Radiotherapy-induced early change in lymphocyte subset counts in patients with recurrent or metastatic hepatocellular carcinoma.

การเปลี่ยนแปลงของระดับเม็ดเลือดขาวในกลุ่มลิมโฟไซต์หลังการฉายรังสีในผู้ป่วยมะเร็งตับที่มีการกลับเป็นซ้ำหรือระยะแพร่กระจาย

Yukihiro Hama, Etsuko Tate

Department of Radiation Oncology, Tokyo-Edogawa Cancer Centre, Edogawa Hospital.

Corresponding author

Yukihiro Hama

Department of Radiation Oncology, Tokyo-Edogawa Cancer Centre, Edogawa Hospital.

2-24-18 Higashikoiwa, Edogawa, Tokyo, 133-0052, Japan

Telephone: +81-3-3673-1221

Email address: yjhama2005@yahoo.co.jp

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Abstract

Background: In addition to the direct effects of radiation therapy on cancer, the subsequent immune response activates the immune system.

Objective: The purpose of this study was to investigate early changes in a subset of peripheral blood lymphocytes in patients with recurrent or metastatic hepatocellular carcinoma (HCC) after radiation therapy.

Materials and methods: Lymphocyte subset analysis of seven consecutive patients with recurrent or metastatic HCC was performed at two time points: immediately before and two weeks after the start of radiation therapy.

Results: Absolute lymphocyte counts decreased two weeks after the start of radiation therapy, but the percentage of CD4+ lymphocytes increased from 30.5 +/- 5.4% before radiation therapy to 42.0 +/- 5.3% two weeks after the start of radiation therapy ($p<0.01$). The CD4+/CD8+ ratio increased significantly from 0.88 +/- 0.32 before radiation therapy to 1.45 +/- 0.62 two weeks after the start of radiation therapy ($p=0.02$).

Conclusion: This is the first report of recurrent or metastatic HCC that radiation therapy induces CD4+ T lymphocytes and increases the CD4+/CD8+ ratio in two weeks. Understanding the lymphocyte subset changes two weeks after the start of radiation therapy may help in developing treatment strategies for recurrent or metastatic HCC.

Keywords: Cancer immunotherapy, radiotherapy, lymphopenia, immunomodulatory effect

บทคัดย่อ

หลักการและเหตุผล: นอกจากผลโดยตรงของการฉายรังสีที่สามารถทำลายเซลล์มะเร็งแล้ว การฉายรังสียังกระตุ้นระบบภูมิคุ้มกันของร่างกายและเกิดการตอบสนองของระบบภูมิคุ้มกันตามหลังอีกด้วย

วัตถุประสงค์: เพื่อศึกษาการเปลี่ยนแปลงของระดับเม็ดเลือดขาวในกลุ่มลิมโฟไซต์หลังการฉายรังสีในคนไข้มะเร็งตับที่มีการกลับเป็นซ้ำหรือระยะแพร่กระจาย

วัสดุและวิธีการ: ตรวจวัดระดับเม็ดเลือดขาวในกลุ่มลิมโฟไซต์ในเลือดของผู้ป่วย 7 คนที่เป็นมะเร็งตับและมีการกลับเป็นซ้ำหรืออยู่ในระยะแพร่กระจาย โดยทำการตรวจวัดก่อนฉายรังสีทันทีและที่ 2 สัปดาห์หลังฉายรังสี

ผลการศึกษา: ระดับลิมโฟไซต์ทั้งหมดลดลงที่ 2 สัปดาห์หลังฉายรังสี แต่ร้อยละของลิมโฟไซต์ ชนิด CD4+ เพิ่มขึ้นจาก 30.5 +/- 5.4 ก่อนการฉายรังสีเป็นร้อยละ 42.0 +/- 5.3 หลังฉายรังสีอย่างมีนัยสำคัญทางสถิติ ($p<0.01$) และอัตราส่วนของ CD4+ ต่อ CD8+ เพิ่มอย่างมีนัยสำคัญจาก 0.88 +/- 0.32 ก่อนการฉายรังสี เป็น 1.45 +/- 0.62 หลังการฉายรังสี ($p=0.02$)

