Data SI. Radiation therapy guidelines (verbatim from protocol)

Simulation. A custom immobilization device was used to minimize setup variability. Oral contrast will be delivered 30 min to 1 h prior to simulation. A radio-opaque anal marker will be placed at the anal verge at the time of simulation. Simulation will be performed with the patient prone or supine with arms at the level of or above the head. CT images will be obtained at a thickness of <5 mm for treatment planning. All tissues to be irradiated must be included in the CT scan.

Treatment volumes. The gross tumor volume (GTV) is defined as all gross disease determined from CT (and MRI and PET if performed), clinical examination, endoscopic findings and biopsy: i) GTVA includes the gross primary anal tumor volumes; ii) GTVN50.4 includes the involved nodal regions (by clinical examination, biopsy, and/or imaging) containing disease measuring <3.0 cm in greatest dimension; iii) GTVN54 includes all nodal disease measuring greater than 3.0 cm in greatest dimension.

The clinical target volume (CTV) is defined as the GTV plus areas considered to contain potential microscopic disease: i) CTVA includes GTVA, the anal canal and a 2.5 cm expansion. Volumes may be adjusted to remove bone or air; ii) CTV45 includes the uninvolved nodal regions with a 1 cm expansion. Volumes may be adjusted to remove, bone, air, genitourinary structures, and muscles); CTV50 includes nodal regions with involved nodes <3 cm with a 1 cm expansion. Volumes may be adjusted to remove, bone, air, genitourinary structures, and muscles); iv) CTV54 includes nodal regions with involved nodes measuring >3 cm with a 1 cm expansion. Volumes may be adjusted to remove, bone, air, genitourinary structures, and muscles).

The planning target volume (PTV) will provide a margin around CTV to account for treatment set up error and organ motion. A minimum margin of 1 cm in all dimensions is required. A nodal PTV should not overlap with PTVA (PTVA takes precedence). PTV should be automatically subtracted from the skin surface with a margin of 5 mm.

Definition of nodal regions. The definition of nodal regions was as follows: i) Mesorectal (includes peri-rectal and presacral); ii) inguinal (left and right); iii) external iliac (left and right); iv) internal iliac (left and right); iv) these volumes will be based on the RTOG consensus for rectal and anal cancer planning.

Normal structures to be contoured are the femoral heads, bladder, external genitalia, iliac crest, small bowel, large bowel (outside CTV) and perianal skin.

Treatment planning. Doses for T1-T2 N0 disease. i) PTVA: 50.4 Gy in 28 fractions (1.8 Gy per fraction); ii) PTV42: 42 Gy in 28 fractions (1.5 Gy per fraction). Will include all nodal regions.

Doses for T3 N0 disease. I) PTVA: 54 Gy in 30 fractions (1.8 Gy per fraction); ii) PTV45: 45 Gy in 30 fractions (1.5 Gy per fraction). Will include all nodal regions.

Doses for N+ disease. I) PTVA: 54 Gy in 30 fractions (1.8 Gy per fraction); ii) PTV45: 45 Gy in 30 fractions (1.5 Gy per fraction). Will include only uninvolved nodal regions; iii) PTV50: 50.4 Gy in 30 fractions (1.68 Gy per fraction). Will include all nodal regions containing involved nodes <3 cm; iv) PTV54: 54 Gy in 30 fractions (1.8 Gy per fraction). Will include all nodal regions containing involved nodes >3 cm.

Heterogeneity corrections should be used for treatment planning.

The dose goals are the following: i) No >5% of any PTV will receive <90% of the prescribed dose; ii) no >2% of the PTV will receive <80% of the prescribed dose; iii) no >2% of PTVA will receive >115% of the prescription dose.

The dose constraint goals are the following: i) Small bowel: <200 cc above 30 Gy, <150 cc above 35 Gy, <20 cc above 45 Gy, none above 50 Gy; ii) femoral heads: <50% above 30 Gy, <35% above 40 Gy, <5% above 44 Gy; iii) iliac crests: <50% above 30 Gy, <35% above 40 Gy, <5% above 50 Gy; iv) external genitalia:<50% above 20 Gy, <35% above 30 Gy, <5% above 40 Gy; v) bladder: <50% above 35 Gy, <35% above 40 Gy, <5% above 50 Gy; vi) large bowel: <200 cc above 30 Gy, <150 cc above 35 Gy, <20 cc above 45 Gy.

Treatment delivery. Treatment will be delivered once daily, Monday through Friday, with the exception of federal holidays. Breaks in treatment should be minimized. Megavoltage equipment capable of delivering intensity modulated radiation therapy will be used.

Treatment breaks. A rest period of <7 days will be allowed for grade 4 skin reactions. Radiation therapy will be held for the following indications if clinically appropriate: i) Platelets <50,000/mm3; ii) absolute neutrophil count <500/mm^3; iii) grade 3 diarrhea (>7 stools per day over baseline); iv) grade 3 vomiting; v) localized or generalized infection secondary to moist desquamation.

Rectal snag biopsy processing and analysis. Biopsies were weighed, placed in 500 μl medium containing RPMI (Mediatech, Inc.) with 10% human serum (Gemini Bio Products). Samples were then digested using 0.5 mg collagenase (Sigma-Aldrich; Merck KGaA) and 250 U benzonase (Novagen) for 40 min at 37˚C before being filtered through a 40 micron screen. After being washed twice with the 10% human serum medium, the resulting cell suspension was counted using a Beckman Coulter Counter to obtain the number of total viable 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 by percentages obtained from flow cytometric analysis (see below) to determine the absolute cell count of the T-cell subsets. 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. Samples were acquired (~200,000 events per sample) and analyzed.