Optimization for better Immunohistochemistry assay and more comprehensive pathological interpretation for PD-L1 expression in Classical Hodgkin lymphoma

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Research Article

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Abstract

Bckgoround: Treating the relapsed or refractory Classical Hodgkin Lymphomas were still challenging, previous studies indicated they can benefit from immune checkpoint inhibitors, over expression of PD-L1 can also be predictive marker for anti -PD -1 therapeutic efficacy, however, harmonization with different IHC assay s and explanation of the PD -L1 immunostaining results remains controversial in CHL. In this study, we sought to optimize the PD-L1 Immunohistochemistry (IHC) assay in classical Hodgkin lymphoma (CHL).

Methods: All tests were performed on tumor tissue microarray established from 54 CHLs. Three IHC assays (405.9A11, SP142, 22C3) for PD-L1 expression were compared semi-quantitatively with RNAscope assay (No. 310035, ACD), and the difference for their expression on background immune cells (ICs), their associations with densities of TIL/TAM makers and their implication on survival were also analyzed.

Results: 405.9A11 assay demonstrated best specificity on HRS cells and sensitivity on ICs. Thus, positive expression of PD-L1 was more frequent on ICs (85.2%) than on HRS cells (48.1%). Different subgroups of background IC cells including tumor associated macrophages (TAMs) were assessed and scored by CD4, CD8, FOXP3, and CD163. PD-L1 expression on ICs were most associated with elevated densities of TAMs, and in concordance with TAMs(P=0.026), PD-L1 expression on ICs (P=0.067)indicated a negative effect on survival.

Conclusions: 405.9A11 assay was proved to be most convincible for demonstrating PD-L1 expression pathologists should report PD-L1 expression in a combined manner including both positive status of HRS cells and positive percentage of ICs.

Background

The Classic Hodgkin's Lymphoma (CHL) is characterized by a minority of malignant Hodgkin and Reed–Sternberg cells (HRS cells) within overwhelming but ineffective background inflammatory infiltrates [1]. Although most CHLs were curable, treating the relapsed or refractory(R/R) CHLs were still challenging.

The programmed death-1 (PD-1) blocking antibody Nivolumab and its mimickers[2], belonging to the so-called immune checkpoint inhibitors, can promote and stimulate an anti-tumor effect via the host immune system rather than directly targeting malignant cells, and has substantial therapeutic activity in R/R CHLs[3–5]. PD-1, which is one of the most important immune checkpoints [6], is reported expressing in the peritumoral activated T- cells [7] rather than HRS cells [8] in CHL. PD-1 has two ligands: programmed death-ligand 1 (PD-L1) and PD-L2 [9]. Binding of the PD-L1/PD-L2 to PD-1 delivers an inhibitory signal, which will prohibit overt activation of T cells physically also confer escape from host immune control in tumor [8, 10, 11]. PD-L1 and PD-L2 had concordant alterations in their encoded gene loci in CHL [12],Expression of PD-L1 and PD-L2 can be measured by immunohistochemical staining (IHC) on formalin-fixed paraffin embedded (FFPE) tissue sections[13–16], the incidence of aberrant PD-L2

expression was similar[12] or less common [16, 17] than PD-L1, and there were more commercial PD-L1 antibodies(Abs) and assays available nowadays [18], so we only focused on PD-L1, which was proposed as a complementary test, to indicate the probability of benefit from anti-PD-1/L1 agents[19].

Expression of PD-L1 can be found not only on tumor cells but also on the peri-tumoral immune cells(ICs) in various tumors including lymphoma[20].Both the expression level of PD-L1 on tumor cells or ICs, are now served as an predictor for therapeutic efficacy [12, 21]. In CHL, over expression of PD-L1 can also be predictive marker for anti-PD-1 therapeutic efficacy [8, 15], however, harmonization with different IHC assays and explanation of the PD-L1 immunostaining results remains controversial [16, 22, 23]. Recently, it was reported that RNAscope assay via in situ hybridization in FFPE [24–26] of tumor might also be a promising method for assessment of PD-L1 mRNA in various types of solid tumors[26–28], which can provide a reference for PD-L1 expression level independent of IHC assays [28], and was seldom descripted in CHL previously.

In various solid tumors, PD-L1 expression were found in relationship with the density of certain specific subtype of ICs, including CD4 + T helper cells(Ths), CD8 + cytotoxic T cells(CTLs), FOXP3 + regulatory T cells (Tregs) and CD163 + tumor associated macrophages(TAMs) [29–33]. TAMs in CHL correlated with poor prognosis[34, 35]. TAMs can express PD-L1 under the activation of IFN-γand PD-1 positive T-cell infiltration [23], or may derive PD-L1 by trogocytosis from HRS cells [27]. Thus, the association between PD-L1 expression and densities of different types of ICs need further investigation in CHL.

In this study, we sought to determine: (1) the best Ab and assay for detecting PD-L1 by comparing three IHC assays with RNAscope assay independently; (2) more accurate expression status of PD-L1 in CHL, both on HRS cells and ICs; (3) analyze the association between PD-L1 expression and different element of background IC cells and their implication on survival.

Materials And Methods

Patient selection and Tissue microarrays

The FFPE specimens of 54 diagnosed nodal CHLs tissue were archived from the department of Pathology in Peking University Cancer Hospital. All cases were reviewed by two hematopathologists (Yunfei Shi and Yumei Lai) to confirm pathological diagnosis. Clinical information including age, sex, Ann Arbor stage, presence or absence of B symptoms, treatment regimen, and survival data was obtained from their medical records. The tissue microarrays (TMA) for further detections were created by fetching duplicate cores (1 mm in diameter) from representative areas in each reviewed block using an arrayer (Alphelys, Plaisir, France) as described previously [36].

