DNA DAMAGE RESPONSES IN HUMAN SKIN AND BLOOD LYMPHOCYTES IN RELATION TO LATE NORMAL TISSUE EFFECTS FOLLOWING RADIOTHERAPY FOR EARLY BREAST CANCER

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A thesis submitted as part of the requirements for the Degree of Doctor of Philosophy at the University College London Faculty of Medical Sciences. 2009–2013

I, Melvin Lee Kiang Chua, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

23th May 2013
For Dad and Mum,
My Dearest Viola
and Elliott
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Acknowledgments

There are a number of people I like to thank for their significant and unwavering support over the years. To my supervisors, John Yarnold, Kai Rothkamm, and Susan Short, thank you for the invaluable mentoring. It seemed not too long ago when we first gathered on a cold wintry morning back in 2009 discussing about the different possibilities for my PhD project. It is incredible recalling the summers and winters that have since passed, and now, I am at the end of this fulfilling journey. Through our intellectual interactions, I have learnt a lot, and for this, I am eternally grateful. To my colleagues at the Health Protection Agency, in particular, Jayne Moquet, Liz Ainsbury, Stephen Barnard, and Donna Lowe, thank you for the wonderful friendships. Lunches became a lot less boring with you all around. To Simon Bouffler, thank you for the generosity extended in hosting me as an external student at the HPA. To Simon Horn, gradually through the years, we have grown to know each other very well. I will treasure memories of the late nights wandering the eerily quiet corridors of the HPA, the occasional nights out in Oxford, not forgetting the vicious moments of intellectual sword play. Thank you for being a dependable colleague and friend. To Sue Davies and Lone Gothard, my collaborators from the Institute of Cancer Research, it is impossible to compliment enough your contributions to my work. Your enthusiasm, support, and assistance are second to none, and quite clearly, you were both integral to the smooth progression of this project. To John Hartley, thank you for agreeing without reservation to be my secondary supervisor. To Julie Olszewski, I cannot ask for a graduate tutor, kinder, more considerate and compassionate than yourself. You were always there to listen when needed, and no matter how dire the situation, you never fail to provide a solution. I am truly indebted for the support you have provided me throughout. To my family, Dad, Mum, Kevin, Joan, Brian, Dad-, Mum-in-law, and Rickie, thank you for supporting me unreservedly through this adventure. Particularly to my Dad and Mum, thank you for taking care of your little boy and never once doubting his choices in life, however unconventional they are. To Kevin and Brian, thank you for being such wonderful brothers. Although miles apart, the constant phone and video conversations kept us close, and you never cease to encourage. To Daniel, my dear friend for the past 20 years, thank you for sharing my convictions and beliefs. Last but not least, to Viola, my dearest wife, the past 4 years have been such a journey, filled with happiness, laughter, tears, and anguish. You are my greatest critic and fan, my trusting confidant, and my lifelong soul mate. Thank you for bringing Elliott into our lives, and the 7th October 2011 will forever remain the happiest day in my life. Your contributions to the fruition of this piece of work is as much as mine, and this thesis is hereby dedicated to you and our son.
Abstract

In this study, we test the hypothesis that an in vivo DNA double-strand break (DSB) repair assay performed in irradiated human skin correlates with severity of late toxicities following breast radiotherapy, as opposed to the same assay performed in ex vivo irradiated blood lymphocytes. Applying a comparative analysis approach, levels of residual DSB were judged to be significantly higher among patients who presented with late adverse effects in their breasts (cases) than controls who had minimal/no effects, but this was only observed in blood lymphocytes. In conjunction with this observation, although DSB repair was comparable between different skin cells, residual DSB levels were not correlated between skin cells and blood lymphocytes of the same patients. These findings suggest that cellular DSB repair may be influenced by factors relating to the tissue microenvironment, and support the notion that blood lymphocytes represent a valid cellular system for testing of predictive markers of normal tissue radiosensitivity. Next, chromosomal radiosensitivity and radiation-induced apoptosis were tested as markers of clinical radiosensitivity among a subset of severe cases and matched controls. Unlike levels of apoptosis in irradiated blood lymphocytes which were comparable between both groups of patients, levels of chromosomal aberrations were significantly higher in irradiated blood lymphocyte metaphases of severe cases, suggesting that likewise to DSB repair, chromosomal radiosensitivity could be a potential marker of clinical radiosensitivity. Crude tests of association were performed to determine if different cellular radiation responses were correlated within the same individuals. Levels of residual DSB were closely related to the formation of excess acentric fragments in the same patients, while separately, induction of apoptosis was found to be independent of DSB repair. Interactions between DSB repair and apoptosis induction were further examined and a mechanistic model linking these radiation responses is proposed.
I

Introduction

I.1 NORMAL TISSUE RESPONSES FOLLOWING RADIOTHERAPY

I.1.1 General principles of radiation-induced effects

Therapeutic efficacy of radiotherapy in the treatment of cancers is determined by its ability to control the primary tumour weighed against the detrimental effects to normal tissues. Based on the principles of radiotherapy planning, it is inevitable that normal tissues surrounding the tumour are irradiated despite the availability of modern radiotherapy techniques capable of greater precision in target irradiation. Depending on the type of tissue, tolerance doses to irradiation vary, and symptoms of radiation-induced injury could range from mild acute effects such as erythema and mucositis in the epithelium to severe late complications including myelitis and necrosis of the spinal cord [1]. Radiation injuries are commonly classified as either acute or late effects. Unlike acute effects which are typically experienced during the course of radiotherapy, late effects occur months to years after completion of treatment, and are irreversible, progressive, and dose-limiting consequences. Rarely, significant aggravation of acute effects can result in the development of consequential late effects [2]. This temporal sequence of clinical changes observed following radiation exposure is assumed to reflect phenotypic differences between tissue systems of differing proliferative kinetics, broadly classified as early (fast) and late (slow) responding tissues. The pathogeneses of radiation effects in these tissues are also represented by different but not mutually exclusive mechanistic models of injury [3]. For example, although primary parenchymal cell injury is responsible for the development of most acute and late effects, damage to the surrounding vasculature is implicated in endarteritis obliterans and atherosclerosis, each responsible for secondary ischemic changes leading to tissue atrophy and fibrosis.

I.1.2 Cellular responses following irradiation of early responding tissues

Although the pathological process of radiation-induced damage begins immediately after irradiation, the onset of frank clinical symptoms in tissues with rapidly proliferating cells such as epithelium of the skin and gastrointestinal tract is only apparent days to weeks later following exposure. This latency corresponds to the turnover duration of superficial differentiated epithelial cells in these early responding tissues, and effects are only observed when depleted progenitors in the deep proliferating layers fail to repopulate the superficial epithelium. In human skin, this loss of cells in
2 Introduction

The basal layer which is dose-dependent, contributes to a dry or moist desquamatory response often associated with an erythematous reaction [4]. Following this early insult, a compensatory repopulation of the basal layer occurs and this is attributed to an increased proliferative capacity of surviving clonogenic cells within the irradiated area (Figure I.1) [5]. The accelerated repopulation of basal cells usually corresponds to the resolution of clinical symptoms approximately 50 days following initial insult [6]. In severe cases where significant losses of basal cells occur following a course of high dose irradiation, non-healing of moist desquamation can potentially progress to necrosis and consequential late effects in the skin [5].

**Figure I.1** Changes in the basal cell population during a 6-week course of skin irradiation in which 2 Gy were given daily except weekends to a total dose of 60 Gy over 42 days. Reprinted from Archambeau et al., 1995 with permission [6].
Hypoplasia of the basal parenchyma as a precipitating mechanism for radiation-induced acute effects occurs primarily through mitotic death or apoptosis of the progenitor cells. Following exposure to ionising radiation, mitotic death occurs as a result of residual chromosomal damage contributing to a loss of cell dividing capacity within the initial or subsequent few cycles of cell division [7, 8]. Apoptosis, on the other hand, follows a sequence of nuclear condensation, fragmentation, phagocytosis, degradation, and is dependent on the ataxia-telangiectasia mutated (ATM)-p53 activation pathway, a key component of the DNA damage response (DDR) [9, 10]. Although it is widely assumed that mitotic death is the predominant mechanism of radiation-induced cell death in the majority of tissues, apoptosis has an unequivocal role in this aspect in certain cell and tissue types. For example, lymphocytes, serous cells of the salivary glands, and proliferating cells within the crypts of the gastrointestinal mucosa have a propensity to undergo apoptosis during interphase following irradiation [11–13]. More recently, the phenomenon of low dose hypersensitivity in basal cells of the human epidermis has been attributed to the induction of growth arrest and apoptosis [14]. Regardless of the mechanism, it is largely acknowledged that cell death following exposure to ionising radiation is often a direct consequence of an inability to repair DNA damage, in particular double-strand breaks (DSB), induced by the production of free radicals secondary to ionisation events within the cell nucleus [15–17].

Inflammatory responses relating to the activation of proteolytic enzymes and proinflammatory cytokines are also induced during the early phases following exposure to ionising radiation [18, 19]. Together with changes in the vasculature and vascular permeability, these processes are involved in the development of tissue inflammation, oedema, and recovery following irradiation. The implication of microvascular endothelial cell death as a precursor to subsequent induction of apoptosis in progenitor cells within the gastrointestinal mucosa further raises the possibility of a potential significance of vascular injury in the pathogenesis of acute effects, arguing against the conventional pathophysiological model of acute gastrointestinal syndrome [20].

I.1.3 Cellular responses relating to late radiation effects

Late effects are characteristic of damage in tissues containing lowly proliferating cellular elements, such as the spinal cord, brain, fatty tissue, and connective tissue. Effects are also diverse, ranging from fibrosis, atrophy to necrosis, with clinical progression and severity strongly dependent on overall radiation dose and fraction size [21]. Commonly, symptoms arise months to years after treatment with the potential to aggravate decades beyond initial onset in a subset of severely affected individuals [22]. This long latency to onset of clinical symptoms has historically been understood to reflect the prolonged cell cycle transition to mitosis, upon which slow proliferating stem cells harbouring residual DSB experience some form of mitotic catastrophe. In the instance of radiation-induced tissue atrophy, the loss of endothelial stem cells through the process of mitotic death or cellular senescence has been postulated as a contributory mechanism for parenchymal cell loss secondary to vascular injury [23].

For some cell types, cellular senescence may be an alternative fate to mitotic death in response to residual DNA damage. Stress-dependent replicative senescence is
associated with telomere shortening, and is induced in human fibroblasts by low levels of radiation-induced DSB [24]. After high therapeutic doses ($\geq$60 Gy), cell replication is severely limited. In the absence of telomere shortening, human fibroblasts enter a phase of prolonged cell cycle arrest resembling senescence, sustained by the expression of p53, p21, and p16 [25, 26]. In the context of late fibrosis, the transformation of mitotic progenitor fibroblasts to a senescent post-mitotic fibrocyte lineage, induced through irradiation, is a key process in the pathogenesis of this late effect. It is well established that these post-mitotic fibrocytes are primarily responsible for the enhanced synthesis and deposition of extracellular matrix proteins leading to the development of radiation-induced fibrosis [27].

Included in the repertoire of cellular radiation responses are a series of paracrine and autocrine signalling events which in addition to the mechanisms of cellular death and senescence, play significant roles in the pathogenesis of late effects. Most notably, transforming growth factor $\beta$1 (TGF\(\beta\)1) produced by a variety of inflammatory, endothelial, and epithelial cell types in response to irradiation, is one of the several cytokines including interleukin-13 (IL-13) and connective tissue growth factor (CTGF), responsible for the activation and expansion of myofibroblast progenitors during the early phases of fibrogenesis [28–30]. Unlike the other cytokines which are largely regulated through gene transcription, active TGF\(\beta\)1 is released following the dissociation of a latency-associated peptide from its latent form, a process catalysed by proteases and reactive oxygen species generated after radiation exposure [31, 32]. Although it is unclear if these mentioned molecular and cellular processes activated days to weeks post-radiotherapy relate to complications manifesting months to decades later, the ability to ameliorate some of these late effects through selective inhibition of these pathways suggests that they are probably integral in the development of late effects [33, 34].

I.1.4 Determinants of acute and late normal tissue responses

Acute and late radiation effects are mostly deterministic, with threshold doses dependent on a spectrum of confounding variables. Broadly, these can be classified under treatment-, organ-, or patient-related factors.

I.1.4.1 Treatment-related factors

Examples of treatment-related factors include total dose and dose per fraction, treatment volume, and the concurrent use of systemic therapy. The conventional wisdom of delivering a high total dose in small fractionated doses was based on the assumption that this would allow for maximal killing of tumour cells while sparing detrimental effects to normal tissues. This pragmatic approach was further supported by findings from early hypofractionation studies where increased rates of normal tissue toxicities were observed from the use of large fraction sizes without the necessary reduction of total treatment dose [35, 36]. From these early clinical studies and empirical radiobiological modelling, it became well established that late effects are generally more sensitive to fraction size and less sensitive to changes in overall treatment time compared to acute effects [37, 38]. Normal tissue responses are also heavily
Radiation responses of normal tissues  5
dependent on total radiotherapy dose as evidenced by the steep dose-response curves
derived for the onset of late effects, implying that a small change in dose is associated
with large differences in toxicities (Figure I.2). This steep dose-response relationship
was nicely illustrated in the results of the UK START Trial A, where based on indirect
estimates generated from a comparison of normal tissue-related outcomes between
different fractionation schedules in the treatment of early breast cancer, a 5.2% effect
on late toxicities was estimated for every 2 Gy change in dose [39]. Separately, volume
of irradiated tissue is also important, with larger volumes more likely to be susceptible
to organ functional damage [40]. Finally, the addition of concurrent systemic therapy
could also potentially exacerbate normal tissue effects since most chemotherapy
agents work by inflicting cellular DNA damage to tumours and normal tissues alike
[41].

Figure I.2 Cumulative frequency dose-response curves illustrating steep dose-gradients for
late effects. The left (dotted) curve shows data for skin telangiectasia [22] and right (solid)
curve is the putative dose-response curve for spinal cord necrosis [42]. Adapted from Barnett
et al., 2009 with permission [43].

I.1.4.2 Organ-related factors
Organ-specific factors relate to regional variability in radiosensitivity and hierarchical
organisation of functional units (serial or parallel) within the organ. The importance
of topographical heterogeneity of radiosensitivity within the organ can be illustrated
by experimental observations of radiation-induced parotid gland dysfunction in
animal models. As reported in rats, a differential effect on salivary production is
observed between irradiation of the cranial and caudal ends of the gland, with the
former being demarcated as the more radiosensitive region [44]. Similarly, in rat models
of radiation-induced heart disease, volume irradiation including the atria of the heart
resulted in cardiac failure at much lower doses than irradiation of the ventricles alone
[45]. Tolerance of normal tissue is also dependent on the functional reserves and
structural organisation of the organ. For example, large doses of radiation delivered to
a small volume of the lung would be far less harmful as opposed to low dose irradiation
to the whole lung. Conversely, a high dose to a small segment of the spinal cord could potentially harbour disastrous consequences whereas low doses to a long segment are largely innocuous.

I.1.4.3 Patient-related factors: Evidence for an intrinsic component in inter-individual variability of normal tissue responses

Patient-related conditions including previous trauma/surgery to the irradiated site and co-morbidities, particularly those associated with impaired vascularity and connective tissue disorders, are potential modulators of radiation effects and wound recovery [46–48]. In the treatment of early breast cancer, the presence of patient co-morbidities such as post-operative breast oedema or hematoma in the setting of high radiotherapy doses significantly influenced the risk of late skin fibrosis [49]. Nonetheless, despite robust control of these treatment- and patient-related variables, as much as 70% of variance in acute and late normal tissue responses between individuals are still unaccounted for [50]. Similarly, observations of acute skin erythema and late telangiectasia in breast radiotherapy patients, having received the same radiation doses and fractionation schedules, revealed significant inter-individual variation in the progression and severity of clinical effects [51, 52]. Collectively, these findings provide supportive evidence to suggest that wide differences in normal tissue responses between individuals are likely due to inter-individual differences in intrinsic radiosensitivity.

The evidence for an intrinsic determinant of normal tissue radiosensitivity dates back to the seminal discovery of the ataxia-telangiectasia (A-T) radiosensitive phenotype [53]. Although severe clinical reactions following radiotherapy and elevated levels of chromosomal damage induced by radiation were observed in patients with this genetic defect [54, 55], it was the seminal finding illustrating the increased radiosensitivity of fibroblast and lymphoblastoid cell lines derived from these patients that provided a direct correlation between genetic susceptibility and clinical radiosensitivity. Since then, through a variety of screening approaches, a number of genetic syndromes, mostly associated with a DDR or DSB repair defect, have been linked to a clinically radiosensitive phenotype (Table I.1). However, intrinsic sources of the wide differences in normal tissue radiosensitivity between non-syndromic individuals remain largely unknown, although high frequency, low penetrance allelic variation and epigenetic modifications have been proposed as possible pathways [43, 56]. Regardless of the genetic or epigenetic source, the concept of a significant intrinsic modulator of normal tissue radiosensitivity proposes the notion that intrinsic radiosensitivity of target cells involved in the cellular processes of early and late effects are perhaps indicative of clinical response in normal tissues after radiotherapy, thereby providing the rationale for a predictive assay of normal tissue radiosensitivity.
I.2 Predicting normal tissue radiosensitivity

I.2.1 Clinical value of predicting intrinsic normal tissue radiosensitivity

Intuitively, the idea of having an assay capable of estimating normal tissue radiosensitivity in a specific patient seems highly appealing. Referring to the proven benefit of improved tumour control with the use of escalated radiotherapy doses in prostate cancers, a possible strategy where predictive testing for normal tissue radiosensitivity could be exploited would be to identify the sensitive tail of the normal distribution of intrinsic radiosensitivities, subjecting these individuals to the conventional or even a reduced dose while treating the remainder with higher doses. This manner of stratifying treatment options as illustrated in Figure I.3, should in theory maintain the overall incidence of normal tissue toxicities and improve therapeutic ratio [61]. Other potential approaches pertaining to the implementation of a predictive assay for normal tissue radiosensitivity include 1) screening for rare individuals with extreme radiosensitivity, treating these cases with a reduced total dose [62, 63], and 2) individualising radiotherapy doses based on the derived in vitro cellular radiosensitivity of each individual such that patients receive an isoeffective treatment with equal likelihood of normal tissue toxicities [64]. Judging from these possible approaches, the clinical benefits of such an assay are self-evident, but the main hurdle remains establishing a reliable and reproducible in vitro assay that is actually clinically applicable. In spite of the confronted challenges to develop a robust assay, the fact that at least 20% of patients are affected by moderate to marked chronic adverse events following radiotherapy reiterates the importance to address this unmet clinical need.

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Protein</th>
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<tbody>
<tr>
<td>Ataxia-Telangiectasia (A-T)</td>
<td>ATM</td>
</tr>
<tr>
<td>A-T-like Disorder (ATLD)</td>
<td>MRE11</td>
</tr>
<tr>
<td>Nijmegen Breakage Syndrome (NBS)</td>
<td>NBS1</td>
</tr>
<tr>
<td>Rad50 defective patient</td>
<td>Rad50</td>
</tr>
<tr>
<td>LIG4 syndrome</td>
<td>Ligase4</td>
</tr>
<tr>
<td>RIDDLE syndrome [58]</td>
<td>RNF168</td>
</tr>
<tr>
<td>RS-SCID</td>
<td>Artemis deficient</td>
</tr>
<tr>
<td>Artemis over-expressed patients (84BR, 175BR cell lines) [59]</td>
<td>Artemis over-expression</td>
</tr>
<tr>
<td>DNA-dependent protein kinase (DNA-PK) exon deletion in a patient with xeroderma pigmentosum [60]</td>
<td>DNA-PK</td>
</tr>
</tbody>
</table>

Abbreviations = MRE11, meiotic recombination element 11; LIG4, Ligase 4; RS-SCID, radiosensitive-severe combined Immunodeficiency.
As a sign of interest on this issue, a global collaborative effort was recently initiated to investigate the suitability of single nucleotide polymorphisms as surrogate markers of normal tissue radiosensitivity [65].

I.2.2 Biological considerations for predictive assays of radiosensitivity

I.2.2.1 In vitro cellular radiosensitivity as a biomarker of clinical response to radiotherapy

Early evidence for a linkage between intrinsic cellular radiosensitivity and normal tissue responses arose from experimental observations of a correlation between in vitro cellular radiosensitivity and clinical phenotype in individuals with rare genetic syndromes. As demonstrated in patients with A-T, germline mutations in the ATM gene resulting in a defective protein and consequential lack of a robust DDR, contributed to an increased radiosensitivity observed in all cells and tissues [62, 66, 67]. Further support for this idea is found in reports describing an increased in vitro radiosensitivity of fibroblasts in non-syndromic patients with an abnormally severe reaction to radiotherapy [68, 69].

