A study on programmed death ligand 1 expression after radiation in colorectal cancer human tissue and in vitro cell line model

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A study on programmed death ligand 1 expression after radiation in colorectal cancer human tissue and in vitro cell line model

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ABSTRACT

A study on programmed death ligand 1 expression after radiation in colorectal cancer human tissue and in vitro cell line model

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Purpose: The aim of this study was to evaluate the programmed death-ligand 1 (PD-L1) expression status before and after radiation in colorectal cancer human tissue and in vitro cell line model.

Materials and methods: In this study, formalin-fixed paraffin-embedded tissue specimens from pre-operative biopsy via sigmoidoscopy and surgical resection of the primary tumor were obtained from 24 rectal adenocarcinoma patients who underwent neoadjuvant CRT between August 2016 and December 2017. Pre- and post-RT PD-L1 expression level on tumor cell and immune cell was assessed by immunohistochemical (IHC) analysis. The human colorectal cancer cell lines, DLD 1, HT-29, and HCT116 were exposed to graded doses of radiation 2, 4, and 8 Gy for 24 h and PD-L1 expression was analyzed using fluorescence-activated cell sorting

analysis.

Results: Patients were classified, according to their tumor regression grade, as responders (grade 2; 9 patients, 37.5%) and non-responders (grade 3, 4, or 5; 15 patients, 62.5%). In the non-responder group, low PD-L1 expression in tumor cells was observed in 13 patients, whereas the reverse pattern was noted in 7 patients (53.9%) after CRT (McNemar test, p = 0.034). There were more patients with advanced T stage in the non-responder group (p = 0.033), and the relative risk of responder after CRT was 2.99 times higher in patients with microsatellite instability (MSI)-high than in patients with microsatellite stable or MSI-low status (relative risk = 2.99, 95% CI = 1.510–5.921). After irradiation at 8 Gy, PD-L1 expression increased 1.5- and 1.7-fold in DLD-1 and HCT-116 cells, respectively. There was no significant change in the low basal level of PD-L1 expression in HT-29 cells.

Conclusion: This prospective study verified the CRT-induced immune-oncologic shift toward increased PD-L1 expression in tumor cells and provided the theoretical framework for a combined treatment strategy that involves CRT with immunomodulation followed by administering an immune checkpoint inhibitor for locally advanced rectal cancer.

Key words: rectal neoplasm, chemoradiation therapy, tumor regression, programmed death-ligand

Programmed death ligand 1 expression after radiation in colorectal cancer human tissue and in vitro cell line model

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I. INTRODUCTION

Preoperative chemoradiation therapy (CRT) is commonly performed for locally advanced rectal cancers in the multimodal era and associated with tumor downstaging, increased sphincter-preservation rate, and improved local control[1-4]. Following CRT, tumor response is influenced by genetic change and epigenetic processes in the tumor and microenvironment with tumor-host interactions[5-7].

CRT delivers ionizing radiation directly to target cells, where the intention is to cause genetic damage, such as radiation-induced DNA double-strand breaks, which are repaired through double-strand break repair mechanisms. In addition to the direct cytotoxic effect of radiotherapy, recent evidence suggests that induced immune-oncologic changes may play an important role in multimodal cancer treatment. These changes may include increased neo-antigen levels, activation of the major

histocompatibility class I system, activation of tumor-infiltrating lymphocytes, and abscopal effect, where localized radiation provokes systemic antitumor effects[8-11].

Immune checkpoints are regulators against antitumor immunity in the tumor microenvironment and play a significant role in inhibiting T-cell-mediated immune response[12,13]. Among various kinds of immune checkpoints, programmed cell death ligand-1 (PD-L1) suppresses the cytotoxicity of CD8-positive T cells, resulting in immune escape and adverse prognosis in several malignancies although the prognostic impact of PD‑L1 expression in colorectal cancer cells is less explicit[14-20]. Furthermore, there have been few studies on PD-L1 expression in rectal cancer cells before and after preoperative CRT. This study aimed to investigate the PD-L1 expression status before and after neoadjuvant CRT in patients with locally advanced rectal cancer and colorectal cell lines.

II. MATERIALS AND METHODS

1. Patients

Tissue specimens, from pre-operative biopsy via sigmoidoscopy and surgical resection of the primary tumor, were obtained from 24 rectal adenocarcinoma patients who underwent neoadjuvant concurrent chemoradiation therapy between August 2016 and December 2017 (Figure 1). Cases of stage 2 to 3 (T3 or T4 and/or node

positive) rectal adenocarcinoma were included. Exclusion criteria were stage 4 tumors, cancer related to familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer, synchronous or previous malignancies, distant metastasis during neoadjuvant CRT, patient refusal of surgery or loss to follow-up, and complete response of tumor. Twenty-four patients meeting these criteria were evaluated.

