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Normal tissue radiosensitivity

Correlation between DNA damage responses of skin to a test dose of radiation and late adverse effects of earlier breast radiotherapy

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ABSTRACT

Aim: To correlate residual double strand breaks (DSB) 24 h after 4 Gy test doses to skin *in vivo* and to lymphocytes *in vitro* with adverse effects of earlier breast radiotherapy (RT).

Patients and methods: Patients given whole breast RT $\geqslant$5 years earlier were identified on the basis of moderate/marked or minimal/no adverse effects despite the absence ('RT-Sensitive', RT-S) or presence ('RT-Resistant', RT-R) of variables predisposing to late adverse effects. Residual DSB were quantified in skin 24 h after a 4 Gy test dose in 20 RT-S and 15 RT-R patients. Residual DSB were quantified in lymphocytes irradiated with 4 Gy *in vitro* in 30/35 patients.

Results: Mean foci per dermal fibroblast were 3.29 (RT-S) vs 2.80 (RT-R) ($p = 0.137$); 3.28 (RT-S) vs 2.60 (RT-R) in endothelium ($p = 0.158$); 2.50 (RT-S) vs 2.41 (RT-R) in suprabasal keratinocytes ($p = 0.633$); 2.70 (RT-S) vs 2.35 (RT-R) in basal epidermis ($p = 0.419$); 12.1 (RT-S) vs 10.3 (RT-R) in lymphocytes ($p = 0.0052$).

Conclusions: Residual DSB in skin following a 4 Gy dose were not significantly associated with risk of late adverse effects of breast radiotherapy, although exploratory analyses suggested an association in severely affected individuals. By contrast, a significant association was detected based on the *in vitro* response of lymphocytes.

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Processes determining cellular radiosensitivity include the recognition and repair of DNA double strand breaks (DSB), inherited defects which account for clinical radiosensitivity in rare individuals [1]. Contrary to the early small case–control clinical studies that suggested a correlation between *in vitro* cellular radiosensitivity and late normal tissue damage after radiotherapy, later studies seeking to test the DNA damage response as a predictor of normal tissue radiosensitivity have been inconclusive [2–6]. A relevant criticism of all these studies has been that *in vitro* cellular responses may correlate poorly with *in vivo* cellular responses due to the modifying influence of tissue environment [7,8]. The reported lack of correlation between the severity of fibrosis and telangiectasia risks after post-mastectomy radiotherapy also suggests that predictive cellular assays need to be endpoint-specific [9]. In the case of subcutaneous fibrosis and cutaneous telangiectasia, for example, this implies the need to measure cellular responses in dermal fibroblasts and dermal endothelial cells, respectively. Given that much of the DNA damage response is common to all cell types, lymphocytes justify investigation by virtue of easy availability and the results of earlier studies [10–12]. Here, we report on residual DSB in different skin cell types 24 h after an *in vivo* test dose of 4 Gy in a group of 35 individuals selected on the basis of their radiation-induced adverse effects following breast radiotherapy. We also report on residual DSB in *in vitro* irradiated G_0 blood lymphocytes from 30/35 individuals, including 16 previously published [13,14].

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Materials and methods

Selection of clinically radiosensitive (RT-S) and radioresistant (RT-R) individuals

All participants were former breast cancer patients enrolled in two breast radiotherapy trials; the START pilot trial ($N = 1410$) and the Royal Marsden breast dosimetry trial ($N = 306$). Both trials delivered treatment according to a predefined protocol and included prospective annual clinical assessments of late adverse effects [15,16]. In addition to clinical assessments, late adverse effects were evaluated using pre- and post-treatment photographs of both breasts collected under predefined conditions at 0, 1, 2, and 5 years [15,17]. Multivariate analysis identified and ranked factors associated with photographic change in breast appearance, including prescribed whole breast radiotherapy dose, radiation dosimetry, radiotherapy boost to tumour bed, breast size, proportion of breast removed at surgery, and axillary treatment, see Table 1. These parameters were used to identify patients with marked adverse effects despite favourable parameters ('radiosensitive' (RT-S)) and unmatched patients ('radioresistant', (RT-R)) with no changes despite unfavourable parameters. This approach attempted to generate maximum separation in terms of intrinsic factors predisposing to the presence or absence of late adverse effects, chiefly breast shrinkage. After identifying potentially eligible individuals according to the above criteria, a final selection was made by two clinicians (JY and NS) to exclude individuals in whom factors omitted by the algorithm were considered to strongly influence clinical response. The commonest reason was breast shrinkage and/or distortion in a patient with an inferior quadrant tumour, where irregular breast contour after wide surgical resection followed by 2D radiation dosimetry was considered sufficient explanation for late changes unaccounted for by multivariate analysis. Ethical approval was obtained from the Royal Marsden Research Ethics Committee, and written consent was obtained from patients prior to participation.

