

Effects of irradiation on human leukocyte antigen class I expression in human papillomavirus positive and negative base of tongue and mobile tongue squamous cell carcinoma cell lines

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Abstract. Human papillomavirus (HPV) infection is a risk factor for oropharyngeal cancer, besides smoking and alcohol. Patients with HPV-positive tumors have a better prognosis than those with HPV-negative tumors. Furthermore, patients with HPV-positive tumors, with high CD8⁺ tumor infiltrating lymphocyte counts or absent/low human leukocyte antigen (HLA) class I expression have the best outcome. The latter is paradoxical, since HLA class I expression is important for tumor recognition. Below, the hypothesis that radiation therapy increases HLA class I expression was tested. HPV16 positive head and neck cancer cell lines UPCI-SCC-154, UPCI-SCC-090 and UM-SCC-47, and the HPV-negative cancer cell line UT-SCC-14, were treated with 2-10 Gray (Gy) and tested for HLA class I expression, cell cycle changes and apoptosis by flow cytometry. HPV16 E5, E7 and HLA-A mRNA expression was tested by quantitative PCR. A dose of 10 Gy resulted in a tendency of increased HLA class I cell surface expression for all cell lines and reached statistical significance for UPCI-SCC-154 and UPCI-SCC-090. There were, however, no significant changes in HLA-A mRNA expression in any of the cell lines, or HPV16 E5, or E7 mRNA

expression for UPCI-SCC-47 and UPCI-SCC-154, while for UPCI-SCC-090 HPV16 E5 mRNA decreased. In all cell lines there was a shift towards G2/M phase and increased apoptosis after irradiation with 10 Gy. To conclude, irradiation with 10 Gy increased HLA class I expression in the HPV-positive cell lines UPCI-SCC-154 and UPCI-SCC-090. A similar tendency was observed for HPV-positive UM-SCC-47 and HPV-negative UT-SCC-14.

Introduction

In 2007, the International Agency for Cancer Research (IACR) announced that human papillomavirus (HPV), in addition to smoking and alcohol consumption, was a strong risk factor for oropharyngeal squamous cell carcinoma (OPSCC) where tonsillar and base of tongue cancer (TSCC and BOTSCC) dominate (1). Studies performed by others and us have shown a steady increased incidence of HPV-positive (HPV⁺) TSCC and BOTSCC from the 1970s, and the past decade ~70% of all TSCC and BOTSCC have been HPV⁺ in Stockholm, Sweden (2-5). Furthermore, patients with HPV⁺ TSCC and BOTSCC have a much better prognosis compared to patients with HPV-negative (HPV⁻) tumors, with ~80 vs. 40-60% 5-year disease-free survival (6-9).

Head and neck cancer (HNSCC) in general has a very poor clinical outcome and, therefore, over the past decade, treatment has been intensified with radiation therapy in combination with chemotherapy, and in some cases with cetuximab, resulting in more severe side-effects (10,11).

Intensified therapy is most likely not necessary for most patients with HPV⁺ TSCC and BOTSCC, that together e.g. in Sweden make up ~35% of all HNSCC (12). In order to de-escalate therapy for the former two patient groups, it is important to identify patients with a very high probability to respond to therapy. By combining additional prognostic markers with HPV status in TSCC and BOTSCC, some biomarkers were found to be of particular interest.

High CD8⁺ tumor infiltrating lymphocyte (TIL) counts, absent/low human leukocyte antigen (HLA) class I expression and absent/low CD44 intensity expression were all positive

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Abbreviations: BOTSCC, base of tongue squamous cell carcinoma; FACS, flow cytometry; FBS, fetal bovine serum; Gy, gray; HLA, human leukocyte antigen; HPV, human papillomavirus; MFI, mean fluorescence intensity; OPSCC, oropharyngeal squamous cell carcinoma; PI, propidium iodine; TIL, tumor infiltrating lymphocyte; TSCC, tonsillar squamous cell carcinoma

Key words: HLA class I, human papillomavirus, oropharyngeal squamous cell carcinoma, base of tongue cancer, mobile tongue cancer, radiation therapy

Table I. Cell lines characteristics.

