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ORIGINAL RESEARCH

Impact of Timing the Combination of Radiotherapy and PD-1 Inhibitors on Outcomes in Patients with Hepatocellular Carcinoma

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Purpose: The optimal timing for combining radiotherapy with immunotherapy in patients with hepatocellular carcinoma (HCC) remains uncertain and affects treatment efficacy and patient outcomes. This study aimed to evaluate and compare the efficacy and treatment-related adverse events (TRAEs) of synchronously administered radiotherapy and programmed cell death protein (PD)-1 inhibitors and sequential administration in patients with HCC.

Patients and Methods: We retrospectively enrolled 67 patients with HCC who were undergoing liver radiotherapy and PD-1 inhibitor therapy at two medical centers between July 2017 and April 2023. Additionally, we created an experimental tumor model using 6-week-old female mice to validate our findings.

Results: In the concurrent group, the median overall survival was indefinite; however, it was 13 months in the sequential group (95% confidence interval [CI] 6.7–19.3 months, P=0.010). The median progression-free survival was significantly longer in the concurrent group (12 months, 95% CI 9.5–14.5 months) than in the sequential group (7 months, 95% CI 1.3–12.7 months; P=0.043). Grade 3/4 TRAEs occurred in 30.4% (concurrent) and 28.6% (sequential) of patients without any treatment-related deaths. In the mouse model, synchronous treatment significantly inhibited tumor growth compared to sequential treatment (293.4±45.18 mm^3 versus 602.7 ±41.68 mm^3; P=0.001). Flow cytometry revealed an increased Tregs/$CD3^+$ T cell ratio and a decreased $CD8^+$/Treg cell ratio post-radiotherapy, suggesting an immunosuppressive tumor microenvironment.

Conclusion: Synchronous treatment demonstrated superior efficacy in treating HCC compared to sequential treatment, with manageable adverse events. The rapid increase in Tregs after radiotherapy may contribute to the reduced efficacy of sequential radiotherapy plus PD-1 inhibitors.

Keywords: immunotherapy, treatment efficacy, adverse events, tumor microenvironment

Introduction

Hepatocellular carcinoma (HCC) is a prevalent and deadly malignant tumor that is frequently diagnosed at advanced stages, preventing radical treatment.[1,2] Advanced HCC is mainly treated with local therapy combined with systemic treatment. Local therapies include radiotherapy, ablation, hepatic arterial infusion chemotherapy, and transarterial chemoembolization (TACE). Systemic treatments include immune checkpoint inhibitors (ICIs) and targeted drugs. Nevertheless, the combination of local therapy and systemic treatment that best improves the prognosis of patients with HCC remains unclear.

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Radiation-induced damage to cancer cells exposes tumor-specific antigens to immune surveillance and facilitates the activation of cytotoxic T cells. Additionally, radiotherapy can activate the tumor immune microenvironment by stimulating the cyclic GMP-AMP synthase/stimulator of interferon genes signaling pathway.[3,4] Hence, radiotherapy is considered an optimal complement to immunotherapy. Combining stereotactic body radiation therapy (SBRT) with programmed cell death-1 (PD-1) inhibitors has yielded favorable clinical outcomes in TACE-refractory patients with intermediate-stage HCC.[5] Five patients with large unresectable HCC treated with SBRT followed by nivolumab showed a 100% objective response rate (ORR) and tolerable toxicity.[6] Therefore, radiotherapy combined with PD-1 inhibitors may serve as a synergistic therapeutic strategy.

One of the key considerations when combining radiotherapy with immunotherapy is the timing of the combination. Although prior studies have examined this aspect, the optimal time window for the combination remains unclear. The PACIFIC trial suggested that patients with unresectable stage III non-small cell lung cancer (NSCLC) receiving devarumab after standard chemoradiotherapy had a significantly improved prognosis.[7] Exploratory analyses revealed that patients randomized ≥14 days post-radiotherapy had significantly extended progression-free survival (PFS) and overall survival (OS) compared to those who were randomized ≤14 days post-radiotherapy. Safety profiles were similar across the subgroups.[8] However, in patients with stage IV NSCLC, no difference in efficacy was observed between the concurrent and sequential dual ICI therapies and SBRT. Concurrent dual ICIs and SBRT were not more toxic than sequential therapy.[9] Multiple studies have shown that radiotherapy combined with ICIs is safe and feasible for melanoma brain metastases (MBM), resulting in a better prognosis than non-synchronous therapy.[10–12] However, in contrast to previous research, the ELEKTRA study found that sequencing radiotherapy followed by ICI treatment led to better immunological responses and clinical outcomes in patients with MBM.[13]

In summary, the results of studies on the timing of radiotherapy combined with ICIs are inconsistent, with some studies showing contradictory results. The optimal timing for combining radiotherapy with PD-1 inhibitors in HCC remains unclear. Therefore, it is crucial to explore the optimal timing of combination therapy in patients with HCC.

This study aimed to compare the efficacy and adverse effects of concurrent and sequential administration of radiotherapy and PD-1 inhibitors in patients with HCC.