Skin irradiation, 53BP1 immunohistochemistry, and foci analyses of skin sections

A test dose of 4 Gy was delivered to an area of buttock skin measuring 2×4 cm using 6 MeV electrons via a purpose-built end-frame, ensuring dose homogeneity to the epidermis and dermis as described previously [18]. Paired 4 mm biopsies were collected from the centre of the irradiated area 24 h post-irradiation and from unirradiated skin on the opposite buttock.

Table 1
Risk factors for late adverse effects following breast radiotherapy established by multivariate analyses of outcomes of two breast radiotherapy trials [15,16]. Odds ratios are presented relative to favourable factors: lower radiotherapy dose, 3D dosimetry, no boost dose, small breast size, minimal surgical cavity and no axillary radiotherapy.

Clinical parameters	Odds ratio for late RT-induced effects (95% CI)
RT dose (39, 42.9 or 50 Gy)[1]	1.09 (1.01–1.17), $p = 0.02$
Radiation dosimetry (3D dosimetry vs standard 2D wedge)	1.71 (1.15–2.54), $p = 0.008$
Boost dose to tumour bed (none, 11.1 or 15.5 Gy)[2]	1.03 (1.02–1.05), $p < 0.001$
Surgical deficit (small, medium, large)	Medium = 2.00 (1.23–3.25), large = 1.38 (0.57–3.37), $p = 0.009$
Axillary treatment (none, surgery, RT)	Surgery = 1.38 (0.72–2.63), RT = 2.49 (1.20–5.18), $p = 0.05$

[1] Allowing for differences in fraction size, assuming $\alpha/\beta = 3$ Gy.
[2] Prescribed to 100% in 5 or 7 fractions (2.0 Gy to 90%), mostly commonly electrons.

Table 2
Characteristics and treatment parameters of 'radiosensitive' (RT-S) and 'radioresistant' (RT-R) patients.

	RT-S	RT-R
Patients ($n = 35$)	20	15
Median age, years (range)	70 (52–83)	68 (54–78)
Median follow-up, years (range)	11 (3–24)	13 (11–24)
Mean breast RT dose, Gy[1]	50.0	50.8
Dosimetry techniques		
3D	10	3
2D	10	12
Number patients prescribed boost dose	15	15
Mean tumour bed boost dose, Gy	9.8	12.7
Breast size		
Small	8	2
Medium	10	13
Large	2	0
Surgical deficit		
Small	8	11
Medium	8	3
Large	4 (1 mastectomy[2])	1
Axillary treatment	15	11
Tamoxifen	14	12
Chemotherapy	8	15

[1] Equivalent total dose assuming $\alpha/\beta = 3$ Gy.
[2] Patient had mastectomy & reconstruction before RT; this was the only patient with <5 yr follow up.

53BP1 foci were scored in dermal fibroblasts, dermal endothelial cells, suprabasal keratinocytes and basal keratinocytes, with 50–100 cells scored for each cell type per biopsy of each patient. Details of tissue processing, 53BP1 immunostaining and foci analyses have been described previously [18]. Residual DSB were corrected for DSB in unirradiated control skin biopsied at the same time.

Dermal fibroblast cultures and in vitro irradiation

Patients were invited to donate a second set of paired skin biopsies from irradiated and unirradiated skin 12 weeks after the test dose. Fibroblast cultures were established from unirradiated skin for studies of *in vitro* sensitivity, as described [19].

Peripheral blood separation, G_0 blood lymphocyte irradiation and residual DSB foci

Thirty patients from the same cohort consented to peripheral blood sampling. As described in an earlier publication, G_0 blood lymphocytes were isolated from whole blood and irradiated to 4 Gy using 250 kV X-rays delivered at 0.69 Gy/min (Pantak, Surrey, UK) [13,14]. DSB in irradiated blood lymphocytes were quantified using γH2AX and 53BP1 immunostaining and co-localising γH2AX and 53BP1 foci were scored 24 h after irradiation [13,14].

