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Evaluating PD-L1 in Head & Neck Squamous Cell Carcinoma: concordance between 22C3 PharmaDx and SP263 assays on whole sections from a multicenter study

Running title: Concordance in PD-L1 scoring on HNSCC

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Conflict of interest statement

The authors declare that they have no competing interests.

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Abstract

Aims: The introduction of immunotherapy in head and neck squamous cell carcinoma (HNSCC) raises the need for harmonization between different types of antibodies and immunohistochemistry platforms for evaluating the expression of (Programmed death-ligand 1) PD-L1 with Combined Positive Score (CPS) in this tumor. We compare the expression of PD-L1, using the CPS and two widely used antibody (22C3 pharma Dx and SP263 assay) in a cohort of HNSCC.

Methods and results: We analyzed forty-three whole sections of HNSCC with two different anti-PD-L1 antibodies, 22C3 pharma Dx and SP263 assay. Results, expressed as CPS, were evaluated by ten trained pathologists and statistical analyses, including interobserver agreement, were performed. We found a very similar distribution for PD-L1 expression between 22C3 pharmaDx assay and SP263 assay in our cohort and a strong significant correlation between the two assays for all specimens (Spearman r= 0.945; p<0.0001). The interobserver reliability among pathologists for the continuous scores of CPS with ICC and the correlation between the two assays was both good. Moreover, the agreement rate between assays was high at all cut-offs and was best for the most relevant cut-off of CPS ≥1, while the kappa values were always in the range of almost perfect.

Conclusions: Two different assays (22C3 pharmaDx assay and SP263 assay) for PD-L1 in HNSCC showed high agreement. This data suggests the interchangeability of these two antibodies in the selection of patients with HNSCC for immunotherapy.

Key words: PD-L1; Head and neck squamous carcinoma; 22C3 and SP263 assays

1. Introduction

Head and Neck cancer represents the sixth most common type of cancer worldwide, with a prevalence of 6% translating into 650,000 new cases per year.[1] Almost 90% of head and neck cancers are of the squamous cell type (HNSCC). To date, the standard of care for locally advanced HNSCC is based on primary resection followed by radio-chemotherapy treatment.[2,3] Despite this intensive multimodality therapy, the survival is poor due to the high frequency of disease recurrence.[4,5] A turning point in the therapy of HNSCC consisted in the introduction of immunotherapy targeting the programmed death-1 (PD-1)/programmed death-ligand-1 (PD-L1) axis, resulting in an improvement in patients' overall survival.[6-8] Recent trials investigating the efficacy of this first-line immune-checkpoint inhibition in recurrent and/or metastatic HNSCC showed that PD-L1 expression is associated with an increased objective response rate in patients with combined positive score (CPS) ≥1, with better response with CPS value ≥20.[9,10] These last studies allowed the Federal Drug Administration (FDA; 2019) to approve Pembrolizumab in combination with platinum and fluorouracil regardless of PD-L1 status in recurrent and/or metastatic HNSCC, and in monotherapy in patients with CPS ≥1 evaluated with an FDA-approved test.[11] The FDA also expanded the intended use for the PD-L1 immunohistochemistry (IHC) 22C3 pharma Dx kit to include use as a companion diagnostic device for selecting patients with HNSCC for treatment with pembrolizumab. In 2020, the European Medicines Agency (EMA https://www.ema.europa.eu/en/documents/variation-report/keytruda-h-c-3820-ii-0065-epar-assessment-report-variation_en.pdf) and UK's National Institute for Health and Care Excellence (NICE) with their guideline documents approved pembrolizumab, both as monotherapy or in combination with chemotherapy, as first-line treatment for metastatic or unresectable recurrent HNSCC in patients whose tumors express PD-L1 with a CPS≥ 1, regardless of the test (antibody and IHC platform) used.[12,13] Unfortunately, several antibody clones and platforms have been used for the evaluation of PD-L1 expression, making comparison amongst these difficult, especially when the literature data regarding the HNSCC are poor.

