Advances in Radiation Oncology (2023) 8, 101159

advances in radiation oncology

www.advancesradonc.org

Scientific Article

# Calreticulin Upregulation in Cervical Cancer Tissues From Patients After 10 Gy Radiation Therapy

**Kohei Okada, MD,[a] Hiro Sato, MD, PhD,[a,*] Takuya Kumazawa, MD, PhD,[a,b] Yasumasa Mori, MD, PhD,[c] Tiara Bunga Mayang Permata, MD, PhD,[d] Yuki Uchihara, PhD,[e] Shin-ei Noda, MD, PhD,[f] Keiji Suzuki, PhD,[g] Hayato Ikota, MD, PhD,[h] Hideaki Yokoo, MD, PhD,[i] Soehartati Gondhowiardjo, MD, PhD,[d] Takashi Nakano, MD, PhD,[c] Tatsuya Ohno, MD, PhD,[a,*] and Atsushi Shibata, PhD[e]**

*[a]Department of Radiation Oncology, Graduate School of Medicine, Gunma University, Maebashi, Gunma, Japan; [b]Department of Radiation Oncology, Saku Central Hospital Advanced Care Center, Nakagomi, Saku, Nagano, Japan; [c]National Institute of Radiological Sciences, National Institute for Quantum and Radiological Science and Technology, Anagawa, Inage, Chiba, Japan; [d]Department of Radiation Oncology, Faculty of Medicine Universitas Indonesia - Dr. Cipto Mangunkusumo National General Hospital, Jakarta, Indonesia; [e]Signal Transduction Program, Gunma University Initiative for Advanced Research (GIAR), Gunma University, Maebashi, Gunma, Japan; [f]Department of Radiation Oncology, Comprehensive Cancer Center, International Medical Center, Saitama Medical University, Saitama, Japan; [g]Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki University, Sakamoto, Nagasaki, Japan; [h]Clinical Department of Pathology, Gunma University Hospital, Maebashi, Gunma, Japan; and [i]Department of Human Pathology, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan*

Received 1 June 2022; accepted 18 December 2022

**Abstract**

**Purpose:** Understanding the immune response during radiation therapy (RT) in a clinical setting is imperative for maximizing the efficacy of combined RT and immunotherapy. Calreticulin, a major damage-associated molecular pattern that is exposed on the cell surface after RT, is presumed to be associated with the tumor-specific immune response. Here, we examined changes in calreticulin expression in clinical specimens obtained before and during RT and analyzed its relationship with the density of $CD8^+$ T cells in the same patient set.

Sources of support: This study was supported by JSPS KAKENHI (grant nos. JP21H03596, JP17H04713, and JP19K08195), the Takeda Science Foundation, the Uehara Memorial Foundation, the Astellas Foundation for Research on Metabolic Disorders, The Kanae Foundation for the Promotion of Medical Science, the Yasuda Memorial Medicine Foundation, and the Nakajima Foundation. This study was also supported by the Program of the Network−Type Joint Usage/Research Centre for Radiation Disaster Medical Science of Hiroshima University, Nagasaki University, Fukushima Medical University, and the grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan for programs for Leading Graduate Schools, Cultivating Global Leaders in Heavy Ion Therapeutics and Engineering.

Disclosures: 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 paper.

Data sharing statement: The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

*Corresponding authors: Hiro Sato, MD, PhD and Tatsuya Ohno, MD, PhD; E-mails: hiro.sato@gunma-u.ac.jp, tohno@gunma-u.ac.jp,

https://doi.org/10.1016/j.adro.2022.101159

**Methods and Materials:** This retrospective analysis evaluated 67 patients with cervical squamous cell carcinoma who were treated with definitive RT. Tumor biopsy specimens were collected before RT and after 10 Gy irradiation. Calreticulin expression in tumor cells was evaluated via immunohistochemical staining. Subsequently, the patients were divided into 2 groups according to the level of calreticulin expression, and the clinical outcomes were compared. Finally, the correlation between calreticulin levels and density of stromal $CD8^+$ T cells was evaluated.

