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Comparative Analysis of the Antitumor Immune Profiles of Paired Radiotherapy-naive and Radiotherapy-treated Cervical Cancer Tissues

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Abstract. *Background/Aim: This study aimed to elucidate the effect of radiotherapy on expression of immune response-related genes in cervical cancer tissues. Materials and Methods: Tumor tissues were obtained from 16 patients with cervical cancer before initiation of radiotherapy and after treatment with 10 Gy X-rays, delivered in five fractions. Expression of 730 immune response-related genes was assessed using an nCounter PanCancer Immune Profiling Panel (NanoString Technologies. Seattle, WA, USA). Results: Of the 730 genes examined, 41 showed significant changes (fold change of >1.5 or <0.66) in expression in post-radiotherapy samples (28 up-regulated and 13 down-regulated). Analysis of immune cell type-specific genes suggested predominant up-regulation of those related to innate immunity postradiotherapy. Interestingly, cytotoxic T-lymphocyte-associated protein (CTLA4), a key negative regulator of T-cell activation, was marked down-regulated in 93.7% of patients, with an average fold-change of 2.0. Conclusion: To our knowledge, this study is the first to show down-regulation of CTLA4 in clinical cervical cancer tissues after treatment with radiotherapy.*

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Radiotherapy (RT) is one of the three pillars of cancer therapy, along with surgery and chemotherapy. Conventionally, it is considered as a local treatment used to shrink or eliminate a tumor inside the irradiated field; however, it can have systemic effects because it induces antitumor immune responses. During the past few decades, immune responses induced by RT have been identified. These include immunogenic cell death, which is a type of cell death leading to dendritic cell recruitment followed by T-cell activation (1, 2), and up-regulation of class I major histocompatibility complex expression by irradiated cancer cells, which contributes to tumor antigen recognition by T-cells (3-5). Recent studies demonstrate induction of more specific immune responses, such as transcription of immunogenic mutant genes and the cyclic GMP-AMP synthase–stimulator of interferon genes pathway in dendritic cells and tumor cells; DNA damage in tumor cells triggers inflammatory responses (6, 7). By contrast, and unlike in basic research, there is little evidence of RT-mediated antitumor immune responses in clinical samples. One of the reasons for this is the difficulty in obtaining tumor tissues. Indeed, most analyses of local immune responses at the clinical level use postoperative specimens that have undergone neoadjuvant RT; therefore, immune responses in directly irradiated lesions are still unclear. Furthermore, from a technical point of view, immunohistochemical staining (which is frequently used in clinical studies) has limited sensitivity with respect to detection of target molecules, as well as the number of molecules that can be analyzed simultaneously.

nCounter is a method that labels arbitrary nucleic acids with sequence-specific fluorescence barcodes and then digitally counts the fluorescence signals, resulting in highly sensitive and comprehensive measurement of mRNA expression in clinical samples (8). The PanCancer Immune Profiling Panel is an nCounter analysis package that includes 730 immune response-related genes. This method

Table I. *Patient characteristics*.

Characteristic	(n=16)	Value
Follow up period, months	Median (range)	63 (4-105)
Age, years	Median (range)	59.5 (35-77)
FIGO stage, n (%)	IB	1 (6.2%)
	II	5 (31.3%)
	III	10 (62.5%)
Tumor diameter	≤40 mm	1 (6.2%)
	41-60 mm	9 (56.3%)
	>60 mm	6 (37.5%)
Pelvic LN involvement, n (%)	Negative	5 (31.3%)
	Positive	11 (68.7%)
PALN involvement, n (%)	Negative	10 (62.5%)
	Positive	6 (37.5%)
HPV type, n (%)	HPV16	7 (43.7%)
	HPV18	2 (12.5%)
	Other	4 (25.0%)
	Not detected	3 (18.8%)
Concurrent chemotherapy	Yes	13 (81.2%)
	No	3 (18.8%)
Tumor mutational burden	Median (range)	11.1 (4.1-68.7)
PIK3CA mutation, n (%)	Negative	10 (62.5%)
	Positive	6 (37.5%)
ARID1A mutation, n (%)	Negative	9 (56.3%)
	Positive	7 (43.7%)
NOTCH1 mutation, n (%)	Negative	8 (50.5%)
	Positive	8 (50.5%)
FBXW7 mutation, n (%)	Negative	11 (68.7%)
	Positive	5 (31.3%)

