의학박사 학위논문

# 수술 전 화학방사선 요법을 시행 받은 직장암 환자에서 재발 위험성과 관련된 면역세포 분석

**Immune infiltrates and prediction of recurrence in rectal cancer after preoperative chemoradiation by quantitative multiplexed immunohistochemistry**

울산대학교대학원

의 학 과

이서현

# 수술 전 화학방사선 요법을 시행 받은 직장암 환자에서 재발 위험성과 관련된 면역세포 분석

지도교수 명 승 재

이 논문을 의학박사학위 논문으로 제출함

2017 년 12 월

울산대학교대학원
의 학 과
이 서 현

이서현의 의학박사학위 논문을 인준함

심사위원장　김상엽　인

심사위원　명승재　인

심사위원　박인자　인

심사위원　홍승모　인

심사위원　차재명　인

울 산 대 학 교 대 학 원

2017 년 12 월

# ABSTRACT

**Purpose:** The aim of this study was to evaluate the prognostic value of tumor-infiltrating lymphocytes (TILs) and checkpoint molecules and their overlapped expression in surgical specimens of rectal cancer from patients who received preoperative chemoradiotherapy (PCRT).

**Methods:** A total of 27 rectal cancer patients with pathological stage II and III were included in this study. We divided the patients into two groups and enrolled only the corresponding patients. The recurrence group was defined as having tumor recurrence within one year, and the non-recurrence group was defined as showing no evidence of tumor recurrence for over three years. We stained formalin-fixed paraffin-embedded slides of surgically resected specimen using multispectral fluorescent immunohistochemistry with a panel including CK, CD3, CD4, CD8, FOXP3, PD-L1, and DAPI. We evaluated tumor microenvironment by visualization and quantification of multiple immune cell types with Vectra 3.0

Automated Quantitative Pathology Imaging System, the inForm software and TIBCO Spotfire (Perkin-Elmer, Waltham, MA).

**Results:** Both in tumor cells and in stromal cells, the densities of CD3+ and CD8+T lymphocytes were significantly higher in the non-recurrence group than in the recurrence group. The proportions and the densities of $CD3^+CD8^+$, $CD3^+CD8^+PD\text{-}L1^-$, and $CD3^+CD8^+PD\text{-}L1^-FOXP3^-$ were significantly greater in the non-recurrence group ($p$ = 0.0013, $p$ = 0.00075 ,and $p$ = 0.00081, respectively). The $CD8^+$ proportion and $CD3^+CD8^+PD\text{-}L1^-$ proportion showed the highest predictability of recurrence ($p < 0.001$). Low $CD3^+CD8^+$ co-expression ($p$ = 0.003) and low $CD3^+CD8^+PD\text{-}L1^-$ co-expression ($p$ = 0.020) were significantly associated with poor recurrence free survival regardless of cytokeratin status both in tumor cells and in stromal cells.

**Conclusion:** Immunological cell expression of rectal cancer after PCRT has prognostic value to predict the recurrence after surgery. Precision medical care

with individualized surveillance may benefit the patients to prevent the recurrence in rectal cancer.

**Keywords:** Rectal cancer, chemoradiotherapy, recurrence, Tumor-infiltrating lymphocytes, Immune markers

**AGENDA**

## Figure Agenda

## INTRODUCTION

Rectal cancer incidence rates are rapidly increasing in areas historically at low risk, including Asia.[1-3] UICC-TNM classification has been shown to be valuable in estimating the outcome of patients.[4] However, this classification provides limited information for prognosis since cancer outcomes can vary significantly among patients within the same histological tumor stage. Accumulating evidence suggests that high levels of tumor-infiltrating lymphocytes (TILs) have been shown to be associated with improved oncological outcomes of colorectal cancer patients. Moreover, the favorable prognosis associated with TILs seems to be independent of tumor stage.[5, 6] An immune classification of tumors was proposed based on an immune score generated by the quantification of two lymphocyte populations (CD3/CD8, CD3/CD45RO, or CD8/CD45RO), in both the core and invasive margin of the tumor, to establish prognosis of clinical outcomes in patients.[7, 8] Interestingly, this immune classification has a prognostic value that may be superior to the AJCC/UICC TNM-classification.[9, 10]

Preoperative chemoradiotherapy (PCRT) may improve local control of locally advanced rectal cancer.[11, 12] This has led to recommendations by the National Comprehensive Cancer Network and the European Society for Medical Oncology

advocating PCRT for stage II and III disease.[13] However, PCRT does not improve survival, and disease recurrence remains the major cause of mortality in rectal cancer patients.[14] Preclinical studies have suggested that cytotoxic agents and radiation may partly deliver their antitumor activities by activating antitumor immune response.[15] The proimmunogenic effects of radiotherapy are known to be counteracted by immunesuppressive ones, for example mediated by induction of TGF-β that suppresses the expression of cytotoxic mediators in CD8 cells, and promotes the generation of regulatory T cells.[16] In a previous study, the number of both $CD4^+$ and $CD8^+$ TIL in pre-PCRT biopsy samples was strongly correlated with tumor reduction ratio evaluated by barium enema.[17] Another study reported that total density of T-cell infiltrates and PD-$L1^+$ cell infiltrates in biopsy samples were main discriminator between patients with total regression and residual disease after PCRT.[18] Other recent studies have demonstrated that radiosensitivity is greatly affected by immune function of the host.[19, 20] However, research focusing on the identification of biomarker-related recurrence after chemoradiation in rectal cancer has not been thoroughly investigated. In addition, most of studies have focused on the immune mechanisms in radiosensitivity. This fact has inspired us to investigate the differences of immune cells and tumor microenvironment in rectal cancer which are resistant to radiation therapy.

In the present study, we aimed to assess the prognostic value of TILs and

checkpoint molecules and their overlapped expression in surgical specimens of rectal cancer from patients who did not achieve complete regression following PCRT.

## METHODS

### Patients

We retrospectively identified locally advanced rectal cancer (pathological stage II and III, based on UICC-TNM classification) patients who received PCRT at an Asan medical center between 2008 and 2015.

Patients were excluded from analysis if they had polyposis syndrome, Lynch syndrome, inflammatory bowel disease, or a positive family history for these diseases. We also excluded patients with a history of cancer other than rectal cancer.

### Pre and post-surgical treatment and follow-up

All patients received concurrent chemoradiation. The median dose of radiation was 50 Gy administered in daily fractions of 1.8 to 2.0 Gy. The most common dose

scheme was 45 Gy administered to the entire pelvis, followed by a 5.4 Gy boost to the primary tumor in a 1.8 Gy daily fraction. All patients received concurrent chemotherapy during radiation therapy. The two most common regimens were oral capecitabine (825 mg/m2) administered twice daily during radiation therapy, or two cycles of a bolus 5-fluorouracil (375 mg/m2/day for three days) with leucovorin during the first and fifth week of radiation therapy. All patients underwent surgical resection, such as total mesorectal excision, 6–8 weeks following the end of preoperative treatment.

Evaluation of pathological tumor response to PCRT was completed using the tumor regression grading system proposed by Dworak et al.[21] Patients with good clinical response after PCRT (total regression or near total regression) were excluded because we could not detect tumor cells on surgical specimens and only fibrotic tissue was present in complete regression patients. Adjuvant chemotherapy with fluoropyrimidine, with or without oxaliplatin, was administered in all study patients. Each patient provided informed consent before the treatment.

After operation, patients received follow-ups approximately every 3–6 months for first 1-2 years, then every 6-12 months until 5 years after surgery. At each follow-up, a complete history, physical examination, and laboratory tests including serum carcinoembryonic antigen (CEA) levels were performed. Chest radiography, and chest, abdominal, and pelvis computerized tomography were performed every 3-6

months. In addition, patients underwent colonoscopy at 6 months to 1 year post operatively, and every 2-3 years thereafter based on the guidelines.[22)]

**Definitions**

Patients were categorized into two groups and enrolled only the corresponding patients. The recurrence group was defined as having tumor recurrence within one year from the operation date, and the non-recurrence group was defined as showing no evidence of tumor recurrence for over three years. Local recurrence was defined as radiological or endoscopic evidence of disease regrowth confined to the regional area around the primary tumor site. Systemic recurrence was defined as a recurrence at any other location, such as systemic lymph node, liver, and lung.[23)] The time until local or systemic recurrence was determined as the interval from the date of surgery to the first date of diagnosis of recurrence (radiological or pathological).[13, 24)] This study was approved by the institutional review board of an Asan medical center; patient informed consent was waived. (IRB No. 2015-1188)

**Multiplexed immunofluorescence**

4-μm sections of rectal cancer-resected specimens were cut from formalin-fixed paraffin-embedded (FFPE) blocks. Slides were heated for at least one hour in a dry oven at 60℃ and dewaxed using xylene, then dehydrated by sequential incubation in 100%, 95%, and 70% ethanol, followed by hydrogen peroxide. Antigen was retrieved by microwave treatment (MWT) for 15 minutes in citrate buffer (pH 6.0).