**Results:** The calreticulin expression significantly increased after 10 Gy (82% of patients showed an increase; $P < .01$). Patients with increased calreticulin levels tended to show better progression-free survival, but this was not statistically significant ($P = .09$). In patients with high expression of calreticulin, a positive trend was observed between calreticulin and $CD8^+$ T cell density, but the association was not statistically significant ($P = .06$).

**Conclusions:** Calreticulin expression increased after 10 Gy irradiation in tissue biopsies of patients with cervical cancer. Higher calreticulin expression levels are potentially associated with better progression-free survival and greater T cell positivity, but there was no statistically significant relationship between calreticulin upregulation and clinical outcomes or $CD8^+$ T cell density. Further analysis will be required to clarify mechanisms underlying the immune response to RT and to optimize the RT and immunotherapy combination approach.



## Introduction

With the development of immune checkpoint inhibitors, multiple clinical trials combining radiation therapy (RT) and immune checkpoint inhibitors are ongoing.[1] However, the clinical outcomes have varied among studies, and not all patients have shown favorable results. Importantly, the underlying mechanisms that affect the results of these clinical trials remain unclear. Therefore, it is crucial to investigate the immune response to RT in a clinical setting, to enable the development and success of the combination strategy.

Immunogenic cell death is a well-known form of cell death that is observed after RT and is characterized by the release of damage-associated molecular patterns.[2] Calreticulin, a major damage-associated molecular pattern, is a chaperone protein that exists in the endoplasmic reticulum (ER) and is involved in protein quality control and regulation of the intracellular calcium concentration, along with other diverse functions.[3] During ER stress triggered by irradiation or chemotherapeutic agents, calreticulin moves from the ER to the cell membrane surface.[4] Calreticulin is known to act as an "eat me" signal and enhances the uptake of cells on which it is expressed, leading to the release of interferons by antigen-presenting cells such as dendritic cells.[5,6] This pivotal step leads to the maturation of dendritic cells by promoting antigen uptake and cross-presentation of antigens to naïve T cells in the lymph nodes, followed by activation and recruitment of mature antigen-specific T cells.[2] Previous in vitro and mouse model studies demonstrated that irradiation, including x-rays and particle beams, induced calreticulin expression on the surface of cancer cells.[7-9] Consistent with the observations in these studies, higher calreticulin expression was observed in clinical tumor specimens from patients with pancreatic cancer or renal cell carcinoma who received preoperative RT[10,11]; however, the specimens analyzed in these studies were obtained several weeks after completing RT. Thus, it remains to be elucidated as to whether upregulation of calreticulin expression is induced in the same patients during RT and is associated with prognosis. Therefore, in this study, we evaluated changes in calreticulin expression in clinical specimens from patients with cervical cancer during RT at the time point of 10 Gy in 5 fractions and further evaluated its correlation with clinical outcomes and density of $CD8^+$ T cells in tumor tissues.

## Methods and Materials

### Patients and tumor characteristics

In this retrospective analysis, 67 patients with cervical squamous cell carcinoma (median age, 60 years; range, 32-87 years) who were treated with definitive RT (in combination with chemotherapy or RT alone) at Gunma University Hospital (Maebashi, Japan) between August 2009 and November 2013 were evaluated. The diagnosis of cervical squamous cell carcinoma was performed by experienced pathologists who were not associated with the present study. Tumor biopsy specimens collected from each patient before treatment (preRT) and after 10 Gy irradiation (hereafter referred to as "10 Gy") were used for immunohistochemical staining. With the written consent of all patients, 10 Gy specimens were collected to evaluate the initial response to RT while the tumor cells were still viable. The present retrospective study was performed on these samples. The interval between RT initiation and biopsy was 5 to 11 days. The biopsy specimens were collected at 0 to 4 days after 10 Gy irradiation of patients. Seventy-five percent of the specimens (50/67) were collected on the same day as the 10 Gy treatment. Patient characteristics were recorded according to the tumor stage (International Federation of Gynecology and Obstetrics classification 2008) and age (Table 1). The Gunma University Clinical Trial Review Board approved the study protocol (approval no. HS2020-015). All patients provided informed consent to participate in the

