

Apoptosis for prediction of radiotherapy late toxicity: lymphocyte subset sensitivity and potential effect of *TP53* Arg72Pro polymorphism

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Published online: 15 November 2014
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Abstract We tested apoptosis levels in in vitro irradiated T-lymphocytes from breast cancer (BC) patients with radiotherapy-induced late effects. Previous results reported in the literature were revised. We also examined the effect of *TP53* Arg72Pro polymorphism on irradiation-induced apoptosis (IA). Twenty BC patients, ten with fibrosis and/or telangiectasias and ten matched controls with no late reactions, were selected from those receiving radiotherapy between 1993 and 2007. All patients were followed-up at least 6 years after radiotherapy. Using the combination of both CD3 and CD8 antibodies the in vitro IA was measured in CD3, CD8 and CD4 T-lymphocytes, and CD8 natural killer lymphocytes (CD8 NK) by flow cytometry. The *TP53* Arg72Pro genotype was determined by sequencing. Patients with late radiotherapy toxicity showed less IA for

all T-lymphocytes except for the CD8 NK. CD8 NK showed the highest spontaneous apoptosis and the lowest IA. IA in patients with toxicity appears to be lower than the control patients only in *TP53* Arg/Arg patients ($P = 0.077$). This difference was not present in patients carrying at least one Pro allele ($P = 0.8266$). Our data indicate that late side effects induced by radiotherapy of BC are associated to low levels of IA. CD8 NK cells have a different response to in vitro irradiation compared to CD8 T-lymphocytes. It would be advisable to distinguish the CD8 NK lymphocytes from the pool of CD8+ lymphocytes in IA assays using CD8+ cells. Our data suggest that the 72Pro *TP53* allele may influence the IA of patients with radiotherapy toxicity.

Electronic supplementary material The online version of this article (doi:10.1007/s10495-014-1056-2) contains supplementary material, which is available to authorized users.

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Keywords Radiotherapy-induced late toxicity · In vitro irradiation-induced apoptosis · T-lymphocytes · Natural killer CD8+ lymphocytes · *TP53* Arg72Pro polymorphism

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Introduction

The success of radiotherapy in eradicating a tumour depends on the total radiation dose given, but the tolerance of the normal tissues surrounding the tumour limits this dose [1]. The majority of patients tolerate well standard radiation therapy, but up to 10 % of patients suffer from acute to life threatening adverse effects arising from the intrinsic sensitivity of normal tissue [2]. As there is a direct relation between radiation dose and tumour control, the development of side effects in a minority limits the dose that can be safely prescribed to the majority of patients [1, 3]. Furthermore, radiotherapy side-effects impact negatively on quality-of-life [4].

Development of a test to predict side effects should enable individualized radiation dose prescription. It would increase cancer cure, while reducing the number of survivors suffering from the consequences of treatment [5]. Since the early 1990s, various cell-based in vitro assays have been developed to quantify cellular radiosensitivity and predict the outcome after radiotherapy [5]. These studies showed a relationship between the biomarkers measured and normal tissue toxicity, although, in general, cell-based assays lack the sensitivity, specificity and reproducibility required for a routine clinical test [1, 5, 6].

Crompton et al. [7] reported lower levels of irradiation-induced apoptosis (IA), measured by flow cytometry on CD4 and CD8 cells, in patients with severe reactions to radiotherapy when compared to healthy and cancer patients who displayed normal toxicity. In a prospective study of 399 patients treated by radiotherapy for different cancer types, Ozahin et al. [8] demonstrated that a lower CD4 or CD8 apoptosis correlated to higher risk of late adverse effects. Recently, other authors have also shown that patients with localized cervical carcinoma or advanced head and neck cancer and late toxicity induced by radiotherapy displayed a low apoptotic response compared with patients with normal tissue response [9, 10].

One of the most important apoptotic regulatory proteins is the tumour suppressor protein p53 (encoded by *TP53* gene). Acting as a transcription factor, p53 responds to diverse cellular stresses to regulate cell cycle arrest, apoptosis, senescence, and DNA repair (reviewed by Riley et al. [11]). *TP53* has a common single-nucleotide polymorphism (SNP), that results in either proline or arginine at amino-acid position 72 (Arg72Pro) (dbSNP: rs1042522) in the p53 protein. This polymorphism is located in a p53 proline-rich domain, which is necessary for the protein to fully induce apoptosis (reviewed by Whibley et al. [12]). Some evidences support that these two p53 variants exhibit different biochemical properties, one of which is that p53-72Arg is a more efficient inducer of apoptosis than p53-72Pro [12, 13].

In our study we tested in ten breast cancer (BC) patients with fibrosis or/and telangiectasias and 10 patients without late lesions induced by radiotherapy: (1) the levels of lymphocyte apoptosis induced by in vitro irradiation using the combination of both CD3 and CD8 antibodies to specifically identify T-lymphocytes; and (2) the effect on the IA of the *TP53* Arg72Pro polymorphism. We also reviewed the previous studies reported in the literature.

