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Overview

Where Do We Look for Markers of Radiotherapy Fraction Size Sensitivity?

N. Somaiah *, K. Rothkamm †, J. Yarnold *

* The Institute of Cancer Research & The Royal Marsden NHS Foundation Trust, London, UK
† University Medical Center, Hamburg-Eppendorf, Germany

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Abstract

The response of human normal tissues to radiotherapy fraction size is often described in terms of cellular recovery, but the causal links between cellular and tissue responses to ionising radiation are not necessarily straightforward. This article reviews the evidence for a cellular basis to clinical fractionation sensitivity in normal tissues and discusses the significance of a long-established inverse association between fractionation sensitivity and proliferative indices. Molecular mechanisms of fractionation sensitivity involving DNA damage repair and cell cycle control are proposed that will probably require modification before being applicable to human cancer. The article concludes by discussing the kind of correlative research needed to test for and validate predictive biomarkers of tumour fractionation sensitivity.

Key words: Cancer; DNA repair; fractionation; hypofractionation; normal tissues; radiotherapy

Statement of Search Strategies Used and Sources of Information

The search strategy included PubMed index terms ionising radiation, radiosensitivity, hypofractionation, normal tissues, tumours.

Introduction

The responses of normal tissues to radiotherapy fraction size have long been assumed to have a basis in cellular recovery, one of the 4 Rs of classical radiobiology [1]. This article reviews the evidence for a cellular basis to fractionation sensitivity and discusses the significance of the close association between fractionation sensitivity and proliferative indices. A molecular model of normal tissue fractionation sensitivity involving DNA repair and cell cycle control is proposed that needs to incorporate genetic and epigenetic modifications before being applicable to cancer. The article concludes by considering how clinicians might investigate the applications of predictive biomarkers of tumour fractionation sensitivity.

Target Theory and the Cellular Basis of Fractionation Sensitivity

The first application of target theory in radiation biology proposed the nucleus as the subcellular target of ionising radiation [2]. In the current article, the cell is considered as the critical target underpinning tissue responses to fraction size (fractionation sensitivity). Oncologists are often introduced to fractionation sensitivity via the linear quadratic equation, an empirical model describing the non-linear relationship between fraction size and tissue response. The relationship is described by the α/β ratio, values $\geq$10 Gy being typical of early-reacting normal tissues and lower values reflecting the greater sensitivity to fraction size of late-reacting normal tissues (see Figure 1) [3–5]. The same model is used to describe the non-linearity or

Author for correspondence: N. Somaiah, Division of Radiotherapy and Imaging, The Royal Marsden, Downs Road, Sutton SM2 5PT, UK. Tel: +44-208-661-3460; Fax: +44-208-661-3107.
E-mail address: navita.somaiah@icr.ac.uk (N. Somaiah).

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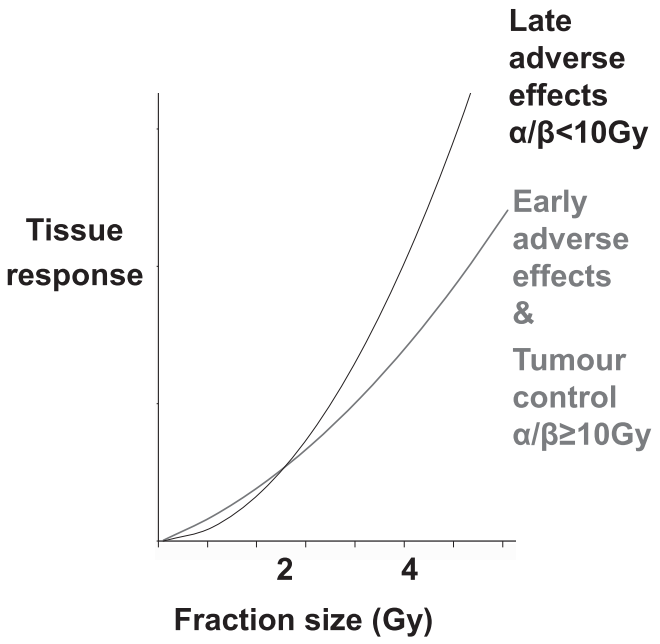

Fig 1. Schema illustrating the traditional model of fractionation sensitivity in normal and malignant tissues, late-reacting normal tissues being more sensitive to fraction size than early-reacting normal tissues and most cancers.

