

# Analysis of radiotherapy-induced alteration of CD8<sup>+</sup> T cells and PD-L1 expression in patients with uterine cervical squamous cell carcinoma

YASUMASA MORI<sup>1,2</sup>, HIRO SATO<sup>1</sup>, TAKUYA KUMAZAWA<sup>1</sup>, TIARA BUNGA MAYANG PERMATA<sup>3</sup>, YUYA YOSHIMOTO<sup>4</sup>, KAZUTOSHI MURATA<sup>1</sup>, SHIN-EI NODA<sup>5</sup>, TAKUYA KAMINUMA<sup>1</sup>, KEN ANDO<sup>1</sup>, TAKAHIRO OIKE<sup>1</sup>, NORIYUKI OKONOGI<sup>6</sup>, KOHEI OKADA<sup>1</sup>, SANGEETA KAKOTI<sup>1,7</sup>, KEIJI SUZUKI<sup>8</sup>, HAYATO IKOTA<sup>9</sup>, HIDEAKI YOKOO<sup>10</sup>, TAKASHI NAKANO<sup>6</sup>, TATSUYA OHNO<sup>1</sup> and ATSUSHI SHIBATA<sup>7</sup>

<sup>1</sup>Department of Radiation Oncology, Graduate School of Medicine, Gunma University, Maebashi, Gunma 371-8511;

<sup>2</sup>Department of Radiotherapy, Saitama Cancer Center, Ina, Saitama 362-0806, Japan; <sup>3</sup>Department of Radiation Oncology, Faculty of Medicine Universitas Indonesia, Dr Cipto Mangunkusumo Hospital, Jakarta 10430, Indonesia;

<sup>4</sup>Department of Radiation Oncology, Fukushima Medical University, Fukushima 960-1247; <sup>5</sup>Department of Radiation Oncology, Comprehensive Cancer Center, International Medical Center, Saitama Medical University, Hidaka, Saitama 350-1298; <sup>6</sup>National Institute of Radiological Sciences, National Institute for Quantum and Radiological Science and

Technology, Chiba 263-8555; <sup>7</sup>Signal Transduction Program, Gunma University Initiative for Advanced Research,

Gunma University, Maebashi, Gunma 371-8511; <sup>8</sup>Department of Radiation Medical Sciences, Atomic Bomb Disease

Institute, Nagasaki University, Nagasaki 852-8523; <sup>9</sup>Clinical Department of Pathology, Gunma University Hospital;

<sup>10</sup>Department of Human Pathology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

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**Abstract.** Radiotherapy induces an immune response in the cancer microenvironment that may influence clinical outcome. The present study aimed to analyse the alteration of CD8<sup>+</sup> T-cell infiltration and programmed death-ligand 1 (PD-L1) expression following radiotherapy in clinical samples from patients with uterine cervical squamous cell carcinoma. Additionally, the current study sought to analyse the association between these immune responses and clinical outcomes. A total of 75 patients who received either definitive chemoradiotherapy or radiotherapy were retrospectively analyzed. CD8<sup>+</sup> T-cell infiltration and PD-L1 expression were determined by immunohistochemistry using biopsy specimens before radiotherapy (pre-RT) and after 10 Gy radiotherapy (post-10 Gy). The PD-L1<sup>+</sup> rate was significantly increased from 5% (4/75) pre-RT to 52% (39/75) post-10 Gy (P<0.01). Despite this increase in the PD-L1<sup>+</sup> rate post-10 Gy, there was no significant

association between both pre-RT and post-10 Gy and overall survival (OS), locoregional control (LC) and progression-free survival (PFS). On the other hand, the CD8<sup>+</sup> T-cell infiltration density was significantly decreased for all patients (median, 23.1% pre-RT vs. 16.9% post-10 Gy; P=0.038); however, this tended to increase in patients treated with radiotherapy alone (median, 17.7% pre-RT vs. 24.0% post-10 Gy; P=0.400). Notably, patients with high CD8<sup>+</sup> T-cell infiltration either pre-RT or post-10 Gy exhibited positive associations with OS, LC and PFS. Thus, the present analysis suggested that CD8<sup>+</sup> T-cell infiltration may be a prognostic biomarker for patients with cervical cancer receiving radiotherapy. Furthermore, immune checkpoint inhibitors may be effective in patients who have received radiotherapy, since radiotherapy upregulated PD-L1 expression in cervical cancer specimens.

## Introduction

Cervical cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer-associated death in women worldwide, with an estimated 570,000 new cases and 311,000 deaths reported in 2018 (1). The number of patients with cervical cancer is increasing, particularly in developing countries; moreover, it ranks second as the cause of mortality among women, after breast cancer (1). Patients with cervical cancer are currently treated with radical hysterectomy in addition to pelvic lymph node dissection, or concurrent platinum-based chemoradiotherapy (CRT), and course of treatment is generally determined by tumor stage and size (2-4). Notably, treatment at early stages is directly associated with

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*Correspondence to:* Dr Hiro Sato or Professor Tatsuya Ohno, Department of Radiation Oncology, Graduate School of Medicine, Gunma University, 3-39-15 Showa, Maebashi, Gunma 371-8511, Japan

E-mail: hiro.sato@gunma-u.ac.jp

E-mail: tohno@gunma-u.ac.jp

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improved clinical outcomes (5). A meta-analysis of patients treated by CRT reported that the 5-year overall survival (OS) rate for stage I and II was >80%, but it was 40-60 and 10-40% for stage III and IVA, respectively (6). Furthermore, current CRT using image-guided brachytherapy (IGBT) achieved significant local control if treatment was started early (7). A multicentre retrospective study (RetroEMBRACE) reported that the 5-year local control rate for definitive external beam radiotherapy (EBRT) with or without chemotherapy followed by IGBT for stage IB, IIB and IIIB was 98, 91 and 75%, respectively (7). However, since clinical outcomes after CRT for advanced diseases remain unsatisfactory, the establishment of biomarkers that affect survival, local control and distant metastasis is a critical issue.

