Personalisation of radiotherapy for breast cancer

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Abstract

The role of radiotherapy is well established in the multidisciplinary management of breast cancer. However, its use could be customised further with the intent of enhancing tumour cell radiosensitisation and reducing normal cell toxicity. The importance of tumour heterogeneity and the microenvironment in the response to radiotherapy is under intense scrutiny and the value of molecular profiling is being increasingly recognised. Genome Wide Association Studies are likely to play an important role in elucidating the molecular pathogenesis of radiotoxicity in the emerging area of radiogenomics. Biomarkers of tumour radiosensitivity should help indicate potentially responsive and unresponsive cancers. Further understanding of the tumour microenvironment and better preclinical models will help identify targets to enhance radiosensitivity or reverse radioresistance.

Keywords: Radiotherapy, Partial breast irradiation, radiosensitivity, tumour heterogeneity, tumour microenvironment

Introduction

Adjuvant radiotherapy continues to play a key role in the multidisciplinary management of breast cancer. Its objective is to eradicate residual tumour after surgery. Adjuvant radiotherapy has traditionally been restricted to the postoperative setting after breast conserving surgery and selectively after mastectomy. It has an established role in locally advanced disease either following systemic therapy where inflammatory changes or peau d'orange preclude mastectomy or postmastectomy. Recently it has been investigated in the preoperative setting in an early phase clinical trial in combination with neoadjuvant chemotherapy [1].

The Oxford overview shows that, after mastectomy and breast conserving therapy, radiotherapy reduces significantly both local recurrence and breast cancer mortality. At 15 years of follow up, for every 4 recurrences prevented in the first 5 years, one breast cancer death can be avoided [2]. As the incidence of breast cancer rises globally, as a consequence of early detection from breast screening programmes and rising life expectancy, demands for adjuvant irradiation are likely to rise. Using models of evidence based recommendations, 83% of patients with breast cancer will require external beam irradiation [3]. Increasing pressures on limited radiotherapy resources pose challenges for better selection of patients predicted to benefit from irradiation. The rationale for personalisation of radiotherapy is therefore strong. There are two possible areas of individual modulation: tumour and normal tissue radiosensitivity. Scope for modulation of the former seems a more realistic prospect than the latter, given how little we understand about the genetic basis of normal tissue radiosensitivity. With the rising population of older patients in whom comorbidities may preclude surgery and chemotherapy, the understanding of normal tissue radiosensitivity and the interaction with comorbidities will become increasingly important [4].

Traditionally the treatment of breast cancer has been based on evidence from patients in clinical trials with a limited range of clinico-pathological characteristics (typically TNM staging, grade, ER status etc). Where data show clinically significant benefit from intervention (surgery/drug/ radiation) which reached a predefined threshold (e.g. 3% in overall survival benefit at 5 years), the results are applied to all patients meeting the eligibility criteria for that trial. In the case of adjuvant radiotherapy for breast cancer, the application of a boost of irradiation to the site of excision after breast conserving surgery (with clear margins) and whole breast irradiation is predominantly applied to women under the age of 50 where most benefit in reducing local recurrence is obtained [5].

The rationale for treatment personalisation

The development of high throughput sequencing and improvements in our understanding of the molecular mechanisms underlying malignant transformation and progress have facilitated a move away from a 'one size fits all' approach to efforts to personalize treatment for breast cancer [6]. One of the major limitations of the 'one size fits all' approach has been the underrepresentation of older patients in clinical trials of adjuvant radiotherapy, in part due to the historical exclusion of such patients due to arbitrary age eligibility caps at around 70 years. Advances in the therapeutic ratio for adjuvant radiotherapy either have to achieve a higher level of local control for the same or lower levels of acute and late radiation induced toxicity or the same level of local control for lower levels of radiotoxicity.

Much of the current focus on the personalisation of the treatment of breast cancer has been driven by two developments. The first has been risk assessment in early stage breast cancer to avoid overtreatment, in particular in node negative disease with the application of molecular profiling [7]. The second is the availability of targeted therapies, particularly for HER2 positive disease. Much less attention and research has been devoted to customising radiotherapy to the biology of the cancer and to the sensitivity of normal tissues to irradiation. This field is still in its infancy. Progress has been hampered in part because radiotherapy is not accepted as a targeted therapy and therefore personalization is not viewed in the same way as targeted therapies such as trastuzumab for HER-2 positive cancers.