ข้อสรุป: งานวิจัยนี้เป็นงานวิจัยแรกที่รายงานการเพิ่มขึ้นของร้อยละของลิมโฟไซต์ ชนิด CD4+ และอัตราส่วนของ CD4+ ต่อ CD8+ ที่สองสัปดาห์หลังฉายรังสี ในผู้ป่วยมะเร็งตับที่มีการกลับเป็นซ้ำหรืออยู่ในระยะแพร่กระจาย โดยที่

การทราบการเปลี่ยนแปลงของระดับเม็ดเลือดขาวในกลุ่มลิมโฟไซต์อาจมีประโยชน์ในการพัฒนาการรักษาในอนาคต

คำสำคัญ: การรักษาด้วยภูมิคุ้มกันบำบัด, การฉายรังสี, ระดับเม็ดเลือดขาว, การกระตุ้นภูมิคุ้มกัน

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Introduction

Radiation therapy and immunotherapy are treatment options for patients with recurrent or metastatic hepatocellular carcinoma (HCC) for whom targeted therapy with multi-kinase inhibitors or transcatheter arterial chemoembolization (TACE) is unsuitable or ineffective[1,2]. Recent advances in radiation therapy have made it possible to irradiate intrahepatic HCC and extrahepatic metastases accurately with minimal adverse events[2,3]. Radiation therapy has been reported to improve T lymphocyte immune responses by altering differentiation pathways and functions[1-3]. Immunotherapy with immune checkpoint inhibitors (ICIs) against programmed death receptor-1 (PD-1) and its ligand (PD-L1) can prolong survival[4], however, many patients with HCC have primary resistance to ICIs, and the response rates have been reported to be 10-30%[4,5]. It is also unclear whether the ICIs in combination with radiation therapy further prolong overall survival.

Radiation therapy has been shown to promote the antitumor immunity, alter the tumor microenvironment, and synergize with ICIs[5-7]. There is also tumor resistance to radiation therapy, with the primary mechanism reported to be the exhaustion of antitumor immunity via CD4+ T lymphocyte suppression and apoptosis of cytotoxic CD8+ T lymphocytes (CTLs)[8]. Thus, knowledge of early changes in lymphocyte subset counts during and after radiation therapy is important in determining when to initiate immunotherapy among patients with recurrent or metastatic HCC. Peripheral blood lymphocyte counts usually decrease within two weeks after the start of radiation therapy, but if antitumor lymphocytes are induced, administration of ICIs may amplify antitumor immunity.

The purpose of this study was to investigate early changes in a subset of peripheral blood lymphocytes in patients with recurrent or metastatic HCC after radiation therapy.

Materials and Methods

This study was approved by the institutional review board of Edogawa Hospital (RO20190503b), and all procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its

later amendments or comparable ethical standards. Written informed consent was obtained from all patients for use of clinical data in research.

Patients

A total of 7 consecutive patients with recurrent or metastatic HCC who underwent intensity-modulated radiation therapy (IMRT) or stereotactic ablative radiotherapy (SABR) using helical tomotherapy (Hi-ART system, Accuray, Sunnyvale, CA, USA) were included in this study. Radiation therapy was given for oligometastases (1-5 metastatic lesions) or in cases of recurrence after secondary targeted therapy or TACE.

Radiation therapy

Helical IMRT was used to treat lymph node metastases and SABR was used to treat liver, pulmonary or adrenal gland metastases. Prescribed doses were 50-60 Gy in 25-30 fractions for IMRT and 40-60 Gy in 4-8 fractions for SABR. Each dose was defined as the minimum dose received by 95% planning target volume (D95%). Most patients received radiation treatments daily, 5 days a week for 2 to 6 weeks. No patients received chemotherapy or immunotherapy concurrently with radiation therapy. Chemo-therapy, immunotherapy, and other treatments were stopped at least 2 weeks prior to the start of radiation therapy and for 2 weeks after the start of radiation therapy.

Lymphocyte subset measurements

Peripheral blood lymphocyte subsets were measured immediately before and 2 weeks after the start of radiation therapy. Whole blood was used, and staining for CD3, CD4 and CD8 was performed after red cell lysis, according to a standard flow cytometric multicolor protocol. For patients previously treated with anticancer drugs (TACE and Lenvatinib), blood samples were collected after a treatment-free period of at least two weeks. The absolute CD3+, CD4+ or CD8+ T lymphocyte counts as well as the percentage of each subset were measured. The ratio of CD4+/CD8+ T cells was also calculated.

