Radiation combined with KRAS-MEK inhibitors enhances anticancer immunity in KRAS-mutated tumor models

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KRAS mutation is a common driver in solid tumors, and KRAS-mutated tumors are relatively resistant to radiotherapy. Therefore, we investigated the combined effect of radiation and KRAS-MEK inhibitors (AMG510 and trametinib) in KRAS-mutated tumors. The expression of programmed death-ligand 1 (PD-L1), major histocompatibility complex (MHC) class I molecules, and cytokines in KRAS-mutated cell lines was assessed using flow cytometry, western blot analysis, quantitative polymerase chain reaction, and enzyme-linked immunosorbent assay. In vivo, tumor growth, T cell infiltration, and gene sequencing analyses were conducted in 2 murine KRAS-mutated models. Both AMG510 and trametinib decreased the radiation-induced increase in PD-L1 expression. Radiation and trametinib additively induced the expression of CXCL10 and CXCL11 cytokines and MHC class I in murine CT26 and LLC cell lines. The combination of trametinib and radiation controlled tumor growth and induced more infiltration of CD4+ and CD8+ T cells in vivo, wherein tumor inhibition function and the survival period of mice could be reduced by CD8+ and/or CD4+ T cell depletion. The expression levels of immune-related genes also increased in the combination therapy group. Our results indicate that KRAS-MEK inhibitors in combination with radiotherapy can enhance antitumor immunity, providing new therapeutic strategies for KRAS-mutated tumors.

Abbreviations: CXCL10, C-X-C motif chemokine ligand 10; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FDA, Food and Drug Administration; HRP, horseradish peroxidase; IHC, immunohistochemistry; IFN, interferon; MEKI, MEK inhibitors; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PD-L1, programmed death-ligand 1; qPCR, quantitative polymerase chain reaction; RT, radiotherapy; SEM, standard error of the mean; VEGFA, vascular endothelial growth factor A

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trtametinib, have been approved by the Food and Drug Administration (FDA). Recently, the novel KRAS inhibitor AMG510 (sotorasib) showed encouraging anticancer activity in pretreated patients with NSCLC harboring the KRAS p.G12C mutation.\textsuperscript{7} Research has shown that both AMG510\textsuperscript{8} and MEK inhibitors (MEKi)\textsuperscript{9} can activate the anticancer immune system. However, it remains unclear whether KRAS-MEK inhibitors can improve radiation-induced anticancer immunity.

In this study, we explored whether the combination of radiation with AMG510 or trametinib can further activate antitumor immunity. The therapeutic efficiency of the combined therapy was evaluated by constructing 2 immunocompetent mouse models harboring KRAS-mutated tumors. These findings can provide insight in developing new therapeutic strategies for patients with KRAS-mutated cancers.

**Material and Methods**

**Tumor cell lines**

NSCLC cell lines (H358, H23, and H1972) and a pancreatic cell line (miapaca2) were purchased from the American Type Culture Collection (Manassas, VA, USA). Mouse cell lines (CT26 and LLC) were purchased from the Cell Resource Center of the Chinese Academy of Sciences (Beijing, China). H358, H23, H1972, and miapaca2 cells have the KRAS G12C mutation; CT26 cells have the KRAS G12D mutation; and LLC cells have both the KRAS G12C and NRAS Q61H mutations. H358, H23, H1972, and CT26 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% dimethyl sulfoxide and 10 nmol/L or 30 nmol/L AMG510. One hour later, the cells were irradiated at a dose of 8 Gy. After 48 hour, the cells were harvested via trypsinization in each group, rinsed with cold phosphate-buffered saline (PBS), and fixed with 70% ice-cold ethanol overnight at \( -20\degree \)C. Fixed cells were rinsed with cold PBS followed by incubation with 0.5 mL PI/RNase staining buffer (BD Biosciences; Cat No. 550825) for 15 minute at room temperature before analysis.

