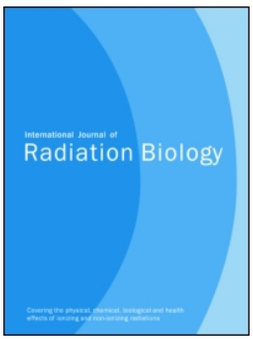

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RESEARCH ARTICLE

DNA repair foci and late apoptosis/necrosis in peripheral blood lymphocytes of breast cancer patients undergoing radiotherapy

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ABSTRACT

Purpose: Double-strand breaks (DSB) repair and apoptosis are assumed to be key factors in the determination of individual variability in response to radiation treatment. In this study we investigated tumor protein p53 (TP53) binding protein 1 (53BP1) and phosphorylated histone 2A family member X (γH2AX) foci, γH2AX pan-staining and late apoptosis/necrosis (LAN) in lymphocytes from breast cancer (BC) patients undergoing radiotherapy.

Materials and methods: BC patients were subjected to local radiotherapy with fractionated doses using linear accelerator. Adverse reactions of patients were classified according to the Radiation Therapy Oncology Group (RTOG)/European Organization for Research and Treatment of Cancer (EORTC) criteria. Blood samples were collected before treatment, at various time-points during and after radiotherapy. Residual 53BP1 and γH2AX foci, γH2AX pan-staining were analyzed in peripheral blood lymphocytes (PBL) using the Metafer system and confocal laser scanning microscopy. LAN cells were counted by the trypan blue (TB) exclusion assay. Statistical analysis was performed using Mann–Whitney test, Spearman rank correlation test and analysis of covariance (ANCOVA).

Results: No statistically significant changes were observed in the levels of γH2AX foci during radiotherapy. In contrast, radiation-induced residual 53BP1 were detected already after the first fraction. Increased individual variability in the 53BP1 focus formation was observed during treatment. The background level of DNA repair foci and its individual variability in response to radiotherapy decreased after the end of radiotherapy indicating successful removal of DNA-damaging effects. A correlation between stage of cancer and 53BP1 focus formation was established which suggests the prognostic value of this test. We show that the fraction of LAN cells negatively correlates with the level of 53BP1 and positively correlates with individual radiosensitivity. Only weak correlation was observed between γH2AX pan-staining and LAN cells. Due to large interindividual variability, both *in vivo* assays, LAN and focus formation, have shown relatively low predictive power at the individual level.

Conclusions: It is likely that radiosensitive patients have less efficient mechanisms of elimination of apoptotic cells with DNA damage resulting in accumulation of LAN cells and facilitating adverse reactions. Our data suggested that the grade of adverse reaction may positively correlate with LAN cells in PBL before and during radiotherapy.

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Introduction

Predicting normal tissue and tumor radiosensitivity has yet to be routinely integrated into radiotherapy (Dikomey et al. 2003). Severe side-effects from radiation therapy of cancerous tumors, particularly breast cancer tumors, can work against optimal radiation therapy for the individual patient. The main objective of predictive testing is to tailor radiotherapy prescriptions to the individual patient. This study examined possible biomarkers for identifying patients for whom standard radiotherapy may be unsuitable and others who may tolerate standard radiation therapy without serious side-effects.

The data published so far about the cellular and the molecular factors underlying the acute or late tissue reactions upon radiotherapy appear to be contradictory (Dikomey et al. 2003; Goodarzi and Jeggo 2012; Henriquez-Hernandez et al. 2012). Lymphocytes, derived lymphoblastoid cell lines or fibroblasts are usually used in attempts to correlate cellular radiosensitivity with the individual radiosensitivity of the patients undergoing radiotherapy (Greve et al. 2012; Henriquez-Hernandez et al. 2012). In general, the analysis of lymphocytes appears to be more promising than studies using fibroblasts (Löbrich and Kiefer 2006). The radiosensitivity is

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often, but not always, correlated with alternations in cell cycle checkpoints, and changes in cell growth and apoptosis (Henriquez-Hernandez et al. 2012). Overall, measurements of double-strand breaks (DSB) and DSB repair have widely been used in order to validate correlation with radiosensitivity (Löbrich and Kiefer 2006).

A recent development of technique based on analysis of proteins that participate in DSB repair allows precise measurement of DSB at doses relevant to radiotherapy (usually 2 Gy in one fraction). The cytological manifestation of nuclear rearrangements in response to ionizing radiation is the formation of so-called ionizing radiation induced foci (IRIF) (Bekker-Jensen et al. 2006). IRIF are dynamic structures containing thousands of copies of proteins involved in various aspects of DSB repair and signaling. These structures, which are also referred to as DNA repair foci, can be microscopically visualized as discrete foci around DSB. Following irradiation, histone H2AX is phosphorylated by ataxia telangiectasia mutated (ATM) and other phosphatidylinositide 3-kinase (PI-3 kinase) family members, ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK) (Redon et al. 2002). Phosphorylated histone 2A family member X (γH2AX) provides a chromatin scaffold structure for DNA repair foci and DSB repair. This scaffold is organized on a megabase-size chromatin domain containing DSB and functions by recruiting proteins involved in the DSB repair (Rogakou et al. 1999; Paull et al. 2000; Mochan et al. 2004). These proteins include tumor protein p53 (TP53) binding protein 1 (53BP1), which is a well-established marker of DSB (Belyaev 2010).

The majority of DSB/DNA repair foci induced by radiation in the dose range relevant to radiotherapy are repaired within a few hours following irradiation. The initial number of DSB and their repair within 24 h post-irradiation did not correlate with acute side-effects of radiotherapy (Fleckenstein et al. 2011; Greve et al. 2012). However, recent studies revealed that some residual DNA repair foci, such as γH2AX and 53BP1, remain in cells for a relatively long time after irradiation and may correlate with cell death (Iwabuchi et al. 2003; MacPhail et al. 2003; Markova et al. 2003; Rothkamm and Löbrich 2003; Kuhne et al. 2004; Olive and Banath 2004; Taneja et al. 2004; Wykes et al. 2006; Yu et al. 2006).

While γH2AX is usually used for enumeration of DSB, some studies reported a lack of correlation between γH2AX and DSB and suggested that some part of γH2AX foci deal with changes in chromatin structure (Hamer et al. 2003; Banath et al. 2004; Warters et al. 2005; Yu et al. 2006). *In vitro* application of γH2AX assay to lymphocytes had a limited scope in predicting individual radiosensitivity of cancer patients (Vasireddy et al. 2010; Werbrouck et al. 2010; Fleckenstein et al. 2011; Brzozowska et al. 2012; Greve et al. 2012). Importantly, various effects of radiation may significantly differ between situations *in vitro* and *in vivo* (Girinsky et al. 1991; Löbrich et al. 2005; Zarybnicka et al. 2011; Park et al. 2014). It is especially relevant to peripheral blood lymphocytes (PBL), because in contrast to *in vitro* irradiation, the *in vivo* situation includes also secondary cytotoxicity released from irradiated tissues, elimination of apoptotic cells by the immune system, and cell repopulation. All these processes may contribute to the individual radiosensitivity (Torudd et al. 2003; Markova et al. 2008). In this study, we investigated the possible correlation between *in vivo* formation of residual γH2AX/53BP1 foci in PBL of breast cancer (BC) patients undergoing radiotherapy and acute side-effects of radiation treatment. Our previous study has suggested that residual 53BP1 foci may be linked to induction of apoptosis in lymphocytes (Torudd et al. 2005). If removal of apoptotic cells is affected in some patients, it may result in accumulation of late apoptotic/necrotic (LAN) lymphocytes followed by acute side-effects. To verify this hypothesis, we have also analyzed correlation of acute side-effects with LAN lymphocytes during radiotherapy.

Materials and methods

Ethics statement

The study was approved by the Institutional Review Board of the National Cancer Institute (NCI), Bratislava. Written informed consent was obtained from each participant prior to collection of peripheral blood samples. The Ethics Committee approved this consent procedure.

Patients and healthy controls

This study involved 38 patients treated for breast carcinoma from February 2006 to February 2014. The patients were undergoing local radiotherapy for treatment of breast cancer at the Department of Radiation Oncology, NCI, Bratislava. Carcinoma *in vivo* was diagnosed in four patients. Other patients had stage I ($n = 21$), stage II ($n = 7$), stage III ($n = 4$) and stage IV ($n = 2$) BC diagnoses. Mean age in the group of BC patients was 61.2 ± 10.2 years (from 29–82 years old). To decrease heterogeneity and avoid difficulties in separating effects of cytostatic treatment from radiotherapy, patients with cytostatic treatment were not enrolled in this study.

