Effect of Radiation on Cell Proliferation and Tumor Hypoxia in HPV-positive Head and Neck Cancer *In Vivo* Models

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Abstract. *Background/Aim: Human papilloma virus-associated head and neck squamous cell carcinomas (HNSCC) represent a distinct subgroup of HNSCC characterized by a favorable prognosis and a distinct molecular biology. There is a range of unresolved questions regarding the different biology and clinical outcome of HPV-positive HNSCC. The purpose of the present project was to obtain insight into the biology of treatment responsiveness of HPV-related HNSCC. Materials and Methods: Tumor xenografts were established from HPV-negative (FaDuDD,) and HPV-positive (UD2 and UMSCC47) HNSCC cell lines. Tumors were treated with 10 Gy or 20 Gy and the effect on the tumor microenvironment was studied at different time points after treatment. Cryosections were imaged for cell proliferation, hypoxia, vessel density and vessel perfusion. Results: In the HPV-positive tumor models the levels of cell proliferation decreased significantly following irradiation. This was not seen in the HPV-negative model ($FaDu_{DD}$). Furthermore, it was found that the tumor hypoxic fraction decreased over time after treatment in irradiated HPV-positive tumors and not in the HPV-negative tumors. Conclusion: The radiosensitivity previously observed in vitro could be applied in vivo in respect to a radiation-induced decrease in proliferating cells. A decreasing hypoxic fraction following irradiation in the HPV-positive tumors could explain the lack of benefit from hypoxic modifiers observed in patients.*

Human papilloma virus (HPV) has been detected in a subset of head and neck squamous cell carcinomas (HNSCC), especially in oropharyngeal tumors (1, 2). HPV-associated HNSCC forms a distinct clinical and molecular entity characterized by a distinct molecular biology, epidemiology and better prognosis (3-6), rendering HPV the most important independent prognostic factor in HNSCC (6).

An association between the favorable outcome of HPV-positive tumors treated with radiotherapy and their hypoxic status has been explored in a range of clinical studies. This has demonstrated a lack of correlation between HPV status and tumor hypoxia measured with gene expression profiles, pO2 measurements or hypoxia positron emission tomography (PET) scanning (7-9). However, randomized trials investigating the effect of hypoxic modification (nimorazole and tirapazamine) in head and neck cancer have shown differentiated responses dependent on the HPV status of the tumors with no measurable effect in HPV positive tumors (10, 11). These rather contradictory results suggest hypoxia and, hence, resistance to radiotherapy to have a differential effect according to HPV status.

The majority of *in vitro* studies addressing the issues of radiosensitivity and hypoxia effects in HPV-positive cell lines have reached the conclusion that HPV-positive cell lines are, overall, more radiosensitive than HPV-negative cell lines (12-17), while the effect of hypoxia and nimorazole during irradiation has been demonstrated to be equal between HPV positive and HPV negative cell lines (15). Therefore, the aim of the present study was to investigate the background concerning the different biology of HPV-positive HNSCC. In vivo tumors were established from HPV-positive and HPV negative HNSCC cell lines and the effect of radiation on a range of microenvironmental factors was determined by immunohistochemistry. This demonstrated that the radiosensitivity previously observed *in vitro* could be applied *in vivo* in respect to a radiation induced decrease in proliferating cells. Furthermore, it was found that the hypoxic fraction was decreasing with time after treatment only in HPV positive tumors in response to irradiation.

Materials and Methods

Cell lines. The HPV negative HNSCC cell line FaDuDD (a subline of FaDu, an undifferentiated hypopharyngeal carcinoma; obtained from Dr. Michael Baumann, University of Technology, Dresden,

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Germany) was used. Two HPV positive (HPV-16) HNSCC cell lines: UMSCC47 (from the lateral tongue; established by Dr. Thomas Carey, University of Michigan (18, 19)) and UDSCC2 (from the hypopharynx; established by Dr. Thomas Hoffmann, University of Düsseldorf (20, 21)) were used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Carlsbad, USA) with 10% fetal calf serum, 100,000 U/l penicillin and 100 mg/l streptomycin, with 5% CO_2 in a well humidified incubator.

