Inhibition of CDK4/6 Overcomes Primary Resistance to PD-1 Blockade in Malignant Mesothelioma

Running title: CDK4/6 inhibitors beat immune resistance

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ABSTRACT

Background: Despite the profound number of malignant pleural mesothelioma (MPM) patients now treated with PD-1 blockade, insight into the underpinnings of rational therapeutic strategies to treat resistance to checkpoint immunotherapy remain unrealized. Our objective was to develop a novel therapeutic approach to overcome primary resistance to PD-1 blockade in MPM.

Methods: We generated a transcriptome signature of resistance to PD-1 blockade in MPM patients treated with nivolumab (4 responders and 4 non-responders). We used the TCGA MPM cohort (N=73) to determine what genomic alterations were associated with the resistance signature. We tested whether regulation of identified molecules could overcome resistance to PD-1 blockade in an immunocompetent mouse malignant mesothelioma model.

Results: Immunogenomic analysis by applying our anti-PD-1 resistance signature to the TCGA cohort revealed that deletion of *CDKN2A* was highly associated with primary resistance to PD-1 blockade. Under the hypothesis that resistance to PD-1 blockade can be overcome by *CDK4/6* inhibition, we tested whether *CDK4/6* inhibitors could overcome resistance to PD-1 blockade in subcutaneous tumors derived from *Cdkn2a*(-/-) AB1 malignant mesothelioma cells, which were resistant to PD-1 blockade. The combination of daily oral administration of *CDK4/6* inhibitors (abemaciclib or palbociclib) and intraperitoneal anti-PD-1 treatment markedly suppressed tumor growth, compared with anti-PD-1 or *CDK4/6* inhibitor alone.

Conclusions: We identified a novel therapeutic target, *CDK4/6*, to overcome primary resistance to PD-1 blockade through comprehensive immunogenomic approaches. These data provide a rationale for undertaking clinical trials of *CDK4/6* inhibitors in the more than 40% of patients with MPM who demonstrate loss of *CDKN2A*.

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INTRODUCTION

Immune checkpoint inhibitors (ICIs) have dramatically changed the treatment approach to various advanced cancers, resulting in durable clinical responses in many cases.¹⁻⁴ Programmed Cell Death 1 (PD-1) inhibitors have recently shown encouraging clinical activity with good tolerability in patients with advanced malignant pleural mesothelioma (MPM) previously treated with chemotherapy. In these single-agent trials, objective response rates, defined as complete response (CR) or partial response (PR), ranged from 15 to 21%; and rates of stable disease (SD) were 33 to 56%, indicating a favorable impact of these drugs on durable clinical benefit.⁵⁻⁷ However, similar to patients with other tumor types treated with anti-PD-1 therapy, most patients with MPM do not respond to PD-1 inhibition, highlighting an unmet need for the development of novel therapeutic approaches to augment the efficacy of immune checkpoint treatment.

Anti-PD-1 monoclonal antibody blocks the interaction between PD-1 on activated T cells and its ligands, which is thought to reinvigorate T cells to kill cancer cells.^{8,9} However, the therapeutic efficacy of PD-1 checkpoint inhibition is limited by primary and acquired resistance.¹⁰⁻¹³ Resistance to ICIs may be classified into two categories: (1) primary resistance, which generally refers to patients who do not respond at all to ICIs and instead progress; and (2) acquired resistance referring to those who have an initial response to ICIs followed eventually by disease progression. The mechanisms responsible for primary resistance continue to be characterized as ineffective T cell priming, lack of tumor recognition due to defective antigen presentation, suppression via other checkpoints, tumor cell resistance to interferon, and local immunosuppressive factors in the tumor-immune microenvironment.¹⁴ Furthermore, there is emerging data pointing to the loss-of-function mutations in JAK1 and JAK2 from the IFN signaling pathway and beta-2 microglobulin (B2M) from the antigen presentation pathway in patients who develop acquired resistance to immune checkpoint blockade therapy.¹⁵⁻¹⁸

To date, there have been no approved therapeutic breakthroughs for thwarting resistance, and there has been a remarkably unmet need to investigate the mechanisms of resistance to ICIs. Understanding the underlying mechanisms of resistance may allow the rational design of combinatorial therapies to overcome

primary resistance. Therefore, mechanism-based strategies to overcome resistance to PD-1 blockade therapy are urgently needed. Our objective was to develop a novel therapeutic approach to overcome resistance to PD-1 blockade in MPM through comprehensive immunogenomic analysis.