Advances in radiation planning and delivery

Already there has been significant progress in personalizing radiotherapy through the application of 3D treatment planning with radiation portals customized to maximize target coverage while minimising irradiation of critical structures such as the heart and the lungs. More recent developments have been the application of intensity modulated (IMRT) and image guided radiation therapy (IGRT) to optimize dose distribution [8]. Much less progress has been made in understanding the biological factors which underlie differences in radiosensitivity between patients. We are therefore some way from an assay which would predict clinical radiation response for individual patients [9]. Gilbert Fletcher, a pioneer in the development of radiotherapy, compared the successful development of a radiotherapy predict assay to the quest for the Holy Grail [9, 10].

Importance of tumour heterogeneity

The heterogeneity within tumours is an acknowledged component in resistance to both radiation and systemic therapy. Studies in renal cancer have demonstrated that each area of a tumour displays specific genomic arrangements [11]; these findings are now being replicated in breast cancer [12, 13]. Such heterogeneity has a significant influence on radio and chemotherapy [14]. Differences in radiation sensitivity are apparent among patients with similar types of breast cancer, which may reflect disparities in the mutational burden of these tumours [15]. Variation in response can also be caused by the presence of radioresistant breast cancer stem cells [16 – 18], and further by the induction of 'stemness' by radiation treatment itself [19, 20].

Enhancing the efficacy of adjuvant radiation therapy

Adjuvant radiotherapy is a potent agent in the multidisciplinary armamentarium of anticancer therapy in breast cancer both in terms of loco-regional therapy and survival.The 2005 Early Breast Cancer metanalysis of adjuvant radiotherapy after breast conserving surgery and mastectomy showed a direct relationship between the absolute reduction in locorregional recurrence at 5 years and an improvement in long term survival at 15 years [2]. This equated to a 4:1 ratio with one breast cancer death being avoided for every 4 locoregional recurrences prevented by radiotherapy. This was the first evidence of a systemic effect of radiation in breast cancer. However this equation proved to be erroneous because of the competing risks of local and distant disease, the lack of definition of time to locoregional recurrence and the systemic effect of RT [21]. As a result, the EBCTCG decided to report the effects of RT on first recurrence whether loco-regional or distant.The Oxford overviews of adjuvant postoperative radiotherapy for breast cancer after breast conserving surgery and mastectomy show that it has a higher effective to loco-regional control and overall survival [22, 23]. It halves approximately the risk of first recurrence at 10 years after breast conserving surgery [22] and improves long term survival. For patients treated by mastectomy and axillary clearance (at least level II) the overview shows that radiotherapy reduced mortality from breast cancer by 20% in women with 1-3 positive lymph nodes (rate ratio [RR], irradiated *vs* not, 0·80, 95% CI 0·67–0·95; 2p=0·01) and by 13% in women with at least 4 positive axillary nodes nodes (RR 0·87, 95% CI 0·77–0·99; 2p=0·04) [23].

It is important to recognize that the benefits of radiotherapy as a local therapy in terms of survival need to be seen in the context of systemic therapy. Systemic therapy reduces the risks of both local and distant recurrence. With the ability of systemic therapy to control micrometastatic disease, loco-regional control assumes even greater importance. However while these meta-analyses provide important insights to the effectiveness of loco-regional radiotherapy, they do not predict individual patient benefit. Indeed the magnitude of benefit might be larger among the radiosensitive population of patients since the benefits in the overview may be diluted by patients with radioresistant tumours. Hence, the dividends of being able to predict radiotherapy benefit by biomarkers may be substantial if they are more accurate than conventional clinico-pathological factors. This is an area of active investigation. Personalisation of breast cancer radiotherapy can be considered in a number of ways: 1) selection for treatment based on clinico-pathological factors and

molecular features 2) Optimising dose distribution within the irradiated volumes 3) Minimising loco-regional morbidity by minimizing dose to critical structures, particularly the lung and heart.