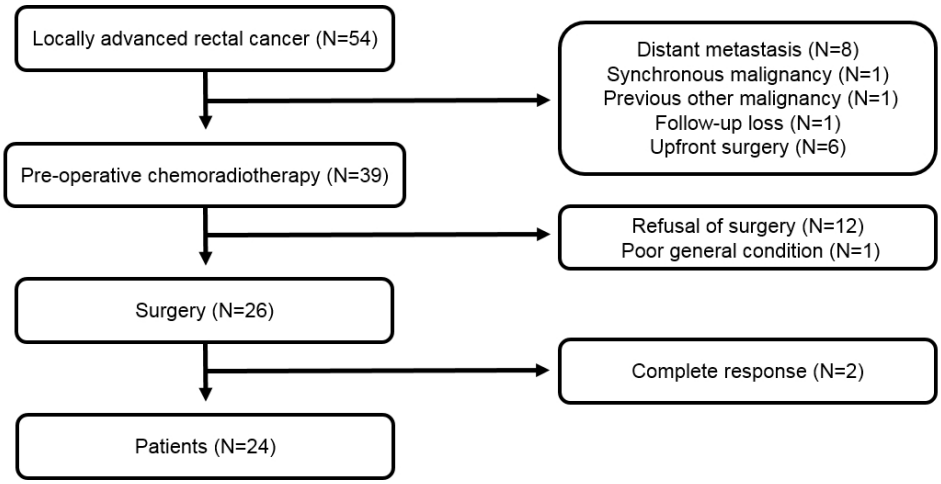

Figure 1. Flow chart of the study

These patients received conventional long-course neoadjuvant CRT (two cycles of fluorouracil-based chemotherapy and 50.4 Gy). All patients underwent a staging

abdomino-pelvic and chest computed tomography scan, rectal magnetic resonance imaging, colonoscopy, biopsy, and positron emission tomography scans. Total mesorectal excision was performed within 6–8 weeks of the final dose of CRT.

2. Evaluation parameters

Patient age, sex, body mass index, tumor location, and preoperative carcinoembryonic antigen (CEA) status were recorded. Additionally, the following factors were evaluated: American Joint Committee on Cancer tumor, node, metastases (TNM) classification; histology; tumor budding; lymphovascular invasion; perineural invasion; extranodal extension; and K-ras and BRAF mutations. Primary tumor regression grade (TRG) was defined according to the Mandard regression grading system [26]. Patients were divided into two groups based on TRG: responders (TRG2) and non-responders (TRG3/4/5).

3. Evaluation of PD-L1 expression by immunohistochemistry (IHC)

PD-L1 expression level was assessed by IHC analysis. All human primary rectal cancer tissue samples were formalin-fixed paraffin-embedded. We built sets of tissue microarrays from biopsy and surgical specimens of rectal cancer tissue for IHC. PD-L1 staining was performed using a BenchMark ULTRA

automated staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA), according to the manufacturer's protocol, and with rabbit polyclonal PD-L1 (1:400, AnaSpec, Fremont, CA, USA) as a primary antibody. Bound antibodies were visualized using the UltraView Universal DAB Detection Kit (Ventana Medical Systems, Inc., Tucson, AZ, USA). Slides were counterstained with Mayer's hematoxylin. Tissues from placenta and tonsil, known to have high PD-L1 expression, were used as an external positive control. Slides incubated without primary antibody were used as a negative control. Positive and negative controls stained appropriately.

Cytoplasmic staining of PD-L1 was considered positive, and expression in both tumor and immune cells was scored according to staining intensity, from 1 to 3, as follows: 1, weak; 2, moderate; and 3, strong staining. Representative examples of immunostaining are shown in Figure 2.

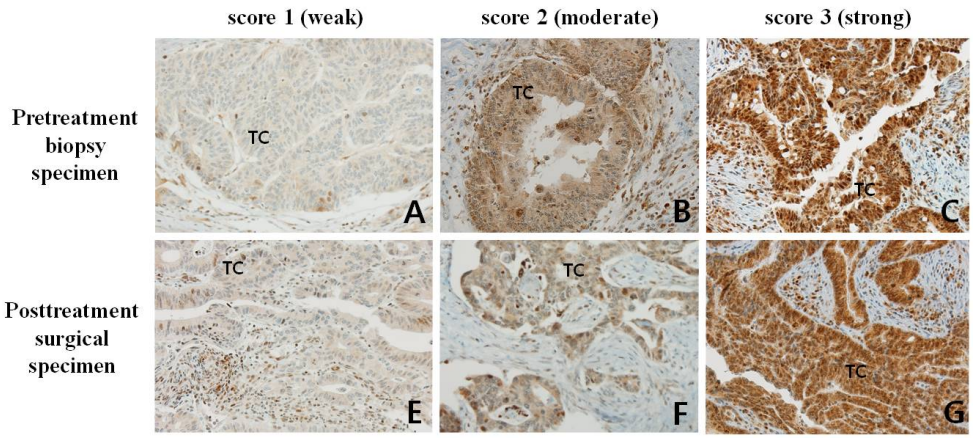

Figure 2. Immunohistochemical analysis of PD-L1 expression in colorectal tissues. PD-L1 staining intensity was divided into low (scores 1 and 2) and high (score 3) expression groups for statistical analysis. (TC, tumor cell)

4. Cell culture

The human colorectal cancer cell lines, DLD 1, HT-29, and HCT116 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; ATCC), 2 mM glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and were maintained in humidified 37 °C, 5% CO_2 incubators.