Statistical methods

The study was designed to recruit 15 RT-S and 15 RT-R patients with the aim of detecting a standardised difference of 1.2 with 85% power (5% two-sided significance level). Comparative analyses of clinical parameters and foci levels between RT-S and RT-R patients were performed using the Mann–Whitney U test. Spearman's rank correlation test was used to test for correlation of residual foci levels in the different cell types and clinical severity of late effects among RT-S patients. However, as these tests of association were secondary analyses undertaken on an exploratory basis, conservative p-values ($p < 0.01$) were employed in their interpretation. Statistical calculations were performed using SPSS version 21.0.

Results

Patients

Of 35 breast radiotherapy patients who consented for the study, 20 and 15 were recruited as RT-S and RT-R patients, respectively. More potential RT-S candidates were available and offered consent, resulting in 5 additional patients in this group. Patient characteristics and treatment-related parameters are summarised in Table 2.

Correlation between residual DSB foci in different skin cell types after a 4 Gy test dose in vivo and to blood lymphocytes and dermal fibroblasts irradiated in vitro

Residual DSB counted in dermal fibroblasts, endothelial cells, basal keratinocytes and suprabasal keratinocytes in skin sections collected 24 h after the 4 Gy *in vivo* test dose, corrected for DSB in unirradiated control skin biopsied at the same time, are shown in Supplementary Table S1, which also includes corrected foci counts for *in vitro* irradiated dermal fibroblasts and blood G_0 lymphocytes. Patients (first column) are numbered according to increasing levels of residual DSB in dermal fibroblasts irradiated *in vivo* (second column). The colour washes identify DSB grouped in quartiles (9 + 9 + 8 + 9 = 35), red identifying the quartile with the highest DSB for each cell type. Correlation between different cell types is shown in Supplementary Table S2, suggesting a significant correlation restricted to *in vivo* irradiated dermal fibroblasts, endothelial cells and suprabasal keratinocytes.

Residual DSB foci levels in irradiated skin and G_0 lymphocytes in RT-S and RT-R patients

Patient-averaged residual DSB 24 h after 4 Gy to skin and measured in epidermal and dermal skin cells did not differ significantly between RT-S and RT-R patients, see Fig. 1. Mean foci per cell were 3.29 (RT-S) and 2.80 (RT-R) for dermal fibroblasts ($p = 0.137$), 3.28 (RT-S) and 2.60 (RT-R) for endothelial cells ($p = 0.158$), 2.50 (RT-S) and 2.41 (RT-R) for suprabasal keratinocytes ($p = 0.633$), and 2.70 (RT-S) and 2.35 (RT-R) for basal keratinocytes ($p = 0.419$). Foci