Cell lines	Origin	HPV status	Stage	Ref.
UM-SCC-47	Lateral tongue cancer	HPV16 mRNA positive	T3N1M0	(26)
UPCI-SCC-154	Base of tongue cancer	HPV16 mRNA positive	T4N2M0	(27)
UPCI-SCC-090	Recurrence of base of tongue cancer	HPV16 mRNA positive	T2N0M1	(27)
UT-SCC-14	Mobile tongue cancer	HPV16 mRNA negative	T3N1M0	(28)

prognostic markers for HPV⁺ TSCC and BOTSCC (13-18). The fact that having high numbers of CD8⁺ TILs was favorable for prognosis was expected and has been shown for several types of tumors (19). However, it was paradoxical that absent/low HLA class I expression was an advantage, since it is assumed that foreign antigens, in this case HPV-derived peptide antigens, are presented to CD8⁺ TILs by HLA class I molecules (20). In HPV⁺ tumors, however, it has been shown that HPV E5 and E7 have the ability to suppress HLA class I expression, thereby assisting the virus to avoid immune recognition (21-24).

In an experiment using mice with HPV⁺ tumors, it was shown that treatment with radiation therapy and chemotherapy was only efficient in immunocompetent and not in immunodeficient mice (25). It is likely that the immune system also plays an important role for tumor clearance in humans, and that radiation therapy in particular, may induce increased HLA class I expression in HPV⁺ tumors, this way facilitating immune recognition after treatment.

In the present study, the hypothesis that radiation therapy increases HLA class I expression was therefore tested *in vitro* in HPV⁺ and HPV⁻ base of tongue and mobile tongue squamous cell carcinoma cell lines.

Materials and methods

Cell lines. Three HPV⁺ cancer cell lines (UM-SCC-47, UPCI-SCC-154 and UPCI-SCC-090) and one HPV⁻ cancer cell line (UT-SCC-14) were studied throughout these experiments (26-28). Cell line characteristics are summarized in Table I. Cell lines UM-SCC-47 and UPCI-SCC-090 were cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories, Inc., Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 1% L-glutamine (Gibco) and 1% penicillin streptomycin solution (Gibco). Cell lines UT-SCC-14 and UPCI-SCC-154 were cultured in minimum essential medium (MEM; Gibco) containing 10% FBS, 1% L-glutamine, 1% non-essential amino acids (Gibco) and 0.1% gentamicin (Gibco). Stocks of all cell lines were grown in t75 cell culture flasks with filter lids (Sarstedt, Nümbrecht, Germany) from which cells were plated into 6-well cell culture plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) for the irradiation experiments, and kept at 37°C with 0.5% carbon dioxide at 100% humidity. Medium was changed regularly; usually every 2-3 days and the cell cultures were split using trypsin EDTA 1x (Gibco) when ~70-100% confluent. All cell lines were tested for mycoplasma using the Takara mycoplasma detection set (Takara Bio, Shiga, Japan).

Irradiation and experimental setup. A Caesium-137 source was used to treat the cells with radiation therapy. Initial experiments and testing different radiation doses, were performed on cell lines UM-SCC-47, UPCI-SCC-154 and UT-SCC-14. Irradiation was tested with doses of 2-10 Gray (Gy), as well as fractionated doses of 2 Gy given for 5 consecutive days, and as a result of this calibration, one dose of 10 Gy was used for the consecutive experiments. Standardized experiments were performed on the four cell lines plated in 6-well plates, with the goal of reaching 75% confluence upon initiation of experiments. To obtain this, 2.5x10⁵ cells/well were plated for UM-SCC-47; 8x10⁵ cells/well for UPCI-SCC-154; 2x10⁵ cells/well for UPCI-SCC-090; and 3.5x10⁵ million cells/well for UT-SCC-14. For cell cycle analysis half the amount of previously indicated cell numbers per cell line were plated.

Flow cytometry for evaluation of HLA class I expression, cell cycle analysis and apoptosis

HLA class I analysis. The cells were stained for HLA class I proteins 48 h after irradiation. The cells were washed, trypsinized and collected in 5 ml tubes for centrifugation. After discarding the supernatant, 2x10⁵ cells/well were collected for staining, re-suspended in 2 ml FACS buffer (DPBS, 30% BSA, 2 mM EDTA) and spun down at 2,000 rpm for 5 min. To distinguish live cells from dead, cells were incubated at 4°C for 10 min with 0.3 µl LIVE/DEAD® Fixable Near-IR Dead Cell Stain kit (Thermo Fisher Scientific) diluted in 40 µl FACS buffer. The cells were then washed, spun down in FACS buffer and incubated at 4°C for 20 min with 3 µl HLA class I (A, B and C) specific antibody, clone W6/32, coupled to an Alexa Fluor 488 (anti-human) fluorescent dye (BioLegend, San Diego, CA, USA). Unstained controls and isotype controls [1 µl IgG2a (mouse) per control] (BioLegend) were included. The samples were then washed and within 1-h run on a NovoCyt™ (ACEA Biosciences, Inc., San Diego, CA, USA) flow cytometer. HLA class I expression for each sample was acquired and further analyzed using NovoExpress™ software (ACEA Biosciences). Initial experiments (Fig. 1) were run on an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Cell cycle analysis. Cells were stained for DNA content in order to analyze their stage in the cell cycle. Floating cells were collected and pooled with adherent cells after trypsin treatment and spun down for 5 min at 2,000 rpm at 4°C, this was done 24 h after irradiation. The cell pellet was then re-suspended in 0.75 ml cold PBS, followed by drop-wise addition of 1 ml cold 100% ethanol, while vortexing. Samples were stored overnight