Statistical analysis

Results are expressed as mean +/- standard deviation (SD) with 95% confidence interval (CI), and a paired t-test was used to compare the results before and 2 weeks after the start of radiation therapy. The CD4+/CD8+ ratios for hepatitis virus-associated HCC and that for non-B, non-C HCC before and 2 weeks after the start of radiation therapy were compared by unpaired t-test. A p-value of less than 0.05 was considered statistically significant.

Results

The baseline characteristics of patients were shown in **Table 1**. The mean age was 59 years with an SD of 8.7 years. Three (43%) patients were positive for hepatitis B e antigen, and four (57%) patients were negative for both hepatitis B and C virus infections. Three (43%) patients underwent liver resection, 3 (43%) patients underwent TACE, and 2 (29%) patients received oral Lenvatinib prior to radiation therapy. Two (29%) patients had intrahepatic recurrences,

three (42%) patients had both intrahepatic and extrahepatic recurrences (lymph nodes: n=2, adrenal glands: n=1), and two (29%) patients had isolated extrahepatic (lung: n=2) recurrences. Absolute lymphocyte counts before and 2 weeks after the start of radiation therapy were 820 +/- 422 (95% CI: 507 to 1133) cells/mm3 and 392 +/- 245 (95% CI: 210 to 573) cells/mm3, respectively ($p < 0.01$) (**Figure 1a**). The absolute counts of CD3+, CD4+ and CD8+ peripheral blood T lymphocytes significantly decreased 2 weeks after the start of radiation therapy (**Table 2**). The percentages of CD3+ and CD8+ T lymphocytes did not change, whereas the percentage of CD4+ T lymphocytes significantly increased 2 weeks after the start of radiation therapy (**Figure 1b**). The CD4+/CD8+ ratio increased significantly 2 weeks after the start of radiation therapy ($p=0.02$) (**Figure 1c**). The CD4+/CD8+ ratio for hepatitis B virus-associated HCC before radiation therapy was 0.61+/-0.06, and the CD4+/CD8+ ratio for non-B, non-C HCC was 1.09+/-0.28 ($p=0.04$, unpaired t-test). The CD4+/CD8+ ratio for hepatitis B virus-associated HCC 2 weeks after the start of radiation therapy was 1.19+/-0.52, and the CD4+/CD8+ ratio for non-B, non-C HCC was 1.65+/-0.68 ($p=0.36$, unpaired t-test).

Discussion

The prognosis for HCC with extrahepatic metastases or recurrence after TACE or administration of the multi-kinase inhibitors (e.g., Sorafenib, Lenvatinib, Regorafenib, Cabozantinib, Ramucirumab) is poor, and no standard treatment has been established[4,5,9]. In recent years, attempts have been made to treat advanced HCC with ICIs[4,10]. The combination of Atezolizumab and Bevacizumab has been shown to improve overall survival compared to Sorafenib[10], and the combination of Durvalumab and Tremelimumab has been shown to improve

Table 1. Baseline characteristics of patients with recurrent or metastatic hepatocellular carcinoma.

No	Age/Sex	PS	Infection	Prior treatment	Site of metastasis	Timing of blood sampling	Site of RT	Dose/Fx
1	64/M	1	HBV	Lenvatinib	No metastasis	14 days after the start	Liver	60 Gy / 30 Fx
2	75/M	0	NBNC	TACE	No metastasis	16 days after the start	Liver	50 Gy / 8Fx
3	65/M	0	HBV	N/A	Liver	14 days after the start	Liver	60 Gy / 30Fx
4	59/M	0	HBV	TACE	No metastasis	14 days after the start	Liver	60 Gy / 25Fx
5	55/M	1	NBNC	Lenvatinib	Lung	16 days after the start	Lung	40 Gy / 4Fx
6	48/F	1	NBNC	N/A	Lung	14 days after the start	Lung	60 Gy / 8Fx
7	56/M	0	NBNC	TACE	Peritoneum	14 days after the start	Peritoneum	50 Gy / 25Fx

