Downregulating MK5 to reduce immune escape induced by the binding of PD-1 with PD-L1 in NSCLC

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Abstract

Lung cancer is the leading cause of cancer death in both men and women worldwide. Previous studies have indicated that MK5 stabilizes Yes-associated Protein (YAP) in LATS1/2-null RPE1 cells, and YAP regulates PD-L1 expression in NSCLC. Since previous studies did not associate MK5 KO with PD-L1 expression in NSCLC, this study investigates whether downregulating MK5 in the H1299 cell line using MK5 CRISPR will reduce the expression of PD-L1 both in vitro and in vivo conditions for NSCLC. The negative control is a scrambled CRISPR targeting vector, and the positive control is the depletion of MK5. Through western blot and MTT in vitro and xenograft in vivo, the paper investigates whether or not downregulating MK5 will reduce immune escape induced by the binding of PD-1 with PD-L1 in NSCLC.

Keywords: MK5, YAP, PD-L1, immune escape, NSCLC, EGFR, targeted therapy

I. Introduction

The American Society of Clinical Oncology (ASCO) estimated that 2,206,771 people were diagnosed with lung cancer globally, and 82% of the patients are affected by non-small cell lung cancer (NSCLC) [1]. The existing treatment with EGFR-tyrosine kinase inhibitors (TKIs) has proven to be effective; however, the durability of the treatment is an issue as nearly half of the EGFR mutant NSCLC patients have acquired resistance to EGFR-TKIs; targetable secondary mutations remain unclear [2]. Without any targetable secondary mutations, patients will not receive adequate treatment, and their condition might worsen. Therefore, prolonging the resistance to EGFR-TKI and exploring effective treatments for NSCLC with EGFR-TKI resistance is necessary.

Mutated EGFR directly correlates with programmed death-ligand 1 (PD-L1) expression. High expression of mutant EGFR upregulates the expression of PD-L1 [3]. PD-L1 has an affinity to bind to programmed cell death protein-1 (PD-1), an immune checkpoint receptor. The binding of PD-L1 with PD-1 results in the failure of T cells to recognize the cancer cell and leads to immune escape [2]. The binding of EGFR-TKI and mutated EGFR would block signal transduction and turn down the entire pathway, reducing the expression of PD-L1 and activating an immune response.

Yes-Associated Protein (YAP) has played an essential role in the progression of human NSCLC. The Hippo pathway regulates YAP. The active Hippo pathway inhibits the activity of YAP by Hippo kinases and leads to degradation; the inactive Hippo pathway enables YAP to translocate into the nucleus, forming a complex with transcriptional enhancer factors (TEAD) that activate the transcription of PD-L1 [2]. MK5 is a kinase that protects YAP from degradation [4]. Thus, the downregulation of MK5 would inhibit the activity of YAP and reduce the expression of PD-L1, offering a potential treatment for NSCLC with EGFR-TKI resistance.

Therefore, in order to test the therapeutic effect of MK5 knockdown in preclinical conditions, a comparative study should be designed. This paper investigates the effect of knocking down MK5 in the H1299 cell line using MK5 CRISPR on the expression of PD-L1 in both in vitro and in vivo conditions.

Research Question: Will downregulating MK5 reduce immune escape induced by the binding of PD-1 with PD-L1 in NSCLC?

Hypothesis: I predict that knocking down MK5 in the H1299 cell line using MK5 CRISPR will reduce the expression of PD-L1 both in vitro and in vivo conditions. Measure PDL1 in MK5 KO and WT with western blot and MTT assay for cell growth in cell culture, develop MK5 KO and WT Xenograft mice and measure tumor size and weight through various time points PDL1 western blot from tumor cells in xenograft. The negative control is a scrambled CRISPR targeting vector, and the positive control is that the depletion of MK5 would reduce the LATS1/2-null RPE1 cell level.

II. Method and Materials

A. Cell culture

LATS1/2-null RPE1 cells are immortalized human nontransformed epithelial cells with exogenous telomerase, and CRISPER knocks down the LATS1/2 gene. It serves as a control. H1299 cell line is developed from a patient with lung carcinoma [9].

B. CRISPR & siRNAs

CRISPR is used to knockout MK5 using CAS9 nickase. siRNAs were purchased as validated or pre-designed from Abcam. The sequence of the siRNA used was MK5 siRNA 5′-GGA AUU AGU GGU CCA GUU A-3. siRNAs were transfected with Lipofectamine LTX and PLUS reagent (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen), respectively, according to the manufacturer's protocol [4].

