research article

Pulsed low dose-rate irradiation response in isogenic HNSCC cell lines with different radiosensitivity

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Background. Management of locoregionally recurrent head and neck squamous cell carcinomas (HNSCC) is challenging due to potential radioresistance. Pulsed low-dose rate (PLDR) irradiation exploits phenomena of increased radiosensitivity, low-dose hyperradiosensitivity (LDHRS), and inverse dose-rate effect. The purpose of this study was to evaluate LDHRS and the effect of PLDR irradiation in isogenic HNSCC cells with different radiosensitivity.

Materials and methods. Cell survival after different irradiation regimens in isogenic parental FaDu and radioresistant FaDu-RR cells was determined by clonogenic assay; post irradiation cell cycle distribution was studied by flow cytometry; the expression of DNA damage signalling genes was assessed by reverse transcription-quantitative PCR.

Results. Radioresistant Fadu-RR cells displayed LDHRS and were more sensitive to PLDR irradiation than parental FaDu cells. In both cell lines, cell cycle was arrested in G_2/M phase 5 hours after irradiation. It was restored 24 hours after irradiation in parental, but not in the radioresistant cells, which were arrested in G_1-phase. DNA damage signalling genes were under-expressed in radioresistant compared to parental cells. Irradiation increased DNA damage signalling gene expression in radioresistant cells, while in parental cells only few genes were under-expressed.

Conclusions. We demonstrated LDHRS in isogenic radioresistant cells, but not in the parental cells. Survival of LDHRS-positive radioresistant cells after PLDR was significantly reduced. This reduction in cell survival is associated with variations in DNA damage signalling gene expression observed in response to PLDR most likely through different regulation of cell cycle checkpoints.

Key words: DNA damage; isogenic cell lines; low dose irradiation; pulsed low dose-rate irradiation; radiosensitivity

Introduction

Low dose hyperradiosensitivity (LDHRS) is a phenomenon of increased radiosensitivity to single doses below 0.5 Gy.[1] LDHRS has been demonstrated in various normal and tumour cell lines, tumour spheroids and human tumours.[2–7] LDHRS was not observed in the intrinsically radiosensitive cell lines, whereas radioresistant cell lines demonstrated the most marked LDHRS.[3,8] LDHRS precedes the occurrence of increased radioresistance (IRR) to cell killing by radiation over the dose range of 0.5 – 1 Gy.[1] Transition from LDHRS to IRR is cell type-dependent and has been typically observed in the dose range of 0.2 Gy to 0.6 Gy.[1,2,9,10]

0.3 Gy ▯

7x0.3 Gy ▯◄►▯◄►▯◄►▯◄►▯◄►▯

2.1 Gy ▮

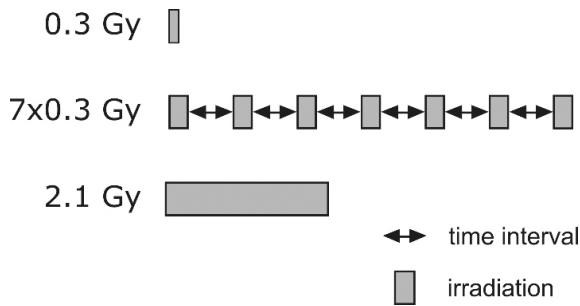◄► time interval

▮ irradiation

FIGURE 1. Schematic diagram of irradiation schedule.

Another phenomenon of increased radiosensitivity, especially in some LDHRS-positive tumor cells, is the inverse dose-rate effect. In contrary to normal tissue sparing due to repair of sublethal DNA damage during low dose-rate irradiation, increased radiosensitivity of tumour cells was observed when the dose-rate was decreased.[11] The inverse dose-rate effect can be observed at dose-rates below 1 Gy/h in cells showing LDHRS.[11,12]

The LDHRS and the inverse dose-rate effect were exploited in pulsed low-dose rate (PLDR) radiotherapy as a treatment strategy combining multiple low doses (hyperfractionation) in a pulsed delivery to reduce the effective dose-rate.[13] Its effectiveness was evaluated first in the radioresistant gliomas.[14] The delivery of low dose fractions in a pulsed fashion significantly reduced surviving fraction of glioma cell lines *in vitro*[13], greatly inhibited tumour growth of orthotopic xenografts, preserved vascular density, caused less neuronal cell death *in vivo*[15,16], and allowed retreatment of recurrent glioma tumors.[14] A similar low-dose fractionated regime significantly increased tumour growth delay in metastatic melanoma, leiomyosarcoma, breast cancer, and non-Hodgkin lymphoma.[6] In the last decade, PLDR irradiation has been used clinically for re-irradiation of recurrent tumours in the previously irradiated areas.[14,17]

Both glioblastoma and head and neck squamous cell carcinoma (HNSCC) are known for tumour recurrences within the previously irradiated area.[18–20] Based on the promising glioblastoma results using PLDR radiotherapy, this approach could be beneficial also to improve HNSCC management, namely to decrease regrowth of recurrent tumours and to reduce normal tissue toxicity. Management of HNSCC remains challenging due to complex anatomy of the region, the need for preserving function of the involved organs, locoregional recurrence of radioresistant tumours, and normal tissue toxicity.[19] In HNSCC cell lines with different radiosensitivity, so far no apparent
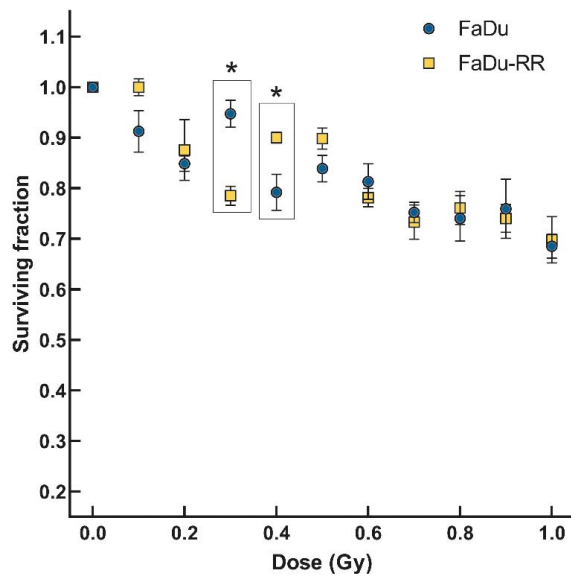

FIGURE 2. Surviving fraction of parental FaDu and radioresistant FaDu-RR cells after exposure to low doses of ionizing radiation. Symbols are mean ± standard error of the mean from four independent experiments. * - significantly different from FaDu cells.