Theories of breast cancer spread

Personalization of radiotherapy must consider the heterogeneity of breast cancer. In the first half of the 20th century, breast cancer was believed to be a disease that spread from the primary site to the regional nodes (the Halstedian hypothesis), giving a rationale for aggressive loco-regional treatment. This was succeeded by the view of breast cancer as a systemic disease (the Fisher hypothesis), based on the observation that patients developed distant metastases despite the primary site being controlled. Fisher argued that patients could be divided into two categories; those tumours with the ability to spread to distant sites and those that lacked that ability [24]. Neither view was valid for all breast cancers [25]. The current 'spectrum' theory advanced by Samuel Hellman is that breast cancer is a 'heterogeneous disease…[with] a spectrum of proclivities extending from a disease that remains local throughout its course to one that is systemic when first detectable' [26, 27]. Although some tumours have not metastasized from the primary site at the time of diagnosis, there is no current, reliable method to detect micrometastatic disease. Failure to achieve local control may facilitate metastatic spread and reduce survival. Hellman's theory acknowledges that the higher the chance that systemic disease is present at the time of diagnosis, the lower the impact of local therapy. This may explain why the survival advantage of loco-regional postemastectomy radiotherapy in the Danish Cooperative Breast Group trials is seen in the subgroup of good prognosis ER or PgR positive, HER2 negative patients and not in ER or PgR negative or HER2 positive patients where the latter have probably developed distant metastases [28]. With the recognition that breast cancer is a heterogeneous range of diseases with distinct molecular subtypes (luminal A, luminal B, HER2/neu), optimal strategies for different subsets of patients may be necessary [29].

Molecular profiling might assist in identifying patients (a) at sufficiently low risk of relapse that radiation might be avoided, (b) at sufficiently high risk of loco-regional recurrence who might benefit from additional radiation dose or combination with systemic therapy, (c)with early breast cancer who would benefit from postmastectomy radiotherapy and (d) suitable for partial breast irradiation [29]. In addition molecular targets that may be related to radiation resistance might be found.

Personalisation of radiotherapy, heterogeneity and tumour/normal tissue radiosensitivity

Improving the therapeutic gain of radiation (ie improving local control for the same level of normal tissue toxicity) for individual breast cancer patients is an enormous challenge because of tumour heterogeneity and the limited understanding of the genetic basis of tumour and normal tissue radiosensitivity. Breast cancer is a spectrum of diseases with heterogeneity at molecular, histopathological and clinical levels [30]. This heterogeneity includes the tumour microvironment and its different cellular components involving many different biological processes including angiogenesis, tumour metabolism and the immune response. New approaches on combining drugs targeting the pH regulatory mechanisms of breast cancer in preclinical models in combination with radiation are discussed later in this chapter.

Selection of patients on clinico-pathological factors

Already we have some degree of personalization of adjuvant radiotherapy based on clinico-pathological factors. For example patients under the age of 50 benefit from an additional boost dose to the site of excision following breast conserving surgery and postoperative whole breast irradiation [5]. The biological mechanism underlying the impact of young age still remains poorly understood.

Biomarkers of tumour radiosensitivity

It is a common clinical experience that local control rates following adjuvant systemic therapy and radiotherapy based on traditional clinicopathological factors (tumour size, grade, nodal status and clinical stage) do not reliably predict clinical outcome. One of the holy grails of radiation

oncology in general and breast cancer in particular has been to identify reliable and clinically applicable biomarkers of tumour radiosensitivity which could select patients likely to benefit and those that would either derive no or minimal benefit. Patients with no evidence of radiosensitivity might be spared the toxicity of radiation therapy and offered alternative systemic approaches. Those with low or moderate radiosensitivity might be subject to combinatorial approaches with drugs increasing radiosensitivity.There are however a limited number of studies,all retrospective showing promise for molecular markers of breast cancer radiosensitivity. Prospective studies and randomised trials will be needed to evaluate their clinical utility.

The variables which determine radiosensitivity can be categorized into three group (i) Intrinsic radiosensitivity (ii) tumour oxygenation status (iii) tumour proliferative potential (Tpot). Clonogenic cell survival assays have been the cornerstone for measuring intrinsic radiosensitivity. However they are difficult to do as the *ex vivo* plating efficiency is around 1% and none of these assays is applicable in the clinic. The Eppendorf probe has been the main method of measuring intratumoral oxygenation status but is impractical to apply in breast cancer and other solid deep seated tumours. Tpot is a measure of tumour doubling time based on flow cytometry from a tumour biopsy stained with bromodeoxyuridine. However, it is a weak predictor of outcome [9].

Gene expression classifiers

Genomic profiling technologies have allowed the stratification of human breast tumors into clinically useful groups and have further aided in the personalization of the treatment of breast cancer. The genomic era has produced an exponential increase in our understanding of cancer biology and has greatly accelerated cancer drug development. With the advent and implementation of microarray expression profiling, it is now possible to evaluate gene expression in tumors on a genome-wide basis. These advances have led to the utilization of gene expression profiling to not only subtype cancers, but to predict prognosis and disease free survival, and determine optimal treatment.