**Table 1 Patient characteristics (n = 67)**

| Characteristic | Value |
|---|---|
| Observation period (mo) | |
| Median (range) | 63 (8-134) |
| Age (y) | |
| Median (range) | 60 (32-87) |
| Treatment | |
| RT alone | 22 (33%) |
| Concurrent CRT | 45 (67%) |
| FIGO stage (2008) | |
| IB | 9 (13%) |
| II | 27 (40%) |
| III | 30 (45%) |
| IVA | 1 (2%) |
| Lymph node metastasis in pelvis | |
| Positive | 36 (54%) |
| Negative | 31 (46%) |
| Para-aortic lymph node metastasis | |
| Positive | 6 (9%) |
| Negative | 61 (91%) |

*Abbreviations:* CRT = chemoradiation therapy; FIGO = International Federation of Gynecology and Obstetrics; RT = radiation therapy.

study and were given the option to opt out of the study at any time.

## Treatment procedures

All patients were treated with definitive RT consisting of external beam RT (EBRT) and intracavitary brachytherapy (ICBT). EBRT was performed using 10 MV x-rays at 2 Gy per fraction with 5 fractions per week. Whole-pelvic irradiation was performed at a total dose of 50 Gy in 25 fractions with the final 20 or 30 Gy delivered through a 3-cm-wide central shielding, depending on the clinical stage. The pelvic field expanded into the metastatic area in patients with para-aortic lymph node metastases. Patients with lymph node metastases received additional irradiation of 6 to 8 Gy in 3 to 4 fractions. ICBT was performed once per week concurrently with central shielding EBRT. EBRT was skipped on the day of ICBT administration. Three-dimensional image guided brachytherapy was performed on all patients using an $^{192}$Ir remote afterloading system (microSelectron; Elekta, Stockholm, Sweden). ICBT was performed to cover 90% of the high-risk clinical target volume, at a total dose of 6 Gy per fraction. Bulky and/or asymmetrical tumors were treated with additional interstitial brachytherapy.[12] ICBT was performed 4 times. Concurrent chemotherapy with cisplatin was administered weekly at a dose of 40 mg/m$^2$ to 67.0% of patients (45/67).

## Immunohistochemical staining and evaluation of clinical samples

Calreticulin expression in tumor cells was evaluated using immunohistochemical staining. Immunohistochemical staining was performed on biopsy samples that were excised from cervical tumors preRT and after 10 Gy. The biopsy samples were fixed in 10% buffered formalin for 24 hours at room temperature (20-25°C) and then dehydrated. Paraffin sections (4-$\mu$m thick) were dewaxed in xylene and rehydrated using a series of graded ethanol. The sections were then incubated for 10 minutes in 0.3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed using a citric acid buffer (pH = 6) in an autoclave (121°C, 10 minutes). The sections were then stained using an anticalreticulin antibody (rabbit monoclonal IgG, cat. no. ab92516; Abcam, Cambridge, UK; 1:100 dilution) and incubated overnight at 4°C. For secondary antibody staining, a Histofine Simple Stain Kit (cat. no. 424142; Nichirei, Tokyo, Japan) was used according to the manufacturer's instructions, and 3,3′-diaminobenzidine was used to visualize calreticulin. The quality of the tumor samples was validated independently by 2 pathologists who are coauthors of the present study.

Calreticulin staining was evaluated as described in a previous study.[13] All immunostaining images were obtained using a Leica DM4000 B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 20x objective lens. To evaluate the staining intensity, ImageJ vl.53a software (National Institutes of Health, Bethesda, MD) was used. The staining results were acquired as a digital image, which was displayed as a combination of 3 colors: red, green, and blue. In the original image, the stained calreticulin area is shown in brown, and nucleus is shown in blue. The acquired images were split into the red, blue, and green channels, respectively. To evaluate the intensity of calreticulin staining, the intensity of the blue signal was subtracted from that of the red signal. We measured the intensity of the signal obtained from 3 areas of the tumor tissues. The rate of increase in calreticulin intensity was obtained by dividing the intensity of calreticulin staining in 10 Gy by that preRT. To evaluate the correlation between calreticulin and the density of stromal CD8$^+$ T cells, we used slides from a previous study that had been stained with a CD8$^+$ T cell antibody (mouse monoclonal IgG, cat. no. M7103; Dako, Glostrup, Denmark; 1:800 dilution), which corresponded to calreticulin-stained slides.[14]

