DIFFERENTIAL EFFECTS OF PROTONTHERAPY AND PHOTONTHERAPY ON HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) POST-TREATMENT AGGRESSIVENESS

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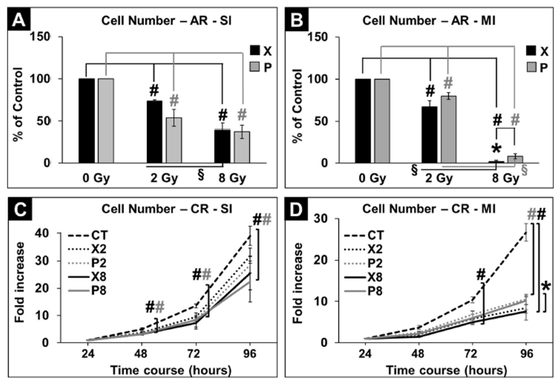
The treatment of squamous cell carcinoma of the aerodigestive tract (SCC) by standard radiotherapy is challenging because of the proximity of organs at risk. Due to its higher precision in tumor targeting, protontherapy could become the treatment of choice for SCC. Additionally, recent studies have shown that proton irradiation suppresses angiogenic genes and impairs tumor cell invasion/growth.

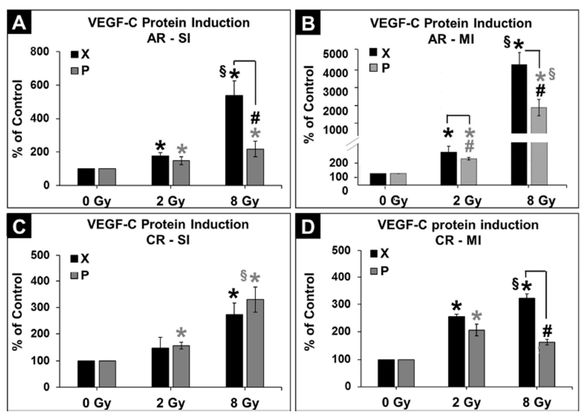
We investigated the effectiveness of single and multiple proton (P) versus photon (X) irradiations in SCC cells on their proliferation potential and on the expression of genes / proteins involved in proliferation, (lymph)angiogenesis, metastasis and anti-tumor immunity.



CELL PROLIFERATION

Cell proliferation after a single or multiple proton (P) or photon (X) irradiations at 2 Gy or 8 Gy.

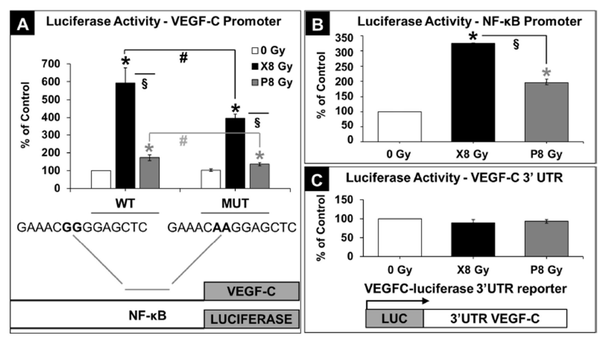
Both P and X irradiations acutely and chronicly affected cell growth and cell viability as measured by cell counting of viable cells 48, 72, 96hours after cell seeding. $p<0.05$



VEGF-C PROTEIN INDUCTION

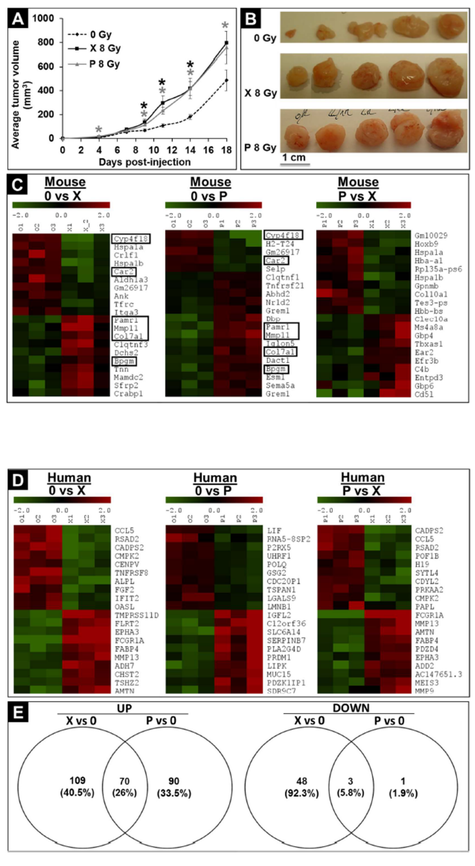
In CAL33 cells, VEGF-C protein levels increased in a dose-dependent manner following both P and X irradiations.

