Effect of preoperative chemoradiotherapy on the immunological status of rectal cancer patients

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ABSTRACT

The aim of the study was to investigate the effect of chemo-radiation on the genetic and immunological status of rectal cancer patients who were treated with preoperative chemoradiotherapy (CRT). The expression of immune response-associated genes was compared between rectal cancer patients treated ($n = 9$) and not-treated ($n = 10$) with preoperative CRT using volcano plot analysis. Apoptosis and epithelial-to-mesenchymal transition (EMT) marker genes were analysed by quantitative PCR (qPCR). Other markers associated with the tumor microenvironment (TME), such as tumor-infiltrating lymphocytes (TIL) and immune checkpoint molecules, were investigated using immunohistochemistry (IHC). The clinical responses of preoperative CRT for 9 rectal cancer patients were all rated as stable disease, while the pathological tumor regression score (TRG) revealed 6 cases of grade2 and 3 cases of grade1. According to the genetic signature of colon cancers, treated tumors belonged to consensus molecular subtype (CMS)4, while not-treated tumors had signatures of CMS2 or 3. CRT-treated tumors showed significant upregulation of EMT-associated genes, such as CDH2, TGF-beta and FGF, and cancer stem cell-associated genes. Additionally, qPCR and IHC demonstrated a suppressive immunological status derived from the upregulation of inflammatory cytokines (IL-6, IL-10 and TGF-beta) and immune checkpoint genes (B7-H3 and B7-H5) and from M2-type macrophage accumulation in the tumor. The induction of EMT and immune-suppressive status in the tumor after strong CRT treatment urges the development of a novel combined therapy that restores immune-suppression and inhibits EMT, ultimately leading to distant metastasis control.

Keywords: chemoradiotherapy (CRT); consensus molecular subtype (CMS); tumor microenvironment (TME); epithelial-to-mesenchymal transition (EMT); tumor-infiltrating lymphocytes (TIL)

INTRODUCTION

Currently, preoperative fluoropyrimidine-based chemoradiotherapy (CRT) followed by radical surgery is one of the standard treatments for locally advanced rectal cancer, and it has been successful in local control with a recurrence rate of ~5% [1–3]. However, the distant recurrence rate after preoperative CRT and surgery remains ~30%, and it is still a crucial clinical problem hindering the improvement of survival rates [4, 5].

Recently, a novel immunotherapy has been developed using specific monoclonal antibodies targeting immune checkpoint molecules, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1)/PD-ligand 1 (PD-L1). This type of therapy has been applied in many clinical trials [6–9] and shows promising clinical benefits for even advanced-stage or metastatic cancer patients. Use of an immune checkpoint antibody is not recommended in colorectal cancers in terms of its overall response rate; however, metastatic microsatellite instability-high (MSI-H) cases, which account for ~15% of all colorectal cancers [10], were approved for anti-PD-1 antibody and pembrolizumab-based treatment. In the future, new preoperative neoadjuvant regimens combined with pembrolizumab will be developed.

Two main parameters might be involved in the overall survival (OS) in rectal cancer patients with regard to the prognosis of locally advanced rectal cancers in terms of distant recurrence control. The first is molecular subtype classification based on genetic expression data; four consensus molecular subtypes (CMS), CMS1–4, were demonstrated to be associated with survival time, and the CMS4 group has the worst prognosis due to features of epithelial-to-mesenchymal transition (EMT) [11, 12]. The second parameter is memory T cell infiltration inside the tumor, which is used as a tumor microenvironment (TME) marker and is reported to be a good prognostic factor for OS and progression-free survival (PFS) [13–15].

In the present study, aimed to dissect the mechanism of distant recurrence developed after neoadjuvant regimen treatment in rectal cancer patients, we compared genetic and immunohistochemistry (IHC) profiling between preoperative CRT-treated and non-treated rectal cancer patients using whole exome sequencing (WES) and gene expression profiling (GEP) analyses conducted under the HOPE project in the Shizuoka Cancer Center (SCC) [16].

MATERIALS AND METHODS
Patient registration

In 2014, the SCC launched Project HOPE, which is based on multi-omics analyses including WES and GEP. Informed consent was obtained from all patients participating in Project HOPE, and the study was approved by the Institutional Review Board of the Shizuoka Cancer Center, Japan. All experiments using clinical samples were carried out in accordance with the Ethical Guidelines for Human Genome and Genetic Analysis Research. Nine rectal cancer patients given preoperative CRT and 10 rectal cancer patients without preoperative CRT participated, and both groups received surgery. The group without preoperative CRT was given CRT after surgery. At our institution, CRT was applied for patients with locally advanced rectal cancers for the purpose of obtaining clear resected margins after surgery and maximizing the chance of preserving anal sphincters and preventing urinary diversion [17]. The characteristics of both rectal cancer patient groups are shown in Table 1. The tumor tissue and surrounding normal tissue were collected by surgeons after surgery.

