INVESTIGATION OF IMMUNE CHECKPOINT INHIBITOR BIOMARKERS IN SOLID TUMORS

Synopsis of the PhD thesis

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1. INTRODUCTION

Cancer remains a major health concern despite declining incidence and mortality. Malignant transformation arises from genetic, epigenetic, and environmental factors. Transcriptomics enables comprehensive gene expression analysis using platforms like microarrays, RNA-sequencing, and NanoString nCounter providing valuable information about tumors.

Immune checkpoints regulate immune responses but can be hijacked by cancer cells. Checkpoint inhibitors, such as CTLA-4 (ipilimumab), PD-1 (pembrolizumab, nivolumab), and PD-L1 (atezolizumab) inhibitors, have been approved for multiple cancers. Recently, the FDA approved LAG-3 inhibitors (e.g., relatlimab) to enhance antitumor responses. Tumors are classified as "hot" (high immune infiltration) or "cold" (immune-excluded). Cold tumors often evade immunity via impaired T cell trafficking, antigen presentation defects, or immunosuppressive environments. Response rates to ICIs range from 15-60%, with over half of melanoma patients showing innate resistance. While biomarkers like PD-L1, TMB, and MSI-H/dMMR guide therapy selection, they

lack precision. Resistance mechanisms involve antigen presentation defects, immunosuppressive microenvironments, and genetic alterations. Combination therapies (e.g., chemotherapy, radiotherapy, targeted therapy) can enhance efficacy. Yes-associated protein (YAP1), regulated by the Hippo pathway kinases MST1/2 and LATS1/2, drives tumor progression. When phosphorylated, YAP/TAZ is sequestered and degraded; when dephosphorylated, it translocates to the nucleus, promoting oncogenic gene expression via TEAD transcription factors. The FDA-approved drug Verteporfin disrupts YAP-TEAD interactions, showing promise in cancer therapy. Melanoma incidence is rising despite declining mortality. It develops from benign nevi or cellular stress and has high metastatic potential. Prognostic and predictive factors include tumor thickness, mutations (BRAF, NRAS, TMB/MSI status), and immune infiltration. Higher YAP/TAZ expression correlates with invasiveness and therapy resistance.

2. OBJECTIVES

I. Establishing a pan-cancer database for immune checkpoint inhibitors.

The first objective was to identify publicly available gene expression datasets of cancer patients treated with anti-PD1, anti-PD-L1, or anti-CTLA-4 immune checkpoint inhibitors.

II. Identifying predictive and pharmacologically targetable biomarkers of immune checkpoint inhibitors.

By using the established database, the second objective was to find biomarkers of resistance to anti-PD1, anti-PD-L1, and anti-CTLA4 therapies, focusing on those with pharmacological applicability.

III. Characterization of a clinically relevant, targetable biomarker in a selected tumor.

The third objective was to characterize a selected biomarker in a specified tumor type, where the target is the most robust and the well-supported by the literature.

IV. *In vivo* validation of the identified biomarker to potentiate immune checkpoint inhibitor responses.

The next aim was to validate the database by testing the previously identified target – demonstrating the benefits of the selected combination therapy in mouse models.

V. Molecular characterization of tumors following immune checkpoint inhibitor potentiation.

Lastly, to investigate the molecular mechanisms observed in mice following treatment with an immune checkpoint inhibitor and inhibition of the selected target.

3. METHODS

We searched NCBI GEO, CRI iAtlas, and literature for ICI-treated cancer datasets with clinical and bulk-tissue gene expression data. Patients were classified as responders or non-responders based on survival or RECIST criteria. Gene expression data were merged, quantile normalized, and analyzed using Mann-Whitney U-test, ROC curves, and survival analysis, with Bonferroni-adjusted significance. We extended ROC Plotter and Kaplan-Meier Plotter platforms for validation. Druggable resistance biomarkers were identified by screening pre-treatment samples from anti-PD1, anti-PD-L1, or anti-CTLA-4 therapies for significant protein-

coding genes with >1.5-fold change in non-responders. A selected biomarker candidate was tested using CellTiter-Glo assay in B16-F10 and YUMM1.7 melanoma cells, cultured under standard conditions, and treated with Verteporfin at 0.1 – 10 μM concentrations. Cell viability was measured via luminescence, and Friedman-test was applied for statistical analyses in GraphPad Prism. In vivo studies followed EU Directive 2010/63/EU, using male C57BL/6JRj mice housed in ventilated cages. 500,000 cells of YUMM1.7 and B16-F10 were subcutaneously injected into 8-week-old mice. Once tumors reached 300 mm^3, mice were randomized into four groups: IgG2a isotype control, anti-PD1, Verteporfin, or combination therapy. Treatments were administered i.p. every other day. Tumor volumes were normalized to body weights, and statistical analysis was performed in SPSS using independent t-tests with Benjamini-Hochberg correction. Histological sections were prepared from FFPE tumor blocks, deparaffinized, rehydrated, and stained with CD3ε and PCNA antibodies, imaged using a Leica LMD6 microscope. RNA was extracted from tumor samples via chloroform/isopropanol precipitation, quantified with a