In this scenario, the availability of a reproducible and robust immunohistochemical test for CPS score is of major importance and the interchangeability of different assays requires appropriate validation and harmonizing studies.[14,15] Another important point when assessing PD-L1 expression, especially with the formulation of CPS, is concordance among pathologists. Indeed, CPS is more complex and perhaps less intuitive than tumor proportion score (TPS), as it requires specific counting of tumor and immune cells to calculate the score. Not surprisingly, training in this regard has been shown to be

important.[16] To date, there are only few studies focusing specifically on CPS evaluation in HNSCC. Both are based on the analysis of tissue microarrays (TMAs) and highlight a variable degree of agreement between the different assays, the reference standard and laboratory developed tests (LDT).[17,18] These results raise concerns about the interchangeability of the available tests. Moreover, the evaluation of whole sections instead of TMAs, that represent the "real life" setting, could further affect the degree of concordance between the different diagnostic tests, and, perhaps, the interobserver variability.

The aim of our study is to compare the diagnostic performance of the two widely diffused assays (22C3 pharma Dx performed on Agilent autostainer link 48 versus SP263 assay performed on Ventana Benchmark XT staining systems) at clinically relevant cut-offs (≥ 1 and ≥ 20) on whole sections from a multicentric cohort of HNSCC.

2. Methods

Sample collection and evaluation

The present study represents a multicenter observational retrospective study of 43 patients with metastatic or unresectable recurrent HNSCC who underwent biopsy or surgical resection (27 biopsies and 16 surgical specimens). A maximum of five samples was collected from each of the 10 participating regional hospitals. Patients undergoing neoadjuvant chemotherapy and/or radiotherapy were excluded from this study. Fixation of the specimens was performed using 10% buffered formalin with and exposure from 12 to 48 hours. Then, biopsies or surgically resected HNSCC samples were paraffin-embedded and, for each case, a representative hematoxylin and eosin (H&E)-stained slide was obtained. HPV status in oropharyngeal SCC and in metastases was assessed by CINtec p16 Histology assay (Roche, Milan, Italy) with strong and diffuse nuclear and cytoplasmic staining in at least 70% of cells used as the cut-point for positivity. All patient data were collected anonymously and written informed consent, as part of the routine diagnosis and treatment procedures, was obtained from patients or their guardians according to the Declaration of Helsinki and the study adhered to Good Clinical Practice guidelines.[19]

PD-L1 IHC and interpretation

PD-L1 immunohistochemistry assay was performed on each specimen (3 μm thick consecutive sections) with two anti–PD-L1 antibodies, clone 22C3 and SP263, according to the manufacturer's instructions. Briefly, we used 22C3 pharmDx (mouse monoclonal primary anti–PD-L1 antibody, prediluted, clone 22C3, Dako, Carpinteria, CA) on the Autostainer Link 48 with EnVision DAB Detection System (Agilent Technologies, Santa Clara, CA), and Ventana SP263 (rabbit monoclonal primary anti–PD-L1 antibody, prediluted, Ventana Medical Systems, Tucson, AZ) on the Benchmark XT staining systems and OptiView Universal DAB Detection Kit (Ventana Medical Systems). Immunohistochemical analysis for both anti-PD-L1 antibodies was centralized and performed at Molecular Pathology Laboratory of Università Cattolica del Sacro Cuore. PD-L1 control slides from 22C3 pharmDx (containing sections of two pelleted, formalin-fixed paraffin-embedded cell lines: NCI-H226 with moderate PD-L1 protein expression and MCF-7 with negative PD-L1 protein expression) were used as positive and negative control for both antibodies (22C3 and SP263). We also used placenta, tonsil, and vermiform appendix tissues as positive controls. All slides (hematoxylin-eosin and PD-L1 stains) were digitized with an Aperio CS2 (Leica Biosystem) at ×40, uploaded on a

shared web platform provided by Nikon, and viewed with NDP.view2 software by head and neck pathologists specifically trained and certified in CPS assessment from each participating Center. The evaluation was performed on whole slides, and CPS was determined as the number of PD-L1 positive tumor cells, lymphocytes and macrophages divided by the total number of viable tumor cells, multiplied by 100. Any perceptible and convincing partial or complete linear membrane staining of viable tumor cells that was perceived as distinct from cytoplasmic staining was considered as positive PD-L1 staining and included in the scoring. Likewise, any membrane and/or cytoplasmic staining of mononuclear inflammatory cells within tumor nests and/or adjacent supporting stroma was considered positive PD-L1 staining and was included in the CPS numerator. Neutrophils, eosinophils, plasma cells, and ICs associated with in situ components, benign structures, or ulcers were excluded from the CPS score. The cut-offs of ≥ 1 and ≥ 20 were considered. Each countable section contained at least 100 viable HNSCC cells. All pathologists (n. 10) received appropriate training course for CPS score evaluation in HNSCC (certified pathologists) and were blinded to clinical information as well as the evaluation results of other pathologists.

