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Famitinib enhances the antitumor effect of radioimmunotherapy in murine lung cancer

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Abstract

Background: Combining antiangiogenic therapy with radioimmunotherapy is believed to further improve antitumor efficacy, but there is still a lack of evidence to support this. This study aimed to investigate the role of the tumor vascular-targeted agent famitinib with a combination of radiotherapy and an immune checkpoint inhibitor in murine lung cancer.

Methods: The effect of VEGFA and HIF1A on clinical prognosis and the tumor immune microenvironment was analyzed using public databases. Enrichment analyses of post-irradiation gene expression and mRNAs related to immunotherapy efficacy were carried out based on GEO datasets. A C57BL/6 mouse subcutaneous tumor model was used to evaluate the antitumor effects of different treatment schemes. The tumor immunophenotyping was identified by flow cytometry.

Results: We demonstrated that high level of VEGFA and HIF1A expression in lung cancer was related to poor prognosis and immunosuppressive tumor microenvironment. In a mouse model, the triple therapy of famitinib, radiotherapy and immunotherapy had the most dramatic antitumor activity. It significantly increased tumor infiltrating lymphocytes and reversed the immunosuppressive state of the tumor microenvironment in mice. Compared with radioimmunotherapy, the addition of famitinib further promoted the infiltration of CD8+ T cells and M1 type tumor associated macrophages, and reduced the number of myeloid suppressor cells. Therefore, triple therapy converted the immunosuppressive tumor microenvironment into an immunostimulatory one.

Conclusion: Famitinib can synergize with radioimmunotherapy by regulating the tumor immune microenvironment in murine lung cancer.

KEYWORDS
immunotherapy, radiotherapy, tumor microenvironment, vascular-targeted therapy

INTRODUCTION

The combination of radiotherapy and immune checkpoint inhibitors (ICIs) has been comprehensively studied and been demonstrated to have a brilliant synergistic effect, but this combination can also encounter treatment resistance. Recent studies have focused on how to further improve the response rate and expand the potential beneficiary population. More and more studies have shown that antiangiogenic therapy not only reconstructs tumor vasculature and alleviates hypoxia, but also eliminates immunosuppressive factors and balances the tumor microenvironment (TME), and may therefore further improve the efficacy of radioimmunotherapy.[1–5] Pilot research has demonstrated that the vascular-targeted agent

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anlotinib might be a potential synergistic treatment for radioimmunotherapy.[6] However, the preclinical and clinical evidence of the antitumor effect of the triple therapy is still limited. The purpose of this study was to verify the impact of angiogenesis and hypoxia on clinical treatment and patient prognosis using public databases, and demonstrate the effects of tumor vascular-targeted therapy on radioimmunotherapy, and assess the tumor immune microenvironment in a C57BL/6 mouse subcutaneous tumor model.

METHODS

Analysis of the effect of VEGFA and HIF1A on clinical prognosis and the tumor immune microenvironment

Patients were divided into two groups according to vascular endothelial growth factor A (VEGFA) or hypoxia-inducible factor-1α (HIF1A) expression, with data from the Gene Expression Omnibus (GEO), the European Genome-phenome Archive (EGA) and the Cancer Genome Atlas (TCGA) repositories processed by Kaplan–Meier Plotter platform (https://kmplot.com/analysis/) and TIMER (http://timer.cistrome.org/), where the expression level of patients above cutoff and below cutoff were selected as VEGFA or HIF1A-high and VEGFA or HIF1A–low groups. Survival analysis of overall survival (OS) in lung adenocarcinoma patients was performed according to the different expression level.[7,8] We used the TIMER web server[8] to quantify the amounts of tumor-infiltrating immune cells and explore the relationship between VEGFA or HIF1A and immune cells (http://timer.cistrome.org/). The purity-corrected partial Spearman's correlation and statistical significance between VEGFA or HIF1A expression and each immune cell subset were used to depict the correlation on the scatter plots (https://cistrome.shinyapps.io/timer/).

Gene set enrichment analysis using public database

The gene expression microarray dataset GSE24876 and mRNA sequencing dataset GSE136961 were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). In the former, lung adenocarcinoma A549 cell lines were divided into a sham irradiation group and 6 Gy irradiation group. Gene expression was analyzed after exposure. In the dataset GSE136961, mRNA of non-small cell lung cancer patients before receiving anti-PD-1 treatment was measured, and patients were divided into two groups according to the clinical efficacy of immunotherapy: the durable clinical benefit group and nondurable benefit group. Differentially expressed genes between groups were determined using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r) and limma package in R-4.2.0 with the following thresholds: p-value <0.05 and $|\text{fold change}| > 2$. Enrichment analyses were performed using clusterProfiler package in R-4.2.0. Infiltration of immune cells was analyzed using CIBERSORT (https://cibersortx.stanford.edu/index.php).