5. Evaluation of PD-L1 expression in cell lines by immunoblotting

Cells were resuspended in RIPA lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, plus proteinase inhibitors) and incubated on ice for 10 min, after which non-soluble material was eliminated by centrifugation. Total protein concentrations were determined using the Bradford assay. Approximately 50 μg of each protein extract were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Whatman, Thermo Fisher Scientific, Inc,). Non-specific binding was blocked with 5% skimmed milk for 1 h, after which the membranes were incubated with primary antibodies: PD-L1 (1:2,000) and β-actin (1:10,000) overnight at 4 °C. Next, the membranes were incubated with secondary antibodies (1:2,000) for 90 min at room temperature, and protein bands were visualized using a chemiluminescent reagent and exposure to X-ray films.

6. Cellular viability assay after radiation in human colorectal cell lines

Cells were incubated in 6-well plates at 1×105 cells per well with different concentrations of photon irradiation. Cell viability was measured by trypan blue

staining after radiation for 72 h. Both adherent and suspended cells were collected and mixed with 0.4% 2× trypan blue buffer. Cell mixture (10 μL at ~106 cells/mL) was transferred to a hemocytometer under a fluorescence-based, automatic cell counter (Luna-FL™ dual fluorescence cell counting, Logos Biosystems, Annandale, Virginia) to accurately measure the number of cells. Dead cells were stained blue.

7. Evaluation of PD-L1 expression using fluorescence-activated cell sorting (FACS) analysis before and after radiation in human colorectal cell lines

For detection of cell surface PD-L1, cells were suspended in 100 μL cell staining buffer (2% FBS in PBS) and incubated with FITC-labeled anti-human CD274 (PD-L1) antibody (#558065, BD Biosciences) at room temperature for 30 min. After washing in the staining buffer, cells were analyzed using a NovoCyte flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) and NovoExpress software. Cells were exposed to graded doses of radiation 0, 2, 4, and 8 Gy for 24 h and PD-L1 expression was analyzed using fluorescence-activated cell sorting analysis.

8. Microsatellite instability (MSI) test

MSI status was evaluated on DNA extracted from FFPE tumor and matched normal tissues. Tumor and adjacent normal areas were separately marked and collected.

Genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), and PCR amplification was performed with fluorescent dye-labeled primers targeting five microsatellite markers recommended by the NCI (D17S250, BAT26, D5S346, BAT25, and D2S123). Differences in amplified PCR fragments between normal and tumor tissues were evaluated using the gene analyzer Qsep100 (BiOptic Inc. Taiwan, China). MSI-H tumors were defined as having instability in two or more microsatellite loci, MSI-low tumors as having instability in only one locus, and microsatellite stable (MSS) tumors as showing no apparent instability in accordance with the NCI criteria.

9. Statistical analyses

Differences in clinicopathological features between responders and non-responders, before and after CRT, were analyzed by Fisher's exact tests for categorical variables. PD-L1 expressions and immune cell reaction before and after CRT were evaluated by McNemar test. The relative risk was calculated for evaluation of pre-CRT immune-oncologic markers to predict the tumor response. All statistical tests were analyzed using IBM SPSS Statistics for Windows version 21.0 (IBM Corp., Armonk, NY, USA). P-values < 0.05 indicated significant differences.

III. RESULTS

1. Clinicopathological characteristics of patients

Clinicopathological characteristics of all patients is presented in Table 1. Regarding TRG, 9 (37.5%) patients were categorized as responders and 15 (62.5%) as non-responders. Demographic characteristics were similar between the two cohorts for age, sex, CEA level, nodal stage, TNM stage, body mass index, nodal stage, histology, tumor budding, lymphovascular invasion, perineural invasion, extranodal extension, and KRAS and BRAF mutations. The only demographic characteristic that differed was tumor stage, which was classified as advanced in more patients in the non-responder group ($p = 0.033$) (Table 1).

Table 1. Characteristics of patients with rectal cancer according to tumor regression grade

	All patients (N=24)	Responder (N =9)	Non-responder (N = 15)	p-value
Age (years), n (%)				1.000
≤ 60	9 (37.5)	3 (33.3)	6 (40.0)	
> 60	15 (62.5)	6 (66.7)	9 (60.0)	