'bendiness' of the *in vitro* clonogenic cell survival curve; an important question to ask is to what extent does the fractionation sensitivity of tissues reflect this cellular response [4,6]. The molecular correlates of cellular recovery focus on DNA double-strand break (DSB) induction and repair, so an even more fundamental question is whether and to what extent does DNA repair explain the fractionation sensitivity of normal tissues.

A central role of DNA damage repair as a determinant of radiation response is shown by the exquisite tissue sensitivity to ionising radiation of rare patients with ataxia telangiectasia, but an association between *in vitro* cellular radiosensitivity and normal tissue response is difficult to detect in non-syndromic patients [7,8]. Among a long list of reasons for past failed attempts to correlate *in vitro* cellular responses to clinical responses in non-syndromic individuals is that *in vitro* assays cannot take into account modifying interactions between different target cell populations and between cells and extracellular matrix [9–11]. This limitation is also relevant to testing the relationship between classical cellular recovery and tissue fractionation sensitivity. If the fractionation sensitivities of all normal tissue target cell populations could be reliably measured *in vivo*, the estimates of α/β for each target cell type contributing to the function of a particular tissue would incorporate the modifying effects of cell–cell and cell–matrix interactions. The challenge would then be to build a biological model describing how the responses of individual target cell populations explain the fractionation sensitivity of the tissue or organ as a whole.

Human skin is a good place to start thinking about this, as responses to radiotherapy are well characterised. For example, the fractionation sensitivity of desquamation can be considered in relation to a single population of target stem cells in the basal epidermis [12]. The low sensitivity of moist desquamation to fraction size (high α/β value) has been accurately quantified for treatment times >10 days, and there is a very close association between desquamation and depletion of basal epidermal cells [13,14]. Human skin biopsies collected during and after 40 Gy in 10 fractions over 5 weeks compared with 50 Gy in 25 fractions over 5 weeks generate values of $\alpha/\beta \geq 10$ Gy for basal cell depletion, consistent with a causal link between basal cell depletion and fractionation sensitivity of desquamation [14]. Acute epidermal responses are probably influenced by interactions with the underlying dermis, which two-dimensional keratinocyte cultures cannot take into account. The capillary dilatation responsible for erythema is clearly a dermal response, sharing the same high α/β value as desquamation [13]. The impossibility of dissociating desquamation from erythema is consistent with a direct relationship between them. The vasodilator vascular endothelial growth factor (VEGF), over-expressed by basal epidermis in some chronic inflammatory diseases and induced by radiation, is one of several paracrine mechanisms that might be responsible [15,16]. If moist desquamation is severe, permanent epidermal stem cell depletion causes healing by secondary intention (granulation) where skin atrophy, fibrosis and telangiectasia are classified as 'consequential' effects characterised by high α/β values [17]. If moist desquamation heals without scarring, late onset skin atrophy, fibrosis and telangiectasia are regarded as 'true' late effects, each having low α/β values. The relevant target cells for telangiectasia include endothelial and myoendothelial cells. Endothelium is also a likely target cell to consider in relation to atrophy, where the latter may be partly a response to tissue ischaemia and hypoxia.

If there are no fibroblasts, there can obviously be no fibrosis [18]. If confluent fibroblast monolayers are irradiated, they enter a prolonged G1 cell cycle arrest and upregulate collagen production rather than undergo apoptosis or suffer mitotic catastrophe [19–21]. High levels of stable chromosomal translocations in fibroblasts cultured from human skin irradiated many years previously suggest that fibrosis can be, at least in part, the product of surviving, irradiated fibroblasts rather than immigrant cells [22]. However, the fractionation sensitivity of fibrosis probably needs to consider more than resident fibroblasts. In systemic sclerosis, dermal fibrosis represents a response to microvascular occlusion (endothelial target cell) and hypoxia [23,24]. Platinum electrodes confirmed cutaneous hypoxia many years after high dose radiotherapy for head and neck cancer, so perhaps a target theory applied to fibrosis needs to consider endothelial cells. Endothelial cells and fibroblasts are not the only putative target stem cells when considering the fractionation sensitivity of fibrosis. Smooth muscle cells differentiate into collagen-producing fibroblasts in several human fibrotic states, including atherosclerosis [25]. Fibroblast progenitors might also be unexposed, immigrant cells, such as marrow-derived fibrocytes [26]. When describing fibrogenic responses to fraction size, a target cell model assumes that the intracellular target is DNA and the relevant

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processes relate to DNA repair. In conclusion, multiple target cell types may need to be considered even when considering the fractionation sensitivity of a single pathological response (fibrosis) within a single organ (skin) [18–22,27,28].

