Supplemental Materials: DMA, a small molecule increases median survival and reduce radiation-induced xerostomia via activation of ERK1/2 pathway in Oral Squamous Cell Carcinoma

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Supplementary Methods

M1. Histological studies of tissue sections of HK-1 xenograft and PDX mice models

The Crl: NU (NCr)-Foxn1^{nu}mice was sacrificed at the end of the study by a*sphyxiation* from *carbon dioxide* (CO_2). Organ perfusion was performed by injecting approximately 20mL of PBS into the left ventricle of the mice. The tumor and the organs such as the intestines, lungs, and spleen were collected after sacrifice, washed with PBS, soaked in blotting paper, and immediately fixed in 10% neutral formalin buffer. Formalin-fixed tissues were trimmed and dehydrated using increasing grades of alcohol (70%, 95%, and 100%), cleared with xylene, and finally infiltrated with paraffin. The 5µm tissues were sectioned using a rotary microtome (Leica RM2245) and stained with hematoxylin and eosin (H&E).

M2. Immunohistochemistry of AQP5, Ki-67, and CD44 positive cells in HK-1 xenograft and PDX mice model

Tissue sections from the tumor, lungs, small intestine, and spleen samples (three animals/group) were evaluated for Ki-67 analysis, the salivary gland was assessed for aquaporin 5 (AQP5) expression and tumor tissue for CD44 by randomly selecting three fields per tissue section. Formalin-fixed paraffin-embedded tumor tissues were sectioned at 4–6 µm thickness using a rotary microtome (Leica RM2245), and the sections were mounted on microscopic slides. Tissue sections were deparaffinized by warming on a flattening table (Leica) at 60°C for 10 min, followed by xylene treatment. Sections were rehydrated with ethyl alcohol at gradient concentrations of 100%, 90%, and 80%, respectively, followed by washing with PBS-T washing buffer (0.3% Triton X-100 in PBS). After rehydration, antigen retrieval was performed by incubation in citrate buffer (0.01M, pH 6.0) at 90–95°C for 20 min. Tissue sections were then subjected to the blocking of endogenous peroxidase activity by incubating the sections in a 3% H_2O_2 solution in PBS at 25°C for 10 min. After washing with buffer for 5 min, the slides were incubated in the blocking solution (1% BSA in PBS) for 1h at 25°C. The sections were incubated for 1h in anti-Ki67 primary antibody (1:300 dilution in 1% BSA in PBS), anti-CD44 and anti-AQP5 primary antibody[1:100 dilution in tris-buffered saline with 0.1% tween 20 detergent (TBST)] solutions in a humidified chamber maintained at 25°C. After incubation, the sections were rinsed, and excess washing buffer was added. Anti-Rabbit IgG and anti-mouse IgG were used as secondary antibodies against the respective primary antibodies and added to the sections at a dilution ratio of 1:800 in PBS and incubated at 25°C for 30 min. After a series of washes with PBS, sections were incubated in IHC Select® Streptavidin-HRP for 30 min at 25°C. After another series of washes with PBS, sections were incubated in the 3, 3′-diaminobenzidine (DAB) solution for 1–2 min. After being washed with PBS (three times for 2 min each), tissue sections were counterstained by immersing them in the hematoxylin solution for <1 min. The slides were rinsed in running tap water to remove excess stain. Sections were dehydrated using 80%, 90%, and 100% ethyl alcohol. Slides were cleared in xylene three times before mounting with coverslips using an appropriate mounting medium. Microscopy was performed with Nikon TE-2000S microscope and DSFi3 camera for Ki-67, AQP5, and CD44, respectively, and NIH ImageJ was used for quantification. Ki-67, AQP5, and CD44 of each group, respectively.

M3. Immunohistochemistry of Integrin β3 in PDX mice model

Immunohistochemistry of Integrin β3 was performed using a kit namely Poly Excel DAB detection system by PathnSitu, Biotechnologies Pvt. Ltd, USA. Tissue sections were deparaffinized in three changes of xylene. The slides were hydrated in a series of graded alcohol solutions in water. Tissue slides were blocked for 5 min with poly excel H_2O_2. The tissue sections were treated with a primary antibody for integrin β3 (1:100) and incubated overnight. The tissue sections were covered with a poly excel Target Binder and incubated for 10 min at 25°°C. After this, the tissue sections were

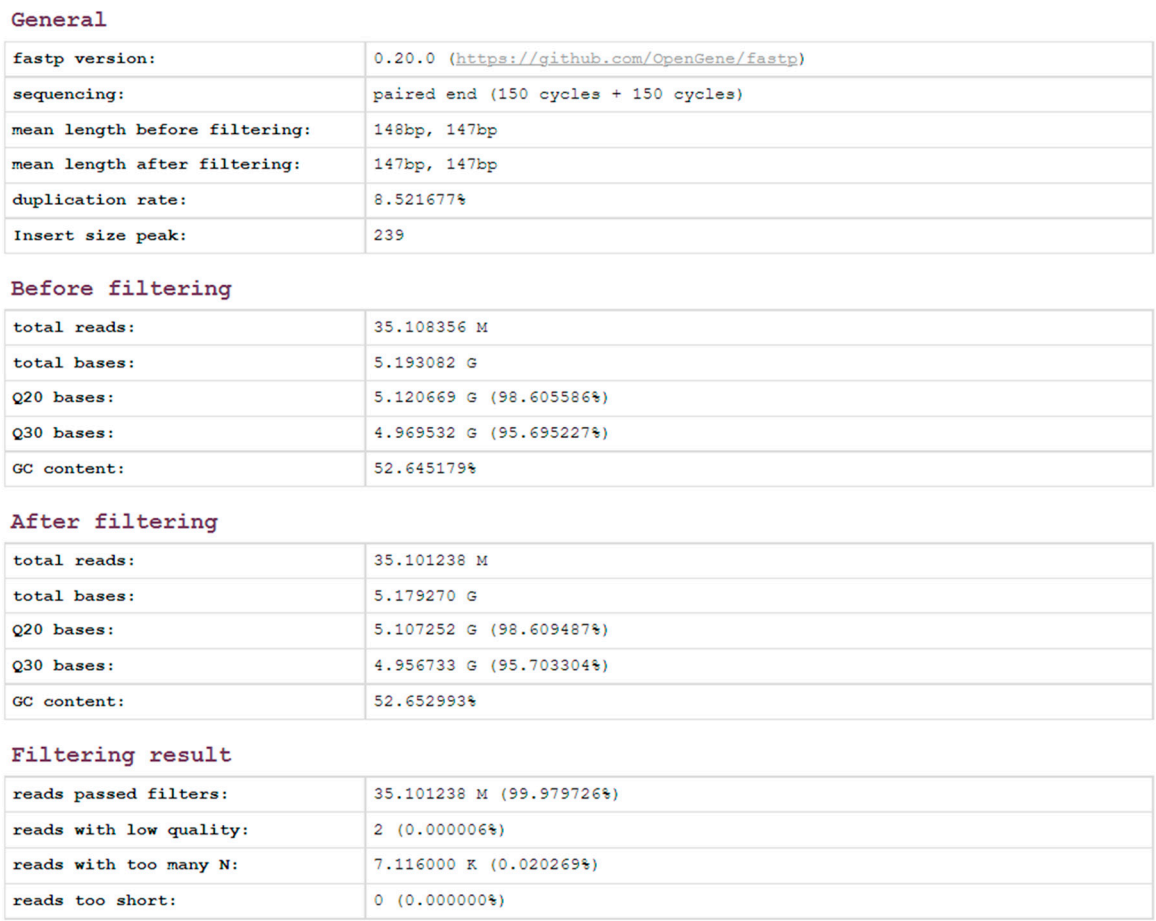
covered with poly excel polyHRP and incubated for 10 min at rt, followed by incubation in the stunn DAB solution for 5 min at room temperature. Next, the tissue sections were dipped in hematoxylin followed by a wash with distilled water. The slides were then dried and mounted with DPX and analyzed under inverted Nikon Microscope TE 2000 with a DSFI3 camera.