NanoPhotometer, and converted to cDNA using SensiFAST kit. RT-qPCR was performed on a LightCycler 480 II, with PPIA and HPRT as housekeeping genes. Relative expression was calculated using the $2^{-\Delta\Delta Cp}$ method, followed by statistical comparisons via ANOVA or Kruskal-Wallis tests.

4. RESULTS

Integrative database of immune-checkpoint inhibitor treated cancer patients

With the utilization of NCBI GEO, CRI iAtlas, and referenced literatures, 246 datasets were involved with 3,823 samples to be screened. After omitting datasets with unfitting or duplicated data, we manually screened 1,502 samples. Of which, 68 samples were excluded due to duplication, irrelevant treatment, or no available data. The final database consists of 1,434 samples from 19 datasets. This database was integrated to www.rocplot.com/immune ROC Plotter Immunotherapy analysis platform consisting of melanoma (n = 570), urothelial cancer (bladder/ureter/pelvis cancer) (n = 438),

head and neck squamous cell carcinoma (*n* = 110), esophageal and gastroesophageal junction adenocarcinoma (*n* = 103), lung cancer (small cell and non-small-cell lung cancer, or squamous and non-squamous non-small cell lung cancer) (*n* = 60), gastric cancer (*n* = 45), renal cell carcinoma (*n* = 44), glioblastoma (*n* = 28), hepatocellular carcinoma (*n* = 22), breast cancer (triple-negative (*n* = 12), and ER+HER2- breast cancer (*n* = 2)). Patients either received anti-PD1 (nivolumab, or pembrolizumab) (all *n* = 877; pre-treatment *n* = 776; on-treatment *n* = 101), anti-PD-L1 (atezolizumab, or durvalumab) (all *n* = 488; pre-treatment *n* = 457; on-treatment *n* = 31), or anti-CTLA-4 (ipilimumab) (all *n* = 124; pre-treatment *n* = 98*;* on-treatment *n* = 26) treatments, or *n* = 55 combination therapy.

Pan-cancer biomarkers of anti-PD1, anti-PD-L1, and anti-CTLA-4 baseline resistance with pharmacological interventions

In the anti-PD1 pre-treatment group, ROC AUC and p-values of 29,755 genes were analyzed. After applying

Bonferroni correction (p < 1.6E-06) to mitigate false discoveries, 912 genes remained significant. We investigated only those genes that showed FC > 1.5 in non-responding patients, including *STK35* (FC = 1.7, AUC = 0.651, p = 1.4E-08), *SPIN1* (FC = 1.6, AUC = 0.682, p = 9.1E-12), *SRC* (FC = 1.6, AUC = 0.667, p = 5.9E-10), *SETD7* (FC = 1.7, AUC = 0.663, p = 1.0E-09), *TEAD3* (FC = 1.7, AUC = 0.649, p = 4.1E-08), *FGFR3* (FC = 2.1, AUC = 0.657, p = 3.7E-09), *YAP1* (FC = 1.6, AUC = 0.655, p = 6.0E-09), and *BCL2* (FC = 2.2, AUC = 0.634, p = 9.7E-08).

In the anti-PD-L1 pre-treatment group, 26,819 genes were analyzed, and 38 hits were significant (p < 1.8E-06). We found no tumor-agnostic, upregulated, druggable genes of resistance.

In the anti-CTLA-4 pre-treatment group, 22,561 genes were analyzed, yielding 80 significant genes. Among them, only *BLCAP* (FC = 1.7, AUC = 0.735, p = 2.1E-06) was identified as a druggable gene overexpressed in the pan-cancer cohort of non-responding patients.

Predictive biomarkers of anti-PD1 resistance in melanoma

We re-ran ROC and Mann-Whitney tests and performed a survival analysis using the anti-PD1 pre-treament melanoma cohorts only. After Bonferroni-correction, twenty-one genes showed significant overexpression in the anti-PD1 resistant melanoma group. Among these, we found available inhibitors for six targets: *YAP1*, spindlin 1 (*SPIN1*), eukaryotic translation initiation factor 4H (*EIF4H*), solute carrier family 25 member 36 (*SLC25A36*), lysophospholipase 1 (*LYPLA1*), and GID complex subunit 4 homolog (*GID4*).

