Development of a Chromogenic *in situ* Proximity Ligation Assay for Detection of PD1/SHP2 Proximity in Human FFPE Tissues

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Background

PD1/PDL1 is a well-known immune checkpoint that when activated inhibits the T-cells. Cancer cells expressing PDL1 can escape the immune system by binding to PD1 on tumor specific T-cells [1, 2]. Anti PD1 and anti PD-L1 treatments are used as immune checkpoint inhibitors in cancer patients, and IHC of PD-L1 is one biomarker used to identify patients eligible for treatments [3]. Not all patients benefits from the treatment and there is a need for better biomarkers as well as better tools to study the underlying mechanisms of this immune checkpoint axis [1, 4].

The activation of PD1 and subsequent inhibiting signaling pathway 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 MHC. In the last step SHP-2 binds to the phosphorylated PD1 via its two SH2 domains, becoming active and dephosphorylates its targets [5, 6] (figure 1, model of signal transduction pathway).

One way of studying these interactions is by using the proximity ligation assay technique, which allows the detection of protein-protein interactions *in situ.* Proximity ligation assay is an antibody-based technique where the use of pairs of antibodies conjugated to unique oligonucleotides allows the visualization of interacting proteins (figure 2, schematic model of proximity ligation assay).

In this work we have developed two target-specific proximity ligation assays for use in tissues: one for visualizing the phosphorylation of PD1 and one for the interaction of PD1 with SHP-2 (figure 3, proximity ligation assay of phospho PD1 and PD1/SHP2).

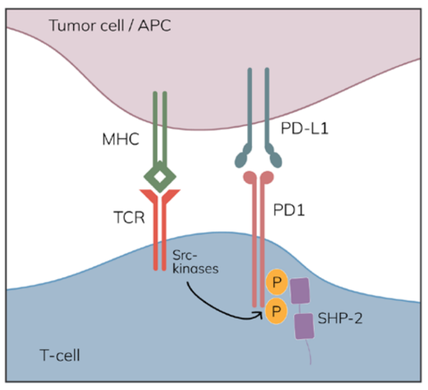

Figure 1. Model of the PD1/PDL1 signaling pathway, resulting in SHP-2 binding to PD1 and thereby becoming active.

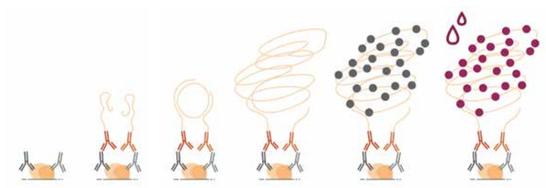

Figure 2. Schematic overview of the Naveni Bright™ proximity ligation assay.

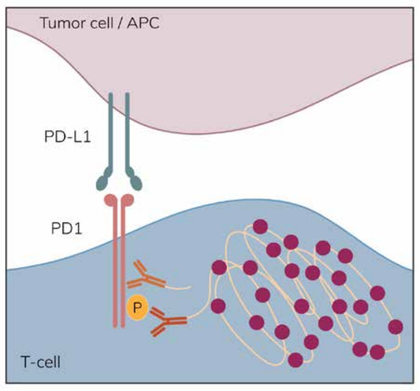

Figure 3. a) Model of proximity ligation assay for phosphorylated PD1.

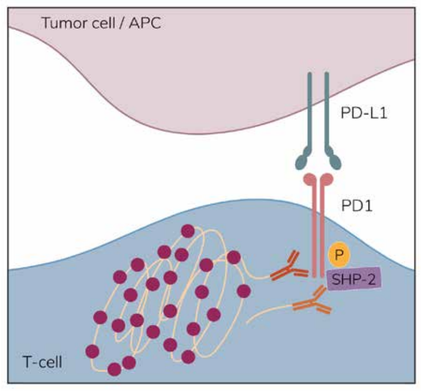

Figure 3. b) Model of proximity ligation assay for SHP-2 interaction with PD1.

Results

The antibodies was first evaluated by IHC in tonsils. For SHP-2 and PD1 there is specific staining in the germinal centers, SHP-2 also resulting in some unspecific background (figure 6 a and b). pTyr results in a general staining (figure 6 c). The phospho specific PD1 antibody resulted in an unspecific staining not correlating with where PD1 staining was seen (figure 6 d).

The SHP2 antibody was paired with the PD1 antibody, and a proximity ligation assay performed on tonsil tissues. Specific staining was seen in some of the germinal centers of the tonsil (figure 4). The pTyr antibody was paired with the PD1 antibody and a proximity ligation assay performed on tonsil tissues. Specific staining was seen in some of the germinal centers of the tonsil (figure 5). The SHP/PD1 interaction and the phosphorylation of PD1 were in general seen in the same germinal centers of the tissue.