Slides were washed with 1X TBST two times, and blocking was performed with antibody diluent (ARD1001EA, Perkin-Elmer, USA) for 10 minutes. The first primary antibodies for FOXP3 (236/E7, ab20034, Abcam, USA, dilution 1:100) were incubated for one hour in a humidified chamber at room temperature, followed by detection using the Polymer HRP Ms+Rb (ARH1001EA, Perkin-Elmer, USA) for 10 minutes. Visualization of FOXP3 was accomplished using Opal 690 TSA Plus (dilution 1:50) for 10 minutes, after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. In a serial fashion, the slide was then incubated with primary antibodies for PD-L1 (E1L3N, #13684, Cell signaling, USA, dilution 1:1000) for one hour in a humidified chamber at room temperature, followed by detection using the Polymer HRP Ms+Rb (ARH1001EA, Perkin-Elmer, USA). Visualization of PD-L1 was accomplished using Opal 650 TSA Plus (dilution 1:50) for 10 minutes, after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with primary antibodies for CD4 (EPR6855, ab133616, Abcam, USA, dilution 1:100) for one hour in a humidified chamber at room temperature, followed by detection using the Polymer HRP Ms+Rb (ARH1001EA, Perkin-Elmer, USA). Visualization of CD4 was accomplished using Opal 620 TSA Plus (dilution 1:50) for 10 minutes, after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with primary antibodies for CD8 (4B11, NB100-65729, Novusbio, USA, dilution 1:100)

for one hour in a humidified chamber at room temperature, followed by detection using the Polymer HRP Ms+Rb (ARH1001EA, Perkin-Elmer, USA). Visualization of CD8 was accomplished using Opal 570 TSA Plus (dilution 1:50) for 10 minutes, after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with primary antibodies for CD3 (2GV6, 790-4341, Ventana, USA, dilution 1:5) for one hour in a humidified chamber at room temperature, followed by detection using the Polymer HRP Ms+Rb (ARH1001EA, Perkin-Elmer, USA). Visualization of CD3 was accomplished using Opal 540 TSA Plus (dilution 1:50) for 10 minutes, after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with the last primary antibody, CK (AE1/AE3, M3515, Dako, USA, dilution 1:700), for one hour in a humidified chamber at room temperature, followed by detection using the Polymer HRP Ms+Rb (ARH1001EA, Perkin-Elmer, USA). Visualization of CK was accomplished using Opal 520 TSA Plus (dilution 1:50) for 10 minutes, after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. Nuclei were subsequently visualized with DAPI (1:2000), and the section was coverslipped using HIGHDEF® IHC fluoromount (ADI-950-260-0025, Enzo, USA).

**Image acquisition and quantitative data analysis**

Slides were scanned using the PerkinElmer Vectra 3.0 Automated Quantitative Pathology Imaging System (Perkin-Elmer, Waltham, MA), and images were

analyzed using the inForm software and TIBCO Spotfire (Perkin-Elmer, Waltham, MA). To acquire reliable unmixed images, representative slides of each emission spectrum and unstained tissue slide were used. Each of the individually stained sections (FOXP3-Opal690, PD-L1-Opal650, CD4-Opal620, CD8-Opal570, CD3-Opal540, CK-Opal520, and DAPI) was used to establish the spectral library of fluorophores required for multispectral analysis. This spectral library formed the reference for target quantitation, as the intensity of each fluorescent target was extracted from the multispectral data using linear unmixing. Each cell was identified by detecting nuclear spectral elements (DAPI). The total number of FOXP3, PD-L1, CD4, CD8, CD3, and CK positive cells was considered as the total immune cell infiltrations identified in the tissue. (Figure 1,2)

**Statistical methods**

Patients' characteristics were summarized using the median (range) for continuous variables and the frequency (percentage) for categorical variables. The probability for predicting the recurrence of immune cell profiling was evaluated using the Receiver Operating Characteristic (ROC) curve. Recurrence-free survival (RFS) was estimated using the method of Kaplan-Meier, where a recurrence-free event was defined as evidence of persistent or recurrent rectal cancer. RFS was calculated as the time from initial diagnosis to the date of first relapse. All statistical analyses were carried out in R software (version 3.4.1; R Foundation for Statistical

Computing, Vienna). A *p* value of less than 0.05 was considered statistically significant for all studies.

## RESULTS

### Patient characteristics

There were 16 (59.3%) men and 11 (40.7%) women in the study cohort. A total of 17 patients had a disease relapse within one year. There were no identified disease-related deaths during the follow-up period. The recurrence group and non-recurrence group showed no difference in age, sex, BMI, level of CEA (pre- and post-chemoradiation), tumor regression grade, tumor differentiation, and pathological stage. Microsatellite status was evaluated in 24 patients, and only one patient in the non-recurrence group showed microsatellite instability. In the recurrence group, only two patients had local recurrence, while the others had systemic recurrence. The median time to recurrence was eight (from 6–11) months (Table 1).

**Table 1.** Clinicopathological characteristics of the study patients

| Characteristics | Non-recurrence (n=10) | Recurence (n=17) | *P* value |
|---|---|---|---|
| Age | | | |
| Median (range), years | 57 (41–69) | 56 (37–71) | 0.086 |
| Sex | | | 1 |
| Male, n (%) | 6 (60.0) | 10 (58.8) | |
| Female, n (%) | 4 (40.0) | 7 (41.2) | |
| BMI | 24.0 ± 2.2 | 24.5 ± 2.8 | 0.643 |
| CEA level (initial) | | | 0.517 |
| ≥5, n (%) | 9 (90.0) | 12 (70.6) | |
| <5, n (%) | 1 (10.0) | 5 (29.4) | |
| CEA level (pre-operative) | 2.5 (1.7-3.8) | 1.8 (1.0-5.6) | 0.451 |
| Tumor regression grade | | | 0.426 |
| Minimal, n (%) | 1 (10.0 ) | 4 ( 23.5) | |
| Moderate, n (%) | 9 (90.0) | 13 (76.5) | |
| Tumor differentiation | | | 0.613 |
| Well, n (%) | 0 (0.0) | 3 (17.6) | |
| Moderate, n (%) | 10 (100.0) | 13 (76.5) | |
| Poor, n (%) | 0 (0.0) | 1 (5.9) | |
| MSI instability | | | 0.413 |
| Stable | 8 (80.0) | 15 (88.2) | |
| Unstable | 1 (10.0) | 0 (0.0) | |
| Unchecked | 1 (10.0) | 2 (11.8) | |
| Post-operative stage , n (%) | | | 0.202 |
| T2N0 | 1 (10.0) | 2 (11.8) | |
| T3N0 | 9 (90.0) | 7 (41.2) | |
| T3N1 | 0 | 5 (29.4) | |
| T3N2 | 0 | 2 (11.8) | |
| T4N1 | 0 | 1 ( 5.9) | |
| Lymphovascular invasion | 0 | 2 (11.8) | 0.260 |
| Perineural invasion | 0 | 3 (17.6) | 0.232 |
| CRM involvement | 0 | 0 | |
| Follow-up duration | | | |
| Median (range), months | 57 (18–70) | | |
| time to recur, months | | 8 (6–11) | |

**Table 1.** Clinicopathological characteristics of the study patients (continued)

| | |
|---|---|
| Site of recurrence | |
| Local , n (%) | 2 (11.8) |
| Systemic, n (%) | 15 (88.2) |

n; number, BMI; Body mass index, CEA; carcinoembryonic antigen, MSI; Microsatellite Instability, CRM; circumferential resection margin

**Difference in peripheral blood lymphocytes between the recurrence versus non-recurrence groups**

We retrospectively examined circulating white blood cells (WBCs) and lymphocyte counts between the recurrence and non-recurrence groups and assessed the possible relationship between these laboratory values and the amount of lymphocyte infiltrations in surgical specimens. The blood data were obtained from samples collected after the end of PCRT (just before surgery). None of the WBCs and lymphocyte counts showed any significant association with the total amount of immune infiltrations in surgical specimens. In addition, there was no difference in WBCs (5.0 ± 1.2 *vs.* 5.1 ± 1.4, $p = 0.753$) and lymphocyte counts (25.1 ± 7.2 *vs.* 20.4 ± 8.3, $p = 0.143$) between the non-recurrence and recurrence groups.