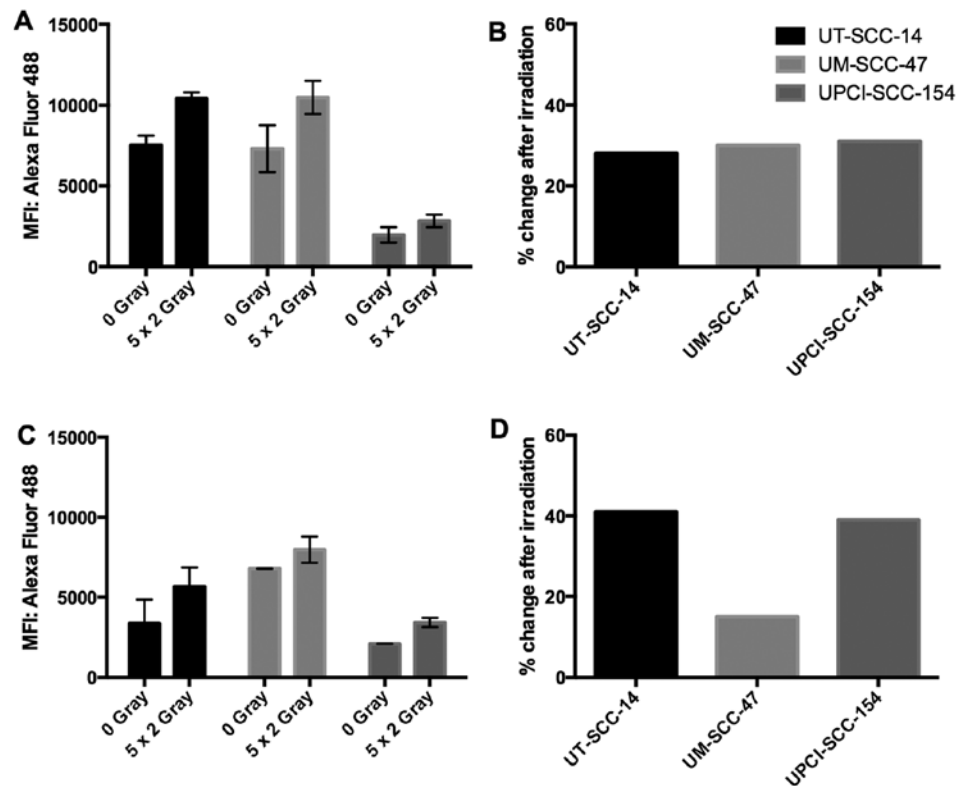


Figure 1. HLA class I expression after irradiation with 5x2 Gray. Hyperfractionating the dose (10 Gy) by irradiating with 2 Gy for 5 consecutive days showed a tendency for HLA class I upregulation in cell lines that were not trypsinized and split during the observation period (A) mean fluorescence intensity (MFI) and (B) relative increase (%). Similar trends were observed in cells that were re-plated on day 2 of the irradiation period (C) MFI, and (D) relative increase (%). P-values were not calculated for the above single experiment.

at 4°C for fixation. The cells were then spun down and stained with 100 μ l propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA)/RNase A solution (Sigma-Aldrich) (0.05 mg/ml PI, 0.25 mg/ml RNase A, diluted in 1X PBS) for 30 min at 37°C in the dark. The cells were then washed in PBS and spun down twice before being re-suspended in 200 μ l PBS and run on the NovoCyte™ flow cytometer. Cell cycle stage was further analyzed with the software NovoExpress™.

Apoptosis assay. To distinguish apoptotic from normal cells, cells were stained with a FITC Annexin V apoptosis detection kit I (BD Biosciences, San Diego, CA, USA), which binds to phospholipid phosphatidylserines, a protein that in apoptotic cells becomes more exposed. This was done 96 h after the irradiation. Floating cells were collected and pooled with adherent cells after trypsin treatment, spun down for 5 min at 2,000 rpm at 4°C, washed in cold PBS containing Mg^{2+} and Ca^{2+} , then spun down and re-suspended in Annexin V binding buffer. Cells/well (10^5) were then added to FACS tubes and incubated for 15 min at room temperature in the dark with 5 μ l Annexin V coupled to a FITC fluorophore and 5 μ l PI. The cells were then re-suspended in Annexin V buffer and within 1 h run on a NovoCyte flow cytometer. Each sample was further analyzed for apoptosis using NovoExpress software.