Emerging gene expression data suggests that breast cancer is a clinically heterogeneous disease. This clinical heterogeneity is driven to a large extent by abnormal gene expression within tumors. Investigators now have the ability to identify the gene-expression fingerprint of an individual's tumor. This information may be used to rationally design treatment regimens for patients in the future, and also to predict the clinical course of an individual's disease, including response to a radiation treatment. Genetic profiles of tumors are now being correlated with clinical outcome, and several prognostic and predictive indicators have emerged based on this research. Additionally, transcriptional and proteomic profiling is advancing our understanding of the RNA and protein alterations in human cancers. Despite early limitations, genomic classifiers are now being used clinically to better risk stratify patients and guide rational therapy decisions by clinicians.

Multiple gene sets have been developed in an attempt to stratify patients based on the gene expression signature of their tumors. One of the first of these was the Rotterdam gene set. It was developed to predict the prognosis of patients with lymph node negative (LNN) breast cancer [31]. Markers were selected separately from ER-negative and ER-positive tumors and were combined into a single 76-gene prognostic signature that was able to predict distant metastatic recurrence with a sensitivity of 93% and a specificity of 48% [31]. This prognostic indicator performed better than standard, clinical variables in a multivariate analysis (hazard ratio [HR], 5.55; 95% confidence interval [CI], 2.46-12.5). Subsequently, this test was also validated using two other sets of patients with early stage breast cancer that were not included in the original study. This test in now FDA-approved and is clinically used to identify patients who should receive chemotherapy. The success of this gene expression profiling approach to address clinically relevant uncertainties underscores the utility of such profiling in the management of breast cancer

Subsequent gene expression profiles have been developed and are now being used clinically to help identify individual tumors that will respond to chemotherapy. One such example is OncotypeDx®. This 21-gene assay was derived from 250 candidate genes chosen from gene-expression profiling experiments, published literature, and genomic databases and then correlated with breast cancer recurrence in 447 patients [32, 33]. Sixteen cancer-related genes and five

reference genes were selected from the candidate genes. The 16 cancer-related genes were then used to develop an algorithm based on the expression levels of these genes, thus allowing a Recurrence Score™ (RS) to be computed for each specimen. This RS correlated with the rate of distant recurrence at 10 years. The OncotypeDx® assay was externally validated in the National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trial B-14, which examined the effect of adjuvant tamoxifen in patients with hormone receptor–positive LNN breast cancer [32, 33]. The results of this analysis showed that 7% of low-risk patients (RS <18) relapsed, whereas 31% of high-risk patients (RS >31) relapsed. Subsequent studies have shown that the RS is independently associated with sensitivity to chemotherapy and mortality [33, 34]. The OncotypeDx® assay is now FDA-approved for use in profiling the risk and need for chemotherapy responsiveness in breast cancers. It is now in wide clinical use by oncologists to determine which patients would benefit from chemotherapy.

OncotypeDx® is not the only prognostic gene profiling test being used clinically. Other tests currently approved include Mammaprint [35], Mammostrat [36], Prosigna [37–39], and CellSearch [40]. These tests, which also mainly rely on the use of gene expression technologies and molecular signatures, underscore the power and utility of such approaches at identifying expression derangements and the potential for treatment personalization for individual patients.

In addition to defining molecular subtypes of cancers and predicting prognosis and disease free survival, gene expression classifiers have been used to determine optimal treatment [41-45]. Several groups have already used expression profiling to identify gene signatures of chemotherapeutic resistance [41, 42, 46]. These studies have identified tumor gene expression profiles associated with response to chemotherapy including docetaxel [41,42] adriamycin/cyclophosphamide [47], paclitaxel, fluorouracil, doxorubicin, cyclophosphamide [48], and epirubicin, cyclophosphamide, paclitaxel [49] in the neoadjuvant setting. Together, these studies indicate the potential to not only determine the likelihood of response to a particular therapy, but may be incorporated into ways to personalize radiation therapy.

As mentioned previously, gene expression assays have been developed to predict clinical outcomes in breast cancer. The main focus of these has been to predict systemic rather than local recurrence. The 70 gene signature was the first to identify patients who are at higher risk of systemic relapse. This signature was developed using a cohort of 78 patients with axillary node negative, ER-positive or -negative early- stagebreast cancer all under the age of 55 years with tumour up to 5cm in size [47]. This approach identified 70 genes that were able to identify women with a significantly increased risk of distant metastasis and death from breast cancer. Not surprisingly, genes associated with cell cycle regulation, invasion, metastasis and angiogenesis predominantly comprised the 70 gene signature. Interestingly, this signature also was able to identify *BRCA1* mutation carriers based on the expression level of these genes. This was one of the first successful applications of the gene expression analysis to guide clinical care. Prior to the development of genomic tools, a number of prognostic approaches to assess tumour radiosensitivity were developed including: hypoxic fraction, DNA strand break and repair, intrinsic radiosensitivity, proliferation cell fraction [50].