Before leaving target theory, early epidermal (parenchymal) and late dermal (stromal) responses are separated in time by years, but in other organs, symptomatic parenchymal and stromal responses may coincide as, for example, in some cases of pneumonitis and lung fibrosis. Whatever target cell types are critical to organ failure, the coordinated response of multiple cell lineages underlies the 'tissue functioning unit' as a concept in radiobiology [29,30]. Target theory merely implies that the fractionation sensitivity (α/β value) of tissue functioning units represents a synthesis of the α/β values of its different target cell components.

Fraction Size Sensitivity and the Proliferative Status of Normal and Malignant Tissues

A strong inverse association between the proliferative indices of normal tissues and their fractionation sensitivity has long been recognised, e.g. tissues with high proliferation indices, such as oral mucosa, are relatively insensitive to fraction size (see Figure 2) [4,31]. Proliferative indices are measured in parenchymal cells, so if stromal cells contribute to normal tissue fractionation sensitivity, it implies a close association between the proliferative indices of both cellular compartments. It is relevant to ask if the same association between proliferative indices and fractionation sensitivity applies to cancer, where the malignant cells are assumed to be the target [32]. Incorporation of tritiated or halogenated pyrimidines identifies cells in S phase and is used to generate a labelling index, the percentage of labelled cells several hours after exposure to drug. Although there are variations in technique, the labelling index is more reproducible than the Ki67 index as a measure of cell proliferation [33]. The median labelling indexes of breast and

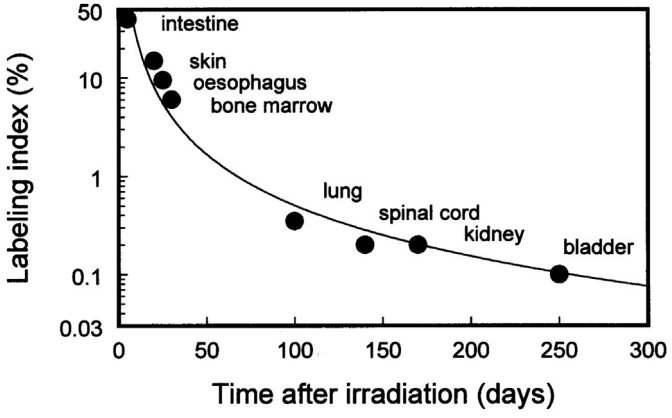

Fig 2. Relationship between labelling index (left vertical axis) and latent interval between irradiation and onset of moderate to severe functional injury in rodents (courtesy of Dr Fiona Stewart, Netherlands Cancer Institute. The figure was first published in the ESTRO Basic Clinical Radiobiology Book (Editor: Gordon Steel, Publisher: Arnold, 1997).

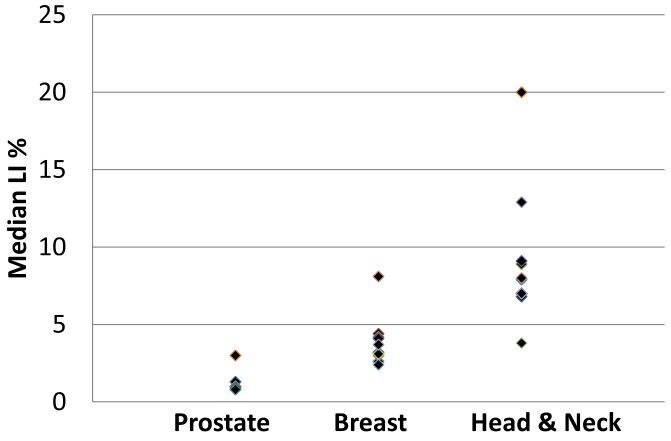

Fig 3. Labelling indices (LI) of human prostate, breast and head and neck cancers [34–58].

prostate cancers are low (see Figure 3) and stand in contrast to squamous carcinomas of the head and neck region, where the median labelling index was >5% in 10/11 published series [59]. These data are consistent with the median labelling index of 8.85% (range 0.6–47.7) reported in a single series of 404 squamous carcinomas of the head and neck contributed by 11 collaborating centres [60]. The association between proliferation index and tissue fractionation sensitivity of normal and malignant tissues is not evidence of a causal relationship, but it does justify thinking about mechanistic links.