RNA extraction, cDNA synthesis and quantitative real-time PCR. Irradiated cells and control cells were collected 48 h after treatment and used immediately, or frozen down at -70°C for later extraction. RNA was extracted using the RNeasy® Mini

kit (Qiagen, Venlo, The Netherlands). Samples were DNase treated using the RNase-free DNase set (Qiagen) to ensure DNA free samples. In total 0.1 μ g of RNA was utilized for first strand cDNA synthesis using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific). For this, random hexamers were used as primers. The product of the cDNA synthesis was subsequently taken for a SYBR-Green based qPCR, as previously described by Lindquist *et al* (9). The following genes were examined: HPV16 E5, E7, with primers as described in Ramqvist *et al* (29) and HLA-A for exon 3 with primers as described by Villabona *et al* (30). GUS B was added as an endogenous control. Triplicates were included from each cell line, treated and non-treated, for each gene of interest and endogenous controls, as well as triple-negative controls. Treated samples were compared to non-treated samples by calculation of $\Delta\Delta C_t$ values.

Statistical analyses. Two-tailed unpaired Student's t-test was used to examine the difference in means between the treated and the non-treated groups. A $P < 0.05$ was considered statistically significant.

Results

Sensitivity to irradiation distributed as a single or several doses was similar. Preparatory irradiation experiments were performed in order to calibrate the dosage and administration frequency. Testing single doses of 2 or 5 Gy compared to untreated cell lines showed no significant difference in

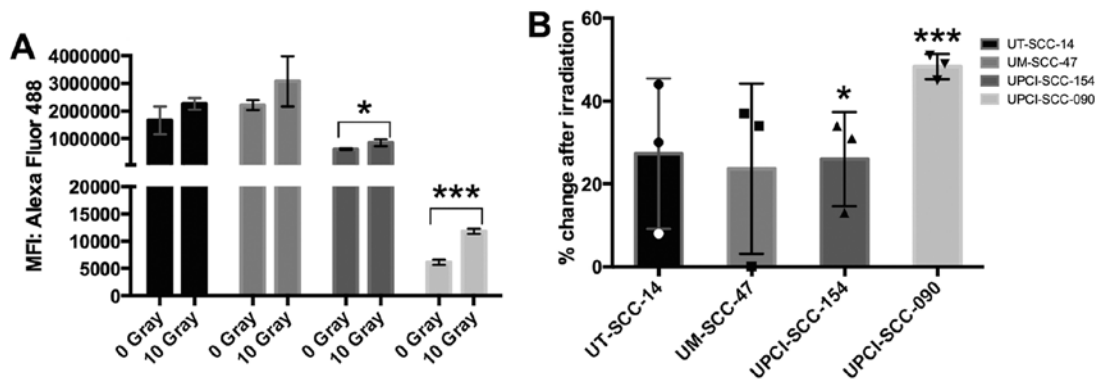


Figure 2. HLA class I expression after irradiation with a single dose of 10 Gray. HLA class I expression increased significantly in cell lines UPCI-SCC-154 ($P=0.035$) and UPCI-SCC-090 ($P<0.001$) calculated from 3 separate experiments, and as shown by mean fluorescence intensity (MFI) (A) and percentage increase (B). Similar tendencies were shown for UM-SCC-47 ($P=0.185$) and UT-SCC-14 ($P=0.129$), but did not reach statistical significance as demonstrated by MFI-values (A) and percentage increase (B). * $P\leq 0.05$, *** $P\leq 0.001$.

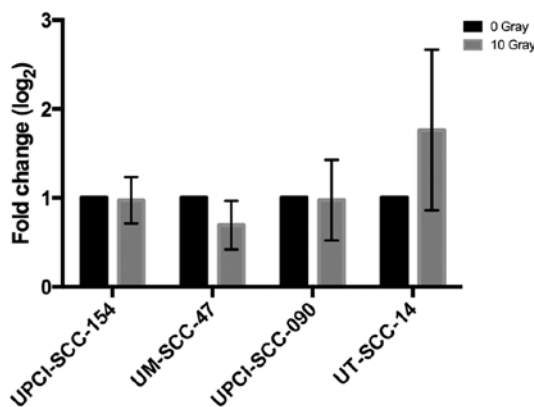


Figure 3. HLA-A mRNA levels after irradiation with a single dose of 10 Gray. For cell lines UPCI-SCC-154 ($P=0.860$) and UPCI-SCC-090 ($P=0.925$) the HLA-A mRNA levels did not change compared with the treated and non-treated group. Whereas there is a tendency of decrease of HLA-A mRNA levels in cell line UM-SCC-47 ($P=0.171$) after irradiation with 10 Gy, as opposed to cell line UT-SCC-14 ($P=0.218$), which tended to increase in HLA-A mRNA in the treated group. However, these findings could not be statistically proven.