The HPV and p16 expression status of the cell lines was confirmed, as previously described (15).

In vivo models. Male non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were bred and maintained in our Institutional SPF animal facility. UMSCC47 (8×10^6 cells), UD2 (5×10^6 cells) and FaDuDD (5×10^6 cells) were implanted subcutaneously into the dorsal region of mice. For tumor mapping experiments mice were randomly assigned to experimental groups when average tumor volumes were 200-400 mm^3.

Radiation. Mice were fixed in a lead jig and tumor only was irradiated. Dose was given as a single fraction locally to the tumor. Tumors were irradiated with 10 Gy (UD2, UMSCC47 and FaDuDD) or 20Gy (FaDuDD). Radiation was delivered using a Pantak-Siefert XRAD320, 300 KeV and 10 mA with 1.5 mm Al, 0.25 mm Cu and 0.5 mm Sn filter. The dose rate was 2.8 Gy/min.

Animals were euthanized and the tumors excised 6 h, 12 h, 24 h and 48 h after irradiation. Unirradiated control tumors were included in all treatment groups.

Experiments described in this manuscript were approved by the Animal Care Committee of the University of British Columbia in accordance with the Canadian Council on Animal Care guidelines.

Reagents. The S-phase marker 5-Bromo-2-deoxyuridine (BrdUrd; Sigma-Aldrich, St. Louis, USA) was administered at 1000 mg/kg along with 60 mg/kg of the hypoxia marker pimonidazole (provided by Dr. J Raleigh, Hypoxyprobe, Burlington, Massachusetts, USA) as an intraperitoneal injection 2 h prior to tissue harvesting. The fluorescent dye Carbocyanine DiOC7(3) for vessel perfusion (Molecular Probes, Life Technologies, Carlsbad, USA), administered as 35 μl of 0.6 mg/ml dissolved in 75% (v/v) dimethyl sulfoxide/25% sterile H_2O, was injected intravenously 5 min prior to euthanasia.

Immunohistochemistry. Immediately after excision, tumors were frozen on an aluminum block held at −20˚C, embedded in cutting medium (OCTTissue-TEK, VWR, Radnor, PA, USA) and stored at −20˚C until sectioning. The general immunohistochemical procedure used has been previously reported (22). Briefly, 10 μm tumor cryosections obtained 2-3 mm from the tumor edge were air-dried, imaged for native DiOC7(3) fluorescence and fixed in 50% (v/v) acetone/methanol for 10 min at room temperature. Vasculature was identified with hamster anti-mouse PECAM/CD31- (Clone 2H8, MAB1398Z, Millipore, Billerica, MA, USA) bound pimonidazole using the Hypoxyprobe™-1 monoclonal antibody1 (MAb1) incorporated BrdUrd using Rat-anti-BrdU (clone BU1/75, Abcam, Cambridge, England), with light counter-staining using Hoechst.

Image acquisition and overlay. The imaging system has been previously described (23). Images of entire tumor cryosections were captured at a resolution of 0.75 μm per pixel. Grayscale CD31 (vessel distribution) and DiOC7(3) images were thresholded and a composite color image was produced using ImageJ (version 139n; http://rsbweb.nih.gov/ij/download.html). The combined vascular image was then overlaid onto grayscale images of BrdU, pimonidazole and Hoechst.

Image analysis. Using the ImageJ software application and user-supplied algorithms, fluorescent images were inverted and combinations of DiOC7(3), CD31, pimonidazole, BrdU and Hoechst images from each tumor section were aligned, cropped to tumor tissue boundaries and staining artifacts removed. Necrosis was cropped away based on Hoechst stained sections and the remaining viable fraction (VF) of tumors was calculated as the ratio between the viable area and the total tissue section area. Percent positive staining was obtained using the proportion of pixels at intensities meeting or exceeding a threshold value above background. Average intensity values represent the average pixel intensity for viable tumor tissue. For distribution analysis of BrdU relative to vasculature, each pixel in an image was sorted based on its distance relative to the nearest CD31-positive vessel and the average intensity in 1.5 μm increments from vasculature was determined.