## Statistical analyses

Statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Japan). The Kaplan-Meier method and the log-rank test were used for survival analysis. Wilcoxon's signed-rank test was used to perform a paired analysis to compare differences in calreticulin expression in the preRT and 10 Gy tumor samples. The correlation between calreticulin expression and stromal $CD8^+$ T cell density was evaluated using Spearman's correlation analysis. Clinical factors between patients with increased or decreased calreticulin expression after 10 Gy RT were compared using an unpaired Student *t* test or Fisher's exact test. Multivariate analyses were performed using Cox proportional hazard regression model. Statistical significance was set at $P < .05$.

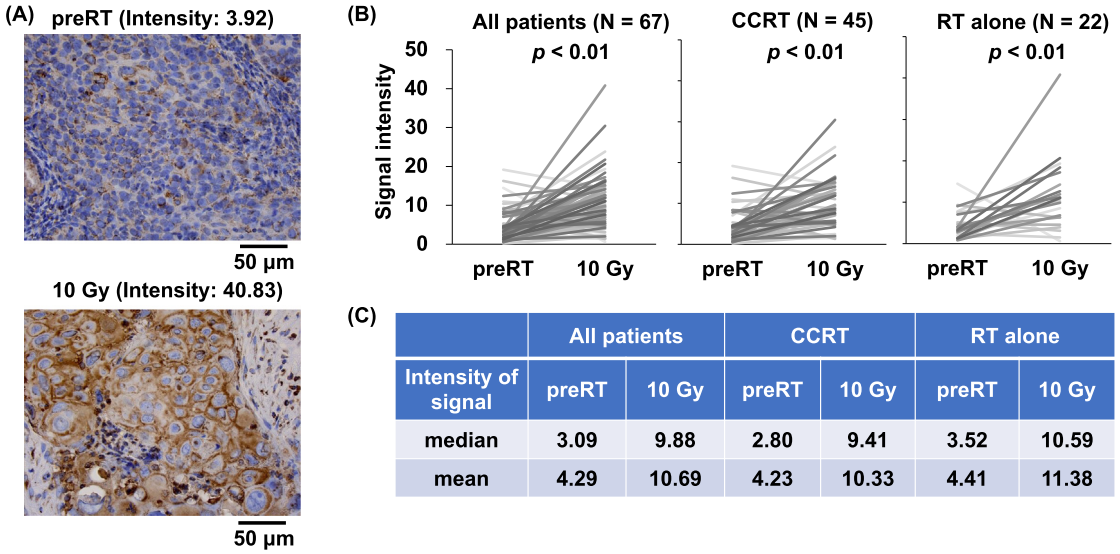
## Results

### RT upregulated the expression of calreticulin in patients with cervical squamous cell carcinoma

To investigate whether RT affects calreticulin expression, we performed immunohistochemical staining on biopsy specimens obtained preRT and after 10 Gy in 5 fractions. Figure 1A shows representative images of the sequentially obtained samples from the same patient. A comparison of calreticulin intensity in preRT and 10 Gy samples revealed an increase in calreticulin intensity during RT in 55 patients (82%), whereas a decrease in calreticulin intensity was observed in 12 patients (18%) ($P < .01$) (Fig. 1B). The increase in calreticulin intensity after 10 Gy treatment was observed regardless of concurrent chemotherapy ($P < .01$ in both groups; Fig. 1B). There was no specific correlation between calreticulin expression and patient characteristics, including age and clinical stage (Table 2). The median and mean of the signal intensity of calreticulin is shown in Fig. 1C (median, 3.09 in preRT, 9.88 in 10 Gy; mean, 4.29 in preRT, 10.69 in 10 Gy). These results demonstrate that calreticulin was upregulated in 80% of cervical cancer cases during RT.

