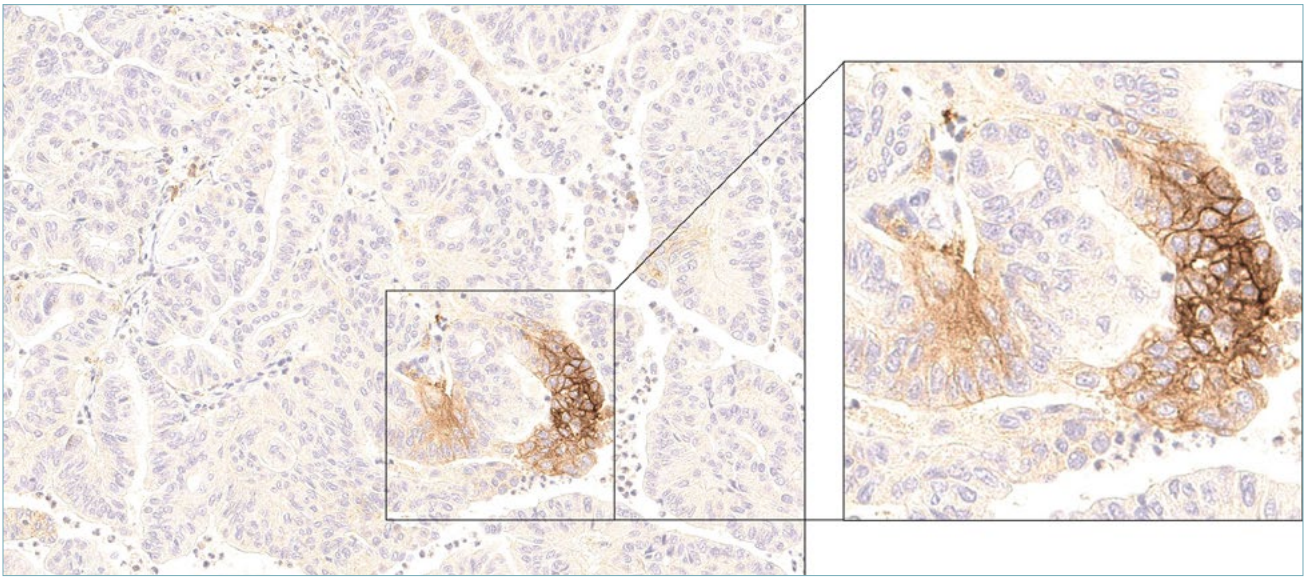

Figure 7. Small tumor area with PD-L1 Staining.

Lymphocytes showing membrane and/or cytoplasmic staining at any intensity (red arrows) should be included in the CPS numerator. PD-L1 stained lymphocytes may be difficult to identify due to their small size and high nuclear/cytoplasmic ratio.

Gastric adenocarcinoma specimen stained with PD-L1 antibody (Dako 22C3) exhibiting staining of tumor associated-immune cells (Fig. 5).

Macrophages showing membrane and/or cytoplasmic staining at any intensity should be included in the CPS numerator. There are conflicting data about whether positive macrophages within the lumen of neoplastic glands should be regarded as negative, if no staining is observed in tumor cells.

Gastroesophageal adenocarcinoma specimen stained with PD-L1 antibody (Dako 22C3) exhibiting

staining of tumor associated-immune cells (Fig. 6).
Plasma cells showing membrane and/or cytoplasmic staining at any intensity should not be included in the CPS numerator. As shown in Figure 6, the presence of a tumor immune infiltrate enriched in positive plasma cells may result in a false high CPS value, if plasma cells are not excluded when evaluating the CPS.

Calculation of CPS on a small tumor area with PD-L1 Staining

Gastric adenocarcinoma biopsy specimen stained with PD-L1 antibody (Dako 22C3) exhibiting staining of a small tumor area (Fig. 7).
In this case, approximately 10% of the tumor shows convincing staining, while the remaining 90% shows absence of staining. The area of staining should be assessed to quantify the number of positive cells to be included in the CPS numerator. In Figure 7 the CPS of the stained area is 70 ([~70 positive cells/100 viable tumor cells]x100). Thus, the CPS of the entire tumor area shown in the figure should be calculated by multiplying the CPS of the stained area by the percentage of the entire tumor area represented by the stained area (CPS = 70 x 10/100 = 7).

Calculation of CPS on a tumor area with heterogenous PD-L1 Staining

Gastroesophageal adenocarcinoma surgical resection specimen stained with PD-L1 antibody (Dako 22C3) exhibiting heterogenous staining (Fig. 8).
In this case the tumor area must be divided into four regions with an approximately equal number of tumor cells. The total number of PD-L1 positive cells and viable tumor cells for every region must be quantified to calculate the CPS. In Figure 8 the CPS calculated for each region is: 15, 20, 80 and 50. The CPS of the entire tumor area shown in the figure is the average value of CPS of the four regions: CPS = (15 + 20 + 80 + 50)/4 = 33.