Statistics. Data from average BrdU and pimonidazole staining across the section were log(x+1)-transformed to improve normality and homogeneity of variance of data. All data were analyzed using one-way analysis of variance (ANOVA) with an All Pairwise Multiple Comparison Procedure (Bonferroni *t*-test) or with a Kruskal-Wallis rank test for data which did not pass the normality test for comparisons between groups. Where appropriate, charts display values for individual tumors as means for analysis of whole tumor sections, normalized to levels in the control group; combined means are reported for 4-5 tumors per group±standard deviation (SD).

Results

Tumor mapping: Vessel distribution and perfusion. Tumor sections were analyzed by immunohistochemistry for vessel distribution (CD31), vessel perfusion (Carbocyanine), cell proliferation (BrdU) and tumor hypoxia (pimonidazole) (Figure 1, A-C). The microvessel density (MVD, number of CD31 objects per 100 μm × 100 μm area) did not change in the different treatment groups following irradiation, whereas the percentage of CD31-positive tissues decreased in UMSCC47 and UD2 tumors, indicating a reduction in vessel size (Figure 2A-C). Following irradiation, at the analyzed timepoints, neither of the tumor models showed any difference in the level of perfused vasculature (PF) (Figure 2A-C).

Cell proliferation. The tumor sections were analyzed for the level and the distribution of proliferating cells based on BrdU labeling. It was observed that the median pre-treatment values for percent of tissue positive for BrdU was 3.41 (±1.4), 1.73 (±0.8) and 2.2 (±1.07), in UMSCC47, UD2 and FaDuDD tumors, respectively. In response to irradiation, the average level of proliferating cells in UD2 tumors was significantly decreased at 6 h ($p=0.009$) and 48 h after irradiation ($p<0.001$) (Figure 3A). For UMSCC47 tumors the average level of proliferating cells displayed a small, but