Immunol staining and evaluations the expressions of PDL1 and other associated biomarkers

The expression of PD-L1 was assessed with 3 different Abs and assays, that is clone 405.9A11 (call 9A11 for short, Cell Signaling Technology) [8], SP142(Ventana, Roche)[37], and 22C3 (Dako, Agilent technologies)[38]. Other antibodies include PD-1(UMAB199, Origen), CD4 (EP204, Origen), FOXP-3(ab20034, Abcam), CD8(SP16, BioCare) and CD163(NCL-CD163, Novocastra). Dilutions were 1:50 or follow the instruction from each supplier. All IHC staining steeps were performed using automated IHC staining instrument (VENTANA, Roche) excluding clone 22C3 of PD-L1, which were done with a Dako Auto Stainer. Negative/positive controls were established as recommended[38].

Pathological Evaluation

After staining, the expressions of PD-L1 and other above-mentioned makers were dual-reviewed independently, on six representative fields at high power (×400) magnification from 2 different TMA cores. The concept of IC staining of PD-L1 was introduced to evaluate PD-L1 expression level on ICs in CHL by assessing the proportion(%) of tumor area occupied by PD-L1-positive ICs as described.[37, 39] The staining of PD-L1 were considered as positive if moderate/strong (yellow to brown signal located) at the membrane and or cytoplasm were seen on targeted cells(either HRS cells or ICs); and positive threshold was 25% for HRS cells [16], while > 10% for ICs [40, 41].

Grading: Scoring cells of PD1+, CD4+, FOXP3+, CD8 + and CD163 + were also performed independently by two pathologists based on visual estimation in reference to the previous study[34, 42, 43], the relative percentage of IHC positively stained cells of tumor cell regions in relation to overall cellularity were calculated as an average of both duplicate cores and graded as: <5% (score 1+), 5–25%(score 2+), > 25% (score 3+). PD-1 was considered as positive if the percentage of PD-1 + cells to total cellularity was ≥ 10% [44, 45].

In situ hybridization for EBV-encoded-RNA

EBV status was determined by in situ hybridization for EBV encoded RNA 1 and 2 (EBER1/2s) using peroxidase labeled probes (Pan Path, Budel, Netherlands). Tissue of a known EBV-positive CHL as positive control. Results were also independently dual-assessed. The positive cut off value for HRS cell was > 10%.

In situ mRNA hybridization for PD-L1 and scoring guidelines

In situ detection of PD-L1 transcripts in the CHL TMA sample was performed using the RNAscope Detection Kit (Cat. No. 310035, ACD, USA), with custom-designed horseradish peroxidase (HRP) labeled probes (from ACD, USA). Briefly, 5μm TMA sections were deparaffinized, boiled with preamplification reagent for 15 minutes, and submitted to protease digestion followed by hybridization for 2 hours with target probes against PD-L1 mRNA, Detection Kit (DAB substrate and solutions) were subsequently pipetted onto the tissue sections to detect hybridization signals enabling RNA molecules to be visualized as brown chromogenic dots, and was finally counterstained with hematoxylin. DapB and PPIB Probe were used as Negative and Positive Control Probe.

Grading: A manual semi-quantitative scoring guideline for PD-L1 mRNA was established according to the estimated number of punctuate dots present within boundary of each HRS cell at 40× magnification: the scores were defined as 0 (less than 1 dot/cell), 1+ (1–4 dots/cell), 2+ (5–10 dots/cell without dot clusters), or 3+ (> 10 dots/cell or with dot clusters)[28]. All cases were double-blind interpreted. Positive expression of PD-L1 mRNA on the background ICs could also be detected as internal positive control, but was difficult to estimate the percentage of occupied aera by positive ICs for the “dot-like” staining pattern.

Statistical analysis and Follow ups

Descriptive statistics were used to summarize co-variates. Categorical co- variates were reported as percentages and counts. Continuous variables were reported as medians and ranges. Pearson chi-square test was used to analyze categorical co-variates. Student’s t (normal distribution) or Mann–Whitney U (non-normal distribution) tests were used to analyze continuous co-variates.

The comparisons between PD-L1 expressions in protein with 3 different IHC assays and RNAscope, and their associations with other characteristics (including PD-1, EBERs, and density scores of TIL/TAM makers) were analyzed using Chi-square test (or Fisher’s exact test when necessary), and the correlation relationships were determined using Pearson’s correlation coefficient (r). The Kappa-Index was determined in all patients [K-Index = (K-FLC × sALB) / (sFLC-K × CSF-ALB)], aiming at evaluating the precision of pathology diagnostic method. Concordances between mRNA levels and antibodies were assessed by Cohen’s Kappa that was calculated as an index of inter-rater agreement. We used the following scale: < 0.50: low concordance; 0.50–0.75: moderate concordance; 0.75–0.90: high concordance; and > 0.9: nearly perfect concordance[28].

The follow-up of living patients (with or without events) was censored at their last follow-up date. Overall Survival (OS) was defined as the interval between the date of diagnosis to death for any cause. Event-free survival (EFS) was defined as the interval between the date of diagnosis and the date of disease progression, relapse, or death from any cause. Median follow-up time was estimated on overall survival by reverse Kaplan-Meier method. Kaplan-Meier estimator was used to estimate survival probability. Survival difference between groups was tested by log-rank test for statistical significance. Confidence interval of survival rate was calculated by Greenwood's formula. Cox hazard regression was performed by analysis of univariable and multivariable risk factors. All statistical tests were two-sided with an alpha level of 0.05 as the significance cutoff. All analyses were performed in statistical software R 4.1.3 (NYC, co.).

Results

Clinical and pathological features

All 54 CHL cases primarily presented with nodal involvement. The mean onset age was 44.3 years (range: 22.0 ~ 68.0 yrs, median age: 45.5 yrs), with a male: female ratio of 1.7: 1(34/20). Of the 45 patients with hospitalized data available, 40.0% (18/45) were with B symptoms, and 53.3% (24/45) were staged as

III IV. All 45 cases were administered with ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) as their first-line treatment, combined with other therapy when necessary: 7 cases were combined with radiotherapy, 3 cases underwent sequential autologous stem cell transplantation (ASCT), 1 relapsed case was applied with chimeric antigen receptor T cell (CAR-T) immunotherapy. All CHL cases were classified according to the WHO 2017 classification of lymphoma: 59.3% (32/54) cases were classified as nodular sclerosis type (NS), 38.9% (21/54) as mixed cellularity type (MC) and 1 (1.9%) as lymphocyte-rich type (LR). The total positive rate of EBER1/2s was 25.9% (14/54): 9.4% (3/32) in NS, 51.4% (11/21) in MC, and 0% (0/1) in LR, with significant difference ($P = 0.002$) for EBV status among different types.