The classification of Tumors, Nodes, Metastases (TNM) was T0, $n = 4$; T1, $n = 21$; T2, $n = 12$; T4, $n = 1$; N0, $n = 32$; N1, $n = 2$; N2, $n = 2$: N3, $n = 2$ and M0, $n = 36$; M1, $n = 2$. The Scarff, Bloom and Richardson (SBR) grade was I, $n = 14$; II, $n = 16$; III, $n = 1$ and ductal carcinoma *in situ* (DCIS), $n = 5$. The mean and median histologic tumor size was 15.75 mm and 13.5 mm (range 4–50), respectively. The incidences of axillary lymph node involvement are presented in Table I. The Karnowsky index of patients was 90–100%. The adverse reactions of all patients were classified according to the Radiation Therapy Oncology Group (RTOG)/European Organization for Research and Treatment of Cancer (EORTC) criteria such as acute skin erythema reactions after radiotherapy, as well as other markers of adverse side-effects (Cox et al. 1995). Eight patients exhibited no adverse reactions (G0); 25 patients had G1 adverse reactions (follicular, faint or dull erythema, dry desquamation). Five patients exhibited severe side-effects and were classified as G2 (tender or bright erythema, patchy moist desquamation, moderate edema), $n = 4$, and G3 (confluent moist desquamation, hyperpigmentation), $n = 1$. A group of healthy controls included 10 individuals without diagnosed cancer and matched by age with a group of cancer patients (58.1 ± 10.3 years, from 48–77 years old).

Table I. Breast cancer patients enrolled in the study.

Patient	Age (years)	TNM	Stage	Histology	Tumor size (mm)	Irradiated volume (ccm)	Dose (Gy)	Boost (Gy)	RTOG
BC1	75	pT2 pN3 M0	3b	IDC G2	35	664	50		G1
BC2	72	pT2 pN1 M1	4	IDC G2-3	30	2634	50		G1
BC3	71	pT1b pN0 M0	1	IDC G2	7	1044	50		G1
BC4	82	pT2 pN3 M1	4	IDC G2	50	592	50		G1
BC5	59	pT1c pN0 M0	1	IDC G1	13 × 8	1600	50		G1
BC6	69	pT1a pN0 M0	1	G1	4	928	50		G1
BC7	57	pT1-2 pN0 M0	2a	DCIS	NA	1196	50		G1
BC8	69	pT4 pN0 M0	3b	G2-3	15	972	50		G1
BC9	68	pT2 pN2 M0	3a	IDC G2	30 × 15	1307	50		G1
BC10	56	pT2 pN0 M0	2a	IDC G1	14 × 20	821	50	10	G1
BC11	72	pT2 pN2 M0	3a	IDC GI2	8	1973	50		G1
BC12	29	pTis NX M0	0	DCIS	40	724	46		G1
BC13	60	pT1 pN0 M0	1	G1	NA	2082	50		G0
BC14	57	pT1 pN0 M0	1	IDC G1	12	1241	50		G0
BC15	69	pT1 pN0 M0	1	IDC G1-2	7	1001	44	9	G1
BC16	66	pT1b pN0 M0	1	G2	9	880	44		G0
BC17	56	pT1 pN0 M0	1	IDC G2-3	10	NA	44	9	G0
BC18	63	pT1-2 pN0 M0	2a	NA	10	NA	44		G0
BC19	58	pT1 pN0 M0	1	IDC G2	15	NA	50		G0
BC20	69	pT1 pN0 M0	1	G3	7	NA	50		G0
BC21	70	pT1 pN0 M0	1	G1-2	14	NA	50		G0
BC22	50	pT2 pN0 M0	2a	G2	22	NA	50	12	G1-2
BC23	68	pT2 pN0 M0	2a	NA	23	1012	50		G1
BC24	63	pTis pNX M0	0	DCIS	15	1076	44		G1
BC25	66	pTis N0 M0	0	DCIS	15	1943	50		G1
BC26	50	pT1b pN0 M0	1	G2	10	709	50	9	G1
BC27	55	pT1b pN0 M0	1	G1-2	8	810	50		G1
BC28	52	pTis pN0 M0	0	DCIS G3	15	2011	50	9	G1
BC30	52	pT1c pN0 M0	1	G1	10	1407	50		G1
BC31	64	pT1b pN0 M0	1	G2	7	1658	50		G1
BC32	56	pT1b pN0 M0	1	G1	7	1055	50		G1
BC33	73	pT2 pN1mi(Sn) M0	2b	G2	27	922	50		G2
BC34	52	pT1c pN0 M0	1	IDC G1	15	674	50	10	G2
BC36	42	pT1b pN0 M0	1	G1	6	498	50		G1
BC37	62	T1 N0 M0	1	G1	7	1379	50		G2
BC38	56	pT1a pN0 M0	1	G1-2	5	1998	50		G3
BC39	50	pT2 pN0 pMX	2a	G2	24	1546	50	10	G1
BC40	67	pT1c pN0 pM0	1	G2	15	1227	50	10	G1

RTOG, Radiation Therapy Oncology Group; BC, breast cancer patient; ccm, cubic centimeter; NA, non-available; Tumors, Nodes, Metastases (TNM): pT, tumors; pN, regional lymphatic nodes; pM, outlying metastases; M0, without metastases; M1, metastases present; MX, impossibly evaluate. Histology: G, histopathology grade; G1, good differentiation; G2, middle differentiation; G3, poorly differentiation; G4, non-differentiation; IDC, invasive ductal carcinoma.

Radiation treatment

A total of 32 BC patients were treated with external beam radiation therapy with radical intent (conventional fractionation 2 Gy per fraction, one daily fraction, five fractions weekly) to a total dose 50 Gy to the whole breast and six patients were treated with daily 2.2 Gy to a total dose 44 Gy. The total dose of 44 Gy (2.2 Gy/fraction) is equivalent dose in 2 Gy fractions (*EQD2*) 50 Gy. Thirty-one patients were exposed to 6 MV X-rays, and seven patients were exposed to 18 MV X-rays. Radiation therapy of most patients was delivered by linear accelerator (Clinac 2300 C/D, Varian, Palo Alto, CA) using two tangential fields ($n = 29$); others were exposed as field in field technique to 5 ($n = 2$), 4 ($n = 1$), and 3 fields ($n = 4$). The dose rate was 300 MU (monitor units) per minute which corresponded to 3 Gy/min delivered to a point at the depth of maximum dose in a water-equivalent phantom whose surface is at the isocentre of the machine. Eight patients received a boost into tumor bed at total dose 9–12 Gy (2 Gy per fraction, one daily fraction, five fractions weekly) with 9 MV electrons. Computed tomography (CT) scanning was used to plan radiation treatment to the specific areas requiring radiation therapy.

Collection of samples

Blood samples were obtained from BC patients locally irradiated during radiotherapy and from a control group of non-irradiated healthy persons. Some BC patients were not able to participate in this study during the whole project and did not donate blood samples at each time-point. Samples were obtained twice before treatment and then 24 h after 1st, 5th and 10th fractions ($n = 33$ for each time-point) during radiation therapy, and also at 1 month ($n = 30$) and 1 year ($n = 11$) after the end of radiation therapy. Mononuclear cells (MNC) were isolated from peripheral blood by density gradient centrifugation in Lymphocyte Separation *Medium* (LSM) (PAA Laboratories GmbH, Pashing, Austria) as described previously (Markova et al. 2005). After isolation, MNC were incubated for 2 h at 37°C to purify PBL from monocytes. LAN cells were counted by Trypan blue (TB) exclusion assay (Gibco, Life Technologies, Grand Island, NY). At least 400 PBL were analyzed in Burker chamber at each data-point. By this assay, loss of membrane integrity, which occurs late in apoptosis and relatively early in necrosis can be detected (Zhivotosky and Orrenius 2001).

Chemicals

Reagent grade chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Merck KGaA (Darmstadt, Germany) and Boehringer Mannheim, division Roche (Basel, Switzerland).

Antibodies and immunostaining

Levels of 53BP1 foci were analyzed in PBL of all patients, whereas level of γH2AX foci were assessed only in a subgroup of patients ($n = 20$). For the analysis of 53BP1 foci following combinations of primary and secondary 53BP1 antibodies were used: (i) 53BP1 mouse monoclonal (1:20, kindly gifted by Prof. T. Halazonetis, University of Geneva, Geneva, Switzerland), or (ii) 53BP1 mouse monoclonal (1:500, #MAB3802, Millipore, MA) with Alexa Fluor 488 goat anti-mouse antibody (1:200, #A11029, Molecular Probes, Invitrogen, Life Technologies, NY) and (iii) 53BP1 rabbit polyclonal (1:800, #NB100-304, Novus Biologicals, Littleton, CO) with Alexa Fluor 488 goat anti-rabbit antibody (1:200, #A11034, Molecular Probes). Respectively, three combinations of primary and secondary γH2AX antibodies were used along with 53BP1 antibodies: (i, ii) γH2AX rabbit polyclonal (1:500, #NB100-384, Novus Biologicals) with Alexa Fluor 555 goat anti-rabbit antibody (1:200, #A21429, Molecular Probes), (iii) γH2AX mouse monoclonal (1:500, #ab18311-100, Abcam, Cambridge, UK) or (iii) γH2AX mouse monoclonal (1:400, #NB100-78356, Novus Biologicals) with Alexa Fluor 555 goat anti-rabbit antibody (1:200, #A21424, Molecular Probes). Levels of DNA repair foci obtained with these antibodies did not differ statistically significantly and had similar mean values and levels of variation. Thus, final statistical analysis was performed in the group of all patients regardless of antibodies used.