ARID1A: AT-rich interaction domain 1A; *FBXW7*: F-box and WD repeat domain-containing 7; FIGO: International Federation of Gynecology and Obstetrics 2009; HPV: human papillomavirus; LN: lymph node; *NOTCH1*: notch receptor 1; PALN: para-aortic lymph node; *PIK3CA*: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

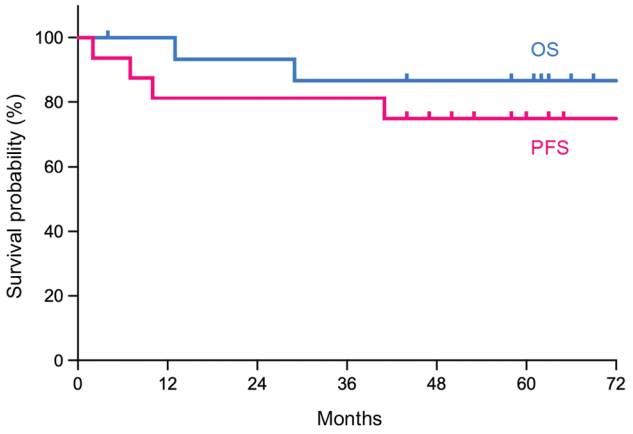

Figure 1. *Kaplan-Meier survival estimates of overall (OS) and progression-free (PFS) survival for the present study cohort (n=16).*

enables comprehensive analysis and comparison of immune-related gene expression levels, *e.g.* between normal and tumor tissues, or before and after treatments containing immune checkpoint inhibitors (9, 10). As mentioned above, the effects of RT on expression of immune response-related genes in tissues subjected to RT have not been fully elucidated. Therefore, we used nCounter technology to examine the effects of RT on expression of immune response-related genes in cervical cancer tissues.

Materials and Methods

Patients. Patients who met the following inclusion criteria were enrolled retrospectively: (i) Newly diagnosed and pathologically confirmed squamous cell carcinoma of the uterine cervix, (ii) treated with definitive RT at Gunma University Hospital from 2006 to 2013, and (iii) available fresh frozen tumor tissues obtained by punch biopsy before initiation of RT (pre-RT) and after treatment with 10 Gy X-rays in five fractions (post-RT). The interval between initiation of RT and biopsy of the specimen after 10 Gy RT was approximately 7 days.

Definitive RT, comprising external beam RT and computed tomography-based high-dose-rate brachytherapy, was performed as described previously (11). Patients with stage III-IV disease, tumor diameter >40 mm, or nodal involvement received cisplatin-based chemotherapy (40 mg/m^2 weekly) concurrently with RT.

Patients were followed up every 1-3 months for the first 2 years after treatment and then every 3-6 months for the subsequent 3 years. Disease status was assessed at each follow-up by gynecological examination and imaging (computed tomography or magnetic resonance). Overall (OS) and progression-free (PFS) survival were recorded from the day of initiation of RT until the last follow-up. Data pertaining to somatic mutations in genes with a mutation prevalence greater than 30% were collected from previous reports (12, 13).

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board of Gunma University Hospital (approval number 1109). The requirement for informed consent was waived by the Institutional Review Board of Gunma University Hospital due to the opt-out design of the study.