HLA class I expression (data not shown). However, 10 Gy given either at one time-point or with 2 Gy given daily for 5 consecutive days tended to show an increase of HLA class I expression for HPV-positive UM-SCC-47, UPCI-SCC-154 and HPV-negative UT-SCC-14 (Fig. 1), while HPV-positive UPCI-SCC-090 was not tested in these first experiments. In addition, since controls and cells irradiated with 2 Gy x 5 were kept for a longer time period, experiments were performed with cells that were split or not split after 2 days of irradiation, to identify possible fluctuations in HLA class I expression depending on culture conditions. HLA class I expression tended to increase in both split and non-split cell lines (Fig. 1). Similar results were observed when 10 Gy was administered as a single dose (data not shown). Due to that no differences were observed in HLA class I expression after giving 10 Gy at one time-point, or during a time period of 5 days, all additional experiments were performed giving 10 Gy as a single dose.

In these initial sets of experiments, regardless if the cells had received irradiation, or not, base line HLA class I expression differed between cell lines and was lowest for

UPCI-SCC-154 and highest for UT-SCC-14 and UM-SCC-47 (Fig. 1). When UPCI-SCC-090 later was tested, it was shown that its HLA class I expression was the lowest of all the cell lines included in this analysis (data not shown).

Irradiation with 10 Gy tends to increase HLA class I expression in all cell lines. Three sets of experiments, with duplicate samples, were performed with 10 Gy administered as a single dose to HPV-positive cell lines UM-SCC-47, UPCI-SCC-154, UPCI-SCC-090 and HPV-negative cell line UT-SCC-14 and HLA-class I expression was compared to non-treated cells. Calculating the mean fluorescence intensity (MFI) values from these three experiments, a significant increase of HLA class I expression was indicated in cell lines UPCI-SCC-154 ($P=0.035$) and UPCI-SCC-090 ($P<0.001$) (Fig. 2A). In cell lines UM-SCC-47 and UT-SCC-14 a similar tendency with an increase of HLA class I expression was observed, although it did not reach statistical significance ($P=0.185$ and $P=0.129$) (Fig. 2A). In addition, the average increase of HLA class I expression for UM-SCC-47, UPCI-SCC-154 and UT-SCC-14 was 20-25%, while UPCI-SCC-090 with a very low initial HLA class I expression disclosed an increased expression of ~50% (Fig. 2B).

To confirm that the results were specific, an isotype antibody was included as a control for each cell line and treatment group. The background MFI of the isotype controls ranged between 2,000 and 16,000 for all non-treated and 10 Gy treated cell lines and were relatively low and negligible for UT-SCC-14, UM-SCC-47 and UPCI-SCC-154 that exhibited high values (MFIs ranging between 400,000 and 3,000,000) with the HLA class I (A, B and C) antibody. For UPCI-SCC-090 the isotype MFI values ranged between 6,000 and 11,000, thus, close to those obtained for the HLA class I antibody (Fig. 2).

HLA-A mRNA expression was analyzed by quantitative PCR in three consecutive experiments for all cell lines. HLA-A mRNA expression did not differ significantly between treated (10 Gy) and non-treated cells (Fig. 3).

HPV16 E5 and E7 mRNA expression after a 10-Gy radiation dose. To examine whether presence of HPV E5 or E7 mRNA expression was correlated to HLA class I expression, mRNA levels of E5 and E7 were measured by RT-qPCR in all HPV+

