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Efficacy of pembrolizumab and comprehensive CD274/PD-L1 profiles in patients previously treated with chemoradiation therapy as radical treatment in bladder cancer

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ABSTRACT

Background Chemoradiation therapy (CRT) has been increasingly reported as a possible alternative to total cystectomy (TC) for localized bladder cancer (BC). Pembrolizumab is the standard of care for platinum-refractory metastatic urothelial carcinoma, although it is unknown whether the efficacy of pembrolizumab in patients previously treated with curative CRT varies from the results of benchmark trials.

Methods We retrospectively assessed whether the survival benefit of pembrolizumab differs between patients previously treated with TC or CRT as radical treatment. A total of 212 patient records were collected for a logistic regression propensity score model. An independent dataset with next-generation sequencing (n=289) and PD-L1 Combined Positive Score (CPS: n=266) was analyzed to assess whether CRT-recurrent tumor harbors distinct CD274/PD-L1 profiles.

Results Propensity score matching was performed using putative clinical factors, from which 30 patients in each arm were identified as pair-matched groups. There was no significant difference in overall survival from the initiation of pembrolizumab (p=0.80) and objective response rate (p=0.59) between CRT and TC treatment groups. In the independent 289 BC cohort, 22 samples (7.6%) were collected as CRT-recurrent tumors. There was no significant difference in CD274 mRNA expression level between CRT-naïve and CRT-recurrent tumors. The compositions of CD274 isoforms were comparable among all isoforms detected from RNAseq between CRT-naïve (n=267) and CRT-recurrent (n=22) tumors. No actionable exonic mutation in CD274 was detected in CRT-recurrent tumors. PD-L1 CPS was positively correlated with CD274 mRNA expression level, and PD-L1 CPS was comparable between CRT-naïve and CRT-recurrent tumors.

Conclusions The efficacy of pembrolizumab for patients previously treated with CRT was similar to those treated with TC. The enhanced tumor regression by combining programmed cell death protein 1/PD-L1 inhibitor and CRT might be expected only in the concurrent administration.

INTRODUCTION

Bladder cancer (BC) is the fourth most common and the eighth lethal cancer among various types of malignancies in men.[1] Approximately 20%–30% of patients present with muscle-invasive BC (MIBC) at diagnosis, requiring intensive treatment compared with non-MIBC (NMIBC). Total cystectomy (TC) with the construction of the urinary tract has been the mainstay of the curative treatment for high-risk NMIBC and MIBC. However, it has been noted that the TC potentially accounts for the severe complications (blood loss, paralytic ileus, infections, and issues with wound healing) and clinical consequences with limited patient's quality of life[2] Emerging evidence for the administration of the chemoradiation therapy (CRT) with curative intent has been increasingly reported in the treatment of the various types of cancers.[3] For the treatment of MIBC, comparable treatment outcomes of CRT in combination with maximal transurethral resection have been demonstrated as the possible alternative to TC, which could allow patients to preserve their own functional bladders.[4 5]

For patients with metastatic urothelial carcinoma (mUC), platinum-based chemotherapy has been widely offered as the first-line treatment. Since GC (gemcitabine and cisplatin) regimen was approved by the

Food and Drug Administration (FDA) with a similar effect for clinical survival and a lower rate of intolerable treatment-related adverse events (AEs) compared with the conventional methotrexate, vinblastine, doxorubicin, and cisplatin regimen,[6] GC regimen became a standard of care for mUC patients. However, the survival benefit for mUC patients had been restricted due to the lack of reliable subsequent therapy after the treatment failure of the first-line chemotherapy for more than a decade. In 2017, the results from KEYNOTE–045 trial demonstrated the survival benefit of pembrolizumab, a monoclonal antibody targeting programmed cell death protein 1 (PD-1), compared with second-line chemotherapy (docetaxel, paclitaxel, and vinflunine) in patients with advanced platinum-refractory UC.[7] Since then, pembrolizumab has been widely used in large numbers of patients worldwide as well as in Japan.[8 9]

To date, a number of studies have indicated that radiotherapy could offer immunogenic effects, such as increased major histocompatibility complex (MHC) class I molecules with released neoantigens from tumors and enhanced tumor infiltration of CD8 +cytotoxic T lymphocytes (CTLs), which raises the hypothesis that radiotherapy exerts the synergistic effect with PD-1 blockage.[10 11] Indeed, there has been a volume of clinical reports showing the augmented abscopal effect by PD-1 inhibition that is characterized by the tumor regression of untreated metastatic lesions following the local radiotherapy.[12 13] We previously reported an increased BUBR1 expression in CRT-resistant UC tumors that offers an enhanced mutagenic non-homologous end joining activity, leading to a higher mutation burden.[14] However, it is still unknown whether the clinical effect of PD-1 blockage varies from the results of benchmark trials in the case of recurrent mUC previously treated with curative CRT. To answer this clinical question, we investigated the clinical outcomes of BC patients who had undergone CRT as a radical treatment followed by the pembrolizumab treatment adopting propensity score-matched analysis. We also explored CD274/PD-L1 profiles in the independent 289 BC patient dataset that includes a corresponding whole-exome sequence (WES) and RNA-sequencing data.

MATERIALS AND METHODS

For propensity score-matched analysis

This study was retrospectively coordinated using a multi-institutional dataset of Osaka Medical and Pharmaceutical University (Osaka, Japan) and The Jikei University School of Medicine (Tokyo, Japan) between January 2018 and December 2020. All the patients enrolled in the dataset were diagnosed with mUC including upper tract UC (UTUC) and BC, following the disease progression using platinum-based chemotherapy. Figure 1 illustrates a schematic of the current study. In short, a total of 212 patient records were collected. Exclusion criteria from the current study were as follows: patients with UTUC (85 patients), de novo M1 (22 patients), and no radical treatment for BC (13 patients). A logistic regression propensity score model stratified by the type of radical treatments including CRT or TC was performed. In this study, the protocol of CRT consists of GC regimen (1000 mg/m^2 gemcitabine on day 1, 8, and 15 and 70 mg/m^2 cisplatin on day 2) for cisplatin-eligible patients or GCarbo regimen (1000 mg/m2 gemcitabine on day1, 8 and 15 and targeted AUC 4.5 of carboplatin on day2) for cisplatin-ineligible patients with a total of 50 Gy radiation (2 Gy x 25 fractions to the whole pelvis) simultaneously performed during the chemotherapy. Dose modification was allowed in each individual according to the comorbidity profiles and general status.