DNA repair foci and H2AX pan-staining

Blind scoring of DNA repair foci were performed in 500 or 1000 cells/variant by two different techniques: Confocal laser scanning microscopy using confocal microscope LSM5/510 Axiovert 100M (Zeiss Microscopy, Jena, Germany) (Markova et al. 2007) and using Zeiss Axio Imager Z1 (Zeiss Microscopy) microscope combined with Metafer system MetaCyte (MetaSystems, Altlussheim, Germany) for automated analysis of samples (Sorokina et al. 2013; Vasilyev et al. 2013). The levels of DNA repair foci assessed with these two methods were compared in healthy control group and no significant differences were found between them. Therefore, statistical analysis was performed on combined group of samples assessed by both techniques. Diffuse nuclear γH2AX pan-staining, which is a feature of early apoptosis, was assessed as previously described (Vasilyev et al. 2013).

Statistics

All data are shown as mean values ± standard deviation (SD) if not indicated otherwise. Statistical analysis was performed using Statistica 8.0 (Statsoft, Tulsa, OK). Differences between groups of patients were assessed using Mann–Whitney test. Wilcoxon matched pairs test were used for analysis of differences between various time-points during radiation therapy. Correlations were tested using the Spearman rank correlation test and analysis of covariance (ANCOVA) was used. Receiver Operating Characteristic (ROC) analysis was performed using the Youden's index in order to assess sensitivity and specificity at the individual level.

Results

Endogenous foci

Background level of 53BP1 foci in BC patients was 0.97 ± 0.57 foci/cell and did not differ significantly in comparison with healthy control group (1.18 ± 0.57 foci/cell, Mann–Whitney test). Level of γH2AX foci in PBL of BC patients (0.92 ± 0.72 foci/cell) was similar to that of 53BP1 foci and also did not differ significantly as compared with group of healthy controls (0.60 ± 0.32 foci/cell). While there was no statistical difference in terms of foci per cell between control and BC patients, the higher variation in foci per cell was observed in cells of BC patients (Figure 1A and B).

During radiation therapy

A small but statistically significant increase in the level of 53BP1 foci was observed 24 h after the first fraction of radiation therapy ($n = 35$, $p = 0.039$, Wilcoxon matched pairs test) (Figure 1A) and in the middle of treatment 24 h after 5 and 10 fractions ($n = 36$, $p = 0.05$ and $n = 36$, $p = 0.037$, respectively). Our previously published data have shown that residual 53BP1 foci detected in PBL 24 h post-irradiation with doses below 2 Gy linearly depend on dose and persist up to 4 weeks after irradiation *in vitro* (Markova et al. 2011). Based on this long-time persistence and linear dose dependence of residual IRIF, it might be expected that residual IRIF measured in PBL of cancer patients 24 h after each irradiation fraction would accumulate according to equation $Y_n = (n - 1)Y_1$, where Y_1 and Y_n is excess of radiation-induced foci after the 1st and nth fraction, respectively. Thus, 4-fold and 9-fold increase in IRIF after the 5th and 10th fraction, respectively, might be expected in comparison to the 1st fraction. However, no such increase radiation-induced 53BP1 was observed during two weeks from the 1st to the 10th fraction (Figure 1A) suggesting efficient elimination of damaged cells *in vivo*. Coefficient of variation (CV) of the 53BP1 foci levels increased from 59.2% before treatment to 95.8% after the first fraction of radiation therapy. Increased variability in the 53BP1 focus formation persisted during whole course of radiotherapy (CV = 75.8% after 5 and CV = 90.8% after 10 fractions of radiation therapy) and at 1 month after the end of radiation therapy (CV = 102.5%). The reason for such increase in variability could be the fact that residual 53BP1 foci were induced by radiation only in the subgroup of patients whereas smaller fraction of patients showed stable level of 53BP1 foci during radiation therapy. However, we found no significant differences in age, irradiated volume, summary dose and stage of cancer between these subgroups of BC patients (data not shown). The level of γH2AX foci did not increase significantly and no accumulation of residual

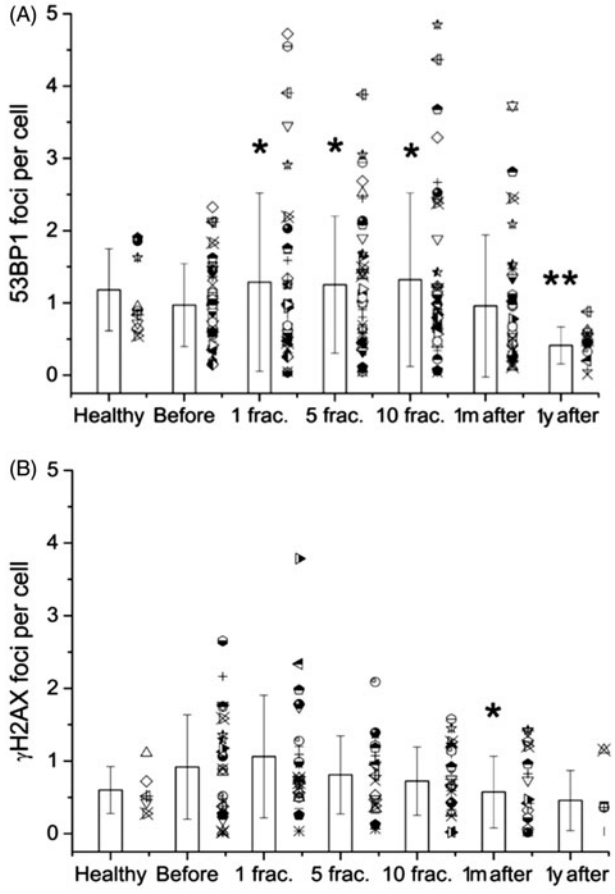


Figure 1. 53BP1 and γH2AX foci at different time-points before, during and after therapy. Number of 53BP1 (A) and γH2AX (B) foci in peripheral blood lymphocytes (PBL) of breast cancer (BC) patients is shown before, during (24 h after the 1st, 5th and 10th fraction) and after the end of radiation therapy (at 1 month and 1 year). Different symbols correspond to individual patients. The level of 53BP1 foci was significantly higher after 1st, 5th and 10th fraction than before radiation therapy (*$p<0.05$) and significantly lower one year after radiotherapy (**$p<0.005$). In general, number of foci was lower after the end of therapy than before radiation therapy reaching statistical significance at 1 month for γH2AX and 1 year for 53BP1 lower (*$p<0.05$). In all figures, error bars show the standard deviation (SD) of the mean for $n=11$–38 independent experiments if not indicated otherwise.

γH2AX foci was observed during entire course of radiation therapy (Figure 1B).

After radiation therapy

The number of 53BP1 foci at 1 month post-irradiation did not differ from pre-irradiation levels (Figure 1). At 1 year after the end of radiation therapy the levels of 53BP1 foci decreased and were significantly lower in comparison with background level before radiotherapy ($n=11$, $p=0.004$) (Figure 1). In line with these data, the level of γH2AX foci decreased after treatment. One month after the end of radiation therapy it was statistically significantly lower than the background level before treatment ($n=17$, $p=0.044$). Moreover, individual variability in 53BP1/γH2AX focus formation measured by CV also decreased reaching the background level identified before treatment started. These data suggested successful removal of DNA damaging effect and its individual component in PBL of patients after the treatment.

Effects of age and stage of cancer

The level of 53BP1 foci before radiation therapy significantly increased with age of BC patients and this age dependence was observed during whole treatment (Figure 2). There was still positive correlation of 53BP1 foci with age of patients 1 month and 1 year after the end of radiation therapy while it was not statistically significant. There was no correlation between γH2AX foci and age of BC patients at any time-point (data not shown).

Stage of cancer correlated with age of patients ($r=0.43$, $p=0.007$) (Figure 3A) and 53BP1 foci ($r=0.50$, $p=0.000001$). Similarly, 53BP1 foci correlated with tumor size (T) ($r=0.49$, $p=0.002$), nodes (N) ($r=0.43$, $p=0.006$), and metastases (M) ($r=0.37$, $p=0.024$). There was no correlation between γH2AX foci and stage of cancer. The rational for this fact may be the different kinetics for retaining of γH2AX and 53BP1 at the locations of DSB. Indeed, all measurements were made 24 h after irradiation when co-localization of γH2AX and 53BP1 foci is up to about 30% as follows from this *in vivo* study and also from our *in vitro* data (Markova et al. 2011).