M4. Hematology analysis of HK-1 xenograft mice model.

Three mice out of each group: control, DMA (50mg/kg), radiation (4 Gy), and DMA+radiation (50mg/kg +4 Gy)were used for blood collection (100µL) from the ventricle, accessed via the left side of the chest. Blood samples were taken without an anticoagulant in a sterile 1.5 mL microcentrifuge tube for the separation of serum, which was used to measure biochemical parameters. Total serum protein (BiOLiS 24i Product No.12002022) and serum albumin levels (BiOLiS 24i Product No.12002001) were measured. The serum globulin level was calculated by subtracting the albumin level obtained from the total serum protein level. The serum aminotransferase activities of aspartate aminotransferase (AST, BiOLiS 24i Product No.12002020) and alanine aminotransferase (ALT, BiOLiS 24i Product No.12002021) were determined calorimetrically, and so were alkaline phosphatase (ALP) levels(24i Product No.12002002).

Supplementary Figures and Table

Figures



General

fastp version:	0.20.0 (https://github.com/OpenGene/fastp)
sequencing:	paired end (150 cycles + 150 cycles)
mean length before filtering:	148bp, 147bp
mean length after filtering:	147bp, 147bp
duplication rate:	8.521677%
Insert size peak:	239

Before filtering

total reads:	35.108356 M
total bases:	5.193082 G
Q20 bases:	5.120669 G (98.605586%)
Q30 bases:	4.969532 G (95.695227%)
GC content:	52.645179%

After filtering

total reads:	35.101238 M
total bases:	5.179270 G
Q20 bases:	5.107252 G (98.609487%)
Q30 bases:	4.956733 G (95.703304%)
GC content:	52.652993%

Filtering result

reads passed filters:	35.101238 M (99.979726%)
reads with low quality:	2 (0.000006%)
reads with too many N:	7.116000 K (0.020269%)
reads too short:	0 (0.000000%)

Figure S1. Fastp report of high quality reads of HNMT1 primary cells after removal of the adapter sequences and reads with more than 10% quality threshold (QV)<20 phred score.

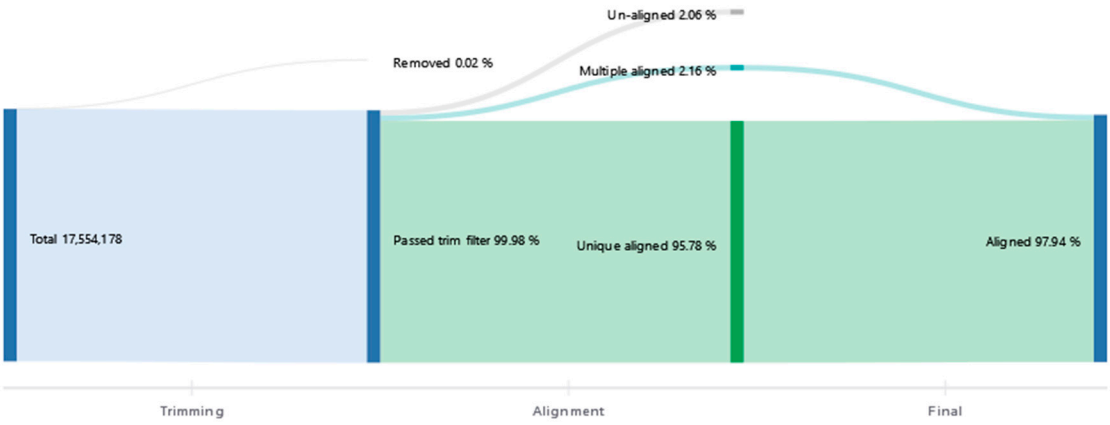


Figure S2. Aligned reads with the mean quality score of high quality reads of HNMT1 primary cells reads using STAR expression count.