MATERIAL and METHODS

Patients and Data Acquisition

This study was performed in accordance with Institutional Review Board protocol at Baylor College of Medicine (H-43208). Informed consent was obtained for the collection of clinical data and biospecimens. We performed transcriptome mRNA arrays using the pre-immunotherapy tumor biopsies of eight MPM patients with advanced and unresectable MPM, whom we treated with nivolumab (anti-PD-1) therapy after they had progressed following treatment with a platinum-based agent and pemetrexed (**Supplementary Table 1**).¹⁹ Original mRNA expression data deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus database (GSE99070) were used to generate a transcriptome-based anti-PD-1 resistance signature. To identify the association of genetic alteration with this anti-PD-1 resistance signature, we utilized mRNA sequencing data from 73 samples in The Cancer Genome Atlas (TCGA) portal (<https://gdc-portal.nci.nih.gov/>).²⁰

Briefly, anti-PD-1 resistance signature was used to generate a support vector machine (SVM) classifier²¹ for estimating the likelihood that a particular MPM tumor belonged to the subgroup in which the anti-PD-1 resistance signature is present (anti-PD-1 resistant subgroup) or the subgroup in which the signature is absent (anti-PD-1 responsive subgroup). The robustness of the classifier was estimated by the misclassification rate determined during leave-one-out cross-validation (LOOCV) of the training set. After LOOCV, the sensitivity and specificity of the prediction models were estimated by the fraction of samples correctly predicted.

Mouse malignant mesothelioma cell line and syngeneic mouse tumor model

AB1 mouse malignant mesothelioma (MM) cell lines, purchased from Sigma-Aldrich, were thawed and cultured at 37°C with 5% CO₂ in RPMI 1640 (with 2mM L-Glutamine+25mM HEPES) supplemented

with 5% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Six-week-old male BALB/cJ mice (Jackson Laboratory) were used under the protocol approved by the Institutional Animal Care and Use Committee, in accordance with all relevant animal-use guidelines and ethical regulations. To develop the immunocompetent mouse model of MM, 5×10^5 tumor cells in 50 µL of the serum-free medium were inoculated subcutaneously on the right flank area, where was prepared with 70% alcohol. Tumor size was monitored three times per week. The greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined to determine by external caliper and used to calculate tumor volume. The tumor volume, based on these caliper measurements, was calculated using the modified ellipsoidal formula: tumor volume = $\frac{1}{2}(\text{length} \times \text{width}^2)$.

Before *in vivo* drug treatment, mice were randomly divided into 6 groups when the length of tumor of each mouse reached 5mm. For anti-PD-1 therapy, anti-mouse PD-1 Abs (10mg/kg, clone 29F.1A12, BioXCell, Cat# BE0273) or isotype control (Clone 2A3, BioXCell, Cat# BE0089) were injected intraperitoneally twice per week. For cyclin-dependent kinase (CDK) 4/6 inhibition, abemaciclib (Cat# S5716) and palbociclib (Cat# S1116) was purchased from Selleckchem. Abemaciclib (75mg/kg) and palbociclib (100mg/kg) dissolved in normal saline were administered by oral gavage daily. Mice were euthanized three weeks after the first treatment to measure tumor burdens.

Methods for Imaging mass cytometry (IMC) and time-of-flight mass cytometry (CyTOF) were described in the supplementary section (**Supplementary Table 2 and 3**).

Statistical Analysis

Student's t-tests or Mann-Whitney U tests were used to compare continuous variables, and Chi-square or Fisher's exact tests were performed to analyze categorical variables. Z values were calculated by subtracting the overall average gene expression from the raw data for each gene and dividing that result by the standard deviations of all of the measured expression. All statistical analyses were performed with SPSS 27.0 (IBM Corp, Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY), Prism 5.0 (GraphPad Software, Inc, San Diego, CA), and R language and software environment version 4.0.3

(<http://www.r-project.org>). Statistical significance was considered at $P < 0.05$, and all tests were two-tailed.

RESULTS

Development of anti-PD-1 resistance mRNA signature.

This study scheme is illustrated in **Figure 1**. We first designed an anti-PD-1 resistance mRNA signature representative of resistance to PD-1 checkpoint immunotherapy by comparing mRNA expression in 4 responders (CR+PR) and 4 MPM patients with progressive disease after nivolumab treatment. Differential gene expression between responders and progressors was found in 158 differential mRNAs with a p-value less than 0.01 and above 2-fold changes, which was defined as our anti-PD-1 resistance signature (**Fig.2A** and **Supplementary Table 4**). Exploratory analysis of upstream regulators through the Ingenuity pathway analysis (<http://www.ingenuity.com>) revealed that the anti-PD-1 resistance gene signature was significantly associated with CDK4/6 and cyclin D1 activation in the cell cycle pathway (**Fig.2B**).