Cellular radiosensitivity is often referred to by the clonogenic potential of a cell following exposure to ionising radiation and is measured using the colony forming assay (Figure I.4). To date, assessment of colony forming ability remains widely regarded as the method of choice for determination of mitotic death, a cellular...
response many consider to be indicative of the effectiveness of radiation-induced cell killing. Standard protocols define a colony to consist of at least 50 cells, thereby implying that the surviving progeny had undergone at least five cycles of cell division, an appropriate cut-off considering that cells are still capable of dividing beyond the initial cycle in the presence of chromosomal damage [8]. Using this assay, in vitro radiosensitivity is typically expressed as a function of the number of surviving progenies after a 2 Gy dose (SF2). Alternatively, D0 and D0.1 which refer to the dose required to reduce surviving fraction (SF) to 0.37 and 0.1, respectively, are also commonly adopted parameters of cellular radiosensitivity. Illustrative definitions of these parameters are provided in Figure I.4 (Right panel).

Figure I.4 (Left panel) Clonogenic assay performed using clones of SW-1573 lung tumour cells. Upper panel represents untreated controls with 70 (left) and 115 (right) colonies after seeding 100 and 200 cells, respectively. Lower panel shows effects of 4 Gy irradiation in reducing colony forming ability after seeding 400 (left) and 800 (right) cells. Adapted from Franken et al., 2006 with permission [70]. (Right panel) Survival curve of CNE-1 nasopharyngeal tumour cells after irradiation. SF is derived using the following formula: SF = number of colonies formed after irradiation/(number of seeded cells x plating efficiency). SF2 is indicated by the extrapolated dotted line on the y-axis while the solid line indicates the manner by which D0.1 is derived.

In spite of concerns regarding the practicality of the colony forming assay in the clinical setting, initial series of case-control studies applying this assay for the estimation of in vitro cellular radiosensitivity reported promising results suggestive of a relationship between in vitro cellular and in vivo normal tissue responses among non-syndromic individuals [71–77]. These studies were designed based on the hypothesis that loss of cellular ‘reproductive’ ability or otherwise, known as mitotic death, is the predominant cellular response contributing to the pathology of most acute and late adverse events following radiotherapy. In the instances of epithelial desquamation and tissue atrophy, an association with loss of clonogenicity in parenchymal cells would be plausible. However, as pointed out earlier, cellular
responses other than mitotic death such as senescence, lethal cell cycle arrest, and terminal differentiation may also be relevant to tissue responses like fibrosis [27, 30]. Nonetheless, in these studies, in vitro clonogenic potential of irradiated primary fibroblasts or lymphocytes isolated from skin biopsies and peripheral blood samples, respectively, were revealed to correlate with a range of acute and late effects, mostly in the human skin. Of greater interest, even though fibroblasts are not the primary target cells responsible for the onset of telangiectasia or acute skin erythema and desquamation, in vitro clonogenicity of this cell type was found to be associated with clinical severity of these late and early clinical end-points, respectively [71–73, 76]. In the same vein, cellular radiosensitivity of lymphocytes was also correlated with acute and late effects in the skin and pelvis, although the relevance of this cell type in the pathology of these normal tissue effects is largely unknown [77, 78]. Conceptually, these findings allude to a model where factors generic in all cells and tissues modulate radiation responses within an individual. If indeed intrinsic host factors were the main determinants of normal tissue effects, it would be logical to expect different cells and tissues within an individual (host) to share a similar radiosensitivity phenotype, implying then that radiosensitivity assays performed using different cellular systems should theoretically yield similar predictive power. With the added advantage of tissue accessibility and experimental practicality, these findings provided the scientific basis justifying the use of lymphocyte-based assays in predicting normal tissue radiosensitivity, despite suggestions of clinical and laboratory data contradictory to this model.

I.2.2.2 Inter-cell/tissue variation in cellular and clinical radiosensitivity

The model of a dominant intrinsic component of normal tissue radiosensitivity, though supported by the phenotypes of rare genetic syndromes, remains inconclusive in non-syndromic individuals. For example, although in vitro cellular radiosensitivity of fibroblasts and lymphocytes have been shown to be independently predictive of normal tissue responses among radiotherapy patients, a lack of correlation of in vitro radiosensitivity between these cell types has also been reported, thus questioning if cellular radiosensitivity of one cell type is representative of sensitivity of any other within the same individual [67, 74, 79, 80]. The relationship between different late effects in the same tissue was also examined in patients who received post-mastectomy radiotherapy to the chest wall and axilla. Although a significant intrinsic host factor was identified for a specific clinical end-point, severities of unrelated clinical end-points of late skin telangiectasia and fibrosis were not correlated in the same patients, suggesting that perhaps in non-syndromic individuals, radiation responses in different tissues are not dominated by a common intrinsic pathway [81]. However, there are caveats pertaining to the conclusions of this study. Firstly, it is generally harder to grade for varying degrees of late fibrosis given that it is a more subjective change relative to telangiectasia. Secondly, the study reported considerable inter-individual differences for telangiectasia, but contrastingly, scores for fibrosis were largely comparable between patients. Although it would seem intuitive that these late effects are not related, in truth, not a lot of clinical information is available from this study on fibrosis as a clinical end-point. Given the wider implications of these laboratory and clinical
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findings, it is still at present unclear if predictive assays of radiosensitivity should be designed aimed at a specific tissue or clinical end-point. Nonetheless, utilising a lymphocyte-based predictive assay of clinical radiosensitivity remains a sensible approach as supported by findings of more recent studies indicating an association between radiation-induced cellular responses in lymphocytes and clinical radiosensitivity in a range of normal tissues [82–86].

I.2.2.3 Association between normal tissue and tumour radiosensitivity

Separately, it has been suggested that a predictive assay for normal tissue radiosensitivity may also be extrapolated to estimating tumour response to irradiation. The background of this proposed relationship lies in the notion that tumour cells likely retain the radiosensitivity of their tissues of origin, and hence an association between radiosensitivity of normal tissue and tumour is in theory possible. Early evidence in support of this notion was largely derived from experimental findings in mouse and human cell lines. In the former, comparative analyses of in vitro and in vivo radiosensitivity between the RS-SCID and control mouse models indicated an increased response to ionising radiation in tumours and normal tissues of the RS-SCID phenotype compared to control [87]. Complementing the findings in mice, evaluation of in vitro radiosensitivity of tumour and fibroblast cell lines derived from the same patients revealed an intra-individual association between both cell types [88]. To validate the laboratory findings in mice and human cells, a correlation analysis of local tumour control and normal tissue complications was performed in head and neck cancer patients treated radically with institutional standardised radiotherapy protocols [89]. Although a study design of such nature is unavoidably inherent with statistical biases and treatment-related confounders, nonetheless these investigators reported an associative trend between tumour control and acute normal tissue complications. In a separate study of cervical cancer patients treated radically with radiotherapy, in vitro radiosensitivities of tumour cells and lymphocytes of these patients were found to be independently predictive of late radiotherapy morbidities [90]. If indeed related, the association between tumour and normal tissue radiosensitivity indirectly proposes a significant clinical value for a predictive assay of the latter. For example, in individuals identified to be intrinsically radiosensitive, a reduction in radiotherapy dose would reduce the probability of complications without necessarily diminishing the chances of cure [91].

I.2.3 Assays for predicting normal tissue radiosensitivity

As mentioned above, the early assays of cellular radiosensitivity tested for their predictive value of normal tissue responses were primarily colony forming assays performed mostly in fibroblasts or lymphocytes isolated from skin biopsies or peripheral blood samples of patients, respectively. Using this assay, there is a reasonable amount of evidence to suggest that in vitro cellular radiosensitivities of these cell types are indicative of in vivo normal tissue responses following radiotherapy. However, testing of cellular clonogenic ability is slow, labour intensive, and requires a high level of expertise rendering it unsuitable for routine clinical use. Hence, other alternative
cellular end-points related to loss of clonogenecity have been considered and tested in case-control studies of predicting normal tissue radiosensitivity. Among them, assays of chromosomal aberration frequency, DNA damage, and radiation-induced apoptosis are few which have demonstrated a certain level of correlation with in vivo normal tissue responses.

1.2.4 Chromosomal radiosensitivity assays

1.2.4.1 Correlation between chromosomal damage and cell survival

The mechanistic linkage between chromosomal aberration and cell survival has long been well appreciated. Apart from the early experimental findings by Dewey et al. implicating the lethal effects of ionising radiation to structural damage of the chromatin [7], further evidence for a causative relationship between chromosomal damage and radiation-induced cell death, specifically in the form of a mitotic catastrophe, was contributed by elegant experiments conducted in live cultured mammalian cells. In these reports by Grote et al. and Joshi et al., presence of acentric fragments during mitosis resulted in a loss of proliferation in as many as 80% of cells with the remaining 20% not progressing beyond a further five cycles [92, 93]. Nonetheless, it has also been opined that the effects of radiation-induced cell killing may not be fully explained by the induction of exchange and deletion type aberrations. As reported in a range of rodent tumour cell lines, the induction of dicentrics, centric rings, and acentric fragments failed to account for the overall proportion of inactivated clones following exposure to different sources of ionising radiation, suggesting that perhaps other forms of cellular damage may play an important role in radiation-induced cell killing [94]. In contrast, it was reported in human and rodent fibroblasts that following irradiation, the proportion of cells without lethal chromosomal aberrations corresponded closely to surviving fraction [95, 96]. It is probable that this variation in experimental findings is attributed to differences in physiological characteristics between individual cell types. As evident in the analysis of surviving fraction and chromosomal aberration frequencies in irradiated human lymphocytes, a correlation between chromosomal damage and surviving fraction was only observed after adjusting for the loss of cells occurring through interphase cell death, a mechanistically distinct pathway of cellular demise common in certain cell types [97]. In summary, there is conclusive evidence indicating a direct association between structural chromosomal damage and cellular radiosensitivity in most, if not all, cellular systems, hence justifying the use of chromosomal aberration frequency as an alternative indirect marker of cellular radiosensitivity.

1.2.4.2 In vitro chromosomal radiosensitivity as a predictive marker of normal tissue responses after radiotherapy

Chromosomal aberrations detected using the micronucleus and conventional fixed metaphase assays in irradiated lymphocytes have been shown to correlate with normal tissue radiosensitivity in radiotherapy patients. Specific to chromosomal but not chromatid-type damage, lethal aberrations namely exchanges (dicentrics, centric
rings) and deletions (acentric fragments) are identified using these techniques (Figure I.5). Early evidence suggestive of a relationship between chromosomal and clinical radiosensitivity was observed in breast cancer patients suffering from severe radiotherapy-induced reactions. Using both micronucleus and metaphase assays, these clinical over-reactors were found to have yields of micronuclei and dicentrics in irradiated lymphocytes which were comparable to A-T heterozygotes [98], indirectly suggesting that clinically radiosensitive breast cancer patients are potential carriers of the A-T gene. Although the validity of the latter hypothesis remains questionable given that subsequent genomic analysis of primary cells obtained from small cohorts of over-reactors to radiotherapy had failed to reveal the presence of mutations in ATM or other DDR-related genes [84, 99], nonetheless applying a novel method of stratifying patients according to their in vitro chromosomal radiosensitivity phenotype (Figure I.6), it was demonstrated rather convincingly in more recent case-control studies that this cellular parameter can be applied as a potential predictor of radiotherapy-induced normal tissue effects [84–86].

Figure I.5 (Left) Metaphase spread prepared using conventional techniques illustrating exchanges (closed symbols) and deletions (open symbols). (Right) Example of a micronucleus seen in the bi-nucleate cell on the left. Micronuclei are representative of acentric fragments or lagging chromosomes formed during anaphase which are not included in the nuclei of the daughter cells. Courtesy of Dr. Jayne Moquet for the micronucleus image.

1.2.5 Assays of DNA damage responses

1.2.5.1 Overview of cellular responses related to ionising radiation-induced DNA damage

Following exposure to ionising radiation, exogenous free radicals produced through a series of ionisation events within the tissue are a major contribution to DNA damage within the cell nucleus. Among the array of DNA lesions induced by ionising radiation,
it is generally agreed that DSB are the most lethal. It is therefore paramount that cells repair DSB accurately as failure to do so can result in mutations or genetic rearrangements, and indeed, a single DSB is sufficient to disrupt the genomic integrity and kill a cell [100, 101]. In response to the induction of a DSB, a complex and co-ordinated set of signalling pathways involving DNA damage sensing, cell cycle arrest, repair, and cell death is triggered within the cell. This chain of events is often collectively termed as the DDR (Figure I.7).

One of the key aspects of these DNA damage signalling events includes the inhibition of cell cycle progression while damage persists, so as to prevent the replication of damaged DNA or segregation of damaged chromosomes during mitosis. ATM, a member of the phosphatidylinositol 3-kinase-related kinases (PI3KK) superfamily, plays a central role in mediating these responses following its recruitment to DSB sites by the MRN complex, comprising of MRE11, Rad50, and NBS1 [103, 104]. A key substrate for ATM is the histone 2A variant (H2AX) which, upon phosphorylation, leads to the formation of γH2AX foci [105]. Phosphorylation of H2AX contributes to the recruitment of mediator and additional damage response proteins, such as mediator of DNA damage checkpoint protein 1 (MDC1) and tumour suppressor p53 binding protein 1 (53BP1), to DSB sites [106]. Additionally, amplification of the DDR signal occurs through MDC1-mediated accumulation of ATM at these sites of damage, facilitating further phosphorylation of H2AX [107].

![Figure I.6](image-url)

**Figure I.6** Association between chromosomal radiosensitivity (acentric fragments) and grade 2 or 3 fibrosis in breast cancer patients ascertained using the log rank test. Patients were stratified according to their *in vitro* chromosomal radiosensitivity, as opposed to clinical phenotype. High sensitivity, ≥ Mean + 1 SD lethal aberrations per lymphocyte after 6 Gy; low/intermediate, < Mean + 1 SD; mean = 5.47, SD = 0.71. SD = standard deviation. Adopted with permission from Hoeller et al., 2003 [86].
regulation of cell cycle checkpoint, activation of ATM leads to the downstream phosphorylation of checkpoint kinase 2 (Chk2) and p53 [108]. While Chk2 functions mainly via its subsequent phosphorylation of cell division cycle 25 (Cdc25) phosphatases, p53 is involved in the transcriptional regulation of p21, a cyclin-dependent kinase (Cdk) inhibitor which modulates G1/S checkpoint arrest. Likewise, ATM-mediated activation of Chk2 and p53 also function redundantly in the induction of apoptosis as a response to unreppaired radiation-induced DNA damage. Perhaps, of

Figure I.7 Schematic representation of how cells respond to DSB. The presence of DSB is recognised by various sensor proteins which in turn initiate a range of downstream signalling pathways that impact a wide variety of cellular processes. Reused with permission from Jackson, 2009 [102].
a greater significance to cellular radiosensitivity, it has been proposed that ATM is required for the repair of approximately 15% of DSB induced by ionising radiation [109]. More recently, mechanistic insights on this ATM-dependent DSB repair indicate that these breaks likely represent damage within the heterochromatin and ATM signalling via KRAB-associated protein 1 (Kap1) overcomes the barrier to DSB repair posed by the more condensed heterochromatin structure [110, 111]. Figure I.8 provides a schematic representation of the recruitment of ATM and ATM-related proteins at DSB sites and their roles in modulating checkpoint control during various phases of the cell cycle.

It is evident from the above mentioned molecular pathways that the prime objective for cells experiencing DSB is to initiate a series of cellular responses conducive for the repair of these toxic lesions. In higher eukaryocytes, DSB repair is known to occur via two largely distinct and complementary pathways, namely non-homologous end-joining (NHEJ) and homologous recombination (HR) [115]. Whilst NHEJ remains the primary mechanism of DSB repair in most circumstances, HR plays a significant role in repair during S and G₂ phases of the cell cycle [116]. Given their relevance to the discussion of this thesis, these DSB repair pathways are further elaborated in the following section.

I.2.5.2 NHEJ and HR in the repair of ionising radiation-induced DSB

NHEJ, as mentioned above, represents the major DSB repair pathway in mammalian cells. Minutes after the induction of DSB, repair takes place primarily via end-joining while other damage signalling processes relating to checkpoint control, onset of apoptosis, and chromatin remodelling initiate simultaneously in the background [117]. The core proteins involved in NHEJ include the Ku sub-units, DNA-PK, LIG4, and its co-factor, X-ray cross complementation group 4 (XRCC4). Together with the MRN complex, Ku70/80 are among the earliest factors rapidly recruited to the ends of DSB [118]. This is soon followed by binding of DNA-PK, generating the DNA-PK holoenzyme complex resulting in activation of its kinase activity [119]. Broken ends of DNA are then acted upon by nucleases, most notably, Artemis, before being ligated by the XRCC4-like factor (XLF)-XRCC4-LIG4 complex [120, 121]. Interestingly, in contrast to defects in other NHEJ core proteins, Artemis-defective cells continue to repair most DSB efficiently which may suggest that Artemis-dependent end-processing is not an integral step in NHEJ [122]. Recent evidence however revealed that Artemis is required in the repair of a small subset of DSB (=15%) and current thinking points to a role for Artemis in the end-processing of breaks located within the heterochromatin [109, 123].

Unlike in NHEJ, initial resection of DSB ends is mandatory in HR, and remains an important regulatory step in the choice between both pathways for the repair of DSB. This resection process is performed by the MRN complex, in conjunction with auxiliary factors, such as C-terminal binding protein interacting protein (CtIP), RECQ family helicases, exonuclease 1 (Exo1), and Dna2 [124–126]. The resulting single strand DNA (ssDNA) overhangs are then coated by ssDNA binding complex replication protein A (RPA) before being replaced by RAD51, promoting invasion of a homologous template on the sister chromatid strand [127]. This need for a homologous template
Figure I.8 (Top) ATM activation is initiated by the recruitment of MRN to sites of DSB. ATM is also recruited to the flanks of the break where at these flanking sites, ATM is partially activated and phosphorylates p53 along with other substrates. Acetylation also contributes to the activation process of ATM. MDC1 plays a central role in protein assembly at DSB sites. It binds γH2AX, NBS1, and recruits RNF8, an E3 ubiquitin ligase which catalyses the ubiquitylation of H2A-type histones, promoting the recruitment of 53BP1 to the site of DSB [112, 113]. RNF168, also an ubiquitin ligase, amplifies this ubiquitylation process and stabilises the DDR protein complex [58, 114]. Adapted with permission from Jeggo et al., 2009 [57]. (Bottom) ATM regulation of checkpoint arrest at different phases of the cell cycle. Arrows indicate stimulation; T-shape lines mark inhibition; inhibitory phosphorylations are indicated by a line through the arrow. The numbers indicate the positions of the phosphorylated residues. Reprinted with permission from Shiloh, 2003 [10].
also explains the availability of HR only during S and G₂ phases of the cell cycle. The search for homology and strand invasion is largely assisted by a protein complex comprising of XRCC2, XRCC3, Rad52, Rad54, Rad51 paralogs b, c, d, breast cancer 1 and 2 (BRCA1, BRCA2) [128]. Following DNA synthesis and ligation by DNA polymerases and ligase 1, respectively, DNA helicase and resolvase enzymes then mediate cleavage and resolution of HR intermediates to yield intact, repaired DNA. Judging from the processes in HR, it is evident that compared to NHEJ which is prone to erroneous repair, HR repairs DSB with a greater degree of fidelity. A schematic illustration of both repair pathways is provided in Figure I.9.

I.2.5.3 Biological principles underlying the association between in vitro DNA damage responses and normal tissue radiosensitivity

The ability to repair DSB is imperative for cell survival as evidenced by the increased radiosensitivity observed in cells of syndromic individuals harbouring an intrinsic

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**Figure I.9** (A) NHEJ, (B) HR. See main text for details of the individual pathways. Reprinted with permission from Downs et al., 2007 [129].
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DSB repair defect [130, 131]. Among non-syndromic patients displaying significant inter-individual variation in DSB repair, a similar association was also observed between residual DSB levels and loss of clonogenicity, confirming once again that these cellular responses are related [15, 132–134]. These findings led to the interpretation that assays of DNA damage may be a sensible alternative to the more cumbersome colony forming assay for predicting normal tissue radiosensitivity [61]. Early methods for measuring DNA damage were predominantly based on pulsed field electrophoresis or the comet assay. In more recent times, the discovery of damage-induced foci formation at DSB sites such as γH2AX and 53BP1 along with their close correlation with DSB repair kinetics has led to their progressive implementation as surrogate markers of DSB [135, 136].