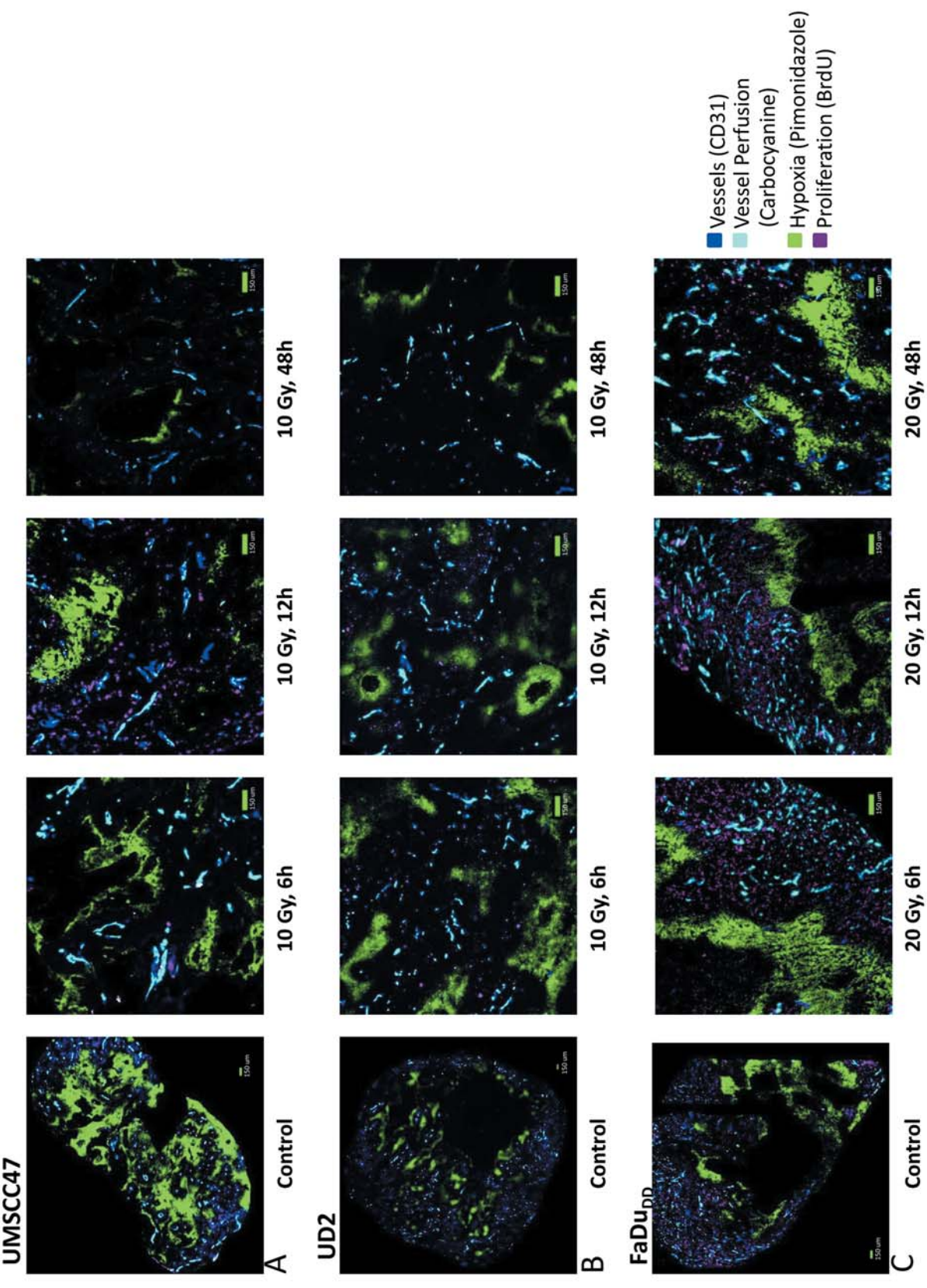

Figure 1. *Staining of (A) UMSCC47, (B) UD2 and (C) FaDuDD tumor models with CD31 for vessels (blue), carbocyanine for vessel perfusion (light blue), pimonidazole for tumor hypoxia (green) and BrdU for cell proliferation (purple). For unirradiated tumors (controls), whole tumor sections are shown. For irradiated tumors (6, 12 and 48 hours after irradiation), an enlargement from a section is shown.*