Evaluation of the PD-L1 expression on HRS cells or ICs by immunostaining with different antibody clones

IF only focused on the HRS cells, PD-L1 positive rates for the assays were 48.1% (26/54) for 9A11,59.3% (32/54) for SP142, and 63.0% (34/54) for 22C3. All three antibodies could delineate the cell membrane of the positive cells but still with difference (Fig. 1A, 1B ,1C) [46]. For the background ICs, and there were still discordances: the percentage of PD-L1 expression ranged from 2.5% 90.0% (mean 42.3%) for 9A11, 1.0% 90.0% (mean 27.5%) for SP142, 1.5% 90.0% (mean 32.5%) for 22C3 (Fig. 1D,1E,1F). Moreover, the positive rate of PD-L1 expression on ICs (> 10% as cutoff value) was 85.2% (46/54) for 9A11, 68.5% (37/54) for SP142 and 74.1% (40/54) for 22C3 (Fig. 2).

Expression of PD-L1 mRNA on HRS cells and its correlation with PD-L1 immunostaining

PD-L1 mRNA expression on HRS cells was detected successfully in 46 cases, 45.7% (21/46) cases expressed PD-L1 mRNA at higher level(2+ 3+, Fig. 3A, 16 cases were 3 + and 5 cases were 2+). The other 54.3% (25/46) cases expressed at a lower level (0 1+, Fig. 3B, 23 cases were 1 + and 2 cases 0). By IHC of above 46 cases defined by 9A11, SP142 and 22C3, 21 cases, 26 cases and 29 cases were positive on HRS cells, 95.23% (20/21), 76.92% (20/26) and 72.41%(21/29) among the positive cases expressed PD-L1 mRNA at higher level(2+ 3+); for the rest PD-L1 protein negative cases subsequently, 96.0% (24/25), 95.0%(19/20), and 100%(17/17) cases expressed mRNA at lower level(0 1+).

9A11 showed the best linear correlation with mRNA expression in detecting PD-L1 expression on HRS cells, with a Kappa-Index of 0.91 for 9A11 (nearly perfect concordance, see in Fig. 3A vs. Figure 3C and Fig. 3B vs. Figure 3D), with 0.72 for SP142, and 0.70 for 22C3 (both belong to moderate concordance), shown in Fig. 4.

Scoring the tumoral Immunol cell sub-population associated biomarkers in CHL including PD-1

When we scored the different groups of background immune cells by IHC, for CD4 + Ths: 3 cases were scored 1+, 33 cases scored as 2 + and 18 cases scored as 3+; while for FOXP3 + Tregs: 9 cases were scored as 1+, 42 cases as 2 + and 3 cases as 3+; for CD8 + CTLs :1 case was scored as 1+, 34 cases were scored as 2 + and 19 cases were scored as 3+. CD163 + TAMs were 12 cases were-scored as 1+, 34 cases

were score as 2 + and 8 cases were scored as 3+, see in FigureS1A-1D&Table 1. PD-1 + cells ranged from 0.0–90.0% (mean7.41%), and 25.9%(14/54) were considered as “Positive” (cut off value ≥ 10%), shown in FigureS1E.

Correlation between PD-L1 expression and densities of different sub-populations of IC

If focused on PD-L1 expression on HRS cells: the PD-L1 positivity showed poor concordance with scoring grades of IC sub-populations by CD163, CD4, CD8, and FOXP3 (Figure S2). But the PD-1 positivity rate decreased significantly in these HRS-PD-L1 positive cases stained with 9A11 and SP142 (P = 0.020 and 0.011) than those HRS-PD-L1 negative cases, that is 11.5% (3/26) vs. 39.3% (11/28), and 12.5% (4/32) vs. 45.5% (10/22) subsequently; but no such difference was found with 22C3 assay (P = 0.337). When for the ICs of PD-L1 expression, it was proved that CD163 + TAM densities (%) correlated best (moderate concordance) among all subtypes of ICs, especially when stained with 9A11 (Kappa index is 0.69), details shown in Fig. 5.

Overall PD-L1 expression status stained with 9A11 and the association with other clinicopathological features

Overall, for immunostaining of PD-L1 with 9A11, 87.0% (47/54) were found with high level of PD-L1 expression: 48.1% (26/54) were evaluated as positive on HRS cells, and 85.2% (46/54) were considered as positive for ICs. The expression of PD-L1, either on HRS cells or ICs, showed poor concordance with other clinical factors (including gender, age, morphological subtypes, EBERs status, Stages). Further analysis can be seen in Figure S3A &S3B.

Survival Analysis for PD-L1 expression and subgroup of ICs

The last follow-up date for CHL were in April 1st, 2022, and the Overall Survival (OS) time ranged from 7.0 to 94.5 months. The 5-year expected OS rate was 87% and 5-year expected Event Free Survival (EFS) rate was 59%. Univariable survival analysis were done for the impacts of CD4+, FOXP3+, CD8+, CD163 + TAMs densities with the clinical outcomes, only CD163 higher density(grouped by < 5%,5%-25%,>25%) indicated worse outcome for OS (P = 0.026, Fig. 6A). Patients with higher level of PD-L1 expression of ICs(cut off value > 25%) will also have obvious adverse effect on OS, although without statistically significance (P = 0.067, Fig. 6B). However, no significant difference of PFS were identified (Figure S4A and S4B). Survival analysis details can be seen in **Table S1.**

