Abstract # P486

Utilization of dual IHC and quantitative image analysis techniques to evaluate LAG-3 positive T cells in the tumor microenvironment of NSCLC tissue

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Background

Immunotherapeutic approaches targeting inhibitory checkpoint molecules expressed on dysfunctional T cells have shown promising responses in non-small cell lung cancer (NSCLC) (Lin and Shaw, 2017). One molecule under investigation is lymphocyte activation gene-3 (LAG-3) which is mainly expressed on exhausted T cells (Anderson *et al.,* 2017). The expression of LAG-3 increases with lung cancer progression, and the presence of LAG-3 positive T cells in the tumor microenvironment is associated with a poor prognosis (He *et al.*, 2017; Thommen *et al.*, 2015). To allow for a for specific evaluation of LAG-3 positive T cells in clinical samples, we implemented chromogenic anti-LAG-3/CD3 dual immunohistochemistry (IHC) and digitally quantified CD3 and LAG-3 immune cell relationships in terms of cell infiltrations and proportions in the tumor microenvironment of NSCLC tissue.

Methods

Immunohistochemistry:

IHC was implemented on the DISCOVERY XT staining platform (Ventana) by Indivumed using anti-LAG-3 clone 17B4 (Abcam), anti-CD3 clone 2GV6 (Ventana), polyclonal anti-pan-Cytokeratin (pan-CK) (#Z062201-2, Dako), and anti-PD-L1 clone SP142 (Ventana). DAB and Purple chromogen-based detection systems (Ventana) were applied. The first of three formalin-fixed paraffin-embedded (FFPE) serial sections was dual stained for LAG-3/CD3, the second for pan-CK and the third for PD-L1.

Digital image analysis:

Image analysis was performed by OracleBio using Indica Labs Halo software. Tumor and stroma regions of interest (ROI) were classified using the pan-CK section (**Figure 1**) and automatically transferred to the co-registered LAG-3/CD3 and PD-L1 sections. Cellular analysis was performed using thresholds established to identify and count CD3, LAG-3 and dual labeled cells (**Figure 2**). For PD-L1, stained area proportions of the ROI were determined (**Figure 3**).

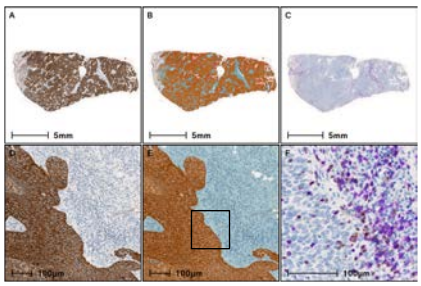

Figure 1: Anti-pan-CK and LAG-3/CD3 dual IHC. A: Pan-CK (brown) original tissue section. **B:** Pan-CK classifier overlay showing tumor (orange) and stroma (blue) ROI. **C:** LAG-3/CD3 original tissue section. **D:** Magnified area of pan-CK section. **E:** Corresponding pan-CK area showing tumor and stroma ROI. **F:** Magnified area showing anti-LAG-3/CD3 dual (brown/purple) IHC.

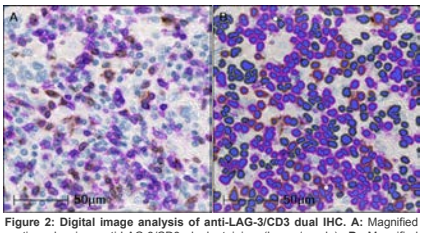

Figure 2: Digital image analysis of anti-LAG-3/CD3 dual IHC. A: Magnified section showing anti-LAG-3/CD3 dual staining (brown/purple). **B:** Magnified section with cellular image analysis overlay showing nuclei (blue nuclear overlay), anti-LAG-3 (brown cytoplasmic overlay), CD3 (purple cytoplasmic overlay) and LAG-3/CD3 dual IHC (brown/purple cytoplasmic overlay).

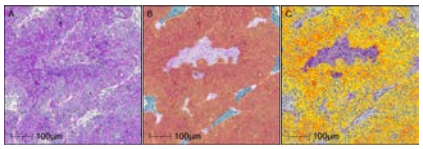

Figure 3: Digital image analysis of anti-PD-L1 IHC. A: Magnified section with anti-PD-L1 (purple) staining. **B:** Corresponding area with classifier overlay showing tumor (orange) and stroma (blue). **C:** Corresponding area with cellular image analysis overlay showing PD-L1 area quantification (yellow/orange/red).