Resistance to PD-1 blockade is associated with CDKN2A deletion.

To determine the genomic alterations associated with the anti-PD-1 resistance signature, our immune signature was applied to predict the subgroups in the TCGA MPM cohort (N=73), utilizing a previously developed model (**Fig.3A**).^{19,22,23} No clear associations of clinical features were observed between two subgroups. BAP1 mutation was observed only in the anti-PD-1 responsive subgroup ($P=0.016$). Furthermore, anti-PD-1 responsive tumors had abundant plasma cells and M1 macrophages, which were calculated using CIBERSORT (<http://cibersort.stanford.edu/>), an analytic deconvolution method of “*in silico* flow cytometry” that accurately estimates the abundances of specific leukocyte subsets using gene expression data.²⁴ In contrast, MPM tumors predicted as anti-PD-1 resistant tumors had a significant deep deletion of Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*) located at 9p21.3. *CDKN2A* loss was found in 47% (N=34) of MPM patients. Among them, 74% (N=25) were classified into anti-PD-1 resistant tumors. Resting memory CD4 T cells were significantly increased in this subgroup (**Fig.3B**). Representative

146 multiplexed images of an MPM patient with intact *CDKN2A* who had a partial response to nivolumab,
147 showed abundant infiltration of CD8 T cells and B cells. In contrast, the other representative images of an
148 MPM patient with *CDKN2A* loss who had progressive disease, showed abundant infiltration of tumor-
149 associated macrophages (TAMs) in the absence of CD8 T cells (**Fig.4**).

150 In addition, antigen presenting machinery including *HLA-A*, *HLA-B*, *HLA-C*, and *B2M* was
151 significantly downregulated in the anti-PD-1 resistant subgroup (**Fig.5A**). The cytolytic activity²⁵ derived
152 from the geometric mean of *GZMA* and *PRFI*, and the *IFN-γ* signaling signature (*CIITA*, *GBP4*, *GBP5*,
153 *IRF1*, *IRF2*, and *JAK2*)²⁶ were significantly lower in the anti-PD-1 resistant subgroup, compared with those
154 of the anti-PD-1 responsive subgroup (**Fig.5B**). On the contrary, *CDK4* and *CDK6* mRNA expression was
155 significantly increased in the anti-PD-1 resistant subgroup (**Fig.5C**), implying cell cycle activation through
156 the depletion of *CDKN2A*.

157 **CDK4/6 inhibitors overcome resistance to PD-1 checkpoint immunotherapy.**

158 *CDKN2A* is a well-known tumor suppressor that inhibits CDK4/6. The *CDKN2A-cyclin D-*
159 *CDK4/6-retinoblastoma* protein pathway promotes G1-S cell-cycle transition, and this pathway is
160 commonly dysregulated in most cancers. CDK4/6 inhibitors leading to cell-cycle arrest, senescence, and/or
161 cell apoptosis are well described in preclinical models of various cancers, including melanoma and breast
162 cancer.^{27,28} Increased activity of CDK4/6 is seen where *CDKN2A* is deleted.^{29,30} Therefore, we hypothesized
163 that CDK4/6 inhibition could sensitize the tumor-immune microenvironment to PD-1 blockade. Whole
164 exome sequencing of fifteen asbestos-induced murine MM tumor cell lines from BALB/c, CBA, and
165 C57BL/6 mouse strains showed homozygous loss of *Cdkn2a* in 14 out of 15 tumors including AB1 MM
166 cells, which demonstrated resistance to PD-1 blockade *in vivo* (**Fig.6A**).³¹ To test our hypothesis, we used
167 a subcutaneous mouse malignant mesothelioma (MM) model using the AB1 cell line. Like human MPM
168 resistant to PD-1 blockade, this mouse MM tumor contained abundant TAMs with less lymphocyte
169 infiltration (**Fig.6B**).

FDA-approved oral CDK4/6 inhibitors, palbociclib (100mg/kg) and abemaciclib (75mg/kg), were administered daily 7 days after tumor inoculation for 3 weeks, and anti-mouse PD-1 antibody (10mg/kg) was administered intraperitoneally twice per week (**Fig.7A**). CDK4/6 inhibition slowed tumor growth compared with untreated and anti-PD-1 treated groups. Abemaciclib or palbociclib single treatment showed modest effects for controlling tumor growth. A combination of CDK4/6 inhibitor and PD-1 blockade markedly suppressed tumor growth (both $P < 0.001$) (**Fig.7B-7C**).