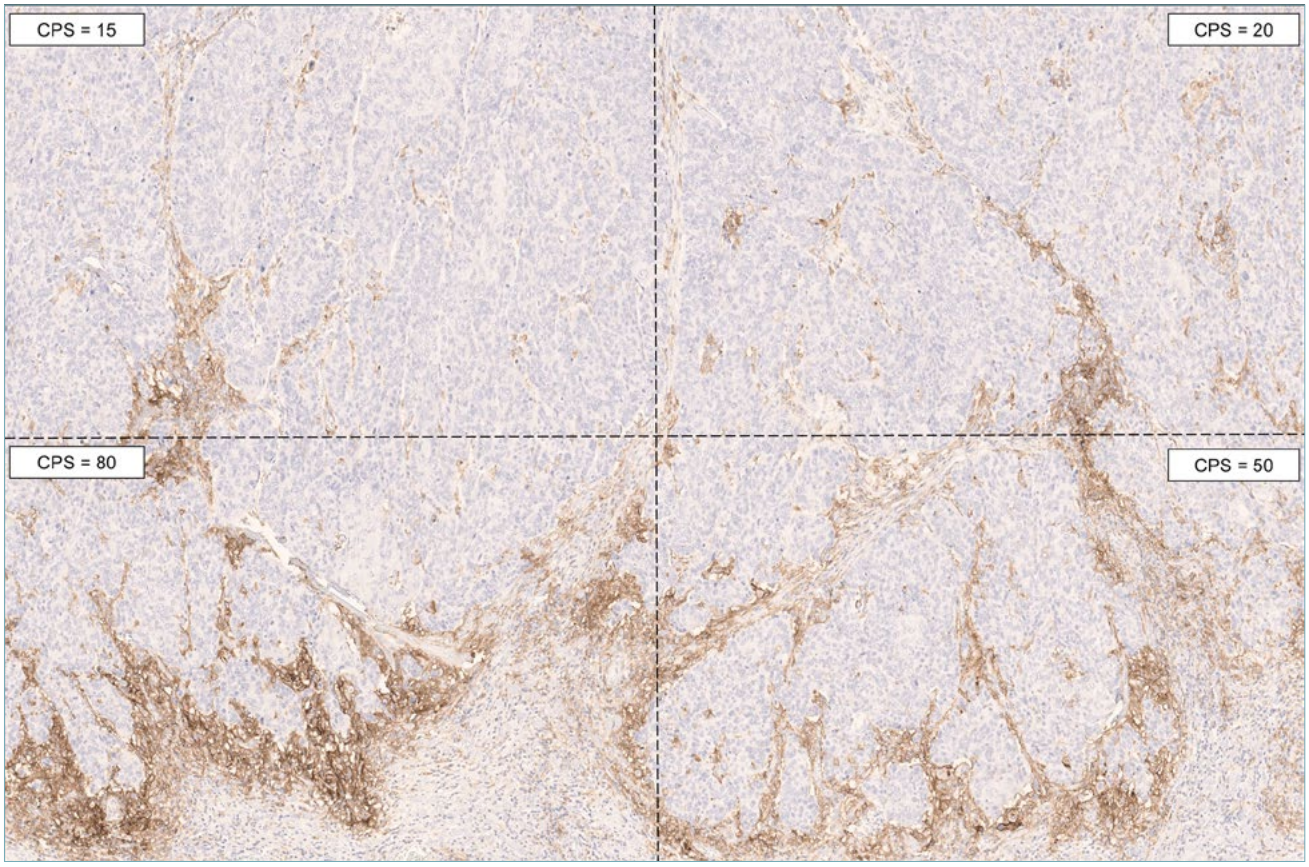

Figure 8. Heterogenous PD-L1 staining.

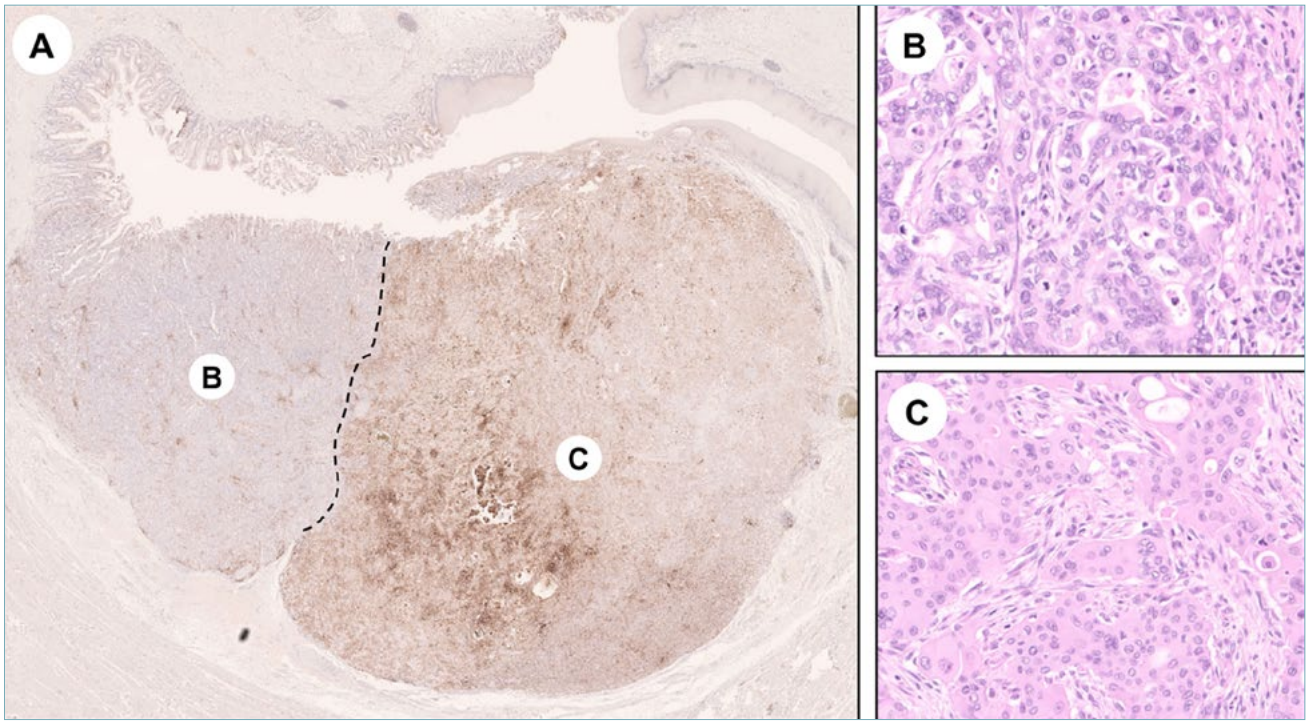

Figure 9. PD-L1 staining and morphologic heterogeneity.