Patients and methods

Patients

The late toxicity after radiotherapy was reviewed in the patient records treated with radiation for BC between 1993 and 2007. About one hundred patients per year received radiotherapy during this period. After breast-conserving surgery, the patients received adjuvant systemic treatment (chemotherapy, hormone therapy or combined). The patients received a standard breast irradiation treatment with two tangential fields, each one with a wedge: a lateral and a medial. Planning was based on 2D simulation, including verification and quality assurance. The patients were given whole breast radiotherapy with conventional fractionation, 50 Gy, 2 Gy/fraction with ^{60}Co unit. Treatment added a tumorectomy electron boost (9–16 MeV) from a lineal accelerator, with doses ranging from 14 to 20 Gy. The dose was 20 Gy at 85 % isodose. All patients have been followed-up at least 6 years after radiotherapy (6–19 years). The occurrence of acute and late effects of radiotherapy were monitored and documented by the physician during the standard patient follow-up along and after radiotherapy using Radiation Therapy Oncology Group toxicity grading system.

For the case–control study it was possible to recruit ten BC patients with fibrosis and/or telangiectasias (Table 1). Each case was matched with one control that showed no late change in breast appearance. There is no statistically significant difference among the two groups of patients for treatment, demographic and tumour characteristics (Table 2). Supplementary Table S1 shows the tumour staging for the ten BC patients with late toxicity and for the ten control patients. Three BC patients with late toxicity had criteria for *BRCA1/BRCA2* analysis, and no mutation was identified (Supplementary Table S1). The ethical committee of the hospital approved the study and all women participating signed an informed consent.

Radiation-induced apoptosis assay

The protocol of IA assay was performed as previously reported [7, 14] with some modifications (Supplementary

Table 1 Acute and late toxicity in the analysed breast cancer patients

Group	Patient ID	Acute toxicity (evaluated ≤ 3 months after radiotherapy)	Late toxicity (evaluated along >3 months up to at least 6 years after radiotherapy)
Without late normal tissue reactions			
	SP-13	Epithelitis	None
	SP-4	Epithelitis	None
	SP-19	Erythema	None
	SP-8	Epithelitis grade 2	None
	SP-18	None	None
	SP-7	None	None
	SP-11	Erythema	None
	SP-6	Epithelitis grade 3	None
	SP-12	Erythema	None
	SP-16	Dermatitis	None
With late normal tissue reactions			
	SP-1	Erythema and Epithelitis	Telangiectasia grade 2 + Fibrosis grade 2
	SP-10	Erythema	Telangiectasia grade 1
	SP-9	Erythema and Edema	Fibroadenoma grade 1
	SP-14	Erythema and Moist Epithelitis grade 3	Fibrosis grade 3
	SP-3	Erythema	Telangiectasia grade 1
	SP-5	Erythema	Telangiectasia grade 1
	SP-15	Epithelitis grade 2 in each bilateral breast cancer	Telangiectasia and Fibrosis grade 3 in each bilateral breast cancer
	SP-17	Erythema	Fibrosis grade 3
	SP-20	Erythema	Telangiectasia grade 2 and Fibrosis grade 3
	SP-2	None	Telangiectasia grade 2 bilateral and Fibrosis grade 2 in right breast

Materials). Briefly, two fresh blood cultures, in RPMI 1640 supplemented with 20 % foetal bovine serum, were set up for each patient. Immediately after, one was treated with 8 Gy at room temperature at a dose rate of 3 Gy/min with a 6 MV photon beam. Both cultures were then placed into a 37 °C incubator with 5 % CO₂. After 48 h of incubation, the cell pellet was labelled with FITC-conjugated anti-CD8 and APC-conjugated anti-CD3 monoclonal antibodies. The erythrocytes were lysed and the cell pellet was stained with propidium iodide and treated with RNase. The cells were subsequently examined using a FACScan flow cytometer. Data from at least 10,000 lymphocytes per culture were acquired.

Apoptosis was measured in the following cell types: (a) total T-lymphocytes (CD3⁺); (b) CD8 T-lymphocytes (CD3⁺ CD8⁺); (c) CD4 T-lymphocytes (CD3⁺ CD8⁻)

Table 2 Clinical and demographic characteristics of analysed patients. The *TP53* genotype is also indicated

Characteristic	Non-toxicity group (n = 10)	Toxicity group (n = 10)
Age at radiotherapy (mean \pm SD)	45.7 \pm 10.01	52.8 \pm 8.6 ^a
Age at study (mean \pm SD)	55.2 \pm 8.01	61.6 \pm 8.63 ^a
BMI (mean \pm SD)	25.42 \pm 3.41 (n = 5)	26.9 \pm 6.21 (n = 4) ^a
Smoking status		
Non-smoker	50 % (n = 8)	70 % ^b
Current smoker	12.5 % (n = 8)	10 % ^b
Former smoker	37.5 % (n = 8)	20 % ^b
Histological type		
Invasive ductal	70 %	80 % ^b
Invasive lobular	10 %	0 % ^b
In situ	10 %	10 %
Other	10 % (mixed ductal and lobular)	10 % (tubule-lobular carcinoma)
Adjuvant treatment		
Neo-adjuvant chemotherapy	10 %	0 % ^b
None	10 %	10 % ^b
Chemotherapy	0 %	20 % ^{b,c}
Hormonotherapy	20 %	50 % ^b
Chemotherapy + Hormonotherapy	60 %	20 % ^b
Radiotherapy		
Basic irradiation dose	50 Gy (n = 9) 46 Gy (n = 1)	50 Gy
Boost dose	14–20 Gy	16–20 Gy
Total prescribed dose Gy (mean \pm SD)	65.8 \pm 0.63 ^a	67.0 \pm 1.69 ^a
Boost with brachytherapy	10 %	10 %
Radiotherapy concomitant to chemotherapy	30 %	10 % ^b
Years of follow-up after radiotherapy until time of publication (mean \pm SD)	12.0 \pm 3.78 ^a	10.4 \pm 4.65 ^a
<i>TP53</i> codon 72 genotype		
GG (Arg/Arg)	n = 6	n = 7
GC (Arg/Pro)	n = 3	n = 3
CC (Pro/Pro)	n = 1	n = 0