**Flow cytometry analysis**

To assess the expression of PD-L1 and major histocompatibility complex (MHC) molecules, tumor cells were treated as described above in the cell cycle analysis. The markers of human and mouse tumor cells, as well as those of mouse T cells, were determined using flow cytometry after staining with specific antibodies conjugated with different fluorochrome molecules. For intracellular staining of interferon (IFN)-\( \gamma \), erethyocyte-excluded spleen cells were first incubated with a cell stimulation cocktail plus protein transport inhibitors (eBioscience; Cat No. 4975-03) at 37\degree \text{C} under 5% CO\(_2\) for 5 hour. The following antibodies were used: PE-anti-human PD-L1 (Biolegend; Cat No. 329708), PE-anti-mouse PD-L1 (Biolegend; Cat No. 124307), PE-anti-mouse H-2D\( ^\beta \) (Biolegend; Cat No. 110607), PE-anti-mouse H-2L\( ^\alpha /\beta \) (Biolegend; Cat No. 114507), PE-anti-mouse H-2K\( ^\text{d} \) (Biolegend; Cat No. 110607), PE-anti-mouse H-2\( ^\text{k} \) (Biolegend; Cat No. 116507), eBioscience Fixable viability dye eFluor 506 (Invitrogen; Cat No. 65-0866-14), APC/Cyanine7 anti-mouse CD45.2 (Biolegend; Cat No. 109824), FITC-anti-mouse CD3 (Biolegend; Cat No. 100203), PerCP5.5-anti-mouse CD4 (Biolegend; Cat No. 100434), APC-anti-mouse CD8 (Biolegend; Cat No. 100712), and PE-anti-mouse IFN-\( \gamma \) (Biolegend; Cat No. 505808). The stained cells were analyzed on a FACS Calibur flow cytometer (BD Bioscience), and data were analyzed using the FlowJo10 software (Tree Star, Inc., Ashland, OR, USA).

**Western blot analysis**

After radiation or drug treatment, the cells were harvested and lysed using a radioimmunoprecipitation assay buffer with a protease inhibitor and phosphatase inhibitor cocktail. After determining the protein concentration of the cell lysates using Quick Start Bradford Protein Assay Reagent (Biolegend; Cat No. 500000), 50 \( \mu \)g of protein sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidine fluoride membranes. The membranes were blocked in a 5% skim milk solution for 1 hour and incubated overnight with corresponding primary antibodies at 4\degree \text{C}. The primary antibodies used were anti-human PD-L1 (1:1000; Cell Signaling; Cat No. 13684), anti-mouse PD-L1 (1:1000; R&D; Cat No. MAB90781), anti-ERK (1:1000; Cell Signaling; Cat No. 9102), anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000; Cell Signaling; Cat No. 4370), anti-AKT (1:1000; Cell Signaling; Cat No. 2920), anti-phospho-AKT (Ser473) (1:1000; Cell Signaling; Cat No. 4060), anti-S6 ribosomal protein (1:1000; Cell Signaling; Cat No. 2217), anti-phospho-S6 ribosomal protein (Ser235/246) (1:1000; Cell Signaling; Cat No. 4858), and anti-\( \beta \)-actin (1:1000; Santa Cruz; Cat No. sc-47778). Subsequently, the membranes were washed and incubated at 25\degree \text{C} with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody for 1 hour. The bands were visualized using an enhanced chemiluminescence detection reagent.

**RNA extraction and real-time quantitative polymerase chain reaction (qPCR)**

Tumor cells were harvested and RNA was extracted using a total RNA extraction kit (Fastagen; Cat No. RNAfast200). Complementary DNA was synthesized from 2 \( \mu \)g of purified total RNA using the HiScript III RT SuperMix for quantitative PCR (Applied Biosystems; Cat No. 4385612) according to the manufacturer’s instructions. The mRNA expression levels of PD-L1, CXCL9, CXCL10, CXCL11, IFN-\( \alpha \), IFN-\( \beta \), IL6, MMP2, MMP9, TGF-\( \beta \), and VEGFA in tumor cells were determined using specific primers, analyzed using the comparative cycle threshold method, and normalized to GAPDH levels. All experiments were performed in triplicate. The primers used are listed in Supplementary Table 1.