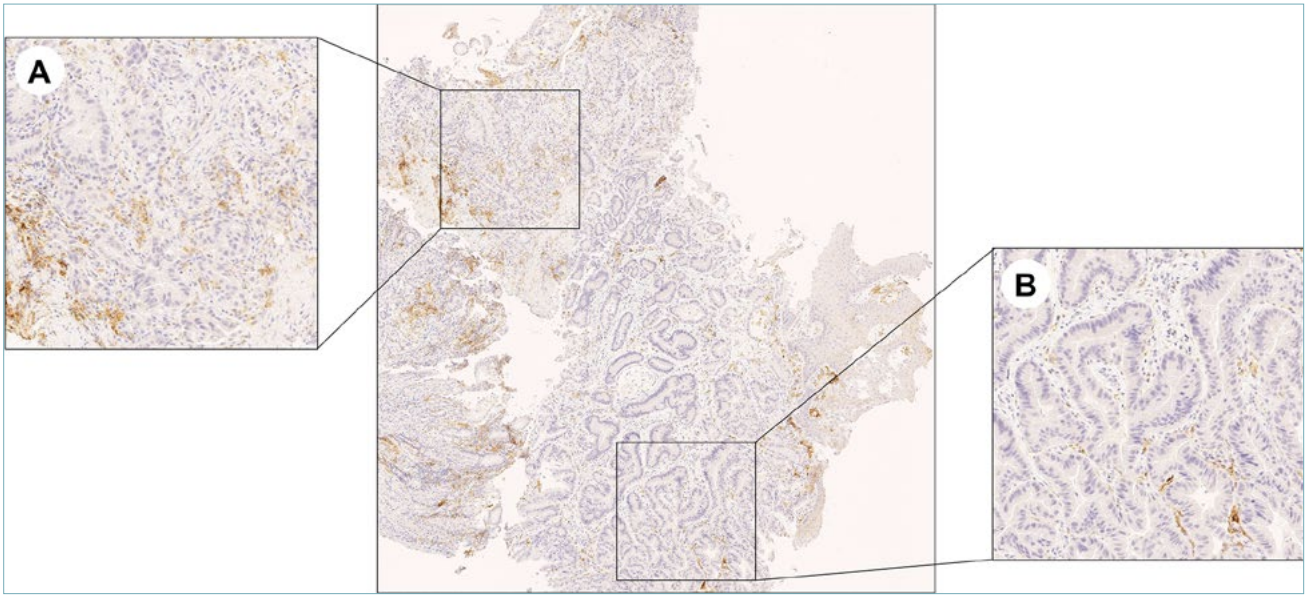

Figure 10. PD-L1 positivity in pre-invasive lesions.

Heterogenous PD-L1 tumor staining of adenosquamous carcinoma of the gastroesophageal junction (A) and hematoxylin and eosin (H&E) staining of the glandular (B) and squamous (C) components (Fig. 10).

Adenosquamous carcinoma is a rare variant of gastroesophageal carcinoma composed of a glandular and squamous component and is characterized by a more aggressive clinical course. Figure 9 shows the

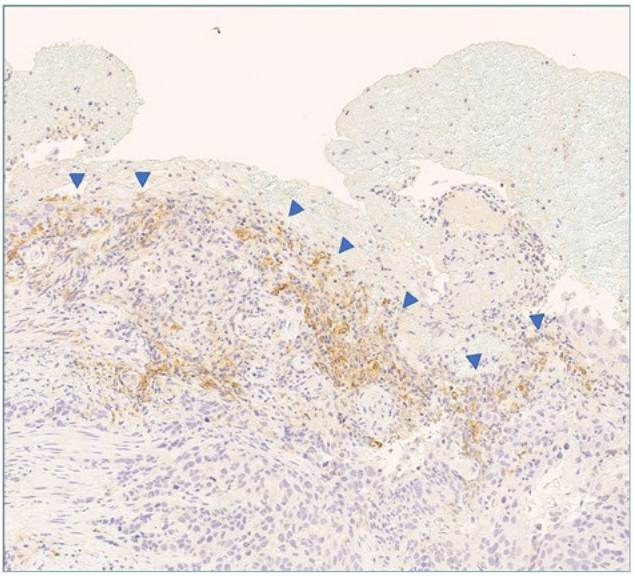

Figure 11. PD-L1 positivity in an ulcerated area.

presence of a different staining pattern within the two components. While the squamous component exhibits strong and diffuse staining in tumor cells, the glandular component exhibits staining only in the immune cell compartment. In the presence of morphological intratumor heterogeneity, both morphological components should be carefully evaluated when assessing the CPS/TPS of the entire tumor area.

Potential pitfalls in PD-L1 evaluation: pre-invasive lesions and ulcers

Gastric adenocarcinoma biopsy specimen stained with PD-L1 antibody (Dako 22C3) showing an invasive component (A) alongside a pre-invasive component (i.e., low grade dysplasia) (B) (Fig. 10).
A work by Fassan and colleagues [19] demonstrated that PD-L1 can be expressed also in pre-invasive gastroesophageal lesions. A significantly higher prevalence