**Difference in immune infiltrates between the recurrence versus non-recurrence groups (single markers)**

Multispectral imaging was captured and quantified multiple immune cell types were

counted. After obtaining the whole scanned image, the tumor region was determined and the immune cells were counted. The number of immune infiltrates in each evaluated specimen varied, the density of immune infiltrates were calculated using the mean number of immune infiltrates of all evaluated specimens. The total density of immune cell infiltrates was higher in the non-recurrence group without statistical significance. The total density of $CD4^+$, $FOXP3^+$, and $PD\text{-}L1^+$ T lymphocytes was not significantly different between recurrence groups. However, the densities of $CD3^+$ T lymphocytes (1972.0 ± 1048.9/$mm^2$ *vs.* 592.5 ± 652.7/$mm^2$, $p = 0.001$) and $CD8^+$T lymphocytes (3420.6 ± 1509.6/$mm^2$ *vs.* 1365.6 ± 910.5/$mm^2$, $p < 0.001$) was higher in the non-recurrence group than in the recurrence group (Figure 3). The proportions of each type of immune infiltrate were also compared. The proportions of $CD3^+$ (9.4 ± 4.2% *vs.* 4.0 ± 4.3%, $p = 0.004$) and $CD8^+$ (16.6 ± 6.2% *vs.* 8.1 ± 5.0%, $p = 0.001$) were significantly higher in the non-recurrence group.

**Difference in immune infiltrates between tumor cells versus stromal cells (single markers)**

Since cytokeratin marker is believed to represent the tumor cells, the expression of CD3, CD4, CD8, FOXP3, and PD-L1 was examined in CK positive cells to evaluate their expression in tumor cells. In tumor cells ($CK^+$), the densities of $CD3^+$ T lymphocytes (139.9 ± 126.7/$mm^2$ *vs.* 30.2 ± 23.3/$mm^2$, $p = 0.023$) and $CD8^+$ T

lymphocytes (190.7 ± 182.5/mm$^2$ *vs.* 48.3 ± 68.8/mm$^2$, $p$ = 0.038) were higher in the non-recurrence group than in the recurrence group. Among stromal cells (CK$^-$), the CD3$^+$ T lymphocytes (1832.1 ± 962.3/mm$^2$ *vs.* 562.2 ± 644.4/mm$^2$, $p$ = 0.002) and CD8$^+$ T lymphocytes (3229.9 ± 1416.3/mm$^2$ *vs.* 1317.3 ± 893.4/mm$^2$, $p$ = 0.002) were dominant in the non-recurrence group (Figure 4).

**Difference in immune infiltrates between the recurrence versus non-recurrence groups (co-expression)**

The proportions of CD3$^+$CD8$^+$ (4.7 ± 2.8% *vs.* 1.6 ± 2.2%, $p$ = 0.005), CD3$^+$CD8$^+$PD-L1$^-$ (4.4 ± 2.7% *vs.* 1.5 ± 2.0%, $p$ = 0.003), and CD3$^+$CD8$^+$PD-L1$^-$ FOXP3$^-$ (4.3 ± 2.6% *vs.* 1.5 ± 1.9%, $p$ = 0.004) were significantly higher in the non-recurrence group. The densities of CD3$^+$CD8$^+$ (989.6 ± 661.1/mm$^2$ *vs.* 258.2 ± 395.3/mm$^2$, $p$ = 0.0013), CD3$^+$CD8$^+$PD-L1$^-$(920.4 ± 601.8/mm$^2$ *vs.* 229.5 ± 339.2/mm$^2$, $p$ = 0.00075), CD3$^+$CD8$^+$FOXP3$^-$ (961.6 ± 643.8/mm$^2$ *vs.* 251.9 ± 389.3/mm$^2$, $p$ = 0.0014), and CD3$^+$CD8$^+$PD-L1$^-$FOXP3$^-$ (896.1 ± 586.3/mm$^2$ *vs.* 225.2 ± 334.2/mm$^2$, $p$ = 0.00081) were significantly greater in the non-recurrence group (Figure 5).

**Difference in immune infiltrates between tumor cells versus stromal cells (co-expression)**

The difference in immune infiltrates was identified based on cytokeratin expression. In tumor cells, higher proportions of CD3$^+$CD8$^+$(1.4 ± 1.2% *vs.* 0.2 ± 0.2%, $p$ =

0.008), $CD3^+CD8^+PD\text{-}L1^-$(1.4 ± 1.1% *vs.* 0.2 ± 0.2%, $p = 0.008$), and $CD3^+CD8^+PD\text{-}L1^-FOXP3^-$(1.3 ± 1.1% *vs.* 0.2 ± 0.2%, $p = 0.009$) were examined in the non-recurrence group. The densities of $CD3^+CD8^+$, $CD3^+CD8^+PD\text{-}L1^-$, $CD3^+CD8^+FOXP3^-$, and $CD3^+CD8^+PD\text{-}L1^-FOXP3^-$ were lower in the recurrence group among tumor cells; however, these did not show statistical significance. In stromal cells, the densities of $CD3^+CD8^+$ (912.2 ± 608.4/$mm^2$ *vs.* 251.3 ± 389.3/$mm^2$, $p = 0.002$), $CD3^+CD8^+PD\text{-}L1^-$ (846.5 ± 558.2/$mm^2$ *vs.* 223.4 ± 333.0/$mm^2$, $p = 0.001$), $CD3^+CD8^+FOXP3^-$ (26.3 ± 26.9/$mm^2$ *vs.* 6.0 ± 9.0/$mm^2$, $p = 0.0021$), and $CD3^+CD8^+PD\text{-}L1^-FOXP3^-$ (885.9 ± 592.2/$mm^2$ *vs.* 245.3 ± 383.1/$mm^2$, $p = 0.0013$) expression were significantly greater in the non-recurrence group (Figure 6).

**Prediction of recurrence and survival analysis based on immune infiltrate profiles**

Data from the entire cohort were used for the ROC curve. As a single marker, CD8 proportion showed the highest predictability of recurrence (optimal cutoff value: 1551, AUC: 0.853, $p < 0.001$). ROC contrast estimation and testing results revealed the highest AUC value with the $CD3^+CD8^+PD\text{-}L1^-$proportion (optimal cutoff value: 218, AUC: 0.859, $p < 0.001$) as a co-expressed marker (Figure 7).

Low $CD3^+CD8^+$ co-expression ($p = 0.003$) and low $CD3^+CD8^+PD\text{-}L1^-$ co-expression ($p = 0.020$) were significantly associated with poor RFS regardless of cytokeratin

status (in tumor cells or in stromal cells) (Figure 8).

## DISCUSSION

We found that immune infiltrations in surgical specimens of rectal cancer may play a meaningful role in predicting recurrence from our current study.

Immunological markers to predict the recurrence of rectal cancer have not been fully established. Although many clinical factors, radiological findings, and molecular markers have been suggested to be related to prognosis, the clinical usefulness of immunological markers remains controversial.

PCRT could result in a lower rate of postoperative local recurrence and a higher rate of sphincter-preserving surgery as well as longer survival.[25] Our study demonstrated that specific immune infiltrates of resected specimens in patients who underwent PCRT were different between the recurrence and non-recurrence groups. Recent studies have reported that high $CD3^+$, $CD4^+$, or $CD8^+$ TIL densities in biopsy samples before PCRT are correlated with chemoradiosensitivity and good prognosis in rectal cancer.[17, 26] Another recent study reported that the good

response group after PCRT had lower $CD4^+$ and higher $PD\text{-}L1^+$ proportions than the residual group. Some previous studies have reported that in unresponsive cases of chemoradiation, PCRT may be assumed to have a disadvantage in immune suppression. In our study, the $CD3^+$ TILs and $CD8^+$ TILs of post-PCRT resected specimens in the non-recurrence group were significantly higher than those in the recurrence group. These results are similar to those of previous studies related to immunoscores and suggest that the role of immune cells does not change according to the treatment outcome regardless of response to chemoradiotherapy. The possible reason for this might be that chemotherapy or radiotherapy per se is generally insufficient to overcome a host's immune tolerance targeting the tumor.[26] Previous studies have shown a correlation between rectal cancer patient survival and density of TILs, including $CD8^+$ and $CD4^+$ TILs[7]. In this study, $CD3^+CD8^+PD\text{-}L1^-$ overlap was the most sensitive predictive marker for recurrence, and high $CD3^+CD8^+PD\text{-}L1^-$ was also associated with better RFS. To summarize our findings, the combinations of simultaneously expressed immune cells are stronger and more powerful predictors than single immune cell markers.

