Original Article

In vitro prediction of breast cancer therapy toxicity

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Background: Understanding the basis of clinical radiosensitivity is a key goal of radiation research. In this study, we used the limiting dilution assay (LDA) to analyze *in vitro* radiosensitivity of cell lines from individuals with breast and other cancers, who had been treated with ionizing radiation, and who either had a non-radiosensitive (RS) radiation response or who were clinically RS.

Methods: Lymphoblastoid cell lines (LCLs) were created from 29 cancer patients including 19 RS patients, 10 controls who had not developed severe normal tissue reactions, and 1 ataxia telangiectasia RS control cell line. The clinically RS patients had grade 3 or grade 4 reactions; one had a grade 2 reaction. All cells were exposed to graded doses of gamma-radiation *in vitro* and cell survival assessed via LDA. Cell survival was expressed on non-linear regression analysis-fitted survival curves and also as the surviving fraction at 2 Gray (Gy) (SF_2).

Results: Our LDA analysis yielded two notable positive results. Firstly, it could distinguish control cells from cells from pooled breast cancer cases with severe reactions of all types (acute reactors, consequential late reactors and late reactors). Secondly, two radiosensitivity outliers were detected on the fitted curves, corresponding clinically to grade 3 and 4 late radiation reactions in breast and head and neck cancer cases respectively. The assay showed considerable cell survival heterogeneity.

Conclusions: The LDA as used here may provide unique clinical utility in detecting potential RS breast cancer patients prior to radiotherapy (RT), a form of personalized medicine. The assay may be especially useful in situations where its results can be temporally available prior to therapy initiation (e.g., those patients not undergoing RT until some months after surgery, typically those having adjuvant chemotherapy prior to RT). Two LCLs from RS outliers could potentially yield insight into the cellular and/or genetic basis of radiosensitivity, for example by undertaking genomic analyses on these cell lines.

Keywords: Ionizing; radiation; radiotherapy (RT); clonogenic survival; cell survival; breast; cancer

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Introduction

Approximately 50% of cancer patients receive radiotherapy (RT) (1) with sub-population (~1–5%) developing significant normal tissue complications within the treatment field, limiting safe dose-escalation across the general RT population. Various assays have been trialled in an attempt to predict excessive normal tissue toxicity prior to the instigation of therapy, with no practical assay useful in the clinical setting. In this study we characterize a unique

bank of cell samples from radiosensitive (RS) patients, utilizing an assay that allows for cross-comparison with patient characteristics, to investigate its predictive clinical outcome power.

Variation in normal tissue reactions in the cancer patient population has been observed as being normally distributed, ranging from RS to radioresistant sub-populations, to extreme over-reactors lying outside the normally distributed curve (2). For a large group of patients treated with the same techniques, a small number show a highly sensitive normal tissue response, with either severe acute or severe late radiation reactions. In some cases, severe acute reactions progress to severe late effects, so-called "consequential late effects". Some variability among individuals in normal tissue response can be explained by Poisson randomness in cell killing (2), with a genetic basis for radiosensitivity suggested by studies using cells from individuals with a RS phenotype, such as ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS) (3,4). Such individuals are readily identified by their abnormal clinical characteristics, including in addition to radiosensitivity, a Mendelian pattern of genetic transmission, immunodeficiency and cancer-proneness, especially lymphoid malignancies. In contrast to such patients, the present study was limited to patients with no additional discernible phenotypes apart from clinical radiosensitivity.

In conjunction with predicting inherent radiosensitivity and hence relieving treatment morbidity, identification of individuals with sensitive normal tissues using a predictive assay could theoretically allow dose escalation in the majority of patients, with the aim of increasing local control and cure rates (5-7) (*Figure 1*). In spite of enormous attempts to develop a predictive assay recapitulating intrinsic clinical radiosensitivity, there is still conflicting evidence regarding the correlation of *in vitro* radiosensitivity and *in vivo* responses to IR, especially in breast cancer (8); the field requires further investigation.