^a No statistically significant differences between both groups: *t* Test

^b No statistically significant differences between frequencies of both groups: Yates corrected Chi square test

^c One of them was also treated with a monoclonal antibody as adjuvant therapy

and; (d) CD8 subset of natural killer (NK) lymphocytes (CD3⁻ CD8⁺).

DNA genotyping

The *TP53* Arg72Pro SNP: c.215G > C was genotyped by Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit, on a 3130xl Genetic Analyzer. PCR amplification was performed using primers designed to include the SNP: 5'-CCTGGTCTCTGACTGCTCT-3' (forward) and 5'-CAGGCATTGAAGTCTCATGG-3' (reverse).

Statistical analysis

The outcome variables analyzed were the percentage of spontaneous apoptotic cells and the IA ratio, calculated as the percentage of apoptotic cells induced by 8 Gy divided by the basal percentage of apoptotic cells. As each cell type was studied in the same patients, the between-group differences in basal apoptosis and IA ratio were analyzed using generalized linear models (GzLMs) for repeated measures. Also, a lognormal distribution was specified as it fitted well both outcome variables. Sequential Bonferroni corrections for multiple comparisons were used for post hoc comparisons if appropriate. The analysis included three factors: (1) cell type; (2) clinical toxicity due to radiotherapy; and (3) *TP53* Arg72Pro polymorphism genotype. The outcome variables are presented as geometric means with 95 % confidence intervals (95 % CIs), since this is the best way when data follow a lognormal distribution.

The demographic and clinical data were compared between the patients without late toxicity and patients presenting late toxicity by using a *t* test for continued variables or Yates corrected Chi square test for categorical variables (Table 2).

In order to estimate the classification properties of the ratios of IA, area under the receiver-operating characteristic curves (AUC ROC) and the respective 95 % CIs were calculated, as well as a cut-off value according to Youden's index (maximum of sensitivity + specificity - 1).

Statistical computations were performed using the packages SAS[®] 9.2 (GzLMs), Stata 11 (AUC), and STATISTICA 8.0 (Graphic charts).

Results

Spontaneous apoptosis

The results of the GzLMs analysis for repeated measures indicated that the clinical toxicity factor does not affect the percentage of spontaneous apoptosis ($P = 0.806$), since

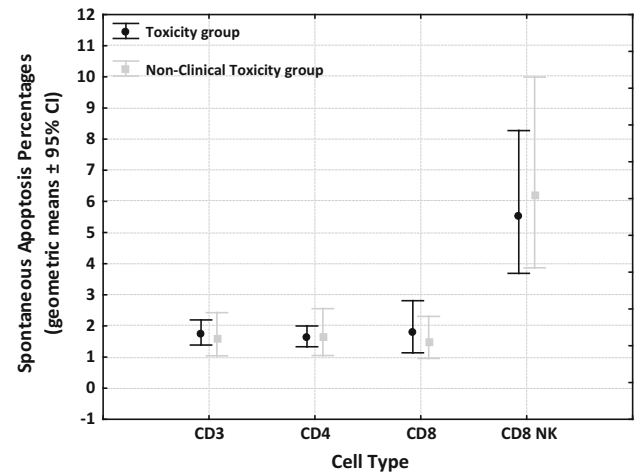


Fig. 1 Spontaneous apoptosis levels (geometric means \pm 95 % CI) in non irradiated cultures compared between patients with and without late effects induced by radiotherapy, and among the different cell types. No differences in the spontaneous apoptosis between patients with and without late effects induced by radiotherapy. The CD8 subset of natural killer lymphocytes (CD8 NK) showed the highest values of spontaneous apoptosis compared with the rest of analyzed cell types

there were no differences between patients with and without late effects induced by radiotherapy (Fig. 1). In contrast, the factor of cell type showed a significant effect on the basal percentage of apoptosis ($P < 0.0001$), as the CD8⁺ subset of natural killer lymphocytes (CD8 NK) showed higher basal percentage of apoptosis than the other cell types, irrespective of clinical toxicity status ($P = 0.0001$ for CD8NK vs. CD3, CD8NK vs. CD4 and CD8NK vs. CD8, Bonferroni test) (Fig. 1). Supplementary Table S2 shows the age, the number of cells evaluated and the percentage of spontaneous apoptosis for the four untreated cell types.