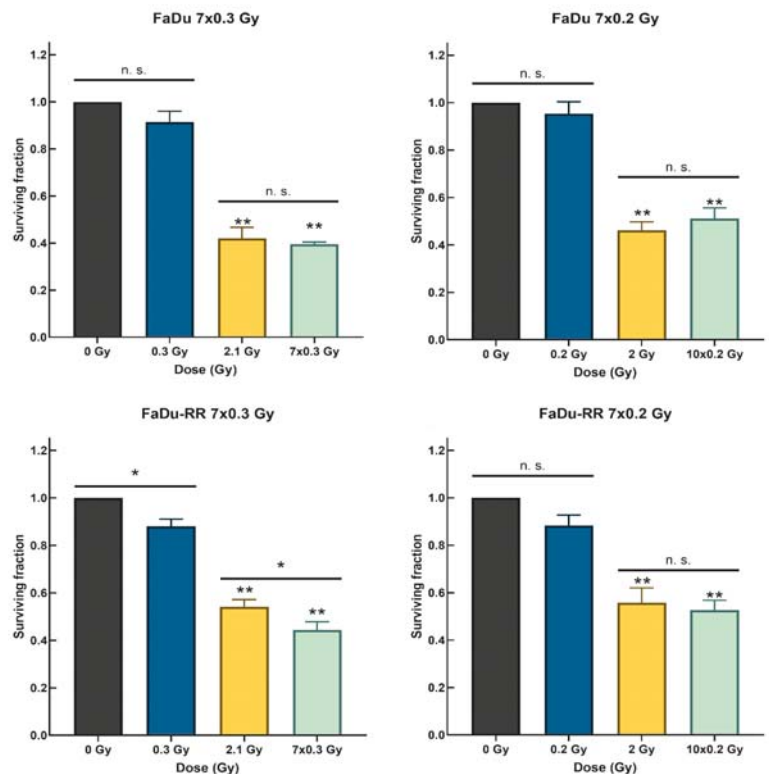**FIGURE 3.** Surviving fraction of parental FaDu and radioresistant FaDu-RR after exposure to different PLDR irradiation regimes. **(A)** Surviving fraction of parental FaDu cells after 7x0.3 Gy PLDR and **(B)** after 10x0.2 Gy PLDR irradiation. **(C)** Surviving fraction of radioresistant FaDu-RR cells after 7x0.3 Gy PLDR and **(D)** after 10x0.2 Gy PLDR irradiation. Bars present mean ± SEM from four independent experiments. ** = significantly different from 0 Gy and low dose IR (0.3 Gy or 0.2 Gy); * = significant difference between the groups; n. s. = non-significant difference.
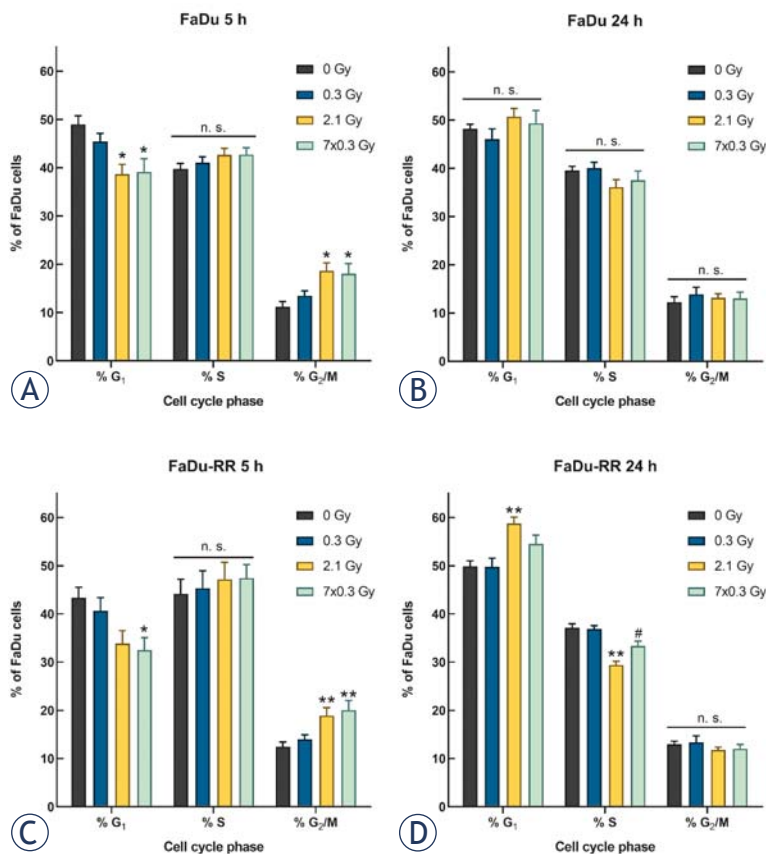
FIGURE 4. Cell cycle distribution in parental FaDu and radioresistant FaDu-RR cells after exposure to different PLDR irradiation regimes. **(A)** Cell cycle distribution in FaDu cells 5 h and **(B)** 24 h after irradiation. **(C)** Cell cycle distribution in FaDu-RR cells 5 h and **(D)** 24 h after irradiation. Bars present mean ± SEM from four independent experiments. * = significantly different from 0 Gy; ** = significantly different from 0 Gy and 0.3 Gy; # = significantly different from 0 Gy, 0.3 Gy and 2.1 Gy; n. s. = non-significant difference.

be attributed to the activation of different cellular mechanisms.

The purpose of this study was first: to evaluate the LDHRS status of two isogenic HNSCC cell lines with different radiosensitivity, followed by the evaluation of cell survival after PLDR irradiation in the isogenic cell lines. Second, with the aim to explore the underlying mechanisms of radiosensitivity of radioresistant cells to PLDR irradiation, we determined cell cycle progression and DNA damage signalling gene expression in response to low dose, conventional and PLDR irradiation.

Materials and methods

Cell lines

Human pharyngeal HNSCC cell line FaDu (ATCC, HTB-43) and 2.6-fold more radioresistant FaDu-RR cells, were established in our laboratory from the parental FaDu cells after repeated exposure to ionizing radiation as previously described.[26] Both cell lines were grown in Advanced Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher, MA, USA) supplemented with 5% fetal bovine serum (FBS, Gibco, Thermo Fisher), 10 mM L-glutamine (GlutaMAX, Gibco), penicillin (100 U/mL) (Grünenthal, Germany) and gentamicin (50 mg/mL) (Krka, Slovenia). Cells were routinely subcultured twice a week and incubated in a humidified atmosphere at 37°C and 5% CO_2.