The development of immune checkpoint therapy using anti-programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) antibodies caused a paradigm shift in cancer therapy. Notably, it highlighted the importance of antitumor immunity in cancer treatment. PD-L1 which is expressed on the surface of various cells, including tumor cells, activated T cells and antigen-presenting cells, such as dendritic cells, macrophages/monocytes and B cells is the major ligand for PD-1 (8,9). In tumor microenvironments, the interaction between PD-L1 on tumor cells and PD-1 on T cells induces T-cell exhaustion, resulting in tumor escape from host immune surveillance (10,11). Indeed, cancers with high PD-L1 expression are associated with a poor prognosis due to the suppression of immune function (12). By contrast, CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs), which serve an important role in immune response for eliminating tumor cells, have been reported as biomarkers for clinical outcomes of cervical cancer (13-15). However, for cervical cancer, conflicting results have been reported for the prognostic value of PD-L1 and CD8<sup>+</sup> TILs and, thus, their utility for prognosis remains unclear (16-18). Additionally, while immune responses induced by radiotherapy (RT) have been characterized in several preclinical models (19), immune responses in patients with cervical cancer have not been sufficiently analyzed. The present study aimed to analyze the alterations and associations between patient outcomes and PD-L1 expression or density of CD8<sup>+</sup> TILs using biopsy specimens before and during RT in patients with cervical cancer. The findings of the present study suggested that CD8<sup>+</sup> TILs density have potential to be a predictive biomarker for patients with cervical cancer treated with CRT/RT.

## Materials and methods

**Patients and tumor characteristics.** In the present retrospective analysis, 75 consecutive patients with uterine cervical squamous cell carcinoma (median age, 62 years; range, 32-87 years) who underwent concurrent platinum-based CRT or RT alone between August 2009 and November 2013 at the Gunma University Hospital (Maebashi, Japan) were enrolled. Herein, the abbreviation 'RT' represents both CRT/RT unless otherwise stated. Paired tumor specimens obtained from all patients before RT (pre-RT) and after 10 Gy RT (post-10 Gy) were used for pathological analysis and immunohistochemistry (IHC). Most specimens in both groups (~89% in the RT alone group and ~96% in the CRT group) were collected on days 8-9

(range, 5-11 days; Table SI). The number of days between application of 10 Gy RT and biopsy of the specimens was in the range of 0-4 days. Of these, 60/75 (80%) were performed on the same day or one day later (Table SII). Specimens were used to examine PD-L1 expression and stromal CD8<sup>+</sup> TILs. Patient characteristics were recorded for tumor stage [stages I B, IIA, IIB, IIIA, IIIB and IVA according to the International Federation of Gynecology and Obstetrics [FIGO classification 2008 (20)], age and lymph node metastasis (Table I). The Institutional Review Board for clinical trials of Gunma University approved the study protocol.

**Treatment.** All patients underwent definitive RT, involving a combination of EBRT and intracavitary brachytherapy (ICBT). EBRT was typically administered using a four-field technique and 10 MV X-ray. The most common EBRT dose and fractionation regimen was 50 Gy in 25 fractions. EBRT was performed with a combination of whole-pelvic irradiation (20-40 Gy) followed by 3-cm-wide central shielding irradiation. In patients with para-aortic lymph node metastases, pelvic irradiation fields were extended to include the gross metastatic region (2 Gy/fraction, 1 fraction/day, 5 fractions/week). For patients with lymph node metastases, additional boost irradiation of 6-8 Gy in 3-4 fractions was administered. ICBT was performed once per week, concurrently with the central shielding EBRT. EBRT was skipped on the day of ICBT administration. Three-dimensional IGBT was performed with a high-dose rate source using an <sup>192</sup>Ir remote afterloading system (microSelectron; Elekta Instrument AB) for all patients. The prescribed dose of each ICBT was determined to cover 90% of high-risk clinical target volume with a 6 Gy total dose. Interstitial brachytherapy was added along with ICBT for bulky and/or asymmetric tumors. ICBT was most commonly performed four times.

Concurrent chemotherapy was administered to 63.0% of the patients (48/75). Cisplatin was typically administered weekly at a dose of 40 mg/m<sup>2</sup> for patients treated with CRT.

**Follow-up and assessment of clinical outcomes.** After the completion of CRT/RT, patients were followed up every 1-3 months for the first two years and every 3-6 months for three subsequent years by radiation oncologists. During each follow-up examination, disease status was assessed in terms of locoregional control (LC) and progression-free survival (PFS). OS was defined as the term from initial RT until death as a result of any cause or the date of last follow up. LC was defined as no evidence of tumor regrowth or recurrence in the pelvic region. PFS was defined as no evidence of tumor regrowth or recurrence in the pelvic region or distant metastasis.