Despite the development of numerous gene expression signatures to predict survival, distant metastasis, and response to chemotherapy in patients with breast cancer (i.e. OncotypeDX®, Mammaprint®, Prosigna®) there has, until recently, been no attempt to develop signatures predictive or radiation response. One of the first attempts to identify such a radiation response came from a group out of the University of Chicago [46]. This group derived an IFN-related DNA damage resistance signature and then applied it to 34 cancer cell lines from the NCI60 panel. The genes in this signature that correlated most robustly with radiation resistance (SF-2Gy) were retained within the signature and then applied to patient datasets. The authors found that the IFN-related DNA damage resistance signature was able to predict response to both chemo- and radiotherapy. Though not further developed into a clinically tranlslateable test, it was the first proof-of-principle attempt to develop a molecular signature predictive of radiation response.

One test recently proposed for clinical practice is borrowed heavily from the existing OncotypeDx® test, a test that was developed to predict reponse of patients with ER-positive, lymph

node-negative breast cancer to adjuvant chemotherapy. This spin-off of the OncotypeDx® test was recently reported to estimate local recurrence risk in the absence of radiotherapy in women with DCIS after breast conserving surgery [51]. While not yet validated in a prospectively run clinical trial, it does suggest the potential power of molecular signatures to personalize treatment decisions regarding the need for adjuvant radiation treatment.

More recently a 10-gene expression signature (AR, cJun, STAT1, PKC, RelA, cABL, SUMO1, CDK1, HDAC1, and IRF1) has been described by Eschrich et al [52]; this signature was developed initially in studies using a panel of 48 cancer cell lines before being validated in 3 independent datasets (rectal, esophageal, and head and neck cancers) [52]. In these initial validation studies, the signature was able to accurately predict which patients were at increased risk of recurrence based on the expression of these 10 genes. Furthermore, when applied to two independent breast cancer datasets (totalling 503 patients) indicated that the radiosensitivity signature may also act as a predictive biomarker for breast cancer, though these studies looked at distant metastasis and not local recurrence [52].

A final and most recent signature has been developed by investigators at the University of Michigan [53]. Previous attempts to identify signatures predictive of response to radiation using gene expression data have relied on expression data from patient tumor samples for signature development and have repeatedly failed external validation. The University of Michigan group instead assessed the intrinsic radiation sensitivity of 21 breast cancer cell lines and identified a panel of 147 genes whose expression was significantly correlated with radiation sensitivity. This 147 gene signature was then trained, locked, and validated on cohorts of patients with early stage breast cancer treated with radiation therapy after breast conserving surgery for whom local recurrence data was available. This radiation sensitivity signature (RSS) was shown to effectively and accurately identify patients with high rates of local recurrence based on predicted radiation resistance as reflected by the RSS score. This was the first signature to specifically identify patients with increased rates of local recurrence, not systemic progression, after radiation treatment. Like the signature developed by Eschrich et al., this signature awaits external validation before it can be effectively translated into clinical care.

Personalising approaches to minimize radiation induced toxicity

In the clinic we see wide variations in the intensity of acute and late radiation reactions (for example skin erythema and breast fibrosis). It is estimated, based on studies of internal mammary irradiation, that patient specific factors account for 49-90% of these differences [54], but the exact reasons for these variations or how they might be modified based on genetic profiles of radiosensitivity are little understood. In older patients, radiotoxicity might also interact with comorbidities such as heart disease and diabetes. Currently, no clinically applicable assays predict normal tissue radiosensitivity. Preclinical tests using lymphocyte and fibroblast radiosensitivity proved too complex and non-replicable to be used clinically [55].

In vitro tests of radiosensitivity in humans demonstrate a nearly normal distribution, implying that normal tissue sensitivity is a polygenic trait; therefore, most of the clinically observed variation in radiosensitivity is likely to be due to low penetrance genetic variants [56]. The radiosensivity phenotype is possibly influenced by multiple loci ranging from rare variants with large effects to common variants with minor effects [57]. The scope for modulating the response of individual patients by any form of drug therapy in the foreseeable future is therefore likely to be limited.

