Correlation between the radiation responses of fibroblasts cultured from individual patients and the risk of late reaction after breast radiotherapy

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Abstract

Late normal tissue toxicity varies widely between patients and limits breast radiotherapy dose. Here we aimed to determine its relationship to DNA damage responses of fibroblast cultures from individual patients. Thirty-five breast cancer patients, with minimal or marked breast changes after breast-conserving therapy consented to receive a 4Gy test irradiation to a small skin field of the left buttock and have punch biopsies taken from irradiated and unirradiated skin. Early-passage fibroblast cultures were established by outgrowth and irradiated *in vitro* with 0 or 4Gy. 53BP1 foci, p53 and p21/CDKN1A were detected by immunofluorescence microscopy. Residual 53BP1 foci counts 24h after *in vitro* irradiation were significantly higher in fibroblasts from RT-sensitive versus RT-resistant patients. Furthermore, significantly larger fractions of p53- but not p21/CDKN1A-positive fibroblasts were found in cultures from RT-sensitive patients without *in vitro* irradiation, and 2h and 6d post-irradiation. Exploratory analysis showed a stronger p53 response 2h after irradiation of fibroblasts established from patients with severe reaction. These results associate the radiation response of fibroblasts with late reaction of the breast after RT and suggest a correlation with severity.

1. Introduction

In women treated with radiotherapy for primary breast cancer after local excision of the primary tumour, breast shrinkage, hardness and pain are common consequences [1-3]. A study of telangiectasia by Turesson [4] showed considerable variation in severity between patients treated under the same conditions. Analysis of these data suggested that if all known extrinsic factors are controlled, those intrinsic to the individual may account for ≥80% of clinical complication risk [5, 6].

Radiation damage to fibroblasts is considered to be a key factor in the pathogenic pathway leading to fibrosis. However, although initial results suggested that intrinsic radiosensitivity of fibroblasts isolated from individual patients may predict the patients' risk of developing fibrosis after radiotherapy [7-10], correlations were weak and subsequent studies did not confirm a significant correlation [10-16]. This has been explained by the hypothesis that *in vitro* cellular responses correlate poorly with *in vivo* responses due to the modifying influence of tissue environment [17-19]. The number of residual radiation-induced DNA double-strand breaks (DSBs) has received less attention. An early study reported a correlation between the severity of fibrosis and the fraction of DNA released by pulse-field gel electrophoresis (PFGE) after irradiation of early-passage fibroblast strains *in vitro* [20], but this was not confirmed in a validation cohort [21] or in a different study using constant-field gel electrophoresis [11]. However, very high doses (up to 150Gy) were used to detect residual DSBs by this technique. Since the latter study found significantly higher numbers of lethal chromosome aberrations in lymphocytes irradiated with a more moderate dose of 6Gy from patients with severe late reaction, there is still a powerful argument for exploring ways of measuring cellular responses to

relevant doses of radiotherapy *ex vivo* using the much more sensitive technique of DNA DSB repair foci.

The purpose of the present study was to explore the relationship between the risk of developing late breast shrinkage/hardening after radiotherapy and the DNA damage response in cultures of dermal fibroblasts from individual patients, established from unirradiated skin and skin given a test irradiation dose *in vivo*..

2. Materials and methods

Patients, *in vivo* irradiation, and establishment of fibroblast cultures

The manner of patient selection had been previously described [22]. Briefly, patients in this study had taken part in two randomised clinical trials organised by the Institute of Cancer Research and The Royal Marsden NHS Foundation Trust, comparing fractionation schemes and irradiation techniques, respectively, with prospective annual clinical assessments of late adverse effects using standard proformas [23, 24]. Thirty-five breast cancer patients who showed no evidence of recurrent cancer 3-10 years after surgical excision of the tumour and post-operative radiotherapy to the whole breast consented to a test irradiation and subsequent biopsies of the skin 24h and 12 weeks after irradiation. Ethical approval for the study was obtained from the Royal Marsden NHS Research Ethics Committee, and written consent was obtained from the patients prior to participation.

The study group comprised of clinically RT-sensitive patients (n=20) and RT-resistant (n=15) patients based on the severity of their reactions versus known clinical risk factors and was enriched for highly sensitive patients. Follow up was 3-24 years for RT-sensitive (median 11 years) and 11-24 years (median 13 years) for RT-resistant patients. Patient characteristics and treatment parameters are shown in Table 1. Overall, RT-sensitive patients had larger surgical deficits but received less tumour-bed boost irradiation and chemotherapy. One RT-sensitive patient withdrew consent prior to the 12-week biopsies, leaving 34 patients for analysis in the present study. For exploratory analysis, RT-

sensitive patients were further subdivided into two subgroups of moderate risk (n=9) and high risk (severe reaction, n=10).

Test irradiation of the skin was performed as previously described [25]. Patients received a single 4Gy radiation dose to a small area of skin on the upper outer quadrant of the buttock. A 6MeV electron beam from a radiotherapy linear accelerator exposed an area of skin 4×2cm, and an 8mm perspex build-up filter was used to ensure dose homogeneity throughout the epidermis and dermis.