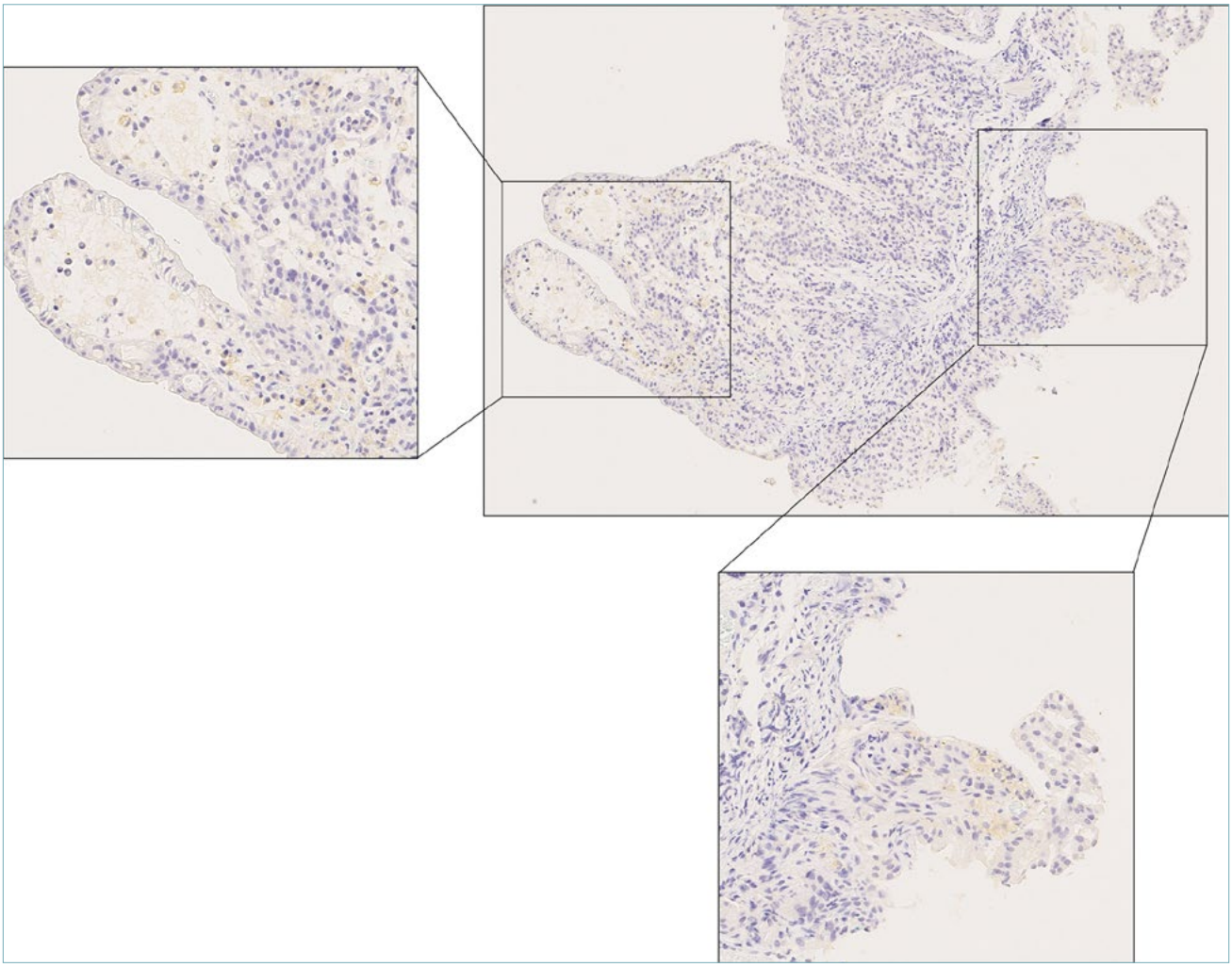

Figure 12. Non-specific staining.

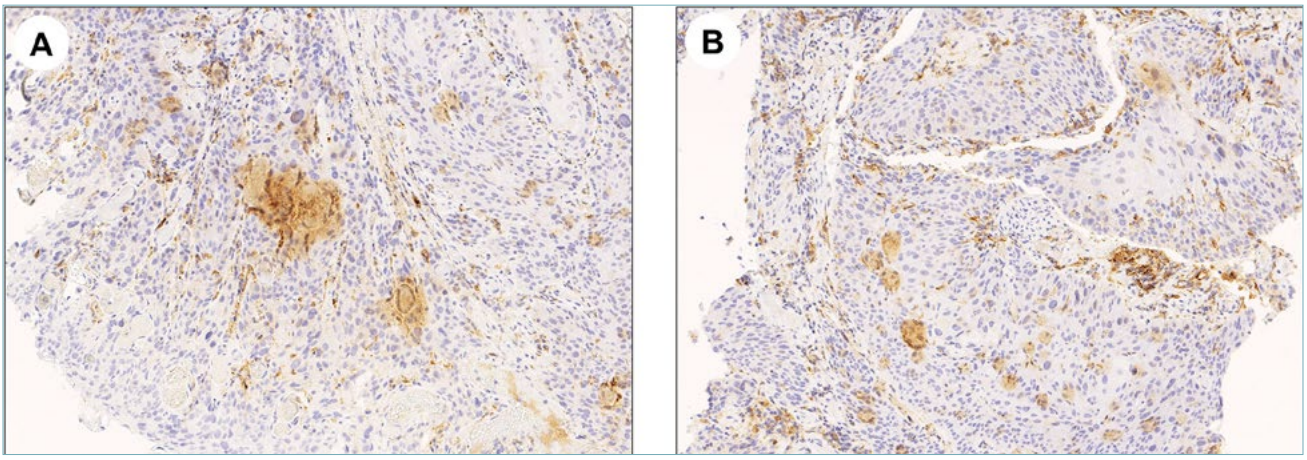

Figure 13. DAB staining.

of PD-L1 positivity was observed among Barrett dysplasia in comparison with gastric dysplasia and during the carcinogenetic cascade from low-grade to high-grade dysplastic lesion and to adenocarcinoma. When assessing CPS/TPS, it is crucial to distinguish pre-invasive lesions (*i.e.*, gastroesophageal dysplasia and squamous dysplasia) from invasive carcinoma and to perform PD-L1 evaluation only in the latter. However, in certain circumstances, especially in biopsy specimens, it may be challenging to make a proper distinction between pre-invasive lesions and carcinomas, leading to an incorrect biomarker assessment with important influences on the therapeutic decision-making process.

ESCC biopsy specimen stained with PD-L1 antibody (Dako 22C3) exhibiting staining in an ulcerated superficial area (blue arrows) (Fig. 11).

PD-L1 stained immune cells associated with ulcers, chronic gastritis or other inflammatory processes of the gastroesophageal mucosa should be excluded from the CPS nominator.

PD-L1 staining artifacts

Gastric adenocarcinoma biopsy specimen stained with PD-L1 antibody (Dako 22C3) exhibiting faint cytoplasmic staining of macrophages (Fig. 12).