To date, clinical and experimental findings arising from a number of small case-control studies as well as mouse models have suggested a promising correlation between residual DNA damage and normal tissue radiosensitivity, regardless of the DNA damage assay being applied [137–142]. Of interest, high levels of residual DSB following irradiation and repair in human fibroblasts and blood lymphocytes were observed in individuals presenting with severe acute and/or chronic effects of radiotherapy [137–140]. The presence of residual DNA damage late into repair may hold mechanistic significance, particularly in the pathogenesis of normal tissue injury following fractionated radiotherapy. In the therapeutic setting where radiation is conventionally delivered in small equal daily doses over a protracted period, unrepaired genomic damage prior to the next fraction could lead to further accumulation of damage with each additional fraction, a phenomenon well documented in studies of fractionated irradiation in a number of tumour and normal tissue cell lines [143, 144]. It is therefore likely that subtle impairments of DNA repair could amount to exceedingly high levels of cellular genomic damage following repeated radiotherapy fractions, thereby potentially having a profound impact on subsequent cell fate and normal tissue responses. On the basis of this notion, it would be within reason to propose an association between intrinsically defined DNA repair capacity and normal tissue radiosensitivity of an individual.

Radiation-induced apoptosis appears to be another promising cellular marker associated with clinical radiosensitivity. By quantifying the levels of apoptosis in blood lymphocytes following in vitro irradiation, investigators have reported that low levels of apoptosis are indicative of overt clinical radiosensitivity, while high levels of apoptosis are specific for normal responders to radiotherapy (Figure I.10) [82, 83, 145–147]. As illustrated in Figure I.11, considering that apoptosis is primarily an ATM-mediated cellular response to ionising radiation, these findings could be interpreted to fit the A-T phenotype, where impaired apoptotic response and clinical radiosensitivity are both characteristic features associated with a loss of ATM function [147]. In further support of this notion, a recent quantification of ATM protein levels within a cohort of non-syndromic breast radiotherapy patients demonstrated an association between low levels of ATM and clinical radiosensitivity [148]. Although the mechanistic basis of increased cellular and clinical radiosensitivity among patients harbouring the A-T phenotype remains uncertain, lately, the inability to repair DSB located within the heterochromatin has been postulated to be a contributing factor [110, 111]. Judging from the clinical data presented in the studies demonstrating an association between apoptosis induction in irradiated lymphocytes and late adverse effects following...
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Radiotherapy, it is likely that other mechanistic models besides A-T may account for the relationship between these cellular and clinical end-points. For example, decreased levels of apoptosis in patients with marked adverse effects could reflect an impaired response mechanism to cellular injury. Separately, it has also been proposed that...

Figure I.10 Cumulative incidence of grade ≥ 2 late adverse effects according to percentage of CD8 T-lymphocyte apoptosis 48 hours after 8 Gy *in vitro* exposure. Upper solid curve, ≤ 16% apoptosis; middle dashed, 16-24%; lower dashed, > 24%. From Ozsahin et al., 2005 with permission [83].

Figure I.11 Apoptotic responses of CD8 and CD4 T-lymphocytes from three cohorts of donors. +, 105 normal donors and 48 radiotherapy patients without late effects; •, 12 radiotherapy patients with late effects; ×, 9 A-T homozygotes. Data are presented as z-scores (number of standard deviations from the mean). Note that patients with adverse effects tend to have lower than average z-scores. From Crompton et al., 1999 with permission [147].
apoptotic cells possess the ability to suppress pro-inflammatory signals associated with tissue injury provoked by radiation exposure [149, 150]. In these models, the relative association between radiation-induced apoptosis and normal tissue radiosensitivity as reported in these studies would be expected.

A comprehensive review of current literature indicates that there are now a range of possible assays for predicting risks of normal tissue effects in radiotherapy patients. As already elaborated, the scientific hypotheses underlying these assays are distinct but not mutually exclusive. In this background, an interesting approach to predicting normal tissue radiosensitivity could entail testing a combination of these assays in a cohort of radiotherapy patients presenting with varying degrees of normal tissue injury. In addition to comparing the robustness of each assay in predicting clinical response, a study of such nature also offers potential insights into the common molecular pathways between cellular and tissue responses following exposure to ionising radiation. For example, current evidence collectively indicates that molecular processes relating to DNA repair and induction of apoptosis may be impaired in clinically radiosensitive individuals [82, 83, 138-141, 145–147]. In this scenario, ATM functionality can be interpreted to be a central determinant of normal tissue injury following radiation exposure, even among non-syndromic individuals, since ATM shares critical roles in both DNA repair and apoptosis induction [10, 109–111].

I.3 REFERENCES

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II

Project overview

II.1 BACKGROUND

As reviewed in the preceding chapter, there is clear evidence to support the notion of an intrinsic determinant of normal tissue radiosensitivity. Over the years, it has therefore been the interest of several research groups to establish in vitro cellular markers which are indicative of radiosensitivity of cells/tissues and consequently, predicts for normal tissue responses following curative radiotherapy. To date, colony forming assay remains the gold standard for assessing cellular radiosensitivity, but owing to its time consuming nature, surrogates assays have been investigated. These include assays of chromosomal and DNA damage, and between the different assays, there are modest levels of correlation with colony formation.

At first glance, early evidence arising from small case-control studies testing these assays to predict for normal tissue radiosensitivity appeared promising. Comparing the in vitro radiosensitivity of cells from carefully selected clinically radiosensitive individuals against those of matched controls, an apparent disparity in cellular responses to radiation exposure was evident between patients presenting with differing degrees of normal tissue effects after radiotherapy. Nonetheless, when a similar hypothesis was extrapolated to larger patient cohorts, results from these wider comparisons have mostly been inconclusive, prompting suggestions that these assays may not be clinically applicable for stratifying radiotherapy patients and perhaps more robust new methods should be sought. On this note, it is appropriate to highlight that a number of other key factors are also contributory to the outcomes of studies testing for predictive assays of normal tissue radiosensitivity. Broadly, these factors relate to patient selection and biological principles underlying the design of these predictive assays.

II.1.1 Clinical considerations in the selection of ‘over-responders’ and ‘controls’

The ability to demonstrate an association between results of an in vitro radiosensitivity assay and the degree of normal tissue damage relies significantly on the accurate identification of clinical phenotype. Although it may not be possible to fully ascertain that the clinical severity of normal tissue effects truly reflects the intrinsic radiosensitivity of an individual, nonetheless clinical parameters that could influence normal tissue response, such as radiation dose, fraction size, the addition of systemic therapy as a radiosensitising agent, have to be considered during patient stratification. Secondly, the duration of follow-up could also affect the relative relationship between cellular and clinical end-points. Based on the work by Jung and colleagues, it is well characterised that the incidence of late effects in several organs occurs with exponential kinetics, and
in some individuals, these effects may only manifest years after radiotherapy [1]. Conversely, individuals presenting early on with late effects do not necessarily imply clinical radiosensitivity. On this basis, it may be prudent to test the chosen cohorts of ‘over-responders’ and ‘controls’ for their rates of developing late tissue effects using the log-linear plots proposed by Jung et al. to confirm that they are indeed phenotypically distinct prior to testing of predictive markers.

Ideally, investigations into the efficacy of an in vitro cellular assay to predict for normal tissue radiosensitivity should preferably include homogenously treated patients with accurate documentation of treatment-related parameters relevant to the normal tissue of interest. The clinical end-point should be amendable to accurate assessment using a well established method of scoring, and should also have a component of significant inter-individual variation [2]. For reasons provided above, patients should also have consistent follow-up periods to ensure that their clinical ranking corresponds to the assigned phenotype [3]. Finally, factors other than the cellular parameter being tested that are known to influence normal tissue responses should be incorporated into a multivariate analysis, but in reality, this is often limited by the small scale of these studies given the low prevalence of clinically radiosensitive individuals.

II.1.2 Are cell-based assays valid systems for predicting normal tissue radiosensitivity?

Pertaining to the design of a predictive assay of normal tissue radiosensitivity, a frequently challenged notion revolves around choosing an ideal cellular system for a predictive assay. The idea for a cell- and tissue-specific approach to predictive assays of normal tissue radiosensitivity was initially put forth in the background of clinical evidence indicating a lack of association between the severity of different normal tissue effects within an individual, therefore proposing that perhaps predictive assays should be designed aimed at a specific tissue/clinical end-point [4–6]. Further support for this idea can be derived separately from laboratory data showing that radiosensitivities of different cell types within the same host are not necessarily correlated, indirectly implying that depending on the choice of cell-based assay, predictive potential of normal tissue radiosensitivity may differ [7–9]. However, arguing against this concept, positive outcomes have been observed in studies using either fibroblasts or lymphocytes to test for biomarkers of radiosensitivity in a range of normal tissues, but again, the results of such an approach have not been consistent [10–22]. The conflicting evidence arising from these studies utilising a cell-based approach has indirectly led to doubts of whether in vitro cellular responses are indeed an accurate indicator of in vivo tissue pathology given the potential modifying influence of the tissue environment [23, 24].

II.2 AIMS AND OUTLINE

This thesis describes a body of work performed in human skin tissues and blood lymphocytes of 35 individuals who displayed either moderate/marked (cases) or
minimal/no radiation-induced (controls) late changes in their breast following previous breast radiotherapy. Briefly, these patients were carefully selected from a group of previous participants of two breast radiotherapy trials, where radiation dosimetry and delivery of breast radiotherapy had withstood quality assurance, and patients were monitored annually for radiation-induced changes in the breasts [25, 26]. For the first part, DSB repair was assessed using 53BP1 immunohistochemistry in human skin tissues which had been exposed to ionising radiation in vivo. As a unique aspect of this study, patients were subjected to a small test dose of 4 Gy delivered using electrons to a small area over their lower back skin. With strict dosimetric verification, a uniform radiation dose was delivered to the epidermis and dermis of the skin, thus ensuring that any biological variation observed between individuals or cells was not a result of variation in radiation dose delivery. Duplicate biopsies of irradiated and unirradiated skin were then obtained 24 hours following irradiation, and residual 53BP1 foci levels were compared between different epidermal and dermal cell types in skin sections of these 35 patients. This analysis was primarily undertaken to determine if either cell-specific physiological factors or host-specific genetic or epigenetic factors play a greater role in modulating DSB repair in vivo.

Next, we proceeded to test if inter-individual variation in DSB repair could account for differences in clinical presentation of late normal tissue effects among breast radiotherapy patients. To this end, residual 53BP1 foci levels 24 hours after 4 Gy were assessed in in vivo irradiated skin tissues and ex vivo irradiated blood lymphocytes of the same patients, and mean residual 53BP1 foci levels of skin cells and lymphocytes were compared between cases and controls. The ability to measure in vivo cellular responses to ionising radiation in our skin model presents the opportunity to compare if cellular responses derived in vivo and ex vivo, given the contribution of tissue microenvironment in the former, produce similar levels of correlation with clinical phenotype. Separately, within this analysis, we also test the hypothesis that a cellular assay based on skin cells better predicts clinical responses in the human skin, as opposed to the same assay performed in blood lymphocytes.

Having then established lymphocytes as a valid cellular system for predicting normal tissue radiosensitivity, at least for late effects in skin, we performed further tests for biomarkers of clinical radiosensitivity using lymphocytes of a subset of eight severe cases from our original cohort of patients. Specifically, we examined if incremental responses of in vitro chromosomal radiosensitivity and radiation-induced apoptosis were indicative of overt and normal clinical responses to radiotherapy, respectively, as suggested by published literature [14–18]. In the attempt to draw the maximum difference in cellular responses between clinically radiosensitive individuals and controls, we consciously selected only the most clinically severe cases and controls with minimal reactions, particularly those who have had a lengthy follow-up, for this subgroup analysis. Based on these experimental findings, we also examined the relationships between chromosomal damage, induction of apoptosis, and residual DSB through crude correlation analyses. In particular, we designed specific experiments using small molecule inhibitors of ATM and DNA-PK to further characterise the mechanistic relationship between DSB repair and induction of apoptosis following radiation exposure. Finally, we identified a clinically radiosensitive individual among our cases who presented with extremely high levels of residual DSB and chromosomal...
damage in her skin tissues and blood lymphocytes. In this individual, preliminary experiments were conducted to determine the nature of DSB repair defect and involved molecular pathway.

Following decades of unsuccessful research in the field of predicting normal tissue radiosensitivity using cell-based assays, this study was designed primarily with the aim of comparing a predictive assay based on an in vivo skin model against the historical approach of in vitro cell-based assays, in this case, blood lymphocytes. In addition to evaluating DSB repair as a potential biomarker of clinical radiosensitivity, we aimed to establish that in vivo cellular responses in skin predict for late adverse skin effects of radiotherapy and may well be the appropriate approach for predictive assays of normal tissue radiosensitivity going forward. Although limited by the small number of study participants, this was overcome by a meticulous selection process of cases and controls, taking into account several clinical parameters which could have contributed to their clinical phenotype. Judging from our study findings as detailed in the following chapters, we were able to derive a number of interesting hypotheses and conclusions regarding the biology of late normal tissue effects following radiotherapy.

II.3 REFERENCES


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III

Original research

III.1 INTER-INDIVIDUAL AND INTER-CELL TYPE VARIATION IN RESIDUAL DNA DAMAGE AFTER IN VIVO IRRADIATION OF HUMAN SKIN

III.1.1 Abstract

The aim of this study was to compare inter-individual and inter-cell type variation in DSB repair following in vivo irradiation of human skin. Duplicate 4 mm core biopsies of irradiated and unirradiated skin were collected from 35 patients 24 hours after 4 Gy exposure using 6 MeV electrons. Residual DSB were quantified by scoring 53BP1 foci in dermal fibroblasts, endothelial cells, superficial keratinocytes, and basal epidermal cells. Coefficients of inter-individual variation for levels of residual foci 24 hours after in vivo irradiation of skin were 39.9% in dermal fibroblasts, 44.3% in endothelial cells, 32.9% in superficial keratinocytes, and 46.4% in basal epidermal cells (p < 0.001, ANOVA). In contrast, the coefficient of inter-cell type variation for residual foci levels was only 11.3% in human skin between the different epidermal and dermal cells (p = 0.034, ANOVA). Foci levels between the different skin cell types were correlated (Pearson’s R = 0.855-0.955, p < 0.001). Collectively, these findings suggest that patient-specific factors appear to be more important than cell type-specific factors in modulating DSB repair following in vivo irradiation of human skin.

III.1.2 Introduction

Wide inter-individual variation in late-onset normal tissue damage has been reported after radiotherapy for early breast cancer delivered under very carefully controlled conditions, suggesting that factors intrinsic to the individual, including genetic determinants of cellular and tissue radiosensitivity, are responsible in explaining clinical responsiveness [1]. Apart from a limited number of rare genetic syndromes, the source of inter-individual variation in the majority of non-syndromic patients is unknown, but high frequency, low penetrance polymorphic alleles in a wide range of genes are potential candidates [2]. Whether sources of variation are genetic or epigenetic in origin, they might influence responses in all cells and tissues, as in A-T, or display some form of cell or tissue specificity, depending on the molecular pathway involved. After radiotherapy for early breast cancer, Bentzen et al. reported a lack of correlation between the frequency and severity of skin fibrosis and telangiectasia within patients [3]. A similar result was also reported by Tucker et al., who presented a lack of correlation between acute and late skin responses following post-mastectomy radiotherapy [4]. Collectively, these findings suggest relative differences in
radiosensitivity between different cell types, at least in the skin of an individual. These clinical findings are consistent with a lack of correlation between in vitro radiosensitivity of dermal fibroblasts, epidermal keratinocytes, and blood lymphocytes from the same patients [5–7]. In non-syndromic patients, the combined clinical and laboratory findings suggest sources of inter-patient variation in radiosensitivity that are cell type- or tissue-specific rather than generic to an individual.

The ability to repair DSB has been shown to correlate with cellular radiosensitivity in rare hyper-radiosensitivity syndromes and non-syndromic healthy controls [8–10]. It is however unclear if differences in intrinsic radiosensitivity between different cells of normal tissues are explained by differences in DSB repair kinetics. While several studies have reported cell-specific and differentiation-dependent DSB repair kinetics [11–14], a recent study of in vivo irradiated mouse tissues indicated similar initial DSB yields and repair kinetics for a range of different cells [15].

Early DSB repair signalling involves the phosphorylation of histone variant H2AX. Using immunofluorescence microscopy for the phosphorylated form of H2AX, discrete nuclear foci can be visualised at sites of DSB [16]. Foci of γH2AX and 53BP1, which are recruited in large numbers to sites of DSB [17, 18], are now widely accepted surrogate markers for DSB induction and repair [19, 20]. In this study, we used 53BP1 immunohistochemistry to compare residual DSB between different cell types in irradiated human skin from 35 breast cancer patients. The hypothesis under test was cell-specific physiological factors, in addition to intrinsic host factors, modulate repair of DSB in skin tissues of different individuals.

III.1.3 Materials and methods

III.1.3.1 In vivo irradiation of skin of breast cancer patients

Breast cancer patients were identified following local tumour excision and post-operative radiotherapy to the whole breast for early invasive ductal carcinoma. Scientific and ethical approval were obtained from research ethics committees at all participating centres, and written informed consent was obtained from patients prior to participation. A single test dose of ionising radiation was delivered to a $4 \times 2$ cm$^2$ area of buttock skin at The Royal Marsden Hospital using a purpose-built endframe cutout. This incorporated a 7 mm Perspex filter to ensure dose homogeneity to the epidermis and dermis of the skin (Figure III.1). A single 4 Gy dose was delivered to 4 mm depth below the skin surface at a dose rate of 4 Gy/minute with 6 MeV electrons using a linear accelerator. At 24 hours following irradiation, duplicate 4 mm punch biopsies were obtained from both irradiated and contralateral unirradiated skin. The biopsies were taken from the centre of the irradiation field to ensure dosimetric consistency. Tissues were then fixed in 10% neutral buffered formalin for 24 hours, embedded in paraffin, and sectioned at an average thickness of 5 μm.

III.1.3.2 53BP1 immunohistochemistry in skin sections

To ensure accurate morphological identification of the different cell types and avoid dermal collagen auto-fluorescence with immunofluorescence techniques, chromogenic
staining method was chosen for 53BP1 immunohistochemistry. 53BP1 was preferred to γH2AX as a marker for DSB as foci obtained using 53BP1 immunostaining were more consistent in staining quality and easier to score in skin sections. Tissue sections were heated to 60°C for 10 minutes, followed by de-waxing, and rehydration in a graded series of alcohol. Following microwave treatment in 10 mM citric acid (pH titrated to 6.0), DAKO peroxidase and protein blocks (DAKO, Cambridgeshire, UK) were applied onto sections for 5 and 2 minutes, respectively. Tissue sections were then incubated for 90 minutes with 53BP1 rabbit polyclonal antibody (IHC-00001, Bethyl Laboratories, Universal Biologicals, Cambridge, UK) diluted to 1:400. The primary antibody was detected using DAKO Envision kit after a further three washes with Tris-buffered saline. The slides were then lightly counter-stained with Gill's No. 1 haematoxylin and mounted in DPX for analysis under light microscopy at 600× magnification. 53BP1 nuclear foci were visually counted in dermal fibroblasts, endothelial cells, and epidermal cells, with 50 to 100 cells scored for each cell type per biopsy. Heat shock protein 47 (Hsp47, Stressgen, York, UK, 1:200) and CD31 (CD31-1A10, Novocastra, Newcastle Upon Tyne, UK, 1:150) immunostaining were used in the initial stages on serial sections to practise and confirm the histomorphological identification of dermal fibroblasts and endothelial cells, respectively (Figures III.2A, D). Basal and suprabasal epidermis were considered separately using the following criteria, 1) superficial keratinocytes (suprabasal epidermis) defined as cells residing in the junction of stratum granulosum and spinosum, 2) basal epidermal cells were keratinocytes in the stratum basale (Figures III.2G, H).

III.1.3.3 Statistical methods

Inter-cell type and inter-individual variation in foci levels were tested using one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests. Inter-biopsy variation was tested using the Paired T-test. Pearson’s correlation method was used to test for correlation in foci levels between the different cells. All statistical calculations were performed using the statistical software SPSS version 15.0 (SPSS, Inc., Chicago, IL). To
account for multiple statistical testing, statistical significance was set at a p-value of < 0.01.

III.1.4 Results

III.1.4.1 Patients

35 breast cancer patients were enrolled into the study. Ages of all patients ranged from 52 to 83 years, with the median age being 68.5 years.