Tumor-infiltrating lymphocytes (TILs) and checkpoint molecules are known to have a crucial effect on tumor progression and outcomes.(Table 2) There are some immunosuppressive TIL subtypes, one example of which is FOXP3. As one of the major players in tumor immune suppression, FOXP3 appears to function as a

Table 2. Immune cell infiltrates with prognosis in colorectal cancer; representative researches

| Author | biomarker | Samle size | Pathologic method | Results | significance |
|---|---|---|---|---|---|
| Galon,J. et al.[7)] | CD3,CD8,GZMB,and CD45RO | 415 | Insitu immunostaining analysis (combination analysis of CT plus IM) | *Good prognosis marker; High density of adaptive immune cells ($CD3^+$, $CD8^+$, $GZMB^+$,and $CD45RO^+$) ¶ Poor prognosis marker; Low density of $CD3^+$ cells and $CD45RO^+$ cells in both tumor regions | The type, density, and location of immune cells in CRCs had a prognostic value that was superior to and independent of those of the UICC-TNM classification. |
| Mlecnik,B. et al.[9)] | CD3, CD8, GZMB, and CD45RO | 559 | Tissue microarray construction and immunohistochemistry | *Good prognosis marker; High density of $CD8^+$ infiltrates ¶ Poor prognosis marker; Low density of $CD8^+$ | Assessment of $CD8^+$ T lymphocytes in combined tumor regions provides an indicator of tumor recurrence beyond that predicted by AJCC/UICC-TNM staging |
| Sinicrope, F.A. et al.[27)] | CD3 and FoxP3 | 185 | Dual immunofluorescence microscopy | *Good prognosis marker; Increase in intraepithelial $CD3^+$ T cell density Poor prognosis marker; Increase in intraepithelial $FoxP3^+$cells and Low $CD3^+/FoxP3^+$ cell ratio ¶ Poor prognosis marker; Increase in intraepithelial $FoxP3^+$ cells and Low $CD3^+/FoxP3^+$ cell ratio | Indicating importance of an effector to Treg cell ratio in colon cancer prognosis |
| Frey, D.M. et al.[28)] | CD3, CD8 and FoxP3 cells stratified by MMR status | 1420 | Tissue microarray construction and immunohistochemistry | *Good prognosis marker; High $FoxP3^+$ expression | Correlation between Treg infiltration and prognosis in MMR-proficent CRC |
| Tosolini,M.et al.[29)] | Regulatory T cell, Th1, Th2 and Th17 | 125 | Tissue microarray construction and immunohistochemistry (CT and IM) | *Good prognosis marker; High Th1 and low Th17 ¶ Poor prognosis marker; Low Th1 and high Th17 | The functional Th1 and Th17 clusters yield opposite effects on patient survival in colorectal cancer. |

Table 2. Immune cell infiltrates with prognosis in colorectal cancer; representative researches (continued)

| | | | | | |
|---|---|---|---|---|---|
| Lee.et al[30] | CD3, CD45RO, FoxP3, and CD25 | 87 | Immunohistochemistry | *Good prognosis marker; High-density of $CD45RO^+$ and $FoxP3^+$ | Assessment of the prognostic role of TIL in stage II colon cancer |
| Deschollmeester V.et al.[31] | CD3, CD8, GZMB and MSI status | 209 | Immunohistochemistry and semiquantitative scoring | *Good prognosis marker; High intra-epithelial $CD3^+$, $CD8^+$ and stromal $CD3^+$ | Irrespective of the MSI status, infiltrating lymphocytes has antitumor immunity |
| Correale P.et al.[32] | FoxP3 | 57 | Immunohistochemistry | *Good prognosis marker; High density of $FoxP3^+$ | Higher $FoxP3^+$ score is a favorable prognostic factor in patients undergoing chemotherapy. |
| Teng F. et al.[15] | CD8, CD4, CD56, FOXP3, CD33, CD11b, PD-L1, and CTLA-4 | 62 | Immunohistochemistry | *Good prognosis marker; High pretreatment $CD8^+$<br>Pre-nCRT $CD8^+$, $CD4^+$, and MDSC-TILs are sensitive predictive marker for response to CRT | Comparison of immune infiltrates between pretreatment biopsy specimens and posttreatment surgically resected specimen; tumor immunity is activated after nCRT by increased infiltrating CD8 and $CD4^+$ |
| Yasuda K. et al[17] | CD4 and CD8 | 48 | Immunohistochemistry | *Good response marker for tumor reduction; $CD4^+$ and $CD8^+$<br>Independent prognostic factor for achieving CR after CRT; $CD8^+$ TIL; | T lymphocyte-mediated immune reactions play an important role in tumor response to CRT. |
| Park et al.[18] | CD4,CD8,PD-L1, FoxP3 and cytokeratin | 75 | Multiplexed immunofluorescence assay ($OPAL^{TM}$) | *Good response marker for PCRT; lower $CD4^+$ and higher $PD\text{-}L1^+$, lower $CD4^+/PD\text{-}L1^+$, $CD8^+/PD\text{-}L1^+$ and $FoxP3^+/PD\text{-}L1^+$ ratio | Immune infiltrate in biopsies before treatment could be a valuable information for the prediction of responsiveness to PCRT. |

GZMB; granzyme B, CT; center of tumor, IM; invasive margin, MMR; mismatch-repair gene, Treg; reculatory T cells, Th; T helper cell, MSI; microsatellite instability, nCRT; neoadjuvant chemoradiotherapy, MDSC; myeloid-derived suppressor cells, TIL; tumor-infiltrating lymphocyte, CR; complete response

master regulator of the regulatory pathway in the development and function of regulatory T cells.[33] $FOXP3^+$ T cells are associated with an unfavorable prognosis in many cancers. However, the prognostic effect of tumor-infiltrating $FOXP3^+$ T cells is controversial in rectal cancers.[34] $FOXP3^+$ showed no statistical significance as a single marker; however, it was thought to be a negative prognostic factor in this study based on its interaction with $CD3^+$ and $CD8^+$.

PD-L1 is a molecule that involves an immune checkpoint; PD-L1 can inhibit T-cell proliferation and cytokine production by binding with PD-1 on T cells, consequently playing an important role in immune tolerance.[35] It has been reported that the expression of PD-L1 in several different cancers is significantly correlated with poor prognosis.[36] The role of PD-L1 in this study was similar to that of FOXP3. When co-expression of PD-L1 with other immune cells was analyzed, it acted as a negative prognostic factor.

The tumor microenvironment comprises a heterogeneous population of cells composed of tumor cells plus nearby endogenous stromal cells recruited by the tumor. In previous studies related to tumor lymphocyte infiltrates, TILs were evaluated in the center and periphery of the tumor and around invasive margins.[9, 37, 38] However, structures around the tumor were destroyed by radiation; we could not analyze immune infiltration in different areas of the tumor microenvironment, such as invasive margins and the centers of cancer cell nests. Instead, we investigated

the tumor microenvironment as tumor cells and stromal cells based on cytokeratin expression because cytokeratin has been identified as a marker for the detection of cancer cells.[39)]

We defined the recurrence group as including any recurrence within one year from the operation date. This was done to ensure that we had patients in two categories (recurrence versus non-recurrence) to show whether distinct differences existed, as the number of patients in this study were quite limited and would not be representative of the rectal cancer population; that said, this approach may be conducive to new biomarker discovery with a small sample size.

Our research using the Opal™ system enabled a more comprehensive and specific view and analysis of the interaction between immune systems at a cellular level. Recently, quantitative IHC methods have shown promise in the development of diagnostic and prognostic cancer biomarkers for tailored therapy.[40, 41)] Multi-color immunohistochemistry could visualize biological interaction by allowing simultaneous phenotyping and functional assessment of multiple cell types. This imaging system provided quantitative results regardless of spectral and spatial overlap among fluorescence markers, enabling tissue segmentation, cellular phenotyping, and spatial analysis.

As a result, multiplex IHC images led to a comprehensive understanding of complex cellular interaction by analysis of overlapping biomarkers within cells and

cellular compartments not accessible by conventional immunohistochemistry. Moreover, by using an automated quantitative pathology imaging system (Vectra), we received fully automated, fast, and efficient digital slide scanning and continuous slide loading that allowed unlimited throughput. This is important because better visualization and identification of biomarkers and immune checkpoints could lead to improved understanding of cancer biology and the development of more effective cancer immunotherapies.

Our study has limitations insofar as analysis to find factors associated with recurrence was not performed because of the small sample size. The small sample size precluded a multivariate analysis. In addition, the retrospective design and limited data did not allow subgroup analysis such as correction for the expression of immune cells according to the stage and tumor regression grades.