C. Western Blot

Western blot was performed using a standard protocol. MK5 antibodies are used in this study. Target proteins were detected using enhanced chemiluminescence western blot detection solution (LumiGlo, KPL; Western Bright, Advansta) [4]. The positive control is the Positope™ Control Protein from ThermoFisher, to which 100 ng would be applied. The negative control is PD-L1 Knockout A549 Cell Lysate. Before being used as the negative control, it should be "spin down briefly and resuspend in 100 uL 1xSDS sample buffer (2% SDS, 60 mM Tris-HCl pH 6.8, 10% Glycerol, 0.02% Bromophenol blue, 60 mM beta-mercaptoethanol)" [8]. After heating the lysate for 3 - 5 minutes, inject the lysate into the well.

D. Xenograft

After NSCLC tumor burden was confirmed in vivo treatment of mice with MK5 antibodies by MRI imaging, each group was treated either with isotype control (untreated) or MK5 antibody on Days 0, 4, 8, and 12 (4 doses). Then on day 12, mice were sacrificed for analysis. Xenograft was hydrated with saline solution as a negative control group.

E. MTT assay

3-(4,5-Di-2-yl)-2,5-tetrazolium bromide (MTT) assay was performed to evaluate the cytotoxic effect and cell viability of PDL1 in MK5 KO cell culture. The cells were seeded in 96-well plates (3×103 cells/well), cultured for 24 h, and treated with the cytotoxic compound. At the end of the incubation period, the cells were incubated with 1 mg/ml MTT solution. SDS will be utilized along with DMSO to resolve the formazan and prevent further conversion to the reagent. After three hours, and ELX-800 spectrometer reader (Bio-Tek Instruments) is used to measure the absorbance at 450 nm [5]

F. Statistics Analysis

The numeric data are gathered through MTT assay, western blot, and in vivo treatment of mice. GraphPad Prism version 6 (GraphPad Software) was used for data analysis [4]. When the p-value was < 0.05, there was a significant difference between the observed and experimental results. All trials are repeated at least three times.

III. Results

Table 1. Possible Results (PR)

	Possible Results	PR 1	PR 2	PR 3	PR 4	PR 5	PR 6	PR 7	PR 8
In vitro	PDL1-WB decreases with MK5 KO?	+	+	-	-				
	MTT death decreases with MK5 KO measured by MTT	+	-	+	-				
In vivo	MK5 KO decreases PDL1 WB?					+	+	-	-
	Tumor size					-	+	-	+
hypothesis		Yes	Partially	Partially	No	Yes	partially	partially	No

Note: "+" represents a significant difference in the experiment following the possible result. "- "represents a significant difference in the experiment against the possible result. "+" represents an increase in tumor size for in vivo model. "- "represents a decrease in tumor size for in vivo model. Blank means no experimental data.

A. Possible Result 1: MK5 KO reduces immune escape in NSCLC.

In vitro cell culture, MK5 KO decreases the expression of PD-L1, as shown in the western blot assay. In the western blot, the experimental group would show a lighter colored band than the control group with MK5. The lighter band indicates a lower concentration. If the protein band runs as a thin line, there might not be a post-transcriptional modification. If the protein band does not have well-defined edges, post-transcriptional modification might occur. A double band would indicate that the protein is phosphorylated. In MTT assay, MK5 KO increases the cell viability with more remarkable cell survival.

B. Possible Result 2: MK5 KO decreases PD-L1 expression but does not impact cell viability.

Western blot suggests a decreased expression in PD-L1 with MK5 KO since a lighter-colored band is observed. The occurrence of other scenarios is enumerated in possible result one and applies to possible result 2. The result of the MTT assay can be classified into two scenarios: MTT death stays the same with MK5 KO, and MTT deaths increase with MK5 KO.

C. Possible Result 3: MK5 KO does not decrease PD-L1 expression but increases cell viability.

In vitro cell culture, MK5 KO does not decrease the expression of PD-L1 as shown in the western blot assay. The result in western blot can be classified into two scenarios: PD-L1 expression stays the same, and PD-L1 expression increases with MK5 KO. The band color will be the same in the first scenario. A darker protein band would occur in the second scenario, indicating greater protein concentration. The occurrence of other scenarios is enumerated in possible result one and applies to possible result 3. In MTT assay, MK5 KO increases the cell viability with more significant cell survival.