Furthermore, they were significantly lower after P irradiation, except for the CR-SI setting where the two types of radiations.



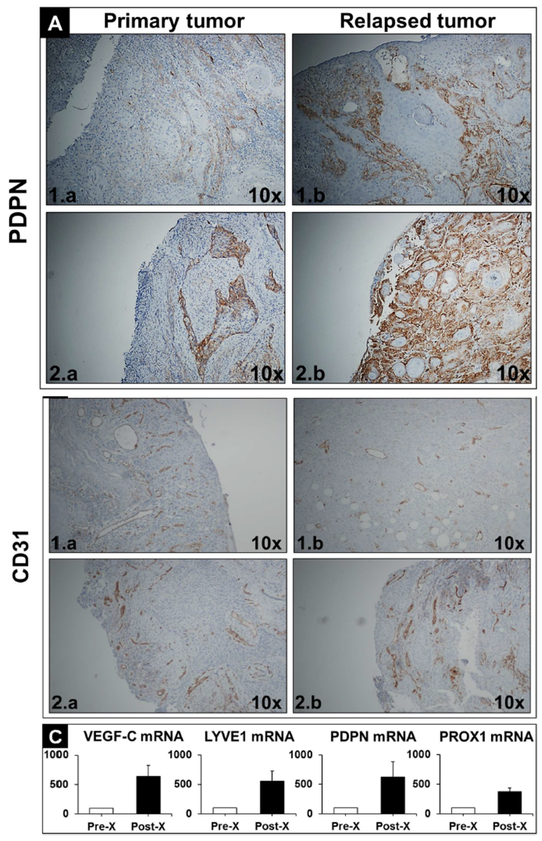
X AND P IRRADIATIONS STIMULATE VEGF-C PROMOTER ACTIVITY

Irradiation by either X or P stimulated the activity of the VEGF-C promoter especially in CAL33 cells surviving to multiple X irradiations (6- and 18-fold increase, respectively, $p<0.001$). Mutation of the NF-κB binding site (MUT) had no effect on the basal VEGF-C promoter activity in non-irradiated cells. However, in cells surviving to MI by P and X, the activity of the MUT, as compared to WT, promoter was significantly decreased (by 33%, $p=0.004$ and by 30%, $p=0.027$, respectively).



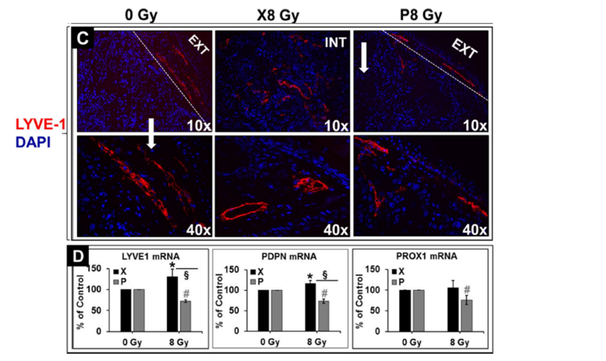
ANALYSIS OF TUMORS GENERATED BY SUBCUTANEOUS XENOGRAFTS

(A) Average tumor volume (mm3); (B) Representative images of tumor xenografts; (C) Heatmap of ten most up- and down-regulated mouse genes in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors; (D) Heatmap of ten most up- and down-regulated human genes in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors; (E) Venn diagrams showing common up-regulated and down-regulated human genes between P and X tumors. Framed genes are commonly expressed in P and X tumors. Selection is adjusted p value < 0.05 and lofFC > 1.



EVALUATION OF LYMPHANGIOGENESIS IN BIOPSIES FROM HNSCC PATIENTS

Representative images of immunohistochemistry for (A) PDPN and (B) CD31 expression: (1) oral and (2) hypopharyngeal localization; Left panels (1.a, 2.a) – primary tumor; Right panels (1.b, 2.b) – relapsed tumor in the same patient after surgery and chemo-X radiotherapy (brown, PDPN/CD31; blue, hematoxylin - nuclei); (C) quantification of PDPN, VEGF-C, LYVE1 and PROX1 mRNA expression.