Ratio of the radiation-induced apoptosis (IA)

The ratio of apoptosis induced by 8 Gy was affected by both the clinical toxicity of the patients and the cell type ($P = 0.049$ and $P = 0.0003$, respectively). The patients with fibrosis and telangiectasias demonstrated a lower rate of IA than the patients without side effects (Fig. 2) for the CD3, CD4 and CD8 T-lymphocytes although this difference was only significant for the CD3 cells ($P = 0.004$ for CD3; $P = 0.060$ for CD4; $P = 0.097$ for CD8, Bonferroni test). In contrast, the IA ratio of the CD8 NK cells was similar between patients with or without late lesions ($P = 0.389$, Bonferroni test) (Fig. 2).

Considering the cell type effect, the CD8 T-lymphocytes showed IA ratios higher than other cells irrespectively of the patient toxicity status (CD3 vs. CD8 $P = 0.0026$; CD4 vs. CD8 $P = 0.0015$; CD8 vs. CD8NK $P = 0.0006$,

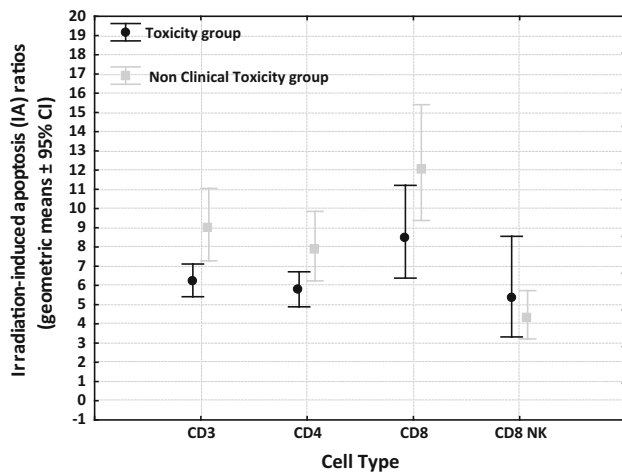


Fig. 2 Irradiation-induced apoptotic (IA) ratios (geometric mean \pm 95 % CI) compared between patients with and without late effects induced by radiotherapy, and among the different cell types. Patients with late radiotherapy toxicity showed less IA for all T-lymphocytes except for the CD8 NK. CD8 NK showed the lowest IA

Bonferroni test), while the ratio values of CD8 NK lymphocytes were lower (CD3 vs. CD8 NK $P = 0.0181$; CD4 vs. CD8 NK $P = 0.0431$; CD8 vs. CD8 NK $P = 0.0006$, Bonferroni test) (Fig. 2). Supplementary Table S3 shows the total number of evaluated cells, the % of apoptotic cells induced by 8 Gy, and the IA ratio per each cell type and patient.

The GzLMs analysis for repeated measures including the Arg72Pro *TP53* genotype factor showed, although marginally non-significant and irrespectively of cell type, that the IA ratio in patients with late toxicity was lower than that of patients without late toxicity only in patients with Arg72Arg genotype (six control patients vs. seven patients suffering late toxicity, $P = 0.0776$; Bonferroni test). No such difference was present in carriers of at least one Pro allele (four control patients vs. three patients with late toxicity, $P = 0.8266$; Bonferroni test) (Fig. 3). Permutation analysis confirmed that the all above obtained P -values were robust (Supplementary Table S4).

The ROC curve analyses of IA ratios of CD3, CD4, CD8, and CD8 NK showed that the area under the curve was greater for CD3 (0.91; 95 % CI, 0.683–0.988) than for CD4 (0.80; 95 % CI, 0.563–0.943), CD8 (0.77; 95 % CI, 0.509–0.913) and CD8 NK (0.47; 95 % CI, 0.231–0.685). The ratio ≥ 6.46 of CD3 cells had the highest sensitivity (100 %) and specificity (80 %) as a cut-off value. Therefore, the 100 % of patients without late radiotherapy toxicity exhibited CD3 IA ratio values ≥ 6.46 , while the 80 % of patients with late lesions had values lower than 6.46 (Fig. 4).

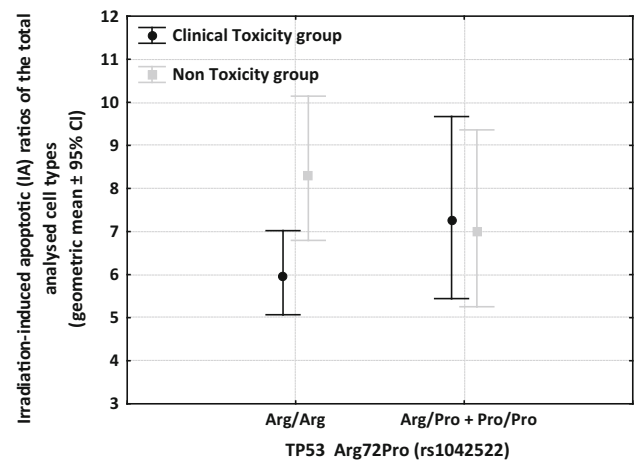


Fig. 3 Irradiation-induced apoptotic (IA) ratios of the all analysed cell types (geometric mean \pm 95 % CI) compared between 72Arg and 72Pro *TP53* allele carriers with or without clinical toxicity. IA in patients with toxicity is lower than the control patients only in *TP53* Arg/Arg patients ($P = 0.077$). This difference was not present in patients carrying at least one Pro allele ($P = 0.8266$)