Fractionation sensitivity is not necessarily a fixed property of tissues. If the overall treatment time is less than 14 days, acute epidermal responses are more sensitive to fraction size (α/β value ~ 4 Gy) than when radiotherapy is delivered over many weeks (α/β values >10 Gy), a difference that coincides with changes in epidermal proliferative indices [13,61]. In a series of patients undergoing skin biopsy once-weekly during and after post-mastectomy chest wall radiotherapy, basal epidermal cell density began to fall in a dose-dependent manner within 1 week of starting treatment, fell to a nadir at the end of 5 weeks of radiotherapy (50 Gy in 25 fractions) and recovered a week or so later [14]. The classical interpretation of these data, supported by work in pig skin, is that epidermal depletion stimulates accelerated repopulation during the fourth and fifth weeks of treatment, coinciding with the increase in α/β value. More recently, it has been shown that accelerated repopulation of epidermis that gets underway in pig skin during fractionated radiotherapy does not start until radiotherapy has finished in humans [62]. In a separate study, serial skin biopsies during a 5 week course of breast radiotherapy confirmed a G2 arrest (gap between DNA synthesis and mitosis) in the basal epidermis for the duration of radiotherapy [63]. This was also associated with a higher proportion and number of basal cells using homologous recombination repair (only active in S and G2 phases), towards the end of a 5 week course of radiotherapy [63]. This coincides with the loss of fractionation sensitivity seen clinically. The possible significance of this observation for molecular models of fractionation is discussed below.

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Molecular Models of Fractionation Sensitivity in Normal Tissues

Molecular processes relevant to classical cellular recovery and the fraction size sensitivity of normal tissues include processing of DNA DSB and cell cycle controls. There is a direct linear relationship between the number of induced DSB and absorbed radiation dose [64], 2 Gy of megavoltage X-rays introducing about 40 DSB per human genome. An initial fast exponential phase of repair deals with most DSB in the first hour or two. This is followed by a slow exponential phase spanning many hours that tackles the remainder, not all of which are necessarily repaired correctly. Two major pathways are used to rejoin radiation-induced DSB, non-homologous end-joining (NHEJ) and homologous recombination repair [65]. NHEJ rejoins break ends in an error-prone manner, frequently causing micro-deletions or -insertions at the breakpoint and, when multiple breaks coincide, joining break ends derived from different DSB [66]. The latter causes large deletions and translocations, examples of misrepair [67–70]. In contrast to error-prone NHEJ repair, homologous recombination uses a homologous DNA sequence as a template for repair. In S and G2 phase cells, the sister chromatid can therefore enable high fidelity DSB repair by homologous recombination to faithfully restore the original DNA sequence [71]. The DSBs considered most important for determining cell fate are tackled in the slow phase. Until recently, it was thought that these might take longer to repair due to higher chemical complexity, but the delay may also reflect the relative inaccessibility of some lesions to the DSB repair machinery [72]. Access requires physical opening of compact heterochromatin, a loosening achieved by ATM-mediated phosphorylation of the Kruppel-associated box (KRAB)-associated co-repressor Kap1. So, ATM works with NHEJ in G0/1 and with homologous recombination as well as NHEJ in G2/S to repair DSB in heterochromatin. It is useful to distinguish this role of ATM from its activation of G1, intra-S and G2 checkpoints in response to radiation-induced DSB [73]. It is also worth remembering that the clinical literature is consistent with a slow component to tissue recovery [74–78]. Breast cancer patients treated via direct electron fields to right and left internal mammary chains with 25 once daily (24 h interval) or twice daily (8 h interval) 2.0 Gy fractions developed a significantly higher incidence and severity of cutaneous telangiectasia on the side irradiated using 8 h inter-fraction intervals [75].