Mice and cell lines

Female C57BL/6 mice (SPF, 6 weeks old) were purchased from HFK Bioscience Co., Ltd. and raised in the Experimental Animal Center of the Chinese Academy of Medical Sciences. Lewis lung cancer (LLC) cells were provided by the CAMS Key Laboratory of Translational Research on Lung Cancer. The study protocols were approved by the Institutional Animal Care and Use Committee (NCC2020A305) at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (NCC/CH, CAMS, Beijing, China).

Establishment of subcutaneous tumor model

LLC cells (6×10^6/mouse) were injected subcutaneously into the left inguinal region of C57BL/6 mice. The tumor size was measured every other day with a caliper. The calculation formula of tumor volume was as follows: volume $= L \times W^2/2$, where L is the length and W the width of the tumor. The tumor-bearing mice were randomly divided into six groups with five mice in each group. The antitumor treatment began when the tumor volume reached 50 ± 20 m^3.

Antitumor therapy

Mice were randomly divided into the following six groups (5 mice in each group, 30 in total): control, irradiation, anti-PD-1, irradiation + famitinib, irradiation + anti-PD-1, irradiation + anti-PD-1 + famitinib (control group and irradiation group were shared groups in another parallel experiment). Irradiation (3 fractions of 8 Gy on days 3, 5 and 7): avertin (300 mg/kg) anesthetized mice were irradiated using the Varian Unique-SN2242 linear accelerator with 6 MV xray, and the source skin distance (SSD) was 1 m. During irradiation, the mice were placed supinely on a fixing frame and covered with a 5-mm bolus. The radiation field was adjusted to include only the tumor area of mice (Figure 1a). Anti-PD-1 (10 mg/kg, HRP00262-020) was administered intraperitoneally on days 3, 6, 9 and 12. Famitinib (5 mg/kg, N685200402) was given daily by intragastric administration from days 1 to 14 (Figure 1b).

Flow cytometry

The tumor tissues were digested with 1 mg/ml collagenase IV and 0.01 mg/ml DNAase, and passed through a 70-μm nylon cell strainer to obtain single cell suspensions. The single cell suspensions were stained with fluorescent-labeled

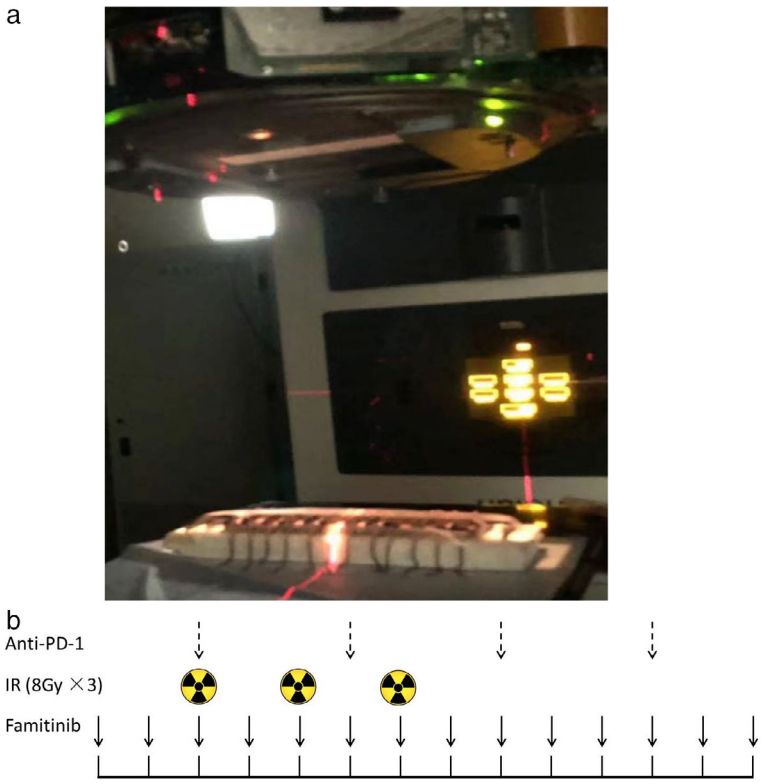

FIGURE 1 Administration of antitumor therapy. (a) Local irradiation scheme of tumor-bearing mice. (b) Schematic showing schedules of radiotherapy, famitinib and anti-PD-1.

antibodies, including anti-CD3 (#100329, Biolegend), anti-CD4 (#100405, Biolegend), anti-CD8 (#100733, Biolegend), anti-CD45 (#103113, Biolegend), anti-CD11b (#101245, Biolegend), anti-Gr-1 (#108407, Biolegend), anti-F4/80 (#123107, Biolegend), anti-CD86 (#105029, Biolegend) and anti-CD206 (#141707, Biolegend). The data were obtained by LSR-II (Becton Dickinson) and analyzed with FlowJo version 10.0 software.