Given the correlation of 53BP1 foci with age in groups of both BC patients and healthy controls and lack of significant difference in numbers of 53BP1 foci between two groups, the correlation between 53BP1 foci and age of patients was taken into consideration using ANCOVA. This analysis showed that both T and stage of cancer significantly affect background level of 53BP1 foci in PBL of BC patients prior radiotherapy regardless to age of patients ($p=0.044$ and $p=0.035$, respectively) (Figure 3B). Similarly, 53BP1 foci depend on age regardless to stage of cancer and T ($p=0.013$ and $p=0.002$, respectively) as analyzed by ANCOVA.

Adverse reactions, H2AX/53BP1 foci, LAN cells and pan-staining

At some time-points, the levels of γH2AX and 53BP1 foci differed between subgroups of BC patients with various level of adverse reaction. Level of γH2AX was significantly lower in G2 subgroup of BC patients than in G0 subgroup after 10 fractions ($p=0.037$) of radiation therapy. Significantly lower number of 53BP1 foci was seen in G2 as compared to G0 group after 1 fraction ($p=0.017$). In clinical practice, G3 acute toxicity would be especially important to predict. Only one G3 patient was enrolled in our study. Thus, all BC patients were divided into groups of responders (patients with G1–G3 adverse reaction to radiation therapy) and non-responders (G0 patients without adverse reaction) for further analysis. Levels of γH2AX were significantly lower in responders than in non-responders after 10 fractions ($p=0.035$) of radiation therapy. Also, the grade of adverse reaction significantly correlated with the level of γH2AX after 5 and 10 fractions (Spearman rank test, $r=-0.51$, $p=0.037$; $r=-0.46$, $p=0.036$, respectively). The grade of adverse reaction negatively correlated with γH2AX foci and γH2AX/53BP1 co-localized foci when combined analysis was performed for all samples from all time points ($r=-0.30$, $p\leq 0.002$; $r=-0.33$, $p\leq 0.047$, respectively). At the same time, the increased LAN level was generally seen with increased side-effects (Figure 4). More LAN cells were observed

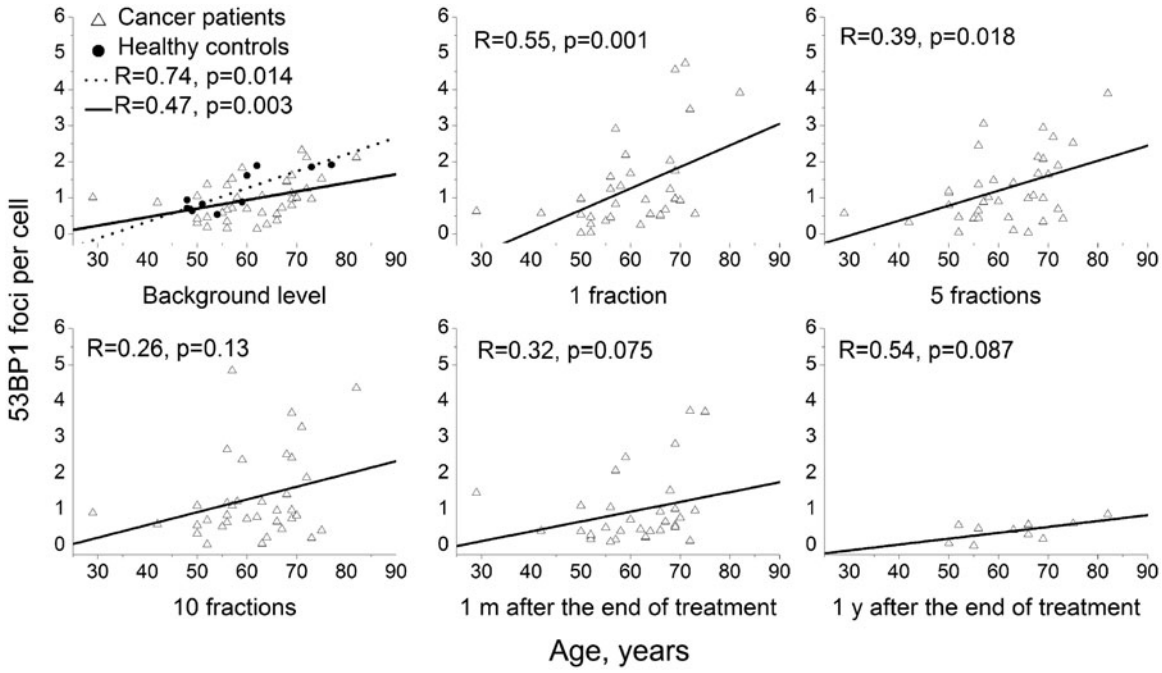


Figure 2. Correlation between 53BP1 foci and age. Correlation between number of 53BP1 foci and age of healthy controls and breast cancer (BC) patients was analyzed before, during (24 h after the 1st, 5th and 10th fractions) and after the end of radiation therapy (at 1 month and 1 year) by the Spearman rank test. Individual points correspond to individual patients. Linear regression is indicated as dashed line for healthy controls and solid line for BC patients. Endogenous 53BP1 foci significantly correlated with age of individuals in group of BC patients and healthy control group ($r = 0.44$, $p = 0.009$ and $r = 0.74$, $p = 0.014$, respectively). In general, radiation-induced 53BP1 foci positively correlated with age reaching statistical significance 24 h after 1st and 5th fractions.

in G1 subgroup of patients than in G0 subgroup after 1 fraction ($p = 0.004$), 5 fractions ($p = 0.005$) of radiation therapy and at 1 month after the end of treatment ($p = 0.004$) (Figure 4). The fraction of LAN cells in the subgroup of five G2–G3 patients was significantly higher than in G0 patients before, during and after radiation therapy (except for after 10 fractions, when the difference was statistically insignificant). Fraction of LAN cells was significantly higher in responders than in non-responders after 1 fraction ($p = 0.004$), 5 fractions ($p = 0.005$) and 1 month after the end of treatment ($p = 0.004$). Also, the grade of adverse reaction significantly correlated with the fraction of LAN cells before radiotherapy, after 1 fraction, and 1 month after the end of radiation therapy ($r = 0.33$, $p = 0.047$; $r = 0.47$, $p = 0.026$; $r = 0.66$, $p = 0.002$, respectively). Fraction of LAN cells correlated with the grade of adverse reaction at high significance level ($r = 0.42$, $p \leq 0.000001$) when combined analysis was performed for all samples from all time points. In line with our data, Severin et al. (2006) found a correlation between proportion of LAN lymphocytes and severe side reactions in leukemic patients undergoing total body irradiation at 4 Gy.

Altogether our data suggested that the grade of adverse reaction may negatively correlate with DNA repair foci and positively correlate with LAN cells in PBL before and during radiotherapy. In line with this suggestion, fraction of LAN cells negatively correlated with level of 53BP1 foci before the start of radiation therapy ($r = -0.37$, $p = 0.023$) and at the 1 and 2 weeks during radiotherapy, $r = -0.57$, $p = 0.010$ and $r = -0.53$, $p = 0.017$, respectively. At 1 month and 1 year after the treatment, this correlation was not statistically significant. However, fraction of LAN cells negatively correlated with level of 53BP1 at high significance level ($r = -0.37$, $p = 0.00004$) when combined analysis was performed for all samples from all time points (Figure 5A).

These data show that LAN cells are accumulated at the expense of impaired elimination of cells with unrepaired or misrepaired DNA damage and in dependence on individual radiosensitivity. Thus, it is likely that radiosensitive patients have less efficient mechanisms for eliminating apoptotic cells with high DNA damage, which result in accumulation of LAN cells and side effects in these patients.

In parallel with analysis of LAN, the fraction of cells with γH2AX pan-staining, which are considered to be early apoptotic cells (Turesson et al. 2010; Wen et al. 2010; Vasilyev et al. 2013), was assessed. Only weak correlation was observed between γH2AX pan-staining and LAN cells (Figure 5B) suggesting that the γH2AX pan-staining may be a worse diagnostic marker. In line with these data, pan-staining did not correlate with individual radiosensitivity showing that late stage of apoptosis/necrosis may have a better prognostic role. It is interesting that mean values for LAN cells and γH2AX pan-staining were similar for all time points during and after therapy (Figure 6).