### Analysis of correlations between clinical outcomes and RT-induced calreticulin expression

To investigate whether RT-induced calreticulin expression is associated with clinical outcomes, patients were categorized into 2 groups according to changes in calreticulin expression. We found that progression-free survival (PFS) rates tended to be higher in patients with increased calreticulin expression, although this difference was not statistically significant ($P = .09$) (Fig. 2). In contrast, there was no significant correlation between calreticulin expression and overall survival (OS) ($P = .79$) or local control (LC) ($P = .30$) (Fig. 2). We also examined whether the status of calreticulin expression at preRT or 10 Gy influenced clinical outcomes. Patients were categorized into 2 groups based on their relation to the median calreticulin staining intensity value, high or low expression; however, no correlation between calreticulin expression and clinical outcomes was observed




(C)

| | All patients | | CCRT | | RT alone | |
|---|---|---|---|---|---|---|
| Intensity of signal | preRT | 10 Gy | preRT | 10 Gy | preRT | 10 Gy |
| median | 3.09 | 9.88 | 2.80 | 9.41 | 3.52 | 10.59 |
| mean | 4.29 | 10.69 | 4.23 | 10.33 | 4.41 | 11.38 |

**Figure 1** Radiation therapy (RT) upregulates calreticulin expression in cervical cancer specimens from the same patient set. (A) Representative images of immunohistochemical staining at preRT and after 10 Gy from the same patient. The numbers indicate the staining intensity. (B) Changes in calreticulin intensity after 10 Gy irradiation. (C) Median and mean values of calreticulin intensity in preRT and 10 Gy samples.

(LC, PFS, and OS) at preRT or after 10 Gy (Fig. E1). In the multivariate analysis, change in calreticulin expression was not a significant prognostic factor for any clinical outcome, while advanced stage (Ⅲ/Ⅳ) and RT alone were significant poor prognostic factors in PFS and OS (Table E1).

## Analysis of the correlation between calreticulin levels and $CD8^+$ T cell density

Our group have evaluated the density of $CD8^+$ T cells in the tumor stroma of the same cohort of patients[14]; therefore, we analyzed the relationship between calreticulin expression and the stromal $CD8^+$ T cell density using the same data set. Although there was no statistically significant correlation, we observed a positive association between increased calreticulin expression and stromal $CD8^+$ T cell density in patients treated with definitive RT ($P$ = .06 in all patients) (Fig. 3, left panel). The patient group treated with RT alone showed a relatively stronger correlation between increased calreticulin expression and stromal $CD8^+$ T cell density compared with patients treated with chemo-RT (Fig. 3, middle and right panels). However, this correlation was also not statistically significant, possibly because of the small sample size (N = 22). In contrast, there was no significant correlation between calreticulin expression and the $CD8^+$ T cell density in either preRT or after 10 Gy in all patient subgroups (Fig. E2A, E2B).

## Discussion

In the present study, we found that calreticulin expression was upregulated in tumor cells of patients with cervical cancer receiving a dose of 10 Gy in 5 fractions of RT. Our analysis showed that patients with increased calreticulin expression tended to have a higher PFS rate, although this difference was not statistically significant ($P$ = .09). We hypothesized that calreticulin expression levels in tumors would show statistically significant differences in LC, but no significant difference was found, likely because of the higher LC in both groups and a limited number of patients. Further studies involving larger sample sizes will be required to clarify the presence of a significant positive correlation between calreticulin expression levels and clinical outcomes. In addition, we found a higher density of stromal $CD8^+$ T cells in patients with higher calreticulin induction, particularly in the group treated with only RT, although this correlation was not statistically significant ($P$ = .06). The underlying mechanisms are unclear, but it is possible that the relatively weak correlations observed in the concurrent chemo-RT group reflect the decreased number of systemic lymphocytes in patients who received concurrent chemotherapy.