Tissues regenerate clonally after irradiation, for example, in the skin (9). Colony-forming assays using fibroblasts are regarded by many as the gold-standard measurement of *in vitro* clonal cell survival (10). Alternative clonogenic assays such as the limiting dilution assay (LDA), as used here and which are suitable for non-adherent lymphoid cells, are based on the same principle as the CFA in its ability to assess clonal survival of a cell population after radiation exposure *in vitro*. Whatever assay is used to measure *in vitro* radiosensitivity, all studies have found a great deal of inter-individual variability. Clinical observation

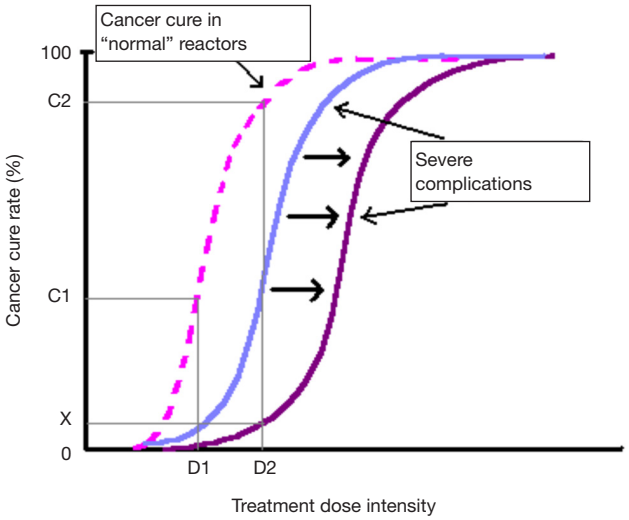

Figure 1 Theoretical dose-response curves of radiotherapy (RT) patient population (5-7). At a radiation dose (D1) given to mammals, a certain cure rate (C1) is achieved. By increasing the therapeutic dose to D2, the cure rate increases greatly to ~90% (C2). However, the improvement in cancer cure rate by dose escalation is not possible in practice due to restriction by a few percentages of patients showing severe complications (X). If these individuals could be identified before the commencement of RT, the severe complication curve would shift to the right (horizontal arrows), thereby enabling dose escalation of the non-RS population ("normal" reactors) with relatively the same proportion of patients suffering severe complications.

of IR-induced normal tissue damage supports the notion that intrinsic differences in late radiation injury to normal tissues may have a genetic basis (11-13).

Directly demonstrating a genetic factor contributing to non-syndromic human radiosensitivity, a large genome-wide association study with requisite power demonstrated a novel candidate radiosensitivity gene, *TANC1*, within a defined genomic region in a RS prostate cancer cohort compared to control (14). Mutations in this gene were associated with late RT toxicity. The gene had previously been linked to neurological phenotypes and in regenerating damaged muscle, the latter compatible with a role in late IR toxicity. The gene is thus a potential marker for RT side-effects in the prostate cancer RT patients of the type studied in this analysis.

Other studies have attempted to identify molecular markers of clinical radiosensitivity (15,16). These include using a candidate gene/protein approach and studying molecules known or suspected to influence radiosensitivity

in other species, but using IR-sensitive patient cells/tissues. There are a number of examples of such studies (17-21). Increasingly, gene expression profiling is giving insights into human disease and such studies are emerging in the field of radiosensitivity (22-24).

If there is a general genetic basis for the observed differences in clinical radiosensitivity in otherwise phenotypically normal cancer patients, then it is reasonable to expect a relationship between radiosensitivity of different normal tissues within an individual and some differences in *in vitro* IR-responses between normal and sensitive patients examined. However, evidence to date does not allow such a conclusion.

Using lymphoblastoid cell lines (LCLs) from patients with the LDA has some advantages over other cellular survival assays. LCLs are easily derived from blood samples, which can be collected from patients with minimal pain and discomfort compared to skin biopsies. Such cells are immortalized, therefore providing a robust source of cells available for use in different assays. The LDA can easily provide many replicates, increasing statistical power.

The aim of this study was to examine normal tissue responses to IR using the LDA on immortalized peripheral B-lymphocytes (LCLs) as a model, with the ultimate and main goal of assessing its suitability as a predictive test for clinical application in RT. The data obtained was related to clinicopathological manifestations of a unique RS patient cohort and was compared with pooled controls. If the severe normal tissue reactions have a genetic basis, clinically sensitive individuals may have radiosensitivity that is distinguishable from the control cohort.