Yes-associated protein 1 (YAP1) as a druggable, predictive, and prognostic biomarker of anti-PD1 resistance in melanoma

In the anti-PD1 pre-treated melanoma cohort, *YAP1* emerged as the most promising target (FC = 1.85, Mann-Whitney p-value = 1.07E-08, AUC = 0.699, ROC AUC p-value = 7.50E-11). Higher *YAP1* expression also correlated with poorer progression-free survival (HR =

2.51, p = 1.2E-06), and overall survival (HR = 2.15, p = 1.2E-05). Based on the robust predictive and prognostic capabilities of *YAP1*, we chose this target for *in vitro* and *in vivo* validation using verteporfin inhibitor.

Yes-associated protein 1 (YAP1) inhibition with verteporfin in melanoma cells

We assessed the effects of verteporfin (VP) on YUMM1.7 and B16-F10 melanoma cell viability using a luminescent assay. Only the highest VP concentration (10 μM) showed a significant reduction in viability after 24 hours, compared to both untreated cells (p = 0.0378) and the vehicle control (p = 0.0019) in B16-F10. In YUMM1.7 cells, 5 μM VP was also effective, significantly decreasing viability compared to untreated (p = 0.0305) and vehicle control cells (p = 0.0001). Extending the incubation period to 48 hours enhanced the treatment in both cell lines: 1 μM VP reduced cell viability in B16-F10 (p = 0.0210) and YUMM1.7 (p = 0.0030) cells. These findings indicate that verteporfin treatment effectively decreases cell viability in our tumor models.

Verteporfin potentiates anti-PD1 therapy in mice bearing $BRAF^{V600E}$ mutations in melanoma

To investigate whether YAP1 inhibition could enhance the efficacy of anti-PD1 therapy in melanoma, we treated C57BL/6J mice bearing YUMM1.7, and B16-F10 tumors with the following regimens: 200 μg isotype control, 200 μg anti-PD1, 50 mg/kg verteporfin, and a combination of verteporfin+anti-PD1. Tumor volumes in the YUMM1.7-inoculated verteporfin+anti-PD1 group were significantly smaller compared to the isotype control ($p = 0.021$, adjusted $p = 0.063$) and the anti-PD1 monotherapy group ($p = 0.008$, adjusted $p = 0.048$). However, neither verteporfin alone ($p = 0.425$) nor anti-PD1 alone ($p = 0.971$) showed a significant advantage over the control group. While verteporfin monotherapy appeared more effective than anti-PD1, the difference was not statistically significant. The combination therapy resulted in the greatest reduction in tumor weight (mean ± SD: 1.738 g ± 0.73) compared to anti-PD1 (2.609 g ± 0.78) ($p = 0.038$). We did not find significant differences between verteporfin (2.238 g ± 0.95), or the isotype control groups (2.630 g ± 1.36) in YUMM1.7 tumor mass.

Mice inoculated with B16-F10 melanoma tumors exhibited rapid tumor progression, rendering them unresponsive to all treatments thus early euthanasia was required, leading to small sample size at the end of the study (n = 9 animals altogether). Thus, data was not evaluated in the B16-F10 group.

Given the strong contrast in treatment responses between the YUMM1.7 and B16-F10 models, we examined *YAP1* expression in these tumors. YUMM1.7 tumors exhibited significantly higher *YAP1* expression compared to B16-F10 tumors, with significant differences observed in both the isotype control groups ($p = 0.003$) and the anti-PD1-treated groups ($p = 0.001$). However, anti-PD1 monotherapy did not directly affect *YAP1* expression in either tumor model.

These show that the presence of *BRAF* in YUMM1.7 tumors contribute to higher *YAP1* expression, enhancing their response to verteporfin while simultaneously driving resistance to anti-PD1 therapy. In contrast, B16-F10 tumors, which lack these mutations, remain unresponsive to the mentioned treatments.

Verteporfin plus anti-PD1 combination therapy shifts immunologically "cold" tumors to "hot"

Next, we analyzed dissected tumor samples from YUMM1.7-inoculated mice to evaluate proliferative and immune infiltrative characteristics. Hematoxylin & eosin staining, along with the proliferation marker PCNA, confirmed a high density of tumor cells across all treatment groups, with no discernible effect on proliferation. Based on RT-qPCR, *CD3ε* mRNA expression was higher in the verteporfin+anti-PD1 group compared to the isotype control ($p = 0.047$).