Materials and Methods

Patients

Retrospective chart reviews were conducted on patients with HCC who underwent radiotherapy and PD-1 inhibitor treatment at two medical centers (Ganzhou People's Hospital and Nanfang Hospital, Southern Medical University) between July 2017 and April 2023. The following were the criteria for inclusion: (1) definitive diagnosis of HCC, (2) radiotherapy administered for primary liver cancer or portal vein (hepatic vein) cancer thrombus, (3) receipt of at least two cycles of PD-1 inhibitors, and (4) based on the response evaluation criteria in solid tumors (RECIST) version 1.1, at least one measurable lesion must have been present. There were several exclusion criteria, including: (1) pathological findings of cholangiocarcinoma or mixed-type HCC from biopsy or surgery, (2) previous history of liver transplantation, (3) intracranial metastases, (4) non-completion of radiotherapy, (5) presence of other malignancies, and (6) incomplete patient information.

Patients were categorized into the following two groups based on the timing of radiotherapy plus PD-1 inhibitors: radiotherapy concurrent with PD-1 inhibitors (concurrent group) and sequential radiotherapy plus PD-1 inhibitors (sequential group). The concurrent group comprised patients who received anti-PD-1 therapy within 7 days of the first administration of radiotherapy or during radiotherapy.[13–16] In the sequential group, PD-1 inhibitors were administered 1–12 weeks after radiotherapy. The Ethics Committee of Ganzhou People's Hospital (approval number: 001, 2020) and Nanfang Hospital, Southern Medical University (approval number: NFEC-2020-226) approved this study and waived the need for informed consent due to its retrospective nature. All study protocols complied with the guidelines of the Declaration of Helsinki. All patient data was kept confidential.

Data Collection

Age, sex, alpha-fetoprotein level, Eastern Cooperative Oncology Group score, radiotherapy, PD-1 inhibitor, prior therapy, targeted agents, and hepatitis B virus infection status were extracted from the patients' medical records. Baseline patient characteristics were documented within 14 days of the first immunotherapy. PD-1 inhibitors include camrelizumab, sintilimab, tislelizumab, pembrolizumab, nivolumab, and toripalimab. Complete response (CR) or partial response (PR), as well as stable disease (SD) or progressive disease (PD), were assessed using contrast-enhanced magnetic resonance imaging or computed tomography according to RECIST v.1.1 or mRECIST. ORR was classified as CR or PR. The disease control rate (DCR) was calculated by adding CR, PR, and SD. OS was measured from the first administration of PD-1 inhibitors to the date of the last follow-up or death. PFS was assessed from the first treatment to the time of PD, last contact, or death. Treatment-related adverse events (TRAEs) were reviewed based on CTCAE v4.03.

Cell Culture

Dulbecco's Modified Eagle's Medium (Corning, USA) with 10% fetal bovine serum was used to culture Hepa 1–6 cells obtained from the Shanghai Institute of Biochemistry and Cell Biology. The cell culture was maintained in a constant-temperature incubator set at 37°C with a 5% CO_2 atmosphere.

Construction of Subcutaneous Tumor Model

Six-week-old female C57BL/6 mice were procured from the Southern Medical University Animal Resource Center (Guangzhou, China) and randomly allocated to groups. Hepa 1–6 cells were implanted subcutaneously into the C57BL/6 mice's right hind flank. The formula for calculating tumor volume was length × width^2/2. Tumor volumes were measured and recorded every 5 days. Animal ethics approval was granted by the Experimental Animal Ethics Committee of Southern Medical University (SMUL2020118). All animal experiments followed the guidelines of the Institutional Animal Care and Use Committee.

Mice Treatment and Group

Treatment was administered when the tumor size reached approximately 400–500 mm^3. In the first, third, and fifth days after the start of the treatment, three fractions of 6 Gy were administered. The small animal radiation research platform (SARRP, Xstrahl) was used for imaging and radiation. The mice in the concurrent group were intraperitoneally administered anti-mouse PD-1 (200 μg, clone RMP1-14, Bio X Cell) as soon as the treatment began on days 1, 4, and 7. Conversely, in the sequential group, 200 μg anti-mouse PD-1 was administered intraperitoneally on days 14, 17, and 20 post-treatment initiation. For the sequential group receiving anti-CD25, mice received intraperitoneal injections of anti-mouse CD25 (500 μg, clone PC-61.5.3, Bio X Cell) simultaneously with anti-mouse PD-1 on days 14, 17, and 20. The mice were monitored until they reached the standard endpoint or died.

Flow Cytometry

After obtaining single cells of the tumor, single-cell suspensions were stained and analyzed following the methods outlined in our previously published articles.[17] All antibodies used were as follows: Brilliant Violet 421TM-FOXP3 (MF-14, 126419, Biolegend, California, USA), PerCP/Cyanine5.5 CD45 (30-F11, 103132, Biolegend, California, USA), Brilliant Violet510TM-CD8a (53–6.7, 100752, Biolegend, California, USA), FITC CD3 (17A2, 100204, Biolegend, California, USA), PerCP/Cyanine5.5 Brilliant Violet 421TM NK-1.1 (PK136, 108741, Biolegend, California, USA), APC F4/80 (BM8, 123116, Biolegend, California, USA), PE/Cy7 CD206 (C068C2, 141719, Biolegend, California, USA), PE CD86 (GL-1, 105008, Biolegend, California, USA), FITC CD11b (M1/70, 101206, Biolegend, California, USA), and PPE–CD25 (PC61, 102008, Biolegend, California, USA).