Low dose irradiation

Irradiation was delivered using a Gulmay MP1-CP225 X-ray unit (Gulmay Medical Ltd, UK) with a filter consisting of Cu thickness of 0.55 mm and Al thickness of 1.8 mm at 200 kV and 1.0 mA to achieve the low dose rate 0.185 Gy/min. The low dose rate was used to allow precise delivery of single low dose (0.1 Gy was delivered in 0.5 min). To determine the radiosensitivity of parental FaDu and radioresistant FaDu-RR cells, cells were exposed to single doses of 0.1 – 1 Gy in steps of 0.1 Gy and plated for clonogenic assay as described below.

Pulsed low dose-rate irradiation

Cells were exposed to three different irradiation schedules (Figure 1). The control, non-irradiated cells, were handled as irradiated samples but were not exposed to any irradiation. The irradiated cells were exposed to either a single dose of 0.3 Gy, a series of seven 0.3 Gy pulses (7x0.3 Gy) or a single dose of 2.1 Gy. A series of 0.3 Gy pulses was sep-

difference was observed between conventional and low dose irradiation, however, LDHRS status of these HNSCC cell lines was unknown.[21] Therefore, proper selection of LDHRS-positive cell lines and tumours is crucial to evaluate the effect of PLDR radiotherapy and/or ultrahyperfractionated irradiation in HNSCC.

The exact mechanisms causing the LDHRS are not clear yet. Most prominently LDHRS appears in G_2-phase cells, where the threshold amount of DNA damage needs to occur to overcome LDHRS and induce IRR.[22,23] DNA damage signalling network is involved in cell cycle checkpoint activation and plays an important role in cellular radiosensitivity.[24,25] Isogenic cell lines with different LDHRS status are an attractive model to study the mechanisms involved in the LDHRS response. Due to the same genetic background, observed difference in the response to PLDR irradiation can

ules, they were plated for clonogenic assay as described below.

Clonogenic assay

For all irradiation doses, 350 cells/dish were plated onto 60-mm tissue culture dish and irradiated with a specific single dose or specific irradiation schedule using Gulmay MP1-CP225 X-ray unit, as described above. After 10 days, the resulting colonies were stained with crystal violet and counted. Surviving fraction was calculated as a ratio of the plating efficiencies for irradiated and control non-irradiated cells. The experiments were repeated 3 to 4 times in triplicates.

Cell cycle

Cell cycle distribution of parental FaDu and radioresistant FaDu-RR cells after irradiation was determined by flow cytometry as previously described.[26] Briefly, the samples were prepared following the standard procedure using fluorochrome DAPI (4',6-diamidino-2-phenylindole-dihydrochloride). The samples were acquired using a flow cytometer Partec PAS II (Partec GmbH, Germany) and at least 30,000 cells per sample were collected during sample acquisition. Results were analyzed with MultiCycle AV DNA analysis software (Phoenics Flow Systems, Inc., CA, USA) and percent of cells in G_1, S and G_2/M phases of the cell cycle were calculated. The experiment was repeated 4 times.
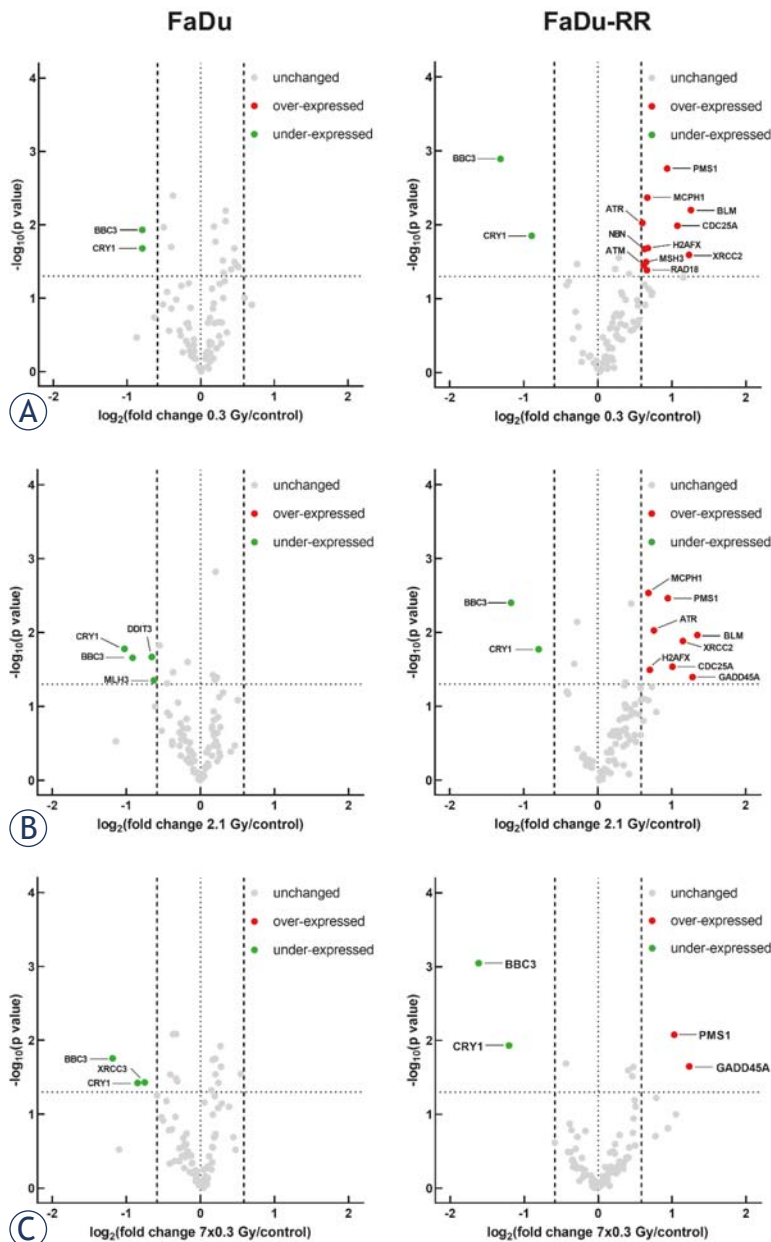
DNA damage signalling gene expression