The importance of metastatic LNs in determining the prognosis after PCRT has been constantly emphasized.[42-44] However, the implications have varied between studies. Some authors reported that LN metastasis had a great impact on the prognosis while others reported that LN metastasis did not affect the prognosis.[24, 45-47] Because of these inconsistent finding, and the absence of standardized guideline on how to best evaluated the regression grade of metastatic LNs, in this current study, the pathologic regression grade and prognostic effect of metastatic lymph node (LNs) after PCRT was not evaluated.

Despite these limitations, this study has noteworthy strength. To our knowledge, our study is the first to report multiplex IHC with CD3, CD4, CD8, FOXP3, and PD-L1 to identify overlapping immunological cell expression in PCRT rectal cancer resected specimens. Importantly, this method provides the ability to examine the spatial interactions and relationships among multiple parameters on a single tissue section. Additionally, this approach will likely allow for the enrichment of data extracted from the tissue microenvironment as well as for the analysis of the relationship between cell subsets for added information.

Our research will be especially useful in the area of tailor medical care at tumor level. The biologic approaches identifying biomarkers will lead to implementation of personalized therapy and offers the opportunity to increase therapeutic efficacy by individualized surveillance. Larger-scale clinical trials will be necessary to produce strong evidence for clinical practice.

## CONCLUSSION

Our study is the first to report for analysis of overlapping immunological cell

expression in PCRT rectal cancer specimens. The combinations of co-expressed immune cells are stronger and more powerful predictors than single immune cell markers. Out data suggest the possibility that immune infiltration in surgical specimens may play a positive role in predicting recurrence.

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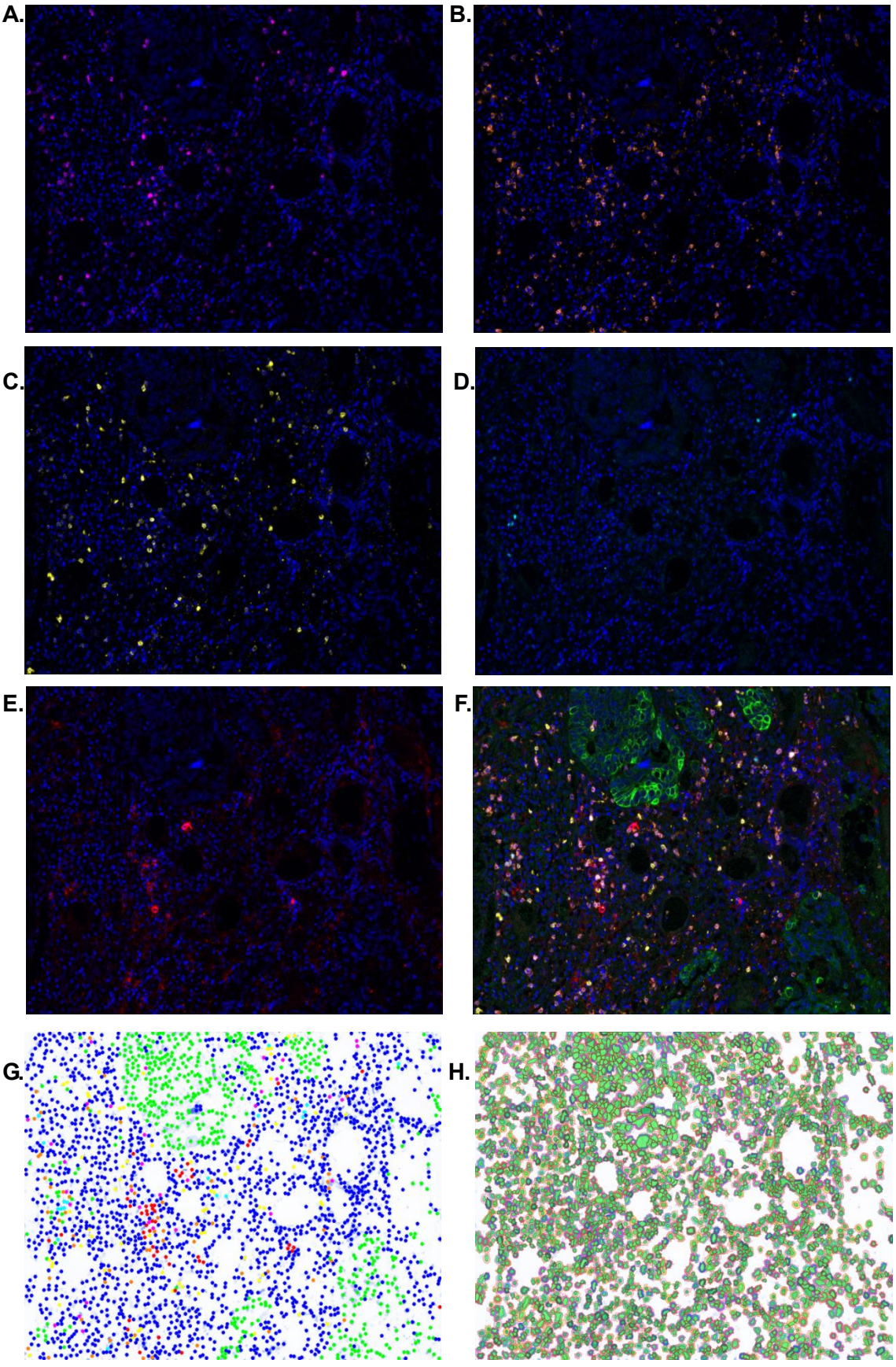
A.
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**Figure 1** Quantification of immunohistochemical staining and phenotype analysis for immune marker in rectal cancer surgical specimen.

(A) $CD3^+$ (Magenta colored with opal 540 fluorescent probe) and DAPI (blue)

(B) $CD4^+$ (Orange colored with opal 620 fluorescent probe) and DAPI (blue)

(C) $CD8^+$ (Yellow colored with opal 570 fluorescent probe) and DAPI (blue)

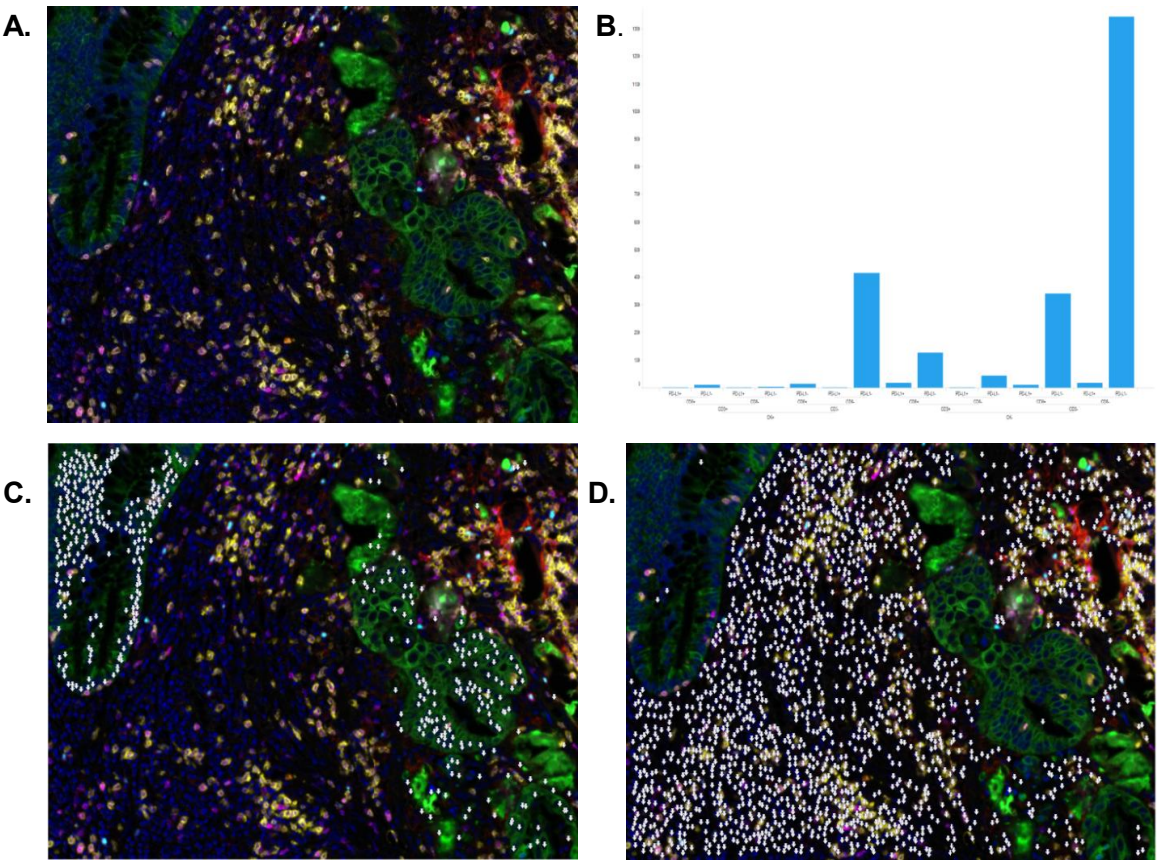
(D) $FOXP3^+$ (Cyan colored with Opal 690 fluorescent probe) and DAPI (blue)