Statistical analysis

The inter-observer reliability of pathologists for the CPS score was determined by calculating intraclass correlation coefficient (ICC) for each assay. Correlation among the continuous values of CPS between the two assays was assessed with ICC based on a single-rating, absolute agreement, two-way mixed model. The level of agreement of PD-L1 expression between assays was determined via Cohen's kappa with confidence intervals (CI) for each cut-off after stratification of cases among the relevant cut-offs. Overall percent agreement (OPA) with 95% CI at each cut-off value (≥ 1, ≥ 20 and for the three categories together) and positive and negative percent agreement (PPA, NPA) were calculated. Statistical analyses were performed using Microsoft Excel 2013, IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.) and R software version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

Patient characteristics and PD-L1 staining by 22C3 pharma Dx and SP263 assay

The sample analyzed included 27 (62.8%) biopsies and 16 (37.2%) surgical specimens of HNSCCs collected from 2020 to 2021 for a total amount of 43 digitized cases. Main clinicopathologic characteristics of our cohort are reported in Table 1. Mean age at the time of diagnosis was 61 years and 72% of patients were male. Thirty-two patients (74.4%) had metastatic disease and 11 (25.6%) had unresectable/recurrent neoplasia. Seventeen out of 43 patients (39.5%) had squamous carcinoma located in the oropharynx, 13 out of 43 (30.2%) in hypopharynx, 8 out of 43 (18.6%) in larynx and 5 out of 43 (11.7%) had metastatic localization. Seven out of 22 (31.8%; including 17 cases of oropharyngeal SCC and 5 cases of SCC metastases) were positive for p16 expression while 15 out of 22 were negative (68.2%). PD-L1 IHC was performed using the 22C3 pharmDx and SP263 assays and the CPS was calculated for each sample. Representative IHC images and boxplot of CPS values of PD-L1, determined with the 22C3 pharmDx and SP263 assay, showed similar staining patterns and distribution for the same samples (Figure 1 and 2). When we considered the cut-off of ≥1, 38 samples (88.4%) had a positive CPS with both 22C3 pharmaDx and SP263 assays. CPS values between 1 and 20 were scored respectively in 21/43 (48.8%) cases with 22C3 pharmDx assay and in 20/43 (46.5%) samples with SP263 assay. CPS values ≥ 20 were diagnosed in 17 out of 43 (39.5%) cases with 22C3 pharmDx assay and in 18 out of 43 (41.9%) cases with SP263 assay. Five samples (11.7%) had a CPS<1 with both assays. We found a strong significant correlation between the two assays for all specimens when comparing the CPS with 22C3 pharmaDx assay and SP263 assay (Spearman r= 0.945; p<0.0001; Figure 3, panel A). The distribution of CPS is shown in the Figure 3, panel B. We found a very similar distribution for the two assays, although the cases with CPS ≥20 were slightly higher based on the SP263 assay results in comparison to 22C3 pharmDx assay results.

Interobserver agreement

To evaluate the interobserver agreement in PD-L1 interpretation, we analyzed the results of CPS evaluation on the 43 HNSCC samples by 10 pathologists (Table 2). Interobserver reliability among pathologists for the continuous scores of CPS with ICC were 0.834 (CI 0.758-0.896) and 0.868 (CI 0.803-0.918) for 22C3 and for SP263 assays, respectively. The correlation between the two assays with ICC was 0.901 (CI 0.885-0.931). At single cut-off of CPS ≥1, the two assays showed a Cohen's kappa of 0.891 (CI 0.825-0.957) with OPA 98% (CI 95-99%), while at cut-off of CPS ≥20, the kappa value was 0.808 (CI 0.753-0.862) with OPA 90% (CI 87-93%), both in the range of almost perfect

agreement. At cut-off CPS ≥1, PPA and NPA between clones were 98% (CI 95%-99%) and 97% (CI 85%-100%), respectively. At cut-off CPS ≥20, PPA and NPA between clones were 95% (CI 90%-98%) and 87% (CI 80%-91%), respectively. When considering the three cut-off categories CPS <1, CPS between 1 and <20 and CPS ≥20, the weighted kappa was 0.878 (CI 0.813-0.943) with OPA 88% (CI 84-92%). The agreement rate between assays was high at all cut-offs and was best for the most relevant cut-off of CPS ≥1, while the kappa values were always in the range of almost perfect (Table 2).