An array of 84 pathway-specific and 5 reference genes (Human DNA Damage Signalling Pathway RT² Profiler™ PCR Array, PAHS-029Z, Qiagen, Germany) was used to study the DNA damage response in parental FaDu and radioresistant FaDu-RR cells after the low dose and PLDR irradiation. Genomic DNA control, reverse transcription control, and positive PCR controls were included in the array. Samples for gene expression analysis were prepared as previously described.[26] Briefly, 5 hours after different irradiation protocols, total RNA was isolated from the cells using RNeasy Plus Mini Kit (Qiagen), and RNA concentration and sample purity ($A_{260/280}$) were determined spectrophotometrically. For cDNA synthesis, 2 µg total RNA was used using the RT² First Strand Kit (Qiagen). Reverse transcription-quantitative PCR was carried out on QuantStudio 3 Real-time PCR
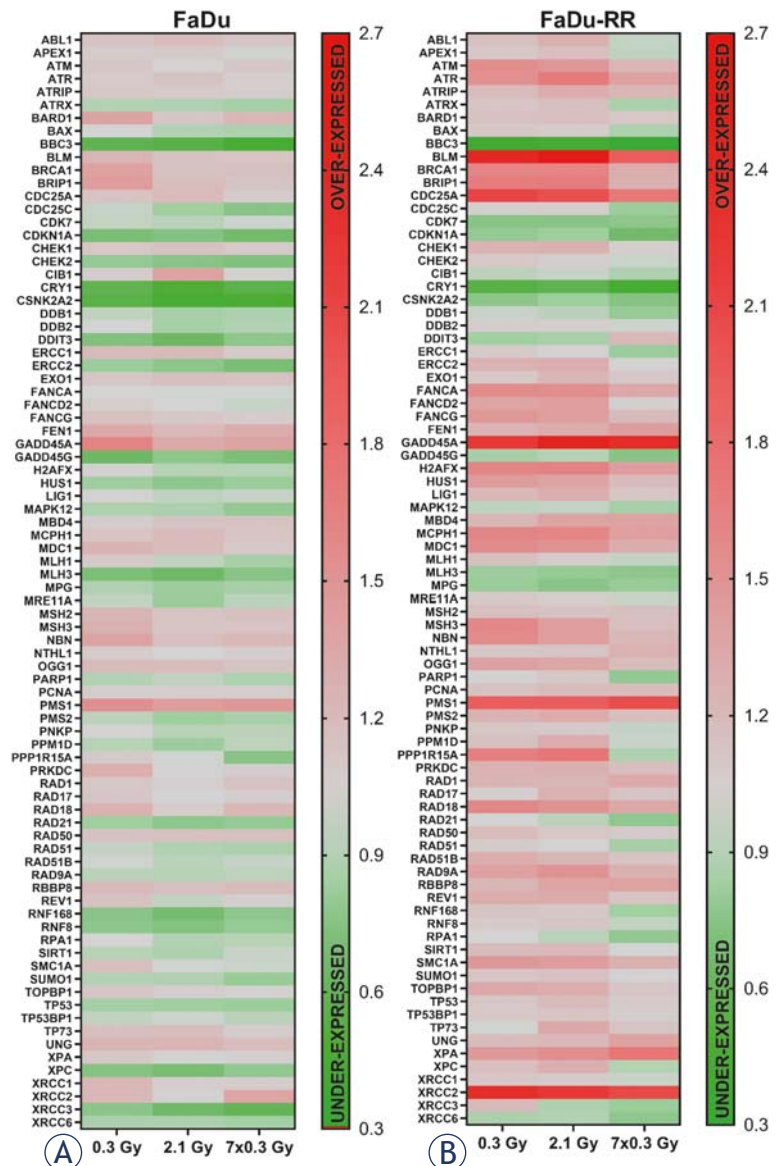
FIGURE 5. Heat maps of DNA damage signalling gene expression in parental FaDu **(A)** and radioresistant FaDu-RR cells **(B)** in 0.3 Gy, 2.1 Gy and 7x0.3 Gy irradiated cells relative to the gene expression in control non-irradiated cells. The magnitude of the fold change in gene expression of each gene from three independent experiments is represented by the colour. Green indicates under-expressed genes, and red indicates over-expressed genes.

arated by 4.5 min intervals to create an apparent dose rate of 0.055 Gy/min. Additionally, a series of 0.2 Gy pulses was separated by 3 min intervals, to create an apparent dose rate of 0.053 Gy/min, and was compared to the effect of single 2 Gy dose. A 4.5-minute and 3 min interval between the doses for each of the above PLDR irradiation protocols, was chosen to create a similar apparent dose-rate as proposed by Tome *et al*.[13] To determine radiosensitivity of the cells to these irradiation sched-

FIGURE 6. DNA damage signalling gene expression in parental FaDu and radioresistant FaDu-RR cells in response to different irradiation protocols. **(A)** Gene expression of FaDu cells in response to 0.3 Gy, 2.1 Gy, and 7x0.3 Gy irradiation relative to the control non-irradiated FaDu cells. **(B)** Gene expression in FaDu-RR cells in response to 0.3 Gy, 2.1 Gy, and 7x0.3 Gy irradiation relative to the control non-irradiated FaDu-RR cells. Volcano plots show the fold change in gene expression and statistical significance (p value). The horizontal line shows the statistical significance threshold (p value < 0.05). Two vertical dashed lines show the threshold of over-expressed (right) and under-expressed genes (left), while the solid vertical line shows no change in gene expression. Symbols represent the mean gene expression of each tested gene in irradiated cells relative to control non-irradiated cells from three independent experiments.

System (Applied Biosystems, USA) using RT² qP-CR Sybr Green ROX Mastermix (Qiagen) and cycling conditions as described previously.[26]

GeneGlobe Data Analysis Center (Qiagen) was used to analyze the results. Data were normalized to the gene expression of the reference gene with the most stable expression (*HPRT1*). Fold change in gene expression was calculated using the $\Delta\Delta CT$ method.[27] We used 1.5 fold-change in gene expression as a threshold and p values less than 0.05 to identify significantly different gene expression.

Statistics

GraphPad Prism 8.1.2 (GraphPad Software, Inc., CA, USA) was used for graphs and statistical analysis. Normal distribution of data was tested using the Shapiro-Wilk test. For normally distributed data, data are shown as the mean ± standard error of the mean (SEM). Differences between parental and radioresistant cells were identified by unpaired two-tailed t-test. One Way ANOVA with Tukey test for posthoc multiple comparisons were used to identify the difference between groups. Differences were considered significant for p values less than 0.05.

For statistical analysis of DNA damage signalling gene expression data, Student's t-test (two-tail distribution and equal variances between the two samples) was used on the replicate $2^{-\Delta\Delta CT}$ values for each gene in each irradiation protocol compared to the control non-irradiated cells from 3 independent experiments.

Results

Low dose irradiation

We observed similar radiosensitivity to single low doses of ionizing radiation in parental FaDu and radioresistant FaDu-RR cells, except at 0.3 Gy and 0.4 Gy doses (Figure 2). Surviving fraction of radioresistant FaDu-RR at 0.3 Gy was significantly lower (p=0.006) than the surviving fraction of parental FaDu cells, exposed to the same irradiation dose. From 0.3 Gy up to 0.5 Gy, an increase in the surviving fraction of radioresistant FaDu-RR cells was observed compared to parental FaDu cells, the difference was significant at 0.4 Gy (p=0.048), but not at 0.5 Gy (p=0.160).