Abbreviations: PS= ECOG performance status scale, HBV= hepatitis B virus, NBNC=non-B, non-C hepatitis, TACE=transcatheter arterial chemoembolization, RT=radiation therapy, Fx=fraction., N/A= not available

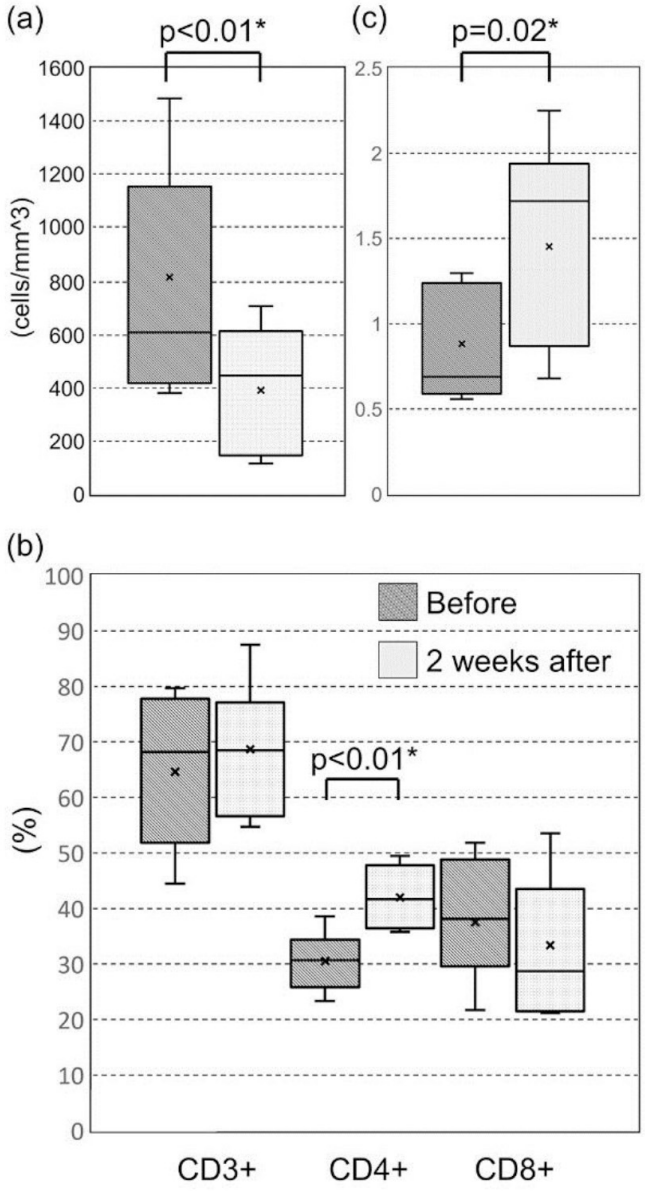

Figure 1. Box plots of absolute lymphocyte counts (a), percentages of CD3+, CD4+ and CD8+ T lymphocytes (b) and CD4+/CD8+ ratios (c) before and 2 weeks after the start of radiation therapy. "X" marks correspond to the means. The central horizontal bars are the medians. The lower and upper limits of the box are the first and third quartiles, respectively. Points above or below the whiskers' upper and lower bounds are outliers. P-values marked with asterisks indicate statistically significant differences

Table 2. Lymphocyte subset counts before and two weeks after the start of radiation therapy. Values are expressed as mean +/- standard deviation.

	Immediate before RT	2 weeks after RT	p-value*
Absolute counts (cells/mm^3)	820 +/- 422 (507-1133)	392 +/- 245 (210-573)	p < 0.01
Lymphocyte counts (cells/mm^3)			
CD3+ T lymphocytes	541 +/- 305 (315-767)	284 +/- 191 (143-426)	p<0.01
CD4+ T lymphocytes	269 +/- 180 (135-402)	170 +/- 119 (81-258)	p=0.03
CD8+ T lymphocytes	299 +/- 156 (184-414)	134 +/- 95 (64-205)	p<0.01
The percentages (%)			
CD3+ T lymphocytes	64.6 +/- 13.0 (54.9-74.2)	68.7 +/- 11.4 (60.3-77.1)	p=0.09
CD4+ T lymphocytes	30.5 +/- 5.4 (26.5-34.5)	42.0 +/- 5.3 (38.1-45.9)	p<0.01
CD8+ T lymphocytes	37.5 +/- 10.8 (29.4-45.5)	33.4 +/- 12.7 (24.0-42.8)	p=0.22
CD4+/CD8+ ratio	0.88 +/- 0.32 (0.64-1.12)	1.45 +/- 0.62 (0.99-1.91)	p=0.02

Numbers in parentheses are 95% confidence intervals.