D. Possible Result 4: MK5 KO does not inhibit immune escape.

In vitro cell culture, MK5 KO does not decrease the expression of PD-L1 as shown in the western blot assay. The result in western blot can be classified into two scenarios: PD-L1 expression stays the same, and PD-L1 expression increases with MK5 KO. The band color will be the same in the first scenario. A darker protein band would occur in the second scenario, indicating greater protein concentration. The occurrence of other scenarios in western blot is enumerated in possible result one and applies to possible result 4. MTT death does not decrease with MK5 KO measured by MTT. The result of the MTT assay can be classified into two scenarios: MTT death stays the same with MK5 KO, and MTT deaths increase with MK5 KO.

E. Possible Result 5: MK5 KO reduces PD-L1 expression in vivo and reduces tumor size.

In vivo cell culture, MK5 KO decreases the expression of PD-L1, as shown in the western blot assay. In the western blot, the experimental group would show a lighter colored band than the control group with MK5. The lighter band indicates a lower concentration. The occurrence of other scenarios in western blot is enumerated in possible result one and applies to possible result 5. MK5 KO reduces the tumor size for NSCLC as measured by gross examination.

F. Possible Result 6: MK5 KO decreases the expression of PD-L1 but increases the tumor size.

In vivo cell culture, MK5 KO decreases the expression of PD-L1 as shown in the western blot assay with a lighter colored protein band compared to the control group. The lighter band indicates a lower PD-L1 concentration. The occurrence of other scenarios in western blot is enumerated in possible result one and applies to possible result 6. MK5 KO increases the tumor size for NSCLC as measured by gross examination.

G. Possible Result 7: MK5 KO does not decrease the expression of PD-L1 but reduces tumor size.

PD-L1 expression does not decrease in xenograft as shown by a darker protein band in western blot, but tumor size decreased as measured by gross examination.

H. Possible Result 8: MK5 KO does not inhibit tumor growth.

PD-L1 expression does not decrease in xenograft, as shown by a darker protein band in western blot, and tumor size increased as measured by gross examination.

IV Discussion

Possible result one fully supports the hypothesis: knocking down MK5 in the H1299 cell line using MK5 CRISPR will reduce the expression of PD-L1. Previous studies have verified the MK5's role in stabilizing and enhancing YAP activity through immunostaining, and overexpression of MK5 demonstrated a higher level of YAP/TAZ nuclear translocation [4]. As YAP translocates into the nucleus, it serves as a transcriptional coactivator, interacting with the TEAD transcription factors and forming a YAP/TEAD complex that enhances the expression of PD-L1[6]. With the knockdown of the MK5, YAP becomes less stable and is more susceptible to degradation. This reduces the YAP/

TEAD complex level as more YAPs are phosphorylated and encounter CK1δ/ϵ-mediated YAP ubiquitination and degradation, so the degree of PD-L1 expression is lowered. MK5 knockdown would alter the shape of the kinases, leading to an altered function or even a loss of function. Without a functioning MK5, YAP will be prohibited from forming YAP/TEAD complex. A decrease in the PD-L1 expression signifies a lower binding with PD1, which reduces the chances of immune escape in NSCLC. T cells would be able to recognize the tumor cells, and levels of tumor-promoting cytokines will be lowered. As a result, cell viability increases, as reflected by the MTT assay. Therefore, MK5 KO should be further investigated to see its effect on other cancer cell lines with the EGFR pathway or YAP-driven cancers.

In possible result 2, PD-L1 expression decreases with MK5 KO, but cell viability did not increase, so it partially supports the hypothesis. The other immune checkpoint might cause a no change in or decrease in cell viability. For instance, the effect of immunosuppression caused by the binding of CTLA4 and B7 might outweigh the effect of reduced PD-L1 expression. This reflects that the clinical application of MK5 KO might be weak. Another potential causes of this unexpected result in MTT, which quantifies the number of living cells, are a random and systematic error. To eliminate random error, researchers should conduct experiments in more trials. The systematic error might be a seeding error, in which an equal number of cells in each well should be guaranteed, and apply Annexin V staining to confirm if there exists a mismatch between MTT assay and Annexin-V [10, 11].