Sex, n (%)				1.000
Male	20 (83.3.)	8 (88.9)	12 (80.0)	
Female	4 (16.7)	1 (11.1)	3 (20.0)	
BMI (kg/m^2), n (%)				1.000
≤ 23	9 (37.5)	3 (33.3)	6 (40.0)	
> 23	15 (62.5)	6 (66.7)	9 (60.0)	
Preoperative CEA (ng/mL)				1.000
≤ 5	19 (79.2)	7 (77.8)	12 (80.0)	
> 5	5 (20.8)	2 (22.2)	3 (20.0)	
Tumor stage, n (%)				0.033
T 0, 1, 2	14 (58.3)	8 (88.9)	6 (40.0)	
T 3	10 (41.7)	1 (11.1)	9 (60.0)	
Nodal stage, n (%)				1.000
N0	18 (75.0)	7 (77.8)	11 (73.3)	
N1,2	6 (25.0)	2 (22.2)	4 (26.7)	
Histology				0.130
Well and moderately	22 (91.7)	7 (77.8)	15 (100)	
poorly	2 (8.3)	2 (22.2)	0 (0)	
Tumor budding	2 (8.3)	0 (0)	2 (13.3)	0.511
Lymphovascular invasion, n (%)	0 (0)	0 (0)	0 (0)	1.000
Perineural invasion	3 (12.5)	0 (0)	3 (20.0)	0.266
Extranodal extension, n (%)	2 (8.3)	0 (0)	2 (13.3)	0.511

K-ras mutation, n (%)	7 (29.2)	3 (33.3)	4 (26.7)	0.657
BRAF mutation, n (%)	2 (8.3)	1 (11.1)	1 (6.7)	1.000

BMI, body mass index; CEA, carcinoembryonic antigen

2. PD-L1 expression by IHC before and after CRT

In the non-responder group, low PD-L1 expression in tumor cells was evaluated in 13 patients, whereas the reverse pattern was noted in 7 patients (53.9%) after CRT (Table 2) (McNemar test, $p = 0.034$). No significant changes were observed in PD-L1 expression in immune cells in paired samples before and after CRT.

Table 2. PD-L1 expression and intratumoral immune cell reaction before and after CRT

After (N = 20) / Before (N = 24)	Low n (%)	High n (%)	P -value	*Missing sample
PD-L1 expression in tumor cells in all patients			0.058	
Low (weak and moderate)	9 (52.9)	8 (47.1)		3
High (strong)	2 (66.7)	1 (33.3)		1
PD-L1 expression in tumor cells in responders			1.000	
Low	3 (75.0)	1 (25.0)		3
High	1 (100.0)	0 (0.0)		1
PD-L1 expression in tumor cells in non-responders			0.034	
Low	6 (46.1)	7 (53.9)		
High	1 (50.0)	1 (50.0)		
PD-L1 expression in immune cells in all patients			0.248	2
Low (weak and moderate)	4 (33.3)	8 (66.7)		2
High (strong)	4 (50.0)	4 (50.0)		
PD-L1 expression in immune cells in responders			0.564	
Low	1 (50.0)	1 (50.0)		2
High	2 (66.7)	1 (33.3)		2

PD-L1 expression in immune cells in non-responders			
Low	3 (30.0)	7 (70.0)	0.096
High	2 (40.0)	3 (60.0)	

3. Cellular viability assay after radiation in human colorectal cell lines

We examined colon cancer cell lines 72 h after radiation (8 Gy) and compared the shape with that observed in pre-radiation samples. Growth inhibition was observed in all three cell lines, although HCT-116 cells were more sensitive to radiation than DLD-1 or HT-29 cells (Figure 2). To further assess the impact of radiation on cell viability, cell counts were performed on colon cancer cell lines 72 h after exposure to 0, 2, 4, and 8 Gy radiation. A radiation dose-dependent reduction in cell number was observed in all three cell lines and most pronounced in HCT-116 cells (Figure 3).

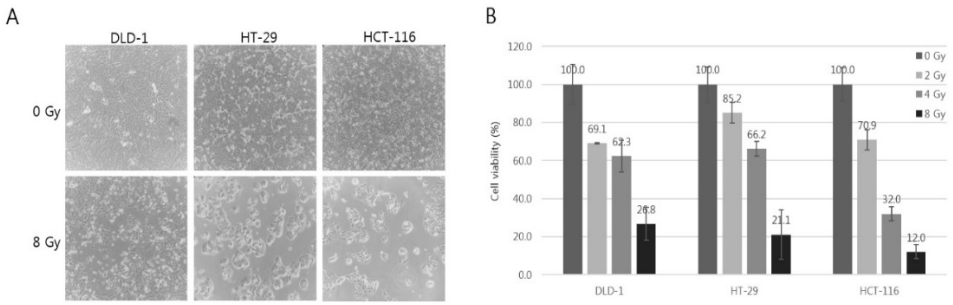

Figure 3. Growth inhibition of colorectal cancer cell lines after irradiation therapy.

(A) Cell morphology of DLD-1, HT-29, and HCT-116 cells was observed under a phase- contrast microscopy (×100). (B) Colorectal cancer cell lines were irradiated for 72 h, followed by irradiation at 0, 2, 4, and 8 Gy and then analyzed by cell viability assay.

4. PD-L1 expression using fluorescence-activated cell sorting analysis before and after radiation in human colorectal cell lines

We tested the basal PD-L1 level of the three colon cancer cell lines by immunoblotting and found that basal PD-L1 expression in DLD-1 and HCT-116 cells was higher than in HT-29 cells (Figure 4). We exposed each of the cell lines to 0, 2, 4, and 8 Gy radiation for 24 h and then stayed with PD-L1 after 4 h of incubation to measure PD-L1 in the cell through flow cytometry. Following 8 Gy radiation, PD-L1 levels were 1.5- and 1.7-fold higher in DLD-1 and HCT-116 cells, respectively. PD-L1 expression remained unchanged in irradiated HT-29 cells (Figure 5).