**Enzyme-linked immunosorbent assay (ELISA)**

The levels of CXCL10 secreted by tumor cells in 6-well plates were detected using an ELISA Kit (Boster; Cat No. EK0736). Briefly, cell-free supernatants from a tumor treatment system after 48 hour were collected, and 100 \( \mu \)L/well of standards and samples were loaded into 96-
well plates. After incubation with biotinylated antibodies, streptavidin-conjugated horseradish peroxidase (HRP) was added to each well and reacted with HRP substrate solution. The optical density at 450 nm values were detected. The concentration levels were adjusted according to every 10^6 living tumor cells.

In vivo studies

Female BALB/c and C57BL/6 mice (6–8 weeks old) were purchased from the Beijing SIPEIFU Biotechnology Company. All animal studies were approved by the Animal Care Committee of Jinan Central Hospital (No. JNCHACUC2021-32) and complied with the current Chinese regulations and standards for laboratory animal use.
Effects of MEKi and radiation on CT26 and LLC cells in vitro. A, MEKi (10 nmol/L or 100 nmol/L) was added 1 hour before radiation, and changes in the PD-L1 signaling pathway and PD-L1 expression were detected 48 hour later. B and C, MEKi (10 nmol/L or 100 nmol/L) was added 1 h before radiation, and the fluorescence signal intensity of cells expressing PD-L1 was detected via flow cytometry 1, 2, and 3 days later. D and E, Fluorescence signal intensity of cells expressing MHC class I molecules (H-2Kd on CT26 cells and H-2Kb on LLC cells) determined via flow cytometry. F and H, MEKi (10 nmol/L) was added 1 hour before radiation, and mRNA expression of cytokines and MMPs in tumor cells was detected 48 hour later. G and I, Concentration of CXCL10 in the supernatant detected 48 hour later. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
In total, $3 \times 10^5$ CT26 or LLC cells were subcutaneously injected into the right flanks of BALB/c or C57BL/6 mice. When the tumor volume reached 50–75 cm$^3$, the mice were randomly assigned to the following 4 groups: control, MEK, RT (radiotherapy alone), and MEK + RT (MEK combined with radiotherapy). B, Tumor growth curves in the 4 groups. C, Comparison of tumor weights after exfoliation. D–G, Proportions of CD3$^+$, CD4$^+$, CD8$^+$, and IFN-γ$^+$ T lymphocytes in the spleen determined via flow cytometry. H–J, Immunohistochemistry of CD3, CD4, and CD8 immune cells infiltrating the tumors. K, Immunohistochemistry of PD-L1 expression. L and M, Immunohistochemistry of IFN-γ$^+$ and Foxp3$^+$ immune cells infiltrating the tumor. N, Immunohistochemical map of the above molecules. Scale, 50 μm. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Tumor volumes were measured every other day using a digital caliper and calculated as $0.5 \times length \times width$. When tumors reached the size limit (2 cm$^3$), mice were euthanized and tumors were isolated and weighed. Immune cells from the spleen were isolated after lysing red blood cells for subsequent flow cytometry analysis.

LLC cells ($3 \times 10^5$) were subcutaneously injected into the bilateral flanks of male C57BL/6 mice to conduct T cell depletion experiments.