Follow-up protocol was described in the previous report.[8] Briefly, follow-up CT for detecting any findings suspected of disease progression was scheduled every 6 weeks during the follow-up, and treatment response of pembrolizumab was assessed by the Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1.[15] Re-evaluation using MRI, bone scintigraphy, and positron emission tomography/CT (PET/CT) were further performed when necessary for the definitive diagnosis of immune-confirmed disease progression.[16] The primary endpoint was overall survival (OS). OS was calculated as the interval from the initiation of pembrolizumab to the date of last follow-up or deaths from any cause. The secondary endpoint was objective response rate (ORR) using the best overall response after pembrolizumab treatment, which was defined as the percentage of patients who achieved complete response (CR) or partial response (PR) according to the RECIST V.1.1 and iRECIST,[15 16] as well as the progression-free survival (PFS) from the initiation of pembrolizumab. Pembrolizumab was administrated intravenously at a dose of 200 mg every 3 weeks as approved by FDA.[7] Discontinuation of pembrolizumab due to the disease progression or treatment-related AE was decided by the physician's discretion.

The distribution of each factor was assessed by a contingency table with a χ^2 analysis. Kolmogorov-Smirnov normality was examined to check normal distribution in continuous variables followed by conducting a Student's t-test, or one-way analysis of variance was examined to assess the difference between the variables. For variables with non-normal distribution, Wilcoxon or Kruskal-Wallis test was performed to assess the difference. For the propensity-score matching, the following variables that could impact survival outcomes were involved in the regression model: age at the initiation of pembrolizumab treatment (continuous variables), sex (male, female), body mass index (continuous variables), Eastern Cooperative Oncology Group Performance Statu (0, 1, 2, 3, 4), pelvic lymph node metastasis at the curative therapy (±), visceral metastasis at the initiation of pembrolizumab treatment (±), serum hemoglobin level and neutrophil-to-lymphocyte ratio (NLR) at the initiation of pembrolizumab treatment (continuous variables), cycles of previous chemotherapy performed before pembrolizumab (continuous

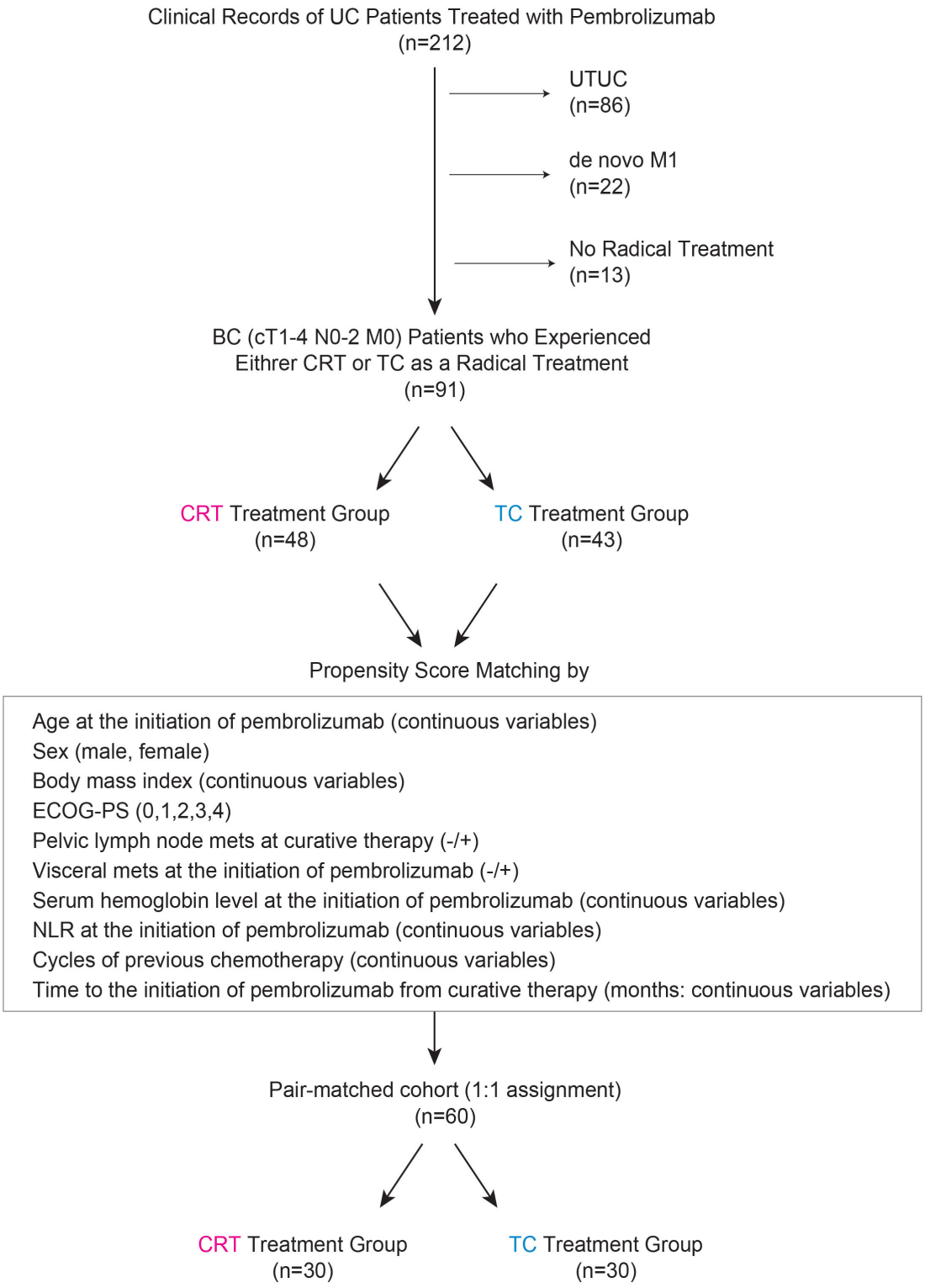

Figure 1 Study design and inclusion criteria of the propensity score-matched analysis in the multi-institutional cohort of urothelial carcinoma treated with pembrolizumab. A 1:1 matching across the two treatment arms (chemoradiation therapy and total cystectomy) was performed using the nearest neighbor method with a 0.5-width caliper of the SD of the logit of the propensity scores. BC, bladder cancer; CRT, chemoradiation therapy; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; NLR, eutrophil-to-lymphocyte ratio; TC, total cystectomy; UTUC, upper tract urothelial carcinoma.

variables), time to the initiation of pembrolizumab from curative therapy (months: continuous variables). A 1:1 matching (without replacement) across the two curative treatment arms was achieved by the nearest neighbor method with a 0.5-width caliper of the SD of the logit of the propensity scores to reduce bias by those potential confounding factors. Kaplan-Meier survival analysis and stratified generalized Wilcoxon test were used to compare