Methods

Ethics approval (96/39) for this study, employing cell lines from cancer patients, was obtained from the Ethics Committee of the Peter MacCallum Cancer Centre, Melbourne, Australia. Patients gave individual consent for the creation of LCLs and for their use in research. A group of 29 RT patients treated between 1987 and 1999 were recruited for this study. Patients with Radiation Therapy Oncology Group (RTOG) grade 3 or 4 toxicities (*Figure 2*) (one case had grade 2 toxicity) were designated as being clinically RS, with those with RTOG scores ≤1 serving as controls. The study cohort consisted of LCLs from 9 patients with severe acute reactions, 4 with consequential late reactions, 6 with severe late reactions only, 10 controls and 1 AT patient. Most controls were matched for a number of factors, including age, sex, ethnicity, cancer type and total radiation dose, with corresponding RS patient cells. The overall group represented 5 cancer subtypes, with a predominance of breast cancer among the radiosensitivity patients (n=14). All patients selected for this study were treated with a fractionated RT schedule using 6 MV photons, 2 Gray (Gy) per fraction, apart from one prostate cancer patient who received 18 MV photons. Total dose and duration varied depending on cancer type and occurrence of acute radiation reactions. Mean date of therapy completion for acute and consequential late reaction patients was 1997, while the mean completion date for patients with late reactions was 1993.

Blood samples were spun down for lymphocyte isolation; B-cells immortalized with Epstein-Barr virus (EBV), expanded in culture and cryo-preserved in liquid nitrogen storage tanks. LCLs were also obtained from an AT patient to serve as an IR sensitive control.

For the LDA assay, LCLs were irradiated at room temperature as a cell suspension with γ-radiation from a ^{137}Cs source, delivered at 1 Gy/1.497 min. Cells from the AT patient were irradiated at 1, 1.5, and 2 Gy, due to the extreme radiosensitivity of AT cell lines, with the other cell lines irradiated at 1, 2, and 3 Gy.

For the LDA, cells were fed 24 hours prior to the assay. A cell count was carried out on cell suspensions using the Sysmex (Bayswater, Victoria, Australia) counter system. Only cell lines with more than 3×10^5 cells/mL were used for experimentation. Cell suspensions were then diluted to 10–15× the final plating cell concentration and 4 mL of the suspension was used for each irradiation dose (*Figure 3A*). An aliquot of 4 mL was mock irradiated (0 Gy) at room temperature. This served as a baseline for cell survival before radiation exposure (100% survival). Samples at each dose level were serially diluted to four concentrations, and 160 µL/well put into 96-well, round-bottomed plates. The outside wells of each plate were filled with 200 µL of phosphate-buffered saline to reduce evaporation from the inside wells. For a preliminary test for each cell line, six different concentrations, each in 10 wells, were used to determine the four most appropriate cell concentrations for plating (*Figure 3A*). After pilot studies were carried out, each of the four concentrations was plated into 20 wells in triplicate. The full experiment was repeated at least in duplicate on a separate occasion to ensure accuracy and reproducibility.

Plates were incubated at 37 ℃ in humidified air with 5% CO_2 for 2 weeks without a medium change. At day 14, 40 µL