Fig 4. MEKi and radiotherapy (RT) inhibit tumor growth in the LLC model. A, LLC cells were inoculated into C57BL/6 mice, and tumor size was measured in the following 4 groups: control, MEKi, RT (radiotherapy alone), and MEKi + RT (MEKi combined with radiotherapy). B, Tumor growth curves in the 4 groups. C, Tumor weights after exfoliation. D–G, Proportions of CD3$^+$, CD4$^+$, CD8$^+$, and IFN-γ$^+$ T lymphocytes in the spleen determined using flow cytometry. H–J, Immunohistochemistry of CD3, CD4, and CD8 immune cells infiltrating the tumor. K, Immunohistochemistry of PD-L1 expression. L and M, Immunohistochemistry of IFN-γ$^+$ and Foxp3$^+$ immune cells infiltrating the tumor. N, Immunohistochemical map of the above molecules. Scale, 50 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. 
Fig 5. AMG510 and radiotherapy (RT) inhibit tumor growth in the LLC model. A, LLC cells were inoculated into C57BL/6 mice, and tumor size was measured in the following 4 groups: control, AMG510, RT (radiotherapy alone), and AMG510 + RT (AMG510 combined with radiotherapy). B, Tumor growth curves in the 4 groups. C, Tumor weights after exfoliation. D–G, Proportions of CD3⁺, CD4⁺, CD8⁺, and IFN-γ⁺ T lymphocytes in the spleen determined via flow cytometry.

Anti-CD8 (Bioxcell, Cat No. BE0061), anti-CD4 (Bioxcell, Cat No. BE0003-1) neutralizing antibody (200 µg), or the isotype (Bioxcell, Cat No. BE0090) was injected intraperitoneally in mice in the different groups.

**Immunohistochemical (IHC) staining and quantification**

Mouse cancer tissues were embedded in paraffin and sectioned into 4 µm slices. The sections were deparaffinized and rehydrated in a series of decreasing ethanol solutions. Following antigen retrieval, the sections were incubated with 3% hydrogen peroxide for 20 minute. Tissue slides were then incubated overnight at 4°C with the following primary antibodies: anti-PD-L1 (1:100; RD Systems; Cat No. MAB90781), anti-CD3 (1:200; Abcam, Cat No. ab5690), anti-IFN-γ (1:50; Affinity; Cat. DF6045), anti-CD4 (1:800; Abcam; Cat No. ab183685), anti-CD8 (1:150; Abcam; Cat No. ab237723), and anti-Foxp3 (1:800; Abcam; Cat No. ab215206). A 2-step immunohistochemical staining kit (Cat No. PV-9000) was used for protein expression analysis according to the manufacturer’s instructions. Finally, the slides were visualized with a 3,3′-diaminobenzidine solution and counterstained with hematoxylin. The numbers of CD3⁺, CD4⁺, CD8⁺, IFN-γ⁺, and Foxp3-positive cells in each specimen were counted at 400 × magnification. PD-L1 was semi-quantitatively scored according to the following scale: 0, <5% tumor cells; 1, 5–25% tumor cells; 2, 25–50% tumor cells; 3, >50–75% tumor cells; 4, >75% tumor cells. Staining intensity was also semi-quantitatively scored as 0 (negative), 1 (weak), 2 (intermediate), or 3 (strong). The final score for each specimen was expressed as the product of the proportion and intensity scores.

**Gene sequencing**

Freshly isolated tumor tissues were cleaned using PBS. Tissue blocks (50 mm³) were cut to avoid the necrotic site, and 3 tumor tissues from different mice were analyzed for each group. Tissues were placed in TRIzol, stored at −80°C, and transported to BGI for gene sequencing on dry ice. Sequencing was started after samples passed quality control.

**Statistical methods**

All data are expressed as the mean ± standard deviation (SD) unless otherwise noted. SPSS 24 and GraphPad Prism 8.0 were used for statistical analysis. A minimum of 3 independent experiments were performed. The differences between groups were analyzed using an unpaired Student’s t-test and considered statistically significant at *P* < 0.05.

**Results**

**KRAS-meki reduces radiation-induced pd-l1 expression in human kras-mutated cell lines**

RT-induced PD-L1 expression is 1 of the main contributors to immune tolerance. We found that the expression of PD-L1 increased in KRAS-mutated cells after irradiation with 8 Gy via qPCR and western blotting (Fig 1A and Supplementary Fig1A).