One promising new approach to understanding the genetic basis of normal tissue radiosensivity is the emerging field of radiogenomics which investigates the influence of genetic variation on radiation response [58]. The long term goal of this research is to develop SNP-based risk models to stratify patients for individualised radiotherapy protocols [59]. Genetic association studies initially focussed on candidate genes involved in known radioresponse pathways and sought to

identify functional SNPs (single nucleotide polymorphisms) that influence normal tissue radiotoxicity [58]. SNPs within the XRCC family of genes, ATM and TGFB1 genes have all been suggested to be involved in radiotoxicity in breast cancer patients [60]. Transforming growth factor Beta 1 (TGFB1) is one of the candidate genes considered to be involved in the genesis of radiation induced breast/chest wall fibrosis [61]. However, data analysis of SNPs in predicting radiation toxicity shows conflicting results. For example, the independent validation of SNPs in 46 genes previously published to be associated with radiosensivity was not confirmed in the UK RAPPER study [61]. The more recently introduced Genome Wide Association Studies (GWAS) could play an important role in elucidating the molecular pathogenesis of radiotoxicity [61]. An important finding in many of the GWAS studies is that the identified SNPs are not in known genes or pathways already considered to be key candidates [58]. Furthermore many of the SNPs are found in non-coding regions without any known or obvious function.

The principal challenge for emerging radiogenomics consortia is the availability of accurately reported toxicity data [62]. Risk models incorporating genetic assays as well as comorbidities, radiation dose and the volume irradiated require development [63, 64].

What is presently achievable on an individual basis is maximising the homogeneity of dose distribution in the breast and reducing where possible exposure of critical structures such as the lungs and the heart to radiation. Acute toxicities have been defined as those occurring within 90 days of treatment, affecting tissues such as the skin which have a rapid renewal rate. Late toxicities occur more than 90 days after radiotherapy [57]. The changing shape of the breast in the transverse and sagittal planes makes it difficult to irradiate homogeneously. There is level I evidence that the application of intensity modulated radiotherapy (IMRT) reduces acute skin toxicity [65] and improves cosmesis [66] compared to standard RT.

Minimising radiation induced cardiac toxicity

Before the advent of 3D planning it was difficult to assess how much of the heart was being irradiated by postoperative radiotherapy after breast conserving surgery or mastectomy. The Oxford overview showed that an approximately 2% 20 year reduction in breast cancer mortality from adjuvant radiation therapy was counterbalanced by a similar percentage of non breast cancer mortality (mainly cardiac) [67]. Much of the cardiac morbidity and mortality may have been accounted for by older radiation techniques where the doses delivered to the heart were higher. However minimising cardiac exposure has been a contemporary priority in adjuvant radiotherapy for breast cancer. A study of two cohorts of women from Denmark and Sweden shows that there is a linear relation between mean heart dose and the rate of major coronary events with an increase of 7.4% per Gy [68]. A prospective trial has shown has shown that active breathing control can reduce mean heart dose by =/> 20% of patients in 88% of cases [69].

Personalisation of radiotherapy - pre-clinical models

Most pre-clinical research in oncology begins with the use of panels of cell lines that reflect the relevant cancer and its diverse subtypes. This is an oversimplistic model that neither replicates the *in vivo* tumour microenvironment or the heterogeneity found in breast and other tumours (see above). Single cell sequencing illustrates that tumours are made up from many distinct clones with varied mutations [70]. Therefore even in cancers of the same subtype, different clones and mutations will be present that will determine the outcome of both systemic and radiation treatment. Cell line derived xenograft models are extremely useful to monitor tumour growth, drug pharmacokinetics, efficacy and toxicity and have the added benefit of modelling the oxygen and pH gradients found in the tumour microenvironment, but the models are still dependent on available cell lines. Genetically engineered mouse models allow investigation of specific mutations, but although they can closely replicate clinical trial results, they are not personalised tumours.

One method that can examine a specific patient's tumour directly is tumour grafting or patient-derived xenografts (PDXs) [71]. In this model, cells from digested patient tumour tissue are transferred to immunodeficient mice, and then passaged from host to host. PDXs of human breast

cancer accurately duplicate receptor expression, growth, metastatic capability and pathology of the actual tumour [72, 73]. However, these models have substantial costs, because PDXs must be sustained in mice, and like other xenografts, tumour growth can take time to establish [74]. Although these and other animal models are extremely useful, all have limitations and none are suitable for fast, high-throughput methodology [75, 76].

However, primary tumour material (explants) from a specific breast cancer patient can be investigated *ex vivo*, using pre-treatment biopsy material [77, 78]. This method can be a useful adjunct in the thrust for personalised treatment, allowing analysis of a heterogeneous tumour, with an intact microenvironment and stromal tissue [79]. In our laboratory, pieces of tissue from all breast cancer subtypes are placed in a collagen matrix and cultured with or without drug or radiation treatment. Changes can be perceived within 5 days in most cases. Therefore this system could be used to investigate tumour responses to a specific therapy. Our studies indicate that these cultures can be maintained with minimum management for at least 30 days [78]. Such cultures can be continuously observed and examined when necessary, and lysed or fixed for analysis when required.