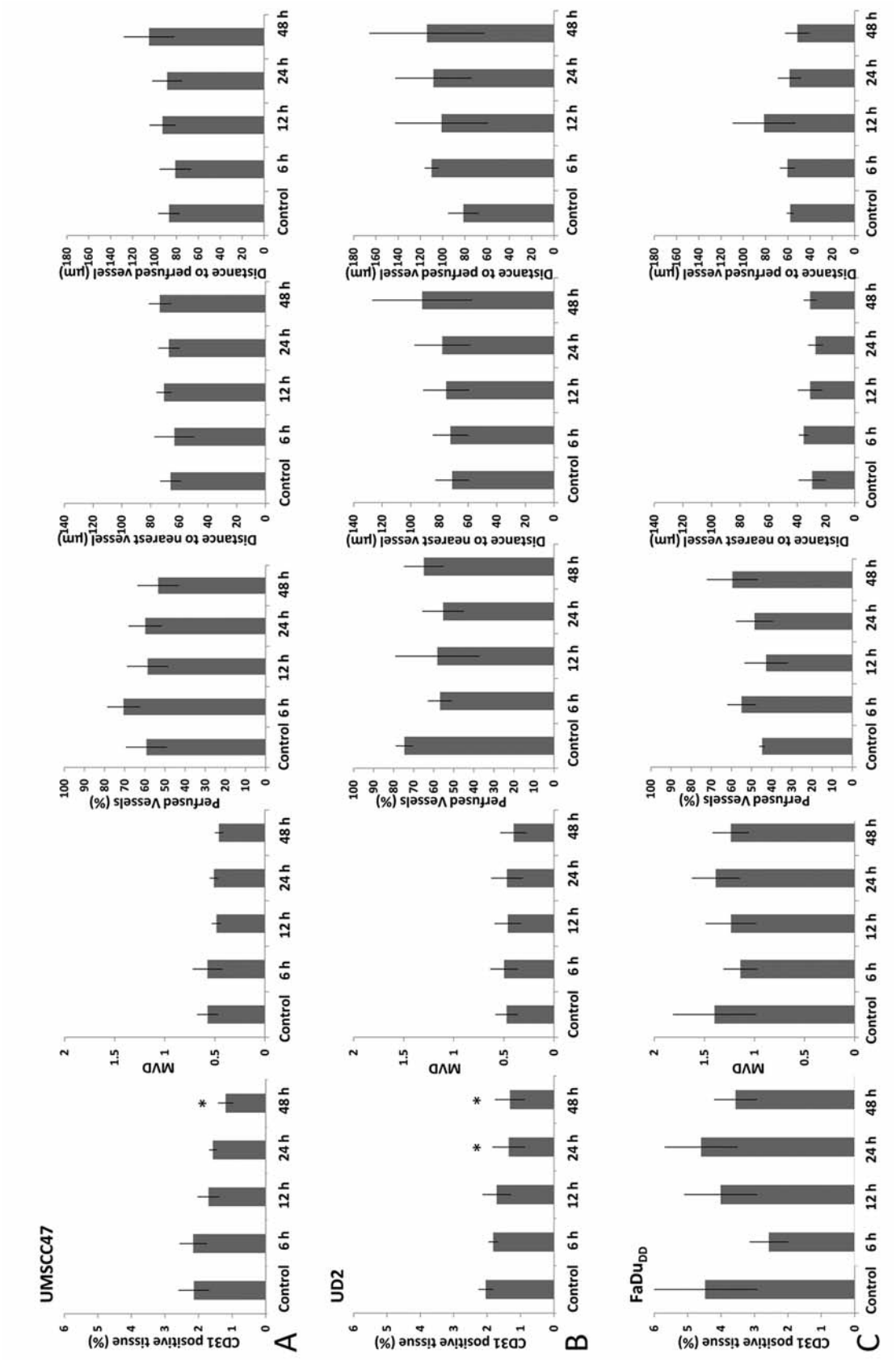

Figure 2. *Vessel distribution and perfusion in (A) UMSCC47, (B) UD2 and (C) FaDuDD tumor models. Tumors were irradiated with 10 (UMSCC47, UD2) or 20 Gy (FaDuDD) and excised at different timepoints following irradiation. The percentage of CD31 positive tissue, figumicrovessel density (MVD, number of CD31 objects per 100 µm × 100 µ m area), perfusion level (% CD31 objects positive for carbocyanine), media tissue distance to nearest vessel (µm) and media tissue distance to nearest perfused vessel (µm) is shown. Levels are average of treatment groups (n=4-5, ±SD) (due to technical issues n=2 for perfusion data in controls in FaDuDD; these data have not been included in the statistical analysis). p-values <0.05 are reported (*).*