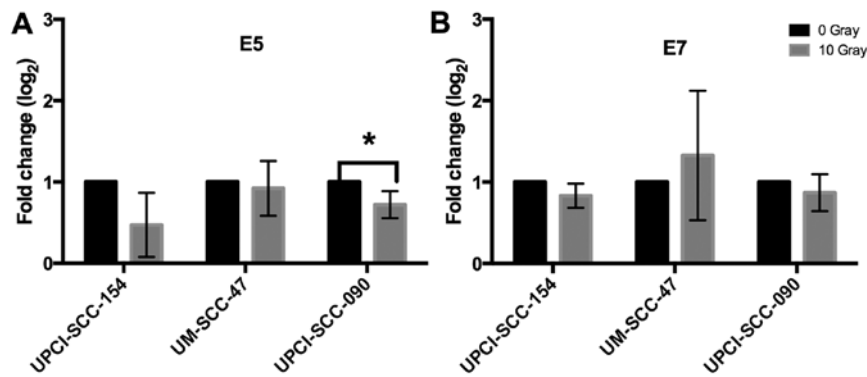


Figure 4. HPV16 E5 and E7 mRNA levels after irradiation with a single dose of 10 Gray. HPV16 E5 mRNA levels (A) tended to decrease marginally in UPCI-SCC-154 ($P=0.083$) and significantly in UPCI-SCC-090 ($P=0.044$), in the irradiated group as compared to the non-irradiated, whereas E5 levels were more stable in UM-SCC47. HPV16 E7 mRNA levels (B) were quite similar, with a slight decrease in UPCI-SCC-154 ($P=0.125$) and UPCI-SCC-090 ($P=0.386$), whereas UM-SCC-47 ($P=0.859$) tended to increase, however, due to large variability this could not be statistically proven. * $P\leq 0.05$.

