Pilot trial of topical MTS-01 application to reduce dermatitis in patients receiving chemoradiotherapy for stage I-III carcinoma of the anal canal

DEBORAH CITRIN[1], LUCA VALLE[2], KEVIN CAMPHAUSEN[1], THERESA COOLEY-ZGELA[1], DEEDEE SMART[1], MICHAEL YAO[3], JAMES B. MITCHELL[4], WILLIAM THOMPSON[5], IRINI SERETI[5] and THOMAS ULDRICK[6,7]

[1]Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD 20892; [2]Department of Radiation Oncology, University of California Los Angeles, Los Angeles, CA 90045; [3]Gastroenterology and Hepatology Section, Washington DC VA Medical Center, Washington, DC 20422; [4]Radiation Biology Branch, Center for Cancer Research, NCI, NIH, Bethesda, MD 20892; [5]Laboratory of Immunoregulation, NIAID, Bethesda, MD 20814; [6]HIV and AIDS Malignancy Branch, Center for Cancer Research, NCI, NIH, Bethesda, MD 20892; [7]Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

Received January 31, 2022; Accepted March 30, 2022

DOI: 10.3892/ijo.2022.5358

Abstract. The purpose of the present trial was to determine the feasibility of the daily topical application of the piperidine nitroxide, MTS-01, combined with chemoradiotherapy in the treatment of patients with anal carcinoma. The secondary study endpoints were the description of the effects of this agent on skin toxicity and rectal-associated lymphoid tissue. The participants received radiotherapy concurrent with mitomycin-C and 5-fluorouracil for carcinoma of the anal canal. MTS-01 was applied to the bilateral inguinal area and the gluteal cleft. Dermatologic and non-dermatologic toxicity was graded throughout the treatment period. Circulating lymphocytes were serially collected for phenotyping. Rectal mucosal snag biopsies were collected at baseline and at 1 year of follow-up. A total of 5 patients received topical MTS-01. Adverse events attributed to MTS-01 included asymptomatic grade 1 hypoglycemia and grade 1-2 diarrhea. Dermatitis within untreated, radiated skin was not more severe than dermatitis in MTS-01-treated, unirradiated skin. Circulating $CD4^+$ lymphocyte suppression was noted at >1 year following treatment in human immunodeficiency virus-negative participants. $CD4^+$ lymphocytes remained suppressed in the irradiated rectal mucosa at 1 year, whereas the $CD8^+$ lymphocyte numbers recovered or increased. On the whole, the present study demonstrates that the MTS-01 topical application was tolerable with minimal toxicity. Chemoradiation for anal cancer led to prolonged $CD4^+$ lymphocytopenia in the circulation and gut mucosa.

Correspondence to: Dr Deborah Citrin, Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute (NCI), National Institutes of Health (NIH), 10 CRC/B2-3500, 10 Center Drive, Bethesda, MD 20892, USA
E-mail: citrind@mail.nih.gov

Key words: anal carcinoma, radiation, dermatitis, MTS-01, Tempol

Introduction

There are ~8,300 cases and 1,280 related deaths due to carcinoma of the anal canal each year in the United Sates (1). The current standard treatment for the majority of anal cancers includes the use of 5-fluorouracil (5-FU) and mitomycin-C (MMC) delivered concurrently with intensity modulated radiation therapy (IMRT), with the aim of anal sphincter preservation (2). The use of IMRT compared to 3-dimensional conformal radiotherapy (3D-CRT) in the treatment of anal carcinoma has been shown to reduce acute grade 3 or higher skin toxicity (from 49 to 23%), reduce grade 3 or higher gastrointestinal toxicity (from 36 to 21%), and reduce grade 2 or higher hematologic toxicity (2,3). Regardless, the morbidity associated with treatment with chemoradiation for anal carcinoma remains substantial. Aside from an impact on the quality of life of patients, severe toxicity during chemoradiotherapy for anal cancer can lead to a need for treatment breaks and an extension in treatment duration, which has been associated with an increased local recurrence and higher colostomy rates (2).

Radiation dermatitis is one of the most common severe toxicities observed during chemoradiation for anal cancer. In Radiation Therapy Oncology Group (RTOG) 0529 (2), 75% of patients with anal carcinoma treated with chemoradiation experienced grade 2 or higher dermatologic toxicity, and 23% experienced grade 3 or higher dermatologic toxicity. The perineal, perianal, genital and inguinal regions are at a higher risk of skin breakdown and radiation dermatitis due to the numerous skin folds contributing to a 'bolus' effect that increases skin dose and the inherent moisture in the area. In addition to causing significant pain and discomfort for patients, severe dermatitis can lead to an environment conducive to superinfection.

MTS-01 (Tempo1; 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a nitroxide oxygen radical scavenger that has been formulated as a topical gel (tempol 70 mg/ml in water, ethanol and hydroxylpropyl cellulose). The nitroxides are a class of stable free radical compounds that exhibit antioxidant activity, protecting mammalian cells against hydrogen peroxide, superoxide and t-butyl hydroperoxide cytotoxicity (4-7). Tempol has been shown to protect against lethal total body radiation exposures, while having no effect on the tumor radioresponse (8-11). The MTS-01 formulation of Tempol has been found to protect against radiation-induced skin toxicity, specifically alopecia, in animal models and clinical studies (12-14). The possible mechanisms of Tempol radioprotection include the oxidation of reduced transition metals, superoxide dismutase-like activity, and the scavenging of oxy- and carbon-based free radicals (15).

The primary objective of the present study was to assess the safety and tolerability of delivering a topical Tempol application on a daily basis prior to irradiation in the inguinal area and gluteal cleft of patients receiving combined therapy with MMC, 5-FU and radiation therapy for carcinoma of the anal canal. The secondary objectives included the description of the severity of skin toxicity with this regimen and the need for treatment breaks.

Patients and methods

Patients with histologically proven invasive primary squamous carcinoma of the anal canal, stage T1-4, N0-3, M0, with no previous therapy for anal cancer were eligible for this National Cancer Institute Institutional Review Board-approved clinical trial (NCT01324141; registered on March 28, 2011). All research was performed in accordance with relevant guidelines and regulations. All studies reported were outlined in an informed consent document signed by all participants. The study subjects were >18 years of age with an Eastern Cooperative Oncology Group (ECOG) performance status ≤2 and adequate bone marrow, renal and hepatic functions. Patients with human immunodeficiency virus (HIV) and a CD4 T-cell count >100 cells/μl and an ECOG performance status <2 were eligible.