4. Discussion

The introduction of immunotherapy with anti-PD1/PD-L1 inhibitors has resulted in a remarkable outcome improvement of several advanced solid tumors.[20] Recently, FDA approved the use of Pembrolizumab as first-line monotherapy in recurrent and/or metastatic HNSCC in those patients with CPS ≥1 evaluated with an FDA-approved companion test, i.e. the 22C3 pharmaDx test. Afterwards, the EMA and UK's NICE have approved Pembrolizumab, both as monotherapy or in combination with chemotherapy, as first-line treatment for metastatic or unresectable recurrent HNSCC in patients whose tumors express PD-L1 with a CPS≥ 1, regardless of the test (antibody and IHC platform) used. The registration of an anti-PD1/PD-L1 inhibitor for clinical practice is associated with a specific diagnostic assay and staining platform, as well as an immunohistochemical score with specific cut-off values for patients' selection. However, the majority of Pathology Departments do not have access to more than one of the staining platforms, resulting in the inability to provide a full screening for all the available checkpoint inhibitor drugs in different tumors. The alternative use of laboratory developed tests (LDTs) can be limited by the difficulty to standardize many of the assay components. Thus, LDTs are likely to be less robust than commercial tests and may be a source of results variability. Whenever a companion diagnostic test is not required, comparative studies between different immunohistochemical assays are needed to assess the interchangeably of the different antibodies and platforms at given cut-off values, as previously reported for some solid tumors such as NSCLC.[21] Moreover, the evaluation of PD-L1 staining may be affected by its heterogeneous expression within tumor samples and by the interobserver variability.[22,23] In the last five years only a small number of studies were published, dealing with the interpretation of PD-L1 staining in HNSCC. Moreover, they were based on different evaluation criteria (considering either immune or neoplastic cells, or both) and variable cut-off values for positivity, affecting the reproducibility of the results. In this study, we aimed to investigate the concordance between the two most common PD-L1 assays (22C3 pharmaDx and SP263 assay), on a cohort of HNSCC. We demonstrated a significant similarity in the results of CPS evaluation when comparing the 22C3 pharmaDx with the SP263 assay ($p<0.0001$). In addition, we found that the interobserver reliability among pathologists for the continuous scores of CPS with ICC and the correlation between the two assays were both good. Moreover, at cut-off values of CPS ≥1 and CPS ≥20, the two assays showed a Cohen's kappa of 0.891 with OPA 98% and 0.808 with OPA 90% respectively, both in the range of almost perfect agreement. The agreement rate between assays was

high at all cut-offs and was best for the most relevant cut-off of CPS ≥1, while the kappa values were always in the range of almost perfect.

Although the study was carried out on a small number of cases, our report presents some substantial novelties. In fact, we restricted our analysis only to two platforms (the Autostainer Link 48 with EnVision DAB Detection System for 22C3 pharmaDx, and Benchmark XT staining systems and OptiView Universal DAB Detection Kit for SP263 assay) with the same immunohistochemistry protocol. This approach has greatly reduced the variability related to the use of different immunohistochemical platforms and protocols, as recently highlighted by the study of Crosta S et al. which compared the performance of five different PD-L1 protocols with the 22C3 pharmDx on 15 cases/30 cores.[18]

A further point of innovation as compared to previous reports is represented by the histological material analyzed. Our samples consisted of whole sections from 27 biopsies and 16 surgical specimens of HNSCCs. This type of samples represents the patient's tumor with the full variability in PD-L1 expression as in the "real world" setting. However, the results of evaluation of PD-L1 on these samples were relatively more homogeneous than those obtained using TMAs. Indeed, a previous report provided evidence that a single TMA core is not representative of the whole tumor section, with a 0% negative predictive value (NPV) of single and double negative biopsies using a CPS cut-off value of ≥1.[24] This could explain at least to some extent the difference between our results and the study of De Ruiter et al. which reported precisely on the ICC and Cohen's kappa for the comparison with CPS among the reference standard and both the SP263 and a LDT with 22C3 on Ventana platform.[17] De Ruiter et al. demonstrated that, in a serial section of TMA containing 147 HNSCC, the concordance between 22C3 pharmDx and SP263 is lower than moderate with ICC and in the range of fair (0.20-0.40) with Cohen's kappa both at cut-off 1 and 20 with no significant increase at the highest cut-off, while the LDT showed an ICC of at least moderate in both the comparisons and concordance kappa from fair to substantial with high variability.[14] The authors did not insist on tumoral heterogeneity, but concordance investigation among TMA cores and whole section was carried out only in a subset of 12 tumors out of 147.