**IHC analysis for PD-L1 expression and CD8<sup>+</sup> TILs.** PD-L1 expression on tumor cells and density of stromal CD8<sup>+</sup> TILs were evaluated with IHC using biopsy samples excised from the cervical cancer samples pre-RT and post-10 Gy. Biopsied samples were fixed in 10% buffered formalin for 24 h at room temperature, then dehydrated, deaged and paraffin-embedded. Paraffin sections (4- $\mu$ m-thick) were dewaxed in xylene at room temperature and rehydrated using a graded ethanol series. Endogenous peroxidase activity was blocked with a 10 min incubation at room temperature in 0.3%

Table I. Characteristics of patients with uterine cervical squamous cell carcinoma enrolled the present study (n=75).

Characteristics	Value
Observation period (range), months	63 (8-120)
Median age (range), years	62 (32-87)
Treatment, n (%)	
RT alone	27 (36)
Concurrent CRT	48 (64)
FIGO stage, n (%)	
IB	11 (15)
II	31 (41)
III	31 (41)
IVA	2 (3)
Lymph node metastasis in pelvis, n (%)	
+	36 (48)
-	39 (52)
Para-aortic lymph node metastasis, n (%)	
+	6 (8)
-	69 (92)

RT, radiotherapy; CRT, chemoradiotherapy; FIGO, International Federation of Gynecology and Obstetrics.

hydrogen peroxide. Subsequently, PD-L1 sections were heated in 1 mmol/l ethylenediaminetetraacetic acid (pH 8.0) and CD8 sections were heated in 0.01 mol/l citric acid (pH 6.0) at 121°C for 10 min for antigen retrieval. After blocking by 10% goat normal serum for PD-L1 sections and 10% rabbit normal serum for CD8 sections with a 20 min incubation at room temperature, sections were incubated overnight with primary antibodies at 4°C. Next day, the sections were incubated with biotin-labeled secondary antibodies followed by peroxidase-labeled streptavidin (Histofine; SAB-PO (rabbit) cat. no. 424032 and SAB-PO (mouse) cat. no. 424022; Nichirei Biosciences Inc.; Nichirei Corporation), both for 20 min each at room temperature. Then, sections were incubated for 5 min with diaminobenzidine at room temperature for detecting the molecules. The following primary antibodies were used: Monoclonal anti-PD-L1 antibody (1:100; clone E1L3N; rabbit IgG; cat. no. 13684; Cell Signaling Technology, Inc.) and monoclonal anti-CD8 antibody (1:800; clone C8/144B; mouse IgG; cat. no. M7103; Dako; Agilent Technologies, Inc.).

All immunostaining images were obtained using a KEYENCE light microscope (BZ-9000; Keyence Corporation; magnification, x400). PD-L1<sup>+</sup> tumor cells and CD8<sup>+</sup> cells were automatically counted using the visual inspection application software BZ-X analyzer JP ver.1.4.1 (Keyence Corporation). Percentages of tumor cells with cell surface staining for PD-L1 were recorded and presented as tumor proportion score (TPS). When the TPS was >1%, the sample was classified as PD-L1<sup>+</sup>. A minimum of 100 tumor cells was evaluated to calculate the TPS. The percentage of CD8<sup>+</sup> TILs among total nucleated cells in the stromal compartments was defined as stromal CD8<sup>+</sup> TIL density (21). To calculate stromal CD8<sup>+</sup> TIL density,

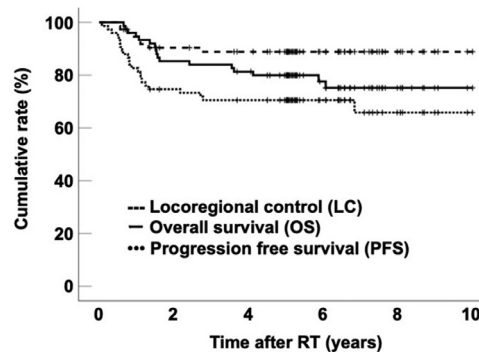


Figure 1. Survival curves for enrolled patients with uterine cervical squamous cell carcinoma. Kaplan-Meier curves for overall survival, locoregional control and progression-free survival. RT, radiotherapy.

the CD8<sup>+</sup> cells in hotspot areas of the specimens were counted; hotspot areas were defined as those containing the highest density of nucleated cells (22). The tumor area was manually excluded. The quality of tumor samples was carefully evaluated and validated independently by 2 co-authors (Department of Human Pathology, Gunma University Graduate School of Medicine) of the present study who were pathologists.

**Statistical analysis.** The OS, LC and PFS rates were calculated using the Kaplan-Meier method, and the log-rank test was used to confirm significant differences. Continuous data were compared with a non-parametric test (Wilcoxon signed-rank test for paired data). Receiver operating characteristic (ROC) curve analyses were performed to determine the optimal cut-off value of CD8<sup>+</sup> TIL density. Univariate analyses were performed using Cox proportional hazards model. P<0.05 was considered to indicate a statistically significant difference. Bonferroni correction was used to adjust the familywise error rate from multiple comparisons for P-values. All statistical analyses were performed using SPSS 26.0 for Mac (IBM Corp.).

## Results

**Clinical characteristics and outcomes.** Median follow-up duration was 63 months (range, 8-120 months). The 5-year OS, LC and PFS rates for all patients were 80.0% (95% CI, 70.9-89.0%), 89.0% (95% CI, 81.6-96.2%) and 70.5% (95% CI, 60.1-80.9%), respectively (Fig. 1). Patient clinical characteristics are shown in Table I.

