# COMBINED IMMUNOHISTOCHEMISTRY AND NGS-BASED PATIENT PROFILING FOR PREDICTING ANTI-PD-1/PD-L1 THERAPY RESPONSE

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## ABSTRACT

There are several different modalities of predictive tests which support response to anti-PD-1/PD-L1 inhibitors therapy, including PD-L1 expression by immunohisto chemistry (PD-L1 IHC), mismatch repair deficiency (dMMR), microsatellite instability (MSI), and recently emerging tumor mutation burden (TMB), and Gene Expression Panels (GEP). Each of these methods capture different facets of the immune system: TMB and MSI evaluates mutational/neoantigen load which can stimulate the immune system; GEP establishes a profile of immune response, and whereas PD-L1 IHC directly evaluates the state of checkpoint inhibition in the tumor and tumor microenvironment (TME). We constructed a compound testing paradigm for immune system monitoring called PredicineX, which combines genome analysis which relies on tissue or blood-derived nucleic acids and advanced tissue context analytics based on PD-L1 IHC in solid tissue biopsies to create a comprehensive patient profile to support anti-PD-1/PD-L1 therapy decision making.

## MATERIALS & METHODS

20 Formalin-Fixed Paraffin Embedded (FFPE) Non-Small Cell Lung Cancer (NSCLC) sections tissue blocks were cut into 4µm-thick tissue sections on to positively-charged glass slides. NSCLC serial tissue sections were stained for PD-L1 using the SP263 clone according to specified protocols. SP263 staining was performed on the Ventana Benchmark Ultra autostainer platform. Images of all stained slides were scanned at 20x magnification in brightfield using Aperio AT2 slide scanners. Scanned images were analyzed using Flagship Biosciences' cTA® digital pathology platform. In brief, cTA® analysis first detects all cells in a slide section, then measures hundreds of descriptive features (Biofeatures™) characterizing every single cell, which includes morphometric, spatial, and IHC-related data points. Additional machine-learning and data-science based workflows allow the cTA® platform to separate the tumor and TME-associated cells to assign biomarker scoring in those compartments. Additional Flagship Phenotype analysis identifies specific cellular phenotypes within the tissues to understand biomarkers in the context of specific cellular subtypes. All data generated during these workflows are fed into Flagship's Scorecard modeling system, which builds predictive models around specified endpoints, such as responder/non-responder, pre/post-dose, or in this case, PD-L1 positivity. The top cellular Biofeatures™ associated with predicting outcomes were identified as the features which explained the most variance in a regression analysis. These features were normalized and displayed as visual representations of the top features in each data set, along with the values of each identified Biofeature™.

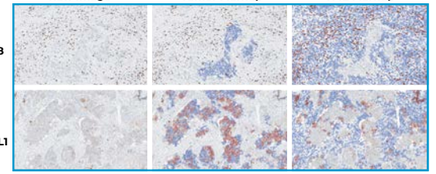
Serial sections of each tissue sample were sent to Predicine for genomic analysis. DNA was extracted from FFPE samples. Minimal 50ng of purified DNA is recommended for downstream NGS assay. UMI (unique molecular identifier)-tagged libraries were enriched by predicine NGS panel using hybrid capture method and deep sequenced by Illumina pair-end sequencing. Raw sequencing data (BCL files) were fed through DeepSea, a Predicine proprietary NGS analysis pipeline. Paired-end reads originated from the same molecules were merged as single strand fragments. Single strand fragments from the same double strand DNA molecules were further combined as double stranded. Both sequencing and PCR errors were deeply suppressed during this process. Detected variants were further filtered based on variant background (defined by normal plasma samples and historical data), repeat regions and other quality metrics. For tissue samples, variants with mutation allele frequency (MAF) 5% and hotspot variants with MAF down to 2.5% were reported. Benign and likely benign variants (clinical significance defined based on public databases) were excluded. All somatic variants (with 1000 genome SNP variants excluded) in coding regions with MAF > 5% were included in TMB score estimation. Thresholds were adjusted for FFPE deamination changes.