Statistical analysis

A two-tailed Student's *t*-test was performed for comparison between two groups. Coefficient of drug interaction (CDI) was calculated to evaluate the effect of combination treatment. One way ANOVA was used for analysis of more than two groups. $p < 0.05$ was statistically significant. *, * *, * * * and * * * * denote $p < 0.05$, <0.01, <0.001 and <0.0001, respectively. Statistical analyses were performed using GraphPad Prism software 9.0.

RESULTS

Transcriptomic data validated impacts of VEGFA and HIF1A on clinical prognosis and the tumor immune microenvironment

First, we studied the clinical impact of VEGFA with data from GEO, EGA and TCGA databases. Survival was compared between the VEGFA-high and VEGFA-low groups through analysis of 1014 lung adenocarcinoma patients. Significant negative correlation was found between VEGFA expression and OS as shown in Figure 2a ($p = 0.0045$). The survival analysis of HIF1A was also performed using a public database. We found that the high HIF1A expression group had poor overall survival compared with the low expression group ($p = 0.0159$), as shown in Figure 2b.

Further research of gene data revealed an obvious relationship between VEGFA or HIF1A expression and certain immunophenotypes. CD8+ T and B cells were negatively correlated with VEGFA expression in lung adenocarcinoma and lung squamous cell carcinoma patients. Myeloid-derived suppressor cells (MDSCs) were positively correlated with VEGFA expression in both lung adenocarcinoma and lung squamous cell carcinoma. These results suggested an inhibitory tumor immune microenvironment in lung cancer with high expression of VEGFA (Figure 2c). We also found that high expression of HIF1A led to decreased CD8+ T and B cell infiltration, and increased macrophage and MDSC infiltration in lung adenocarcinoma. In lung squamous cell carcinoma, high expression of HIF1A was related to decreased infiltration of CD8+ T and B cells. These also indicated that high expression of HIF1A had a negative impact on the tumor immune microenvironment (Figure 2d).

Bioinformatic analysis revealed gene expression and tumor immune microenvironment changes after irradiation

To investigate expression changes induced by radiotherapy, bioinformatic analysis was performed. There were 6324 differentially expressed genes determined between the