Participants were simulated in the supine position at 1 h following oral contrast administration with a marker placed at the anal verge. CT images were obtained through the pelvis and inguinal regions. Contouring or targets and critical structures was performed using Eclipse software (v4, Varian Medical Systems, Inc.) and based on the RTOG Consensus guidelines for rectal and anal cancer planning (16). Radiation fractionation, the total dose to target structures and normal tissue constraints (described in Data S1) were based on RTOG 0529. As per these guidelines, the anal canal with a 2.5 cm expansion received a dose of 50.4-54 Gy in 28 fractions and the elective nodal regions (mesorectal, inguinal, external iliac and internal iliac) received 42-45 Gy in 28 fractions. Radiation was delivered as a single daily fraction, 5 days per week. Treatment breaks were allowed for grade 4 skin reactions, absolute neutrophil counts <500/mm^3, grade 3 diarrhea, grade 3 vomiting, or localized or generalized infection.

MMC was delivered intravenously at a dose of 10 mg/m^2 (maximum 20 mg) on days 1 and 29. 5-FU was delivered at the dose of 1,000 mg/m^2/day as a 96-h continuous venous infusion on days 1 and 29. Radiotherapy commenced concurrently with chemotherapy (day 1) using IMRT.

To guide the MTS-01 application, an anterior projection of the body surface with the prescription isodose volumes [primary planning target volume (PTV) and nodal PTV] was generated using Eclipse software v4 (Fig. 1). Treatment planning images were reviewed to assess skin dose and to guide MTS-01 application. The corresponding areas were outlined with markers on the skin surface prior to the first MTS-01 application and maintained throughout treatment. The gluteal cleft region and a 3 cm radius from the anal verge border were also marked. Up to 100 ml MTS-01 (70 mg/ml, Mitos Pharmaceuticals, Inc.) was applied uniformly to the patient's targeted skin area 15-30 min prior to each fraction of radiation by trained personnel. Tempol was withheld if the patients were unable to tolerate the agent, if moist desquamation occurred within the treatment area, or if other grade 3 or 4 toxicities deemed related to Tempol manifested. In total, two control sites (2x2 cm each) were marked (Fig. 1), including a site receiving radiation only without MTS-01 (left inguinal area, C1) as well as a site outside of the treatment field receiving Tempol only (umbilical area, C2). The use of Aquaphor and Biafine® cream was allowed; however, these agents were not applied prior to daily treatment.

Adverse events (AE) were assessed throughout treatment and until 4 weeks of follow-up using the Common Terminology Criteria for Adverse Events (CTCAE v4.0, https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf). The attribution of AEs from standard chemoradiation, MTS-01 or research other components were assessed. Skin toxicity was evaluated at five different sites (L inguinal, R inguinal, gluteal cleft, C1 and C2) weekly using the RTOG Acute Radiation Morbidity Scoring Schema provided by the study principal investigator, DC. All five sites were professionally photographed weekly during treatment and at multiple intervals during follow-up to allow for toxicity scoring by a second blinded radiation oncologist. The validated brief pain inventory questionnaire (17) was administered over the same time points, and additional exploratory laboratory tests and clinical lymphocyte phenotyping assay were conducted throughout treatment and follow-up.

Optional rectal mucosal snag biopsies were obtained during flexible sigmoidoscopy performed at baseline and at 12 months following the completion of treatment, and processed to a single cell suspension as previously described (18,19) and as detailed in Data S1. Cells extracted from the biopsy specimens were stained for 30 min at room temperature with the following antibodies: anti-CD3 PE-Cy7 (Clone: SK7, cat. no. BD-341101, Becton, Dickinson and Company), anti-CD4 APC-Cy7 (Clone: SK3, cat. no. BD341105), anti-CD8 PacBlue (Clone: RPA-T8, cat. no. MHCD0828, Invitrogen; Thermo Fisher Scientific, Inc.), anti-CD8 PerCP (Clone: SK1, cat. no. MABF1687 EMD Millipore). All antibodies were used at 50% of the manufacturer's recommended dilution. The proportion of CD4$^+$ and CD8$^+$ T-cells in tissue was assessed after gating in CD3$^+$ cells. Absolute numbers of CD4$^+$ and CD8$^+$ T-cells per gram of gut tissue were calculated by dividing the viable cell count by the tissue weight. This number was then multiplied