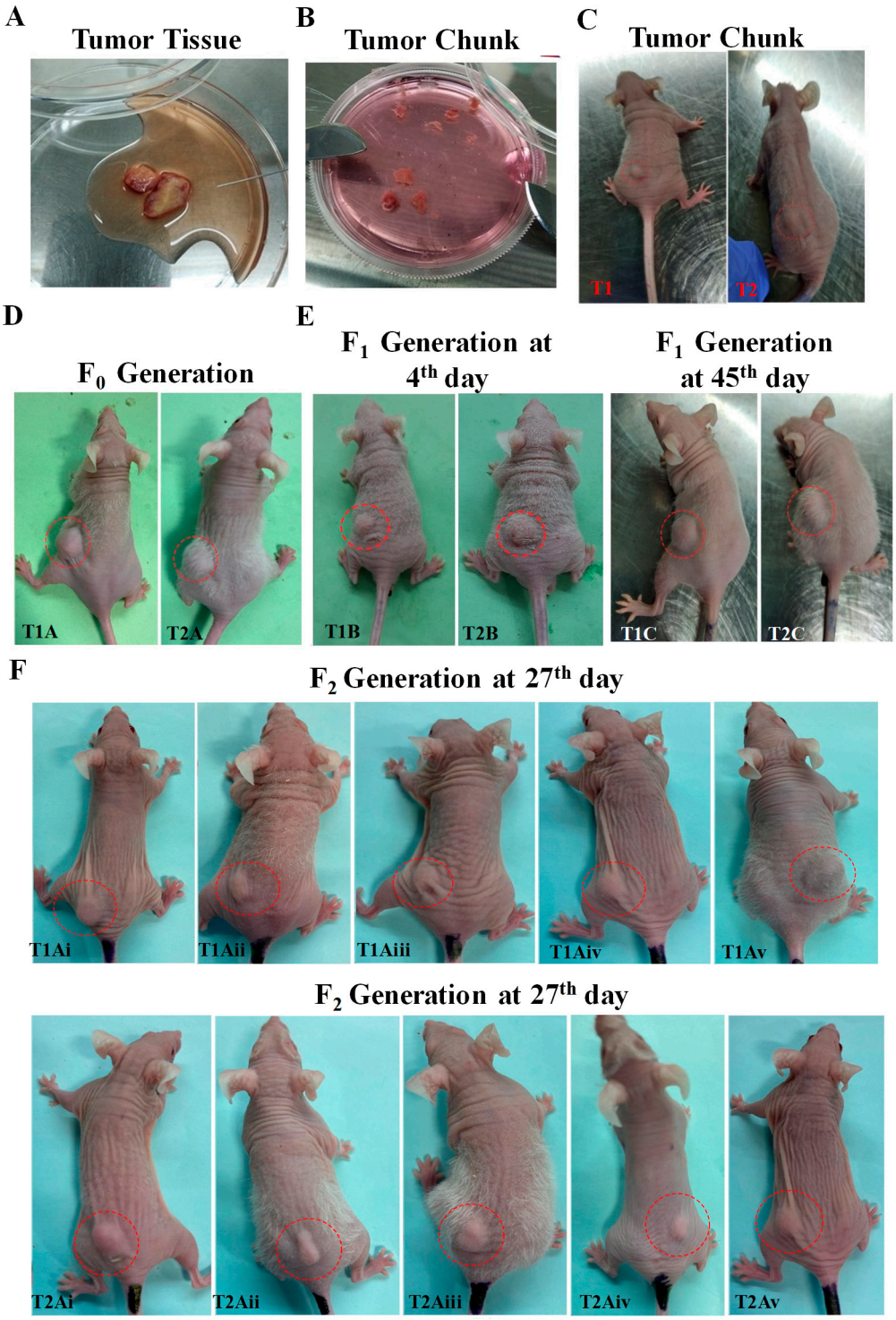


Figure S3. PDX mice model. Schematic outline of the development of three different generations of PDX, (**D**) F_0 generation, (**E**) F_1 generation, and (**F**) F_2 generation using tongue squamous carcinoma patient1 tumor implanted on the back of the nude mice

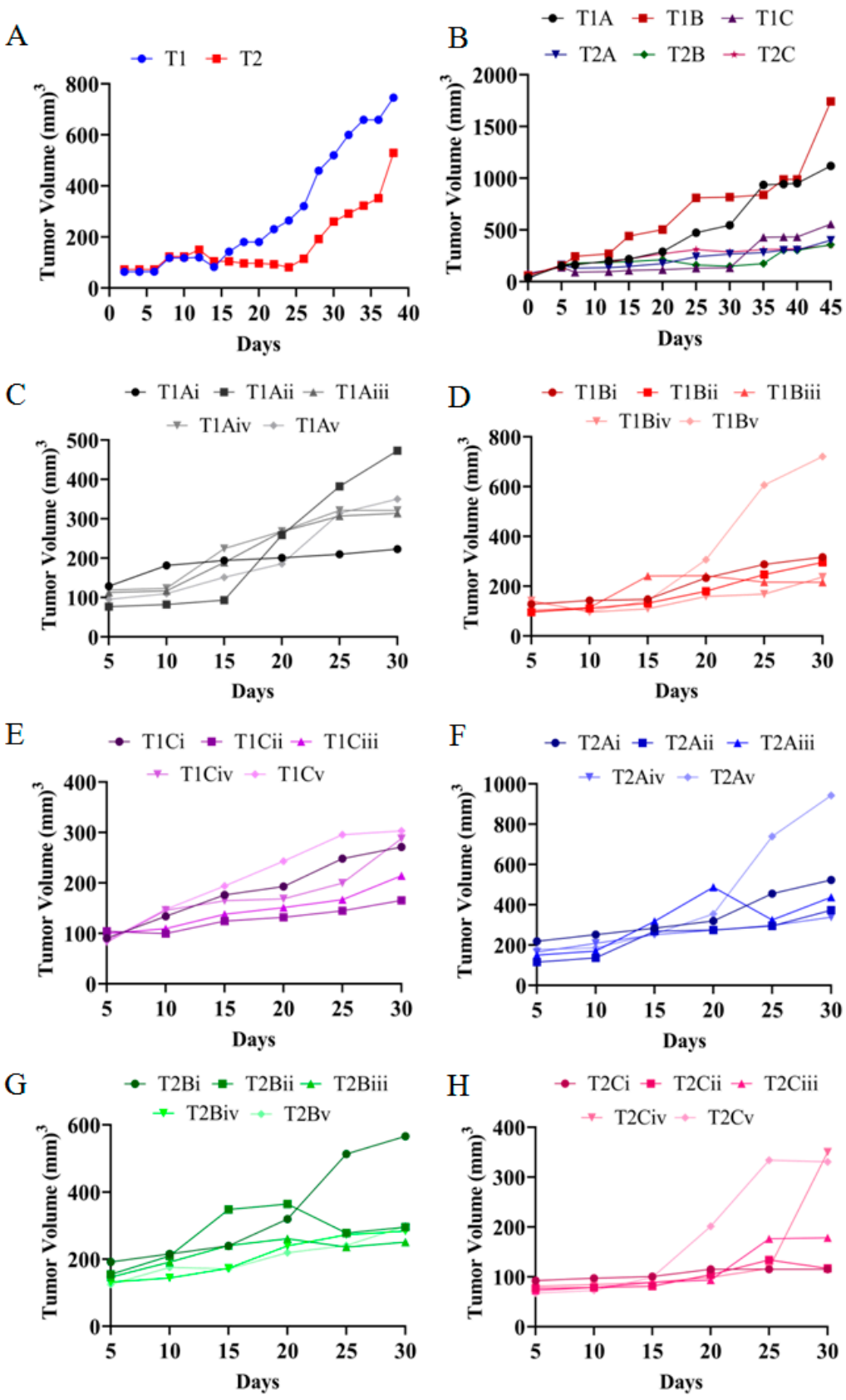


Figure S4. Graphical representation of the tumor formation rate of (**A**) F_0 generation (**B**) F_1 generation and (**C-H**) F_2 generation.