Significant variation in LAN cells was observed inside each group of patients. To estimate sensitivity and specificity of LAN cells for prediction of G2–G3 acute adverse reactions at the individual level, ROC analysis was performed and the optimal cut-off value was determined using the data obtained before treatment and 24 h after one fraction of radiation therapy (Figure 7). When a fraction of LAN cells before treatment of 1.83% was used as the cut-off value, the sensitivity and specificity in predicting G2–G3 acute adverse reactions were 80% and 56.3%, respectively. The area under the curve

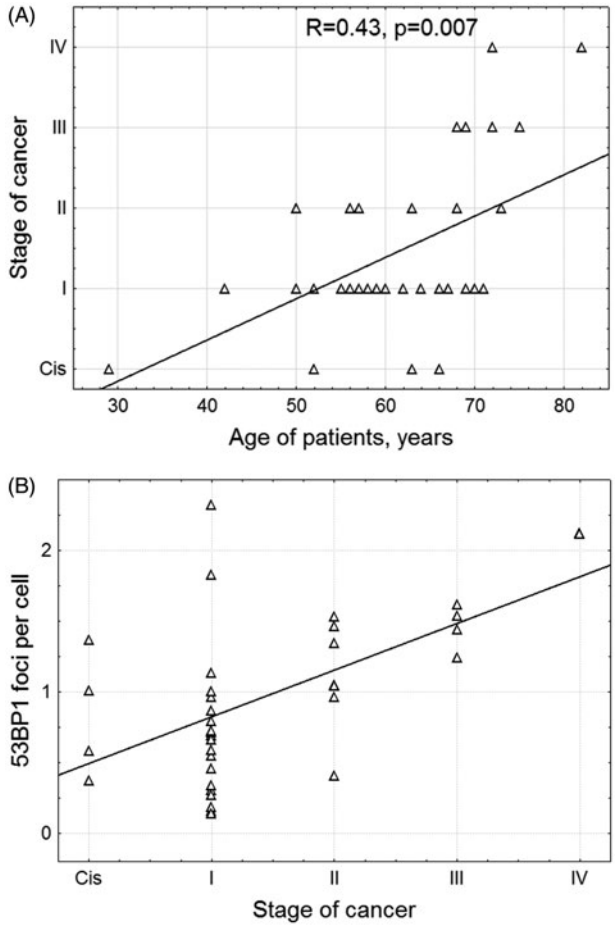


Figure 3. Correlation between stage of cancer, age and 53BP1 foci. Stage of cancer correlated with age of patients (A) and number of 53BP1 foci (B). Analysis was performed from 53BP1 data obtained prior to irradiation. Linear regression is indicated as solid line.

(AUC) was 0.688 (standard error [SE] = 0.143). Similar AUC values were obtained after 1st, 5th and 10th fraction of radiotherapy indicating relatively low predictive power of LAN cells for assessing G2–G3 acute adverse reactions at the individual level. Even lower predictive power for assessment of individual radiosensitivity was obtained using IRIF (data not shown).

Breast irradiated volume varied from 298–2634 cm^3. However, there was no significant effect of irradiated volume on number of IRIF, fraction of LAN cells and level of nuclei with γH2AX pan-staining. The boost did not affect the results either.

Discussion

Endogenous DNA damage in PBL of BC patients

The level of endogenous 53BP1 foci in PBL of healthy controls obtained in this study was comparable with those in our previous studies (Torudd et al. 2005; Markova et al. 2011; Vasilyev et al. 2013). In the group of BC patients, the level of 53BP1 foci before radiation therapy did not differ significantly from that in the control group. To our knowledge, only one study has assessed the level of γH2AX and 53BP1 foci in lymphocytes of BC patients before and during radiation therapy (Djuzenova et al. 2013). In comparison to present study, lower 53BP1 foci levels in both controls and patients have been reported by Djuzenova et al. (2013). This discrepancy between studies does not exceed usual interlaboratory variations in focus enumeration (Greve et al. 2012), which may be accounted for various experimental conditions but should not affect the intralaboratory data analysis because the same conditions are applied to all samples within each study (Belyaev 2010). In line with our results, the combined data did not differ between BC patients and healthy subjects in the study by Djuzenova et al. (2013), while these authors have found significant differences between patients with adverse clinical reaction to radiotherapy and controls. While endogenous γH2AX focus formation was slightly higher, about 0.1 γH2AX foci/cell, in lymphocytes of breast/rectal cancer patients above 40 years of age in comparison to that of healthy individuals, similar values were found in cancer patients and healthy individuals younger than 40 years of age by Wenger et al. (2014).

DNA damage during and after the treatment

To the best of our knowledge, no clinical data have previously been published regarding the long-term monitoring of 53BP1 foci in lymphocytes of cancer patients during and after radiation therapy. We monitored the number of 53BP1 and γH2AX foci in lymphocytes of BC patients during whole course of radiation therapy, 1 month and 1 year after radiotherapy. Significant increase of 53BP1 foci number 24 h after the first fraction of radiation therapy and its persistence during two weeks of treatment (Figure 1A) suggested the presence of misrepaired or unrepaired DSB. Given the high variability of radiation-induced 53BP1 foci levels between patients during the treatment, this effect was accounted for increased 53BP1 foci level in a subgroup of patients whereas other patients (29–42%, depending on time-point) demonstrated levels of 53BP1 foci during radiation therapy lower or similar to background. In line with our data, Djuzenova et al. (2013) reported that 26% of patients had lower number of 53BP1 foci at one week of radiation therapy than before treatment. We analyzed correlation of γH2AX foci during radiotherapy with background γH2AX level, patients' age, tumor size or variations in treatment. However, no significant association was found. Our data show that γH2AX foci did not accumulate in PBL during the whole course of radiotherapy.

A new finding of this study is significantly decreased levels of 53BP1/γH2AX foci and individual variability in 53BP1 focus formation 1 year after the end of radiotherapy. This data indicate elimination of DNA damaging factors most likely due to successful treatment of tumor.

In vivo versus in vitro IRIF data

It was found, that residual 53BP1/γH2AX foci detected in PBL 24 h following irradiation *in vitro* linearly depended on dose up to 2 Gy, the dose at which radiation-induced apoptosis in PBL is saturated (Torudd et al. 2005; Markova et al. 2011). Moreover, these dose-dependent residual foci persisted up to 4 weeks

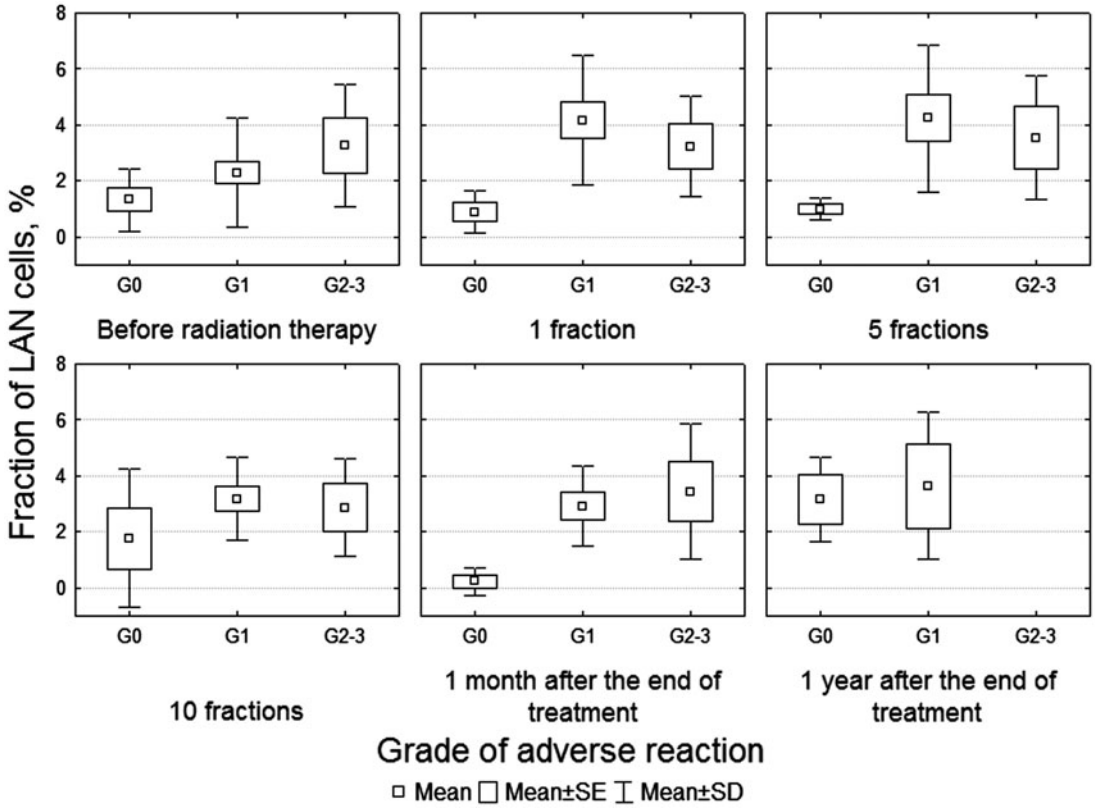


Figure 4. Late apoptosis/necrosis (LAN) cells in correlation with the grade of adverse reaction according to the Radiation Therapy Oncology Group (RTOG). Fraction of LAN cells in peripheral blood lymphocytes (PBL) of breast cancer (BC) patients before, during (24 h after the 1st, 5th and 10th fractions) and at 1 month and 1 year after the end of radiation therapy. The boxes show mean values and standard errors, and whiskers indicate mean ± SD.