Statistical Analysis

Data were analyzed using the IBM Statistical Package for Social Sciences (SPSS) version 26.0 (IBM Corp., Armonk, New York, USA). The Mann–Whitney *U*-test was used to compare continuous covariates. Categorical variables were compared using the chi-square test or Fisher's exact test. The Cochran–Mantel–Haenszel test was used to compare ORR

and DCR between treatment groups using a Clopper–Pearson method with 95% confidence intervals (CIs). An analysis of survival was conducted using the Log rank test and Kaplan–Meier method to estimate OS and PFS. Using a mixed-effects model and Tukey's multiple comparison test, we analyzed mouse subcutaneous tumor volume. We used analysis of variance combined with Tukey's multiple comparison test to analyze the comparisons between three or more groups. A two-tailed P-value of < 0.05 was considered statistically significant. Plots were made with GraphPad Prism V.8.0.2 (San Diego, CA, USA).

Results

Patient Characteristics

Sixty-seven patients with HCC undergoing radiotherapy and taking PD-1 inhibitors at two medical centers were enrolled. Figure 1 depicts the study's flow diagram. Patients were categorized into two groups based on the timing of combination therapy: 46 and 21 patients in the concurrent and sequential groups, respectively. Table 1 illustrates the similarities in the patient characteristics between the two groups.

Clinical Outcome of Concurrent and Sequential Groups

The median follow-up duration was 15 months (range: 4–40 months) and 18 months (range: 1–36 months) in the concurrent and sequential radiotherapy groups, respectively, by the cutoff date of April 2023. Using Kaplan–Meier curves, the OS and PFS were calculated for each group (Figure 2). The median OS was not achieved in the concurrent group. However, it was 13 months (95% CI 6.7–19.3 months) in the sequential group (P=0.010). The survival rates at 6 and 12 months were 94.6% and 84.8%, respectively, in the concurrent group and 78.5% and 49.0%, respectively, in the sequential group. Additionally, the median PFS (12 months, 95% CI 9.5–14.5 months) in the concurrent group was

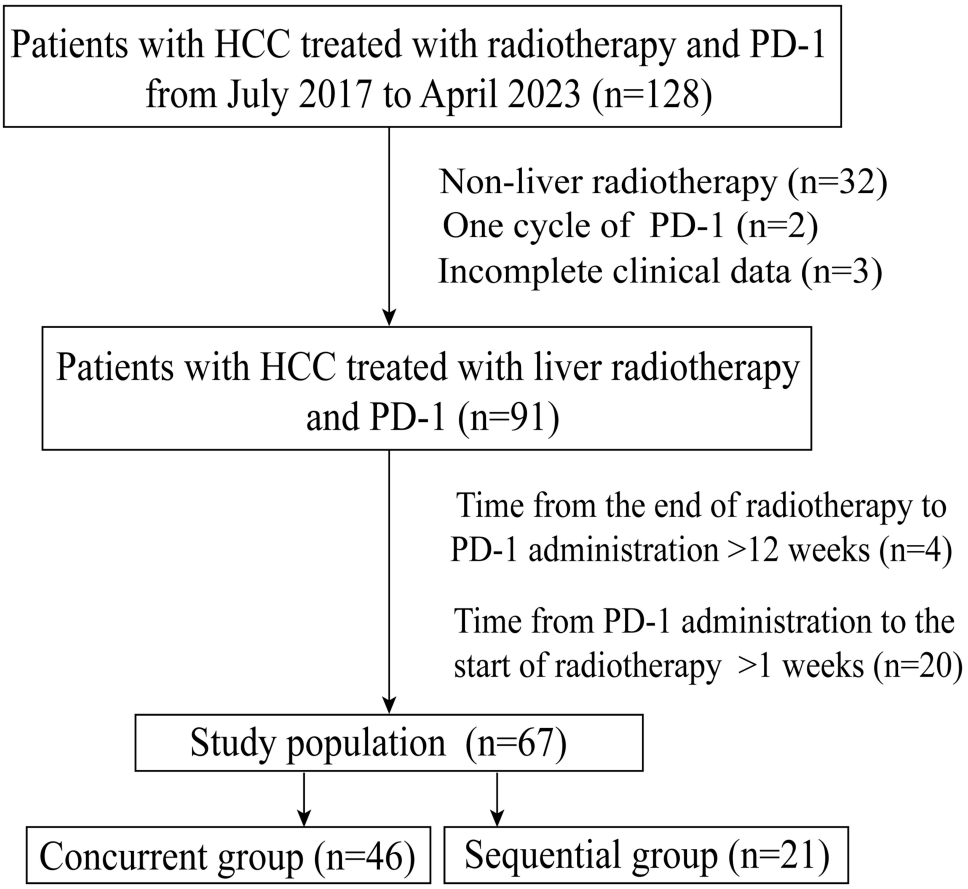

Figure 1 Flow diagram for the present study.

Table 1 Patient Characteristics and Treatments

Characteristics	Concurrent Group (%) (n=46)	Sequential Group (%) (n=21)	*P*
Age (year)			
Median(range)	54 (25–83)	56 (30–84)	0.598
Gender			
Male	43 (93.5)	18 (85.7)	
Female	3 (6.5)	3 (14.3)	0.368
ECOG score			
0–1	44 (95.7)	18 (85.7)	
2	2 (4.3)	3 (14.3)	0.315
Serum AFP level, ng/mL			
<400	21 (45.7)	10 (47.6)	
≧400	25 (54.3)	11 (52.4)	0.881
Child-Pugh class			
A	40 (87.0)	15 (71.4)	
B	6 (13.0)	6 (28.6)	0.124
BCLC stage			
A-B	12 (26.1)	4 (19.0)	
C	34 (73.9)	17 (81.0)	0.758
HBV infection	42 (91.3)	17 (81.0)	0.247
Macrovascular invasion	29 (63.0)	8 (38.1)	0.057
Extrahepatic spread	15 (32.6)	11 (52.4)	0.123
Course of PD-1 inhibitors[a]			
Median(range)	9 (2–43)	7 (2–41)	0.081
Combined with targeted agents	45 (97.8)	19 (90.5)	0.229
Radiotherapy dose (BED Gray)			
Median(range)	73.2 (46.8–100.8)	72 (48–86.4)	0.919
Radiotherapy technique			
SBRT	14 (30.4)	7 (33.3)	
Hypofractionated radiotherapy	18 (39.1)	7 (33.3)	
Conventional radiotherapy	14 (30.4)	7 (33.3)	0.902
Prior treatment			
Hepatectomy	5 (10.9)	6 (28.6)	0.070
Ablation	9 (19.6)	8 (38.1)	0.106
TACE or HAIC	41 (89.1)	21 (100.0)	0.173