Finally, we believe that a strength of our study is the number of pathologists who evaluated the expression of PD-L1 on HNSCC cohort. Contrariwise to other scientific reports where the number of the pathologists involved in the PD-L1 evaluation in HNSCC is small and often without a specific expertise in the field, this study involves 10 pathologists from 10 different centers who, after taking a training course on the evaluation of CPS in HNSCC, had an almost perfect agreement, best for the most relevant cut-off of CPS ≥ 1 and with kappa values always in the range of almost perfect.[17,18,25]

The reproducibility of pathologists in the CPS evaluation was excellent and implies that when a pathologist is adequately trained in reading CPS the choice of the assay does not affect the assessment. This is in line with studies from a Canadian group which investigated concordance among pathologists with several clones also in a cohort of HNSCC, showing high agreement values on evaluation of PD-L1 even with separate evaluation of tumor and immune cells, and highlighting how the SP263 clone achieves the best performance in terms of interobserver reproducibility.[25-27]

The patients of our cohort were all untreated. Although studies seem to indicate a role of cisplatin-based therapeutic regimens in the induction of PD-L1 expression in HNSCC, other studies investigating the effects have provided controversial data.[28-30] Moreover, most recent studies have demonstrated that radiotherapy could deeply affect the tumor microenvironment and the immune response against tumor also influencing the expression of PD-L1 (abscopal effect).[31]

Our study had some limitations: firstly, the low number of cases analyzed and the relative under-representation of some specific head and neck district and of metastatic sites. Secondly, the cases were assessed remotely by pathologists using their own personal workstations. This implies a potential limited standardization of viewing displays and network bandwidth.

In conclusion, our data demonstrates the substantial interchangeability between SP263 assay and 22C3 pharmaDx in the PD-L1 evaluation of HNSCC patients. Standardized methods (immunohistochemical protocol, antibody and IHC platform), whole section tissue samples and training could deeply impact on PD-L1 evaluation and the concordance between different anti-PD-L1 antibodies in HNSCC patients. Further studies in other independent cohorts are needed to confirm our data and definitively support the harmonization of the different PD-L1 assays.

List of abbreviations

HNSCC: head and neck squamous cell carcinoma; CPS: combined positive score; TMA: tissue microarrays; LTD: laboratory developed test; FDA: Federal Drug Administration; EMA: European Medicines Agency; IHC: immunohistochemistry; TPS: tumor proportion score; ICC: intraclass correlation coefficient; OPA: Overall percent; PPA: positive percent agreement; NPA: negative percent agreement; NPV: negative predictive value.

Declarations

Ethics approval and consent to participate

All patient data were collected anonymously and written informed consent, as part of the routine diagnosis and treatment procedures, was obtained from patients or their guardians according to the Declaration of Helsinki and the study adhered to Good Clinical Practice guidelines.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

B.C., I.G., G.d.A and M.M. were the principal authors and the main contributors in writing the manuscript. B.C., L.C., S.T., R.S., M.B., T.L., P.L.A., F.S., M.G.P., G.P. and M.M. analyzed and interpreted the data. I.G and A.E. performed the statistical analysis. M.M. and B.C. performed the immunohistochemistry analysis. M.G.P. digitized the slides and uploaded the file on a shared web platform. B.C., I.G., A.E., G.d.A and M.M. read and corrected the manuscript. All authors read and approved the final manuscript.

Consent for publication

All authors reached an agreement to publish the study in this journal.

Competing interests

The authors declare that they have no competing interests.