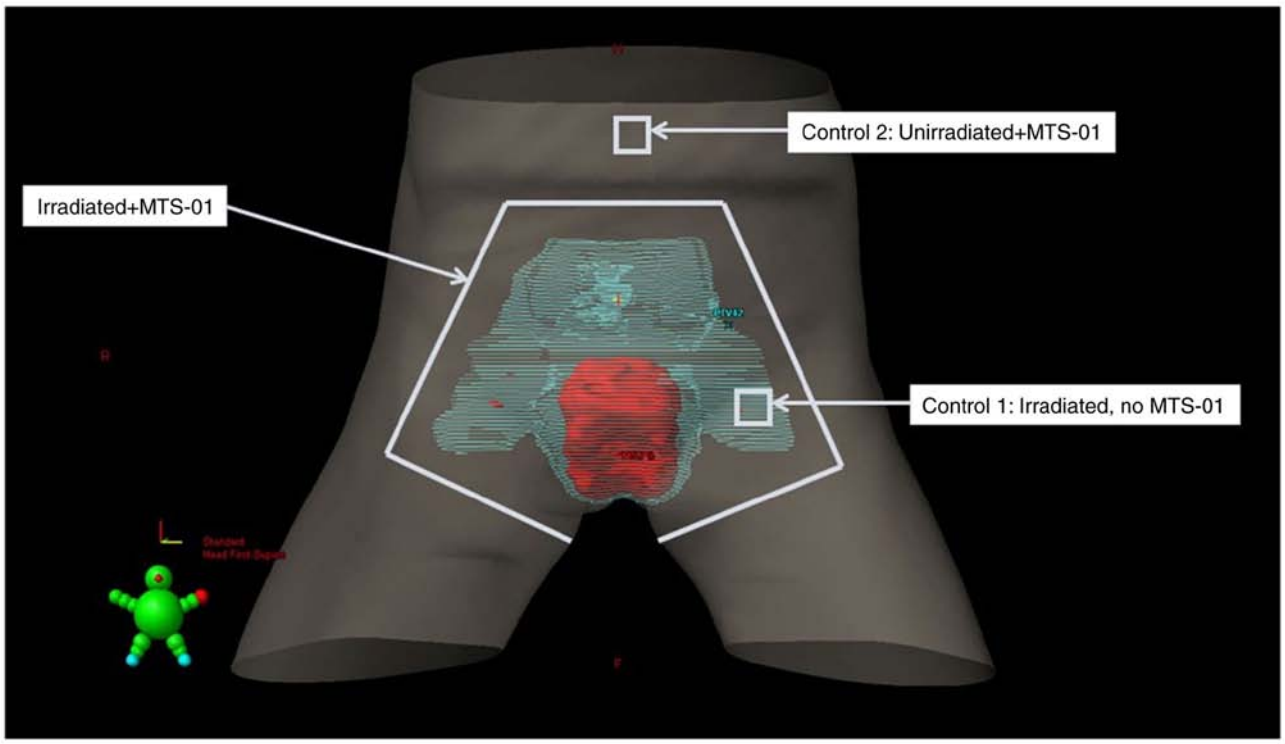

Figure 1. MTS-01 application site. A surface rendering of the patient's body was generated in the Eclipse treatment planning system. The planning target volume for the pelvic lymph nodes (blue) and the primary tumor planning target volume (red) were overlaid and the dose to the skin was reviewed. An MTS-01 treatment area was selected based on these volumes and the dose received by the skin in this area. A 2x2 cm control site was selected in the inguinal area (Control 1) where no MTS-01 was applied. A second control site was selected near the umbilicus outside of the radiation field where MTS-01 was applied was also selected (Control 2). MTS-01 was applied to the areas outlined in white.

by percentages obtained from flow cytometric analysis (LSRII Flow Cytometer, Becton, Dickinson and Company), to determine the absolute cell count of the T-cell subsets.

Aliquots of plasma were collected prior to treatment (baseline) and course 1 (day 28) and stored at -80˚C until use. The concentrations of interleukin (IL)-7, transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor A (VEGF-A) in plasma were determined using Meso Scale Discovery multiplex chemiluminescent assays as per the manufacturer's recommended protocol, and analyzed using a S6000 Instrument (Meso Scale Diagnostics LLC).

Results

A total of 5 patients were enrolled in the study. All participants completed chemoradiation and MTS-01 treatment. The patient demographics are summarized in Table I. The median age of the study participants was 57 years (range, 49-63 years). In total, 1 patient was African-American and 4 were Caucasian, and 1 patient had HIV. In addition, 4 patients had stage II and 1 patient had stage III disease [American Joint Committee on Cancer (AJCC) 7th edition (20)] (Table I). The study was closed early due to slow accrual.

AEs attributed to MTS-01 were rare, with the majority of AEs being attributed to chemotherapy or radiation. There were no dose-limiting toxicities. In all cases, toxicities possibly attributed to MTS-01 were also possible toxicities of chemoradiotherapy or concurrent medications. For example, the only grade 3 toxicities possibly attributable to MTS-01 were a decrease in the $CD4^+$ T cell count in a single patient, and a single brief episode of grade 3 fatigue, which were also attributable to chemoradiotherapy. Another patient experienced grade 2 diarrhea, and all remaining toxicities were grade 1, including fatigue and hypoglycemia (Table II).

There were several grade 3 or higher AEs attributed to either IMRT, MMC or 5-FU treatment that are summarized in Table II. Radiation dermatitis was more severe in the perianal area where MTS-01 was not applied, with all patients developing grade 2 or higher dermatitis in that area. Other common non-hematologic AEs of chemoradiotherapy included diarrhea, perianal and perineal pain, nausea, vomiting and dysuria. There was one incidence of a skin infection and one urinary tract infection. All participants had hematologic AEs, including grade 3+ leukopenia, lymphocytopenia and CD4 count decreases, while 3 patients had neutropenia, including an episode of febrile neutropenia. None of the 5 patients required a treatment break.

As this trial aimed to assess gut-associated lymphoid tissue as an exploratory endpoint, the serial lymphocyte phenotyping of blood was performed as a comparator for tissue studies throughout treatment and follow-up. As expected with chemoradiotherapy, leukopenia was pronounced soon following treatment initiation (Fig. 2A). Leukocyte counts recovered to a normal range at 12 months of follow-up in only 2 patients. A similar trend was observed in lymphocyte counts following treatment initiation, with increasing lymphocyte counts over time (Fig. 2B); however,

Table I. Patient demographics.

Characteristic	No. of patients (%)
Sex	
Male	2 (40)
Female	3 (60)
Age, years	
Range	49-63
Median	57
Race	
African-American	1 (20)
Caucasian	4 (80)
ECOG status	
0	3 (60)
1	2 (40)
2	0 (0)
3	0 (0)
4	0 (0)
5	0 (0)
HIV status	
Positive	1 (20)
Negative	4 (80)
HPV status (anal swab)	
Positive	0 (0)
Negative	5 (100)
T stage (AJCC 7th edition)	
T1	0 (0)
T2	2 (40)
T3	3 (60)
T4	0 (0)
N stage (AJCC 7th edition)	
N0	4 (80)
N1	0 (0)
N2	0 (0)
N3	1 (20)
Disease stage (AJCC 7th edition)	
I	0 (0)
II	4 (80)
IIIA	0 (0)
IIIB	1 (20)
IV	0 (0)

AJCC, American Joint Committee on Cancer (20); HIV, human immunodeficiency virus; HPV, human pappilomavirus.

none of the 4 remaining patients in the study recovered to a normal lymphocyte range at 1 year after completing treatment. A more profound decrease in circulating lymphocytes was observed in the $CD4^+$ lymphocyte subset relative to the $CD8^+$ lymphocytes (Fig. 2C and D).

In total, 1 patient (HIV-positive, stage T4 disease) developed rapid disease progression outside of the radiation field following treatment and was removed from the study. The remaining 4 patients are alive and relapse-free with no evidence of disease throughout the duration of follow-up.

All 5 patients experienced radiation dermatitis in the radiation treatment field. Radiation dermatitis within the MTS-01-treated areas and the control areas was assessed at each time point during examination by the treating physician (Fig. 3A) or by a blinded observer reviewing professional medical images (Fig. 3B). The mean RTOG acute skin toxicity score at each site per time point was similar with both techniques of assessment. In the umbilicus control (C2), there was no noticeable reaction at any timepoint in any patient with either examiner.

Toxicity in the MTS-01-treated gluteal cleft was more severe than that in other assessed sites (Fig. 3C), with 1 patient developing grade 3 dermatitis in the gluteal cleft, 2 patients developing grade 2 dermatitis and 2 patients developing grade 1 dermatitis only. Toxicity in the radiation-treated (no MTS-01) area in the left inguinal region was less than that observed in the remainder of the MTS-01-treated left inguinal area. The most severe dermatologic toxicity in the inguinal regions tended to be the most medial areas, and the control site (C2) was generally situated more laterally in the inguinal region, possibly explaining the slightly reduced toxicity scoring at that site. In general, the severity of dermatitis increased until peaking at 6 weeks following treatment initiation, and the time of maximum toxicity was not obviously different between the sites (Fig. 3D). A total of 3 patients developed grade 2 dermatitis in the inguinal regions, whereas 2 patients developed grade 1 only.