**PD-L1 expression and clinical outcomes.** To investigate the alteration of PD-L1 expression post-10 Gy, the rate of PD-L1<sup>+</sup> cells in the IHC samples pre-RT and post-10 Gy was analyzed (Fig. 2A). In pre-RT samples, 4 patients (5%) were positive and 71 patients (95%) were negative; in post-10 Gy samples, 39 patients (52%) were positive and 36 patients (48%) were negative (Fig. 2B). In all the patient groups, the percentage of PD-L1<sup>+</sup> patients significantly increased from 5 to 52% between pre-RT and post-10 Gy (P<0.01; Fig. 2B). In patients treated with CRT, the percentage of PD-L1<sup>+</sup> patients significantly increased from 6 to 48% between pre-RT and post-10 Gy (P<0.01; Fig. 2B). In patients treated with RT alone, the percentage of PD-L1<sup>+</sup>

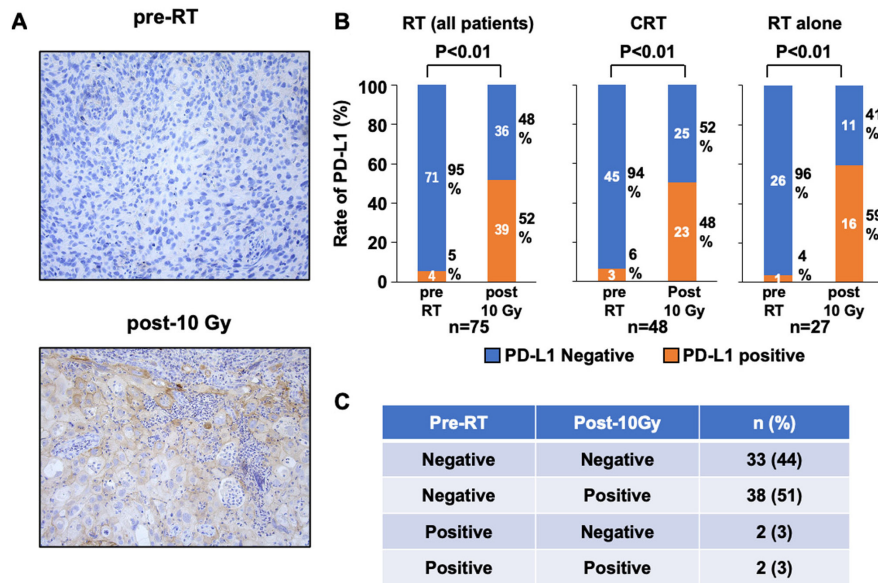


Figure 2. PD-L1 staining and alterations in PD-L1 expression on tumor cells induced by CRT/RT. (A) Representative images of immunohistochemical PD-L1 staining pre-RT (negative) and post-10 Gy (positive) from the same patient (magnification, x400). (B) PD-L1 expression was upregulated by RT (all patients), CRT and RT alone. The number within the bar represents the number of patients. The percentage is displayed next to the bar graph. (C) Summary of PD-L1 status pre-RT and post-10 Gy in all patients. PD-L1, programmed death-ligand 1; RT, radiotherapy; CRT, chemoradiotherapy.

patients significantly increased from 4 to 59% between pre-RT and post-10 Gy ( $P<0.01$ ; Fig. 2B). Therefore, RT significantly upregulated PD-L1 expression in patients with uterine cervical squamous cell carcinoma. When compared with PD-L1 expression before RT, 38 patients (51%) exhibited an increase, two patients (3%) exhibited a decrease and 35 patients (47%) exhibited no change post-10 Gy RT (Fig. 2C).

Subsequently, the association between pre-RT and post-10 Gy PD-L1 expression and clinical outcome was examined. OS, LC and PFS did not display significant differences with positive and negative PD-L1 expression for both pre-RT and post-10 Gy samples (Fig. S1A-F). Furthermore, patients were categorized into three groups according to changes in PD-L1 expression. It was revealed that PD-L1 alteration exhibited no significant association with clinical outcome (Fig. S1G-I).

**CD8<sup>+</sup> TILs and clinical outcomes.** To investigate the alteration of CD8<sup>+</sup> TILs in tumor tissues post-10 Gy, the density of stromal CD8<sup>+</sup> TILs in the IHC samples both pre-RT and post-10 Gy was analyzed (Fig. 3A). IHC staining revealed that 31 patients (41.3%) exhibited an increase, while 44 patients (58.7%) exhibited a decrease in stromal CD8<sup>+</sup> TIL density after 10 Gy (Fig. 3B). Stromal CD8<sup>+</sup> TIL density was significantly decreased in post-10 Gy samples (median density, 23.1% pre-RT vs. 16.9% post-10 Gy;  $P=0.038$ ; Fig. 3B and E). According to the treatment type, stromal CD8<sup>+</sup> TIL density was significantly decreased after CRT (median density, 29.6% pre-RT vs. 13.4% post-10 Gy;  $P=0.001$ ; Fig. 3C and E). By contrast, stromal CD8<sup>+</sup> TIL density tended to increase after RT alone (median, 17.7% pre-RT vs. 24.0% post-10 Gy;  $P=0.400$ ; Fig. 3D and E). No significant difference was observed in the density of pre-RT CD8<sup>+</sup> TILs between the CRT and RT alone groups (data not shown). Furthermore, to evaluate the optimal cut-off value for CD8<sup>+</sup> TIL density in the present study, a

ROC curve analysis was performed. The association between clinical outcome and stromal CD8<sup>+</sup> TIL density was assessed in all 75 patients. ROC curve analysis revealed that both pre-RT and post-10 Gy CD8<sup>+</sup> TIL density exhibited significant prognostic value for predicting death and tumor recurrence, with optimal cut-off values of 32.2 and 16.9%, respectively (Fig. S2). Notably, patients with high stromal CD8<sup>+</sup> TIL density (based on the ROC optimal cut-off values) both pre-RT and post-10 Gy exhibited significantly improved OS, LC and PFS compared with patients with low stromal CD8<sup>+</sup> TIL density (Fig. 4A-F).