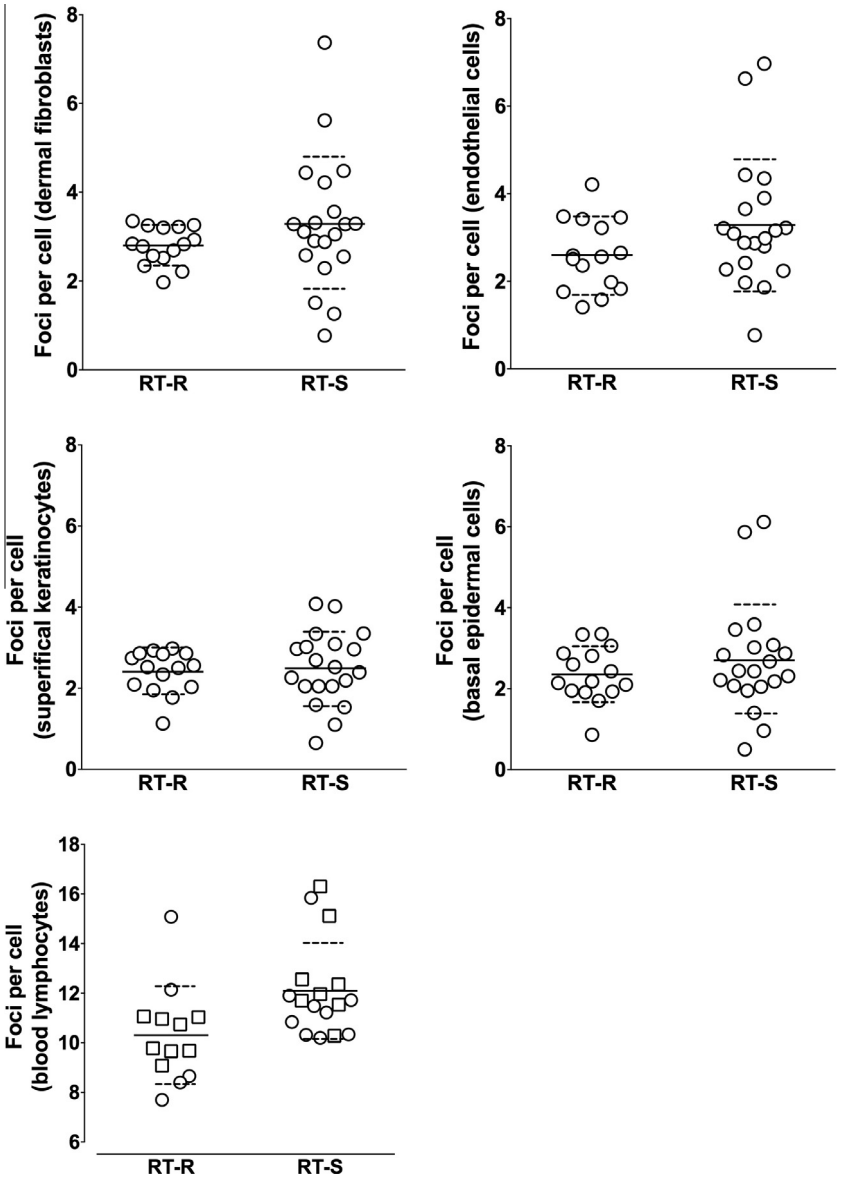

Fig. 1. Individual DSB foci levels scored in irradiated skin of RT-S and RT-R patients 24 h after 4 Gy *in vivo*. Horizontal lines represent patient-averaged residual foci levels with dashed lines representing one standard deviation. The squared symbols in fifth panel represent previously published data [13,14].

levels fell almost to baseline levels in 12-week biopsies with no significant difference between RT-S and RT-R patients (data not shown).

We previously reported that DSB foci levels of G_0 blood lymphocytes 24 h after 4 Gy X-rays *ex vivo* were higher in 8 RT-S compared to 8 RT-R patients from the current cohort ($p = 0.01$), selected on the basis that these 16/35 individuals appeared to represent the two extremes of the response distribution [13,14]. We therefore proceeded to test if foci levels in lymphocytes correlated with severity of late radiation-induced skin changes in all 30 patients offering blood samples. The association between residual DSB in lymphocytes and adverse effects was significant, based on mean foci levels of 12.1 (RT-S) and 10.3 (RT-R) ($p = 0.0052$),

Exploratory analyses of irradiated skin and G_0 lymphocytes in RT-S1, RT-S2 and RT-R patients

Although patient-averaged residual foci levels in *in vivo* irradiated skin cells were not significantly different between 'sensitive' and 'resistant' groups, the variance was much larger among RT-S than RT-R individuals, as seen in Fig. 1. This stimulated an exploratory analysis of residual foci levels according to the severity of late radiation-induced skin changes in the 20 RT-S patients. Two equal-sized subgroups, RT-S1 (less severe) and RT-S2 (more severe), were selected by JY and NS blind to the residual DSB data. Illustrative examples of patients classified under the respective groups are shown in Fig. 2. The levels of association between residual foci levels in the different skin cells and clinical severity in RT-S1 and RT-S2 patients are shown in Fig. 3. Residual foci levels in dermal fibroblasts were correlated with change in breast appearance in RT-R and RT-S1 and RT-S2 patients (Spearman's $R^2 = 0.248$, $p = 0.002$). There was a trend for association between residual foci levels in endothelial cells and clinical severity for the same groups, but this was not statistically significant (Spearman's $R^2 = 0.158$, $p = 0.018$). No association was observed between residual foci levels in keratinocytes and adverse effects (suprabasal keratinocytes, Spearman's $R^2 = 0.028$, $p = 0.334$; basal keratinocytes, Spearman's $R^2 = 0.019$, $p = 0.433$).

As for dermal fibroblasts, a positive patient-specific association was observed between residual foci levels 24 h after 4 Gy X-irradiation in blood lymphocytes and severity of adverse effects in RT-R, RT-S1 and RT-S2 patients, see Fig. 3 (Spearman's $R^2 = 0.365$, $p < 0.001$).

