Unraveling the tumor microenvironment with spatial proteomics: *in situ* detection of immune checkpoint interactions in cancer patient tissues

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Background

Immune checkpoints (ICs), such as the PD-1/PD-L1 axis, are responsible for down-regulation of the immune responses of T cells and play a crucial role in maintaining immune self-tolerance in peripheral tissues. IC pathways can be hijacked by cancer cells to evade immune surveillance, thus making ICs excellent immunotherapy targets. However, the success of immune checkpoint inhibitors (ICIs) has been hampered by poorly defined patient groups, relying in part on the use of PDL1 in IHC as a biomarker. Expression of PDL1 alone has proved not to be informative enough when it comes to stratifying patients. In the search for better biomarkers, and better tools to study underlying mechanisms of the IC axis, there is a need to look at the bigger picture – including interactions in the axis and co-staining.

The activation of PD1 and subsequent inhibiting signaling pathways requires PD1 to be both bound to PDL1 as well as phosphorylated. The phosphorylation of PD1 is dependent on the T-cell receptor (TCR) binding to MHC. In the last step, SHP-2 binds to the phosphorylated PD1 via its two SH2 domains, becoming active and dephosphorylates its downstream targets (figure 1, model of signal transduction pathway). Using Navinci's highly sensitive and specific proximity ligation technology technology, it is possible to visualize not only the interaction between PD1-PDL1 but also pPD1, and PD1/SHP2. Moreover, by combining the power of the PD-1/PD-L1 interaction with the concomitant visualization of relevant markers (such as CD20, CD3d, CD8a and cytokeratin), it is possible to create an informative immune profile, improving the likelihood of successful immunotherapy treatment. Here, the tumor microenvironment (TME) in Hodgkin's lymphoma, head and neck squamous cell carcinoma (HNSCC), and non-small-cell lung cancer (NSCLC) are visualized.

Fig. 1.