## FLAGSHIP COMPREHENSIVE TISSUE ANALYSIS

**Working with Flagship Biosciences**

| | | |
|---|---|---|
| 1 | IHC / ISH Assays | ActionAssays™, custom assays, CLIA assays or existing slides |
| 2 | Digital Slide Scanning | Discovery, translational research, or clinical trials |
| 3 | AI-Powered cTA Image Analysis | Intuitive endpoints (% POS, etc) or advanced Flagship endpoints |
| 4 | Data Analysis and Reporting | Typical statistics or Flagship Scorecards |
| 5 | Consultation with Scientific Experts | Pathology, biology, image analysis, data science, artificial intelligence |

Flagship Biosciences' flexible tissue analysis workflow integrates tissue analysis. Discovery, clinical, and clinical trial projects ranging from IHC assay development to intricate data science reports driven by an AI-powered image analysis platform can all be performed in Flagship's CAP/CLIA certified labs.

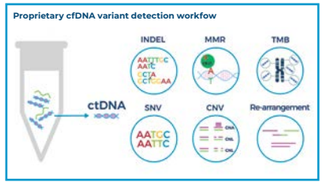


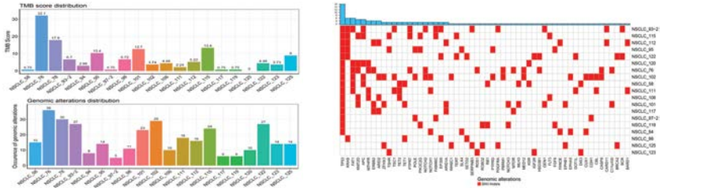

Flagship's cTA image analysis was run on serial NSCLC sections to investigate PD-L1 and CD8 expression in whole tissue slides. Flagship's AI algorithms separate and score both tumor and tumor microenvironment compartments in the tissue (above) and report endpoints such as percent positive in each tissue compartment (right)

| HID | SP263 % Positive | SP263 % Positive | SP263 % Positive | CD8 % Positive | CD8 % Positive | CD8 % Positive All |
|---|---|---|---|---|---|---|
| NSCLC 101 | 19.147 | 19.645 | 18.853 | 15.635 | 3.496 | 8.944 |
| NSCLC 102 | 10.894 | 4.078 | 7.654 | 6.415 | 4.182 | 5.671 |
| NSCLC 106 | 29.208 | 26.615 | 28.659 | 19.68 | 7.354 | 15.895 |
| NSCLC 111 | 3.861 | 2.337 | 3.643 | 6.163 | 2.245 | 2.766 |
| NSCLC 112 | 20.651 | 23.181 | 21.281 | 13.963 | 5.388 | 7.723 |
| NSCLC 115 | 62.158 | 64.526 | 63.08 | 27.264 | 15.595 | 22.355 |
| NSCLC 117 | 24.366 | 19.769 | 22.475 | 12.684 | 7.465 | 10.351 |
| NSCLC 119 | 0.357 | 0.032 | 0.113 | 1.659 | 0.153 | 0.49 |
| NSCLC 120 | 9.033 | 13.611 | 13.736 | 2.84 | 0.272 | 1.054 |
| NSCLC 122 | 8.873 | 1.576 | 5.407 | 10.92 | 5.913 | 7.907 |
| NSCLC 123 | 20.884 | 10.5 | 15.4 | 18.618 | 3.937 | 8.73 |
| NSCLC 125 | 14.694 | 9.836 | 13.049 | 8.83 | 5.35 | 7.348 |
| NSCLC 58 | 12.512 | 7.627 | 10.096 | 3.242 | 0.451 | 1.719 |
| NSCLC 76 | 3.516 | 1.57 | 3.062 | 6.561 | 3.563 | 5.236 |
| NSCLC 78 | 35.773 | 57.301 | 44.739 | 14.471 | 6.947 | 11.457 |
| NSCLC 90-2 | 25.143 | 22.966 | 24.311 | 11.586 | 3.128 | 8.281 |
| NSCLC 94 | 3.004 | 1.513 | 2.596 | 3.295 | 0.529 | 2.484 |
| NSCLC 95 | 38.411 | 66.916 | 48.946 | 8.993 | 6.144 | 7.758 |
| NSCLC 97-2 | 10.104 | 5.657 | 8.451 | 4.906 | 1.143 | 3.878 |
| NSCLC 98 | 20.602 | 12.929 | 17.935 | 6.286 | 4.998 | 5.695 |