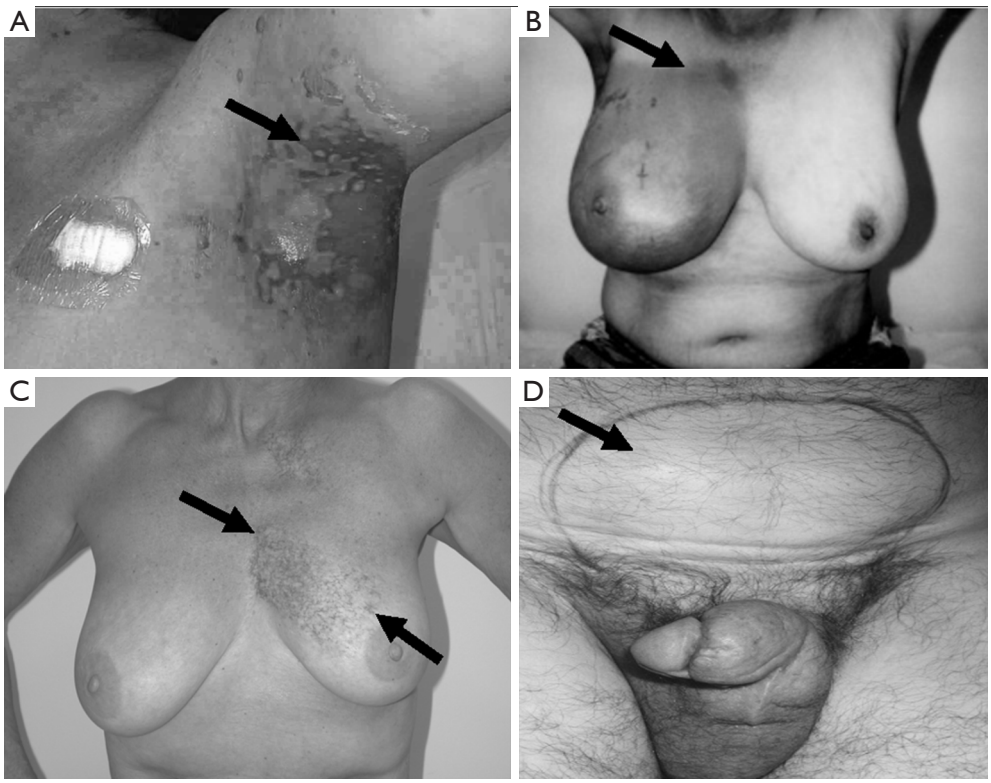

Figure 2 Post-radiotherapy (RT) patients, all with Radiation Therapy Oncology Group (RTOG) grade 3 toxicity. Patients A and B had acute toxicity, and C and D, late toxicity. (A) Resolving skin toxicity. Regenerating individual skin clonogens are evident (arrow), as observed by others after IR (McKay and McKay, 2015); (B) swollen breast with regions of moist desquamation. The upper limit of the radiation fields are clearly demarcated (arrow); (C) severe telangiectasia in the area of a RT boost for breast cancer (arrows). The breast is slightly swollen and retracted upwards; (D) abdominal wall fibrosis. Arrow points to the affected region.

of a 0.5 mg/mL solution of MTT dissolved in PBS was added to each well, then plates were reincubated for 2–4 hours to allow the mitochondrial enzymes to reduce the MTT to an insoluble dark blue formazan salt (25). Wells with dark blue cellular aggregates containing more than 50 cells were scored as a colony (*Figure 3B*).

LDA analysis was performed on six of 30 LCLs using 10% human serum in place of 10% FBS. These cell lines were RS10 (an AT line), CL20, CL29, RS9, RS29 and RS127. There is some evidence that human serum gives higher cloning efficiency than FBS (10). However, with repeat experiments on these cell lines using FBS as serum no significant difference in cell survival was seen. Therefore, media supplemented with 10% FBS was subsequently used throughout the study. The effect of FBS itself on cell growth may vary from batch to batch; therefore the same batch of FBS was used for all experiments to minimize this variation.

Most samples were coded and blinded, including RS6, RS12, RS18, RS21, RS28, RS43, RS106, RS112, CL7, CL21, CL22, CL32, CL38, CL39, CL44 and CL103. This protocol was suggested after a number of LDAs had been performed on some cell lines, with the aim of reducing bias in data collection.

Results

The LDA, a surrogate for clonogenic survival, was performed on a total of 30 LCLs in at least two repeat assays on separate occasions except for two cell lines, RS28 and RS38, which were tested just once. The mean SFs at all irradiation doses were obtained from each of 28 LCLs in replicate, and single values of SF from the other two LCLs. Thirty survival curves were generated using these values.

LCL radiosensitivity was determined as both Gray [the surviving fraction at 2 Gray (SF_2)] and D_0 (the latter being

