

# Decay of $\gamma$ -H2AX foci correlates with potentially lethal damage repair in prostate cancer cells

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**Abstract.** To determine the relationship between ionizing radiation-induced levels of  $\gamma$ -H2AX foci and cell survival in cultured prostate cancer cell lines, three prostate cancer cell lines: LNCaP (wt TP53), DU145 (mut TP53) and PC3 (TP53 null), were studied. For  $\gamma$ -H2AX foci induction, cells were irradiated with a single dose of 2 Gy and foci levels were studied at 30 min and 24 h after irradiation. Cell survival was determined by clonogenic assay, directly and 24 h after irradiation with doses ranging from 0 to 8 Gy. Irradiation was performed with a Siemens Stabilipan 250 KeV X-ray machine at a dose rate of approximately 3 Gy/min. Survival curves were analyzed using the linear-quadratic model  $S(D)/S(0) = \exp(-\alpha D + \beta D^2)$ . LNCaP cells clearly demonstrated potentially lethal damage repair (PLDR) which was assessed as increased survival levels after delayed plating as compared to cells plated immediately after irradiation. DU145 cells demonstrated only a slight PLDR and PC3 cells did not show PLDR at all. Levels of  $\gamma$ -H2AX foci were significantly decreased in all cell lines at 24 h after irradiation, compared to levels after 30 min. The LNCaP cells which demonstrated a clear PLDR also showed the largest decay in the number of  $\gamma$ -H2AX foci. In addition, the PC cells which did not show PLDR had the lowest decay of  $\gamma$ -H2AX foci. A clear correlation was demonstrated between the degree of decay of  $\gamma$ -H2AX foci and PLDR.

## Introduction

DNA double-strand breaks (DSBs) are generally assumed to play a major role in radiation-induced cell death (1).

Phosphorylation of the histone protein H2AX ( $\gamma$ -H2AX) is one of the earliest markers of DNA damage after ionizing radiation. These  $\gamma$ -H2AX ionizing radiation-induced foci (IRIF), which appear already at 3 min after irradiation and increase in time reaching a maximum at 20-30 min after irradiation, have been reported to mark the locations of DNA DSBs (2-7). After the breaks are rejoined,  $\gamma$ -H2AX is dephosphorylated again. The disappearance of the foci is related to repair of the DNA (8).

An important factor in responses of cells to irradiation is potentially lethal damage repair (PLDR). Repair of PLD is usually complete between 6 and 12 h after irradiation (9). PLDR can be studied with delayed plating experiments of plateau-phase cultures. Survival of cells plated after a delay of 24 h following irradiation is compared with survival of cells plated directly after irradiation (10-13). In cells demonstrating PLDR, cell survival is enhanced if the cells are allowed to remain undisturbed for some time after irradiation before they are assayed for colony formation (10,11). Several studies have demonstrated that PLDR can be influenced by the status of the tumor protein 53 (TP53) (14-18). However, some studies have suggested that PLDR does not depend on functional TP53 (19-21).

Survival curves are commonly described and analyzed using the linear-quadratic (LQ) model:  $S(D)/S(0) = \exp(-\alpha D + \beta D^2)$  (22-24). Studies investigating the repair of potentially lethal damage are critical as factors influencing PLDR may alter tumor radiocurability. The advantage of using the LQ model is that changes in PLDR can be determined quantitatively by analyses of the linear parameter  $\alpha$ , describing the low dose range of the survival curve, separately from the parameter  $\beta$  dominating the high dose range (10,25-27). Analysis of survival curves from numerous studies has shown that PLDR is most clearly demonstrated by changes of the linear parameter  $\alpha$  (24,28-30).

The relationship between radiation sensitivity and the induction of  $\gamma$ -H2AX IRIF is not always clear. Foci of  $\gamma$ -H2AX are also induced by factors other than ionizing radiation, such as during the process of replication. Not all cell lines have similar numbers of  $\gamma$ -H2AX foci after equal radiation doses. Several studies showed that the induction of  $\gamma$ -H2AX

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foci is not directly correlated with the cellular survival after radiation (4,31). However, it has been demonstrated that there is a correlation between the number of residual DNA DSBs at 10 h after irradiation and cell survival (32,33). Previous studies suggested that the induction of  $\gamma$ -H2AX after single and fractionated irradiation appears to be a useful marker of cellular radiosensitivity (8,34).

