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A Novel Tumor Adenosine Signature to Guide Indication Selection for Adenosine Pathway Inhibitors

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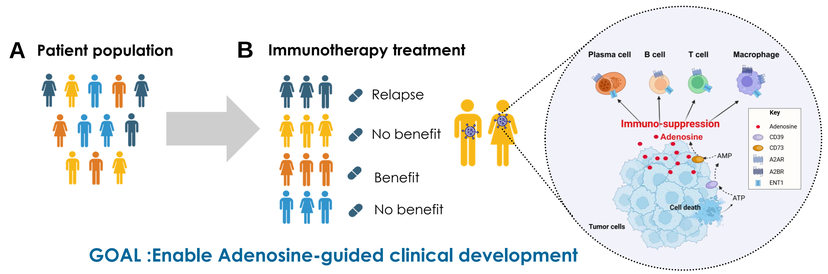
Introduction

Adenosine (ADO) is highly immuno-suppressive in tumor micro-environment but hard to measure in clinical settings

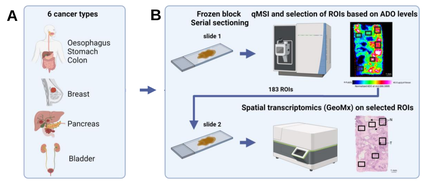


GOAL :Enable Adenosine-guided clinical development

- **Immunotherapy** has revolutionized cancer care, but a large proportion of patients do not benefit (**A**).
- **Adenosine** (ADO) is a major **immunosuppressive** player within tumor micro-environment 1(TME; **B**). ADO exerts these effects via engagement of **A2A** and A2B receptors or via transport through the **Equilibrative Nucleoside Transporter 1 (ENT1)**[1,2,3].
- Agents targeting ADO pathway (e.g. A2AR inhibitors) have entered clinical trials and hold promise to unlock immunotherapy responses[4]. Quantifying ADO in the TME could target these agents to the right tumor types and patient segments.
- **ADO measurement** is **impractical in clinical trials.** Several ADO-associated gene signatures have been proposed in the past, but none truly reflects ADO content in the TME[5,6].
- Here, we propose the **first ADO gene signature (qADO signature) based on quantification of ADO and gene expression in human tumors,** and we showcase its application for indication selection.

Figure 1

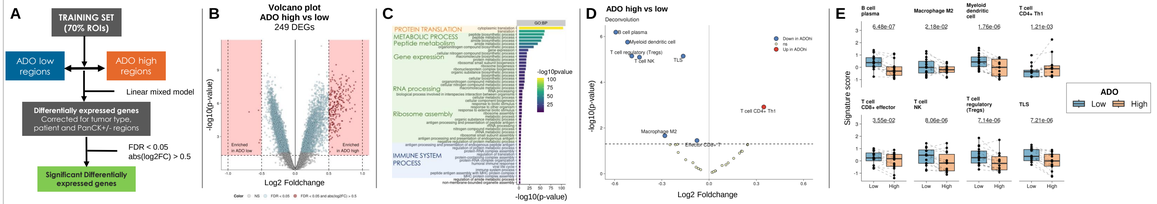
qMSI and Spatial Transcriptomics were used to derive the 1st ADO gene signature (qADO signature) based on overlay of quantification of ADO and gene expression analysis on selected regions of human tumors



A. Human tumor biopsies (n=13) were collected from six cancer types and immediately flash frozen. **B.** Frozen blocks were sectioned and tumor slides were analyzed by quantitative Mass Spectrometry Imaging (qMSI) to determine adenosine levels. 183 Regions of Interest (ROIs) were selected based on their high or low quantified ADO content. Similar ROIs were then selected on adjacent slides and RNA extracted and checked for quality by RNA-scope. Gene expression was analyzed using spatial transcriptomics (GeoMx).

Figure 2

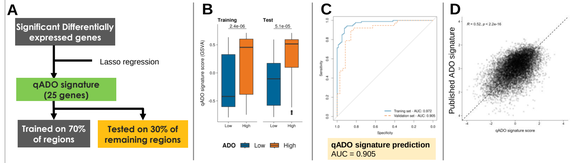
High ADO regions are associated with a specific gene expression pattern and contain fewer immune cells compared to low ADO regions



A. ROIs with matched ADO content and gene expression were divided in a training (70%) and a Test (30%) set: a linear mixed model corrected for tumor type and patient was applied to the training set and identified 249 significantly differentially expressed genes (DEGs) in ADO high vs ADO low regions (FDR <0.05), most of them over-expressed. **B.** Volcano plot showing the genes upregulated in ADO high (right) or ADO low (left) regions. **C.** Gene ontology enrichment analysis on genes upregulated in ADO high regions revealed associations with protein translation, metabolic processes and immune activation. **D.** Volcano plot displaying the difference (Log2FC) in the abundance of immune cell populations between ADO high and ADO low regions. Immune cell signatures[7,8] were used to deconvolute spatial transcriptomic data. Linear mixed model adjusted for tumor type, patients and PanCK +/- regions was used to compare immune signatures in ADO high vs low regions. ADO high regions are poor in immune infiltrate compared to ADO low regions. **E.** Box plots for each immune cell population showing significantly lower expression of the associated gene signature in ADO high vs ADO low regions. N = 13 paired samples

Figure 3

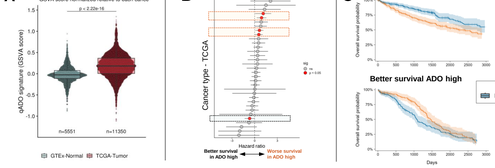
The qADO signature predicts ADO levels in a test set and correlates with another adenosine signaling signature



A. Lasso regression was applied to identify DEGs with the highest predictive power and derive the qADO signature. **B.** Boxplot showing the significative difference between ADO low and ADO high ROIs in the training set as well as the test set. The qADO signature can stratify regions with high and low ADO content (N=128 Training ROIs; N= 55 Test ROIs; Mann-Withney U test). **C.** ROC analysis showing that the predictive power of the qADO signature is confirmed in the test set. **D.** Correlation plot showing the relative expression of the qADO signature and an independent adenosine signaling signature[6] in TCGA dataset. The qADO signature correlates with the independent adenosine signaling signature.