Analysis of immune response-related gene expression. Expression of immune response-related genes was analyzed using an nCounter Analysis System with a PanCancer Immune Profiling Panel (NanoString Technologies, Seattle, WA, USA) (8). This panel consists of 730 immune response-related genes, covering 24 different immune cell types, and both the adaptive and innate immune responses (14). Analysis was performed as reported previously (9, 14-17). Briefly, total RNA was extracted from frozen tumor tissues using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). The quality of the extracted RNA was tested by absorbance measurements and electrophoresis using the following cutoffs: >60 ng/μl for total RNA concentration, >300 ng for total RNA quantity, and ≥50% for the electropherogram area corresponding to >300-nucleotide fragments. Using 300 ng of RNA per sample, hybridization was performed at 65˚C for 16 h using nCounter Prep Station (NanoString Technologies). Hybridized probes were bound

Table II. *Genes whose expression was changed by more than 1.5-fold post-radiotherapy.*

	Gene	Encoded protein	Fold change	*p*-Value	*q*-Value
Up-regulated	*CXCR2*	C-X-C Motif chemokine receptor 2	2.10	0.004	0.076
	IFNA17	Interferon alpha 17	2.02	0.002	0.065
	CXCL2	C-X-C motif chemokine ligand 2	2.00	0.009	0.092
	TREM1	Triggering receptor expressed on myeloid cells 1	1.99	0.002	0.059
	CXCL5	C-X-C Motif chemokine ligand 5	1.85	0.006	0.082
	SELL	Selectin L	1.83	0.005	0.082
	MME	Membrane metalloendopeptidase	1.77	<0.001	**0.022**
	CEACAM8	CEA Cell adhesion molecule 8	1.76	<0.001	**0.008**
	CCL3L1	C-C Motif chemokine ligand 3 like 1	1.73	<0.001	0.053
	CXCL3	C-X-C Motif chemokine ligand 3	1.73	0.004	0.076
	SSX1	SSX Family member 1	1.72	0.028	0.157
	CCL18	C-C Motif chemokine ligand 18	1.71	0.042	0.193
	IL1A	Interleukin 1 alpha	1.67	0.028	0.157
	LILRB2	Leukocyte immunoglobulin like receptor B2	1.62	0.007	0.088
	CR1	Complement C3b/C4b receptor 1	1.61	0.013	0.108
	CT45A1	Cancer/testis antigen family 45 member A1	1.61	0.008	0.088
	CEACAM1	CEA cell adhesion molecule 1	1.61	0.012	0.103
	CCL11	C-C Motif chemokine ligand 11	1.59	0.041	0.193
	CCL22	C-C Motif chemokine ligand 22	1.58	0.009	0.092
	CCL25	C-C Motif chemokine ligand 25	1.57	0.005	0.081
	AIRE	Autoimmune regulator	1.56	0.038	0.188
	FPR2	Formyl peptide receptor 2	1.55	0.018	0.128
	AMMECR1L	AMMECR1-like	1.54	0.024	0.146
	IL1B	Interleukin 1 beta	1.54	0.002	0.059
	GAGE1	G Antigen 1	1.53	0.010	0.098
	ULBP2	UL16-binding protein 2	1.52	0.021	0.137
	CCL14	C-C Motif chemokine ligand 14	1.51	0.029	0.159
Down-regulated	*S100A12*	S100 Calcium-binding protein A12	1.51	0.003	0.076
	TNFRSF18	Tumor necrosis factor receptor superfamily member 18	0.48	<0.001	**0.010**
	CTLA4	Cytotoxic T-lymphocyte associated protein 4	0.50	<0.001	**0.022**
	GNLY	Granulysin	0.58	0.011	0.102
	ITGA4	Integrin subunit alpha 4	0.61	0.007	0.088
	NOTCH1	Notch receptor 1	0.61	0.001	0.056
	LAG3	Lymphocyte activating 3	0.62	0.010	0.097
	TTK	TTK protein kinase	0.62	0.001	0.053
	CD274	N/A	0.63	0.040	0.193
	NLRC5	NLR family CARD domain-containing 5	0.64	0.006	0.083
	BLNK	B-Cell linker	0.64	0.029	0.161
	TLR5	Toll-like receptor 5	0.64	0.015	0.115
	IGF2R	Insulin-like growth factor 2 receptor	0.66	0.025	0.150
	HRAS	HRas proto-oncogene	0.66	0.008	0.090