Notes: [a]Including camrelizumab (n=26), tislelizumab (n=18), sintilimab (n=11), toripalimab (n=7), pembrolizumab (n=3), nivolumab (n=2).

Abbreviations: ECOG, Eastern Cooperative Oncology Group; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; BED, biological equivalent dose; SBRT, stereotactic body radiotherapy; TACE, trans-arterial chemo-embolization; HAIC, hepatic arterial infusion chemotherapy.

significantly better than that in the sequential group (7 months, 95% CI 1.3–12.7 months; *P*=0.043). The PFS at 6 months was 87.8% and 55.8% in the concurrent and sequential groups, respectively.

Treatment efficacy was evaluated in 66 of the 67 patients. The treatment response was not assessed during weeks 6–8 following the first administration of immunotherapy in one patient. As shown in Table S1, the ORRs were 58.7% (95% CI 43.2–73.0%) and 30.0% (95% CI 11.9–54.3%) in the concurrent group and sequential groups, respectively, based on independent assessment using RECIST 1.1 (*P*=0.036). The DCR was 97.8% (95% CI 88.5–99.9%) in the concurrent group compared with that in the sequential group (75.0%, 95% CI 50.9–91.3%, *P*=0.017). The best overall response waterfall plots (RECIST 1.1) are shown in Figure S1. According to the HCC-specific mRECIST, 35 (76.1%) patients in the concurrent group, as compared to 9 (45.0%) in the sequential group, had a CR or PR (Table S2). The best overall response waterfall plots (mRECIST) are shown in Figure S2.

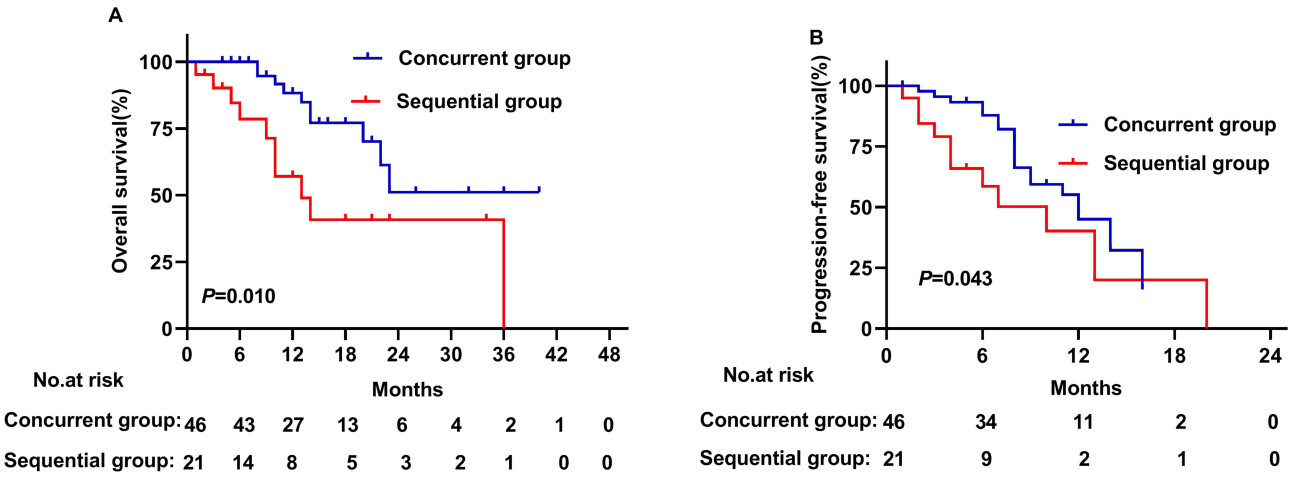

Figure 2 Kaplan–Meier curves of (**A**) overall and (**B**) progression-free survival between the concurrent radiotherapy group and sequential radiotherapy group.