Non-specific staining occurs when the primary antibodies bind to off-target proteins, resulting in clinically meaningless data [20]. Non-specific staining can be caused by pre-analytic issues, including poor fixation, improper deparaffinization, improper drying and incomplete rinsing of slides and analytic issues, such as antigen retrieval. In Figure 12, non-specific staining can be seen as faint cytoplasmic staining of macrophages. In similar cases showing an off-target and diffuse staining pattern, PD-L1 staining should be repeated.

ESCC biopsy specimen stained with PD-L1 antibody (Dako 22C3) exhibiting non-specific DAB (Diaminobenzidine) staining (Fig. 13).

Non-specific DAB staining, recognizable as patches of color not related to specific cellular figures, should be identified and excluded from the CPS/TPS evaluation.

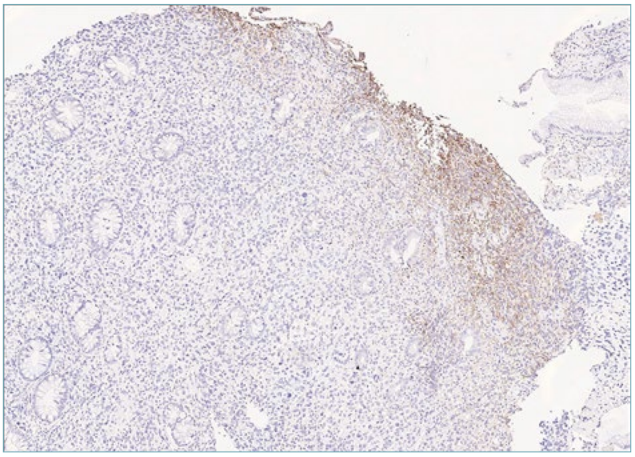

Figure 14. Edge effect.

Metastatic gastric adenocarcinoma biopsy specimen stained with PD-L1 antibody (Dako 22C3) exhibiting edge effect (Fig. 14).

The edge effect consists in a ring of non-specific

staining at the edge of the tissue, with the central part of the tumor showing absent or faint staining. In the presence of such an artifact, edge staining must be excluded from the CPS/TPS, to avoid false positivity or overestimation. In these cases, PD-L1 staining should be repeated.

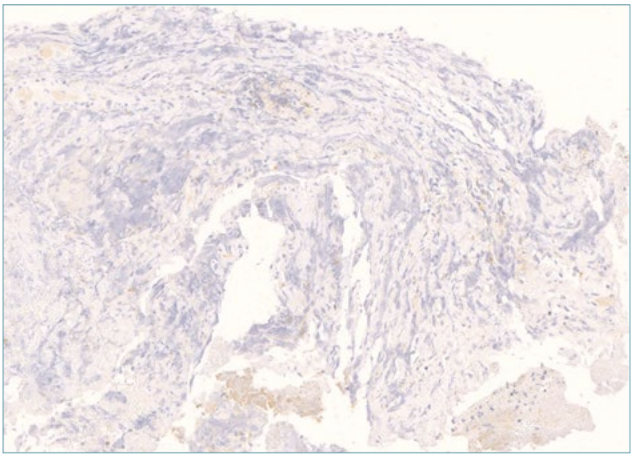

Figure 15. Electrocution artifacts.

Gastric adenocarcinoma biopsy specimen stained with PD-L1 antibody (Dako 22C3) exhibiting extensive electrocution artifacts, also known as cautery artifacts (Fig. 15).

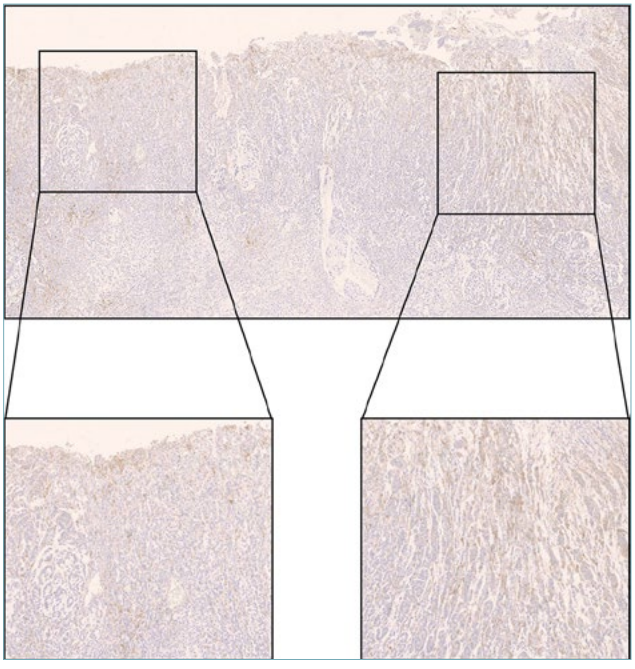

Figure 16. Poor fixation artifacts.

Tissue subject to electrocautery microscopically appears as torn and coagulated. Antigen expression may also be impaired and electrocuted areas should be excluded from PD-L1 evaluation.

Gastroesophageal adenocarcinoma surgical specimen resection stained with PD-L1 antibody (Dako 22C3) exhibiting poor fixation artifacts (Fig. 16). Poor fixation of tissue specimen may hamper PD-L1 evaluation due to morphologic alterations and unreliable PD-L1 staining. While hyperfixation can be often identified by clefts between epithelium and surrounding, tissue hypofixation artifacts include fading nuclei due to autolysis and overstained cytoplasm. Poor fixation can influence the staining pattern of IHC biomarkers, including PD-L1, causing false-negative staining, edge effect and non-specific cytoplasmic staining, as shown in Figure 16.

PD-L1 evaluation in biopsy specimens with less than 100 cells

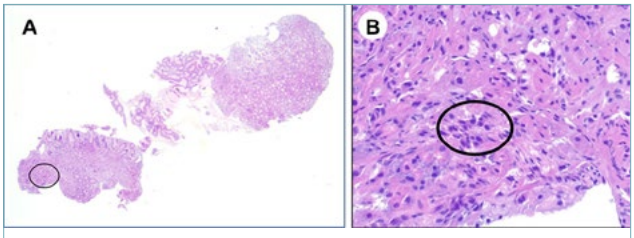

Figure 17. Biopsy specimen with less than 100 cells, from a patient with marked thickening of the stomach walls. Out of a total of 6 biopsies submitted (2 are shown here) only one had a small focus of neoplastic cells. The only area with diagnostic tumor cells is seen in the oval in A and this is shown at higher magnification in the oval in B, where approximately 50 neoplastic cells are seen in total.