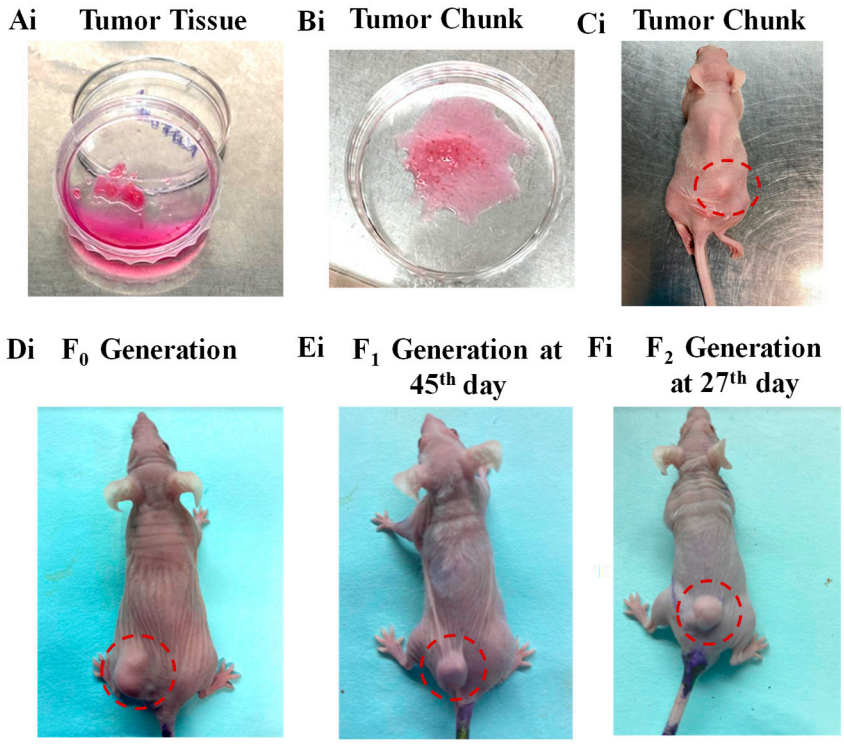


Figure S5. PDX mice model. Schematic outline of the development of three different generations of PDX, (**Di**) F_0 generation, (**Ei**) F_1 generation, and (**Fi**) F_2 generation using head and neck squamous carcinoma patient 2 tumor implanted on the back of the nude mice.

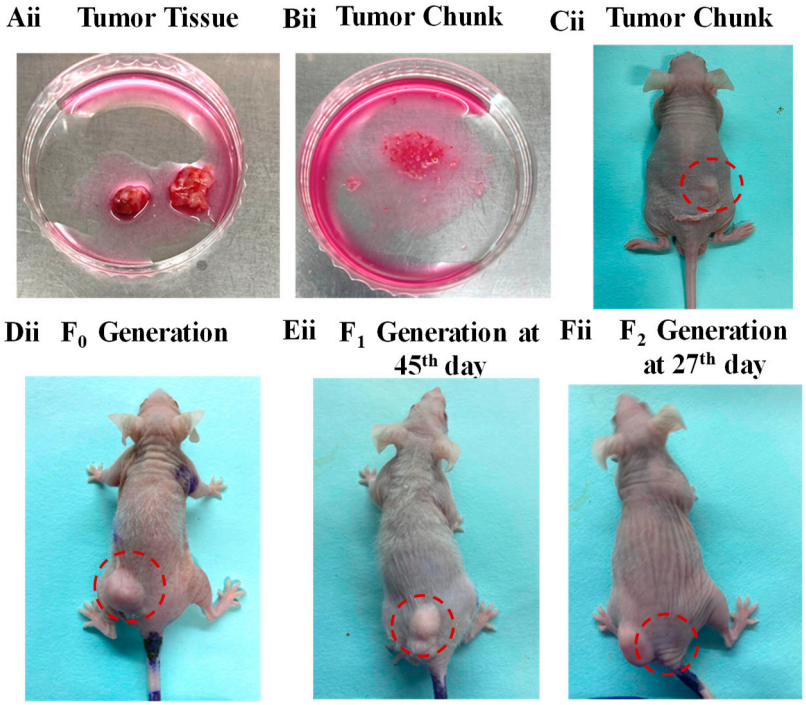


Figure S6. PDX mice model. Schematic outline of the development of three different generations of PDX, (**Di**) F_0 generation, (**Ei**) F_1 generation, and (**Fi**) F_2 generation using head and squamous carcinoma patient number 3 tumor implanted on the back of the nude mice.

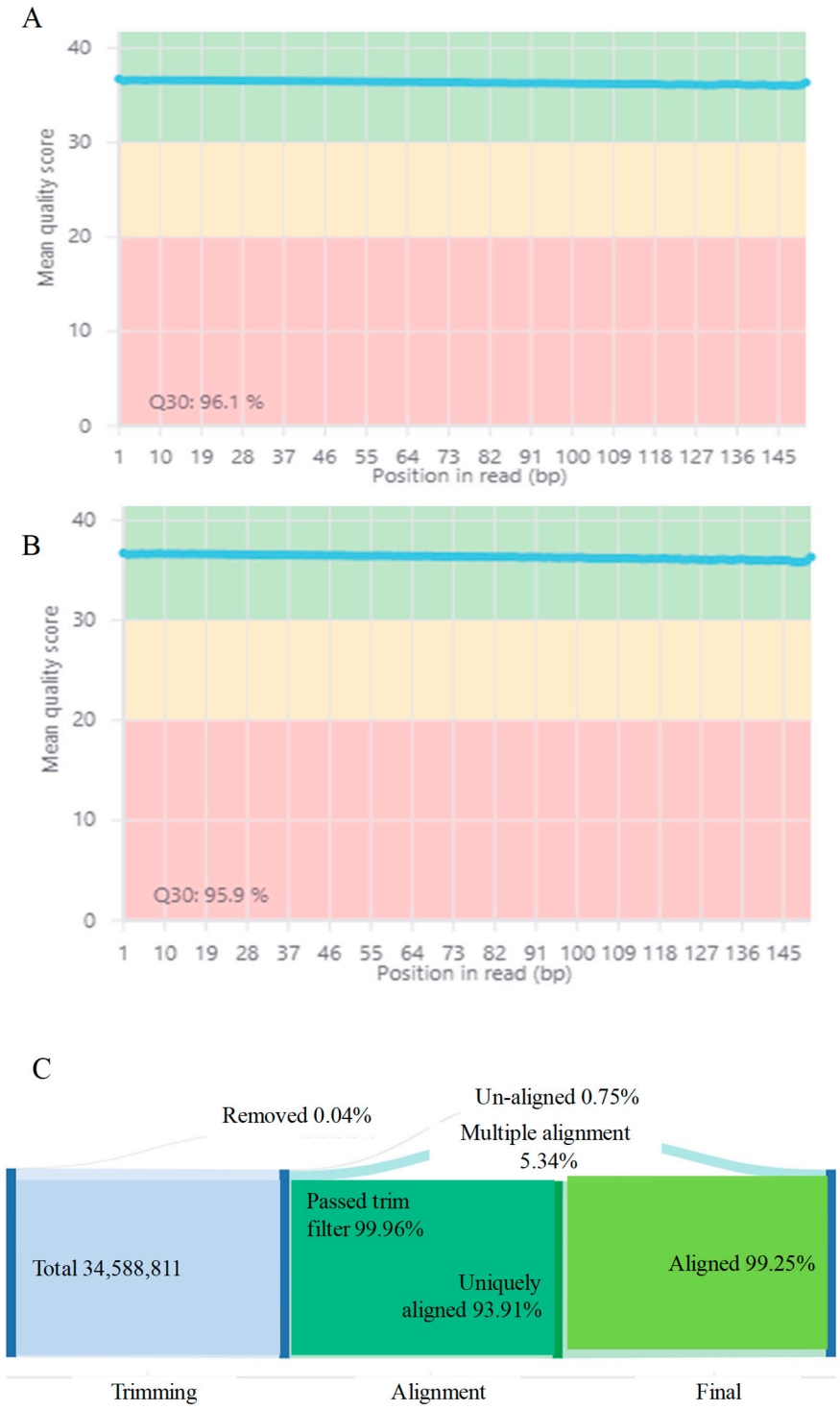


Figure S7. Aligned reads with the mean quality score (**A, B**) of tumor chunk 1 (T1) procured from the PDX mice model of F0 generation using STAR expression count (**C**).