We are seeking to understand normal tissue fractionation sensitivity in terms of cell and molecular processes by focusing on DSB repair. In genetic systems, NHEJ is sensitive to fraction size [66]. As NHEJ accounts for both fast and slow phases of DSB repair in all phases of the cell cycle [79], this sensitivity to fraction size might explain the fractionation sensitivity of late-reacting normal tissues with very low proliferative indices. Why should NHEJ be sensitive to fraction size? A non-linear increase in the risk of DSB misjoining with radiation dose (fraction size) could be relevant. A physical model that assumes availability of all break ends to each other predicts that the rate of misjoined break ends would rise very steeply with dose (fraction size), a relationship that recalls the beta (D^2) component of the linear quadratic model for chromosome aberration formation, cell survival and normal tissue damage. In practice, all break ends are not freely available to each other (proximity influences interaction probability), but misrepair leading to unbalanced chromosome exchanges may be important enough to form the basis of a mechanistic model. Mammalian fibroblasts genetically defective in NHEJ are exquisitely radiosensitive, as shown by the SCID phenotype in mice [80,81] and they are not spared by low dose rate irradiation that mimics hyperfractionation [82]. We have shown that sensitivity to fraction size is undetectable in cells lacking functional NHEJ and thereby relying on homologous recombination for repair of radiation-induced DSBs [83]. DNA-PK-deficient cells accumulate in S/G2 phase of the cell cycle with fractionated irradiation. It is tempting to speculate that in the absence of the conventional DNA-PK-dependent NHEJ pathway, homologous recombination may operate as a high fidelity repair pathway regardless of fraction size, a property that is helped by the action of cohesin protein complexes that encircle and bind sister chromatids [84]. It is probably not as simple as this, as there is evidence that backup NHEJ mechanisms operating in the absence of the normal NHEJ pathway contribute more than homologous recombination to overall DSB repair [85]. Although the relative importance of these three DSB repair pathways is still somewhat unclear, all of them seem to operate more efficiently in S and G2 than in the G1 phase [86,87]. Sister chromatid cohesion may contribute to this effect by not only aligning homologous DNA sequences to promote homologous recombination but also by acting as a scaffold to hold DSB ends together for efficient repair by NHEJ [88]. Both of these functions may reduce the interaction probability of DSB to form rearrangements, which increases with dose and is therefore sensitive to fraction size.

The foregoing suggests a model of tissue fractionation sensitivity based on the use of error-prone NHEJ for DSB repair in G0/1 cells (sensitive to fraction size) and the dominance of high fidelity NHEJ and homologous recombination for DSB repair in the presence of sister chromatids in S/G2 phase cells (insensitive to fraction size) (see Figure 4) [63,86]. If the proliferative indices measured in tissue sections reflect the proliferative status of constituent target stem cells, self-renewal tissues would be expected to rely more than late-reacting normal tissues on high fidelity repair in G2/S phase. The corollary would be that the high fractionation sensitivity of late-reacting normal tissues reflects the dependence on NHEJ to repair G0/1 phase cells.

Do These Molecular Models Apply to Human Cancers?

Molecular mechanisms relevant to fractionation that differ between normal and malignant tissues include genetic

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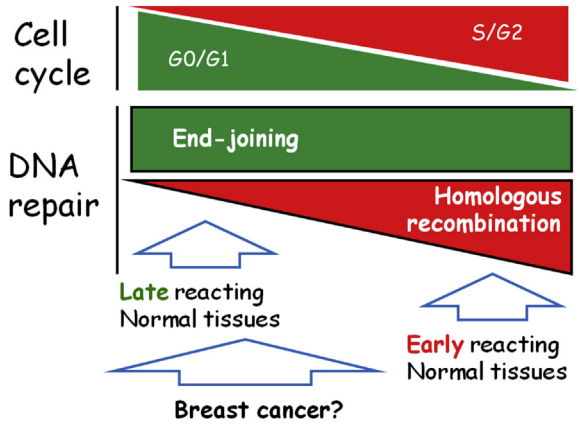

Fig 4. Schema illustrating in qualitative terms how homologous recombination and end-joining repair vary according to the cell cycle phase, and how this variation might be causally linked to the fractionation sensitivity of normal tissues. The inclusion of breast cancer ignores the potential modifying effects of genetic/epigenetic modifications.