A minimum of 100 viable tumor cells should be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation. In case of low tumor content or in the presence of artifacts, a H&E slide should be evaluated to assess the presence of an adequate number of cells. IHC for cytokeratins may also be helpful in the evaluation of the exact number of epithelial cancer cells. If less than 100 viable tumor cells are present, tissue should be recovered from another block or a deeper level of the same block.

PD-L1 expression after neoadjuvant therapy

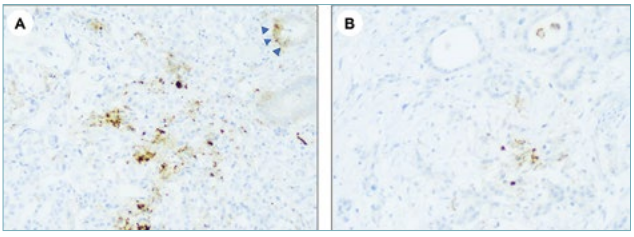

Figure 18. PD-L1 staining in post-neoadjuvant therapy specimen.

Gastroesophageal adenocarcinoma post-neoadjuvant surgical resection specimen stained with PD-L1 antibody (Ventana SP263) (Fig. 18).
Figure 18 shows residual neoplastic glands negative for PD-L1, while tumor-associated macrophages and mononuclear inflammatory cells are positive. Non-neoplastic epithelium exhibits staining for PD-L1 (blue arrows) and it should not be included in the evaluation. The effect of cytotoxic chemotherapy and chemoradiotherapy on PD-1 and PD-L1 expression has been investigated in several malignancies, including G/GEA [21]. Both increase and decrease of PD-L1 expression has been described for gastroesophageal junction adenocarcinoma, depending on cancer subtype and neoadjuvant regimen used (chemo *vs* chemoradiotherapy). Data are still too scarce to draw any meaningful considerations: the mechanism at the basis of PD-L1 expression variations in gastroesophageal junction adenocarcinoma is not well elucidated and its possible role in resistance to treatment is under discussion. Considering this, a suggestion for practicing pathologists may be that, in case of relapse of cancer after neoadjuvant therapy, PD-L1 evaluation should be preferably evaluated (when available) on new biopsies derived from the recurrence/metastatic site.

Different staining patterns of Ventana SP142 and Dako 22C3

Gastric surgical resection specimen stained with two different PD-L1 antibodies: Ventana SP142 (A) and Dako 22C3 (B) (Fig. 19).
Dako 22C3 is approved for Pembrolizumab in patients with several solid tumors, including gastroesophageal adenocarcinoma, while Ventana SP142 is approved for Atezolizumab in patients with urothelial carcinoma, triple-negative breast cancer or non-small-cell lung cancer [22]. Figure 19 shows the different staining patterns of two clones, which may demonstrate different scores, as in this case. Accordingly, some authors state that the two clones should not be considered interchangeable [23].

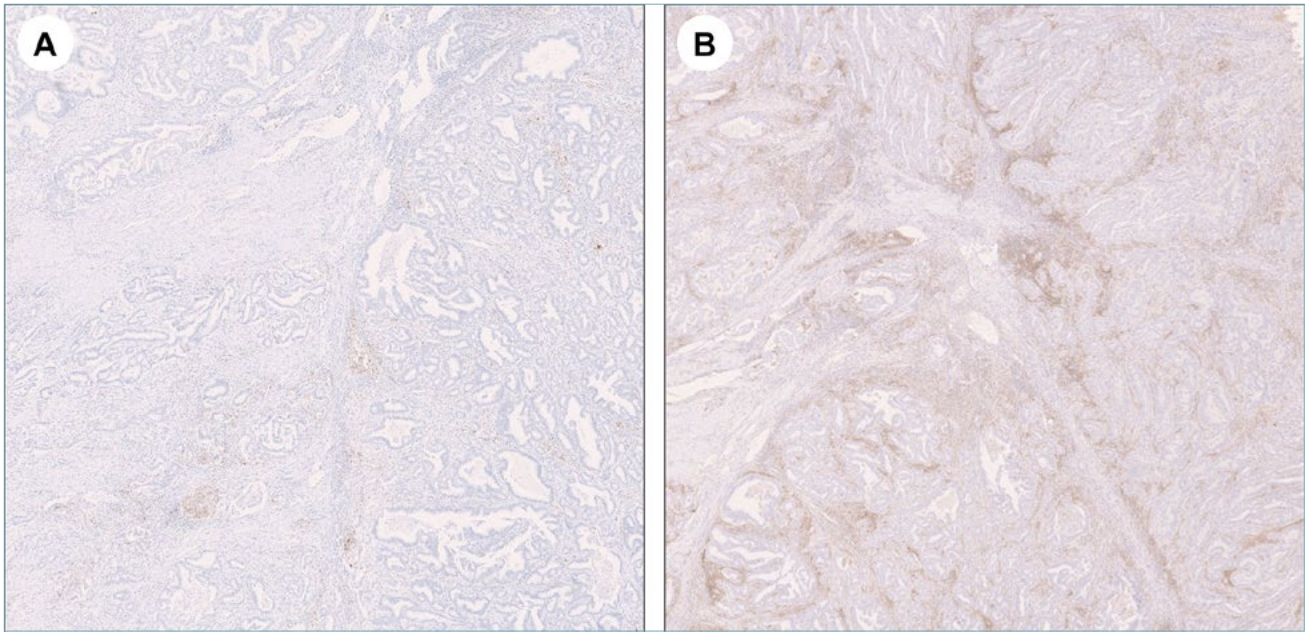

Figure 19. Same tissue specimen showing different staining patterns of Ventana SP142 and Dako 22C3.