post-irradiation *in vitro*. Given similar persistence of residual foci in PBL *in vivo*, accumulation of residual IRIF might be expected in PBL after each fraction during whole course of radiotherapy. Additional accumulation might be possible because small areas of the bone marrow in the ribs behind the breast, about 5%, are irradiated during radiotherapy of BC patients (Huber et al. 1999). In line with the reported *in vitro* data (Torudd et al. 2005; Markova et al. 2011), residual 53BP1 were detected in PBL of cancer patients 24 h after the first fraction while no significant increase was seen in γH2AX. Based on long-time persistence and linear dose dependence of residual IRIF *in vitro* (Markova et al. 2011), 4-fold and 9-fold increase after the 5th and 10th fractions, respectively, might be expected in comparison to the 1st fraction. However, no such increase of IRIF was detected. This finding provide further evidence for the notion that radiation response *in vitro* and *in vivo* may differ significantly (Girinsky et al. 1991; Löbrich et al. 2005; Zarybnicka et al. 2011). The mechanism of the observed differences between maintenance of cells with IRIF *in vitro* and *in vivo* is likely based on efficient elimination of damaged cells from human peripheral blood. While this mechanism has not completely been elucidated, it is supported by a faster drop in cell counts after irradiation *in vivo* than *in vitro* in studies where radiation response of human lymphocytes (Girinsky et al. 1991) and lymphocytes from large white pig (Zarybnicka et al. 2011) was followed post-irradiation *in vitro* and *in vivo*.

Effects of stage of cancer

While both 53BP1 foci and tumor stage correlated with age, ANCOVA revealed statistically significant correlation of endogenous 53BP1 foci with stage of cancer regardless of the age of patients. Interestingly, we found no correlation with stage of cancer for γH2AX. These data may provide evidence for better prognostic value of 53BP1 focus analysis. To our knowledge, this is the first report showing correlation between 53BP1 foci in PBL and stage of cancer. Djuzenova et al. (2013) reported correlation of tumor size with neither 53BP1 nor γH2AX foci. In Djuzenova's study, nodes and metastases were not taken into account and only two patients were analyzed with the advanced tumor sizes T3 and T4. These two patients were at lower ages, 54 and 53 years, in comparison to the group of stage III–IV patients in our present study, ≥68 years. The aforementioned differences in analysis and study groups may underlie the discrepancy in results. More data from extended groups of patients are needed to validate dependence of DNA damage in PBL on stage of cancer.

Individual radiosensitivity

In healthy tissues of BC patients, DNA damage induced by the fractionated radiotherapy varies between individuals (Huber et al. 1999). In general, the white blood cell count tends to decrease with time during radiotherapy (Catena et al. 1997; De Ruyck et al. 2004). Lifespan of lymphocytes is estimated

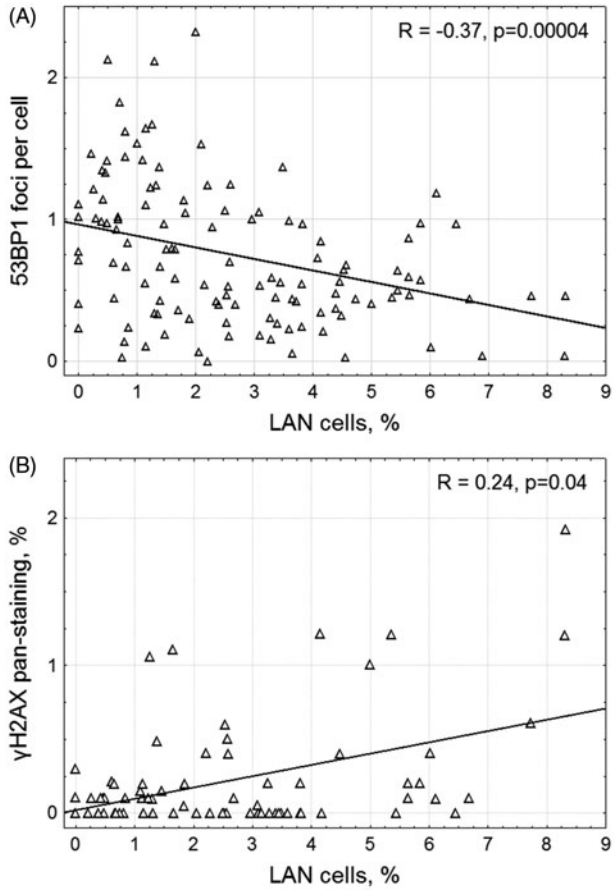


Figure 5. Correlation of late apoptosis/necrosis (LAN) cells with 53BP1 foci and γH2AX pan-staining. Negative correlation of LAN cells with number of 53BP1 foci (A) and positive correlation with γH2AX pan-staining (B) was observed in combined samples from all BC patients and all time-points before, during and after radiation therapy. Linear regression is indicated as solid line.

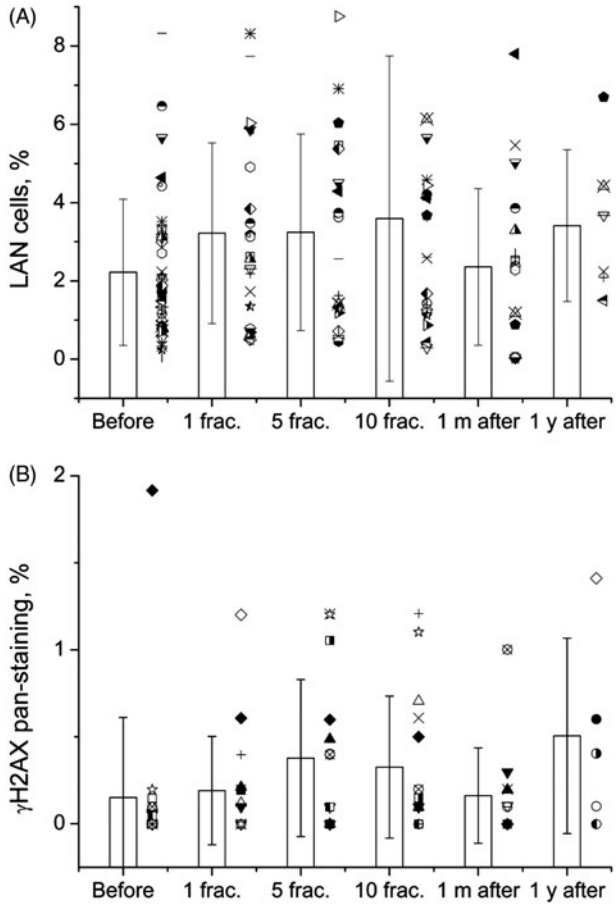
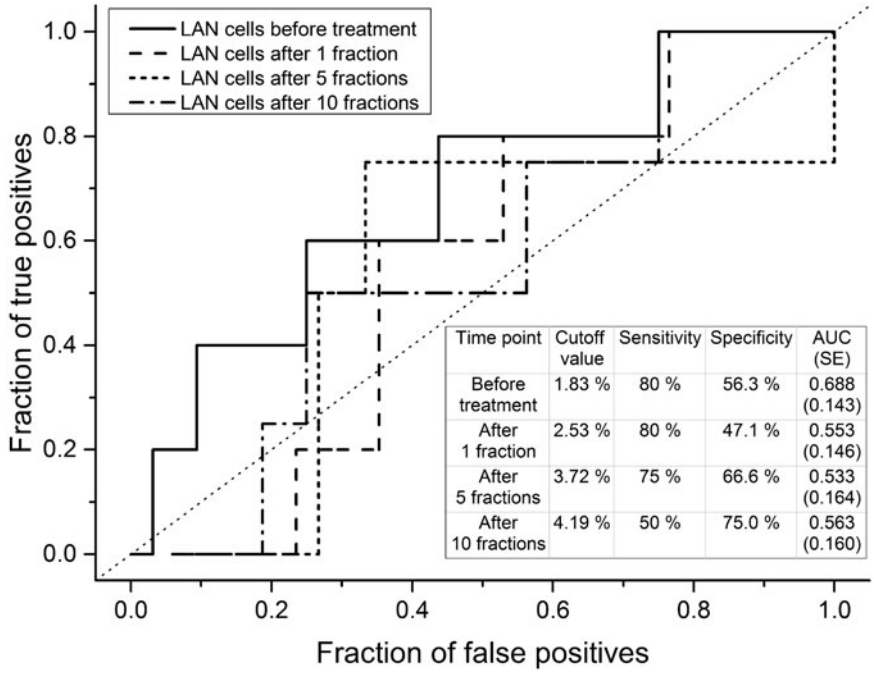