DNA microarray-based GEP and WES using next-generation sequencing

The method used to perform GEP and WES analyses were described previously [16, 18]. Briefly, for GFP the ratio of the expression intensity between the tumor tissue (T) and the surrounding normal tissue (N) was calculated from the normalized values. The expression values for all probes were log (base 2)-transformed before performing the statistical analysis. In WES, all the variants called by the variant caller were filtered to discard sequences with a quality <30, variant allele frequency <10% or depth of coverage <20. Those mutations that were identified in tumor samples and not observed in matched normal samples were extracted as somatic mutations. Single-nucleotide variants (SNVs) of the total exonic mutations for each sequenced tumor included nonsynonymous, synonymous and indels/frameshift mutations.

Determination of tumor mutation burden and copy number variation

Tumor mutation burden (TMB), representing the number of somatic mutations per megabase, was defined as follows:

$$\mathrm{TMB} = \frac{N}{L}$$

where N is the number of somatic mutations identified in RefSeq exons targeted by the WES amplicon, and L is the length of the target region with a depth of coverage $\geq$20 in both the tumor and normal samples. N and L are calculated for each sample. On average, L is 33.16 Mb in our cohort; therefore, a TMB value of 20 corresponds to 660 mutations in the exons of 18 835 protein-coding genes in the human genome.

A joint segmentation-based approach that accounts for the relative ratio of the read depth in tumor and matched normal samples and variant allele frequency was applied for copy number variation (CNV) analysis. SAAS-CNV (a joint segmentation approach on aggregated and allele specific signals for the identification of somatic copy number alterations with next-generation sequencing data) by Zhang and Hao [19] is the method we utilized in the current study. The following information was extracted from the output of Torrent Variant Caller (TVC) and used as input for SAAS-CNV: (i) chromosome, (ii) position, (iii) reference sequence, (iv) alternative sequence, (v) quality score, (vi) genotype in normal sample, (vii) reference read depth in normal sample, (viii) variant read depth in normal samples, (ix) genotype in tumor samples, (x) reference read depth in tumor samples and (xi) variant read depth in tumor samples. SNVs with a quality score $\geq$100 and depth of coverage $\geq$20 were used in the CNV analysis. Those regions were extracted as CNVs.

Immune response-associated genes and SCC820 gene panel expression profiling

An updated list of immune response-associated genes is shown in Supplementary Table 1, see online supplementary material. In total, 203

Table 1. List of rectal cancer patients with or without preoperative chemoradiation

Case	Age, years	Sex	Clinical stage	Preoperative therapy (RT dose)	Pathological effect (TRG)	Relapse
REC-001	60	Male	IIIC	CRT (66 Gy)	2	-
REC-002	71	Male	IIIC	CRT (50.4 Gy)	2	-
REC-003	63	Male	IIIC	CRT (50.4 Gy)	1a	-
REC-004	44	Male	IIIC	CRT (50.4 Gy)	2	-
REC-005	47	Male	IIIC	CRT (50.4 Gy)	2	-
REC-006	65	Male	IIIB	CRT (50.4 Gy)	2	+ (Liver)
REC-007	51	Male	IIC	CRT (50.4 Gy)	2	+ (Bone)
REC-008	60	Female	IIIC	CRT (50.4 Gy)	1b	-
REC-009	47	Male	IIIC	CRT (50.4 Gy)	1a	-
REC-010	40	Male	IIIB	-	-	-
REC-011	59	Male	IIA	-	-	-
REC-012	65	Female	IIIC	-	-	-
REC-013	71	Male	IIIB	-	-	-
REC-014	60	Male	IIIC	-	-	-
REC-015	57	Male	IIIC	-	-	-
REC-016	43	Female	IIIC	-	-	-
REC-017	70	Male	IIIC	-	-	-
REC-018	63	Male	IVA	-	-	+ (Liver)
REC-019	61	Male	IIA	-	-	-

immune response-associated genes [97 antigen-presenting cell (APC)-associated and T-cell-associated genes, 34 cytokine- and metabolism-associated genes, 47 tumor necrosis factor (TNF) and TNF receptor superfamily genes and 25 regulatory T-cell-associated genes] were used for comparing gene expression levels between the CRT-treated and not-treated group. Additionally, the expression levels of genes of the SCC820 cancer-associated panel, which was reported previously [16], were also compared between the CRT-treated and not-treated groups.