The 4 human cancer cell lines with the KRAS G12C mutation had different sensitivities to AMG510, and the CCK-8 assay showed that miapaca2 and H358 cells were more sensitive to AMG510 than the other cell lines (Fig 1B) and were also sensitive to MEKi (Fig 1C). The activation of the KRAS-MEK-ERK signaling pathway has been reported to induce PD-L1 expression. We found that the expression of PD-L1 decreased in miapaca2 and H358 cells upon AMG510 administration on the second and third days (Supplementary Figure 1B and C); thus, we chose the treatment time of 48 hour in subsequent experiments. The downstream signaling molecules of KRAS were analyzed, and we found that pERK and pS6 expression levels were decreased upon AMG510 and MEKi administration after 48 hour (Fig 1D). Both MEKi and AMG510 decreased the expression of PD-L1 induced by radiation at both the mRNA and protein levels (Fig 1D and E). The expression of PD-L1 on the surface of miapaca2 and H358 cells was also analyzed using flow cytometry (Fig 1F and G), and the result was consistent with western blotting results. After 48 hour, radiation induced PD-L1 expression in the miapaca2, H358, and H1792 cell lines, and AMG510 decreased this expression (Fig 1H).

**Statistical methods**

All data are expressed as the mean ± standard deviation (SD) unless otherwise noted. SPSS 24 and GraphPad Prism 8.0 were used for statistical analysis. A minimum of 3 independent experiments were performed. The differences between groups were analyzed using an unpaired Student’s t-test and considered statistically significant at *P* < 0.05.
The combined effect of the 2 factors on the cell cycle was also studied. AMG510 and radiation increased the proportion of sub-G1 and apoptotic cells (Supplementary Figure 1D).

Influence of radiation and meki on ct26 and llc cells in vitro

The 2 murine cell lines, CT26 and LLC, were found to be sensitive to MEKi (Fig 1C). MEKi treatment decreased pERK and PD-L1 expression (Fig 2A). The expression of PD-L1 was verified via flow cytometry (Fig 2B and C), and we found that MEKi decreased PD-L1 expression after the second day. The expression of MHC class I molecules in CT26 (H-2Kd, H-2Dd, and H-2Ld) and LLC (H-2Kb and H-2Db) cells significantly increased after MEKi and radiation treatment (Fig 2D and E, and Supplementary Figure 1E−G). We also analyzed the transcription of cytokines and matrix metalloproteinases (MMPs) (Fig 2F and H). Radiation reportedly induces MMPs to facilitate tumor invasion; however, MEKi could not decrease the expression of MMP2 and MMP9. The combination of MEKi and radiation induced the expression of CXCL10 and CXCL11 in an additive manner. The increase in CXCL10 expression in the MEKi + RT group was also confirmed using ELISA (Fig 2G and I).
RT and MEKi activate immunity in ct26 murine models

We performed in vivo experiments to demonstrate the efficacy of the combined therapy. CT26 cells were inoculated into BALB/C mice, and when the tumor grew to 50−75 mm³, the mice were divided into 4 groups. The MEKi + RT group had the smallest tumor volume (Fig 3A). Growth curves showed that combination therapy consistently controlled tumor growth (Fig 3B). The weights of the tumors reflected their volumes (Fig 3C).

We measured the proportion of lymphocytes in the spleens of mice, and found that the proportions of CD3⁺, CD4⁺, and CD8⁺ T cells in the MEKi + RT group were higher than those in other groups; those in the control group were the lowest (Fig 3D−F). However, there was no difference in the IFN-γ⁺ T cell number among the 4 groups (Fig 3G). The gating strategy for the analysis of the immune cells in the spleen is shown in Supplementary Figure 2. IHC was used to detect the tumor infiltration of CD3⁺, CD4⁺, and CD8⁺ T lymphocytes, and the results showed a