PD-L1 expression in rare histotypes and relationship with other biomarkers

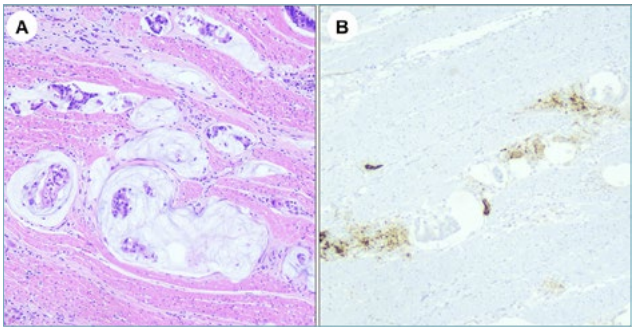

Figure 20. Mucinous adenocarcinoma.

Mucinous gastroesophageal adenocarcinoma stained with H&E (A), PD-L1 (Ventana SP263) (B) (Fig. 20).

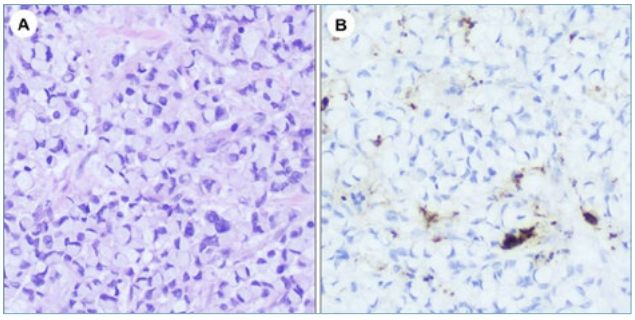

Figure 21. Signet ring cell carcinoma.

Mucinous adenocarcinoma accounts for 2.1-8.1% of gastric cancers and is composed of extracellular mucin pools (> 50% of tumor area) and malignant epithelium (either glandular structures or chains, nests or single tumor cells). Gastric mucinous adenocarcinoma is associated with higher PD-L1 expression, due to the high rates of microsatellite instability (MSI) (see below) (Fig. 20).

Signet ring cell gastric carcinoma stained with H&E (A), PD-L1 (Ventana SP263) (B) (Fig. 21).

Signet ring cell carcinoma is classified as a poorly cohesive carcinoma composed predominantly of tumor cells with prominent cytoplasmic mucin and an eccentrically placed nucleus. A systematic evaluation of PD-L1 expression has not been performed yet. A recent report indicates similar levels of expression to that of other subtypes of gastric adenocarcinoma [24].

Gastric carcinoma with lymphoid stroma stained with H&E (A), EBER in situ hybridization (B) and PD-L1 (Dako 22C3) (C) (Fig. 22).

Gastric carcinoma with lymphoid stroma (also known as medullary carcinoma and lymphoepithelioma-like carcinoma) is a rare histological variant of gastric cancer, characterized by prominent peri- and intra-tumoral infiltration of immune cells, mainly lymphocytes and plasma cells with sparse aggregates of pleomorphic tumor cells [25]. Gastric carcinoma with lymphoid stroma is frequently associated with EBV infection and is characterized by high levels of PD-L1 immunoreactivity (Fig. 22) [26].

Gastric hepatoid carcinoma stained with H&E (A) and PD-L1 (Ventana SP263) (B) (Fig. 23).

Gastric hepatoid carcinoma is a rare variant that histologically resembles hepatocellular carcinoma, with large polyhedral cells with eosinophilic cytoplasm, central nuclei, and prominent nucleoli [27].

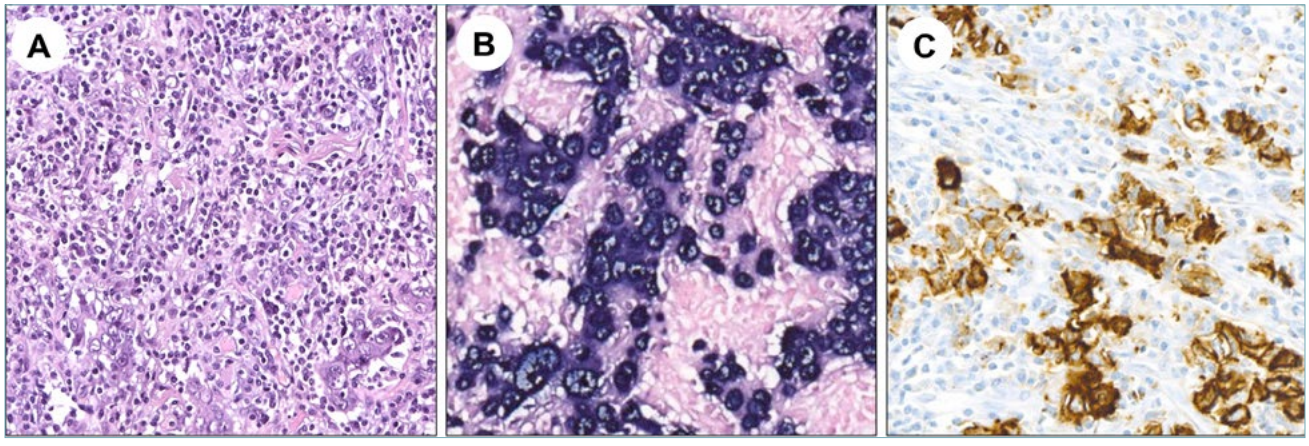

Figure 22. Carcinoma with lymphoid stroma.