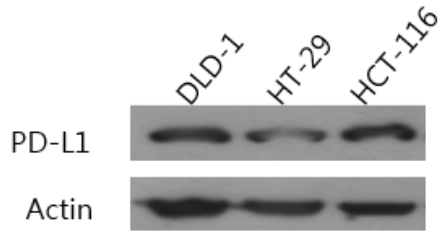

Figure 4. Basal level of PD-L1 expression in colorectal cancer cell lines as determined by immunoblot. The PD-L1 expression level appeared to be higher in DLD-1 and HCT116 than in HT-29.

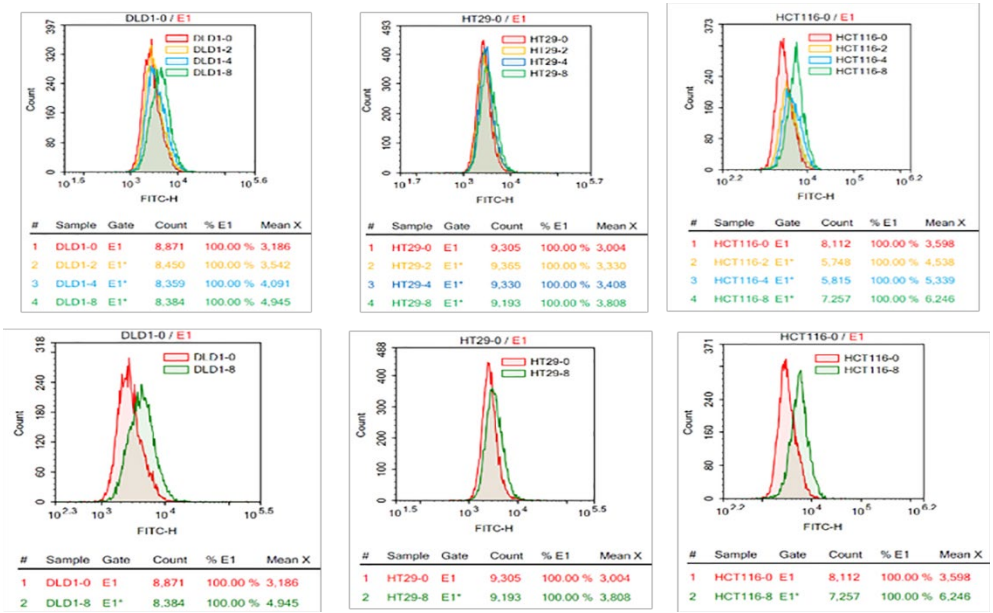

Figure 5. PD-L1 expression using fluorescence-activated cell sorting analysis after stimulation with irradiation colorectal cancer cell lines. Cells were exposed to graded doses of radiation 0, 2, 4, and 8 Gy for 24 h and PD-L1 expression was increased all three cell lines.

5. PD-L1 expression, immune cell reaction, and MSI according to tumor regression grade.

There were no significant differences between TRG categories in pre- and post-CRT PD-L1 expression in tumor and immune cells by IHC, pre- and post-CRT immune cell reaction, and pre- and post-CRT MSI status (Table 3). However, both patients with MSI-H status were classified as responders, and there were no patients with pre-CRT MSI-H status in the non-responder group (relative risk = 2.99, 95% CI = 1.510–5.921).

Table 3. PD-L1 expression, intra-tumoral immune cell reaction, and MSI status according to tumor regression grade

	Responder (N =9)	Non-responder (N = 15)	p-value	Relative risk	95% CI
Pre-CRT PD-L1 expression on tumor, n (%)			0.615		
Low	7 (77.8)	13 (86.7)		0.700	0.222-2.206
High	2 (22.2)	2 (13.3)		1	
Pre-CRT PD-L1 expression on immune cell, n (%)			0.403		
Low	4 (44.4)	10 (66.7)		0.571	0.203-1.608
High	5 (55.6)	5 (33.3)		1	
Pre-CRT intra-tumoral immune cell reaction, n (%)			0.403		
No and mild	4 (44.4)	10 (66.7)		0.571	0.203-1.608
Moderate and severe	5 (55.6)	5 (33.3)		1	
Pre-CRT MSI status, n (%)			0.130		
MSI-high	2 (22.2)	0 (0)		3.143	1.705-5.794
MSS-low and MSS	7 (77.8)	15 (100.0)		1	
Post-CRT PD-L1 expression on tumor, n (%)			0.319		
Low	4 (80.0)	7 (46.7)			
High	1 (20.0)	8 (53.3)			