## PREDICINE GLOBALLY HARMONIZED NGS PLATFORM

To address the diverse needs in clinical genomics, Predicine has developed a biology-oriented portfolio of liquid biopsy NGS panels (ranging from 25-600 genes) for measurement of TMB, DNA damage repair, fusion, and common cancer variants. Combined with its in-house proprietary computational algorithms, Predicine's harmonized assays are designed to support biomarker-driven global clinical trials in US, EU, APAC including China. PredicineATLAS NGS panel is the ultimate liquid biopsy panel for TMB in immuno-oncology, using a 600-gene panel tailored to identify biomarkers relevant to cancer immunotherapy.




Serial sections of 20 NSCLC samples were analyzed via the PredicineATLAS panel for mutations in 600 key cancer genes. Specific genetic mutations, indels, amplifications, and rearrangements are reported per sample as well as an overall Tumor Mutation Burden (TMB) score.

## PREDICINEX: A COMPOSITE BIOMARKER SOLUTION FOR TISSUE ANALYSIS AND TMB ASSESSMENT TO SUPPORT IMMUNO-ONCOLOGY DRUG DEVELOPMENT

Flagship Biosciences and Predicine have partnered to join their complementary tissue diagnostic platforms into one composite biomarker solution called PredicineX. This collaboration between the two platforms enables incredibly powerful tissue analysis, merging contextual IHC data and morphometric analysis with robust genomic analyses such as genomic alteration panels and TMB measurements. Data from each platform can be combined to assess any number of combinations of tissue/IHC and genomic data. In a cohort of tissues with clinical data such as response or survival, we will get a comprehensive view of the tissue and protein architecture complete with the underlying genomic mechanisms which are indicative of response or non-response. In this study, we analyzed the relationships between PD-L1 and CD8 expression in the Tumor and Tumor Microenvironment (TME) and asked how the expression of these markers alone and combined will correlate with the Tumor Mutation Burden (TMB) Score. While there is a general trend seen in PD-L1 and CD8 expression when arranged by TMB Score, the markers themselves alone or in combination in each compartment do not correlate well with TMB. By adding in SNV/Indel data from genomic analysis, we see increasing correlative value of these tissue and genomic composite markers with TMB. Additionally, we find panels of 8 genomic SNV/Indels which are highly correlative with the expression of either PD-L1 or CD8. Notably, four of those eight genes are expressed in predicting both PD-L1 and CD8 presence. As a final analysis of tissue or genomic alterations which result in higher TMB, we run all data through the Flagship Scorecard process, which analyzes all tissue and genomic data generated to find intricate data points in each tissue which result in the given clinical outcomes. Taken together, this data indicates that composite tissue and genomic biomarkers are more robust in assessing clinical outcomes than pathology or genomic data alone.

**The PredicineX Composite Tissue and Genomic Biomarker**

Cohort of Patient Tissue Samples

↓

Comprehensive Tissue IHC and Cellular Morphology Analysis
Robust Genomic Alteration Panels and TMB Score

↓

Composite Biomarker to Inform Drug Development and Treatment Strategies

↓

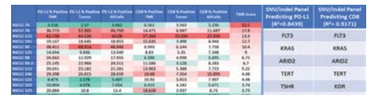
Add in Clinical Outcomes Data to Build Predictive Models Around Composite Markers

Continue Monitoring Patients for Composite Biomarkers of Interest via ctDNA/ctRNA Liquid Biopsy

**Increasing Outcome Correlations With a Composite Biomarker Strategy**

Combined computational tissue analysis with genomic alteration profiles shows increasing correlation with TMB when PD-L1 or CD8 alone are assessed in combination with genomic alteration data. These results indicate that PD-L1 or CD8 status alone is not indicative of TMB status. While PD-L1 tumor status and HSD3B1 expression was the highest correlated composite marker with TMB, 5 out of the top 6 correlated composite markers were due to CD8 expression alongside a genomic alteration. Flagship Scorecard analysis, which looks at specific tissue morphology and IHC features can be combined with genomic panels to increase predictive accuracy of composite biomarkers.