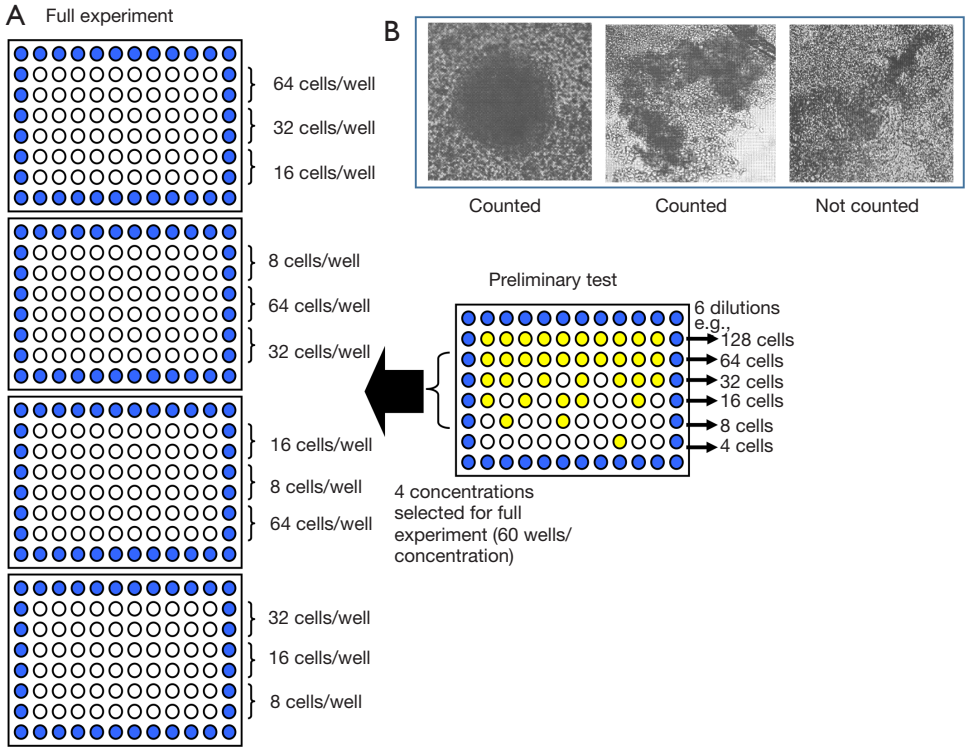

Figure 3 Limiting dilution assay (LDA) used for determining cellular radiosensitivity. (A) Schematic representation of dilution and plating method for the LDA at a particular dose level. Plates are represented by rectangles with 96 blue and white circles in each one. Blue circles are wells filled with PBS. White circles are wells containing cells. Six different cell densities were plated out into a total of 10 wells for a preliminary test. Four cell concentrations were selected if numbers of wells containing colonies (yellow circles) were around 50%. Most full experiments consisted of triplicates at each concentration. Note that the cell concentrations shown in the diagram are examples only; each cell line was independently optimized for cell densities appropriate for their growth rates; (B) examples of appearance of colonies which were counted or not.

the slope of cell survival curve), both obtained from the fitted survival curves. SF_2 values for all cell lines ranged from 0.0245 to 0.3894 (mean 0.2521±0.0791 SD). The D_0 values ranged from 0.539 to 2.071 Gy (mean 1.463±0.3727 SD), using Spearman's rank correlation test (26). Both IR sensitivity parameters of cell survival equally well represented the cellular radiosensitivity of LCLs ($P<0.0001$). Thereafter the subsequent description of radiosensitivity was also expressed in terms of SF_2.

A range of sensitivities was observed across LCLs exposed to graded doses of IR (*Figure 4*). The mean radiosensitivity of the breast cancer patients was greater than, and clearly distinguishable from, that of the controls. Both occupied their own distinctive zones on the cell survival curves. When the patients were grouped according to their cancer types, control breast cancer patients (n=8) had a higher mean SF_2 than breast cancer patients who were clinically sensitive to RT (n=14) ($P=0.011$). Other cancer types did not have enough controls for statistical analysis. However, SF_2 appeared to be related to the cancer type, with prostate cancer patients having more resistant LCLs and head and neck cancer patient LCLs being the most *in vitro* sensitive. However, the significance of these differences could not be evaluated because of an inadequate number of samples.

There were also two notable patient RS strains, RS20 and RS9, from patients with breast cancer and head and neck cancer, respectively. When comparing the difference of the outliers and the mean of all controls, RS20 was marginally more sensitive ($P=0.0682$), whilst RS9 was significantly more sensitive than the controls ($P=0.0329$). None of the samples equalled the RS control line, the AT homozygote, in sensitivity as may have been expected.