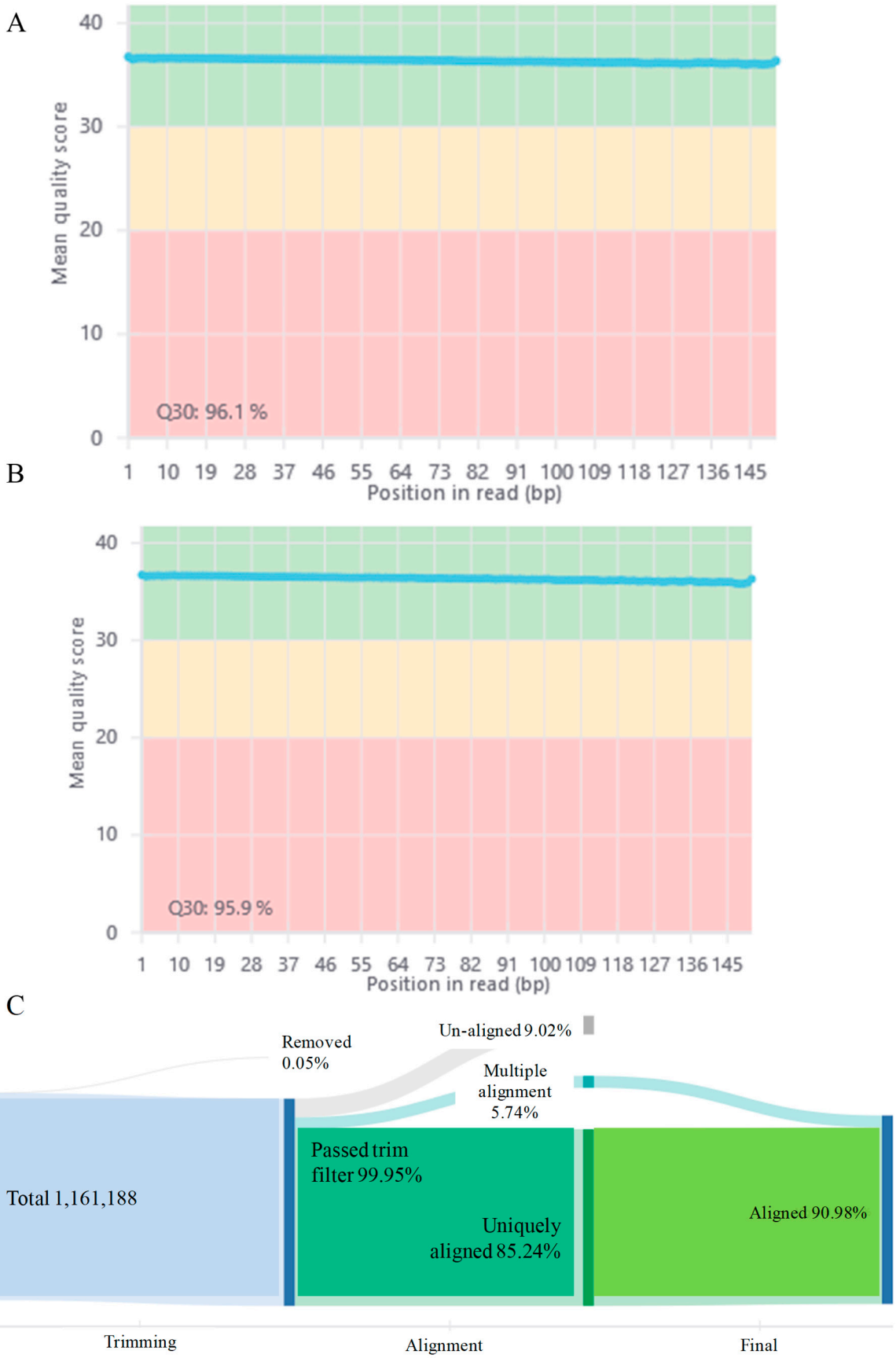


Figure S8. Aligned reads with the mean quality score (**A, B**) of tumor chunk 2 (T2) procured from the PDX mice model of F0 generation using STAR expression count (**C**).