To describe global pain during treatment, the brief pain inventory was administered weekly during treatment and in the subsequent follow-up period. As demonstrated in Table III, pain was most severe at the completion of treatment, 6 weeks after initiating therapy. At this time point, pain was also most refractory to relief from medication and interfered most with daily activities (Table III).

Rectal mucosal biopsies were obtained from 3 consenting patients at baseline and at 1 year following the completion of treatment. The analysis of lymphocyte subsets in these biopsy tissues revealed a reduction in $CD3^+$ and $CD4^+$ T-cells in all patients at the 1-year follow-up (Fig. 4A). By contrast, the numbers of $CD8^+$ cells largely recovered at the 1-year follow-up time point. Simultaneously collected blood was analyzed, demonstrating a consistent decline in $CD4^+$ lymphocytes in tissue and circulation at 1 year following treatment relative to baseline levels (Fig. 4B). By contrast, although $CD8^+$ lymphocytes were reduced in the circulation relative to baseline, $CD8^+$ lymphocytes were similar or increased relative to baseline levels. This association was more clearly demonstrated when comparing the $CD4^+/CD8^+$ ratio (Fig. 4C) and suggests that the $CD8^+$ lymphocyte subset was more effectively regenerated in irradiated tissue compared to the circulation and compared to $CD4^+$ lymphocyte subsets (representative flow cytometry plots for these data are available from the corresponding author on reasonable request).

As aforementioned, leukopenia and lymphopenia were rapid and often profound with the chemoradiotherapy delivered in this trial. The evaluation of cytokines known to play a role in lymphopoiesis were analyzed at baseline vs. the

Table II. Adverse events observed in the present study trial.

Type of adverse event	MTS-01 Grade 1	MTS-01 grade 2	MTS-01 Grade 3	5-FU/MMC/IMRT Grade 2	5-FU/MMC/IMRT Grades 3-4
Non-hematologic					
Radiation dermatitis				2	3
Nausea				3	
Vomiting				2	
Abdominal pain				1	
Diarrhea	1	1		1	2
Gastroesophageal reflux				1	
Mucositis				1	
Urinary tract pain				2	
Bladder spasm				1	
Urinary incontinence				1	
Fatigue	1		1	1	
Hypoglycemia	1				
Transaminitis				1	
Hypoalbuminemia				1	
Hypocalcemia				1	
Myalgia				1	
Headache					1
Syncope					1
Infection				1	1
Insomnia				1	
Pain				2	2
Hematologic					
Leukopenia					5
Lymphopenia					5
Neutropenia				2	3
Febrile neutropenia					1
Thrombocytopenia				2	
Anemia				3	
CD4 count decrease			1		

5-FU, 5-fluorouracil; MMC, mitomycin-C; IMRT, intensity modulated radiation therapy.

Table III. Brief pain inventory scores.

	Baseline		Treatment week 3		Treatment week 6		1-Month follow-up		3-Month follow-up	
Pain inventory	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Worst pain	4.8	0-10	3.5	0-8	7.2	4-10	3.1	0-9	2.1	0-5
Least pain	1.4	0-4	1.5	0-4	3.6	1-5	1.2	0-3	2.8	0-5
Average pain	1.8	0-4	1.9	0-3.5	5.0	2-7	2.0	0-6	3.0	0-6
Pain at time of survey	1.6	0-6	1.3	0-5	5.4	2-9	2.2	0-6	2.2	0-5
% Pain relief after medication	90.0	60-100	93.8	85-100	44.0	10-90	89.0	75-100	83.0	50-100
Pain interference	1.73	0.33-3.89	3.00	0.44-8.00	6.70	2.89-9.44	3.17	0-8.33	1.87	0-4.56

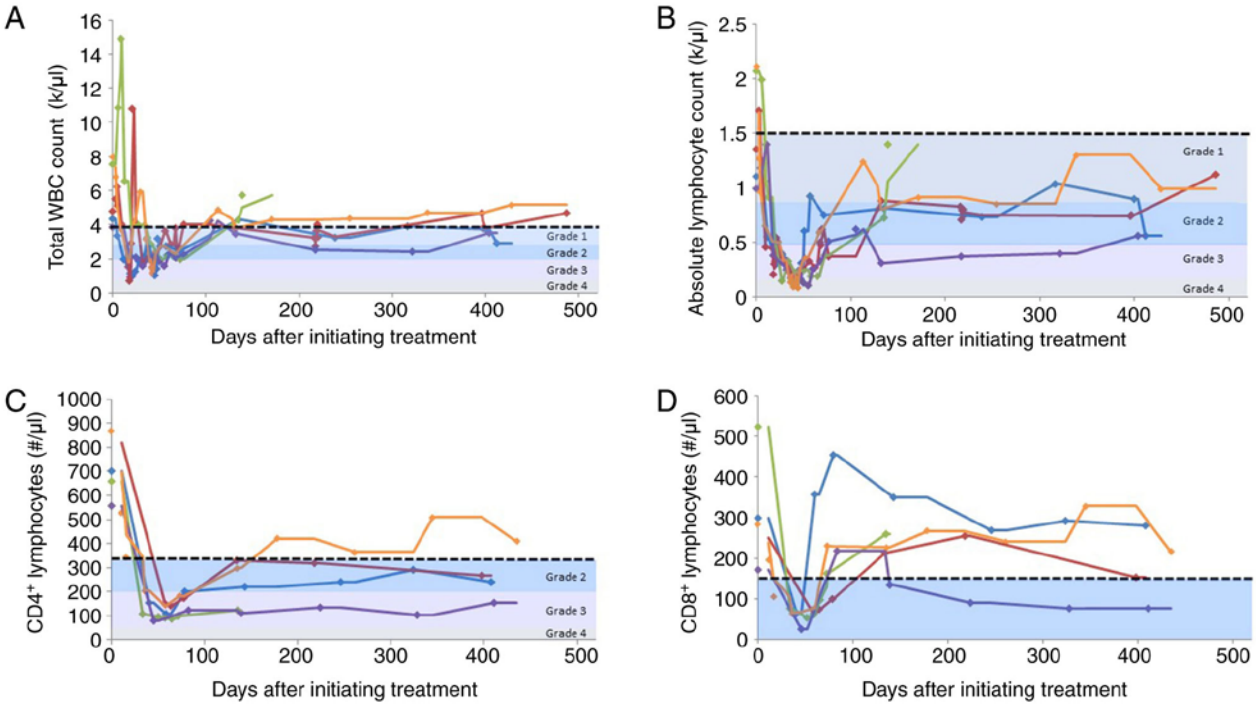

Figure 2. Circulating leukocyte and lymphocyte counts during and after treatment. Complete blood counts and lymphocyte phenotyping by a clinical laboratory was conducted weekly during chemoradiation and then at varying intervals throughout the duration of follow-up. Individual patients are color-coded consistently across the graphs for (A) total white blood cell count, (B) absolute lymphocyte count, (C) $CD4^+$ lymphocyte count, and (D) $CD8^+$ lymphocyte count. The lower limit of normal (based on the clinical laboratory that conducted the assay) for each measure is noted as a hashed line. CTCAE v4.0 toxicity grades are noted, with the exception of $CD8^+$ lymphocytes, where CTCAE toxicity was not defined. The data of the 1 patient who was positive for human immunodeficiency virus are represented by the green-colored line. CTCAE, Common Terminology Criteria for Adverse Events.

