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Combined blockade of Tim-3 and MEK inhibitor enhances the efficacy against melanoma

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ABSTRACT

Insights into the role of the mitogen-activated protein kinase (MAPK) pathway and immune checkpoints have led combined targeted therapy and immunotherapy to be a promising regimen. Trametinib, as a mitogen-activated extracellular signal-regulated kinase (MEK) inhibitor, has demonstrated effectiveness in patients with advanced melanoma. T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3), an immune checkpoint molecule, participates in multiple negative regulation of antitumor immunity. We for the first time to our knowledge reported the combination of trametinib and anti-Tim-3 monoclonal antibody (mAb) in treating B16-F10 melanoma mice. We discovered that trametinib remarkably promoted apoptosis and inhibited cell proliferation while inhibition of MEK improved the expression of Tim-3 and caused the decrease of $CD8^+$ T cells; to the contrary, anti-Tim-3 mAb enhanced antitumor immunity by stimulating $CD8^+$ T cells, thus the combined therapy produced potent antitumor effect cooperatively. Taken together, our study provides compelling evidence for combining trametinib and anti-Tim-3 mAb as a potential valuable regimen in treating melanoma.

1. Introduction

Melanoma, the fifth most common cancer in men and seventh in women in USA, accounts for 70% of skin cancer-related deaths [1,2]. Approximately 132,000 melanoma skin cancers were diagnosed in the year of 2015 [3]. The prevalence of the disease is increasing globally and it is estimated that 2.1% of humans will be diagnosed with melanoma during their lifetime [4]. Although early stage of melanoma is treatable with surgery, the 5-year survival for patients with advanced melanoma is still less than 5%.

The molecular pathogenesis of melanoma involves a complex interaction of numerous cellular circuits including the mitogen-activated protein kinase (MARK) pathway, a critical promoter of cell proliferation and survival [5]. In addition, the inhibition of MAPK pathway possesses the potential of the treatment in BRAF-mutant melanoma [6,7]. In the last several years, successful targeted therapies have revolutionized the treatment of advanced melanoma by targeting the MAPK pathway. Trametinib is a mitogen-activated extracellular signal-regulated kinase (MEK) inhibitor that has recently gained Food and Drug Administration (FDA) approval for unresectable melanoma. In patients receiving trametinib treatment and chemotherapy, the overall survival (OS) was 81% and 67%, respectively [8]. Although these new drugs increase overall response rate and extend survival dramatically, treatment of advanced melanoma is still a challenge.

Immunotherapy now has demonstrated efficacy in advanced melanoma and gained recommendations as first-line therapeutic

Abbreviations: MAPK, mitogen-activated protein kinase; MEK, mitogen-activated extracellular signal-regulated kinase; OS, overall survival; CTLA-4, cytotoxic T lymphocyte antigen-4; PD-1, programmed cell death-1; Tim-3, T cell immunoglobulin- and mucin-domain-containing molecule-3; IFN, interferon; PFS, progression free survival; MDSCs, myeloid-derived suppressor cells; IL, interleukin; mAb, monoclonal antibody.

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option in the 2016 National Comprehensive Cancer Network Guidelines. Several immune checkpoint inhibitors have already been approved in the clinical treatment, such as nivolumab (anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) mAb) and ipilimumab (anti-programmed cell death-1 (PD-1) mAb). Nevertheless, the autoimmune toxicity associated with anti-CTLA-4 treatment [9] or anti-PD-1 treatment [10] has impeded the evaluation and development of reagents that target other molecules. Recent preclinical models demonstrated T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) was another attractive candidate for clinical development. Tim-3 is an inhibitory receptor initially identified in $CD4^+$ interferon (IFN)-γproducing T cells [11]; and it's also found to be expressed on cytotoxic $CD8^+$ lymphocytes, NK cells, dendritic cells and monocytes [12]. Blockade of Tim-3 restores the antigen-specific effector activities of $CD8^+$ T lymphocytes. Therefore, these data indicated exciting avenues for new therapies targeting Tim-3 in immunotherapy of melanoma.

As immunotherapy has exhibited positive results in melanoma [9], it was supposed to synergistically enhance the antitumor efficacy by combining immune checkpoint blockades with MAPK inhibition [13,14]. Accumulating studies indicated that anti-Tim-3 mAb and trametinib are both potential therapeutic strategies in treatment of melanoma, thus in this study we tested the hypothesis that the combined therapy can generate potent synergistic antitumor effect and explored the possible mechanisms underlying this combination medication.

2. Materials and methods

2.1. Ethics statement

All animal care and experiments complied with the guidelines provided by Institution of Laboratory Animals of Huazhong University of Science and Technology and were approved by the Committee on the Ethics of Animal Experiments of Huazhong University of Science and Technology.