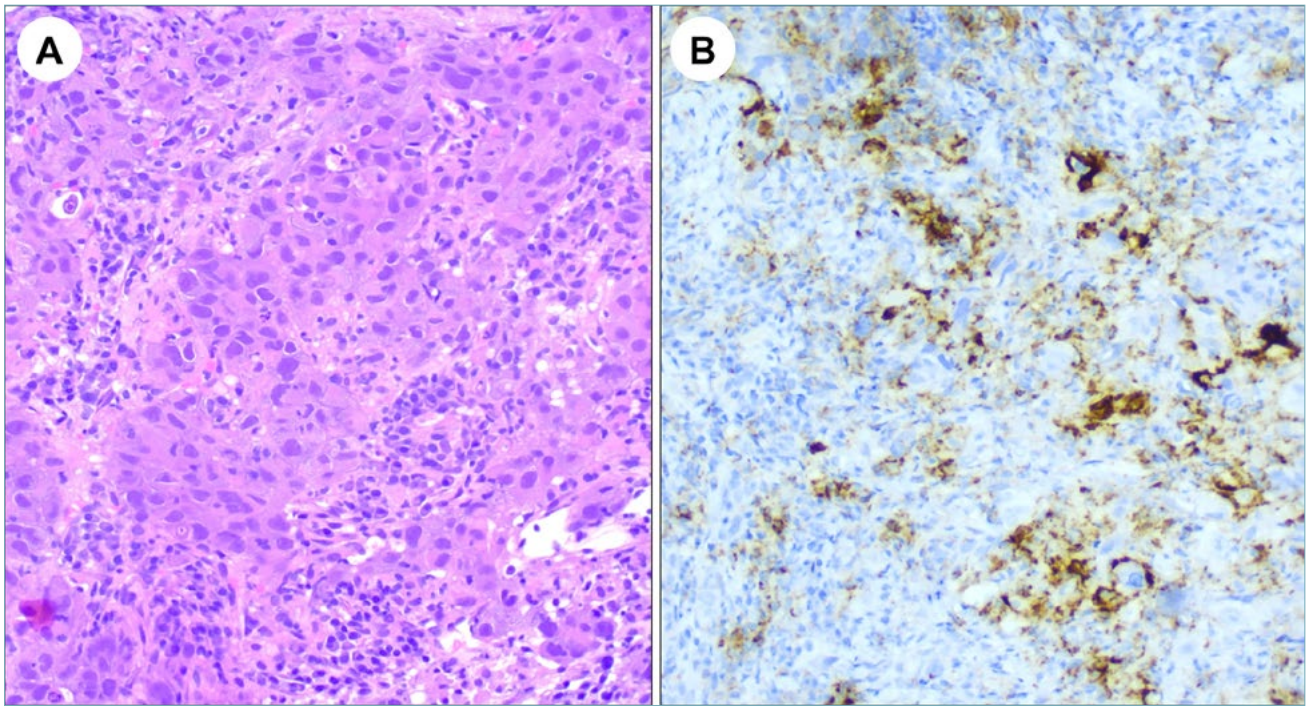

Figure 23. Hepatoid carcinoma.

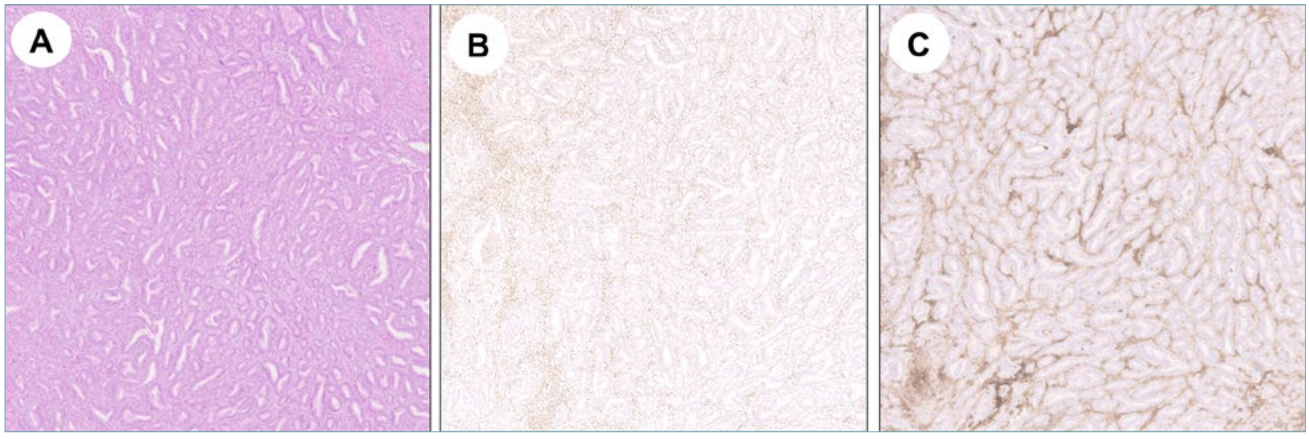

Figure 24. Mismatch repair deficient (MMRd)/microsatellite instable (MSI) gastric adenocarcinoma with high PD-L1 expression.

Gastric adenocarcinoma stained with H&E (A) exhibiting loss of MLH1 (B) and high PD-L1 expression (Dako 22C3) in tumor associated immune cells (B) (Fig. 24). MMRd is the phenotypic fingerprint of MSI and is defined by the loss of one or more of the four MMR proteins (MLH1, MSH2, PMS2, MSH6) [5]. MMRd/MSI has a well-established predictive value of response to immune checkpoint inhibitors and is associated with high levels of lymphocytic infiltration and PD-L1 expression [28].

Conclusions

The understanding of the role of immune checkpoint inhibitors in treating gastrointestinal neoplasms is currently expanding, alongside the need for reliable predictive biomarkers. The evaluation of PD-L1 expression requires that the pathologist plays a crucial role in the predictive selection of advanced gastroesophageal cancer patients who should undergo immunotherapeutic regimens. An accurate, objective

and reproducible assessment of PD-L1 is necessary to provide patients with the best therapeutic option. In this context, the implementation of training programs and incorporation of digital pathology and automation into the workflow may help pathologists navigate the constantly changing scenario of PD-L1 assessment.

Conflicts of interest

The authors declare no conflict of interest related to the present work.

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Authors' contributions

Conceptualization: MF, LM, AV; methodology, GP and FG; data curation, AV and PP; writing-original draft preparation, AV, PP, CR, IG, MLS, MC; writing-review and editing, AV, MF, LM, FG. All authors have read and agreed to the published version of the manuscript.

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