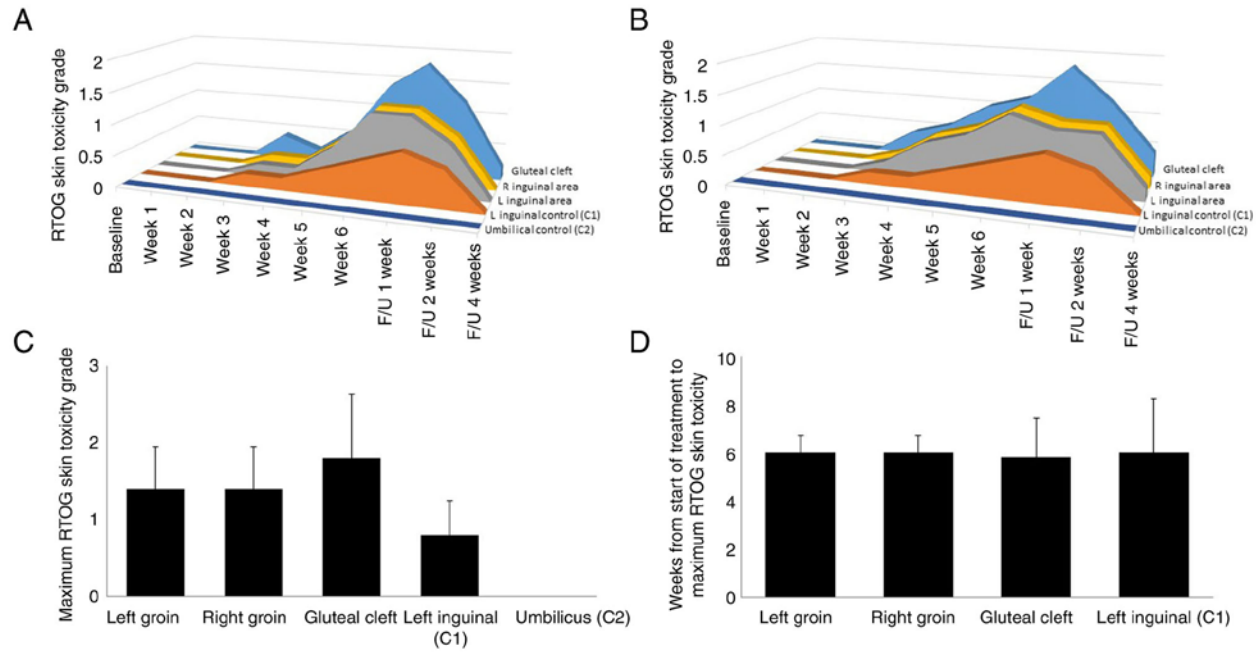

Figure 3. RTOG Skin Toxicity Grading. RTOG acute skin toxicity in MTS-01 treatment and control areas was graded at baseline and weekly during treatment, and then at weeks 1, 2 and 4 of follow-up. Each site was scored separately by (A) a single treating physician at the point of care or (B) by a blinded radiation oncologist via review of professionally acquired medical photographs and presented as a mean of scores for all participants at each time point. Maximum grade of RTOG skin toxicity (C) and time to highest grade acute skin toxicity (D) are graphed for each site as a mean of all patients with standard deviation. RTOG, Radiation Therapy Oncology Group.

end of the first course of chemotherapy (course 1, day 28). In 4 of the 5 participants, the IL-7 levels increased at course 1 (day 28) relative to baseline levels, whereas the TGF-β1 concentrations in the circulation decreased universally. No clear patterns were observed in the plasma concentrations of TNF-α and VEGF (Fig. 4D).