IHC

For the tumor-infiltrating immune cells, antibodies against CD4 and CD8 (Thermo Fisher Scientific, Waltham, MA, USA), CD204 (Transgenic Inc., Kobe, Japan), IL-6 (Abcam, Cambridge, UK), TGF-beta1 (R and D Systems, Inc., Minneapolis, MN, USA), cytokeratin (AE1 and AE3, Nichirei Bio., Tokyo, Japan), FoxP3 (Abcam), Granzyme B (Dako, Glostrup, Denmark) and PD-L1 (Abcam) were purchased and used for the IHC and immuno-fluorescence analyses. For reference, the staining level of tumor-infiltrating immune cells was assessed by a semiquantitative estimation of the density of $CD8^+$ T cells inside the tumor site as follows: score 0, no or sporadic $CD8^+$ T cells; score 1, moderate number of $CD8^+$ T cells; score 2, abundant $CD8^+$ T cells; and score 3, highly abundant $CD8^+$ T cells [20].

For immuno-fluorescence staining, the TGF-beta1, IL-6 and CD204 stains were conducted using an Opal 4-color IHC kit (PerkinElmer Inc., Waltham, MA, USA) and evaluated on a fluorescent Zeiss imager Z1 microscope (Carl Zeiss, Oberkochen, Germany).

Real-time PCR analysis

Real-time PCR analysis of the apoptosis genes (BAX, BCL2, BCL2L1 and TP53), cytokine genes (STAT3, VEGFA and IL-6), stem cell genes (CD44, OCT3/4 and SOX2) and EMT-associated genes (CDH1, CDH2, COL1A2, MMP2, CTNNB1, RSPO1, TCF4, TGFB1, TGFB3, SMAD2, SNAIL2, TWIST1, NOTCH1, WNT5A and ZEB1) was performed using a QuantStudio 12 K Flex Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) as described previously [21]. Total RNA was isolated from formalin-fixed and paraffin-embedded (FFPE) rectal cancer specimens using a High Pure RNA Paraffin Kit (Roche Diagnostics, Indianapolis, IN, USA).

Statistical analysis

The genes from the 203 immune response-associated gene panel that were upregulated in the CRT-treated group compared to the not-treated group were identified using the volcano plot method. The proportion of categorical variables that were different between the CRT-treated and not-treated group was calculated with Pearson's chi-square test and the unpaired two-tailed t-test. Values with $P < 0.05$ were considered significant. Data analysis was performed using GeneSpring GX software version 13.1.1 (Agilent Technologies) and Microsoft Excel.

RESULTS
Characterization of rectal cancer patients

The number of locally advanced rectal cancer patients with and without preoperative CRT was 9 and 10, respectively (Table 1). We did not observe a significant difference in age, sex or clinical staging between the group with therapy and without therapy. The preoperative therapy regimen consisted of a combination of FOLFIRI (5-FU + l-LV + CPT-11) or Capecitabine and local radiation with a dose of 50.4 to ∼66 Gy.
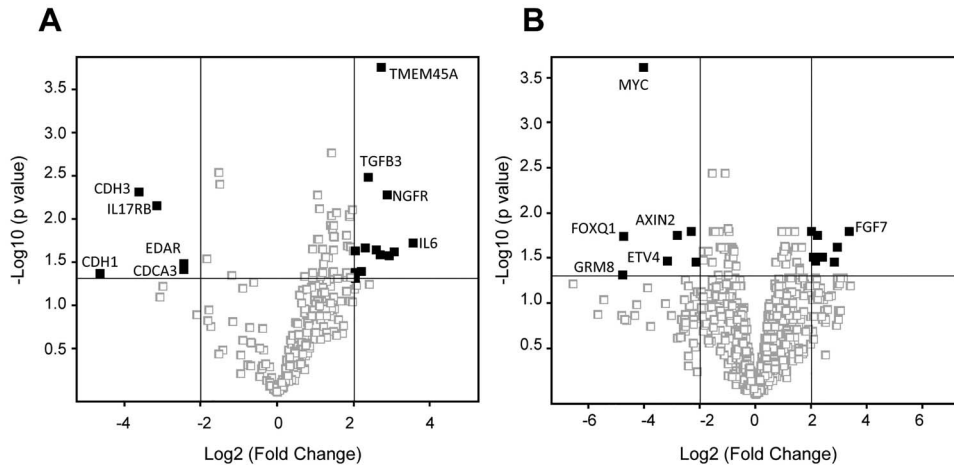
Fig. 1. Immune response-associated gene and SCC820 cancer-associated gene expression profiling in CRT-treated rectal cancers. Upregulated or downregulated immune response-associated genes (**A**) and SCC820 cancer-associated genes (**B**) with changes >4-fold identified using a volcano plot with Benjamini–Hochberg correction. The horizontal grey line represents a *P* value of 0.05. The vertical gray lines show 4- and 0.25-fold changes in gene expression. The closed squares represent the upregulated and downregulated genes with changes >4-fold, respectively.