III.1.4.2 Quantification of 53BP1 foci in different skin cell types

Examples of 53BP1 immunostaining in skin sections are illustrated in Figure III.2. Using 53BP1 immunohistochemistry, multiple 53BP1 foci could be visualized in the nuclei of dermal fibroblasts (C), endothelial cells (F), superficial keratinocytes, and basal epidermal cells (H) in the irradiated skin sections. Few, if any, foci were seen in unirradiated dermal fibroblasts (B), endothelial cells (E), and superficial keratinocytes, other than in basal epidermal cells (G) where foci were more frequent. Mean background foci levels per cell in unirradiated skin were 0.48 in dermal fibroblasts, 0.37 in endothelial cells, 0.27 in superficial keratinocytes, and 2.18 in basal epidermal cells.

![Figure III.2 Immunohistochemical staining of 5 μm-thick skin sections from formalin-fixed, paraffin-embedded skin biopsies obtained 24 hours after treatment with 4 Gy. Hsp47 staining in dermal fibroblasts (A) and CD31 staining in endothelial cells (D). 53BP1 staining in unirradiated and irradiated dermal fibroblasts (B, C), endothelial cells (E, F), superficial keratinocytes, and basal epidermal cells (G, H). Grey arrows indicate superficial keratinocytes and black arrows indicate basal epidermal cells.]
III.1.4.3 Correlation of 53BP1 foci quantification in duplicate skin biopsies

To test for possible sources of variation in radiation dosimetry, tissue collection, fixation, block preparation, tissue sectioning, immunostaining, and/or foci scoring, quantification of 53BP1 foci for the different cell types was repeated in duplicate skin biopsies from the same patients. Figure III.3 shows similar levels of 53BP1 foci in both biopsies ($p = 0.378$, Paired t-test; Pearson’s correlation $R = 0.944$, $p < 0.001$). The high correlation validates the above procedures, which were performed independently on duplicate core biopsies.

![Figure III.3 Correlation of 53BP1 foci levels in duplicate biopsies obtained 24 hours after 4 Gy exposure. Each circle represents average foci per cell for a single cell type in a single patient. Trendline was generated using linear regression and intercepted the y-axis at 0.23 with a corresponding $R^2$ value of 0.891.](image)

III.1.4.4 Inter-individual variation in residual foci levels

Table III.1 lists patient-specific levels of uncorrected 53BP1 foci scored for all the different cell types at 24 hours after 4 Gy irradiation. These levels varied significantly between patients, with coefficients of inter-individual variation of 39.9% for dermal fibroblasts, 44.3% for endothelial cells, 46.4% for basal epidermal cells, and 32.9% for superficial keratinocytes after normalizing against background foci levels in unirradiated skin ($p < 0.001$, one-way ANOVA for all cell types).

Patients in Table III.1 were ordered according to ascending levels of foci measured in dermal fibroblasts. Average foci levels were divided into quartiles for each cell type and colour codes applied as follow; white for the 25% of patients with lowest number of foci, and increasing shades of grey for higher quartiles. Mostly, colour codes tend to correspond for dermal fibroblasts, endothelial cells, and epidermal cells.

In view of the patient-specific clustering of quartiles of foci levels across different skin cell types at 24 hours (Table III.1), a test of association of foci levels between the
different cell types within the same patients was performed (Table III.2). Positive correlation for foci levels was observed between all the skin cell types from the same patients. 

Table III  153BP1 foci levels in skin measured 24 hours after 4 Gy delivered using 6 MeV electrons with full build-up. At least fifty cells were scored for each cell type per patient and repeated in duplicate biopsies. Indicated foci levels represent averages of duplicate biopsies, and are colour-coded in quartiles of increasing shades of grey.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Dermal fibroblasts</th>
<th>Endothelial cells</th>
<th>Basal epidermal cells</th>
<th>Superficial keratinocytes</th>
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Table III.2 Correlation of patient-specific foci levels between the different skin cell types at 24 hours after a single dose of 4 Gy to human skin using 6 MeV electrons.

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<th>Patients</th>
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<td>Endothelial cells</td>
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<td>p-value (2-tailed)</td>
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</tr>
<tr>
<td>Basal epidermal cells</td>
<td>Pearson correlation 0.855</td>
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<td>p-value (2-tailed)</td>
<td>35</td>
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III.1.4.5 Inter-cell type variation in residual foci levels

Comparable levels of patient-average residual foci 24 hours after 4 Gy irradiation were observed in four epidermal and dermal cell types. After normalizing against background foci levels, mean foci per cell were 3.14 in dermal fibroblasts, 3.06 in endothelial cells, 2.51 in superficial keratinocytes, and 2.63 in basal epidermal cells \((p = 0.034, \text{one-way ANOVA, Figure III.4})\).

![Figure III.4](image)

**Figure III.4** Patient-averaged 53BP1 foci levels in different cell types. Foci levels were normalized against background levels from the same patients to derive levels of residual damage. Error bars indicate one standard deviation of foci levels for the whole cohort.

### III.1.5 Discussion

Significant variation between women in the level of residual 53BP1 foci 24 hours after a single *in vivo* dose of 4 Gy to healthy skin was observed in this study of 35 former breast cancer patients. The coefficients of inter-individual variation for the different skin cell types ranged from 32.9% to 46.4% in this cohort of patients and are generally comparable with previous studies \([10, 21]\). In contrast, coefficient of inter-cell type variation was only 11.3% between epidermis, dermal fibroblasts, and dermal endothelium.

Levels of induced DSB per unit of radiation dose in different individuals appear to be similar, as judged by the absence of detectable inter-individual variation in induced DSB yields following irradiation in patients with rare hyper-radiosensitivity syndromes, non-syndromic cancer patients, and healthy controls \([22]\). In a separate study involving the same patients, we observed only limited inter-individual variation in foci levels of *ex vivo* irradiated blood lymphocytes 30 minutes after exposure to 0.5 Gy X-rays, which would be consistent with this notion \([23]\). We conclude that the significant inter-individual variation in residual (24 hours) foci levels in skin cells is therefore indicative of significant differences in DSB repair kinetics between individuals.
It may be argued that outliers (patients 34 and 35, Table III.1) who had extremely high levels of residual foci in the different skin cells compared to other members of the study cohort might have provided a major contribution to inter-individual variation in residual foci. With the exclusion of these outliers, coefficients of inter-individual variation remained significant for the different skin cell types, 33.0% in dermal fibroblasts, 33.9% in endothelial cells, 33.6% in superficial keratinocytes, and 34.9% in basal epidermal cells.

In this study, we demonstrated the reliability of 53BP1 immunohistochemistry using chromogenic techniques for scoring of residual DSB in skin tissues which had been exposed to a test dose of ionising radiation in vivo. Although foci analysis with immunofluorescence staining would invariably be more sensitive compared to chromogenic staining, the presence of significant collagen auto-fluorescence in the dermis limited the use of this technique in the study (Figure III.5). Using chromogenic immunohistochemistry, we observed few, if any, 53BP1 foci in unirradiated skin cells, with the exception of cells residing in the basal epidermis. The higher than expected background foci levels in basal epidermal cells were probably attributed to endogenous generation of DSB during DNA replication in these proliferating cells [24, 25]. After normalising against background levels, comparable residual foci levels were observed between the different epidermal and dermal cells, which are consistent with similar DSB repair kinetics in different cell types within the skin of an individual, as reported in the mouse [15, 26].

Figure III.5 γH2AX/53BP1 immunofluorescence staining of unirradiated and irradiated (4 Gy) skin sections. Although co-localising γH2AX/53BP1 foci (yellow) were readily observed in irradiated dermal fibroblasts 24 hours post-irradiation, significant auto-fluorescence (red) in the adjacent collagen fibres often interfered with foci scoring in these cells. Scale (top left panel) = 10 μm.
An apparent lack of correlation between the severity of skin fibrosis and telangiectasia despite good control of radiotherapy doses in patients with early breast cancer suggests that, in non-syndromic patients, inter-individual differences in clinical radiosensitivity are perhaps cell type- and tissue-specific [3]. Our findings, together with that of Rübe et al. [15, 26], suggest that DSB repair may not be the predominant factor contributing to these cell type- and tissue-specific differences. In the case of radiation fibrosis, although DDR is important in the activation of myofibroblasts, there are other possible pathways leading to fibrogenesis which are independent of DDR [27]. For example, latent TGFβ1 sequestered in extracellular matrix is hydrolytically cleaved by reactive oxygen species with the release of active cytokines [28]. In this model of radiation-induced fibrosis, no correlation with other end-points relating to post-radiation late skin effects would be expected.

### III.1.6 Conclusions

In conclusion, the findings from this study suggest that inter-individual variation in DSB repair is more pronounced than inter-cell type variation, at least in skin. It is possible that in phenotypically normal patients, genetic and epigenetic factors specific to the individual play a more important role than cell type-specific factors in modulating DSB repair *in vivo*. If so, a lack of association between telangiectasia and fibrosis in the same patient despite good control of dose would suggest different cell type-specific responses to similar levels of residual DNA damage that are independent of DSB repair.

### III.1.7 REFERENCES


III.2 DSB REPAIR FOLLOWING IN VIVO AND EX VIVO IRRADIATION OF SKIN TISSUES AND BLOOD LYMPHOCYTES IN RELATION TO LATE EFFECTS OF BREAST RADIOTHERAPY

III.2.1 Abstract
To test if residual DSB in skin tissues and $G_0$ blood lymphocytes 24 hours following 4 Gy in vivo and ex vivo exposure, respectively, correlated with late radiation-induced skin effects of breast radiotherapy patients. In the preceding study, residual DSB were quantified using 53BP1 immunostaining in irradiated skin tissues of 35 breast radiotherapy patients. These patients were selected on the basis of late radiation-induced changes in their breast and individuals with moderate/marked or minimal/no change were classified as cases and controls, respectively. Among them, 30 patients agreed to peripheral blood sampling. $G_0$ blood lymphocytes of these patients were irradiated with 4 Gy X-rays ex vivo, and residual DSB were quantified by $\gamma$H2AX/53BP1 immunofluorescence microscopy 24 hours later. Residual foci levels in dermal and epidermal cell types were not significantly different between cases ($n = 20$) and controls ($n = 15$). Mean foci per cell were 3.29 in cases and 2.80 in controls for dermal fibroblasts ($p = 0.07$), 3.28 in cases and 2.60 in controls for endothelial cells ($p = 0.08$), 2.87 in cases and 2.41 in controls for superficial keratinocytes ($p = 0.45$), and 2.32 in cases and 2.35 in controls for basal epidermal cells ($p = 0.27$). Mean residual foci levels in blood lymphocytes were however significantly higher among cases ($n = 17$, foci per cell = 12.1) compared to controls ($n = 13$, foci per cell = 10.3, $p = 0.01$). Of the different cell types, only residual foci levels of in vivo irradiated dermal fibroblasts and ex vivo irradiated blood lymphocytes correlated with severity of late effects among cases (Spearman $R = 0.722$, $p < 0.001$; 0.593, $p = 0.01$, respectively). These findings suggest that DSB repair ex vivo in $G_0$ blood lymphocytes may be predictive of late normal tissue responses after breast radiotherapy.

III.2.2 Introduction
Processes relevant to cellular radiosensitivity include the recognition and repair of DSB, as evident by the increased cellular and clinical radiosensitivity observed in individuals harbouring a defective DSB repair phenotype [1]. Nonetheless, contrary to the early successes of small case-control studies indicating a correlation between in vitro cellular radiosensitivity and clinical responses following radiation exposure, subsequent studies seeking to establish DSB repair as a predictive marker of normal tissue radiosensitivity have largely been inconclusive [2–6]. Critical opinions for the lack of success with this assay include the argument that in vitro cellular responses correlate poorly with in vivo responses due to the modifying influence of tissue environment [7, 8]. Hence, there is a powerful argument to exploring ways of measuring
cellular responses to radiotherapy \textit{ex vivo}. Separately, the choice of an ideal cellular system for a predictive assay is also a contentious issue. For example, the lack of correlation between late fibrosis and telangiectasia in breast radiotherapy patients suggests that predictive assays should be aimed at a specific normal tissue end-point \cite{9}. In the case of late fibrosis and telangiectasia, this would imply measuring cellular responses in dermal fibroblasts and endothelium of the skin, respectively. Nonetheless, opposing evidence to this model can be derived from studies demonstrating a modest correlation between \textit{in vitro} cellular responses in lymphocytes and \textit{in vivo} late tissue effects \cite{10–12}.

In the previous study, 53BP1 foci analyses were performed \textit{ex vivo} in test-irradiated buttock skin of 35 breast cancer patients. These patients were selected on the basis of late adverse effects to previous breast radiotherapy, comprising of individuals with moderate/marked (cases) or little/no radiation-induced change (controls) in their breast. In an attempt to select cases and controls whose phenotype most likely represents their intrinsic radiosensitivity, we employed a manner of patient stratification where clinical parameters of normal tissue response following breast radiotherapy were considered. Here, we examined if DSB repair measured in skin following \textit{in vivo} irradiation discriminates between non-syndromic individuals varying in severity of radiation-induced late skin effects. Additionally, DSB repair was assessed in \textit{ex vivo} irradiated $G_0$ blood lymphocytes of the same individuals to determine if lymphocytes are a valid alternative cellular system for predicting normal tissue responses following radiation exposure.

\textbf{III.2.3 Materials and methods}

\textbf{III.2.3.1 Selection of cases and controls}

The volunteers in our study comprised of patients who were previously diagnosed with early invasive ductal carcinoma of the breast and had enrolled into two breast radiotherapy randomised trials, involving treatment under standard conditions and prospective annual clinical assessments of late adverse effects using standard proformas \cite{13, 14}. In addition to conventional scoring methods (common terminology criteria for adverse events (CTCAE) version 3.0), late adverse effects in the breasts were further evaluated by comparing pre- and post-radiotherapy photographs of both breasts, collected under predefined conditions before radiotherapy and at 1, 2, and 5 years post-radiotherapy. Specifically, the late effects graded with comparisons of pre- and post-radiotherapy photographs include 1) breast asymmetry, 2) telangiectasia, 3) hyper- or hypo-pigmentation, 4) increased breast density, 5) oedema, 6) subcutaneous fibrosis, and 7) aesthetic sequelae. Patients were then selected in the following manner. Initial selection was based on clinical and photographic assessments indicating moderate/marked radiation-induced change (case) or very little/no change (control) (Figure III.6). Following which, clinical parameters associated with an elevated risk of radiation-induced change in breast appearance were assessed in these patients to derive the most informative individuals. These factors were generated by multivariate analyses of outcomes in the breast radiotherapy trials and namely, they were 1) prescribed whole breast radiotherapy dose, 2) radiation dosimetry, 3) radiotherapy
boost to tumour bed, 4) breast size, 5) proportion of breast removed at surgery, and 6) axillary treatment (Table III.3). Taking into account these clinical parameters, an unmatched case-control study design was attempted, such that selected cases, in spite of favourable treatment conditions which would have gone against the risk of developing late adverse effects in the breasts, presented with moderate/marked changes, whereas controls had minimal/no change despite their predicted risk of increased late events following unfavourable treatment conditions. As an added consideration, the cases selected would have shown changes in their breast at early time points after radiotherapy, while controls would exhibit little/no symptoms out to a minimum of 5 years post-radiotherapy. After the selection of two potential groups according to the above criteria, the final selection was made by two experienced clinicians (JY and NS1) with primary reference to the photographic scoring of pre- and

---

1 JY, John Yarnold; NS, Navita Somaiah
post-radiotherapy changes, excluding individual patients for whom factors omitted by
the algorithm were considered to influence response. An illustrative example would be
tumour location in the inferior quadrants of the breast where breast contour is very
irregular and accurate radiotherapy dosimetry was difficult to ensure. Ethical approval
was obtained from research ethics committees at all participating centres. Informed
consent was obtained from patients prior to participation.

III.2.3.2 Skin irradiation, 53BP1 immunohistochemistry, and foci analyses of skin
sections
A test dose of 4 Gy was delivered to a small area of buttock skin using 6 MeV electrons.
Duplicate biopsies of irradiated and unirradiated skin were obtained and processed
for 53BP1 immunohistochemistry. 53BP1 foci were scored in four epidermal and
dermal cell types, namely superficial keratinocytes, basal epidermal cells, dermal
fibroblasts, and endothelial cells, with 50 to 100 cells scored for each cell type per
biopsy of each patient. Details entailing irradiation, 53BP1 immunostaining, and foci
analyses of skin were described earlier in Chapter III.1.3.
III.2.3.3 Peripheral blood separation and $G_0$ blood lymphocyte irradiation

Thirty patients from the same cohort also consented to repeated peripheral blood sampling. $G_0$ blood lymphocytes were isolated from whole blood samples using Histopaque-1077 (Sigma-Aldrich, Dorset, UK). Whole blood samples, delivered overnight from the Royal Marsden Hospital, Sutton, to the Health Protection Agency, Chilton, were diluted in equal volumes (1:1) of Hank’s buffered solution (Invitrogen, Paisley, UK) before layering onto Histopaque-1077. Samples were then spun at $350 \times g$ for 40 minutes, $21^\circ C$, and upon completion, the opaque interface (containing the blood lymphocytes) between the upper plasma layer and Histopaque-1077 was carefully aspirated. After washings with Hank’s buffered solution, blood lymphocytes were exposed to 0.5 and 4 Gy using 250 kV X-rays delivered at 0.69 Gy/min (Pantak, Surrey, UK). They were then incubated in minimum essential medium (Invitrogen, Paisley, UK), supplemented with 1% L-glutamine, 1% streptomycin, 10% fetal calf serum, and kept for 0.5 (0.5 Gy) and 24 hours (4 Gy) in a $37^\circ C, 5\% CO_2$ incubator prior to immunostaining.

III.2.3.4 DSB in ex vivo irradiated $G_0$ blood lymphocytes

DSB in irradiated blood lymphocytes were quantified using $\gamma H2AX$ and 53BP1 immunostaining and co-localizing $\gamma H2AX$ and 53BP1 foci were scored (Figure III.7). For immunostaining, cells were plated on glass slides, fixed and permeabilised for 10 minutes with 2% paraformaldehyde and 0.5% Triton X-100 in PBS, respectively, washed with PBS, and blocked for 30 minutes with PBS + 1% BSA fraction V (Sigma-Aldrich, Dorset, UK). Cells were then incubated with primary antibody diluted in PBS + 1%

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**Figure III.7** $\gamma H2AX$ and 53BP1 immunofluorescence staining 24 hours after 4 Gy X-irradiation in $G_0$ blood lymphocytes. Blood lymphocytes were co-stained with DAPI (top left panel), anti-$\gamma H2AX$ antibody (green), and anti-53BP1 antibody (red). $\gamma H2AX$ and 53BP1 foci co-localise in the cell nuclei as evident by the yellow-coloured foci (bottom right panel), product of the merging of green and red colours. Scales = 10 μm.
BSA fraction V for 1 hour at room temperature, washed twice, and incubated with secondary antibody and DAPI (4’,6-diamidino-2-phenylindole) diluted in PBS + 1% BSA fraction V for 1 hour at room temperature in the dark. After final washes with PBS and drying, slides were mounted in Vectashield (Vector Laboratories Limited, Peterborough, UK) and visualized using a Nikon Eclipse TE200 epifluorescence microscope (Nikon UK Limited, Surrey, UK). At least 50 cells were scored per slide for each patient. The following antibodies were used: anti-53BP1 (mab3802, Millipore, Watford, UK, 1:400 and ab36823, Abcam, Cambridge, UK, 1:400) and anti-\(\gamma\)H2AX (05-636, Millipore, 1:500, ab18311 and ab26350, Abcam, 1:500) primary antibodies, Alexa-Fluor 488 goat anti-mouse, Alexa-Fluor 555 goat anti-rabbit (Invitrogen, Paisley, UK, 1:200), and TRITC (Tetramethylrhodamine-5-(and-6)-isothiocyanate, Jackson immunoresearch, Suffolk, UK, 1:200) donkey anti-rabbit secondary antibodies.

III.2.3.5 Statistical methods
To avoid biases in foci scoring, the following blinding process was employed. Skin tissue blocks and peripheral blood samples of each patient were randomly recoded by an independent third party prior to transfer from The Royal Marsden Hospital. As an added measure of blinding, skin tissue blocks and peripheral blood samples of each corresponding patient were also assigned different codes such that it would not be known to the scorer if the skin and blood samples came from the same source. The manner of coding, along with the patients’ clinical phenotype (case/control) were revealed for data analysis only upon the completion of foci scoring.

The study was originally designed to recruit 15 cases and 15 controls to detect a standardised difference of 1.2 with 85% power (5% two-sided significance level). Comparative analyses of clinical parameters and foci levels between cases and controls 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 cases. 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 15.0.