Pulsed low-dose rate irradiation

Based on our experimental results, the radioresistant FaDu-RR cells showed the highest radiosensitivity at 0.3 Gy, therefore we used this dose to deliver PLDR irradiation. In both parental FaDu

and radioresistant FaDu-RR, the surviving fraction of cells irradiated with either a single dose of 2.1 Gy or a PLDR dose of 7x0.3 Gy was significantly reduced in comparison to control non-irradiated cells or cells irradiated with a single dose of 0.3 Gy (p<0.0001). However, no difference in surviving fraction was observed between parental FaDu cells irradiated with a single dose of 2.1 Gy or PLDR irradiation of 7x0.3 Gy (p=0.607) (Figure 3A). On the contrary, surviving fraction of radioresistant FaDu-RR cells irradiated with PLDR dose of 7x0.3 Gy was significantly reduced (p=0.028) in comparison to cells irradiated with a single dose of 2.1 Gy (Figure 3C). Similarly, a significant reduction of surviving fraction after irradiation with 0.3 Gy was observed in radioresistant FaDu-RR cells (p=0.020), but not in parental FaDu cells (p=0.178) compared with the control non-irradiated cells. Modifying PLDR irradiation to 10x0.2 Gy abolished the difference in cell survival between PLDR and single-dose irradiation in radioresistant FaDu-RR cells (p=0.951) (Figure 3B and 3D).

Cell cycle

Differences in cell cycle distribution in parental FaDu and radioresistant FaDu-RR were evaluated at 5- and 24-hour time point after different irradiation protocols (Figure 4). Asynchronous populations of non-irradiated FaDu and FaDu-RR cells did not differ in the cell cycle distribution. In response to different irradiation schemes, perturbations of cell cycle were observed in both FaDu and FaDu-RR cells. Namely, 5 hours after irradiation with a single dose of 2.1 Gy and a PLDR dose of 7x0.3 Gy, the percent of G_1-phase FaDu cells was significantly reduced (p=0.021 and p=0.027, respectively), while the percent of G_2/M-phase FaDu cells was significantly increased (p=0.023 and p=0.035, respectively) in comparison to control, non-irradiated FaDu cells. Contrary to FaDu cells, the percent of G_1-phase radioresistant FaDu-RR cells was significantly reduced only after PLDR irradiation (p=0.047), while the percent of G_2/M-phase cells was increased after both, a single dose of 2.1 Gy and a PLDR dose of 7x0.3 Gy (p=0.037 and p=0.014, respectively). No difference was observed in S-phase in FaDu nor FaDu-RR cells in all treatment groups. Cell cycle phase distribution was restored 24 hours after different irradiation protocols in FaDu cells, but not in FaDu-RR cells where an increase in G_1-phase and a decrease in S-phase cells was observed after 2.1 Gy irradiation regimen (p=0.007 and p=0.0001). A similar increase in G_1-
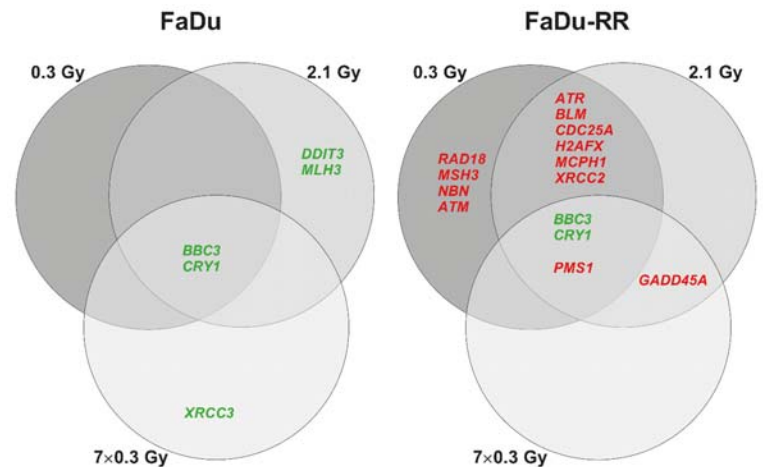

FIGURE 7. Venn diagrams of DNA damage signalling gene expression in parental FaDu and radioresistant FaDu-RR cells showing overlapping and differential gene expression. Only genes significantly over-expressed or under-expressed relative to control non-irradiated cells are shown. Genes in bold red are over-expressed, genes in bold green are under-expressed.

phase and decrease in S-phase cells was observed also in PLDR-irradiated FaDu-RR cells, however not as prominent as after a single dose of 2.1 Gy. No difference in G_2/M-phase of FaDu-RR cells was observed in any group.