N/A: Not available. *p*-Values were calculated by paired *t*-test. *q*-Values were calculated by Benjamini–Hochberg method and those below 0.05 are shown in bold.

and aligned to an nCounter Cartridge (NanoString Technologies) and counted using a Digital Analyzer (NanoString Technologies) by scanning 555 fields of view. Raw gene count data were processed using nSolver 3.0 (NanoString Technologies). The background count, determined as the average count of the negative controls, was subtracted from the raw gene counts. The raw gene counts were then normalized to the geometric mean of the positive control counts and to that of internal reference genes.

Statistical analysis. Differences in gene expression between pre-RT and post-RT samples from the same patient were examined using a paired *t*-test. The outcomes of the paired *t*-test were analyzed using the Benjamini–Hochberg method (18), with a false-discovery rate (*q*) of 0.05. Differences in gene expression between patients were stratified according to categorical variables and examined using the Mann-Whitney *U*-test. Correlations between gene expression and clinical or genetic factors (numerical variables) were examined using Spearman's rank-sum test. The probability of OS and PFS was estimated using the Kaplan-Meier method. All statistical analyses were carried out using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), with statistical significance set at $p=0.05$.

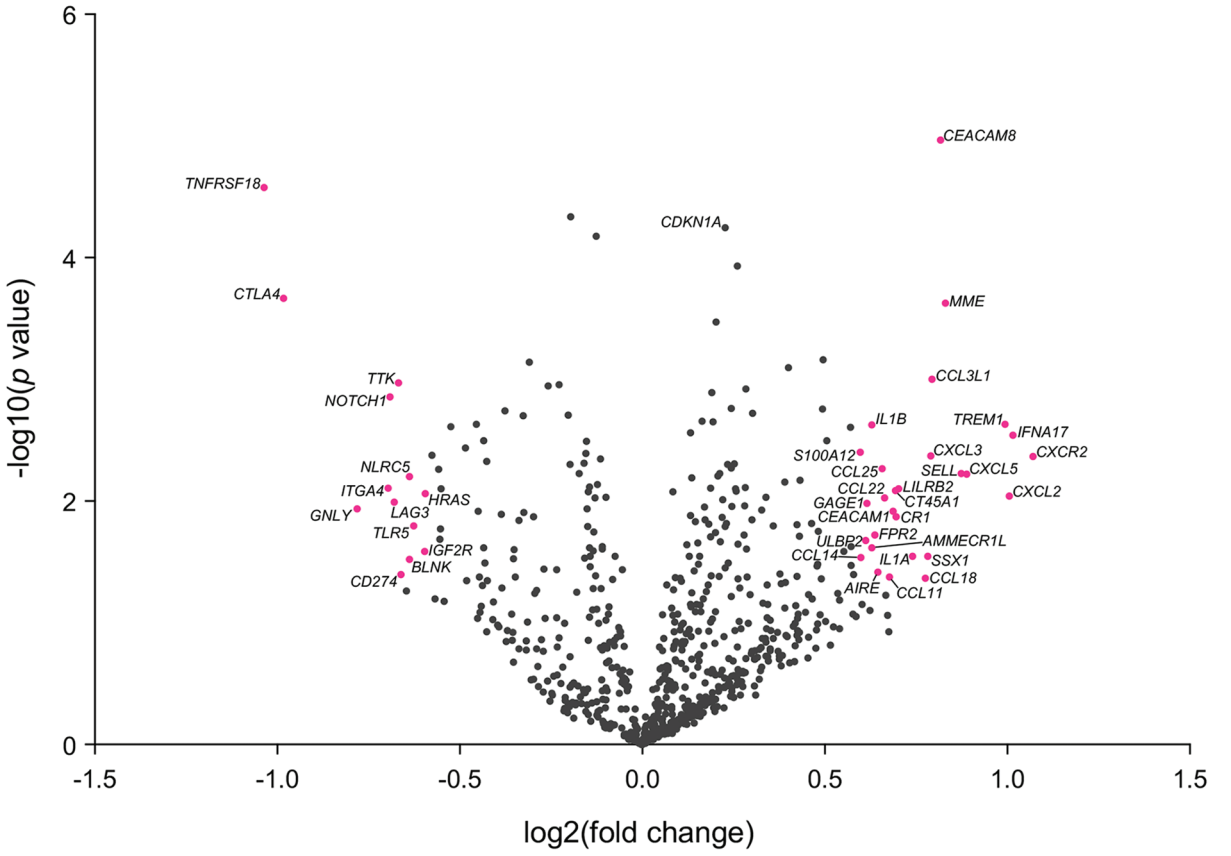

Figure 2. *Volcano plot showing the statistical significance and the magnitude of postradiotherapy changes in expression of immune response-related genes (n=730). Genes with a >1.5-fold or <0.66-fold change in expression and a value of p<0.05 (by paired t-test) and are indicated in magenta, namely: AIRE: Autoimmune regulator; AMMECR1L: AMMECR1-like; BLNK: B-cell