III.2.4 Results
III.2.4.1 Cases and controls
Of 35 breast radiotherapy patients who volunteered for the study, 20 and 15 patients were recruited as cases and controls, respectively. Among them, 17 cases and 13 controls consented to repeated peripheral blood sampling. Median age of cases and controls was 70 and 68 years, respectively. Patients’ characteristics, including treatment-related parameters, are summarised below in Table III.4. It is evident from the table that treatment characteristics of the selected cases and controls did not match the original intent of stratifying these patient cohorts based on favourable and unfavourable clinical determinants of late effects following breast radiotherapy, respectively (Table III.3). Nonetheless, it is still noteworthy that these factors were comparable between both groups of patients.
Table III.4 Parameters of cases and controls*

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
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<tr>
<td>Patients (n = 35)</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>70 (52–83)</td>
<td>68 (54–78)</td>
</tr>
<tr>
<td>Median follow-up, years (range)</td>
<td>11 (3–24)</td>
<td>13 (11–24)</td>
</tr>
<tr>
<td>Mean breast RT dose, Gy (±SD)</td>
<td>47.2 (±6.26)</td>
<td>48.6 (±2.94)</td>
</tr>
<tr>
<td>Dosimetry techniques</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>2D</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Mean tumour bed boost dose, Gy (±SD)</td>
<td>9.78 (±6.11)</td>
<td>12.66 (±2.03)</td>
</tr>
<tr>
<td>Breast size</td>
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<td></td>
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<tr>
<td>Small</td>
<td>8</td>
<td>2</td>
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<tr>
<td>Medium</td>
<td>10</td>
<td>13</td>
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<tr>
<td>Large</td>
<td>2</td>
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<tr>
<td>Surgical deficit</td>
<td></td>
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<tr>
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<td>8</td>
<td>11</td>
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<tr>
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<td>12</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>8</td>
<td>5</td>
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</table>

* There were no significance differences between groups. All cases and controls received breast radiotherapy for treatment of early invasive ductal carcinoma of the breast. SD = One standard deviation.

III.2.4.2 Residual foci levels of in vivo irradiated skin in cases and controls

Patient-averaged levels of residual foci 24 hours after 4 Gy in epidermal and dermal skin cells were not significantly different between cases and controls (Figure III.8). Mean foci per cell were 3.29 in cases and 2.80 in controls for dermal fibroblasts (p = 0.07), 3.28 in cases and 2.60 in controls for endothelial cells (p = 0.08), 2.87 in cases and 2.41 in controls for superficial keratinocytes (p = 0.45), and 2.32 in cases and 2.35 in controls for basal epidermal cells (p = 0.27).

Figure III.9 illustrates the individual foci levels in dermal fibroblasts, endothelial cells, superficial keratinocytes, and basal epidermal cells for all cases and controls within the cohort. Although patient-averaged residual foci levels in these skin cells were not significantly different between both patient groups, in a few selected cases, foci levels present in the dermis and superficial keratinocytes 24 hours after 4 Gy were significantly higher compared to the majority of controls.
Figure III.8 Patient-averaged residual foci levels in the different skin cells 24 hours after 4 Gy. Error bars represent one standard deviation.

Figure III.9 Individual foci levels scored in irradiated skin of cases and controls 24 hours after 4 Gy. Horizontal lines represent patient-averaged residual foci levels.
To ascertain the significance of these increased residual foci levels in a subset of cases, we proceeded to test if residual foci levels of in vivo irradiated skin correlated with severity of late radiation-induced skin changes in our cohort of 20 cases. As mentioned earlier, late radiation-induced changes in the breasts were scored based on a combination of clinical and serial photographic assessments pre- and post-radiotherapy (Chapter III.2.3.1). For the purpose of this exploratory analysis, clinical symptoms of cases were further graded using an arbitrary scale of one to three, in ascending order of severity. This clinical classification of cases was performed with primary reference to photographic assessments of late adverse effects in the breasts, taking into account parameters such as 1) breast asymmetry, 2) telangiectasia, 3) hyper- or hypo-pigmentation, 4) increased breast density, 5) oedema, 6) subcutaneous fibrosis, and 7) aesthetic sequelae. On the basis of clinical impression of the severity of the aforementioned, two experienced oncologists (JY and NS), while blinded to the foci scores and patients’ identification on the pre- and post-radiotherapy photographs, then independently assigned ten, four, and six cases to groups one, two, and three, respectively. Illustrative examples of cases classified under the respective groups are presented in Figure III.10.

Of the different skin cells, only residual foci levels in dermal fibroblasts were correlated with clinical symptoms of the same cases (Spearman’s $R = 0.722$, $p < 0.001$). Although a positive association was observed between residual foci levels in endothelial cells and clinical severity for the same cases, this was not statistically significant (Spearman’s $R = 0.506$, $p = 0.023$). No association was observed between residual foci levels in the epidermis and clinical severity of cases (superficial keratinocytes, Spearman’s $R = 0.062$, $p = 0.794$; basal epidermal cells, Spearman’s $R = 0.121$.

**Figure III.10** Post-surgical photographs of cases with varying degree of late effects in the irradiated breast (grey arrow) at 5 years post-radiotherapy. Compared to the cases assigned to group one (least severe), cases categorised into groups two (moderately severe) and three (most severe) displayed enhanced toxicities from breast radiotherapy, including marked breast hardening, significant loss of breast volume, and overlying skin telangiectasia. Consent was obtained from patients for the use of these photographs.
III.2.4.3 Foci levels of ex vivo irradiated $G_0$ blood lymphocytes in cases and controls

In contrast to residual foci levels of in vivo irradiated skin, foci levels of $G_0$ blood lymphocytes 24 hours after 4 Gy X-rays ex vivo were significantly higher in cases compared to controls. Mean foci per cell were 12.1 in cases versus 10.3 in controls (Figure III.12, $p = 0.01$). Foci levels in $G_0$ blood lymphocytes 0.5 hour after 0.5 Gy
X-rays *ex vivo* were however comparable between both groups of patients (mean foci per cell = 7.70, cases versus 8.05, controls, p = 0.16, Figure III.12).

Given the difference in residual foci levels of *ex vivo* irradiated G₀ blood lymphocytes between cases and controls, we proceeded to test if foci levels of this cell type correlated with severity of late radiation-induced skin changes for the same cases. Like dermal fibroblasts, a positive patient-specific association was observed between residual foci levels 24 hours after 4 Gy X-irradiation in blood lymphocytes and severity of clinical symptoms among cases (Figure III.13, Spearman’s $R = 0.593$, p = 0.01).

### III.2.5 Discussion

*Ex vivo* measurements of residual DSB levels in test-irradiated buttock skin of breast radiotherapy patients failed to discriminate between individuals with varying severity of late radiation-induced skin effects. On the contrary, DSB repair assessed in G₀ blood lymphocytes of the same patients indicated higher levels of residual $\gamma$H2AX/53BP1 foci among clinically radiosensitive individuals compared to matched controls. Exploratory secondary analyses suggest a positive association between residual foci levels and severity of late effects among cases (patients with moderate/marked late radiation-induced change in their breast), albeit only in dermal fibroblasts and blood lymphocytes. Together, these findings propose that cellular responses in blood lymphocytes are potentially indicative of normal tissue radiosensitivity in non-syndromic individuals.

The basis of testing residual DSB as a marker of clinical radiosensitivity in this study was extrapolated from consistent reports of a slow component to tissue recovery between fractions during radiotherapy [15–17]. For example, in breast radiotherapy patients treated using once daily or twice daily (8 hours interval) fractions to the breasts, increased incidence and severity of late telangiectasia in skin irradiated twice daily were equivalent to a 10% increase in total dose [16]. Likewise, long repair
halftimes have been estimated for a number of late normal tissue end-points, such as skin telangiectasia and fibrosis, based on clinical outcomes of altered fractionated radiotherapy regimes in head and neck cancers [17]. Judging from these findings, it is plausible to hypothesise that residual DSB levels prior to the next fraction of radiotherapy have a profound influence on cell fate, including cell cycle arrest, senescence, apoptosis, and inter-individual variation in DSB repair could therefore account for variation of late normal tissue responses between radiotherapy patients.

As reported in the preceding chapter, residual 53BP1 foci levels in different epidermal and dermal cells 24 hours after 4 Gy in vivo irradiation varied significantly among patients from our study cohort. Nonetheless, a comparison of residual foci levels in these irradiated skin tissues of cases and controls failed to yield a discernible difference between both patient groups. Judging from individual foci levels in the different skin cells (Figure III.9), this outcome was not unexpected since a significant proportion of foci levels of cases and controls overlapped one another. Perhaps, prior to rejecting the hypothesis that in vivo cellular responses measured in human skin predict for radiation-induced skin effects, it is pertinent to consider if the patients selected in this study were indeed representative of their assigned phenotype. At the outset, this study was designed to recruit cases representing the 5% most damaged individuals among a cohort of approximately 1000 patients on follow-up, from whom controls have been highly selected [13, 14]. Clinical determinants of radiation-induced late changes in the breasts were also considered during patient selection such that on the basis of these clinical factors alone, cases and controls would have a predicted low and high risk of late adverse events following breast radiotherapy, respectively, thereby implying that their clinical phenotype rightly reflects their intrinsic radiosensitivity. As it turns out,

![Graph showing correlation between grade of clinical symptoms and foci per cell in blood lymphocytes.](image)

**Figure III.13** Individual residual foci levels in blood lymphocytes of 17 cases, stratified according to an arbitrary clinician-defined grading of late radiation-induced effects in the breast (Figures III.10, 11). Horizontal lines represent patient-averaged foci levels for all cases classified within the same group. $R^2$ value was generated using Spearman’s rank correlation test with corresponding p-value indicated in brackets.
strict adherence to the stipulated criteria was extremely difficult to ensure, which is in part due to the low prevalence of clinically radiosensitive individuals, even among breast radiotherapy patients who had been closely monitored over a prolonged period of time. Nonetheless, it was equally important that treatment characteristics remained balanced between both patient groups. All things considered, it is unlikely these clinical factors could have confounded the selection of cases and controls and in turn, obscured a relationship between in vivo DSB repair and radiation-induced late effects in the skin.

Conversely, residual foci levels (24 hours post-4 Gy) in ex vivo irradiated G₀ blood lymphocytes of cases were significantly higher compared to controls, albeit this comparative analysis was performed in a subset of cases and controls from the original cohort of 35 patients. Among these patients, foci levels shortly after irradiation (0.5 hour post-0.5 Gy) did not vary between them. On this basis, it is within reason to attribute the difference in residual foci levels between cases and controls to inter-individual variation in DSB repair processes. Along with existing literature, the findings of this study also support the notion of testing cellular end-points in lymphocytes as predictive markers of clinical radiosensitivity [10–12, 18].

Strikingly, although a fixed dose of irradiation was delivered to skin and blood lymphocytes of the same patients, scoring of residual foci levels 24 hours post-irradiation were significantly higher in the latter cell type (Patient-average foci per cell \(= 11.32\) in blood lymphocytes versus \(3.14\) in dermal fibroblasts, \(3.06\) in endothelial cells, \(2.51\) in superficial keratinocytes, and \(2.63\) in basal epidermal cells, \(p < 0.001\), ANOVA). While this may suggest that DSB repair is influenced by tissue environment, in this case, skin, the higher number of residual foci in ex vivo irradiated blood lymphocytes compared with in vivo irradiated skin cells could actually, for the most part, be explained by different experimental conditions rather than cell-specific characteristics. In mice, a comparison of DSB repair between ex vivo and in vivo irradiated blood lymphocytes revealed slightly higher levels of residual foci after ex vivo exposure [19]. Separately, the use of different radiation sources, tissue processing, and immunostaining protocols could also collectively contribute to the detection of lower residual foci levels in skin cells.

A number of interesting observations were made from tests of association between residual foci levels and clinical severity of late effects among cases. Using an unconventional grading system generated on the bases of clinical and photographic scores for radiation-induced changes in the breast, ten cases were considered the least severe (‘Grade 1’), while four and six patients were deemed to be moderate (‘Grade 2’) and most severe (‘Grade 3’) cases, respectively. The clinical impression at the time of patient stratification was that cases presenting with ‘Grade 2 and 3’ symptoms in our study likely represent the 1% and 0.1% most damaged individuals, respectively. Whilst it is important to highlight the blinding processes undertaken to ensure an unbiased sub-classification of cases, it is also prudent to note that these analyses were simply exploratory, and any observed measure of association must be perceived with a generous level of caution. Nonetheless, there are two findings of worthy mention arising from this exploratory analysis. Firstly, although the process of cases stratification may seem subjective, the fact that grading of cases concurred between two independent assessments suggests for an element of objectiveness in this scoring system. Moreover, through photographic comparisons pre- and post-radiotherapy,
clinicians were not simply limited to conventional normal tissue end-points in their assessment of late radiation-induced effects, but rather, other parameters such as breast shrinkage and shape, which are indicative of fat necrosis and tissue atrophy following radiation exposure, were also integrated in the determination of clinical radiosensitivity. In support, the robustness of such a method of assessment had been tested and validated in a large randomised study of 1410 patients [13]. Secondly, positive associations were observed between clinical severity and residual foci levels in blood lymphocytes, dermal fibroblasts, and endothelial cells for the same cases, albeit statistical significance was not reached for endothelial cells. Closer scrutiny of individual foci levels (Figures III.11, 13) suggests that these positive associations were mostly a result of extremely high levels of residual foci among a select few cases bearing ‘Grade 2 and 3’ symptoms. Considering the relevance of dermal fibroblasts and endothelial cells in the pathology of late skin fibrosis and telangiectasia, respectively, these observations do suggest a role for residual DNA damage following irradiation of the skin dermis in the pathogenesis of late radiation-induced skin effects, at least in a small cohort of severely radiosensitive patients harbouring an impaired DSB cellular phenotype.

III.2.6 Conclusions

In conclusion, the findings of this study propose that ex vivo DSB repair of irradiated blood lymphocytes may be more useful than in vivo DSB repair of irradiated skin tissues in predicting normal tissue responses in patients following breast radiotherapy. In a small subset of extremely radiosensitive individuals, residual DNA damage may have a role in the pathogenesis of late adverse effects, at least in the human skin.

III.2.7 REFERENCES


III.3 RESIDUAL DNA AND CHROMOSOMAL DAMAGE IN EX VIVO IRRADIATED BLOOD LYMPHOCYTES CORRELATED WITH LATE NORMAL TISSUE RESPONSES TO BREAST RADIOThERAPY

III.3.1 Abstract
To test the association of DSB repair and chromosomal radiosensitivity in ex vivo irradiated blood lymphocytes with late-onset normal tissue responses following breast radiotherapy. Patients with minimal (controls) or extremely marked late radiotherapy changes (cases with ‘grade 2/3’ clinical reactions) were selected from our cohort of 35 breast radiotherapy patients. DSB induction and repair were quantified by γH2AX/53BP1 immunofluorescence microscopy 0.5 and 24 hours after exposure of G0 blood lymphocytes to 0.5 and 4 Gy X-rays, respectively. Chromosomal aberrations
were scored in blood lymphocyte metaphases after 6 Gy X-rays. Despite similar foci levels at 0.5 hour in cases (n = 7) and controls (n = 7), foci levels 24 hours after 4 Gy irradiation differed significantly between them (Foci per cell were 12.8 in cases versus 10.2 in controls, p = 0.004). Increased chromosomal radiosensitivity was also observed in cases (Aberrations per cell were 5.84 in cases versus 3.79 in controls, p = 0.001), with exchange and deletion type aberrations contributing equally to the difference between cases and controls. Residual foci correlated with formation of deletions (Spearman's $R = 0.589$, $p = 0.027$) but not exchanges ($R = 0.367$, $p = 0.197$) in blood lymphocytes from the same patients. The higher levels of exchange type aberrations observed among radiosensitive breast cancer patients suggest a role for DSB misrepair, in addition to residual DNA damage, as determinants of late normal tissue damage. Separately, correlation of residual foci levels with deletion type aberration yields in the same cohort confirms their mechanistic linkage.

### III.3.2 Introduction

In a small subset of patients, manifestations of radiation-induced late normal tissue damage can be significantly marked and potentially physically debilitating. While mechanisms underlying late normal tissue effects following exposure to radiotherapy remain unclear, it is probable that a combination of various molecular processes are involved in the pathogeneses of these late effects. A suitable example illustrating this would be the activation of myofibroblasts by TGF-β1 in the pathogenesis of radiation-induced fibrosis, where activation of TGF-β1 following radiation exposure could result from either direct activation by reactive oxygen species or through a series of transcriptional responses to DNA damage [1, 2]. Given the complexity of pathogenetic mechanisms involved in the development of radiation-induced late normal tissue damage, it is not surprising that studies looking at predictive markers of clinical radiosensitivity have so far yielded conflicting results [3–12]. Of the proposed assays to date, single nucleotide polymorphism analysis [7–10], colony forming [13, 14], DSB repair [15, 16], lymphocyte apoptosis [17, 18], and chromosomal aberration assays [3, 4] are few which had shown modest correlations with clinical phenotype.

Within our cohort of 35 breast radiotherapy patients, we had identified a small subset of extremely radiosensitive individuals whom had significantly high levels of residual DNA damage in skin and blood lymphocytes 24 hours after 4 Gy in vivo and ex vivo irradiation, respectively. In this study, we aimed to compare the efficiency of DSB repair and chromosomal radiosensitivity in ex vivo irradiated blood lymphocytes of these severe cases and matched controls. As this was a subset analysis, we adopted the strategy of recruiting only the most informative cases with the intent of detecting the maximum difference in cellular responses between cases and controls.

### III.3.3 Materials and methods

#### III.3.3.1 Selection of severe cases and controls

Among our cohort of 20 cases, 10 cases were considered extremely severe (‘Grade 2/3’, Figures III.10, 11, 13) based on clinical and photographic assessments of pre- and post-
radiotherapy changes in their breast, conducted independently by two experienced clinical oncologists (JY and NS). Of these cases, seven agreed for repeated blood sampling over an 18-month period. Seven carefully matched controls with minimal/no late effects were then selected from our original cohort of 15 controls for comparison. Ethical approval for the study was obtained from the research ethics committees from all the participating centres. Informed consent was obtained from the patients prior to participation.

III.3.3.2 Isolation, ex vivo irradiation, and γH2AX/53BP1 co-immunostaining of G0 blood lymphocytes

Separation of G0 blood lymphocytes from peripheral blood samples was performed as described earlier (Chapter III.2.3.3). After washings with Hank’s buffered solution, cells were exposed to 0.5 and 4 Gy using 250 kV X-rays delivered at 0.5 Gy/minute (AGO X-ray Ltd, Reading, UK). They were then incubated in minimum essential medium, supplemented with 1% L-glutamine, 1% streptomycin, and 10% fetal calf serum, at 37°C, 5% CO2 for 0.5 (0.5 Gy) and 24 hours (4 Gy) prior to immunostaining. γH2AX/53BP1 co-immunostaining in blood lymphocytes was performed as previously described in Chapter III.2.3.4. A minimum of 50 cells were scored for co-localising γH2AX/53BP1 foci per patient sample.

III.3.3.3 Chromosomal radiosensitivity in blood lymphocyte metaphases after ex vivo irradiation

Heparinised whole blood (0.4 ml) diluted in minimum essential medium supplemented with 20% fetal calf serum, was irradiated to 6 Gy using 250 kV X-rays delivered at 0.5 Gy/minute. Irradiated blood was then incubated with 2% phytohaemagglutinin (Invitrogen, Paisley, UK) and 0.05 µg/ml colcemid (Sigma-Aldrich, Dorset, UK) at 37°C, 5% CO2 for 72 hours before hypotonic treatment with 0.075 M potassium chloride, fixation in methanol:acetic acid (3:1), slide making by conventional techniques, and staining in 2% Giemsa (VWR International Limited, Poole, UK) prepared in pH 6.8 buffer. Slides were mounted in Shandon medium (ThermoScientific, Hertfordshire, UK) and visualized under light microscopy. Between 50 to 75 metaphase spreads per patient were analysed for exchange (dicentrics and rings) and deletion (excess acentric fragments) type aberrations. Examples of exchanges and deletions are illustrated in Figure III.14.

III.3.3.4 Statistical methods

Blinding processes as elaborated in Chapter III.2.3.5 were similarly applied to peripheral blood samples of cases and controls in this sub-group analysis. Comparative analyses of foci and chromosomal aberration levels between cases and controls were performed using the Mann-Whitney U test. Spearman’s rank correlation test was used to test for correlation of foci and chromosomal aberration levels for the same patients. All statistical calculations were performed using SPSS version 15.0. To account for the small study sample size and multiple statistical comparisons, statistical significance was set at a p-value of < 0.01.
III.3.4 Results

III.3.4.1 Patients

Clinical parameters of the seven selected cases and controls are summarized in Table III.5 (presented at the end of Results). Median age of cases and controls were 69 and 67 years, respectively. Median time of follow-up was 9 and 16 years for cases and controls, respectively.