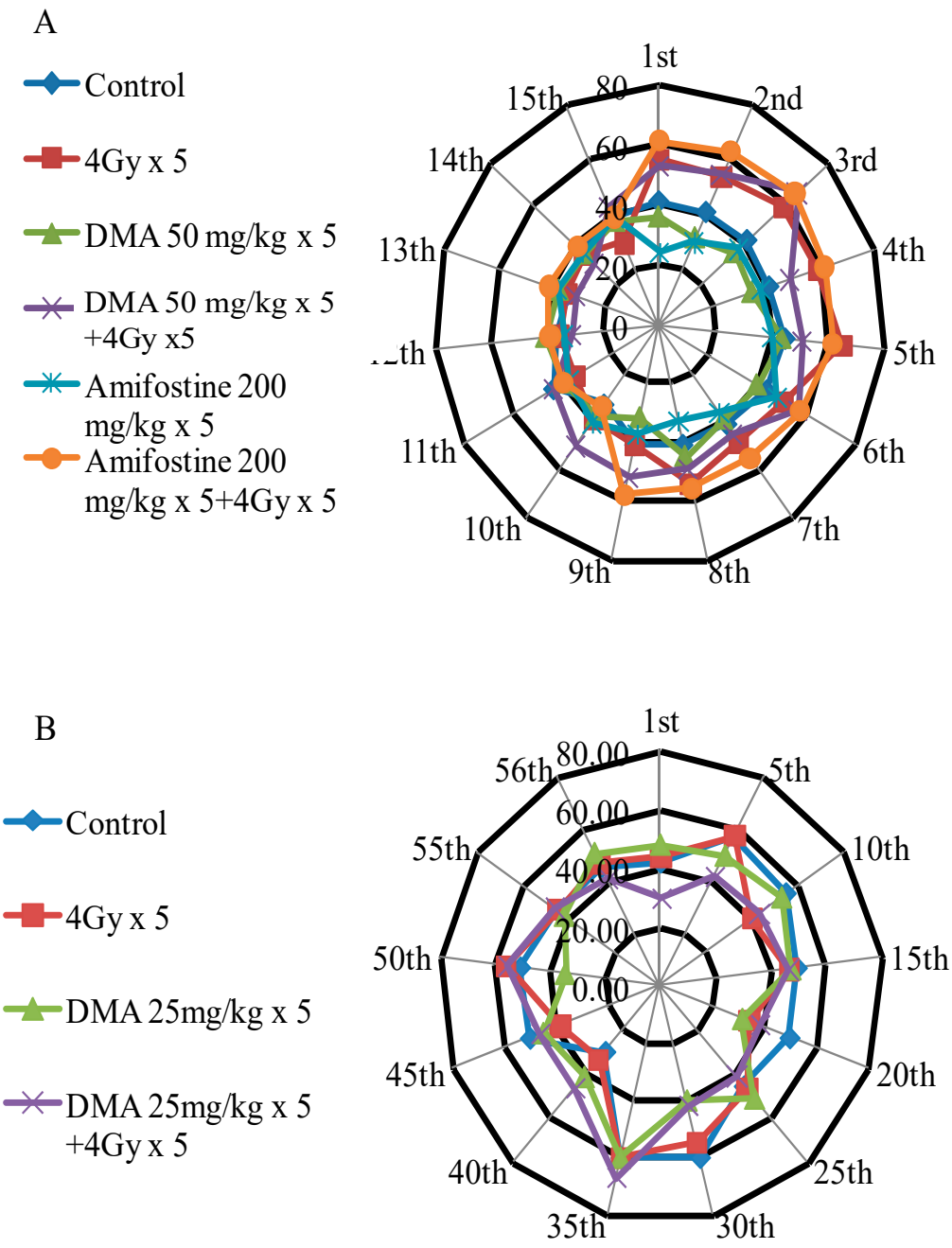


Figure S9. The radar plot represents the water intake (ml) in each group of tumor-bearing mice (**A**) HK-1 xenograft (**B**) PDX. Groups of 4-5 week old male and female mice were treated with DMA, focal radiation, and DMA+radiation with a dose rate of 1.2 Gy/min for consecutive 5 days.

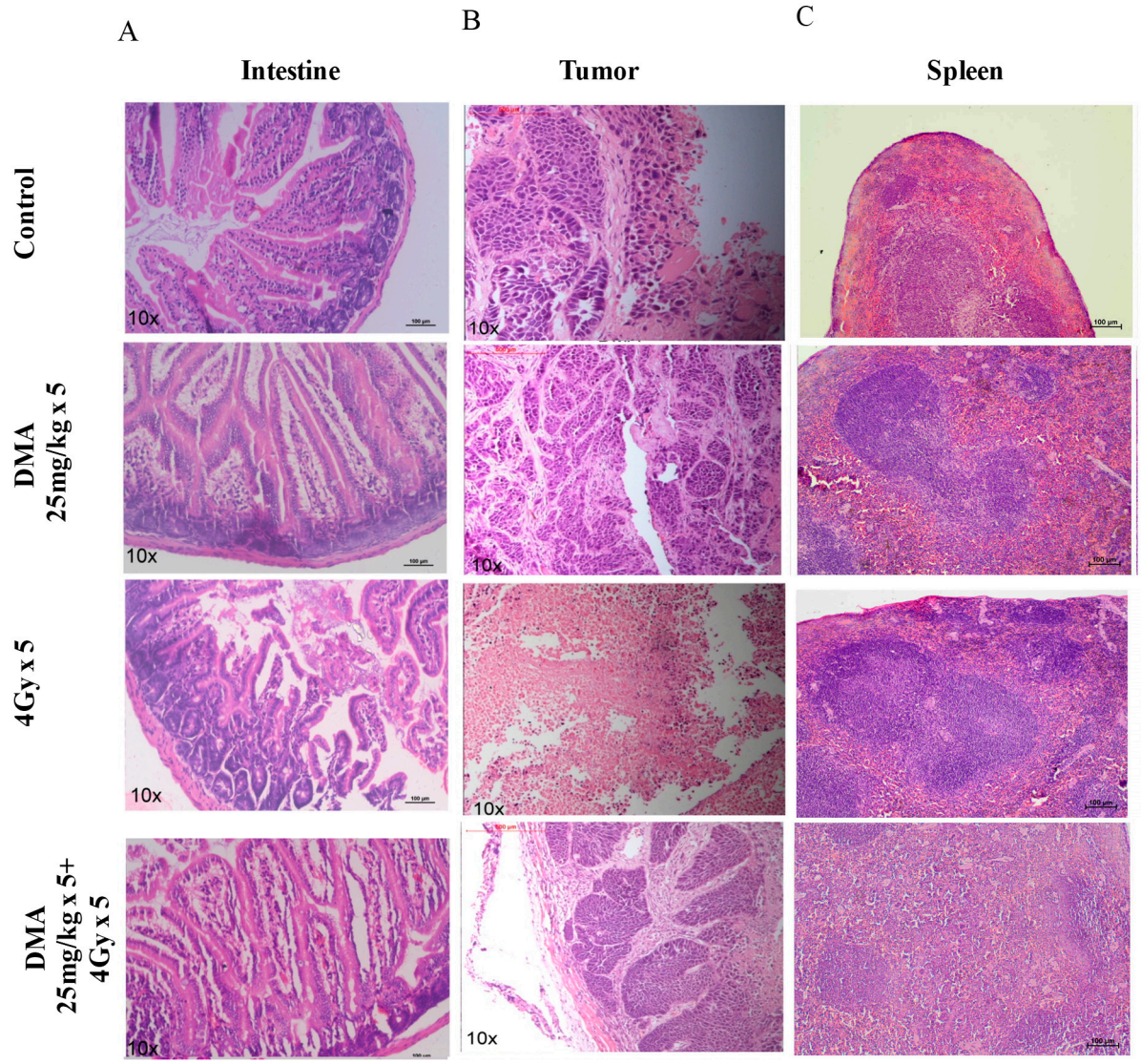


Figure S10. Histology of PDX tumor-bearing mice tissue sections with H&E staining on the day (60 days) of completion of the experiment. (H&E, magnification×10); Spleen, Intestine and tumor tissue of control, DMA, radiation, radiation+DMA of nude mice. Represented images were captured in Nikon 80*i* bright field microscope