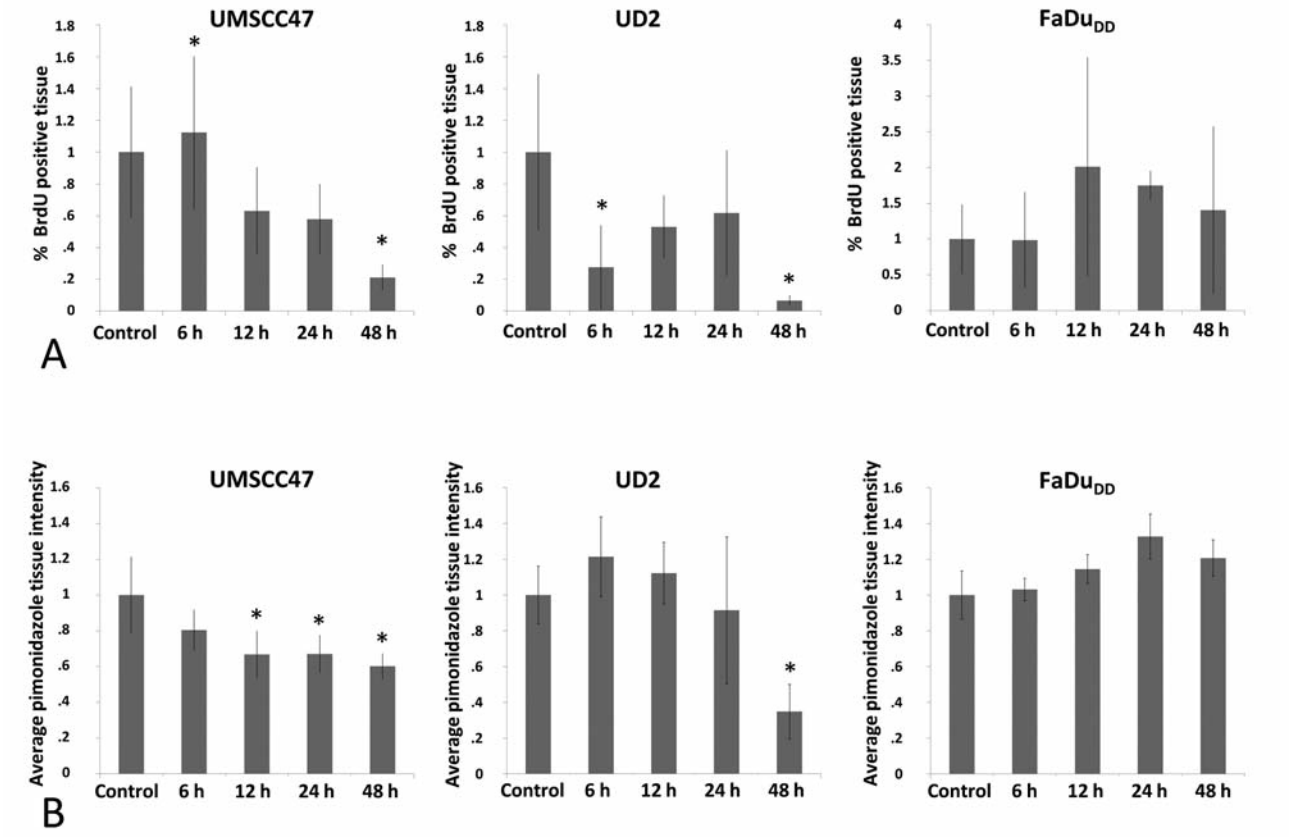

Figure 3. *Level of BrdU staining (A) and pimonidazole (B) as average on whole section viable fraction (VF). Tumors were irradiated with 10 (UMSCC47, UD2) or 20Gy (FaDuDD) and excised at different timepoints following irradiation. For BrdU the level is determined as percent positive staining obtained using the proportion of pixels at intensities meeting or exceeding a threshold value above background. Pimonidazole levels are the average intensity values. For both BrdU and pimonidazole, levels are average of treatment groups (n=4-5) normalized to the levels in the control group, ±SD. p-values <0.05 are reported (*).*

significant, increase 6 h following irradiation, whereas the level was decreased 48 h after irradiation, compared to non-irradiated controls (p=0.002).

In previous *in vitro* experiments, including the same cell lines, it was found that the HPV positive models were more radioresistant (15) with a 1.7-time higher dose at a 10% survival fraction for FaDuDD. It was therefore expected that FaDuDD would require a higher dose in order to reach the isoeffect of the HPV negative models. Hence, FaDuDD tumors were irradiated with both 10 (selected timepoints only) and 20Gy. Although not statistically significant, there was an apparent increase in the levels of cell proliferation following irradiation with 20Gy (Figure 3A).

The level of BrdU staining was measured as a function of the distance to the nearest vessel (Figure 4A-C). In the unirradiated controls, the highest level of proliferation is found around 30-40 μm from the vessels and is then decreasing with distance. The observed lower proliferation rate closer to the vessels most likely represents stromal tissue. In the two HPV-positive models, UMSCC47 and UD2, decreasing levels of cell proliferation following irradiation can be observed, whereas in FaDuDD, the HPV-negative model, there is an increase in the levels of cell proliferation following irradiation, corresponding to the increase in the average level in Figure 2A.