Discussion

Mean levels of residual DSB foci in dermal fibroblasts measured 24 h after a 4 Gy *in vivo* test dose to buttock skin did not differ significantly between RT-S and RT-R groups identified on the basis of normal tissue effects, mainly breast shrinkage, >5 years after breast radiotherapy. There were interesting trends, though: mean residual foci levels were 3.29 (RT-S) and 2.80 (RT-R) ($p = 0.07$) in dermal fibroblasts, 3.28 (RT-S) and 2.60 (RT-R) ($p = 0.08$) in endothelial cells. Only the association between residual DSB in lymphocytes and adverse effects was significant, based on mean foci levels of 12.1 (RT-S) and 10.3 (RT-R) ($p = 0.0052$), extending the results of an earlier series including 16 of the current cohort [13,14]. Notably, the relative difference between RT-S and RT-R was identical (17–18%) in fibroblasts and lymphocytes, and larger (26%) in endothelial cells, supporting the notion that the differences may be real.

The main strength of the investigation lies in controlling for potential effects of tissue microenvironment on cell responses, measured using residual DSB as a surrogate endpoint that was relevant to cell fate and late onset deterministic effects of treatment. As such, it is the only clinical study known to us that has attempted

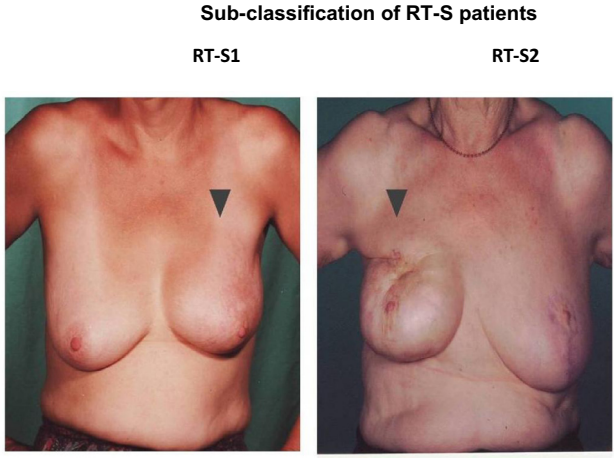

Fig. 2. Post-surgical photographs of two equal-sized ($n = 10$) exploratory RT-S subgroups with moderate (left) and marked (right) degree of late effects in the irradiated breast (grey arrow) at 5 years post-radiotherapy. Consent was obtained from patients for the use of these photographs.

this approach. A second strength is recruitment of patients under prospective follow up according to standardised criteria that allowed change in photographic breast appearance to be used as an endpoint of proven sensitivity to small randomised differences in dose [15,20]. In principle, the attempt to identify subgroups of individuals whose change in breast appearance was unexpectedly marked (RT-S) or unexpectedly mild/absent (RT-R) was a further strength in that it avoided the need to identify matched controls and selected individuals from opposite ends of the population sensitivity distribution. In practice, however, patient selection proved very difficult, as noted from the patient characteristics in Table 2, for reasons including the relative crudeness of the algorithm, as well as more practical issues related to consent. At a time when RT-S patients were particularly difficult to recruit, one exceptional patient was entered with a very marked radiation phenotype only 3 years after post-mastectomy latissimus dorsi flap reconstruction and radiotherapy; but all other participants were trial patients as planned. The marked differences in phenotype between RT-S patients, as well as marked inter-patient variation in residual DSB in this population, prompted an exploratory subgroup analysis that suggests an association between residual DSB in dermal fibroblasts irradiated *in vivo* and late adverse effects (Spearman's $R = 0.498$, $p = 0.002$) in addition to the association found for lymphocytes. We consider that the RT-S1 subgroup represents the 1–5% most responsive, i.e. dose-limiting, patients, while the RT-S2 subgroup includes 6/10 patients whom we consider represent <1% of the population of radiotherapy patients. We could not attempt any ranking of the RT-R group. It is clear from our results that the reported associations are dependent on the most severely affected individuals, albeit consistent with lymphocyte responses as predictors of clinical risk reported by other colleagues [10–12,21]. However, the findings of our study raise no expectation that we can identify dose-limiting subgroups, a conclusion consistent with Bentzen's predictions almost 20 years ago [22].

A final comment might be made concerning the associations between residual DSB in different skin cell types after *in vivo* or *in vitro* irradiation, which are interesting in themselves, independently of the lack of significant association with clinical outcome. The colour wash in Supplementary Table S1 illustrates this most clearly, suggesting that cell types with the lowest proliferative indices, including dermal fibroblasts, dermal endothelial cells, suprabasal keratinocytes and G_0 blood lymphocytes stand in contrast to basal keratinocytes irradiated *in vivo* and *in vitro* cultured dermal fibroblasts cultured from the same patients.