Post-CRT PD-L1 expression on immune cell, n (%)			0.347
Low	3 (60.0)	5 (33.3)	
High	2 (40.0)	10 (66.7)	
Post-CRT peri-tumoral immune cell reaction, n (%)			0.266
No and mild	9 (100.0)	12 (80.0)	
Moderate and severe	0 (0)	3 (20.0)	
Post-CRT MSI status, n (%)			0.348
MSI high	1 (11.1)	0 (0)	
MSS-low and MSS	8 (88.9)	15 (100.0)	

PD-L1, programmed death-ligand 1; CRT, chemoradiotherapy; MSI, microsatellite instability; MSS, microsatellite stable

IV. DISCUSSION

CRT delivers ionizing radiation to target malignant cells, where the aim is to induce cell death by imposing a variety of genetic damage[22]. CRT also causes damage to immunogenic cell death by stimulating damaged tumor cells to release danger-associated molecular patterns that boost immune response[10,23]. Some studies have demonstrated that irradiation on cells has the following impact on the immune system: an increase in neo-antigen level, activation of major histocompatibility complex molecules, and activation of tumor-infiltrating lymphocytes, which affect the response of immune checkpoint inhibitors[11,24]. Chiang et al.[25] demonstrated that

neoadjuvant CRT triggered PD-L1 upregulation in tumor cells in patients with locally advanced rectal cancer. Moreover, they reported that IFN-γ was released from cancer cells after radiation using ELISA and therefore suggested that radiation induced IFN-γ production, leading to increased PD-L1 expression in tumor cells. In the present study, low PD-L1 expression in tumor cells was observed in 13 patients in the non-responder group, whereas the reverse pattern was noted in 7 patients (53.9%) after CRT (Table 2) (McNemar test, $p = 0.034$). We believe that antigenicity with neo-antigen was more increased and the immune system was more activated in the non-responder group, which can, hence, be used as a good candidate of combined treatment with immune checkpoint inhibitor.

Saigusa et al.[26] analyzed the change in PD-L1 gene expression between pre- and post-radiation in colon cancer cell lines using quantitative real-time PCR and reported that irradiation decreased PD-L1 gene expression in multiple cell lines. Conversely, Chiang et al.[25] demonstrated, using immunoblotting, that PD-L1 expression was significantly upregulated by radiation in cell culture. In our study, increased PD-L1 expression was observed when analyzed using FACS analysis in two of three cell lines tested. Further research using material with tumor microenviornment, such as organoid and patient derived xenograft models, is still needed.

Since PD-L1 can provide a mechanism of immune tolerance and escape through suppression of the CD8 cytotoxic immune response, the combination of neoadjuvant CRT and inhibition of PD-L1 expression may synergistically enhance tumor response.

Recently, some authors have reported that a combination of radiation and PD-1/PD-L1 blockade synergistically inhibited tumor growth in animal models with an "abscopal effect," i.e., tumor control in distant contralateral non-irradiated tumors[8,10]. Additionally, several clinical outcomes in bladder, lung, and esophageal cancer also support the synergistic effects of CRT and immune checkpoint inhibitors[9,27,28]. In the present study, we showed an increase in the proportion of high PD-L1 expression in tumor cells, especially in the non-responder group. Considering the sequence of combined treatments, CRT with immunomodulation followed by an immune checkpoint inhibitor may offer a reasonable combined treatment strategy for locally advanced rectal cancer.

Approximately 12% to 15% of colorectal carcinomas are MSI-high tumors. These tumors have some characteristics, including higher numbers of tumor-infiltrated lymphocytes, more favorable prognosis compared to that of mismatch repair (MMR)-proficient tumors, right-sided location, and poorly differentiated histology. Previous studies have reported that MMR proteins are associated with various aspects of DNA metabolism, including not only DNA MMR but also DNA damage response with double-strand break repair29,30. However, the relationship between MSI status and response to CRT in rectal cancer is not well known. Previous studies demonstrated that MSI cancers may exhibit radiosensitivity due to possible direct effects of MMR proteins on DNA damage repair in response to ionizing radiation, affecting cell cycle arrest, double-strand break repair, and apoptosis, although there is some controversy regarding this issue[22,31-34]. In this study, both patients with MSI-high status were classified as responders, and there was no patient with pre-CRT MSI-high status

classified as non-responder, where the relative risk for prediction as a responder was 2.99. Therefore, we believe that MSI-high status might be used as a predictive biomarker of good response to CRT for locally advanced rectal cancer.

Our study has several limitations, including the small number of patients; lack of investigation on immune-oncologic biomarkers, such as IFN-γ, TGF-β, and various immune cell populations; and lack of in vivo models, such as animal or organoid models that preserve the TME. Therefore, further studies are needed to investigate the diverse immune-oncologic changes after CRT in patients with locally advanced rectal cancer. Another limitation is that the tumor areas where PD-L1 expression was observed before and after radiotherapy were different. Although it is better to compare the mucosal surface of the same patient before and after CRT, this is not possible with real patient samples. Previous studies have shown that PD-L1 expression tends to be greater in the tumor invasive front, especially in colorectal cancer35,36. Therefore, despite this bias, we believe that the reduction in PD-L1 expression is a meaningful result.