Discussion

In our study, the BC patients suffering from severe late complications due to radiotherapy exhibited lower levels of IA in total T-lymphocyte (CD3) compared to patients without complications. A similar trend was obtained from CD4 and CD8 T-lymphocytes, although statistically not significant. Regarding the prediction of patients at risk for late complications after radiotherapy, ROC analysis showed that a cut-off value of 6.46 of CD3 IA ratio correctly classifies 90 % of the patients. These results together with others previously reported [7–10, 15–17, 24] seem to support that IA determined in blood lymphocytes can be a reliable method to predict late tissue toxicity due to radiotherapy. However, in our study two patients (SP-3 and SP-15) with late skin lesions displayed IA higher than 6.46 (Fig. 4). Tumour staging of both patients are similar to those of the other patients (Supplementary Table S1). The SP-15 patient, that presented bilateral BC and telangiectasia and fibrosis grade 3 in both events (Table 1), exhibited a high level of IA (9.76) (Fig. 4). Moreover, no pathogenic *BRCA1/BRCA2* variant was identified in the SP-15 patient (Supplementary Table S1). The severe sensitivity to radiotherapy in this patient could be due to other mechanisms than those leading to a low IA. Larger groups of patients, including other tumour sites, are needed to study these IA differences.

Some other studies in cancer patients reported no correlation between individual response to radiotherapy and IA. Table 3 shows the studies published to date and the differences in experimental conditions that could in part, explain these contradictory results. Thus, there was no

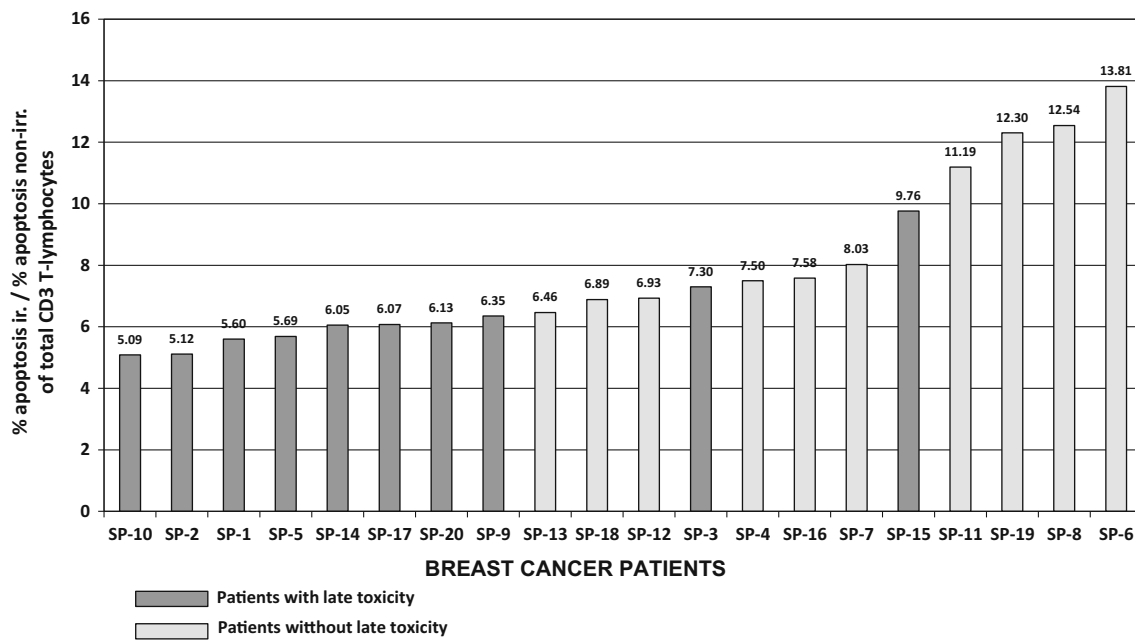


Fig. 4 Values of irradiation-induced apoptosis (IA) ratios of CD3 T-lymphocytes for each analysed patient. The 100 % of patients without late radiotherapy toxicity exhibited CD3 IA ratios ≥ 6.46 , while the 80 % of patients with late lesions had values lower than 6.46

relation between low apoptosis and clinical toxicity when lymphoblastoid cell lines (LCLs) or frozen lymphocytes rather than fresh ones were used [18–21], when the cells were irradiated with doses lower than 8 Gy [18–24] and, when the TUNEL assay was employed to analyze apoptosis [21, 22] (Table 3).

The mechanism responsible for low apoptotic response in lymphocytes of cancer patients with late side effects remains unclear. Lymphocytes from individuals with radiation hypersensitivity diseases caused by germ-line mutations in genes implied in DNA repair as ataxia telangiectasia and Nijmegen breakage syndrome also display a reduced apoptotic induction after in vitro irradiation [7, 15, 22, 25]. An inverse association between initial number of DNA double-strand breaks (DSBs) induced by in vitro irradiation and the IA has also been described in patients with locally advanced BC [26, 27]. Therefore, there is a potential link between cellular response to DNA DSBs and decreased IA [27]. Likewise, Henríquez-Hernández et al. [28] compared in a group of 12 locally advanced BC patients the significantly regulated pathways and biological process of differentially expressed genes and found common routes between clinical toxicity and DNA DSBs, clinical toxicity and apoptosis, and DNA DSBs and apoptosis. Thus, initial DNA damage, IA and clinical toxicity seem to share a common genetic background [28].