and epigenetic modifications of DNA repair and cell cycle control networks. Deregulation of the DNA damage response is a very common feature of human cancer, reflecting the importance of induced genome instability in accelerating mutagenesis [89–91]. Illustrative examples offer some idea on how mutational deregulation of cancer cells could, in principle, affect fractionation sensitivity. Functional BRCA defects inactivate the cell's homologous recombination repair machinery in a few per cent of breast cancer patients with inherited defects in BRCA genes, but epigenetic silencing of the same genes is much more common in triple negative (ER-PR-HER2-) tumours, which achieve high levels of pathological complete response after treatment with anthracyclines, taxanes or platinum compounds [92–94]. Tumours deficient in homologous recombination may be expected to rely disproportionately on error-prone NHEJ repair of radiation-induced DSB and to show greater sensitivity to fraction size, a phenotype that might be reinforced or neutralised by mutational or epigenetic deregulation of other pathways. However, the pathways for homologous recombination are complex and although BRCA2 defective cells show a 300-fold lower rate of homologous recombination repair by gene conversion, they show less than a 2-fold lower rate of sister chromatid exchange than normally induced by DSB [95], using a similar type of homologous recombination to that induced by ionising radiation [96]. Hence, the homologous recombination defect in BRCA defective cancers may sensitise tumours to cisplatin or PARP inhibitors, but not to the same extent to ionising radiation.

A second example is over-expression of epidermal growth factor receptor (EGFR), which is a common feature of squamous cancers of the head and neck and which contributes to tumour radiation resistance via stimulation of NHEJ [97]. At least two mechanisms of resistance to ionising radiation have been described. The first involves dimerisation of EGFR and HER2 in response to irradiation, followed by activation of the ras/AKT1 pathway and activation of NHEJ repair [98]. The second mechanism describes internalisation of EGFR and transport to the nucleus, where it stimulates relaxation of compact chromatin and increases access of NHEJ proteins to DSB [99]. Increased effectiveness of NHEJ repair is a cause of radiation resistance, but it may also enhance the fractionation sensitivity of cancers over-expressing EGFR. Squamous carcinomas of the head and neck are, on average, insensitive to fraction size, a feature associated in normal tissue with high fidelity repair achieved by homologous recombination and perhaps, as discussed above, by NHEJ repair of DSB in sister chromatids bound by cohesins. This may be a good example of the complexities expected when considering how to individualise fractionation to tumour subpopulations. The contribution of EGFR over-expression to high proliferation indices in squamous carcinomas is expected to increase the relative importance of homologous recombination repair (reduce fractionation sensitivity and increase α/β value), whereas the enhancement of NHEJ repair would be expected to enhance fractionation sensitivity and reduce α/β value. Some challenges for correlative research are considered below, but three further examples of differences between normal and malignant tissues that might affect fractionation sensitivity will be described first.

DNA damage by ionising radiation activates cell cycle checkpoints in G1, S, early G2 and late G2, in order to, among other functions, facilitate DSB repair [100,101]. All four checkpoints are activated by ATM protein – ATR may also be involved – leading to phosphorylation of p53, CHK1/2 and modulation of downstream substrates, including p21, cdc25, cyclin/CDK complexes and retinoblastoma protein. The justification for listing these proteins is that several are targets for activation or inactivation by mutation in cancers in ways that might, conceivably, affect fractionation sensitivity [100,102]. If checkpoints are leaky or deregulated in cancers, allowing cell cycle progression despite elevated levels of unrepaired DSB, this would be expected to reduce fractionation sensitivity and to increase radiation sensitivity. This would be consistent with studies of ATM-deficient cells, which suffer both cell cycle checkpoint abrogation and persistent residual DSBs even at very low doses, and show hardly any delayed plating recovery and little low dose rate (LDR) sparing [103]. However, the relationship between leaky or incompetent checkpoints and cellular sensitivity is not straightforward. The radiosensitivity of ATM-deficient cells may have more to do with its regulation of NHEJ or homologous recombination repair than with its checkpoint function [104]. In addition, the threshold for triggering the late G2 checkpoint in wild-type fibroblasts seems to be as high as 10–20 DSB, with implications for cell survival as well as genomic instability [19,73].

The second further example describes deregulated tumour angiogenesis as a source of tumour hypoxia and a classic cause of radiation resistance [105,106]. Several groups have reported that the expression and function of homologous recombination proteins, including RAD51, BRCA1 and BRCA2 are compromised under hypoxic conditions [106–108]. These studies also observed that the decreased homologous recombination gene expression was independent of p53 and cell cycle distribution. In addition

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there are recent data suggesting activation of DNA-PK by hypoxia [109]. Based on the models proposed above for normal tissues wherein cells relying on NHEJ are more sensitive to fraction size, it is possible that hypoxic cells may in actual fact be sensitive to fraction size while being radioresistant due to the oxygen effect. Poor tumour oxygenation has also been shown to affect DNA damage checkpoint activation and suppress DNA mismatch repair [110]. The final further example concerns tumour vasculature, which has been postulated to play a critical role in determining radiocurability of cancer [32]. According to this model, the doses delivered as part of curative radiotherapy are capable of ablating all tumour endothelium, recovery of which relies on the HIF1-induced release of bone marrow monocytes into the circulation that restore tumour vasculature and rescue surviving tumour clonogens. On this model, fractionation sensitivity would be trumped by stromal cells repopulating the tumour bed after radiotherapy delivery.