Figure 6. Late apoptosis/necrosis (LAN) cells and pan-staining before during and after therapy. Different symbols correspond to individual patients. Both fraction of LAN cells (A) and level of nuclei with γH2AX pan-staining (B) in peripheral blood lymphocytes (PBL) of breast cancer (BC) patients did not change significantly before, during (24 h after 1st, 5th and 10th fractions) and after the end of radiation therapy (at 1 month and 1 year).

about 1 year for short-lived and about 6 years for long-lived cell subsets (Michie et al. 1992; Bogen 1993). However, they can be earlier eliminated by radiation-induced apoptosis during radiotherapy. Apoptosis may be either directly induced by irradiation of small part of lymphocytes circulating throughout irradiated breast or indirectly induced by cytotoxic agents released from the irradiated tissue into the blood. Presently, no standardized method exists to assess accurately the absorbed dose to the irradiated lymphocytes after partial-body exposure because only a fraction of blood cells is exposed to irradiation. Assuming that the irradiated tissue is 1 kg, the weight of BC patient is 75 kg, and the blood is equally distributed, the maximal dose in total blood can be estimated as about 3 cGy after one irradiation fraction. Thus, the total maximum dose in blood would be about 75 cGy during whole course of radiotherapy. This estimate is within the total biological dose, which averaged 0.33 Gy and varied between 0.1 and 1.1 Gy in blood lymphocytes of similarly irradiated BC patients as measured with chromosomal aberrations (Legal et al. 2002). Given the limitations of our assumption on blood content in the irradiated tissue, the aforementioned estimates may suggest that the indirect effect of irradiation is negligible in blood of most BC patients.

In parallel with the reduced cell count, apoptosis is increased during radiation therapy (Catena et al. 1997). The physiologic importance of complete apoptosis and removal of apoptotic cells by phagocytosis should not be overlooked because this process might be critical to avoid the persistence and release of toxic cellular components. The phagocytosis of apoptotic cells occurs prior to their lysis, thereby preventing the release of potentially proinflammatory intracellular contents (Savill 2000). The acute side-effects may be induced in some individuals if phagocytosis is not able to remove all apoptotic cells and they reach the stage of lysis becoming membrane-damaged LAN cells, which are usually detected by the TB exclusion assay. Finally, cell repopulation is accomplished by the bone marrow. This repopulation may also be individually affected by radiation because about 5% of bone marrow might be irradiated during radiotherapy (Huber et al. 1999).

An interesting new finding of this study is the increased number of LAN cells with increased radiosensitivity. This increase was accompanied by a decreased number of cells with residual IRIF. The rational for selective removal of cells with residual foci follows from our previous studies, which show that PBL containing at least one residual IRIF undergo apoptosis (Torudd et al. 2005). Thus, LAN cells may accumulate in radiosensitive patients due to cells with residual IRIF that likely contain unrepaired or misrepaired DNA damage.



Time point	Cutoff value	Sensitivity	Specificity	AUC (SE)
Before treatment	1.83 %	80 %	56.3 %	0.688 (0.143)
After 1 fraction	2.53 %	80 %	47.1 %	0.553 (0.146)
After 5 fractions	3.72 %	75 %	66.6 %	0.533 (0.164)
After 10 fractions	4.19 %	50 %	75.0 %	0.563 (0.160)

Figure 7. Validation of sensitivity and specificity of late apoptosis/necrosis (LAN) cells for prediction of G2-3 acute adverse reactions of breast cancer patients in radiotherapy. Receiver Operating Characteristic (ROC) curve of the level of LAN cells before treatment and 24 h after the 1st, 5th and 10th fractions of radiation therapy is shown along with the calculated data for sensitivity, specificity and area under the curve (AUC). The ordinate and abscissa represents the true and false positive rate, respectively. AUC was used to estimate the predictive value of LAN cells.

Such factors as phagocytosis of apoptotic cells and cell repopulation may also contribute to mechanistic relationship between radiosensitivity and residual foci/LAN cells measured *in vivo*. On the other hand, the early apoptotic cells detected by the γH2AX pan-staining did not correlate with individual radiosensitivity showing that the late stage of apoptosis/necrosis may have a better prognostic role. Altogether our data suggest that: (i) LAN cells may accumulate due to incomplete removal of apoptotic cells with unrepaired or misrepaired DNA damage; (ii) more radiosensitive patients may have less efficient mechanisms for elimination of cells with DNA damage resulting in accumulation of LAN cells and facilitating side-effects.

Both assays, LAN and IRIF, have shown large interindividual variability inside each group of patients and relatively low predictive power at the individual level. Thus, each of these assays is not reliable test for individual radiosensitivity. However, due to revealed correlations, these assays may constitute the complementary tests.

To conclude, our data suggested that both endogenous and radiation-induced 53BP1 foci increased with age of BC patients and healthy controls while γH2AX focus formation did not correlate with age in the studied group. The stage of cancer correlated with the number of 53BP1 foci prior radiotherapy. The yield of residual 53BP1 foci increased 24 h after the first fraction of radiation therapy but did not accumulate upon delivering further fractions. The level of 53BP1 foci significantly decreased after the end of radiation therapy. This decrease, also observed in γH2AX foci, is likely to be caused by disappearance of genotoxicity upon treatment. This study has shown that kinetics of radiation-induced residual foci in PBL of BC patients undergoing radiotherapy cannot be predicted based on *in vitro* data. The difference between *in vitro* and *in vivo* data is most likely due to immune and hematopoietic systems, which may significantly contribute to individual radiosensitivity by removal of apoptotic cells and cell repopulation. The data suggested that the grade of adverse reaction may positively correlate with LAN cells and negatively correlate with IRIF in the *in vivo* tests, however, these measures alone are unlikely to be sufficiently robust for clinical use.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

References

Banath JP, MacPhail SH, Olive PL. 2004. Radiation sensitivity, H2AX phosphorylation, and kinetics of repair of DNA strand breaks in irradiated cervical cancer cell lines. Cancer Res 64:7144–7149.

Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, Lukas J. 2006. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol 173:195–206.

Belyaev IY. 2010. Radiation-induced DNA repair foci: Spatio-temporal aspects of formation, application for assessment of radiosensitivity and biological dosimetry. Mutat Res 704:132–141.

Bogen KT. 1993. Reassessment of human peripheral T-lymphocyte lifespan deduced from cytogenetic and cytotoxic effects of radiation. Int J Radiat Biol 64:195–204.

Brzozowska K, Pinkawa M, Eble MJ, Muller WU, Wojcik A, Kriehuber R, Schmitz S. 2012. In vivo versus *in vitro* individual radiosensitivity analysed in healthy donors and in prostate cancer patients with and without severe side effects after radiotherapy. Int J Radiat Biol 88:405–413.

Catena C, Parasacchi P, Conti D, Sgura A, Trenta G, Righi E, Trinci MM, Trinci M. 1997. Peripheral blood lymphocyte decrease and micronucleus yields during radiotherapy. Int J Radiat Biol 72:575–585.

Cox JD, Stetz J, Pajak TF. 1995. Toxicity criteria of the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC). Int J Radiat Oncol Biol Phys 31:1341–1346.

De Ruyck K, Lambert B, Bacher K, Gemmel F, De Vos F, Vral A, De Ridder L, Dierckx RA, Thierens H. 2004. Biologic dosimetry of 188Re-HDD/lipiodol versus 131I-lipiodol therapy in patients with hepatocellular carcinoma. J Nuclear Med 45:612–618.

Dikomey E, Borgmann K, Peacock J, Jung H. 2003. Why recent studies relating normal tissue response to individual radiosensitivity might have failed and how new studies should be performed. Int J Radiat Oncol Biol Phys 56:1194–1200.

Djuzenova CS, Elsner I, Katzer A, Worschech E, Distel LV, Flentje M, Polat B. 2013. Radiosensitivity in breast cancer assessed by the histone gamma-H2AX and 53BP1 foci. Radiat Oncol (London, UK) 8:98.

Fleckenstein J, Kuhne M, Seegmuller K, Derschang S, Melchior P, Graber S, Fricke A, Rube CE, Rube C. 2011. The impact of individual *in vivo* repair of DNA double-strand breaks on oral mucositis in adjuvant radiotherapy of head-and-neck cancer. Int J Radiat Oncol Biol Phys 81:1465–1472.

Girinsky T, Socie G, Cosset JM, Malaise EP. 1991. Blood lymphocyte subsets after the first fraction in patients given hyperfractionated total body irradiation for bone marrow transplantation. Br J Cancer 63:646–647.

Goodarzi AA, Jeggo PA. 2012. Irradiation induced foci (IRIF) as a biomarker for radiosensitivity. Mutat Res 736:39–47.