Twelve weeks following irradiation, four 4mm punch biopsies were obtained from both irradiated and contralateral unirradiated skin. Two of the replicate biopsies were used to establish primary fibroblast cultures for the present study. The skin samples were shipped to Mannheim in serum-free basal medium at ambient temperature where they were cut into smaller pieces, placed in T25 tissue culture flasks and left to adhere for 2h. After attachment, cells were incubated with cell culture medium (Gibco AmnioMax C100 basal medium; Life Technologies GmbH, Darmstadt, Germany) supplemented with 7.5% AmnioMax C100 supplement (Life Technologies), 7.5% fetal bovine serum (FBS; Biochrom AG, Berlin), 2mM glutamine, and penicillin/streptomycin. Primary cultures established by outgrowth were expanded by passaging twice and cryopreserved in the 3rd passage. For the present in vitro experiments, frozen vials of fibroblasts in passage 3 were thawed, expanded by passaging twice and used for the present experiments in passage 5.

Fibroblast cultures were successfully established from all patients and showed similar levels of the human proliferation marker Ki-67 (MKI67) in cultures established from skin

irradiated *in vivo* (clinically) 12 weeks earlier as well as cultures from unirradiated skin (Supplementary Figure S1).

The study design is shown in Figure 1. Residual 53BP1 (TP53BP1) foci were determined 24h after *in vitro* irradiation of fibroblasts established from unirradiated skin biopsies taken at the 12-week time point. Furthermore, 53BP1 foci were determined in unirradiated parallel fibroblast cultures established from biopsies taken from unirradiated and *in vivo* irradiated skin 12 weeks after irradiation. p53 (TP53) and p21/CDKN1A were determined 2h, 2d and 6d after irradiation *in vitro* and in unirradiated cultures from unirradiated and *in vivo* (clinically) irradiated skin (in parallel with day 2 samples).

Irradiation and immunofluorescence microscopy of fibroblasts *in vitro*

5×10^3 cells were seeded per well in chamber slides (BD Falcon), incubated overnight and irradiated the next day with 4Gy of 6MV X-rays from a linear accelerator (Synergy, Elekta, Crawley, UK) at a dose rate of 6 Gy/min. After irradiation, cells were incubated at 37°C under CO_2 and fixed at different time points. The cells were rinsed with PBS, fixed for 15 minutes at room temperature with 3.7% paraformaldehyde and permeabilised for 5 minutes with 0.5% Triton X-100 in PBS at 4^oC. Slides for detection of residual γH2AX and 53BP1 foci 24h after irradiation were shipped in PBS to the PHE Centre for Radiation, Chemical and Environmental Hazards, Chilton, UK and processed as previously described [26]. For detection of Ki-67, p53 and p21/CDKN1A, fibroblasts were treated and fixed at 2h, 2d and 6d after irradiation using 3.7% formaldehyde in PBS with 0.2% Triton X-100 (PBST) for 10 min. Details of antibodies and staining protocols are given in Supplementary Material.

Statistical analysis

Differences between fibroblasts from RT-sensitive and RT-resistant patients were analysed by the non-parametric Wilcoxon/Mann-Whitney test. Correlations were analysed by linear regression or the non-parametric Spearman's ρ rank correlation test. A linear model was used to test the effect of measured parameters on clinical radiosensitivity. All tests were performed using the JMP.v11 Statistical Discovery software package (SAS Institute GmbH, Boeblingen, Germany). $P < 0.05$ was considered statistically significant for the planned analysis, and $P < 0.01$ for exploratory analysis.

3. Results

Quantification of residual DSB in skin fibroblasts *in vitro*

The mean number of residual 53BP1 foci per cell in the nuclei 24h after *in vitro* irradiation of fibroblasts established from unirradiated skin was significantly increased over the background. The median for all patients was 2.07 [quartiles: 1.73; 2.46] for 4 Gy versus 0.64 [quartiles: 0.51; 0.83] for 0 Gy ($P<0.0001$). We compared these values with the residual and background numbers of foci in fibroblasts scored *in situ* in sections of skin biopsies 24h after the clinical test irradiation of the skin *in vivo* (Somaiah et al., submitted). Overall, the two sets of values for irradiation with 4 Gy *in vitro* or *in vivo,* and their corresponding background values without irradiation, compared quite well although the difference in mean foci numbers between irradiated and unirradiated cells was larger *in situ* (Supplementary Figure S2). However, within each dose level no correlation between the numbers of foci scored in fibroblast cultures and in skin sections from individual patients was observed.

Because foci levels with and without irradiation might be informative in their own right, it was decided to analyse the data without background subtraction. Residual foci levels after *in vitro* irradiation of fibroblasts established from unirradiated skin were significantly higher ($P=0.007$, $n=34$) in cultures from RT-sensitive than from RT-resistant patients (Figure 2a). In contrast, no significant difference was seen either for unirradiated fibroblast cultures established from unirradiated skin ($P=0.27$, $n=31$) or from skin irradiated *in vivo* with a test dose of 4 Gy 12 weeks earlier ($P=0.18$, $n=34$). A moderate, positive correlation (linear regression: $R^2=0.34$; Spearman's $\rho=0.60$, $P=0.0004$;

n=31) was observed between residual 53BP1 foci and the background levels in the unirradiated fibroblast cultures from individual patients (Figure 2b). However, subtraction of the background levels for individual patients did not enhance the difference between RT-sensitive and RT-resistant patients (not shown).