Furthermore, to analyze the effects of each factor on prognosis, univariate analyses using Cox proportional hazards model were performed (Tables II-IV). Univariate analyses revealed that FIGO stages III-IVA and lymph node metastasis in the pelvis were significantly associated with unfavorable OS and PFS (Tables II and IV). Univariate analyses revealed that para-aortic lymph node metastasis in the pelvis were significantly associated with unfavorable LC (Table III). Notably, high stromal CD8<sup>+</sup> TILs in both pre-RT and post-10 Gy groups exhibited a significant association with improved OS and PFS (Tables II and IV).

Finally, patients were classified into four groups according to stromal CD8<sup>+</sup> TIL density pre-RT and post-10 Gy [i] High (pre-RT CD8 density)-high (post-10 Gy CD8 density) defined as CD8 High-High; ii) high (pre-RT CD8 density)-low (post-10 Gy CD8 density) defined as CD8 high-low; iii) low (pre-RT CD8 density)-high (post-10 Gy CD8 density) defined as CD8 low-high and iv) low (pre-RT CD8 density)-low (post-10 Gy CD8 density) defined as CD8 low-low (Fig. 5A). Notably, the group displaying low stromal CD8<sup>+</sup> TIL density both pre-RT and post-10 Gy (CD8 Low-Low group) exhibited a significantly lower OS, LC and PFS compared with the other groups (Fig. 5B-D). Overall, the current data suggested that low stromal CD8<sup>+</sup> TIL density either pre-RT or post-10 Gy may be

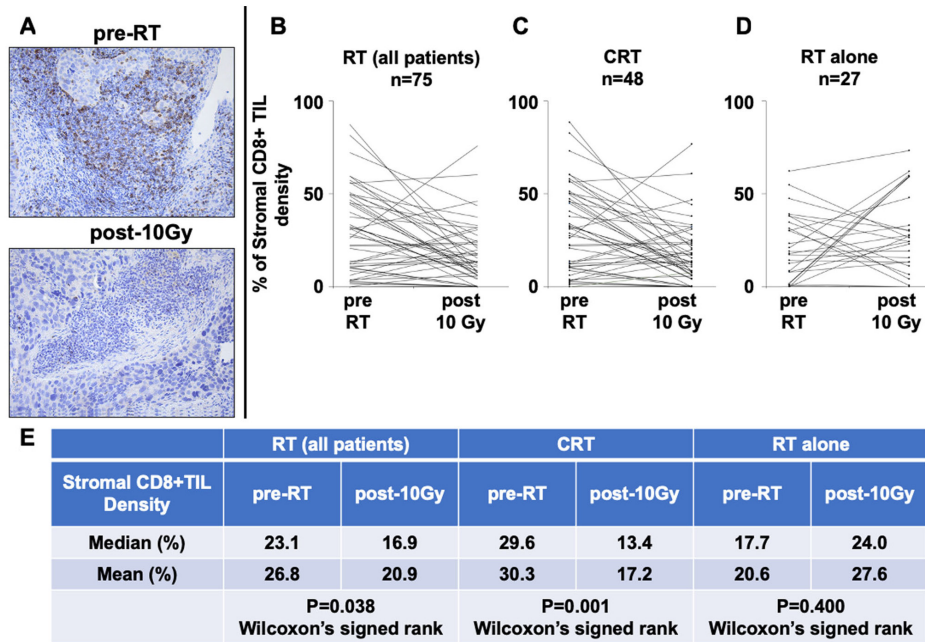


Figure 3. CD8 staining and alterations in CD8<sup>+</sup> TILs induced by CRT/RT. (A) Representative images of immunohistochemical CD8<sup>+</sup> staining alterations pre-RT (low) and post-10 Gy (high) from the same patient (magnification, x400). Alterations in stromal CD8<sup>+</sup> TIL density pre-RT and post-10 Gy in (B) RT (all patients), (C) CRT and (D) RT alone samples. (E) Summary of the alterations. TIL, tumor-infiltrating lymphocyte; CRT, chemoradiotherapy; RT, radiotherapy.