Table 1 Clinical characteristics of relapsed bladder cancer patients after curative therapy

Variables	Total (n=91)			Pair-matched (n=60)		
	Chemoradiation therapy (n=48)	Surgical therapy (n=43)	P value	Chemoradiation therapy (n=30)	Surgical therapy (n=30)	P value
Gender (%)						
Male	41 (85.4)	33 (76.7)	0.29	23 (76.7)	22 (73.3)	0.77
Female	7 (14.6)	10 (23.3)		7 (23.3)	8 (26.7)	
Age (median (IQR))	71 (62–76)	74 (66–79)	0.23	70 (60–78)	72 (59–78)	0.74
ECOG-PS at pembrolizumab (%)						
0–1	41 (85.4)	40 (97.7)	0.24	27 (90.0)	28 (93.3)	0.64
>2	7 (14.6)	3 (2.3)		3 (10.0)	2 (6.7)	
Smoking history (%)						
Never	17 (35.4)	13 (30.2)	0.6	11 (36.7)	7 (23.3)	0.26
Former/current	31 (64.6)	30 (69.8)		19 (63.3)	23 (76.7)	
Body mass index (%)						
≥25	8 (16.7)	4 (9.3)	0.3	3 (10.0)	4 (13.3)	0.69
<25	40 (83.3)	39 (90.7)		27 (90.0)	26 (86.7)	
Pelvic lymph node metastasis at curative therapy (%)						
Yes	16 (33.3)	7 (16.3)	0.06	10 (33.3)	7 (23.3)	0.39
No	32 (66.4)	36 (83.7)		20 (66.7)	23 (76.7)	
Liver metastasis at pembrolizumab therapy (%)						
Yes	7 (17.1)	3 (2.3)	0.25	2 (6.7)	3 (10.0)	0.64
No	41 (82.9)	40 (97.7)		28 (93.3)	27 (90.0)	
Visceral metastasis at pembrolizumab therapy (%)						
Yes	35 (72.9)	18 (41.9)	0.003*	20 (66.7)	15 (50.0)	0.19
No	13 (27.1)	25 (58.1)		10 (33.3)	15 (50.0)	
Hemoglobin concentration at pembrolizumab therapy (%)						
>100 g/L	22 (45.8)	33 (76.7)	0.003*	17 (56.7)	22 (73.3)	0.18
≤100 g/L	26 (54.2)	10 (23.3)		13 (43.3)	8 (26.7)	
NLR at pembrolizumab therapy (%) (median (IQR))	4.80 (3.50–7.84)	3.67 (2.36–5.5)	0.01*	4.05 (2.85–6.12)	3.6 (2.34–6.33)	0.42
Previous chemotherapy cycles (median (IQR))	3 (1–4)	3 (2–6)	0.009*	3 (2.5–4)	3 (2–4)	0.91
Time to pembrolizumab therapy from curative therapy (months) (median (IQR))	16.5 (7.58–32.5)	11.9 (6.33–28.3)	0.29	19.9 (8.28–37.2)	11.2 (5.69–29.0)	0.18

*p<0.05
ECOG-PS, Eastern Cooperative Oncology Group Performance Status; NLR, neutrophil-to-lymphocyte ratio.

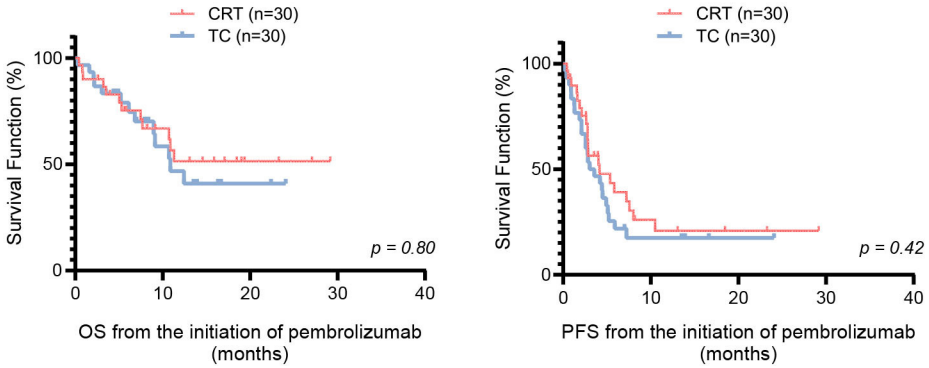

Figure 2 Kaplan-Meier curves for overall survival (OS) and progression-free survival (PFS) from the initiation of pembrolizumab according to the radical treatment. CRT, chemoradiation therapy; TC, total cystectomy.

OS and PFS between the assigned groups. All the statistical tests were two tailed, and a threshold of p<0.05 was considered significant for statistical analyses. All analyses were performed using JMP V.13 (SAS Institute).

Cell lines, proliferation assay

The BC cell lines including T24 and 5637 cells were obtained from the American Type Culture Collection (ATCC). The authentication was obtained by Human STR Profiling Cell Authentication Service, and the mycoplasma test was tested in all cell lines. These cells were maintained in 10% fetal bovine serum supplemented with 2 mM of L-glutamine at 37°C in 5% CO2. Cells treated in individual experiments were assessed for cell viability using Cell Titer-Fluor Assay and Caspase-Glo 3/7 Assay (Promega, Madison, WI) following the manufacturer's protocol.

Quantitative PCR

RNA was isolated using TRIzol (Invitrogen, Carlsbad, California, USA) and Direct-zol RNA Prep Plus (Zymo Research, Irvine, California, USA) according to the manufacturer's protocol, followed by quantification using a NanoDrop spectrophotometer, and 100–500 ng of RNA was reverse-transcribed using a SuperScript IV VILO Master Mix (Invitrogen, Carlsbad, California, USA). The sequences of primers are as follows: CD274: 5'GGTCATCCCAGAACTACCTC3' and 5'CATCCATCATTCTCCCTTTTC3', GAPDH: 5'AGCCACATCGCTCAGAGACC3' and 5'GCCCAATACGACCAAATCCG3'. Quantitative PCR was performed on an ABI Quant Studio 5 detector. Product formation was detected by incorporation of SYBR Green I using ROX as a passive reference. The expression data were normalized with GAPDH in each sample. Experiments were independently repeated and analyzed three times.