The aim of the present study was to establish whether PLDR is correlated with repair of DNA DSB. As the status of TP53 is important for PLDR (16), the level of PLDR was determined in three prostate cell lines with different TP53 status. Then, the induction of  $\gamma$ -H2AX foci after a 2 Gy dose was determined directly and 24 h after radiation. The data indicate correlations between TP53 status and PLDR, and decay of  $\gamma$ -H2AX foci and the level of PLDR.

## Materials and methods

**Cell cultures.** Human prostate cancer cell lines with different status of TP53 were used: LNCaP, wt TP53; PC3, TP53 null; and DU145, mut TP53, as previously described (35-37). All three cell lines were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin and 1 mM glutamine in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The doubling time of all three cell lines was 24 h.

**Western blotting.** Western blotting of TP53 induction at 4 h after 4 Gy is shown in Fig. 1. In LNCaP cells, TP53 was induced at 4 h after 4 Gy; in DU145 cells, the mutated TP53 protein was present before and after irradiation; and in PC3 cells, no TP53 was detected. Erk-2 protein was used for loading control.

**Radiation treatment.** Confluent cultures of cells growing in monolayers were irradiated at 37°C in a waterbath and 5% CO<sub>2</sub>/95% air was supplied during irradiation. Irradiation was performed with a Stabilipan 250 KeV X-ray machine (Siemens, Germany). For determination of  $\gamma$ -H2AX foci, a radiation dose of 2 Gy was applied and for the survival experiments cells were exposed to single doses of 0, 2, 4, 6 and 8 Gy. The distance between the focus and the culture dish was 9 cm. A 0.5-mm Cu filter was used and the dose rate was ~3 Gy/m.

**Clonogenic survival.** Directly and 24 h after irradiation, cells were trypsinized and replated for clonogenic survival assay in appropriate cell numbers in 6-well macroplates (38,39). Subsequently, cells were incubated for 10 days. Surviving colonies were fixated and stained with glutaraldehyde-crystal violet solution and counted. Survival curves were analyzed using SPSS statistical software (Chicago, IL, USA) by means of fit of data by weighted linear regression, according to the linear-quadratic formula:  $S(D)/S(0) = \exp(-\alpha D + \beta D^2)$  (10,27,40,41). In the formula, the  $S(D)$  is the survival at dose  $D$  and  $S(0)$  is the survival at dose 0. As a measure of PLDR, the ratio PLD- $\alpha$  is calculated as the ratio of the value of linear parameter  $\alpha$  of cells immediately plated (ip) after irradiation and cells delayed plated (dp) 24 h after irradiation.

**Immunohistochemistry for  $\gamma$ -H2AX.** We counted the number of  $\gamma$ -H2AX foci in cells that were grown on cover slides (42,43). The

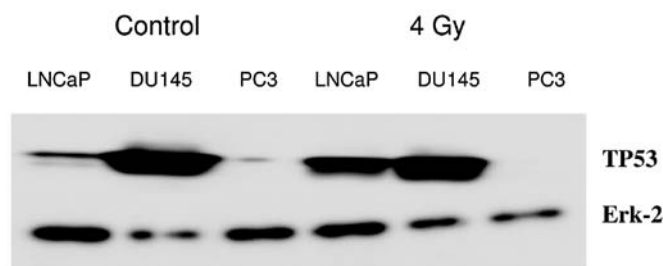


Figure 1. Western blot analysis of TP53 status in LNCaP, DU145 and PC3 prostate cancer cells. Induction of TP53 was measured at 4 h after 4 Gy. Clear induction of TP53 in LNCaP (wt TP53), no induction TP53 in DU145 (mut TP53) and absence of TP53 in PC3 (TP53 null) cells.

cover slides (21x26 mm) were sterilized with alcohol and were placed in 60-mm cell culture dishes. The cells were reseeded at a density of  $2.5 \times 10^5$  cells on cell culture dishes containing sterile cover slides and were grown until a confluent layer was obtained. The cells were then irradiated. The number of  $\gamma$ -H2AX foci was determined 30 min and 24 h after irradiation.

Following irradiation, cells were washed with phosphate-buffered saline (PBS) and fixed in PBS containing 2% paraformaldehyde for 15 min. After three further washes with PBS, cells were treated with PBS containing 0.1% Triton X-100 and 1% FCS (TNBS) for 30 min to permeabilize the cells.

A primary mouse monoclonal anti- $\gamma$ -H2AX antibody (Millipore) was diluted 1:100 in TNBS. Fixed, permeabilized cells on the cover slides were incubated with 50  $\mu$ l primary antibody under a parafilm strip for 90 min at room temperature. Cells were then washed with PBS for ~5 min and the parafilm strip was removed. Subsequently, cells were washed 2 times with TNBS.