DNA damage signalling gene expression

Different DNA damage signalling gene expression pattern was observed in response to different irradiation protocols relative to the control non-irradiated cells. In parental FaDu cells, more DNA damage signalling genes were under-expressed (Figure 5A), while in the radioresistant FaDu-RR cells, DNA damage signalling genes were predominantly over-expressed in response to irradiation (Figure 5B). In parental FaDu cells, significant under-expression of 2, 4, and 3 genes was observed in response to 0.3 Gy, 2.1 Gy, and 7x0.3 Gy irradiation, respectively (Figure 6A). In radioresistant FaDu-RR cells, significant under-expression of 2 genes and over-expression of 11, 8, and 2 genes was observed in response to 0.3 Gy, 2.1 Gy, and 7x0.3 Gy irradiation, respectively (Figure 6B). Specifically, *BBC3* and *CRY1* genes were under-expressed in both parental FaDu and radioresistant FaDu-RR cells in response to all irradiation schedules (Figure 7). *PMS1* was over-expressed in radioresistant FaDu-RR cells in response to all three irradiation schedules, while *ATR*, *BLM*, *CDC25A*, *H2AFX*, *MCPH1*, and *XRCC2* were over-expressed in 0.3 Gy and 2.1 Gy irradiated FaDu-RR cells.
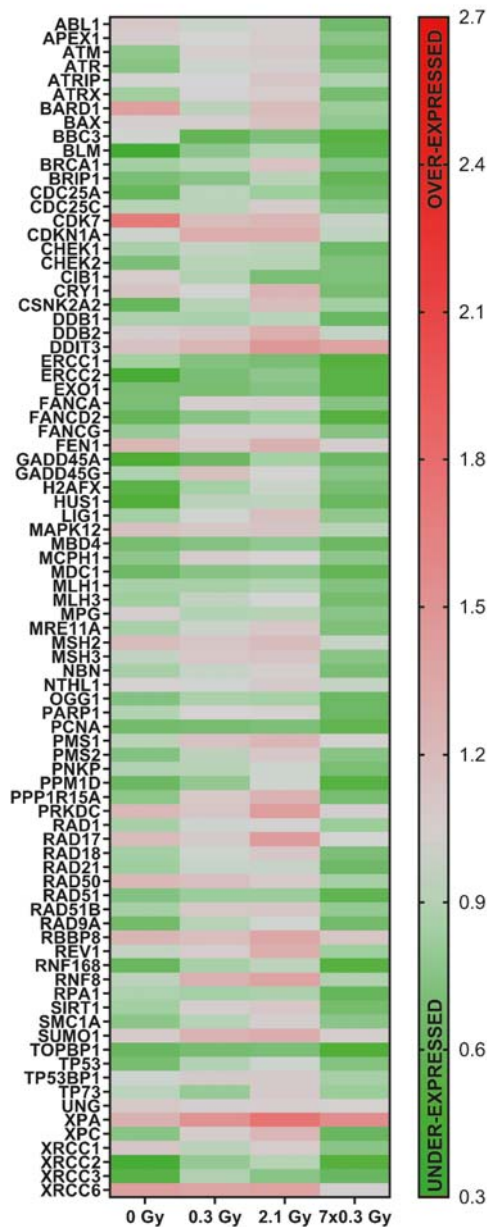
FIGURE 8. Heat maps of DNA damage signalling gene expression in radioresistant FaDu-RR cells relative to the gene expression in parental FaDu cells. The magnitude of the fold change in gene expression of each gene from three independent experiments is represented by colour. Green indicates under-expressed genes, and red indicates over-expressed genes.

GADD45A was over-expressed in 2.1 Gy and 7x0.3 Gy irradiated FaDu-RR cells, while *ATM*, *MSH3*, *NBN*, and *RAD18* were over-expressed in 0.3 Gy irradiated FaDu-RR cells only.

Direct comparison of the DNA damage gene expression in radioresistant FaDu-RR relative to parental FaDu cells identified differences in gene expression profile in non-irradiated cells and 7x0.3 Gy irradiated cells, but not 0.3 Gy and 2.1 Gy irradiated cells (Figure 8). Specifically, 71% of the tested DNA damage signalling genes in the control non-irradiated FaDu-RR cells were under-expressed, of which 7 genes (*BLM*, *ERCC2*, *H2AFX*, *HUS1*, *RNF168*, *TOPBP1*, *XRCC3*) were significantly under-expressed (Figure 9A). No significant difference in gene expression was observed in 0.3 Gy (Figure 9B) and 2.1 Gy (Figure 9C) irradiated FaDu-RR cells relative to FaDu cells. In 7x0.3 Gy irradiated FaDu-RR cells, 6 genes (*ERCC1*, *EXO1*, *MBD4*, *PCNA*, *PPM1D*, *TOPBP1*) were under-expressed and 1 gene (*XPA*) was over-expressed relative to parental FaDu cells irradiated with the same irradiation scheme (Figure 9D). TOPBP1 was the only gene under-expressed in both non-irradiated and PLDR-irradiated radioresistant FaDu cells relative to parental FaDu cells (Figure 10).

Discussion

Understanding molecular mechanisms of cellular response to low dose irradiation is important in order to evaluate risks and benefits of such exposure.[28] In radioresistant tumors this could provide the basis for a more tailored and effective radiotherapy. Re-irradiation of recurrent tumours in the previously irradiated areas is a feasible approach that improves survival, but is limited due to normal tissue toxicity.[19] However, altered fractionation regimen could improve the therapeutic outcome of re-irradiated tumours and reduce normal tissue toxicities.[14,15,29,30]

In this study, we confirmed the presence of LDHRS in the experimentally established radioresistant FaDu-RR cells *in vitro*, but not in its parental FaDu cells. Furthermore, radioresistant FaDu-RR cells were more sensitive to PLDR irradiation than parental FaDu cells, likely due to the observed perturbations of the cell cycle and changes in the expression of DNA damage signalling genes observed in these cells.

The use of PLDR irradiation in local recurrent HNSCC has been recently tested in a clinical trial in order to evaluate safety and treatment efficacy.[31–33] PLDR irradiation was initially proposed for the treatment of recurrent radioresistant gliomas.[13,16] It exploits two phenomena, LDHRS, and the inverse dose-rate effect. First, the low dose fractions used in this approach fall within the LDHRS region, generally observed in the more radioresistant tu-

mour cells.[3,8] Second, the short intervals between low dose pulses create an apparently reduced dose-rate, which contributes to the normal tissue sparing and results in increased radiosensitivity of tumour cells.[11] PLDR irradiation can be delivered over multiple days to increase the total irradiation dose, and improve the antitumor effects compared to conventional fractionation.[4]

LDHRS has to be confirmed prior to PLDR irradiation. Tailoring the PLDR parameters, such as the low dose and time intervals between the low doses, can further increase radiosensitivity.[34] Low dose pulses should be applied within the LDHRS range of specific cell type, however, this might not be straightforward. The transition dose from LDHRS to IRR is cell type-specific and has been observed in the range of 0.2 Gy to 0.6 Gy for different tumour cells.[1,2,9,10] In the clinics, this transition dose might differ between tumours as well as within the tumour due to tumour cell heterogeneity; identification of specific LDHRS markers is needed to select patients, which could benefit from PLDR irradiation. Deciphering mechanisms contributing to the LDHRS could provide a better starting point to determine the efficient low irradiation doses used for PLDR clinically. In our study, dose reduction from 0.3 Gy to 0.2 Gy abolished the difference in cell survival between PLDR and single-dose irradiation in parental FaDu and radioresistant FaDu-RR cells.