Immunoblotting

Whole-cell lysates were collected and lysed in radioimmunoprecipitation assay lysis buffer with proteinase inhibitor mixture (Thermo Scientific, Waltham, Massachusetts, USA) and sonicated using a Bioruptor Standard. After centrifugation at 13 200 rpm for 10 min, the supernatant was collected. Proteins were subjected to NuPAGE Bis-Tris Gels or NuPAGE Tris-Acetate Gels before being transferred onto the PVDF membrane (Millipore, Darmstadt, Germany). The antibodies used in this study include rabbit monoclonal PD-L1 antibody (Abcam: ab205921) and mouse monoclonal B-actin antibody (Sigma Aldrich: A-5316). Detection of the protein was performed using the Fusion FX imaging system, and Fusion-Capt Advance analyzing system was used to quantify the protein expression levels.

Biospecimen collection and PD-L1 immunohistochemistry in the independent cohort

Biospecimens from patients diagnosed with UC by the tumor resection with either transurethral resection or radical cystectomy performed at Osaka Medical and Pharmaceutical University hospital were collected in RNAlater reagent (Thermo Fisher Scientific). All the H&E-stained cases were reviewed by a board-certified pathologist to confirm that the tumor specimen was histologically consistent with UC. Tumor sections were required to contain an average of 60% tumor cell nuclei equal to or less than 20% necrosis for inclusion in the study. RNA and DNA were extracted from tumor-adjacent normal tissue specimens using the DNA/RNA AllPrep kit (QIAGEN). Quantification of nucleic acids was performed using NanoDrop Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). RNA was analyzed via the Agilent 2100 Bioanalyzer (Agilent) for the determination of an RNA Integrity Number (RIN), and only the cases with RIN >7.0 were included in this study.

PD-L1 protein expression in immunohistochemistry (IHC) was evaluated in obtained tumor samples from the independent cohort using the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies, Santa Clara, California, USA) and the 22C3 ant-PD-L1 antibody (Merck & Co., Kenilworth, New Jersey, USA).[17] In short, the PD-L1 protein expression is determined by Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. The CPS was evaluated by a board-certified pathologist.

Table 2 Clinical characteristics of relapsed bladder cancer patients after curative CRT

Variables	Total (n=48)		
	<12 months (n=16)	≧12 months (n=32)	P value
Gender (%)			
Male	14 (87.5)	27 (84.4)	0.77
Female	2 (12.5)	5 (15.6)	
Age (median (IQR))	72(63-75)	70(61-78)	0.75
ECOG-PS at pembrolizumab (%)			
0–1	13 (81.2)	28 (12.5)	0.57
>2	3 (18.8)	4 (87.5)	
Smoking history (%)			
Never	6 (37.5)	11 (34.4)	0.83
Former/current	10 (62.5)	21 (65.6)	
Body mass index (%)			
≥25	3 (18.8)	5 (15.6)	0.79
<25	13 (81.2)	27 (84.4)	
Pelvic lymph node metastasis at curative therapy (%)			
Yes	7 (43.8)	9 (28.1)	0.28
No	9 (56.2)	23 (71.9)	
Liver metastasis at pembrolizumab therapy (%)			
Yes	2 (12.5)	5 (15.6)	0.77
No	14 (87.5)	27 (84.4)	
Visceral metastasis at pembrolizumab therapy (%)			
Yes	10 (62.5)	25 (78.1)	0.26
No	6 (37.5)	7 (21.9)	
Hemoglobin concentration at first pembrolizumab therapy (%)			
>100 g/L	8 (50.0)	14 (43.8)	0.68
≤100 g/L	8 (50.0)	18 (56.2)	
NLR at first pembrolizumab therapy (%) (median (IQR))	5.50 (4.24–12.1)	4.31 (2.72–7.47)	0.14

ECOG-PS, Eastern Cooperative Oncology Group Performance Status; NLR, neutrophil-to-lymphocyte ratio.

WES and RNA sequence

All the procedures for the library preparation were conducted by humanoid robotic crowd biology with Maholo LabDroids at Robotic Biology Institute (Tokyo, Japan).[18] For the WES library, exome capture was performed using xGen series with Exome Research Panel V.1.0 (Integrated DNA Technologies) and libraries were generated using KAPA Hyper plus kit (KAPA Biosystems) according to the manufacturer's protocol. For the RNA-seq library, NEBNext rRNA Depletion Kit was used for the rRNA depletion, followed by the library amplification using NEBNext Ultra IIDirectional RNA Library Prep Kit for Illumina (New England Biolabs) according to manufacturers' protocol. All WES and RNA sequencing was performed on the Illumina Novaseq6000 platform with a paired-end flow cell platform (2×150 bp for WES and 2×100 bp for RNA sequence). All the analyses were performed using the supercomputing resource provided by SHIROKANE (https://gc.hgc.jp/) (Human Genome Center, the Institute of Medical Science, the University of Tokyo).

For the WES analysis, mutation calling was conducted through GenomonPipeline:2.6.3 (https://github.com/Genomon-Project/GenomonPipeline). In short, bwa:0.7.8 (https://github.com/lh3/bwa) was used for the mapping of FASTA on GRCh38. Detection of duplicate alignment was performed by samtools:1.2 (https://github.com/samtools/samtools). Mutation calling was performed using GenomonFisher:0.2.1 (https://github.com/Genomon-Project/GenomonFisher), followed by filtering false-positive somatic mutations from cancer genome sequencing data by Genomon Mutation Filter (https://github.com/Genomon-Project/GenomonMutationFilter). Mutation annotation format was conducted through Genomon Mutation Annotator (https://github.com/Genomon-Project/GenomonMutationAnnotator) and ANNOVAR:20210202.[19]

For the RNA-seq analysis, STAR:2.5.2a (https://github.com/alexdobin/STAR) was used for the mapping of FASTA on GRCh38. Then, featureCounts (SUBREAD): 2.0.1 (http://subread.sourceforge.net/) was adopted to determine the read counts in Transcripts Per Kilobase

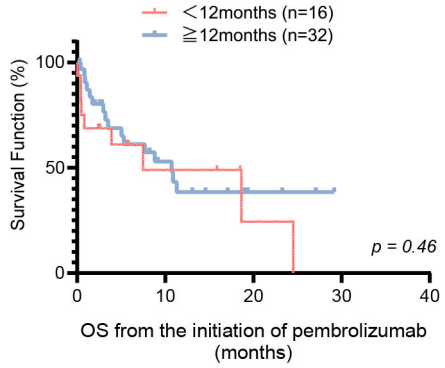

Figure 3 Kaplan-Meier curve for overall survival (OS) from the initiation of pembrolizumab according to the duration from the administration of CRT to pembrolizumab treatment. CRT, chemoradiation therapy.