Possible result three partially supports the hypothesis as it increases cell viability, but the influence on PD-L1 expression is abnormal. If PD-L1 expression does not change, it suggests that MK5's influence on YAP degradation is low. In particular, MK5 might not exhibit a decisive role in YAP nucleus translocation. Despite MK5's crucial role in cytoplasmic translocation in YAP regulation, MK5's impact on YAP nucleus translocation remains unclear. In possible result 3, likely, MK5 did not reduce the YAP nucleus translocation. The YAP/TEAD complex level stays the same, leading to no change in PD-L1 expression or high expression of PD-L1. The experiment requires further research on MK5's role in YAP nucleus translocation. The cause of increases in cell viability under high PD-L1 expression requires further investigation of the pathway and function of MK5.

Possible result 4 contradicts the hypothesis as PD-L1 expression did not decrease and MTT cell death did not decrease. As aforementioned in possible result 3, MK5's insignificant role in YAP nucleus translocation might result in the unexpected PD-L1 expression. In possible result 2, the abnormality in MTT cell death is explained. If the two data coincided, MK5 KO does not demonstrate a high clinical potential to reduce immune escape in NSCLC. Since MK5 does not directly regulate the function of PD-L1 or its binding with PD1, the effectiveness of making MK5 a target point in targeted therapy is low. However, it elucidates that MK5 does not influence PD-L1 in the signaling pathway shown in Figure 1.

Possible result five supports the hypothesis as the experimental results in vivo reinforced MK5 KO's ability to reduce immune escape and inhibit the proliferation of NSCLC. If results 1 and 5 are observed simultaneously, it is reasonable to conclude that the hypothesis stands since both in vitro and in vivo models illustrate hindrance to tumor growth. The experiment can be improved for a prolonged period and observe the long-term impact of MK5 KO on the tumor.

Possible result six partially supports the hypothesis as PD-L1 expression decreases but tumor size increases. A plausible explanation resembles the one presented in possible result 2, where the immunosuppression caused by the binding of CTLA4 and B7 might outweigh the effect of reduced PD-L1 expression. Thus, immune escape stays the same or increases. Failure to detect cancer cells, T cell failed to inhibit tumor growth. Therefore, the experiment should be repeated with the addition of CTLA4 and B7 immune checkpoint inhibitors (ICI). It will investigate the potential of combined treatment with ICIs.

Possible result seven partially supports the hypothesis as PD-L1 expression did not decrease, but tumor size decreased. The current reason behind this phenomenon is unclear, so further research on the signaling pathway and MK5 is required.

Possible result eight contradicts the hypothesis as PD-L1 expression did not decrease, and tumor size increased. Result 8 strengthens the reasoning presented in result four and the opposite position against the established hypothesis.

Any combination of results 2 and 3 with results 6 and 7 requires further investigation to conclude.

A recent study has suggested that activation of YAP enhances the downstream gene expression of TGF-α, AREG, and EGFR [7]. Amphiregulin binding with EGFR on T-reg cells would promote its function and indirectly promote immunosuppression. It offers insight into how YAP cell contributes to immune escape in NSCLC through their interaction with the EGFR pathway. This experiment can be further improved by using different cell lines to verify the direct relationship between YAP and PD-L1 expression.

V Conclusion

This study explores the potential of MK5 knockdown as a therapeutic target for NSCLC with EGFR-TKI resistance. The result of our study will indicate whether or not MK5 knockdown has an ideal therapeutic effect in reducing tumor growth and immune escape in preclinical conditions. Potential cell lines for further research include HCC1975, HCC1650, HCC827, and PE089. MK5's role in YAP nucleus translocation requires further investigation. The combined effect of MK5 and ICIs in treating NSCLC should be investigated.

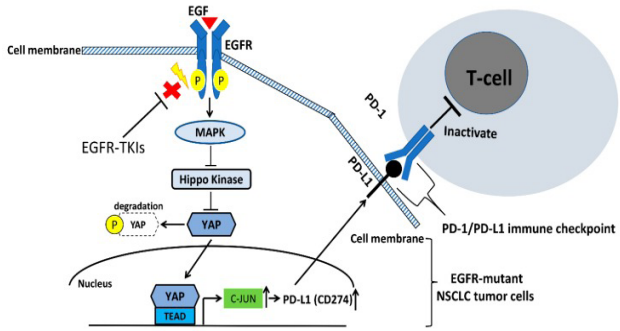

Figure 1. EGFR pathway increases PD-L1 expression. EGFR-TKIs inhibit EGFR pathway to reduce PD-L1 expression. Hippo Kinase inhibit YAP and results in YAP degradation. Activation of EGFR inhibit Hippo kinase. PD-L1 binds with PD1 and deactivates T-cell, leading to immune escape.

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