Results

Ten individual NSCLC tissue samples were stained with anti-LAG-3/CD3 dual IHC and LAG-3, CD3 and LAG-3/CD3 dual positive cells in the tumor and stroma regions of interest (ROI) were quantified by digital image analysis. Only low numbers of LAG-3 single positive cells were observed, whereas high numbers of CD3 single positive T cells were detected in the tumor and especially the stroma ROIs (**Figure 4**). The numbers of LAG-3/CD3 dual positive cells (LAG-3 positive T cells) were low in most samples, but three non-adenocarcinoma samples showed markedly elevated numbers (**Figure 4**). Across the ten NSCLC samples, numbers of T cells were independent of the histological subtype (**Figure 4**), but a significantly higher proportion of T cells in the stroma was LAG-3 positive (LAG-3 / CD3 dual positive) in non-adenocarcinoma compared to adenocarcinoma samples (**Figure 5**). The ten NSCLC tissue samples were additionally stained with anti-PD-L1 IHC, and the stained area proportions of the tumor and stroma ROIs were digitally quantified (**Figure 6**). The PD-L1-stained area proportions of both tumor and stroma exhibited a positive correlation with the numbers of intratumoral and stromal LAG-3 positive T cells (LAG-3/CD3 dual positive cells) ($\rho \geq 0.76$; $P \leq 0.015$; Spearman rank correlation).

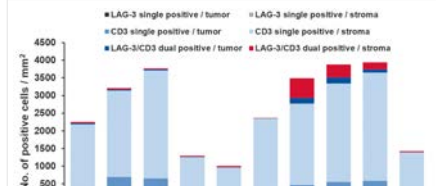

Figure 4: LAG-3 and CD3 positive cell populations in ten NSCLC tissue samples. The non-adenocarcinoma samples P1413-Tp14, P2609-Tp11 and P2613-Tp12 showed elevated numbers of LAG-3/CD3 dual positive cells in the tumor and stroma ROIs.

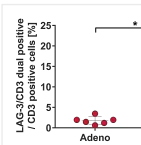

Figure 5: Proportions of LAG-3 positive T cells in the stroma of ten NSCLC tissue samples. A higher proportion of T cells was LAG-3 positive in non-adenocarcinoma than in adenocarcinoma samples (P = 0.019; Mann-Whitney U test).

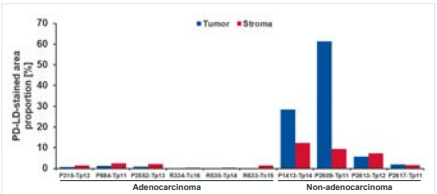

Figure 6: PD-L1 expression in ten NSCLC tissue samples

As observed for the proportions of LAG-3 positive T cells, the proportions of PD-L1 stained areas in the tumor and the stroma were higher in non-adenocarcinoma than in adenocarcinoma samples (**Figure 7**).

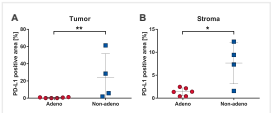

Figure 7: Proportions of PD-L1 stained cells in the tumor and stroma of ten NSCLC tissue samples. A higher proportion of PD-L1 staining was detected in non-adenocarcinoma than in adenocarcinoma samples in the tumor (P = 0.001; Mann-Whitney U test) and the stroma (P = 0.038; Mann-Whitney U test).

Conclusions

We implemented dual IHC and digital image analysis to specifically evaluate LAG-3 positive T cells in a set of ten NSCLC tissue samples. We showed that a higher fraction of tumor-associated T cells is LAG-3 positive in non-adeno-carcinoma compared to adenocarcinoma NSCLC tissue, which correlated with higher expression levels of PD-L1 in non-adenocarcinoma tissue. The obtained results were in agreement with published literature (He *et al.*, 2017). These data highlight the benefits of dual IHC and digital image analysis for characterizing immune cell relationships within the tumor microenvironment.

References:

Anderson, A.C., *et al.* (2017). Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity 44, 989-1004.

He, Y., *et al.* (2017). LAG-3 Protein Expression in Non-Small Cell Lung Cancer and Its Relationship with PD-1/PD-L1 and Tumor-Infiltrating Lymphocytes. J Thorac Oncol 12, 814-823.

Lin, J.J., and Shaw, A.T. (2017). Raising the bar on first-line immunotherapy in lung cancer. Lancet Oncol 18, 2-3.

Thommen, D.S., *et al.* (2015). Progression of Lung Cancer Is Associated with Increased Dysfunction of T Cells Defined by Coexpression of Multiple Inhibitory Receptors. Cancer Immunol Res 3, 1344-1355.