Million. Gene set enrichment analysis (GSEA) software was used to compare whether gene expressions for the specific pathway are significantly different between CRT-naïve and CRT-recurrent tumors.[20] For the acquisition of immune-related pathways, IMMUNE_RESPONSE (235 genes: GO0006955) and IMMUNE_SYSTEM_PROCESS (332 genes: GO0002376) were extracted from the Molecular Signatures Database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). The CD274 mRNA expression among the isoforms was assessed by StringTie (https://github.com/gpertea/stringtie) platform that assembles a new transcript isoform from the mapped data and quantifies the expression level of each isoform in Fragments Per Kilobase of exon per Million mapped reads format, followed by visualizing the box-plots among the isoforms using Ballgown R package (https://github.com/alyssafrazee/ballgown).

RESULTS

Figure 1 represents the study design in this study. Out of 212 patients, we first extracted the clinical records of 91 patients who had undergone the radical treatment including CRT and TC for the treatment of localized BC (cT1-4N0-2M0) excluding patients with UTUC (86 patients), de novo M1 (22 patients), and no radical treatment to BC (13 patients). There were 48 and 43 patients who had undergone CRT and TC with curative intent for their localized BC (cT1-4N0-2M0), respectively. Of 91 patients, 41 patients were deceased during their follow-up. OS from the initiation of pembrolizumab in the CRT treatment group was significantly shorter than TC treatment group (the median OS of 10.7 and 13.0 months in CRT and TC groups, respectively, p=0.034). ORR and PFS were 27.1% and 4.5 months in the 48 CRT group, and 37.2% and 7.2 months in the 43 TC group. Patients treated with CRT were more likely to have visceral metastasis (p=0.003), lower hemoglobin level (p=0.003), and higher NLR (p=0.01) than those with TC, implying aggressive profiles in the CRT group (table 1).

Therefore, to reduce biases due to potential confounders that could affect treatment outcomes between CRT and TC treatment groups, propensity score matching was performed using putative factors as shown in figure 1, from which 30 patients in each arm were identified as pair-matched groups. In the pair-matched cohort (60 patients), all the variables had no significant difference between CRT and TC treatment groups. Twenty-five patients (41.7%) died during their follow-up. As shown in figure 2, Kaplan-Meier curves exhibited no significant difference in OS from the initiation of pembrolizumab between CRT and TC treatment groups (the median OS of 'not reached' and 10.9 months in CRT and TC groups, respectively: p=0.80). Similarly, there was no significant difference in PFS between the treatment groups (the median PFS of 4.0 and 3.3 months in CRT and TC groups, respectively: p=0.42). One-year OS rates were 51.2% in the CRT group and 46.8% in the TC group. With regards to the ORR, patients treated with CRT exhibited the ORR of 40.0% (12/30 patients: CR in one patient and PR in 11 patients), which showed no significant difference with the ORR of 33.3% in patients treated with TC (10/30 patients: CR in three patient and PR in seven patients) (p=0.59).

In the 48 patients who underwent CRT, the time from CRT to the initiation of pembrolizumab extensively ranged from 3.3 to 89.1 months (median of 16.5 months). Thus, to investigate whether the time from CRT to the initiation of pembrolizumab affects treatment outcomes, we divided 48 patients into two groups according to the cut-off of 12 months. There were 16 (33.3%) and 32 (66.6%) patients who were offered pembrolizumab <12 months and ≥12 months after the CRT, respectively. The median time from CRT to the initiation of pembrolizumab was 4.4 months in 16 patients and 28.5 months in 32 patients. Table 2 shows the patient characteristics in the two groups, and no significant difference was observed between the groups. Kaplan-Meier curves showed no significant difference in OS from the initiation of pembrolizumab between the two groups (the median OS of 7.5 and 10.7 months in <12 and >12 months groups, respectively: p=0.46) (figure 3). Similarly, no significant difference in PFS between the two groups was observed (the median PFS of 3.5 and 4.2 months in <12 and >12 months groups, respectively: p=0.52). For the ORR, patients who started pembrolizumab within 12 months from the CRT exhibited the ORR of 31.3% (5/16 patients: CR in no patients and PR in 5 patients), which showed no significant difference with the ORR of 25.0% in patients who started pembrolizumab ≥12 months after the CRT (8/32 patients: CR in one patient and PR in 7 patients) (p=0.65). We also assessed the shorter cut-off of 6 months from the CRT (online supplemental table 1) and confirmed the similar OS between the patients with the cut-off of 6 months from the CRT to the initiation of pembrolizumab (p=0.28) (online supplemental figure 1).

We next assessed whether CRT-resistant tumor cells have distinct PD-L1 expression level compared with the