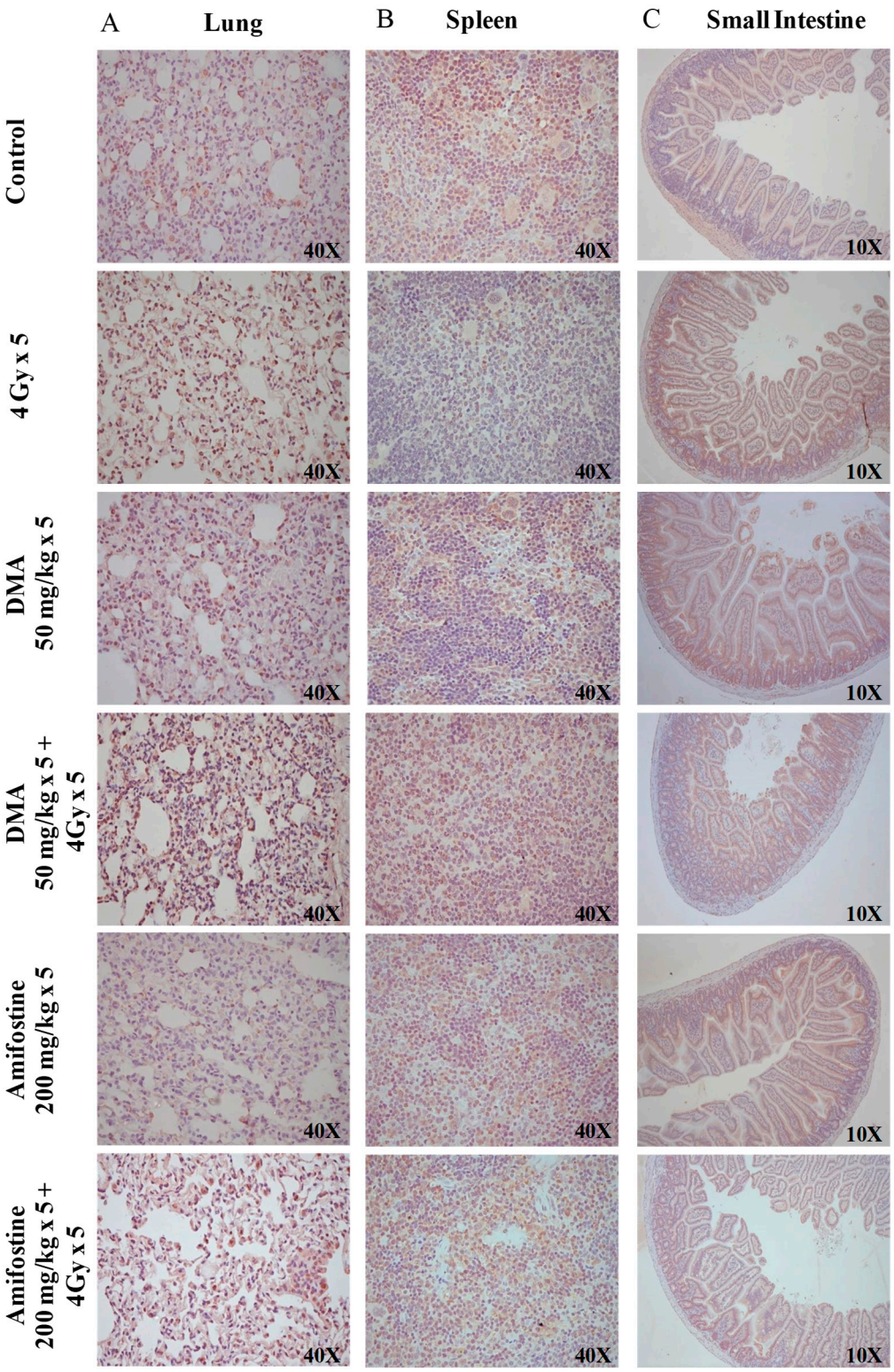


Figure S11. Representative images of IHC of tissue procured from HK-1 xenograft stained with Ki-67 antibody as a marker for cellular proliferation in **(A)** lung, **(B)** spleen, and **(C)** small intestine from each group and quantitation using the IHC profiler plug-in from the NIH Image J software (**Figure 6D**). The overall score is considered as the number of Ki-67 positive cells. Images were captured at 40X and 10X magnification, scale bar of 100µm.

Tables

Table S1. List of the antibodies, software, and the reagent used in the study with their Source (Company name), Catalog number, and Unique RRID code.

Reagent	Source	Identifier
Antibodies		
Ki-67	Cell Signaling Technology, Inc.	Cat# 12790, RRID: AB_2631166
AQP5 (D-7)	Santa Cruz Biotechnology, Inc.	Cat# sc-514022, RRID: AB_10891784
CD44	Becton Dickinson (BD) India Private limited	Cat#, RRID: AB_2229289
Cleaved caspase 3	Cell Signaling Technology, Inc.	Cat#2922S, RRID: AB_2228523
Anti-Ki-67	BioLegend	Cat# 652402, RRID:AB_11204254
Anti-Ki-67 antibody	Sigma-Aldrich	Cat# AB9260, RRID: AB_2142366
Ras (D2C1) Rabbit mAb	Cell Signaling Technology, Inc	Cat# 8955, RRID:AB_2797685
Cyclin B1 (V152)	Cell Signaling Technology, Inc	Cat# 4135S
Cleaved Caspase-3	Cell Signaling Technology, Inc	Cat#9664S
p44/42 MAPK	Cell Signaling Technology, Inc	Cat#4696S
GAPDH	Santa Cruz Biotechnology	Cat# sc-47724
Anti-Rabbit IgG (whole molecule)–Biotin antibody produced in goat	Sigma-Aldrich	Cat# B8895, RRID: AB_258649
Anti-Mouse IgG	Sigma -Aldrich	Cat# A9044, RRID: AB_258431
Anti-Integrin β3 ITGB3 Rabbit Monoclonal Antibody	Biosource	Cat#M00587-1
Softwares		
Sigma Plot	Systat Software, Inc.	RRID:SCR_010285
Image J	National Institute of Health (NIH)	RRID: SCR_003070
GraphPad 8.0	GraphPad Software, Inc.	RRID: SCR_002798
Chemicals and reagent		
AMG510 (Sotorasib)	Allianz Bioinnovation	CAS No. 2296729-00-3
Dulbecco's Modified Eagle's	Sigma-Aldrich	Cat# D5523-10L

Medium (DMEM)		
Minimum Essential Medium Eagle (EMEM)	Sigma-Aldrich	Cat# M0643-10L
RPMI-1640	Sigma-Aldrich	Cat# R6504-10L
McCoy's 5A	Sigma-Aldrich	Cat# M4892-10L
Fetal Bovine Serum (FBS)	ThermoFisher Scientific-US	Cat# 10270-106
1X antibiotic, antimycotic solution	Sigma-Aldrich	Cat# A5955-100ml
MTT	Sigma-Aldrich	Cat# M2128-1G
FITC Annexin V Apoptosis Detection Kit I	Becton Dickinson (BD) India Private limited	Cat# 556547
PolyExcel HRP/DAB Detection System	PathnSitu Biotechnologies Pvt Ltd	Cat# OSH001
Trypsin	Sigma-Aldrich	Cat# T4799-5G
Bradford	Sigma-Aldrich	Cat# B6916-500ML
DMSO	Merck-Life Science	Cat# 1.07046.0521
DNase	HiMedia Laboratories Pvt. Ltd.	Cat# ML068
Vetbond 3M adhesive	3M	Cat# 1469SB
Antifade reagent	ThermoFisher Scientific-US	Cat# PM10144
Matrigel Matrix	Corning Inc.	Cat# 354234
Citric acid	Rankem	C2859
DPX mountant liquid	HiMedia Laboratories Pvt. Ltd.	GRM655
Ethyl Alcohol	CSHS fine chemicals	64-17-5
Hematoxylin (Harris)	HiMedia Laboratories Pvt. Ltd.	S034
HEPES	Sigma-Aldrich	H3375
Hydrogen peroxide	AVRA Synthesis Pvt. Ltd	ASH1579
IHC select Streptavidin-HRP pre-diluted	Sigma-Aldrich	20774
L-Glutamine	Sigma-Aldrich	59202C
D-Glucose	Merck-Life Science	CAS# 50-99-7