References

1. Botticelli A, Mezi S, Pomati G *et al.* The 5-Ws of immunotherapy in head and neck cancer. *Review Crit Rev Oncol Hematol* 2020; **153**: 103041.
2. Pignon J.P, Bourhis J, Domenge C, Designé L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-analysis of chemotherapy on head and neck cancer. *Lancet* 2000; **355**: 949-955.
3. Pignon J.P, le Maître A, Maillard E, Bourhis J, MACH-NC Collaborative Group. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomized trials and 17,346 patients. *Radiother. Oncol* 2009; **92**: 4–14.
4. Marur S, Forastiere A.A. Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment. *Mayo Clin Proc.* 2016; **91**: 386-396.
5. Siegel R.L, Miller K.D, Jemal A. Cancer statistics, 2019. *CA Cancer J. Clin.* 2019; **69**: 7-34.
6. Yang B, Liu T, Qu Y *et al.* Progresses and perspectives of anti-PD-1/PD-L1 antibody therapy in head and neck cancers. *Front Oncol.* 2018; **8**: 563.
7. Chow LQM, Haddad R, Gupta S *et al.* Antitumor activity of pembrolizumab in biomarker-unselected patients with recurrent and/or metastatic head and neck squamous cell carcinoma: results from the phase Ib KEYNOTE-012 expansion cohort. *J Clin Oncol.* 2016; **34**: 3838-3845.
8. Mehra R, Seiwert TY, Gupta S *et al.* Efficacy and safety of pembrolizumab in recurrent/metastatic head and neck squamous cell carcinoma: pooled analyses after long-term follow-up in KEYNOTE-012. *Br J Cancer.* 2018; **119**: 153-159.
9. Seiwert TY, Burtness B, Mehra R *et al.* Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): An open-label, multicentre, phase 1b trial. *Lancet Oncol* 2016; **17**: 956-965.
10. Burtness B, Harrington KJ, Greil R *et al.* Pembrolizumab alone or with chemotherapy versus cetuximab with chemotherapy for recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-048): A randomised, open-label, phase 3 study. *Lancet* 2019; **394**: 1915-28.
11. [Internet]. FDA approves pembrolizumab for first-line treatment of head and neck squamous cell carcinoma. [Accessed November 6, 2019]: Available from: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-

pembrolizumab-first-line-treatment-head-and-neck-squamous-cell-carcinoma#:~:text=squamous%20cell%20carcinoma-,FDA%20approves%20pembrolizumab%20for%20first%2Dline%20treatment%20of,and%20neck%20squamous%20cell%20carcinoma&text=On%20June%2010%2C%202019%2C%20the,squamous%20cell%20carcinoma%20(HNSCC).

12. [Internet]. EMA Committee for Medicinal Products for Human Use (CHMP) assessment report EMEA/H/C/003820/II/0065. [Accessed October 17, 2020]: Available from: https://www.ema.europa.eu/en/documents/variation-report/keytruda-h-c-3820-ii-0065-epar-assessment-report-variation_en.pdf
13. [Internet]. Pembrolizumab for untreated metastatic or unresectable recurrent head and neck squamous cell carcinoma. [Accessed November 25, 2020]: Available from: https://www.nice.org.uk/guidance/ta661
14. Ionescu DN, Downes MR, Christofides A, Tsao MS. Harmonization of PD-L1 testing in oncology: a Canadian pathology perspective. *Curr Oncol.* 2018; **25**: e209–e216.
15. Torlakovic E, Lim HJ, Adam J *et al.* "Interchangeability" of PD-L1 immunohistochemistry assays: a meta-analysis of diagnostic accuracy. *Mod Pathol.* 2020; **33**: 4–17.
16. Eccher A, Fontanini G, Fusco N *et al.* Digital slides as an effective tool for programmed death ligand 1 combined positive score assessment and training: Lessons learned from the "Programmed death ligand 1 key learning program in Head-and-Neck squamous cell carcinoma". *J Pathol Inform* 2021; **12**: 1.
17. De Ruiter E.J, Mulder F.J, Koomen B.M *et al.* Comparison of three PD-L1 immunohistochemical assays in head and neck squamous cell carcinoma (HNSCC). *Mod. Pathol.* 2020.
18. Crosta S, Boldorini R, Bono F *et al.* PD-L1 Testing and Squamous Cell Carcinoma of the Head and Neck: A Multicenter Study on the Diagnostic Reproducibility of Different Protocols. *Cancers (Basel).* 2021; **13**: 292.
19. Teofili L, Giona F, Martini M *et al.* The revised WHO diagnostic criteria for Ph-negative myeloproliferative diseases are not appropriate for the diagnostic screening of childhood polycythemia vera and essential thrombocythemia. *Blood.* 2007; **110**: 3384-3386.
20. Gong J, Chehrazi-Raffle A, Reddi S, Salgia R. Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. *J Immunother Cancer.* 2018; **6**: 8.