It was noted that the variance of residual 53BP1 foci was 5.5-fold larger in the RT-sensitive than in the RT-resistant group (0.908 and 0.166, respectively). To explore whether the higher mean number of residual 53BP1 foci and larger variance might be associated with the degree of severity, the correlation with risk score was tested. A significant correlation with severity (ρ=0.45, P=0.008, n=34) was observed after *in vitro* irradiation (Supplementary Figure S3b). However, a trend for a correlation (ρ=0.33, P=0.07, n=31 and ρ=0.35, P=0.04, n=34, respectively) was also observed in unirradiated cultures established from unirradiated and *in vivo* test-irradiated skin (Supplementary Figure S3a,c). In fact the background numbers were very similar in the two cultures and showed an increase in samples from patients with severe reaction (risk group 2). Therefore, it seemed justified to treat these cultures as independent determinations of the background 53BP1 foci. When the two cultures were analysed together, the correlation with severity was highly significant (ρ=0.33, P=0.007, n=34+31). After background subtraction, the correlation of residual foci after *in vitro* irradiation showed only a trend (ρ=0.35, P=0.05, n=31) (Supplementary Figure S3d). Taken together, these data suggest that the higher numbers of 53BP1 foci in RT-sensitive patients with severe reaction may be associated with higher background levels before irradiation.

Radiation response markers p53 and p21/CDKN1A

Irradiation of fibroblasts *in vitro* increased the fraction of cells scoring positive for p53 within 2h (Figure 3a). p53 stayed at a similar level on day 2 but decreased on day 6 to a level slightly but not significantly higher than the basal level without irradiation. The p53 level in cultures established from *in vivo* irradiated skin was not significantly increased compared with the basal level in cultures from unirradiated skin (P=0.62). The fraction of p53-positive fibroblasts was significantly higher in cultures from RT-sensitive patients compared with RT-resistant patients before and 2h after *in vitro* irradiation, and was marginally significant on day 6 (Figure 3b). The overall difference in p53 levels between RT-sensitive versus RT-resistant patients for all conditions was highly significant (P=0.0013). When basal p53 levels (0 Gy *in vitro*) in cultures from unirradiated and *in vivo* irradiated skin were analysed together, the basal level in fibroblasts from RT-sensitive patients was significantly increased (P=0.009, n=34+34).

The variance of p53 levels in unirradiated fibroblasts (0 Gy *in vitro*) from unirradiated skin was 3.0-fold larger in the RT-sensitive than the RT-resistant group (0.00448 and 0.00147, respectively) and 5.2-fold larger 2h post-irradiation (0.0617 and 0.0119, respectively). The latter was related to an early, strong induction of p53 in fibroblasts from RT-sensitive patients with severe reaction compared to RT-resistant patients or RT-sensitive patients with more moderate risk. Whereas the basal level of p53-positive cells in unirradiated fibroblast cultures was increased for RT-sensitive versus -resistant patients, p53 in fibroblasts from patients with severe reaction (risk group 2) showed a strong, early increase 2h after irradiation. By contrast, fibroblasts from patients with moderate risk score (risk group 1) reached the highest value at 2d post-irradiation similar to fibroblasts from RT-resistant patients (Figure 3c).

The fraction of cells scoring positive for p21/CDKN1A (a p53 downstream target) was unchanged 2h after irradiation but was significantly increased at 2d and 6d whereas no significant increase was observed in fibroblasts from *in vivo* irradiated skin (Figure 4a). However, no significant difference in p21/CDKN1A between fibroblasts from sensitive and resistant patients was detected in any of the groups (Figure 4b).

The increased basal p53 level for RT-sensitive patients was observed in both risk groups (1 and 2) whereas the positive correlation of early p53 response with clinical severity (ρ=0.47, P=0.005, n=34) was due to patients with severe reaction only (Supplementary Figure S4a,b). Furthermore, early upregulation of the downstream target p21/CDKN1A at 2h was indicated for the RT-sensitive patients with severe reaction (Supplementary Figure S4d).

A weak correlation (ρ=0.38; P=0.03) was observed between the *in vitro* parameters residual 53BP1 foci (4Gy, 24h) and p53 response (4Gy, 2h), whereas the correlation of residual 53BP1 with basal p53 (0 Gy) showed a trend (ρ=0.31; P=0.08). However, in a linear model, only residual 53BP1 foci and basal p53 were predictive of RT-sensitive versus RT-resistant patients (P= 0.010 and P=0.03, respectively) with a weak trend for an interaction (P=0.16). With respect to severity (risk score), predictive modelling showed a strong trend (P=0.04) for early p53 response (2h) and a weak trend (P=0.18) for background 53BP1 foci (0Gy) with no significant interaction (P=0.58). In fact, early p53 response alone was a more significant predictor of severity (P=0.0014). These results reflect that residual 53BP1 foci and basal p53 levels were increased for both RT-sensitive risk groups (1 and 2) while only fibroblasts from RT-sensitive patients with severe

reaction (risk group 2) showed a strong early increase of p53. Taken together, this indicates that 53BP1 and p53 may be partly independent predictors.