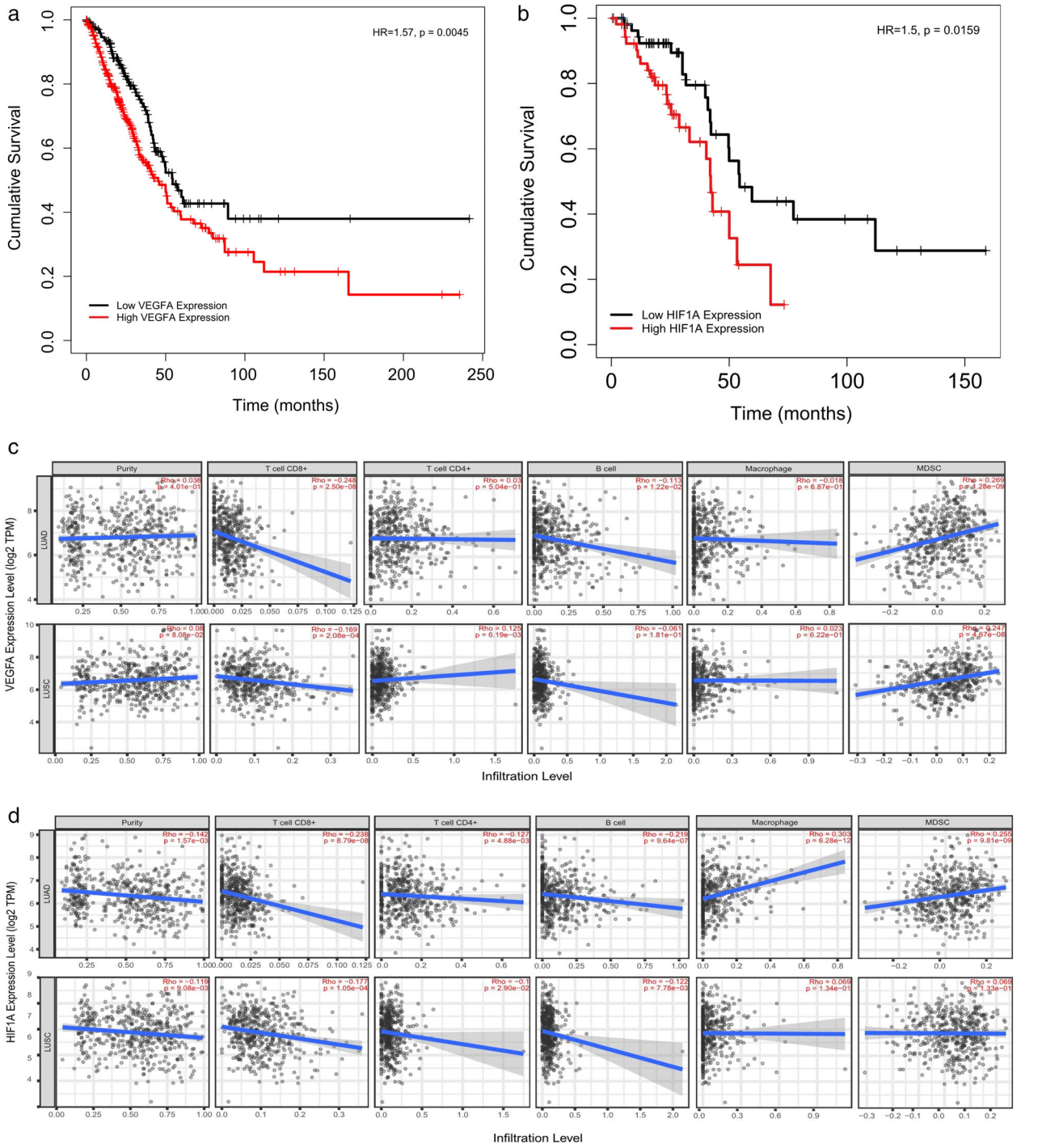

FIGURE 2 Impact of VEGFA and HIF1A on clinical prognosis and the tumor immune microenvironment. (a) Negative correlation between the expression level of VEGFA and overall survival (OS) in lung carcinoma. (b) Negative correlation between the expression level of HIF1A and OS in lung carcinoma. (c) Relationship between VEGFA expression and immune cells in lung cancer. (d) Relationship between HIF1A expression and immune cells in lung cancer. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma

nonirradiation and 6 Gy irradiation groups in GSE24876. The differentially expressed genes were then subjected to functional enrichment analyses. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the HIF-1 and VEGF signaling pathways were among the most significantly enriched pathways (Figure 3a), which suggested that hypofractionated radiotherapy could lead to hypoxia and hyperangiogenesis. Other top enriched pathways included PI3K−Akt signaling pathway, MAPK signaling pathway, Ras signaling pathway, mTOR signaling pathway, FoxO signaling pathway, NF − kappa B signaling pathway and TGF − beta signaling pathway.