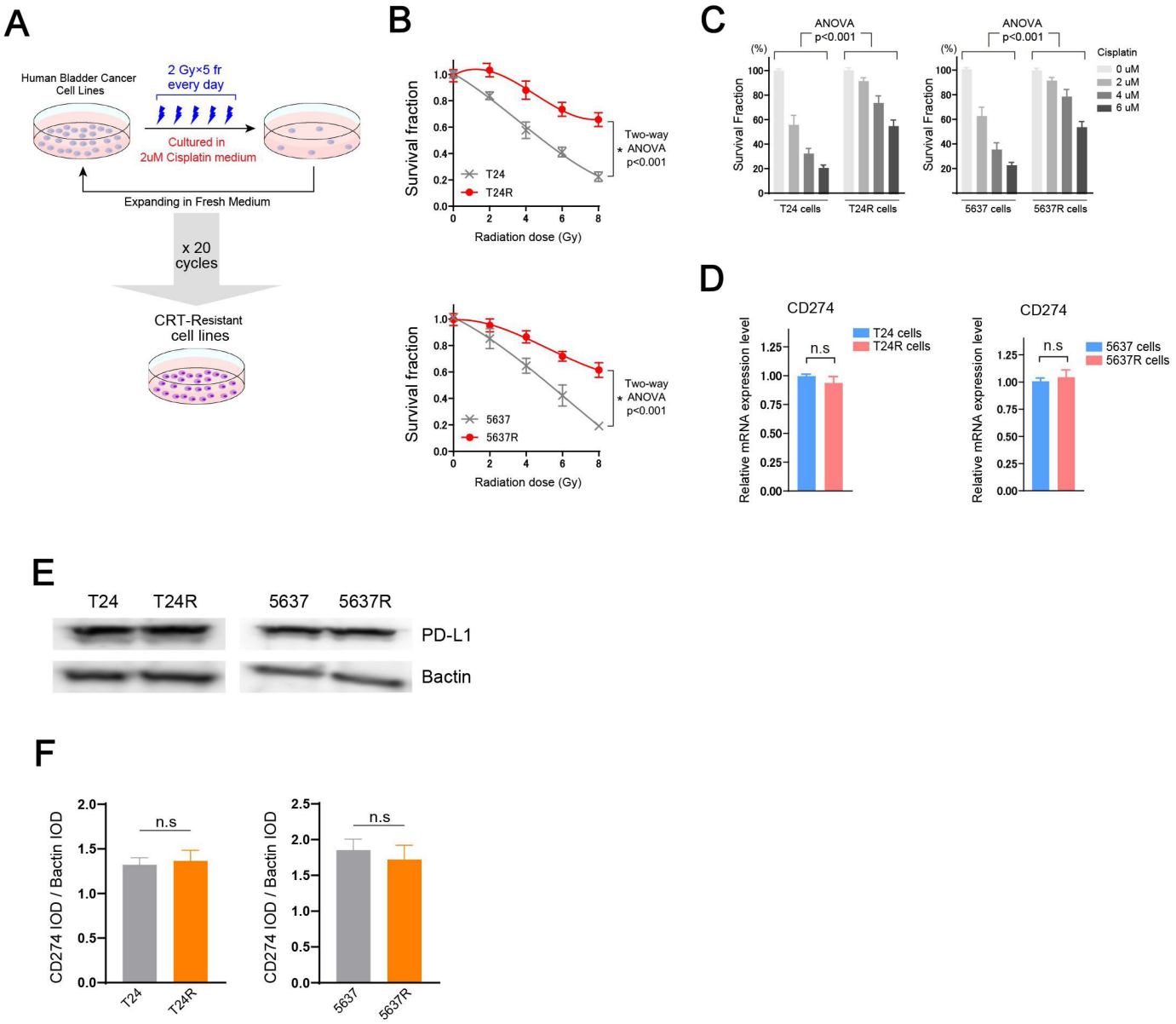

Figure 4 (A) Schematic representation of the protocol for establishing T24R and 5637R CRT-resistant BC cell lines. Parent T24 and 5637 BC cell lines were treated with IR (2 Gy / 5 Fraction ×20 cycle: Total 100 Gy) and 2 µM of cisplatin. (B) BC cell lines were treated with IR in the indicated dose, followed by the measurement of cell viability assay after 6 days. The inhibitory effect on cell growth by the IR is presented as a relative value (mean±SD) compared with control (0 Gy) as 100%. (C) Cells were treated with cisplatin at the indicated concentration for 96 hours and then collected to measure cell viability. Survival fraction was determined using the value without the treatment (0 µM of cisplatin) in each cell as a control. Results are shown as mean±SD. (D) The quantitative PCR for the mRNA expression level of CD274 in indicated BC cells. Data are shown as mean±SD. (E) Immunoblotting of PD-L1 antibody in T24, T24R, 5637, and 5637 R cells. B-actin was loaded as an internal control. (F) Quantitative evaluation by integrated optical density (IOD) for the immunoblotting was performed in three independent experiments, and results are shown as mean+SD.

CRT-naïve tumor cells. We first sought to develop CRT-resistant BC cell lines. T24 and 5637 BC cells were treated with IR (2 Gy / 5 Fraction ×20 cycle: Total 100 Gy) and 2 µM of cisplatin, and CRT-resistant clone was established as T24R and 5637 R cell lines (figure 4A). These resistant cells exhibited significantly decreased sensitivity to IR and cisplatin treatment (figure 4B,C). Importantly, CD274 mRNA expression level was comparable between parent and resistant cells (figure 4D). PD-L1 protein expression level in the resistant cells was also similar to the parent cells (figure 4E,F), suggesting that resistance to CRT does not affect the PD-L1 expression level in the tumor cells.

To further explore the association between CRT-resistant tumor and CD244/PD-L1 expression profiles, we used an independent clinical cohort (n=289) that offers a corresponding next-generation sequencing of WES and RNA-sequence in each individual (table 3). There were 22 clinical samples (7.6%) collected at the disease progression after CRT. There was no significant difference in CD274 mRNA expression level between

Table 3 Clinicopathological characteristics in 289 BC patients at the collection of biospecimens

Variables	n=289
Sex (%)	
Male	231 (79.6)
Female	58 (20.0)
Age (median (IQR))	71(62–79)
Median follow-up period (months)	21
Clinical stage (%)	
cN0M0	237 (82.0)
cN1M0	35 (12.1)
cNxM1	17 (5.9)
Muscle invasion (%)	
NMIBC	91 (31.5)
MIBC	198 (68.5)
Histological variants (%)	
No	259 (89.6)
Yes	30 (10.4)
Concomittant CIS (%)	
No	242 (83.7)
Yes	47 (16.3)
Pathological grade (WHO2004)	
Low	36 (12.5)
High	253 (87.5)
Lymphovascular invasion	
No	128 (44.3)
Yes	161 (55.7)
Prior BCG therapy (%)	
No	261 (90.3)
Yes	28 (9.7)
Prior CRT (%)	
No	267 (92.4)
Yes	22 (7.6)

BC, bladder cancer; CIS, carcinoma in situ; CRT, chemoradiation therapy; MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer.

CRT-naive (267 cases) and CRT-recurrent (22 cases) tumors (figure 5A). For exonic mutation analysis, CD274 mutation was called in nine cases (3.1%), of which seven cases were synonymous mutation (L50L). There were two cases of R260C non-synonymous mutation for CD274 in CRT-naive BC tumors, whereas no actionable mutation was detected in CRT-recurrent tumors. Of 289 clinical samples, six patients offered match-paired samples (pre-CRT and post-CRT). When comparing the CD274 mRNA expression level between those paired samples, the mRNA expression level was comparable regardless of the pre-CRT and post-CRT tumors (figure 5B). We next assessed the composition of CD274 isoforms in CRT-naïve and CRT-recurrent tumors (figure 5C, online supplemental table 2). Seven types of CD274 isoforms were detected in 289 tumor samples. The compositions of CD274 isoforms were comparable among the v1–v7 isoforms between CRT-naïve (n=267) and CRT-recurrent (n=22) tumors (figure 5D). We also assessed whether the synonymous mutation (L50L of CD274) affects the regulation of the splicing process, causing distinct CD274 isoforms. There was no significant difference in the ratio of the CD274-isoform expression level between the L50L (n=7) and the other (n=282) samples in all isoforms (v1–v7) (online supplemental figure 2A, table 3). We further investigated GSEA to assess whether immune-related pathways are modified in CRT-recurrent tumors. As shown in figure 5E, we referred to two immune-related gene sets (IMMUNE_RESPONSE of 235 genes: GO0006955 and IMMUNE_SYSTEM_PROCESS of 332 genes: GO0002376), and no significant difference was observed in both gene sets.