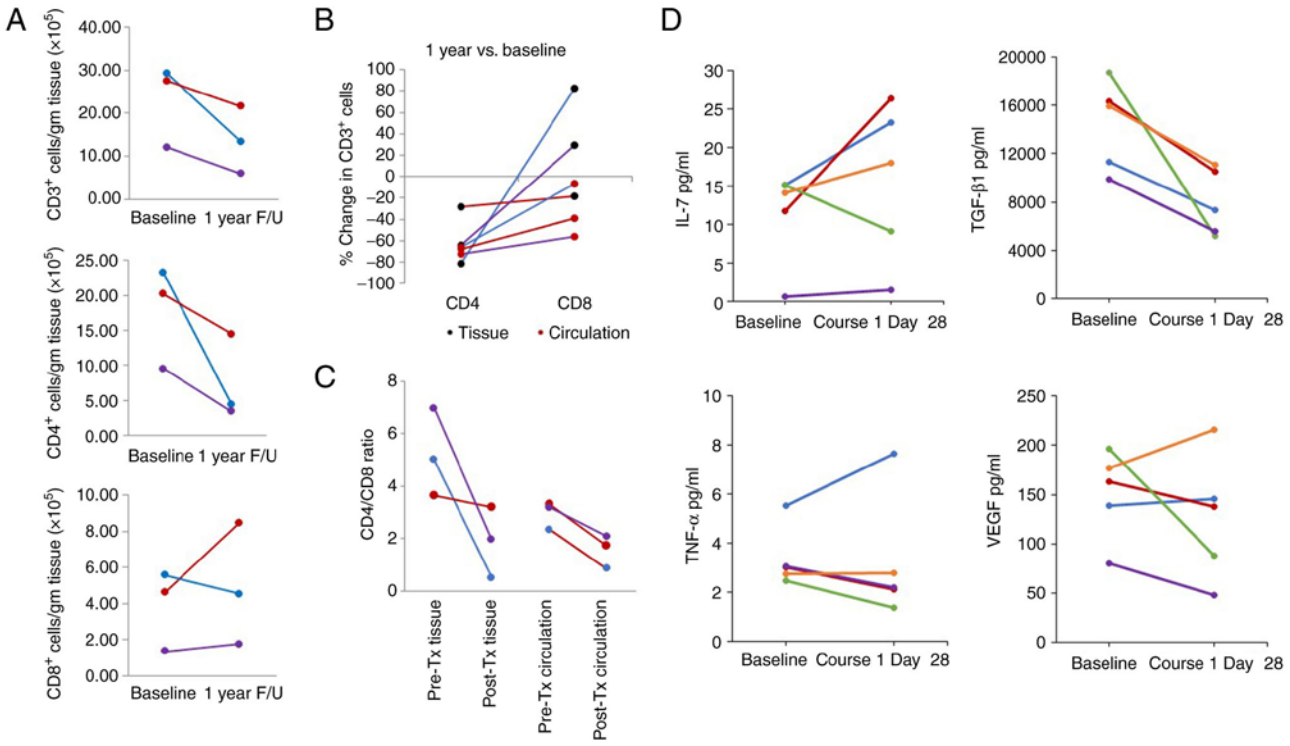

Figure 4. Circulating and peripheral lymphocyte subsets. Biopsy tissue from the colon and blood was collected at baseline and at 1 year of follow-up. Tissue was dissociated to individual cells, fixed and subjected to flow cytometric analyses for cell surface markers (representative flow cytometry plots for these data are available from the corresponding author on reasonable request). All patients who consented to the tissue biopsy were HIV-negative. Colors correspond to individual patients, and those reported in Fig. 1. (A) The numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cell subsets were determined per gram of rectal mucosal tissue obtained in the biopsy using flow cytometry. The proportion of $CD4^+$ and $CD8^+$ T-cells in tissue was assessed after gating in $CD3^+$ cells. (B) The percentage change in $CD4^+$ and $CD8^+$ lymphocytes between baseline and 1 year post-treatment was compared between tissue and the circulation. (C) The ratio of $CD4^+$ lymphocytes to $CD8^+$ lymphocytes was calculated in baseline tissue and blood relative to 1 year of follow-up. (D) Plasma concentrations of IL-7, TGF-β1, TNF-α and VEGF were measured in plasma obtained at baseline and at day 28 following the initiation of treatment. The data of the 1 patient who was HIV-positive are represented by the green-colored line. F/U, follow-up; HIV, human immunodeficiency virus; IL-7, interleukin 7; TGF-β1 transforming growth factor-β1; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

Discussion

The primary objective of the present study was to assess the safety and efficacy of delivering topical MTS-01 daily prior to irradiation in the bilateral inguinal area and gluteal cleft of patients receiving combined therapy with MMC, 5-FU and radiation therapy for carcinoma of the anal canal. In the present phase I study on 5 patients, minimal toxicity was noted with the application of MTS-01. A strong signal of efficacy was not demonstrated, although there are significant limitations to this observation in this small-scale study. The present study reported a long-term decrease in leukocyte counts, specifically $CD4^+$ lymphocytes, associated with standard chemoradiation for anal carcinoma, with evidence of $CD8^+$ lymphocyte persistence or recovery in tissue relative to circulation and relative to $CD4^+$ lymphocytes. Chemoradiotherapy combined with MTS-01 led to a universal decrease in TGF-β1 levels.

The present study employed several methods to increase the likelihood of determining the efficacy of radioprotection. Dermatitis was scored in real-time by a single trained physician using control sites in each patient. A blinded observer assessed response using deidentified professionally captured medical photographs of the sites scored for toxicity. These images were collected in identical locations, with identical lighting conditions and identical photography equipment.

However, there were also several limitations to the ability to demonstrate the efficacy of MTS-01 as a radioprotector of skin in the present study. An inherent challenge in the evaluation of topical radioprotectors is the difficulty of predicting locations of severe dermatitis in an individual patient. The inguinal control site (no MTS-01 applied) was situated in the center of the inguinal region, below the inguinal fold, and was specifically selected to reduce the chance of MTS-01 contamination of the site during hip flexion when patients rose to walk to radiation treatment following the MTS-01 application. Although the skin surrounding this control site, where MTS-01 was not applied, often had less dermatitis than the more medial portions of the inguinal region, toxicity was scored based on the most severe toxicity within the inguinal region, which may have resulted in toxicity grading spuriously appearing to reflect less toxicity at the inguinal control site (MTS-01 not applied, radiated) relative to the remaining inguinal areas. Thus, even if zones of toxicity can be accurately predicted, control sites must be carefully selected to ensure accurate comparisons of efficacy, the simultaneous goals of both minimizing contamination and ensuring toxicity grading accounts for regional variation in dermatitis. The inclusion of only 5 patients prior to study closure also prevented firm conclusions regarding the efficacy of MTS-01. Regardless, the lessons learned from the

techniques utilized in the present study may be useful in designing future studies assessing topical radioprotectors or mitigators.

Despite an inability to demonstrate a reduction in dermatitis with MTS-01, there was minimal toxicity to its application, even in the setting of evolving dermatitis. Consistent with the findings of other clinical trials in cancer patients receiving topical formulations of Tempol, the most commonly reported AEs were gastrointestinal, constitutional, dermatological and metabolic (14). These toxicities of MTS-01 are also commonly associated with chemoradiation for carcinoma of the anal canal, such as diarrhea, mild hypoglycemia and fatigue. With the caveat of a limited sample size, the minimal MTS-01-associated systemic toxicities, and the consistent lack of AEs at the umbilical control site, suggest that the topical application of MTS-01 has limited toxicity in this clinical setting. Prior clinical and preclinical studies have suggested that systemic exposure is negligible following the topical application of this formulation (12-14). Out of concern that desquamation at the site of application may increase the likelihood of systemic absorption, MTS-01 was withheld in any region with moist desquamation; thus, the safety in the setting of severe skin toxicity remains uncertain.

The lack of systemic absorption of MTS-01 in previous research (14) is encouraging, not only as it limits potential toxicity, but also as it is unlikely to adversely impact tumor control. Although the assessment of Tempol on the tumor response to radiation was not an end point of the present study, 4 out of the 5 patients were alive, with no evidence of disease at the extended follow-up. Future studies on MTS-01 are required however, to address this important issue in a larger group of patients.

Although hematologic toxicity is frequently described as a consequence of chemoradiation for carcinoma of the anal canal, a strength of the present study was a more comprehensive evaluation of lymphocytopenia in a small patient subset. All participants underwent the serial assessment of blood counts and lymphocyte phenotyping. In addition, the four HIV seronegative participants without progression who were follow-up for 1 year following the completion of therapy were noted to have prolonged lymphocytopenia. Lymphocytopenia was largely due to prolonged decreases in the numbers of $CD4^+$ T-cells, while circulating $CD8^+$ lymphocytes recovered in the majority of patients to a normal range within weeks following treatment. These finding were true even in the setting of increases in the levels of the homeostatic cytokine, IL-7, suggesting that physiological responses to lymphocytopenia may be inadequate to promote CD4 reconstitution over a period of 1 year after standard chemoradiation for anal cancer.