Discussion

We chose 3 representative clone of PD-L1 antibody for IHC assay comparison, 405.9A11 (9A11) was first used by Ansell et al[8] in their outstanding study in CHL, 22C3 was considered as a sensitive assay[38] and was the first FDA approved and most widely used Ab recently[18] ,and SP142 were the 1st clinically validate assay for both TC and IC[37, 39]. The RNAscope assay, which was an antibody independent

assay on FFPE sections, was recently invented to detect PD-L1 expression at mRNA level and applied recently in breast, lung and gastric tumors [24–26]. Compared to IHC, its specificity, reproducibility and interpretative objectivity has been reported in gastric cancer [25, 26]. Because both HRS cells and reactive IC cells in CHL can express PD-L1 mainly in a membrane pattern, sometimes the PD-L1 positive IC cells are so crowded around the HRS cells, it might be difficult to tell whether the HRS cells expressed PD-L1 or not (as showed in Fig. 3A). In this situation, the PD-L1 mRNA detected by RNAscope as a “dot-like” pattern distributed in the cytoplasm of HRS cells is much easier identified and better evaluated (Fig. 3C). RNAscope assay was successfully applied in our CHL cases and a provisional scoring system was also developed.

After compared with RNAscope assay, for PD-L1 expression on HRS cells, we proved that 9A11 was the most accurate IHC assay, showing nearly perfect correlation with RNAscope assay. 9A11 is a PD-L1 Ab clone binding cytoplasmic domain and thus was more selective for membranous PD-L1, with stronger, more membrane and less cytoplasmic staining [46], made it easier for 9A11 to distinguish the membranous staining of the HRS cells with surrounding ICs for higher intensity and specificity(shown in Fig. 3A).

For PD-L1 expression on HRS cells, nearly half of the cases expressed high level protein or mRNA (48.1% detected by IHC with clone 9A11 and 45.7% detected by RNAscope), and was nearly the same to the results published recently from Veldman et al.[16], although much lower than R/R cases showing 100% positive from Ansell [8]. But for the study from Roemer et al[12], alterations of the 9p24.1 gene encoded PD-L1 in HRS cell of CHL included copy gain (56%) and amplification (36%), patients with amplification was found with significant increased PD-L1 expression and shorter PFS[12]. However, only 4 out of 10 cases were found with gene amplification from the R/R CHL series of Ansell[8]. Discordance in PD-L1 positive rate might be caused by different evaluation criteria among researchers.

While for PD-L1 expression on ICs, in the study from Veldman et al,69% CHL cases was positive, with Ab clone E1L3N [16]. In our study, we found the PD-L1 positive rates on ICs were 85.2% (9A11), 68.5% (SP142) and 74.1% (22C3) respectively, and were most likely to be expressed by TAMs proved by statistical analysis. The majority of CHL cases express high level of PD-L1 on ICs, however, it should take note that different antibody clone would affect the positive rate. 9A11 was most sensitive, SP142 assay was lowest in sensitivity, as reported previously in lung cancer by Tsao et al [38].

In our study, PD-L1 expression, either on tumoral HRS cells or on ICs, showed poor concordance with clinicopathological factors including gender, age morphological subtypes, and even with EBER infection status. Although it was reported by Green et al [1] that EBV-positive CHL would up-regulate PD-L1 expression [1, 40]. But Paydas et al. did not find this association [47] ,which was supported by our study. In another word, high level of PD-L1 expression was so prevalent in CHL, that it was impossible caused solely by EBV.

There were plenty of T-cells and TAMs s subclassified by IHC makers in the microenvironment of our series of CHLs. However, PD-L1 expression status on HRS cells (by 9A11 assay) correlated poorly with the

density score of Ths (CD4+), Tregs (FOXP3+), CTLs (CD8+) and TAMs (CD163+). Increase level of PD-L1 expression on ICs correlated best with higher density of CD163 + TAMs, same finding was seen in a study from lung cancer [33], the percentage of PD-L1-positive ICs was significantly higher in TAM-high group than in TAM-low group. In addition, PD-L1 expression on TAM may be derived from HRS cells [27]. There were controversial results about PD-1 + cell densities [9], from small [48] to huge amount [49]. In our study, only about 1/4 cases presented with “high” Level of PD-1 positive cells (cut off value ≧ 10%), in those cases with “high-level(Positive)” of PD-L1 on HRS cells, the PD-1 positivity rate decreased significantly, indicating a reverse correlation to stop over activation of PD-1 pathway and keep the immunosuppressive balance in CHL. Although there were various amount of FOXP3 + Tregs as reported previously [50, 51], different from other tumors[31, 32, 52], they were not associated with PD-L1 expression level (either on HRS cells or ICs) in our series.

Patients with higher density (> 25%) of TMA was adverse predictor of clinical outcome by univariate survival analysis, which was in concordance with previous report [34]. In concordance of the closest relationship between CD163 positive TAM density, patients with higher level of PD-L1 on the ICs was associated with apparently worse OS. In fact, the CD163 positive TAM represent M2 macrophage[34], increased number of tumor-associated macrophages was strongly associated with shortened survival[42] and was also found in various tumors including diffuse large B-cell lymphoma[32, 45, 53].

Conclusions

405.9A11 assay was most convincible to evaluate the expression of PD-L1 on HRS cells, as proved independently by RNAscope assay, and was also most sensitive to detect PD-L1 expression on ICs. High level of PD-L1 expression was prevalent in CHL, and was more frequent on ICs than on HRS cells. Increased expression of PD-L1 on ICs was most associated with elevated densities of CD163 positive tumor associated macrophages (TAMs). In concordance with TAMs, may have negative effect on survival of CHLs. Thus, pathologists should report PD-L1 expression in a combined manner, including both positive state of HRS cells and positive percentage of ICs.

Abbreviations

CHL, Classic Hodgkin’s Lymphoma

HRS cells, Hodgkin and Reed–Sternberg cells

R/R, relapsed or refractory

PD-1, programmed death-1

PD-L1, programmed death-ligand 1

FFPE, formalin-fixed paraffin embedded

ICs, immune cells

CTLs, cytotoxic T cells

Tregs, regulatory T cells

TAMs, tumor associated macrophages

TMA, tissue microarrays

EBER1/2s, EBV encoded RNA 1 and 2

HRP, horseradish peroxidase

OS, Overall Survival

EFS, Event-free survival

NS, nodular sclerosis type

MC, mixed cellularity type

LR, lymphocyte-rich type

Declarations

Ethics approval and consent to participate

All procedures above were approved by the Ethical Committee of our institution, and written consensus was obtained from each individual in this study.