Using the explant model we are currently investigating the radioresponsiveness of individual breast cancer patient tumours to radiotherapy (Figure 1). This figure illustrates the use of tumour tissue obtained from pre-treatment breast cancer patientbiopsies. In Figure 1A the invasive growth of an untreated single explant is monitored for 10 days. This control explant could be treated with drug or radiation and the effects on further growth or invasion monitored. Figure 1B shows a radiosensitive explant in which no further growth or invasion has occurred in tissue treated with 5GY radiation, compared with control explants, where invasive growth has increased by approximately 500%. Contrast this with Figure 1C, where 5GY radiation treatment has had no effect on explant growth in comparison to untreated control explants, showing that tumour material from this patient is radiation resistant. Use of this experimental system could a) allow personalisation of radiation treatment and b) permit therapeutic strategies for radiation sensitisation to be assessed in a more physiological model using actual breast cancer tissue.

The radioresistant tumour microenvironment – novel therapeutic targets

Hypoxia causes significant resistance to both radio- and chemotherapy and is found in approximately 40% of all breast cancers and 50% of advanced breast cancers demonstrating oxygen concentrations under 0.3% [80]. Tumour cells use aerobic glycolysis to provide fuel and components for growth, even in the presence of sufficient oxygen [81, 82]. This leads to the excess production of CO_2, lactate and protons, which lowers the intracellular pH (pHi). However, several ion pumps, enzymes and transporters preserve pHi between 7.0 - 7.4 [81]. These include carbonic anhydrase (CA) IX, a Hypoxia-inducible factor-1 (HIF-1) induced enzyme that catalyses the conversion of CO_2 and H_2O to hydrogen ions and $HCO3^-$, the hypoxia-activated Na+/H+ exchanger 1 (NHE1), which extrudes hydrogen for sodium ions, monocarboxylate transporters (MCTs), which are H+/lactate transporters, and finally V-ATPases, which act as H^+-transporters [81]. However these proteins, while contributing to the alkaline pHi found in hypoxic cancer cells, lead to acidosis in the tumour microenvironment, facilitating invasion and metastasis and also increasing resistance to both systemic and radiation treatment [83]. Radiation resistance increases because hypoxia decreases cell proliferation and DNA damage is augmented in proliferative cell populations; lactate, via its antioxidant properties, has also been linked to resistance mechanisms in several tumours [83-85]. Therefore targeting the adapation mechanisms induced by the changing microenvironment may offer new targeted therapies. Currently, preclinical strategies are exploring novel compounds that disrupt pH regulation in concert with radiation and systemic drug therapies [86-88].

Pre-clinical research in our laboratory suggested that NHE1 inhibitors could sensitize breast cancer cells to radiation (unpublished data), however phase III clinical trials of these drugs reported increased levels of stroke [89]. Several xenograft models have shown enhanced sensitivity to radiation in colorectal and small cell lung cancer using MCT1 inhibitors [90, 91]. MCT1 and MCT4 require an accessory molecule, CD147, for correct situation in the plasma membrane. Silencing of CD147 in breast cancer cells caused robust inhibition of lactate production and glycolysis [92].

Strategies that reduce lactate concentration in the tumour microenvironment should reduce radiation resistance [84, 93, 94]. Invasion and metastasis of breast cancer cells can be inhibited by blocking V-ATPase activation [95, 96]. Some inhibitors are too toxic to be used clinically, but several proton pump inhibitors (PPIs) are used therapeutically, with negligible contra-indications, and these compounds also act as V-ATPase inhibitors [97]. Radiation resistance may be partly influenced by the induction of autophagy, which is V-ATPase-dependent [98], therefore PPIs may increase sensitivity to radiation.

Currently, CAIX inhibition is a promising therapeutic objective. This enzyme is often overexpressed in breast cancer, where it correlates with poor prognosis [99], but it is infrequently found in normal breast tissue. CAIX knockdown in murine models show that the consquences are limited to gastric hyperplasia implying limited toxicity issues [100, 101]. Knockdown of CAIX has been linked to radiation sensitization [102], and a novel class of sulfamate CAIX inhibitors enhanced the effects of radiation in a colorectal cancer model, both in vitro and in vivo [87, 88]. Pre-clinical research in our laboratory suggests that these novel inhibitors may sensitise breast cancer to radiation using several models.

