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PAK4 as a potential marker for poor response to immunotherapy in melanoma patients

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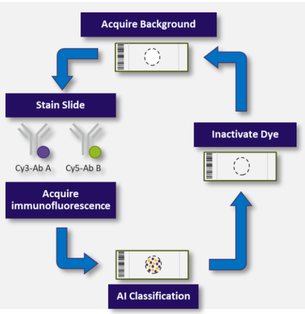
Background: Despite remarkable clinical success with ICI therapy targeting the PD-1 pathway for the treatment of melanoma some patients still remain unresponsive to immunotherapy. Using an unbiased mass spectrometry (LC-MS) discovery-based approach we identified a panel of protein biomarkers that correlated with ICI therapy response, one of which was the p21-activated kinase 4 (PAK4) which we found to be significantly elevated in non-responding tumor biopsies. For an in-depth analysis of PAK4 in these same patient samples we followed up with spatial tissue analysis using a multiplexed immunofluorescence (mIF) platform to gain information on PAK4 mechanisms in melanoma patients treated with ICI therapy.

Methods: FFPE tumor samples were firstly used for unbiased whole proteome profiling using TrueDiscovery™, a data-independent acquisition (DIA) LC-MS technology. Baseline patient samples were classified as responders (R; n=9) or non-responders (NR; n=15) based on the response at 3 months post ICI-treatment. Subsequently, the same patient samples were analyzed by an 18-marker custom panel using MultiOmyx™, a multiplexed immunofluorescence (mIF) assay utilizing a pair of conjugated Cyanine dye-labeled antibodies per round of staining coupled with the deep-learning based cell classification platform NeoLYTX.

Results: PAK4 was found to be elevated in tumor samples from non-responder patients by both unbiased proteomics analysis, as well as MultiOmyx mIF analysis. Furthermore, we observed a clear difference in the correlation to other immune cells between the two patient groups by MultiOmyx analysis, PAK4 density being negatively correlated to T cells and TAMs only in non-responders, while positively correlated to MDSCs only in responders.

MultiOmyx™ workflow and multiplexing IF panel



18-Marker mIF Panel	
CD3	HLA-DR
CD4	PD-1
CD8	PD-L1
FoxP3	CTLA-4
Tbet	LAG3
GranzymeB	TIM3
CD11b	CD31
CD68	PAK4
CD163	SOX10

Figure 1. For multiplexing two conjugated fluorescent antibodies are applied per round, followed by image acquisition of the stained slides. The dye is erased, enabling a subsequent round of staining. **Table)** 18-marker protein panel composition.

TrueDiscovery™ whole proteome profiling

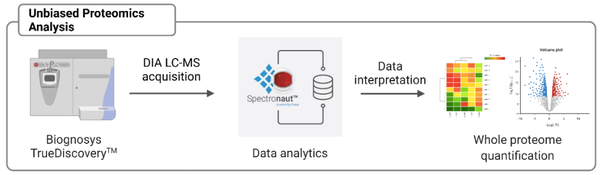


Figure 2. Proteome profiling of 23 patient FFPE melanoma tumor samples using TrueDiscovery™ LC-MS technology resulted in quantification of close to 10,000 proteins. Of these 76 proteins were changed significantly between non-responders and responders.

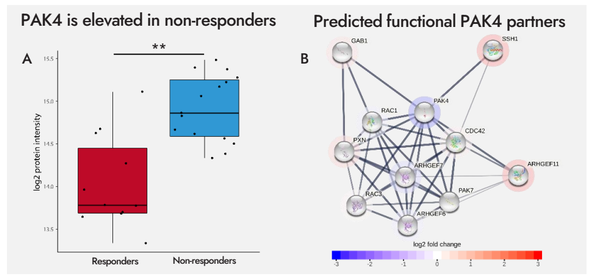


Figure 3. A. Intensity of PAK4 measured by proteome profiling is significantly elevated in responder compared to non-responder samples (p = 0.001). **B.** Protein-protein **interaction network of PAK4** with quantitative log2 fold change (R/NR) displayed as halo.

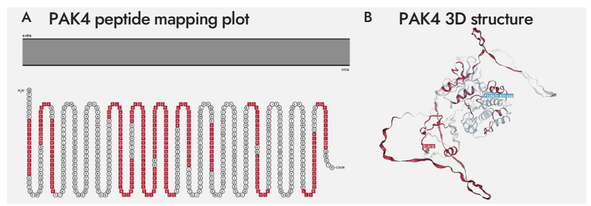


Figure 4. A. PAK4 is measured with 17 distinct peptides spanning the protein amino acid sequence. **B. Predicted 3D structure of** PAK4 with protein kinase and CRIB domain, and detected peptides shown in red.

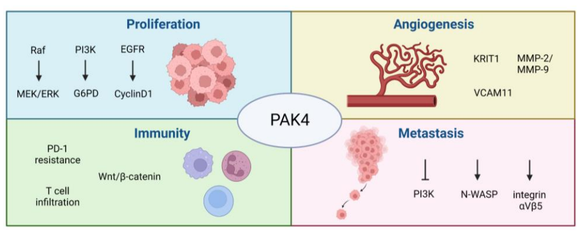


Figure 5. Key roles and pathways of PAK4 in cancer. Created with BioRender.com.