III.3.4.2 γH2AX/53BP1 foci levels in cases and controls

Comparable foci levels were observed between cases and controls 0.5 hour after 0.5 Gy X-rays. In contrast, foci levels 24 hours after 4 Gy X-rays were significantly higher in cases compared to controls. Mean foci levels per cell were 7.89 in cases and 8.25 in controls 0.5 hour after 0.5 Gy X-rays (p = 0.097), and 12.78 in cases and 10.15 in controls 24 hours after 4 Gy X-rays (p = 0.004, Figure III.15).

Figure III.16 shows individual foci levels at both dose- and time-points for all patients. Low inter-individual variation of foci levels 0.5 hour after 0.5 Gy was observed for all cases and controls, with a coefficient of variation of 5.0%. Inter-individual variation of foci levels 24 hours after 4 Gy was significantly higher, specifically among cases. Coefficients of variation were 16.8% in cases and 7.7% in controls. All cases, with the exception of one, had higher levels of foci 24 hours after 4 Gy compared to controls.
III.3.4.3 Chromosomal aberration levels in cases and controls

Increased chromosomal radiosensitivity was observed in cases compared to controls after 6 Gy X-rays (Figure III.17). Mean aberrations per cell were 5.84 in cases and 3.79 in controls ($p = 0.001$). Exchange and deletion type aberrations were on average higher in cases. Mean exchanges per cell were 2.74 in cases and 1.83 in controls ($p = 0.017$), and mean deletions per cell were 3.10 in cases and 1.95 in controls ($p = 0.026$).

![Figure III.15](image1.png)

**Figure III.15** Patient-averaged foci levels in irradiated $G_0$ blood lymphocytes for cases and controls. Error bars represent one standard deviation.

![Figure III.16](image2.png)

**Figure III.16** Foci levels in blood lymphocytes 0.5 hour after 0.5 Gy and 24 hours after 4 Gy X-rays for the same patients, ordered according to ascending foci levels in blood lymphocytes 24 hours after 4 Gy.
Figure III.18 shows individual exchange and deletion type aberration levels after 6 Gy X-rays for all patients. Between cases and controls, exchanges and deletions contributed equally to the overall increase of chromosomal radiosensitivity in cases. Notably, all cases had higher total aberration yields than controls.

III.3.4.4 Association between residual foci in G₀ lymphocytes and chromosomal aberrations in lymphocyte metaphases

Residual foci levels 24 hours after 4 Gy X-rays correlated with levels of chromosomal aberrations measured 72 hours after 6 Gy X-rays for the same patients (Spearman’s...
$R = 0.719, p = 0.004$). Separate tests of association suggested a patient-specific association between residual foci and deletion type aberrations ($\text{Spearman's } R = 0.589, p = 0.027$) but not exchange type aberrations ($\text{Spearman's } R = 0.367, p = 0.197$, Figure III.19).

### III.3.5 Discussion

Comparative analyses of DSB repair and chromosomal radiosensitivity in \textit{ex vivo} irradiated blood lymphocytes between women whom had displayed either extremely marked or minimal late radiotherapy changes in their breast indicated higher levels of residual DNA and chromosomal damage among the clinically radiosensitive patients. Exchange or deletion type aberrations contributed equally to the overall increase in chromosomal radiosensitivity for this group of patients. Residual $\gamma$H2AX/53BP1 foci 24 hours after 4 Gy in G$_0$ blood lymphocytes were correlated with chromosomal aberrations after 6 Gy in blood lymphocyte metaphases for the same patients. Specifically, residual foci correlated with the formation of deletions but not exchanges from the same patients.

Limited inter-individual variation of foci levels in \textit{ex vivo} irradiated blood lymphocytes 0.5 hour after 0.5 Gy (Figure III.15) supports the notion of similar yields of induced DSB following a fixed dose of radiation, as had been reported in patients with rare hyper-radiosensitivity syndromes, non-syndromic cancer patients, and healthy controls [19]. Likewise, wide inter-individual variation of DSB repair following a fixed test dose of ionizing radiation to human skin from non-syndromic individuals is consistent with our findings of significant inter-individual variation of residual foci levels in lymphocytes 24 hours after 4 Gy [Chapter III.1, 20]. It is therefore within reason to conclude that differences in levels of residual foci between cases and controls are indicative of significant differences in DSB repair.

A previous comparison between dose intensities of 3 and 6 Gy for determining \textit{in vitro} chromosomal radiosensitivity of blood lymphocytes had indicated a better association between chromosomal radiosensitivity and clinical phenotype if the former was measured after 6 Gy irradiation [21]. Using similar irradiation conditions, we observed significantly higher levels of chromosomal aberrations in blood lymphocyte metaphases among women presenting with marked late radiotherapy changes. Interestingly, although all cases had higher overall levels of chromosomal aberrations compared to controls following a fixed radiation dose, a detailed characterisation of exchange and deletion type aberrations (Figure III.18) indicated that individually, cases did not necessarily possess consistently higher levels of exchanges and deletions compared to controls, and the increment of chromosomal radiosensitivity could be a result of either increased exchange or deletion formation in cases.

The association between residual foci in unstimulated blood lymphocytes and deletions in blood lymphocyte metaphases for the same patients (Figure III.19) is consistent with the notion that deletion type chromosomal aberrations arise from unrepaired DSB [22, 23]. Similarly, a lack of association between residual foci and exchanges is not unexpected given that residual foci are primarily determined by overall DSB repair kinetics, whereas DSB misrepair is implicated in the formation of exchanges.
Figure III.19 Correlation of total aberrations, deletions, and exchanges per blood lymphocyte metaphase after 6 Gy with foci levels per G₀ blood lymphocyte 24 hours after 4 Gy for the same patients. Trendlines were generated using linear regression. $R^2$ values were generated using Spearman’s rank correlation test with corresponding p-values indicated in brackets.
Table III.5  Clinical parameters of cases and controls, including determinants of late effects in the breasts generated from multivariate analyses of outcomes from two breast radiotherapy trials (Chapter III.2.3.1, Table III.3).\textsuperscript{*}

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\textsuperscript{*}Partial and whole breast radiotherapy were delivered simultaneously using intensity modulated techniques in this patient.
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* There were no significant differences between both patient groups. Abbreviations = #, fraction; RT, radiotherapy.
Considered together with the independent contributions of exchanges and deletions to the increased chromosomal radiosensitivity of clinically radiosensitive patients, these findings suggest a plausible role for DSB misrepair, in addition to residual damage, in the development of late normal tissue damage following radiotherapy.

Severe ‘over-reactors’ to radiotherapy are extremely rare, constituting less than 1% of non-syndromic individuals [24, 25]. The intention of selecting only cases with severely marked late radiotherapy changes inevitably led to the small study numbers and limited the statistical robustness of the study. To account in part for this limitation of the study, a modest p-value of < 0.01 was set for statistical significance. In spite of this strict criterion, differences in residual foci levels and chromosomal radiosensitivity between cases and controls remained statistically significant.

A recognised obstacle in the development of reliable assays for predicting clinical radiosensitivity remains the wide inter-individual biological variation of radiation responses within a non-syndromic cohort of patients [26]. Often, a suitable robust cut-off point for the prediction of clinical radiosensitivity is not possible given the presence of high and low ‘outliers’ from the non-radiosensitive and radiosensitive groups of patients, respectively. This phenomenon is similarly evident within our cohort of 14 breast cancer patients where scatter plots of individual patient data (Figures III.16, 18) indicate that levels of residual foci and chromosomal aberrations were only marginally higher in some cases compared to controls. This, despite having selected for patients who differed significantly in their clinical symptoms, suggests to us the limitations associated with single assay studies for predicting clinical radiosensitivity. Perhaps, the combination of multiple assays may prove to be a more successful approach eventually.

III.3.6 Conclusions

In conclusion, higher levels of residual DSB and deletion type aberrations in ex vivo irradiated blood lymphocytes from clinically radiosensitive breast cancer patients support a mechanistic framework involving DSB repair in the development of radiation-induced late normal tissue damage. The presence of higher levels of exchange type aberrations observed in these radiosensitive patients raises the possibility of DSB misrepair influencing late normal tissue responses as an independent mechanism.

III.3.7 REFERENCES


Original research


III.4 DSB REPAIR AND INDUCTION OF APOPTOSIS FOLLOWING EX VIVO IRRADIATION OF BLOOD LYMPHOCYTES IN RELATION TO LATE NORMAL TISSUE RESPONSES OF BREAST RADIOTHERAPY PATIENTS

III.4.1 Abstract

To test if induction of apoptosis following ex vivo irradiation of G0 blood lymphocytes correlated with clinical radiosensitivity and DSB repair in breast radiotherapy patients and healthy volunteers. Using small molecule inhibitors, we examined the relationship between DSB repair and radiation-induced apoptosis. Sixteen breast cancer patients with minimal (controls, n = 8) or extremely marked late radiation-induced change (cases, n = 8) and eight healthy volunteers were selected. These included the highly selected seven cases and controls from the preceding study. DSB were quantified by γH2AX/53BP1 immunostaining, and apoptosis levels were assessed using a fluorogenic inhibitor of caspases (FLICA) assay. Apoptotic cells were defined by small cell size and positive FLICA signal measured using flow cytometry. DNA-PK (Nu7441) and ATM (Ku55933) inhibitors were used at concentrations of 1 and 10 μM, respectively. Mean γH2AX/53BP1 foci levels 24 hours after 4 Gy were higher in cases (12.7) than in controls (10.3, p = 0.002). In contrast, the mean proportion of apoptotic cells 48 hours after 8 Gy was comparable, 37.2% in cases and 34.7% in controls (p = 0.442). Residual foci and apoptosis levels were not correlated within individuals (Spearman’s R = −0.0059, p = 0.785). However, Nu7441-treated cells had higher foci and apoptosis levels 48 hours after 1 Gy compared to mock-treated cells, suggesting that apoptosis induction following irradiation is modulated by DSB repair. This effect required functional ATM since cells treated simultaneously with Nu7441 and Ku55933 were resistant to apoptosis despite high levels of residual foci. Among our clinical cases, one displayed an impaired DNA-PK-dependent end-joining cellular phenotype. In summary, clinical radio-
sensitivity may be associated with impaired DSB repair in some patients. Although pharmaceutical inhibition of ATM and DNA-PK affected apoptosis induction and DSB repair, no association was observed between apoptosis and residual foci levels in patients and volunteers.

**III.4.2 Introduction**

The most common factor limiting dose escalation in radiotherapy treatment of cancers remains the risk of late normal tissue effects [1, 2]. Apart from rare instances when severe acute reactions result in consequential late effects, often, the onset and severity of acute effects are not necessarily predictive of late events occurring months or years after treatment [3, 4]. In individuals who lack characteristic phenotypes of rare clinical radiosensitivity syndromes, this temporal sequence of clinical changes prevents the identification of individuals at elevated risk of severe late toxicities. Recently, a rapid lymphocyte-based apoptosis assay has been proposed to predict clinical radiosensitivity rather reliably [5, 6]. Stratifying patients according to their *in vitro* apoptotic responses, high levels of radiation-induced apoptosis were predictive of normal responses to radiotherapy, while individuals with low levels of apoptosis were likely to be clinically radiosensitive. Given that radiation-induced apoptosis is primarily an ATM-mediated cellular response to ionising radiation, these findings could be interpreted to fit the A-T model where clinical radiosensitivity is a phenotype associated with loss of ATM function.

Following exposure to ionising radiation, critical cellular responses to DSB include the induction of apoptosis, a form of programmed cell death triggered largely by the recruitment and activation of ATM protein at these break sites. ATM-mediated apoptosis occurs primarily through controlling post-translational modifications of p53 and phosphorylation of Mdm2, an ubiquitin ligase which in its native unphosphorylated state binds to p53, thereby targeting the tumour suppressor protein for degradation [7–9]. In addition, ATM also regulates apoptotic signalling indirectly via a redundant pathway involving Chk2 phosphorylation of p53 [10]. Although much is known about the signalling events surrounding ATM-mediated induction of apoptosis, the mechanistic regulation between DNA damage signalling and cell death following exposure to ionising radiation is unclear. Lately, it has been suggested that the balance between these cellular processes is governed by the phosphorylation of serine 139 and tyrosine 142 on H2AX. Phosphorylation of H2AX on serine 139 (γH2AX), most notably by ATM, promotes the recruitment and accumulation of MDC1 and other damage signalling proteins, while phosphorylation on tyrosine 142 by the kinase activity of Williams-Beuren syndrome transcription factor (WSTF) prevents MDC1 focus formation and instead promotes binding to pro-apoptotic proteins, such as JNK1 [11–14]. Concerning DSB repair and induction of cell death, early experimental data had long implicated the presence of persistent DNA damage as a trigger of apoptosis, supporting a mechanistic linkage between these cellular endpoints [15]. Nonetheless, the validity of this notion was recently brought into question on the basis of laboratory findings demonstrated in wild-type thymocytes where inhibition of DNA-PK-dependent end-joining failed to modulate levels of apoptosis despite high levels of residual DNA damage in these cells [16].
In this study, DSB repair and apoptosis induction in \textit{ex vivo} irradiated blood lymphocytes were assessed as markers of normal tissue radiosensitivity in 16 women selected on the basis of late adverse effects to breast radiotherapy. With the inclusion of a further eight healthy individuals, we proceeded to test the relationship between these cellular responses within our cohort of radiotherapy patients and volunteers using small molecule inhibitors of DNA-PK and ATM kinase.

\textbf{III.4.3 Materials and methods}

\textbf{III.4.3.1 Severe cases, controls, and healthy volunteers}

Eight cases and controls, including the seven severe cases and controls from the preceding study, were recruited. Median age of cases and controls was 66 and 67.5 years, respectively. Median duration of follow-up for cases and controls was 8 and 14.5 years, respectively. Eight healthy volunteers were also accrued for the study. Age of healthy volunteers ranged from 25 to 64 years. Ethical approval for the study was obtained from research ethics committees at all participating centres. Informed consent was obtained from the patients and healthy volunteers prior to participation.

\textbf{III.4.3.2 \textit{Ex vivo} irradiation and immunostaining of G_0 blood lymphocytes}

G_0 blood lymphocytes were isolated from whole blood using similar protocols (Chapter III.2.3.3). Blood lymphocytes were irradiated using 250 kV X-rays delivered at 0.5 Gy/minute (AGO X-ray Ltd, Reading, UK), and immunofluorescence staining was performed as previously described at selected time-points (Chapter III.2.3.4). The following primary antibodies were used for immunostaining: anti-53BP1 (mab3802, Millipore, Watford, UK, 1:400 and ab36823, Abcam, Cambridge, UK, 1:400), anti-\(\gamma\)H2AX (05-636, Millipore, 1:500, ab18311 and ab26350, Abcam, 1:500), and anti-phosphoS1981-ATM (ab81292, Abcam, 1:100). Appropriate secondary antibodies were conjugated with Alexa-Fluor 488, 555, and 594 (Invitrogen, Paisley, UK, 1:200). Co-localising \(\gamma\)H2AX/53BP1 foci were scored in a minimum of 50 cells per slide for each patient sample.

\textbf{III.4.3.3 Radiation-induced apoptosis in irradiated G_0 blood lymphocytes}

Radiation-induced apoptosis was assessed using a carboxyfluorescein fluorochrome inhibitor of caspases assay (FLICA, AbD Serotec, Oxford, UK). Blood lymphocytes were incubated with FLICA (1:150) for 1 hour prior to irradiation, and the proportion of apoptotic cells was measured by flow cytometry 48 hours following exposure to X-rays. Apoptotic cells were defined by small cell size and positive cellular FLICA uptake. For apoptosis measurements in CD8 and CD4 T-lymphocyte subsets, cells were further incubated with anti-CD8 (ab4055, Abcam, 1:100) and anti-CD4 (ab1046, Abcam, 1:100) antibodies for 1 hour, washed with PBS, and incubated with appropriate secondary antibodies conjugated with Alexa-Fluor 660 (Invitrogen, Paisley, 1:200) for 1 hour. Following final washes, lymphocytes were analysed using a Millipore Guava EasyCyte flow cytometer (Millipore UK Ltd, Watford, UK). Information was obtained
from 10,000 lymphocytes per sample and characterised by cell size, granularity, and fluorescent signal intensity using Guava InCyte software (Millipore UK Ltd, Watford, UK).

III.4.3.4 Treatment with small molecule inhibitors

Blood lymphocytes were treated with Nu7441 and Ku55933 to inhibit the activity of DNA-PK and ATM, respectively. Drugs were purchased from Tocris Bioscience, Bristol, UK, reconstituted in DMSO as 10 mM stocks, and stored in –20°C. Nu7441 and Ku55933 were added to lymphocytes for 1 hour prior to irradiation and kept in situ until harvest at concentrations of 1 (0.01% DMSO) and 10 μM (0.1% DMSO), respectively. All results were compared against controls incubated with 0.1% DMSO alone.

III.4.3.5 Statistical methods

Blinding processes as elaborated in Chapter III.2.3.5 were similarly applied to peripheral blood samples of cases and controls in this study. Comparative analyses of foci and apoptosis levels between cases and controls were performed using the Mann-Whitney U test. Spearman’s rank correlation test was used to test for correlation of foci and apoptosis levels for the same individuals. All statistical calculations were performed using the statistical software SPSS version 15.0. Given the small sample size, statistical significance was set at a modest p-value of < 0.01.

III.4.4 Results

III.4.4.1 Increased γH2AX/53BP1 foci levels 24 hours after 4 Gy in cases compared to controls

Significant inter-individual variation of foci levels in G₀ blood lymphocytes 24 hours after 4 Gy was observed for cases and controls (coefficient of variation of the entire cohort was 16.9%). Compared to controls, foci levels were higher in all cases, with the exception of one. Mean foci per cell were 12.7 in cases and 10.3 in controls (Figure III.20, p = 0.002).

III.4.4.2 Comparable apoptosis levels 48 hours after 8 Gy between cases and controls

Among cases and controls, there was significant inter-individual variation of apoptosis levels in G₀ blood lymphocytes 48 hours after 8 Gy (coefficient of variation of the entire cohort was 30.4%). In contrast to residual foci levels 24 hours after 4 Gy, similar mean levels of apoptosis were observed in cases (37.2%) and controls (34.7%, p = 0.442, Figure III.21).

Apoptosis levels in the CD8 and CD4 T-lymphocyte subsets were also comparable between them. Mean proportion of CD8 and CD4 apoptotic cells was 37.7% in cases, 38.3% in controls (p = 0.721) and 23.6% in cases, 23.8% in controls (p = 0.878, Figure III.22), respectively.
III.4.4.3 Induction of apoptosis is dependent on DSB end-joining and ATM kinase activation in G₀ blood lymphocytes

A test of association indicated that apoptosis levels 48 hours after 8 Gy were not correlated with residual foci levels 24 hours after 4 Gy in G₀ blood lymphocytes of radiotherapy patients and healthy volunteers (Figure III.23, Spearman $R = -0.059$, $p = 0.785$).
To determine if apoptosis levels were modulated by DSB signalling, we proceeded to measure apoptosis levels in ex vivo irradiated G0 blood lymphocytes treated with Nu7441 for the purpose of inhibiting DNA-PK-dependent end-joining in these cells. Figure III.24 illustrates the foci levels measured at different times (1, 2, 4, 24, and 48 hours) after 1 Gy in mock- and Nu7441-treated cells of a healthy volunteer. At 48 hours post-irradiation, 73% of DSB were repaired in mock-treated cells in contrast to Nu7441-treated cells where only 33% of DSB were repaired, thereby confirming the
efficacy of Nu7441. In addition, modulation of ATM-driven induction of apoptosis in irradiated $G_0$ blood lymphocytes was also tested by treating these cells with Ku55933, an ATM kinase-specific small molecule inhibitor. To validate the efficacy of Ku55933, we confirmed the absence of phosphorylated ATM in irradiated $G_0$ blood lymphocytes following treatment with Ku55933, as well as the failure to observe $\gamma$H2AX/53BP1 foci formation post-irradiation when cells were treated with a combination of Ku55933 and Nu7441 (Figure III.25).

![Figure III.24](image.png)

**Figure III.24** Foci levels at different times post-1 Gy X-irradiation in mock- and Nu7441-treated cells. Error bars represent one standard error of mean of three independent experiments. Abbreviation = IR, irradiation.

In accordance with the increased residual foci levels in Nu7441-treated cells, we observed significantly higher levels of apoptosis in these cells compared to mock-treated controls 48 hours after 1 Gy (Figure III.26). However, when ATM was inhibited simultaneously using Ku55933 in these cells, low levels of apoptosis, comparable to levels in cells treated with Ku55933 alone, were observed. Experimental repeats in cases and controls were consistent with observations in healthy volunteers (Figure III.27).