(E) $PD\text{-}L1^+$ (Red colored with Opal 650 fluorescent probe) and DAPI (blue)

(F) Seven-plex image ($CK^+$,$CD3^+$, $CD4^+$, $CD8^+$, $FOXP3^+$, $PD\text{-}L1^+$, and DAPI)

(G) A representative image of cell phenotyping using inForm software for analysis of single marker expression

(H) A representative image of cell segmentation using inForm software for analysis of overlapped markers



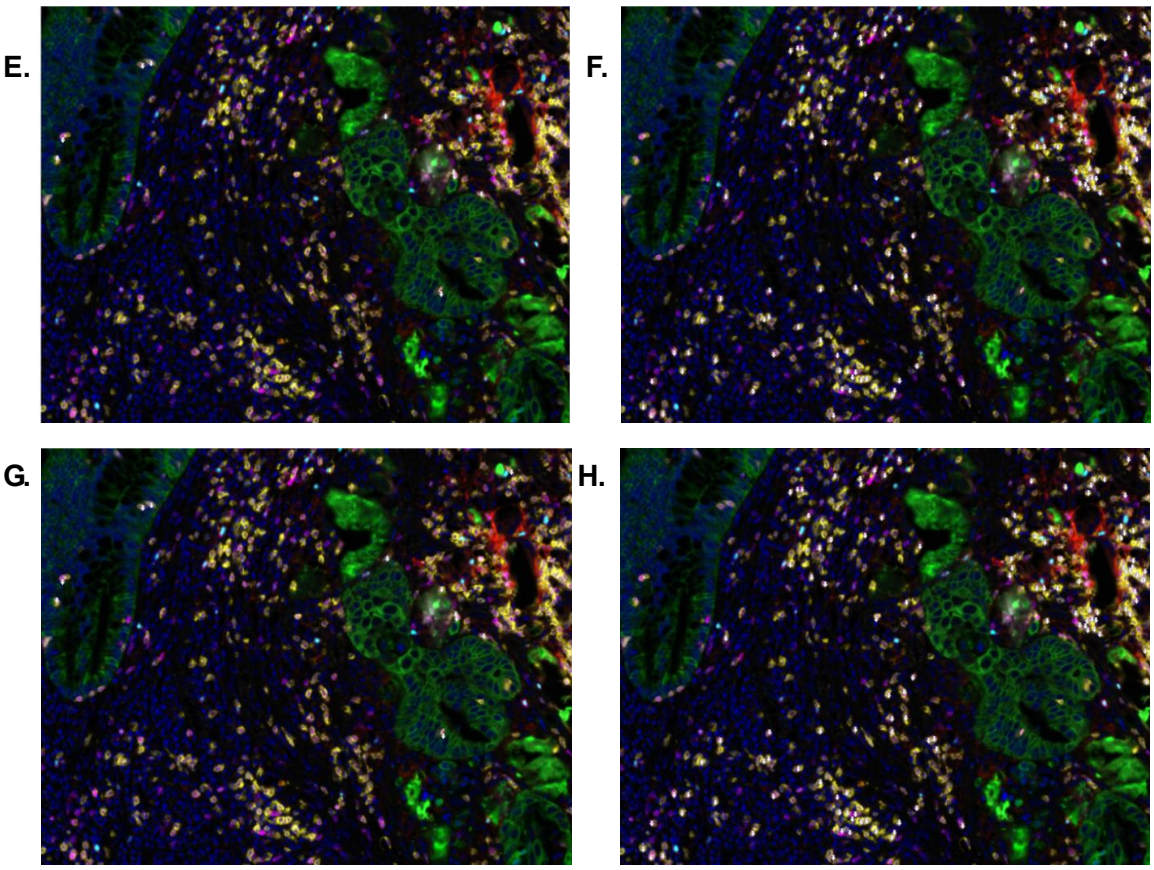



**Figure 2** Representative OPAL™ image of overlapping immunological cell expression

(A) A spectral composite image of 7-plexed image (same as previously designated color; Magenta, CD3; Orange, CD4; Yellow, CD8; Cyan, FOXP3; Red, PD-L1; Green, CK; Blue, DAPI)

(B) A representative density plot of concurrently expressed cell

(C) The CK positive cells were marked with arrow.

(D) The CK negative cells were marked with arrow.

(E) The $CK^{+}CD3^{+}CD8^{+}$ coexpressed cells were marked with arrow.

(F) The $CK^{-}CD3^{+}CD8^{+}$ coexpressed cells were marked with arrow.

(G) The $CK^{+}CD3^{+}CD8^{+}PDL1^{-}$ coexpressed cells were marked with arrow.

(H) The $CK^{-}CD3^{+}CD8^{+}PDL1^{-}$ coexpressed cells were marked with arrow.