**Table 2 Characteristics of patients showing increased and decreased calreticulin after 10 Gy RT**

| Characteristic | Calreticulin increased (n = 55) | Calreticulin decreased (n = 12) | *P* value |
|---|---|---|---|
| Age (y) | | | .405 |
| Median (range) | 59 (33-81) | 63.5 (32-87) | |
| Treatment | | | .510 |
| RT alone | 17 (31%) | 5 (42%) | |
| Concurrent CRT | 38 (69%) | 7 (58%) | |
| FIGO stage (2008) | | | .095 |
| IB | 6 (11%) | 3 (25%) | |
| II | 24 (42%) | 3 (25%) | |
| III | 25 (38%) | 5 (42%) | |
| IVA | 0 (0%) | 1 (8%) | |
| Lymph node metastasis in pelvis | | | .761 |
| Positive | 29 (53%) | 7 (58%) | |
| Negative | 26 (47%) | 5 (42%) | |
| Para-aortic lymph node metastasis | | | .582 |
| Positive | 6 (11%) | 0 (0%) | |
| Negative | 49 (89%) | 12 (100%) | |

*Abbreviations:* CRT = chemoradiation therapy; FIGO = International Federation of Gynecology and Obstetrics; RT = radiation therapy.

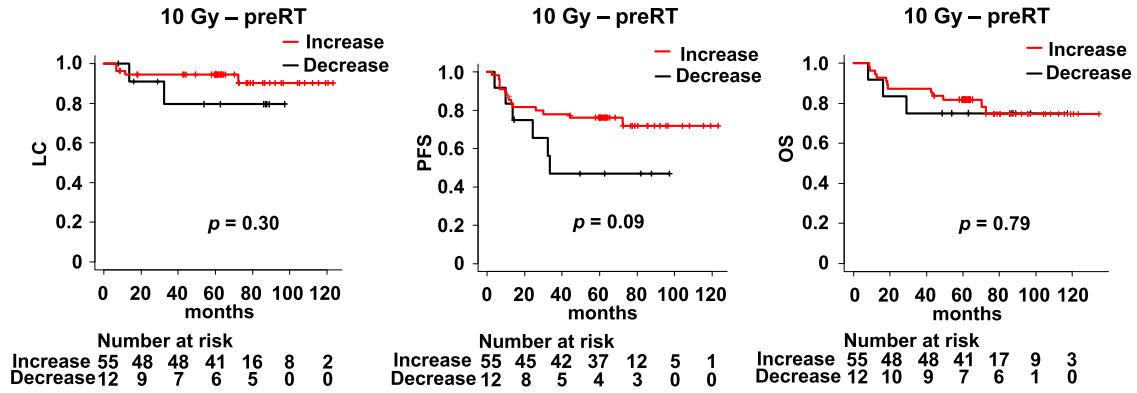



**Figure 2** Change in calreticulin expression and clinical outcomes. Kaplan-Meier curves of clinical outcomes between patients with increased and decreased calreticulin expression in 10 Gy samples. (A) Local control (LC), (B) progression-free survival (PFS), and (C) overall survival (OS).

The details of this relationship should be examined in future studies.

Preoperative RT upregulates calreticulin expression in tumor cells. Murakami et al[10] analyzed 84 patients with pancreatic cancer who underwent surgical resection with or without neoadjuvant chemo-RT (NACRT). A significant upregulation of calreticulin expression was observed in 65% of patients treated with NACRT (33/51) compared with 42% of patients who were not treated with NACRT (14/33) ($P = .045$). In addition, Singh et al[11] reported that calreticulin expression on the tumor cell surface was significantly increased in samples resected from patients treated with stereotactic body RT with 15 Gy compared with that in control samples ($P < .01$). However, it must be noted that in these studies, specimens from different patient groups were compared, and a few weeks passed between the completion of irradiation and the collection of specimens. In contrast, we examined the expression of calreticulin at the timepoint of 10 Gy in 5 fractions from the same patient, which has rarely been analyzed till date. In the present study, we showed that calreticulin expression is upregulated in tumor tissue from the same patient via irradiation starting at an early stage of RT (10 Gy/5 fractions) in clinical settings.