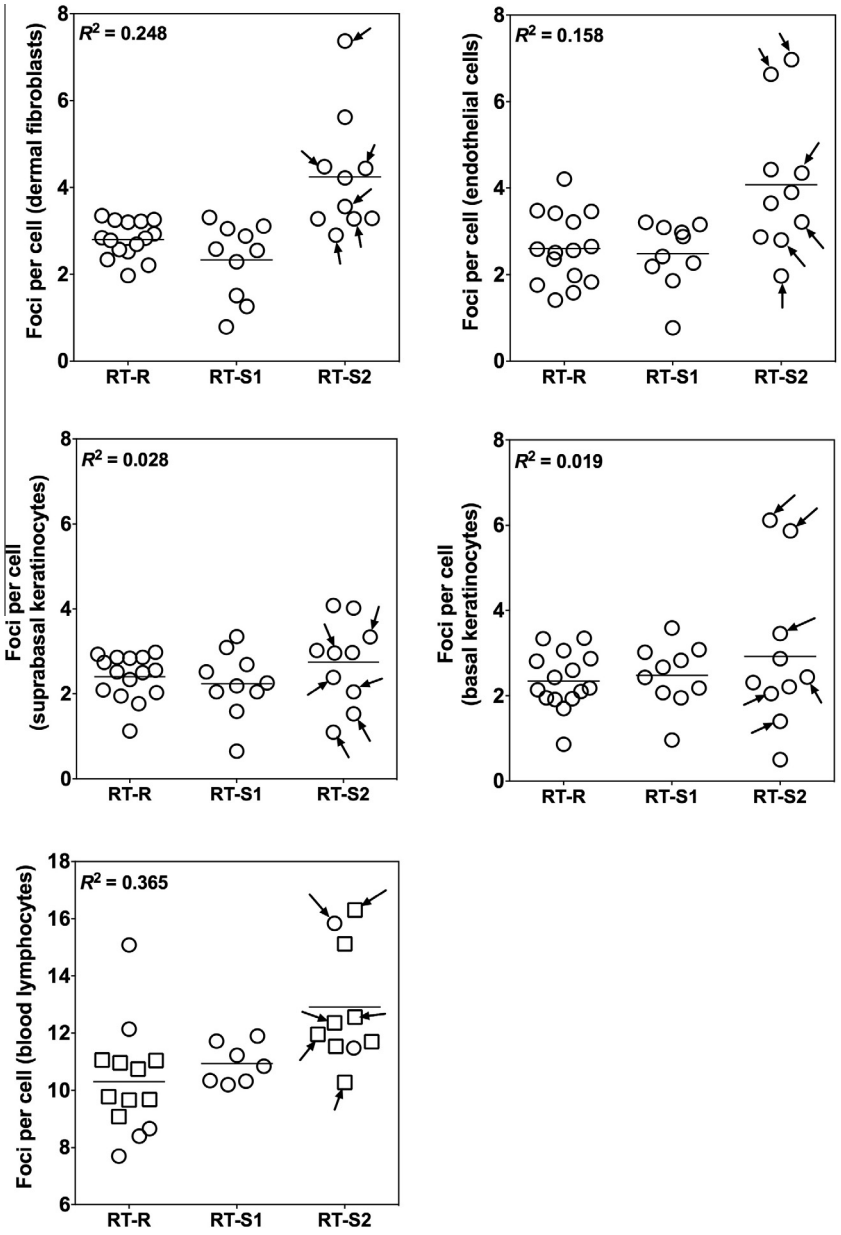

Fig. 3. Individual residual foci levels in the different skin cells for all RT-R and RT-S patients, with the latter divided into 2 equally-sized subgroups (RT-S1 & RT-S2) based on severity of late effects in exploratory analyses. Horizontal lines represent patient-averaged foci levels for all patients classified under the same grade of clinical severity. R^2 values were generated using Spearman's rank correlation test. Squared symbols in fifth panel identify the patients for whom lymphocyte sensitivities have been previously published [13,14]. Six individuals with exceptionally severe effects are marked with arrows.

Conclusions

This study suggests that residual DSB in skin irradiated *in vivo* are weakly associated with late normal tissue response to breast radiotherapy, but that residual DSB of blood G_0 lymphocytes irradiated *in vitro* have a closer relationship with clinical outcome.

Role of funding source

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Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2016.04.012.

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