The clinical responses of preoperative CRT for 9 rectal cancer patients were all rated as stable disease, while the pathological tumor regression score (TRG) revealed 6 cases of grade2 and 3 cases of grade1.

Characterization of genetic signatures from rectal cancer patients

There was a significant reduction in exon SNV and Vogelstein driver gene mutation numbers in the preoperative CRT-treated rectal cancer group (Table 2). Similarly, there was a significant reduction in tumor mutation burden (TMB) number and CNV size. The decrease in tumor mutation frequency might be a result of preoperative CRT; however, the difference in tumor content was not statistically significant.

Expression profiling of immune response-associated and SCC820 gene panels in rectal cancers treated with CRT

Volcano plot analysis demonstrated that the expression of 13 and 5 immune response-associated genes was upregulated and downregulated, respectively, in preoperative CRT-treated rectal cancers (Fig. 1A, Supplementary Table S1). Additionally, the expression analysis of SCC820 cancer-associated genes revealed that 8 were upregulated and 7 were downregulated (Fig. 1B, Supplementary Table S2, see online supplementary material). Among the genes with altered expression, 7 upregulated genes (FGF7, HGF, IL-6, PTGS2, TGFB3, CDH2 and NGFR) and 2 downregulated genes (CDH1 and CDH3) were closely associated with EMT occurrence.

Interestingly, TMEM45A, which belongs to a large family of genes encoding predicted transmembrane (TMEM) proteins, was obviously upregulated in preoperative CRT-treated rectal cancers.

Apoptosis and EMT marker gene expression measured by quantitative PCR

Quantitative PCR revealed that the expression of apoptosis-associated genes (BCL2) and the EMT-associated genes (STAT3, SOX2, CDH2, COL1A2, MMP2, CTNNB1, RSPO1, TCF4, SNAIL2 and TWIST1) was upregulated in preoperative CRT-treated rectal cancers (Fig. 2). Meanwhile, the expression of TP53, c-Myc, CDH1 and NOTCH1 was downregulated. In Fig. 3, a scheme of gene groups that were upregulated in CRT-treated rectal cancers and promoted EMT mediated by SNAIL and TWIST signaling, is provided.

B7 Family and cytokine gene expression profiling in rectal cancers treated with chemoradiation

The heatmap analysis of the B7 family and cytokine gene profiling indicated upregulation of immune-suppressive cytokines (IL-6, IL-10), upregulation of immune checkpoint genes (B7-H3, B7-H5), upregulation of the M2-type macrophage-associated gene (CD204) and downregulation of the immune-activating B7 family genes (B7-H6) (Fig. 4).

Tumor-infiltrating immune cell characterization in the TME from rectal cancer patients using IHC

The number of CD4^{+} T-cells tended to decrease and the number of FoxP3^{+} regulatory T-cells tended to increase inside the tumor nest between CRT-treated and not-treated rectal cancers. CD8^{+} T-cell infiltration was neither remarkable nor different between groups with and without therapy. However, CD204^{+} macrophage infiltration was higher in CRT-treated rectal cancers (Fig. 5A, Supplementary Table S3, see online supplementary material). Meanwhile, immuno-fluorescence staining showed that TGF-beta was derived from tumor cells and IL-6 might be produced from macrophages (Fig. 5B).

Table 2. Genetic characteristics of rectal cancer patients with or without preoperative chemoradiation