On the other hand, Crompton et al. [15], showed a decreased IA of CD4 and CD8 T-lymphocytes in two individuals with germ-line heterozygous mutation in the

NF1 gene that causes the Neurofibromatosis I Syndrome (NF1). The loss of the *NF1* gene product, neurofibromin, results in the presence of activated RAS, which in turn inhibits apoptosis and stimulates cell proliferation [29]. Further, it has been described that individuals with NF1 are at increased risk of developing secondary malignant neoplasms after radiotherapy [29]. In addition, a recent study demonstrates that *Nf1* \pm mutant mice develop more malignancies after irradiation than matched wild type mice [30]. All these findings support that the decreased apoptosis after in vitro irradiation found in patients with late complications induced by radiotherapy could be attributed to impaired response mechanisms to radiation cell exposure such as DNA DSBs repair and RAS signal transduction pathway.

Given that a subset of NK cells express CD8 on their surface, the analysis with only anti-CD8 mixes up two distantly related lymphoid lineages [31, 32]. We thus chose double surface immunostaining using the combination of anti-CD3 and anti-CD8 for distinguishing T and NK cells expressing CD8 on their surface. Using this approach we found two clear different responses to in vitro irradiation: the CD8 T-lymphocytes showed the greatest sensitivity to radiation while the CD8 NK cells subset were the most radio-resistant cells (Fig. 2). These data concur with previous reports showing that the total NK cells (without differentiating the subset expressing CD8) were the most radio-resistant cells [33–35] and that CD8⁺ lymphocytes were more sensitive to irradiation than CD4⁺ cells [8, 15,

Table 3 Reports published to date of association's studies between radiation-induced apoptosis (IA) and clinical toxicity induced by radiotherapy

Reference	Study type	Patients	Toxicity	Sample	In vitro irradiation	Incubation at 37 °C before/after irradiation	Apoptosis	Used method	Cell type	Endpoint	Result
Crompton et al. [7]	R	105 healthy donors; 48 cancer patients with normal response to radiotherapy; 12 hypersensitive cancer patients to radiotherapy; 9 AT patients	Enhanced early and late toxicity	Fresh heparinised blood	2 and 9 Gy X-rays; 3.4 Gy/min	–/48 h	Late	FLC + PI	CD8 and CD4 lymphocytes	^a Background-adjusted IA, CD4 and CD8 integrated z-scores	Reduced IA in hypersensitive and AT patients
Barber et al. [22]	^b R	13 healthy donors; 5 AT homozygotes; 9 AT Het; 31 BC patients	Late	PBMC	4 Gy 137Cs gamma rays; 3.15 Gy/min	16 h/24 h	Late	TUNEL Assay + FLC	Total PBMC	Background-adjusted IA	No correlation between IA and any clinical toxicity parameter assessed. Reduced IA in AT, AT Het and BC patients
Crompton et al. [15]	R	105 healthy donors; 48 cancer patients without side effects; 12 hypersensitive cancer patients to radiotherapy; 12 AT patients; 1 ICF; 2 neurofibromatosis; 1 NBS donor	Enhanced early and late toxicity	Fresh heparinised blood	2 and 9 Gy X-rays; 3.4 Gy/min	–/48 h	Late	FLC + PI	CD8 and CD4 lymphocytes	Background-adjusted IA, CD4 and CD8 z-scores	Reduced IA in hypersensitive, AT, 1 NBS, 2 NF1, and 1 ICF patients
Ozsohlin et al. [8]	P	393 cancer patients: 38 % BC; 19 % head and neck; 11 % genitourinary; 10 % gastrointestinal; 22 % other locations	Early and late	Fresh heparinised blood	8 Gy X-rays; 3.4 Gy/min	–/48 h	Late	FLC + PI	CD8 and CD4 lymphocytes	Background-adjusted IA	Reduced IA predicted late toxicity grade 2 and 3. No association with early side effects
Wistop et al. [21]	R	LCLs: 5 healthy donors; 3 AT; 1 AT het, 2 NBS, 1 NBS rectal cancer patients with late effects grade 3–4; Blood: 21 healthy, 18 cancer patients with different primary tumors; 7 with late effects grade 3, 11 without toxicity	Late	LCLs and PBMC	2 Gy; 5 Gy; X-rays; 2.2 Gy/min	–/48 h	Late	TUNEL Assay + fluorescence microscope	LCLs and CD3 T-Lymphocytes Isolated by magnetically labelled antibodies	Background-adjusted IA	Reduced IA in 2 out of 3 AT LCLs and in 2 out of 5 cancer patients LCLs with ≥ 3 grade lymphocytes: 3 out of 7 patients with ≥ 3 grade toxicity with reduced IA