To describe the survival curve of LDHRS-positive cell lines, the linear-quadratic model fails in the low dose region and has to be adjusted to account for the increased radiosensitivity and IRR bellow 1 Gy. To take account for these specific processes, the induced repair model was proposed by Joiner et al.[35] In addition to the induced repair model, alternative models have been proposed[5,36,37], such as the variable induced repair, which is more complex, but does not account for the dose rate effect.[5] As a proof of LDHRS, different approaches can be considered. Namely, the condition $\alpha_S > \alpha_R$, confidence limits of α_S and α_R not overlapping, and D_C value significantly greater than zero can be used to deduce the presence of LDHRS.[4,5] Due to the variability in the measurements made by conventional clonogenic assay, which is typical at such high survival levels[35], the experimental data fit the induced repair model in a variable extent. Because LDHRS is prevalent in radioresistant tumour cell lines[3,8], we first evaluated the LDHRS status in the isogenic FaDu and radioresistant FaDu-RR cells, which was confirmed in the latter but not in the parental cell line. The observed transition from LDHRS to IRR in the 0.3 to 0.4 Gy dose range is similar to obser-
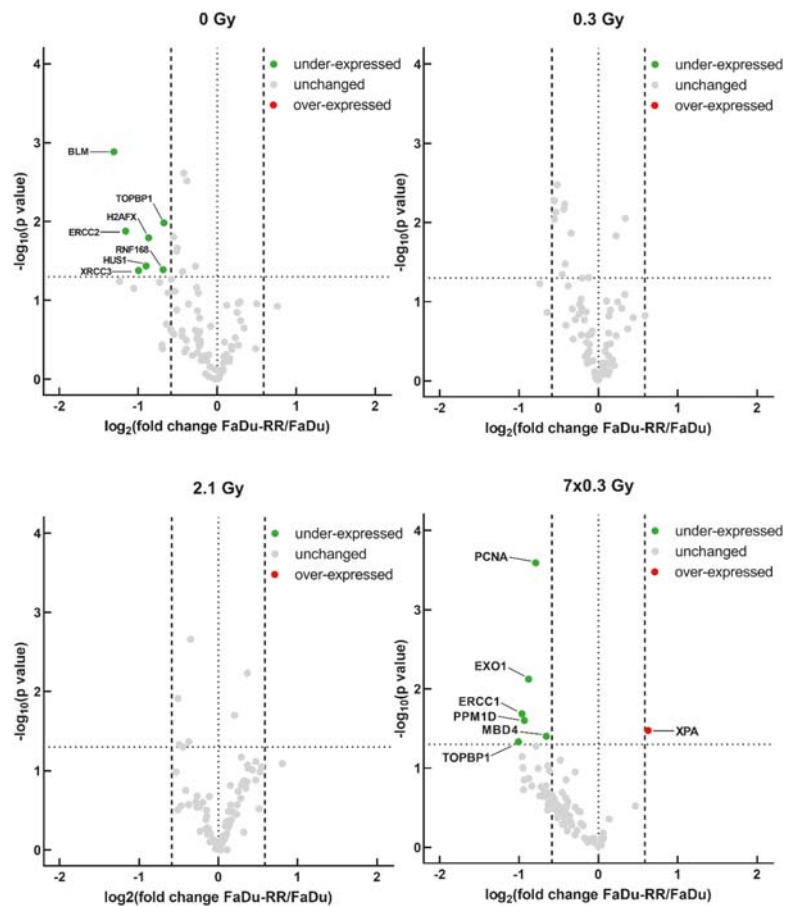
FIGURE 9. DNA damage signalling gene expression in radioresistant FaDu-RR cells relative to parental FaDu. **(A)** Gene expression in control non-irradiated cells. **(B)** Gene expression in 0.3 Gy irradiated cells. **(C)** Gene expression in 2.1 Gy irradiated cells. **(D)** Gene expression in 7x0.3 Gy irradiated cells. Volcano plots show the fold change in gene expression in radioresistant FaDu-RR relative to parental FaDu cells and statistical significance (p value). The horizontal line shows the statistical significance threshold (p value < 0.05). Two vertical dashed lines show the threshold of over-expressed (right) and under-expressed genes (left), while the solid vertical line shows no change in gene expression. Symbols represent the mean gene expression of each tested gene in radioresistant FaDu-RR cells relative to parental FaDu cells from three independent experiments.

vations in other reports.[1,2,9,10] In this preliminary experiment, we focused on the low dose response and did not evaluate cell survival in response to doses above 1 Gy due to technical limitations of our X-ray unit. Fitting these experimental data to the induced repair model is not balanced due to the lack of high dose response, and the parameters describing LDHRS (α_S, α_R, and D_C) cannot be estimated with confidence intervals, which is a drawback of this study. In addition, the model-derived D_C and the experimental dose with the lowest survival are not always the same. Mathematically, D_C is defined as the dose required for 63% induction of radioresistance,[35] therefore variations are expected
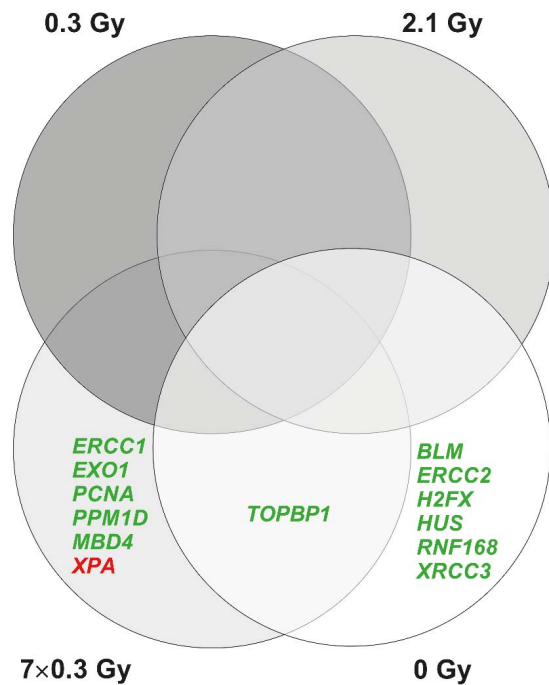
FIGURE 10. Venn diagrams of DNA damage signalling gene expression in radioresistant FaDu-RR cells showing overlapping and different gene expression after different irradiation protocols relative to parental FaDu cells. Only genes significantly over-expressed or under-expressed relative to parental FaDu cells are shown. Gene in bold red is over-expressed, genes in bold green are under-expressed.

between model-derived and experimentally observed transition points from increased radiosensitivity to increased radioresistance.[4]

LDHRS can be efficient, if the proposed pulsed irradiation scheme delivers pulses of smaller dose than the transition dose from LDHRS to IRR.[13] In our study, the PLDR irradiation scheme therefore consisted of a series of 0.3 Gy pulses. While surviving fraction of parental FaDu cells did not differ between single-dose and PLDR irradiation, a significantly lower survival was observed in PLDR-irradiated radioresistant FaDu-RR cells in comparison to single-dose irradiation. Similarly, several *in vivo* studies showed PLDR irradiation tumour volume reduction, resulting in a longer tumour growth delay in comparison to continuous irradiation.[14,15]

Ample scientific evidence supports an important role of cell cycle checkpoints and DNA damage signalling networks in the mechanisms of LDHRS.[2] Cellular repair processes are induced above a certain threshold dose as described by the induced repair model.[9] Below this threshold dose, cells can

show increased radiosensitivity, while above this dose cell survival is increased due to induced signalling and repair. In the IRR range, DNA double-strand break (DSB) repair is reportedly more efficient than in the LDHRS dose range.[38] Evaluation of LDHRS in isogenic cell lines has not been studied extensively and therefore the isogenic cell lines with different LDHRS statuses are an attractive model to study the mechanisms of LDHRS in more detail. Novel insights into the unknown mechanisms of LDHRS could thus be gained.