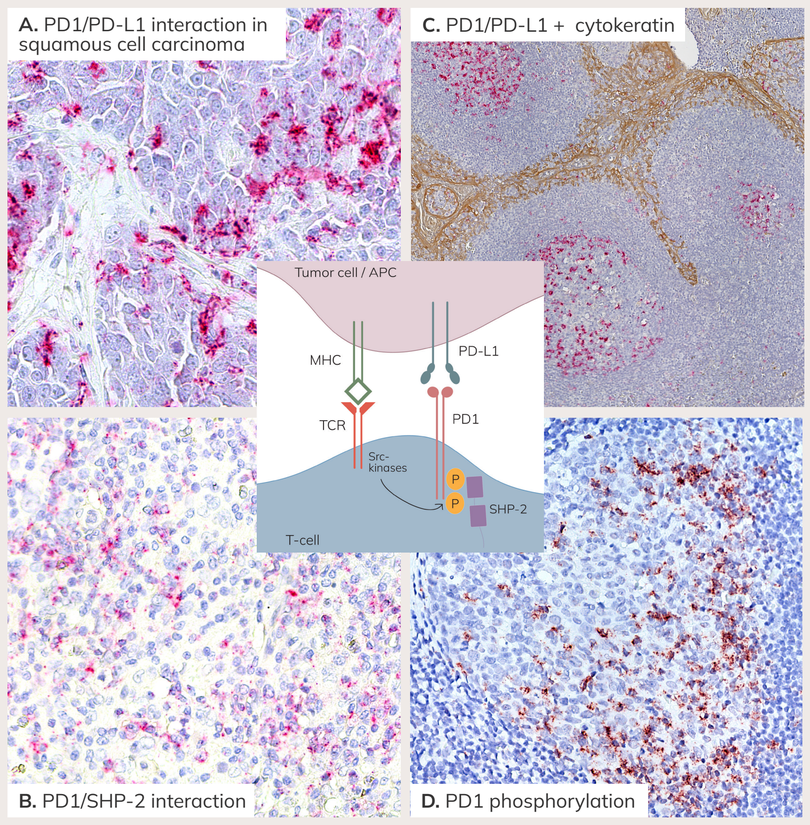
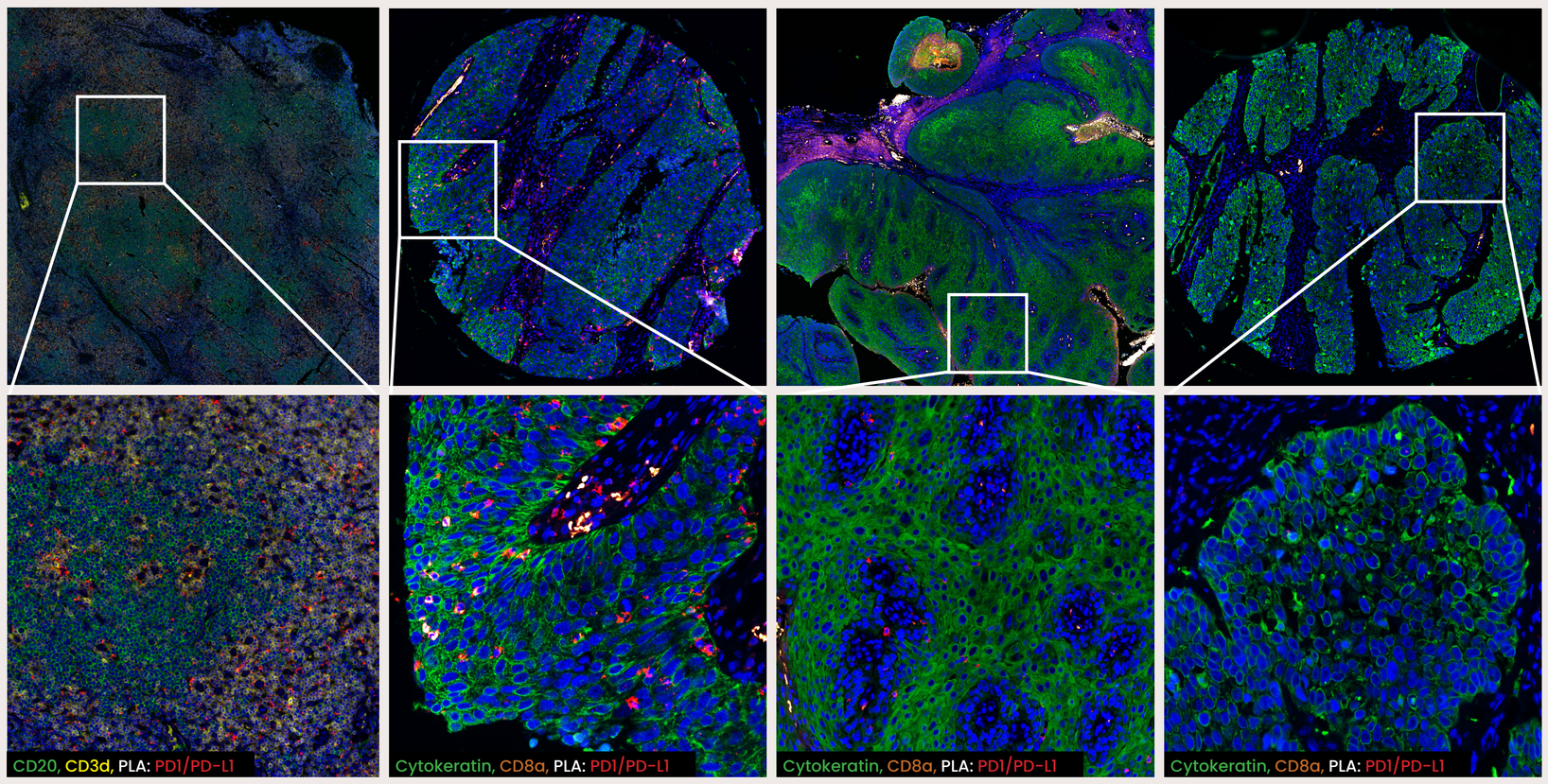


Fig. 2. **A:** Hodgkin's lymphoma **B:** NSCLC **C:** HNSCC **D:** NSCLC



Results

Fig. 1: **1A.** PD1/PD-L1 interaction using Naveni® PD1/PD-L1. Directly visualizes the interaction between PD-1 and PD-L1 in squamous cell carcinoma. **1B.** PD1/SHP-2 interaction, using the NaveniBright™ AP kit with primary antibodies against PD1 and SHP-2 in a lymph node (diffuse T cell lymphoma of right neck). PD1/SHP2 is a biological highly relevant interaction and indicative of PD1/PD-L1 interaction as well as pPD1. **1C.** PD1/PD-L1 interaction + cytokeratin using Naveni® PD1/PD-L1 AP and a cytokeratin-HRP co-stain in tonsil. **1D.** PD1 phosphorylation using Naveni® pY PD1 HRP in tonsil resulting in a specific staining compared to IHC using a phospho-PD1 antibody (result not shown).