| Case	Preoperative therapy	Duration (days)	SNV (exon)	SNV (nonsynonymous)	Driver gene mutations	TMB (mutations/Mb)	CNV size (Mb)	Tumor content (%)
REC-001	CRT	86	9	6	None	0.122	2.27	18.5
REC-002	CRT	42	3	1	None	0.091	175.9	93.5
REC-003	CRT	41	4	1	None	0.06	66.4	23.4
REC-004	CRT	50	7	5	None	0.091	14.6	18.4
REC-005	CRT	56	9	8	None	0.12	131.5	19.2
REC-006	CRT	42	7	5	LMO1	0.06	833.2	20.4
REC-007	CRT	47	11	8	None	0.325	NA	NA
REC-008	CRT	43	19	13	None	0.565	NA	NA
REC-009	CRT	56	4	3	None	0.117	NA	NA
REC-010	-	-	65	38	APC, NOTCH1, NOTCH2, TP53	1.982	2447	68.6
REC-011	-	-	101	65	APC, FBXW7, KRAS	3.249	655.5	47.1
REC-012	-	-	107	73	BRAF, TP53	3.025	611.5	53.8
REC-013	-	-	146	108	APC, CREBBP, TP53	4.371	2545	28.5
REC-014	-	-	155	107	APC, BRCA2, KRAS	4.702	835.0	36.1
REC-015	-	-	144	89	APC, FBXW7, KRAS, TP53	4.399	785.3	39.6
REC-016	-	-	94	61	APC, BRAF, PTEN	2.845	1087	27.4
REC-017	-	-	70	47	TP53	2.136	1700	33.7
REC-018	-	-	79	52	AMER1, APC, KDM6A, KMT2C, TP53	2.238	1874	83.3
REC-019	-	-	80	54	APC, ATM, KRAS, PTEN	2.41	1668	47.5
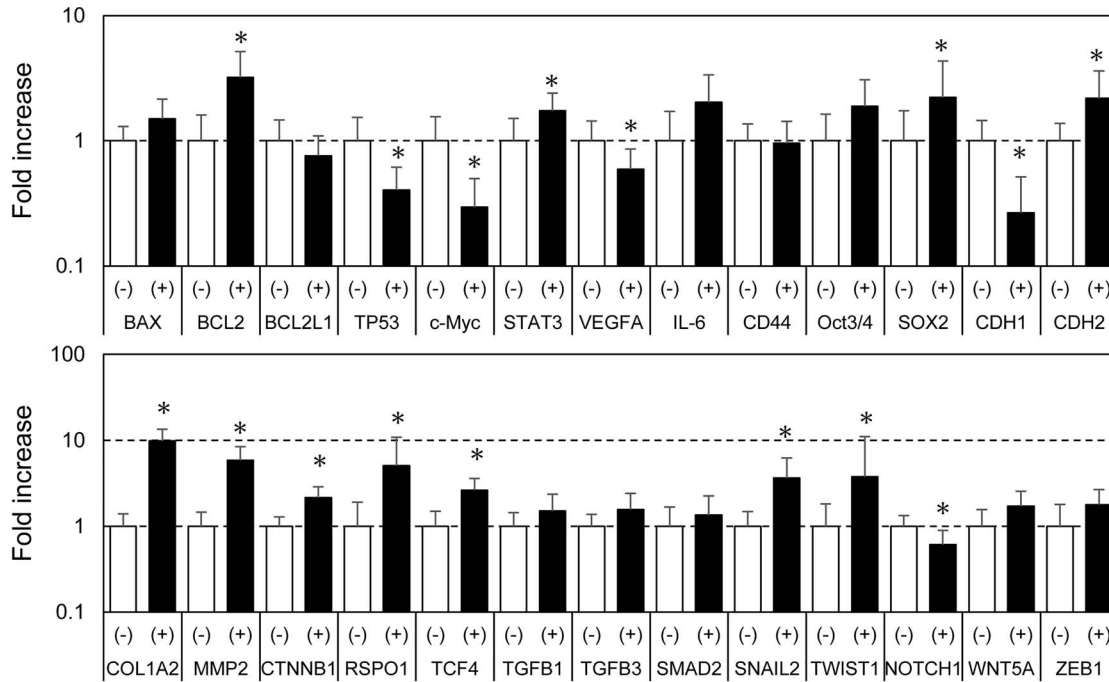
Fig. 2. Apoptosis and EMT-associated gene expression profiling using real-time PCR. Total RNA was isolated from the formalin-fixed paraffin-embedded (FFPE) rectal cancer specimens and real-time PCR was performed using a QuantStudio 12 K Flex Real Time PCR System. Open columns represent the control group, and shaded columns the CRT-treated group. Each column represents the mean value of triplicate experiments. ∗$P < 0.05$, statistically significant.
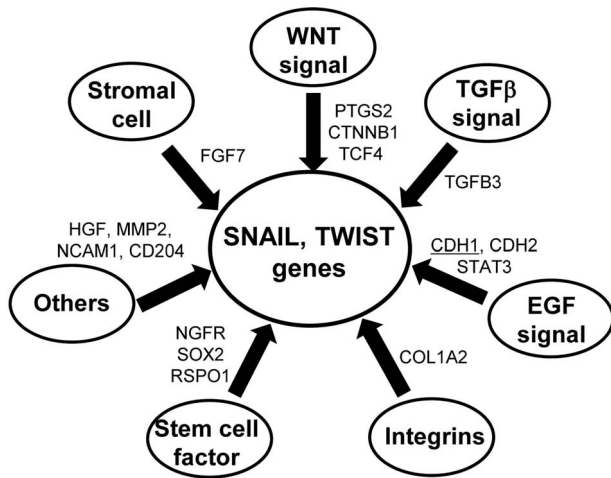

Fig. 3. Scheme of differentially expressed genes in CRT-treated rectal cancers leading to EMT induction. The upregulated genes (FGF7, PTFS2, CTNNB1, TCF4, TGFB3, CDH2, STAT3, COL1A2, NGFR, SOX2, RSPO1, HGF, NCAM1 and CD204) and downregulated genes (underlined, CDH1) were associated with EMT events, as determined from the gene profiling of immune response-associated and SCC820 panel genes.