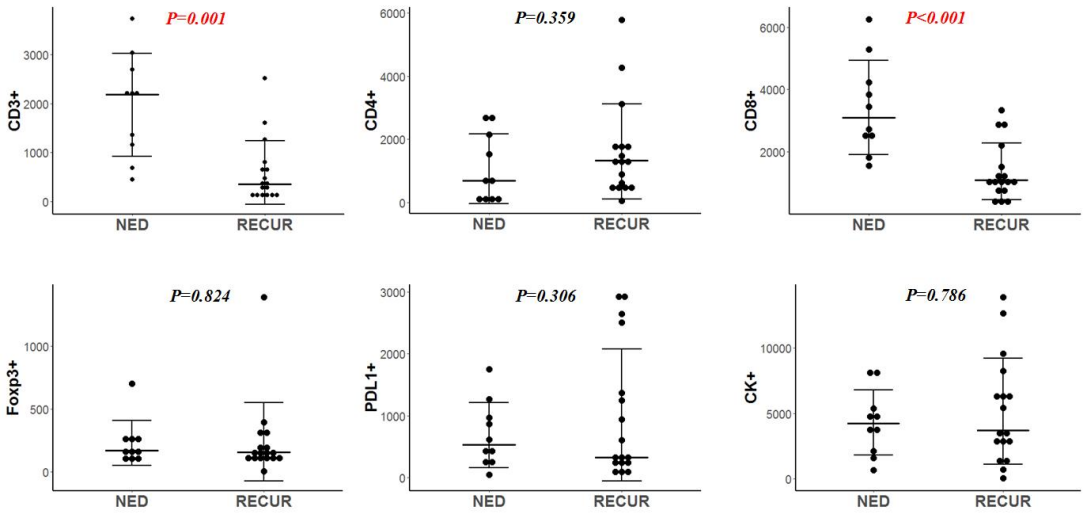



**Figure 3** Differences of immune infiltrates between recurrence and non-recurrence group

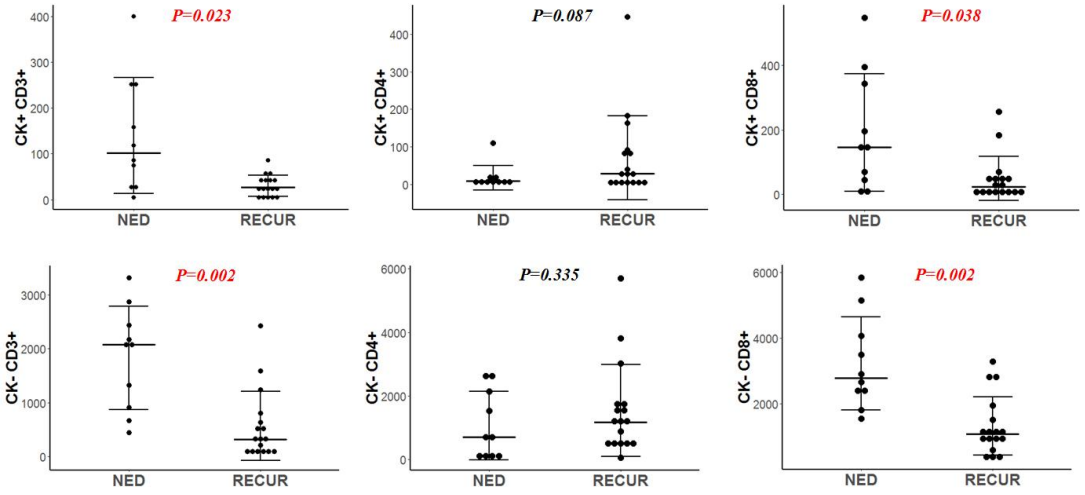



**Figure 4** Differences of immune markers in tumor cells versus stromal cells

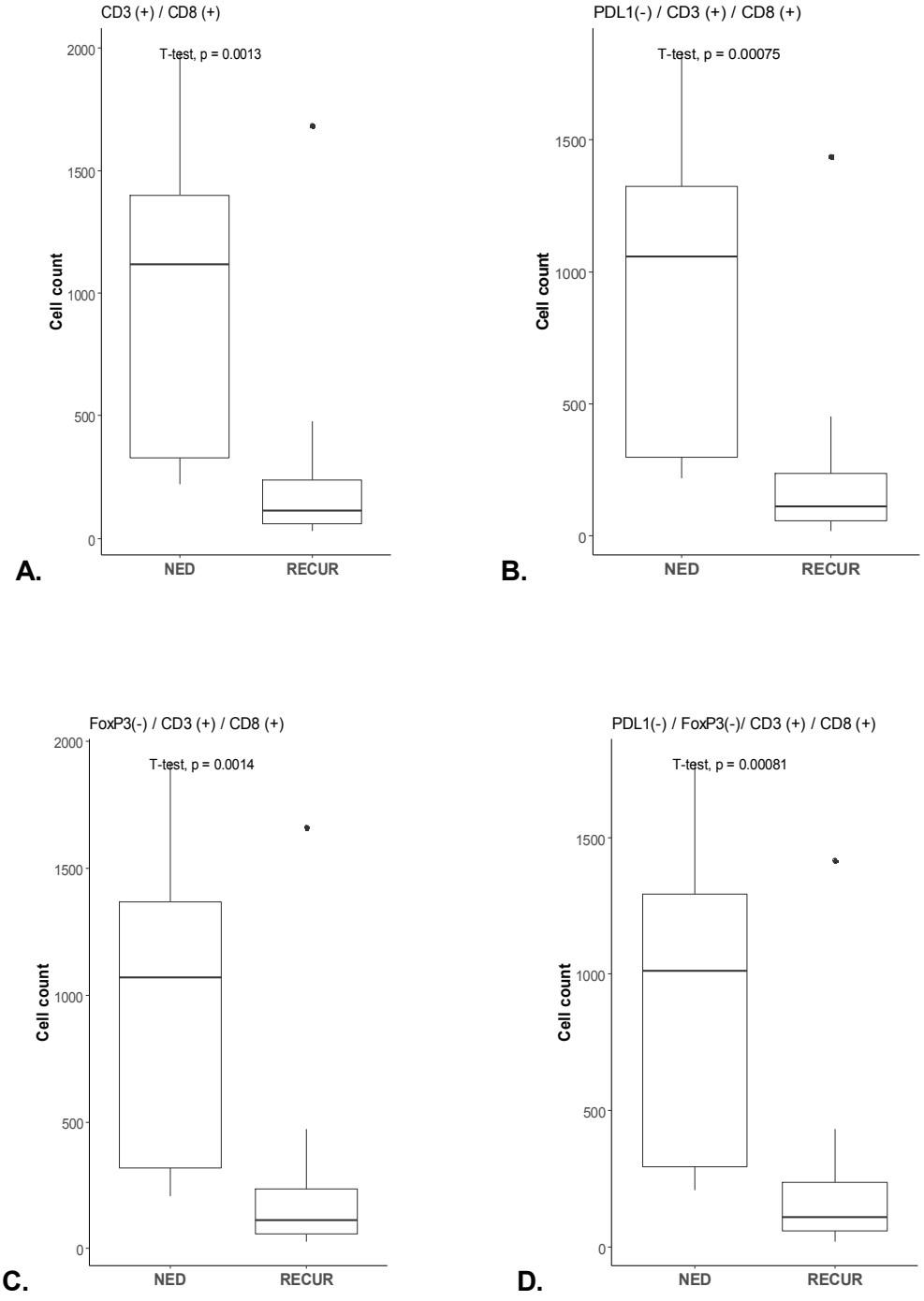



**Figure 5** Differences of co-expressed immune markers in rectal cancer resected specimen

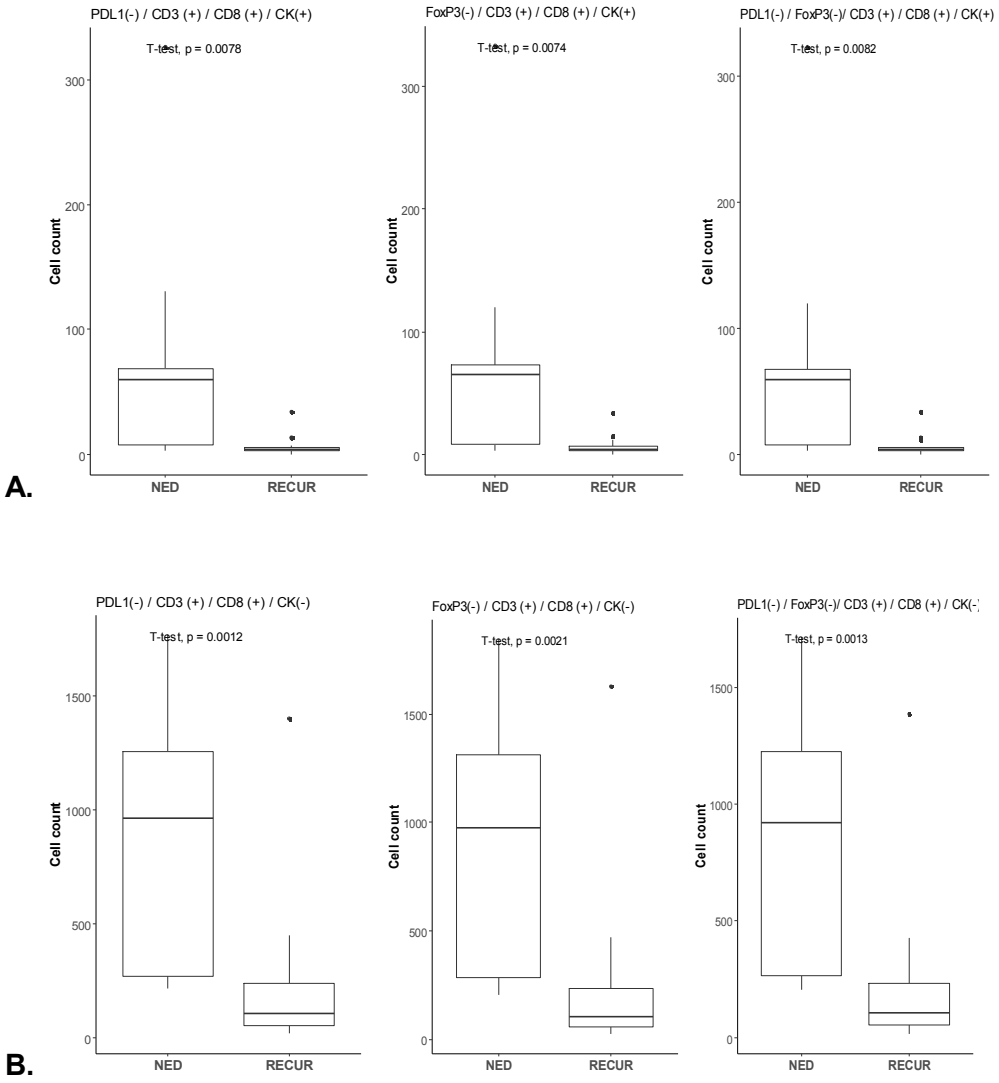



**Figure 6** Differences of co-expressed immune infiltrates in tumor cells versus in stromal cells (A) In tumor cells (B) In stromal cells