4. Discussion

The present study tested the relation between individual patients' late normal tissue reaction (changes in breast appearance) after whole-breast radiotherapy and the radiation response of their individual fibroblast cultures *in vitro*. The major findings for RT-sensitive versus RT-resistant patients are (I) a higher number of residual DSBs after *in vitro* irradiation and (II) an increased fraction of p53 positive cell without *in vitro* irradiation. These results associate the radiation response of fibroblasts, i.e. the functional cells of connective tissue, with the development of radiation-induced breast shrinkage/hardening. Further exploratory analysis showed a vigorous, early p53 response to radiation in the subgroup of patients who developed severe reaction despite few identified risk factors. Since the RT-sensitive group was enriched in such patients, the association with radiation response may be important only in a minority of the patients.

The study design involved a test irradiation to previously unirradiated skin in order to determine residual and longer-term damage. Residual DSBs scored *in situ* will be presented elsewhere (Somaiah et al., submitted). However, the cultures established 12 weeks after the test irradiation from *in vivo* irradiated and unirradiated skin were rather similar with respect to the proliferation marker Ki-67, background 53BP1 foci, and basal p53 levels. Therefore, the fibroblasts growing out from these skin biopsies appeared to have largely recovered from radiation damage.

Radiation-induced oxidative stress is observed in tissue not only during irradiation but may persist many weeks after irradiation, and may even affect tissues outside the irradiated field via non-targeted effects [27, 28]. These reactions are considered to be

propagated by NF-κB-mediated cytokine production and inflammatory reactions [29], leading to further oxidative DNA damage [30, 31]. Thus the immune system plays an important role in maintaining a vicious circle involving persistent activation of the DNA damage response [32]. Residual 53BP1 foci mark unrepaired DSBs after irradiation. On the other hand, increased p53 levels without irradiation suggest an increased basal stress level which might be caused by systemic or genetic factors. Support for a patient-related factor (systemic or genetic) comes from a previous study on lymphocytes from seven RT-sensitive and seven RT-resistant patients belonging to the present cohort, which showed significantly increased residual 53BP1 foci 24h after irradiation *in vitro* [25]. Furthermore, chromosome aberrations were significantly increased, and residual 53BP1 foci correlated with deletion type of aberrations indicating a deficiency in DSB repair. Together with the findings from the present study, this suggests a hypothesis in which a defect in the p53 stress response pathway may contribute to late reaction in RT-sensitive patients, possibly via increased misrepair and, genomic instability leading to premature terminal differentiation of fibroblasts. Thus, it is well established from work with tumour cells, that overexpression of p53 frequently represents a defect in p53 function. However, the partial independence of basal p53 levels and residual 53BP1 foci may indicate that unrepaired DSBs and genomic instability may be caused by a DSB repair deficiency in some patients.

For all significant endpoints, the increased levels in RT-sensitive compared with RT-resistant patients were associated with a three to five-fold larger variance. Thus only a proportion of RT-sensitive patients showed increased levels of 53BP1 foci or p53 while the rest were in the same range as RT-resistant patients (Supplementary Figures S3 and

S4). Exploratory analysis suggested a correlation with severity of late reaction supporting the hypothesis that subgroups of patients characterised by different mechanisms of late reaction may exist [Herskind et al., in revision]. Bioinformatic analysis can be used to identify genetic factors of clinical and cellular radiation responses [33] and thus combining the two approaches might help characterise pathways associated with different subgroups [Herskind et al., in revision].

In contrast with the present work, recent studies on the relation between DSB induction and repair by moderate doses *in vitro* and patients' normal-tissue reaction after radiotherapy have been performed with lymphocytes. Expression of γH2AX protein measured by flow cytometry (FACS) showed higher sustained levels in over-reactors with different acute or chronic toxicity after RT for different tumours [34] while a similar study of mixed endpoints after prostate RT showed no significant difference [35]. Three studies on late reaction after breast [22, 36] or prostate RT [37] showed significant associations with residual DSBs while two studies of late toxicity in breast [38] or various RT patients [39] were not significant. Recent large studies (n=54 to 89) on early toxicity to RT showed a clearer picture with significant associations of DSB repair in breast [40] and various mixed cancers [41-43]. This supports the hypothesis that residual DSBs are associated with the risk of normal tissue reaction to RT although the association may be influenced by the clinical endpoint and cell type studied.

Upon induction of cell stress by DNA damaging agents such as ionising radiation, phosphorylation processes lead to stabilisation of p53 by dissociation from the E3 ubiquitin-protein ligase MDM2 which normally inactivates p53 and targets it for cytoplasmic translocation and proteasomal degradation. Stabilised p53 undergoes post-

translational modification, nuclear translocation and tetramerisation to act as a transcription factor, and has multiple functions including induction of cell-cycle arrest in G1 via p21/CDKN1A, DNA repair, and apoptosis [44-46]. Recent studies have emphasised the role of pulsed p53 expression in response to different levels of DNA damage suggesting that short pulses lead to transient cell-cycle arrest while sustained p53 signalling leads to apoptosis or permanent cell-cycle arrest [47, 48]. Furthermore, p53 may induce or suppress differentiation in different cell types [49].