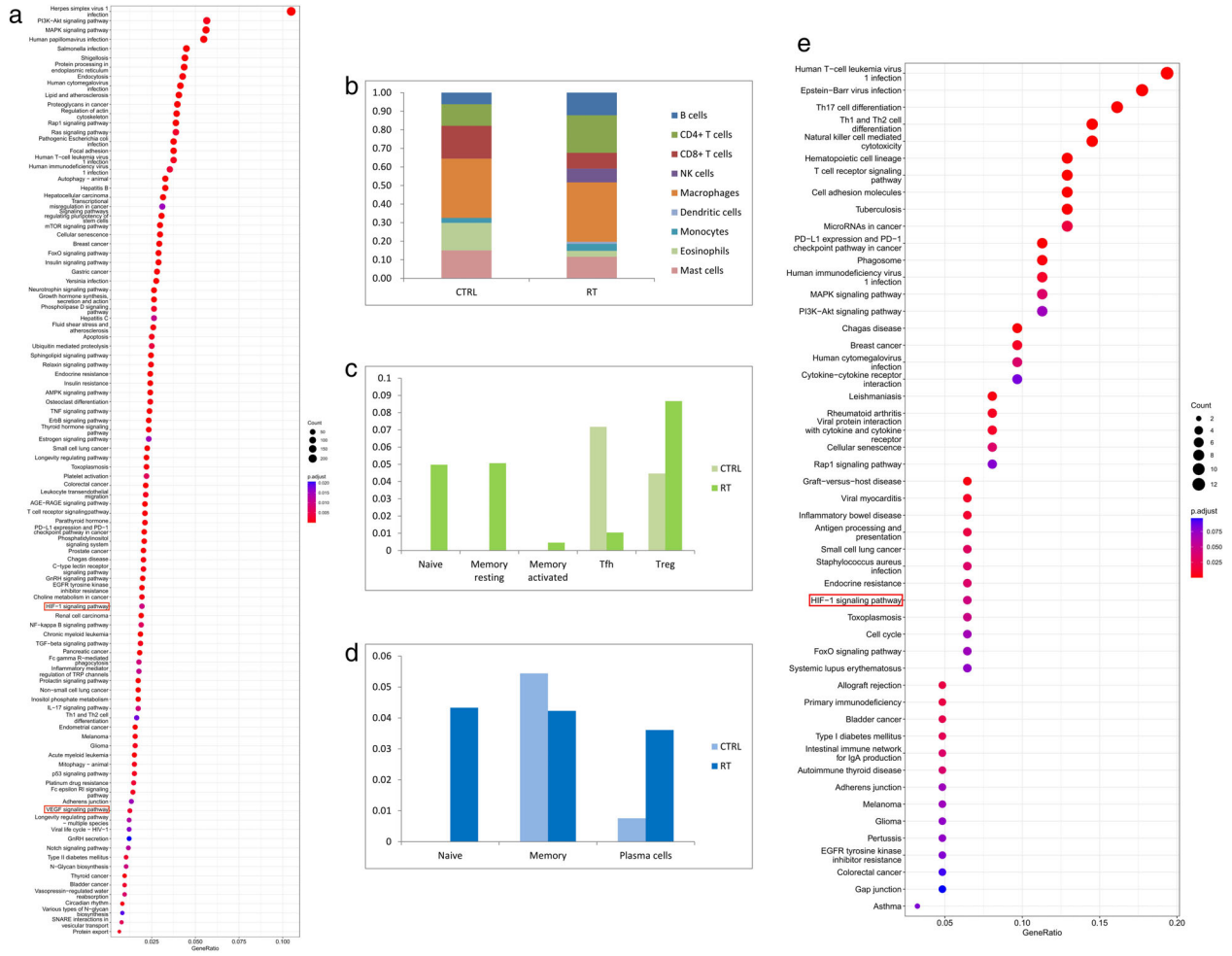

FIGURE 3 Bioinformatic analysis revealed gene expression changes after irradiation and predicted the efficacy of immunotherapy. (a) Enrichment analysis of differentially expressed genes between nonirradiation and 6 Gy irradiation groups. (b) An overview of estimated immune cell abundance in the irradiation and control groups. (c) Estimated CD4+ T cell subsets in each group. (d) Estimated B cell subsets in each group. (e) Enrichment analysis of differentially expressed genes between the immunotherapy durable clinical benefit and nondurable benefit groups in lung cancer patients. RT, radiotherapy; CTRL, control; Tfh, follicular helper T cell; Treg, regulatory T cell.

Hypofractionated radiotherapy can have certain impacts on the tumor immune microenvironment. To gain an insight into the different composition of immune cells between groups, CIBERSORT, a method for estimating the fractions of immune cell types, was engaged. The general immune cell abundance of each group is displayed in Figure 3b. We confirmed an increase of total B cell, CD4+ T cell, monocytes, and natural killer (NK) cell, and a decrease of CD8+ T cell and eosinophil after irradiation. Further analysis of CD4+ T cell revealed that regulatory T cell (Treg) was the dominant type of increased CD4+ T cell (Figure 3c). Plasma cells dramatically increased in B cells (Figure 3d).

Bioinformatic analysis predicted clinical efficacy of immunotherapy

In the immunotherapy cohort of dataset GSE136961, 84 differentially expressed genes were identified between the durable clinical benefit and nondurable benefit groups. Enrichment analyses were carried out to increase our understanding of the dysregulated genes. We found that the HIF−1 signaling pathway was also significantly enriched when comparing the immunotherapy nondurable benefit group with the durable clinical benefit group (Figure 3e), indicating that hypoxia may cause resistance to immunotherapy afterwards.

Famitinib enhanced the efficacy of radioimmunotherapy, and antitumor activity of triple therapy was the most significant

To verify whether famitinib, a vascular-targeted agent, can increase the efficacy of radioimmunotherapy, a C57BL/6 mouse subcutaneous tumor model was established (total rates of tumor formation of the subcutaneously implanted tumor cells were 75%), and several treatment combinations which can be applied clinically were administered respectively.

LLC cells were injected into the left inguinal region of mice and allowed to grow for 10 days when the mean tumor

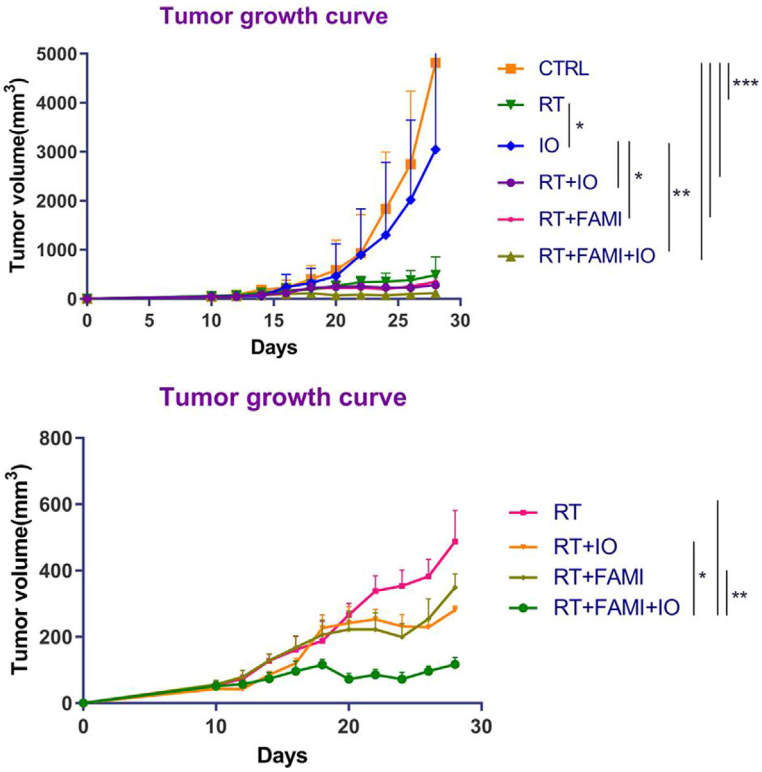

FIGURE 4 Radiotherapy combined with anti-PD-1 and famitinib had the best antitumor activity. (a) Tumor growth curve of mice in control group and experimental groups; (b) tumor growth curve of mice in irradiation group and irradiation combined treatment groups. CTRL, control; RT, radiotherapy; IO, immunotherapy; FAMI, famitinib