Previous research has demonstrated the suppression of $CD4^+$ lymphocytes in HIV-positive individuals following chemoradiotherapy for anal cancer (21). Indeed, a lower post-treatment CD4 count has been associated with an increased risk of local recurrence following chemoradiation for anal cancer in HIV-positive patients (22). However, there are limited data available on the prevalence or impact of CD4 suppression following treatment in patients without HIV. The majority of trials demonstrating lymphopenia and a decrease in the CD4 count following chemoradiation in HIV-negative patients have no follow-up of patients beyond 4-12 weeks (23-25); thus, the prolonged suppression observed herein is notable. If validated in larger patient cohorts, this observation may have important implications for the long-term monitoring and care of patients who receive chemoradiotherapy for anal cancer. Further studies are required to determine the reproducibility and relevance of this additional therapeutic toxicity.

A notable component of the present study is the assessment of $CD4^+$ and $CD8^+$ lymphocytes in rectal mucosal biopsies at 1 year following irradiation compared to baseline levels. Although both circulating CD4 and CD8 cells remained suppressed at 1 year, only the numbers of $CD4^+$ lymphocytes were reduced in rectal tissue at 1 year. Preclinical studies and limited human tissue studies suggest that although T-cells do not account for the majority of accumulated cells in irradiated tissue (26), they are capable of orchestrating immune responses through effector mechanisms that drive chronic inflammatory diseases with pathologies similar to those observed after irradiation (27). An altered balance in T-cell subsets has been implicated as a possible contributor to radiation injury (27), such as in animal models of radiation proctitis (28). The capacity to observe these changes in the rectal mucosa acquired from asymptomatic individuals suggests that these changes occur as a consequence of therapy even in asymptomatic individuals and are not merely a marker of proctitis.

The design of the present study does not allow for the ruling out of the possibility that these profound and sustained immunosuppressive effects were related to MTS-01 administration. However, other clinical studies evaluating MTS-01 have not identified prolonged immune effects, and the available literature that has described the lymphocyte count and CD4 count suppression when combining chemotherapy and radiotherapy for the treatment of other cancers further supports that the causative agents are chemotherapy and radiation (29).

Another observation was that treatment with chemoradiation combined with MTS-01 led to a decrease in plasma levels of TGF-β1. TGF-β1 has been implicated in the pathogenesis of human papillomavirus-associated malignancies (30), with elevated levels being associated with a poor prognosis of patients with cervical cancer treated with chemoradiation (31), as well as radiation lung toxicity in non-small cell lung cancer (32). The impact of MTS-01 on these findings is unknown and the further evaluation of plasma TGF-β1 as a biomarker is thus warranted in anal cancer and studies evaluating MTS-01.

In conclusion, as demonstrated herein, MTS-01 is tolerable when used to manage dermatotoxicity in patients with localized anal cancer undergoing chemoradiation. The lack of an efficacy signal that was noted to be due to the inadequate sample size and control site selection, were significant factors in the decision to close the study to accrual early. A more suitable control site that would not be subject to easy cross-contamination with MTS-01, but would be expected to develop severe dermatitis, could not be identified. Regardless, there are important lessons to be learnt for future studies evaluating a topical radiation protector and attempting to integrate a control site. Despite the sample size, there are several interesting hypotheses generating findings related to treatment induced CD4 lymphocytopenia and TGF-β1 that provide subjects for future studies.

Acknowledgements

The authors are grateful to Ms. Luz Giordano and Ms. Debbie McNally, both from the National Cancer Institute, for their contributions to the conduct of this trial. Both were involved in the application of the investigational agent and the conduct of research-related assessments. The trial registration number for the study is NCT01324141 (registered on March 28, 2011).