linker; CCL: C-C motif chemokine ligand CCL3L1: C-C motif chemokine ligand 3-like 1; CEACAM: CEA cell adhesion molecule; CR1: complement C3b/C4b receptor 1; CT45A1: cancer/testis antigen family 45 member A1; CTLA4: cytotoxic T-lymphocyte-associated protein 4; CXCL: C-X-C motif chemokine ligand; CXCR2: C-X-C motif chemokine receptor 2; FPR2: formyl peptide receptor 2; GAGE1: G antigen 1; GNLY: granulysin; HRAS: HRas proto-oncogene; IFNA17: interferon alpha 17; IGF2R: insulin-like growth factor 2 receptor; IL1A/B: interleukin 1 alpha/beta; ITGA4: integrin subunit alpha 4; LAG3: lymphocyte-activating 3; LILRB2: leukocyte immunoglobulin-like receptor B2; MME: membrane metalloendopeptidase; NLRC5: NLR family CARD domain-containing 5; NOTCH1: notch receptor 1; S100A12: S100 calcium-binding protein A12; SELL: selectin L; SSX1: SSX family member 1; TLR5: toll-like receptor 5; TNFRSF18: tumor necrosis factor receptor superfamily member 18; TREM1: triggering receptor expressed on myeloid cells 1; TTK: TTK protein kinase; ULBP2: UL16-binding protein 2.*

Results

Data and samples from 18 patients were analyzed. Of these, 16 passed the quality test for RNA samples extracted from pre-RT and post-RT tumor tissues. The median age of the 16 patients was 59.5 years (Table I), and the median follow-up period was 63 months. Sixty-two percent of the patients had stage III disease, as assessed by the International Federation of Gynecology and Obstetrics classification, 2009 (19). Ninety-three percent of the primary tumors were over 40 mm in diameter. The 5-year OS rate was 86.6%, and the 5-year PFS rate was 75.5% (Figure 1), which is consistent with the outcome of a previous benchmark study (20). Thus, these data suggest that the present study cohort is representative of patients with locally advanced cervical cancer treated with definitive RT.