volume reached 50 mm^3. The treatment groups included irradiation, anti-PD-1, irradiation + famitinib, irradiation + anti-PD-1, irradiation + anti-PD-1 + famitinib. Figure 4 shows the tumor growth curve of mice. Tumor tissues were collected on the 18th day after the start of treatment. Compared with the control group, the irradiation, irradiation + famitinib, irradiation +- anti-PD-1 and irradiation + anti-PD-1 + famitinib groups could significantly inhibit tumor growth (Figure 4a). Compared with the irradiation group, only the irradiation + anti-PD-1 - + famitinib group significantly inhibited tumor growth (Figure 4b). Among all treatment regimens, the triple therapy showed the strongest antitumor activity (on the 28th day after tumor grafting, tumor volumes of each group: control: 4814 ± 1022 mm^3, irradiation: 487.5 ± 94.39 mm^3, anti-PD-1: 3046 ± 664.5 mm^3, irradiation + famitinib: 349.2 ± 41.17 mm^3, irradiation + anti-PD-1: 280.9 ± 11.99 mm^3, irradiation + anti-PD-1 + famitinib: 116.7 ± 20.96 mm^3). In addition, compared with other combined treatments, the addition of famitinib was well tolerated without a significant decrease in weight in mice. The above data show that triple therapy had the best antitumor activity and good tolerance in a mouse tumor model.

Combination of famitinib and radioimmunotherapy had a synergistic antitumor effect

To elucidate whether combining famitinib and radioimmunotherapy presents a synergistic antitumor effect, we assessed the CDI of this triple therapy. CDI < 1 indicates synergism, especially CDI < 0.7 indicates a significant synergistic effect, CDI = 1 indicates additivity, and CDI > 1 indicates antagonism. With the CDI of the triple therapy being 0.519, we concluded that the combination of famitinib and radioimmunotherapy had a significant synergistic antitumor effect, not another kind of treatment addition.

Triple therapy promoted remodeling of the tumor immune microenvironment

Studies have shown that radiotherapy, immunotherapy, antiangiogenic therapy and different combinations of these treatments can have certain impacts on the tumor immune microenvironment. To explore which strategy has the potential to weaken and reverse the suppressive immune microenvironment, we analyzed the tumor infiltrating immune cells, including CD4+ T cells (CD45+, CD3+, CD4+), CD8+ T cells (CD45+, CD3+, CD8+), MDSCs (CD45+, CD11b+, Gr-1+), and M1 type tumor associated macrophages (M1 cells) (CD45+, CD11b+, F4/80+, CD86+) in the different treatment groups. The results of flow cytometry showed that irradiation + anti-PD-1 - + famitinib produced the most positive tumor immune microenvironment, and immune cell infiltration was significantly increased compared with other groups (Figure 5a). Compared with the control group, the triple therapy group significantly increased the CD8+ T cell infiltration ($p = 0.006$), with the percentage of 27.1%, which was significantly higher than that in any other treatment group (Figure 5b). M1 cell infiltration (26.6%) was also significantly higher than other treatment groups (compared with