Normal saline	Infutec	OC90003
Pilocarpine	Sigma-Aldrich	P6503
Sodium Bicarbonate	Sigma-Aldrich	S8875
Sodium chloride	Sigma-Aldrich	S5886
Triton X-100	Sigma-Aldrich	X100
Trypan Blue Stain	Sigma-Aldrich	T8154
Xylene	Rankem	X0092
3,3′-Diaminobenzidine tetrahydrochloride hydrate (DAB)	Sigma-Aldrich	D5637
Potassium chloride	Merck-Life Science	CAS# 7447-40-7
Disodium hydrogen phosphate	Merck-Life Science	CAS# 7558-79-4
Potassium dihydrogen phosphate	Merck-Life Science	CAS# 7778-77-0
EGTA	BioBasic Inc.	D0077
Collagenase	Roche	10103578001
HBSS	Gibco	13150-016
Polyvinyl alcohol	Sigma-Aldrich	341584-25G
Albumin Bovine fraction V (Bovine Serum Albumin, BSA)	Sisco Research Laboratories Pvt. Ltd.	CAS# 83803
40µm Nylon Cell Strainer	Corning	352340

Table S2: Incidence Scoring of Radiation Induced Histopathological Changes in HK-1 Xenograft

Organ	HP findings	Control	4Gy x 5	DMA 50 mg/kg x 5	DMA 50 mg/kg x 5 +4Gy x 5	Amifostine 200 mg/kg x 5	Amifostine 200 mg/kg x 5+ 4Gy x 5
Spleen	Extra-medullary Hematopoiesis Increased / Decreased	0	4+	0	2+	0	3+
Lung	Degeneration, Bronchiolar	0	3+	0	4+	0	4+
Tumour	Degeneration/ Necrosis, Neoplastic cells	0	5+	0	5+	0	5+

Lesion with percent incidence: 0: No incidence; 1+: <10, 2+: 11-25, 3+: 26-50, 4+: 51-75, 5+: >75

NOTE: Tabulated scoring of the histological analysis. Control means i.v. administration of saline to the group of mice for five consecutive days, focal radiation of 4 Gy @ per day single dose x 5 = 20 Gy (total dose) through X ray irradiator with a dose rate of 1.2Gy/min.DMA 50mg/kg @ per day single i.v. dose x 5= 250 mg/kg (total dose). DMA 50 mg/kg @ per day single i.v. dose 2h before focal irradiation x 5 and 4Gy focal radiation was given for 5 days=250 mg/kg with 20 Gy (total dose).Amifostine 200mg/kg @ per day single i.v. dose = 1000 mg/kg (total dose).Amifostine 200mg/kg @ per day single i.v. dose 2h before irradiation x 5 and 4Gy focal radiation was given for 5 days = 1000 mg/kg with 20 Gy (total dose).

Table S3: Incidence Scoring of Radiation-Induced Histopathological Changes in PDX

Organ	HP findings	Control	4Gy x 5	DMA 25 mg/kg x 5	DMA 25 mg/kg x 5 +4Gy x 5
Spleen	Extra-medullary Hematopoiesis Increased / Decreased	3+	5+	3+	0
Small Intestine	Amyloidosis, Autolysis	0	4+	3+	2+
Tumour	Degeneration/ Necrosis, Neoplastic cells	0	4+	0	5+

Note: Lesion with percent incidence: 0: No incidence; 1+: <10, 2+: 11-25, 3+: 26-50, 4+: 51-75, 5+: >75

Table S4. Liver and Kidney Function Tests in HK-1 Xenograft*

Parameters	Normal	Control	R (4Gy x5d)[ζ]	DMA50mg/kg[Ϯ]	DMA 50 mg/kg + 4Gy[#]	Amifostine, 200 mg/kg[ϰ]	Amifostine, 200 mg/kg + 4Gy[ϒ]
LFT Parameters							
Bilirubin Total (mg/dl)	0-0.9	0.25	0.12	0.10	0.12	0.08	0.09
Bilirubin Direct (mg/dl)		0.14	0.01	0.03	0.03	0.05	0.02
Bilirubin Indirect (mg/dl)		0.11	0.11	0.07	0.09	0.03	0.07
SGOT(AST) U/L	54-298	946.5	292.33	604.74	541.74	768	810
SGPT(ALT) U/L	17-77	82.2	23.83	55	45.43	38.6	45.5
Alkaline phosphatase U/L	35-96	131.6	156.66	94	83.35	128	150
Total Protein (g/dl)	3.5-7.2	7.15	6.8	5.12	6.37	6.8	7.3
Albumin (g/dl)	2.5-3.0	3.45	3.03	3.0	2.88	2.4	3.1
Globulin (g/dl)	1.0-4.2	3.7	3.76	2.12	3.48	4.2	4.2
A/G ratio		0.94	0.8	1.415	0.84	0.57	0.74
KFT Parameters							
Blood Urea (mg/dl)	17.1-70.6	88.5	59	45.31	49.31	54	64
Creatinine (mg/dl)	0.2-0.9	0.38	0.44	0.72	0.54	0.34	0.29
Uric acid (mg/dl)	1.2-7.5	3.32	2.16	3.15	2.78	4.2	3.88
Calcium (mg/dl)	7.1-10.1	8.7	9.56	8.2	9.96	9.0	9.5

Phosphorous (mg/dl)	5.7-9.2	7.55	6.8	6.32	7.42	8.1	6.7
Sodium (mEq/l)	140-160	146.1	148.36	152.56	147.56	150.3	149.3
Potassium (mEq/l)	5.0-7.5	5.8	6.16	6.76	5.77	5.6	7
Chloride (mEq/l)	88-110	101	102.33	92	103.25	89	104

NOTE:*Blood for liver function and kidney function test was collected without anticoagulant in 1.5 ml micro centrifuge tube, kept for 15 mins on ice after the completion of the experiment (30 days) and centrifuged at 1,4000rpm for 15 mins. ˜Control indicates administration of saline to the group of mice, ϟR =radiation through X ray with a dose rate of 1.2Gy/min, 4Gy radiation was given for 5 days; 4 Gy @ per day single dose x 5 = 20 Gy (total dose). ϯ DMA 50mg/kg @ per day single i.v. x5 =250 mg/kg(total dose). ♯ DMA 50mg/kg @ per day single i.v. dose 2h before irradiation x 5 and radiation 4Gy radiation was given for 5 days; 4 Gy @ per day single dose x 5 = 250 mg/kg with 20 Gy (total dose). ϰAmifostine 200mg/kg @ per day single i.v. dose x 5= 1,000 mg/kg (total dose). ϒ Amifostine 200mg/kg @ per day single i.v. dose 2h before irradiation x 5 and radiation 4Gy radiation was given for 5 days; 4 Gy @ per day single dose x 5) = 1.000 mg/kg with 20 Gy (total dose) ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.