Figure 4

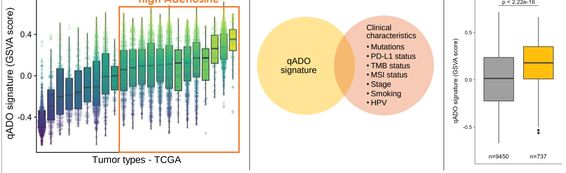
The qADO signature is enriched in tumor vs healthy tissue and is associated with poor survival in selected cancer types



A. Violin plot showing the expression of the qADO signature in normal and tumor tissues. The qADO signature is enriched in tumor (TCGA) vs healthy (GTex) tissue (Mann-Whitney U test). **B.** Forest plot depicting the Hazard ratio and 95% confidence for overall survival for each tumor type in TCGA datasets. The qADO signature is associated with worse survival in several cancer types (Cox regression using ADO score as continuous variable). **C.** Representative survival curves for a tumor type displaying worse (top) or better (bottom) survival in ADO high vs ADO low patients. Patients were stratified according to ADO content using the qADO signature and divided in 2 groups based on the median.

Figure 5

The qADO signature enables tumor type prioritization

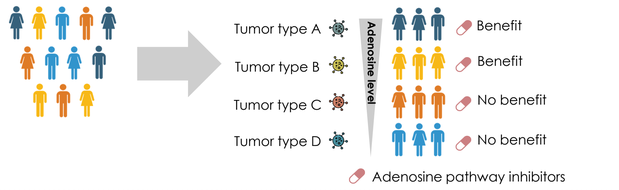


A. Boxplot showing the relative expression of the qADO signature in TCGA. The qADO signature can stratify tumor types by ADO content. **B.** Using TCGA, we can identify actionable patients segments with high ADO. **C.** Boxplot showing the relative expression of the qADO signature in WT (grey) and Kras mutated (yellow) tumor types. Patients with KRAS mutations have a high score in qADO signature. (N= 9450 WT; N=737 MUT; Mann-Whitney U test)

Conclusions

Mixed patient population

Assign the right treatment to the right tumor



- Using spatial quantification of ADO and gene expression measurements, we developed **the qADO signature,** the 1st ADO signature based on quantified ADO content in human tumors.
- This new tool can be used for **tumor type prioritization** for adenosine pathway inhibitors
- Further exploration of the qADO signature may lead to:
  - Deeper understanding of ADO immunosuppressive mechanisms
  - Novel patient selection strategies

Methods

- **Human tumor collection.** Thirteen tumor tissue resections from six tumor types (Pancreatic, Stomach, Colon, Oesophagus, Bladder, and Breast cancer) were collected by Fidelis Research. Tissue were snap frozen on dry ice and stored at -80°C.
- **ADO quantification on human tumor.** Tumor blocks were sectioned at -20°C in serial sections. Sections were dried in a dessicator and immediately sprayed with a mixture of 6 inhibitors of ADO pathway. Sections were analyzed for ADO content using quantitative mass spectrometry imaging (qMSI). A total of 183 regions of interest (ROIs) were selected based on ADO content (high vs low) and profiled by spatial transcriptomics (GeoMx).
  - **qMSI**: All the biological sections were analyzed by 7T MALDI-FTICR with following methods: CASI negative mode m/z 267 Da with an isolation window of 30Da at 90 µm spatial resolution for the tissue sections and 200 µm for the calibrants on tissue.
  - **RNAscope**: Mild expression model transcript PPIB and low expression model transcript POLR2A were analyzed by RNAscope to evaluate sample's transcriptome integrity before GeoMx whole transcriptome analysis.
  - **WTA**: transcripts were analyzed by GeoMx DSP using a Whole Transcriptome Atlas (WTA) panel and off the shelf morphological markers CD45, PanCK and DNA counterstain. Transcripts detected from PanCK$^+$ and PanCK$^-$ tissues areas were sequenced using a NovaSeq 6000 sequencer and compared between areas enriched in ADO and low in ADO.
- **Bioinformatic analysis.** ROIs were divided into training (70%) and test (30%) sets. ADO high and low regions were defined based on the overall ADO level median across all ROIs. Immune cell[7] and tertiary lymphoid structure (TLS)[8] signature scores were interred using GSVA[9]. A linear mixed model adjusted for tumor type and patient was applied to the training set to identify differentially immune cell signatures and expressed genes (DEGs) in ADO high vs ADO low regions (FDR <0.05). Gene ontology enrichment analysis were performed on DEGs using g:profiler[10]. Lasso regression was then used to identify DEGs with the highest predictive potential i.e. qADO signature. qADO signature scores were computed using GSVA in our in-house GeoMX dataset, tumor samples from The Cancer Genome Atlas (TCGA) and healthy tissue from the Genotype-Tissue Expression dataset (GTEx). The predictive power of the resulting gene signature was evaluated in the test set using the Area Under the Receiver Operating Characteristics (AUROC). Mann-Whitney U test was used for all pairwise comparisons of qADO signature scores. An adjusted p-value of 0.05 was defined as significative. All analysis were performed using R 4.4.0.

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Declaration of interest : T.S. is employee of and owns stock options and/or shares in iTeos Therapeutics.

Ethics Approval : This study was approved by Local Ethics Committee.