DISCUSSION

From the results of past clinical trials that targeted locally advanced rectal cancers, preoperative CRT has been reported to show better control of local recurrence; however, it showed no significant difference in cumulative incidence of distant metastasis compared with postoperative CRT [2]. With the aim of improving overall survival after chemoradiation therapy, it is therefore important to evaluate the patient status after treatment in terms of genetic and immunological observations. Few studies have investigated the direct effect of radiation or chemoradiation on rectal cancers using NGS-based genetic analysis [22–24].

Molecular subtype classification based on genetic expression data has been reported by Guinney *et al.* to facilitate large-scale data sharing and analysis [25]. They found that CMS 1–4 were associated with survival time.

In the present study, most rectal cancers without preoperative CRT are considered CMS2 or 3, and rectal cancers with preoperative CRT show CMS4 typing with obvious EMT. Specifically, many EMT marker genes were recognized as upregulated (STAT3, SOX2, CDH2, COL1A2, MMP2, CTNNB1, RSPO1, TCF4, SNAIL2 and TWIST1) and downregulated (CDH1) (Fig. 2). Such an EMT-promoting genetic signature induced by CRT might lead to immunological escape, resulting in the occurrence of distant metastasis. In particular, TGF-beta is known to perform a central role in constructing the immune-suppressive TME by inducing regulatory T cells and MDSCs in the tumor site and attracting M2-type macrophages into the tumors [24]. Additionally, elevated TGF-beta production can promote IL-11 and
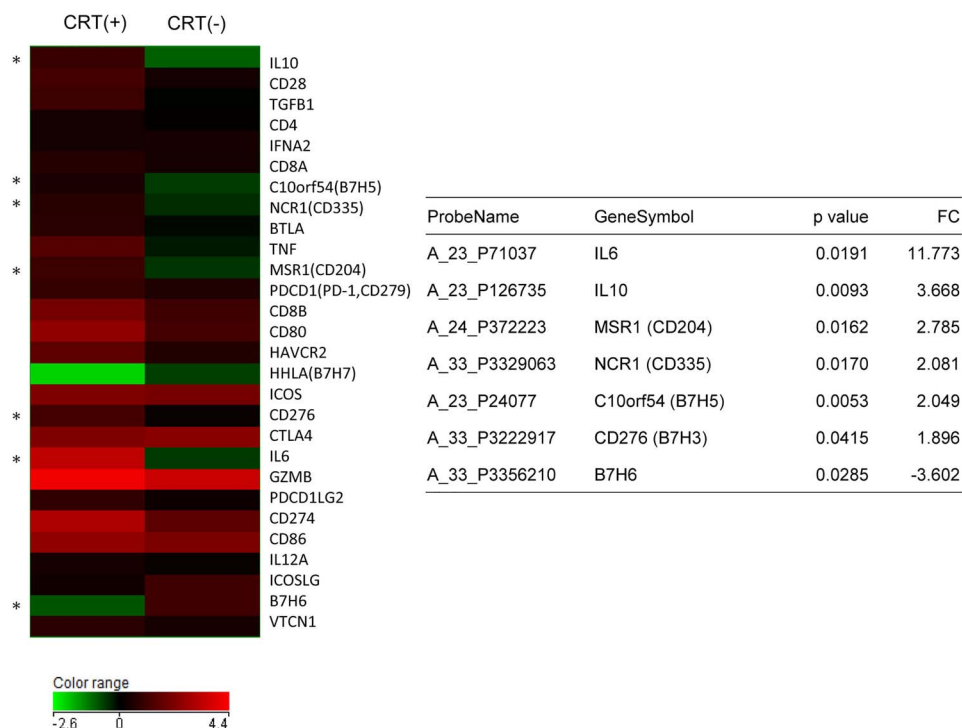
CRT(+) CRT(-)

* IL10
CD28
TGFB1
CD4
IFNA2
CD8A
* C10orf54(B7H5)
* NCR1(CD335)
BTLA
TNF
* MSR1(CD204)
PDCD1(PD-1,CD279)
CD8B
CD80
HAVCR2
HHLA(B7H7)
ICOS
* CD276
CTLA4
* IL6
GZMB
PDCD1LG2
CD274
CD86
IL12A
ICOSLG
* B7H6
VTCN1

ProbeName	GeneSymbol	p value	FC
A_23_P71037	IL6	0.0191	11.773
A_23_P126735	IL10	0.0093	3.668
A_24_P372223	MSR1 (CD204)	0.0162	2.785
A_33_P3329063	NCR1 (CD335)	0.0170	2.081
A_23_P24077	C10orf54 (B7H5)	0.0053	2.049
A_33_P3222917	CD276 (B7H3)	0.0415	1.896
A_33_P3356210	B7H6	0.0285	-3.602