Fibroblasts do not normally undergo radiation-induced apoptosis [50]. Instead they arrest permanently in a state which is sometimes termed senescent but is more appropriately described as premature differentiation since the cells stay metabolically active with increased synthesis and deposition of extracellular matrix proteins [51-54]. The present results suggest that p53 is increased in RT-sensitive patients even without irradiation and may be sustained at a higher level at 6d after *in vitro* irradiation. This would be consistent with a higher propensity to undergo permanent cell-cycle arrest leading to premature differentiation or senescence. In addition, patients with more severe reaction showed strong early upregulation of p53 suggesting a more vigorous response of transient cell-cycle arrest and DNA repair. However, expression of the endogenous cdk inhibitor p21/CDKN1A which is a major transcriptional target of p53 and part of the stress-induced G1/S cell-cycle checkpoint, was not associated with late reaction. Instead, we speculate that another p53 transcriptional target GADD45, which plays important roles in genomic stability and differentiation [55-57], might be involved, in which case it might target the G2/M rather than the G1/S checkpoint [58].

In conclusion, we have found a small but significant increase in the number of residual DSBs and a consistently higher fraction of p53-positive cells without irradiation, as well as 2h and 6d after *in vitro* irradiation of fibroblasts from RT-sensitive versus RT-resistant patients. This establishes an association between the radiation response of fibroblasts and late reaction of the breast after RT. Exploratory analysis of ranked risk groups suggested that vigorous, early upregulation of p53 is prominent in a small subgroup of patients with severe reaction. Residual 53BP1 foci at 24h and basal p53 levels or p53 at 2h post-irradiation showed only weak correlations, and prospective analysis suggested they may be independent markers, possibly representing different aspects of the radiation response. Although the present study does not allow prediction of RT-sensitive patients, the identification of rare patients at risk for severe reaction after radiotherapy might be feasible based on the early p53 response. However, validation and further studies on mechanisms and genetic factors will be required to establish a reliable test and search for additional surrogate markers.

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Figure legends

Figure 1. Experimental design of the present study. Residual foci were also scored in sections of skin biopsies 24h after irradiation *in vivo* and will be presented elsewhere (Somaiah et al. submitted).

Figure 2. (a) Comparison of 53BP1 foci in fibroblast cultures from RT-sensitive and RT-resistant patients. Cells established from irradiated or unirradiated skin 12 weeks after the *in vivo* test-irradiation were fixed 24h after *in vitro* irradiation with 0 Gy or 4 Gy. . The mean number of residual 53BP foci 24h after *in vitro* irradiation was significantly increased for RT-sensitive versus -resistant patients (P=0.007, n=34). Background levels of 53BP1 in the cultures established from irradiated and unirradiated skin from the same patients did not differ significantly (pairwise non-parametric test: P=0.71, n=14, and P=0.22, n=17 for RT-resistant and -sensitive patients, respectively). Mean values and standard errors are shown. n=19 (RT-sensitive) and n=15 (RT-resistant) except for 0Gy fibroblasts from unirradiated skin (n= 17, and n=14, respectively). (b) For cultures from unirradiated skin, the mean number of residual 53BP1 foci per fibroblast 24h after *in vitro* irradiation correlated with the background numbers in unirradiated cells (R^2=0.34; Spearman's ρ=0.60, p=0.0004; n=31, samples from RT-sensitive and RT-resistant patients were included).

Figure 3. (a) The fraction of p53 positive cells was significantly increased 2h (P=0.012) and 2d (P=0.0001) after *in vitro* irradiation of fibroblast cultures (n=34) from unirradiated skin while a trend was observed at 6d (P=0.10). No significant increase

(P=0.62) was observed for unirradiated cultures of fibroblasts established from *in vivo* irradiated skin. Box plot shows median, and 25/75% and 10/90% percentiles. P-values were calculated by Wilcoxon comparison of each pair. (b) Increased positive fractions in cultures from RT-sensitive relative versus RT-resistant patients. P-values were calculated by the Wilcoxon/Mann-Whitney test. Mean values and standard errors are shown. (c) Kinetics of radiation-induced p53 induction for different risk groups (0: RT-resistant, n=15; 1: RT-sensitive with moderate risk, n=9; 2: RT-sensitive with severe reaction (high risk, n=10). Mean values and standard errors are shown.

Figure 4. (a) The fraction of p21 positive cells was significantly increased 2d and 6d (P<0.0001) after *in vitro* irradiation of fibroblast cultures (n=34) from unirradiated skin but not at 2h (P=0.61). No significant increase (P=0.71) was observed for unirradiated cultures of fibroblasts established from *in vivo* irradiated skin. Box plot shows median, and 25/75% and 10/90% percentiles. P-values were calculated by Wilcoxon comparison of each pair. (b) No significant differences between positive fractions in cultures from RT-sensitive and RT-resistant patients (Wilcoxon/Mann-Whitney test; mean values and standard errors are shown).

Table 1. Patient characteristics and treatment parameters[1]. Radiotherapy (RT) dose to the whole breast, RT technique (3D or standard 2D wedge), tumour bed boost, surgical deficit, and axillary treatment, were significant risk factors.