Safety of Concurrent Group and Sequential Group

Overall, 67 patients had at least one TRAE (Table 2). The most common TRAEs of any grade were decreased platelet count (23.9%), increased aspartate aminotransferase (AST) levels (23.9%), increased alanine transaminase (ALT) levels (22.4%), decreased appetite (17.9%), decreased white blood cell (WBC) count (14.9%), and rash (14.9%). Grade 3/4

Table 2 Treatment Related Adverse Events in Concurrent Group and Sequential Group

Adverse Events	Any Grade n(%)			Grade 3/4 n(%)		
	Concurrent Group (n=46)	Sequential Group (n=21)	*P*	Concurrent Group (n=46)	Sequential Group (n=21)	*P*
Decreased platelet count	11(23.9%)	5(23.8%)	0.993	1(2.2%)	2(9.5%)	0.229
Aspartate aminotransferase increase	10(21.7%)	6(28.6%)	0.543	2(4.3%)	1(4.8%)	>0.999
Alanine transaminase increase	10(21.7%)	5(23.8%)	0.850	2(4.3%)	1(4.8%)	>0.999
Decreased appetite	9(19.6%)	3(14.3%)	0.858	1(2.2%)	0	>0.999
Decreased white blood cell count	7(15.2%)	3(14.3%)	>0.999	1(2.2%)	1(4.8%)	0.532
Rash	7(15.2%)	3(14.3%)	>0.999	1(2.2%)	0	>0.999
Fatigue	7(15.2%)	2(9.5%)	0.804	0	0	–
Nausea	6(13.0%)	1(4.8%)	0.550	1(2.2%)	0	>0.999
Pruritus	4(8.7%)	1(4.8%)	0.946	0	0	–
Blood bilirubin increase	4(8.7%)	1(4.8%)	0.946	2(4.3%)	0	>0.999
Diarrhea	2(4.3%)	2(4.3%)	0.784	0	0	–
Gastrointestinal hemorrhage	2(4.3%)	1(4.8%)	>0.999	0	1(4.8%)	0.313
Dental ulcer	2(4.3%)	1(4.8%)	>0.999	0	0	–
Abdominal pain	2(4.3%)	1(4.8%)	>0.999	0	0	–
Vomiting	2(4.3%)	1(4.8%)	>0.999	0	0	–
Infusion-related reaction	2(4.3%)	0	>0.999	0	0	-
Hypothyroidism	1(2.2%)	1(4.8%)	0.532	0	0	-
Immune nephritis	1(2.2%)	0	>0.999	1(2.2%)	0	>0.999
Immune encephalitis	1(2.2%)	0	>0.999	1(2.2%)	0	>0.999
Immune colitis	1(2.2%)	0	>0.999	1(2.2%)	0	>0.999
Epistaxis	1(2.2%)	0	>0.999	0	0	-

TRAEs occurred in 14 (30.4%) of the 46 patients who received synchronous radiotherapy plus PD-1 inhibitors, primarily including liver dysfunction (elevated AST, ALT, and bilirubin levels), decreased platelet count, and decreased WBC count. Grade 3/4 TRAEs occurred in six (28.6%) of the 21 patients in the other group of sequential radiotherapy plus PD-1 inhibitors, including increased AST, ALT, decreased WBC, and gastrointestinal hemorrhage. Notably, no treatment-related deaths were reported. Compared with the sequential group, synchronous radiotherapy plus PD-1 inhibitors did not significantly increase the incidence of TRAE.

Mice Model of Concurrent Group and Sequential Group

To further validate the efficacy of the concurrent and sequential treatments, we constructed a subcutaneous tumor model. Figure 3 shows the subcutaneous tumor growth curves for both groups. Radiotherapy combined with concurrent PD-1