Tumor hypoxia. In the previous *in vitro* experiments using the same cell lines, it was demonstrated that the HPV-positive and the HPV-negative cell lines displayed the same sensitivity to hypoxia with comparable oxygen enhancement ratios (15). In the present *in vivo* study, the level of tumor hypoxia was assessed by pimonidazole staining (Figure 3A-C). Pimonidazole was in all treatment groups administered to mice 2 h prior to being euthanized. The percentage of pimonidazole-positive tissues prior to treatment was 8.29 (±1.8), 9.6 (±1.6) and 3.7 (±1.0) in UMSCC47, UD2 and FaDuDD tumors, respectively. Following irradiation with 10 Gy, the level of average pimonidazole tissue intensity was

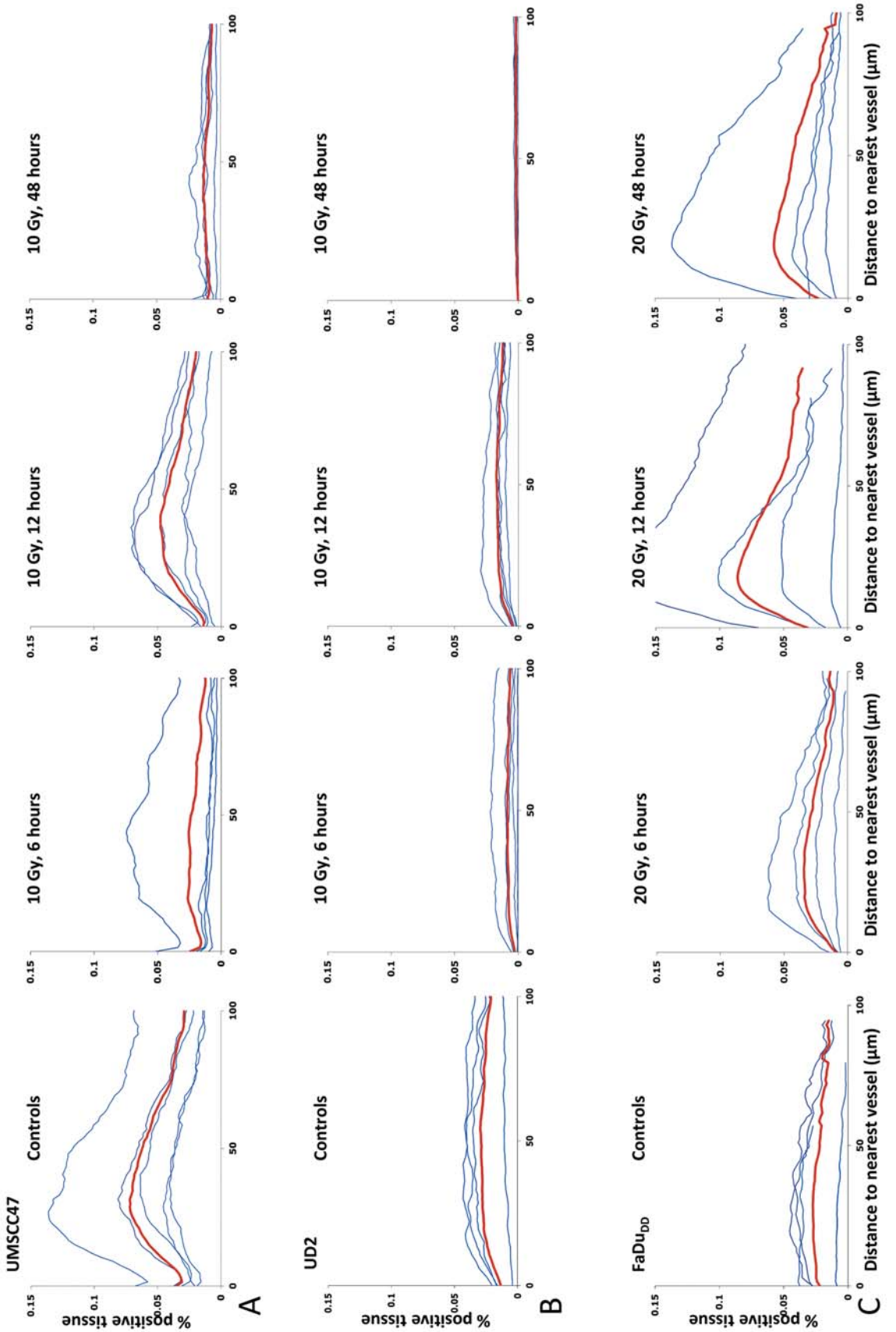

Figure 4. *The fraction of BrdU staining (percent positive staining exceeding a threshold value above background) as a function of the distance to the nearest vessel for UMSCC47 (A), UD2 (B) and FaDuDD (C) following irradiation (10Gy for UMSCC47 and UD2, 20Gy for FaDuDD). Data are shown as individual measurements (blue lines) and as average of the treatment group (red line).*

significantly decreased in UMSCC47 at the 12-, 24- and 48-h timepoints following treatment, and 48 h following treatment in UD2. In FaDuDD tumors there was no difference in the hypoxic fraction following irradiation, neither with 10 (data not shown) or 20 Gy (Figure 3C).

Discussion

In the present study we addressed the issue of increased radiosensitivity and impact of tumor hypoxia in pre-clinical head and neck cancer tumor models. It was found that cell proliferation is decreasing with time in response to radiation in two of the models, UMSCC47 and UD2, whereas no impact of radiation was observed in the HPV-negative model, FaDu, even at double the dose the HPV-positive tumors where treated with. This result confirms the majority of previous *in vitro* data (12-17) demonstrating HPV-positive cell lines to be distinctly more radiosensitive compared to HPV-negative cell lines, whereas a few studies have indicated the radio-response not to be affected by HPV status *in vitro* (24) or HPV-positive cell lines to be less sensitive to radiotherapy (25). The current data, demonstrating a HPV-dependent influence of radiation on proliferating cells, confirms results previously reported *in vitro*, where HPV-positive cells were found to be arrested in the G_2 phase of the cell cycle (14, 16).