Table 3 continued

Reference	Study type	Patients	Toxicity	Sample	In vitro irradiation	Incubation at 37 °C before/after irradiation	Apoptosis	Used method	Cell type	Endpoint	Result
Azria et al. [16]	^c R	Group A: 9 BC and 6 HNC with grade 3 subcutaneous fibrosis, 1 meningioma with severe intracerebral edema. Group B: 10 BC, 7 HNC and 1 meningioma without long term side effects after radiotherapy	Late	Fresh heparinised blood	8 Gy X-rays; 3.4 Gy/min	–/48 h	Late	FLC + PI	CD8 lymphocytes	Background-adjusted IA	Reduced IA in late toxicity group. 94 % of these patients carried 4 or more SNPs associated with clinical radiosensitivity
Schnarr et al. [24]	^d R	45 prostate cancer patients	Late	PBMC	2, 4, 8 Gy; 137Cs; 0.1 Gy/min	–/44 h	Early	Annexin V-FITC and DIOC ₆ + FLC	Total, CD4 ⁺ , CD8 ⁺ , and CD4 ⁺ CD8 [–] lymphocytes	Background-adjusted IA	Reduced IA at 8 Gy in total and CD4 ⁺ CD8 [–] lymphocytes. Weaker correlation with late toxicity at lower dose points
Bordon et al. [9]	P	94 cervical cancer patients, 4 healthy donors	Late	PBMC	1, 2, 8 Gy; X rays; 50 cGy/min	–/24 h, 48 h and 72 h	Early and late	Annexin V, PI + FLC	CD45 lymphocytes	Background-adjusted IA, β constant values of integrated late and acute apoptosis	Reduced IA associated with late toxicity
Greve et al. [19]	^e R	10 BC patients and 5 HNC with acute radiation; 10 BC patients and 5 head and neck carcinomas non-sensitive patients as controls	Acute and late	PBMC in nitrogen liquid. LCLs from each patient	0.4 Gy and 0.8 Gy; 60 Co or 137Cs; 0.6–2.0 Gy/min	Upon thawing: lymphocytes 24 h/48 h; LCLs 8 days/48 h	Early and late	Annexin V kit, FLC + PI	CD3 T-lymphocytes; CD19 B-lymphocytes; LCLs	Irradiated-induced cell death (ICD) : $CD_{ind} = \frac{(CD_{8Gy} - CD_{0Gy})}{(100\% - CD_{0Gy})} \times 100\%$	No differences of ICD between radiosensitive and non-radiosensitive patients
Bordon et al. [10]	P	79 HNC patients	Late	PBMC	1, 2, 8 Gy X rays; 50 cGy/min	–/24 h	Early and late	Annexin V, PI + FLC	CD45 lymphocytes	Background-adjusted IA, β constant values of integrated late and acute apoptosis	Reduced IA associated with late toxicity
Bordon et al. [33]	P	94 cervical cancer patients, 4 healthy donors	Late	PBMC	1, 2, 8 Gy; X rays; 50 cGy/min	–/24 h, 48 h and 72 h	Early and late	Annexin V, PI + FLC	CD45 lymphocytes; CD4 (CD3 ⁺ CD4 ⁺); CD8 (CD3 ⁺ CD8 ⁺); NK (CD3 [–] CD16 ⁺); CD56 ⁺ ; B (CD20 ⁺)	Background-adjusted IA, β constant values of integrated late and acute apoptosis	Reduced IA of CD8 and B-lymphocytes associated with late toxicity

Table 3 continued

Reference	Study type	Patients	Toxicity	Sample	In vitro irradiation	Incubation at 37 °C before/after irradiation	Apoptosis	Used method	Cell type	Endpoint	Result
Hernandez et al. [26]	P	26 patients with locally advanced/inflammatory breast cancer	Late	PBMC	1, 2, 8 Gy; X rays; 50 cGy/min	–/24 h	Early and late	Annexin V, PI + FLC	CD45 lymphocytes	Background-adjusted IA, β constant values of integrated late and acute apoptosis	Those patients who presented lower levels of initial DNA damage and higher levels of IA were at low risk of suffering severe subcutaneous late toxicity
Brzozowska et al. [23]	R	25 prostate cancer patients with severe side effects after radiotherapy and 25 prostate cancer patients without side effects. 23 age-matched male healthy donors	Acute and late	PBMC	1 Gy gamma rays; 0.74 Gy/min	Overnight/0.5, 5 and 24 h	Early and late	Annexin V, PI + FLC	Total lymphocytes	No clearly stated	No significant differences of early nor late IA between patients with and without clinical side effects
Greve et al. [20]	^e R	10 BC patients and 5 head and neck carcinomas with acute radiation; 10 BC patients and 5 HNC non-sensitive patients as controls	Acute and late	PBMC in liquid. LCLs from each patient	0.4 and 0.8 Gy; 60Co or 137Cs; 0.6–2.0 Gy/min	Upon thawing: lymphocytes 24 h/48 h; LCLs 8 days/48 h	Early and late	Annexin V kit, FLC + PI	CD3 T-lymphocytes; CD19 B-lymphocytes; LCLs	ICD values Analysis combining the ICD, DSB induced and repair endpoints	No differences of combined ICD/DSB/repair endpoints between radiosensitive and non-radiosensitive patients
Fimmon et al. [18]	^f R	31 cases of BC with long-term adverse effects and 28 matched BC controls without adverse effects	Late	PBMC stored in liquid nitrogen	4 Gy X-rays	–/48 h	Late	FLC + PI	CD4 and CD8 lymphocytes	Total IA without background adjusts	No differences between cases and controls
Foro et al. [17]	P	198 prostate cancer patients	Late	Fresh blood and PBMC after in vitro irradiation	8 Gy X-rays	–/48 h	Early	Annexin V binding FLC + PI	CD4 and CD8 lymphocytes	Background-adjusted IA	Reduced IA of CD4 T-lymphocytes associated with late genitourinary toxicity