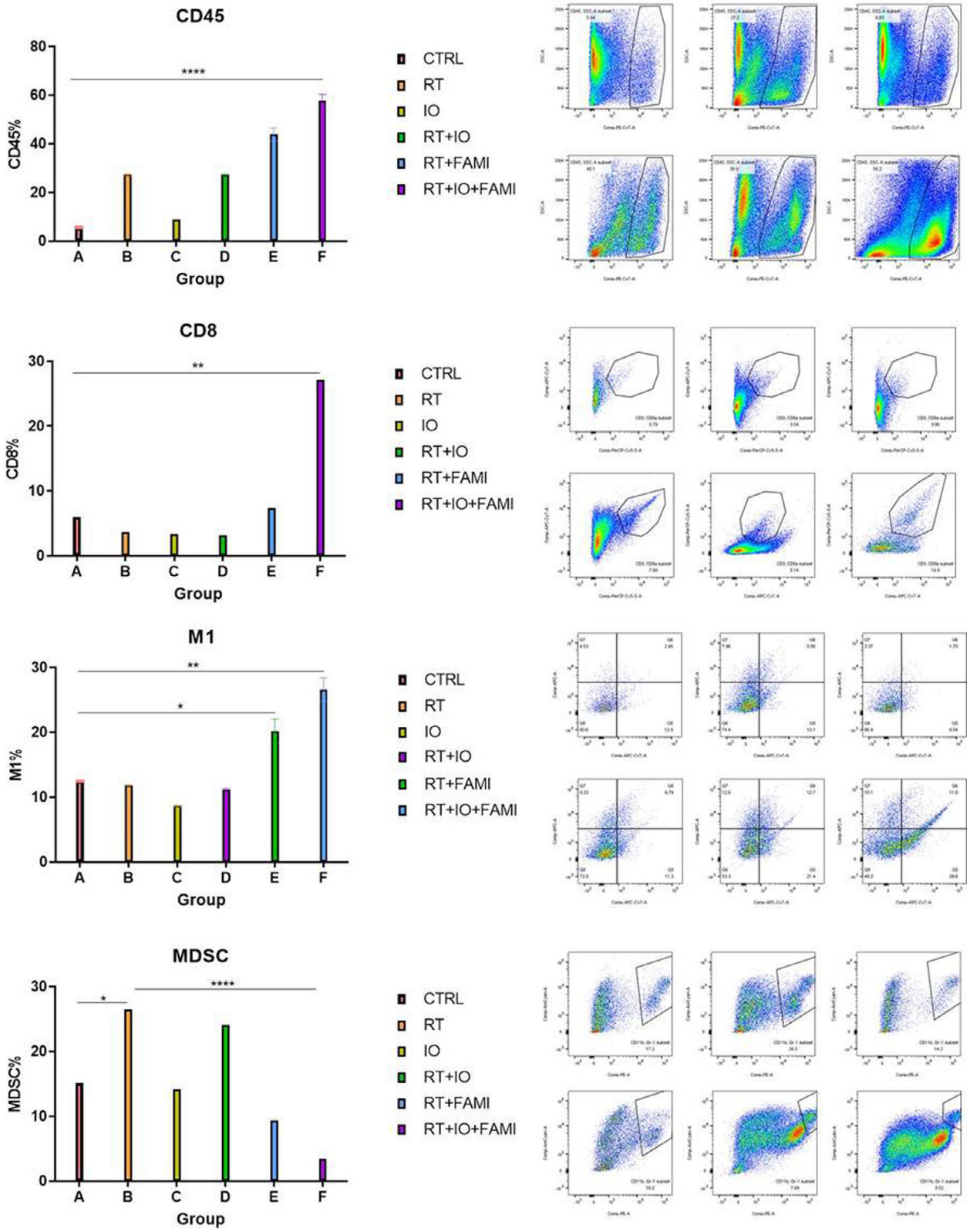

FIGURE 5 Compared with other combination treatments, radiotherapy combined with anti-PD-1 and famitinib promoted the positive tumor immune microenvironment. The infiltration of (a) CD45+ T cells, (b) CD8+ T cells, (c) M1 cells and (d) myeloid-derived suppressor cells (MDSCs). CTRL, control; RT, radiotherapy; IO, immunotherapy; FAMI, famitinib

the control group, $p = 0.0015$) (Figure 5c). Compared with the irradiation group, MDSCs in the triple therapy group was significantly reduced ($p < 0.0001$), and the proportion of MDSC infiltration (3.41%) was the lowest in all groups (Figure 5d). In conclusion, the triple therapy significantly promoted the infiltration of CD8+ T cells and M1 cells, and

reduced MDSCs. Therefore, the triple therapy strategy of radiotherapy, anti-PD-1 and famitinib not only had the best antitumor activity, but also reversed the suppressive tumor immune microenvironment, and significantly enhanced the positive tumor immune microenvironment compared with all other treatments.

DISCUSSION

Our study revealed that both VEGFA overexpression and HIF1A are negatively correlated with lung cancer patient survival, and that the presence of hyperangiogenesis and hypoxia in the TME may compromise the effect of antitumor treatments. The vascular-targeted agent famitinib can enhance the efficacy of radioimmunotherapy, and triple therapy has the best antitumor activity compared with all other treatments. Compared with other treatment combinations, the strategy of combining antiangiogenic therapy with radioimmunotherapy can reverse the suppressive tumor immune microenvironment, significantly promote the infiltration of lymphocytes and produce the most positive tumor immune microenvironment.

The combination of radiotherapy and ICIs has been proven to have obvious synergistic effects and may shift the tumor immune balance toward "elimination", and can therefore effectively suppress tumor growth and metastasis, which is better than radiotherapy or ICIs alone.[9,10] However, there are still some challenges in the combination of radiotherapy and immunotherapy, including the aggravation of tissue hypoxia caused by hypofractionated radiotherapy which is recommended to be combined with immunotherapy, and a large number of abnormal blood vessels counteracting the positive factors in the microenvironment, aggravating immunosuppression and seriously affecting drug delivery, tissue perfusion and lymphocyte infiltration.