III.4.4.4 Impaired DSB repair cellular phenotype in a case with severe clinical radiosensitivity

Of the cases in our cohort, we identified an individual with an exceptionally severe phenotype who had markedly elevated levels of residual foci in $G_0$ blood lymphocytes 24 hours after 4 Gy (mean foci per cell $= 16.3$, Figure III.20). The foci level in this patient was highest among all in the study cohort and at least 1.5-fold higher compared to controls. Figure III.28 illustrates foci levels measured at different times (1, 2, 4, 24, and 48 hours) after 1 Gy in $G_0$ blood lymphocytes of this unique case and a control.
The control was selected on the basis of scoring for the lowest level of residual foci among the breast cancer patients. Four hours following irradiation, a 21.5% difference in foci levels was observed between case and control and the difference increased to 46.3% 48 hours post-irradiation, confirming a slower rate of DSB repair in this individual.

To broadly determine the impaired DSB repair process in this severely radiosensitive individual, we designed a series of experiments based on the following principle. In non-cycling G₀ blood lymphocytes where NHEJ is the primary mode of DSB repair, ATM and DNA-PK have been determined to be key drivers of the slow and fast components of a bi-exponential model of DSB end-joining, respectively [17, 18]. Hence, depending on the pathway (slow/fast repair) contributing to the difference in foci levels post-irradiation between our case and control, inhibition of the involved process should in theory abolish any observed difference in foci levels between them, indirectly implying that the inhibited pathway is the source of the impaired DSB repair phenotype in this radiosensitive patient. To this end, foci measurements were repeated in G₀ blood lymphocytes of the same case and control at identical time-points after 1 Gy X-irradiation. Separately, cells of both patients were also treated with Ku55933 or Nu7441 to inhibit ATM kinase or DNA-PK activity, respectively.
Figure III.29 illustrates the foci levels in irradiated lymphocytes of case and control following mock, Ku55933, and Nu7441 treatment. Expectedly, inhibition of ATM and DNA-PK affected rates of foci loss in both case and control, as evident by the higher levels of foci scored in drug-treated cells compared to cells which were not exposed to the kinase inhibitors. However, unlike after ATM inhibition where foci levels remained higher in cells of the clinically radiosensitive individual (upper panel, Figure III.29), comparable foci decay kinetics were observed between case and control following...
Figure III.27 Repeats of the experiments shown in Figure III.26 were performed in two cases, controls, and healthy volunteers. Bar plots indicate patient-averaged percentages of apoptotic cells corrected for levels in unirradiated mock- or drug-treated samples. Error bars represent one standard deviation. Abbreviation = IR, irradiation.

Figure III.28 Foci levels at different time-points over 48 hours after 1 Gy X-irradiation in G₀ blood lymphocytes of a severely radiosensitive case and control. Error bars represent one standard error of mean of three independent experiments. Abbreviation = IR, irradiation.
inhibition of DNA-PK activity (lower panel, Figure III.29), suggesting that in this specific patient, fast component DSB end-joining may be impaired.

**III.4.5 Discussion**

A study of DSB repair and induction of apoptosis in *ex vivo* irradiated G₀ blood lymphocytes of breast radiotherapy patients revealed higher levels of residual γH2AX/53BP1 foci among highly selected individuals with marked late adverse effects
of breast radiotherapy compared to controls, but comparable levels of apoptosis between them. Among the most severely affected cases was an individual with the highest levels of residual DNA damage in blood lymphocytes 24 hours after 4 Gy X-irradiation and an impaired DNA-PK-dependent DSB end-joining cellular phenotype. Supporting the findings ex vivo, foci measurements of in vivo irradiated skin of this patient also revealed high levels of residual DNA damage among a cohort of 35 breast radiotherapy patients [Table III.1, Patient 32]. In that analysis, foci levels 24 hours after 4 Gy in epidermal and dermal cells (fibroblasts and endothelium) of this patient were on average 1.5-fold higher compared to patient-averaged foci levels of the whole cohort. Collectively, these findings suggest an association between clinical radiosensitivity and impaired DSB repair in this and perhaps other patients.

We have already reported similar observations of increased levels of residual DNA damage among patients presenting with severe late normal tissue effects following previous radiotherapy [Chapters III.2 and 3]. The significance of our current findings thus stems from the potential identification of an impaired DNA-PK-dependent end-joining cellular phenotype in a non-syndromic individual. Interestingly, treatment records of this patient did not indicate the presence of severe acute skin reactions during radiotherapy. Apart from the late adverse effects of radiotherapy, she is well and does not harbour clinical features characteristic of a defective DSB end-joining phenotype, such as immunodeficiency, developmental delay, or susceptibility to other haematological and solid tumours [19]. To investigate the nature of the DSB repair impairment, we had explored the use of kinase-specific inhibitors targeted at ATM and DNA-PK. The rationale of our experimental design was based on the idea that inhibition of the impaired DSB repair process in this clinically radiosensitive individual and likewise in a control would abolish any difference in repair kinetics between both subjects. Although the study findings suggest that DNA-PK-dependent DSB end-joining is affected in this unique individual, some limitations associated with this experimental approach ought to be highlighted. Foremost, a key flaw with the experimental design stems from the reliance on γH2AX/53BP1 foci formation as the surrogate of DSB repair. Despite the fact that ATM and DNA-PK have been established to partake in the phosphorylation of H2AX with some degree of functional redundancy between both pathways [20], it is uncertain if inhibiting either kinases would have altered the read-out of a γH2AX/53BP1 foci assay. To augment and validate our study findings, it is therefore necessary to repeat the experiments using DSB repair assays such as pulsed field electrophoresis or the comet assay where assay read-outs are independent of ATM and DNA-PK phosphorylation of H2AX. As such, one could argue that on the basis of our study findings alone, there is no conclusive evidence to support the proposal of a novel DNA-PK-dependent DSB repair defect in this unique patient. Moreover, the broad and perhaps crude nature of our experimental design did not allow for the identification of a specific defect within the NHEJ signalling cascade. To investigate further, we have henceforth embarked on exome sequencing of genes encoding for proteins relevant to NHEJ in this patient. Hopefully, through these analyses, we are able to derive further definitive evidence supporting a novel DNA-PK-dependent DSB end-joining defect in this clinically radiosensitive individual.

Comparative analysis of radiation-induced apoptosis in blood lymphocytes between cases and controls did not reveal a difference. Additional analyses of apoptosis
in specific T-lymphocyte subsets also indicated a lack of difference between the patient groups. Although experimental conditions, such as irradiation dose and time-point, were consistent between this and previous reports showing a correlation between lymphocyte apoptotic responses and clinical radiosensitivity [5, 6], it is perhaps noteworthy that a different assay was adopted for assessing apoptotic lymphocytes in this current study. In the studies performed by Azria and colleagues, apoptosis induction was measured through quantification of the proportion of a distinct population of cells bearing features of a small cell size and significant subgenomic DNA content, while the assay described in this report utilises the presence of caspase activation as a surrogate for induction of apoptosis following radiation exposure. A plausible explanation for the discrepancy in study findings could then relate to the variation in experimental end-points, particularly since caspase-independent mechanisms of apoptosis induction would not have been captured with the latter assay [21]. In this regard, it would be necessary to validate our assay against conventional apoptosis assays designed to test for cellular morphological changes associated with apoptosis, such as terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and test of annexin A5 affinity. Individually, these assays measure DNA fragmentation and loss of plasma membrane asymmetry, respectively. Separately, it is also known that assays of radiation-induced apoptosis are exquisitely liable to multiple physiological sources of variation [22]. It is therefore also possible that subtle experimental variations such as the use of an isolation procedure to derive blood lymphocytes from whole blood samples and small sample cohorts explained the failure to detect differences between cases and controls as reported by Azria and colleagues.

Although pharmacological inhibition of DNA-PK-dependent end-joining in G0 blood lymphocytes reduced DSB repair and enhanced apoptosis induction, residual foci and apoptosis levels were not correlated in the same radiotherapy patients and healthy volunteers. Our experimental findings suggest that apoptosis is a process dominated by ATM kinase activation (Figure III.26), whereas NHEJ in G0 blood lymphocytes is largely driven by DNA-PK and to a lesser extent, ATM (Figure III.29). Taken together, the lack of correlation between residual foci and apoptosis levels in our cohort could conceivably be explained by inter-patient variation in ATM protein levels or kinase function. In a recent study by Fang et al., quantitative analyses of ATM levels among a cohort of breast cancer patients revealed significant inter-patient differences of up to 12.5-fold, which would be consistent with this notion [23].

A separate point of difference raised from our experimental findings is the modulation of apoptosis levels following inhibition of DSB repair, contrasting recent findings by Callén et al [16]. In their study, induction of apoptosis was monitored by caspase 3 activation 24 hours after 5 Gy. To address these minor experimental variations between both studies, we repeated apoptosis measurements in Nu7441-treated blood lymphocytes 24 and 48 hours after 5 Gy. In accordance with our observations 48 hours after 1 Gy, apoptosis levels were similarly increased in Nu7441-treated cells 24 and 48 hours following exposure to a higher radiation dose (Figure III.30).

In light of these conflicting findings, it must be said that the regulation of apoptosis in the presence of persistent DNA damage remains open to question. It would be interesting to resolve the inter-play of signalling events regulating DSB repair and apoptosis given that these processes are largely governed by different protein kinases.
Judging from our findings, it is plausible to suggest a mechanistic model involving the additional recruitment of ATM to the flanks of these persistent DSB leading to further activation of p53-dependent apoptosis since inhibition of ATM phosphorylation obliterates this phenomenon [24].

III.4.6 Conclusions

In summary, we have demonstrated that an impaired DSB repair cellular phenotype may be associated with clinical radiosensitivity in some radiotherapy patients presenting with severe late normal tissue effects. Nonetheless, the small sample size of this study precludes support for a lymphocyte-based DSB repair assay for predicting normal tissue radiosensitivity. Impairment of DSB end-joining increases the propensity for cells to undergo apoptosis, but this phenomenon is primarily regulated by an active ATM function.

III.4.7 REFERENCES

Original research

IV

General discussion

IV.1 PROJECT SUMMARY

A test of DSB repair in healthy skin tissues and primary G₀ blood lymphocytes of 35 breast radiotherapy patients reveals that this cellular process is impaired among a subset of clinically radiosensitive patients, in particular, individuals with severely marked late effects to breast radiotherapy. Although comparative analyses of residual DSB levels between highly selected cohorts of cases (patients with moderate/marked radiotherapy changes) and controls (patients with minimal effects to radiotherapy) indicated higher levels of residual DSB among the former patient group, this was only observed when the assay was applied in *ex vivo* irradiated blood lymphocytes but not *in vivo* irradiated skin tissues. The disparity in results of these comparisons of patient-averaged levels of residual DSB could be attributed to observed differences in DSB repair between skin cells and blood lymphocytes following exposure to a fixed dose of ionising radiation. Between different skin cells in the epidermis and dermis, comparable levels of residual DSB were observed 24 hours after irradiation, suggesting that repair of cellular DSB is a molecular process independent of cell-specific physiological characteristics. Higher levels of cellular DNA damage sustained in blood lymphocytes as compared to skin cells despite similar doses of irradiation would propose a modifying influence of the tissue microenvironment on DSB repair, although the perceived slower rate of DSB repair may be explained in part by experimental variations. Taken together, it can be concluded that assessment of cellular responses to ionising radiation exposure in blood lymphocytes constitutes a valid cell-based approach to predicting normal tissue radiosensitivity, and an *in vivo* tissue-specific model does not appear to be a more robust alternative.

In a subset analysis comprising of only individuals with severe late reactions to breast radiotherapy (likely representing the 0.1% and 1% most damaged individuals) and matched controls, chromosomal radiosensitivity and radiation-induced apoptosis were compared against DSB repair as potential cellular markers of clinical radiosensitivity. Higher levels of chromosomal aberrations were uniformly observed in blood lymphocyte metaphases of all selected cases compared to controls, a phenomenon contributed by the increased formation of both exchange (dicentrics and centric rings) and deletion type (excess acentric fragments) aberrations among cases. These findings add to the existing literature supporting the application of chromosomal radiosensitivity as a biomarker of normal tissue responses to radiotherapy. Separately, the lack of correlation between formation of exchanges and levels of residual DSB in the same patients further raises the possibility that presence of chromosomal translocations, formed as a result of incorrect joining of DSB ends, may have an impact on critical tissue responses involved in the pathogeneses of late adverse effects to radiotherapy.
Contrary to reported literature, we were unable to demonstrate differences in apoptotic responses of irradiated blood lymphocytes between cases and controls. As elaborated in the preceding chapter (Chapter III.4.5), this discrepancy could be in part related to the use of different apoptosis assays between this and previous reports. Moreover, considering the relative size of our patient cohort to the reported studies, it must be cautioned that the lack of association between this cellular parameter and clinical phenotype bears no implication on the potential usefulness of this assay for predicting normal tissue radiosensitivity. It is interesting that levels of apoptosis induction and residual DSB were not correlated in irradiated blood lymphocytes of the same individuals, although through pharmacological inhibition of DSB repair, it was demonstrated that cells harbouring higher levels of DNA damage had an increased propensity to undergo apoptosis. Nonetheless, it was also observed that induction of apoptosis, even in the presence of significant DNA damage, was hugely reliant on ATM kinase activation. It is therefore conceivable that if individuals possess differing levels of functional ATM kinase activity, a lack of association between these molecular processes within the individual can be expected.

Finally, we highlight a clinically radiosensitive individual who presented to us with extremely severe late effects shortly after undergoing breast radiotherapy. Treatment parameters for this patient did not include prescription of a high radiotherapy dose or other significant unfavourable factors which may have predisposed her to an increased risk of late adverse effects. DSB repair assay performed in irradiated skin and blood lymphocytes of this patient indicated markedly high levels of residual DSB in these tissues, approximately 1.5-fold higher compared to mean DSB levels of the whole patient cohort. To broadly characterise the defective molecular pathway leading to DSB repair impairment in this individual, $G_0$ blood lymphocytes of this patient and a control were treated with small molecule inhibitors of DNA-PK (Nu7441) or ATM (Ku55933) to inhibit components of DSB repair relating to DNA-PK-dependent NHEJ (fast) or ATM-dependent repair of heterochromatin-related DSB (slow). The rationale underlying the design of this assay was such that if the pathway involved was simultaneously inhibited in both case and control, similar kinetics of DSB repair would be observed in both patients. Based on the findings of these tests, we proposed that DNA-PK-dependent NHEJ is impaired in this unique individual. Nonetheless, alluding to reasons as discussed earlier (Chapter III.4.5), it is imperative to recognise the limitations associated with our experimental design, particularly with regard to the possibility of interference of assay end-point read-out. Considering the absence of other clinical features associated with a defective DSB repair phenotype in this clinically radiosensitive patient, it would be interesting to determine the specific genetic mutation or epigenetic modification contributing to this cellular phenotype. Given that NHEJ is essentially a multi-step process driven by a variety of proteins, the initial steps of identifying the lesion currently involve exome sequencing to characterise the genomic composition of the different proteins involved in this repair mechanism. Short of clonogenic assessment studies to confirm an increased radiation sensitivity of primary cells of this patient, it is within reason to conclude that our findings allude to an indirect association between an impaired DSB repair cellular phenotype and clinical radiosensitivity, once again, highlighting the pivotal role of this molecular process in modulating normal tissue radiosensitivity, as suggested by historical data [1, 2].
IV.2 CHALLENGES AND CLINICAL IMPACT OF PREDICTIVE TESTING OF NORMAL TISSUE DAMAGE

IV.2.1 Controversies of case-control studies testing for predictive markers of normal tissue radiosensitivity

For decades, developing a robust predictive assay of normal tissue radiosensitivity has been the subject of intensive research within the radiotherapy community. However, as many are also aware, much of these efforts have been rewarded with limited success, often confined to small case-control pilot studies. Typically, a promising predictive marker of normal tissue radiosensitivity, proposed on the basis of correlation between cellular and clinical end-points within a small cohort of radiotherapy patients, fails to reproduce comparable levels of association between both parameters when tested in a larger and more heterogeneous patient cohort [3–6]. Other times, conflicting results would arise from independent studies investigating the same predictive assay of normal tissue radiosensitivity, thus questioning the reproducibility and reliability of the tested assay [7–10]. As raised in the earlier chapters, the potential to establish a correlation between a cellular marker and clinical radiosensitivity is heavily influenced by a number of factors [11]. Briefly, these factors include selection of cases and controls while ensuring that modifying factors of normal tissue responses are considered, adopting an appropriate clinical end-point as an indicator of clinical radiosensitivity, quality and consistency of clinical follow-up, employing an assay with a well established laboratory protocol, along with choosing the optimal experimental conditions for dose, dose rate, time-point, and cell type.

In addition to these factors, flaws pertaining to study designs and result analyses have also been proposed for the lack of success of case-control studies testing for predictive markers of normal tissue radiosensitivity. Most notably, in a 2003 editorial by Dikomey and colleagues from the Hamburg group [12], basing their analysis on data reported by Peacock et al. [6], it was elegantly demonstrated that although comparative analysis of mean cellular radiosensitivity between cases and controls did not reveal a difference between them, a positive association between cellular radiosensitivity and late clinical effects may have been observed in that study had the authors undertook a different approach with result analysis. Perhaps, before elaborating further, it must be mentioned in the same vein that the methods proposed by Dikomey et al. are based on the assumption that incidence of late normal tissue effects occurs at a constant annual rate in radiotherapy patients across all spectrums of intrinsic radiosensitivity, a concept recently put forth by Jung and colleagues [13]. Figure IV.1 provides an illustrative explanation of this concept. From this example, it is apparent that ‘resistant’ patients are also liable to severe late effects of radiotherapy. In this context, it would be unsurprising why comparison of mean cellular radiosensitivity between two groups of patients, selected based on their clinical presentation of late adverse effects alone, is likely to yield a negative result, since there is a strong possibility that selected ‘cases’ may not necessarily correspond to an intrinsically radiosensitive population.

The method of analysis proposed by Dikomey et al. is presented in Figure IV.2. Contrary to comparing mean cellular radiosensitivity between cohorts of cases and
controls (Figure IV.2, bottom panel), it was suggested that tests of association between the predictive test and clinical end-point should be performed on data stratified according to individual intrinsic radiosensitivity, as determined by the former (Figure IV.2, top and middle panels). Applying this method, it is evident that an association exists between both cellular and clinical parameters, given that a higher proportion of intrinsically ‘sensitive’ individuals suffers from grade 2/3 reactions, whereas ‘resistant’ individuals mostly present with grade 1/no reaction.

Figure IV.1 Kinetics of Grade 3 late effects following radiotherapy based on estimates generated from the model proposed by Jung et al. [13]. Data is presented for each patient sub-group, classified according to their intrinsic radiosensitivity (determined in vitro). For each group, proportion of complication-free patients was plotted against time after radiotherapy. Dashed line represents kinetics calculated for the entire cohort. Reused with permission from Dikomey et al., 2003 [12].

Figure IV.2 Theoretical distribution of ‘resistant’, ‘normal’, and ‘sensitive’ patients in relation to clinical severity of late normal tissue effects (grade ≤ 1, 2, 3) calculated partially based on plots presented in Figure IV.1 and an assumption of a normal distribution of intrinsic radiosensitivity among radiotherapy patients. Data is generated for a follow-up period of 6 years post-radiotherapy. (Top) Relative fractions of ‘resistant’, ‘normal’, and ‘sensitive’ patients with grade ≤ 1, 2, 3 reactions. (Middle) Absolute number of patients (based on a cohort of 200 patients) for each category derived from data presented in the top panel. (Bottom) Absolute number of patients classified according to their clinical severity. Reiterating an earlier point made regarding possible flaws relating to a case-control study design, it is evident that an analysis of mean cellular radiosensitivity between cohorts of patients with marked or minimal clinical reactions is unlikely to yield a positive difference between them. It is nonetheless important to note that this argument is primarily based on the concept that incidence of late reactions occurs at a constant annual rate, albeit at different kinetics in individuals of differing intrinsic radiosensitivity. Reused with permission from Dikomey et al., 2003 [12].
Challenges of predicting clinical radiosensitivity

![Bar chart showing the distribution of patients across different grades and radiosensitivity levels.](chart_image)
At this point, it is probably relevant to address rightful concerns of the methods employed in our study. Although a comparative analysis approach was adopted for data analyses in the study, additional critical steps pertaining to patient selection were undertaken, taking into account some of the arguments presented by Dikomey and colleagues. Firstly, cases in the study were chosen among patients who had presented with late adverse effects at an early time-point after breast radiotherapy, while it is required that controls with minimal symptoms have had a lengthy duration of follow-up. In selecting controls who had been monitored over a lengthy period ranging from 11 to 24 years (Table III.4), we aimed to reduce the likelihood of a false-negative selection. Similarly, identifying cases among patients who present with moderate to severe late effects soon after radiotherapy (ideally within 1 to 2 years post-radiotherapy) lessens the probability of a false positive selection, especially since such a patient population is likely to comprise mostly of intrinsically radiosensitive individuals (Figure IV.1). To further ensure that clinical phenotype of the patients truly reflects their intrinsic radiosensitivity, we attempted added measures of selecting cases and controls who had been exposed to favourable and unfavourable clinical factors known to influence normal tissue response after breast radiotherapy, respectively. However, as already explained, adhering to the stipulated criteria proved to be extremely difficult. Separately, considering the relatively small sample size of our cohort and the skewed nature of the patient selection process adopted in this study (where selected cases and controls were assumed to reflect extreme spectrums of intrinsic radiosensitivity), it would have been inappropriate analysing our data using the methods proposed by Dikomey et al. Ultimately, there are grounds to affirm the validity of the methods employed in our study, since after all, a comparative analysis of residual DSB levels in blood lymphocytes of cases and controls reveals higher levels of residual DNA damage among the clinically radiosensitive cohort.