The effect of the, possibly temporary, decrease in cell proliferation on the overall tumor growth has been shown in an *in vivo* study from Kimple *et al*. who investigated the radioresponse in HPV-positive tumor models in respect to tumor growth time (14) using limited fractionation. Herein, a significant effect in the HPV-positive tumor models was shown, which includes the same tumor models as from the present study, whereas no effect of the radiation scheme on the HPV-negative models was found. This observed biological effect on cell proliferation may be overcome by fractionation, as shown in the clinical Dahanca 6 and 7 studies, where patients with HPV positive and HPV negative tumors benefitted equally from accelerated radiotherapy (26).

Comparing the original, untreated characteristics in the included tumor models in the present study, it was observed that the HPV-negative tumor model, FaDuDD, was less hypoxic than the HPV-positive models. This should lead to the effect that HPV-negative tumors are more radiosensitive; however, the data showed the opposite, that the HPV-positive tumors were more radiosensitive. The vessel size was observed to decrease in the two HPV-positive models, which could be speculated to be due to a change in the vascular endothelial growth factor (VEGF) signaling or could be a response to the decrease in proliferating cells. These issues should be further studied. It was also found that the hypoxic fraction of the HPV-positive tumor models decreased in response to radiotherapy; a trend that was not seen in the HPV-negative tumors. The mechanism for the decreasing hypoxic fraction could be reoxygenation, as has previous been observed following irradiation (27).

As this study only included three tumor models, 2 HPV-positive and one HPV-negative, further studies on a broader range of tumor models are needed to draw definitive conclusions that may link the present data to future clinical approaches. Nevertheless, it is tempting to speculate that if HPV-positive tumors are becoming less hypoxic during a course of radiotherapy, a decrease in hypoxic fraction in response to radiotherapy could explain why HPV-positive tumors do not respond to hypoxic modification.

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