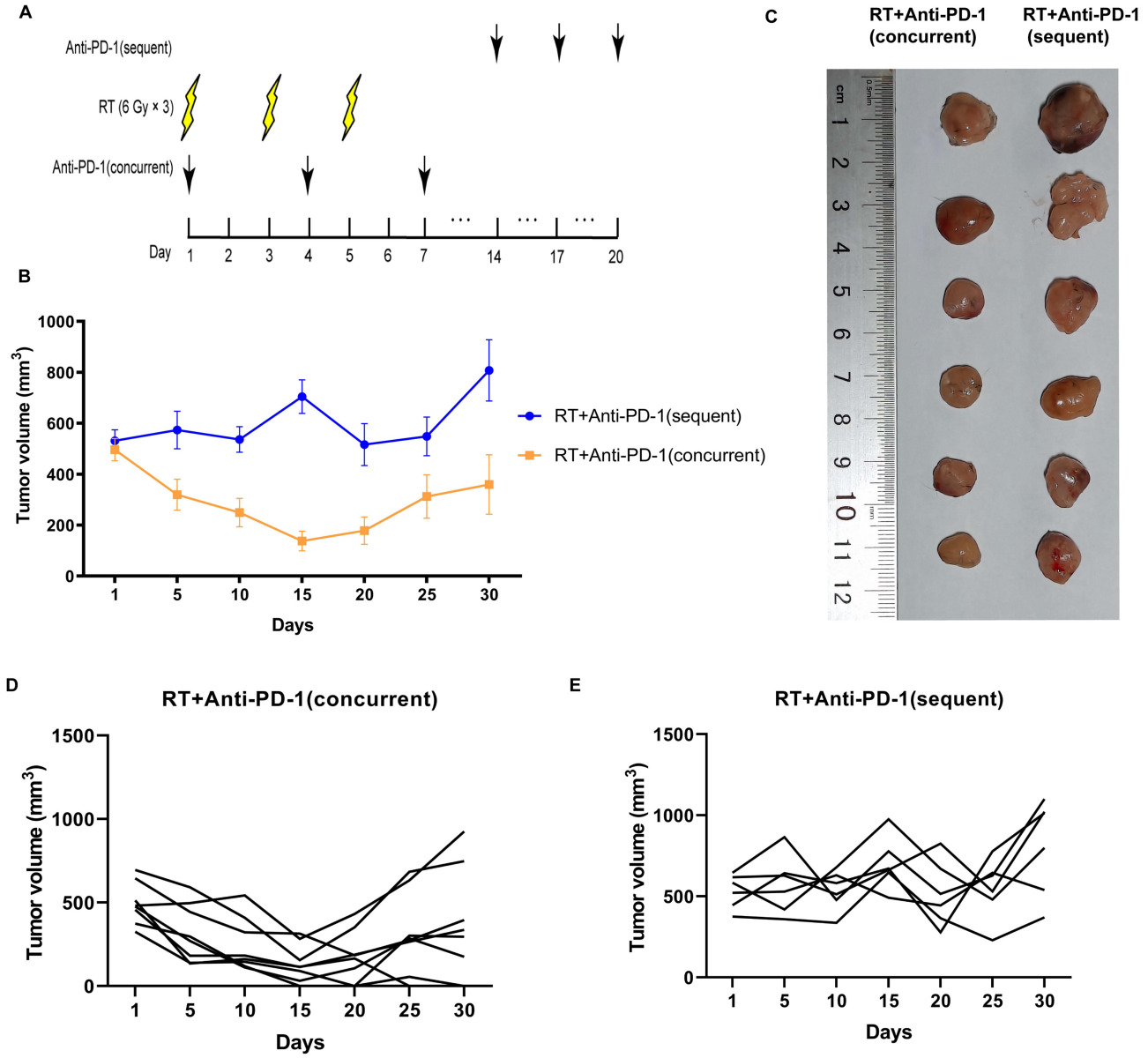

Figure 3 In a mouse model with subcutaneous graft tumors, concurrent radiotherapy (RT) plus anti-PD-1 therapy significantly inhibited tumor growth compared with sequential RT plus anti-PD-1 therapy. (**A**) Schematic showing schedules of RT and anti-PD-1 therapy. (**B**) Response of the subcutaneous tumors to the indicated treatment regimens. (**C**) A representative picture of subcutaneous tumors at the endpoint of the experiment. Two mice in the concurrent group had complete tumor remission on day 30. (**D**)Response of the individual tumor volumes in the concurrent RT plus anti-PD-1 therapy group (n=8). (**E**) Response of the individual tumor volumes in the sequential RT plus anti-PD-1 therapy group (n=6).

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inhibitors significantly inhibited tumor growth compared to the sequential approach (293.4±45.18 mm^3 vs 602.7 ±41.68 mm^3; P=0.001).

Changes of Tumor-Infiltrating Immune Cells After Radiotherapy

To understand why concurrent radiotherapy with PD-1 inhibitors was more efficacious than sequential radiotherapy plus PD-1 inhibitors, tumor tissues were harvested on days 7, 14, 21, and 28 after the initiation of radiotherapy. Flow cytometry analysis of tumor tissues was conducted to assess changes in tumor-infiltrating immune cells after radiotherapy. The tumor-infiltrating lymphocyte (TIL) Tregs/$CD3^+$ T cell ratio increased on day 7 (6.252%±1.490%) and peaked on day 14 (12.04%±7.028%, P=0.0183), as shown in Figure 4. Conversely, the Tregs/$CD3^+$ T cell ratio gradually decreased on days 21 and 28. On day 7, the number of TIL $CD8^+$ T cells was 99.90±84.23, indicating a decreasing trend with time, reaching the lowest on day 28 (45.20±13.07, P=0.189). Although the difference in TIL $CD8^+$ T cells did not reach statistical significance, the TIL $CD8^+$/Tregs ratio on day 14 significantly declined compared to that on day 7 (9.005 ±6.422 vs 23.10±7.625; P=0.0134). From day 21 to 28, the TIL $CD8^+$/Tregs ratio decreased further (P=0.5629). Therefore, the TIL Tregs/$CD3^+$ T cell ratio significantly increased, whereas the TIL $CD8^+$ T cell and TIL $CD8^+$/Tregs ratio decreased on day 14. This may explain why tumor control was worse in the sequential group (PD-1 inhibitor administered on day 14 after radiotherapy initiation) than in the concurrent therapy. Additionally, we analyzed the