Color range
-2.6   0   4.4

Fig. 4. B7 Family and cytokine gene expression profiling in CRT-treated rectal cancers. The relative expression ratio of each tumor tissue versus surrounding normal tissue was calculated from normalized values. The color log scale indicates the average of each group. Genes with significantly altered expression between CRT-treated (6 cases) and not-treated (8 cases) rectal cancers were selected. ∗P < 0.05, statistically significant. A summary of the statistical analysis is shown in the table.

IL-6 secretion from cancer-associated macrophages and fibroblasts, and this secretion is associated with distant metastasis of colorectal cancers by activating STAT3 signaling [26]. On the other hand, changes in FGF signaling-associated gene, such as FGF7, was identified, and this is an important observation because CAFs are associated with poor prognosis and resistance to radiotherapy in untreated colorectal cancers [27]. One more upregulated gene, TMEM45A, was verified in CRT-treated rectal cancers. Functionally, little is known about the TEMM45A gene; however, several studies with siRNA-based inhibition indicated similar results of strong inhibition of cancer cell proliferation and invasion, which partially suppressed in vivo tumors [28–30]. These results suggest that TMEM45A might be a novel target for anti-EMT therapeutics development.

Interestingly, another genetic observation was noted in CRT-treated rectal cancer patients. The numbers of SNV, TMB and Vogelstein driver gene mutations were remarkably decreased after CRT, as shown in Table 2. The reduction in c-MYC and ETV4 gene expression might be further evidence of the decrease of cancer cells with driver gene mutations. The tumor content reduction after CRT was partially responsible for this effect because tumor content with CRT (32.2%) tended to decrease compared to that without CRT (46.5%).

The IHC study showed similar results from genetic expression profiling of rectal cancer tissues. Upregulation of immune-suppressive cytokines (TGF-beta and IL-6) and immune checkpoint proteins (B7-H3 and B7-H5) and higher infiltration of M2-type macrophages were observed. The frequency of other tumor-infiltrating lymphocytes (TILs) ($CD4^+$, $CD8^+$ and $FoxP3^+$) was not significantly different between the rectal cancer groups with and without CRT (Supplementary Table S3). Matsutani et al. investigated $CD8^+$ TIL numbers before and after neoadjuvant CRT in the same rectal cancer patients and demonstrated that the density of $CD8^+$ TILs in post-treatment resected tumors was significantly increased compared with that in pretreatment biopsy samples [31]. Additionally, Shinto et al. used multivariate analyses to demonstrate that high levels of stromal $CD8^+$ T cells post-CRT and a high pretreatment intraepithelial CD8/FoxP3 ratio were associated with better prognosis in CRT-treated rectal cancers [32]. Our study demonstrated that $CD4^+$ T-cells tended to decrease and $FoxP3^+$ regulatory T-cells tended to increase inside the tumor nest between the CRT-treated and not-treated rectal cancers, which could reflect the immunological effect on TME of CRT; observations of patient survival time are necessary in the near future. These observations suggest that the timing of posttreatment resection and the dose of radiation might be important factors for evaluating TIL status.