We demonstrated that a high level of VEGFA and HIF1A expression in lung cancer is related to poor prognosis in patients and also the immunosuppressive TME. Additionally, the characteristic of TME hypoxia was relevant to decreased efficacy of immunotherapy based on our analysis of a public dataset, which has been elucidated in previous studies.[11–13] It has also been determined from bioinformatic analysis that hypoxia and heperangiogenesis can occur after high-dose irradiation, further resulting in radiation resistance.[14–16] Preclinical studies have demonstrated that HIF-1α can independently promote radiation resistance,[17] and VEGF being reactively upregulated in tumors from the beginning of radiotherapy can also be a contributor to the development of radioresistance.[18] One probable route for radioresistance could be the activation of the PI3K/Akt/mTOR pathway which in turn helps activate the HIF-1α-VEGF pathway in tumor cells.[19] In summary, VEGFA overexpression and TME hypoxia can be a negative prognostic factor and also an efficacy predictor in lung cancer.

Antiangiogenic therapy can reduce abnormal angiogenesis, reconstruct and regulate the tumor vascular system, promote the normalization of the TME, reduce leakage, tumor hypoxia and tumor tissue hydraulic pressure, and improve drug delivery, so as to make the tumor more sensitive to radiotherapy, chemotherapy and immunotherapy.[20,21] In addition, previous studies have reported that angiogenesis inhibitors may downregulate TGF-β1 induced by radiotherapy and other inflammatory molecules, and therefore reduce the degree of radiation-induced lung injury to a certain extent,[22] which can enable more patients to benefit from this combination strategy. Therefore, the combination of radiotherapy, immunotherapy and tumor vascular-targeted therapy has a rich theoretical basis. This study innovatively added the antiangiogenesis agent famitinib to radioimmunotherapy to investigate whether a triple therapy strategy could further improve the antitumor efficacy. The results showed that radiotherapy combined with immunotherapy and antiangiogenic therapy exhibited the most significant antitumor effect in all treatment combinations, and this combination had a significant synergistic antitumor effect.

Previous studies have shown that the infiltration of immune cells in the tumor microenvironment is closely related to the therapeutic effect and prognosis of patients.[23] CD8+ T cells or cytotoxic T lymphocytes (CTL) play a critical role in tumor killing, while regulatory T cells (Treg) weaken the activity of effector T cells and promote the formation of an immunosuppressive microenvironment.[24,25] Most studies have reported that M1 cells play a role in promoting inflammation and antitumor effect, while M2 cells, namely tumor associated macrophages (TAM), promote angiogenesis and tumor invasion by secreting Th2 cytokines.[26,27] Additionally, an important external mechanism of primary resistance to ICIs lies in the suppressive tumor immune microenvironment, characterized by lacking CTL infiltration, T cell exhaustion and phenotypic changes, aggregation of immunosuppressive cell population (Tregs, MDSCs, M2 cells), and the release of inhibitory cytokines and metabolites (CSF-1, tryptophan metabolite, TGF-β, adenosine) in the tumor microenvironment.[28] The type and enrichment of immune cells infiltrating in the tumor stroma determine the characteristics of the tumor immune microenvironment, which can further affect tumor progression and antitumor immune response.

In the tumor immune microenvironment analysis based on a public dataset, we found that even though the subtypes of immune cells were enriched after radiation, there was still a decrease of CD8+ T cells and increase of Tregs in the radiation group, which might lead to a compromised efficacy of combined immunotherapy therapy due to the immunosuppressive microenvironment.

We detected immunophenotyping of the tumor microenvironment in different treatment groups using flow cytometry in an effort to explain the difference of therapeutic effect through the change of tumor immune microenvironment. We found that the triple treatment strategy produced the most positive tumor immunophenotyping compared with other treatment schemes, which is

consistent with its notable antitumor effect. Also, the addition of famitinib to radioimmunotherapy significantly promoted the infiltration of CD8+ T cells in tumor tissues, and the number of M1 cells also reached a peak in the triple therapy group, which are crucial to antitumor immune responses. On the other hand, triple therapy significantly reduced the number of suppressive immune cells. We found that triple therapy reduced MDSC infiltration compared with radiotherapy and other radiotherapy combination treatments. Altogether, radiotherapy combined with immunotherapy and antiangiogenic therapy significantly increased the positive factors in the tumor immune microenvironment and reduced the negative ones, produced the most positive immunophenotyping, and showed the best antitumor effect.

In conclusion, we found that angiogenesis and hypoxia could weaken the efficacy of antitumor therapies, and was associated with a poor prognosis. In the Lewis lung cancer mouse model, the triple strategy of radiotherapy, immunotherapy and antiangiogenic therapy could further increase the infiltration of CD8+ T cells and M1 cells, reduce MDSCs, and significantly improve the tumor immune microenvironment. Compared with radioimmunotherapy, the addition of famitinib showed a more significant antitumor effect. This study has certain clinical significance. Using a mouse model, it was confirmed that tumor vascular-targeted therapy can have a synergistic effect with radioimmunotherapy, which provides a basis for optimizing radioimmunotherapy in clinical research.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

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