Next, we analyzed expression of 730 immune response-related genes using the nCounter PanCancer Immune Profiling Panel (NanoString Technologies) (8). We found that 182 genes were significantly differentially expressed between the pre-RT and post-RT samples (Figure 2). Among them, cyclin-dependent kinase inhibitor 1 (*CDKN1A*) was significantly up-regulated in post-RT samples, with the fourth-lowest *p*-value ($p=5.6\times10^{-5}$; fold change=1.17). *CDKN1A* encodes p21, which induces cell-cycle arrest in response to ionizing radiation (21). In accordance with this, increased expression of p21 after RT has been reported in clinical cervical cancer specimens (22). Thus, these data suggest the technical robustness of the assays

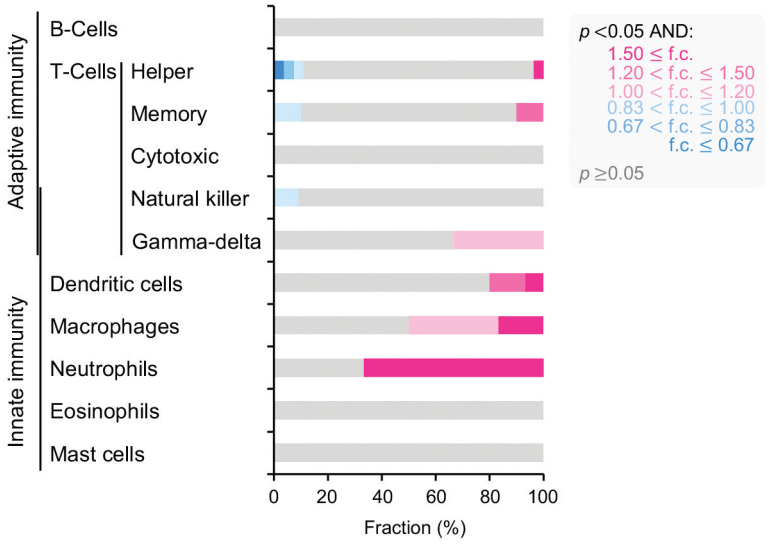

Figure 3. *Postradiotherapy changes in the expression of immune cell type-specific genes (n=97). The values indicate the fraction of genes in a given cell type whose level was significantly altered post-RT. p-Values were assessed using a paired t-test. f.c.: Fold change.*

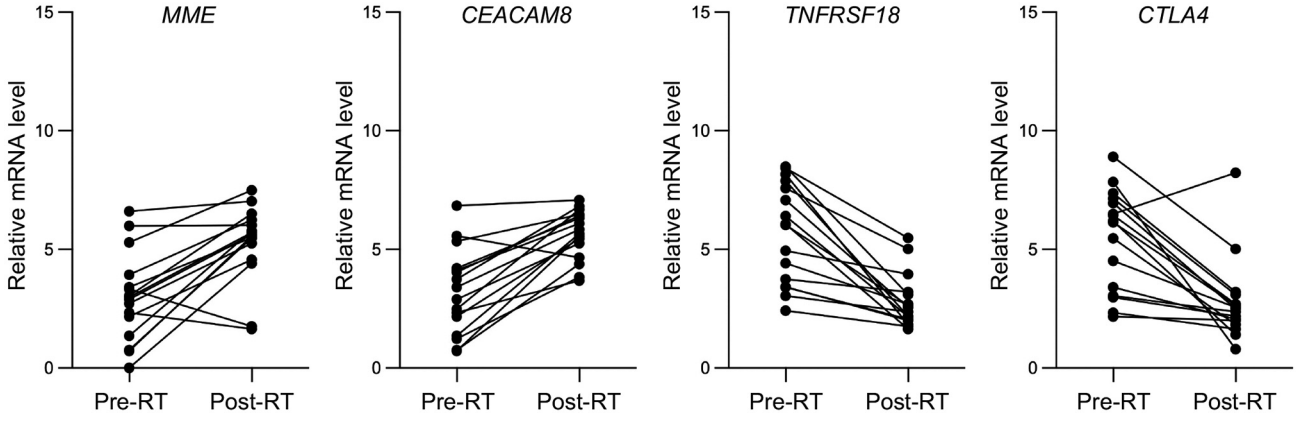

Figure 4. *Change in expression of four genes, namely CEA cell adhesion molecule 8 (CEACAM8), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), membrane metalloendopeptidase (MME) and tumor necrosis factor receptor superfamily member 18 (TNFRSF18), with q<0.05 (by Benjamini–Hochberg method) and a >1.5-fold or <0.66-fold change between pre- and post-radiotherapy (RT) values in individual patients.*