Fig 7. Gene sequencing analysis A and B. Gene set enrichment analysis (GSEA) revealed significant enrichment of antigen processing and presentation and T cell receptor signaling pathways in the MEKi + radiotherapy (RT) group compared with the control group in the CT26 model. C and D, GSEA revealed significant enrichment of chemokine signaling pathway and cytokine–cytokine receptor interaction in the MEKi + RT group compared with the control group in the LLC model. E and F, GSEA revealed significant enrichment of PD-L1 expression and the PD-1 checkpoint pathway in the RT group compared with the MEKi group in the CT26 and LLC models. G, The treatment groups had differentially expressed genes enriched in the immune system compared with the control group. H−J, Heat map analysis of immune-related gene expression in the CT26 and LLC models.
trend consistent with that of the flow cytometry results (Fig 3H–J and N). Similarly, we measured the expression of PD-L1, which was higher in the RT group than that in the MEKi group; there was no significant difference between the other groups (Fig 3K and N). There was no significant difference in the proportion of IFN-γ+ and Foxp3+ T cells among all groups (Fig 3L–M).

**RT and MEKi/AMG510 activate immunity in LLC murine models**

LLC cells were also inoculated into C57BL/6 mice. The MEKi + RT group had the smallest tumor volume (Fig 4A). The growth curve showed that combination therapy consistently controlled tumor growth (Fig 4B). The weights of the tumors reflected their volumes (Fig 4C).

We detected the expression of lymphocytes in the spleens of mice and found that the proportions of CD3+, CD4+, and CD8+ T cells in the MEKi and MEKi + RT groups were higher than those in the other 2 groups (Fig 4D–F). There was no difference in the number of IFN-γ+ T cells among the 4 groups (Fig 4G). IHC was used to detect the infiltration and activation of CD8+ T cells (Fig 4H). The weights of the tumors in the MEKi + RT group had the smallest tumor volume (Fig 4A). The growth curve results were consistent with those of flow cytometry. The infiltration of T lymphocytes was higher in the MEKi + RT group than that in the MEKi group (Fig 4H–J and 4N). Similarly, the expression of PD-L1, IFN-γ, and Foxp3 was detected, but no significant difference was found among the groups (Fig 4K–N).

AMG510 was also used for the combination therapy. As LLC cells had both KRAS G12C and NRAS Q61H mutations, they were not that sensitive to AMG510 (Supplementary Figure 1H). The AMG510 + RT group also had the smallest tumor volume and weight (Fig 5A–C). We further investigated the number of lymphocytes in the spleens of mice, demonstrating that the proportions of CD3+, CD4+, and CD8+ T cells in the AMG510 + RT group were higher than those in the other groups (Fig 5D–F).

**CD4+ and CD8+ T cells are necessary for the combined therapy**

To confirm the roles of CD4+ and CD8+ T cells in the antitumor immune response following combined therapy, we depleted these 2 cell populations in vivo and analyzed the effects on transplanted tumor growth and survival in the subcutaneous transplanted tumor model (Fig 6A). Depletion of CD4+ and CD8+ T cells (P = 0.031 vs combined therapy) or CD8+ T cells (P = 0.001 vs combined therapy) weakened the inhibitory effect of the combined therapy on the growth of transplanted tumors (Fig 6B). The growth curves of every tumor in the 5 groups are shown in Fig 6C–G. Furthermore, depletion of CD4+ and CD8+ T cells (P = 0.018 vs combined therapy) or CD8+ T cells (P = 0.004 vs combined therapy) shortened the survival time of mice (Fig 6H). Depletion of CD4+ T cells weakened the inhibitory effect and shortened the survival time, but the difference was not statistically significant. These findings suggest that the antitumor immunity induced by the combined therapy is mainly achieved through the infiltration and activation of CD8+ T cells.

**RT and MEKi lead to the activation of immune-related signaling pathways in vivo**

The tumor tissues of the mice were analyzed via gene sequencing. Gene set enrichment analysis (GSEA) demonstrated significant enrichment in gene signatures associated with antigen processing and presentation, T cell receptor signaling pathway, chemokine signaling pathway, and cytokine-cytokine receptor interaction in the MEKi + RT group compared with the control group (Fig 7A–D). In addition, the activation of immunity in the RT + MEKi group was multifaceted (Supplementary Table 2 and 3), involving B cell receptor, Toll-like receptor, and tumor necrosis factor signaling pathways. We also analyzed PD-L1 expression and the PD-1 checkpoint pathway, and found enrichment in the RT group compared with the MEKi group in both the CT26 and LLC models (Fig 7E and F).