Cells on cover slides were incubated with 50  $\mu$ l secondary antibody anti-Mouse Cy3 (Jackson) (1:100 in TNBS) under a parafilm strip for 30 min at room temperature. Cells were then washed 2-3 times with TNBS for ~5 min and the parafilm strip was removed. Nuclei were stained with DAPI (2.5  $\mu$ g/ml) for 5 min and embedded in Vectashield. Then, cover slides were sealed to microscope slides. Rubber cement was used to seal the whole construct.

**$\gamma$ -H2AX foci scoring.** Digital image analysis was performed to determine the number of  $\gamma$ -H2AX IRIF. Fluorescent photomicrographs of  $\gamma$ -H2AX foci were obtained using Image Pro Plus software. Stack images of cells were obtained using a Leica DM RA HC Upright Microscope equipped with a CCD camera. Stack images of 50 cells/sample were captured using Image Pro Plus software. One stack image consisted of 23 slices with a 300-nm interval between the slices along the Z-axis. Images were then processed and the number of foci in cells was scored using custom-made software (42-45).

All experiments were carried out in triplicates, independently from each other. Numbers of foci in unirradiated control cells were subtracted from numbers in irradiated samples. S-phase cells were excluded using an EdU (5-ethynyl-2'-deoxyuridine) staining (Invitrogen, Eugene, OR, USA) to mark these cells. The ratio of the number of  $\gamma$ -H2AX foci at 30 min and 24 h after irradiation was calculated as a measure of foci decay resulting from repair of DNA DSBs.

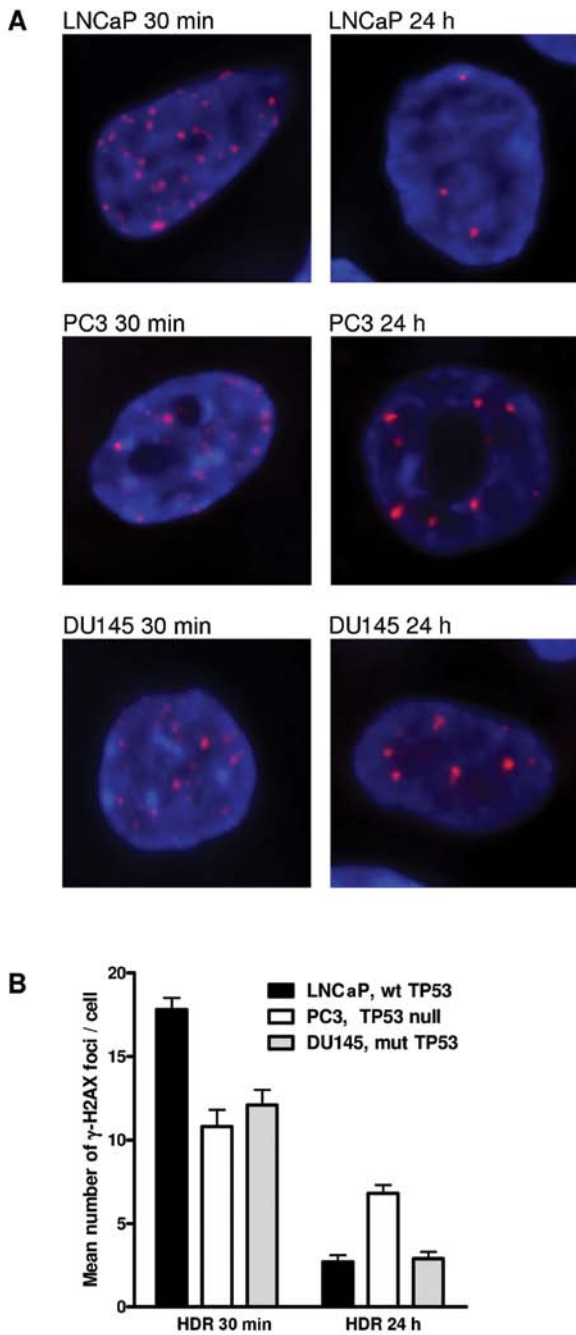


Figure 2. (A) Representative nuclei of LNCaP, DU145 and PC3 cells with  $\gamma$ -H2AX foci at 30 min and 24 h after 2 Gy. (B) Quantification of the number of  $\gamma$ -H2AX foci. At least 150 cells are counted in 3 different experiments; error indicates standard error of the mean (SEM) N=3.