performed in the present study. Of the 182 differentially expressed genes, 41 showed a >1.5-fold or <0.66-fold change in expression; in the post-RT samples, 28 and 13 genes were up-regulated and down-regulated, respectively (Table II).

Next, we analyzed immune cell types activated by RT, focusing on 97 population-specific genes included in the panel (23). Interestingly, we found evident up-regulation of population-specific genes corresponding to neutrophils (66.6%, 2/3 genes), macrophages (50.0%, 3/6 genes), and dendritic cells (20.2%, 3/15 genes), whereas no genes corresponding to these cell types were down-regulated (Figure 3). These data indicate that RT induces innate immune responses in cervical cancer tissues.

To further detect genes that showed robust changes in expression in response to RT, we analyzed the expression data using the Benjamini–Hochberg method (18). As a result, four genes showed $q<0.05$ and >1.5-fold or <0.66-fold change (Table II). Notably, cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), a negative regulator of T-cell activation, was markedly down-regulated in the post-RT samples; down-regulation was observed in 15 out of 16 patients, with an average 2.0-fold change (Figure 4). Nevertheless, we were not able to identify clinical or genetic factors associated with either steady-state expression or down-regulation of *CTLA4* (Table III). The influence of *CTLA4* status on treatment outcomes was not assessable due

Table III. *Association of expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA4) with clinical and genetic factors.*

	p-Value		
Factor	Pre-RT	Post-RT	Fold change
Age	0.74	0.20	0.05
FIGO stage (IB+II *vs.* III)	0.11	0.95	0.71
Tumor diameter	0.73	0.11	0.64
Pelvic LN involvement	0.66	0.05	0.50
PALN involvement	0.63	0.42	0.11
Tumor mutational burden	0.18	0.33	0.93
PIK3CA mutations	0.21	0.87	0.71
ARID1A mutations	0.40	>0.99	0.17
NOTCH1 mutations	0.79	0.57	0.15
FBXW7 mutations	0.91	0.91	>0.99

ARID1A: AT-rich interaction domain 1A; *FBXW7*: F-box and WD repeat domain-containing 7; FIGO: International Federation of Gynecology and Obstetrics 2009; LN: lymph node; *NOTCH1*: notch receptor 1; PALN: para-aortic lymph node; *PIK3CA*: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; RT: radiotherapy. *p*-Values were calculated using the Mann-Whitney *U*-test (age, lymph node involvement, and mutational status) or those using Spearman's rank-sum test (other factors).

to the small number of events (*i.e.* two events for OS and four events for PFS). In addition, no clinical or genetic factors were found to be associated with the kinetics of membrane metalloendopeptidase (*MME*), CEA cell adhesion molecule 8 (*CEACAM8*), or tumor necrosis factor receptor superfamily member 18 (*TNFRSF18*).

Discussion

Here, we describe comprehensive profiling of immune response-related genes, which identified a significant decrease in *CTLA4* expression in cervical cancer tissues post-RT. To the best of our knowledge, this is the first report to demonstrate down-regulation of *CTLA4* in response to RT in clinical cervical cancer tissues. In addition, the results suggest up-regulation of *MME* and *CEACAM8*, and down-regulation of *TNFRSF18*, in response to RT.