	RT-sensitive	**RT-resistant**
Patients (n=35)	20[2]	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*	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[3])	1
Axillary treatment	15	11
Tamoxifen	14	12
Chemotherapy	8	15

[1] Equivalent total dose given in 2 Gy fractions assuming $\alpha/\beta=3$ Gy
[2] One RT-sensitive patient (risk score 1) withdrew consent before biopsies were taken at 12 weeks.
[3] Patient had mastectomy & reconstruction before RT; this was the only patient with <5yr follow up

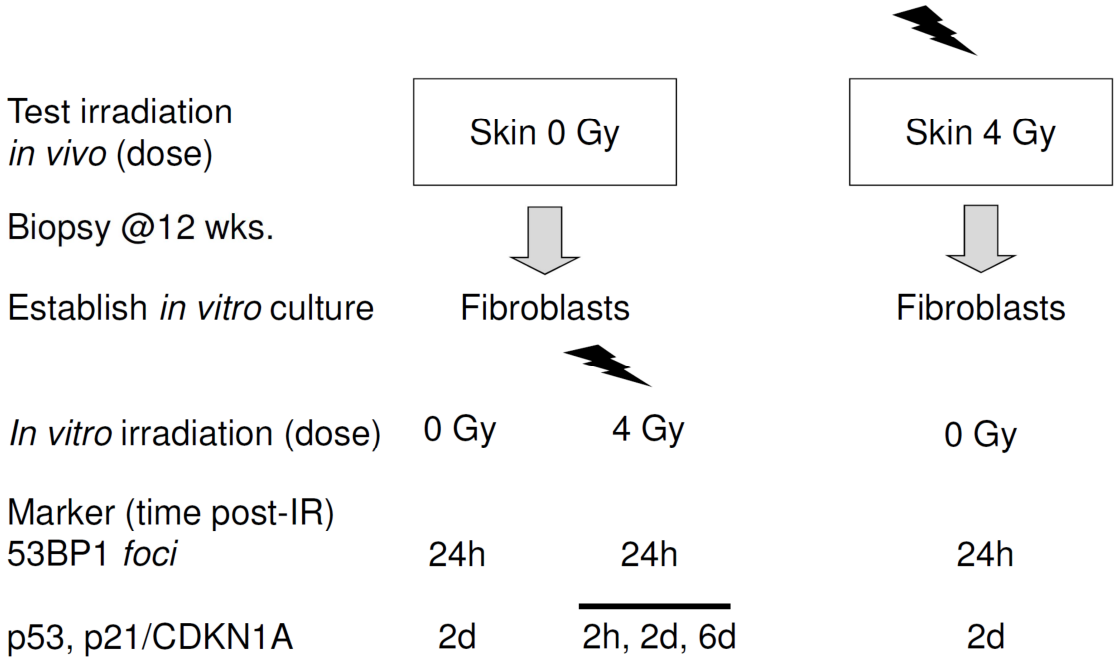

Test irradiation
in vivo (dose)
Skin 0 Gy
Skin 4 Gy
Biopsy @12 wks.
Establish in vitro culture
Fibroblasts
Fibroblasts
In vitro irradiation (dose)
0 Gy
4 Gy
0 Gy
Marker (time post-IR)
53BP1 foci
24h
24h
24h
p53, p21/CDKN1A
2d
2h, 2d, 6d
2d

Figure 1

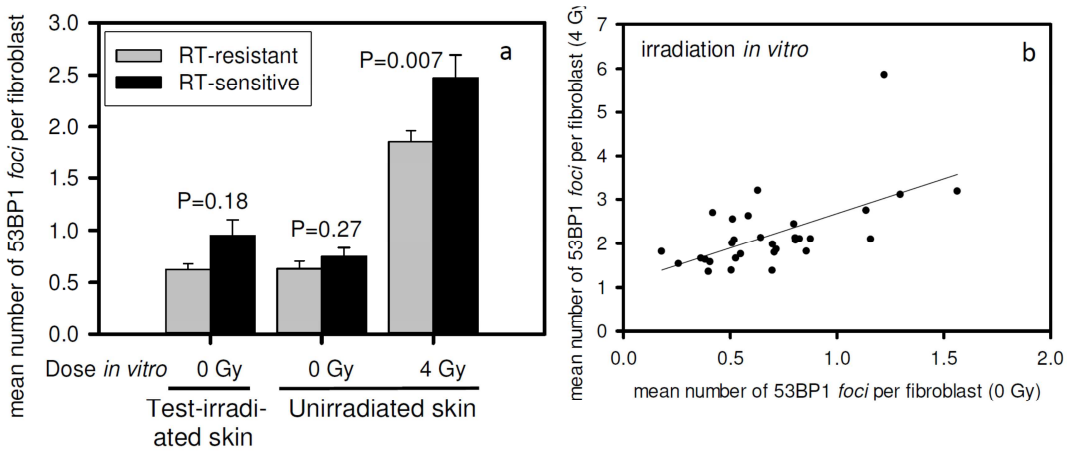

mean number of 53BP1 foci per fibroblast
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RT-resistant
RT-sensitive
a
P=0.007
P=0.18
P=0.27
Dose in vitro
0 Gy
0 Gy
4 Gy
Test-irradi-
ated skin
Unirradiated skin
mean number of 53BP1 foci per fibroblast (4 Gy)
irradiation in vitro
b
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
mean number of 53BP1 foci per fibroblast (0 Gy)