Funding

The present study was supported in part by the intramural research program of the National Cancer Institute, Center for Cancer Research (grant no. ZIA BC 010850).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DC and TU were involved in the conception and design of the study, in the acquisition of data, data analysis, interpretation of the data and in manuscript preparation. LV was involved in data analysis, interpretation of the data and in manuscript preparation. KC was involved the acquisition of data, data analysis and in manuscript preparation. TCZ, DS and MY were involved in the acquisition of data and in manuscript preparation. JBM was involved in the conception of the study and in manuscript preparation. WT was involved in data analysis and in manuscript preparation. IS was involved in the acquisition of data, data analysis, interpretation of the data and in manuscript preparation. DC and KC confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All analyses reported in the present study relating to human subjects were reviewed and approved by the National Cancer Institute Institutional Review Board. All studies reported were outlined in an informed consent document signed by all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fuchs HE and Jemal A: Cancer statistics, 2021. CA Cancer J Clin 71: 7-33, 2021.
2. Kachnic LA, Winter K, Myerson RJ, Goodyear MD, Willins J, Esthappan J, Haddock MG, Rotman M, Parikh PJ, Safran H and Willett CG: RTOG 0529: A phase 2 evaluation of dose-painted intensity modulated radiation therapy in combination with 5-fluorouracil and mitomycin-C for the reduction of acute morbidity in carcinoma of the anal canal. Int J Radiat Oncol Biol Phys 86: 27-33, 2013.
3. Ajani JA, Winter KA, Gunderson LL, Pedersen J, Benson AB III, Thomas CR Jr, Mayer RJ, Haddock MG, Rich TA and Willett C: Fluorouracil, mitomycin, and radiotherapy vs fluorouracil, cisplatin, and radiotherapy for carcinoma of the anal canal: A randomized controlled trial. JAMA 299: 1914-1921, 2008.
4. Mitchell JB, Samuni A, Krishna MC, DeGraff WG, Ahn MS, Samuni U and Russo A: Biologically active metal-independent superoxide dismutase mimics. Biochemistry 29: 2802-2807, 1990.
5. Samuni A, Krishna CM, Mitchell JB, Collins CR and Russo A: Superoxide reaction with nitroxides. Free Radic Res Commun 9: 241-249, 1990.
6. Samuni A, Godinger D, Aronovitch J, Russo A and Mitchell JB: Nitroxides block DNA scission and protect cells from oxidative damage. Biochemistry 30: 555-561, 1991.
7. Samuni A, Mitchell JB, DeGraff W, Krishna CM, Samuni U and Russo A: Nitroxide SOD-mimics: Modes of action. Free Radic Res Commun 12-13 Pt 1: 187-194, 1991.
8. Hahn SM, Tochner Z, Krishna CM, Glass J, Wilson L, Samuni A, Sprague M, Venzon D, Glatstein E, Mitchell JB, *et al*: Tempol, a stable free radical, is a novel murine radiation protector. Cancer Res 52: 1750-1753, 1992.
9. Hahn SM, Sullivan FJ, DeLuca AM, Krishna CM, Wersto N, Venzon D, Russo A and Mitchell JB: Evaluation of tempol radioprotection in a murine tumor model. Free Radic Biol Med 22: 1211-1216, 1997.
10. Cotrim AP, Hyodo F, Matsumoto K, Sowers AL, Cook JA, Baum BJ, Krishna MC and Mitchell JB: Differential radiation protection of salivary glands versus tumor by Tempol with accompanying tissue assessment of Tempol by magnetic resonance imaging. Clin Cancer Res 13: 4928-4933, 2007.
11. Cotrim AP, Yoshikawa M, Sunshine AN, Zheng C, Sowers AL, Thetford AD, Cook JA, Mitchell JB and Baum BJ: Pharmacological protection from radiation +/-cisplatin-induced oral mucositis. Int J Radiat Oncol Biol Phys 83: 1284-1290, 2012.
12. Goffman T, Cuscela D, Glass J, Hahn S, Krishna CM, Lupton G and Mitchell JB: Topical application of nitroxide protects radiation-induced alopecia in guinea pigs. Int J Radiat Oncol Biol Phys 22: 803-806, 1992.
13. Cuscela D, Coffin D, Lupton GP, Cook JA, Krishna MC, Bonner RF and Mitchell JB: Protection from radiation-induced alopecia with topical application of nitroxides: Fractionated studies. Cancer J Sci Am 2: 273-278, 1996.
14. Metz JM, Smith D, Mick R, Lustig R, Mitchell J, Cherakuri M, Glatstein E and Hahn SM: A phase I study of topical Tempol for the prevention of alopecia induced by whole brain radiotherapy. Clin Cancer Res 10: 6411-6417, 2004.
15. Mitchell JB, DeGraff W, Kaufman D, Krishna MC, Samuni A, Finkelstein E, Ahn MS, Hahn SM, Gamson J and Russo A: Inhibition of oxygen-dependent radiation-induced damage by the nitroxide superoxide dismutase mimic, tempol. Arch Biochem Biophys 289: 62-70, 1991.
16. Myerson RJ, Garofalo MC, El Naqa I, Abrams RA, Apte A, Bosch WR, Das P, Gunderson LL, Hong TS, Kim JJ, *et al*: Elective clinical target volumes for conformal therapy in anorectal cancer: A radiation therapy oncology group consensus panel contouring atlas. Int J Radiat Oncol Biol Phys 74: 824-830, 2009.
17. Cleeland CS and Ryan KM: Pain assessment: Global use of the brief pain inventory. Ann Acad Med Singap 23: 129-138, 1994.
18. Sereti I, Estes JD, Thompson WL, Morcock DR, Fischl MA, Croughs T, Beq S, Lafaye de Micheaux S, Yao MD, Ober A, *et al*: Decreases in colonic and systemic inflammation in chronic HIV infection after IL-7 administration. PLoS Pathog 10: e1003890, 2014.
19. Ciccone EJ, Greenwald JH, Lee PI, Biancotto A, Read SW, Yao MA, Hodge JN, Thompson WL, Kovacs SB, Chairez CL, *et al*: $CD4^+$ T cells, including Th17 and cycling subsets, are intact in the gut mucosa of HIV-1-infected long-term nonprogressors. J Virol 85: 5880-5888, 2011.
20. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A (eds). AJCC Cancer Staging Manual. 7th edition. Springer, New York, NY, 2010.
21. Alfa-Wali M, Allen-Mersh T, Antoniou A, Tait D, Newsom-Davis T, Gazzard B, Nelson M and Bower M: Chemoradiotherapy for anal cancer in HIV patients causes prolonged CD4 cell count suppression. Ann Oncol 23: 141-147, 2012.
22. Bryant AK, Mudgway R, Huynh-Le MP, Simpson DR, Mell LK, Gupta S, Sharabi AB and Murphy JD: Effect of CD4 count on treatment toxicity and tumor recurrence in human immunodeficiency virus-positive patients with anal cancer. Int J Radiat Oncol Biol Phys 100: 478-485, 2018.

23. Cattin S, Fellay B, Calderoni A, Christinat A, Negretti L, Biggiogero M, Badellino A, Schneider AL, Tsoutsou P, Pellanda AF and Rüegg C: Circulating Immune cell populations related to primary breast cancer, surgical removal, and radiotherapy revealed by flow cytometry analysis. Breast Cancer Res 23: 64, 2021.
24. Xi J, Hassan B, Katumba RGN, Khaddour K, Govindan A, Luo J, Huang J and Campian JL: The predictive value of absolute lymphocyte counts on tumor progression and pseudoprogression in patients with glioblastoma. BMC Cancer 21: 285, 2021.
25. Chen Y, Jin Y, Hu X and Chen M: Effect of Chemoradiotherapy on the proportion of circulating lymphocyte subsets in patients with limited-stage small cell lung cancer. Cancer Immunol Immunother 70: 2867-2876, 2021.
26. Citrin DE and Mitchell JB: Mechanisms of normal tissue injury from irradiation. Semin Radiat Oncol 27: 316-324, 2017.
27. Schaue D and McBride WH: T lymphocytes and normal tissue responses to radiation. Front Oncol 2: 119, 2012.
28. Linard C, Strup-Perrot C, Lacave-Lapalun JV and Benderitter M: Flagellin preconditioning enhances the efficacy of mesenchymal stem cells in an irradiation-induced proctitis model. J Leukoc Biol 100: 569-580, 2016.
29. Ellsworth SG: Field size effects on the risk and severity of treatment-induced lymphopenia in patients undergoing radiation therapy for solid tumors. Adv Radiat Oncol 3: 512-519, 2018.
30. Strauss J, Gatti-Mays ME, Cho BC, Hill A, Salas S, McClay E, Redman JM, Sater HA, Donahue RN, Jochems C, *et al*: Bintrafusp alfa, a bifunctional fusion protein targeting TGF-β and PD-L1, in patients with human papillomavirus-associated malignancies. J Immunother Cancer 8: e001395, 2020.
31. Dickson J, Davidson SE, Hunter RD and West CM: Pretreatment plasma TGF beta 1 levels are prognostic for survival but not morbidity following radiation therapy of carcinoma of the cervix. Int J Radiat Oncol Biol Phys 48: 991-995, 2000.
32. Anscher MS, Kong FM and Jirtle RL: The relevance of transforming growth factor beta 1 in pulmonary injury after radiation therapy. Lung Cancer 19: 109-120, 1998.