By analyzing differentially expressed genes and comparing the treatment groups with the control group, we found that immune-related gene expression was higher in the MEKi + RT group than that in the other groups (Fig 7G). The heatmap also showed higher immune-related gene expression in the MEKi + RT group than in the other groups (Fig 7H and I).

**Discussion**

KRAS-mutated cancers are thought to be resistant to anti-EGFR therapy and conventional RT. In our study, we found that KRAS inhibitors or MEKi can decrease radiation-induced PD-L1 expression, and hypofractionated radiation therapy combined with targeted drugs can further increase chemokine secretion and MHC class I expression in vitro and can increase T lymphocyte infiltration and immune-related gene expression in vivo. Taken together, our results show that radiation and KRAS or MEK inhibitors can further activate anticancer immunity within the tumor microenvironment.

In KRAS-mutated cancer cells, KRAS persistently activates downstream signaling pathways. Several MEKi have entered clinical research to restrain the downstream RAS-MEK-ERK signaling pathway, among which trametinib has been approved by the FDA for the treatment of NSCLC23 and melanoma24 with BRAF mutations. Owing to tumor heterogeneity, and secondary or primary drug resistance, MEKI alone is not effective against KRAS-mutated lung cancer. The novel direct covalent KRAS-G12C inhibitor, AMG510, was first reported in 2019.25 However, as with other targeted drugs, the clinical activity of AMG510 is limited by the development of resistance.14,15 Nevertheless, it was reported that both MEKi and AMG510 can improve and promote the antitumor activity of T cells.25-27 In a lung cancer mouse model with KRAS mutation, intermittent MEKI administration could improve antitumor immunity and T cell function, and combination with CTLA4 antibody treatment achieved good efficacy.19 Therefore, combination treatment is a promising strategy to achieve better efficacy of KRAS-MEK inhibitors.

RT is a highly accessible local therapy for tumors, and its immunomodulatory effects have gradually been recognized in recent years. However, the exact mechanism underlying the effect of RT on tumor immunity is complicated.20 The expression of the immunomodulatory molecule PD-L1 is influenced by radiation. We found that hypofractionated radiation significantly increased the expression of PD-L1 in KRAS-mutated cell lines. In the LLC model, radiation alone could not increase T lymphocyte infiltration nor control tumor growth in vivo. Whether inhibition of the KRAS signaling pathway combined with RT can improve anticancer efficiency is unknown.

In a series of in vitro experiments with human and mouse KRAS-mutated cell lines, both AMG510 and trametinib inhibited proliferation and increased the expression of pERK and radiation-induced PD-L1. PD-L1 inhibits the T cell activation of tumor cells, and several immune checkpoint inhibitors that block this target have been widely applied in clinical practice.21 KRAS can stabilize PD-L1 mRNA without degradation and increases the expression of PD-L1 through the MEK-ERK signaling pathway. We also found that pAKT expression increased after MEKI treatment in the H358 and CT26 cell lines, which indicated that MEKI inhibited the MAPK signaling pathway and in turn caused activation of the PI3K-AKT pathway. There is a complex crosstalk and feedback in the PI3K/AKT/mTOR signaling network.25