Personalisation of treatment through monitoring strategies

Because of the erratic tumour vasculature, tumour hypoxia is discontinuous. If tumour oxygenation could be monitored in real time, radiation treatment could be given when oxygen concentrations in the tumour were at their highest to achieve maximal efficiency. Alternatively, hypoxic areas could be treated with larger radiation doses to increase efficacy. Oxygen and metabolic indicators can be observed in real time using microphysiometry equipment in *in vitro* research [65], and currently biosensors are being developed that would permit personalised monitoring of tumour hypoxia in real-time [http://www.see.ed.ac.uk/drupal/impact], and allow optimal radiation treatment strategies to be scheduled with the greatest clinical benefit for each patient.

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Figure 1. Personalised assessment of radiosensitivity using pre-treatment breast cancer patient biopsy material

A) Tumour biopsy material was trimmed to remove fat. 1mm3 pieces were cultured in a collagen matrix and growth monitored over 10 days. B) illustration of a radiosensitive tumour treated with 5GY radiation and monitored for 15 days. Data shown indicates changes in growth normalised to Day 0 area (n=4 ± SD). C) illustration of a radioresistant tumour treated with 5GY radiation and monitored for 15 days. Data shown indicates changes in growth normalised to Day 0 area (n=4 ± SD).

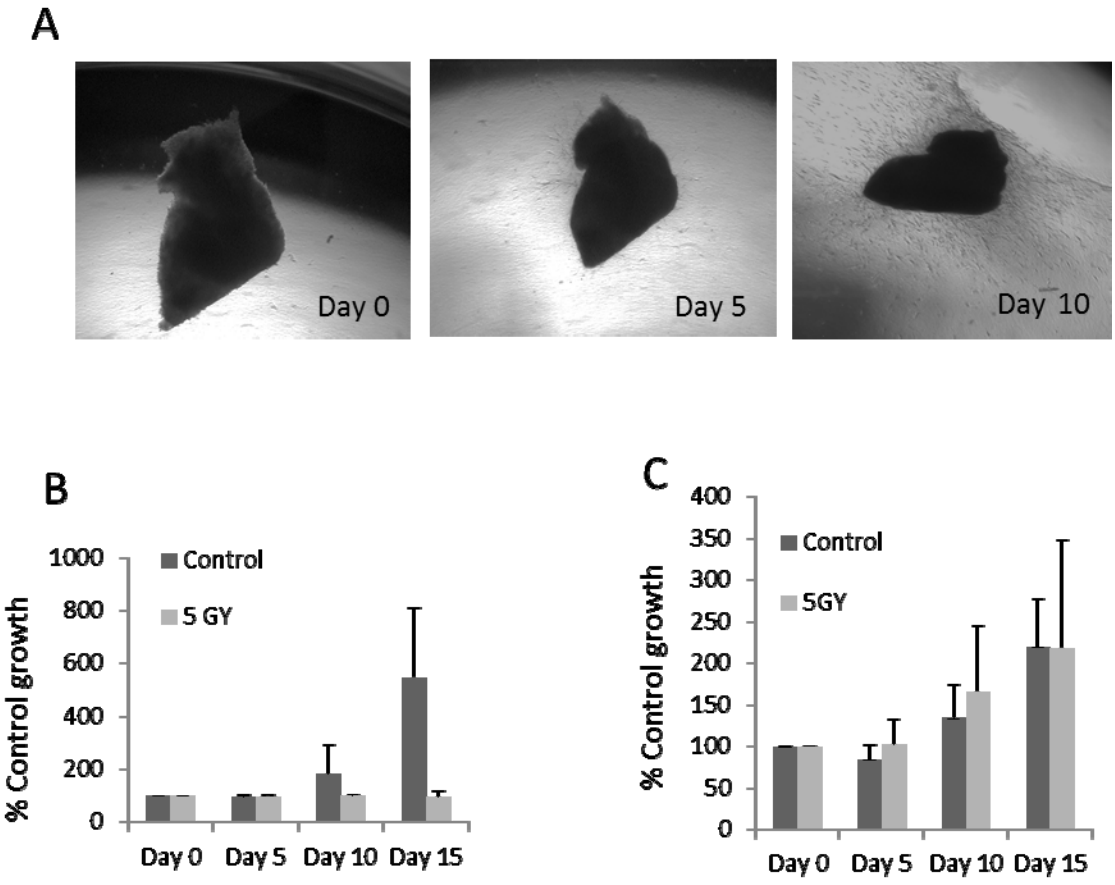

Figure 1