CytoF displayed that the majority of immune compositions in untreated AB1 tumors were TAMs (**Fig.8**). Treatment of anti-PD-1 antibody led to a modest increase of T cells and a slight decrease of TAMs. Palbociclib treatment led to a significant increase of both B cells and CD4 T cells, and the significant decrease of TAMs. Combination therapy resulted in abundant infiltration of B cells and CD4 T cells and a dramatic decrease of TAMs. Taken together, a combination of CDK4/6 inhibitors (palbociclib or abemaciclib) and a PD-1 inhibitor eradicated anti-PD-1-resistant Cdkn2a(-) AB1 MM tumors with tumor cells through the reprogramming of the tumor-immune microenvironment.

COMMENT

Immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have changed the standard-of-care treatment for a variety of advanced cancers. In MPM, PD-1 inhibitors have recently shown encouraging clinical activity with good tolerability in patients previously treated with chemotherapy.⁵⁻⁷ However, the mechanisms of response and resistance to immune checkpoint blockade in human tumors are only beginning to be understood and are still unknown in MPM. Through comprehensive immunogenomic analysis, we uncovered that *CDKN2A* loss is associated with primary resistance to PD-1 blockade. Our *in vivo* experiment using immunocompetent mouse MM model with primary resistance to anti-PD-1 treatment supported that suppressing CDK4/6 activated by *CDKN2A* loss overcomes primary resistance to checkpoint immunotherapy.

Deletions in the 9p21 area containing *CDKN2A* and *CDKN2B* genes are found in 45-52% of

MPMs.^{20,32,33} Targeting proliferative cancer cells by CDK4/6 inhibitors may directly eliminate cancer cells, which results in the sensitization of the tumor immune microenvironment to PD-1 blockade by abundant neoantigen exposure. Furthermore, a potential link between CDK4/6 activity and PD-L1 protein stability was recently reported.³⁴ In addition to the anti-proliferative effect of CDK4/6 inhibitors on cancer cells, recent studies have indicated that CDK4/6 inhibitors may influence immune cells in the tumor microenvironment, and CDK4/6 inhibitor-induced senescence may enhance the production of a functional chemoattractant for T cells.³⁵ A strong relationship between copy number loss of a large region of chromosome 9p and decreased lymphocyte estimates was also reported in melanoma, pancreatic, and head/neck cancers.³⁶ The analysis of specimens before and after nivolumab treatment suggests that genetic alterations in *SOX2* and *CDKN2A/CDKN2B* and changes in the tumor microenvironment could be reasons for the acquired resistance to nivolumab observed in the patient with lung cancer.³⁷

To face the challenge of primary resistance, constant effort has been made on combination strategies to broaden the responders.^{12,38,39} Furthermore, there has been an extensive search for predictive biomarkers for initial ICI response. PD-L1 expression, tumor mutational burden, tumor-infiltrating lymphocytes, or related gene expression signatures have been explored as potential predictors, and various other markers are currently under investigation.⁴⁰ Our study also provides the potential role of homozygous deletion of *CDKN2A* by fluorescence in situ hybridization as a mechanistic predictive marker to recommend the combination treatment of CDK4/6 inhibitors with ICIs.

Our study limitations include the small number of patients with more advanced disease receiving ICIs to generate the immunologic profile that was applied to the TCGA cohort with earlier disease, requiring further mechanistic investigation with multiple strains to determine the effect of CDK4/6 inhibitors on multiple cellular compositions as well as cancer cells, and uncovering the cause and effect between *CDKN2A* and the alteration of tumor milieu.

217 CONCLUSIONS

218 Through comprehensive immunogenomic approaches, we have identified CDK4/6 as a novel
219 therapeutic target for overcoming primary resistance to PD-1 blockade. These data provide the rationale for
220 undertaking clinical trials of CDK4/6 inhibitors combined with PD-1 blockade in the more than 40% of
221 patients with malignant pleural mesothelioma who demonstrate loss of *CDKN2A*.

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FIGURE LEGENDS

Figure 1. Study scheme.

A transcriptome signature of resistance to PD-1 blockade was generated from malignant pleural mesothelioma (MPM) patients treated with nivolumab. To identify target candidates to overcome resistance, the upstream regulator analysis was performed. Next, the TCGA MPM cohort was used to determine what genomic alterations are associated with immune resistance signature. Finally, to test if targeting candidate molecules can increase the efficacy of PD-1 blockade, we performed *in vivo* experiment.

(CR, complete response; PD, progressive disease; PD-1, programmed cell death 1; PR, partial response; TCGA, The Cancer Genome Atlas)

Figure 2. Development of anti-PD-1 resistance mRNA signature.