Lastly, PD-L1 protein expression was explored. In the independent cohort (289 BC samples), 266 samples were eligible to evaluate the PD-L1 CPS. There was a positive correlation between CD274 mRNA expression level and PD-L1 CPS in 266 BC tumors (spearman's rank correlation coefficient: 0.381, 95% CI 0.270 to 0.482, p<0.0001) (figure 5F). The ratio of PD-L1 CPS >10 was 22.2% (59/266 samples) (figure 5G). Importantly, there was no significant difference in PD-L1 CPS between CRT-naïve (n=248) and CRT-recurrent (n=18) tumors. Of the 266 CPS-determined samples, three patients had match-paired (pre-CRT and post-CRT) samples, and all six samples were diagnosed as CPS=0 (online supplemental figure 2B), suggesting no change of PD-L1 protein expression level in CRT-recurrent tumors.

DISCUSSION

The results from the KEYNOTE–045 trial with >2 years follow–up exhibited a longer median OS of 10.1 months (95% CI 8.0 to 12.3 months) with pembrolizumab treatment than the second-line chemotherapy (median OS of 7.3 months).[21] The trial enrolled 270 patients in the pembrolizumab treatment group, in which the primary site of the tumor was the upper tract (renal pelvis or ureter) in 38 patients (14.1%) and lower tract (bladder or urethra) in 232 patients (85.9%). Of note, 61 of 270 patients (22.6%) had undergone prior TC or nephroureterectomy, whereas it is not described how many patients had experienced prior CRT as a radical treatment in the trial. In this study, we focused on 91 patients who had previously undergone CRT (48 patients) or TC (43 patients) for the treatment of cT1-4N0-2M0 BC. We sought to assess whether the survival benefit from pembrolizumab treatment differs between the two types of radical treatments including TC and CRT in our multi-institutional dataset of UC patients. Adjusting the effect of confounding factors between treatment arms by propensity score matching identified the pair-matched cohort of 60 patients with no significant differences among all variables between treatment arms, which allowed us to compare the survival outcomes from the initiation of