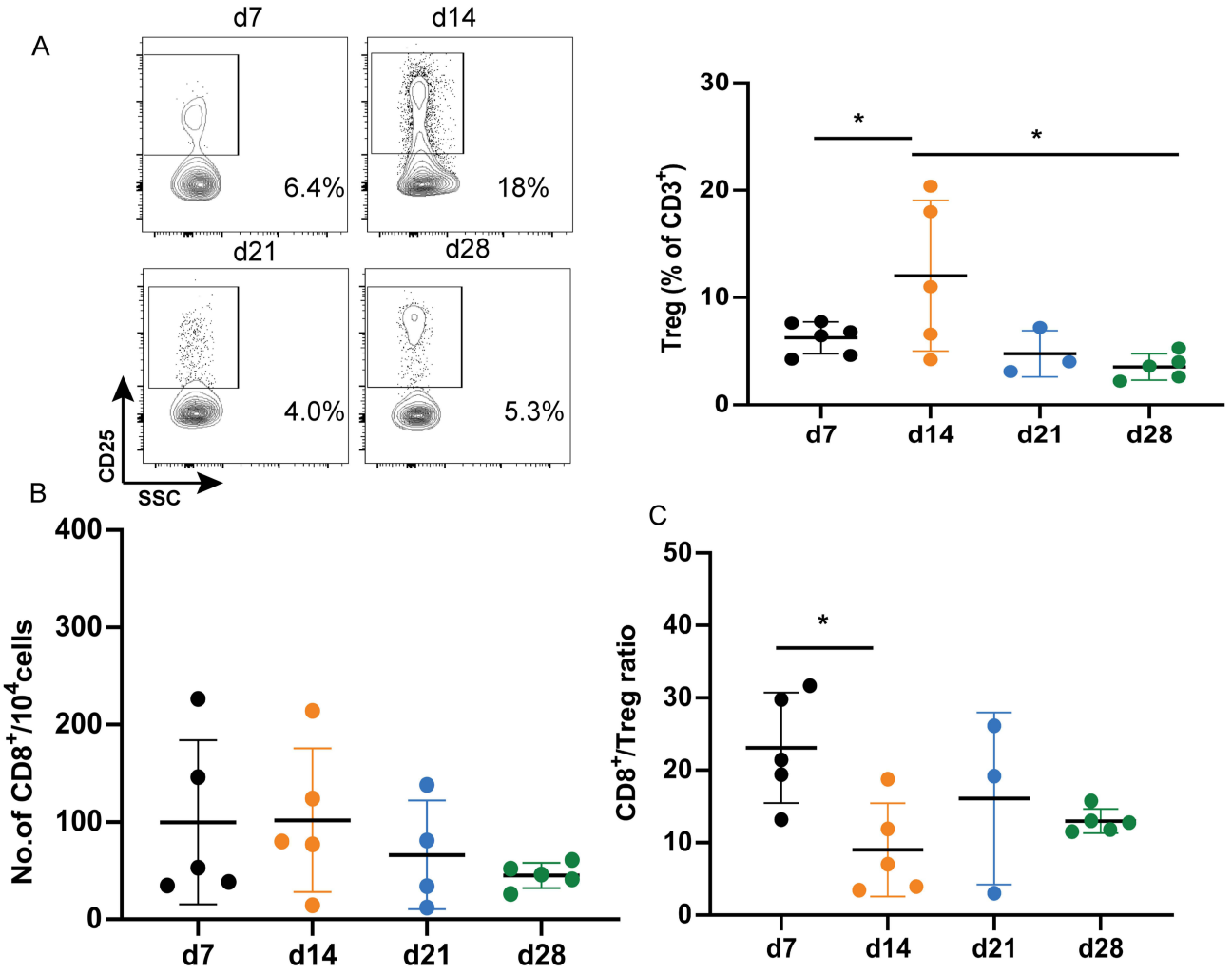

Figure 4 Change of T cell infiltration levels in tumors by different periods for mice receiving radiotherapy. (**A**) Quantitation of Tregs/CD3 + T ratios in tumors starting radiotherapy on days 7, 14, 21, and 28. (**B**) Quantitation of the number of CD8 + T cells per 10 4 cells in tumors starting radiotherapy on days 7, 14, 21, and 28. (**C**) Quantitation of CD8 + /Tregs ratios in tumors starting radiotherapy on days 7, 14, 21, and 28. *P < 0.05, statistically significant.

changes in natural killer cells, M1-type macrophages, M2-type macrophages, and myeloid-derived suppressor cells after radiotherapy (Figure S3).

Anti-CD25 reversed tumor growth in the group receiving radiotherapy sequentially plus PD-1 inhibitors (666.9 ±22.01 mm^3 vs 701.3±51.92 mm^3; P=0.0064) and achieved tumor control effects similar to those of radiotherapy administered synchronously with PD-1 inhibitors (666.9±22.01 mm^3 vs 531.6±46.91 mm^3; P=0.0681) (Figure S4).

Discussion

This is the first study to investigate the optimal timing of the combination of radiotherapy and ICIs in HCC. Our findings revealed that radiotherapy administered concurrently with PD-1 inhibitors had better efficacy than sequential radiotherapy plus PD-1 inhibitors alone. Additionally, the synchronous group exhibited tolerable toxic side effects.

Kaplan–Meier analysis and Log rank test indicated better OS and PFS in the concurrent group. These results are consistent with the findings from a study in 2021, which observed that concurrent radiotherapy of brain metastasis combined with ICIs significantly improved OS and PFS compared with the non-concurrent group and that simultaneous combination was an independent prognostic factor.[18] Furthermore, a meta-analysis published in *Radiotherapy and Oncology* demonstrated prolonged OS with concurrent radiotherapy and immunotherapy in patients with brain metastasis.[19] According to the RECIST 1.1 criteria, the ORR of the concurrent group (58.7%) surpassed that of the sequential group (30.0%, P=0.036). A Phase II clinical study reported an ORR of 52.4% with SBRT combined with carlizumab for HCC, which is consistent with our findings.[20] In this study, no additional toxic effects or safety events were identified. Notably, in both the concurrent and sequential treatment groups, most patients received targeted therapy. Hence, TRAEs caused by targeted drugs were not excluded. Radiotherapy combined with atezolizumab and bevacizumab for HCC has shown that the most common TRAEs include neutropenia, fatigue, and hypertension.[21] Additionally, we demonstrated that radiotherapy combined with a PD-1 inhibitor and anti-vascular targeted therapy is safe and feasible for HCC.[16]

In a mouse model of subcutaneous graft tumors, we demonstrated that synchronous radiotherapy plus PD-1 inhibitors significantly inhibited tumor growth compared to the sequential approach. This finding is consistent with our clinical results. Concurrent PD-L1 inhibitors and radiotherapy are more effective in controlling tumor growth than sequential treatments, as shown in an animal model of colorectal cancer.[22] Additionally, another animal experiment in pancreatic cancer found that anti-PD-L1 synchronously combined with radiotherapy significantly improved the antitumor response; however, radiosensitization completely disappeared when anti-PD-L1 was delayed to 7 days after radiotherapy.[23] Synchronous radiotherapy combined with anti-PD1/PD-L1 therapy may induce a synergistic antitumor host immune response and improve treatment response.