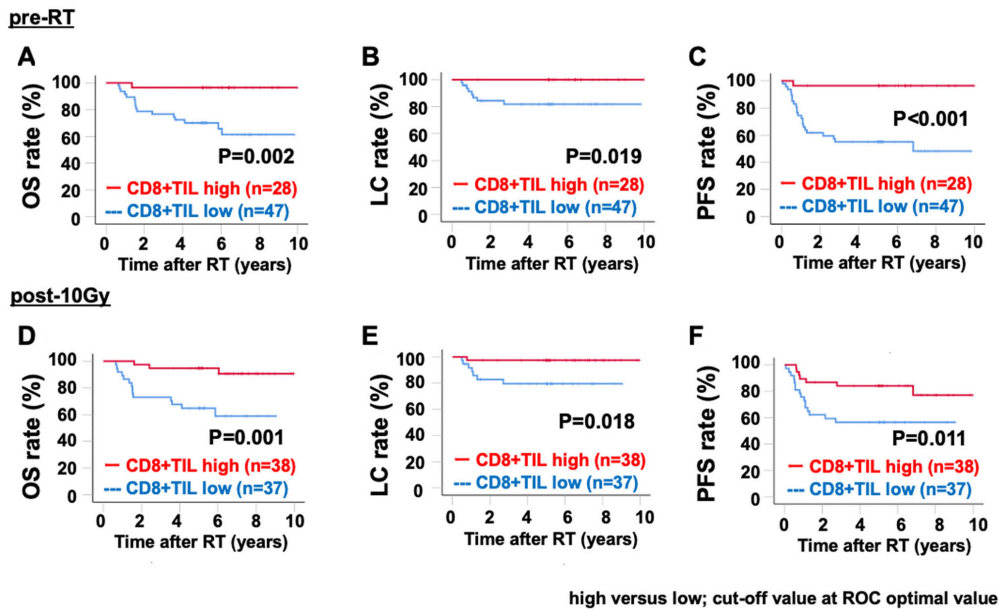


Figure 4. Survival curves of patients with cervical cancer according to stromal CD8<sup>+</sup> TIL density. (A) OS, (B) LC and (C) PFS curves of patients with high (n=28) or low (n=47) CD8<sup>+</sup> TIL density in pre-RT samples. (D) OS, (E) LC and (F) PFS curves of patients with high (n=38) or low (n=37) CD8<sup>+</sup> TIL density in post-10 Gy samples. TIL, tumor-infiltrating lymphocyte; RT, radiotherapy; OS, overall survival; LC, locoregional control; PFS, progression-free survival; ROC, receiver operating characteristic.

a critical predictive biomarker for patients with cervical cancer treated with definitive RT.

*Association between PD-L1 expression and CD8<sup>+</sup> TILs.* To clarify the association between PD-L1 expression and CD8<sup>+</sup> TIL density, the changes in PD-L1 expression and CD8<sup>+</sup> TIL density in the 10-Gy irradiated samples were analyzed. It was revealed that 68% (21/31) of the increased CD8<sup>+</sup> TIL density group also exhibited an increase in PD-L1 expression, whereas 61% (27/44)

of the decreased CD8<sup>+</sup> TIL density group exhibited no increase in PD-L1 expression (Table V). Thus, there was a significant association between an elevation in CD8<sup>+</sup> TIL density and the induction of PD-L1 expression (P=0.013; Table V). Furthermore, the present study investigated whether changes in CD8<sup>+</sup> TILs and PD-L1 expression in response to 10 Gy affected clinical outcomes in patients. However, a log-rank test did not indicate a significant association between changes in CD8<sup>+</sup> TIL density and PD-L1 expression with prognosis (Fig. S3).

Table II. Univariate analysis of overall survival.

Factor	Univariate analysis	
	Hazard ratio (95% CI)	P-value
Age (≥62 years vs. <62 years)	0.868 (0.334-2.252)	0.770
Concurrent chemotherapy (no vs. yes)	0.223 (0.211-1.423)	0.548
FIGO stage (I+II vs. III+IV)	7.041 (2.020-24.541)	0.002
PeLN (positive vs. negative)	0.277 (0.090-0.851)	0.025
PALN (positive vs. negative)	0.365 (0.105-1.273)	0.114
CD8 <sup>+</sup> TILs (pre-RT) (low vs. high)	0.086 (0.110-6.46)	0.017
CD8 <sup>+</sup> TILs (post-10 Gy) (low vs. high)	0.160 (0.046-0.560)	0.004

PeLN, lymph node metastasis in pelvis; PALN, para-aortic lymph node metastasis; RT, radiotherapy; TIL, tumor-infiltrating lymphocyte; FIGO, International Federation of Gynecology and Obstetrics.

Table III. Univariate analysis of locoregional control duration.

Factor	Univariate analysis	
	Hazard ratio (95% CI)	P-value
Age (≥62 years vs. <62 years)	0.630 (0.105-3.792)	0.614
Concurrent chemotherapy (no vs. yes)	0.321 (0.053-1.935)	0.215
FIGO stage (I+II vs. III+IV)	2.104 (0.350-12.638)	0.416
PeLN (positive vs. negative)	1.403 (0.233-8.457)	0.711
PALN (positive vs. negative)	0.126 (0.021-0.757)	0.024
CD8 <sup>+</sup> TILs (pre-RT) (low vs. high)	0.337 (0.037-3.053)	0.334
CD8 <sup>+</sup> TILs (post-10 Gy) (low vs. high)	0.187 (0.021-1.703)	0.137

PeLN, lymph node metastasis in pelvis; PALN, para-aortic lymph node metastasis; RT, radiotherapy; TIL, tumor-infiltrating lymphocyte; FIGO, International Federation of Gynecology and Obstetrics.

Table IV. Univariate analysis of progression-free survival.