V. CONCLUSION

This prospective study verified the chemoradiation-induced immune-oncologic shift toward increased PD-L1 expression in tumor cells, and_provided the theoretical rationale for combined treatment strategy, CRT with immunomodulation followed by an immune checkpoint inhibitor for locally advanced rectal cancer.

REFERENCES

1.Minsky BD, Cohen AM, Kemeny N, et al. The efficacy of preoperative 5-fluorouracil, high-dose leucovorin, and sequential radiation therapy for unresectable rectal cancer. Cancer. 1993;71:3486-3492.

2.van Gijn W, Marijnen CA, Nagtegaal ID, et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. Lancet Oncol. 2011;12:575-582.

3.Lim DR, Bae SU, Hur H, et al. Long-term oncological outcomes of robotic versus laparoscopic total mesorectal excision of mid-low rectal cancer following neoadjuvant chemoradiation therapy. Surg Endosc. 2017;31:1728-1737.

4.Kim NK, Kim MS, Al-Asari SF. Update and debate issues in surgical treatment of middle and low rectal cancer. J Korean Soc Coloproctol. 2012;28:230-240.

5.Liersch T, Grade M, Gaedcke J, et al. Preoperative chemoradiotherapy in locally advanced rectal cancer: correlation of a gene expression-based response signature with recurrence. Cancer Genet Cytogenet. 2009;190:57-65.

6.Williamson JS, Harris DA, Beynon J, Jenkins GJ. Review of the development of DNA methylation as a marker of response to neoadjuvant therapy and outcomes in rectal cancer. Clin Epigenetics. 2015;7:70.

7.Lee YJ, Kim WR, Han J, et al. Prognostic Impact of Immunonutritional Status Changes During Preoperative Chemoradiation in Patients With Rectal Cancer. Ann

Coloproctol. 2016;32:208-214.

8.Demaria S, Ng B, Devitt ML, et al. Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys. 2004;58:862-870.

9.Vatner RE, Cooper BT, Vanpouille-Box C, Demaria S, Formenti SC. Combinations of immunotherapy and radiation in cancer therapy. Front Oncol. 2014;4:325.

10.Wennerberg E, Vanpouille-Box C, Bornstein S, Yamazaki T, Demaria S, Galluzzi L. Immune recognition of irradiated cancer cells. Immunol Rev. 2017;280:220-230.

11.Kalbasi A, June CH, Haas N, Vapiwala N. Radiation and immunotherapy: a synergistic combination. J Clin Invest. 2013;123:2756-2763.

12.Mlecnik B, Bindea G, Angell HK, et al. Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. Immunity. 2016;44:698-711.

13.Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27:450-461.

14.Ostrand-Rosenberg S, Horn LA, Haile ST. The programmed death-1 immune-suppressive pathway: barrier to antitumor immunity. J Immunol. 2014;193:3835-3841.

15.Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell. 2015;160:48-61.

16.Hirano F, Kaneko K, Tamura H, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res. 2005;65:1089-1096.

17.Wu P, Wu D, Li L, Chai Y, Huang J. PD-L1 and Survival in Solid Tumors: A Meta-Analysis. PLoS One. 2015;10:e0131403.

18.Droeser RA, Hirt C, Viehl CT, et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer. 2013;49:2233-2242.

19.Song M, Chen D, Lu B, et al. PTEN loss increases PD-L1 protein expression and affects the correlation between PD-L1 expression and clinical parameters in colorectal cancer. PLoS One. 2013;8:e65821.

20.Park J-S, Baek J-H, Lee W-S, et al. Long-term oncologic outcomes in pathologic tumor response after neoadjuvant chemoradiation for locally advanced rectal cancer. Korean J Clin Oncol. 2018;14:37-42.

21.Mandard AM, Dalibard F, Mandard JC, et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. Cancer. 1994;73:2680-2686.

22.Shin JS, Tut TG, Yang T, Lee CS. Radiotherapy response in microsatellite instability related rectal cancer. Korean J Pathol. 2013;47:1-8.

23.Showalter A, Limaye A, Oyer JL, et al. Cytokines in immunogenic cell death: Applications for cancer immunotherapy. Cytokine. 2017;97:123-132.

24.Eckert F, Gaipl US, Niedermann G, et al. Beyond checkpoint inhibition -

Immunotherapeutical strategies in combination with radiation. Clin Transl Radiat Oncol. 2017;2:29-35.

25.Chiang SF, Huang CY, Ke TW, et al. Upregulation of tumor PD-L1 by neoadjuvant chemoradiotherapy (neoCRT) confers improved survival in patients with lymph node metastasis of locally advanced rectal cancers. Cancer Immunol Immunother. 2018. DOI:10.1007/s00262-018-2275-0.

26.Saigusa S, Toiyama Y, Tanaka K, et al. Implication of programmed cell death ligand 1 expression in tumor recurrence and prognosis in rectal cancer with neoadjuvant chemoradiotherapy. Int J Clin Oncol. 2016;21:946-952.

27.Wu CT, Chen WC, Chang YH, Lin WY, Chen MF. The role of PD-L1 in the radiation response and clinical outcome for bladder cancer. Sci Rep. 2016;6:19740.