IV 2.2 DSB repair as a biomarker of clinical radiosensitivity

On the basis of experimental findings demonstrating an increased cellular radiosensitivity among syndromic and non-syndromic individuals presenting with an aggravated normal tissue response to radiotherapy, it was proposed that determination of cellular radiosensitivity using an in vitro cell-based assay could provide an estimate of an individual risk of normal tissue damage following radiotherapy [14–17]. To date, colony forming assay remains the gold standard approach to evaluating cellular radiation sensitivity, but performing this assay is time-consuming and laborious, notwithstanding the fact that culturing of primary cells requires an added level of expertise. Given the disadvantages, several surrogate cellular markers have been suggested and DSB repair is included among them.

It is widely agreed that DSB induced by ionising radiation if left unrepaired have an incredibly toxic effect on cells. In response to the induction of this lesion following radiation exposure, a range of molecular processes relating to checkpoint arrest and DSB repair is triggered within the cell nucleus to preserve the genomic integrity and physiological function of the cell. Nonetheless, in the event that DSB repair is impaired, a common assumption is that termination of cell viability through a variety of mechanisms such as apoptosis, autophagy, necrosis, or some form of mitotic...
catastrophe would ensue, thus preventing the erroneous replication of damaged genome. In this background, it would be intuitive to expect a close correlation between levels of residual DSB and loss of clonogenicity. This mechanistic relationship was also confirmed in experimental studies demonstrating a significant level of correlation between both cellular parameters in cell lines of radiotherapy patients and syndromic individuals, although in some of these reports, a positive association was only observed when certain experimental conditions were fulfilled [18–24].

Regardless of a correlation between cellular radiosensitivity and DSB repair, the verdict remains at large if assays of the latter cellular end-point predict for normal tissue responses in radiotherapy patients. Biological principles underlying the potential of the DSB repair assay for this purpose had been elaborated in the earlier chapters, and mostly they relate to the target cell model of normal tissue damage where tissue effects are a direct consequence of radiation-induced cell killing leading to a loss or inactivation of functional structural units within the irradiated tissue. Nonetheless, for several tissues, models not directly related to intrinsic radiosensitivity have been developed, showing the involvement of cytokine-mediated multi-cellular interactions in the radiation response [25, 26]. A fine example would be the release of extracellular TGFβ1, a cytokine with an established role in fibrogenesis, via oxidative and proteolytic cleavage from its latency-associated peptide following irradiation of mammary tissue [27]. In this model of radiation-induced fibrosis, no correlation with DSB repair or other related cellular end-points would be expected.

In breast radiotherapy patients who represented the 5% most damaged individuals within a cohort of women treated for early breast cancer using standardised radiotherapy protocols, we observed raised levels of residual DSB in blood lymphocytes 24 hours after 4 Gy irradiation compared to that of controls. A similar observation was also noted in test irradiated skin tissues, albeit only in a subset of patients who likely represent the 0.1% and 1% most damaged population. Collectively, these study findings are in agreement with a mechanistic role of DSB repair in the pathogenesis of late normal tissue effects in radiotherapy patients. However, considering the complex inter-play of several molecular processes contributing to the onset of late radiation-induced adverse effects, whether this cellular marker alone predicts for normal tissue radiosensitivity remains to be seen, and certainly the strength of this assay in predicting clinical radiosensitivity ought to be tested in a large scale prospective study.

IV.2.3 Potential clinical impact of predictive testing of normal tissue radiosensitivity

IV.2.3.1 Screening for ‘over-responders’ to radiotherapy

At first glance, it would appear that if a predictive assay of normal tissue radiosensitivity exists, such an assay would be of great value in clinical practice with various potential applications as had been discussed in Chapter I.2.1. The proposed applications are somewhat overlapping, and for the most part relate to the identification of individuals who are intrinsically radiosensitive, treating these patients with a lower radiotherapy dose or perhaps referring them to a different treatment option. In this scenario, the routine use of an assay to screen for ‘over-responders’ to radiotherapy would require
an extremely precise assay with a high positive predictive value. A positive predictive value refers to the probability that an individual is intrinsically radiosensitive if he or she receives a positive test result. Mathematically, it is defined by the formula shown below.

Positive predictive value = \frac{\text{Number of true positives}}{\text{Number of positive results}}

From the above formula, it is evident that in order to achieve an assay with a strong positive predictive value, conditions of a high detection of true positive cases coupled with a low false detection rate (i.e. false positives) have to be fulfilled. However, as often encountered in the screening of lowly prevalent conditions, in this case clinical radiosensitivity, a common problem associated with an assay designed for this purpose is a low positive predictive value. To illustrate this argument, a reference is made to Figure IV.3 which is adopted from a 1997 editorial by Bentzen [28]. In this example, theoretical normal distributions of SF$_2$, the parameter used to define individual cellular radiosensitivity, were plotted for cohorts of ‘over-responders’ (stippled) and the remaining normal population (full line), keeping in mind that the prevalence of the former patient group is probably about 1% of all radiotherapy patients [16, 17]. Means and coefficients of variation of SF$_2$ were estimated to be 0.15 in ‘over-responders’, 0.37 in normal individuals, and 20% in ‘over-responders’, 25% in normal individuals, respectively. A modest SF$_2$ of 0.19 was then chosen as the threshold for classifying an individual as an ‘over-responder’, and true positive and false positive detection rates of ‘over-responders’ are represented by areas under the stippled and full line plots to the

Figure IV.3 Lognormal distributions of SF$_2$ values in two hypothetical cohorts of ‘over-responders’ (stippled) and normal individuals (full line). For the clarity of illustration, only the left hand tail of the latter distribution is shown in the graph. Reused with permission from Bentzen, 1997 [28].

1 Positive results include true and false positives.
left of this threshold, respectively. Based on these estimates, a predictive assay of clinical radiosensitivity with this level of capability would yield a relatively high rate of true positive detection ($\approx 90\%$), but only a fairly modest positive predictive value ($\approx 65\%$) owing to the significant false positive detection rate. From this example, it is obvious that to derive an assay with a high positive predictive value of clinical radiosensitivity, the cellular parameter under test must be able to yield a wide separation between the two curves. Specifically, there should be a significant difference in cellular responses between ‘over-responders’ and the remaining normal population. The degree of inter-individual variation of this cellular end-point should ideally also be limited in both patient subsets.

Perhaps, a different approach can be undertaken with predictive assays of clinical radiosensitivity. Instead of applying the assays for the screening of ‘over-responders’, a more sensible and viable approach could entail using them as a negative predictive test to identify individuals who belong to the normal population. With reference once again to Figure IV.3, applying $SF_2 = 0.19$ as the cut-off for defining an individual as a normal responder to radiotherapy, the negative predictive value of an assay can be easily determined by the areas under the stippled (false negative) and full line (true negative) plots to the right of this value. Quite evidently, contrary to the rather modest positive predictive value associated with an assay for predicting clinical radiosensitivity, it would seem that a strong negative predictive value is feasible for such a test. Given this possibility, it may be more practical implementing the assays for the screening of normal rather than clinically radiosensitive individuals.

IV.2.3.2 Modifying radiotherapy dose prescriptions according to individual intrinsic radiosensitivity

Another related rationale for a predictive assay of normal tissue radiosensitivity is to identify individuals within the sensitive tail of the distribution of normal tissue radiosensitivities. Empirically, this population of radiotherapy patients can be defined in relation to the dose at which incidence of late normal tissue complications among all patients would not exceed 5% at 5 years post-radiotherapy, a dose also commonly referred to as the TD$_{5/5}$. Hence, individuals presenting with late adverse effects having received doses to normal tissues lower than the stipulated TD$_{5/5}$ are classified as the 5% most clinically radiosensitive, and this would include the 1% most damaged ‘over-responders’ mentioned in the earlier scenario. Again, the idea behind the identification of these clinically radiosensitive individuals rests on the notion that by referring these patients to other modalities of treatment, such as surgery and/or systemic therapy, a meaningful dose escalation may be possible in the remaining normal population. In theory, this should result in an improvement of tumour control while maintaining a comparable rate of late complications in the whole cohort.

To test this concept, we refer to a typical example of a dose-response curve for late normal tissue end-points, illustrated in Figure IV.4. As reported in the literature, dose-response in late responding tissues is typically characterised by a steep dose gradient, implying that a small change in dose leads to a significant increment in response in these tissues [29–31]. In the graphs below, the stippled plots represent dose-response curves for an unselected population, while the super-imposed full line plots are
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Generated after excluding the 5% most clinically radiosensitive individuals. From these plots, it can be deduced that even with the exclusion of these intrinsically radiosensitive sub-population, total dose to normal tissue could only be increased by 3.1 Gy, corresponding to a mere 5.4% dose increment. The limited dose escalation is primarily due to the steep dose-response curve for late normal tissues. Based on this example, it is obvious that such a strategy is sub-optimal unless more patients are removed from the sensitive tail, which would allow for a more clinically meaningful dose escalation.

Figure IV.4 Hypothetical example of a dose-response curve for late responding tissues generated based on a $\gamma_{50}$ value of 4. Stippled and full line plots represent curves for an unselected population and a population cohort when 5% most clinically radiosensitive patients were excluded, respectively. Shaded areas indicate the 5% response level. Top and bottom (zoomed image of the shaded area) panels illustrate the possible dose increment from 57.1 to 60.2 Gy to normal tissues without exceeding the TD$_{5/5}$ in a population where the 5% most radiosensitive subset is excluded from treatment. Reused with permission from Bentzen, 1997 [28].
IV.2.3.3 Improving therapeutic ratio: relationship between tumour control and late normal tissue complication probability models

Ultimately, the goal of a predictive assay of clinical radiosensitivity is to improve therapeutic ratio, either by lowering the rate of normal tissue complications through screening of clinically radiosensitive individuals or raising the tumour control probability by escalating doses in the remainder. It is therefore also important to scrutinise the possibility of achieving gains in tumour control probability as claimed in the latter context. For this exercise, an analysis of the dose-response curves for tumour control and late normal tissue complications was performed (Figure IV.5). Unlike the curves for late responding normal tissues, clinical dose-response curves for tumours are generally less steep. For example, in head and neck tumours, estimated $\gamma_{37}$ values ranged between 0.5 to 2.8, which is in stark contrast to values generated for late effects [33]. The difference in dose gradients between these curves impacts significantly on the potential therapeutic gains with dose escalation. Considering that a clinically detectable dose escalation would probably need to be in the order of 10% or more, it can be easily inferred from Figure IV.5 that the benefits of improving tumour control with a 10% increment in dose is only modest at best, whereas a disproportionate increment in late adverse events can be expected. This loss in therapeutic ratio is further amplified in tumours with a high tumour control probability where the steepness of the slope approaches zero. On this account, it is discouraging to note that the expected gain with dose escalation, even if a reliable and robust predictive assay of clinical radiosensitivity were available, is marginal. Unfortunately, it would appear that the
maximum benefit from dose escalation is mainly derived at low levels of tumour control probability, but in such instances, one would even question the indication of radiotherapy in the first place.

IV.2.3.4 Potential strategies for the use of normal tissue radiosensitivity assays

Given the arguments put forth, it is clear that in order to exploit the clinical usefulness of normal tissue radiosensitivity assays, a knowledge on tumour radiosensitivity is required. Interestingly, findings arising from historical laboratory and clinical studies designed to examine a possible relationship between cellular radiosensitivities of tumours and normal tissues have suggested a significant host factor in modulating radiation responses of both tissues [34–37]. For example, tumours and normal tissues originating from mice harbouring the RS-SCID phenotype were significantly more radiosensitive compared to the wild-type counterparts [34]. Similarly in humans, A-T patients having received a sub-optimal dose due to the onset of severe clinical reactions to radiotherapy demonstrated remarkable tumour control with treatment which is usually considered inadequate [36]. In a study by West et al. from the Manchester group, tests of cellular radiosensitivity of tumour cells and lymphocytes from non-syndromic cervical cancer patients were shown to be independently predictive of late normal tissue complications in these patients [37]. Collectively, these studies support the notion that intrinsic radiosensitivity of tumours and normal tissues are correlated, although it must be cautioned that findings inconsistent with the proposed notion have also been reported [38–40].

Assuming that an association between normal tissue and tumour radiosensitivity exists, a clinical situation where predictive testing of the former parameter may be useful relates to the modification of radiotherapy doses in clinically radiosensitive individuals. In this subset of patients, it is possible that dose-response of tumours differs from the average curves derived for the whole population. Although not a lot of information is available on this, it is probably within reason to postulate steep dose-responses in both tumours and normal tissues of these patients. In this hypothetical situation, patients who are on the flat portion of the dose-tumour control probability curve are likely to benefit from a dose reduction if they are on the steep part of the dose-normal tissue complication curve, since this would only lead to a minor change in tumour control probability while impacting a marked change in their risks of late effects (Figure IV.6).

With the advent of modern radiotherapy techniques, such as intensity modulated radiotherapy (IMRT), image guided adaptive radiotherapy (IGRT), and stereotactic radiosurgery (SRS), radiation oncologists are progressively able to design treatment plans entailing prescription of high radiation doses to tumour targets while minimising irradiation of normal tissues. Conceptually, these new modalities aim to deliver a vast improvement in therapeutic ratio, and in the instance of head and neck cancers, the use of IMRT has been proven to reduce the incidence of late toxicities relating to dysfunction of salivary glands and swallowing [41, 42]. In this background, it is prudent to reconsider the potential advantages of predicting normal tissue responses to radiotherapy. Alluding to an earlier point made regarding the modest effects of dose escalation in a population of non-clinically radiosensitive individuals, a favourable
gain in therapeutic ratio would certainly be achievable if radiation doses in different orders of magnitude can be delivered to tumours and normal tissues.

IV.3 FUTURE PERSPECTIVES

The predictive assays of normal tissue radiosensitivity described in this thesis were mostly designed and tested in the early 90s. As alluded to in the earlier chapters, the results obtained with these traditional assays have been inconsistent, which may be unsurprising considering the complexity of pathological processes involved in the development of late clinical reactions to radiotherapy. In more recent times, interests have however shifted to establishing genetic profiles that may predict a patient’s probability of suffering toxicity following radiotherapy [43]. With the seminal discovery of the A-T phenotype, researchers have long postulated that genetic variation likely contributes to inter-individual differences in radiotherapy complications. This was later supported by small pilot studies reporting an association between variations in genes encoding proteins involved in the DDR and clinical radiosensitivity [44]. By the end of 1990s, it is largely agreed among researchers that individual radiosensitivity represents an inherited, polygenic trait. On this basis, a collective effort to screen for novel genetic variants associated with clinical radiosensitivity was initiated, and since the inception of a radiogenomics consortium in 2009, a flourish of studies investigating the relationship between genetic variants and normal tissue effects have been reported [45, 46]. Among the different possible variants, single nucleotide polymorphisms
(SNPs) are most well characterised, although copy number variations (CNVs) also represent an important source of variation. Up until recently, SNPs association studies have been limited to the candidate gene approach, but with the advent of high throughput genotyping, it is now possible to approach the analyses of SNPs in a form of a single, large genome-wide association study (GWAS). Unlike the candidate gene approach where a gene will only be analysed if its function is well understood, GWAS avoids the need for prior knowledge, allowing for identification of novel disease loci within the human genome, even in non-protein coding regions. It is also important to note that large sample sizes are absolutely critical in studies investigating for an association between genotype and phenotype. Currently, a number of large scale studies based on a GWAS design are being conducted, and hopefully the approach undertaken by these studies will eventually yield a promising genotype which is predictive of normal tissue radiosensitivity [45, 47, 48].

On our end, DSB repair, chromosomal radiosensitivity, and radiation-induced apoptosis in ex vivo irradiated blood lymphocytes are currently being tested for an association with normal tissue responses of 400 breast and prostate cancer patients. Patients will be recruited among participants of two randomised trials, who are being prospectively monitored for late toxicities to radiotherapy [49, 50]. Although an association between radiation-induced apoptosis in lymphocytes and clinical radiosensitivity was not observed in our study, Azria and colleagues from the Montpellier group have been engaged as collaborators in this new project given their successes with the assay [51]. Through this large scale prospective study, we hope to conclusively address the value of these singular cellular end-point assays for predicting normal tissue radiosensitivity.

IV.4 FINAL CONCLUSIONS

Predictive assays of normal tissue radiosensitivity have often been referred to as the ‘holy grail of radiotherapy’, which bears the connotation of something intensely sought but never found [52]. While the current in vitro assays have at times demonstrated a statistically significant correlation between a cellular end-point and clinical radiosensitivity, the real obstacle is to develop an assay which is reproducible and possesses the power to precisely predict individual normal tissue outcomes following radiotherapy. With the introduction of newer and more robust approaches involving multi-centre collaborations in GWAS and large scale well designed prospective studies, one can only hope that we are now better equipped in the pursuit of the ‘holy grail’. Otherwise, it may well be that after years of research into predicting normal tissue radiosensitivity, the only real gain, at the end of it all, is merely an improved understanding of normal tissue radiobiology.

IV.5 REFERENCES


ANOV A Analysis of variance
A-T Ataxia-telangiectasia
ATLD A-T line Disorder
ATM Ataxia-telangiectasia mutated
BRCA1/2 Breast cancer 1/2
Cdc25 Cell division cycle 25
Cdk Cyclin-dependent kinase
Chk2 Checkpoint kinase 2
CNV Copy number variation
CtIP C-terminal binding protein interacting protein
CTCAE Common terminology criteria for adverse events
CTGF Connective tissue growth factor
D0.1 Dose required to reduce survival fraction to 0.1
DDR DNA damage response
DNA-PK DNA-dependent protein kinase
DSB DNA double-strand break
Exo1 Exonuclease 1
\gamma_{37} Normalised slope of the dose-response curve at 37% response level
\gamma_{50} Normalised slope of the dose-response curve at 50% response level
GWAS Genome-wide association study
H2AX Histone 2A variant
\gamma H2AX Phosphorylated histone 2A variant
HR Homologous recombination
HSP4 Heat shock protein 47
IL-13 Interleukin-13
IGRT Image guided adaptive radiotherapy
IMRT Intensity modulated radiotherapy
KAP1 KRAB-associated protein 1
LIG4 Ligase 4
MDC1 Mediator of DNA damage checkpoint protein 1
MRE11 Meiotic recombination element 11
MRN MRE11/Rad50/NBS1
NBS1 Nijmegen breakage syndrome 1
NHEJ Non-homologous end-joining
PI3KK Phosphatidylinositol 3-kinase-related kinases
RPA Replication protein A
RS-SCID Radiosensitive-Severe combined immunodeficiency
RT Radiotherapy
SNP Single nucleotide polymorphism
SRS Stereotactic radiosurgery
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SF</td>
<td>Surviving fraction</td>
</tr>
<tr>
<td>SF₂</td>
<td>Surviving fraction at 2 Gy</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single strand DNA</td>
</tr>
<tr>
<td>TD₅/₅</td>
<td>Threshold dose at which incidence of normal tissue complications does not exceed 5% at 5 years post-radiotherapy</td>
</tr>
<tr>
<td>53BP1</td>
<td>Tumour suppressor p53 binding protein 1</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor β1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>XRCC2/3/4</td>
<td>X-ray cross complementation group 2/3/4</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
</tbody>
</table>
Appendix

Peer-reviewed publications and abstracts

Peer-reviewed publications

3. Chua ML, Rothkamm K. Biomarkers of radiation exposure: can they predict normal tissue radiosensitivity? Review article for Clinical Oncology 2013 Special Issue on Radiobiology [In press].

Peer-reviewed abstracts selected for presentations at conferences