Figure 2

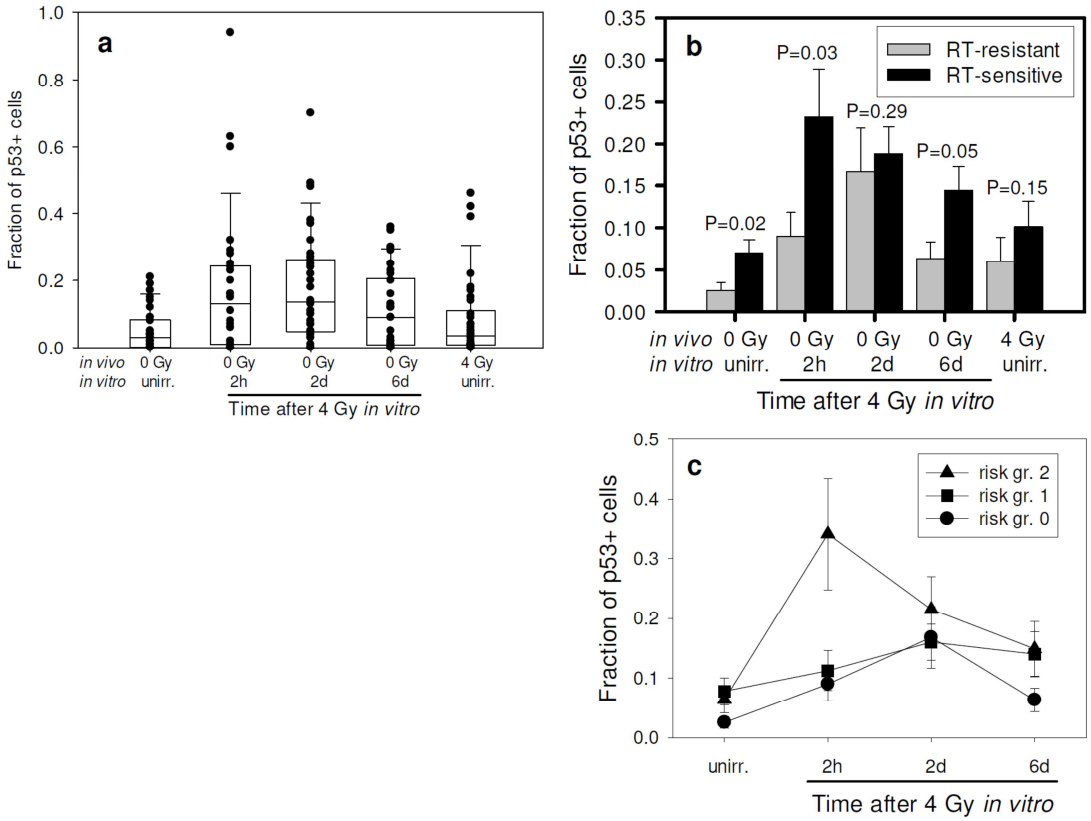

a
Fraction of p53+ cells
1.0
0.8
0.6
0.4
0.2
0.0
in vivo
in vitro
0 Gy
unirr.
0 Gy
2h
0 Gy
2d
0 Gy
6d
4 Gy
unirr.
Time after 4 Gy in vitro
b
Fraction of p53+ cells
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
RT-resistant
RT-sensitive
P=0.02
P=0.03
P=0.29
P=0.05
P=0.15
in vivo
in vitro
0 Gy
unirr.
0 Gy
2h
0 Gy
2d
0 Gy
6d
4 Gy
unirr.
Time after 4 Gy in vitro
c
Fraction of p53+ cells
0.5
0.4
0.3
0.2
0.1
0.0
risk gr. 2
risk gr. 1
risk gr. 0
unirr.
2h
2d
6d
Time after 4 Gy in vitro

Figure 3

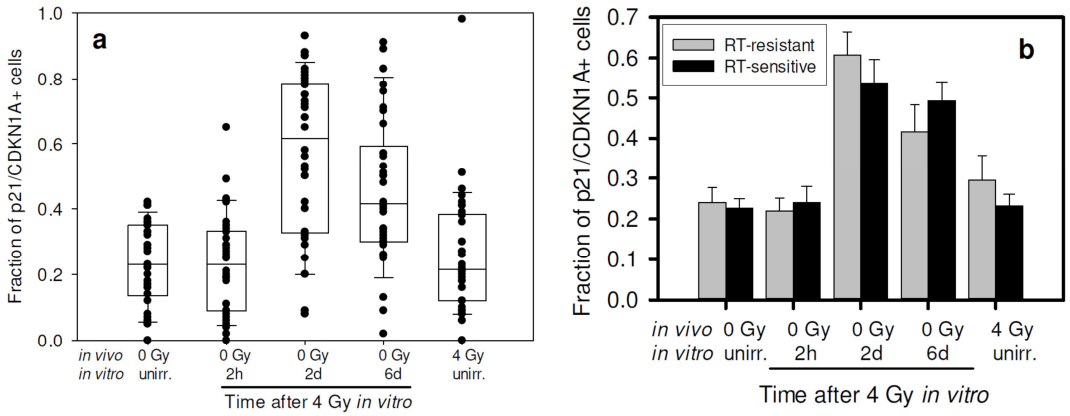

a
Fraction of p21/CDKN1A+ cells
1.0
0.8
0.6
0.4
0.2
0.0
in vivo 0 Gy 0 Gy 0 Gy 0 Gy 4 Gy
in vitro unirr. 2h 2d 6d unirr.
Time after 4 Gy in vitro
b
RT-resistant
RT-sensitive
Fraction of p21/CDKN1A+ cells
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
in vivo 0 Gy 0 Gy 0 Gy 0 Gy 4 Gy
in vitro unirr. 2h 2d 6d unirr.
Time after 4 Gy in vitro