Some Implications for Biomarker Research

Biomarker research aims to identify molecular markers evaluable in tumour biopsies that are predictive of fractionation sensitivity. Such research requires access to large collections of paraffin-embedded pre-treatment tumour samples linked to treatment outcome in patients treated with hypofractionated regimens matched to conventionally fractionated regimens in terms of dose-limiting adverse effects. Hypofractionated schedules would also, ideally, be matched with the comparators for overall treatment time to avoid confounding effects of tumour repopulation, as in recent breast trials [111]. The outcome of such research may not be successful for many technical and biological reasons. An example of the latter is the current requirement to damage DNA in order to test the functionality of homologous recombination in tissue sections, for example, using RAD51 foci as an endpoint. This involves collecting core biopsies a few hours after exposure with ionising radiation, anthracycline or platinum compound. Alternatively, some success in testing homologous recombination activation has been gained by exposing fresh tumour core biopsies to irradiation *ex vivo* [112]. Biomarkers measuring markers of proliferation and checkpoint proteins would also be included in a list of candidates.

If an index were to be validated as a predictor of fractionation sensitivity, based on the kind of correlative research described above, one of the questions to ask is 'how broad is the distribution of fractionation sensitivity around the average in tumours of a particular type/anatomical site'? At one extreme, the distribution of α/β values of breast cancer may be very narrow, in which case biomarkers are not needed; a breast cancer diagnosis may be enough to justify hypofractionation. If the distribution is broad, with significant overlap of inter-quartile ranges between tumours arising at different anatomical sites, it may be justified to test the value of predictive biomarkers in the clinical setting. If labelling indices and fractionation sensitivity are associated in cancers as they are in normal tissues,

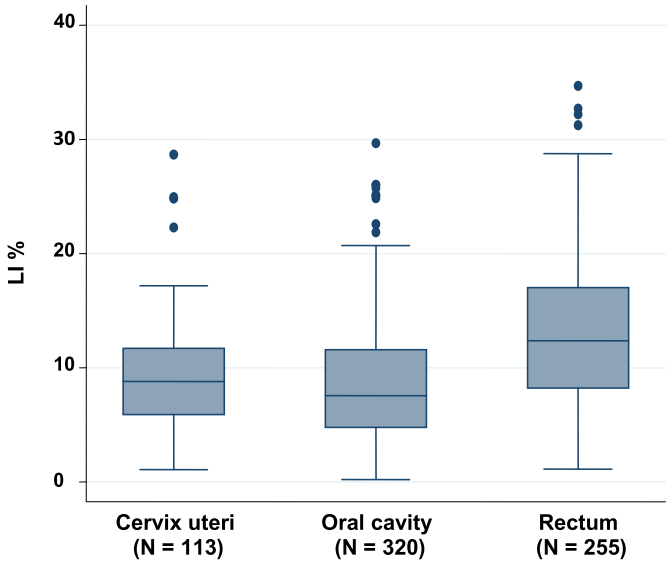

Fig 5. Variation in labelling index (LI) of three human tumour types (cancers of cervix uteri, oral cavity and rectum). 102/111 cancers of the cervix and all 320 cancers of the oral cavity were squamous carcinomas; the other 11 cancers of the cervix uteri and all 265 cancers of the rectum were adenocarcinomas. Shaded boxes encompass 25th–75th percentiles, including the median value (horizontal line) and whiskers approximate to lower and upper quartiles (solid symbols represent outside values). Data provided courtesy of Dr George Wilson, Beaumont Health System.

the broad distribution of labelling indices reported in some human cancers is consistent with a broad range of α/β values within tumour types (see Figure 5). Evaluation of a biomarker approach within a randomised clinical trial could, in principle, include patients with different tumour sites. This scenario describes a major challenge, but one that could bring significant gains in treatment outcome.

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