2.2. Drugs and antibodies

Trametinib, MEK1/2 inhibitor (GSK1120212) was purchased from Selleckchem (S2673). Anti-Mouse Tim-3 Functional Grade Purified (16-5871) was purchased from affymetrix eBioscience. Polymethyl acrylate (PMA) was purchased from sigma-Aldrich (St Louis, MO).

2.3. Cell lines and animals

Mouse B16-F10 melanoma cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. B16-F10 cells were cultured in Dulbecco's Modified Eagle medium (HyClone), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) with 5% CO2 at 37 °C. Jurkat T cells were cultured in 10% fetal bovine serum-supplemented RPMI 1640 (Gibco). C57BL/6 mice (6–8 week old female) were purchased from Beijing HFK Bioscience Co. Ltd.

2.4. CCK8 assay

Cell viability was evaluated by CCK8 assays. The B16-F10 melanoma cells (1×10^3 cells/well) were cultured in 96-well plates. Following one day of culture, the media was substituted by DMEM containing 10% FBS and concentrated trametinib. At the end of the indicated time-periods (4, 8, 12, 24 and 36 h), cell proliferation was measured using a Cell Counting Kit-8 (CCK-8 kit), in accordance with the manufacturer's instructions. In brief, CCK-8 solution was added to the cells and incubated for 2 h. Absorbance was subsequently measured at a wavelength of 450 nm using a micro-plate reader. The relative number of cells was calculated using comparable standard curves of the obtained optical density values. The assay was performed in five replicate wells, and three parallel experiments for each sample were conducted.

2.5. In vivo tumor therapy

C57BL/6 mice were injected subcutaneously in the flank at day 0 with 1.0×10^6 B16-F10 cells. The mice were randomly divided into four groups ($n = 5$; control group, anti-Tim-3 mAb group, trametinib group and combination of trametinib and anti-Tim-3 mAb group). When the tumors reached a size of 20–25 mm^2, 100 μL anti-Tim-3 mAb (concentration: 0.5 μg/μL) was injected *in situ* into the tumor of the anti-Tim-3 mAb group on alternate days for three times. As to the trametinib group, 100 μL trametinib was filled into the stomach daily for a total of 6 times. Mice in combined therapy group received both treatments above while in control group drugs were replaced by phosphate buffer saline (PBS). Tumor size was measured and indicated as length × width (mm^2).

2.6. Immunohistochemistry (IHC)

The melanoma tumors were resected from the mice at the time of sacrifice (Day 14 after B16-F10 cell inoculation) and were then fixed in 4% formaldehyde and embedded in paraffin. IHC staining was performed using tumor samples following deparaffinization. Primary antibodies against Ki-67 and caspase3 were applied. Unbound primary antibodies were washed off and the sections were incubated with a secondary biotinylated immunoglobulin G antibody. The stained cells were visualized using the microscope.

2.7. Cell isolation and FACS analysis

For flow cytometry, tumors were surgically removed from the mice at Day 14 after the tumor cell inoculation and Jurkat T cells were harvested after stimulating by PMA. Single-cell suspensions were made from digested tumor tissues. The cells were stained extracellularly with the specific antibodies, including mouse APC-Tim-3, FITC-CD8, and PE-CD335.PerCP-Cy5.5-CD4, Samples were analyzed by using Canto II (BD).

2.8. si-MEK2

One pair of MEK2 siRNA and one pair of negative control-siRNA were designed and synthesized by Guangzhou RiboBio Co. Ltd. The sequences of MEK2 siRNA were 5′-GAAGAGGUAUUGAAU GCUAdTdT-3′ (sense) and 5′-UAGCAUUCAAUACCUCUUCdTdT-3′ (anti-sense).

Jurkat T cells were electroporated with 2 μg green fluorescent protein (GFP) plasmid and the indicated Nucleofector kits by using the Amaxa Nucleofector II instrument. The Nucleofector program for electroporation was X-001. GFP-expressing cells were observed in the fluorescence microscope.

For transfection of Jurkat T cells, 5×10^6 cells/reaction were used. The cells were washed twice with 1× PBS. For each electroporation reaction, 100 μL Nucleofector V-Kit and 3.75 μL of 20 μM si-MEK2 or negative control-siRNA were prepared. The cell pellets were resuspended with the siRNA duplex suspension; then, cells/siRNA duplex oligo suspensions were transferred into cuvettes and electroporated. Immediately after electroporation, 400 μL of the pre-equilibrated culture medium to the cuvette was added and transferred to a 6-well plate. Eight hours later, Jurkat T cells were stimulated with PMA (50 ng/ml). 24 h later, the cells were