A. Development of transcriptome-based anti-PD-1 resistance signature. The data are presented in a matrix format in which rows represent individual gene and columns represent individual experiment. The red and blue colors in the cells reflect relative high and low expression levels, respectively, as indicated in the scale bar (log2 transformed scale). **B.** Upstream regulator analysis through the Ingenuity pathway analysis revealed that anti-PD-1 resistant gene signature was significantly associated with Cyclin-dependent kinase 4/6 and cyclin D1 activation in cell cycle pathway.

Figure 3. CDKN2A deletion is associated with α PD-1 resistance.

A. The anti-PD-1 resistant mRNA signature was used to predict the subgroup in 73 TCGA MPM cohort. **B.**

MPM tumors predicted as anti-PD-1 resistant tumors had a significant deletion of CDKN2A located at 9p21.3. CDKN2a loss was found in 47% of MPM patients.

Among them, 74% were classified into anti-PD-1 resistant tumors.

(LOOCV, leave-one-out cross validation; SVM, support vector machine; TCGA, The Cancer Genome Atlas)

Partial Response to nivolumab: *CDKN2A* intact

Progressive Disease to nivolumab: *CDKN2A* loss

Figure 4. Representative images to show the association of *CDKN2A* status with the tumor-immune microenvironment.

Representative images of an MPM patient with intact *CDKN2A* who had a partial response, showed abundant infiltration of CD8 T cells and B cells. In contrast, the other representative images of an MPM patient with *CDKN2A* loss who had progressive disease, showed abundant infiltration of TAMs with the absence of CD8 T cells.

(Ir, iridium; Pan-CK, pan-cytokeratin.)

Figure 5. Comparison of mRNA expression related to anti-PD-1 response and resistance.

A. The expression of B2M and MHC I molecules was significantly reduced in the anti-PD-1 resistance group. **B.** Cytolytic activity and IFN- γ signature. Subset predicted as anti-PD-1 resistance demonstrated significantly decreased cytolytic activity and the IFN- γ signaling signature. **C.** Cell cycle-related CDK4/6 expression.

(RPKM, Reads Per Kilobase of transcript, per Million mapped reads.)

Figure 6. Anti-PD-1 resistant mouse malignant mesothelioma model using *Cdkn2a*(-) AB1 cell line.

A. AB1 MM tumors were resistant to PD-1 blockade *in vivo*. **B.** Like human MPM resistance to PD-1 blockade, this mouse mesothelioma tumor contained abundant tumor-associated macrophages (TAMs) with less lymphocyte infiltration.

Figure 7. CDK4/6 inhibitors overcome resistance to PD-1 blockade.

A. Treatment scheme. FDA-approved oral CDK4/6 inhibitors, palbociclib and abemaciclib, were daily administered 7 days after tumor inoculation for 3 weeks, and anti-mouse PD-1 antibody was administered intraperitoneally twice per week (each group n=5). **B, C.** Both abemaciclib and palbociclib showed modest effects for controlling tumor growth. A combination of CDK4/6 inhibitor and PD-1 blockade markedly suppressed tumor growth.

Figure 8. CDK4/6 inhibitors alter the tumor-immune microenvironment.

Time of flight mass cytometry showed that most immune compositions in untreated AB1 tumors were tumor-associated macrophages (TAMs). A single treatment of anti-PD-1 led to a modest increase of T cells and a slight decrease of TAMs. Palbociclib treatment led to a significant increase of B cells and CD4 T cells, and a significant decrease of TAMs. Combination therapy resulted in abundant infiltration of B cells and CD4 T cells and a dramatic decrease of TAMs.

GLOSSARY OF ABBREVIATIONS

- B2M: beta-2-microglobulin
- CDKN2A: Cyclin Dependent Kinase Inhibitor 2A
- CDK4/6: Cyclin Dependent Kinase 4/6
- CR: complete response
- CyTOF: time-of-flight mass cytometry
- FFPE: formalin-fixed paraffin-embedded
- GEO: gene expression omnibus database
- ICIs: immune checkpoint inhibitors
- IMC: imaging mass cytometry
- LOOCV: leave-one-out cross validation
- MM: malignant mesothelioma
- MPM: malignant pleural mesothelioma
- NCBI: the National Center for Biotechnology
- PD: progressive disease
- PD-1: Programmed Cell Death 1
- PD-L1: Programmed Cell Death 1 Ligand 1
- PR: partial response
- RPKM: reads per kilobase of transcript, per million mapped reads
- SD: stable disease
- SVM: support vector machine
- TCGA: The Cancer Genome Atlas
- TAMs: tumor-associated macrophages