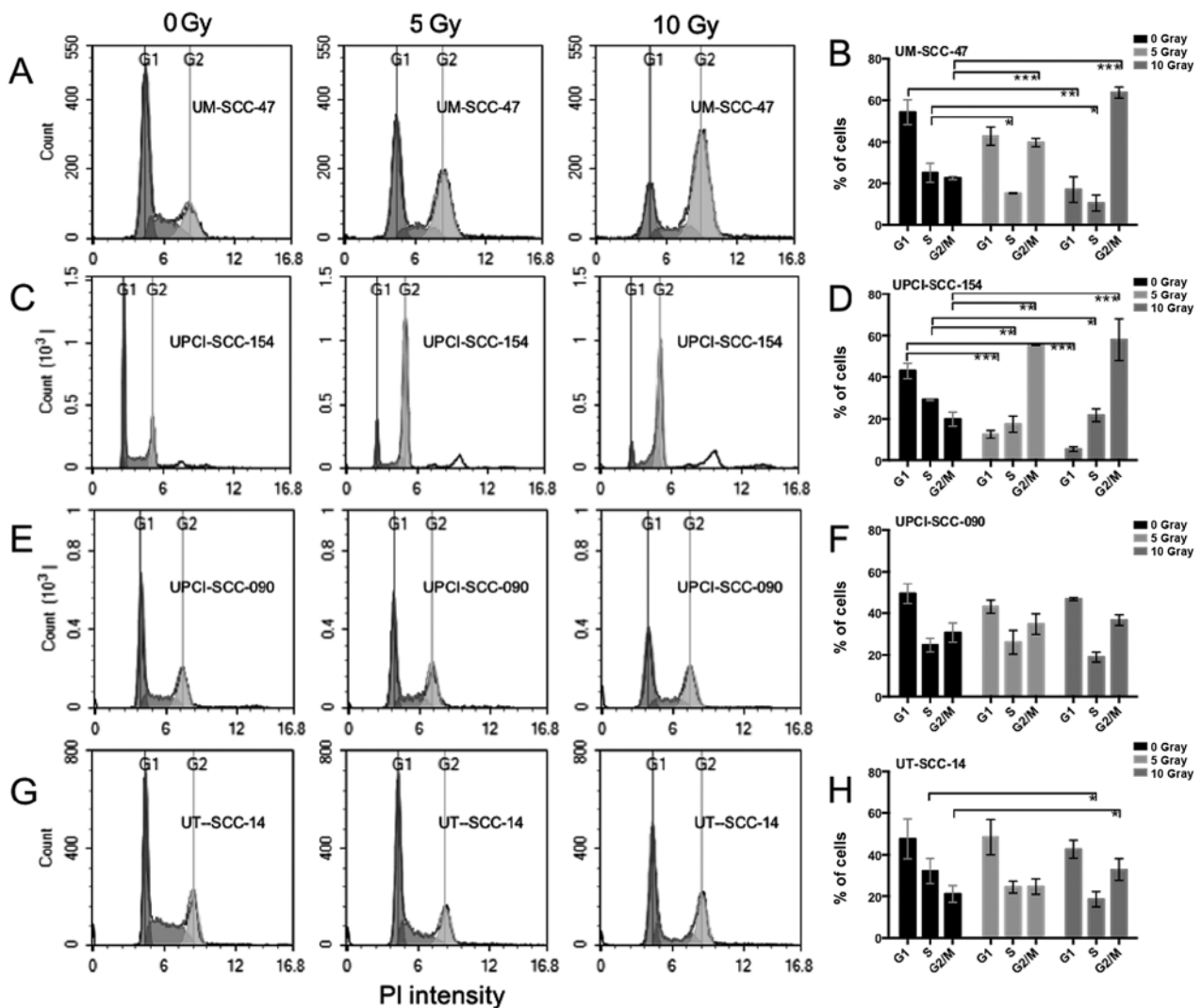


Figure 5. Cell cycle analysis after irradiation with 5 or 10 Gray. (A and B) Cell lines UM-SCC-47 and (C and D) UPCI-SCC-154 show a major shift in proportion of cells in the G1 phase to G2/M phase, and a decrease of cells in S phase, the higher irradiation dose was given. Whereas, the shift from G1 to G2/M was much less pronounced in cell lines UPCI-SCC-090 (E and F) and in UT-SCC-14 (G and H), after irradiation. * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$.

cell lines. Three different experiments were performed with triplicate samples 48 h after irradiation in treated (10 Gy) and non-treated cells. E5 mRNA expression was significantly

decreased in cell line UPCI-SCC-090 after irradiation compared to non-treated cells, and a similar tendency was observed for UPCI-SCC-154, but here there was a greater variability between

Table II. P-values from the cell cycle analysis.

	UM-SCC-47			UPCI-SCC-154			UPCI-SCC-090			UT-SCC-14		
	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
5 Gy	0.055	0.020	<0.001	<0.001	0.007	<0.001	0.127	0.738	0.353	0.915	0.115	0.327
10 Gy	0.002	0.013	<0.001	<0.001	0.014	0.003	0.509	0.122	0.204	0.458	0.029	0.037

P<0.05 are marked in bold.

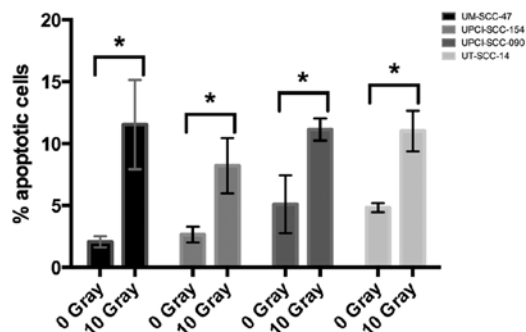


Figure 6. Apoptosis after irradiation with 10 Gray. The amount of apoptotic cell increased significantly in cell lines UM-SCC-47 (P=0.036), UPCI-SCC-154 (P=0.014), UPCI-SCC-090 (P=0.014) and UT-SCC-14 (P=0.003), compared to the corresponding non-treated controls. Notably, since a large proportion of the cells 96 h after radiation already had fragmented, and hence not included in this analysis, the amount of cell death is most likely much higher. *P≤0.05.

experiments and statistical significance was not reached (Fig. 4). No other significant changes were observed for HPV16 E5 and E7 mRNA expression (Fig. 4).

Irradiation induces cell cycle changes. After irradiation with 5 and 10 Gy, compared to non-treated cells, cell cycle changes were observed in all four cell lines, where an increasing dose generally lead to G2/M arrest and a decrease of cells in S-phase with some variation between the different cell lines. UM-SCC-47 and UPCI-SCC-154 showed a more drastic increase of cells in G2/M with less cells in G1 compared to UPCI-SCC-090 and UT-SCC-14 (Fig. 5). P-values are presented in Table II.

Irradiation with 10 Gy induces apoptosis. Apoptosis was estimated using Annexin V staining 96 h after irradiation with 10 Gy and the percentage of cells undergoing apoptosis was compared to non-treated controls. All cell lines showed a significant increase of apoptosis in the treated groups (Fig. 6). However, since a large proportion of the cells were already fragmented 96 h after radiation and were not included in this analysis, the amount of cell death was likely higher.

Discussion

In the present study, the hypothesis that HLA class I expression is upregulated upon radiation therapy was examined *in vitro* in HPV⁺ cancer cell lines UM-SCC-47, UPCI-SCC-154 and UPCI-SCC-090, and the HPV⁻ cancer cell line UT-SCC-14. A

single dose of 10 Gy tended to increase HLA class I expression on a protein level for all four tested cell lines. This increase was statistically significant for UPCI-SCC-154 and UPCI-SCC-090, and was accompanied by a significant drop in HPV16 E5 mRNA expression, for UPCI-SCC-090, with a similar tendency for UPCI-SCC-154. There was, however, no significant change in HLA-A mRNA expression in any of the cell lines, or in HPV16 E7 mRNA expression in any of the HPV⁺ cell lines after irradiation. A shift from G1 to G2/M and decrease in S-phase after irradiation was observed in 3/4 cell lines and irradiation-induced apoptosis in all cell lines.