REPRESENTATIVE FLUORESCENCE IMAGES IN MURINE XENOGRAFTS

LYVE1 (lymphatic endothelial cells, red) / Hoechst (nuclei, blue), showing different patterns of lymphatic vessels development in X (both periphery and interior of the tumor), P and CT (periphery of the tumor) groups; dashed white lines delimit the tumor edge; CT, control (tumors generated by non-irradiated cells); (D) Murine LYVE1, PDPN and PROX1 mRNA levels in xenografts.

A mRNA levels (% of Control) - AR - SI

Role	Gene	X 2 Gy	X 8 Gy	P 2 Gy	P 8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	132	*191	143	160
	VEGF-C	130	*227	159	*209
	VEGF-D	103	96	105	98
Inflammation	IL6	129	136	119	#72
	IL8	147	*436	162	*401
	CCL2	104	123	#78	*136
Proliferation	TRF-2	88	*112	93	102
	Plk-1	85	*108	89	*99
Anti-tumor immunity	PD-L1	108	103	91	112
Gene score		4	6	0	3

B mRNA levels (% of Control) - AR - MI

Role	Gene	X 2 Gy	X 8 Gy	P 2 Gy	P 8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	127	155	136	#233
	VEGF-C	227	*1328	#196	*1019
	VEGF-D	124	*209	134	156
Inflammation	IL6	136	*269	#92	*#172
	IL8	133	*542	138	*#360
	CCL2	117	117	109	#167
Proliferation	TRF-2	121	#94	125	*#155
	Plk-1	123	#64	126	#119
Anti-tumor immunity	PD-L1	136	*192	122	*204
Gene score		9	6	8	8

C mRNA levels (% of Control) - CR - SI

Role	Gene	X 2 Gy	X 8 Gy	P 2 Gy	P 8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	113	*187	117	#141
	VEGF-C	136	*568	150	*449
	VEGF-D	109	115	116	116
Inflammation	IL6	100	99	120	*#57
	IL8	94	97	97	84
	CCL2	146	216	125	#135
Proliferation	TRF-2	104	*116	101	*115
	Plk-1	102	101	#85	*99
Anti-tumor immunity	PD-L1	122	*171	127	#132
Gene score		3	6	4	4

D mRNA levels (% of Control) - CR - MI

Role	Gene	X 2 Gy	X 8 Gy	P 2 Gy	P 8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	118	*208	93	*#107
	VEGF-C	281	*626	228	*#215
	VEGF-D	127	134	#95	79
	VEGFR-1	96	#73	#72	73
	VEGFR-2	88	88	#86	77
	VEGFR-3	116	91	100	80
	NRP1	105	90	#92	85
	NRP2	133	*169	#102	#129
Inflammation	IL6	120	126	#78	*#100
	IL8	27	*7916	#75	*#96
	CCL2	112	*751	#83	*#94
Proliferation	TRF-2	101	92	86	106
	Plk-1	91	97	89	*#117
Anti-tumor immunity	PD-L1	119	140	#92	*#116
Gene score		4	6	-8	-4

QUANTITATIVE GENE EXPRESSION, AS PERCENTAGE OF CONTROL (0 GY), IN EITHER P OR X IRRADIATED CAL33 CELLS BELONGING TO (A) AR-SI, (B) AR-MI, (C) CR-SI AND (D) CR-MI GROUPS

Highlighted values – significantly different ($p<0.05$) expression levels, as compared to control, for genes associated to favorable (dark grey) and non-favorable (black) outcomes; *, significantly different expression levels after low, as compared to high dose(s) of either P or X irradiation; #, significantly different expression levels after either low or high dose(s) of P, as compared to X irradiation.

CONCLUSIONS:

In vitro results:

- Protontherapy leeds to lower expression of factors involved in (lymph)angiogenesis, inflammation and immune tolerance.
- Acquisition of less aggressive phenotype after protontherapy

In vivo results:

- VEGF-C mRNA levels increase in a dose-dependent manner and with the irradiation number, except in the cells surviving after three proton irradiations.
- Subcutaneous xenograft of proton-irradiated cells generate tumors with less lymphatic vessels than the tumors obtained by photon-irradiated cell xenograft

Patient studies:

- Relapsed tumors (after photontherapy) present a marked expression of genes involved in lymphangiogenesis/metastasis.

These observations suggest that photontherapy would lead to less pronounced lymphangiogenesis/metastasis, as compared to photontherapy.