**Results**

To assess TP53 status of the used cell lines, western blot analysis was performed of TP53 induction after 4 Gy radiation dose (Fig. 1). In LNCaP cells, wt TP53 induction was visible; in the DU145 cells, mutant TP53 was present; and in the PC3 cells, no TP53 was observed.

Fig. 2 shows the radiation-induced number of  $\gamma$ -H2AX foci in the different prostate cancer cells. At 30 min after irradiation, LNCaP cells had the highest number and PC3 cells had the lowest number of  $\gamma$ -H2AX foci. On the contrary,

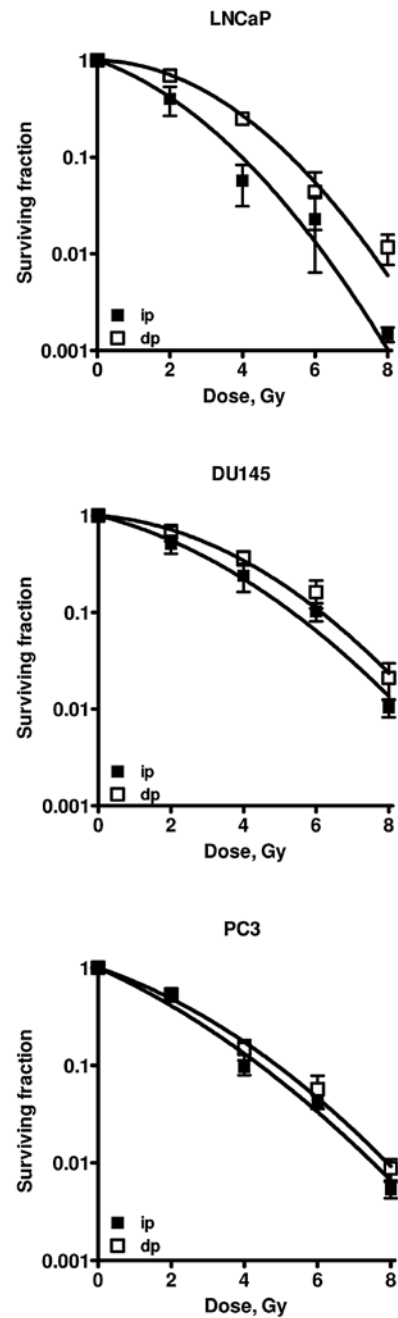


Figure 3. Survival curves of the different prostate cancer cell lines immediately plated (ip) after irradiation and delayed plated (dp) 24 h after irradiation. LNCaP show clear PLDR, DU145 show only slight PLDR and PC3 do not show PLDR. PLDR is the increase in survival after dp as compared to ip. Error bars indicate standard error of the mean (SEM) N=3.

24 h post-treatment PC3 cells had the highest number and LNCaP cells had the lowest number of foci. The DU145 cells had intermediate numbers of foci for both post-irradiation conditions. Initial number of foci at 30 min after irradiation ranged between 10 and 18 foci/cell. At 24 h after treatment, the numbers ranged between 2.7 and 7 foci/cell. The decline in foci number was the highest for LNCaP cells and the lowest for PC3 cells.

Survival curves of the different cell lines are presented in Fig. 3. LNCaP and DU145 cells clearly showed increased survival after dp as compared to ip cells after irradiation.

Table I. LQ parameters  $\alpha$  and  $\beta$ , PLD- $\alpha$ , ratio of foci decay and the TP 53 status of the different prostate cancer cell lines.

LQ parameter cell line	$\alpha$ , Gy <sup>-1</sup>	$\beta$ , Gy <sup>-2</sup>	PLDR- $\alpha$	Ratio foci decay	TP53 status
LNCaP					
ip	0.31±0.09	0.08±0.03	10.3±3.9	6.9±0.3	TP53 <sup>+</sup>
dp	0.03±0.01	0.08±0.02			
DU145					
ip	0.22±0.06	0.04±0.01	3.1±1.6	4.2±0.7	TP53 mutated
dp	0.07±0.03	0.05±0.01			
PC3					
ip	0.39±0.04	0.03±0.01	1.3±0.22	1.6±0.2	TP53 null
dp	0.30±0.04	0.04±0.01			

LQ, linear-quadratic; PLDR, potentially lethal damage repair; ip, cells immediately plated after irradiation; dp, cells delayed plated 24 h after irradiation. Error indicates standard error of the mean (SEM) N=3.

Table II. Surviving fraction and the number of radiation-induced  $\gamma$ -H2AX foci after 2 Gy.