Factor	Univariate analysis	
	Hazard ratio (95% CI)	P-value
Age (≥62 years vs. <62 years)	0.822 (0.355-1.906)	0.648
Concurrent chemotherapy (no vs. yes)	0.463 (0.200-1.070)	0.072
FIGO stage (I+II vs. III+IV)	4.264 (1.664-10.927)	0.003
PeLN (positive vs. negative)	0.318 (0.124-0.816)	0.017
PALN (positive vs. negative)	0.293 (0.098-0.874)	0.280
CD8 <sup>+</sup> TILs (pre-RT) (low vs. high)	0.302 (0.102-0.894)	0.031
CD8 <sup>+</sup> TILs (post-10 Gy) (low vs. high)	0.368 (0.150-0.905)	0.030

PeLN, lymph node metastasis in pelvis; PALN, para-aortic lymph node metastasis; RT, radiotherapy; TIL, tumor-infiltrating lymphocyte; FIGO, International Federation of Gynecology and Obstetrics.

## Discussion

In the present study, it was demonstrated that the PD-L1<sup>+</sup> rate was significantly increased from 5% pre-RT to 52% post-10 Gy,

and the median density of stromal CD8<sup>+</sup> TILs was significantly decreased from 23.1% pre-RT to 16.9% post-10 Gy in patients with cervical squamous cancer. However, stromal CD8<sup>+</sup> TIL density tended to increase from a median of 17.7% pre-RT to

Table V. Alterations in PD-L1 expression and CD8<sup>+</sup> TIL density.

CD8 <sup>+</sup> TIL density	PD-L1 expression		P-value
	Unchanged/decreased, n (%)	Increased, n (%)	
Decreased	27 (61)	17 (39)	0.013
Increased	10 (32)	21 (68)	

PD-L1, programmed death-ligand 1; TIL, tumor-infiltrating lymphocyte.

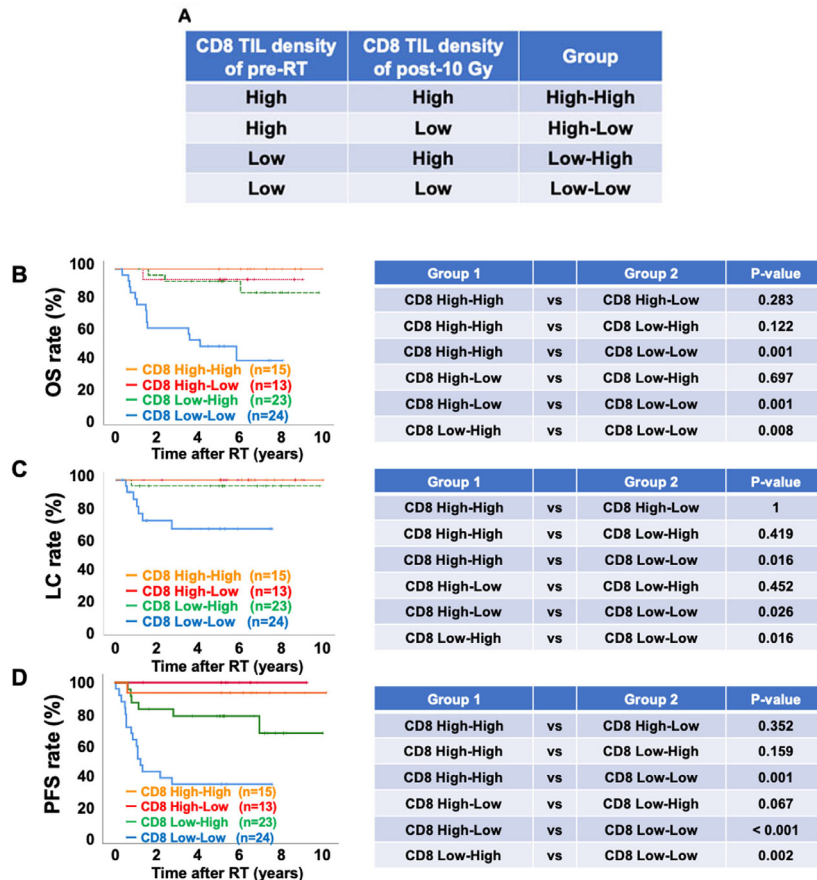


Figure 5. Survival curves of patients with cervical cancer according to stromal CD8<sup>+</sup> TIL alteration between pre-RT and post-10 Gy. (A) patients were classified into 4 groups according to stromal CD8<sup>+</sup> TIL density pre-RT and post-10 Gy. (B) OS, (C) LC and (D) PFS curves among CD8 High-High (n=15), CD8 High-Low (n=13), CD8 Low-High (n=23) and CD8 Low-Low (n=24) groups. Results of statistical analyses are presented on the right. TIL, tumor-infiltrating lymphocyte; RT, radiotherapy; OS, overall survival; LC, locoregional control; PFS, progression-free survival.

24.0% post-10 Gy in patients who received RT alone, suggesting that chemotherapy may be involved in the alternation. With regard to the association of stromal CD8<sup>+</sup> TIL density with prognosis, univariate analyses revealed that stromal CD8<sup>+</sup> TIL density pre-RT and post-10 Gy was significantly associated with improved OS and PFS. By contrast, PD-L1 expression did not show any significant association with the clinical outcomes, despite its upregulation post-10 Gy. To the best of our knowledge, the present study was the first to assess CD8<sup>+</sup> TILs and PD-L1 expression of biopsy specimens by IHC during RT (post-10 Gy).