21. Buttner R, Gosney JR, Skov BG *et al.* Programmed death-ligand 1 immunohistochemistry testing: a review of analytical assays and clinical implementation in non-small-cell lung cancer. *J Clin Oncol.* 2017; **35**: 3867–3876.
22. Munari E, Zamboni G, Marconi M *et al.* PD-L1 expression heterogeneity in nonsmall cell lung cancer: evaluation of small biopsies reliability. *Oncotarget.* 2017; 8: 90123–90131.
23. Brunnstrom H, Johansson A, Westbom-Fremer S *et al.* PD-L1 immunohistochemistry in clinical diagnostics of lung cancer: inter-pathologist variability is higher than assay variability. *Mod Pathol.* 2017; **30**: 1411–1421.
24. Rasmussen JH, Lelkaitis G, Håkansson K *et al.* Intratumor heterogeneity of PD-L1 expression in head and neck squamous cell carcinoma. *Br J Cancer.* 2019; **120**: 1003-1006.
25. Downes MR, Slodkowska E, Katabi N, Jungbluth AA, Xu B. Inter- and intraobserver agreement of programmed death ligand 1 scoring in head and neck squamous cell carcinoma, urothelial carcinoma and breast carcinoma. *Histopathology.* 2020; **76**: 191-200.
26. Wang C, Hahn E, Slodkowska E *et al.* Reproducibility of PD-L1 immunohistochemistry interpretation across various types of genitourinary and head/neck carcinomas, antibody clones, and tissue types. *Hum Pathol.* 2018; **82**: 131-139.
27. Hodgson A, Slodkowska E, Jungbluth A *et al.* PD-L1 Immunohistochemistry Assay Concordance in Urothelial Carcinoma of the Bladder and Hypopharyngeal Squamous Cell Carcinoma. *Am J Surg Pathol.* 2018; **42**: 1059-1066.
28. Ock C-Y, Kim S, Keam B *et al.* Changes in programmed death-ligand 1 expression during cisplatin treatment in patients with head and neck squamous cell carcinoma. *Oncotarget.* 2017; **8**: 97920–97927.
29. Naruse T, Yanamoto S, Okuyama K *et al.* Immunohistochemical Study of PD-1/PD-L1 Axis Expression in Oral Tongue Squamous Cell Carcinomas: Effect of Neoadjuvant Chemotherapy on Local Recurrence. *Pathol Oncol Res POR.* 2020; **26**: 735–742.
30. Pflumio C, Thomas T, Salleron J *et al.* Expression of immune response biomarkers (PD-L1, p16, CD3+ and CD8+ TILs) in recurrent head and neck squamous cell carcinoma within previously irradiated areas. *Oncol Rep.* 2021; **45**: 1273–1283
31. Zhao X, Shao C. Radiotherapy-Mediated Immunomodulation and Anti-Tumor Abscopal Effect Combining Immune Checkpoint Blockade. *Cancers (Basel)* 2020; **12**: 2762.

Figure legend

Figure 1

The figure shows two HNSCC samples (panel A and D, respectively, E&E) analyzed with 22C3 pharmaDx kit (panel B and E, respectively) and SP263 assay (panel C and F, respectively). The PD-L1 expression with 22C3 and SP263 antibodies, shows a similar value of CPS in the two cases (CPS≥20 for above case panels A-C, and <20 for below case, panel D-F).

Figure 2

The figure shows the boxplot of PD-L1 CPS values distribution (clear box for 22C3 and colored box for SP263) for all samples. The smallest value and largest value are found at the end of the 'whiskers', while the interquartile range (IQR) is the box. The two dotted lines in the plot indicates the CPS cut-off of 1 (near the Y axis) and 20, respectively.