Greve B, Bolling T, Amler S, Rossler U, Gomolka M, Mayer C, Popanda O, Dreffke K, Rickinger A, Fritz E, Eckardt-Schupp F, Sauerland C, Braselmann H, Sauter W, Illig T, Riesenbeck D, Konemann S, Willich N, Mortl S, Eich HT, Schmezer P. 2012. Evaluation of different biomarkers to predict individual radiosensitivity in an inter-laboratory comparison — lessons for future studies. PLoS One 7:e47185.

Hamer G, Roepers-Gajadien HL, Van Duyn-Goedhart A, Gademan IS, Kal HB, Van Buul PP, De Rooij DG. 2003. DNA double-strand breaks and gamma-H2AX signaling in the testis. Biol Reprod 68:628–634.

Henriquez-Hernandez LA, Bordon E, Pinar B, Lloret M, Rodriguez-Gallego C, Lara PC. 2012. Prediction of normal tissue toxicity as part of the individualized treatment with radiotherapy in oncology patients. Surg Oncol 21:201–206.

Huber R, Braselmann H, Kulka U, Schumacher-Georgiadou V, Bayerl A, Molls M, Bauchinger M. 1999. Follow-up analysis of translocation and dicentric frequencies, measured by FISH-chromosome painting in breast cancer patients after partial-body radiotherapy with little bone marrow exposure. Mutat Res 446:103–109.

Iwabuchi K, Basu BP, Kysela B, Kurihara T, Shibata M, Guan D, Cao Y, Hamada T, Imamura K, Jeggo PA, Date T, Doherty AJ. 2003. Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. J Biol Chem 278:36487–36495.

Kuhne M, Riballo E, Rief N, Rothkamm K, Jeggo PA, Löbrich M. 2004. A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. Cancer Res 64:500–508.

Legal JD, De Crevoisier R, Lartigau E, Morsli K, Dossou J, Chavaudra N, Sanfilippo N, Bourhis J, Eschwege F, Parmentier C. 2002. Chromosomal aberrations induced by chemotherapy and radiotherapy in lymphocytes from patients with breast carcinoma. Int J Radiat Oncol Biol Phys 52:1186–1195.

Löbrich M, Kiefer J. 2006. Assessing the likelihood of severe side effects in radiotherapy. Int J Cancer 118:2652–2656.

Löbrich M, Rief N, Kuhne M, Heckmann M, Fleckenstein J, Rube C, Uder M. 2005. In vivo formation and repair of DNA double-strand breaks after computed tomography examinations. Proc Natl Acad Sci USA 102:8984–8989.

MacPhail SH, Banath JP, Yu TY, Chu EH, Lambur H, Olive PL. 2003. Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays. Int J Radiat Biol 79:351–358.

Markova E, Hillert L, Malmgren L, Persson BR, Belyaev IY. 2005. Microwaves from GSM mobile telephones affect 53BP1 and gamma-H2AX foci in human lymphocytes from hypersensitive and healthy persons. Environ Health Perspect 113:1172–1177.

Markova E, Lackova A, Pobijakova M, Belyaev I, 2008, DNA repair foci in lymphocytes of breast cancer patients during and after radiotherapy: 5th DNA Repair Workshop, p. 53.

Markova E, Schultz N, Belyaev IY. 2007. Kinetics and dose-response of residual 53BP1/gamma-H2AX foci: Co-localization, relationship with DSB repair and clonogenic survival. Int J Radiat Biol 83:319–329.

Markova E, Schultz N, Torudd J, Harms-Ringdahl M, Protopopova M, Selivanova G, Khakimov H, Belyaev I, 2003, Validation of novel DSB-co-localizing foci assay for radiosensitivity: 12th International Congress of Radiation Research, p. 235.

Markova E, Torudd J, Belyaev I. 2011. Long time persistence of residual 53BP1/gamma-H2AX foci in human lymphocytes in relationship to apoptosis, chromatin condensation and biological dosimetry. Int J Radiat Biol 87:736–745.

Michie CA, Mclean A, Alcock C, Beverley PC. 1992. Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature 360:264–265.

Mochan TA, Venere M, Ditullio RA , Halazonetis TD. 2004. 53BP1, an activator of ATM in response to DNA damage. DNA Repair (Amst) 3:945–952.

Olive PL, Banath JP. 2004. Phosphorylation of histone H2AX as a measure of radiosensitivity. Int J Radiat Oncol Biol Phys 58:331–335.

Park H, Choi DH, Noh JM, Huh SJ, Park W, Nam SJ, Lee JE. 2014. Acute skin toxicity in Korean breast cancer patients carrying BRCA mutations. Int J Radiat Biol 90:90–94.

Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Current Biol 10:886–895.

Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. 2002. Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev 12:162–169.

Rogakou EP, Boon C, Redon C, Bonner WM. 1999. Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. J Cell Biol 146:905–916.

Rothkamm K, Löbrich M. 2003. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low X-ray doses. Proc Natl Acad Sci USA 100:5057–5062.

Savill J. 2000. Apoptosis in resolution of inflammation. Kidney Blood Pressure Res 23:173–174.

Severin E, Greve B, Pascher E, Wedemeyer N, Hacker-Klom U, Silling G, Kienast J, Willich N, Gohde W. 2006. Evidence for predictive validity of blood assays to evaluate individual radiosensitivity. Int J Radiat Oncol Biol Phys 64:242–250.

Sorokina S, Markova E, Gursky J, Dobrovodsky J, Belyaev I. 2013. Relative biological efficiency of protons at low and therapeutic doses in induction of 53BP1/gammaH2AX foci in lymphocytes from umbilical cord blood. Int J Radiat Biol 89:716–723.

Taneja N, Davis M, Choy JS, Beckett MA, Singh R, Kron SJ, Weichselbaum RR. 2004. Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. J Biol Chem 279:2273–2280.

Torudd J, Harms-Ringdahl M, Tapper L, Belyaev I, 2003, Radiation induced apoptosis in human lymphocytes as a possible tool for estimation of individual radiosensitivity for radiotherapy: 12th International Congress of Radiation Research, p. 337.

Torudd J, Protopopova M, Sarimov R, Nygren J, Eriksson S, Markova E, Chovanec M, Selivanova G, Belyaev IY. 2005. Dose-response for radiation-induced apoptosis, residual 53BP1 foci and DNA-loop relaxation in human lymphocytes. Int J Radiat Biol 81:125–138.

Turesson I, Nyman J, Qvarnstrom F, Simonsson M, Book M, Hermansson I, Sigurdardottir S, Johansson K-A. 2010. A low-dose hypersensitive keratinocyte loss in response to fractionated radiotherapy is associated with growth arrest and apoptosis. Radiother Oncol 94:90–101.

Vasilyev SA, Kubes M, Markova E, Belyaev I. 2013. DNA damage response in CD133 + stem/progenitor cells from umbilical cord blood: Low level of endogenous foci and high recruitment of 53BP1. Int J Radiat Biol 89:301–309.

Vasireddy RS, Sprung CN, Cempaka NL, Chao M, Mckay MJ. 2010. H2AX phosphorylation screen of cells from radiosensitive cancer patients reveals a novel DNA double-strand break repair cellular phenotype. Br J Cancer 102:1511–1518.

Warters RL, Adamson PJ, Pond CD, Leachman SA. 2005. Melanoma cells express elevated levels of phosphorylated histone H2AX foci. J Invest Dermatol 124:807–817.

Wen WH, Zhu F, Zhang JS, Keum YS, Zykova T, Yao K, Peng C, Zheng D, Cho YY, Ma WY, Bode AM, Dong ZG. 2010. MST1 Promotes apoptosis through phosphorylation of histone H2AX. J Biol Chem 285:39108–39116.

Wenger B, Schwegler M, Brunner M, Daniel C, Schmidt M, Fietkau R, Distel LV. 2014. PML-nuclear bodies decrease with age and their stress response is impaired in aged individuals. BMC Geriatr 14:42.

Werbrouck J, De Ruyck K, Beels L, Vral A, Van Eijkeren M, De Neve W, Thierens H. 2010. Prediction of late normal tissue complications in RT treated gynaecological cancer patients: Potential of the gamma-H2AX foci assay and association with chromosomal radiosensitivity. Oncol Rep 23:571–578.

Wykes SM, Piasentin E, Joiner MC, Wilson GD, Marples B. 2006. Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. Radiat Res 165:516–524.

Yu T, MacPhail SH, Banath JP, Klokov D, Olive PL. 2006. Endogenous expression of phosphorylated histone H2AX in tumors in relation to DNA double-strand breaks and genomic instability. DNA Repair 5:935–946.

Zarybnicka L, Sinkorova Z, Sinkora J, Blaha V, Vavrova J, Pejchal J, Osterreicher J. 2011. Sensitivity of porcine peripheral blood leukocytes to gamma irradiation *in vivo*, *in vitro* and ex vivo. Int J Radiat Biol 87:491–498.

Zhivotosky B, Orrenius S. 2001. Assessment of apoptosis and necrosis by DNA fragmentation and morphological criteria. Curr Protocols Cell Biol Supplement 12:18.3. 1–18.3. 23.