28.Lim SH, Hong M, Ahn S, et al. Changes in tumour expression of programmed death-ligand 1 after neoadjuvant concurrent chemoradiotherapy in patients with squamous oesophageal cancer. Eur J Cancer. 2016;52:1-9.

29.Beiner ME, Rosen B, Fyles A, et al. Endometrial cancer risk is associated with variants of the mismatch repair genes MLH1 and MSH2. Cancer Epidemiol Biomarkers Prev. 2006;15:1636-1640.

30.Zhang Y, Rohde LH, Wu H. Involvement of nucleotide excision and mismatch repair mechanisms in double strand break repair. Curr Genomics. 2009;10:250-258.

31.Hasan S, Renz P, Wegner RE, et al. Microsatellite Instability (MSI) as an Independent Predictor of Pathologic Complete Response (PCR) in Locally Advanced

Rectal Cancer: A National Cancer Database (NCDB) Analysis. Ann Surg. 2018. DOI:10.1097/SLA.0000000000003051.

32.Franchitto A, Pichierri P, Piergentili R, Crescenzi M, Bignami M, Palitti F. The mammalian mismatch repair protein MSH2 is required for correct MRE11 and RAD51 relocalization and for efficient cell cycle arrest induced by ionizing radiation in G2 phase. Oncogene. 2003;22:2110-2120.

33.Barwell J, Pangon L, Hodgson S, et al. Biallelic mutation of MSH2 in primary human cells is associated with sensitivity to irradiation and altered RAD51 foci kinetics. J Med Genet. 2007;44:516-520.

34.Charara M, Edmonston TB, Burkholder S, et al. Microsatellite status and cell cycle associated markers in rectal cancer patients undergoing a combined regimen of 5-FU and CPT-11 chemotherapy and radiotherapy. Anticancer Res. 2004;24:3161-3167.

35.Llosa NJ, Cruise M, Tam A, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov. 2015;5:43-51.

36.Marginean EC, Melosky B. Is There a Role for Programmed Death Ligand-1 Testing and Immunotherapy in Colorectal Cancer With Microsatellite Instability? Part II-The Challenge of Programmed Death Ligand-1 Testing and Its Role in Microsatellite Instability-High Colorectal Cancer. Arch Pathol Lab Med. 2018;142:26-34.

ABSTRACT(IN KOREAN)

인체 직장암 조직과 대장암 세포주 모델에서 방사선 조사 전과 후의 세포사멸 수용체-1의 발현에 관한 연구

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목적: 방사선 치료는 직접적으로 종양에 방사선을 가함으로써, 종양의 크기를 줄이고, 종양의 국소 치료 효과를 높인다. 본 연구는 진행성 직장암 환자들의 암조직과 대장암 세포주에서 방사선 치료 조사 전과 후의 세포사멸 수용체-1의 발현에 대해 알아보고자 하였다.

재료 및 방법: 2016년 8월부터 2017년 12월까지 국소성 진행성 직장암으로 진단되어 수술 전 항암방사선 치료를 시행 받은 환자들의 치료 전후 암조직을 채취하여 종양세포 및 면역세포들에 대한 면역조직화학염색을 시행하여 세포사멸 수용체-1 발현의 정도를 분석하였다. 대장암 세포주 DLD 1, HT-29, HCT116에 대하여 2,4,8 Gy의 방사선을 조사한 24시간 후 형광이용세포분류를 이용하여 단일세포 표면의 세포사멸 수용체-1 발현 분석하였다.

결과: 환자들을 종양의 사멸정도에 따라 분류하였을 때 반응군은 37.5%였고 비 반응군는 62.5%였다. 항암방사선 치료 전 비 반응군의 종양에서 낮은 세포사멸 수용체-1 발현을 보였던 13명의 환자 중 7명 (53.9%) 의 환자에서 치료 후 높은 발현으로 역전되었다 (McNemar test, p = 0.034).

방사선 24시간 후 형광이용세포분류 분석에서 DLD-1과 HCT-116 세포주에서는 8 Gy의 방사선 조사 후에 각각 1.5배와 1.7배로 세포사멸 수용체-1의 발현량이 증가하였으나, HT-29 세포주에서는 변화가 없었다. 방사선 치료 전 후의 종양 및 염증세포에서 높은 세포사멸 수용체-1 발현군이 차지하는 비율과 염증반응 정도가 종양사멸 정도에 따라 통계학적으로 유의한 차이를 보이지 않았다.

결론: 본 연구는 항암방사선 치료 후에 종양-면역학적으로 종양세포에서의 세포사멸 수용체-1가 증가한다는 것을 보여주었으며, 이것은 방사선에 의한 면역조절과 관련이 있는 것으로 사료되며 국소성 진행성 직장암의 치료전략에 있어서 방사선에 의한 종양-면역학적 조절 반응 이후 방사선 치료와 면역관문차단제의 병합요법에 대한 이론적 틀을 제공하였다.

핵심되는 말: 세포사멸 수용체-1, 직장암, 방사선치료, 면역 조절