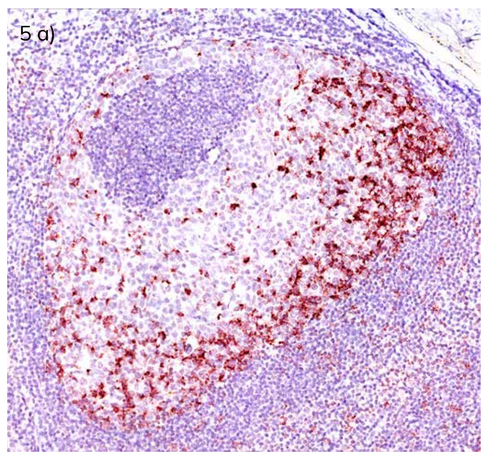

Figure 5: a) Proximity ligation assay in human tonsils visualizing the phosphorylation of PD1. Staining seen primarly on the edge of the germinal centers. b) technical control of pTyr, using only the pTyr antibody in the proximity ligationassay. c) technical control of PD1, using only the PD1 antibody in the proximity ligation assay.

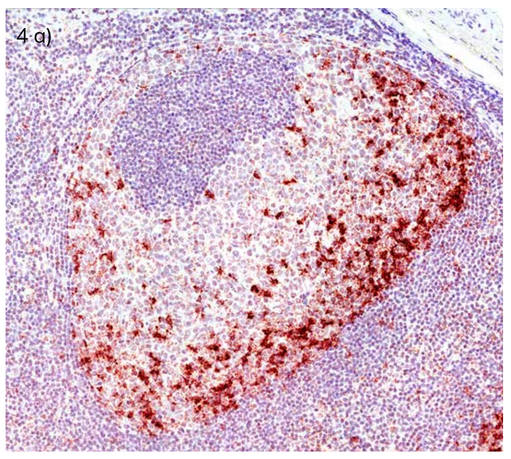

Figure 4: a) Proximity ligation assay in human tonsils visualizing the interaction between PD1 and SHP-2. Staining is seen primarly on the edge of the germinal centers.

b) Technical control of SHP-2, using only the SHP-2 antibody in the proximity ligation assay. c) Technical control of PD1, using only the PD1 antibody in the proximity ligation assay.

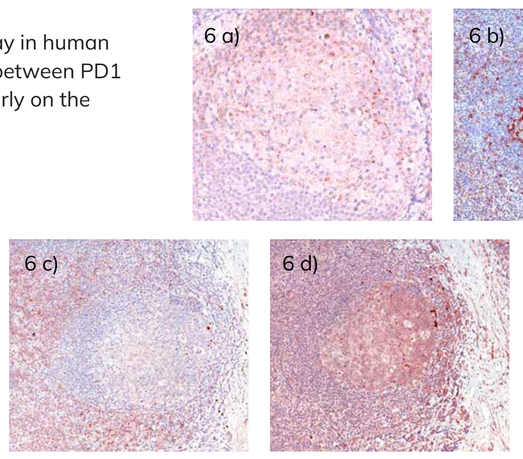

Figure 6: Total protein expression visualized with IHC.

a) IHC of SHP2 in human tonsils.
b) IHC of PD1 in human tonsil.
c) IHC of pTyr in human tonsils.
d) IHC of pPD1 in human tonsils.

Materials and methods

Two specific *in situ* proximity ligation assays were developed based on the **NaveniBright™** (anti-Mouse/anti-Rabbit) kit, one for PD1 phosphorylation and one for PD1/SHP2 interaction. The phosphorylation of PD1 as well as the interaction between PD1 and SHP2 was visualized in FFPE tissues acquired from Acovos. In short, the tissue was incubated with pairs of monoclonal antibodies (anti PD1/anti pTyr or anti PD1/anti SHP2), followed by ligation of oligonucleotides and subsequent rolling circle amplification. The RCA product was then detected using chromogens and the proximity ligation assay signal assessed by brightfield microscopy.

Conclusions

The specific *in situ* proximity ligation assays developed at Navinci Diagnostics can be used to visualize the phosphorylation of PD1 and the interaction of PD1 with SHP2 in human tissues. These assays can potentially be useful tools for researchers investigating the signaling pathways and developing molecular antagonists disrupting the PD1/SHP2 interaction. The PD1/SHP2 assay could have a possible clinical use in the future as a biomarker for identifying patients eligible for treatment with PD1/PDL1 checkpoint inhibitors.

References

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