MultiOmyx™ multiplexed IF & NeoLYTX analysis

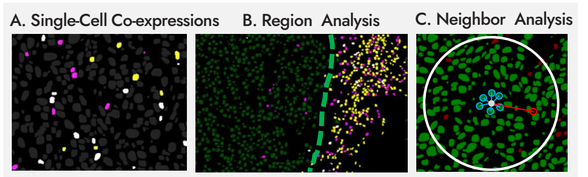


Figure 6. A. Cell phenotypes are identified from biomarker staining patterns using AI-based classification algorithms. Cytotoxic, helper, and regulatory T cells are shown in magenta, yellow and white, respectively. B. Cells are assigned to either the tumor or stromal regions based on SOX10 staining. Infiltrating T cells are shown on the left side of the image. C. Neighbor analysis is used to quantify spatial relationships between nearby cells. Cyan indicates immediate neighbors, and white encircles all TAMs within 100 microns.

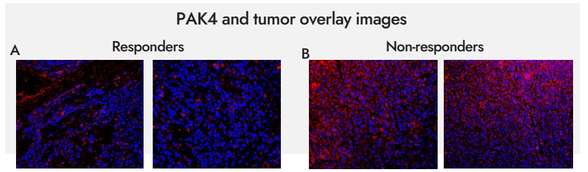


Figure 7. Representative color overlay images of PAK4 and SOX10+ tumor cells. In **A+B** SOX10 is blue and PAK is red.

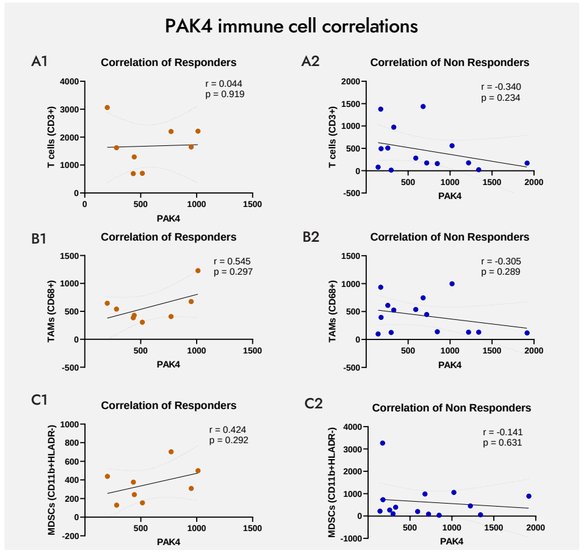


Figure 8. Pearson correlation coefficients (r) and p-values (p) for total densities of PAK4+ cells and T cells (**A1+A2**), TAMs (**B1+B2**), and MDSCs (**C1+C2**), for responders and non-responders.

MultiOmyx™ multiplexed IF & NeoLYTX analysis

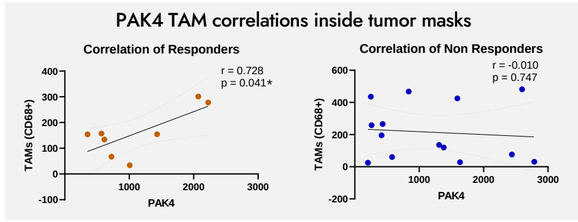


Figure 9. Pearson correlation coefficients (r) and p-values (p) for densities inside the tumor masks of PAK4+ cells and TAMs for responders and non-responders.

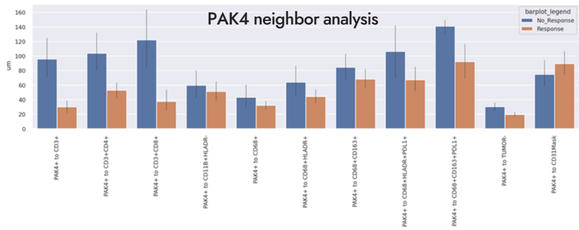


Figure 10. Bar graphs depict the distance of PAK4+ cells to immune cells generated by applying the AI platform NeoLYTX described in figure 6.

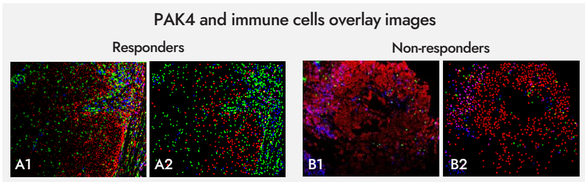


Figure 11. Representative color overlay images of tumors from a responding (**A**) and non-responding (**B**) patient. **A1+B1** are IF overlay images, while **A2+B2** show the labelmaps generated using the AI platform NeoLYTX.
PAK4 is red, CD3 (T cells) is green, and CD68 (macrophages) is blue.

Conclusion & Key Take-Aways

- We compared the protein landscape of tumor biopsies from melanoma patients in non-responders versus responders to ICI therapy, and found PAK4 to be elevated in tumor samples from non-responder patients.
- PAK4 density correlates positively with TAMs, and PAK4+ cells are found in closer proximity to T cells in responders only, suggesting that the immune mechanisms of PAK4 are different in melanoma patients responding to ICI therapy.

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