In the present study, we found that MEKI combined with RT increased the number of MHC class I molecules on the tumor cell surface and changed the expression of cytokines. Cytotoxic T cells can only recognize antigens bound to MHC class I molecules. MHC class I molecules not only contribute to the activation of T cells but also play an important role in the maturation of T cells in the thymus.28 One of the mechanisms of tumor immune escape is the loss of MHC class I expression in tumor cells, leading to the failure of T cells to recognize them. As the key chemokines, CXCL10 and CXCL11 not only attract leukocytes to tumor sites but also shape their biological properties.29,30 CXCL10 is beneficial for inducing the recruitment of tumor-infiltrating lymphocytes into tumors and potentiates effector T cells, mostly CD8+.
effectector-cytotoxic T cells. CXCL11 has a variety of functions, including inhibiting angiogenesis, affecting the proliferation of different cell types, playing a role in fibroblast-targeted cancer invasion, increasing adhesion properties, and inducing CD4+ T regulatory-1 cells. Sequencing analysis also showed that there was an increase in chemokine and antigen processing and presentation signaling pathway. These in vitro results suggest that MEKi + RT is more likely to recruit T cells, improving the ability of T cells to recognize and kill tumor cells.

We found that AMG510/MEKi combined with RT could achieve a better antitumor effect and benefit the activation of antitumor immunity in vivo. Both CD8+ and CD4+ T cell numbers increased upon combined therapy, with no increase in the proportions of Foxp3+ T cells. The T cell depletion experiment showed that both CD8+ and CD4+ T cells were necessary for immune activation, whereas CD8+ T cells were the main functional population. Sequencing analysis showed that there was an increase in the T cell receptor signaling pathway. An increasing trend was observed for IFN-γ-producing T cells in tumors, but the percentage of spleen T cells that produced IFN-γ was not significantly changed, which may be due to the fact that T cells in the spleen could not be adequately exposed to tumor antigen. Other cytotoxicity markers such as granzyme B, perforin, and CD107a may be helpful for the tumor infiltration of lymphocytes. We will continue to explore the effects of combined therapy on T cell proliferation, activation, cytotoxicity, and differentiation. Sequencing analysis showed that in the 2 tumor models, the PD-L1 signaling pathway in the MEKi group was significantly inhibited compared with that in the RT group. However, PD-L1 expression in the tumor, determined using IHC, was also not significantly different. In vivo, the tumors were irradiated 12–14 days before IHC analysis; therefore, the changes in PD-L1 may not be as obvious as those detected in vitro. An earlier detection time point or the use of flow cytometry may be helpful in finding the difference. Combined therapy achieved impressive tumor control in the first 2 weeks and prolonged the survival of the mice; nevertheless, the combined therapy was not effective in the long term. Previous research showed that alteration of the immune landscape of tumors and the network of immune-suppressive cellular interactions are complex, therefore, it is possible to fight cancer by aiming at more immune pathways. Adding immunotherapy such as anti-PD-1 therapy may help achieve long-term control.

Enhancing RT-induced immunological effects and determining the optimal combination therapy mode, such as the RT dose and application sequence, have been explored. When treating tumor-bearing mice, we also considered the RT dose and treatment sequence. Considering that the trametinib blood concentration can reach a certain level during radiation and plays a continuing role after RT, trametinib was administered orally 2 days before radiation in our study. The selection of RT at a dose of 8 Gy was based on the consideration that conventional segmentation (1.8–3 Gy) is not conducive to the activation of CD8+ T cells, and a high radiation dose (>10 Gy) would induce angiogenesis. The 8 Gy dose also showed favorable results in in vitro experiments; therefore, 8 Gy was selected as the ideal dose for animal experiments. This combination model can provide a reference for future translational studies and clinical trials.

Conclusion

KRAS or MEK inhibitors can decrease radiation-induced PD-L1 expression, and hypofractionated radiation therapy combined with targeted drugs can further increase chemokine secretion and MHC class I expression in vitro and increase T lymphocyte infiltration and immune-related gene expression in vivo. This study explored new therapeutic strategies for KRAS-mutated tumors, providing a basis for the treatment of this particularly recalcitrant tumor type.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data Availability

The RNA-seq data was uploaded to the GEO repository. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE209767

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Author contributions are as follows: YZ and LX conceived and designed the work and wrote the manuscript. YZ, YL, FZ, XC, CS, and MZ performed the experiments and analyzed and interpreted the data. MS and YS performed the RNA-seq data analysis. All authors read and approved the final manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.trsl.2022.08.005.

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