Further investigation of tumor-associated immune cell types revealed overexpression of *PTPRC* (CD45) in the verteporfin+anti-PD1 group ($p = 0.045$), a pan-leukocyte marker essential for lymphocyte activation. Additionally, verteporfin monotherapy downregulated *FOXP3* (forkhead box protein 3) expression compared to anti-PD1 treatment ($p = 0.031$), with a similar but non-significant trend observed after combination therapy, suggesting that VP may help to counteract immune-suppressive, tumor-promoting signals. In the combination therapy group, *CD68* was elevated ($p = 0.009$ vs. isotype control, $p =$

0.029 vs. anti-PD1), along with *CD86* ($p = 0.026$ vs. isotype control, $p = 0.048$ vs. VP) and *CD80* ($p = 0.030$ vs. anti-PD1), markers typically expressed on pro-inflammatory, tumor-eliminating M1 macrophages. *PDCD1* (PD-1) expression increased after anti-PD1 monotherapy and combination therapy compared to VP monotherapy ($p = 0.013$, $p = 0.024$, respectively), while *CD274* (PD-L1) showed no differences across groups. No differences were detected in the immunosuppressive neutrophil marker, *LY6G*. These findings indicate that anti-PD1 monotherapy is entirely ineffective in immunologically cold, exhausted tumors like YUMM1.7 and B16-F10. In YUMM1.7, after anti-PD1 therapy, the therapeutic efficacy was hindered despite optimal conditions, such as the presence of immune cells, high PD-1 and PD-L1 expression, likely due to YAP1 overactivation. Adding verteporfin to checkpoint-arrested, immune-infiltrated, YAP1-overexpressed tumors enhance anti-PD1 therapy by improving immune recognition.

5. CONCLUSIONS

I. Robust database of immune checkpoint inhibitor-treated cancer patients

a. A comprehensive database was set up using 1,434 tumor tissue samples from 1,323 patients from solid tumors, collected before or after treatment with nivolumab, pembrolizumab, atezolizumab, durvalumab, or ipilimumab, were included in the final version.

b. The database was integrated into ROC Plotter Immunotherapy and Kaplan-Meier Plotter Immunotherapy web platforms.

II. Predictive, tumor-agnostic biomarkers of immune checkpoint inhibitor resistance

a. Predictive markers of baseline immune checkpoint inhibitor resistance was identified in the anti-PD1 group: *YAP1*, *TEAD3*, *SRC*, *SETD7*, *FGFR3*, *BCL2*, *STK35*. While *BLCAP* was overexpressed in anti-CTLA-4-resistant samples and was found to be a potential druggable target.

III. YAP1 as a druggable biomarker of anti-PD1 resistance in melanoma

a. In the melanoma anti-PD1 pre-treatment group (n = 415), we identified *YAP1* as the strongest predictive and prognostic biomarker, associated with therapy resistance (ROC AUC = 0.699, FC = 1.8, p = 1.1E-08), and poorer survival outcomes (PFS: HR = 2.51, p = 1.2E-06, FDR = 1%, and OS: HR = 2.15, p = 1.2E-05, FDR = 1%).
b. We chose YAP1 for *in vivo* validation with its inhibitor, verteporfin.

IV. Targeting YAP1 with verteporfin to overcome anti-PD1 resistance

a. Verteporfin reduced tumor cell viability in a dose,- and time-dependent manner in B16-F10 and YUMM1.7 melanoma cell lines. YUMM1.7 cells were more sensitive to VP treatment than B16-F10 cells.
b. In C57BL/6J mice with $BRAF^{V600E}$ YUMM1.7 tumors, the combination of verteporfin and anti-PD1 therapy resulted in the greatest reduction in tumor size and weight. Wild-type B16-F10 tumors, which exhibited lower *YAP1* expression both before and after treatment, remained unresponsive to all treatments.

c. *YAP1* overexpression is a major driver of anti-PD1 resistance in melanoma.

V. Immune modulation by verteporfin: from cold tumors to hot tumors

a. RT-qPCR and immunostaining of YUMM1.7 tumors showed that verteporfin+anti-PD1 therapy enhanced immune infiltration, shifting tumors from a “cold" phenotype to a more infiltrated state:

b. Increased *CD3ε*, *CD45* , *CD68*, *CD80*, and *CD86* levels, while decreased *FOXP3* levels, suggesting anti-tumoral immune responses.

c. No significant differences were observed in PD-L1 or *LY6G* expression.

d. *YAP1* may act as a master regulator of ICI-resistance in melanoma by influencing IFN pathways.

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