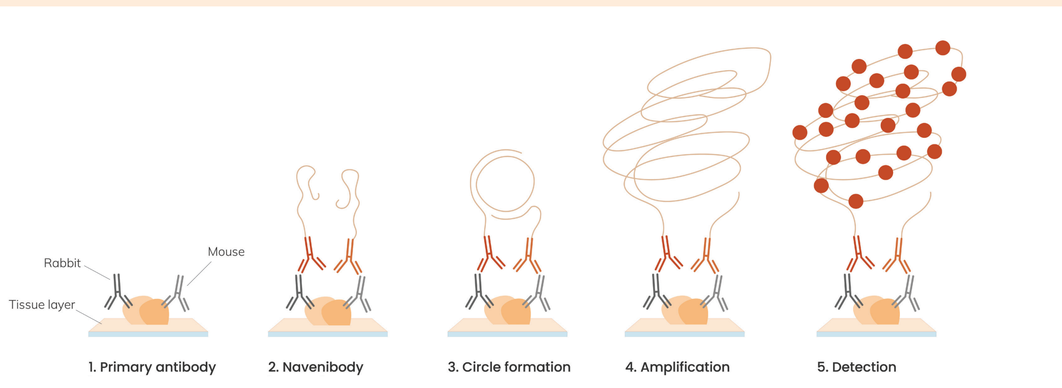
Fig. 2: Four different samples were assessed with Naveni® PD1/PD-L1 Atto647N and co-stained for two other markers. **2A.** Hodgkin's Lymphoma co-stained for CD20 and CD3d revealing a complex TME. The staining pattern of the PD-1/PD-L1 interaction is indicative of cancer cell clusters and the co-localization of CD3d positive T cells around these sites is visible.; **2B** and **2D.** NSCLC co-stained for CD8a and cytokeratin. Sample B has higher level of PD1/PD-L1 interaction when compared to Sample D. Crucially, infiltration of cytotoxic T cells (CD8a positive) in the tumour region (cytokeratin positive) could be observed, and the interaction was concentrated around the intersection of the two cell types.

2C: HNSCC co-stained for CD8a and cytokeratin. PD-L1 expression was consistent across the whole tissue section (assessed with IF, results not shown), the incidences of interaction were much lower. Few interactions were detected in cytokeratin positive cells and no CD8a-postive cells were observed.

Technology and Methods

Optimized *in situ* proximity ligation methods based on the Naveni® Technology were developed for the detection of immune checkpoint interactions. To visualize the target interaction, FFPE tissues were incubated with monoclonal antibodies specific to target, followed by incubation with Navenibodies (affinity reagents conjugated to proprietary oligo arms). A strong and distinct signal is generated only if the Navenibodies are in close enough proximity to generate a rolling circle amplification reaction.

For detection of PD1/SHP2 and phosphorylated PD1 NaveniBright™ kits (brightfield readout) were used. Naveni® PD-1/PD-L1 AP was used for analysis of PD1/PD-L1 interactions in brightfield. For co-staining an HRP-labeled antibody targeting cytokeratin was added. Naveni® PD1/PD-L1 Atto647N kit was used for fluorescent detection of PD1/PDL1 and combined with fluorescently labelled antibodies against relevant biomarkers such as pan-cytokeratin, CD8a, CD20, and CD3d, added during the detection step.



Conclusions

Our study demonstrates the power of Naveni® technology in unraveling the intricacies of immune checkpoint pathways, particularly the PD-1/PD-L1 axis. Relying solely on PD-L1 expression as a biomarker is insufficient for patient stratification, highlighting the need for a more comprehensive approach that considers various aspects of the immune response within the tumor microenvironment. By visualizing PD-1/PD-L1 interactions, phosphorylation of PD-1, and co-staining with relevant biomarkers, we have gained deeper insights into the immune landscape in Hodgkin's lymphoma, HNSCC, and NSCLC. This approach not only enhances our understanding of the underlying mechanisms but also provides a promising tool for improving patient selection and treatment outcomes in immunotherapy.

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