The obtained data could partially explain why HPV⁺ TSCC/BOTSCC with low HLA class I expression still could have a good clinical response after radiation therapy. An increase in HLA-class I expression could definitely trigger or improve a cytotoxic T cell response, and explain the often excellent tumor clearance observed after radiation therapy especially of HPV⁺ TSCC/BOTSCC.

It is possible, that the increase in HLA class I expression, at least in UPCI-SCC-090 and possibly in UPCI-SCC-154 could in part be due to a decrease in HPV16 E5 mRNA expression, since it has been postulated that HPV16 E5 can downregulate HLA class I (21-24). Nevertheless, other mechanisms can also be involved in the upregulation of HLA class I after irradiation. This is supported by the fact that a similar tendency with an increased HLA class I expression was also found in the HPV⁻ cell line, suggesting that this propensity is not necessarily unique for HPV⁺ tumors. Nonetheless, our data must be considered with caution, especially for HPV⁻ cancer, since only one HPV⁻ tumor cell line was included. In addition, although HLA class I expression tended to be upregulated in the HPV⁻ cancer cell line in this study, the effect on tumor clearance may not be as great as for patients with HPV⁺ cancer, since HPV⁻ cancer is not virus driven and viral antigens cannot be presented to the immune system.

One can further speculate as to why HLA class I expression is upregulated after radiation therapy. The amount of intracellular peptides is a limiting factor for HLA class I expression and recently Reits *et al* (31) have shown, that radiation therapy increases the intracellular peptide pool, which in turn leads to an upregulation of MHC class I molecules. For HPV⁺ cancer this may be of great benefit since increasing the peptide pool of e.g. the HPV16 E6 and E7 nucleoproteins and increasing HLA class I expression can be very useful for potentiating the immune response against such tumors, with the result of a more efficient rejection of them.

Nevertheless, it is possible that irradiation also targets other mechanisms than the above in viral induced tumors, where

downregulation of HLA class I molecules is often induced in different ways to escape immune recognition (21-24,32). The decrease, or tendency of a decrease of HPV16 E5 mRNA expression after irradiation in this study, in two of the HPV⁺ cell lines indicates that this could be the case. To resolve this further, additional specific investigations are necessary.

Although an increase in HLA class I at the protein level on the cell surface was observed, this was not correlated to rise in HLA-A mRNA expression. However, HLA class I expression on the cell surface, does not necessarily reflect HLA-A mRNA expression and it has been reported that E5 inhibits surface expression of HLA class I proteins by retaining them in the Golgi apparatus (21).

The fact that a considerable amount of cells underwent apoptosis and a G1 phase to G2/M phase transition was obtained was not unexpected following irradiation with 10 Gy, and has been reported also by others (33,34). Still, there could be other intrinsic differences with regard to radio-sensitivity of the different cell lines irrespective of HPV status or HLA class I expression, however, this needs to be investigated further. A study by Gupta *et al* (35) for example showed that UPCI-SCC-47 was several-fold more sensitive to radiation as compared to UPCI-SCC-090.

There are several limitations in the present study. First of all the analysis was performed *in vitro* on cell lines, thus, lacking the possible cytokine burst accompanied after irradiation *in vivo* (36). Secondly, we only tested a limited number of HPV⁺ cell lines and only one HPV⁻ cell line and the data for UPCI-SCC-090 were due to its very low initial HLA class I expression problematic with regard to background isotype staining. Nevertheless, there was a consistent tendency for HLA class I expression to increase after irradiation in all cell lines, thus, indicating that indeed HLA class I expression could increase after radiation therapy. Furthermore, notably for the UPCI-SCC-090 line E5 mRNA expression dropped significantly, and since E5 has the capability to decrease HLA class I surface, the observed increase in HLA class I expression in this cell line could in fact be a result of this effect.

Another limitation of this study was that at least one cell line was not derived from the tonsils or base of tongue, which is commonly where HPV-driven tumors occur. Whether the HPV-positive cell line, derived from lateral tongue, is from the mobile or base of tongue is not published, but since it expresses HPV E6 and E7 mRNA, this indicates that it is HPV-driven.

To conclude, in the present study, irradiation with 10 Gy induced an increased HLA class I expression in two HPV⁺ cell lines, with a similar tendency in the remaining HPV⁺ and HPV⁻ cell lines. Although further studies are needed on additional HPV⁺ cell lines our findings suggest that radiation may possibly potentiate the immune response to HPV⁺ tumors, where viral antigens contribute to immune recognition.

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