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Multiplexed proximity ligation-based method uncovers immune checkpoint activation in the context of the tumor microenvironment

Navinci

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Background

Immune checkpoints (ICs), such as the PD-1/PD-L1 axis, are inhibitory signaling pathways that down-regulate the immune responses of T cells and play a crucial role in maintaining immune self-tolerance in peripheral tissues. IC pathways can be manipulated by cancer cells to evade immune surveillance, thus making them excellent immunotherapy targets. However, success of these therapies has been hampered by poorly defined patient groups. By combining the power of the PD-1/PD-L1 interaction with the concomitant visualization of relevant biomarkers (such as CD20, CD3d, CD8a and cytokeratin), it is possible to create an informative immune profile, improving the likelihood of successful immunotherapy treatment. Using Navinci's highly sensitive and specific proximity ligation technology combined with additional biomarker staining, it was possible to uncover tumour microenvironments (TME) in Hodgkin's lymphoma, head and neck squamous cell carcinoma (HNSCC) and non-small-cell lung cancer (NSCLC). A comparison of PD1/PD-L1 interaction with PD-L1 staining in immunofluorescence also showed that the presence of PD-L1 does not equate to the presence of interaction.

Technology and methods

An optimized in-situ proximity ligation method for the detection of PD-1/PD-L1 protein-protein interaction was developed for the Naveni PD1/PD-L1 Atto647N kit (fluorescent readout). To visualize the target interaction, FFPE tissues of Hodgkin's lymphoma, head and neck carcinoma, and NSCLC (Discovery Life Sciences, TissueArray) were incubated with monoclonal antibodies specific to PD-1 and PD-L1, 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. To observe relevant biomarkers such as pan-cytokeratin, CD8a, CD20, and CD3d, fluorescently labelled antibodies were added during the detection step, and the signal was assessed by epifluorescence microscopy.

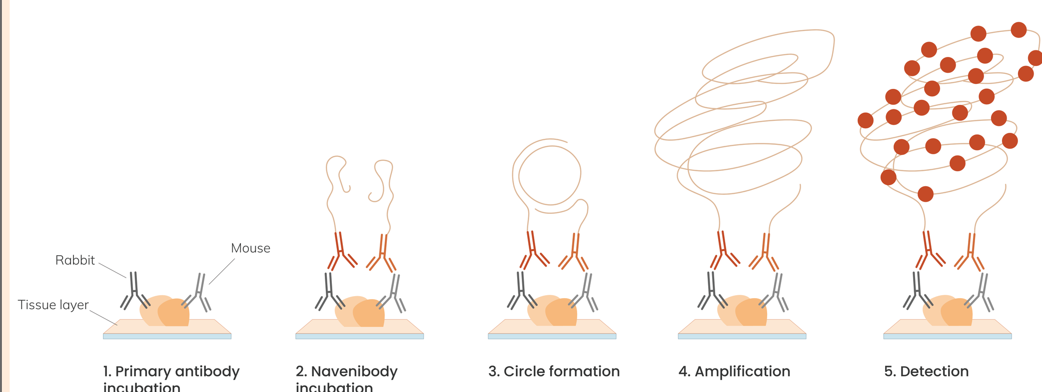

Figure 1: Schematic illustration of the Naveni™ technology workflow.

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A: Hodgkin's lymphoma | B: NSCLC | C: HNSCC | D: NSCLC

Row 1–5

Row 2: CD20, CD3d, PLA: PD1/PD-L1 | Cytokeratin, CD8a, PLA: PD1/PD-L1 | Cytokeratin, CD8a, PLA: PD1/PD-L1 | Cytokeratin, CD8a, PLA: PD1/PD-L1

Row 3: CD3d | CD8a | CD8a | CD8a

Row 4: PLA: PD1/PD-L1 | PLA: PD1/PD-L1 | PLA: PD1/PD-L1 | PLA: PD1/PD-L1

Row 5: IF: PD1/PD-L1 | IF: PD1/PD-L1 | IF: PD1/PD-L1 | IF: PD1/PD-L1

Figure 2: Four different samples (**A.** Hodgkin's Lymphoma; **B. & D.** NSCLC; **C:** HNSCC) were assayed with Naveni PD1/PD-L1 Atto647N and co-stained for two other markers. PD-L1 expression was also evaluated using immunofluorescence **(Row 5).** Nuclei shown in blue in all images.