CTLA4, which is expressed constitutively by effector regulatory T-cells (Tregs), is a member of the CD28 family of receptors that binds competitively to CD80/86 (24-26). Therefore, the present data suggest that RT reduces the number of Tregs, or suppresses the activity of Tregs, in cervical cancer tissues. Consistent with the present data, exposure of isolated human T-cells to 10 Gy irradiation reduced CTLA4 expression by $CD4^+$ Tregs (27). Importantly, Tregs surviving 10 Gy irradiation had a weakened ability to suppress $CD8^+$ T-cells. Similarly, Takenaka *et al.* reported that a single dose of 10 Gy X-rays, or 24 Gy delivered in three fractions, reduced Treg numbers in tumor tissue in a tumor transplant mouse model (28). These studies suggest that our results may be due to a reduction of CTLA-4 expressing Tregs in response to X-rays. Reduced expression of CTLA4 may be an immune activation response to RT (29).

Changes in expression of three other genes in response to RT were also observed. MME, also known as CD10, is expressed by normal and pathological tissues, including normal cervix stroma and endometriosis (30, 31). The effect of RT on $CD10^+$ cells in cervical cancer tissues, and its involvement in prognosis, is unknown. A previous study on a head and neck squamous cell carcinoma cell line suggests that $CD10^+$ cells are radioresistant; therefore, the increase in MME expression observed herein may reflect an RT-induced decrease in $CD10^-$ cells, and relatively high survival of $CD10^+$ cells (32). CEACAM8 is also known as CD66b. A previous immunohistochemistry study reported that CD66b was expressed by tumor-associated neutrophils and was observed in 78.8% of cervical cancer samples (33). Intratumoral density of $CD66b^+$ cells in cervical cancer specimens affects the prognosis of patients who received RT or surgery, but the underlying mechanism remains unclear (33, 34). TNFRSF18 is a co-stimulatory factor expressed by various cells, including T-cells, natural killer cells, and neutrophils (35). Taken together, the results reported herein are consistent with the post-RT kinetics of immune-related genes in cancer tissues. However, a recent study showed that irradiation with Cs-137 induced TNFRSF18 expression by human mesothelioma cell lines (36). Interestingly, sarcomatoid mesothelioma cell lines expressing the ligand of TNFRSF18 show radioresistance. In the present study, *TNFRSF18* mRNA levels decreased significantly in samples exposed to 10 Gy RT, which may reflect lymphocyte depletion rather than *TNFRSF18* up-regulation or a relative increase in the number of radioresistant T-cells. Changes in TNFRSF18 expression in cervical cancer tissues after RT should be addressed in the future.

This study has some limitations: (i) Evaluation was conducted at only two time points (pretreatment and after exposure to 10 Gy in five fractions), and (ii) the entire cervical cancer tissue was analyzed. Although we found up-regulation of innate immune responses at 10 Gy, it is possible that induction of adaptive immunity may be observed later. While the present analysis of whole tissue allowed us to evaluate the actual response of the tissue to 10 Gy irradiation, additional analysis of cells isolated from the same tissues may be valuable for detecting changes in mRNA expression in a more detailed cell population *e.g.*, such analyses can distinguish Tregs from other CTLA4 activated T-cells (37). In addition, it would be useful to perform nCounter analysis on a larger number of patient samples to clarify the prognostic impact. Lastly, other than CTLA4, we were not able to investigate the genes whose expression was significantly changed. This was due to the

small sample size, *i.e.*, detailed statistical analysis on those genes might have increased the risk of false discovery. Validation of the results of this study with larger cohort is warranted.

In summary, we demonstrate for the first time the down-regulation of *CTLA4* in cervical cancer tissues in the clinical setting of RT, indicating a potential role of RT in the induction of an antitumor immune response in these tumors.

Conflicts of Interest

All Authors declare no conflicts of interest.

Authors' Contributions

AI analyzed the data, performed the literature review, and wrote the article. YY collected clinical samples and performed the experiments. T. Oike analyzed the data and reviewed the article. HS finalized the data and article. KA managed clinical sample collection. T. Ohno supervised the entire project and acquired funding. All Authors read and agreed to publication of the article

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