DNA repair is tightly coordinated with the cell cycle checkpoints.[9] In our study, low dose irradiation did not affect cell cycle in isogenic cells, while irradiation with a higher single dose and PLDR irradiation resulted in cell cycle perturbations. Following G_2/M arrest 5 hours after single and PLDR irradiation in both FaDu and FaDu-RR cells, the cell cycle was restored 24 hours after irradiation in FaDu, but not in FaDu-RR cells. This indicates a differential regulation of the cell cycle in radioresistant FaDu-RR cells in comparison to parental cells. Differences in cell cycle checkpoints in LDHRS-positive and LDHRS-negative cells have been observed previously. Most notably, in LDHRS-positive cells G_2/M checkpoint was activated at irradiation doses higher than transition dose.[39] Because LDHRS is associated with the G_2-phase enriched populations[40], it is likely that the observed LDHRS is due to inactive G_2/M checkpoint in response to irradiation below the threshold dose.[39]

This data indicate on important role of DNA damage signalling mechanisms in LDHRS. Activation of G_2/M checkpoint in cells with damaged DNA prevents entry into mitosis and provides an opportunity for DNA repair during the cell cycle delay. Increased radiosensitivity, observed in the LDHRS-positive cells, could be associated with inactive DNA damage-induced cell cycle checkpoints. Functional DNA damage signalling and repair mechanisms constitute DNA damage recognition, recruitment of specific signalling and repair proteins to the damage site and effective repair. LDHRS is not associated with reduced recognition of DSB breaks as seen by the same extent of phosphorylated H2AX.[10,41] Persistent gammaH2AX foci after low dose irradiation despite the functional DNA repair mechanisms support different DSB repair kinetics.[39,41] The unchanged level of phosphorylated ATM in response to low dose irradiation indicates an inactive ATM signalling cascade.[38]

In the present study we focused on the expression of DNA damage signalling and repair genes in

isogenic cell lines with different LDHRS status. The gene panel included DNA repair, apoptosis and cell cycle-associated genes. In LDHRS-negative parental FaDu cells, under-expression of DNA damage signalling genes was observed, while over-expression of DNA damage signalling genes was observed in LDHRS-positive radioresistant cells in response to irradiation. Specifically, DNA damage sensor genes (*ATM*, *ATR*, and *H2AFX*), cell cycle checkpoint regulator genes (*CDC25A*, *BLM*, *GADD45A*, *MCPH1*) and genes involved in homologous recombination (*BLM*, *XRCC2*) were over-expressed in response to 0.3 Gy and 2.1 Gy irradiation. On the other hand, after PLDR irradiation the expression of DNA damage sensor genes and homologous recombination genes was not increased, indicating inactive DNA repair mechanisms and decreased cell survival after PLDR. The reduction in cell survival can be associated also with aberrant regulation of cell cycle checkpoints. The observed G_1 cell cycle arrest after 2.1 Gy and PLDR irradiation is likely mediated by over-expression of GADD45A in radioresistant FaDu-RR cells.[42] Inactivation of GADD45A was also associated with chemosensitization and radiosensitization.[43,44]

Exact mechanisms of PLDR irradiation contributing to reduced cell survival of radioresistant cells are not clear yet. The role of GADD45A in the observed G_1 cell cycle arrest should be further confirmed by RNA interference. Differential DNA damage signalling gene expression analysis demonstrated an early radiation-induced expression of various genes involved in the recognition of DNA damage, DNA repair and cell cycle regulation in radioresistant cells. However, after PLDR irradiation only 2 genes were over-expressed indicating inactive DNA damage response. To support the results of this preliminary study, the response to PLDR irradiation should be evaluated in other radioresistant and LDHRS-positive tumour cell lines. Furthermore, since PLDR irradiation is a promising approach for re-irradiation of previously irradiated tissues, *in vivo* analysis of the effects of PLDR irradiation would greatly contribute to the promotion of PLDR irradiation scheme in the clinical setting. However, *in vivo* studies using human HNSCC tumours are limited by the use of immunocompromised animals to enable engraftment of human xenografts. In addition, the antitumor effects of PLDR irradiation might differ from the effects of PLDR irradiation observed in the clinical settings, because immunostimulatory effects of low dose irradiation would be limited in immunocompromised animals.[45] Also, the role of tumour

microenvironment should be taken into account, as cell-cell and cell-microenvironment interactions importantly contribute to the radiosensitivity of cells.[46]

Modifications of irradiation schemes to improve the therapeutic index in the clinical management is an emerging approach for the treatment of HPV-positive oropharyngeal tumors.[47–50] Considering the prevalence of LDHRS in radioresistant cells and tumours, PLDR irradiation could be more effective in radioresistant tumours than conventional radiotherapy. Modifications of irradiation schemes to reduce the effective dose rate and increase daily treatment time, such as PLDR irradiation, allow safer retreatment of previously irradiated areas, including recurrent radioresistant tumours of different origin.[6,16,17] In this respect, by using PLDR irradiation, a normal tissue damage could be minimised, and tumour control elevated.[51] Benefits of PLDR irradiation, such as less normal tissue damage, were confirmed in *in vivo* studies of human orthotopic xenografts in nude mice.[14,15]

A limit of the PLDR irradiation is the prolonged radiation delivery of one fraction composed of several pulses, which would lead to a larger burden of medical facilities. Although enhanced cytotoxic effects were observed with shorter intervals of several minutes between low dose fractions, it is possible to introduce variations in time intervals between consecutive doses, dose per fraction and dose rate.[34] Reduced cell survival can be observed also when low doses are separated by intervals of several hours, and additional benefit can be observed when combining this approach with chemotherapy.[52–55]

In this study, we demonstrated LDHRS in isogenic radioresistant cells, but not in the parental cells. Cell survival of LDHRS-positive radioresistant cells after PLDR was significantly reduced in comparison to parental cells. This reduction in cell survival of LDHRS-positive radioresistant cells was associated with variations in DNA damage signalling gene expression observed in response to PLDR. Variations in the DNA damage signalling response could be further exploited for the development of combined treatment approaches to radiosensitizing recurrent and radioresistant HNSCC to improve the therapeutic index.

Acknowledgements

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