*Statistical analysis was performed by paired t-test.

overall survival compared to Sorafenib[4]. Despite their efforts to improve response rates and prolong overall survival, the results were unsatisfactory[4,6,10,11]. In this study, the CD4+ T lymphocyte count was lower than that of normal population. The reason for CD4+ lymphopenia is that blood monocytes gradually change due to factors derived from the primary tumor and cannot differentiate into functional dendritic cells[12]. Radiation therapy will prime T lymphocytes in the primary tumor, reducing TGF-β levels and enhancing the antitumor effect of ICIs, while CD4+ T lymphocyte depletion reduces the effect of ICIs, so knowing CD4+ T lymphocyte changes is important in determining the timing and indication for ICIs[13,14]. The results of this study showed a significant increase in the CD4+ T lymphocyte percentage and CD4+/CD8+

ratio 2 weeks after the start of radiotherapy, suggesting that administration of ICIs at this time may enhance antitumor immunity. The efficacy of radiation therapy combined with ICIs has been shown in non-small cell lung cancer, head and neck squamous cell carcinoma, and cervical cancer, but has not been studied in HCC[3,6,7,13]. Before undergoing radiation therapy combined with ICIs, it is necessary to know the early changes in lymphocyte subsets in order to predict the efficacy of ICIs. To our knowledge, this is the first report investigating early changes in lymphocyte subsets after 2 weeks of radiation therapy for patients with recurrent or metastatic HCC.

The study had several strengths. First, radiation therapy was performed using a state-of-the-art helical tomotherapy system. Although the volume of low-dose radiation delivered was larger than that of conventional radiation therapy, the tumor could be accurately irradiated with higher doses of radiation. In addition, low-dose radiation therapy promoted T lymphocyte infiltration and elicits CD4+ cells with features of exhausted effector cytotoxic cells, thus supporting the rational combination of helical tomotherapy with ICIs[14]. Second, lymphocyte subsets were analyzed at a fixed time 2 weeks after the start of radiation therapy. Since the absolute lymphocyte count at week two was the most relevant factor related to survival in cervical cancer patients treated with concurrent chemoradiotherapy[15], changes in lymphocytes at two weeks after the start of radiation therapy were also presumed important in HCC. Although other types of cancers and other combination therapies cannot be directly applied to HCC, the increased percentage of CD4+ peripheral blood lymphocytes in patients with HCC is a notable and characteristic finding. Moreover, the increase in the CD4+/CD8+ ratio was consistent with the results of radiation therapy for other types of cancer[16].

This study had some limitations. First, the spread of HCC and the treatment methods given in the past were varied from patient to patient. However, since all treatment was discontinued two weeks prior to radiation therapy and no combination therapy was given concurrently for two weeks after the start of radiation therapy, bias due to different combinations of pretreatment was considered minimal. Second, with the exception of patients who underwent surgical resection, not all patients underwent pathological evaluation. However, the purpose of this study was to find specific early changes in a subset of peripheral blood lymphocytes from patients with HCC, and we believe that adequate results for our purpose could have been obtained without detailed pathological studies. Third, the number of patients evaluated in this study is small (seven). However, the number of patients is sufficient for statistical analysis, and the small number of patients in this study is not a major limitation of the proof of concept.

Conclusion

In conclusion, a single pilot study cannot be generalized to others without further scientific validation, however, it was demonstrated for

the first time in recurrent or metastatic HCC that radiation therapy induces CD4+ T lymphocytes and increases the CD4+/CD8+ ratio in 2 weeks. Understanding the lymphocyte subset changes 2 weeks after the start of radiation therapy may help in developing treatment strategies for recurrent or metastatic HCC.

Competing Interests

All authors declare no competing financial interests.

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