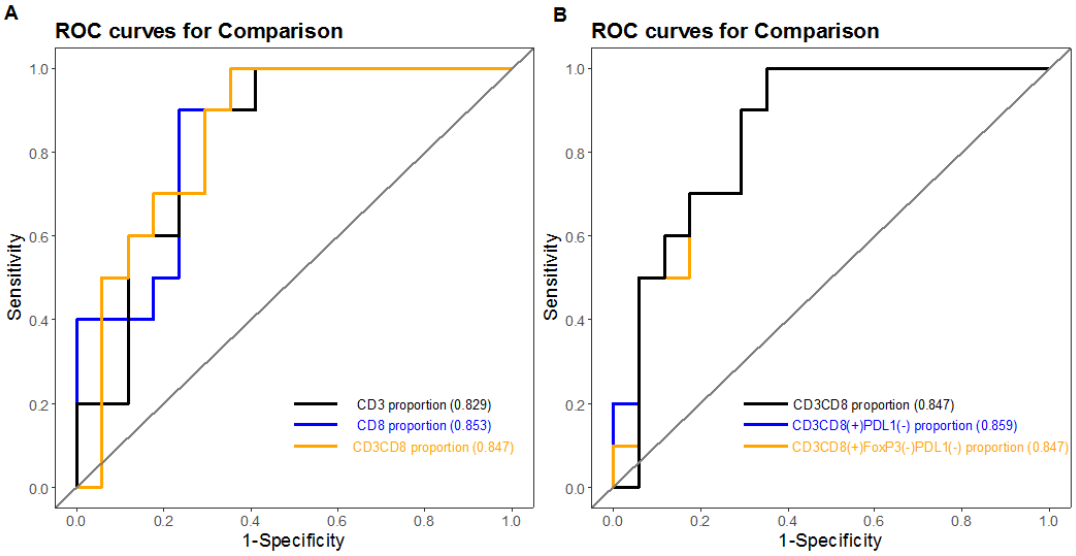



**Figure 7** ROC curve of co-expressed immune markers value for predicting tumor recurrence. (A) CD3, CD8 and CD3+CD8+co-positivity (B) CD3+CD8+, CD3+CD8+PDL1- and CD3+CD8+FOXP3-PDL1- co-expression

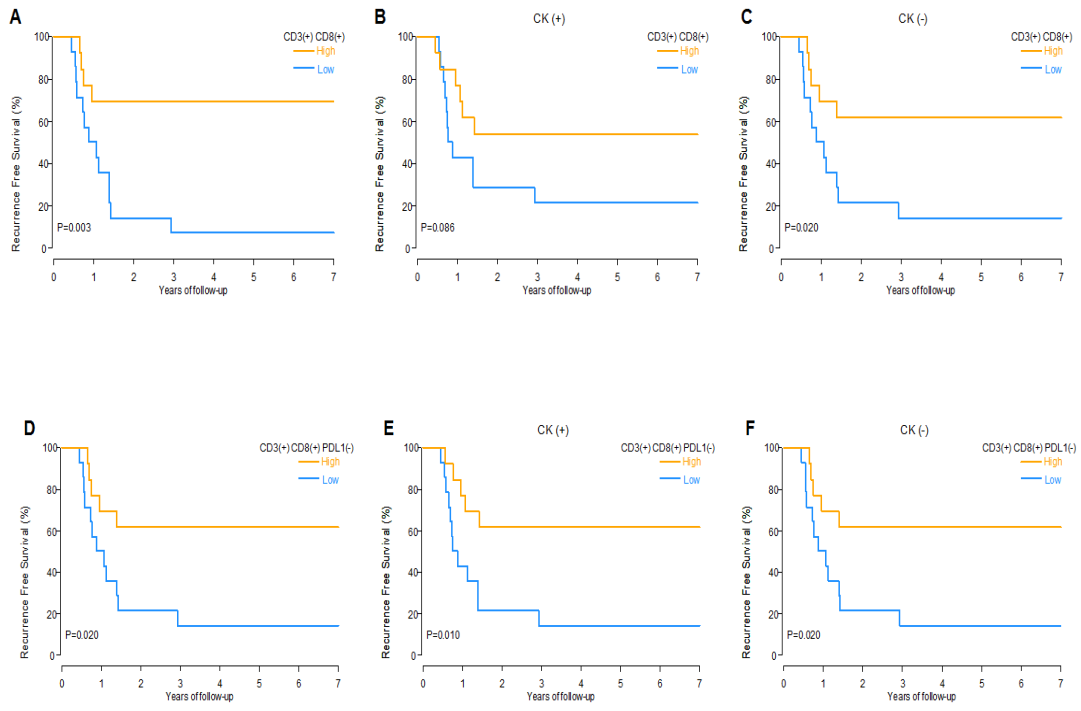


**Figure 8** Kaplan-Meier curve of recurrence free survival (RFS) according to co-expression of immune markers; high and low immune markers densities were plotted according to the cutoff value of immune cell densities defined at the median of the cohort (50% of patients with high cell density and 50% of patients with low cell density).

A. Kaplan-Meier curves illustrate the recurrence free survival according to the density of $CD3^+CD8^+$ as entire cohort [high $CD3^+CD8^+$ expression, yellow line (n=13 patients); low $CD3^+CD8^+$ expression, blue line (n=14)]

B. Kaplan-Meier curves illustrate the recurrence free survival according to the density of $CD3^+CD8^+$ in tumor cell regions [high $CD3^+CD8^+$ expression, yellow line (n=13 patients); low $CD3^+CD8^+$ expression, blue line (n=14)]

C. Kaplan-Meier curves illustrate the recurrence free survival according to the density of $CD3^+CD8^+$ in stromal regions [high $CD3^+CD8^+$ expression, yellow line (n=13 patients); low $CD3^+CD8^+$ expression, blue line (n=14)]

D. Kaplan-Meier curves illustrate the recurrence free survival according to the density of $CD3^+CD8^+$PD-$L1^-$ as entire cohort [high $CD3^+CD8^+$PD-$L1^-$ expression, yellow line (n=13 patients); low $CD3^+CD8^+$PD-$L1^-$ expression, blue line (n=14)]

E. Kaplan-Meier curves illustrate the recurrence free survival according to the density of $CD3^+CD8^+$PD-$L1^-$ in tumor cell regions [high CD3+CD8+PD-L1- expression, yellow line (n=13 patients); low CD3+CD8+ PD-L1- expression, blue line (n=14)]

F. Kaplan-Meier curves illustrate the recurrence free survival according to the density of CD3+CD8+ in stromal regions [high $CD3^+CD8^+$PD-$L1^-$ expression, yellow line (n=13 patients); low $CD3^+CD8^+$PD-$L1^-$ expression, blue line (n=14)]

# 국문요약

**연구 목적** : 본 연구의 목적은 수술 전 항암 화학 방사선 치료를 받은 직장암 환자의 예후에 종양 침윤 림프구 및 면역체크포인트에 관여하는 면역세포의 역할에 대해 확인하고자 함이다.

**대상 및 방법**: 수술 전 항암화학방사선 치료를 받은 직장암 2 기와 3 기 환자 중에서 1 년 이내의 재발을 보인 10 명의 환자(재발군)와 3 년이상 재발소견을 보이지 않은 17 명의 환자(비재발군)가 연구 대상으로 포함되었다. 직장암 수술 검체를 다중 형광 영상시스템을 이용하여 염색하였고 사용된 패널은 CK, CD3, CD4, CD8, FOXP3, PD-L1 그리고 DAPI 로 구성하였다. 이후 면역 염색 결과를 Vectra 를 이용하여 획득하고 inForm 과 TIBCO spotfire 소프트 웨어를 사용하여 이미지 분석과 면역세포 정량화를 시행하였다.

**결과**: 종양세포와 기질세포 모두에서 CD3 양성, CD8 양성 T 림프구의 밀도가 비재발군에서 의미 있게 높았다. 면역세포의 동시발현을 분석한 결과 CD3 와 CD8 동시양성, CD3 양성 CD8 양성과 PD-L1 음성 을 함께 보인 경우, 그리고 CD3 양성 CD8 양성 PD-

L1 음성과 FOXP3 음성을 동시에 보인 세포들의 비율과 밀도가 모두 재발군에서 높았다 ($p = 0.0013$, $p = 0.00075$ , 그리고 $p = 0.00081$). CD8 T 림프구의 비율과 CD3 양성 CD8 양성 PD-L1 음성을 동시에 보이는 세포의 비율이 재발을 예측하는데 가장 의미있는 인자였다 ($p < 0.001$). 종양세포와 기질세포모두에서 CD3 와 CD8 을 동시에 발현하는 세포가 적은 경우 ($p = 0.003$), 그리고 CD3 와 CD8 는 동시에 양성이지만 PDL1 은 발현하지 않는 세포가 적은 경우에는 ($p = 0.020$) 무재발 생존률이 낮았다.

**결론**: 본 연구는 수술 전 항암 화학 방사선 치료를 받은 직장암 환자의 수술 검체에서 면역세포들의 발현과 그 의미를 분석하고자 하는 최초의 연구이며 이 면역세포들에 대한 분석결과가 직장암의 재발예측에 긍정적인 도움을 줄 수 있을 것으로 기대된다.

**중심단어:** 대장암, 화학방사선요법, 재발, 종양침윤림프구, 면역마커