Consent for publication

All authors have read and approved the final manuscript

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Yunfei Shi, and Xianghong Li designed the study, Yunfei Shi was a major contributor in writing the manuscript, Lan Mi analyzed and interpreted the clinical and pathological data. Yunfei Shi and Yumei Lai

reviewed all pathological slides and newly stained TMA slides, Min Zhao constructed the TMA, Ling Jia performed all tests on the TMA slides including immunohistochemistry staining, EBER and RNA scope tests. Tingting Du finished the follow-ups of the patients. Yuqin Song and Xianghong Li revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Table

Table 1 is available in the Supplementary Files section

Figures

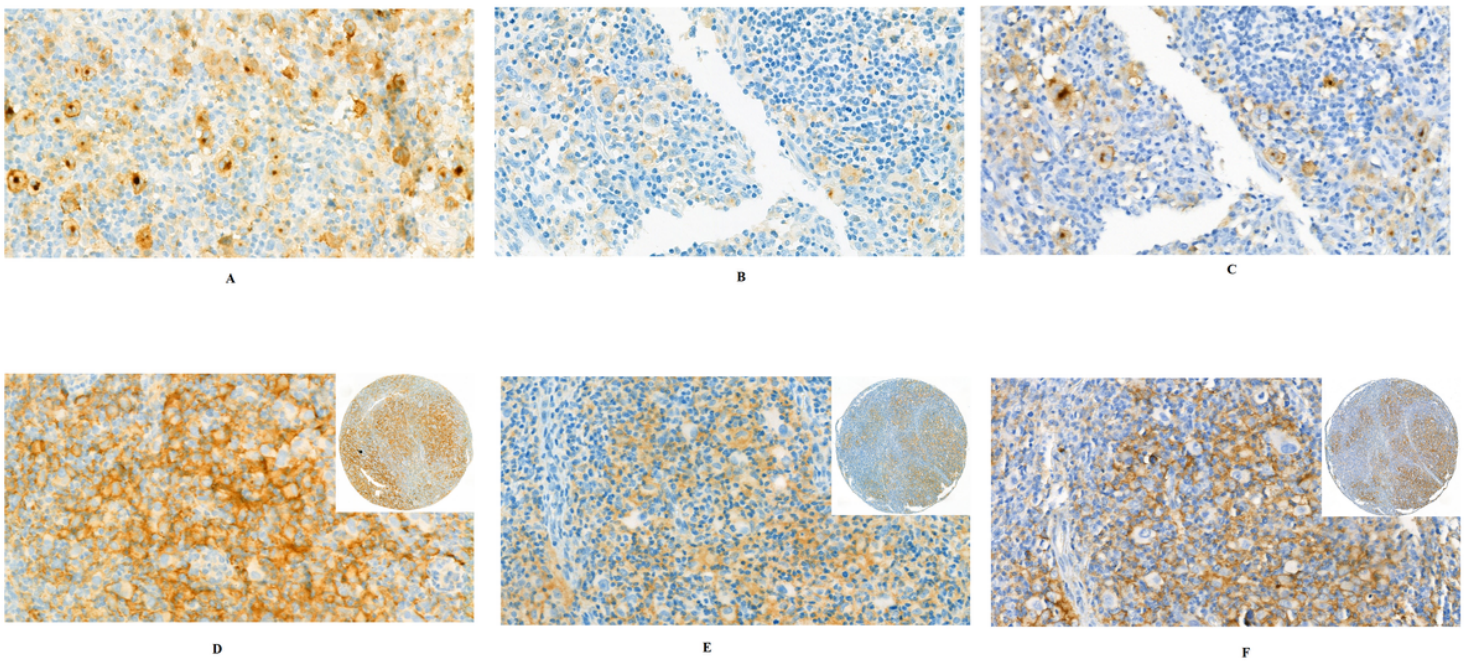

Figure 1

Positive expression of PD-L1 on the cell membrane of HRS cells detected by immunostaining with different clones of antibodies 9A11(1A), SP142(1B) and 22C3(1C) , from high power field of the same CHL case under microscope, there were difference in decorating the positive cell membrane and more background IC cells for positive for PD-L1 in stained by 9A11. Figure1D-1F showed prominent positive expression of PD-L1 on the background immune cells(ICs) from the same CHL case of HPF with 9A11(1D), SP142(1E) and 22C3(1F), in this case the HRS cells were negative for PD-L1 and there were obviously more PD-L1 positive background IC cells stained with 9A11 than other assays from low power field of the whole tissue core (on the upper right).