2 Gy	LNCaP		DU145		PC3	
	Surviving fraction	No. of foci	Surviving fraction	No. of foci	Surviving fraction	No. of foci
ip	0.39±0.10	17.8±0.7	0.55±0.08	12.1±0.9	0.41±0.05	10.8±1.0
dp	0.70±0.15	2.7±0.4	0.71±0.10	2.9±0.4	0.46±0.05	6.8±0.5

ip, cells immediately plated after irradiation and foci after 30 min; dp, cells delayed plated and foci at 24 h after irradiation. Error indicates standard error of the mean (SEM) N=3.

Survival curves of PC3 cells plated immediately and 24 h after irradiation do not show any difference. Values of the linear and quadratic parameters,  $\alpha$  and  $\beta$ , the PLD- $\alpha$  ratio ( $= \alpha_{ip}/\alpha_{dp}$ ) as a measure of PLDR, the ratio of the number of  $\gamma$ -H2AX foci at 30 min and 24 h after irradiation, and the TP53 status of the different cell lines are presented in Table I. The decay of foci correlates well with PLDR. In Table II, the surviving fractions and the number of foci after 2 Gy are given for immediately and delayed plated cells. It can be observed that in almost all cases, high survival levels correlated with low residual foci numbers and, vice versa, low survival levels correlated with high residual numbers of foci.

## Discussion

The three prostate tumor cell lines examined in this study differ in their TP53 status. The TP53 status was confirmed with western blotting. In LNCaP cells, TP53 was induced 4 h after 4 Gy irradiation; in DU145 cells, the mutated TP53 protein was present before and after irradiation; in PC3 cells, TP53 was not detected at all. Earlier studies reported that an intact TP53 status is required for repair of potentially lethal damage (14-17). Therefore, the level of PLDR was investigated in the three cell lines. The LNCaP cells with wt TP53 protein clearly demonstrated PLDR. As expected, in the DU145 cell line harbouring mutated TP53 PLDR was reduced and in the PC3 cells (TP53 null) PLDR was not seen at all.

Furthermore, the present data demonstrates that the decay of  $\gamma$ -H2AX foci after 2 Gy radiation dose correlates with PLDR. Phosphorylation of H2AX occurs rapidly after induction of DNA DSB. The  $\gamma$ -H2AX foci have been suggested to be a valid measure for radiosensitivity and the disappearance of the foci might be related to the repair of DNA damage following radiation treatment (8). It has already been shown by MacPhail *et al* (46) that the decay of  $\gamma$ -H2AX foci is associated with cell survival and repair of DSB. Yoshikawa *et al* (31) suggested that there was no close correlation between residual foci and radiosensitivity in some tumor cell lines. However, Yoshikawa *et al* (31) only studied survival of cells plated immediately after irradiation. In our study, we irradiated cells and plated them both directly and 24 h after irradiation in order to study PLDR. The cell line with the highest PLDR was also found to have the largest decay in number of foci, resembling a more proficient repair of DNA DSB. This is also corroborated by our observation that higher surviving fractions after 2 Gy correlated with a lower number of residual  $\gamma$ -H2AX foci in the prostate cell lines (Table II).

The linear-quadratic model is based on well-accepted biophysical concepts, involving the assumption that lethal damage can be induced by single-particle tracks and by interaction of damage from multiple particles.

In a review of published data on the dependence of different types of lethal damage on the linear energy transfer of ionizing particles, Barendsen (23,25,26) derived evidence that sublethal lesions and potentially lethal lesions show similar RBE-LET

(relative biological effect-linear energy transfer) relationships as DNA DSBs, with only a relatively low RBE at the optimal LET (27). This is evidently different from the high RBE values commonly derived for unreparable lethal lesions and chromosome aberrations. The hypothesis was proposed that sublethal lesions and potentially lethal lesions are both DNA-DSBs. The present results on the decay of  $\gamma$ -H2AX foci, which mark DNA DSBs, and the correlation with PLD repair are consistent with this hypothesis. Potentially lethal lesions contribute only a part to the linear parameter  $\alpha$  in the LQ model and are similarly repairable as sublethal damage. In earlier studies, we demonstrated a correlation between survival, chromosomal aberrations and PLDR, which was shown by a decrease in the value of  $\alpha$  with higher survival and lower number of chromosomal aberrations (12,13). Herein, we further demonstrated that there is an association between PLDR  $\alpha$  and a decrease of DNA DSB, which strengthens the biological basis of the LQ model. However, our study remains to be confirmed in an isogenic system with cells only different in TP53 status.

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