Calreticulin presentation on the plasma membrane facilitates phagocytosis by dendritic cells and activates antigen presentation, resulting in the initiation of adaptive T cell-mediated immunity.[15] Previous clinical studies showed that higher calreticulin expression in resected tumors correlates with better clinical outcomes in patients with non-small cell lung cancer[16] and endometrial cancer.[17] However, in the current study, there was no significant relationship between calreticulin upregulation and clinical outcomes, although PFS rates tended to be higher in patients with increased calreticulin expression levels. This weak correlation could be attributed to the limited

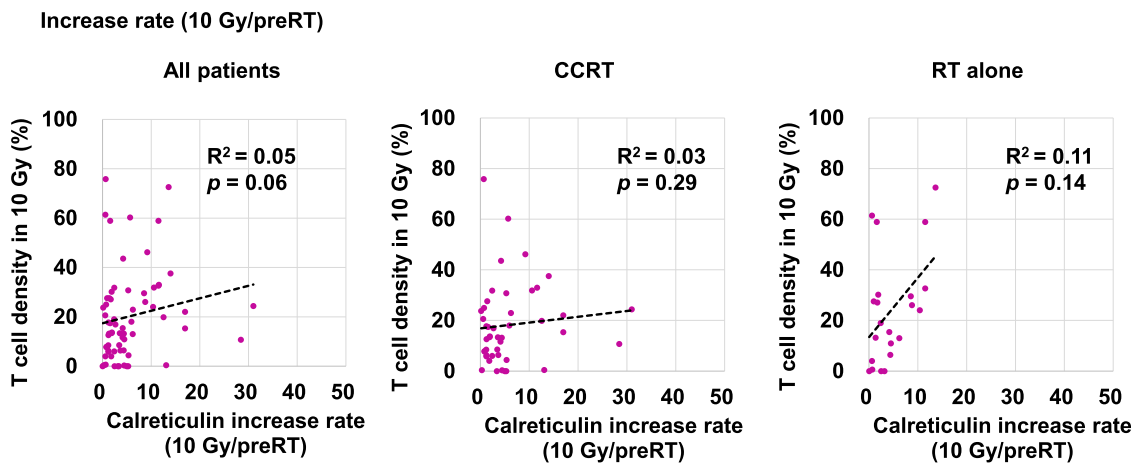



**Figure 3** Relationship between calreticulin expression and density of $CD8^+$ T cells.
A scatter plot showing the relationship between increased calreticulin expression levels and $CD8^+$ T cell density in 10 Gy samples. *Abbreviation:* CCRT = concurrent chemoradiation therapy.

immune activation by immunosuppressive effects of RT, as confirmed by upregulation of programmed cell death-ligand 1 (PD-L1) expression after 10 Gy in an overlapping cohort of patients with cervical squamous cell carcinoma.[14,18] Therefore, programmed cell death-1/PD-L1 blockade therapy may enhance calreticulin-dependent immune responses and improve clinical outcomes. In addition, in the patient group treated with RT alone, RT-induced upregulation of calreticulin expression and stromal $CD8^+$ T cell density were positively correlated but not statistically significant ($R^2 = 0.11$, $P = .14$). The lack of statistical significance may be dependent on the limited number of patients and PD-L1 upregulation by RT.[14] Further analysis with large numbers of patients will clarify the relationship between RT-induced upregulation of calreticulin expression, clinical outcomes, and T-cell recruitment. Calreticulin-dependent antitumor immune effects require further steps to act, such as cross-priming of antigen-presenting cells and lymphocytes in the draining lymph nodes, infiltration of lymphocytes into the tumor microenvironment, and elimination of cancer cells by immune responses at the tumor site.[2] Therefore, the $CD8^+$ T cell density in calreticulin-upregulated tumors may be enhanced at later time points after RT. In a recent mouse study, low-dose irradiated ovarian cancer cells showed calreticulin upregulation within 20 hours and T cell infiltration after 7 days, implying the interval of immune responses to RT.[19] The timing of T cell activation and relationship with calreticulin expression needs to be examined in the future.

## Conclusion

We report that upregulation of calreticulin expression is induced during RT in clinical samples from the same patient set. However, there was no significant correlation between calreticulin upregulation and clinical outcomes. Thus, the results of the current study cannot support the usefulness of calreticulin for stratifying patient prognosis or targeting it as part of the RT and immunotherapy combination strategy. Further analysis will be required to clarify the specific mechanisms underlying the immune response to RT and to optimize the RT and immunotherapy combination approach.