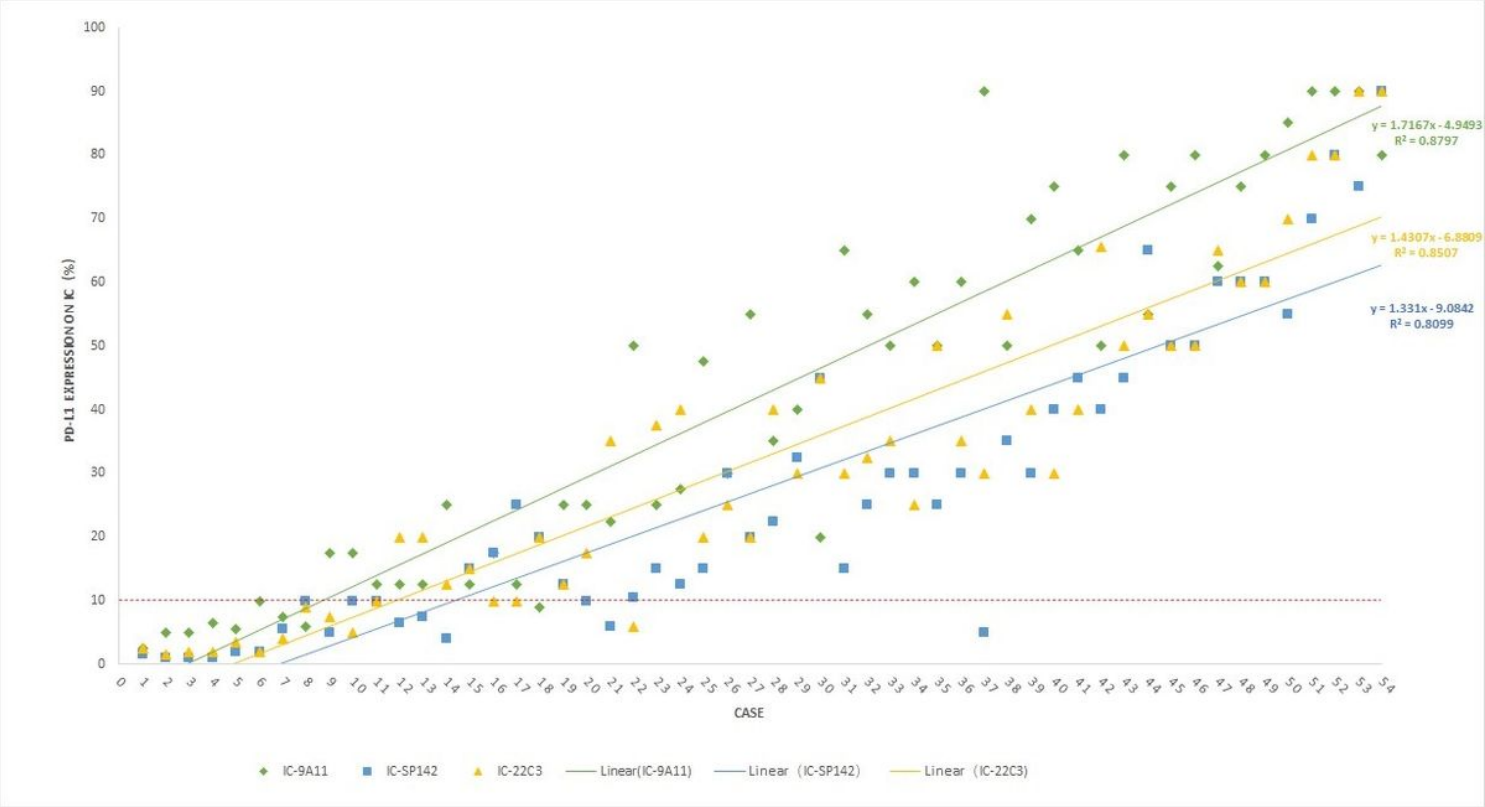

Figure 2

Comparison for of PD-L1 level expression (%) on background immune cells (ICs) in CHL, by IHC with different assays, all 54 cases were ranked and coordinate according to the mean value of the percentage of PD-L1 positive IC cells of each case, scoring IC by 9A11 show the best sensitivity and linear correlation.