Figure 3

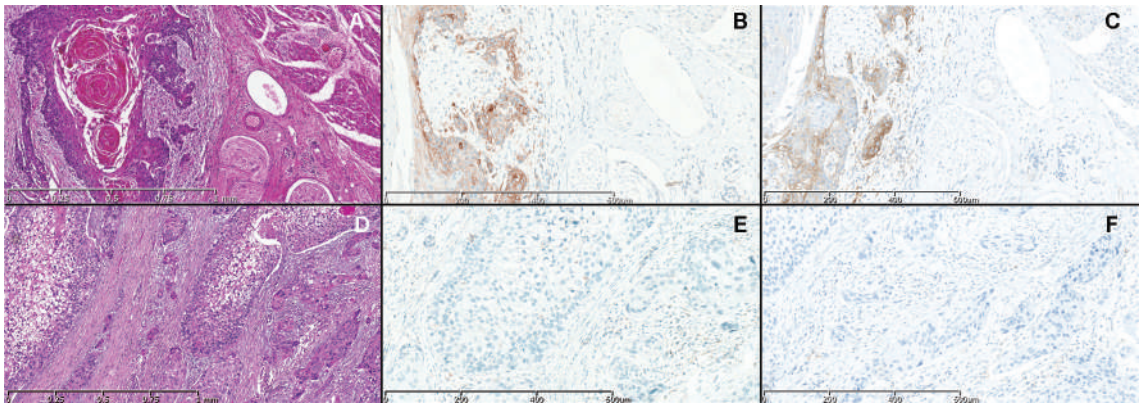
Panel A: the figure shows the direct and significant correlation between CPS evaluated with 22C3 and Sp263 antibodies (Spearman r= 0.945; p<0.0001); Panel B: the figure shows the PD-L1 expression distribution of the 22C3 pharmaDx and SP263 assay for the CPS.

Table 1. Patient characteristics.

	n = 43
Age, mean (±SD)	61 (8.7)
Gender, n (%)	
Male	31 (72.1)
Female	12 (27.9)
Stage, n (%)	
metastatic	32 (74.4)
unresectable recurrent	11 (25.6)
Tumor location, n (%)	
Oropharynx	17 (39.5)
Hypopharynx	13 (30.2)
Larynx	8 (18.6)
Metastatic sites	5 (11.7)
HPV status (p16), n (%)	
Positive	7 (31.8)
Negative	15 (68.2)
PD-L1 expression 22C3, n (%)	
<1	5 (11.7)
1-<20	21 (48.8)
≥20	17 (39.5)
PD-L1 expression SP263, n (%)	
<1	5 (11.7)
1-<20	20 (46.5)
≥20	18 (41.8)

Table 2. Measure of agreement

Measure of agreement	Results
ICC among pathologists 22C3	0.834 (CI 0.758-0.896)
ICC among pathologists SP263	0.868 (CI 0.803-0.918)
ICC between clones	0.911 (CI 0.885-0.931)
Kappa at CPS≥1 between clones	0.891 (CI 0.825-0.957)
OPA at CPS≥1 between clones	98% (CI 95%-99%)
PPA at CPS≥1 between clones	98% (CI 95%-99%)
NPA at CPS≥1 between clones	97% (CI 85%-100%)
Kappa at CPS≥20 between clones	0.808 (CI 0.753-0.862)
OPA at CPS≥20 between clones	90% (CI 87%-93%)
PPA at CPS≥20 between clones	95% (CI 90%-98%)
NPA at CPS≥20 between clones	87% (CI 80%-91%)
Kappa for three categories (CPS<1, CPS≥1 and CPS≥20) between clones	0.878 (CI 0.813-0.943)
OPA for three categories (CPS<1, CPS≥1 and CPS≥20) between clones	88% (CI 84%-92%)



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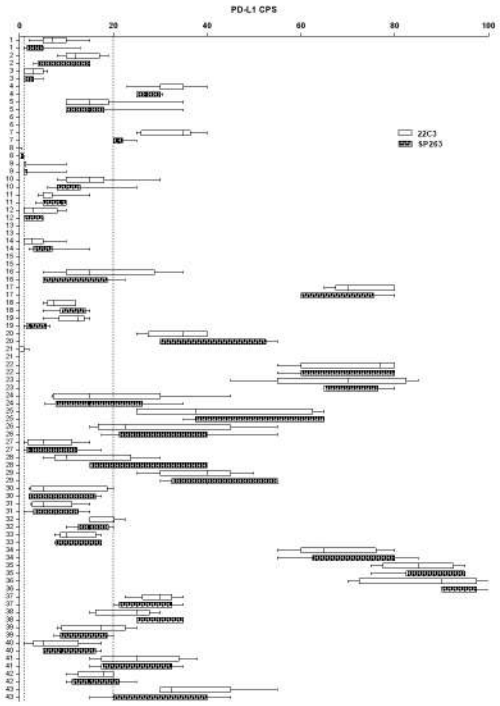

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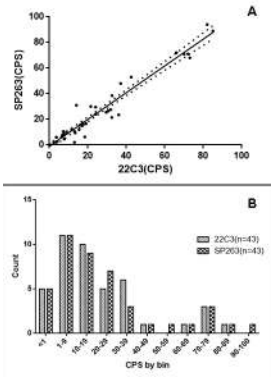

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