Considering the immunological observations in the current study, some EMT markers may be therapeutic targets for the development of an efficient combination regimen with preoperative CRT. The first potential treatments for combination are the immune checkpoint blockade molecules like anti-PD-1/PD-L1 antibodies. Recently,
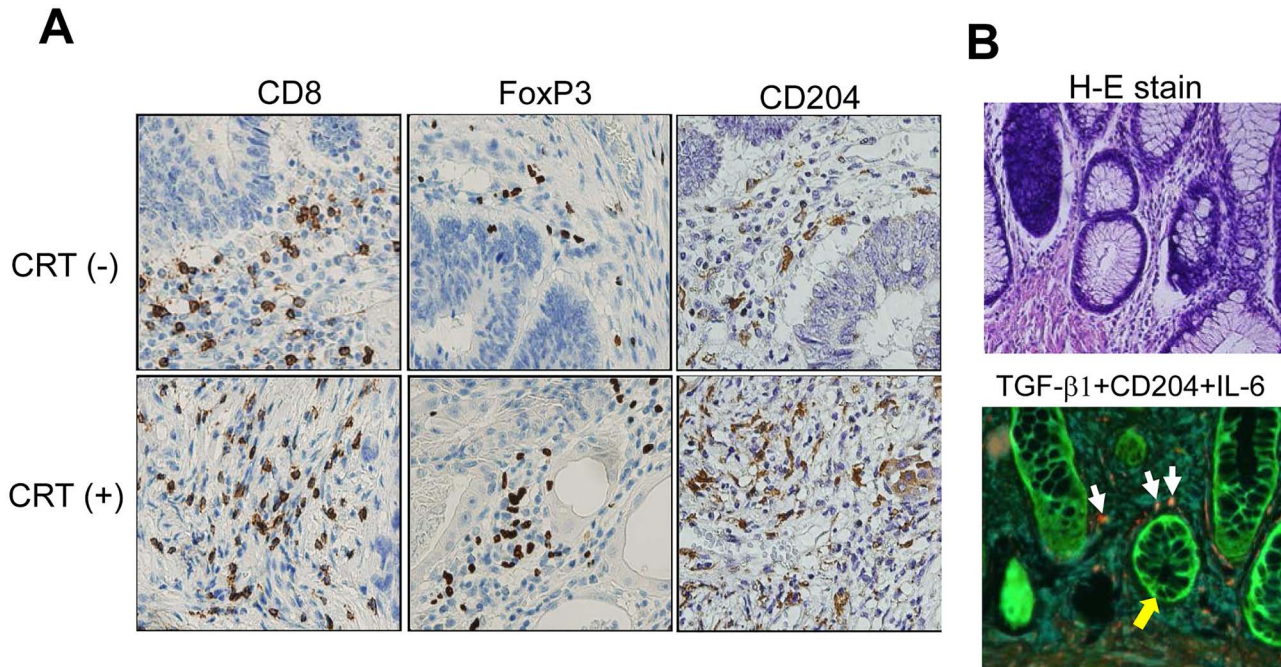
Fig. 5. Tumor-infiltrating immune cell characterization using IHC and immuno-fluorescence staining. (**A**) IHC staining against CD8, Foxp3 and CD204. CRT(−) and CRT(+) specimens were derived from casees REC011 and REC007, respectively. (**B**) H-E staining and immuno-fluorescent staining for TGF-beta1 (green), IL-6 (blue) and CD204 (red) using the Opal 4-color kit. The white arrows show CD204$^+$ macrophages producing IL-6. The yellow arrows show TGF-beta1$^+$ rectal cancer cells. The specimen was derived from case REC006 with CRT(+) which showed the upregulation of IL-6 gene in a quantitative PCR. Magnification x400.

based on the evidence that radiotherapy might upregulate the PD-L1 expression level in the tumor, the combination therapy of radiotherapy with anti-PD-L1 antibody has been performed for recurrent non-small-cell lung cancer and other cancers [33]. Considering that our study demonstrated that the expression of immune checkpoint genes like B7-H3 and B7-H5 was upregulated after CRT, the combination of RT and anti-immune checkpoint blockade could be a possible regimen.

The second target is the TGF-beta signaling pathway. Anti-TGF-beta monoclonal antibody (fresolimumab) [34] and the small molecule compounds regorafenib [35] and galunisertib are under development. Fresolimumab was used in a phase I clinical study against advanced malignant melanoma or renal cell carcinoma [34]. Regorafenib prevents TGF-beta1-induced EMT by activating SH2-domain-containing phosphatase 1 (SHP-1)-dependent p-STAT3 Tyr705 suppression [35]. Galunisertib is an oral small molecule inhibitor of TGF-beta receptor I kinase and has recently been studied in glioblastoma and pancreatic cancer patients.

The third target is tumor-associated macrophages producing inflammatory cytokines like IL-6 and 1L-10, which can contribute to rectal cancer metastasis through cytokine-mediated COX-2/β-catenin signal-induced EMT [36]. However, an efficient therapeutic device has not been available for clinical trial to date.

There were several limitations to this study. It was a retrospective study conducted in a single institution with a small number of patients. In Japan, lateral lymph node dissection is the standard treatment for patients with locally advanced low rectal cancer [37] and therefore

a limited number of patients received neoadjuvant CRT at our institution. This made it difficult to enroll more patients and compare these patients with those of well-matched patients who underwent surgery alone. Also, the chemotherapy regimens were not unified. In addition, patients with a clinically complete response after CRT were not enrolled in this study. This might affect assessments of the microenvironment after CRT.

CONCLUSION

The genetic and immunological features were compared between preoperative CRT-treated and non-treated rectal cancer patients. CRT-treated tumors showed significant upregulation of EMT-associated genes and cancer stem cell-associated genes, and a suppressive immunological status, which was derived from the upregulation of inflammatory cytokines and immune checkpoint genes and from M2-type macrophage accumulation in the tumor. The induction of EMT and immune-suppressive status in the tumor after strong CRT might be one of the mechanisms leading to the development of distant recurrence.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Radiation Research* online.

CONFLICT OF INTEREST

None declared.

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