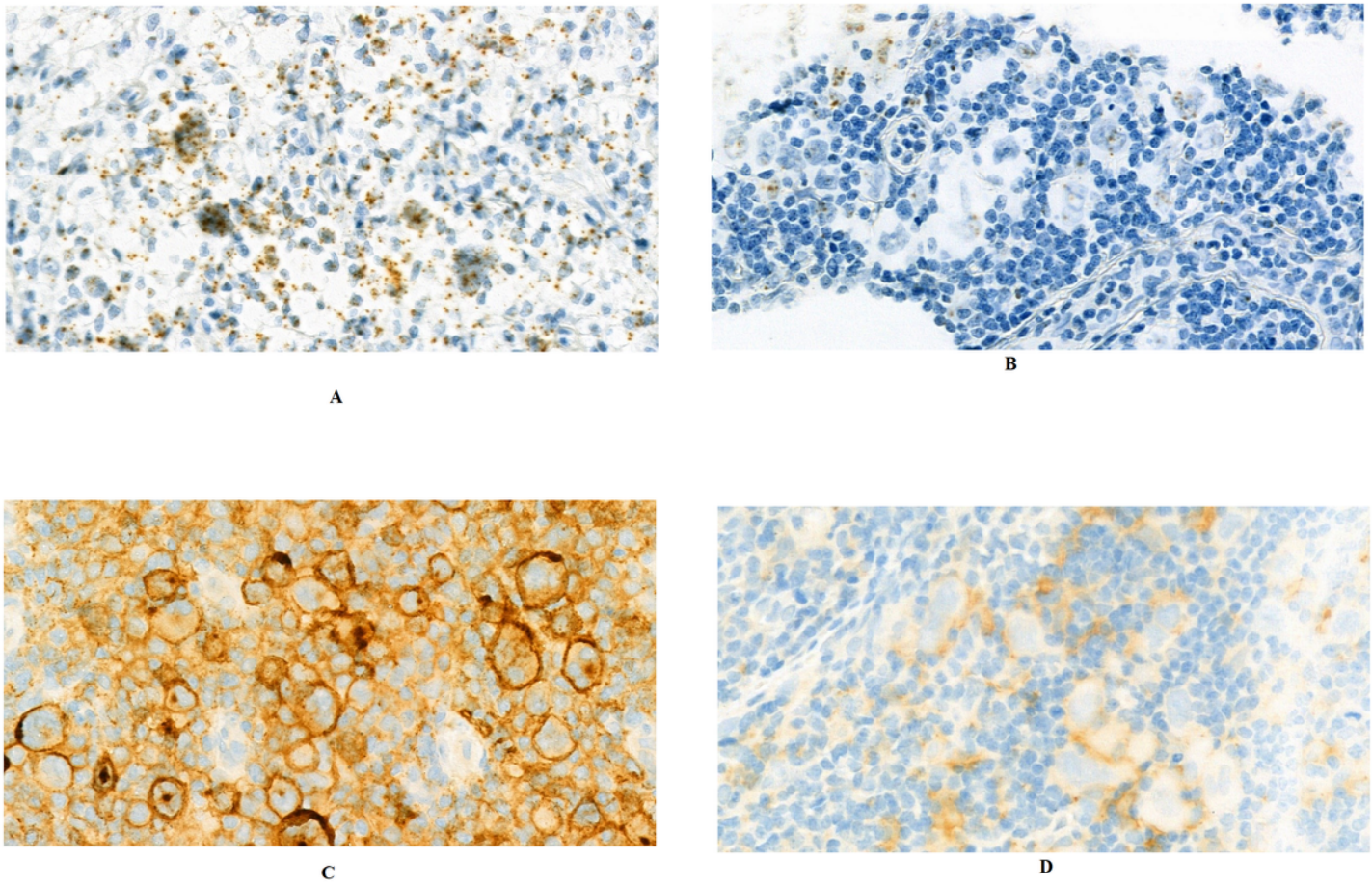

Figure 3

PD-L1 mRNA expression detected by RNAscope in HRS cells: a case with high level expression (scored 3+ 3A) and another case with low level of expression (scored 1+ 3B) were shown. The PDL1 immunostaining by 9A11assay showed the best concordance with mRNA expression: Figure 3C showed PD-L1 protein positive of the same case of Figure 3A, and Figure3D was PD-L1 protein negative and was from the same case of Figure 3B.

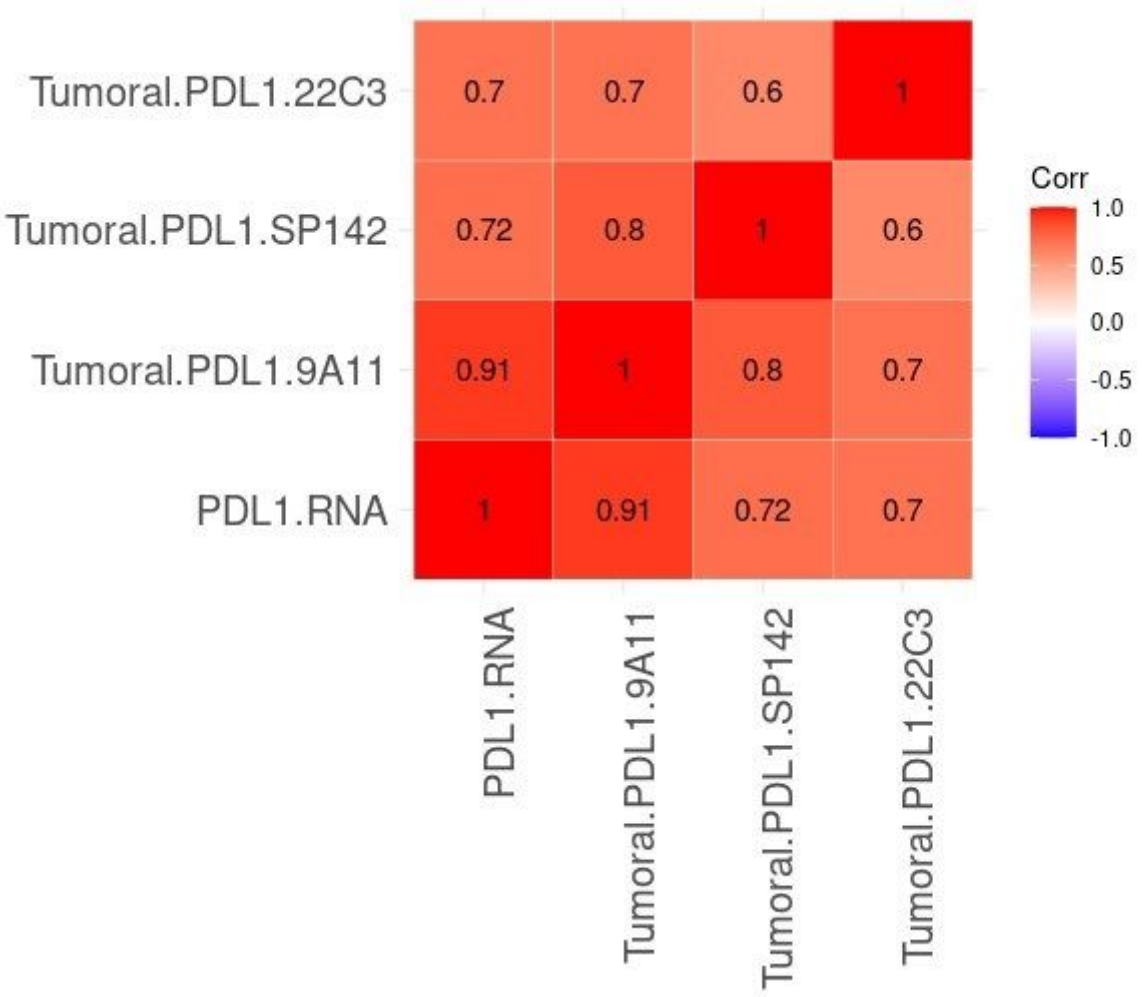

Figure 4

Comparison the concordance of PD-L1 expression on HRS cells (tumoral PD-L1) between protein level detected by with 3 different clones of Antibodies and mRNA level detected by RNAscope as reference platform. Assessed by Cohen's Kappa that was calculated as an index of inter-rater agreement.