Previous research by our group showed that simultaneous radiotherapy combined with anti-PD-L1 treatment has a strong immunostimulatory effect, including increasing the number of tumor $CD8^+$ T cells and enhancing the activity of $CD8^+$ T cells, as well as reducing the infiltration of Tregs.[17] This study conducted a comprehensive analysis of the alterations in tumor-infiltrating immune cells at various time points following radiation therapy to determine the optimal timing for intervention with ICIs. The ratio of Tregs to $CD3^+$ T cells exhibited a rapid increase from days 7 to 14 post-radiation therapy, whereas the TIL $CD8^+$ T cell and TIL $CD8^+$/Tregs ratio decreased on day 14. Consequently, targeting Tregs may be a critical factor influencing the suboptimal efficacy of sequential PD-1 inhibitor therapy after radiation treatment. Furthermore, we discovered that administration of CD25 antibodies could reverse the diminished effectiveness of sequential anti-PD-1 therapy following radiation. In the immune evasion mechanism of HCC, Tregs suppress the ability of immune cells to kill HCC cells through various pathways, thereby promoting the immune evasion of HCC cells. Tregs are closely related to poor prognosis in patients with HCC. They not only inhibit excessive immune responses to prevent normal function from being impaired but also promote the formation of immune tolerance to protect HCC cells from systemic attacks. Additionally, studies have shown that excessive Tregs in patients with HCC are associated with poor treatment outcomes, as Tregs limit the effect of immunotherapy and weaken the efficacy of ICIs.[24] Tregs play a pivotal role in the regrowth of head and neck tumors following radiotherapy combined with ICIs. The depletion of Tregs can enhance antitumor immune responses and induce antitumor memory immunity, leading to tumor shrinkage.[25] Clinical studies have associated Tregs with tumor progression, recurrence, and treatment resistance in various malignancies, including colorectal cancer, soft tissue sarcomas, endometrial cancer, and head and neck squamous carcinoma.[26–29] Tumors with high oxidative stress promote the enrichment of Tregs,

fostering an immunosuppressive microenvironment and resistance to ICIs.[30] Radiotherapy not only has positive regulatory effects on the tumor immune microenvironment but also increases the infiltration of Tregs into the tumor microenvironment to produce immunosuppressive effects.[31–33] In 2011, a study that evaluated the effects of radiation on Treg cells was published in *International Journal of Radiation Oncology, Biology, Physics*. The results suggested that Treg cells have a stronger resistance to radiation than other lymphocytes, leading to a prior increase in their numbers and the induction of an immunosuppressive microenvironment.[34] This is similar to our findings. Fukushima reported that Treg-directed therapy synergistically improved the efficacy of PD-1 inhibitors in syngeneic murine tumor models.[35] Therefore, targeting Tregs may improve the therapeutic potential of radiotherapy in conjunction with immunotherapy.

In our study, we had some limitations. First, the clinical data were constrained by the small sample size and retrospective design. Second, the substantial heterogeneity of the patient population and treatment regimen may affect the interpretation of our findings. Moreover, our in vivo efficacy experiments solely utilized an HCC subcutaneous xenograft model, limiting the generalizability of our findings. Various animal models, including orthotopic models, can provide more robust therapeutic insights. Therefore, further investigation of the underlying molecular mechanisms is warranted to develop a thorough understanding of the therapeutic effects of this treatment regimen.

Conclusion

Synchronous radiotherapy plus PD-1 inhibitors may achieve better tumor control than sequential radiotherapy plus PD-1 inhibitors with manageable TRAEs. Additionally, Treg cell enrichment may contribute to the varying efficacies of the two approaches. Large randomized controlled clinical studies are required to validate the safety and efficacy of synchronous radiotherapy and PD-1 inhibitors.

Abbreviations

PD-1, Programmed Cell Death Protein 1; HCC, Hepatocellular Carcinoma; TRAEs, Treatment-Related Adverse Events; CI, Confidence Interval; RECIST, Response Evaluation Criteria in Solid Tumors; TACE, Trans-Arterial Chemo-Embolization; ICIs, Immune Checkpoint Inhibitors; SBRT, Stereotactic Body Radiation Therapy; ORR, Objective Response Rate; DCR, Disease Control Rate; OS, Overall Survival; PFS, Progression-Free Survival; CTCAE, Common Terminology Criteria for Adverse Events; C57BL/6, A common strain of laboratory mouse; SARRP, Small Animal Radiation Research Platform; TIL, Tumor-Infiltrating Lymphocyte; NSCLC, Non-Small Cell Lung Cancer; mRECIST, Modified Response Evaluation Criteria In Solid Tumors; PC-61.5.3, A monoclonal antibody; NCI, National Cancer Institute; ALT, Alanine Transaminase; WBC, White Blood Cell; Gy, Gray (unit of radiation dose); SPSS, Statistical Package for the Social Sciences; CR, Complete Response; PR, Partial Response; SD, Stable Disease; PD, Progressive Disease; OR, Odds Ratio; C57BL/6, A common strain of laboratory mouse; MBM, Melanoma Brain Metastases; AST, Aspartate Aminotransferase.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests that are relevant to the study.

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