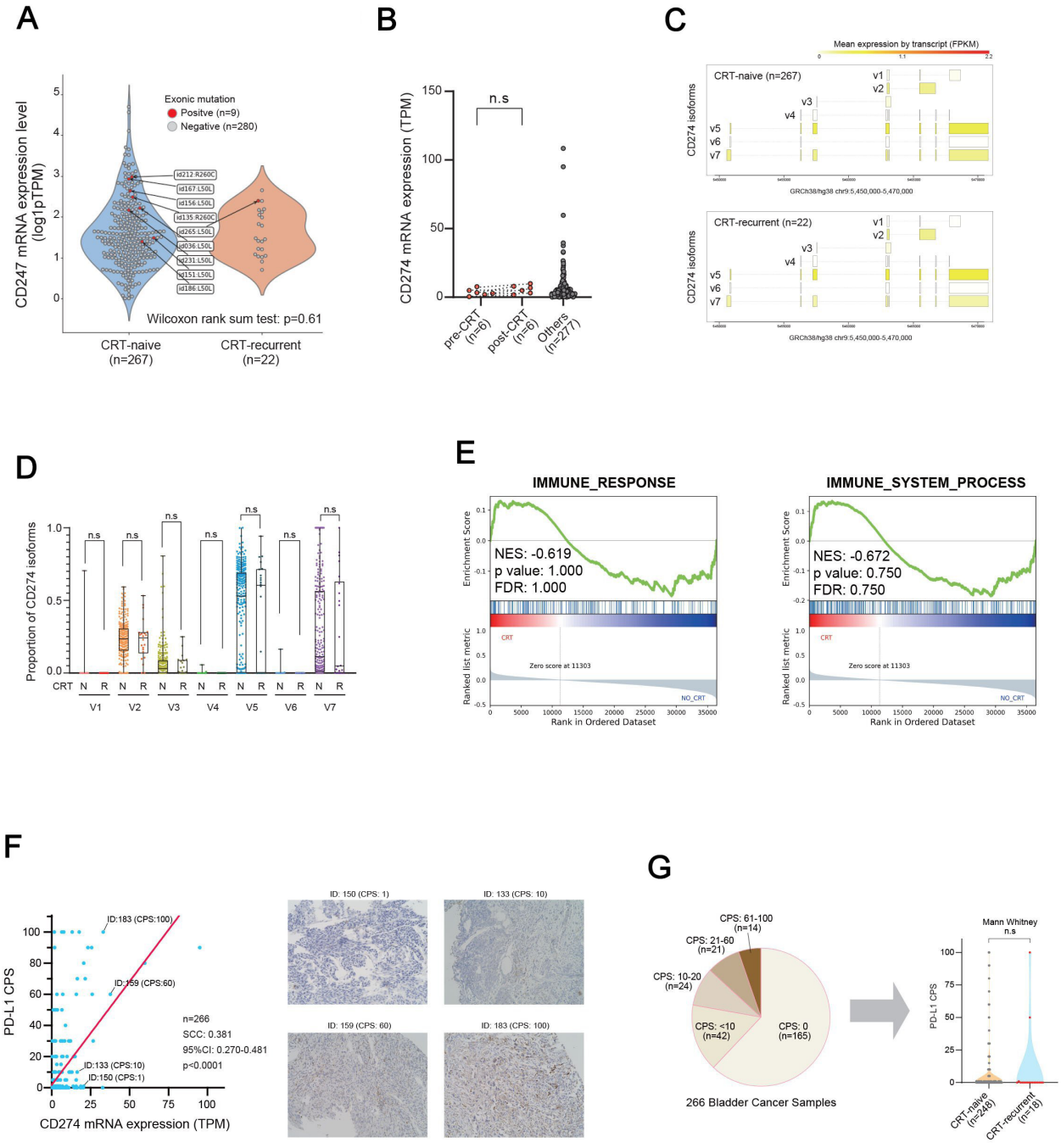

Figure 5 (A) Violin plots of CD274 mRNA expression level with exonic mutations in 289 BC clinical samples. (B) CD274 mRNA expression (Transcripts per million) in six patients with pair-matched samples (pre-CRT and post-CRT: 12 samples) and 277 UC samples. Wilcoxon matched-pairs signed rank test was used to assess the difference between the paired-matched samples (p=0.06, 'n.s' denotes 'not significant'). (C) Schematic of the CD274 isoforms (v1–v7) in 289 UC tumors. Each mRNA expression of CD274 isoforms is colored by the mean expression of FPKM (Fragments Per Kilobase of exon per Million mapped reads) format in CRT-naïve (n=267) and CRT-recurrent (n=22) tumors. (D) Proportion of each CD274 isoform (v1–v7) was compared between CRT -N (naïve: 267 samples) and -R (recurrent: 22 samples) from the RNAseq dataset. The Box-and-whisker plot represents the median, quartile, and range values. Mann-Whitney U test was used to assess the difference in isoform expression level between CRT-N and CRT-R tumors ('n.s' denotes 'not significant'). (E) GSEA enrichment profiles of IMMUNE_RESPONSE (235 genes: GO0006955) and IMMUNE_SYSTEM_PROCESS (332 genes: GO0002376) pathway as annotated in MSigDB between CRT-naive and CRT-recurrent BC. (F) The correlation of PD-L1 CPS (combined positive score) and CD274 mRNA expression level in 266 clinical BC samples. Spearman's rank correlation coefficient (SCC) with 95% CI was calculated. The right panel shows the representative images of PD-L1 CPS. (G) Left panel: pie chart of the PD-L1 CPS (combined positive score) in 266 BC samples. Right panel: The PD-L1 CPS in CRT-naïve (n=248) and CRT-recurrent (n=18) tumors. Mann-Whitney U test was performed to assess the difference (p=0.09). BC, bladder cancer; CPS, Combined Positive Score; CRT, chemoradiation therapy; GSEA, gene set enrichment analysis; MSigDB, Molecular Signatures Database; TPM, transcripts per million.

pembrolizumab between the two sets of patients treated with CRT or TC as their radical treatments before the disease progression. Our results indicated no significant difference in the survival outcomes between the two patient groups.

The abscopal effect is characterized by the metastatic tumor regression observed at a distant site from the irradiated tumor,[22] which was first reported in 1956.[23] To date, a number of studies have reported this phenomenon in various types of cancer.[24] Mechanistically, several premises have been proposed for the abscopal effect. In short, radiation therapy induced the elevation of MHC class I molecules with neo-antigens from dying tumor cells and cytokine stimulation, which leads to the enhanced tumor infiltration of CD8 +CTLs.[25 26] With the emergence of PD-1/PD-L1 inhibitors, the augmented abscopal effect by modulating antitumor immunosuppression has been recognized in the real-world experience.[12 27] Sundahl *et al* reported the results of a randomized phase 1 trial combining pembrolizumab with either sequential or concomitant stereotactic body radiotherapy (SBRT) in mUC patients, in which pembrolizumab (200 mg, 3 weekly) was combined with SBRT (3×8 Gy, to one metastatic lesion), administered either sequentially (nine patients: prior to the first pembrolizumab cycle) or concomitantly (nine patients: prior to the third pembrolizumab cycle).[28] In their trial, the best overall response was defined as the secondary endpoint. Intriguingly, ORR of 0% and 44% at non-irradiated metastatic lesions were observed in sequential and concomitant SBRT groups, respectively. The Median OS of each group was 4.5 months for the sequential SBRT group and 12.0 months for the concomitant SBRT group. Despite the small sample size, they concluded that the effect of SBRT timing on efficacy is worth exploring further. With regard to the treatment of localized MIBC, KEYNOTE-992 (NCT04241185), a phase 3 randomized trial that evaluates the efficacy and safety of pembrolizumab +CRT versus placebo +CRT in patients with previously untreated MIBC, is now ongoing.[29] This study revealed that the treatment outcomes of pembrolizumab for patients previously treated with CRT as a radical treatment are comparable to patients treated with TC. Furthermore, when stratifying the CRT patients group according to the time from CRT to the initiation of pembrolizumab (cut-off: 6 and 12 months), the survival outcomes did not differ between the groups. These findings collectively suggest that the enhanced tumor regression by the combination of PD-1 inhibitor and CRT might be expected only in the concurrent administration.

PD-L1 expression level in the tumor has been considered a biomarker for predicting the treatment outcome of PD-1 and PD-L1 inhibitors. Technically, for mUC patients, the result of PD-L1 protein expression is determined by using CPS, which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. Recently, the result of KEYNOTE-052 investigating the first-line pembrolizumab in cisplatin-ineligible patients with locally advanced or mUC showed a significantly higher ORR of 47.3% in 110 patients with CPS ≥10 than ORR of 20.3% in 251 patients with CPS<10.[30] These results led to the approval of pembrolizumab by the FDA as the first-line treatment for cisplatin-ineligible patients with CPS ≥10. However, whether CRT-recurrent UC has a higher PD-L1 expression level than the primary tumor has been controversial. For example, Hecht *et al* reported that neoadjuvant CRT is associated with an increased PD-L1 expression in rectal adenocarcinoma patients.[31] In contrast, Fujimoto *et al* investigated the PD-L1 expression level in pre-CRT and post-CRT clinical samples in non-small cell lung cancer and revealed that the percentage of PD-L1-positive tumor cells significantly decreased after CRT.[32] In this study, we explored the next-generation sequencing data of WES and RNA-seq in 289 UC clinical samples. There seemed to be no difference in CD274 mRNA expression level between CRT-naïve and CRT-recurrent tumors. We also demonstrated neither actionable mutations nor the variant isoforms of CD274 in CRT-recurrent tumors, indicating that CD274/PD-L1 status is not affected by CRT in UC. We further revealed that PD-L1 protein expression is positively correlated with mRNA expression level in BC, consistent with the data in lung and colorectal cancers.[33] We confirmed that PD-L1 CPS does not change in CRT-recurrent tumors. These findings are consistent with our propensity-score matched analysis that the treatment outcomes of pembrolizumab are not associated with the type of radical treatments (TC or CRT) for mUC patients.

The results from the current research should be interpreted considering several limitations. First, this study was conducted in a retrospective design. In addition, since the analyses in this study did not include de novo M1 UC patients, the median follow-up of those patients from the initiation of pembrolizumab treatment was relatively short compared with the previous studies. Second, the findings in the current study were still subject to selection bias, which we sought to address by using a propensity score-matched model to approximate random assignment. Residual unmeasured confounding factors may have affected the clinical outcomes in this study. Third, radiographic and pathological diagnoses were not centralized. Fourth, discontinuation of pembrolizumab was not standardized among the institutes throughout the study.

In conclusion, we assessed whether the survival benefit of pembrolizumab differs between mUC patients previously treated with TC or CRT as radical treatment. The efficacy of pembrolizumab for mUC patients previously treated with CRT was similar to those treated with TC. When stratifying patients treated with CRT into two groups according to the duration from CRT of >12 months, the survival outcomes also did not differ between the two groups. Large-scale and prospective studies are further warranted to prove the result from the current study.

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