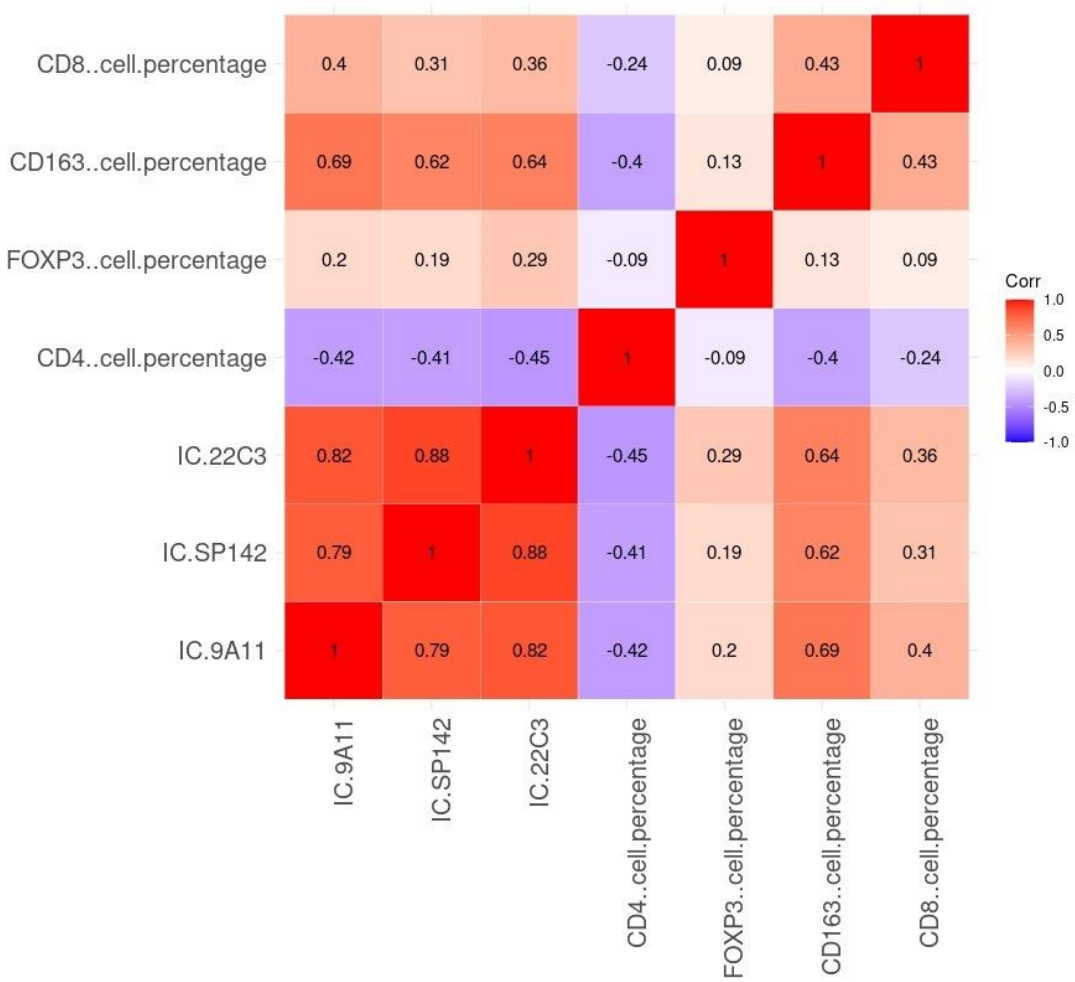

Figure 5

Comparison between the concordance of PD-L1 expression percentage on background immune cells (ICs) with different assays and the densities of different subgroups of immune cells, relationships were determined using Pearson's correlation coefficient (r).

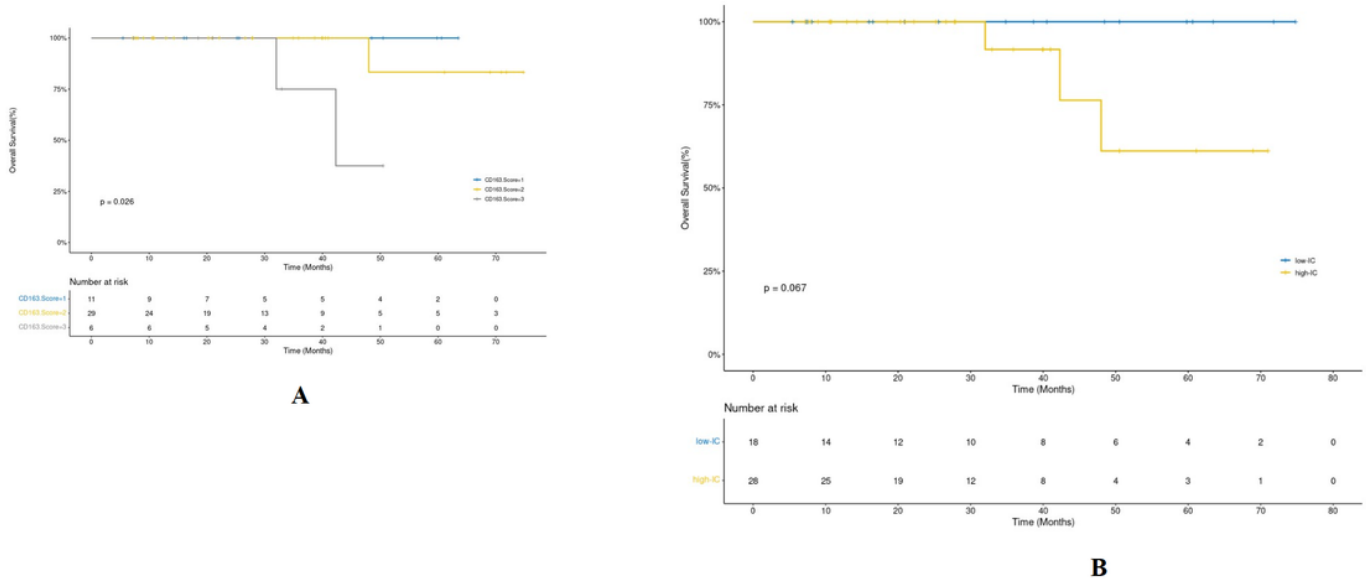

Figure 6

Overall survival Analysis for the impact of CD163+ tumor associated macrophages (Figure 6A, grouped as 1+ <5%, 2+:5-25% and 3+: >25%) and PD-L1 expression level on immune cells (ICs) with 9A11 assay (Figure 6B, cut off value as >25% named “high-IC”, or else “low-IC”), univariable survival analysis, Kaplan-Meier method.

Supplementary Files

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