Table 3 continued

Reference	Study type	Patients	Toxicity	Sample	In vitro irradiation	Incubation at 37 °C before/after irradiation	Apoptosis	Used method	Cell type	Endpoint	Result
This present study	R	10 BC patients with fibrosis or/and telangiectasia and 10 BC patients without late effects	Late	Fresh heparinised blood	8 Gy X-rays; 3 Gy/min	–/48 h	Late	FLC + PI	CD3 T-lymphocytes (CD3 ⁺); CD8 T-lymphocytes (CD3 ⁺ CD8 ⁺); CD4 T-lymphocytes (CD3 ⁺ CD8 ⁻) and; CD8 NK lymphocytes (CD3 ⁻ CD8 ⁺)	% of apoptotic cells induced by 8 Gy divided by the basal % of apoptotic cells obtained in the unirradiated samples	Reduced IA of total CD3 T-lymphocytes in cases with late lesions compared with controls without lesions

R retrospective, P prospective, AT Ataxia telangiectasia, AT Het ATM mutation carriers in heterozygous, NBS Nijmegen breakage syndrome, NBS het NBS mutation carriers in heterozygous, NF1 neurofibromatosis I Syndrome, BC breast cancer, HNC head and neck cancer, PBMC peripheral blood mononuclear cells isolated from heparinised blood by density gradient centrifugation, LCLs lymphoblastoid cell lines, FLC flow cytometry, PI propidium iodide, IA irradiation-induced apoptosis, ICD irradiated-induced cell death, DSB DNA strand break

- Percentage of apoptotic cells induced by irradiation minus percentage of spontaneous apoptotic cells
- Selected from 300 women followed prospectively
- Patients with or without clinical toxicity specifically selected from a previous prospective analysis
- Invited to participate from a previous prospective analysis
- Selected from a previous prospective study for acute reactions
- Selected from a randomised trial

24, 33]. In contrast, Vokurkova et al. [32] analyzing the proportion of live intact cells, described that the CD3⁻CD8⁺ NK subset was more radiosensitive than CD4 and CD8 T-lymphocytes.

In addition, in our study the subset of CD8 NK cells showed the highest level of spontaneous apoptosis compared to the remaining cell types (Fig. 1), while the IA ratios of this type of NK cells in toxicity and non-toxicity patients were similar (Fig. 2). According to our results, and in order to avoid a source of variation, it would be advisable to perform the IA assay in CD8 cells with the combination of anti-CD3 and anti-CD8 to distinguish the CD8 NK subset from the pool of CD8⁺ lymphocytes.

The *TP53* Arg72Pro polymorphism encodes two p53 variants that significantly affect the biological activity of p53 [12, 13]. The apoptotic potential of the different forms of the protein depends on the cell type and cellular environment [36]. The current consensus is that p53-Arg72 is more effective at inducing apoptosis and protecting stressed cells from neoplastic development than p53-Pro72 [12]. However, it is not yet understood how universal these functional differences between p53-Pro72 and p53-Arg72 might be in different cell types or whether they are relevant in vivo [12]. In particular, the apoptotic activity of the codon 72 variants in human lymphocytes irradiated in vitro is not known.

Although slightly non-significant, the IA ratio of the total analysed cells in patients with late toxicity was lower than that of patients without late toxicity only in patients with Arg72Arg genotype. This difference was not present in patients carrying at least one Pro allele (Fig. 3). Our finding, even though based on a low number of individuals, suggests that the Pro allele may influence the lymphocyte apoptotic response to in vitro irradiation in patients suffering from late toxicity after radiotherapy.

In summary, our results show that radiotherapy-induced late toxicity is associated to low levels of IA. Given that the response to irradiation of CD8 NK cells is different from that of the T-lymphocytes, it would be advisable to distinguish the CD8 NK lymphocytes from the pool of CD8⁺ lymphocytes in IA assays using CD8⁺ cells. Because of the potential functional role of the *TP53* Arg72Pro polymorphism on apoptosis, larger and prospective studies are needed to validate the influence of 72Pro *TP53* allele on IA.

Acknowledgments This study was supported by the “Fondo de Investigación Sanitaria” [FIS 05/2181] from the Spanish Health Ministry and the “Fundación de Investigación Médica Mutua Madrileña” [FMMA 2008–2012] grants. Dr Sara Gutiérrez-Enríquez is currently funded by a Miguel Servet contract awarded by the Instituto de Salud Carlos III of the “Ministerio Español de Economía y Competitividad”. We are grateful to the BC donor patients involved in this study.

Conflict of interest The authors declare that they have no conflict of interest.

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