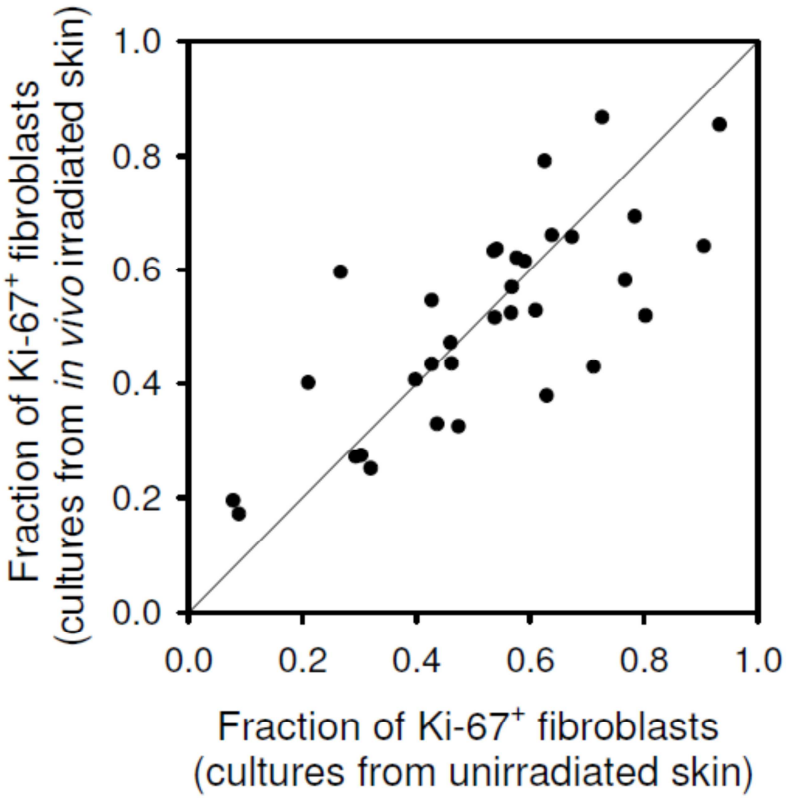
Figure 4

Nuta et al., Supplementary Materials and Methods

Immunostaining for fluorescence microscopy

Upon arrival at the PHE, Chilton, samples for γH2AX and 53BP1 staining were blocked for 30 minutes with PBS containing 3% w/v BSA Fraction V (Sigma-Aldrich, Dorset, UK) and incubated with primary antibody diluted in PBS + 2% FBS for 1 hour at room temperature. Cells were then washed three times with PBS + 2% FBS , incubated with secondary antibody and DAPI (4´,6-diamidino-2-phenylindole) diluted in PBS + 2% FBS for 1 hour at room temperature in the dark, followed by three further washes with PBS + 2% FBS. After drying, slides were mounted using Vectashield and visualized using a Nikon Eclipse TE200 epifluorescence microscope. Co-localizing γH2AX and 53BP1 foci were scored and a minimum of 50 cells were scored for each patient. The following antibodies were used: anti-53BP1 (mab3802, Millipore, Watford, UK, 1:400 and ab36823, Abcam, Cambridge, UK, 1:400), anti-γH2AX (05-636, Millipore, 1:200 and ab26350, Abcam, 1:500). Appropriate secondary antibodies were conjugated with Alexa-Fluor 488 (Invitrogen, Paisley, UK, 1:200) and TRITC (Tetramethylrhodamine-5-(and-6)-isothiocyanate, Jackson Immunoresearch, Suffolk, UK, 1:200).

Samples for detection of Ki-67, p53, and p21, were processed at the UMM in Mannheim. After rinsing, cells were incubated with 1% BSA in PBST for 10 min and incubated with primary antibody in PBST for 45 min. The following primary antibodies were used: rabbit polyclonal anti-Ki-67 (Abcam ab15580, 1:500), rabbit polyclonal anti-p53 (Cell Signaling #9282, 1: 300), mouse monoclonal anti-p21/CDKN1A (Cell Signaling #2946, 1:300). Cells were washed 3×5 min with PBST and incubated with secondary FITC-conjugated antibody (goat anti-rabbit IgG, Millipore AP307F or goat anti-mouse-IgG, Santa Cruz, 1:500).



Supplementary Figure S1. Correlation of Ki-67 positive fraction of fibroblasts in cultures established by outgrowth from unirradiated and *in vivo* irradiated skin. The solid line represents equal values in the two cultures. Proliferation activities were similar in the two cultures suggesting that recovery of surviving cells during the twelve weeks between irradiation and biopsy was complete, allowing outgrowth of sufficient numbers of fibroblasts from the explants. The mean values±standard errors were 0.54±0.04 and 0.53±0.03, respectively (P=0.74, paired t-test). The correlation between the two different cultures from individual patients was good (R^2 = 0.56; P<0.0001, n=33; one value of Ki-67 was missing in the 2×34 cultures). The cultures had not been irradiated *in vitro*.