**PD-L1, CD8, and Genomic Alterations for Predicting TMB Status in NSCLC**




| Single IHC | Model Fit To TMB (R²) | Duplex IHC | Correlation with TMB (p) | Duplex IHC + Gene SNV/Indel | Correlation with TMB (p) | Duplex IHC + Gene SNV/Indel | Correlation with TMB (p) |
|---|---|---|---|---|---|---|---|
| PD-L1 % Pos All Cells | 0.0836 | PD-L1 % Pos Tumor+ CD8 % Pos All Cells | 0.3225 | HSD3B1 + PD-L1 % Pos Tumor | 0.5641 | PD-L1 Tumor + CD8 All Cells + HSD3B1 | 0.4281 |
| PD-L1 % Pos Tumor | 0.0915 | PD-L1 % Pos Tumor + CD8 % Pos TME | 0.3209 | PBRM1 + CD8 % Pos TME | 0.5559 | PD-L1 Tumor + CD8 Tumor + HSD3B1 | 0.3889 |
| PD-L1 % Pos TME | 0.0608 | PD-L1 % Pos Tumor + CD8 % Pos All Cells | 0.3133 | ME012 + CD8 % Pos TME | 0.5559 | PD-L1 Tumor + CD8 TME + HSD3B1 | 0.3879 |
| CD8 % Pos All Cells | 0.1035 | PD-L1 % Pos All Cells + CD8 % Pos All Cells | 0.2908 | CASP8 + CD8 % Pos TME | 0.5559 | PD-L1 Tumor + CD8 Tumor + PIK3C2G | 0.2679 |
| CD8 % Pos Tumor | 0.1167 | PD-L1 % Pos All Cells + CD8 % Pos TME | 0.2878 | PBRM1 + CD8 % Pos All Cells | 0.5211 | PD-L1 Tumor + CD8 Tumor + KRAS | 0.2669 |
| CD8 % Pos TME | 0.0697 | PD-L1 % Pos All Cells + CD8 % Pos Tumor | 0.2863 | ROS1 + CD8 % Pos Tumor | 0.5138 | PD-L1 Tumor + CD8 All Cells + PIK3C2G | 0.2657 |

**Flagship Scorecard Predictive Model: PD-L1 Features Predictive of TMB (R=0.829)**


## CONCLUSIONS

**Combining contextual tissue and IHC analysis with comprehensive genomic alteration profiling and TMB Scoring creates an intricate composite biomarker profile for each tissue sample which can inform and predict clinical endpoints**

- Using Flagship Biosciences cTA® tissue image analysis technology, we created an artificial intelligence (AI) based PD-L1 IHC scoring and morphometric analysis platform which provides both current PD-L1 IHC scoring paradigms and novel computational scores from the rich tissue context data profile created from PD-L1 IHC slides
- Using Predicine's PredicineATLAS NGS technology, we combined contextual tissue data with a tissue-based NGS assay to capture genomic alterations in cancer genes including tumor mutation burden (TMB) and gene mutation data
- We demonstrate the synergistic value of combining genomic based TMB and GEP immune profiles with contextual information from PD-L1 IHC slides in patient biopsies. The high complexity gene profile, combined with the rich tissue context data, provided a novel means to stratify patients into 4 categories:
  1) mutation high/immune competent;
  2) mutation low/immune deficient;
  3) mutation high/immune deficient; and
  4) mutation low/immune competent

*Author is also employed by Roche and research was abided by requisite confidentiality for both Roche and Flagship Biosciences.