## Acknowledgments

The authors thank Dr Yuya Yoshimoto (Fukushima Medical University, Fukushima; Gunma University, Maebashi, Japan) for his assistance in performing the immunohistochemical analysis. The authors thank Mr Koji Isoda (Gunma University, Maebashi, Japan) for his technical assistance in performing the immunohistochemical analysis.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.adro.2022.101159.

## References

1. Sato H, Demaria S, Ohno T. The role of radiotherapy in the age of immunotherapy. *Jpn J Clin Oncol*. 2021;51:513-522.
2. Galluzzi L, Vitale I, Warren S, et al. Consensus guidelines for the definition, detection and interpretation of immunogenic cell death. *J Immunother Cancer*. 2020;8: e000337.
3. Lu YC, Weng WC, Lee H. Functional roles of calreticulin in cancer biology. *Biomed Res Int*. 2015;2015: 526524.
4. Panaretakis T, Kepp O, Brockmeier U, et al. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J*. 2009;28:578-590.
5. Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med*. 2007;13:54-61.
6. Chen X, Fosco D, Kline DE, Kline J. Calreticulin promotes immunity and type I interferon-dependent survival in mice with acute myeloid leukemia. *Oncoimmunology*. 2017;6: e1278332.
7. Golden EB, Frances D, Pellicciotta I, Demaria S, Formenti SC. Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death. *Oncoimmunology*. 2014;3:e28518.
8. Gameiro SR, Jammeh ML, Wattenberg MM, Tsang KY, Ferrone S, Hodge JW. Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing. *Oncotarget*. 2014;5:403-416.
9. Huang Y, Dong Y, Zhao J, Zhang L, Kong L, Lu JJ. Comparison of the effects of photon, proton and carbon-ion radiation on the ecto-calreticulin exposure in various tumor cell lines. *Ann Transl Med*. 2019;7:542.
10. Murakami T, Homma Y, Matsuyama R, et al. Neoadjuvant chemoradiotherapy of pancreatic cancer induces a favorable immunogenic tumor microenvironment associated with increased major histocompatibility complex class I-related chain A/B expression. *J Surg Oncol*. 2017;116:416-426.
11. Singh AK, Winslow TB, Kermany MH, et al. A pilot study of stereotactic body radiation therapy combined with cytoreductive nephrectomy for metastatic renal cell carcinoma. *Clin Cancer Res*. 2017;23:5055-5065.
12. Wakatsuki M, Ohno T, Yoshida D, et al. Intracavitary combined with CT-guided interstitial brachytherapy for locally advanced uterine cervical cancer: Introduction of the technique and a case presentation. *J Radiat Res*. 2011;52:54-58.
13. Kumazawa T, Mori Y, Sato H, et al. Expression of non-homologous end joining factor, Ku80, is negatively correlated with PD-L1 expression in cancer cells after X-ray irradiation. *Oncol Lett*. 2022;23:29.
14. Mori Y, Sato H, Kumazawa T, et al. Analysis of radiotherapy-induced alteration of CD8(+) T cells and PD-L1 expression in patients with uterine cervical squamous cell carcinoma. *Oncol Lett*. 2021;21:446.
15. Fucikova J, Kepp O, Kasikova L, et al. Detection of immunogenic cell death and its relevance for cancer therapy. *Cell Death Dis*. 2020;11:1013.

16. Fucikova J, Becht E, Iribarren K, et al. Calreticulin expression in human non-small cell lung cancers correlates with increased accumulation of antitumor immune cells and favorable prognosis. *Cancer Res*. 2016;76:1746-1756.
17. Xu Q, Chen C, Chen G, Chen W, Zhou D, Xie Y. Significance of calreticulin as a prognostic factor in endometrial cancer. *Oncol Lett*. 2018;15:8999-9008.
18. Sato H, Niimi A, Yasuhara T, et al. DNA double-strand break repair pathway regulates PD-L1 expression in cancer cells. *Nat Commun*. 2017;8:1751.
19. Herrera G, Ronet C, Olza O, et al. Low-dose radiotherapy reverses tumor immune desertification and resistance to immunotherapy. *Cancer Discov*. 2022;12:108-133.