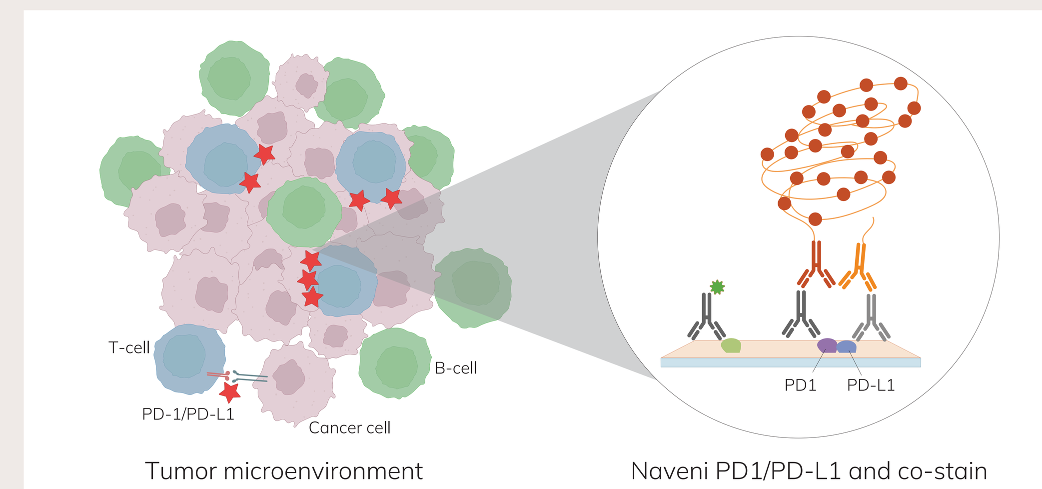

Figure 3: To gain better visualization of the complex TME, Naveni PD1/PD-L1 Atto647N can be multiplexed with fluorophore-conjugated primary antibodies against biomarkers of interest.

Results

Three cancer types – Hodgkin's Lymphoma, Head and Neck Squamous Cell Carcinoma (HNSCC), and Non-Small Cell Lung Cancer (NSCLC)– were assessed using Naveni PD1/PD-L1 Atto647N kit in combination with multiplex immunofluorescence antibodies **(Fig. 3).** Hodgkin's lymphoma is characterized by the presence of Reed-Sternberg cells, which are large, abnormal B cells (CD20). Hodgkin's lymphoma tissue was stained with additional biomarkers CD20 and CD3d **(Fig 2 A1-4)**, 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. Immunofluorescence staining of PD-L1 **(Fig 2 A5)** was consistent with the localization of PD1/PD-L1 interaction.

Anti-PD1/PD-L1 therapy is a first line treatment strategy in certain cases of NSCLC, as such the expression of PD-L1 as a predictive biomarker has been extensively investigated. Two cases of NSCLC were evaluated using Naveni PD1/PD-L1 assay for the presence of interaction and co-stained for cytokeratin and CD8a **(Fig 2 B1-4 and D1-4)**. Immunofluorescence was also performed to assess the expression of PD-L1 **(Fig 2 B5 and D5)**. Sample B has a higher expression of PD-L1 and 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, indicative of possible immune evasion.

Recurrent or metastatic HNSCC could be treated with anti-PD1/PD-L1 immunotherapy; however, response rate is limited. Similar to NSCLC, the expression of PD-L1 is used as a predictive marker for patient stratification. While PD-L1 expression was consistent across the whole tissue section **(Fig C5)**, the incidences of interaction were much lower. Few interactions were detected in cytokeratin-positive cells and no CD8a-postive cells were observed.

Conclusions

Studies have determined that the expression of PD-L1 on Hodgkin's lymphoma cells is associated with a poor prognosis and resistance to therapy. However, the co-expression of PD-L1 and CD3 in the TME may indicate a more favorable prognosis and a better response to immunotherapy. Similarly, the presence of CD8 positive T cells in NSCLC tumors is associated with improved clinical outcomes, likely due to their ability to recognize and kill cancer cells. PD-L1 expression is commonly used to determine treatment strategy for HNSCC, yet response to IC treatments remained low, suggesting that PD-L1 alone is not sufficient as a predictive biomarker. Comparing IF and Naveni PD1/PD-L1 Atto 647N results demonstrates that the expression of PD-L1 may not always indicate the presence of PD1/PD-L1 interaction. Using Navinci technology, it is possible to multiplex proximity ligation technology with co-marker staining for the visualisation of complex TME for improved patient stratification and ultimately appropriate therapy.