Notably, the present study revealed that stromal CD8<sup>+</sup> TIL density tended to increase only in patients receiving RT alone. Consistent with the current data, neoadjuvant RT alone increased stromal CD8<sup>+</sup> TIL density in patients with rectal

cancer (23). In addition, the induction of CD8<sup>+</sup> TILs into the irradiated field after RT alone has been demonstrated in a mouse model (24). In contrast to the results of RT alone, there was a significant decrease in stromal CD8<sup>+</sup> TIL density in the overall RT and CRT groups. This decrease may have been caused by a decrease in the number of systemic immune cells due to chemotherapy. However, increased CD8<sup>+</sup> TIL ratio after neoadjuvant CRT has been observed in patients with colorectal cancer (23), non-small-cell carcinoma (25) and esophageal cancer (26). Thus, at present, changes in CD8<sup>+</sup> TIL levels in tumor tissues caused by CRT/RT are controversial. Indeed, preclinical models have revealed that chemotherapy induces immunogenic cell death, which may recruit CD8<sup>+</sup> TILs into the tumor tissues (27-29). The elucidation of the

molecular mechanism underlying CD8<sup>+</sup> TILs migration into tumors is required to understand the current controversial results dependent on the modality, tissue specificity, type of cancer and other factors.

The present study demonstrated that high density of CD8<sup>+</sup> TILs may contribute to improved outcomes, whereas CRT/RT was less effective in tumors with low density of CD8<sup>+</sup> TILs before treatment. Consistent with the present results, clinical studies have indicated that high levels of CD8<sup>+</sup> TILs in tumor tissues before treatment are associated with improved outcomes in patients with cervical cancer (13,14). Similarly, post-CRT stromal CD8<sup>+</sup> TILs have been associated with improved OS in patients with rectal cancer (30). The idea that tumors harboring high levels of CD8<sup>+</sup> TIL exhibit improved outcomes with RT is also supported by our previous data indicating that the depletion of CD8<sup>+</sup> T cells using an anti-CD8 antibody significantly suppresses the effect of RT on tumor growth delay (31). Thus, the present study confirmed the aforementioned previous findings.

PD-L1 upregulation by RT has been demonstrated in several preclinical models (32,33). A recent similar clinical study revealed PD-L1 upregulation after RT in patients with squamous cervical cancer (14). The novel aspect of the present study is that PD-L1 and CD8<sup>+</sup> TILs were assessed in the biopsied specimens during RT. Our previous *in vitro* study indicated that PD-L1 upregulation after DNA damage by X-ray irradiation was not maintained for >14 days (28). Thus, the current post-10 Gy samples may reflect the DNA damage-induced PD-L1 upregulation more directly. In particular circumstances involving CD8<sup>+</sup> TILs and PD-L1 upregulation after RT, anti-PD-1/PD-L1 antibodies in combination with RT may be more effective, because PD-L1 expression is considered as a predictive biomarker of response rate for anti-PD-1/PD-L1 antibody (34). Based on this idea, several studies have started clinically testing the combined use of an anti-PD-1/PD-L1 antibody during or after RT, suggesting promising outcomes (35-37).

A limitation of the present study is its retrospective evaluation of 75 cases from a single institute. The number of cases is small, and various clinical stages (stages I-IV) were included. Therefore, a larger analysis is required to clarify the association between clinical outcomes and PD-L1 expression or CD8<sup>+</sup> TILs. In addition, to identify novel prognostic biomarkers, evaluation of other factors affecting the immune response in tumor microenvironments, such as HLA class I expression, myeloid-derived suppressor cells, M2 tumor-associated macrophages and regulatory T cells, may be important. Furthermore, knowledge of patients' characteristics leading to PD-L1 upregulation and CD8<sup>+</sup> TILs alteration after CRT/RT will be valuable. The present data suggest the potential of PD-1/PD-L1 blockade immunotherapy for patients with increased PD-L1 expression after 10 Gy. However, it is currently not appropriate to use anti-PD-1/PD-L1 antibodies instead of cisplatin, which is the current standard of care for patients with cervical cancer (38,39). Future studies should also explore this treatment method for patients with other types of cancer.

In summary, the present study revealed that CRT/RT induced PD-L1 upregulation in patients with cervical cancer and affected the density of stromal CD8<sup>+</sup> TILs. Stromal CD8<sup>+</sup> TIL density may be a predictive biomarker both pre-RT and

post-10 Gy. Therefore, careful follow-up may be crucial for patients with cervical cancer with low CD8<sup>+</sup> TILs before RT or no increase in CD8<sup>+</sup> TILs during RT. To investigate the clinical significance of radiation-induced PD-L1 upregulation and CD8<sup>+</sup> TILs alteration, additional studies with large cohorts are required.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

YM summarized the patient information. YM, HS, TKu, TOi, KS, HI, HY and AS performed the experiments and analyzed the data. YM, HS, TBMP, SK and AS were involved in drafting the manuscript and made substantial contributions to analysis and interpretation of data. YM, KM, SEN, TKu, KA, YY, NO, TKa, KO, TN and TOh coordinated the clinics, performed the treatment, participated in the follow-up of the patients, obtained specimens and acquired data. TN and TOh contributed reagents/materials/analysis tools and gave final approval of the manuscript version to be published. The authenticity of all the raw data was assessed by YM and HS. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The Institutional Review Board for clinical trials of Gunma University (Maebashi, Japan) approved the study protocol (approval no. HS2020-015). This study is a retrospective and observational study. All patients provided their informed consent to participate in the study using the opt-out approach



by public notice at internet site of the Institutional Review Board for clinical trials of Gunma University (Maebashi, Japan).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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