The coefficient of variation (CV) for SF_2 (the ratio of

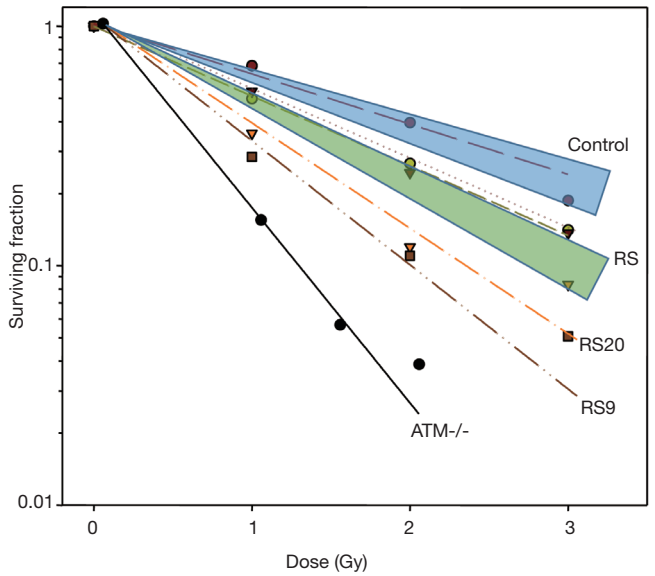

Figure 4 Survival curves fitted by non-linear regression analysis representing the mean of control LCLs (n=10) and LCLs from clinically radiosensitive (RS) breast cancer patients (n=14), with two outliers (RS9 and RS20) graphed independently, and an AT cell line ($ATM^{-/-}$). The outliers were removed from the radiosensitivity cell line pool before analysis of means. Individual means of controls and RS fall within the shaded areas.

standard error and the mean, that indicates the degree of variation among parameters in question, in this case, SF_2 observed in two experiments per cell line) for the whole group of 30 cell lines was 31%, while the CV for D_0 was 22%. *Figure 5* demonstrates the SF_2 variation between different cell lines. Variation between individuals was greater than the variation between experiments for most cell lines, with the most dramatic example being CL20, whose SF_2 in one experiment was the most radioresistant but which was quite RS in a repeat experiment. The radiosensitivity variation in control cell lines, on average, was greater than the radiosensitivity lines.

The role of patients' age, cancer type and total dose received in influencing *in vitro* radiosensitivity was examined. The mean age of patients when the samples were collected (age at sampling) was 59.72 years old, and ranged from 32–82 years. The age at sampling did not influence their LCL radiosensitivity (r=0.0019; P=0.9927). Five cancer types were included in this study with most having breast cancer. Regarding RT schedules, most breast cancer patients received 50 Gy in total, compared to 66 Gy for 2 of 3 prostate cancer patients. The lowest total dose was 16.5 Gy given to a patient with seminoma. Correlation between total dose and SF_2 was not significant (r=0.02759; P=0.8913).

Discussion

Using the LDA, this study investigated the cellular response to IR of LCLs from cancer patients who had or had not experienced severe clinical reactions to RT. Our aim was to determine whether cells from clinically RS patients also manifested *in vitro* IR sensitivity. The rationale was 2-fold: firstly to identify any case of *in vitro* radiosensitivity for further characterization (and potentially to shed light on the cellular and molecular basis of radiosensitivity) and secondly to determine whether the assay could be viable as a predictive assay for radiosensitivity in the clinic. Clinical radiosensitivity is a major issue for the 50% of cancer patients who will undergo RT. For those who suffer severe acute or late toxicities, the morbidity is significant and in some cases debilitating. Fortunately, such reactions are uncommon. Predicting such reactions to IR prior to treatment based on *in vitro* responses could allow improvement of the therapeutic ratio in RT and may have application in a clinical setting to guide therapy, for example, by allowing IR dose de-escalation (as has been used previously in highly IR-sensitive AT patients) or the use of cancer therapies other than radiation.

We created cell survival curves using non-linear regression analysis. Comparison with SF_2 values showed that the latter could substitute for the former, simplifying future use of this assay for LCLs.

Across all tested samples, we found a statistical trend towards separation of radiosensitivity and control cell survival curves. More importantly, we found that the LDA could distinguish between controls and LCLs from clinically RS cases of breast cancer. This suggests that the LDA may provide unique clinical utility as a predictive assay in breast cancer, especially when patients were undergoing neoadjuvant or adjuvant chemotherapy, when LCLs could be created and LDA-tested during the period of chemotherapy. Such data could potentially facilitation of IR schedules based on the LDA results, or patients could be treated with alternative therapeutic modalities. The number of cases in this study could be criticized based on small sample size. However, such severe RS cases are rare, and the statistics we used, robust. These data should nevertheless be confirmed in larger studies with prospectively-accessioned cases, although the relative rarity of severely RS cases may

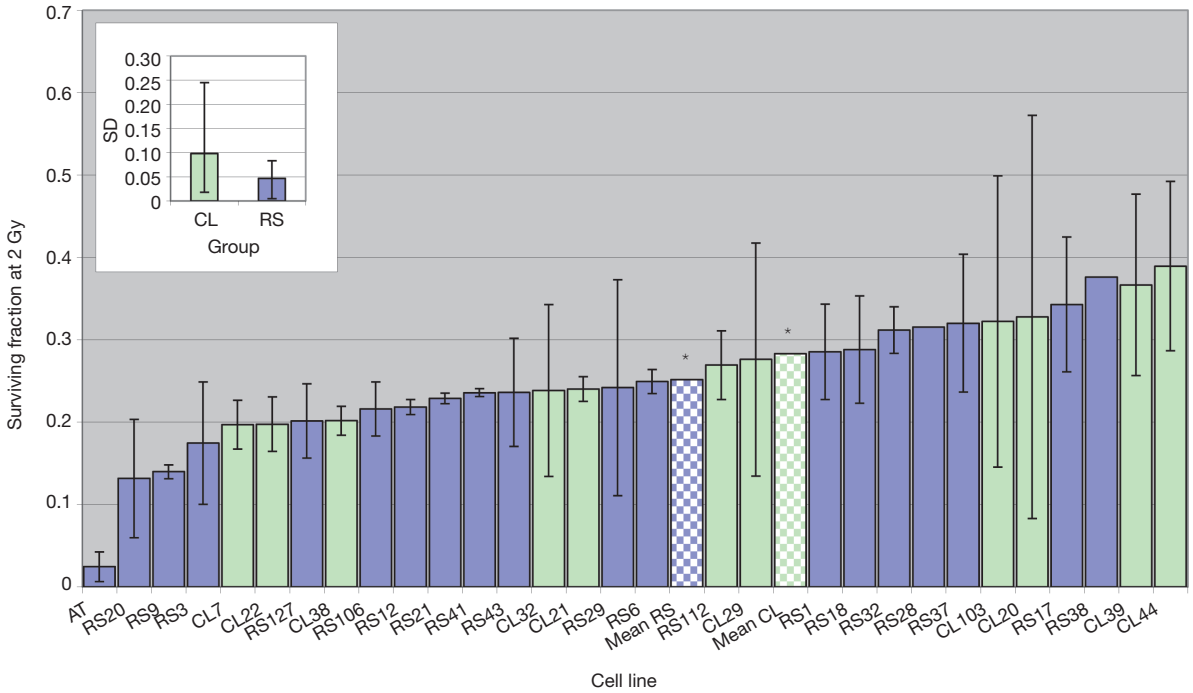

Figure 5 SF_2 of 30 LCLs ranked according to increasing SF_2 (left to right). Dark columns represent RS individuals and light columns are the controls; error bars = SD. Bars with asterisks represent the mean SF_2 of controls (right) and RS cases (left). RS9 is more sensitive than most cell lines even when the error bars are taken into account. A wide range of variation is evident between different cell lines, with smaller variations within cell lines (shown as error bars). A comparison of the overall SDs of LCLs from control and RS patients is shown at the top left.

impede this approach.

The utility of the LDA in cases of hereditary breast cancer, where survival curves after radiation exposure failed to distinguish controls from BRCA1 or BRCA2 carriers (27), has yet to be determined.

In this study two distinctively *in vitro* IR sensitive cases were also found. The lymphocytic cells from these two patients are clearly of a different type from their corresponding cells and tissues showing clinical radiosensitivity, suggesting that IR sensitivity in these cases was constitutive, although tissue-specific differences in radiosensitivity of mammals are described (28). These cell lines may be useful for further molecular and other studies of radiosensitivity.

In summary, our main finding was that the LDA distinguished control cell lines from breast cancer patient-derived lymphoblasts and may be a new predictive assay to allow personalization of RT in breast cancer.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the Ethics Committee of the Peter MacCallum Cancer Centre (96/39) and written informed consent was obtained from all patients.

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